Photoperiodism, melatonin and the pineal

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Photoperiodism, melatonin and the pineal
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Photoperiodism, melatonin and the pineal: it's only a question of time

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Notably absent from the participants at this symposium on Photoperiodism, Melatonin and the Pineal is a present-day Copernicus or Galileo, and yet it is to astronomy that we must turn if we are to understand one of the basic dilemmas faced by all forms of life on our planet. Our days and our seasons are determined by two entirely independent astronomical events, and yet both circadian and circannual rhythms have profound effects on every living organism. How can these two independent rhythms be timed, so that their different biological consequences can be anticipated and prepared for in advance? In the Economy of Nature, has one clock been found that will predict both events with precision? What is the mechanism that makes this clock tick? How does it time the days, and the years?

It is as well to be reminded at the outset of the astronomical origin of the events with which we are dealing (Thomas 1982). The length of a day represents the time taken for the earth to complete one revolution about its vertical axis. But since the sun regulates the hours of daylight, what we are really concerned with from a biological standpoint is the time that it takes for the earth to rotate once *with respect to the sun.* Since the earth is orbiting the sun whilst it rotates, the relative positions of earth and sun are constantly changing, and a *solar day* corresponds to a rotation of the earth through about 361°.

A year represents the time it takes for the earth to orbit the sun. But once again, biologically speaking, it is the timing of the seasons that is important. Since the seasons of the year are determined by the angle of tilt of the earth's axis with respect to its plane of rotation about the sun, and the earth is slowly wobbling, or precessing, about this axis, a *tropical year*, mea-

sured as the time between successive spring equinoxes, is 365.24219879 days, or about 20 min shorter than the true orbital time, which is referred to as a sidereal year.

What clocks was man able to devise to measure time? Perhaps the easiest events to monitor were the annual summer and winter solstices, when the sun rises and sets furthest to the north or south, and reaches its greatest or least angle in the sky at midday. Stonehenge, on Salisbury Plain, is one of the greatest megalithic monuments apparently oriented around the summer solstice, and it was constructed in about 2800 B.C. (Daniel 1980).

Absolute measurement of the time of day presented a more formidable problem (Lloyd 1968). The Egyptians used shadow clocks to record the passing of the daylight hours, and water clocks or clepsydrae for the night hours. The best example of the latter comes from the temple of Karnak; it was of the outflow variety, with its inner surfaces graduated for the hours of the night, with variations to take into account the seasonal differences. The water outflow from such clepsydrae was usually guarded by an effigy of Thoth, the baboon (*Papio cynocephalus*), God of the night hours. Simple outflow clocks were used in classical times in the law courts of Greece and Rome to limit the length of speeches, and the Saxons measured time by means of a bowl with a hole in the bottom which sank in a prescribed time when placed in a bath of fluid. Graduated candles, shielded from draughts, were another popular device, but they all measured time in arbitrary units, and man lacked an exact standard, transferable from place to place.

The first true clocks, driven by water, represented a notable advance, and a Chinese example has been found dating back to the eighth century A.D. The first weight-driven clocks had to await the development of a device that would prevent them from unwinding instantaneously. The first of these, known as the verge escapement, was developed in Western Europe at the end of the 13th century. It was not until about the middle of the 15th century that the driving force of weights was replaced by the unwinding of a coiled spring. But it was Galileo's discovery of the isochronism of the pendulum, which would swing with a constant frequency determined by its length, regardless of the amplitude of the swing, that paved the way for Christian Huygens, the Dutch physicist, to incorporate the pendulum as the timing mechanism for a clock in 1657. This, coupled with William Clement's brilliant design of the anchor escapement, resulted in the development of the grandfather clock, whose 39-inch pendulum with its precise one-second beat could keep time to within a few seconds a day. The subsequent development of temperature and pressure compensation devices in the 18th and 19th centuries resulted in clocks that could keep time to within a hundredth of a second per day; now, we are perhaps approaching the ultimate with the caesium atomic clock, which has an error of less than 0.00001 s/day.

Man's ability to measure the time of day with increasing precision has not helped in the timing of a year, which for convenience must be composed of a finite number of days. Thus today we have the lunar calendar of Islam, based on 12 lunar months or 354⅓ days to the year and hence bearing no relationship to the solar calendar. Then there are luni-solar calendars, such as the Jewish calendar with its 12 lunar months and an additional month added seven times in each 19-year cycle to bring it back in step with the solar cycle. The Western world has adopted the solar Gregorian calendar, first promulgated by Pope Gregory XIII in March 1582 as a variant of the earlier Julian calendar, introduced by Julius Caesar on 1 January 45 B.C.; the seventh month of our year is still named in Caesar's honour. The Gregorian calendar, with its leap years, and the suppression of leap years in 1700, 1800, 1900, 2100, 2200 and 2300 (but not in 2000), results in a mean calendar year of 365.2425 days, a close approximation to the tropical year of 365.2422 days.

Animals have solved the problem of timing the days and the years in a far more ingenious fashion. They have opted for a very imprecise 24 h clock, that in the absence of any entraining cues from the environment free-runs with a periodicity of about 25 or 26 h. This circadian clock is apparently located in the suprachiasmatic nuclei of the hypothalamus, and is normally entrained to 24 h by daily photoperiodic cues from the environment, such as dawn or dusk, which are perceived by the retina and transmitted to the suprachiasmatic nuclei via the retinohypothalamic tract. During the hours of darkness, nerve impulses pass from the suprachiasmatic nuclei to the pineal gland via the cranial sympathetic nervous system, and β-adrenergic stimulation of the pinealocytes causes them to release their principal secretory product, the hormone melatonin, into the systemic circulation. We now know that hamsters and sheep are able to determine the progression of the seasons by sensing the duration of the nocturnal elevation in melatonin concentrations. This 'hourglass effect' has enabled them to exploit their circadian clock to time seasonal circannual events.

The central problem of how to time the days and the seasons of the year is well illustrated in Ph. Galle's 1574 engraving *The Triumph of Time*, thought to be from one of the lost works of Pieter Bruegel the Elder (Fig. 1). Father Time, represented by Saturn, is seen as the central figure, eating his child—a reminder of the destructive nature of time. In his left hand he holds aloft a serpent, biting its tail, an illustration of the endless cycles of time. Saturn is riding in a chariot which also carries the globe, encircled by the signs of the zodiac, representing the motion of the stars in our firmament, against which man has always measured time. The chariot is drawn by two horses, one bearing the symbol of the Sun, and one the Moon, Although Nicolai Copernicus had put forward his heliocentric view of the universe in his brilliant treatise *De revolutionibus orbium coelestium*, published in 1543, this

FIG. 1. *The Triumph of Time.* Engraving by Ph. Galle, 1574, thought to be after one of the lost works of Pieter Bruegel the Elder. The Metropolitan Museum of Art, Harris Brisbane Dick Fund, 1939 (39.94.7). Reproduced by permission. All rights reserved, The Metropolitan Museum of Art.

was regarded as a heresy by the Catholic Church, and was suppressed. It even led to Galileo's downfall in 1633 for having promoted Copernican views in his great work *Dialogo dei Massimi Sisterni* of 1632. So perhaps the lunar horse was added to give the engraving a measure of religious respectability.

Growing out from the globe is the Tree of Life, its leafless branches on the left representing autumn and winter, and the foliage on the right, spring and summer. The problem of the measurement of time is central to the entire composition, for in a fork of the tree there is a weight-driven clock, its hammer poised to strike the hours. Significantly, the clock lacks a pendulum, since this engraving was made a century before the discoveries of Galileo, Huygens and Clements. Immediately beneath Saturn can be seen an alternative form of timekeeping, an hourglass.

The theme of the changing seasons is taken up in the background of the picture. To the left, we have an autumnal equinoctial gale, with those mackerel skies and mare's tails that make tall ships carry small sails. To the right, there is the tranquillity of spring; birds fly high, the windmill's sails are unfurled, and in the village square men and women dance around a maypole, an ancient fertility ritual. But whereas Alfred, Lord Tennyson may have been correct when he said that in the spring a young man's fancy lightly turns to thoughts of love, there is no evidence to suggest that humans are in any sense seasonal breeders. If tropical Africa was the cradle of human evolution, as all the recent archaeological evidence suggests, there would have been no advantage for our early ancestors to restrict their births to certain seasons of the year; none of the great apes do so, even to this day. Perhaps man's colonization of the temperate and polar regions of the globe has been too recent, and our ability to control our environment so successful, that seasonality of reproduction has never had occasion to evolve. Furthermore, it should be remembered that spring matings are only appropriate for mammals with short gestation lengths, like many of the rodents, or long gestation lengths of about a year, like horses and camels, enabling their offspring to be born in time to profit from the summer. Spring matings would be most inappropriate for humans, since they would result in winter births.

To complete the picture, we must return to the foreground, with its symbolism of the progression of time. The wheels of Time's chariot are mandala, archetypal Hindu symbols of completeness, and as time passes they crush the artifacts of man's making beneath them, destroying all things, even the books of scholars. The procession of life is succeeded by death, the great reaper. But all is not lost with death, for last of all comes a Cherubim, mounted on an elephant, trumpeting perhaps the ultimate triumph of wisdom accumulated over time.

We need to be constantly reminded of the many incredible physiological adaptations that animals have developed to cope with seasonal environmental

extremes. Since reproductive fitness is the ultimate key to the survival of a species, we have naturally tended to orient our studies of circannual rhythms around the reproductive system, but this cannot function in isolation. It requires the support of other body systems, such as those concerned with the ingestion and digestion of food, metabolism, thermoregulation, migration and behaviour. Nowhere is this better illustrated than in the case of the Emperior penguin, *Aptenodytes forsteri*, which breeds in Antarctica between the latitudes of 66 °S and 77 °S, probably the most extreme environment colonized by any bird (Le Maho 1977). It is the largest of all the penguins, with a body mass of 20–40 kg, and is remarkable for the fact that it has chosen to lay its single egg, incubate it, and hatch the chick during the depths of an Antarctic winter, when the average minimal ambient temperature is around −48 °C, and the average daily wind speed may reach 40 m/s! It is probably the only bird, and certainly the only penguin, to have opted for winter nesting, a strategy forced upon it primarily by the seasonal changes in the sea ice which constitutes its nesting ground. That it can cope successfully with such an incredibly harsh environment is testimony to the phenomenal powers of adaptation of its physiological life-support systems, and is an indication that, in Nature, all things are possible—given time.

The penguin's year begins at the start of the Austral summer in November, when the sea ice begins to break up, and air temperatures range from −5 to +1 °C. In the ensuing months, all the birds in the colony undergo their annual moult, although for any one bird the process only lasts for 30–40 days. During this time, the birds are confined to the unbroken sea ice, or to islands along the Antarctic coast. They must starve, because lacking both buoyancy and insulation they could never survive the plunge into the icy waters in search of their food. As a result, they show an abrupt weight decline from 35 kg to about 20 kg. Although their thermal insulation against the chill summer winds is severely reduced during the moult, accounting for the drastic loss of weight, the effect is somewhat mitigated by the fact that the old feathers remain attached to the new ones until the latter are about 1 cm long.

Their moulting complete, the Emperors go to sea for two to three months to feed on the rich summer harvest of krill and fish, accumulating body fat and protein against the rigours of the oncoming winter. They are known to dive as deep as 265 m and stay submerged for as long as 18 min in search of their prey, and by the end of the summer, when in peak bodily condition, they will weigh about 40 kg, of which 10 kg is fat.

In the beginning of March, the seas start to freeze once more, and by the end of the month, they are covered by ice. The Emperors then depart south, seeking their traditional rookeries which are located on permanent sea ice around the shores of the Antarctic continent; the largest rookery consists of about 100 000 birds at the height of the breeding season. This southward

migration is an amazing sight, the birds marching in single file, occasionally tobogganing down slopes, at speeds of no more than 2 km/h for 100 km or more to reach their chosen destination. It has been calculated that they utilize about 1.5 kg of their precious fat reserves in a 200 km walk.

After arrival at the rookery, courting and copulation take place in late April, and at the beginning of May each female lays a single egg that weighs about 450 g. By this time, since the female has also been fasting for 40–50 days, she has lost about 25% of her body weight, and so it is imperative that she returns once more to the open sea to replenish her depleted energy reserves, leaving the task of incubation exclusively to the male. He carefully positions the egg on the upper surfaces of his feet, covering it from above with a pendulous flap of skin, the brood pouch, and balancing himself on his heels and his tail with his feet off the snow. Thus encumbered, the male's mobility is severely impaired. Should an egg fall off the flippers, it must be recovered instantly, since it would soon freeze, killing the developing chick.

The key to survival in this forbidding, benighted environment is huddling. The incubating males huddle close together in groups of up to 1000 birds as protection against the howling Antarctic gales, and this has necessitated the abandonment of all territorial behaviour—a male's only defended territory is his brood pouch. The huddles move very slowly down-wind, as the exposed birds in the rear and on the flanks creep to the front, seeking greater protection. Huddling can almost halve the rate of depletion of the fat reserves, thus doubling the bird's powers of endurance.

The male incubates the egg for about 65 days, from the beginning of May to mid-July, enduring a fast that lasts for a total of about 115 days; during this time, he loses about 40% of his body weight. By the time the chick hatches, the male is near to the limits of his powers of endurance, and his survival, and that of the chick, depend on the timely return of his mate from her fishing expedition. He is able to sustain the newly hatched chick for a few days with penguin's milk, an oesophageal secretion similar to pigeon's milk, but then the female must take over the feeding, regurgitating the undigested catch from her stomach. The male goes back to the sea to feed, returning in August, while the female broods the growing chick until it acquires the ability to thermoregulate on its own, when it may be abandoned in a huddle of other chicks for up to three months whilst its parents are away feeding. The chicks moult in the summer, replacing their down with feathers, and eventually leave the rookery to enter the sea in December or January, although their parents may continue to feed them at irregular intervals until they are about a year old. They will not begin to breed until they are four to six years old.

This exhausting life cycle of fast and feast, migration and starvation is critically dependent on the correct timing of a host of physiological responses

to meet the changing demands of the harshest environment in the world. We cannot be sure how the Emperor penguin times these events with such precision, but photoperiod must surely be the signal. The incredible life history of this bird should therefore serve as a stimulus to all of us to search far more widely for effects of photoperiod on other bodily systems, such as those involved in nutrition, metabolism, thermoregulation and behaviour.

There is now increasing evidence that in mammals such as hamsters, ferrets, sheep, deer, horses, mink, skunks, voles and wallabies, all seasonal breeders, the pineal is involved in the timing of reproductive events. Is melatonin simply a timing hormone, exerting its effect indirectly by regulating the circadian and circannual rhythms of secretion of other hormones such as prolactin and the gonadotropins, or does it have direct effects of its own, for example on hair pigmentation or gonadal activity? Where and how does it act? Can it exert a feedback effect directly on the suprachiasmatic nucleus, the presumed site of origin of all circadian rhythms including that of melatonin itself? There is certainly growing evidence to suggest that, in birds, rats and even humans, exogenous melatonin may be able to re-entrain disturbed circadian activity rhythms.

We have come a long way in our understanding since Herophilus of Alexandria first proposed in the third century B.C. that the pineal gland was responsible for controlling the flow of memories from the fourth ventricle into more anterior parts of the brain, or Rene Descartes (1677) thought of it as the seat of the soul and Edmond King (1686) associated pineal calcification with schizophrenia, late in the 17th century. Aaron Lerner first isolated melatonin from the pineal in 1958 (Lerner et al 1958), and yet it is only within the last decade that we have accumulated solid experimental evidence to accord melatonin its true role—the Hormone of Darkness, the Hourglass of Time.

REFERENCES

Daniel G 1980 Megalithic monuments. Sci Am 243:64-76

Descartes R 1677 Tractatus de homine et de formatione foetus. Elsevirium, Amstelodami

King E 1686 A relation of a petrified Glandula Pinealis, lately found in the dissection of a brain: communicated by Sr Edmond King Knt MD and Reg. Soc. S. Philos Trans R Soc Lond 185:228-231

Le Maho Y 1977 The Emperor penguin: a strategy to live and breed in the cold. Am Sci 65:680-693

Lerner AB, Case JD, Lee TH, Takahashi Y, Mori W 1958 Isolation of melatonin, the pineal factor that lightens melanocytes. J Am Chem Soc 80:2587-2594

Lloyd HA 1968 Timekeepers—an historical sketch. In: Fraser JT (ed) The voices of time. Allen Lane, London, p 388-400

Thomas G 1982 Making a firm date. New Sci 93(1298):770-772

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Mammalian pinealocytes: ultrastructural aspects and innervation

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Abstract. In the mammalian pineal gland it is notoriously difficult to relate structure to function. The pineal-specific cells, the pinealocytes, contain only inconspicuous numbers of secretory granules, and the variable amounts of smooth and rough endoplasmic reticulum also do not point to a particular function. In addition to these widely known cellular components, pinealocytes contain organelles, the so-called 'synaptic' ribbons, histophysiological studies of which provide important insights into the structural and functional complexity of the organ. As synaptic ribbons may be involved in neuronal functions of pinealocytes it is notable that these organelles are structurally heterogeneous. Ribbons fall into at least two categories: rod-like (RSR) and sphere-like (SS) structures. RSR and SS usually do not lie within the same pinealocyte profile and appear to be regulated by different mechanisms. It is conceivable that they are important components of the biological clock system. These findings are related to our knowledge of the innervation of the mammalian pineal gland and to electrophysiological characteristics of pinealocytes.

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At first sight, the histological structure of the mammalian pineal gland is not very exciting. The gland consists of only two major cell types, pinealocytes and immature astrocytes. Occasionally, the pinealocytes are arranged in follicles surrounding narrow or wide spaces. The gland is richly innervated by postganglionic sympathetic nerve fibres, most of which are found in the perivascular spaces of capillaries. In addition, pinealopetal fibres of central origin are present (cf. Vollrath 1981). Observed under the light microscope the pineal-specific cells, the pinealocytes, lack prominent and differential staining properties. Special staining reactions such as silver impregnation are necessary to demonstrate their complete outlines. Then it becomes apparent that pinealocytes are nerve cell-like, consisting of a perikaryon and an unknown number of cytoplasmic processes. The large, pale nucleus with its prominent nucleolus is also reminiscent of a large ganglion cell. As cytoplasmic basophilia is vir-

tually non-existent, there is no satisfactory explanation for the high metabolic activity of pinealocyte nuclei.

At the ultrastructural level it is also difficult to relate structure to function. In most mammalian species investigated the pinealocytes contain few granules that can be regarded as morphological correlates of secretory products, and the circadian behaviour of the few dense-core vesicles present does not support the assumption that they contain melatonin. According to Juillard & Collin (1980) dense-core vesicles act as storage sites for serotonin. Pinealocyte perikarya house a prominent Golgi apparatus and relatively small amounts of smooth and rough endoplasmic reticulum. In some species cisternae of the endoplasmic reticulum contain flocculent material which may represent a form of secretory substance. Highly pleomorphic mitochondria tend to form clusters reminiscent of the ellipsoids of inner segments of photoreceptor cells. That the pinealocytes are responsible for the conversion of serotonin to melatonin has been demonstrated histochemically by showing that it is these cells that contain serotonin (Bertler et al 1963) and melatonin (Freund et al 1977), and not the astrocyte-like interstitial cells.

When we probe deeper into the morphology of the gland it is sobering to see that there are many features (microtubular sheaves, mitochondria with plate-like and tubular crests, subsurface cisternae, 'synaptic' ribbons, unusual gap junctions etc.) which cannot be interpreted yet in terms of function. Should we eventually clarify the functional meaning of these unusual features we may have to revise the current pineal concept. The aim of this paper is to focus on the important unresolved problem of why mammalian pinealocytes are equipped with processes, and to consider some implications.

Why are mammalian pinealocytes equipped with processes?

It is generally accepted that mammalian pinealocytes are phylogenetically derived from pineal photoreceptor cells. In lower vertebrates pineal photoreceptors are similar to retinal cones and show outer and inner segments as well as synapses with afferent pinealofugal nerve fibres. During phylogenesis the outer segments regress as do the pinealofugal nerve fibres, but the synaptic ribbons of the afferent synapses persist. In view of the phylogenetic regression and the current concept that it is the function of mammalian pinealocytes to synthesize melatonin and to release it into the systemic circulation, both the shape of the pinealocytes and the architecture of the gland are surprising.

As beautifully demonstrated by Del Rio Hortega, human pinealocytes are equipped with long cytoplasmic processes. Comparable processes are present in all mammalian species investigated ultrastructurally. When we compare these process-bearing cells with the pineal photoreceptor cells of lower verte-

brates it is difficult to envisage that mammalian pinealocytes represent regressed photoreceptor cells. Instead it appears that mammalian pinealocytes are highly differentiated cells similar to nerve cells, the processes of which receive messages and pass on signals. A puzzling feature is that, although the possible morphological correlates of pineal secretory products are particularly prominent in terminal swellings of pinealocyte processes in many mammalian species, only a few terminals are close to blood vessels. Instead, the perivascular spaces are filled with large bundles of postganglionic sympathetic nerve fibres. In fact, according to recent quantitative studies in the rat, 91.1% of the nerve fibres have a perivascular location, the remainder lying between pinealocytes (A. Meyer & L. Vollrath, unpublished work 1985). It is enigmatic that sympathetic nerve fibres predominate in the perivascular spaces since the nervi conarii reach the pineal gland independently of blood vessels, as separate nerves. Apparently sympathetic fibres join the blood vessels secondarily. In view of what we know about the functional interrelationships of pinealocytes and blood vessels on the one hand and sympathetic nerve fibres and pinealocytes on the other, it would be logical to envisage a gland in which most pinealocyte processes terminate in the perivascular spaces to release their secretory products directly into the systemic circulation. The sympathetic nerve fibres, on the other hand, should lie predominantly among pinealocytes. Why the reverse applies is a challenging problem. As many pinealocytes lie distant from the function-regulating sympathetic nerve fibres, the question arises of whether they have structures that they may use for intercellular communication.

Intercellular communication of pinealocytes

Two structures have to be considered: gap junctions and 'synaptic' ribbons.

Gap junctions

Gap junctions are known to be responsible for electrical and metabolic coupling of adjacent cells. In the pineal gland they have been little investigated. The few detailed studies show that gap junctions in the pineal are most interesting structures exhibiting a number of unusual features. First, they are not universally present as illustrated by their absence from the pinealocytes of Syrian hamsters (Huang et al 1984). Secondly, in those species in which they have been found (rat: Taugner et al 1981, guinea-pig: Huang & Taugner 1984) most of them are unlike typical gap junctions. In organs other than the pineal, freeze-fracture studies reveal that gap junctions are macular struc-

tures consisting of densely packed particles termed 'connexons.' In most gap junctions in pinealocytes the junctional particles form (1) interrupted rows that consist of a single layer of connexons, (2) uninterrupted rows of double-row connexons or (3) ring-like or honeycomb-like structures in which the connexons are arranged in one to five layers surrounding a connexon-free central area ('fenestrated gap junctions'). Although the basic structure of these gap junctions is well known, we have an incomplete picture of their precise location both within the pinealocytes and within the parenchyma. In both rat and guinea-pig pineal glands they are most readily seen in pinealocytes forming follicles. Here they occupy the collicular area near the wide or rudimentary lumens. In guinea-pigs they have also been seen in pinealocyte processes. However, nothing is known about their presence in pinealocytes not forming follicles. From the available results one can only conclude that electrical coupling of pinealocytes is apparently not present in all mammalian species. In those species in which gap junctions are present, electrical coupling may occur in certain areas of the pineal only.

'Synaptic' structures

In some sensory organs afferent synapses are characterized by the presence of peculiar structures, the so-called synaptic ribbons (SR). Each consists of a rod-like, plate-like, ring-like or solid-sphere-like electron-dense centre surrounded by electron-lucent synaptic vesicles 40–60 nm in diameter. It has been suggested that it is the function of the central core-structure to channel the surrounding synaptic vesicles to the presynaptic membrane. Similar synaptic structures are present in mammalian pinealocytes. Here they are functionally enigmatic as afferent synapses are absent. Currently two hypotheses are prevailing. One is that the function of SR is similar to that in other organs, i.e. that they are involved in intercellular communication, not between neurons, but between adjacent pinealocytes (cf. Vollrath 1973). Another view is that they regulate the number of β-adrenoceptors of pinealocytes, e.g. by internalizing parts of the receptor-bearing cell membrane (King & Dougherty 1982). As the number of SR changes under various physiological and experimental conditions (cf. Vollrath 1981), it has been suggested that SR may be devices for establishing intrapineal circuits, similar to neuronal circuits, between pinealocytes (Vollrath & Huss 1973). It should be recalled that the present concept of secretory pineal organs 'runs the chance of excluding the still potent possibilities that... sensory information gathering or other strictly neural functions may be accomplished by the machinery contained in parenchymal pineal organs' (Wurtman et al 1968). In view of this possibility we are studying SR in detail, and recent results have indicated that they are

highly complex organelles; their day/night rhythms and changes in number illustrate how little we know about the structure and function of the mammalian pineal gland.

It is now clear that pineal SR show a regular 24 h rhythm characterized by low SR numbers during day-time and high numbers at night. Recently it has become apparent that in some species the structural complexity of SR has a functional counterpart. In the Chinese hamster rod-like SR (RSR) and round-oval SR were found to have the commonly observed day/night rhythm, whereas ring-like SR were more abundant during day-time than at night (Matsushima et al 1983). Subsequently, an 'inverse' day/night rhythm was also found in guinea-pigs, for solid round SR, the so-called 'synaptic' spherules (SS) (Vollrath et al 1983). More detailed studies have revealed that SS show prominent inter-species differences. They are commonly found in guinea-pigs (Vollrath et al 1983) and rabbits (Martinez Soriano et al 1984), but are rare in rats (Karasek & Vollrath 1982, Kosaras et al 1983). A detailed topographical analysis of RSR and SS in guinea-pigs has revealed that the two types of organelle are usually not present within the same pinealocyte profile and, perhaps even more important, occupy different parenchymal domains (L. Vollrath, and A. Meyer & L. Vollrath, unpublished work). Our working hypothesis is that RSR and SS may be present in different types of pinealocytes, previously characterized electrophysiologically (guinea-pig: Semm & Vollrath 1980, rat: Reuss & Vollrath 1984). In the guinea-pig, RSR-bearing pinealocytes may correspond to cells that are electrophysiologically highly active at night and relatively inactive during the day, whereas the SS-containing pinealocytes may be those that are electrically highly active during the day and relatively inactive at night. In accordance with this assumption, RSR-bearing and nocturnally active pinealocytes have been found in the rat pineal, but SS are rare and diurnally active pinealocytes have not been detected (Reuss & Vollrath 1984).

That RSR and SS may play a pivotal role in pineal function is suggested by results obtained *in vitro* (L. Vollrath et al, unpublished work 1985). The aim was to assess to what extent the daily changes of RSR and SS numbers may be influenced by intrapineal melatonin and serotonin concentrations. Rats were killed at different times of day and night (1300 h, 1700 h and 0100 h) and their pineal glands were removed and cultured for different lengths of time with or without melatonin or serotonin added to the incubation medium. The glands were processed for electron microscopy and RSR and SS were counted in an area of 20 000 μm^2 pineal tissue. The results show that melatonin affects SR numbers only at certain times of the 24 h cycle and that melatonin and serotonin affect RSR and SS differently. Melatonin added in the afternoon or in the morning has no effect on RSR numbers. It increases RSR numbers when given during the first half of the night, when RSR numbers normally

increase. Serotonin given in the morning depresses RSR numbers and increases SS numbers. These findings suggest that melatonin and serotonin may play a role in regulating RSR and SS numbers, at least under *in vitro* conditions. Should the indole amines play a similar role *in vivo*, then interesting possibilities for the function of the pineal gland as a biological clock open up. The time of day could be morphologically encoded in the form of RSR and SS numbers and, as RSR and SS numbers change with a 24 h rhythm, the varying RSR/SS ratios could make the clock fairly precise. Another possibility is that night-active and day-active pinealocytes could stimulate or inhibit each other, form circuits etc.

A detailed topographical analysis of RSR, SS and gap junctions, assisted by injections of tracers into pinealocytes, may be extremely valuable for understanding how pinealocytes communicate with each other and how this intercellular communication relates to the function of the pineal as a whole. At the same time there is the question of how the structural and functional complexity of the pinealocytes relates to the innervation of the gland.

Innervation of pinealocytes

There is now ample morphological and electrophysiological evidence that the mammalian pineal gland is not exclusively sympathetically innervated. Pinealopetal fibres of central origin reaching the gland via the commissures are also highly developed.

Sympathetic innervation

This type of innervation has been clearly defined, both morphologically and functionally. The fibres originate in the superior cervical ganglia (SCG) of the sympathetic trunk, continue in the internal carotid nerve and enter the pineal gland as nervi conarii (Zigmond et al 1981, Bowers et al 1984). Their importance for the regulation of melatonin synthesis has been demonstrated by biochemical studies after sympathectomy (cf. Vollrath 1981) or electrical stimulation of the SCG (10 Hz, 1 h), the latter leading to an approximately 50-fold increase of serotonin *N*-acetyltransferase (NAT) activity (Bowers & Zigmond 1980, 1982). That we are far from fully understanding the influence of the sympathetic nerve fibres on mammalian pinealocytes has recently been demonstrated by studying the electrical properties of rat pinealocytes after electrical stimulation of the SCG (Reuss et al 1985). Some pinealocytes did not appear to be influenced by SCG stimulation, a second group responded with enhanced electrical activity and in a third group electrical activity was

depressed. In view of the continuing controversy about whether NAT or hydroxyindole *O*-methyltransferase (HIOMT) is the rate-limiting enzyme for melatonin synthesis, and the lack of a clear-cut day/night rhythm of HIOMT, in contrast to NAT, it is relevant to recall that as early as 1972 Heller reported that preganglionic electrical stimulation of sympathetic nerves resulted in an increase of pineal NAT activity but a decrease of HIOMT activity. These experiments should be repeated in the light of what we currently know about the two enzymes and the sympathetic innervation of the pineal gland. Moreover, immunocytochemical studies should be done to localize NAT and HIOMT and to find out whether they are indeed present in the same pinealocytes.

Central innervation

That nerve fibres reach the pineal gland via the habenular and posterior commissures has long been known (cf. Vollrath 1981). However, it is only with modern neurobiological techniques available that it is becoming apparent that these fibres may be of functional importance. As this evidence has been thoroughly reviewed by Møller & Korf (1984), only a brief description is given here. Lesion and horseradish-peroxidase studies have revealed that central pinealopetal nerves fibres originate in diverse brain regions including the habenular, paraventricular and suprachiasmatic nuclei as well as the preoptic area, amygdala, olfactory centres, lateral geniculate bodies and the sites of origin of the stria medullaris. From a functional point of view it is important to note that the central fibres contain a variety of peptides such as oxytocin, vasopressin, luteinizing hormone-releasing hormone, vasoactive intestinal polypeptide and substance P. Interestingly, the fibres are unevenly distributed in the gland, some lying in the periphery and others in the centre. How this relates to a possible subdivision of the parenchyma into cortex and medulla is an open question. It is also not known whether the neuropeptides contained in the nerve fibres are released into intrapineal blood vessels and/or whether they act locally, e.g. by regulating pinealocyte function. Other important unresolved issues are whether the electrophysiological differences between pinealocytes are due to differences in their innervation, i.e. sympathetic versus central, and what role a possible parasympathetic innervation plays.

Conclusions

Using modern neurobiological techniques we are finding that the structure and function of the mammalian pineal gland are more complex than previously

thought. In particular it is becoming apparent that a conceptual approach that probes the possible neuronal nature of mammalian pinealocytes is very promising. Because of their unusual structural features, mammalian pinealocytes may become highly favoured objects of research for morphologically oriented cell biologists.

REFERENCES

Bertler A, Falck B, Owman C 1963 Cellular localization of 5-hydroxytryptamine in the rat pineal gland. K Fysiogr Sallsk Lund Forh 33:13-16

Bowers CW, Zigmond RE 1980 Electrical stimulation of the cervical sympathetic trunks mimics the effects of darkness on the activity of serotonin:*N*-acetyltransferase in the rat pineal. Brain Res 185:435-440

Bowers CW, Zigmond RE 1982 The influence of the frequency and pattern of sympathetic nerve activity on serotonin *N*-acetyltransferase in the rat pineal gland. J Physiol (Lond) 330:279-296

Bowers CW, Dahm LM, Zigmond RE 1984 The number and distribution of sympathetic neurons that innervate the rat pineal gland. Neuroscience 13:87-96

Freund D, Arendt J, Vollrath L 1977 Tentative immunohistochemical demonstration of melatonin in the rat pineal gland. Cell Tissue Res 181:239-244

Heller A 1972 Neuronal control of brain serotonin. Fed Proc 31:81-90

Huang S-K, Taugner R 1984 Gap junctions between guinea-pig pinealocytes. Cell Tissue Res 235:137-141

Huang S-K, Nobiling R, Schachner M, Taugner R 1984 Interstitial and parenchymal cells in the pineal gland of the golden hamster. A combined thin-section, freeze-fracture and immunofluorescence study. Cell Tissue Res 235:327-337

Juillard M-T, Collin J-P 1980 Pools of serotonin in the pineal gland of the mouse: the mammalian pinealocyte as a component of the diffuse neuroendocrine system. Cell Tissue Res 213:273-291

Karasek M, Vollrath L 1982 'Synaptic' ribbons and spherules of the rat pineal gland: day/night changes in vitro? Exp Brain Res 46:205-208

King TS, Dougherty WJ 1982 Effect of denervation on 'synaptic' ribbon populations in the rat pineal gland. J Neurocytol 11:19-28

Kosaras B, Welker HA, Vollrath L 1983 Pineal 'synaptic' ribbons and spherules during the estrous cycle in rats. Anat Embryol 166:219-227

Martinez Soriano F, Welker HA, Vollrath L 1984 Correlation of the number of pineal 'synaptic' ribbons and spherules with the level of serum melatonin over a 24-hour period in male rabbits. Cell Tissue Res 236:555-560

Matsushima S, Morisawa Y, Aida I, Abe K 1983 Circadian variations in pinealocytes of the Chinese hamster, *Cricetulus griseus*. A quantitative electron-microscopic study. Cell Tissue Res 228:231-244

Møller M, Korf H-W 1984 The innervation of the mammalian pineal gland with special reference to central pinealopetal projections. Pineal Res Rev 2:41-86

Reuss S, Vollrath L 1984 Electrophysiological properties of rat pinealocytes: evidence for circadian and ultradian rhythms. Exp Brain Res 55:455-461

Reuss S, Semm P, Vollrath L 1985 Changes in the electrical activity of the rat pineal gland following stimulation of the cervical sympathetic ganglia. J Auton Nerv Syst 12:281-288

Semm P, Vollrath L 1980 Electrophysiological evidence for circadian rhythmicity in a mammalian pineal organ. J Neural Transm 47:181-190

Taugner R, Schiller A, Rix E 1981 Gap junctions between pinealocytes. A freeze-fracture study of the pineal gland in rats. Cell Tissue Res 218:303-314

Vollrath L 1973 Synaptic ribbons of a mammalian pineal gland. Circadian changes. Z Zellforsch Mikrosk Anat 145:171-183

Vollrath L 1981 The pineal organ. Springer-Verlag, Berlin (Handbuch der mikroskopischen Anatomie, vol VI-7)

Vollrath L, Huss H 1973 The synaptic ribbons of the guinea-pig pineal gland under normal and experimental conditions. Z Zellforsch Mikrosk Anat 139:417-429

Vollrath L, Schultz RL, McMillan PJ 1983 'Synaptic' ribbons and spherules of the guinea-pig pineal gland: inverse day/night differences in number. Am J Anat 168:67-74

Wurtman RJ, Axelrod J, Kelly DE 1968 The pineal. Academic Press, New York

Zigmond RE, Baldwin C, Bowers CW 1981 Rapid recovery of function after partial denervation of the rat pineal gland suggests a novel mechanism for neural plasticity. Proc Natl Acad Sci USA 78:3959-3963

DISCUSSION

Illnerová: What percentage of pinealocytes contain spherules and what percentage ribbons? And how do these percentages correlate with the numbers of light-active and dark-active cells?

Vollrath: In the guinea-pig about 50% of these synaptic structures are ribbons and 50% are spherules, but this depends greatly on the time of day. Whether all pinealocytes contain 'synaptic' bodies is an open question. There are large inter-species variations; for instance, in the rat, in contrast to the guinea-pig and rabbit, there are normally very few spherules in the pineal. We don't yet know how many cells are electrically active during the day and how many are nocturnally active because it is not possible to record from a large number of pinealocytes to estimate percentages. I wish we had a simple way of telling after 10 min of recording whether a cell is day-active or night-active.

Short: Were your unit recordings from single cells after excision from the animal or were they *in situ* recordings?

Vollrath: In situ recordings. We made rather few because it is not possible to record from many cells over long periods of up to 26 h.

Tamarkin: Have you applied melatonin to the pineal while you are recording from cells *in situ*?

Vollrath: Not during long-term recordings, but we have applied melatonin by microiontophoresis during short-term recordings (Semm et al 1981a). There are different kinds of pinealocytes: some are depressed by melatonin, others are activated and some show no response. There is also a difference between responses during the day and those at night. During night-time fewer pinealocytes are stimulated and more are inhibited.

Reiter: You suggest that serotonin and melatonin may be involved in regulating the number of synaptic ribbons. In normal light/dark cycles the number of

synaptic ribbons increases during darkness, but the number also increases with constant light exposure. However, in constant light serotonin and melatonin concentrations are relatively stable and don't show rhythms. How do you reconcile this with a change in the number of synaptic ribbons?

Vollrath: It is premature to be dogmatic about these results. We might expect pineal glands *in vivo* to atrophy under continuous illumination, but they do not (L. Vollrath, unpublished work, guinea-pig). In fact the pinealocytes look as active as ever, so I am not prepared to extrapolate yet from our *in vitro* results to the situation *in vivo*. The increases of ribbon numbers at night and under continuous light are compatible if one assumes that ribbons are stimulatory. Under a light/dark cycle they are thought to enhance melatonin secretion at night, and under constant light they are high in number perhaps to overcome the light-induced depression of melatonin formation.

Klein: I don't see that it is incongruous that synaptic ribbon numbers increase both in constant light and at night. The increase during darkness could simply be an effect of light that takes about 12 h to be expressed.

Zucker: Do you have any idea what, if anything, the pineal gland does in the guinea-pig?

Vollrath: No.

Zucker: In the 1930s Young and collaborators maintained guinea-pigs in constant light for months at a time and found no effects on reproduction of females. The animals had normal oestrous cycles and delivered young (see Young 1969, p 36).

Vollrath: And we have found that continuous illumination has no effect on testicular weight (L. Vollrath, unpublished work).

Turek: Could you comment on the central nervous system innervation of the pineal gland? Is the pineal stalk the main pathway into the pineal from the central nervous system in rodents?

Vollrath: One should be careful when using the word 'stalk' because people think of the pineal stalk in different terms. In the rat the pineal stalk is between the deep pineal and the superficial pineal, but in the guinea-pig it is the connection between the habenular nuclei and the pineal, below the habenular commissure (Semm et al 1981b, Schneider et al 1981). In the rat the central innervation comes via the habenular and posterior commissures into the deep pineal and then through the stalk to the superficial pineal.

Turek: We have looked at the reproductive response of the hamster in a variety of different photoperiods and conditions after lesions of the deep pineal, and we have found no effect, even after making massive lesions that probably obliterated much of the central nervous system innervation of the pineal.

Short: What does pineal stalk section do to pineal function?

Vollrath: This has not often been done. Quay (1971) found a reduction in size

of the superficial pineal after stalk sectioning, and minor changes with respect to phase-shifting of locomotor activity.

Menaker: In sparrows stalk section does nothing to the function of the pineal in supporting circadian rhythmicity (Zimmerman & Menaker 1975).

Lewy: Is it possible that there is a visual input to the pineal through the direct central innervation?

Vollrath: From the anatomical point of view it is quite feasible. Møller & Korf (1983) have recently shown connections between the lateral geniculate body and the pineal gland, and this indicates very clearly that there may be diverse inputs from the visual system, via the brain and not only via the sympathetic nervous system.

Bittman: What is the effect of superior cervical ganglionectomy on the peptide content of the pineal? This might tell us whether the peptides come from the brain or the superior cervical ganglion or are endogenous to the pineal.

Vollrath: This has not been studied in detail yet, but some of these peptides are still present after superior cervical ganglionectomy (Rønnekleiv & Kelly 1984), indicating that they definitely come from the brain directly through the stalk.

Bittman: Unless they are made in the pineal.

Vollrath: I don't think so, since the neuropeptides are apparently restricted to axons.

Rollag: Have you found any morphological correlate that would distinguish the deep pineal component, so prevalent in rodents, from the superficial component? Is there any morphological difference that would suggest different functions for these two components?

Vollrath: The cell nuclei are smaller in the deep pineal than in the superficial pineal (Boeckmann 1980) and there are more astrocytes (Hewing 1981); there are only slightly fewer synaptic ribbons per unit area (Hewing 1980, 1981), so from a morphological point of view we do not see many large differences.

Rollag: In the deep pineal gland, but not in the superficial, pinealocyte processes project into the cerebrospinal fluid (CSF), penetrating the ependymal lining. Do you think this particular relationship between the deep pineal and the CSF is significant?

Vollrath: I'm quite sympathetic towards the idea that there is some sort of communication between the CSF and pinealocytes. This does not fit into our current concept of pineal function, but the idea is worth exploring carefully. Maria Hewing (1984) has shown that in the hamster the contact area between the deep pineal and the third ventricle changes with the season, so this area may be functionally very important.

Menaker: You have emphasized communication among pinealocytes, but is there any physiological evidence for this?

Vollrath: Only very few pinealocytes seem to come into direct contact with the sympathetic nerve fibres, so I think there must be some form of communication.

Menaker: That's interesting because in birds of course there is physiological evidence for such communication.

Goldman: You commented that your observations do not seem to fit with our current ideas about the pineal. Is it the sparse innervation of a gland that we think is totally driven by the nervous system which seems inconsistent to you?

Vollrath: The location of the nerve fibres does not fit our present concept, but there are a number of other things. Day-active pinealocytes are puzzling (Semm & Vollrath 1980), and although continuous illumination has been reported to depress melatonin formation (cf. Vollrath 1981), we see no atrophy of guinea-pig pinealocytes (L. Vollrath, unpublished work).

Goldman: Although we think of melatonin as the functional product of the pineal, the gland also exhibits a rhythm in serotonin levels with higher concentrations during the day, so the activity that you see during the day may be related to that. The sparse innervation might also be simply explained. In lower vertebrates the pineal has an endogenous oscillatory property, and one would expect connections between pinealocytes. Perhaps innervation is less important in these animals. In making use of the pineal system that they inherited from lower vertebrates, mammals perhaps did not require a dense innervation because communications between cells could magnify the effects of the neuronal input.

Vollrath: It's quite possible.

Klein: On the basis of the gap junction studies you mentioned and Taugner's observation of a basement membrane 'insulation' around groups of pineal cells (Huang & Taugner 1984, Huang et al 1984, Taugner et al 1981), it appears that we should think of the mammalian pineal gland as being organized into plastic bags containing interconnected grapes (pinealocytes), with only one or two cells at the open end of each bag communicating with the sympathetic nervous system and the gap junctions amplifying the initial response.

Vollrath: Yes, but before we can be certain that the pineal functions like a bag of grapes we must look at the exact topography of the gap junctions and the synaptic ribbons.

Klein: I agree, and I'm wondering why nobody has injected Lucifer yellow into pineal cells. It is an elegant technique because you can not only map the distribution of connecting cells but also quantitate changes in the degree of communication, which I think we will see in the pineal gland. I've always suspected that some kind of communication is going on from our *in vitro* studies. We work with both individual cells and whole organs. The dose–response data we get with cyclic nucleotides in individual cells are tight and smooth over several orders of magnitude; the curves are highly reproducible

with very small standard errors. However, the organs are frustrating to work with because each one shows an all-or-none response. We may have two organs from the same group of animals treated identically in the same dish, but one will show a complete response and the other will show no response. We have always wondered whether only a few cell clusters or areas have to be activated to turn on the whole gland.

Vollrath: We have been planning to inject dyes into single cells for some time because we would like to correlate the electrical activity of single pinealocytes with the length and course of the processes.

Klein: You mentioned that the histology of the pineal does not hold great interest because there are few unusual features, but I think that interest in this topic will increase as new antisera become available. Antisera against several retinal proteins of special interest have been prepared; one is the S-antigen and another is the intra-photoreceptor retinoid-binding protein which has now been found in high concentrations not only in the monkey retina but also in the pineal gland (Rodrigues et al 1965). Another interesting and unusual protein involved in phototransduction is transducin. Some has been purified from the bovine retina by Bob Somers at the National Eye Institute, and highly specific antiserum against the α-subunit of transducin, which was originally found only in the retina, has been prepared by Peter Giershick and Allen Speigel (National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases). Theo van Veen (University of Lund), Horst Korf (University of Giessen) and I have started to look at pineal organs with this antiserum and have found that all vertebrate photosensitive pineal organs contain the α-subunit in photoreceptor outer segments. These new specific immunohistochemical tools may be valuable not only in identifying pineal cells but also in tracing pineal processes out of the deep pineal area.

Vollrath: I think immunocytochemical studies are going to be very important. You have recently shown that the retinal S-antigen is not evenly distributed in the pineal in some mammalian species (Korf et al 1985). In some species you see lots of immunoreactive cells in the periphery and it is interesting that we find more sympathetic nerve fibres between the pinealocytes in the periphery than in the centre of the organ. It may turn out that wherever there is direct innervation there is a different type of pinealocyte.

Klein: A correlate of the idea that there are different cells in the mammalian pineal gland may be the observation that the autoimmune damage to the pineal gland caused by injecting retinal S-antigens appears to occur predominantly in certain areas high in S-antigen. Igal Gery's group in the National Eye Institute has studied this and has also looked at the damage caused by injection of the retinoid-binding protein, which produces similar results. Accordingly, there may be important regional differences between pinealocytes.

REFERENCES

Boeckmann D 1980 Morphological investigation of the deep pineal of the rat. Cell Tissue Res 210:283-294

Hewing M 1980 Synaptic ribbons in the pineal system of normal and light deprived golden hamsters. Anat Embryol 159:71-80

Hewing M 1981 Topographical relationships of synaptic ribbons in the pineal system of the vole (*Microtus agrestis*). Anat Embryol 162:313-323

Hewing M 1984 Seasonal variations in the cerebrospinal fluid-contacting area of the pineal gland in the golden hamster (*Mesocricetus auratus*). Anat Embryol 169:91-96

Huang S-K, Taugner R 1984 Gap junctions between guinea-pig pinealocytes. Cell Tissue Res 235:137-142

Huang S-K, Nobiling R, Schachner M, Taugner R 1984 Interstitial and parenchymal cells in the pineal gland of the golden hamster *Mesocricetus auratus*: a combined thin section freeze fracture and immunofluorescence study. Cell Tissue Res 235:327-338

Korf H-W, Møller M, Gery I, Zigler JS, Klein DC 1985 Immunocytochemical demonstration of retinal S-antigen in the pineal organ of four mammalian species. Cell Tissue Res 239:81-85

Møller M, Korf H-W 1983 The origin of central pinealopetal fibers in the Mongolian gerbil as demonstrated by the retrograde transport of horseradish peroxidase. Cell Tissue Res 230:273-287

Quay WB 1971 Dissimilar functional effects of pineal stalk and cerebral meningeal interruptions on phase shifts of circadian activity rhythms. Physiol & Behav 7:557-567

Rodrigues M, Gaskins R, Wiggert B, Redman M, Chader G 1965 Immunocytochemical localization of intraphotoreceptor retinoid binding protein in the primate retina and pineal gland. Invest Ophthalmol & Visual Sci 26(suppl):340

Rønnekleiv O, Kelly MJ 1984 Distribution of substance P neurons in the epithalamus of the rat: an immunohistochemical investigation. J Pineal Res 1:355-370

Schneider T, Semm P, Vollrath L 1981 Ultrastructural observations on the central innervation of the guinea-pig pineal gland. Cell Tissue Res 220:41-49

Semm P, Vollrath L 1980 Electrophysiological evidence for circadian rhythmicity in a mammalian pineal organ. J Neural Transm 47:181-190

Semm P, Demaine C, Vollrath L 1981a Electrical responses of pineal cells to melatonin and putative transmitters. Evidence for circadian changes in sensitivity. Exp Brain Res 43:361-370

Semm P, Schneider T, Vollrath L 1981b Morphological and electrophysiological evidence for habenular influence on the guinea-pig pineal gland. J Neural Transm 50:247-266

Taugner R, Schiller A, Rix E 1981 Gap junctions between pinealocytes: a freeze fracture study of the pineal gland in rats. Cell Tissue Res 218:303-314

Vollrath L 1981 The pineal organ. Springer-Verlag, Berlin

Young WC 1969 Psychobiology of sexual behavior in the guinea pig. In: Lehrman DS et al (eds) Advances in the study of behavior. Academic Press, New York, vol 2:1-110

Zimmerman NH, Menaker M 1975 Neural connections of sparrow pineal: role in circadian control of activity. Science (Wash DC) 190:477-479

Photoperiodism, melatonin and the pineal
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Circadian rhythms and photoperiodism

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Abstract. The circadian timing system plays a critical role in the regulation of seasonal modifications in reproductive function. By detecting and transducing changes in the day-length (photoperiod), the neural substrates of the circadian system, including the supra-chiasmatic nuclei of the hypothalamus, trigger reproductive activity or quiescence at the appropriate seasons of the year in photoperiodic species. The circadian system also plays a role in the expression of endocrine changes that occur with seasonal breeding. Surges in luteinizing hormone secretion in female hamsters, for example, are either expressed daily during reproductive quiescence or suppressed on three out of the four days of the cycle during the breeding season. By such mechanisms a daily timer can be used in the regulation of cyclic events of much longer period.

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Of all the rhythmic processes that are observed in living organisms, the most precisely regulated are those which are used to measure and predict periodic changes in the environment. These 'circa-rhythms' (circadian, circatidal and circannual) are generated by internal pacemaking systems which are synchronized to their periodic counterparts in the outside world. Two of the classes of circa-rhythms (circadian and circannual) rely for their synchronization on changes in the illumination of the environment with either the time of day or the season of the year. In this paper we discuss an elegant economy of nature whereby the same neural substrates are used for detecting and processing temporal information on these two different time scales.

The characteristics of the 24 h day–night cycle of environmental illumination that are important to timekeeping processes are its period (cycle length), its phase (timing of dawn and/or dusk), the photoperiod (hours of light per day) and the intensity and wavelength of the incident light. Of primary importance to the synchronization of the circadian timing system are the period and phase of the daily light–dark (LD) cycle, while the circannual system utilizes the photoperiod to ensure synchronization to the season of the year.

Synchronization to environmental day–night and seasonal cycles is essential to survival, especially in temperate zones. Since the nocturnal and day-time environments differ in illumination, temperature, food supplies and predators, many species have developed highly specialized temporal niches so that they tend to be better adapted to activity either during the night or during the day. Similarly, in temperate zones, the environmental conditions can vary markedly with the season of the year. While only some species show dramatic behavioural responses such as hibernation, many species synchronize breeding, and therefore the birth of their offspring, to the most advantageous time of year for survival.

Photoperiodism in mammals

That the photoperiod is the most important environmental factor controlling seasonal breeding was not appreciated until relatively recently (Ortavant et al 1964, Turek & Campbell 1979). On the basis of length of gestation period, photoperiodic species can be classified as long-day or short-day breeders. Animals such as the sheep and goat with moderately long gestation periods begin to breed as daylength decreases (Hafez 1950, Ortavant et al 1964). The breeding seasons of mammals with very short gestation lengths such as hamsters (Bruce & Hindle 1934), mice (Petterborg & Reiter 1980) and some species of rats (Everett 1942) begin as daylength increases. Horses, with very long gestation lengths, are also long-day breeders (Frost & Zucker 1983). In both long-day and short-day breeders, there is a critical amount of light that determines whether an animal becomes and/or remains reproductively competent or whether it becomes anoestrous (or aspermatogenic). In long-day breeders, the critical stimulatory photoperiod length must be exceeded for oestrous cycles to persist, while in short-day breeders, the environmental LD cycle must not exceed the critical photoperiod. This critical photoperiod requirement can be quite stringent. In the adult male hamster for example, a long-day breeder, Gaston & Menaker (1967) reported that at least 12.5 h of light per 24 h were required to maintain testicular weight and full spermatogenesis; 12 h of light per day were not sufficient. Once an animal is exposed to a photoperiod that does not meet the critical length, regression of the testes or anoestrus ensues irrespective of the length of the non-stimulatory photoperiod. In female hamsters, blinding (Reiter 1968) was as effective as LD 10:14 (Seegal & Goldman 1975) in causing anovulation.

Reproductive photoperiodism appears to be a genetic trait. Granados (1951) reported that hamsters had been selected for breeding at the NIH hamster colony on the basis of their ability to breed during the usual period of anoestrus (November to May). In this way, the seasonality trait was bred out of the

hamsters. In the rat, the inbred DA strain, but not the Vanderbilt strain, is responsive to alterations in photoperiod (Everett 1942). In sheep, the duration of the breeding season is related to the latitude and altitude of the origin of the breed (Hafez 1950). The most restrictive breeding season has been observed in ewes whose ancestral origin was in the mountains of Scotland and Wales, while the longest occurred in merino sheep from Spain. Latitude of origin also appears to influence reproduction in the deer mouse (Dark et al 1983).

Although photoperiodism does not seem to be important for human reproduction, many clinical reports suggest that some psychiatric disorders have photoperiodic components. Studies of patients in Europe and the United States have shown a seasonal increase in the number of psychiatric admissions for affective disorders (mania and major depression), with a peak in spring (Parker & Walter 1982). A subpopulation of bipolar manic-depressive patients becomes depressed as photoperiods shorten toward winter and manic when photoperiods lengthen toward spring (Rosenthal et al 1984). That photoperiodism is involved in the affective disorder of the latter group of patients is suggested by the observation that extending the photoperiod artificially with bright lights leads to clinical improvement.

Mechanisms for measuring environmental photoperiod

There are several features of the LD cycle that could be used to measure the environmental photoperiod. The detected characteristic of the LD cycle could be the duration of light (or dark), a change in the number of hours of light per day, or the presence of light at a critical phase during the 24 h day. This question has been addressed in mammals by four techniques: (a) by housing animals in resonant light cycles, (b) by pulsing animals with light at different phases of the day, (c) by driving the period of the circadian system to values other than 24 h and (d) by exposing hamsters to 'skeleton' photoperiods. These studies have indicated that Bünning's hypothesis (1936) that the endogenous circadian system is responsible for daylength measurement is correct. Photoperiodic reproductive responses are triggered by light falling at a critical phase of the circadian cycle that normally lies within the hours of darkness during the extended nights of late autumn and winter.

'Resonance' light cycles consist of a fixed short photoperiod coupled to intervals of darkness of different durations (i.e. 6:18, 6:24, 6:30, etc.) (Turek & Campbell 1979). The light stimulus falls at different phases of the circadian day depending on the particular resonance cycle. If the photoperiodic system was sensitive to light at critical phases and not to the duration of the light, some of the resonance cycles would be interpreted as stimulatory photo-

periods. This hypothesis has been supported by the results of studies using resonance cycles in hamsters, each dealing with a different facet of the photoperiodic response. Male hamsters transferred from a stimulatory photoperiod to a regimen of four resonance cycles with a 6 h photoperiod had regressed testes when light did not coincide with the period of time during which the animals were active in their subjective night (Elliott et al 1972). After male hamsters had regressed in another experiment, precocious recrudescence (resumption of reproductive function) was induced with 6 h photoperiods that illuminated animals during their active phases (Stetson et al 1975). Thus, whenever light fell on a specific phase of the circadian day, the limited photoperiod supported reproductive competence.

Resonance photoperiods have also been used to study reproductive activity in sheep, an example of short-day breeders (Almeida & Lincoln 1982). The testes of rams exposed to LD 16:8 regressed within 16 weeks. Resumption of function occurred more quickly following exposure to LD 8:40 than to LD 8:28, presumably since only the former was interpreted as a short day, even thought both photoperiods provided 8 h of light. Thus, in both long-day and short-day breeders, light falling within a critical phase stimulates the photoperiodic response.

A critical period of photosensitivity within the circadian day has also been examined through the use of timed light pulses. Hoffman & Melvin (1974) and Rudeen & Reiter (1980) housed male and female golden hamsters in LD cycles consisting of 15 min of light every 6 h, repeated four times. Under this environmental regimen animals did not have regressed testes, while control hamsters exposed to the same 1 h photoperiod once every 24 h did regress. One (or more) of the 15 min pulses appears to have fallen on the photosensitive period. In male Djungarian hamsters, 1 min of light in the middle of the dark phase of LD 8:16, a non-stimulatory photoperiod, was sufficient to induce testicular growth (Hoffmann 1979). Even less light may be required than 1 min. Earnest & Turek (1984) exposed male hamsters to 10 s of light every two, four or seven days during the dark phase and significantly prevented gonadal regression.

The ability of light pulses to entrain the circadian timing system to light cycles with periods other than 24 h (T-cycles) has been used to determine the width of the photosensitive period during the circadian day (Elliott 1976). Because the phase of entrainment is a function of the period of the applied T-cycle, it is possible to vary the phase at which the entraining light pulse falls on the rest–activity cycle of the animal. When 1 h light pulses were applied at the beginning or end of the daily active phase (i.e. during the phase of the cycle that is illuminated during the long days of summer but is in darkness during the short days of winter), the testes of the hamsters no longer regressed. When the light pulse fell during subjective day, however, regression occurred.

The duration of the photosensitive phase was calculated to be slightly less than 12 h, close to the critical photoperiod length that supports continued testicular function.

'Skeleton' photoperiods have also been used to determine the circadian components of the photoperiodic response. In female hamsters, two 1 h exposures to light separated by 12 h of darkness mimicked a 14 h photoperiod and were sufficient to maintain ovarian cycles (Goldman & Darrow 1983).

The results of the resonance, light-pulse and T-cycle studies and experiments with 'skeleton' photoperiods demonstrate that the circadian timing system is involved in photoperiodic time measurement. A more direct approach in determining the role of the circadian system is to lesion the suprachiasmatic nuclei (SCN), the hypothalamic pacemakers which are directly innervated from the retina by the retinohypothalamic tract, and which have been demonstrated to be important in generating many of the observed circadian rhythms in physiological function (Rusak & Zucker 1979).

After SCN lesions, male hamsters* retained functional testes even when they were housed in constant darkness (Stetson & Watson-Whitmyre 1976), blinded or exposed to short photoperiods (Lehman et al 1984). If male hamsters had undergone gonadal regression previously, SCN lesions restored testicular weight (Rusak & Morin 1976). SCN lesions in male hamsters also prevented an increase in sensitivity to the inhibitory effects of testosterone on the release of luteinizing hormone (LH) after regression in LD 6:18 (Turek et al 1980, Turek et al 1983). Although these effects of SCN lesions are similar to those of pinealectomy (Morin et al 1977), Rusak (1980) demonstrated that the two procedures were not equivalent. In pinealectomized male hamsters, exogenous melatonin implants induced testicular regression, while similar implants in SCN-lesioned animals did not. These results suggest that SCN lesions may induce a wider spectrum of deficiencies than pinealectomy.

Lesions of the hypothalamic paraventricular nuclei (PVN), like SCN lesions, will prevent the effects of blinding or exposure to short photoperiods in male hamsters (Lehman et al 1984). The PVN lesions may interrupt projections from the SCN that are involved in the photoperiodic response. Alternatively, the PVN may be associated with the effects of the pineal on reproduction, since PVN lesions disrupt circadian pineal function to a greater extent than SCN lesions do. Clearly, more work is required to elucidate the role of different hypothalamic nuclei in photoperiodism.

* Similar lesions in female hamsters produce persistent vaginal oestrus (no cycles), so the role of the SCN in photoperiodism is difficult to confirm in females.

Hormonal basis of reproductive photoperiodism

Once a photoperiod has been 'interpreted' as non-stimulatory, changes in hormone secretion ensue. Because the circadian system plays a key role in the timing of endocrine signals for reproduction, its role in seasonal breeding extends beyond the measurement of photoperiods. Take, for example, the four-day oestrous cycles in hamsters. On the day of pro-oestrus, the circadian system is responsible for discrete and precisely timed surges of LH, follicle-stimulating hormone (FSH) and prolactin several hours before the onsets of behavioural oestrus and locomotor activity (Stetson & Anderson 1980, Moline 1981). The gonadotropin surges (1) cause ovulation 8–10 h after the LH surge on the early morning of oestrus, (2) lead to production of luteal progesterone (which in part serves to terminate the surges) and (3) stimulate a new wave of follicular maturation. As the follicles mature during dioestrus I and II, oestradiol is produced. As the serum titres of this hormone increase, there is positive feedback on LH release so that peak levels of oestradiol in pro-oestrus lead to the gonadotropin surges. Then the cycle repeats.

During the photoperiodic season of reproductive quiescence, the hormones associated with the normal four-day cycle in hamsters show changes in their patterns of secretion. Gonadotropins and progesterone exhibit daily diurnal patterns, and oestradiol and prolactin levels fall. Thus, in anoestrous hamsters, serum LH and FSH levels were elevated during the afternoon on a day selected at random in LD 10:14 (Bridges & Goldman 1975, Seegal & Goldman 1975, Bridges et al 1976, Bittman & Goldman 1979, Goldman & Brown 1979). Moline (1981) measured LH surges in anoestrous hamsters over three days and confirmed that the surges occurred daily and were lower in magnitude than those that occurred once per four-day cycle during stimulatory photoperiods. In addition, a change in phase of the LH surge occurred so that the 'regressed' surge began closer to activity onset than the pro-oestrous surges either in stimulatory photoperiods or after short-term exposure to non-stimulatory photoperiods (before regression occurred). These diurnal changes did not require the presence of the ovaries (Bridges & Goldman 1975, Seegal & Goldman 1975, Moline 1981) or the adrenal glands (Bittman & Goldman 1979). These results suggest that the potential for daily surges in gonadotropin release was being expressed because the facilitating effects of oestradiol needed to obtain the pro-oestrous LH surge were not required in short photoperiods (Norman 1975).

In males of many seasonal breeding species, serum gonadotropin levels also fall during seasonal quiescence. These mammalian species include the lynx, bontebok, spring hare, thar, cheetah (Millar & Aehnelt 1977), mongoose (Soares & Hoffman 1980), snow-shoe hare (Davis & Meyer 1973), ram (Lincoln et al 1977) and hamster (Berndtson & Desjardins 1974, Tamarkin et

al 1976, Turek et al 1976, Goldman & Brown 1979, Jackson et al 1984). A sex difference has been observed in the rhythm of LH and FSH; in the regressed male hamster these hormones do not exhibit diurnal fluctuations (Turek et al 1976, Goldman & Brown 1979, Albers et al 1984). The decrease in serum LH in regressed males is not due to a decrease in the response of the pituitary to exogenous LH-releasing hormone (Pickard & Silverman 1979, Jackson et al 1984).

The changes in hormone rhythms after exposure to non-stimulatory photoperiods appear to be the result of altered interactions between LH and oestradiol in females or LH and testosterone in males. In both sexes, the response to gonadectomy is reduced and the hypothalamus becomes more sensitive to inhibitory feedback by steroids. Castrating regressed male snow-shoe hares (Davis & Meyer 1973) or hamsters (Turek et al 1975, Tamarkin et al 1976, Pickard & Silverman 1979, Ellis & Turek 1980, Tate-Ostroff & Stetson 1980) did not uniformly produce an increase in LH levels or a change in the pattern of LH release. Thus, the castration response (i.e. increased LH levels after gonadectomy) was attenuated (Turek et al 1983). Turek (1982) has shown that serum FSH levels rise rapidly from their suppressed values during regression in castrated male hamsters when the animals are exposed to a single long day, thus emphasizing the inhibitory effects of short photoperiods on gonadotropin release. The hypothalamic–pituitary LH-release mechanism also becomes more sensitive to the inhibitory effects of testosterone on LH release after exposure to short photoperiods. Less testosterone was required to suppress LH levels in the hamster (Tamarkin et al 1976, Turek 1977) and rat (Wallen 1980) in short photoperiods. These results suggest that the hypothalamus of the male may become more sensitive to the inhibitory effects of gonadal steroids during regression.

In female hamsters, the daily LH surges during anoestrus apparently do not require the facilitating effects of oestradiol or progesterone. Since the surges persist in the absence of both the ovaries and the adrenal glands, the major steroid-producing organs, the surges cannot be explained by a supersensitivity to the facilitating effects of oestradiol in low concentrations (Bittman & Goldman 1979). This does not imply, however, that the animal has become insensitive to the steroid hormones. Moline (1981) placed silastic implants of oestradiol benzoate into regressed female hamsters housed in LD 6:18 and showed a significant suppression of the daily LH surges within two days of exposure to the exogenous oestradiol. In contrast, the same dose of oestradiol *facilitated* LH surges in ovariectomized hamsters housed in long, stimulatory photoperiods (LD 14:10).

The effects of gonadal steroids on LH have also been examined in sheep. Although oestradiol could stimulate LH release during anoestrus or during the breeding season within a given time interval, the rise in LH concen-

trations was much smaller during anoestrus (Land et al 1976). Scaramuzzi & Baird (1977) observed that the frequency of LH pulses was decreased during anoestrus and attributed their finding to a hypersensitivity to oestradiol. In ovariectomized ewes, continuous-release implants of oestradiol decreased LH to undetectable levels during the anoestrous season but did not suppress LH concentrations as much during the breeding season (Legan et al 1977). Similar studies of castrated rams with testosterone implants showed comparable season-dependent effects of LH release (Lincoln & Almeida 1982). During the transition periods between anoestrus and breeding, the response to oestradiol was intermediate. These results suggest that a change in response to the inhibitory effects of gonadal steroids may account for seasonal breeding in sheep (Bittman et al 1983, Karsch et al 1980).

In summary, the circadian system fulfils two critical roles in reproductive photoperiodism in many mammalian species. First, it measures the photoperiod length so that, by a mechanism as yet not completely understood, an animal can interpret the environmental photoperiod as stimulatory or non-stimulatory. Second, it provides timing signals for hormone rhythms such as the gonadotropin surges. Although there are profound changes in these hormonal rhythms during seasonal quiescence as the result of alterations in the hypothalamic–pituitary–gonadal axes in both sexes, the circadian system provides time cues for reproductive cycle events which are expressed daily during seasonal quiescence in some species or are selectively suppressed to generate oestrous cycles of several days in length.

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REFERENCES

Albers HE, Moline ML, Moore-Ede MC 1984 Sex differences in circadian control of LH secretion. J Endocrinol 100:101-105

Almeida O, Lincoln G 1982 Photoperiodic regulation of reproductive activity in the ram: evidence for the involvement of circadian rhythms in melatonin and prolactin secretion. Biol Reprod 27:1062-1075

Berndtson WE, Desjardins C 1974 Circulating LH and FSH levels and testicular function in hamsters during light deprivation and subsequent photoperiodic stimulation. Endocrinology 95:195-205

Bittman EL, Goldman BD 1979 Serum levels of gonadotropins in hamsters exposed to short photoperiods: effects of adrenalectomy and ovariectomy. J Endocrinol 83:113-118

Bittman EL, Dempsey RJ, Karsch FJ 1983 Pineal melatonin secretion drives the reproductive response to daylength in the ewe. Endocrinology 113:2276-2283

Bridges RS, Goldman BD 1975 Diurnal rhythms in gonadotropins and progesterone in lactating and photoperiod induced acyclic hamsters. Biol Reprod 13:617-622

Bridges R, Tamarkin L, Goldman B 1976 Effects of photoperiod and melatonin on reproduction in the Syrian hamster. Ann Biol Anim Biochim Biophys 16:399-408

Bruce HM, Hindle E 1934 The golden hamster, *Cricetus* (*Mesocricetus*) *auratus Waterhouse*. Notes on its breeding and growth. Proc Zool Soc Lond 104:361-366

Bünning E 1936 Die endogene Tagesrhythmik als Grundlage der photoperiodischen Reaktion. Ber Dtsch Bot Ges 54:590-607

Dark J, Johnston P, Healy M, Zucker I 1983 Latitude of origin influences photoperiodic control of reproduction of deer mice (Peromyscus maniculatus). Biol Reprod 28:213-220

Davis FC, Meyer RK 1973 Seasonal variation in LH and FSH in bilaterally castrated snowshoe hares. Gen Comp Endocrinol 20:61-68

Earnest DJ, Turek FW 1984 Periodic exposure to a brief light signal stimulates neuroendocrine-gonadal activity in golden hamsters. J Androl 5:64-69

Elliott JA 1976 Circadian rhythms and photoperiodic time measurement in mammals. Fed Proc 35:2339-2346

Elliott JA, Stetson MH, Menaker M 1972 Regulation of testis function in golden hamsters: a circadian clock measures photoperiodic time. Science (Wash DC) 178:771-773

Ellis GB, Turek FW 1980 Photoperiodic regulation of serum luteinizing hormone and follicle-stimulating hormone in castrated and castrated-adrenalectomized male hamsters. Endocrinology 106:1338-1344

Everett JW 1942 Certain functional interrelationships between spontaneous persistent estrus, 'light estrus', and short-day anestrus in the albino rat. Anat Rec 82:409

Frost D, Zucker I 1983 Photoperiod and melatonin influence seasonal gonadal cycles in the grasshopper mouse (Onychomys leucogaster). J Reprod Fertil 69:237–244

Gaston S, Menaker M 1967 Photoperiodic control of hamster testis. Science (Wash DC) 158:925-928

Goldman BD, Brown S 1979 Sex differences in serum LH and FSH patterns in hamsters exposed to short photoperiod. J Steroid Biochem 11:531-535

Goldman BD, Darrow JM 1983 The pineal gland and mammalian photoperiodism. Neuroendocrinology 37:386-396

Granados H 1951 Nutritional studies on growth and reproduction of the golden hamster (*Mesocricetus auratus auratus*). Acta Physiol Scand Suppl 24:87

Hafez ESE 1950 Sexual season of the ewe and daylight environment. Nature (Lond) 166:822-823

Hoffman RA, Melvin H 1974 Gonadal responses of hamsters to interrupted dark periods. Biol Reprod 10:19-23

Hoffmann K 1979 Photoperiodic effects in the Djungarian hamster: one minute of light during darktime mimics influence of long photoperiods on testicular recrudescence, body weight and pelage colour. Experientia 35:1529-1530

Jackson F, Heindel J, Preslock J, Berkowitz A 1984 Alterations in hypothalamic content of luteinizing hormone-releasing hormone with pineal-mediated testicular regression in the golden hamster. Biol Reprod 31:436-445

Karsch FJ, Goodman RL, Legan SJ 1980 Feedback basis of seasonal breeding: test of an hypothesis. J Reprod Fertil 58:521-535

Land RB, Wheeler AG, Carr WR 1976 Seasonal variation in the oestrogen induced LH discharge of ovariectomized Finnish landrace and Scottish blackface ewes. Ann Biol Anim Biochim Biophys 16:521-528

Legan SJ, Karsch FJ, Foster DL 1977 The endocrine control of seasonal reproductive function

in the ewe: a marked change in response to the negative feedback action of estradiol on luteinizing hormone secretion. Endocrinology 101:818-824

Lehman MN, Bittman EL, Newman SW 1984 Role of the hypothalamic paraventricular nucleus in neuroendocrine responses to daylength in the golden hamster. Brain Res 308:25-32

Lincoln GA, Almeida OFX 1982 Inhibition of reproduction in rams by long daylengths and the acute effect of superior cervical ganglionectomy. J Reprod Fertil 66:417-423

Lincoln GA, Peet MJ, Cunningham RA 1977 Seasonal and circadian changes in the episodic release of follicle-stimulating hormone, luteinizing hormone and testosterone in rams exposed to artificial photoperiods. J Endocrinol 72:337-349

Millar RP, Aehnelt C 1977 Application of ovine luteinizing hormone (LH) radioimmunoassay in the quantitation of LH in different mammalian species. Endocrinology 101:760-768

Moline ML 1981 Luteinizing hormone rhythms in female golden hamsters: circadian, photoperiodic and endocrine interactions. PhD Thesis, Harvard University

Morin LP, Fitzgerald KM, Rusak B, Zucker I 1977 Circadian organization and neural mediation of hamster reproductive rhythms. Psychoneuroendocrinology 2:73-98

Norman RL 1975 Estrogen and progesterone effects on the neural control of the preovulatory LH release in the golden hamster. Biol Reprod 13:218-222

Ortavant R, Mauleon P, Thibault C 1964 Photoperiodic control of gonadal and hypophyseal activity in domestic mammals. Ann NY Acad Sci 177:157-193

Parker G, Walter S 1982 Seasonal variation in depressive disorders and suicidal deaths in New South Wales. Br J Psychiatry 140:626-632

Petterborg LJ, Reiter RJ 1980 Effect of photoperiod and melatonin on testicular development in the white-footed mouse, Peromyscus leucopus. J Reprod Fertil 60:209-212

Pickard GE, Silverman AJ 1979 Effects of photoperiod on hypothalamic luteinizing hormone releasing hormone in the male hamster. J Endocrinol 83:421-428

Reiter RJ 1968 Changes in the reproductive organs of cold-exposed and light-deprived female hamsters (Mesocricetus auratus). J Reprod Fertil 16:217-222

Rosenthal NE, Sack DA, Gillin JC et al 1984 Seasonal affective disorder. Arch Gen Psychiatry 41:72-80

Rudeen PK, Reiter RJ 1980 Influence of a skeleton photoperiod on reproductive organ atrophy in the male golden hamster. J Reprod Fertil 60:279-283

Rusak B 1980 Suprachiasmatic lesions prevent an antigonadal effect of melatonin. Biol Reprod 22:148-154

Rusak B, Morin LP 1976 Testicular responses to photoperiod are blocked by lesions of the suprachiasmatic nuclei in golden hamsters. Biol Reprod 15:366-374

Rusak B, Zucker I 1979 Neural regulation of circadian rhythms. Physiol Rev 59:449-526

Scaramuzzi RJ, Baird DT 1977 Pulsatile release of luteinizing hormone and the secretion of ovarian steroids in sheep during anestrus. Endocrinology 101:1801-1806

Seegal RF, Goldman BD 1975 Effects of photoperiod of cyclicity and serum gonadotropins in the Syrian hamster. Biol Reprod 12:223-231

Soares MJ, Hoffman JC 1980 Seasonal variations in serum androgen and luteinizing hormone levels in the male mongoose, *Herpestes auropunctatus*. Soc Stud Reprod Annu Meet, p 130

Stetson MH, Anderson PJ 1980 Circadian pacemaker times gonadotropin release in free-running female hamsters. Am J Physiol 238:1223-1227

Stetson MH, Watson-Whitmyre M 1976 Nucleus suprachiasmaticus: the biological clock in the hamster? Science (Wash DC) 191:197-199

Stetson MH, Elliott JA, Menaker M 1975 Photoperiodic regulation of hamster testis: circadian sensitivity to the effects of light. Biol Reprod 13:329-339

Tamarkin L, Hutchison JS, Goldman BD 1976 Regulation of serum gonadotropins by photoperiod and testicular hormone in the Syrian hamster. Endocrinology 99:1528-1533

Tate-Ostroff B, Stetson MH 1980 Testicular regression, steroid feedback sensitivity and photorefractoriness in golden hamsters. Soc Stud Reprod Annu Meet, p 124

Turek FW 1977 The interaction of the photoperiod and testosterone in regulating serum gonadotropin levels in castrated male hamsters. Endocrinology 101:1210-1215

Turek FW 1982 Rapid increase of serum FSH levels in castrated male hamsters following transfer from short to long days or exposure to a single long day. Endocrinology 111:332-334

Turek FW, Campbell CS 1979 Photoperiodic regulation of neuroendocrine-gonadal activity. Biol Reprod 20:32-50

Turek FW, Elliott JA, Alvis JD, Menaker M 1975 The interaction of castration and photoperiod in the regulation of hypophyseal and serum gonadotropin levels in male golden hamsters. Endocrinology 96:854-860

Turek FW, Alvis JD, Elliott JA, Menaker M 1976 Temporal distribution of serum levels of LH and FSH in adult male golden hamsters exposed to long or short days. Biol Reprod 14:630-631

Turek FW, Jacobson CD, Gorski RA 1980 Lesions of the suprachiasmatic nuclei affect photoperiod-induced changes in the sensitivity of the hypothalamic-pituitary axis to testosterone feedback. Endocrinology 107:942-947

Turek FW, Losee-Olson SH, Ellis GB 1983 Pinealectomy and lesions of the suprachiasmatic nucleus affect the castration response in hamsters exposed to short photoperiods. Neuroendocrinology 36:335-339

Wallen EP 1980 Remnants of photoperiodicity in the male albino laboratory rat. Soc Stud Reprod Annu Meet, p 27

DISCUSSION

Short: You said that you didn't really think that photoperiodism was important for reproduction in humans, but Bob Edwards (1981) has shown that the timing of the LH surge is markedly circadian, starting in 75% of women in the morning. That is strong evidence for a circadian rhythm in the timing of ovulation.

Follett: It may well be that the LH surge in women is dependent in part upon a circadian oscillator and, if so, the precise phase-angle will probably be influenced by photoperiod, but that is not quite the same as the reproductive cycle being driven photoperiodically. In this last situation changing daylength fundamentally alters the way in which LH and FSH are secreted.

Short: Certainly nobody has yet shown conclusively that by changing time schedules you can interfere with ovulation, although we do know that in people who frequently travel in an east–west direction ovulation is inhibited, and the effect is greatest if the time-zone change is in the preovulatory phase of the cycle (Preston et al 1973).

Follett: I would not argue with the proposition that upsetting the circadian system may cause reproductive failure, but I would differentiate that from regulation of the reproductive cycle by seasonally based changes in daylength, which may or may not act through the circadian system.

Menaker: Yes. It is important to distinguish between photoperiodism, which really means seasonal breeding controlled by daylength, and circadian involvement. But it is interesting that, because the phase of the circadian system is sensitive to daylength, possession of that mechanism makes it possible for a species to become seasonal very easily if it is not already. People have not paid enough attention to the variability among species and the ease with which these control mechanisms can be turned on and off by natural selection.

Moore-Ede: It's interesting that in humans Edwards found that the success of artificial implantation was far higher in late evening than during the rest of the day. At one time he had four successful implants out of a total of 79 and all four occurred in a two-hour interval between 2200 h and midnight (Elliott 1979). I think that this, coupled with results showing that the timing of the LH surge is remarkably precise, suggests that the human system is very much dependent on circadian phase, but I agree that it is not photoperiodic in the true sense of the word, in terms of being seasonally modulated.

Short: So how do we interpret the Ehrenkranz (1983) data on seasonal reproduction in Eskimos?

Zucker: Part of the seasonality may be due to social and cultural practices. As Ehrenkranz points out, in the Labrador settler population men traditionally spent long periods alone trapping between October and December. This could impose seasonal cycles, and consequently the correlation between the duration of photoperiod and the timing of births may not reflect a causal relation. However, Ehrenkranz (1983) thinks it unlikely that there is a cultural basis to the seasonal birth cycle of the Labrador Eskimo population and instead implicates seasonal changes in food availability, daylength and temperature.

Short: And the peak number of births seems to be at the wrong time, March, to be of adapative significance.

Herbert: I think it is time we questioned the standard explanation of circadian involvement in photoperiodism. You mentioned the Bünning hypothesis, which we have accepted for a long time, and all the experiments are consistent with it. They are also consistent with another interpretation. If we assume firstly that the mammalian pineal gland acts as an interval timer, and that the duration of its output signal is important, and secondly that there must be a melatonin-free period of some critical length between two interval signals, we can explain many experiments in a different way. Night-interruption experiments, ahemeral experiments and T-cycle experiments can all be explained by the effect of light interrupting the continuity of the melatonin signal. If this is right, then we have an hourglass mechanism in the mammalian neuroendocrine system for determining photoperiodism, and we should look upon the idea of external coincidence differently.

Moore-Ede: It is certainly an intriguing idea and it is interesting that there is now some debate about what is controlling the sleep–wake cycle for example,

and whether we are dealing with something that, although not quite an hourglass mechanism, is certainly a relaxation oscillator process. But you would have to think about what the role of the suprachiasmatic nuclei would be if you are not going to use circadian phase information.

Bittman: In several animals photoperiodism could involve a hybrid system in which there is a circadian generation of the signal, which could fully account for the results of resonance experiments, and a separate mechanism for measuring the duration of the melatonin signal, which could be based on an interval timer. In some situations, for example in poikilotherms, the animal does not really 'want' to have a temperature-compensated system and it may be advantageous to have an interval timer that does not have some of the properties that a circadian system does. Linking two timers together to give a hybrid between pure circadian and pure hourglass mechanisms for measuring daylength may confer a selective advantage (Silver & Bittman 1984).

Moore-Ede: In several species circadian systems have the property of conditionality, where combinations of high or low light intensity and temperatures outside a permissive range appear to stop the circadian pacemaker. This has been best documented in unicellular organisms like *Gonyaulax* but it is not inconceivable that the circadian system of invertebrates could display this sort of behaviour.

Follett: If the pineal is indeed the primary transducer of photoperiodic signals and if it is driven from the suprachiasmatic nuclei through a circadian rhythmic system, then one suspects that by definition photoperiodic time measurement is driven in part by circadian mechanisms.

Herbert: I am certainly not denying the involvement of the circadian system. All I am saying is that it is not involved in the way we thought it was in the coincidence model.

Follett: My only worry about our belief that circadian rhythms are involved in photoperiodism comes from those clearly negative experiments which many of us have carried out. For example, David Ellis and I free-ran hamsters in constant darkness and then gave them a single 15 min light pulse every five days at different circadian times. This was done very carefully indeed but none of the schedules induced gonadotropin secretion!

Zucker: Published results support what you found. Butler & Donovan (1971) have shown that in guinea-pigs surgical isolation of the suprachiasmatic nuclei, which presumably eliminates normal circadian organization, is consistent with continued oestrous cycles. It appears one can dissociate oestrus from the circadian system. In rhesus monkeys also, normal circadian organization is unnecessary for provoking cyclic ovulation. I don't think that it is appropriate to talk in general or blanket terms about *the* mammalian pineal gland in relation to seasonal reproduction; for example, mammals that have true circannual organization can dispense with the pineal gland completely and still have

appropriate seasonal oestrous cycles, as has been shown in ground squirrels (Zucker 1985).

Hoffmann: None of these species is photoperiodic; there is no evidence for photoperiodicity in the rhesus monkey or in the guinea-pig. But in photoperiodic mammals there *is* evidence for a circadian signal that is translated by an hourglass mechanism.

Turek: None of the new melatonin results are in disagreement with the original Bünning hypothesis. This hypothesis states that the circadian system is somehow involved in photoperiodic time measurement. The 'sensitive phase' in the Bünning hypothesis may be the time during which melatonin is secreted. We are only now starting to link physiological mechanisms with the formal analysis of photoperiodic time measurement. Bünning's hypothesis makes no attempt to delineate physiological mechanisms.

Follett: Another point worth making is that the pineal melatonin story does not seem to apply to photoperiodism in birds. However, the circadian system may be involved in photoperiodic time measurement in birds and we can show a sensitive phase during the middle of the night (see Follett et al, this volume). So although both the mammal and the bird fit Bünning's original hypothesis they may use completely different physiological mechanisms.

Herbert: That was my point. The formal properties fit, but when you look at the mechanisms they are different. Once you start talking about periods of sensitivity to light you are making an assumption about the nature of the neural mechanism, but it seems to me that this assumption is not justified any more. I disagree with Fred Turek; I think Bünning's hypothesis and the alternative explanation I suggested are compatible only on the descriptive level but not when you get into the mechanisms.

Zucker: But we cannot contradict the statement that in all photoperiodic species the pineal is the transducer.

Herbert: I agree.

Lewy: Dr Moore-Ede, in your hamsters on short days was there a phase-advance in the LH surge?

Moore-Ede: No, on short days the LH surge became phase-delayed before the animals stopped cycling. Subsequently the LH surge occurred on a 24 h cycle instead of a 96 h cycle.

Lewy: And what was the τ of these animals?

Moore-Ede: About 24.5 h.

Lewy: Could you comment on the relationship between the phase–response curve of an organism and the circadian change in phase position as daylength changes? Would you disagree with the proposition that animals with τ greater than 24 h would phase-advance as the days get longer and phase-delay as the days get shorter?

Turek: You can't make any prediction unless you know something about the

shape and amplitude of the phase–response curve. You cannot predict phase-angle changes only on the basis of τ.

Lewy: But would you say there is a relationship between the amplitude and shape of the illuminated portion of the phase–response curve and the animal's τ?

Turek: No. There is no reason to think that they are necessarily correlated.

Moore-Ede: But an animal whose τ is greater than 24 h would have to use its phase–response curve more often to phase-advance than to phase-delay.

Turek: But that does not tell you what its shape is.

Lewy: It tells you that the area under the illuminated advance portion of the curve is relatively large compared to the area under the illuminated delay portion.

Moore-Ede: Remember how malleable the phase–response curve actually is. Jeffrey Elliott (personal communication) has shown major changes in the amplitude and width of the curve in different photoperiods.

Lewy: But there is a certain logic to expecting a shift in circadian phase position at different times of the year according to which portions of an animal's phase–response curve are being illuminated.

Turek: You can take an animal whose phase–response curve has a particular shape and entrain that animal to daylengths that are either longer than its free-running period or shorter. Yet the phase–response curve may be similar in waveform under both conditions.

REFERENCES

Butler JEM, Donovan BT 1971 The effect of surgical isolation of the hypothalamus upon reproductive function in the female guinea pig. J Endocrinol 50:507-514

Edwards RG 1981 Test-tube babies, 1981. Nature (Lond) 293:253-256

Ehrenkranz JRL 1983 Seasonal breeding in humans: birth records of the Labrador Eskimo. Fertil Steril 40:485-489

Elliott J 1979 Finally: some details on in vitro fertilization. JAMA (J Am Med Assoc) 241:868-869

Follett BK, Foster RG, Nicholls TJ 1985 Photoperiodism in birds. In: Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Found Symp 117) p 93-105

Preston FS, Bateman SC, Short RV, Wilkinson RT 1973 Effects of flying and of time changes on menstrual cycle length and on performance in airline stewardesses. Aerosp Med 44:438-443

Silver R, Bittman EL 1984 Reproductive mechanisms: interaction of circadian and interval timing. Ann NY Acad Sci 423:488-514

Zucker I 1985 Pineal gland influences period of circannual rhythms of ground squirrels. Am J Physiol 249:R111

Photoperiodism, melatonin and the pineal
Editors: David Evered and Sarah Clark

Photoneural regulation of the mammalian pineal gland

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Abstract. Mammalian pineal function appears to be controlled primarily through the release of noradrenaline from the terminals of nerves whose cell bodies lie in the superior cervical ganglia. This is the final segment of the following neural pathway: retina → retinohypothalamic projection → suprachiasmatic nuclei → paraventricular nuclei → intermediolateral cell column → superior cervical ganglia → nervi conarii → pineal gland.

Noradrenaline acts on pinealocytes through α- and β-adrenoceptors in an atypical manner. β-Adrenergic activation is an absolute requirement for the stimulation of both cyclic AMP and cyclic GMP production, and by itself produces a sixfold increase in the former and a twofold increase in the latter. α-Adrenergic activation potentiates the β-adrenergic stimulation of cyclic AMP production 10-fold, and that of cyclic GMP production about 200-fold.

The mechanism of α- and β-adrenergic interaction is being examined, and progress is being made in understanding the adrenergic control of cyclic AMP. It appears that α-adrenergic agonists act through the α_1-subclass of adrenoceptors to stimulate phospholipid turnover and the production of a breakdown product of phosphatidylinositol, diacylglycerol. This compound promotes the association of protein kinase C with membranes, which leads to the marked phosphorylation of one protein. The precise identity of this protein remains a mystery. This interaction leads to a larger cyclic AMP response but does not appear to be involved in the mechanism of potentiation of the cyclic GMP response. Changes in chronic neural stimulation produce reciprocal changes in the magnitudes of cyclic AMP and cyclic GMP responses. Chronic denervation results in a supersensitive cyclic AMP response and nearly complete disappearance of the cyclic GMP response. This is termed 'see-saw' signal processing.

All the available evidence indicates that melatonin production is regulated by cyclic AMP. This nucleotide not only increases the activity of serotonin *N*-acetyltransferase (more correctly called arylalkylamine *N*-acetyltransferase) but also stabilizes the enzyme and prevents its inactivation.

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The mammalian pineal gland evolved from a well-differentiated photoreceptive organ in lower vertebrates, a functional third eye (Collin 1971, Dodt

1973, Oksche 1984). However, the mammalian gland does not have the capacity to respond directly to light. Rather, light controls it through a system which includes the lateral eyes, central and peripheral neural structures and neurochemical transduction mechanisms within the gland, which have replaced the photochemical transduction mechanisms of light-sensitive pineal organs. Although there have been evolutionary changes in the way in which light controls the pineal gland, all vertebrates appear to synthesize melatonin rhythmically on a 24 h basis. This paper is about the system responsible for the precise rhythmic synthesis of melatonin in mammals, the mammalian melatonin rhythm-generating system (Fig. 1).

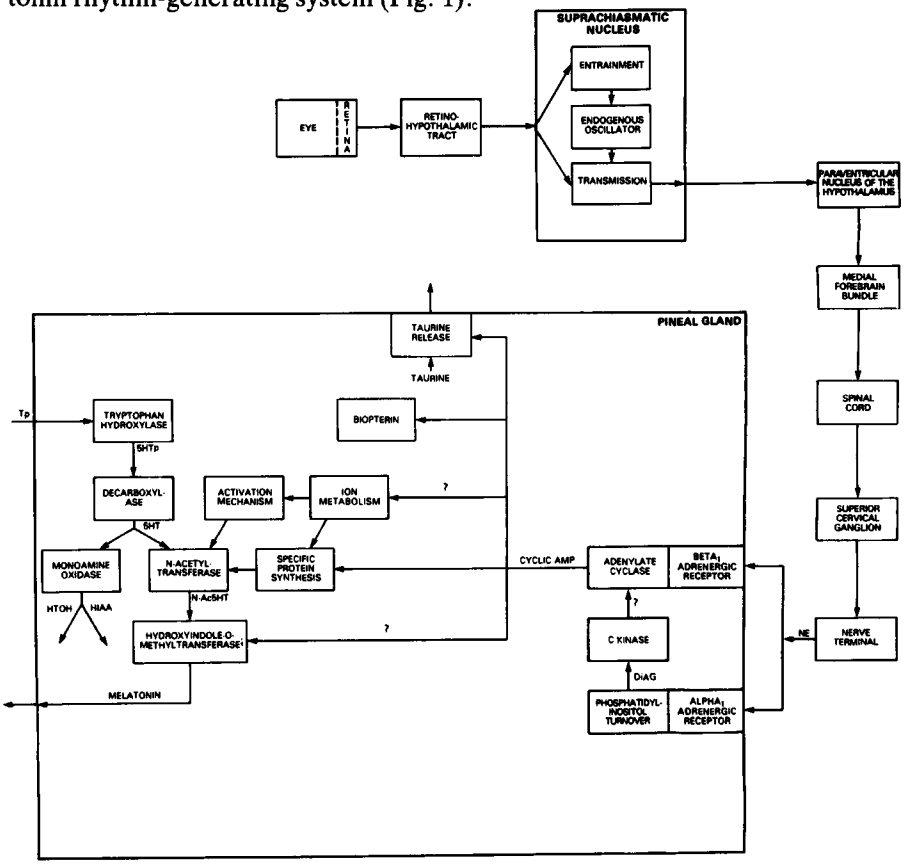

FIG.1. The mammalian melatonin rhythm-generating system. A schematic representation of the system which generates the circadian rhythm in melatonin synthesis. NE, noradrenaline; cyclic AMP , adenosine 3',5'-cyclic monophosphate; Tp, tryptophan; 5HTp, 5-hydroxytryptophan; 5HT, 5-hydroxytryptamine (serotonin); HIAA, 5-hydroxyindole acetic acid; HTOH, 5-hydroxytryptophol; N-Ac5HT, *N*-acetylserotonin; DiAG, diacylglycerol. The question marks indicate unproved hypotheses. Modified from Klein et al (1981a).

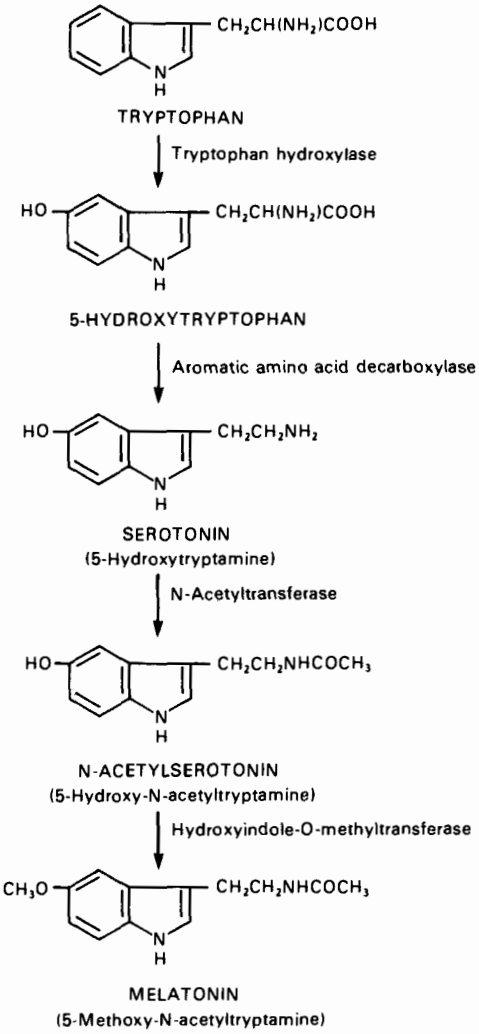

FIG. 2. The conversion of tryptophan to melatonin.

Melatonin synthesis

Melatonin is synthesized from circulating tryptophan as follows: tryptophan → hydroxytryptophan → serotonin → *N*-acetylserotonin → melatonin (Fig. 2) (Klein et al 1981a). The main perturbation causing the daily rhythm in melatonin synthesis is the increase in the rate of *N*-acetylation of serotonin

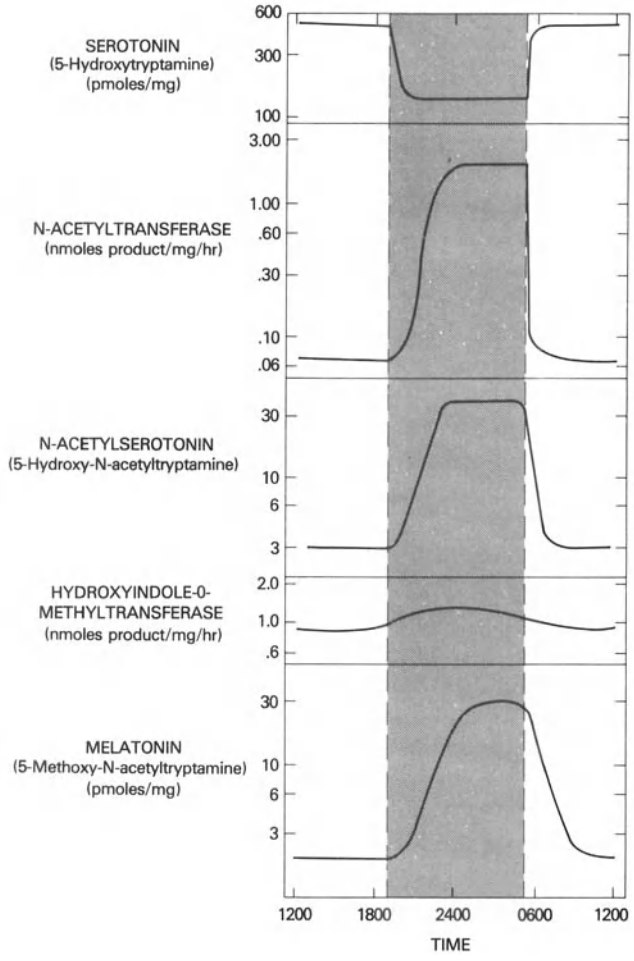

FIG. 3. Rhythms in indole metabolism in the rat pineal gland. The metabolic pathway from 5-hydroxytryptamine to melatonin is on the left. The daily variations in the concentrations of metabolites and activities of enzymes are on the right. The shaded portion indicates the dark period of the lighting cycle.

(Fig. 3). The enzyme responsible for this step, arylalkylamine *N*-acetyltransferase (EC 2.3.1.87) or serotonin *N*-acetyltransferase (Voisin et al 1984), increases in activity at night 70-fold to 100-fold in the rat (Klein & Weller 1970) but to a lesser degree in some other species (Namboodiri et al 1985). This increase is regulated by a photoneural mechanism.

The neural pathway

Retinohypothalamic projection

Light acts on the pineal gland through the retina (Klein & Weller 1972). The effects of light are mediated by a monosynaptic pathway from the retina which leaves the optic nerves at the level of the optic chiasm and terminates in the suprachiasmatic nuclei (SCN). This retinohypothalamic pathway, which was the subject of some debate among pioneering neuroanatomists, was proved to exist unequivocally by Robert Moore, who traced the pathway in a number of mammals by injecting radioactive amino acid labels into the eye, and tracing their transport to the SCN (Moore & Lenn 1972, Moore 1973).

The SCN

The SCN, which are located at the base of the hypothalamus where it contacts the optic chiasm, are of special importance in the rhythmic function of the mammalian pineal gland. These structures contain the circadian timing system which controls pineal and other rhythms. The SCN are remarkable because they continue to oscillate on a 24 h basis (± 0.5 h) in animals that are maintained in constant darkness or are blind (Klein & Moore 1979, Reppert et al 1981b). Thus, the clock is autonomous, not a simple reflection of the environmental lighting cycle.

The effects of light on the melatonin rhythm-generating system are properly thought of as effects on the SCN. Although the SCN can function in a cyclic manner autonomously, environmental lighting has strong effects on the clock, as reflected by changes in pineal *N*-acetyltransferase activity and melatonin production. These effects can be discussed usefully as three seemingly separate functions. First, light can reset the clock, and shift it forward or backward (Reppert et al 1981a, Illnerova & Vanecek 1982b). This is important for keeping the clock entrained to the environmental lighting schedule, and ensures that an animal's activity schedule is optimally synchronized to the environmental lighting cycle. Second, light can alter the duration for which the clock is stimulating the pineal gland and other functions it controls. Elegant work by Helena Illnerova and Jiri Vanecek with the pineal gland has provided compelling evidence that the SCN clock is actually composed of two dependent clocks, and the degree to which their pineal stimulatory periods overlap is determined by the environment (Illnerova & Vanecek 1982a). Long nights appear to allow the clocks to drift apart, so that the pineal gland will be stimulated for a longer period than in animals kept in short nights. A third

effect of light is rapidly to terminate the neural stimulation of the pineal gland (Klein & Weller 1972). This results in a rapid decrease in *N*-acetyltransferase activity and melatonin production (Namboodiri et al 1985).

SCN→ pineal gland

The neural pathway from the SCN to the pineal gland passes first to the paraventricular nuclei and then, perhaps by a single monosynaptic projection, through the brain to the intermediolateral cell column of the spinal cord (Klein et al 1983a). Synaptic connections are made there with preganglionic cell bodies which innervate the superior cervical ganglia of the sympathetic chain. Nerve cells in the superior cervical ganglia send projections back to the brain via the inferior carotid nerve and nervi conarii, which pass along the surface of the hypothalamus and then, with the tentorium cerebelli, to the pineal gland (Moore 1977).

Trans-synaptic regulation of the pineal gland

The transmitter regulating the pineal gland is noradrenaline, which is released at night, in response to stimulatory signals originating in the SCN, from the dense network of fibres pervading the tissue (Brownstein & Axelrod 1974). These fibres are rarely seen to form distinct terminals. Rather, it appears that the transmitter is released into the perivascular space and diffuses to the surface of pineal cells.

The neural network in the pineal gland also controls the level of adrenergic agonists in the extracellular space in a less obvious but equally important way. Sympathetic nerves can take up and concentrate, like a sponge, catecholamines which diffuse from the circulation into the perivascular pineal space (Kvetnansky et al 1979). This protective effect has been well demonstrated in experiments in which highly stressful treatments, known to elevate concentrations of circulating catecholamines, have little effect on the pineal as long as the uptake process is normal. However, when uptake is blocked or if the nerves are removed, stress has profound stimulatory effects on pineal function (Parfitt & Klein 1976).

Adrenergic regulation of melatonin production

Noradrenaline is a mixed α- and β-agonist, and can act via α_1-, α_2-, β_1- and β_2-receptors. The α_1- and β_1-adrenoceptors on pinealocytes have been fully

characterized (Auerbach et al 1981, Sugden & Klein 1983), and both are important in the regulation of the best-studied indicators of pineal function, including cyclic AMP, cyclic GMP, *N*-acetyltransferase and melatonin production (Klein et al 1983b, Vanecek et al 1985).

Cyclic AMP

Noradrenaline increases the cyclic AMP concentration 60-fold in pinealocytes (Strada et al 1972). It activates adenylate cyclase through an action on β_1-adrenoceptors. This activation, which is mediated by GTP-binding adenylate

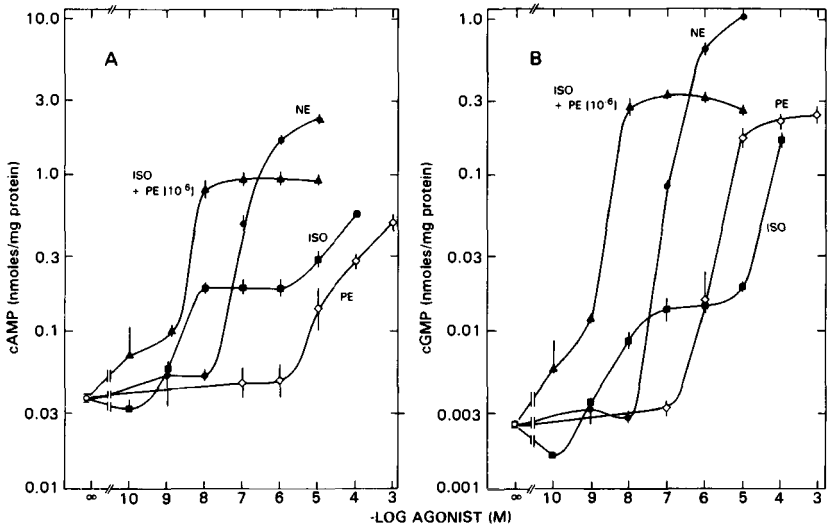

FIG. 4. Agonist-stimulated increases in the concentrations of (A) cyclic AMP (cAMP) and (B) cyclic GMP (cGMP). Pineal cells were incubated (100 000 cells, total volume 500 μl) for 15 min at 37 °C with the indicated concentrations of noradrenaline (NE), phenylephrine (PE), isoprenaline (ISO) or ISO + PE (1 μM). Each point is the mean for triplicate samples assayed in duplicate. Vertical lines represent the SEM; where not shown, the SEM is smaller than the symbol (Vanecek et al 1985).

cyclase stimulatory protein in the membrane, results in a 10-fold increase in cyclic AMP levels 10 min after stimulation; the effect gradually disappears as a result of desensitization mechanisms (Vanecek et al 1985).

Noradrenaline also acts through α_1-adrenoceptors to potentiate the effect of β-adrenergic stimulation on cyclic AMP levels (Vanecek et al 1985). This results in a 10-fold to 20-fold further increase in the production of cyclic AMP (Fig. 4). Interestingly, α_1-adrenergic activation by itself does not alter cyclic

AMP concentrations. The time course of the stimulation of cyclic AMP production is similar to that seen with pure β-adrenergic stimulation (Fig. 5).

The α_1-adrenergic mechanism through which noradrenaline acts to potentiate the effect of β-adrenergic stimulation on cyclic AMP levels appears to involve phospholipids. Noradrenaline stimulates phosphatidylinositol turnover (Berg & Klein 1972); it has been found that this involves α_1-adrenoceptors (Smith et al 1979). For a long time this remained the only known effect of α_1-adrenergic agonists.

More recently, evidence has been presented indicating that a product of the increased phosphatidylinositol turnover, diacylglycerol (DG), is probably a second messenger mediating the α-adrenergic potentiation of β-adrenergic

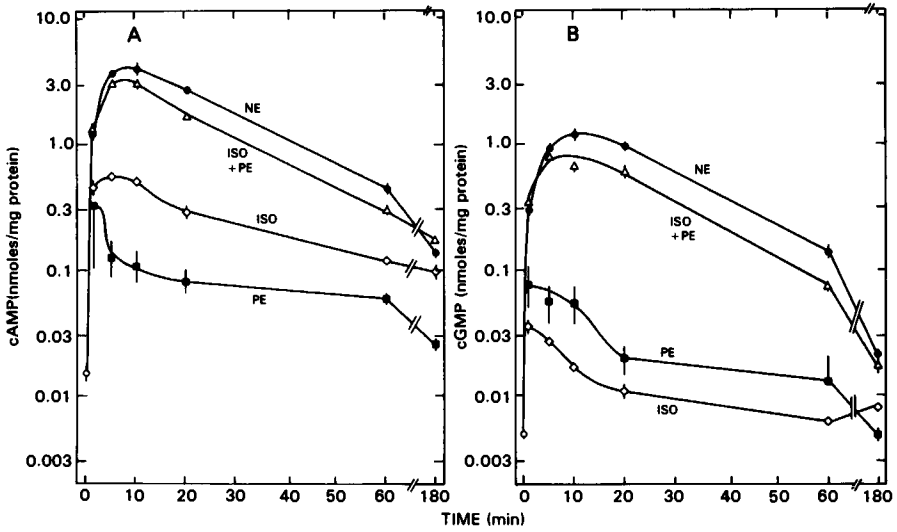

FIG. 5. The time course of accumulation of (A) cyclic AMP (cAMP) and (B) cyclic GMP (cGMP) after treatment of cultured pinealocytes with (−)-noradrenaline (NE, 10 μM), (−)-isoprenaline (ISO, 10 μM), phenylephrine (PE, 10 μM), or (−)-ISO (10 μM) + PE (10 μM). See legend to Fig. 4 for further details (Vanecek et al 1985).

stimulation of adenylate cyclase (Sugden et al 1985b). It has been found that a synthetic diacylglycerol, 1-oleoyl-2-acetylglycerol, mimics the effects of α_1-adrenergic stimulation. In addition it has been found that phorbol esters, tumour-promoting agents which have some structural analogy to DG, mimic the potentiating effect of the α-adrenergic agonist phenylephrine (Fig. 6). Phorbol esters act to promote membrane association of a calcium- and phospholipid-dependent protein kinase (protein kinase C). Both phorbol esters and phenylephrine promote membrane association of protein kinase C in

pinealocytes (Sugden et al 1985b). The association of protein kinase C is thought to bring the enzyme in close association with a substrate, and in this manner to initiate phosphorylation of a membrane protein involved in regulating cyclic AMP generation.

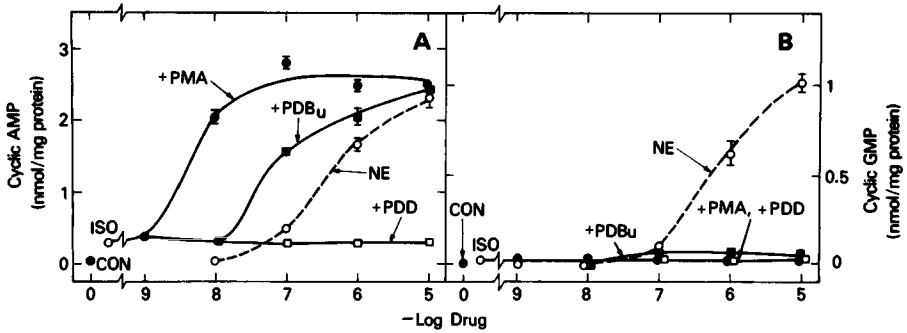

FIG. 6. Potentiation of β-adrenergic cyclic nucleotide responses by phorbol esters (A, cyclic AMP; B, cyclic GMP). Pinealocytes (10^5 cells per tube) were treated for 15 min with (−)-isoprenaline (ISO, 10^{-6} M) either alone or in combination with 4-β-phorbol 12-myristate 13-acetate (PMA), 4-β-phorbol 12,13-dibutyrate (PDBu) or 4-α-phorbol 12,13-didecanoate (PDD). The dose–response curve to (−)-noradrenaline (NE) is shown for comparison. CON, control. Phorbol esters (Sigma) were added as concentrated solutions (100×) in ethanol. All points represent the mean ± SEM of three samples. The lack of error bars indicate that the SEM was less than the area covered by the symbol (Sugden et al 1985b).

The site of potentiation of β-adrenergic stimulation of cyclic AMP production by α-adrenergic agonists appears to be after β-adrenoceptor activation, because potentiation can be produced by phenylephrine in cells treated with cholera toxin, which acts on the adenylate cyclase regulatory protein (J. Vanecek et al, unpublished work). Similarly, phorbol esters potentiate the effects of cholera toxin. The potentiating mechanism might involve only the enhanced activation of adenylate cyclase. Alternatively, inhibition of phosphodiesterase might be involved.

Cyclic GMP

Cyclic GMP, whose concentration is elevated 400-fold in pinealocytes by noradrenaline, is regulated in a manner somewhat similar to cyclic AMP (Fig. 4) (Vanecek et al 1985). β-Adrenergic activation is a requirement, and α-adrenergic stimulation, which by itself has no significant effect, potentiates β-adrenergic stimulation. However, there are some marked differences. First, whereas β-adrenergic stimulation produces a 10-fold increase in cyclic AMP levels, it produces only a twofold increase in those of cyclic GMP. Second, whereas α-adrenergic potentiation produces a sevenfold to 10-fold increase

in cyclic AMP concentrations, it produces a 200-fold increase in those of cyclic GMP. Thus, stimulation of cyclic GMP production by noradrenaline is quantitatively more dependent on α-adrenergic activation.

Most details of the regulation of cyclic GMP are unknown. However, it is interesting to note that the protein kinase C mechanism does not appear to be involved (Sugden et al 1985b). Perhaps the increased phospholipid turnover stimulated by noradrenaline yields, in addition to DG, a second messenger which acts on cyclic GMP levels. It should be noted that the function of cyclic GMP in the pineal gland is a mystery and no evidence is available indicating that it participates in the regulation of melatonin production or in any other function. However, because the regulation of this cyclic nucleotide is so different from that of cyclic AMP, these comments on cyclic GMP are included.

Subsensitivity and supersensitivity in cyclic nucleotide responses: see-saw signal processing

A fascinating feature of the pineal gland is that there are reciprocal changes in the magnitudes of cyclic AMP and cyclic GMP responses produced by changes in chronic neural stimulation (Deguchi & Axelrod 1973, Klein et al 1981b). Following long periods of stimulus deprivation, produced by keeping animals in constant lighting or denervating the pineal gland, the cyclic AMP response increases twofold, an example of denervation supersensitivity. This is similar to what is generally seen in neural and hormonal regulation. In contrast, the pineal cyclic GMP response exhibits the opposite, a denervation subsensitivity. Following constant light, superior cervical ganglionectomy or decentralization, the cyclic GMP response to noradrenaline falls from a nearly 80-fold increase in concentration to less than a fourfold increase. The combined effects of a supersensitive cyclic AMP response and a subsensitive cyclic GMP response are termed 'see-saw' signal processing.

The importance of this has not been established. However, it has potentially important implications because it shifts the pineal gland between a mono- and a bi-cyclic nucleotide second messenger system. Assuming that each regulates different processes, one can imagine that the two cyclic nucleotides might be responsible for turning on and off different hormone systems. Cyclic AMP, as detailed in the following section, controls melatonin production. Cyclic GMP might regulate another hormone.

Cyclic AMP regulation of indole amine metabolism

All the available evidence indicates that melatonin production is regulated by cyclic AMP (Klein & Weller 1973, Klein et al 1978). This compound appears

to be responsible for causing large increases in melatonin production by increasing *N*-acetyltransferase activity. This also decreases the tissue content of serotonin and serotonin oxidation products. The increase in serotonin *N*-acetylation leads to an increase in the concentration of *N*-acetylserotonin. This elevates melatonin production by a mass-action effect. The increase in melatonin production leads to an increase in the release and circulating levels of melatonin.

Serotonin N-*acetyltransferase*

Serotonin *N*-acetyltransferase has been thought to be similar to liver arylamine *N*-acetyltransferase (EC 2.3.1.5), an enzyme that has broad substrate specificity. However, this has recently been found to be incorrect (Voisin et al 1984). Serotonin *N*-acetyltransferase, which has also been termed indolamine *N*-acetyltransferase, is more properly called arylalkylamine *N*-acetyltransferase (EC 2.3.1.87). It has been found to have narrow specificity for arylalkylamines (aromatic compounds having a primary amine on a side-chain), e.g. phenylethylamine and serotonin, and little activity towards arylamines (aromatic compounds with amines on the ring), e.g. phenetidine and phenylamine.

In the rat and sheep pineal gland there is both an arylalkylamine *N*-acetyltransferase, which is involved in controlling indole amine metabolism in general and melatonin synthesis specifically, and an arylamine *N*-acetyltransferase, which is present at unchanging levels, and probably acts as a detoxification mechanism, acetylating amines (Voisin et al 1984).

The regulation of rat pineal arylalkyl *N*-acetyltransferase activity has been studied in cell and organ culture and in whole animals. All available evidence indicates that it is regulated by cyclic AMP (Klein & Weller 1973). Cyclic AMP has two distinct effects on the enzyme. First, it stimulates activity through a mechanism involving the synthesis of new protein and new mRNA. It has not been established whether this involves new synthesis of the enzyme and *N*-acetyltransferase mRNA. It is possible that it actually reflects the induction of an activating enzyme which might recycle existing *N*-acetyltransferase molecules (Klein et al 1981a).

The second effect of cyclic AMP is to stabilize *N*-acetyltransferase and prevent inactivation (Klein et al 1978). This stablilization is not thoroughly understood, but available observations point to the possibility that a redox mechanism is involved, which might maintain a critical thiol group on the enzyme in a reduced state. If the thiol group undergoes thiol:disulphide exchange with a disulphide compound, the enzyme is rapidly inactivated (Klein & Namboodiri 1982). It is interesting that some disulphide-containing peptides are far more potent inactivators than others. Of the peptides tested, insulin

has been found to be the most potent (Namboodiri et al 1981). It is possible that there is a specific inactivation peptide in the pineal gland, with high specificity towards *N*-acetyltransferase, and that, in the presence of a critical concentration of cyclic AMP, this peptide is maintained reduced in an innocuous dithiol form. When cyclic AMP falls below a critical level, this peptide could be oxidized and converted into a potent inactivating disulphide compound.

A possibly related aspect of the regulation of *N*-acetyltransferase is the strong requirement for cellular hyperpolarization. Hyperpolarization is caused by adrenergic agonists, and if prevented by low concentrations of ouabain, stimulation of the enzyme is blocked (Parfitt et al 1975). Perhaps hyperpolarization is related to the redox state of the cell, and hyperpolarization acts on the activation/inactivation mechanism.

Final comment

The description of the mammalian melatonin rhythm-generating system presented is based primarily on studies in the rat. When tested in other mammals, the main features of the system are found to apply: there is a nocturnal increase in melatonin synthesis, the neural pathway appears to include the SCN and superior cervical ganglia and there is evidence for adrenergic regulation of melatonin production. However, variations do exist. We have found that the shapes of the curves for the nocturnal increase in melatonin production in the sheep, the rhesus monkey, the rat and the hamster are different (Reppert et al 1979, Tamarkin et al 1979, 1980, Namboodiri et al 1985). There are marked differences in the kinetic characteristics of the rat and sheep pineal serotonin *N*-acetyltransferase (Voisin et al 1984). There appears to be a greater dependency in the sheep upon α-adrenergic stimulation than in the rat (Sugden et al 1985a), where β-adrenergic stimulation seems to be more important.

Comparative physiological and biochemical studies of the melatonin rhythm-generating system certainly indicate differences exist among species. It would be of interest to determine how these differences have evolved and have enabled each species to adapt to its photic environment and to survive, and what the selecting factors were.

REFERENCES

Auerbach DA, Klein DC, Woodward B, Aurbach GD 1981 Neonatal rat pinealocytes: typical and atypical characteristics of ^{125}I-iodohydroxybenzylpindolol binding and adenosine 3′5′-monophosphate accumulation. Endocrinology 108:559-567

Berg GR, Klein DC 1972 Norepinephrine increases the (^{32}P) labelling of a phospholipid fraction of postsynaptic pineal membranes. J Neurochem 19:2519-2532

Brownstein M, Axelrod J 1974 Pineal gland: 24 hour rhythm in norepinephrine turnover. Science (Wash DC) 184:163-165

Collin JP 1971 Differentiation and regression of the cells of the sensory line in the epiphysis cerebri. In: The pineal gland. Churchill Livingstone, Edinburgh & London (Ciba Found Symp) p 79-125

Deguchi T, Axelrod J 1973 Superinduction of serotonin N-acetyltransferase and supersensitivity of adenyl cyclase to catecholamines in denervated pineal glands. Mol Pharmacol 9:612-619

Dodt E 1973 The parietal eye (pineal and parapineal organs) of lower vertebrates. In: Jung R (ed) Handbook of sensory physiology 8/3B. Springer, Berlin & New York, p 113-140

Illnerova H, Vanecek J 1982a Two oscillator structure of the pacemaker controlling the circadian rhythm of N-acetyltransferase in the rat pineal gland. J Comp Physiol 145:539-548

Illnerova H, Vanecek J 1982b Complex control of the circadian rhythm in N-acetyltransferase activity in the rat pineal gland. In: Aschoff J et al (eds) Circadian systems: structure and function. Springer, Berlin, p 285-296

Klein DC, Moore RY 1979 Pineal N-acetyltransferase and hydroxyindole-o-methyltransferase: control by the retinohypothalamic tract and the suprachiasmatic nucleus. Brain Res 174:245-262

Klein DC, Namboodiri MAA 1982 Control of the circadian rhythm in pineal serotonin N-acetyltransferase activity: possible role of protein thiol:disulfide exchange. Trends Biochem Sci 7:98-102

Klein DC, Weller JL 1970 Indole metabolism in the pineal gland: a circadian rhythm in N-acetyltransferase. Science (Wash DC) 169:1093-1095

Klein DC, Weller JL 1972 Rapid light-induced decrease in pineal serotonin N-acetyltransferase activity. Science (Wash DC) 177:532-533

Klein DC, Weller JL 1973 Adrenergic-adenosine 3',5'-monophosphate regulation of serotonin N-acetyltransferase activity and the temporal relationship of serotonin N-acetyltransferase activity to synthesis of ^{3}H-N-acetylserotonin and ^{3}H-melatonin in the cultured rat pineal gland. J Pharmacol Exp Ther 186:516-527

Klein DC, Buda M, Kapoor C, Krishna G 1978 Pineal serotonin N-acetyltransferase activity: abrupt decrease in adenosine 3',5'-monophosphate may be signal for 'turnoff'. Science (Wash DC) 199:309-311

Klein DC, Auerbach D, Namboodiri MAA, Wheler GHT 1981a Indole metabolism in the mammalian pineal gland In: Reiter R (ed) The pineal gland: anatomy and biochemistry. CRC Press, Boca Raton, p 199-227

Klein DC, Auerbach D, Weller JL 1981b Seesaw signal processing in pineal cells: homologous sensitization of adrenergic stimulation of cyclic GMP accompanies homologous desensitization of β-adrenergic stimulation of cyclic AMP. Proc Natl Acad Sci USA 78:4625-4629

Klein DC, Smoot R, Weller et al 1983a Lesions of the paraventricular nucleus area of the hypothalamus disrupt the suprachiasmatic→spinal cord circuit in the melatonin rhythm generating system. Brain Res Bull 10:647-652

Klein D, Sugden D, Weller JL 1983b Postsynaptic α-adrenergic receptors potentiate the β-adrenergic stimulation of pineal serotonin N-acetyltransferase. Proc Natl Acad Sci USA 80:599-603

Kvetnansky R, Kopin I, Klein DC 1979 Stress increases pineal epinephrine. Commun Psychopharmacol 3:69-72

Moore RY 1973 Retinohypothalamic projection in mammals. A comparative study. Brain Res 49:403-409

Moore RY 1977 The innervation of the mammalian pineal gland. In: Reiter RJ (ed) The pineal and reproduction. Karger, Basel, p 1

Moore RY, Lenn NJ 1972 A retinohypothalamic projection in the rat. J Comp Neurol 146:1-14

Namboodiri MAA, Favilla JT, Klein DC 1981 Pineal N-acetyltransferase is inactivated by disulfide-containing peptides: insulin is the most potent. Science (Wash DC) 213:571-573

Namboodiri MAA, Sugden D, Klein DC, Tamarkin L, Mefford IN 1985 Serum melatonin and pineal indolamine metabolism in a species with a small day/night N-acetyltransferase rhythm. Comp Biochem Physiol B Comp Biochem 80:731-736

Oksche A 1984 Evolution of the pineal complex: correlation of structure and function. Ophthalmic Res 16:88-95

Parfitt AG, Klein DC 1976 Sympathetic nerve endings in the pineal gland protect against acute stress-induced increase in N-acetyltransferase (E.C. 2.3.1.5) activity. Endocrinology 99:840-851

Parfitt A, Weller JL, Klein DC, Sakai KK, Marks BH 1975 Blockade by ouabain or elevated potassium ion concentration of the adrenergic and adenosine cyclic 3'5'-monophosphate-induced stimulation of pineal serotonin N-acetyltransferase activity. Mol Pharmacol 11:241-255

Reppert SM, Perlow MJ, Tamarkin L, Klein DC 1979 A diurnal melatonin rhythm in primate cerebrospinal fluid. Endocrinology 104:295-301

Reppert SM, Perlow MJ, Tamarkin L, Orloff D, Klein DC 1981a The effects of environmental lighting on the daily melatonin rhythm in primate cerebrospinal fluid. Brain Res 223:313-323

Reppert SM, Perlow MJ, Ungerleider L et al 1981b Effects of damage to the suprachiasmatic area of the anterior hypothalamus on the daily melatonin and cortisol rhythms in the Rhesus monkey. J Neurosci 1:1414-1425

Smith TL, Eichberg J, Hauser G 1979 Postsynaptic localization of the alpha receptor-mediated stimulation of phosphatidylinositol turnover in pineal gland. Life Sci 24:2179-2184

Strada S, Klein DC, Weller J, Weiss B 1972 Effect of norepinephrine on the concentration of adenosine 3',5'-monophosphate of rat pineal gland in organ culture. Endocrinology 90:1470-1475

Sugden D, Klein DC 1983 Rat pineal $alpha_1$-adrenoreceptors: identification and characterization using (125I)iodo-2-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone ((125I)HEAT). Endocrinology 114:435-440

Sugden D, Namboodiri MAA, Klein DC, Pierce JE, Grady RK Jr, Mefford I 1985a Ovine pineal α_1-adrenoreceptors: characterization and evidence for a functional role in the regulation of serum melatonin. Endocrinology 116:1960-1967

Sugden D, Vanecek J, Klein DC, Thomas TP, Anderson WB 1985b Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. Nature (Lond) 314:359-361

Tamarkin L, Reppert SM, Klein DC 1979 Regulation of pineal melatonin in the Syrian hamster. Endocrinology 104:385-389

Tamarkin L, Reppert SM, Klein DC, Pratt B, Goldman BP 1980 Studies on the daily pattern of pineal melatonin in the Syrian hamster. Endocrinology 107:1525-1529

Vanecek J, Sugden D, Weller J, Klein DC 1985 Atypical synergistic $alpha_1$- and $beta_1$-adrenergic regulation of adenosine 3',5'-monophosphate in cultured rat pinealocytes. Endocrinology 116:2167-2173

Voisin P, Namboodiri MAA, Klein DC 1984 Arylamine N-acetyltransferase and arylalkylamine N-acetyltransferase in the mammalian pineal gland. J Biol Chem 254:10913-10918

DISCUSSION

Bittman: I'd like to comment on the question of the equivalence of lesions of the suprachiasmatic nuclei (SCN) and paraventricular nuclei (PVN) and their consequences for the pineal. We found that lesions to either SCN or PVN

interrupted the photoperiodic testicular response of the Syrian hamster (Lehman et al 1984). However, when we looked at the melatonin content of glands from lesioned animals at the time when we expected melatonin levels to peak (i.e. 3–5 h before lights were due to come on) we found a significant difference: although both lesions significantly reduced the melatonin content relative to non-lesioned or sham-lesioned controls, SCN lesions produced a 50% reduction whereas the lesions of the PVN resulted in a much more dramatic decline, which we interpreted as a probable absence of the melatonin rise. We are now processing brains to analyse a study in which we killed groups of animals every 4 h to compare the effects of SCN and PVN lesions. Perhaps these lesions are not completely equivalent; although destroying either the SCN or the PVN will eliminate the normal phasing and rhythmicity of pineal indole synthesis, the absolute levels of melatonin might be affected to different extents.

Turek: We too have found that lesions of the PVN abolish the short-day response (Pickard & Turek 1983), as do knife cuts between the SCN and PVN (Inouye & Turek 1985). However, knife cuts about a millimetre dorsal to the PVN also abolish short-day-induced testis regression. These cuts may have severed fibres connecting the PVN to the spinal cord, but it is also possible that melatonin is acting back on the area of the brain dorsal to the PVN, or that projections from the SCN actually pass dorsal to and through the PVN *en route* to the spinal cord. Watts & Swanson (1984) have reported that there are few terminals from the SCN in the PVN; instead, projections from the SCN pass dorsally beyond the PVN and actually terminate in the thalamic paraventricular nucleus. The fibres may run through the PVN but few seem to synapse there.

Klein: I think that studying gonadal regression in PVN-lesioned animals and implying a direct involvement of the pineal gland is dangerous. You don't know whether the lesions are directly affecting the normal hypothalamic control of the gonads or whether they are acting via the pineal gland.

Bittman: You need to do the appropriate controls. But I agree that you can compromise pineal function and photoperiodism with lesions in different places and perhaps be doing it in diffeent ways. Others lesions may alter melatonin responsiveness, steroid feedback or gonadotropin secretion mechanisms. If you look carefully enough you can see the pineal is not behaving in the same way in animals with these different brain insults.

Klein: Another important point to consider in lesion studies is the impressive ability of the brain to compensate for lesions. In the monkey as few as 5% or 3% of SCN cells can do the job of the whole nucleus. We were surprised to see nearly normal rhythms in monkeys with seemingly complete SCN lesions and we had to go back to search for surviving SCN cells, which we found only by identifying labelled neural projections from the eye (Reppert et al 1981). One must make sure that any lesions made are complete before drawing conclusions.

Zucker: I think we need to clarify what we mean by recovery of function after brain lesions. As far as I know there is *no* evidence for recovery of function after SCN ablation; what you are implying is that there is great redundancy in the system and that a very small number of cells may be capable of mediating the function of the entire nucleus.

Klein: Don't you get compensation?

Zucker: No. Mosko & Moore's results (1979a,b) suggest that damage to the SCN within the first few days of life produces effects comparable to the effects of lesions in the adult animal. The early insult is the one that creates a situation most conducive to recovery of function, but even in this case there is no evidence for sparing or recovery of function.

Illnerová: When only β-adrenoceptors in the pineal are activated, you get a sixfold to eightfold increase in cyclic AMP concentration; α-adrenergic stimulation causes about a 10-fold further increase. However, the effects of α-adrenergic stimulation on *N*-acetyltransferase (NAT) activity may not be so pronounced. It seems that stimulation of β-adrenoceptors gives about a 30-fold or 70-fold increase in NAT activity, but α-adrenergic stimulation causes only about a twofold to fourfold further increase. When NAT activity has attained only about one-tenth of its maximal night-time value during its evening rise, the melatonin concentration has already reached 60–70% of its maximum (Illnerová et al 1983). There may be a cascade: you don't need maximal cyclic AMP to get maximal NAT and you don't need maximal NAT to get maximal melatonin. What is the physiological significance of this?

Klein: It's true that 10% activation of NAT is enough to get full melatonin production (Wheler et al 1979). However, although we can get pretty good stimulation of NAT without full stimulation of cyclic AMP, I'm not sure if it is correct to say that we can get maximal NAT activity without maximal stimulation of cyclic AMP. This is not clear and we have not been sufficiently interested in the question to pursue it in detail. It is difficult to study because you are comparing two different time courses: cyclic AMP peaks before NAT. The question arises of how much cyclic AMP is required, and when, to produce a specific level of NAT at a particular time. We think that the early spike in cyclic AMP may be important for turning on the machinery leading to an increase in NAT activity, but that lower levels of cyclic AMP are required to maintain NAT in an active form. Another enigma is that the shape of the time course of the NAT response appears to depend on dose and other factors. For example, we have found that as we increase the dose of noradrenaline we reach a maximum response, as measured at a single time, 6 h after the start of drug administration. However, higher doses appear to produce smaller responses. This probably reflects acute subsensitization; we don't know if it is at the receptor level, the cyclic AMP level or the NAT level and we don't know if it is due to early events or late events. Also, we think the response to lower doses may peak earlier than the response to middle doses of agonists.

The physiological significance of this is not at all clear to me. What is clear is that we are ignorant of a lot of the details of the adrenergic regulation of melatonin production. Although significant strides have been made in understanding the major characteristics of the system, there is still a lot to be learned. I have postponed these questions until we have better tools to study NAT, including molecular probes to use to measure the components of the mechanism regulating enzyme activity. My feeling is that measuring enzyme activity is not direct enough. My co-workers and I have been working on these tools; they are hard to make.

Illnerová: When studying the increase in cyclic AMP levels as a function of isoprenaline concentration you get a biphasic curve, with a plateau after which cyclic AMP concentrations start to rise again and α-adrenoceptors probably begin to be activated too as a result of high concentrations of isoprenaline. When rats are maintained under 12 h light:12 h dark, the curve depicting the evening NAT rise as a function of time may be also biphasic (Illnerová & Vaněček 1982). At first, following a lag period after lights-off, the curve goes up quite quickly; there is then a half-hour plateau and after this it goes up again. It may be that, at the beginning when not much noradrenaline is released, only β-adrenoceptors are stimulated to induce and activate NAT, and that only after enough noradrenaline is released are the α-receptors also stimulated and the enzyme activated further. Is anything known about the affinity of β- and α-adrenoceptors in the pineal towards noradrenaline?

Klein: We have characterized the receptors for α-agonists in the sheep and the rat pineal, so we know a lot about them (Sugden et al 1985, Sugden & Klein 1984); their affinity for noradrenaline is about 100-fold greater than for isoprenaline. But I can't see how these receptors have anything to do with the unusual time course you see. You are proposing that one receptor is activated first and the other one only after a lag in time. However, we know that there is an immediate response for both receptors and we have never seen any lag (Vaněček et al 1985). Even when we see no response or observe subsensitivity we cannot explain the results on the basis of a time lag. It is just that the quantitative response is lower. I think there must be another explanation for your biphasic curve; perhaps the first increase reflects the presence of small amounts of the required mRNA and the second the synthesis of new mRNA.

Rollag: I was struck by your observation that levels of cyclic AMP and cyclic GMP go up together in response to noradrenaline. You have interpreted your experiments in terms of the regulation of cyclic nucleotide *generation*, but I assume you are just measuring content. If transducin, which affects cyclic nucleotides via phosphodiesterase, is present in these cells, perhaps you are regulating cyclic AMP by degradation rather than by generation.

Klein: We have not found transducin in the mammalian pinealocyte, but only in the photosensitive pinealocytes of lower vertebrates. None-the-less, we have been thinking about phosphodiesterase involvement in adrenergic control

mechanisms and have done a number of experiments designed to implicate it in the acute adrenergic regulation of cyclic nucleotides. These have not yielded evidence indicating phosphodiesterase is involved.

Reiter: I subscribe to the notion that noradrenaline is the neurotransmitter, and yet in several species, including the human and the Syrian hamster, it is notoriously difficult to stimulate either NAT activity or melatonin production in the pineal or to increase blood levels of melatonin with isoprenaline. You could argue that isoprenaline is not a specific β-agonist, but we have subsequently tried to do the same thing with noradrenaline in the hamster and have failed. What do you think the reason is for these repeated failures?

Klein: Giving an animal noradrenaline is a naive way to stimulate the pineal gland. The nerves in the pineal act as a sponge for noradrenaline; they will rapidly take up and destroy any noradrenaline coming in. That is why you don't get an effect.

Reiter: If you remove the SCN there are no nerves left, and you might even expect the receptors to be supersensitive. We have done this a dozen times but we have still not been able to stimulate pineal melatonin production with any drugs. Additionally, isoprenaline is not taken up by the nerve endings.

Klein: Under those conditions my explanation is invalid and I really don't know why you get no response. The time course of the response is long in the hamster but I'm sure you have taken that into account. As you know, we have been able to stimulate melatonin production in the hamster pineal gland with isoprenaline (Tamarkin et al 1979).

Reiter: In that particular experiment the day-time levels of melatonin were 30–40 pg per pineal gland, which is relatively low, and they went up to 170 pg per gland in response to two injections of isoprenaline. That is a stimulation, but we would anticipate that normal values of about 100 pg per gland should go up to around 800–1000 pg per gland if we were dealing with the rat. We have repeated the experiments exactly, thinking that we were doing something wrong, but we have not been able to confirm the results.

Arendt: Depending on how we sample, we can observe very high frequency spiking of plasma melatonin concentrations in the sheep, and I think you can see this in some of Eric Bittman's records as well. Is this explicable in terms of your cascade effect?

Klein: How fast are your spikes?

Arendt: Every 2 min in some sheep. We take samples every 30 s.

Klein: Is this in the day-time?

Arendt: No, at night.

Klein: This is very interesting because the half-life of melatonin is almost that short.

Arendt: That's right. These concentration profiles don't look a bit like the pharmacokinetics of melatonin given intravenously, but perhaps the high intravenous doses used are not comparable.

Klein: In sheep we can, under some circumstances, get large changes in melatonin levels without large changes in measurable NAT activity (Namboodiri et al 1985). We can't explain this. There may be a very rapid activation of hydroxyindole *O*-methyltransferase, or an activation of NAT that we do not see after we homogenize the tissue. Or the concentration of circulating melatonin could change rapidly if the circulation through the pineal gland was suddenly cut off.

REFERENCES

Illnerová H, Vaněček J 1982 Two-oscillator structure of the pacemaker controlling the circadian rhythm of *N*-acetyltransferase in the rat pineal gland. J Comp Physiol 145:539-548

Illnerová H, Vaněček J, Hoffmann K 1983 Regulation of the pineal melatonin concentration in the rat (*Rattus norvegicus*) and in the Djungarian hamster (*Phodopus sungorus*). Comp Biochem Physiol 74:155-159

Inouye ST, Turek FW 1985 Horizontal knife cuts either ventral or dorsal to the hypothalamus paraventricular nucleus block testicular regression in golden hamsters maintained in short days. Biol Reprod, in press (abstr)

Lehman MN, Bittman EL, Newman SW 1984 Role of the hypothalamic paraventricular nucleus in neuroendocrine responses to daylength in the golden hamster. Brain Res 308:25-32

Mosko SS, Moore RY 1979a Neonatal ablation of the suprachiasmatic nucleus. Neuroendocrinology 29:350-361

Mosko SS, Moore RY 1979b Neonatal suprachiasmatic nucleus lesions: effects on the development of circadian rhythms in the rat. Brain Res 164:17-38

Namboodiri MAA, Sugden D, Klein DC, Tamarkin L, Mefford IN 1985 Serum melatonin and pineal indoleamine metabolism in a species with a small day/night N-acetyltransferase rhythm. Comp Biochem Physiol B Comp Biochem 80:731-736

Pickard GE, Turek FW 1983 The hypothalamic paraventricular nucleus (PVN) mediates the photoperiodic control of reproduction but not the effects of light on the circadian rhythm of activity. Neurosci Lett 43:67-72

Reppert SM, Perlow MJ, Ungerleider L et al 1981 Effects of damage to the suprachiasmatic area of the anterior hypothalamus on the daily melatonin and cortisol rhythms in the rhesus monkey. J Neurosci 1:1414-1425

Sugden D, Klein DC 1984 Rat pineal α_1-adrenoceptors: identification and characterization using [^{125}I]iodo-2[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone. Endocrinology 114:435-441

Sugden D, Namboodiri MAA, Klein DC, Pierce JE, Graddy R Jr, Mefford IN 1985 Ovine pineal α_1-adrenoreceptors: characterization and evidence for a functional role in the regulation of serum melatonin. Endocrinology 116:1960-1967

Tamarkin L, Reppert SM, Klein DC 1979 Regulation of pineal melatonin in the syrian hamster. Endocrinology 104:385-389

Vaněček J, Sugden D, Weller J, Klein DC 1985 Atypical synergistic α_1- and β-adrenergic regulation of adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate in rat pinealocytes. Endocrinology 116:2167-2173

Watts AG, Swanson LW 1984 Evidence for a massive projection from the suprachiasmatic nucleus to a subparaventricular zone in the rat. Neurosci Abstr 10:611

Wheler GHT, Weller JL, Klein DC 1979 Taurine: stimulation of pineal N-acetyltransferase activity and melatonin production via a beta-adrenergic mechanism. Brain Res 166:65-74

Photoperiodism, melatonin and the pineal
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Melatonin and the brain in photoperiodic mammals

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Abstract. The reproductive cycle of photoperiodic species is driven by seasonal changes in daylength. The pineal gland transduces photic information into an endocrine signal. The duration of the nocturnal bout of melatonin secretion is a direct indicator of night-length. The circadian rhythm of melatonin production is driven by a multisynaptic pathway from the suprachiasmatic nuclei (SCN), via the parvocellular portion of the paraventricular nucleus to the preganglionic sympathetic neurons of the thoracic spinal cord. The melatonin signal acts as an interval timer. The cellular basis of the detection of the signal is unknown. The site of detection is possibly within the anterior hypothalamus. The SCN are not essential components of the system that responds to the pineal interval timer. Photoperiod and the pineal melatonin signal have pronounced effects on the function of endogenous opioids, which are probably related to changes in the neuroendocrine mechanisms that regulate gonadotropin release.

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Seasonal changes in daylength are used in two major ways to ensure that the offspring of photoperiodic mammals are conceived (and hence born) in the most opportune season. Firstly, they stimulate reproductive activity at the optimum time and, secondly, they suppress reproduction in unfavourable seasons. We have argued that species differ in their use of these two neuroendocrine mechanisms to time their breeding seasons (Hastings et al 1985a) Some, such as the hamster, seem to depend principally upon the inhibitory effects of daylength to terminate periods of fertility, whereas others, for example ferrets, have a reproductive cycle driven mainly by the stimulatory effects of daylength upon the gonadal axis. It is probable that most species use elements of both control mechanisms, although their relative importance will differ depending upon the particular requirements of the environmental cycle

and reproductive strategy (Herbert 1981). A consequence of these various seasonal strategies is that species differ in relation to the effects of a particular photoperiod upon the gonadal axis. Sheep, for instance, are brought into breeding condition by shortening photoperiods, whereas hamsters are driven out. A coherent account of the neuroendocrine basis for these findings must take into account such inter-species variability.

Removal of the pineal or disruption of its afferent autonomic innervation abolishes photoperiodic control of reproduction under both natural and artificial photoschedules. Pinealectomized animals may remain permanently in one breeding condition or display recurrent cycles of fertility unrelated to the photoperiod. Any study of the neural mechanisms responsible for photoperiodism thus resolves into two questions. Firstly, which neural pathways transmit information about the duration of photoperiod to the pineal gland? Secondly, how is the signal, emitted by the gland, read by the brain and interpreted into changes in the rate of release of gonadotropic hormones by the anterior pituitary?

The signal from the pineal

We support the hypothesis that the duration of the nocturnal pulse of melatonin secretion is the indicator of daylength. This is supported by strong experimental evidence from both sheep and rodents showing (1) that the duration of endogenous nocturnal melatonin production increases as the photoperiod shortens (Fig. 1) (Hoffmann 1981, Kennaway et al 1983, Roberts et al 1985a) and (2) that programmed infusions of exogenous melatonin may drive the gonadal axis, provided that the pulses are of an appropriate duration, irrespective of the time of day when they are given (Carter & Goldman 1983, Bittman 1984). It seems to be essential that the period of exposure to melatonin be uninterrupted if it is to be read effectively as a long night (short day); light falling on the retina immediately suppresses melatonin production, interrupting the long-night signal and leading to a long-day response. This physiological model of photoperiodic time measurement incorporates features of the earlier internal and external coincidence models used to explain the relationship between daily and photoperiodic rhythms, but has the advantage of being open to experimental test. It is now possible to interpret the results of the effects of pulses of light given during subjective day or subjective night according to whether they interrupt the continuity of the melatonin signal, rather than whether they coincide with some other presumed internal event. These points are discussed more fully by Hastings et al (1985a).

On these grounds, it appears that the pineal functions as an interval timer. Interval timers are well established as important control mechanisms in other

types of neuroendocrine regulation (Silver & Bittman 1984), for example in the induction by oestrogen of either the mid-cycle surge in luteinizing hormone (LH) secretion or lordosis behaviour in females. The important differ-

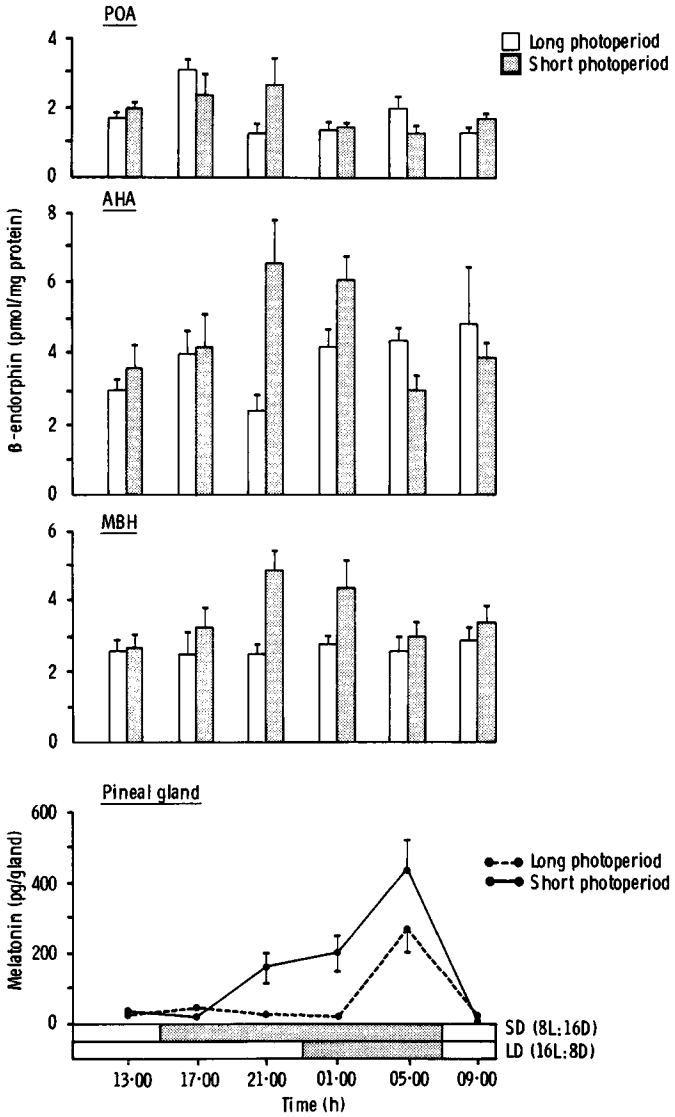

FIG. 1. Tissue content (mean ± SEM) of β-endorphin in preoptic (POA), anterior (AHA) and medio-basal (MBH) hypothalamus, and melatonin content of pineal gland (mean ± SEM) of adult male Syrian hamsters exposed to long or short photoperiods for eight weeks. SD, short days (8 h light: 16 h dark); LD, long days (16 h light: 8 h dark). After Roberts et al (1985a).

ence between these steroid effects and those of melatonin is that it appears that melatonin concentrations have to be elevated continuously to be read as a single period of darkness, whereas oestradiol can be given in separate injections, provided they are not spaced too far apart. This distinction may be more apparent than real. The tissue half-lives of the two hormones may be very different; furthermore, if oestrogen induces an intervening process which decays only slowly, then it might be that this is the process that has to continue without interruption. Melatonin may have a more direct effect upon whatever mechanism it is that requires a continuous signal.

There is a second parallel between the actions of melatonin and those of gonadal steroids on the brain. Although long bouts of melatonin secretion signal short days, it is essential that the bouts should be separated by intervals during which melatonin production is very low or even absent. The maintenance of continuously high levels of melatonin by release from subcutaneous capsules implanted into intact animals appears to block the detection of the endogenous melatonin signal. The reproductive axis operates a default system, often, though not always, behaving as if the animal were pinealectomized (Reiter et al 1974, Hoffmann 1981, Lincoln & Ebling 1985). Thus, if the melatonin signal is to be interpreted accurately by its target site, it would appear that there must be a period free of the compound. It is not known whether this period also has a critical duration.

These observations allow us to specify the properties of the neural system that reads the melatonin signal from the pineal. Similar features apply to the steroid-sensitive neural system, as indicated by studies of the ability of single pulses of oestrogen to trigger a positive feedback on LH production, and hence the LH surge, and by the timing of steroid administration required to induce sexual receptivity in female rodents.

Distinguishing neural pathways regulating circadian rhythms from those regulating photoperiodic rhythms

Besides pinealectomy (or superior cervical ganglionectomy), two other procedures reliably prevent photoperiodic responses: optic enucleation (including section of the optic nerves) (Herbert 1981), or bilateral lesions of the suprachiasmatic nuclei (SCN) (Rusak & Morin 1976). This suggests that the retinohypothalamic tract is a major afferent pathway for photoperiodic information. Since much evidence now indicates that the same pathway is critical for the photic regulation of circadian rhythms, it is clear that there is an anatomical as well as functional overlap between the neural pathways controlling circadian and photoperiodic effects. The daily activity/rest cycle, a widely used marker of the circadian clock, shows photoperiod-dependent changes in the relative

duration of its two phases, indicating that a component of the circadian control system (perhaps the SCN) also transmits information of a photoperiodic nature.

There is both behavioural and neurochemical evidence in favour of separable subunits in the circadian system that are responsive to either 'lights-on' or 'lights-off' (Pittendrigh & Daan 1976, Ralph & Menaker 1985). Interactions between such units may be the basis of the photoperiodic component of the circadian signal. Since the pineal is driven by the circadian control system, such a dual oscillator model for the circadian clock could explain how nocturnal melatonin reflects both circadian (time of day) and photoperiodic (duration of night) variables (Illnerova & Vanecek 1982). In this model, where circadian information and photoperiodic information are integrated in one signal, the pineal differs little from any other effector driven by the circadian system. The alternative model is that the pineal gland receives photoperiodic information that is not present in the signal controlling the circadian effector mechanism.

Recent immunohistochemical evidence shows that the microstructure of the SCN is not homogeneous. The nuclei have been divided into dorso-medial and ventro-lateral components on the basis of the distribution of neurons containing various neurotransmitters including substance P, vasopressin and vasoactive intestinal peptide (VIP), and also the distribution of 5-hydroxytryptamine-containing terminals from the raphe and terminations of the retinohypothalamic tract (Card & Moore 1984). Therefore, there may be regions within the SCN that are specialized for either photoperiodic or circadian information, and dissociation of circadian and photoperiodic information may occur within the nuclei. Alternatively, the specifically photoperiodic component of the circadian signal destined for the pineal may separate from the rest of the circadian signal at a point distal to the SCN. Nevertheless, in both of these models, the pineal would be qualitatively different from the other circadian effector systems. Photoperiodic effects observed in other circadian rhythms such as the activity/rest cycle would be attributed to a secondary action of the pineal upon them (S. M. Armstrong & J. Redman, this volume).

There is a dorsally directed pathway from the SCN to the paraventricular hypothalamic nucleus (PVN), and electrolytic lesions in the area of the PVN prevent both the gonadal response to altered photoperiods and the nocturnal rise in melatonin concentrations (Pickard & Turek 1983, Lehman et al 1984). The PVN is not a homogeneous structure; the medial parvocellular part receives the input from the SCN, whereas the lateral parvocellular area projects to the intermediolateral cell column (IMLT) of the spinal cord in which lie the preganglionic autonomic neurons (Sofroniew et al 1981). Both are distinct from the magnocellular part, which projects to the posterior pituitary. It is therefore interesting that neurotoxic lesions of the lateral parvocellular

PVN, sparing both the magnocellular region and fibres passing through the nucleus, also inhibit the nocturnal melatonin surge in the pineal (Fig. 2). Presumably, photoperiodic information reaching the medial parvocellular PVN is relayed to the lateral part by intranuclear connections before being

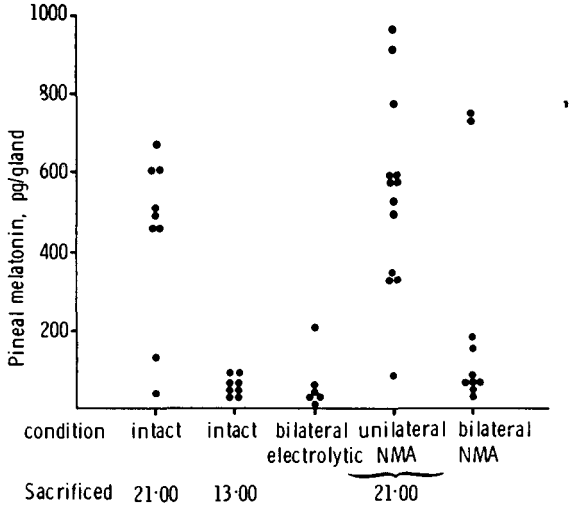

FIG. 2. Pineal melatonin content of adult male Syrian hamsters maintained under 16L:8D (lights-off at 1500 h, lights-on at 2300 h), and killed at 1300 or 2100 h. Animals were intact or received electrolytic or neurotoxic lesions (7.5 μg *N*-methyl aspartic acid (NMA) in 0.5 μl phosphate buffer) of the hypothalamic paraventricular nucleus one week before being killed. M. H. Hastings, unpublished data.

transmitted to the spinal cord. Electrophysiological recordings show that oxytocin can suppress neuronal firing in the IMLT (Gilbey et al 1982). It is therefore possible that oxytocin-containing fibres that project to the cord from the PVN transmit the inhibitory effects of light on melatonin production. However, in view of the inhibitory effect of lesions of the PVN upon pineal melatonin production, it seems likely that PVN neurons also exert a stimulatory effect upon the sympathetic innervation of the pineal.

The SCN have also been suggested as a site of melatonin action. However, bilateral SCN lesions do not prevent the gonadal response to melatonin: regimens of administration that are effective in pinealectomized hamsters remain so after destruction of the nuclei (Bittman et al 1979). Thus, the neural system that reads the pineal signal does not lie in the SCN, despite claims that melatonin implants into these nuclei suppress reproduction. Neurotoxic lesions of the anterior hypothalamus, which spare the SCN, nevertheless prevent short photoperiods from inhibiting reproduction in male and female hamsters (Table

TABLE 1 Reproductive condition of adult Syrian hamsters exposed to 8 h light:16 h dark for 10 weeks

	Males		*Females*	
Experimental treatment	*Mean testis weight (g) ± SEM*	*n*	*Displaying oestrous cyclicity*	*Displaying anoestrus*
None	0.41 ± 0.05	9	0	10
Pinealectomy	2.54 ± 0.22	5	—	—
Unilateral AHAX	0.37 ± 0.02	14	0	15
Bilateral AHAX	2.75 ± 0.17	6	10	0

Animals were intact, pinealectomized or received neurotoxic lesions of the anterior hypothalamic nucleus (AHAX). Pinealectomized female hamsters were not included in the study. After Hastings et al (1985b).

1) (Hastings et al 1985b). Both circadian and oestrous rhythms remain intact in such animals, showing that the SCN are functionally unimpaired and that appropriate lesions in the hypothalamus may disrupt photoperiodic effects without altering other systems under circadian control. To determine whether such lesions interfere with the input to the pineal or with the interpretation of the melatonin signal, it will be necessary to examine the response of such lesioned hamsters to exogenous melatonin.

In conclusion, the major role for the SCN in photoperiodism probably resides in the essential part these nuclei play in the generation and entrainment of circadian rhythms. Damage to the SCN disturbs photoperiodic responses only because melatonin release is dependent upon a circadian signal.

Neurons detecting melatonin

The cellular mechanisms responsible for detecting melatonin are unknown. Given the lipophilic nature of melatonin, it seems likely that all areas of the brain will be exposed to the indole as a consequence of its secretion into the systemic circulation (as is the case for steroids). Attempts have therefore been made to identify melatonin-sensitive neurons as evidence of the hormone's site of action. Neurons within the hypothalamus have been shown to be electrically responsive to melatonin, and the response pattern may vary with time of day (Demaine 1983).

However, it is unclear whether melatonin acts directly upon excitable cell membranes. Reports of specific saturable binding sites have not been substantiated and it is possible that, as with the lipophilic steroids, regulation of neuronal activity may be mediated via the cytoplasm or nucleus. It is perhaps interesting to consider the phylogenetically primitive role of melatonin in

the control of pigment migration within chromatophores and photoreceptors. Such effects are exercised via the microtubule system of the cell. This action may be modified in the neuron to control the passage of transmitter vesicles or other structures along the axon. The requirement for persistent, repeated exposure to melatonin to induce neuronal change may be related to such an effect.

β-Endorphin-containing neural systems and the pineal

Although the site and mechanism of action of melatonin remain uncertain, there is no doubt that photoperiodic regulation leads to changes in activity of the pulse generator controlling the release of luteinizing hormone-releasing hormone (LHRH) (Lincoln & Short 1980). Recent evidence from non-photoperiodic species implicates the β-endorphin-containing neurons of the arcuate/periventricular area of the hypothalamus as regulators of LHRH pulse frequency (Grossman & Rees 1983). Unlike other opioid systems, which are distributed widely throughout the central nervous system, most neurons containing β-endorphin (and its associated family of peptides derived from pro-opiomelanocortin) are restricted to the medio-basal hypothalamus, a well-known site of steroidal feedback. Infusions of β-endorphin into the cerebral ventricles suppress both gonadotropin secretion and the display of sexual behaviour (Kinoshita et al 1980, Meyerson & Terenius 1977). The first effect is synergistic with that of testosterone; the second is not reversed by it. Two questions follow from these findings. Firstly, is the β-endorphin system involved in the action of the pineal on reproduction, since the effects of opioids bear at least a superficial resemblance to those of inhibitory photoperiods? Secondly, if this is so, is such a system specific to pineal-mediated effects, or can it be influenced by other neural mechanisms suppressing reproduction? This is important because there are many other circumstances that call for reproductive inhibition for longer or shorter periods (for example social subordination, sexual immaturity, pregnancy and lactation) and they may operate via a final common pathway.

A role for β-endorphin in photoperiodic regulation is intimated by the observation that gonadal regression in the Syrian hamster is associated with changes in the amount of this peptide within the hypothalamus (Roberts et al 1985a). In photostimulated animals, β-endorphin levels in the anterior hypothalamus (AHA) and medio-basal hypothalamus (MBH) remain constant throughout 24 h. In photoinhibited animals, a marked increase in β-endorphin levels in these areas occurs, particularly in the early dark phase (Fig. 1). Levels in the pre-optic hypothalamus do not show a significant response to daylength. An increase in AHA and MBH levels of β-endorphin is also seen in pinealecto-

mized animals exposed to long days but given thrice daily injections of melatonin, which induce gonadal regression (Fig. 3). These results indicate a role for the pineal melatonin signal in determining regional hypothalamic β-endorphin levels, which may be related to control of the gonadal axis.

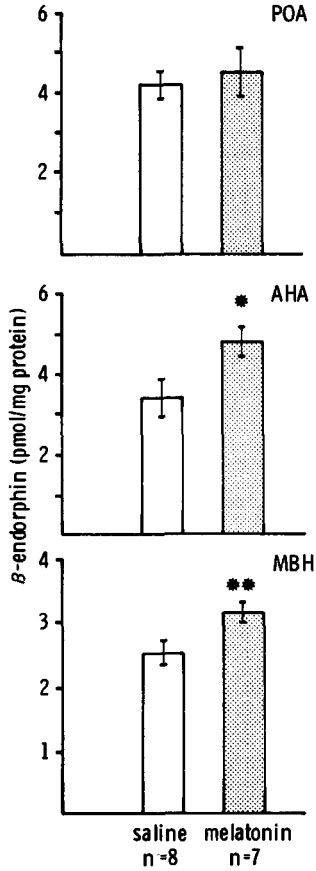

FIG. 3. Tissue content (mean ± SEM) of β-endorphin in preoptic (POA), anterior (AHA) and medio-basal (MBH) hypothalamus of pinealectomized adult male Syrian hamsters maintained under 16L:8D. Animals received thrice daily injections of saline or melatonin for eight weeks. $^{*}P < 0.05$, $^{**}P < 0.01$ (t test). A. C. Roberts, unpublished data.

Another way of testing endogenous opioid function is to observe the effect of drug-induced receptor blockade, although it is important to bear in mind that several populations of opioid receptors exist and pharmacological agents may have a wide spectrum of activity. Nevertheless, it is well established that administration of the opioid receptor blocker naloxone can induce surges

in LH release in both rodents and primates. Such findings have been taken to indicate a tonic suppression of gonadotropin release by the endogenous opioids. Acute administration of naloxone or the benzomorphan antagonist MR 2266 (which is said to have particular affinity for the κ receptor) to photostimulated hamsters leads to surges in LH secretion (Roberts et al 1985a,b).

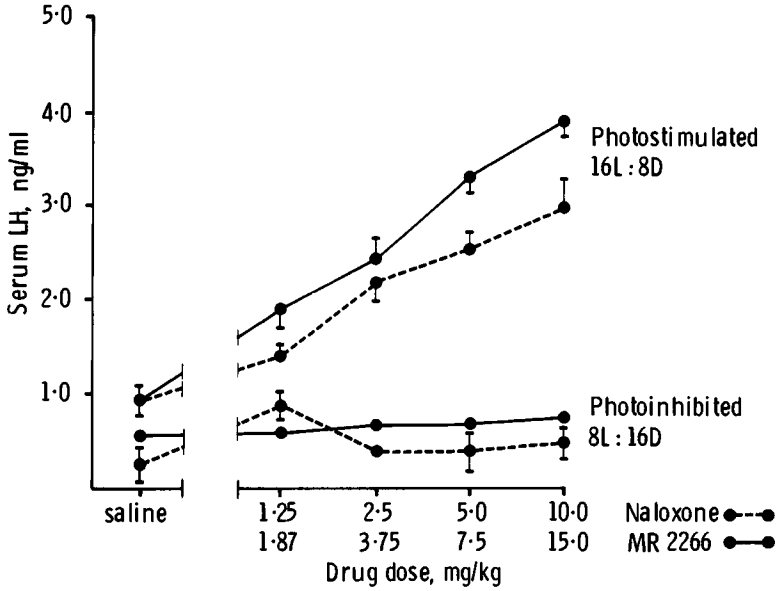

FIG. 4. Serum luteinizing hormone levels (mean ± SEM) of adult male Syrian hamsters 20 min after a subcutaneous injection of saline or a dose of the opioid receptor antagonist naloxone or MR 2266. Animals were maintained in 16L:8D or 8L:16D for eight weeks prior to sampling. After Roberts et al (1985b) and M. H. Hastings, unpublished data.

However, the same dose ranges of naloxone or MR 2266 do not result in the release of LH in the photoinhibited animal (Fig. 4). Similar results for naloxone have been reported for sheep out of breeding condition (G. A. Lincoln, personal communication). In hamsters, either removal of the pineal gland or development of photorefractoriness after exposure to an inhibitory photoschedule for 20 weeks restores sensitivity to naloxone in animals maintained in short daylengths (Fig. 5). Thus, insensitivity to opioid receptor blockade correlates with photoinhibition of the reproductive axis, not with photoperiod. Under such conditions, peripheral steroid production by the gonads is at a low level. However, maintenance of high titres of steroids in photoinhibited animals does not restore their sensitivity to naloxone (Fig. 5). The effect of photoperiod upon opioid function is therefore a pineal-

mediated (and presumably melatonin-dependent) central effect, independent of peripheral steroid levels.

Taken together, do these results suggest an enhanced suppression by endogenous opioids of LHRH release in the photoinhibited animal? It is not clear how far the tissue levels of a neuropeptide can be extrapolated to indicate

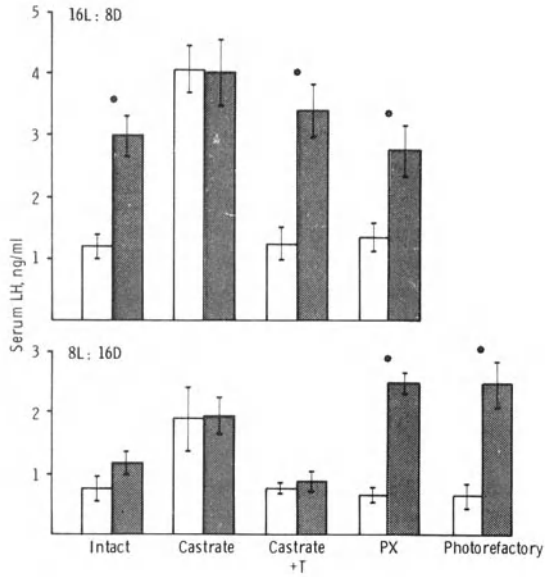

FIG. 5. Serum luteinizing hormone levels (mean ± SEM) of adult male Syrian hamsters 20 min after a subcutaneous injection of saline (open bars) or naloxone at 5 mg/kg body weight (shaded bars). Animals were maintained in 16L:8D or 8L:16D for eight weeks or 8L:16D for 20 weeks (photorefractory group) prior to sampling. Animals were intact, castrated, castrated with subcutaneous implants of testosterone (T) or pinealectomized (PX). $^*P < 0.05$ vs. saline (analysis of variance and *post hoc* Duncan's test). After Roberts et al (1985b).

its release rate; for example, alterations in the pattern of LHRH release can undoubtedly occur without obvious fluctuations in the amounts assayable in the hypothalamus. In view of what we know of their endocrine effects, elevated hypothalamic β-endorphin levels might plausibly be taken to indicate heightened activity during gonadal inhibition by appropriate photoperiods. However, opioid blockade in such circumstances might then be expected to discharge extra quantities of gonadotropins, since an inhibitory mechanism would be counteracted. The converse is found. It may be, of course, that chronic inhibition of the LHRH pulse system, such as that occurring during several weeks of exposure to inhibitory photoperiods, renders it incapable

of response to the brief stimulus represented by a single injection of naloxone, or even several injections given a few hours apart. Alternatively, and equally probably, increased β-endorphin levels may indicate reduced neuronal activity. The control of LHRH release normally exerted by β-endorphin in the photostimulated animal may be suppressed either directly or indirectly through some intervening process during photoinhibition. Consequently, blockade of endogenous opioid activity would not be expected to affect the release of LH.

TABLE 2 Tissue content of β-endorphin-like immunoreactivity in the medio-basal hypothalamus of adult male Syrian hamsters mantained under 16 h light:8 h dark (A. C. Roberts, unpublished data)

Group	*Tissue β-endorphin (pmol/mg protein, mean ± SEM)*
Intact	4.03 ± 0.45
Castrated	7.07 ± 1.36*
Castrated + testosterone	3.84 ± 0.49

*$P < 0.05$ (analysis of variance and *post hoc* Duncan's test).
Animals were intact, castrated or castrated and implanted subcutaneously with testosterone-filled capsules. The MBH was dissected and β-endorphin-like immunoreactivity measured as described by Roberts et al (1985a).

How, then, does altered opioid function contribute to the neuroendocrine basis of seasonal reproduction? The endogenous opioids are intimately involved in the steroidal regulation of gonadotropin secretion. β-Endorphin (as well as morphine) can potentiate the negative feedback effects of steroids on the pituitary. Experiments on rats suggest that morphine is more effective in suppressing gonadotropin output in castrated animals given steroids than in castrates without steroids. Altering the levels of testosterone in photostimulated hamsters has marked effects upon hypothalamic β-endorphin levels. Castration leads to an increase in tissue content within the MBH. Restoration of steroid levels after implantation of subcutaneous capsules filled with testosterone blocks this effect (Table 2). There is clearly a close interdependence of opioid activity and steroid negative feedback.

Enhanced negative feedback can be demonstrated in the photoinhibited hamster and sheep, and such steroid-dependent effects are held to play a part in gonadal control by the photoperiod (Turek & Ellis 1981, Karsch et al 1984). However, since LH output from the pituitary is less in castrated photoinhibited animals than in their photostimulated counterparts, negative feedback may not be the only control system altered by light and responsible for suppressing gonadotropin release (Urbanski & Simpson 1982). Taken together, these findings indicate that it is the steroid-dependent component

of seasonal breeding (in so far as this can be clearly separated from the steroid-independent one) which should be most sensitive to opioid control. We still lack clear information about whether these two mechanisms, steroid dependent and independent, have separate identities. Their relative contributions to the photoperiodic control of reproduction are poorly understood, and it is not known whether these change during the phases of initiation and maintenance of either a breeding or a non-breeding condition. Although an alteration in the sensitivity to steroidal feedback may be of importance in the initiation of reproductive quiescence, once this state is established endogenous steroid levels are very low and steroid feedback may be less important for the maintained suppression of gonadotropin release. Consequently, during reproductive quiescence, the β-endorphin system regulating the release of LHRH may be inactive. The importance of the opioids in seasonal reproduction may therefore lie in the initial period of gonadal regression.

Conclusions

It is apparent that the neuronal activity of the photoperiodic time-measuring system is exquisitely sensitive to environmental illumination. Photoperiodic information, processed in parallel with the circadian signal, is relayed to the pineal gland via the parvocellular portion of the paraventricular nucleus. The duration of the nocturnal bout of melatonin production acts as a marker of scotophase, which is read by neurons probably within the hypothalamus. The cellular basis of this process is unclear. Photoperiodic regulation of the gonadal axis is exercised via changes in the frequency of the LHRH pulse generator. The pineal melatonin signal has a dramatic effect upon the role of endogenous opioids in the regulation of the pulse generator.

Acknowledgements

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REFERENCES

Armstrong SM, Redman J 1985 Melatonin administration: effects on rodent circadian rhythms. In: Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Found Symp 117) p 188-207

Bittman EL 1984 Melatonin and photoperiodic time measurement: evidence from rodents and ruminants. In: Reiter RJ (ed) The pineal gland. Raven Press, New York, p 155-192

Bittman EL, Goldman BD, Zucker I 1979 Testicular responses to melatonin are altered by lesions of the suprachiasmatic nuclei in golden hamsters. Biol Reprod 21:647-656

Card JP, Moore RY 1984 The suprachiasmatic nucleus of the golden hamster: immunohistochemical analysis of cell and fiber distribution. Neuroscience 13:415-431

Carter DS, Goldman BD 1983 Antigonadal effects of timed melatonin infusions in pinealectomized male Djungarian hamsters (*Phodopus sungorus*): duration is the critical parameter. Endocrinology 113:1261-1267

Demaine C 1983 Modification of hypothalamic electrical activity by pineal indoles. In: Axelrod J et al (eds) The pineal gland and its endocrine role. Plenum, London (NATO Adv Study Inst Ser Ser A Life Sci 65) p 417-436

Gilbey MP, Coote JH, Fleetwood SF, Peterson DF 1982 The influence of the paraventriculo-spinal pathway, and oxytocin and vasopressin on sympathetic preganglionic neurones. Brain Res 251:283-290

Grossman A, Rees LH 1983 The neuroendocrinology of opioid peptides. Br Med Bull 39:83-88

Hastings MH, Herbert J, Martensz ND, Roberts AC 1985a Annual reproductive rhythms in mammals: mechanisms of light synchronization. In: Medical and biological effects of light. Ann NY Acad Sci, in press

Hastings MH, Roberts AC, Herbert J 1985b Neurotoxic lesions of the anterior hypothalamus disrupt the photoperiodic but not the circadian system of the Syrian hamster. Neuroendocrinology 40:316-324

Herbert J 1981 The pineal gland and the photoperiodic control of the ferret's reproductive cycle. In: Follett BK, Follett DE (eds) Biological clocks in seasonal reproductive cycles. John Wright, Bristol, p 261-276

Hoffmann K 1981 The role of the pineal gland in the photoperiodic control of seasonal cycles in hamsters. In: Follett BK, Follett DE (eds) Biological clocks in seasonal reproductive cycles. John Wright, Bristol, p 237-250

Illnerova H, Vanecek J 1982 Two-oscillator structure of the pacemaker controlling the circadian rhythm of *N*-acetyltransferase in the rat pineal gland. J Comp Physiol 145:539-548

Karsch FJ, Bittman EL, Foster DL, Goodman RL, Legan SJ, Robinson JE 1984 Neuroendocrine basis of seasonal reproduction. Recent Prog Horm Res 40:185-232

Kennaway DJ, Sandford LM, Godfrey B, Friesen HG 1983 Patterns of progesterone, melatonin and prolactin secretion in ewes maintained in four different photoperiods. J Endocrinol 97:229-242

Kinoshita F, Nakai Y, Katrakami H, Kato Y, Yajima H, Imura H 1980 Effect of β-endorphin on pulsatile luteinising hormone release in conscious castrated rats. Life Sci 27:843-846

Lehman MN, Bittman EL, Newman SW 1984 Role of the hypothalamic paraventricular nucleus in neuroendocrine responses to daylength in the golden hamster. Brain Res 308:25-32

Lincoln GA, Ebling FJP 1985 Effect of constant-release implants of melatonin on seasonal cycles in reproduction, prolactin secretion and moulting in rams. J Reprod Fertil 73:241-253

Lincoln GA, Short RV 1980 Seasonal breeding: Nature's contraceptive. Recent Prog Horm Res 36:1-52

Meyerson BJ, Terenius L 1977 β-Endorphin and male sexual behaviour. Eur J Pharmacol 42:191-192

Pickard GE, Turek FW 1983 The hypothalamic paraventricular nucleus mediates the photoperiodic control of reproduction but not the effects of light on the circadian rhythms of activity. Neurosci Lett 43:67-72

Pittendrigh CS, Daan S 1976 A functional analysis of circadian pacemakers in nocturnal rodents. J Comp Physiol 106:333-335

Ralph MR, Menaker M 1985 Bicuculline blocks circadian phase delays but not advances. Brain Res 325:362-365

Roberts AC, Martensz ND, Hastings MH, Herbert J 1985a Changes in photoperiod alter the daily rhythms of pineal melatonin content and hypothalamic β-endorphin content and the luteinizing hormone response to naloxone in the male Syrian hamster. Endocrinology, 117:141-147

Roberts AC, Hastings MH, Martensz ND, Herbert J 1985b Naloxone-induced secretion of LH in the male Syrian hamster: modulation by photoperiod and gonadal steroids. J Endocrinol 106:243-248

Reiter RJ, Vaughan MK, Blask DE, Johnson LY 1974 Melatonin: its inhibition of pineal antigonadotrophic activity in male hamsters. Science (Wash DC) 185:1169-1171

Rusak B, Morin LP 1976 Testicular responses to photoperiod are blocked by lesions of the suprachiasmatic nuclei in golden hamsters. Biol Reprod 15:366-374

Silver R, Bittman EL 1984 Reproductive mechanisms: interaction of circadian and interval timing. In: Timing and time perception. Ann NY Acad Sci 423:488-514

Sofroniew MV, Weindl A, Schrell U, Wetzstein R 1981 Immunohistochemistry of vasopressin, oxytocin and neurophysin in the hypothalamus and extrahypothalamic regions of the human and primate brain. Acta Histochem (suppl) 24:79-85

Turek FW, Ellis GB 1981 Steroid-dependent and steroid-independent aspects of the photoperiodic control of seasonal reproductive cycles in male hamsters. In: Follett BK, Follett DE (eds) Biological clocks in seasonal reproductive cycles. John Wright, Bristol, p 251-260

Urbanski HF, Simpson SM 1982 Photoperiodic suppression of gonadotrophin secretion in castrated male hamsters. J Reprod Fertil 66:299-303

DISCUSSION

Short: The concept of interval timing is very important, but how do you interpret the inhibitory or stimulatory responses that one sees in animals with melatonin implants, which are clearly not interval timers in that they are releasing the melatonin continuously?

Herbert: Because the melatonin release is continuous, in most cases you get an effect equivalent to pinealectomy.

Zucker: But in some species an implant works as effectively as do timed injections (Johnston & Zucker 1980).

Herbert: That's the problem. Most species behave as I've said but there are one or two exceptions.

Reiter: I would say that in most species the response to continuously available melatonin is *not* equivalent to the response to pinealectomy. Certainly in the Syrian hamster continuous melatonin availability acts like pinealectomy in terms of preventing gonadal regression. But in many other species the response is clearly different, and it depends on whether the animals are long-day or short-day breeders.

Herbert: One problem is that if you are doing these experiments in intact animals you also have endogenous production of melatonin. This adds to what you are giving and you may produce some kind of interval timer if you are not careful. So I would worry about drawing conclusions unless the animals are

pinealectomized. Even if they are pinealectomized the situation is complicated because many circadian metabolic systems, for example those concerned with the clearance of melatonin, will show dramatic changes. Alcohol, it is said, is cleared much more rapidly at certain times of day. So you should monitor very carefully what you *think* is a continuous melatonin infusion. I would only accept some of these findings with a degree of reservation.

Zucker: Has anybody satisfied these criteria and found that a pinealectomized animal given a continuous release capsule of melatonin undergoes gonadal regression?

Turek: If you transfer pinealectomized hamsters with melatonin implants from short days to long days, testicular growth is inhibited (Turek 1977). Serum melatonin levels should be constant unless there is a diurnal change in metabolism.

Lincoln: We have given pineal-intact rams continuous melatonin therapy using a silastic sheet implant, and have measured the blood plasma levels of melatonin at hourly intervals throughout the day. The levels stay relatively constant at 200–400 pg/ml with a small increase at night presumably due to endogenous melatonin secretion. If the implants are introduced into the animals on long days, the reproductive axis, moult cycle, pattern of prolactin secretion and body weight all respond as normally seen following short days. However, in the long term the animals behave more like pinealectomized rams, failing to respond to changes in photoperiod. It is as if the constantly available melatonin initially signals a short day, but subsequently blocks the interpretation of the endogenous melatonin signal (Lincoln & Ebling 1985).

We have also given melatonin implants to ganglionectomized rams, which have little or no endogenous melatonin secretion. The animals were held on long days, and the implants were left in place for 16 weeks, removed for 16 weeks and then replaced for 16 weeks. The cycle was repeated three times. In this way it was possible to reintroduce into these animals cyclical changes in the reproductive and moult characteristics that had been lost in the long term as a result of ganglionectomy. The removal of the melatonin implant (no melatonin period) seemed to allow the animals to re-establish their short-day responses to the continuous melatonin signal.

*Hoffmann:*Pinealectomy and melatonin implants often have similar effects, but these effects may differ depending on whether the animals are in long or in short photoperiods. The species that behaves differently is *Peromyscus*, in which melatonin implants induce regression while pinealectomy prevents it (Lynch & Epstein 1976, Johnston & Zucker 1980, Glass & Lynch 1981). This species might not use an hourglass mechanism.

Zucker: Melatonin implants also produce significant gonadal regression in *Microtus pennsylvanicus* (Dark et al 1983).

*Goldman:*It is important to keep in mind that the concentrations of melato-

nin produced in the blood by the implants used in rodents are 20-fold to 100-fold higher than the peak levels produced by the pineal in normal animals. It is likely to be a waste of time to try to draw sophisticated conclusions by comparing the effects of implants, injections, pinealectomy and so on. There are clearly differences in the way various species respond to a pharmacological stimulus, but I am not sure that we can learn much from these differences— at least not until we have a better understanding of normal pineal function.

Herbert: One thing we have forgotten about is the passage of melatonin into the brain. We all assume that there will be a direct correlation between the concentration of melatonin that we put into the blood and the concentration of melatonin in the brain because melatonin is lipid soluble. Let me warn you against that; it's not true for several steroids that are very lipid soluble.

Goldman: I agree that we should not make any assumptions about the ease with which melatonin crosses from the blood into the brain. But unless it can be established that melatonin does not act via the peripheral circulation, we must assume that treatments that produce very high plasma melatonin concentrations are at least potentially pharmacological.

Rollag: I've injected melatonin into the bloodstream and measured its appearance in the cerebrospinal fluid (Rollag et al 1978). The two compartments equilibrate within about half an hour.

Reppert: We've done similar studies (Reppert et al 1979), measuring melatonin in the blood and cerebrospinal fluid of rhesus monkeys, and have also found that the melatonin quickly equilibrates in the two compartments.

Reiter: According to your studies, when you put hamsters in long nights you get an apparent prolongation of the melatonin peak, but you also get a higher peak (Fig. 1, p 59). Why do you select the prolongation rather than the elevated peak as an explanation for the effects on the reproductive axis? We have taken Syrian hamsters, kept outdoors all year in an environment presumably reminiscent of their natural habitat, and have looked at melatonin levels at the equinoxes and solstices, comparing melatonin patterns in animals with atrophic gonads and in those with normally functioning gonads (Brainard et al 1982). We don't see a nocturnal 'shoulder' like you do, but we do see a higher melatonin peak in the winter than in the summer. That could theoretically account for the gonadal changes, so why did you select the prolongation of the melatonin peak as the important factor?

Herbert: We don't know enough about the critical variables for melatonin (or indeed for oestrogen) to be able to evaluate the contributions of duration or peak to its effects. We don't even know whether the peak is measured in absolute terms or only relative to day-time levels. The same problem is being faced by those studying the LH response and oestrogen. I'm a bit wary of drawing conclusions from our results because there are problems in estimating secretion from the pineal on the basis of levels in the gland, and the sampling

intervals were fairly long. But I am certainly not suggesting that duration is the only thing that matters.

Bittman: I have had the opportunity to measure melatonin in some ewes in which Gary Jackson at the University of Illinois has performed frontal hypothalamic deafferentation. We have used hourly samples from animals with complete or incomplete cuts and correlated the melatonin patterns with the consequences of these cuts on reproduction (unpublished work). Ewes with complete frontal hypothalamic deafferentation show reproductive activity at inappropriate times of year; radioimmunoassay of progesterone indicates that such animals can ovulate in June and that the negative feedback potency of oestradiol is no longer driven by photoperiod (Pau et al 1982). When we tried to correlate this with melatonin patterns, we found to our surprise that it is difficult to eliminate the night-time rise in melatonin in sheep by complete frontal hypothalamic deafferentation. The only animals in which the melatonin rise was eliminated were those with a knife cut that just grazed the caudal border of the suprachiasmatic nuclei, and this is consistent with some of Reiter and Sorrentino's hamster results (Reiter & Sorrentino 1972). What is particularly fascinating is that, although most of the ewes were generating fairly normal melatonin patterns, they seemed unable to respond to photoperiod. There are two possible explanations. Frontal hypothalamic deafferentation may be disrupting circannual rhythmicity or, and perhaps more likely, these cuts may, without interfering with the melatonin rhythm-generating system, be severing the link between a melatonin receptor system and the medio-basal hypothalamus. This second interpretation is consistent with earlier work in the hamster (Reiter et al 1981).

Moore-Ede: I would like to pick up the question of whether the effects of light in T-cycle experiments could be mediated purely by changes in the melatonin rhythm. Fred Turek has shown that 1 s of light can prevent gonadal regression in male hamsters if given at the appropriate phase. Could that 1 s of light shut off and thereby sufficiently manipulate the melatonin rhythm?

Hoffmann: One minute can (Illnerová et al 1979, Hoffmann et al 1980, 1981).

Reiter: One second does not.

Turek: But remember that these animals were exposed to 1 s of light daily over a 10-week period, and it is not necessary to photostimulate animals every day to maintain testicular activity (Earnest & Turek 1983, 1984).

Menaker: We have measured the amount of light necessary for phase-shifting the locomotor rhythm in golden hamsters (Takahashi et al 1984) and the amount of light necessary for suppressing melatonin synthesis (D.J. Hudson & M. Menaker, unpublished work). The curves that describe these two relationships are quite different; the amounts of light required differ by 2.5 orders of magnitude. Melatonin synthesis is much more sensitive to light than is

phase-shifting. If you calculate how much light is necessary to suppress melatonin production by 50% and apply this at a time in the circadian cycle when it is too low in intensity to phase-shift the circadian rhythm, you can affect the reproductive system and inhibit regression of the gonads without touching the circadian system at all. In other words, the circadian system free-runs despite periodic light pulses that suppress melatonin production and affect the reproductive system. You can separate these two things completely.

Moore-Ede: So the answer from the short light-pulse experiments is that it is plausible to have a mechanism that does not involve the circadian system.

Goldman: We have considered whether the results of T-cycle experiments in rodents can be explained on the basis of the effects of T-cycles on the melatonin rhythm. Janet Darrow has used stimulatory and non-stimulatory T-cycles in Djungarian hamsters and in neither case is there evidence for an acute suppressive effect of light on melatonin secretion (Darrow & Goldman 1985). In both cases melatonin concentrations began to rise only 2 h or 3 h after the light pulse, but with the stimulatory T-cycle the melatonin pulse only lasted for about 5 h or 6 h whereas with the non-stimulatory cycle it lasted 9 h or 10 h. The duration of the melatonin signal correlated with whether or not the gonads were stimulated, even though there was no indication of an acute effect of light. These observations would appear to underscore the importance of the circadian system in the regulation of pineal activity.

Rollag: I did similar experiments in Syrian hamsters, and showed that in T-cycles that stimulated the gonads melatonin production was correlated with activity onset (M.D. Rollag & M.H. Stetson, unpublished work).

Hoffmann: There are other reports that, in the golden hamster, changes in the T-cycle involve changes in the duration of melatonin secretion (J.A. Elliott, unpublished paper, Timberline Symp on Biological Clocks, July 1984). To my knowledge there is no clearcut evidence in mammals that light can have photoperiodic effects on reproduction without influencing the melatonin pattern.

Sizonenko: Some of the recent work of Dr Michel Aubert in our laboratory confirms Dr Herbert's conclusions about β-endorphin. In our juvenile male rat model, naloxone does not block the inhibitory action of injected melatonin, but the [Met^5]enkephalin analogue FK-33-824 has exactly the same effect as melatonin (unpublished work). So melatonin may potentiate or mimic the tonic inhibition of LH secretion by endogenous opioids during sexual maturation.

Herbert: It is important to make a distinction between β-endorphin and the enkephalins. The problem is that it is very difficult to pick out the β-endorphin receptors without the others. If you infuse β-endorphin into the brain you will activate all sorts of opioid receptors.

Turek: I'm not sure why you think naloxone is not working through the steroid feedback system. To look at that thoroughly you have to use many

different doses, not only of steroids but also of naloxone. For example, you may not see an effect of naloxone in an intact animal on short days because the system is so sensitive to the negative feedback effects of steroids that a small amount of naloxone is not enough to overcome the inhibition.

Herbert: But we went up to three or four times the maximum dose of naloxone and there was still no release of LH. We thought that perhaps SD made the dose–response curve shift to the right, but it's not true. We went up to an enormous dose of naloxone and nothing happened. We also tried changing steroid concentrations in photoinhibited animals. We thought that if we reproduced the normal concentrations of a reproductively active male hamster, and steroids were affecting β-endorphin levels, we ought to be able to reconstitute the effect of naloxone in photostimulated animals. But we couldn't.

Lincoln: Do high β-endorphin levels in the hypothalamus mean high release of β-endorphin in the brain and into the portal system?

Herbert: That's a critical question. There may not be physiologically significant release into the portal system because the evidence is against β-endorphin acting directly on the anterior pituitary. It may act somewhere in the median eminence. If high hypothalamic levels are reflecting high activity, this would fit with the known actions of β-endorphin, but the problem is that the effects of β-endorphin on prolactin are not consonant with its effects on LH in the context of seasonal breeding. The second problem is that it is difficult to reconcile the effects of opioid blockade with the effects of high levels of β-endorphin in the brain unless you make a number of assumptions. This brings us to the much larger question of what measuring static levels of anything in the brain tells you about activity. The answer is, very little. For example, you can get marked changes in LHRH release in hamsters without any alteration in LHRH levels in the brain. The important change is probably one in pulsatile release patterns, so there is no real reason why that should be reflected in brain levels of LHRH.

Lincoln: We have tried chronic treatment with naloxone (50 mg i.v. every 4 h for seven days) in photoperiodically inhibited sexually regressed rams, with the idea that opioid suppression might be so profound in the regressed state that it would be necessary to give naloxone in the long term to override the inhibition. In our animals photoperiodic inhibition of LH secretion is associated with a delayed rise in LH after castration, not starting until after 24 h; thus, if an opioid mechanism is involved in relaying the sex steroid feedback inhibition we would not expect to see a rapid response to naloxone in the sexually regressed state. However, we got absolutely nowhere with the chronic naloxone treatment; there was a small increase in LH pulsatility in response to the first injection, but no response to the second or subsequent injections all the way through to day 7. There was still a low level of pulsatile LH secretion typical of the quiescent state, but this was not affected by the naloxone; perhaps animals become tolerant to naloxone as they become tolerant to opiates like morphine.

REFERENCES

Brainard GC, Petterborg LJ, Richardson BA, Reiter RJ 1982 Pineal melatonin in Syrian hamsters: circadian and seasonal rhythms in animals maintained under laboratory and natural conditions. Neuroendocrinology 35:342-348

Dark J, Zucker I, Wade GN 1983 Photoperiodic regulation of body mass, food intake and reproduction in meadow voles. Am J Physiol 245:R334-R338

Darrow JM, Goldman BD 1985 Effect of light on reproduction is correlated with rhythms of pineal melatonin content and secretion in the Djungarian hamster. J Biol Rhythms, in press

Earnest DJ, Turek FW 1983 Effects of one-second light pulses on testicular function and locomotor activity in the golden hamster. Biol Reprod 28:557-565

Earnest DJ, Turek FW 1984 Periodic exposure to a brief light signal stimulates neuroendocrine-gonadal activity in golden hamsters. J Androl 5:64-69

Glass JD, Lynch GR 1981 The effect of superficial pinealectomy on reproduction and brown fat in the adult white-footed mouse, *Peromyscus leucopus*. J Comp Physiol 144:145-152

Hoffmann K, Illnerová H, Vaněček J 1980 Pineal N-acetyltransferase activity in the Djungarian hamster: effect of one minute light at night. Naturwissenschaften 67:408

Hoffmann K, Illnerová H, Vaněček J 1981 Effect of photoperiod and one minute light at night-time on the pineal rhythm of N-acetyltransferase activity in the Djungarian hamster *Phodopus sungorus*. Biol Reprod 24:551-556

Illnerová H, Vaněček J, Křeček J, Wetterberg L, Sääf J 1979 Effect of one minute exposure to light at night on rat pineal serotonin N-acetyltransferase and melatonin. J Neurochem 32:673-675

Johnston PG, Zucker I 1980 Antigonadal effects of melatonin in white-footed mice (*Peromyscus leucopus*). Biol Reprod 23:1069-1074

Lincoln GA, Ebling FJP 1985 Effect of constant-release implants of melatonin on seasonal cycles in reproduction, prolactin secretion and moulting in rams. J Reprod Fertil 73:241-253

Lynch GR, Epstein AL 1976 Melatonin induced changes in gonads, pelage and thermogenic characters in the white-footed mouse, *Peromyscus leucopus*. Comp Biochem Physiol C Comp Pharmacol 53:67-68

Pau KYF, Kuehl DE, Jackson GL 1982 Effect of frontal hypothalamic deafferentation on luteinizing hormone secretion and seasonal breeding in the ewe. Biol Reprod 27:999-1009

Reiter RJ, Sorrentino S 1972 Prevention of pineal-mediated reproductive responses in light deprived hamsters by partial or total isolation of the medial basal hypothalamus. J Neuro-Visc Relat 32:355-367

Reiter RJ, Dinh DT, de los Santos R, Guerra JC 1981 Hypothalamic knife cuts suggest a brain site for the antigonadotrophic action of melatonin in the Syrian hamster. Neurosci Lett 23:315-318

Reppert SM, Perlow MJ, Tamarkin L, Klein DC 1979 A diurnal melatonin rhythm in primate cerebrospinal fluid. Endocrinology 104:295-301

Rollag MD, Morgan RJ, Niswender GD 1978 Route of melatonin secretion in sheep. Endocrinology 102:1-8

Takahashi JS, DeCoursey PJ, Bauman L, Menaker M 1984 Spectral sensitivity of a novel photoreceptive system mediating entrainment of mammalian circadian rhythms. Nature (Lond) 308:186-188

Turek FW 1977 Antigonadal effect of melatonin in pinealectomized and intact male hamsters. Proc Soc Exp Biol Med 155:31-34

Photoperiodism, melatonin and the pineal
Editors: David Evered and Sarah Clark

Eyes—the second (and third) pineal glands?

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Abstract. The pineal gland, the retinas and perhaps other tissues as well may in some species produce melatonin that appears in significant quantities in the circulation. In at least one species, Japanese quail, the circadian rhythm in the levels of circulating melatonin reflects contributions from both the pineal and the retinas; in other species circulating melatonin may come exclusively from the pineal or perhaps only from the eyes. Comparative behavioural and physiological data from several bird and lizard species indicate that retinas and pineal glands fulfil similar endocrine roles. Current evidence suggests that in iguanid lizards either retinas or pineal glands, but not both in the same species, have important regulatory influences on circadian organization. This suggests that it should be relatively easy to influence the melatonin-forming ability of a tissue by natural selection, an interpretation bolstered by our finding that the ability to synthesize melatonin has been inadvertently eliminated in the pineal glands of laboratory mice, presumably by the selection involved in producing inbred strains. The genetics of melatonin synthesis in mice is briefly discussed.

1985 Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Foundation Symposium 117) p 78-92

The title of this symposium—Photoperiodism, Melatonin and the Pineal—suggests that melatonin produced in the pineal gland is unique in some important way. I should like to argue that this view is incorrect, that in fact if one looks broadly among the vertebrates one finds that other melatonin-forming tissues, particularly the retina, are indistinguishable from the pineal in the biochemistry of melatonin synthesis, in the temporal pattern of melatonin synthesis, in the effects of light on that pattern and in the physiological function that they perform. Indeed, viewed from this perspective, the retina and the pineal gland appear to be so similar that the eyes could well be called the second and third pineal glands. In an attempt to maintain the distinction between retina and pineal it might be argued that while the pineal glands of all vertebrates synthesize melatonin, the retinas of only some species do so. Even this distinction fails however; in spite of several unpublished attempts

to measure it, no one has yet reported detecting melatonin in the pineal glands of Urodeles and, as I shall discuss, the ability of the mouse pineal to synthesize melatonin can be rapidly abolished by selection.

Although the pineal glands and retinas of vertebrates are indistinguishable as a whole, each of these organs is nonetheless highly variable among species even of the same family. In the pineal this variability has been primarily assessed in terms of function. Pinealectomy of some birds and lizards abolishes circadian locomotor rhythmicity, while in other species of both classes pinealectomy has little or no effect (Menaker 1982, Underwood 1984). Pinealectomy of the golden hamster (*Mesocricetus auratus*) stimulates gonad growth, whereas pinealectomy of the closely related Turkish hamster (*M. brandti*) inhibits gonadal function (Carter et al 1982). Perhaps because we know so little about the endocrine function of the retina, variability in this tissue is usually described in terms of its ability to synthesize melatonin. The retinas of some fish (Gern & Ralph 1979), reptiles (see below) and birds (Hamm & Menaker 1980, Voisin et al 1982, Pang et al 1983, Underwood et al 1984) have been shown to contain melatonin. Many of these measurements have been made in such a way that it is reasonable to conclude that melatonin found in the retina has been synthesized there. In at least one case there is direct evidence that retinal melatonin enters the blood and accounts for a significant fraction of circulating melatonin (Underwood et al 1984).

Small amounts of melatonin have been detected by radioimmunoassay in the mammalian retina by Yu et al (1981); however, other workers have failed to find measurable amounts. Both hydroxyindole *O*-methyltransferase (HIOMT; Cardinali & Rosner 1971) and serotonin *N*-acetyltransferase (NAT; Miller et al 1980) have been detected in the mammalian retina. Dubocovich (1983) discovered that picomolar concentrations of exogenous melatonin selectively inhibited calcium-dependent release of [^{3}H]dopamine from superfused rabbit retina but not from striatum. Her work suggests that in mammals melatonin acts as a neuromodulator in the retina. If that were its only role one might expect it to be present in small quantities and be difficult to detect; this could perhaps account for the discrepancies noted above.

It seems reasonable to conclude that in vertebrates in general retinal melatonin plays both a *local* role as a neuromodulator and/or regulator of photoreceptor metabolism (Besharse & Dunis 1983) and a classical *hormonal* role requiring that it be produced in relatively large amounts and transported to distant target organs by the circulation. The local role might well be very common or even universal, while the hormonal role is probably less so. The possibility that *pineal* melatonin plays a local role, modulating events within the pineal itself, has not been investigated; pineal melatonin is known to play a hormonal role in some vertebrates. Of course pineal melatonin could act hormonally on the retina as a target and perform the same functions

(e.g. modulation of dopamine release) that under other circumstances are performed by retinally synthesized melatonin acting locally. Whatever the details of its synthesis, its circulatory levels or its particular physiological actions, and regardless of its source, it is likely that melatonin always acts as an internal chemical symbol of the external light cycle; wherever it is found its synthesis oscillates with a daily or circadian rhythm, and with a single exception (Gern et al 1978) melatonin levels are higher at night than during the day.

Let us consider melatonin only in its role as a hormone and examine the evidence for the view that the retina and the pineal are interchangeable sources of hormonally active circulating melatonin. Pinealectomy of house sparrows (*Passer domesticus*) abolishes free-running circadian locomotor rhythmicity. Several lines of evidence demonstrate that the pineal organ of this bird is photosensitive and contains circadian oscillators that regulate its synthesis of melatonin (Menaker 1982). Strong circumstantial evidence supports the view that the pineal of the house sparrow, and probably of other passerine birds as well, regulates behavioural rhythmicity by rhythmic secretion into the circulation of melatonin which acts on hypothalamic target sites (Takahashi & Menaker 1979, Zimmerman & Menaker 1979).

Pinealectomy of Japanese quail and of pigeons (species which are not closely related either to each other or to the passerine group) fails to abolish behavioural rhythmicity. However, this rhythmicity can be abolished by removal of the eyes in addition to the pineal (Ebihara et al 1984, Underwood et al 1984). In quail, Underwood et al (1984) have demonstrated that circulating melatonin shows rhythmic changes in concentration and that 54% of the nighttime peak comes from the pineal, 33% from the eyes and 13% from unidentified sources. While quail that have lost either their eyes or their pineal glands retain robust rhythms in the levels of circulating melatonin, such rhythmicity is barely discernable when both organs have been removed. Thus the limited data available for birds suggest that the maintenance of behavioural circadian rhythmicity depends on rhythmic changes in the concentration of circulating melatonin, different proportions of which come from the eyes and the pineal gland in different phylogenetic groups. Why in a particular species one organ rather than the other has been favoured as a source of melatonin by natural selection remains obscure.

Additional evidence of the equivalence of eyes and pineal glands comes from work with lizards. Underwood (1984) has shown that pinealectomy has profound effects on the circadian locomotor rhythms of three species of iguanid lizards: in *Anolis carolinensis* pinealectomy abolishes free-running circadian rhythmicity; in *Sceloporus olivaceus* and in *S. occidentalis* pinealectomy has a variety of effects on the rhythm including causing period change, splitting of the rhythm into multiple components, modification of the phase–response curve for light pulses and arrhythmicity.

We have cultured the pineal organ of *Anolis carolinensis* (Menaker & Wisner 1983) and of *S. occidentalis* in a superfusion system that allows us to collect timed samples of superfusate and assay them for melatonin. The cultured pineal glands of both species produce melatonin with a circadian rhythm in constant darkness (Fig. 1A & B). Underwood's data taken together

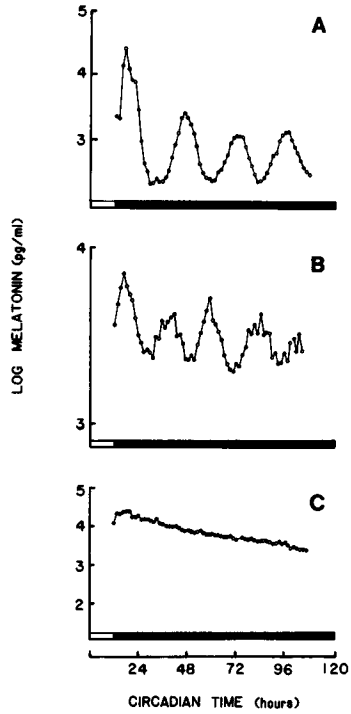

FIG. 1. Temporal patterns of melatonin secretion by single, representative cultured pineal glands of three species of iguanid lizards. The glands were removed from the lizards between hours 11 and 12 and placed in superfused culture in constant darkness (A, *Anolis carolinensis*; B, *Sceloporus occidentalis*; C, *Dipsosaurus dorsalis*). Note that, while glands from both *Anolis* and *Sceloporus* oscillate, the amplitude of the rhythm is greater in *Anolis* and its period is shorter in *Sceloporus*. *Dipsosaurus* glands produce large quantities of melatonin but fail to oscillate in constant darkness (M. Menaker, S. Wisner & D. S. Janik, unpublished work).

with our own suggest that iguanid lizards resemble passerine birds in the role that pineal melatonin plays in their circadian organization, i.e. the pineal rhythmically produces melatonin which enters the circulation and acts on unknown target sites to maintain behavioural circadian rhythmicity. Recently we have begun experiments with a fourth species of iguanid lizard, *Dipsosaurus dorsalis* (D. S. Janik & M. Menaker, unpublished work). To our initial surprise, pinealectomy of these lizards had little or no effect on their free-running circadian locomotor rhythms. Furthermore, when cultured under the

same conditions as the pineal glands of *Anolis* and *Sceloporus*, *Dipsosaurus* pineal glands completely fail to oscillate in constant darkness although they produce large quantities of melatonin (Fig. 1C).

Underwood has measured melatonin in the retinas of *Anolis* at frequent intervals throughout the day and night. He finds very low or non-detectable levels at all times (Underwood, 1985). We have confirmed his report at two time points (mid-light and mid-dark) and in addition have measured melatonin at these time points in the retinas of *S. occidentalis* and *D. dorsalis*. Only the retinas of *Dipsosaurus* contain large amounts of melatonin, as expected, at night (Table 1). Although our results are preliminary and there are still

TABLE 1 Melatonin content of lizard retinas

Species	*Midday*	*Midnight*
Anolis carolinensis	8.16 ± 2.31 (7)	11.20 ± 0.84 (7)
Sceloporus occidentalis	30.43 ± 6.67 (6)	33.66 ± 3.91 (5)
Dipsosaurus dorsalis	16.29 ± 0.05 (2)	215.65 ± 110.65 (2)

Values given are in pg/retina ± SE. Numbers in parentheses are numbers of retinas.

several important pieces of the picture missing, it is hard to escape the conclusion that *Dipsosaurus* is an iguanid lizard that happens to get an important fraction of the rhythmically changing portion of its circulating melatonin from its eyes (cf. Besharse & Iuvone 1983). If this is true, then bilateral enucleation should significantly reduce the amplitude of or perhaps abolish the rhythm in the levels of circulating melatonin and furthermore, either alone or in combination with pinealectomy, should have profound effects on behavioural rhythmicity. In short, while circadian organization in *Anolis* and *Sceloporus* closely parallels that in passerine birds, circadian organization in *Dipsosaurus* may well be analogous to that in pigeons and quail. If so, the entire range of variability discovered so far in birds exists within one group of lizards. Such variability is phylogenetically incoherent and must be the result of selective factors that we do not as yet appreciate, acting in response to pressures in specific environmental niches. Although it is almost certain to have important ecological meaning, such variability obscures the physiological core of the vertebrate circadian system; at least in birds and lizards this core probably involves rhythmic changes in the concentration of circulating melatonin, whatever its source may be.

If the retinas of some species of iguanid lizards synthesize large amounts of melatonin rhythmically while the retinas of others synthesize it only at very low levels or not at all, then perhaps the transition between the two states is easily made. If so we might expect to find retinas, pineal glands and perhaps other melatonin-forming organs switching in and out of melatonin production at the whim of selective forces we have yet to understand. Such

lability might go a long way toward explaining the inter-species variability in the effects of pinealectomy on behavioural and reproductive variables that has plagued the study of this organ since its inception. We have recently discovered a model system that may help in understanding how selection, in this case artificial selection, is able to modify rapidly the melatonin-synthesizing capacity of pineal glands and retinas (S. Ebihara et al, unpublished work).

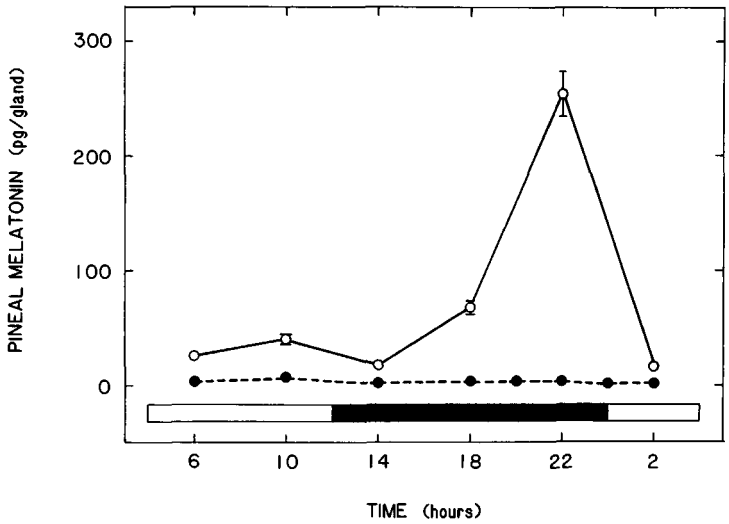

FIG. 2. Pineal melatonin content of C57BL mice (filled circles) and of the field-derived strain (open circles) as a function of time relative to the light cycle on which the animals were held (diagrammed at the bottom of the figure). Unless shown, the standard error bars lie within the points (S. Ebihara, T. N. Marks, D. J. Hudson & M. Menaker, unpublished work).

The pineal gland of the commonly used inbred laboratory mouse C57BL/6J does not synthesize melatonin at any time of the day or night. This is a remarkable fact especially since melatonin is synthesized in the pineal glands of every other mammalian species so far examined. On the other hand, wild mice, that is mice of the same species (*Mus domesticus*) collected in the field and bred under laboratory conditions*, hereafter referred to as the field-derived strain (FDS), synthesize melatonin in their pineal glands with normal rhythmicity. Pineal melatonin content in the two strains over the course of 24 h is compared in Fig. 2.

* About 50 of these mice were collected in granaries in Edmonton, Alberta, Canada, by Dr Frank Bronson in 1979. Since then they have been maintained under laboratory conditions at the University of Texas, Austin, with no deliberate selection; they are now in their fifth generation.

Control of pineal melatonin synthesis in mice is in part genetic as we have demonstrated by crossing C57 mice to the FDS. The pineal glands of the F_1 progeny synthesize about one-quarter of the amount of melatonin at hour 22 (the time of peak synthesis in the FDS) that the pineal glands of their FDS parents do. Reciprocal crosses give the same result. When the F_1 progeny are crossed to the FDS, the pineal glands of all the offspring produce melatonin (at hour 22) at levels somewhat below those of the FDS. When the F_1 progeny are back-crossed to C57 about one-quarter of the offspring produce melatonin (at hour 22) at roughly the same levels as the F_1 animals, while the other three-quarters produce no melatonin. These results suggest that the ability to synthesize melatonin depends on two independently assorting Mendelian genes, both of which are homozygous recessive in C57 mice and homozygous dominant in the FDS. This suggestion is supported by the results of an F_1 cross: of the 54 F_2 mice produced by this cross, 33 had melatonin in their pineal glands (at hour 22) whereas 21 did not have melatonin. These numbers give a very good fit ($\chi^2 = 0.51$) to the 9:7 phenotypic ratio predicted for a dihybrid cross on the basis of the above assumptions.

Both of the enzymes involved in the synthesis of melatonin from serotonin [NAT(EC 2.3.1.5) and HIOMT (EC 2.1.1.4)] are found in the pineal glands of FDS mice. NAT is rhythmic, with the highest activity at hour 22, whereas HIOMT activity remains constant throughout the day and night. The activity of both enzymes is very low or undetectable in the pineal glands of C57 mice; in the F_1 animals the activity of both enzymes is about half its level in FDS mice (Fig. 3).

The genetic and the biochemical data are consistent with, although they do not prove, the hypothesis that each of two genes involved in the ability to synthesize melatonin regulates one of the two enzymes critical to this process. The hypothesis is supported by our finding of a laboratory strain, NZB, which shows NAT activity in its pineal but no HIOMT activity or melatonin synthesis. Our data are incomplete and can be accounted for by other models; we do not wish at this point in our work to subscribe too earnestly to the interpretation outlined above. Our results however do make it quite clear that the selection involved in producing the inbred C57 strain has resulted in rapid genetic changes that have completely eliminated the melatonin-synthesizing capacity of the pineal glands of these animals. Furthermore, these events may have occurred often and independently in the production of inbred mouse strains since we have failed to find melatonin in the pineal glands of several other strains of laboratory mice (BALB/c, AKR, CAST) and, in the two strains for which we have some enzyme data (C57 and NZB), blockage of melatonin synthesis has been accomplished differently. It is hard to escape the surmise that melatonin reduces the fecundity of mice under laboratory conditions to a degree that may become apparent to the artificial

selection process only when it is added to the other reductions in fecundity produced by vigorous inbreeding. Under these circumstances melatonin synthesis may have been inadvertently eliminated by the hand of the breeder looking for mice that 'do well' in the laboratory.

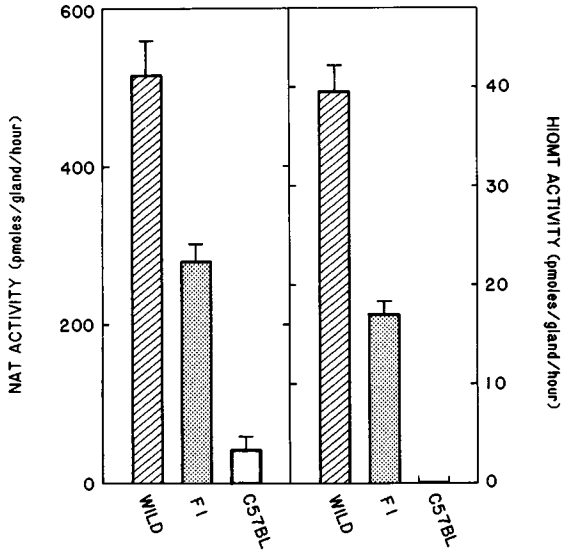

FIG. 3. NAT and HIOMT activity in the pineal glands of three genetically different groups of mice at hour 22. The F_1 mice are the offspring of matings between C57BL and field-derived ('wild') individuals (S. Ebihara, T. N. Marks, D. J. Hudson & M. Menaker, unpublished work).

There are of course other mechanisms by which selection could alter effective melatonin production. One of the simplest of such mechanisms would be to degrade the hormone before it enters the circulation. That may be occurring in the retinas of chickens, which synthesize melatonin rhythmically and in large quantities but contribute very little of it to the circulation. This paradox has been well documented (Pelham 1975, Cassone et al 1983, Reppert & Sagar 1983); it requires, but has not yet received, explanation. It is especially puzzling since the retinas of Japanese quail (Galliformes, like chickens) make melatonin rhythmically and contribute it to the circulation, from which it appears to exert behavioural effects.

Underwood et al (1984) have measured melatonin production by the quail retina and compared the amount synthesized by the two eyes with circulating levels in pinealectomized birds. They conclude that more melatonin is manufactured by the retinas than is found in the circulation and that melatonin must therefore be either stored or metabolized in the eye. Because indefinite

storage of the large amounts of melatonin produced by the retinas of chickens seems unlikely, it is hard to escape the conclusion that this melatonin is degraded before it reaches the circulation. Surprisingly, experimental tests of this possibility appear to have been neglected; there are no reports of searches, with either positive or negative results, for melatonin breakdown products in chicken retinas.

Because retinal melatonin is likely to play a local neuromodulatory role in addition to its hormonal one, there may well be retinal mechanisms already in place for its rapid inactivation. Perhaps these mechanisms have been amplified in the chicken retina by artificial selection during inbreeding. Such selection may have accomplished, by a different mechanism, the same functional result that we have reported for the pineal glands of laboratory mice. In the one case the retina and in the other the pineal may have been inadvertently eliminated as a functional endocrine organ by artificial selection. These examples do not exhaust the possible ways in which selection, either natural or artificial, might modify the melatonin economy of a species. They do suggest that if new and different patterns of melatonin synthesis become adaptive, these can be quickly realized. Since the circadian and reproductive responses of organisms to the particular photic environments in which they live directly affect their fitness, the observed variability in synthetic patterns and physiological effects of melatonin may not be surprising.

Acknowledgements

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REFERENCES

Besharse JC, Dunis DA 1983 Methoxyindoles and photoreceptor metabolism: activation of rod shedding. Science (Wash DC) 219:1341-1343

Besharse JC, Iuvone DM 1983 Circadian clocks in *Xenopus* eye controlling retinal serotonin *N*-acetyltransferase. Nature (Lond) 305:133-135

Cardinali DP, Rosner JM 1971 Retinal localization of the hydroxyindole-O-methyl transferase (HIOMT) in the rat. Endocrinology 89:301-303

Carter DS, Hall VD, Tamarkin L, Goldman BD 1982 Pineal is required for testicular maintenance in the Turkish hamster (*Mesocricetus brandti*). Endocrinology 111:863-871

Cassone VM, Lane RF, Menaker M 1983 Daily rhythms of serotonin metabolism in the medial hypothalamus of the chicken: effects of pinealectomy and exogenous melatonin. Brain Res 289:129-134

Dubocovich ML 1983 Melatonin is a potent modulator of dopamine release in the retina. Nature (Lond) 306:782-784

Ebihara S, Uchiyama K, Oshima I 1984 Circadian organization in the pigeon, *Columbia livia*: the role of the pineal organ and the eye. J Comp Physiol A Sens Neural Behav Physiol 154:59-69

Gern WA, Ralph CL 1979 Melatonin synthesis by the retina. Science (Wash DC) 204:183-184

Gern WA, Owens DW, Ralph CL 1978 Synthesis of melatonin by the trout retina. J Exp Zool 206:263-270

Hamm HE, Menaker M 1980 Retinal rhythms in chicks: circadian variation in melatonin and serotonin *N*-acetyltransferase activity. Proc Natl Acad Sci USA 77:4998-5002

Menaker M 1982 The search for principles of physiological organization in vertebrate circadian systems. In: Aschoff J et al (eds) Vertebrate circadian systems. Springer-Verlag, Berlin-Heidelberg, p 1-12

Menaker M, Wisner S 1983 Temperature-compensated circadian clock in the pineal of *Anolis*. Proc Natl Acad Sci USA 80:6119-6121

Miller L, Stier M, Lovenberg W 1980 Evidence for the presence of *N*-acetyltransferase in rat retina. Comp Biochem Physiol C Comp Pharmacol Toxicol 66:213-216

Pang SF, Chow PH, Wong TM, Tso ECF 1983 Diurnal variations of melatonin and *N*-acetylserotonin in the tissues of quails (*Coturnix* sp.), pigeons (*Columbia livia*), and chickens (*Gallus domesticus*). Gen Comp Endocrinol 51:1-7

Pelham RW 1975 A serum melatonin rhythm in chickens and its abolition by pinealectomy. Endocrinology 96:543-552

Reppert SM, Sagar SM 1983 Characterization of the day-night variation of retinal melatonin content in the chick. Invest Ophthalmol & Visual Sci 24:294-300

Takahashi JS, Menaker M 1979 Brain mechanisms in avian circadian systems. In: Suda M et al (eds) Biological rhythms and their central mechanism. Elsevier/North Holland Biomedical Press, New York, p 95-109

Underwood H 1984 The pineal and circadian rhythms. In: Reiter RJ (ed) The pineal gland. Raven Press, New York, p 221-251

Underwood H 1985 Pineal melatonin rhythms in the lizard *Anolis carolinensis*: effects of light and temperature cycles. J Comp Physiol A Sens Neural Behav Physiol 157:57-65

Underwood H, Binkley S, Scopes T, Mosher K 1984 Melatonin rhythms in the eyes, pineal bodies, and blood of Japanese quail (*Coturnix coturnix japonica*). Gen Comp Endocrinol 56:70-81

Voisin P, Geffard M, Delaage M, Collin JP 1982 Melatonine dans l'organe pineal, la retine et le plasma. Etude immunologique chez le pigeon. Reprod Nutr Dev 22:959-971

Yu HS, Pang SF, Tang PL 1981 Increase in the level of retinal melatonin and peristence of its diurnal rhythm in rats after pinealectomy. J Endocrinol 91:477-481

Zimmerman NH, Menaker M 1979 The pineal: a pacemaker within the circadian system of the house sparrow. Proc Natl Acad Sci USA 76:999-1003

DISCUSSION

Arendt:Four of our apparently perfectly normal human volunteers have no detectable melatonin. Do you think there is a real disadvantage in that?

Menaker: I don't know how far our observations in mice apply to humans. S. Ebihara, who is currently working in my laboratory, is very excited about the correlation between ease of breeding and lack of melatonin in the pineal glands

of several strains of mice. I asked him why rats, which are also domesticated but have lots of melatonin in their pineal glands, are nevertheless easy to breed. He said that in general rats are not highly inbred, and apparently inbreeding makes it very difficult to maintain strains in the laboratory. It is possible that, if on top of the difficulties of keeping a highly inbred strain you add melatonin from the pineal as a reproductive brake on the system, the animals won't reproduce well. It is important to look at inbred strains of rats to investigate this.

Turek: The function of the pineal gland is most readily apparent in photoperiodic species, and indeed most of the papers presented at this symposium on the physiology of the pineal involve experiments on seasonal breeders. Yet the neurochemistry and enzymic control of the pineal have primarily been worked out in the rat, a non-photoperiodic species. Is it possible that the biochemical pathways that have been worked out in the rat are misleading us about what is going on in animals that have a functional pineal gland?

Goldman: We must be careful about deciding whether an animal has a use for its pineal simply on the basis of whether we can demonstrate a photoperiodic effect on reproduction.

Menaker: I don't think that we can possibly understand the central core of the vertebrate system without looking at a wide variety of species. As far as we know mice are completely non-photoperiodic; Bronson (1979) has shown that they do not respond to light in the field. But we don't yet know whether light acutely suppresses melatonin synthesis in the mouse pineal. I would not be surprised if the control mechanisms in the mouse have been pared down to a very basic level. It may turn out to be a very useful model system.

Vollrath: When I published my results on the day–night rhythm in 'synaptic ribbons' in the pineal (Vollrath 1973), I put forward the hypothesis that these ribbons are morphological prerequisites for melatonin formation, but I was very puzzled to find that ribbons are extremely rare in mouse pineal glands. I am now wondering what these structures look like in wild mice. They could be a morphological correlate to follow in genetic studies.

Goldman: Is there any particular reason why some of the strains of mice you mentioned are difficult to breed?

Menaker: I don't know yet; we have not had enough experience trying to breed them. Perhaps the babies get cold; the adults may not make nests properly or keep the babies warm. It is possibly something to do with temperature regulation.

Tamarkin: We have seen something similar in other strains. We were unable to detect melatonin in pineal glands from nude mice, which are derived from a Swiss strain, and there are difficulties in breeding these animals. As in your mice, the problem is not inability to breed but inability to mother the pups. One can get around the problem by fostering the pups to other mothers.

Menaker: But we find that the strains that *have* melatonin are the ones that are difficult to breed.

Illnerová: I would like to comment on breeding behaviour and the pineal in silver foxes. At a farm in Siberia silver foxes have been selected since about 1920 according to whether they behave like domestic animals or not. There are now two groups of animals, one of which is domesticated, but both groups are raised in exactly the same conditions outdoors with extreme cold during the winter. Foxes in the domesticated group have partly lost their photoperiodic behaviour. The wild foxes can breed only during one month, but some of the domesticated foxes can breed during longer periods or even twice a year. The foxes selected for their domesticated behaviour have lower pineal weights than the wild ones (Kolesnikova 1981). It might be interesting to compare melatonin rhythms in the domesticated and wild groups since many domesticated animals selected originally on behavioural grounds have lost their photoperiodism.

Hoffmann: I am a bit concerned about this correlation between lack of melatonin and high fertility and I am glad Dr Tamarkin has provided a counter-example. One can't generalize at this stage.

Menaker: It's only a correlation at the moment.

Hoffmann: But there are certainly several species that reproduce very well but that secrete a lot of melatonin.

Menaker: To study this you would have to look within a species, in fact within a strain, and ask whether the level of melatonin is one of a number of factors that influence fecundity.

Zucker: So far the discussion has been almost exclusively about artificial selection, but we now have results from several laboratories showing the same phenomenon in natural populations of animals (e.g. Dark et al 1983). This is work on latitudinal gradients and reproduction. In temperate zones the further north you go the later the onset of breeding and the shorter its duration; in southern portions of their distribution animals may be reproductive throughout the year. There is evidence that animals from more southerly latitudes, who may be reproductive all year in the field, don't respond to melatonin administered exogenously, whereas animals from the more northerly populations do (Dark et al 1983). So this phenomenon occurs in animals in the field, and is not just a product of breeding practices.

Menaker: Is there any indication that in those animals selection has been operating on the production of melatonin?

Zucker: No, but it is worth reiterating that selection is for outcomes and not for mechanisms. There are probably many different pathways that can produce the same outcome: in some cases melatonin may not be secreted; in others the target tissues may not be responsive to melatonin. This occurs in lab rats, which are not photoperiodic. However, if you do something as bizarre as to remove the olfactory bulbs from these rats, they become photoperiodic (Nelson & Zucker 1981).

Tamarkin: With Bob Lynch, we have looked at the white-footed mouse from different latitudes in the United States (Lynch et al 1982). Bob has studied mice

from Connecticut that do have a seasonal reproductive cycle and mice from Georgia that do not. We have looked at the melatonin profiles of animals from both groups in short and long photoperiods and they are virtually the same.

Short: You North Americans are sitting on an unexploited gold mine in the white-tailed deer whose distribution extends from New York State down across the equator to South America. All you need to do to measure its breeding seasons is to use a pair of binoculars and record what the antlers are doing. There are no really good data on what happens to breeding season across such a large range of latitude, and it would be fantastic to look at this in a totally wild species.

Goldman: There is a difference between these examples and Mike Menaker's. He is working with animals derived from a stock that was already non-photoperiodic and then lost the capacity to synthesize melatonin as well, whereas Irv Zucker is talking about animals that are at first photoperiodic but have demes at certain latitudes that have become non-photoperiodic.

Zucker: We don't know that. The original stock from which such animals were derived may have been non-photoperiodic, and as the animals invaded more northerly zones they may have become photoperiodic. Also, although mice may not be photoperiodic, they do manifest seasonal breeding in the field under some circumstances. I don't know what wild mice Mike Menaker is using, but some of Bronson's strains are not that 'wild' as they have been in the laboratory a number of years.

Menaker: The mice we use were collected quite recently, about five years ago, but even some of the inbred strains still retain melatonin. It is clear that such changes can be selected for over long time intervals in the field, but our results illustrate how labile the system is, how easy it is to change a normal pineal into one that does not make any melatonin at all. It is helpful, for example, when you consider the variability in retinal melatonin synthesis among species of lizards because we now know that selection pressures can act very quickly on melatonin-synthesizing enzymes.

Zucker: It would be very interesting to see whether this correlation with melatonin levels holds only for reproduction. If you look at end points such as huddling, development of the pelage or behavioural thermoregulation you might find that in some circumstances the relation applies and in others it does not.

Hoffmann: When we breed animals in captivity we automatically select against reproductive barriers such as photoperiodic reactions. But golden hamsters, all of which are derived from one litter caught in Syria in 1930 (Adler 1948), have maintained their photoperiodic capacity and their ability to produce melatonin for over 50 years in spite of this selection. So in some species it is not that easy to change the genetic structure.

Short: Do your findings on the absence of pineal melatonin in certain strains of mice also apply to retinal melatonin?

Menaker: I don't know; we haven't looked at the eyes yet.

Lewy: It is very tempting to implicate melatonin in at least one of the many observed retinal and ocular circadian rhythms. In my laboratory, Krauss et al (1985) have studied intraocular pressure in humans around the clock, and have documented a night-time decrease; it is not a function of sleep because the subjects were sleep-deprived. We can abolish the night-time fall in intraocular pressure with bright-light exposure, which also abolishes the rise in plasma melatonin concentrations. Although there are other possible explanations, melatonin may control that particular circadian rhythm. We have looked at post-mortem specimens of about 30 human retinas and have not been able to measure any melatonin (G. Krauss, unpublished work). Moreover, with Duane Denney and others in my laboratory, we have not been able to identify retinal melatonin in pinealectomized pigmented and albino rats (unpublished work). We use the gas chromatographic–negative chemical ionization mass spectrometric assay (Lewy & Markey 1978), which uses deuterated melatonin as an internal standard (and therefore corrects for recovery) and has a sensitivity of less than 1 pg/ml. Consequently, if melatonin is playing a role in the circadian rhythm in intraocular pressure, it may originate in the pineal. The retina may be a target tissue for the pineal hormone.

Reppert: What experience has anyone else had with measuring melatonin in the mammalian retina?

Menaker: We have never managed to convince ourselves that the retinas of any of the mammals we have had in the lab had any melatonin in them.

Reppert: We have found the same. We have measured retinal melatonin rhythms in a variety of vertebrates (chicks, chameleons, frogs) but in the mammalian species we have looked at, including cats, several species of rats (pigmented and albino) and hamsters, we have failed to find much melatonin (unpublished work).

Pévet: By gas chromatography–mass spectrometry we have demonstrated the presence of melatonin in the retina of the hamster (Beck & Pévet 1984). We found between 100 and 200 pmol/retina; this has to be compared with the 2 pmol/pineal, at night, detected in the same animals with the same technique.

Menaker: Considering how sensitive to melatonin Dubocovich (1983) has found the rabbit retina to be in terms of dopamine release, it would be very surprising if there were not any melatonin in the mammalian retina. It seems reasonable that melatonin is made in the retinas of at least some mammals, but I suspect that the enzymes for chewing it up are different from those in the pineal. If melatonin operates as a neuromodulator within the retina, then there is the same need to get rid of it rapidly as there is to get rid of neurotransmitters. Perhaps the way people treat pineal glands when they want to measure melatonin is not a sufficiently cautious way to treat retinas. If one tried to be very careful with the retina, its melatonin content might be preserved. We and others have reported that melatonin from the chicken retina does not get into

the circulation; after you pinealectomize a chicken you do not measure much melatonin in the blood.

Reppert: We have found the same thing (Reppert & Sagar 1983).

Menaker: Perhaps this melatonin is broken down in the retina. There is a tremendous amount of melatonin in the chicken retina; it must go somewhere.

REFERENCES

Adler S 1948 Origin of the golden hamster *Cricetus auratus* as a laboratory animal. Nature (Lond) 162:256-257

Beck O, Pévet P 1984 Analysis of melatonin, 5-methoxytryptophol and 5-methoxyindole acetic acid in the pineal gland and retina of hamster by capillary column gas chromatography mass spectrometry. J Chromatogr Biomed Appl 311:1-9

Bronson FH 1979 The reproductive ecology of the house mouse. Q Rev Biol 54:265-299

Dark J, Johnston PG, Healy M, Zucker I 1983 Latitude of origin influences photoperiodic control of reproduction of deer mice (*Peromyscus maniculatus*). Biol Reprod 28:213-220

Dubocovich ML 1983 Melatonin is a potent modulator of dopamine release in the retina. Nature (Lond) 306:782-784

Kolesnikova LA 1981 Morpho-functional condition of pineal glands of silver-black foxes with the different types of behaviour. Izv Sib Otd Akad Nauk SSSR 1:135-139

Krauss G, Samples JP, Lewy AJ 1985 Melatonin: a potential regulator of intraocular pressure. Invest Ophthalmol & Visual Sci (ARVO Suppl) 26:109

Lewy AJ, Markey SP 1978 Analysis of melatonin in human plasma by gas chromatography negative chemical ionization mass spectrometry. Science (Wash DC) 201:741-743

Lynch GR, Sullivan JK, Heath HW, Tamarkin L 1982 Daily melatonin rhythm in photoperiod sensitive and insensitive white-footed mice (*Peromyscus leucopus*). In: Reiter RJ (ed) The pineal and its hormones. A.R. Liss, New York, p 67-73

Nelson RJ, Zucker I 1981 Photoperiodic control of reproduction in olfactory-bulbectomized rats. Neuroendocrinology 32:266-271

Reppert SM, Sagar SM 1983 Characterization of the day-night variation of retinal melatonin content in the chick. Invest Opthalmol & Visual Sci 24:294-300

Vollrath L 1973 Synaptic ribbons of a mammalian pineal gland. Circadian changes. Z Zellforsch Mikrosk Anat 145:171-183

Photoperiodism, melatonin and the pineal
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Photoperiodism in birds

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Abstract. Birds show a circadian rhythm in melatonin secretion and, as expected, the pattern of output changes with photoperiod. Somewhat surprisingly then, in view of the mechanisms in mammals, birds do not seem to use this seasonal message in the photoperiodic control of reproduction. Some further experiments are needed, however, because in birds the pineal gland is not the only source of melatonin. Another difference from mammals is that birds detect the photoperiodic light not with the retina but by brain photoreceptors, which probably lie in the hypothalamus. An action spectrum for these receptors has now been obtained for the quail and this shows a peak absorption at 492 nm, suggesting that the photoreceptor is rhodopsin-based. The sensitivity of the brain receptors to 500 nm light was calculated at 2×10^4 photons $mm^{-2} s^{-1}$. For light to induce the photoperiodic response it must be interpreted by the bird's clock as a long day. This happens if the light falls 12–20 h after dawn and coincides with a rhythm of photosensitivity. The subsequent neuroendocrine response to the light signal is both precise and relatively long-term. A single 4 h light pulse initiates a wave of gonadotropin secretion lasting for 10 days. The light stimulus can be replaced by a brief (2 min) daily electrical stimulus given to the hypothalamus 10–12 h after dawn. Over the next few years it should be possible to disentangle further the neural processes involved.

1985 Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Foundation Symposium 117) p 93-105

The pineal gland, melatonin and the photoperiodic response in birds

Birds possess a well-developed pineal gland which, as in mammals, secretes melatonin in a rhythmic fashion, normally confining both synthesis and release to the hours of darkness. This rhythm is circadian and is under the control of at least two clock systems. One lies in the suprachiasmatic nuclei and regulates the pineal via the usual multisynaptic pathway through the superior cervical ganglion; the other lies within the pineal itself and, whilst present in other vertebrates, is absent from mammals. When the avian pineal gland is isolated from all its neural inputs either *in vivo* or *in vitro* it is still perfectly capable of secreting melatonin rhythmically under a light–dark cycle. The relative importance of these two clock systems in the normal bird is still unresolved

(see Cassone & Menaker 1984), but together they provide firm circadian control over the pineal gland. As would be expected from such a circadian-driven system (Pittendrigh 1981), the melatonin rhythm alters a number of its characteristics (phase, duration and amplitude) as the photoperiod changes and so, just as in mammals, the melatonin signal delivered into the circulation reflects closely the prevailing daylength: it is a natural transducer of photoperiod (Fig. 1).

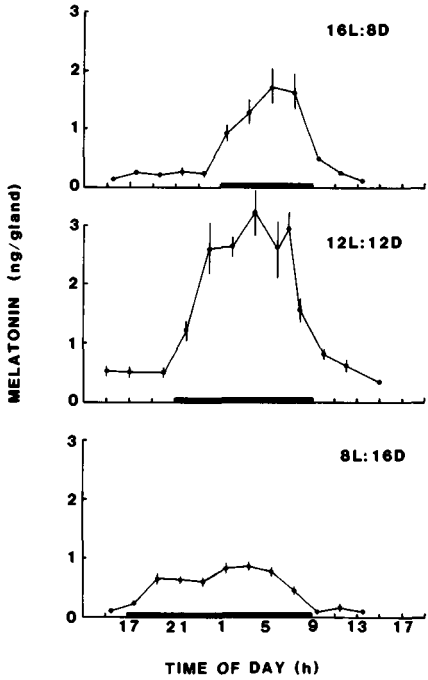

FIG. 1. Rhythms of pineal melatonin content ($n = 6$, mean ± SEM) in quail killed at different times through a 24 h period. The quail were on three different photoperiods with light (L) to dark (D) ratios of 16L:8D, 12L:12D or 8L:16D. Darkness is indicated by the solid black bar. Redrawn from Cockrem (1983).

Given the fact that the photoperiodic control of reproduction is particularly highly evolved in birds, it is surprising that birds do not seem to use this tailor-made seasonal signal to regulate their breeding. Pinealectomy has been performed on more than a dozen species of birds but, with the possible exception of the Indian weaver *Ploceus philippinus*, in no species does the operation have more than a transitory effect upon reproduction. This applies to all the major aspects of the avian photoperiodic response: the inhibition of gonadal growth under short days; its induction with long days and the regression

that follows return to short days; the acquisition of refractoriness under long days and its dissipation with short days. Even when certain aspects are analysed in detail they are not affected by removal of the pineal gland (e.g. Simpson et al 1983). Recently it has become clear, though, that in some birds melatonin is secreted not just from the pineal but also from the retina, and so many of the earlier experiments must be regarded as flawed. Fig. 2, which has been taken from Underwood & Siopes (1984), shows that the pineal contributes just over half of all circulating melatonin in Japanese quail; the rest

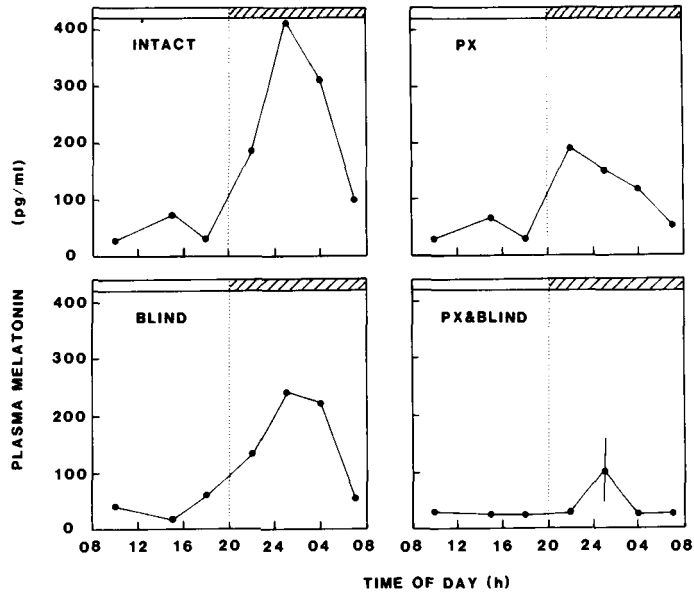

FIG. 2. Plasma melatonin levels in Japanese quail exposed to 12L:12D (darkness indicated by hatched bars). Four groups of quail were studied: intact, pinealectomized (PX), blinded, or pinealectomized and blinded (PX & blind). Note how melatonin continues to circulate after pinealectomy. Redrawn from Underwood & Siopes (1984).

comes from the eye. Therefore, to test the hypothesis that melatonin is not mediating the photoperiodic signal, experiments must be performed with quail that have been both blinded and pinealectomized. Such an experiment would be impossible in mammals since the eye is also the photoreceptor for the photoperiodic response. However, this does not apply in birds (see below) so the experiment can be undertaken; the results published by Siopes & Wilson (1974) are summarized in Fig. 3. The long-day photoperiodic response in blinded, pinealectomized quail is indistinguishable from that in the controls. Such a result certainly does seem to exclude a role for melatonin in this aspect

of the quail's photoperiodic response, a conclusion supported by the absence of any effects of melatonin injections or implants upon gonadal function (e.g. Homma et al 1967). We obtained a typical negative result when we injected melatonin (25 μg) into intact quail at hour 10 after transfer from 8L:16D (8 h light:16 h dark) to 13L:10D. The obvious idea was to mimic a short-day pattern of melatonin, such treatments being highly effective in both hamsters (Tamarkin et al 1976) and sheep (Arendt et al 1983). Ovarian mass after

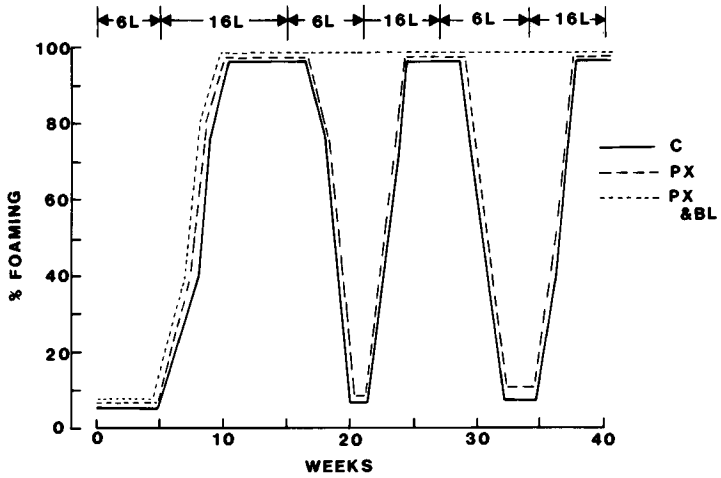

FIG. 3. The photoperiodic response in Japanese quail transferred back and forth between short (6L:18D) and long (16L:8D) days over a period of 40 weeks. In this particular experiment reproductive function was assessed by measuring the percentage of male quail showing foaming cloacal glands. This gland is a secondary sexual character completely dependent upon androgens. Three groups are shown: intact quail (C), pinealectomized quail (PX) and birds that had been both pinealectomized and blinded (PX & BL). Redrawn from Siopes & Wilson (1974).

three weeks was 129.8 ± 28.7 mg ($n = 8$) in the control quail and 97.1 ± 14.0 mg ($n = 8$) in those treated with melatonin. Taken together, such experiments provide more evidence that the regulation of seasonality by pineal melatonin, which is now known to occur in a range of mammals (rodents, ungulates, carnivores and marsupials), does not apply to birds.

Two caveats remain. The first is clear from Fig. 3: blinded, pinealectomized quail cannot regress their testes upon transfer to short days. If this capacity were only restorable with melatonin, then a role for this hormone would be discovered. This is unlikely to be the case, however, because blinded quail that do have a functional pineal also do not undergo gonadal regression on short days (Siopes & Wilson 1980). As an aside it might be mentioned that one reason why such quail do not regress is that they may not become photore-

fractory under long days. Thyroidectomy of quail produces a syndrome remarkably similar to that of blinding (Follett & Nicholls 1984), and this has been interpreted in terms of a need for thyroid hormones to allow the expression of photorefractoriness (see also Nicholls et al 1984). Could it be that a retinal photoreceptor is involved in the acquisition of photorefractoriness in birds even if it is not needed for other aspects of the photoperiodic response? The second caveat is more speculative. The retinal, pineal and hypothalamic photoreceptors in birds share a common embryonic origin. Since both the eye and the pineal are closely associated with mechanisms to secrete melatonin rhythmically, is it possible that the hypothalamus also possesses this capacity and that there is a local secretion of melatonin within the brain which influences the photoperiodic response? This sounds slightly far-fetched, and our efforts to show a day–night difference in the *N*-acetyltransferase content of the quail's hypothalamus have not been successful, although there is so much of this enzyme in the brain that local differences may be obscured. The idea may be worth pursuing, if only because we still remain slightly sceptical of the view that mammals and birds have fundamentally different photoneuroendocrine mechanisms.

The brain photoreceptors involved in the photoperiodic response

The photoreceptors in the brains of birds, first discovered by Benoit over 50 years ago, appear to be the primary receptors both for photoperiodic responses (review, Oliver & Baylé 1982) and for the entrainment of circadian rhythms (McMillan et al 1975). The results of implanting single optic fibres and radioluminous beads into various regions of the brain suggest that the photoreceptors lie in the hypothalamus, but their exact location is still unknown. Indeed, a primary problem has been the lack of an adequate action spectrum that could point the way towards their chemical basis. Experiments in which birds have been exposed to long days of overhead illumination usually show that orange/red light is more effective than blue/green at inducing gonadal growth (summary by Oishi & Lauber 1973), and this suggests that the photopigment is maximally sensitive in the region of 600–650 nm. This is certainly possible, but none of the experiments took into account any differential absorption of the various wavelengths of light as they pass through the skull and brain tissues to reach the photoreceptors. Work by Hartwig & Van Veen (1979) showed clearly that in eels red light passes more easily through the brain than does blue/green, so that when equal intensities of light are applied overhead different numbers of photons reach the hypothalamus.

In the quail (Foster et al 1985) we have measured the relative absorption of light over wavelengths ranging between 350 nm and 700 nm using a micro-

spectroradiometer. Light from a quartz–halogen source was applied via a 2 mm bundle of optic fibres to the surface of the skull and the per cent transmission measured at 10 nm intervals in 12 birds. The shape of the transmission curve is dominated by absorption of light by haemoglobin and as a result transmission of wavelengths around 500 nm is about 30 times less than that of wavelengths around 690 nm. Such quantitative calculations allowed us to correct for relative absorption and so to ensure that the basal hypothalamus received the same relative number of photons at any wavelength. This provided the first prerequisite for determining an action spectrum, but it had to be combined with a quantitative measure of photoinduction. For this we used castrated quail that show a rise in luteinizing hormone (LH) secretion after exposure to a single long day (Nicholls et al 1983). The quail were normally maintained on short daylengths (8L:16D) and were each fitted with a small 'head-cap' on the surface of the skull to which could be attached a fibre-optic lead (2 mm diameter) from a monochromator. On the day of an experiment a quail was moved from its cage just before dusk and attached to the fibre-optic lead for 12 h during which it received monochromatic light (15 nm half-peak bandwidth) of a particular intensity and wavelength. It was then returned to its home cage. Blood samples (200 μl) were collected one day before and three days after photostimulation and the LH concentrations measured by radioimmunoassay, the difference between the two sets of samples representing the degree of photoinduction. An individual bird was used every three weeks and with a batch of 55 castrated quail a regular flow of experiments could be sustained.

Two types of action spectra were determined. The first tested the photoperiodic response at a single intensity of light at the level of the hypothalamus and used nine wavelengths ranging from 410 nm to 650 nm (Fig. 4). A clear maximum was obtained with light around 500 nm. The second measured the relationship between light intensity and the degree of photoinduction at four wavelengths (470, 500, 590 and 650 nm). The four dose–response curves were parallel, suggesting that only a single photosensitive step is involved. Again light of 500 nm was the most inductive and was considerably more effective than orange/red light of 590 or 650 nm. The peak at 500 nm is highly reminiscent of the peak sensitivity of a rhodopsin, and since all rhodopsins show the same general absorption curve, though with differing maxima, it is possible to use the methods of Dartnall (1975) to obtain a best fit between a rhodopsin curve and our data points. The curve obtained suggests a rhodopsin with a maximum absorption at 492 nm. Our results, therefore, suggest that the brain photoreceptors have a rhodopsin pigment with peak sensitivity in the blue/green region of the spectrum. This seems to apply also to the pineal photopigment in chickens (Deguchi 1981).

This conclusion is reinforced by calculating the absolute sensitivity of the

brain photoreceptors, assuming that they lie in the hypothalamus. It was estimated that the amount of 500 nm light that must reach the hypothalamus to trigger a significant photoperiodic response is 2×10^{10} photons m^{-2} s^{-1}. This is a level of sensitivity matched only by higher vertebrate photopigments. The approximate surface area at the level of the hypothalamus in a quail is 4 mm^2, suggesting that induction occurs at a flux rate of 8000 photons s^{-1}.

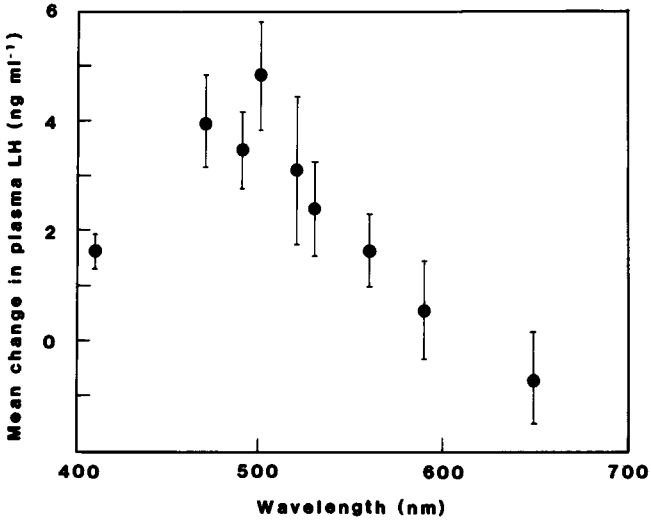

FIG. 4. An equal-intensity action spectrum for the photoperiodic response in castrated Japanese quail. Mean ± SEM ($n = 9$–16). The degree of photoinduction was measured by the change in circulating LH induced by exposing the brain to light of differing wavelengths. The intensity of each wavelength at the skull surface was adjusted to compensate for the differential absorption through the brain. This means that for any wavelength the same relative number of photons reached the hypothalamus. Redrawn from Foster et al (1985).

The results are not inconsistent with the earlier findings that overhead illumination with red light is most effective at causing photoinduction. The rhodopsin photopigment is approximately 10 times more sensitive to blue/green than to red light (see Fig. 4), but the absorption of blue/green wavelengths is 30 times greater than absorption of orange/red.

The subsequent photoneuroendocrine response

The reception of light is, of course, only the first step in triggering the photoperiodic response. We already know from earlier experiments in birds that the photoperiodic 'clock' is based upon a daily rhythm in sensitivity to light. So-called 'night interruption' experiments (e.g. Follett & Milette 1982) show

that quite brief light pulses trigger testicular growth when given 12–15 h after dawn but are ineffective at other times of the night. Such experiments are often interpreted as indicating that the actual time of maximum sensitivity to light is also 12–15 h after dawn, but this conclusion is not justified. A schedule such as 8L-5D-1L-10D, which causes photoinduction when given continuously for some weeks, certainly shows that a time of sensitivity exists, but since one cannot be sure which light pulse is effective it is not possible to define the actual sensitive period. This is not an esoteric matter (see Saunders

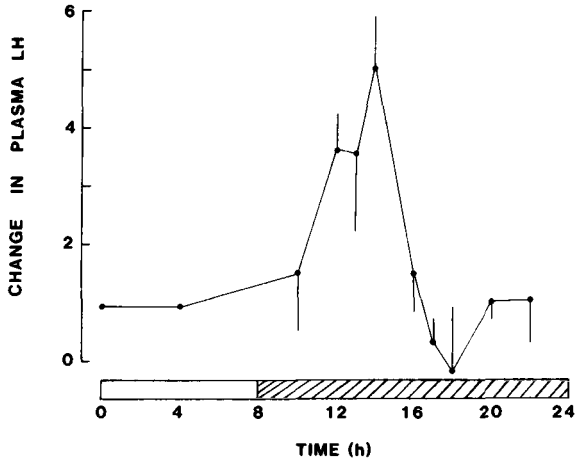

FIG. 5. An experiment in Japanese quail to demonstrate the exact position of the rhythm of photosensitivity that underlies the photoperiodic clock. Castrated quail living on 8L:16D (shown by bar beneath figure) were given a single 4 h 'night-interruption' light pulse during the 16 h dark period. The birds were subsequently retained in darkness until a blood sample was taken 54 h after the original dawn signal. The LH content in this sample was compared with the level of plasma LH before photostimulation and the difference is shown on the ordinate. The times on the abscissa represent the mid-points of the 4 h night-interruption pulses. Mean ± SEM (n = 6–9). Redrawn from Nicholls et al (1983).

1982) because defining the period of sensitivity is important in determining how the long day causes photoinduction. Indeed, it was for this very reason that we developed systems whereby quail would respond to a single long day (Nicholls & Follett 1974, Nicholls et al 1983). With such quail it *is* possible to show directly when the birds are photosensitive. One experiment in which we gave a single 4 h night-break at different times during the night is shown in Fig. 5. It turns out that the actual time of maximum photosensitivity in quail is 12–20 h after dawn. At some point during this first day the quail must actually 'conclude' that it has been exposed to a stimulatory daylength.

So far, the earliest event that we have been able to uncover in the photoneuroendocrine response is the rise in LH which begins at about hour 20 (Follett et al 1977).

The neural events that must begin at about hour 15 and that result a few hours later in a stimulation of secretion of LH-releasing hormone from the median eminence remain obscure, although the means of analysing them may be to hand. Certainly they are both precise and relatively long-term. A single pulse of light leads not to a brief rise in LH secretion beginning at hour

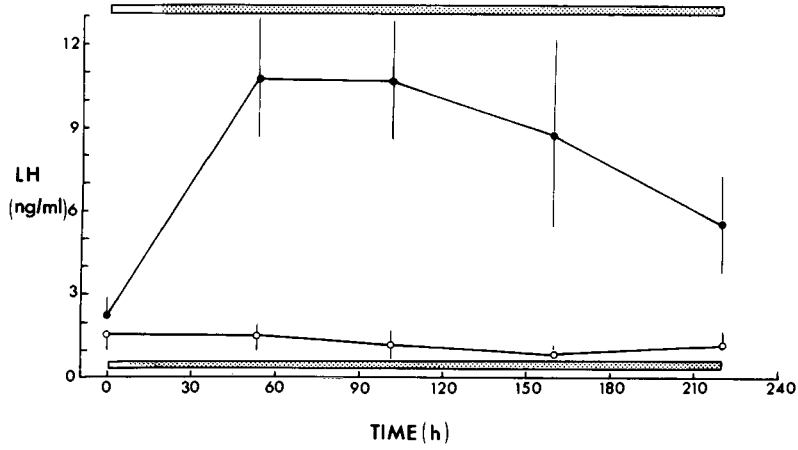

FIG. 6. The duration of the photoperiodic response induced by a single long day (20 h light) given to a group of castrated Japanese quail (solid circles, $n = 24$). After the 20 h of light the birds were confined to darkness for the next 200 h. A control group of quail (open circles, $n = 24$) was exposed to 8 h of light rather than 20 h and then also placed in darkness. Blood samples were collected in dim red light and then assayed for their LH content (mean ± SEM). Redrawn from Nicholls et al (1983).

20 but to a prolonged secretion lasting many days (Fig. 6); the brief photic event interacting with the clock seems to trigger a long-lasting neural process. This has also been found in a quite different type of experiment carried out by Ohta et al (1984). These workers implanted a chronic electrode into the quail's hypothalamus and stimulated it for 2 min per day for three weeks. Different birds received the electrical stimulation at different times in the light–dark cycle. The results are impressive and reinforce our view that time measurement involves a rhythm in sensitivity. Stimulation 10–12 h after dawn induced full testicular maturation (Fig. 7). The reader will note that the most effective time of electrical stimulation does seem to occur a little earlier than the most effective time for light stimulation (Fig. 5). The reason for this

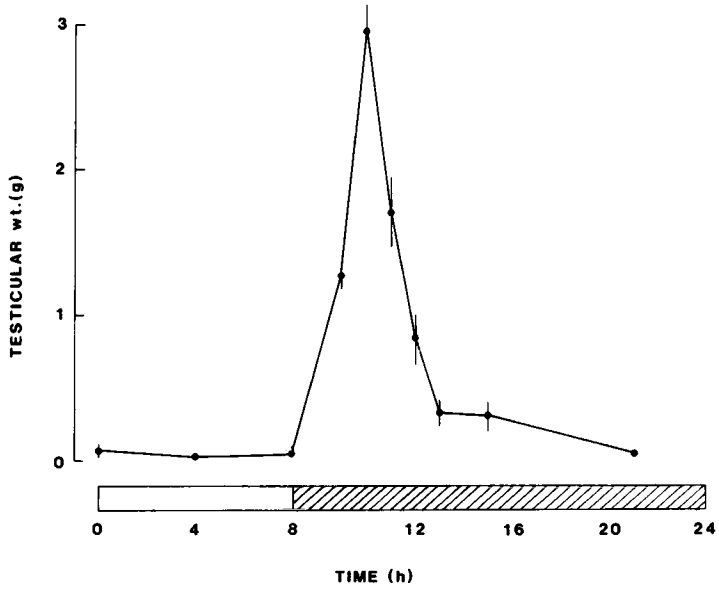

FIG. 7. A dramatic experiment carried out by Ohta et al (1984) in Japanese quail in which the hypothalamus was stimulated electrically for 2 min daily for three weeks. Different groups received electrical stimulation at different times during the short day (8L:16D). Note how such a brief period of daily stimulation at hour 11 is capable of inducing full testicular maturation. Drawn from data given in tabular form by Ohta et al (1984).

is unclear, but at this stage we do not even understand how and why the electrical signal is so effective, although we suspect it must be acting on or through the rhythm in photosensitivity. Perhaps that would be a useful next step to analyse in unravelling this complex system.

REFERENCES

Arendt J, Symons AM, Laud CS, Pryde SJ 1983 Melatonin can induce early onset of the breeding season in ewes. J Endocrinol 97:395-400

Cassone VM, Menaker M 1984 Is the avian circadian system a neuroendocrine loop? J Exp Zool 232:539-550

Cockrem JF 1983 Circadian rhythms of melatonin secretion from the pineal gland of the Japanese quail. PhD Thesis, University of Bristol

Dartnall HJA 1975 Assessing the fitness of visual pigments for their photic environment. In: Alis MA (ed) Vision in fishes. Plenum Press, New York, p 543-563

Deguchi T 1981 Rhodopsin-like photosensitivity of the isolated chicken pineal gland. Nature (Lond) 282:94-96

Follett BK, Milette JJ 1982 Photoperiodism in quail: testicular growth and maintenance under skeleton photoperiods. J Endocrinol 93:83-90

Follett BK, Nicholls TJ 1984 Photorefractoriness in Japanese quail: possible involvement of the thyroid gland. J Exp Zool 232:573-580
Follett BK, Davies DT, Gledhill B 1977 Photoperiodic control of reproduction in Japanese quail: changes in gonadotrophin secretion on the first day of induction and their pharmacological blockade. J Endocrinol 74:449-460
Foster RG, Follett BK, Lythgoe JN 1985 Rhodopsin-like sensitivity of extra-retinal photoreceptors mediating the photoperiodic response in quail. Nature (Lond) 313:50-52
Hartwig H-G, Van Veen T 1979 Spectral characteristics of visible radiation penetrating into the brain and stimulating extra-retinal photoreceptors. J Comp Physiol 130:277-282
Homma K, McFarland LZ, Wilson WO 1967 Response of the reproductive organs of the Japanese quail to pinealectomy and melatonin injections. Poult Sci 46:314-319
McMillan JP, Keatts HC, Menaker M 1975 On the role of eyes and brain photoreceptors in the sparrow; entrainment to light cycles. J Comp Physiol 102:251-256
Nicholls TJ, Follett BK 1974 The photoperiodic control of reproduction in *Coturnix* quail. The temporal pattern of LH secretion. J Comp Physiol 93:301-313
Nicholls TJ, Follett BK, Robinson JE 1983 A photoperiodic response in gonadectomized quail exposed to a single long day. J Endocrinol 97:121-126
Nicholls TJ, Goldsmith AR, Dawson A 1984 Photorefractoriness in European starlings: associated hypothalamic changes and the involvement of thyroid hormones and prolactin. J Exp Zool 232:567-572
Ohta M, Wada M, Homma K 1984 Induction of rapid testicular growth in Japanese quail by phasic electrical stimulation of the hypothalamic area. J Comp Physiol 154:583-589
Oishi T, Lauber JK 1973 Photoreception in the photosexual response of quail. II. Effects of intensity and wavelength. Am J Physiol 225:880-886
Oliver J, Baylé J-D 1982 Brain photoreceptors for the photoinduced testicular response in birds. Experientia (Basel) 38:1021-1029
Pittendrigh CS 1981 Circadian organization and the photoperiodic phenomena. In: Follett BK, Follett DE (eds) Biological clocks in seasonal reproductive cycles. John Wright & Sons, Bristol, p 1–36
Saunders DS 1982 Insect clocks, 2nd edn. Pergamon, Oxford
Simpson SM, Urbanski HF, Robinson JE 1983 The pineal gland and the photoperiodic control of luteinizing hormone secretion in intact and castrated Japanese quail. J Endocrinol 99:281-287
Siopes TD, Wilson WO 1974 Extraocular modification of photoreception in intact and pinealectomized *Coturnix*. Poult Sci 53:2035-2041
Siopes TD, Wilson WO 1980 Participation of the eyes in the photosexual response of Japanese quail (*Coturnix coturnix japonica*). Biol Reprod 23:352-357
Tamarkin L, Westrom WK, Hamill AI, Goldman BD 1976 Effect of melatonin on the reproductive systems of male and female syrian hamsters: a diurnal rhythm in sensitivity to melatonin. Endocrinology 99:1534-1541
Underwood H, Siopes TD 1984 Circadian organization in Japanese quail. J Exp Zool 232:557-566

DISCUSSION

Short: We have heard much about variability in mammals, but how much do we know about birds? We have information on quail, chickens, sparrows and starlings, but what about other birds? Can we generalize?

Follett: Of course there are always other orders of birds that we could look at,

but the range of bird species in which the physiology of photoperiodism has been studied may exceed that of mammals; the systems seem similar in all those studied. Except for your own work on pineal function in marsupials, most work in mammals is quite narrowly based around a couple of orders.

Short: Do you see great variability in birds, Dr Menaker?

Menaker: We have not looked at photoperiodism comparatively in birds, but the role of the pineal in the circadian system is certainly highly variable.

Turek: Although there are a number of differences between birds and mammals, many experiments yield exactly the same results in both classes of animals. For example, with the possible exception of the quail, exposure to resonance light cycles results in the stimulation of the reproductive system in birds as well as mammals. I was struck by the similarity between the electrical stimulation experiments Professor Follett discussed in birds (Fig. 7, p 102) and some of our recent work in hamsters. Single injections of carbachol, which is a cholinergic agonist, can mimic the phase-shifting effects of light on the circadian system. If we give repeated injections in constant darkness we can prevent testicular regression, and we can stimulate the entire long-day response if carbachol injections are coincident with a certain phase of the circadian rhythm of sensitivity to light (Earnest & Turek 1983, 1985). Rusak & Groos (1983) have found that electrical stimulation of the suprachiasmatic nucleus (SCN) causes phase-shifts in circadian rhythms that are similar to those produced by light and carbachol. So I am convinced that we could put an electrode down into the SCN of the hamster and, by stimulating at certain phases of the circadian cycle, initiate the photoperiodic response exactly as Ohta et al (1984) did in the bird. In the mammal, electrical stimulation of the SCN may ultimately work through the pineal gland, but in the bird the same result can be produced by a totally different mechanism.

Follett: Yes, one suspects there is evolutionary convergence. The same formal photoperiodic experiments can be used to show a circadian rhythm of photoperiodic sensitivity not only in birds and mammals, but also in insects and plants. However, our ability to do a good resonance experiment in a Chrysanthemum is hardly indicative of a role for the pineal!

Menaker: The two big differences between the photoperiodic systems of birds and mammals are in the role of the pineal and in the location of the photoreceptors, which are in the brain in birds and in the retina in mammals. Clocks, for example, are involved in both birds and mammals.

Turek: And it's remarkable how similar the clock properties are.

*Goldman:*Do SCN lesions destroy photoperiodicity in birds?

Follett: I don't think I can answer that. We can certainly destroy the photoperiodic response with anterior hypothalamic midline lesions, but it is not clear that the suprachiasmatic nuclei in Galliformes are in the midline, and the lesions may be effective because they interrupt the luteinizing hormone-

releasing hormone (LHRH) circuits to the median eminence. In contrast with SCN-lesioned hamsters, which continue to have large gonads, any hypothalamic perturbation in birds invariably stops reproduction. Hence our problem is that we cannot disentangle an effect of a lesion on the clock from an effect on the rest of the LHRH system.

Bittman: Do you think there is any correlation between the susceptibility of various kinds of birds to manipulation by melatonin and the role of the pineal and melatonin in regulating circadian rhythmicity? What, for example, would be the outcome of trying to manipulate reproduction with melatonin in a pinealectomized house-sparrow? Would it tell us anything about the normal mechanisms of photoperiodic regulation of reproduction in these birds? In other words, do light-induced changes in melatonin secretory patterns normally account for reproductive responses of birds, and does it depend upon whether or not the pineal behaves as a master oscillator in the species under study?

Menaker: All I can tell you is that pinealectomized house-sparrows grow their gonads perfectly well.

Bittman: But if the animals were pinealectomized and kept in constant darkness, what would be the effect of giving melatonin?

Menaker: There is no way of predicting. Melatonin has not been shown to influence reproductive state in passerine birds, but of course it might do so under some circumstances.

Bittman: But there is evidence that melatonin may affect general circadian organization in the sparrow and not in the quail, so it might produce different effects on gonadal growth in the two types of bird. Perhaps in birds one could use species differences in the type of circadian organization to answer a question we often ask of mammals: does the circadian system participate in the response to melatonin?

REFERENCES

Earnest DJ, Turek FW 1983 Role for acetylcholine in mediating effects of light on reproduction. Science (Wash DC) 219:77-79

Earnest DJ, Turek FW 1985 Neurochemical basis for the photic control of circadian rhythms and seasonal reproductive cycles: role for acetylcholine. Proc Natl Acad Sci USA 82:4277-4281

Ohta M, Wada M, Homma K 1984 Induction of rapid testicular growth in Japanese quail by phasic electrical stimulation of the hypothalamic area. J Comp Physiol 154:583-589

Rusak B, Groos G 1982 Suprachiasmatic stimulation phase shifts rodent circadian rhythms. Science (Wash DC) 215:1407-1409

General discussion I

Local and cellular actions of melatonin

Short: One of the important questions about melatonin is whether it can act as a neurotransmitter. Does melatonin have effects at the sites where it is produced in the retina, in the pineal and perhaps elsewhere in the brainstem? Could it actually be a locally active neurotransmitter as well as a systemically active hormone feeding back elsewhere in the brainstem?

Menaker: Dubocovich (1983) has good evidence that melatonin is a locally acting neuromodulator: in the retina it has a very specific effect on the release of dopamine at a concentration of about 10^{-9} M. But it may not fulfil the strict criteria for being a neurotransmitter.

Klein: Chick pigment epithelial cells seem to be even more sensitive to melatonin; Tsukahara's group (Ogino et al 1983) have reported effects at concentrations down to 10^{-11} or 10^{-12} M. But I would be careful about calling melatonin a neurotransmitter; neuromodulator or neurosecretion is a better description. For a transmitter one would expect to see granules of some sort, a release mechanism that is independent of synthesis and a strong charge on the molecule. However, melatonin may well have important local actions, especially if its production or release is limited to very discrete areas of the brain or to specific cell types, for example in the retina.

Sizonenko: We have investigated where melatonin concentrates after being injected subcutaneously into male rats, and find a high concentration in the hypothalamus and in the eye. Ursula Lang has looked for binding sites for melatonin both in the cytosol and in the cell membrane and has found the greatest numbers of receptors also in the hypothalamus and in the eye (Lang et al 1981). So, although the binding sites have yet to be characterized, it seems probable that melatonin acts as a local modulator in these areas.

Lewy: We should remember that the retina can be a target organ for (pineal) melatonin without necessarily being a site for extrapineal production of melatonin. Furthermore, even if melatonin is synthesized in the retina, we do not think that this contributes to circulating melatonin in the rat, human and non-human primate (Lewy et al 1980a, Markey & Buell 1982, Tetsuo et al 1982).

Bittman: Does unlabelled melatonin compete for the specific binding sites? Is the binding saturable?

Sizonenko: Yes. The binding sites are specific for melatonin, but 6-hydroxymelatonin or 5-hydroxytryptamine will also compete to some extent.

Short: The melanocyte is one of the primeval target organs for melatonin, so should we be thinking about the possible roles of melatonin in controlling the distribution of melanin within the retina?

Menaker: It seems reasonable to suggest that melatonin evolved originally as a local neuromodulator to deal with events involved in the light cycle within the retina, that is, retinomotor movements of rods or cones and pigment migration. The one action of melatonin at the molecular level that is consistent with this is inhibition of the assembly of microtubules. If this is a general property of melatonin, it could control a variety of processes. One could imagine an evolutionary sequence that starts with the use of melatonin as a local modulator of light and dark adaptation through the control of movements of cells and parts of cells. The second step might involve the use of melatonin as a hormone at some distance from its site of synthesis to control the migration of pigment granules within melanocytes. Subsequently, since melatonin would then have become a circulating hormone, it could be used to control a variety of responses to the environment, for example by regulating the release of neurotransmitters or the assembly of microtubules (Menaker 1982).

Rollag: To get an effect on microtubules you need at least micromolar concentrations of melatonin (Banerjee et al 1972, Cardinali & Freire 1975, Piezzi & Cavicchia 1981). Normally, the concentrations of circulating melatonin are in the nanomolar range.

*Menaker:*But one could easily imagine increased sensitivity in cells normally exposed to melatonin.

Rollag: I thought you were suggesting that melatonin might act like colchicine in inhibiting microtubule assembly, an action which is not universally accepted (Poffenbarger & Fuller 1976).

Menaker: I am, but in specific cases where perhaps amplified response systems have evolved.

Retinal rhythms

Reiter: Could melatonin be involved in disk shedding in the retina, which occurs a couple of hours after lights-on?

Vollrath: There are species differences in this; in the cat disk shedding occurs in the evening.

Reiter: It depends on whether you are talking about rods or cones. Rods uniformly shed disks in the early part of the light phase but cones shed at various times in a 24 h period.

Klein: Paul O'Brien studied this in pinealectomized animals and showed that it is not dependent upon the pineal, but that doesn't mean that there is not a local effect of melatonin that is generated within the retina (Teirstein et al

1980). Animals with lesions in their suprachiasmatic nuclei (SCN) still showed a rhythm in disk shedding, so the control mechanism may be located within the eye. Outer disk shedding can occur as a direct response to light but there is also this rhythm in constant darkness that is independent of the SCN. In fact, it is possible to shift the rhythm in one eye so that it is 180° out of phase with the other.

Reiter: I think we must be cautious about such experiments until we know how complete the SCN lesions are. It would be interesting to see whether *any* of the rhythms in the retina exist after SCN lesions, i.e. whether the pacemaker for all the rhythms is in the SCN.

Menaker: In *Xenopus* it is clear that the pacemaker is in the eye because the eye is rhythmic when it is isolated (Besharse & Iuvone 1983).

Reppert: In the chick, the retinal melatonin rhythm is still maintained after optic nerve transection, so neural connections from the SCN to the retina are not required for expression of this rhythm (Reppert & Sagar 1983).

Lewy: And Michael Terman (Terman & Terman 1985) has shown that in the rat in constant darkness a robust circadian rhythm in light sensitivity persists after SCN lesion.

Zucker: Although after SCN damage this particular rhythm is maintained, the circadian drinking and/or activity rhythms disappear. These results were subjected to spectral analysis and are convincing.

Reiter: So the implication is that there is a pacemaker within the eye.

Zucker: We can say only that there is a pacemaker other than the SCN.

Light sensitivity and melatonin synthesis

Short: Dr Illnerová, would you like to comment on how the light sensitivity of the eye changes and affects nocturnal melatonin secretion? We know that in the rat light exposure during the night produces a tremendous decline in melatonin secretion, and Dr Lewy has shown that in humans very high light intensities bleach out nocturnal melatonin production.

Illnerová: The intensity of light may be critical. When Takahashi et al (1984) tried to use light to phase-shift circadian rhythms in the hamster (*Mesocricetus auratus*), they found that what was important was the total number of photons the animal received. This means that if the intensity of light is very low it is necessary to prolong the time of exposure. The same holds true for suppression of melatonin synthesis in the rat pineal at night: if you use a very high intensity light then 6 s exposure may be sufficient to suppress high melatonin production but with low intensities you may need 1–20 min (Vaněček & Illnerová 1982). It depends on how sensitive the animal is to light. The rat is more sensitive to light in the middle of the night than during the evening transition from light to

darkness, so it may be important to consider an animal's light history. In this respect, diurnal animals, nocturnal animals, those kept in fields and those kept in the laboratory may differ (Reiter 1983). In some experiments animals are exposed to red light, but one must be very careful using any red light at night, even if it is very faint, because during complete darkness animals may be much more sensitive to it than during the evening.

Short: How do you think changes in light sensitivity are regulated, Professor Reiter?

Reiter: There are a couple of possibilities, one of which is that previous lighting history affects sensitivity. It is possible that the adjustment occurs at the level of the retina, but it could also be controlled in the SCN, more peripherally at the level of the superior cervical ganglia or even in the pineal gland itself.

Hoffmann: Lynch et al (1981) have shown that in the rat the same light intensity can be read as night or as day depending on the contrasting illumination. This and pre-adaptation may play a big role. I don't know whether there are any fundamental differences between nocturnal and diurnal species, but one should carefully compare previous light exposure for each animal. It may be important whether one takes them from outside or whether one keeps them in artificial light conditions before testing.

Reppert: Deguchi (1975) reported that rats born and reared in constant light still had a very nice *N*-acetyltransferase rhythm. When they were first exposed to light–dark cycles they became sensitive to light, in terms of suppression of pineal function, within a few cycles. So even in animals that have no early history of seeing day–night changes, you can get suppression of pineal function by light after exposure to a light–dark cycle.

Reiter: There are remarkable species differences in the sensitivity of the pineal melatonin-generating system to light at night. One extreme is the Richardson's ground squirrel (*Spermophilus richardsonii*), which requires 1850 μW of light at night to inhibit pineal melatonin production (Reiter et al 1983). At the other end of the spectrum, the albino rat responds to much lower intensity light, in the range of 0.0005 $\mu W/cm^2$ (Webb et al 1985). That means that the two extremes differ by a factor of 3.6×10^7. The human falls somewhere between the two.

Lewy: In our study of suppression of melatonin production by light, we suggested that the reason why humans required brighter light than other species was adaptation. That is, our human subjects were tested at night following a day of exposure to substantially brighter light (outside sunlight is 20 to 200 times brighter than ordinary room light). Most previous studies of laboratory animals were done on animals who had never been exposed to outside sunlight (Lewy et al 1980b).

Zucker: There is little point in trying to struggle with these different light

thresholds without taking into consideration the normal ecology of the species. Why determine the threshold for inhibiting pineal melatonin synthesis in the Richardson's ground squirrel when the animal is almost exclusively diurnally active and is never going to see light during the night? I don't doubt the numbers, but what would be the meaning of even several orders of magnitude difference between the Richardson's ground squirrel and a rat? I also worry about doing this kind of study in albino animals, with their unusual visual systems and changed sensitivity to light.

Reiter: Of course, but there does not seem to be an appreciable influence of pigmentation of the eye *per se* on sensitivity. Both rats with pigmented eyes and albinos are relatively sensitive (Webb et al 1985).

Zucker: But pigmented and albino rats differ markedly in certain responses. For example, constant light will suppress water drinking in an albino animal, but with a hooded rat you will get no effect.

Reiter: Yes, but I was only talking about inhibition of melatonin synthesis at night.

Zucker: So what is the relevance of specifying threshold light levels for a diurnal animal that never sees light at night?

Reiter: These animals also spend most of the day-time underground, but they emerge very quickly into bright light, and what we want to know is whether that light will inhibit pineal melatonin synthesis. It is theoretically possible for a diurnally active animal living in a dense forest, where the light is dim, to have its melatonin peak during the day-time.

Short: Can we explain the extreme sensitivity of the rat on the basis that it is a nocturnally active species?

Reiter: That's an interesting question; it could be a matter of whether the animal is diurnal or nocturnal.

Zucker: In some nocturnally active species, such as the bannertail kangaroo rat and the beach mouse, surface activity is inhibited during moonlight (Lockard & Owings 1974).

Tamarkin: The monkey is diurnally active, but we have found that monkeys kept in laboratory situations for many years are exquisitely sensitive to artificial illumination at night. One rhesus monkey that we received from our outdoor colony was not sensitive to light at night the first time we examined him, but after a year in an artificial facility that same animal showed a suppression of night-time melatonin synthesis in response to light. However, it is not just a question of whether the animal is wild or lab-bred; marmots caught in the wild are exquisitely sensitive to artificial illumination at night and respond with a rapid decline in plasma levels of melatonin.

Klein: It would be very helpful to find out to what extent sensitivity to light varies within a species, and by how many orders of magnitude you can get an animal to change its sensitivity. We could then differentiate between species

and define the dynamic range for a single species, for example for the monkey or human. The second question is, how long are these periods of adaptation? I suspect the process could go on for months until an animal has achieved a new level of sensitivity.

Goldman: What is the physiological relevance of the ability of light to suppress melatonin synthesis acutely? It happens in most species, and Dr Illnerová has suggested that it may reflect some aspect of the phase-shifting mechanism. But in the laboratory, under photoperiods with about the same length as a natural day, melatonin levels fall before lights-on in the morning. So what is the role of acute suppression?

Lewy: We think that the suppressant effect of light may be important in nature in regulating the duration and the timing of night-time melatonin production. Specifically, in animals with τ greater than 24 h under sufficiently long photoperiods, light in the evening could suppress the signal that results in the onset of night-time melatonin production (Lewy 1983). Because of the lag time between sympathetic stimulation and the observed increase in melatonin production (Romero et al 1975), the suppressant effect of light at this time of night may have been underestimated.

Reiter: It is certainly important to think about the natural physiology of these species and the significance of the responses we observe. In its natural habitat the Syrian hamster spends its daylight hours underground in darkness and emerges at night; so it may be exposed to more darkness during the day than during the night. Under those circumstances the peak melatonin concentrations could actually occur during the day, when the animal is in its underground burrow. Yet we know from experience with laboratory-maintained hamsters that moonlight is insufficient to inhibit melatonin production at night (Brainard et al 1984). So perhaps these animals come up from their underground burrows periodically during the day and emerge briefly into the light. We know that even 5 s of light is more than adequate to inhibit melatonin production, and these brief exposures might keep melatonin in check during the day when the animals are normally in total darkness in their underground burrows. It is possible that Syrian hamsters come out once every 6 h to 'sample' the photoperiod during the day. This may be the physiological significance of the acute inhibition of melatonin synthesis by light; even if the intensity is not high, it would delay the rise in melatonin levels for another 4 h or 5 h.

Goldman: I would interpret this slightly differently. I think these animals are essentially exposing themselves to a skeleton photoperiod. If this were done artificially in a standard cage, I would expect the animal's melatonin concentrations to peak at the appropriate time, i.e. during the period defined as 'night' by the skeleton photoperiod. Even in constant darkness there is a rhythm in melatonin synthesis. Thus, it is not that the light received when the animal briefly emerges from the burrow in the middle of the day suppresses melatonin

synthesis; it is more probable that the dawn and dusk cues entrain melatonin secretion so that it occurs at night. So the question is, does the acute suppressive effect of light by contrast with the entraining effect, have any relevance for animals in the field?

Turek: A very simple experiment could be done to investigate this. One could look at animals that are relatively insensitive to the acute effects of light to see what sort of response their circadian system shows to light. I would be surprised if these animals don't entrain to light cues at intensities that do not have an acute suppressive effect on melatonin synthesis.

Menaker: You are all talking about what animals *probably* see in the field, but nobody is measuring it except for Pat DeCoursey. She is monitoring flying squirrels in a relatively natural environment with photocells mounted on their heads. A telemeter device sends information back to the laboratory, so the results should show minute by minute what light the animals are exposed to. Our discussions would be more meaningful if we looked at more species in this way.

Klein: I don't know whether the rapid turn-off of melatonin synthesis at night has any physiological significance, but it would be valuable to determine whether or not the photoreceptors and the light intensity involved are the same as those mediating the entraining effects of light. If we discover that discrete photoreceptors or neural systems control the turn-off effect, we will have a real impetus for thinking about its physiological significance. If not, it may be just a quirk of a system that is physiologically involved in shifting the clock rather than terminating transmission.

Illnerová: With Syrian hamsters, Hudson & Menaker (1984) needed a higher intensity of light to phase-shift the circadian system than to suppress the increase in melatonin formation at night. They measured phase-shifts only in locomotor activity and not in the melatonin rhythm, but if we could show that the light intensities needed to suppress and to phase-shift melatonin production are different, then we could draw some conclusions.

Menaker: We are getting ready to do those experiments, to see whether the dim lights that acutely suppress melatonin secretion also shift the melatonin rhythm. One interesting point about the photoreceptors involved is that RCS rats, which have retinal degeneration that essentially wipes out the rods by the time they are 100 days old, may be as sensitive as normal rats to the acute suppressive effects of light on melatonin synthesis (D. Hudson & M. Menaker, unpublished work).

Klein: Similarly, Russ Reiter and I found that white rats were still sensitive to light even after we had kept them for 9.5 weeks in intense white light, which produced complete retinal degeneration (Reiter et al 1971).

Reppert: We looked at the effects of light on melatonin and other rhythms in cats that were kept in artificial illumination for several years before they were

studied (Reppert et al 1982). By subjecting animals to 8 h phase-delays in the photoperiod we got very nice phase-shifts in melatonin and other circadian rhythms, but we did not get a complete suppression of melatonin secretion. So in this species we have been able to differentiate between the acute and phase-shift effects.

Lewy: However, Mike Menaker reports (p 74) the opposite in the golden hamster. These animals can suppress their melatonin production at intensities of light that cannot shift the phase of circadian rhythms.

Lincoln: How closely coupled are the generating systems for the melatonin rhythm and the sleep–wake cycle? Surely this will dictate whether or not animals expose themselves to light during subjective night.

Klein: In the hamster the two are chained together and free-run in parallel (Tamarkin et al 1980).

Menaker: Is there any animal in which the two are not tied together? Even for humans our original ideas are falling apart; we once thought it was possible to uncouple circadian sleep–wake behaviour from body temperature rhythmicity, but I understand that these classic results are now being critically re-examined.

Arendt: In a very few experiments with Dr R.A. Wever in Munich we seem to be able to dissociate melatonin and temperature rhythms and the sleep–wake cycle in humans.

Zucker: Are you using electrographic measures of sleep?

Arendt: No, we use under-the-bed activity recordings.

Lewy: We have also made some observations on this in humans (Lewy et al 1984). We shifted the light–dark cycle while holding the activity–rest cycle as constant as possible, and found we could shift the melatonin rhythm just by changing the light–dark cycle.

REFERENCES

Banerjee S, Kerr V, Winston M, Kelleher JK, Margulis L 1972 Melatonin: inhibition of microtubule-based oral morphogenesis in Stentor coeruleus. J Protozool 19:108-113

Besharse JC, Iuvone PM 1983 Circadian clock in *Xenopus* eye controlling retinal serotonin *N*-acetyltransferase. Nature (Lond) 305:133-135

Brainard GC, Richardson BA, Hurlbut EC, Steinlechner S, Matthews SA, Reiter RJ 1984 The influence of various irradiances of artificial light, twilight and moonlight on the suppression of pineal melatonin content in the Syrian hamster. J Pineal Res 1:105-109

Cardinali DP, Freire F 1975 Melatonin effects on brain. Interaction with microtubule protein, inhibition of fast axoplasmic flow and induction of crystaloid and tubular formations in the hypothalamus. Mol Cell Endocr 2:317-330

Deguchi T 1975 Ontogenesis of a biological clock for serotonin: acetyl coenzyme A *N*-acetyltransferase in the pineal gland of rat. Proc Natl Acad Sci USA 72:2914-2920

Dubocovich ML 1983 Melatonin is a potent modulator of dopamine release in the retina. Nature (Lond) 306:782-784

Hudson DJ, Menaker M 1984 Pineal melatonin synthesis in the hamster: sensitivity to light. Soc Neurosci Abstr 10:820

Lang U, Aubert ML, Sizonenko PC 1981 Location of melatonin receptors. Pediatr Res 15:80 (abstr)

Lewy AJ 1983 Biochemistry and regulation of mammalian melatonin production. In: Relkin RM (ed) The pineal gland. Elsevier North-Holland, New York, p 77-128

Lewy AJ, Tetsuo M, Markey SP, Goodwin FK, Kopin IJ 1980a Pinealectomy abolishes plasma melatonin in the rat. J Clin Endocrinol & Metab 50:204-205

Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Markey SP 1980b Light suppresses melatonin secretion in humans. Science (Wash DC) 210:1267-1269

Lewy AJ, Sack RL, Singer CM 1984 Assessment and treatment of chronobiologic disorders using plasma melatonin levels and bright light exposure: the clock-gate model and the phase response curve. Psychopharmacol Bull 20:566-568

Lockard RB, Owings DH 1974 Moon-related surface activity of bannertail (*Dipodomys spectabilis*) and Fresno (*D. nitratoides*) kangaroo rats. Anim Behav 22:262-273

Lynch HJ, Rivest RW, Ronsheim PM, Wurtman RJ 1981 Light intensity and the control of melatonin secretion in rats. Neuroendocrinology 33:181-185

Markey SP, Buell PE 1982 Pinealectomy abolishes 6-hydroxymelatonin excretion by male rats. Endocrinology 111:425-426

Menaker M 1982 The search for principles of physiological organization in vertebrate circadian systems. In: Aschoff A et al (eds) Vertebrate circadian systems. Springer-Verlag, Berlin-Heidelberg, p 1-12

Ogino N, Matsumura M, Shirakawa H, Tsukahara I 1983 Phagocytic activity of cultured retinal pigment epithelial cells from chick embryo: inhibition by melatonin and cyclic AMP and its reversal by taurine and cyclic GMP. Ophthalmic Res 15:72-89

Piezzi RS, Cavicchia JC 1981 Effects of cold and melatonin on the microtubules of the toad sciatic nerve. Anat Rec 200:115-120

Poffenbarger M, Fuller GM 1976 Is melatonin a microtubule inhibitor? Exp Cell Res 103:135-141

Reiter RJ 1983 The role of light and age in determining melatonin production in the pineal gland. In: Axelrod J, Fraschini F (eds) The pineal and its endocrine role. Plenum, New York, p 227-241

Reiter RJ, Klein DC 1971 Observations on the pineal gland, the Harderian glands, the retina, and the reproductive organs of adult female rats exposed to continuous light. J Endocrinol 51:117-125

Reiter RJ, Hurlbut EC, Brainard GC, Steinlechner S, Richardson BA 1983 Influence of light irradiance on hydroxyindole-O-methyltransferase activity, serotonin N-methyltransferase activity and radioimmunoassayable melatonin levels in the pineal gland of the diurnally active Richardson's ground squirrel. Brain Res 288:151-157

Reppert SM, Sagar SM 1983 Characterization of the day-night variation of retinal melatonin content in the chick. Invest Ophthalmol & Visual Sci 24:294-300

Reppert SM, Coleman RJ, Heath HW, Keutmann HJ 1982 Circadian properties of vasopressin and melatonin rhythms in cat cerebrospinal fluid. Am J Physiol 243:E489-E498

Romero JA, Zatz M, Axelrod J 1975 Beta-adrenergic stimulation of pineal N-acetyltransferase: adenosine 3′,5′-cyclic monophosphate stimulates both RNA and protein synthesis. Proc Natl Acad Sci USA 72:2107-2111

Takahashi JS, DeCoursey PJ, Bauman L, Menaker M 1984 Spectral sensitivity of a novel photoreceptive system mediating entrainment of mammalian circadian rhythms. Nature (Lond) 308:186-188

Tamarkin L, Reppert SM, Klein DC, Pratt B, Goldman BD 1980 Studies on the daily pattern of pineal melatonin in the Syrian hamster. Endocrinology 107:1525-1529

Teirstein PS, Goldman AI, O'Brien PJ 1980 Evidence for both local and central regulation of rat rod outer segment disc shedding. Invest Ophthalmol & Visual Sci 19:1268-1273

Terman M, Terman J 1985 A circadian pacemaker for visual sensitivity? Ann NY Acad Sci, in press

Tetsuo M, Poth M, Markey SP 1982 Melatonin metabolite excretion during childhood and puberty. J Clin Endocrinol & Metab 55:311-313

Vaněček J, Illnerová H 1982 Night pineal *N*-acetyltransferase activity in rats exposed to white or red light pulses of various intensity and duration. Experientia 38:1318-1320

Webb SM, Champney TH, Lewinski AK, Reiter RJ 1985 Photoreceptor damage and eye pigmentation: influence on the sensitivity of rat pineal N-acetyltransferase activity and melatonin levels to light at night. Neuroendocrinology 40:205-209

Photoperiodism, melatonin and the pineal
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Photic influences on the developing mammal

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Abstract. In adult mammals, the daily light–dark cycle acts via the retinohypothalamic pathway to entrain the circadian clock in the suprachiasmatic nuclei (SCN) and to communicate information about daylength to photoperiodic species. Studies in rats show that during late fetal and early neonatal life, before the retinohypothalamic pathway has innervated the SCN, the maternal circadian system entrains the timing of the developing clock to prevailing lighting conditions. Although the nature of the maternal output signal(s) used to entrain the developing clock has not been elucidated, the maternal SCN are a necessary component of maternal entrainment during both prenatal and postnatal life. Maternal entrainment of the fetal and neonatal clock thus ensures that the developing circadian system is synchronized to the outside world until maturation of the retinohypothalamic pathway permits direct photic entrainment. The maternal circadian system is not only necessary for entrainment of the developing circadian system, but recent studies suggest it may also provide the immature mammal with important photoperiodic information. In the montane vole (*Microtus montanus*) and the Djungarian hamster (*Phodopus sungorus*), the prenatal photoperiod affects postnatal photoperiodic responses, and cross-fostering experiments show that this information about daylength is perceived by the fetus. This prenatal information, in conjunction with postnatal perception of photoperiod, allows the developing animal to determine which way the season is changing and to modify the rate of reproductive maturation accordingly.

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The daily light–dark cycle acts on the circadian timing system to synchronize (entrain) circadian rhythms to the 24 h day and to provide photoperiodic species with information about daylength (Aschoff 1981). The entrainment function of light ensures that circadian rhythms are manifested in proper relationship to each other and to the 24 h day. The photoperiodic function of light enables animals to anticipate predictable seasonal changes and to vary biological responses, such as reproductive function, accordingly.

In mammals, a direct retinohypothalamic pathway transmits photic information for these two functions from the retina to a circadian pacemaker in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Moore 1983). During late fetal and early neonatal life in rodents, before the retinal projection innervates the SCN, studies show that the developing animal is already perceiving lighting changes for entrainment and photoperiodic responses. Interestingly, it is the maternal circadian system that provides the immature animal with entraining information, and this system is also probably the provider of photoperiodic information to the young animal. This paper reviews the evidence that the mother communicates both entraining and photoperiodic signals to her offspring.

Maternal entrainment of the developing circadian system

Prenatal entrainment

Even though circadian rhythms are not overtly expressed in rats until well into the postnatal period, it is now known that the circadian clock in the SCN is oscillating well before that time. Deguchi (1975) first provided evidence that the developing biological clock might be functional and entrainable by the mother during fetal life; the phase of an overt rhythm was monitored under constant conditions during the postnatal period to infer what had happened to the central oscillator underlying the rhythm at an earlier developmental stage. The problem with such indirect studies is that they do not rule out the possibility that some aspect of the birth process itself starts and influences the timing of the developing clock. The possible influence of birth on this system is suggested by the fact that time of day of birth in the rat is coupled to the photoperiod and controlled by a circadian mechanism (Lincoln & Porter 1976, Reppert 1983).

One method that is useful for directly monitoring the oscillatory activity of the SCN themselves in adult rats is the ^{14}C-labelled deoxyglucose (DG) technique (Schwartz et al 1980). This method allows for the simultaneous determination of the rates of glucose utilization of individual brain structures *in vivo* after intravenous injection of tracer amounts of DG (Sokoloff et al 1977). Reppert & Schwartz (1983, 1984b) used the DG procedure to study the function of the SCN in fetal rats directly and proved that an entrainable circadian clock oscillates in the nuclei during late fetal life (Fig. 1).

The DG technique was also used to reveal how the fetal clock receives information about the light–dark cycle to set its timing to the outside world. Light that penetrates the maternal abdomen and uterus does not directly act on the fetal SCN; instead, photic information has to be transduced by

the maternal circadian system to influence the phase of the fetal clock (Reppert & Schwartz 1983). Studies in rats (Reppert & Schwartz 1984a) and hamsters (Davis & Gorski 1983) have shown that the maternal SCN are essential for maternal entrainment since destruction of these nuclei disrupts the timing

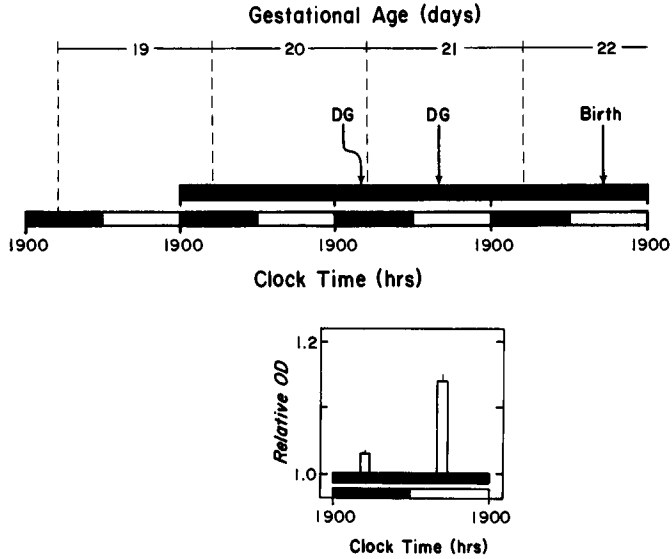

FIG. 1. Deoxyglucose (DG) experiment showing that the fetal SCN manifest a day–night rhythm of metabolic activity. Four pregnant Sprague-Dawley rats were housed in diurnal lighting (lights on from 0700 to 1900 h) throughout pregnancy and outfitted with intra-atrial catheters on gestational day 10. On gestational day 19, the animals were placed in constant darkness in preparation for the DG injection (experimental paradigm depicted in upper panel). Two animals were then injected with DG during the time when the lights would have normally been off (subjective night) on gestational day 20 and the other two were injected during the time when the lights would have normally been on (subjective day) on gestational day 21. At 45 min after injection, the animals were killed and four fetal brains from each pregnant animal were randomly chosen, sectioned and processed for autoradiography. The optical density (OD) of each fetal suprachiasmatic nucleus was measured and the optical density of adjacent hypothalamus was used as an internal reference standard for each fetal brain. The data are thus expressed as relative OD (OD of SCN/OD of adjacent hypothalamus). Each vertical bar in the lower panel gives mean relative OD (±SEM) for the SCN of eight fetal brains. The fetal SCN exhibit a clear day–night rhythm of metabolic activity ($P < 0.01$); the nuclei are metabolically active during the mother's subjective day and inactive during the mother's subjective night. Modified from Reppert & Schwartz (1983).

of the fetal clock. The developing SCN continue to oscillate after maternal SCN ablation but in this case the fetal SCN rhythm is not coordinated to the day–night cycle in the outside world. Thus the maternal circadian system actually entrains an endogenous clock in the fetal nuclei (Fig. 2).

Investigations into the nature of the maternal circadian signal(s) used to entrain the fetal clock have focused on a humoral factor. The basic criterion for such a factor is that it accurately reflects the circadian output of the maternal SCN. Several lines of evidence suggested that the pineal hormone, melatonin, might fill this role (reviewed by Reppert 1982). The rhythm in the concentration of circulating melatonin is of high amplitude, appears to be entrained solely by environmental lighting and is not affected by most forms of stress or end-product feedback. Furthermore, studies have shown that melatonin is rapidly transferred across both the placenta and the blood–brain

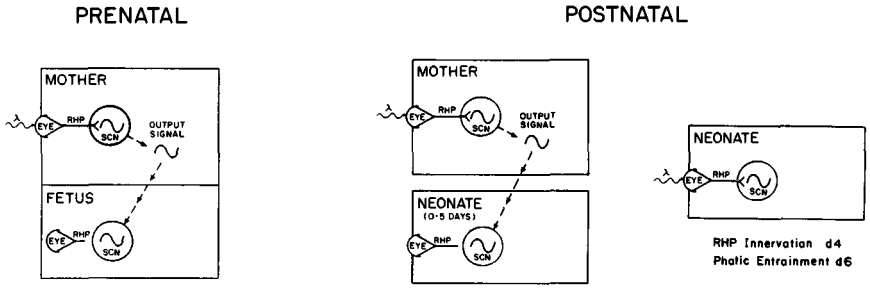

FIG. 2. Entrainment mechanisms during prenatal and postnatal life in the rat. During prenatal and early postnatal life, the maternal circadian system is the source of entraining information to the developing animal. By postnatal day 4 the retinohypothalamic pathway (RHP) has started to innervate the developing SCN. By postnatal day 6 (box on far right), maternal entrainment in waning and retina-mediated photic entrainment commences.

barrier, and that a daily change in maternal melatonin concentrations would be precisely reflected in the fetus; placental transfer of melatonin occurs when the immature rat is incapable of producing its own melatonin. However, melatonin does not appear to be the entraining signal since maternal pinealectomy does not disrupt maternal entrainment of the fetal clock (S. M. Reppert, unpublished work).

Other maternal hormones that are secreted rhythmically into the maternal circulation, such as corticosterone and prolactin, have been investigated as candidates for the entraining signal. Extirpations of the maternal adrenals, pituitary, thryoid, parathyroids and ovaries have been performed but none of these procedures disrupts fetal timing (S. M. Reppert & W. J. Schwartz, unpublished work).

Additional possibilities for the maternal signal centre around neural and behavioural mechanisms. It is conceivable that a neural pathway from the maternal SCN to the placenta (via the autonomic nervous system) may be involved in maternal entrainment of the developing clock, although maternal innervation of the placenta appears sparse.

Maternal behaviour might provide a mechanism for maternal entrainment of the fetal clock in two ways. First, a behaviour such as activity (of the rest–activity cycle) might set the phase of the developing clock by being directly sensed by the fetus. Second, maternal behaviour might influence the fetal clock through a behaviourally initiated but chemically mediated mechanism. For example, a rhythmic behavioural event like feeding initiates increases in the plasma concentrations of substances like glucose and amino acids (along with changes in a variety of gut hormones) that might act to set the time of the developing circadian clock. One other possibility, although not behavioural in nature, is that the daily rhythm of maternal core body temperature might set the phase of the developing SCN.

Postnatal entrainment

Studies in which pineal *N*-acetyltransferase activity, locomotor activity and plasma corticosterone concentrations are used to monitor the developing circadian system in rodents all show that maternal entrainment extends into the postnatal period (Fig. 2; reviewed by Reppert 1985). For these studies, the postnatal rhythm is monitored under constant conditions. Then, by fostering the pups with a mother whose circadian time is out of phase with that of the original mother, one can assess the postnatal influence of the maternal circadian system on the timing of the developing circadian system. Interestingly, the reported potency of the postnatal maternal influence is quite variable among the various laboratories that have studied this issue. However, studies have consistently shown that the magnitude of the postnatal maternal influence changes throughout development, being greatest during the first week of life (Reppert et al 1984, Takahashi & Deguchi 1983).

Maternal SCN lesions abolish not only the prenatal maternal entrainment of the fetal clock, but also the postnatal maternal influence (S. M. Reppert & W. J. Schwartz, unpublished work). The nature of the output signal(s) from the maternal SCN used to influence the timing of the developing clock during the postnatal period is also not known. It is possible that different output signals are used during the prenatal and postnatal periods.

In the rat, by postnatal day 4 the retinohypothalamic pathway has started to innervate the developing SCN (Stanfield & Cowan 1976), and by day 6 retina-mediated light–dark entrainment is functional and capable of overriding any remaining postnatal maternal influence (Reppert 1984). Therefore, when the postnatal maternal influence is decreasing (during the first postnatal week), retina-mediated entrainment commences, leading to the continued presence of an entrainment mechanism (Fig. 2).

Significance of maternal entrainment

Maternal entrainment ensures that the developing circadian system is synchronized to prevailing lighting conditions until maturation of the retina-mediated pathway permits direct photic entrainment. One important function of maternal entrainment might be to synchronize developing processes to the light–dark cycle so that the young animal can assume its ecological niche. This is clearly exemplified in burrow-dwelling mammals whose pups are probably not directly exposed to the daily light–dark cycle for the first few weeks of life, until they can crawl out of the burrow (Pratt 1981). If the pups were not coordinated to the prevailing environmental conditions by the mother, they might emerge from the burrow at an inappropriate time of day, which would render them more susceptible to predation.

Maternal entrainment would also provide the developing animal with a state of internal temporal order, as photic entrainment does in the adult. Without maternal entrainment, circadian rhythms would develop uncoordinated until direct contact with the environment acted to synchronize the phase of the rhythms. Since lack of entrainment and the ensuing desynchronization impair the ability of adults to respond to environmental insults (Fuller et al 1978), such a state might render the neonate especially vulnerable.

A possible reason why the developing clock is functional and entrainable specifically during fetal life is that it may be involved in the initiation of the birth process. As previously mentioned, the time of day of birth in the rat is coupled to the daily light–dark cycle by a circadian mechanism. Since the circadian timing of parturition has been reported in a variety of other mammals, including horses (Rossendale & Short 1967), monkeys (Jolly 1972) and humans (Kaiser & Halberg 1962), the fetal SCN may play a role in this process in many species.

Influence of the prenatal photoperiod on sexual maturation

Daylength, or photoperiod, strongly influences sexual maturation in several seasonally breeding species, including the vole, Djungarian hamster and sheep (Goldman & Darrow 1983). For example, in the montane vole (*Microtus montanus*) and Djungarian hamster (*Phodopus sungorus*) short photoperiods, which simulate winter daylengths, inhibit testicular growth in juvenile males, while longer photoperiods stimulate rapid reproductive development (Horton 1984, Carter & Goldman 1983, Yellon & Goldman 1984).

In the Djungarian hamster, the lactating mother provides her pups with entraining information similar to that discussed above for rats. However, earlier studies suggested that the hamster mothers did not transmit daylength information to the pups; rather, the pups' testicular responses seemed to be

determined entirely by the photoperiod to which they were directly exposed (Pratt 1981, B. L. Pratt & B. D. Goldman, unpublished work). These studies showed that testicular development was not influenced by the photoperiod experienced between birth and 15 days of age, but that Djungarian hamsters became very strongly photoperiodic after day 15. Since hamster pups become mobile and active by about 15 days of age, it seemed that daylength information obtained from the mother would be of little value; the pups probably begin to leave the burrow and expose themselves to the photic environment in time to assess the season and make a rapid 'decision' regarding pubertal development.

In these earlier studies comparisons were made between only very long and very short photoperiods (e.g. 16 h and 10 h daylengths). What would happen if a young Djungarian hamster first encountered an 'intermediate' photoperiod of, for example 14 h? The Djungarian hamster originates from a region of the USSR where a daylength of 14 h would be encountered twice each year, once in mid-April and again in late August. Because of the harsh winter climate in this area, it would clearly be advantageous for the hamster to be able to assess the time of year quickly and to make appropriate reproductive responses.

The elegant experiments of Horton (1984) in the montane vole reveal one way in which a young animal might be able to respond rapidly and appropriately to a potentially ambiguous photoperiod. In Horton's experiments, pregnant voles were exposed to daylengths of 8 h or 16 h throughout gestation. On the day of birth, the mothers and their litters were transferred to an intermediate daylength of 14 h. The young voles were weaned at about 18 days of age and remained in the 14 h daylength until they were killed at 74 days of age. In this experiment, male voles that had undergone gestation in a 16 h photoperiod had smaller testes and weighed less (i.e. were more similar to voles under normal winter conditions) than voles whose mothers had experienced an 8 h daylength during gestation. Thus, when the prenatal photoperiod was longer than the photoperiod experienced after birth, the young vole showed a response appropriate for the autumn season, while an increase in daylength between prenatal and postnatal life elicited a summer-type response. These results suggest that when encountering a daylength of 14 h, young voles can distinguish between 'spring' and 'autumn' depending on whether the photoperiod experienced prenatally was longer or shorter than 14 h, and that appropriate reproductive responses can be made.

In further experiments pregnant female voles were again subjected to different photoperiods during gestation and the litters were transferred to a 14 h daylength after birth. In this study, litters were cross-fostered by mothers who had been exposed to different photoperiods during pregnancy. The results showed that the testicular growth of young voles housed from birth in a 14 h

TABLE 1 Effect of prenatal photoperiod on testicular development in the Djungarian hamster (B. D. Goldman, unpublished work)

Prenatal daylength (h)	*Postnatal daylength (h)*	*Cross-fostered*	*Testis weights*[a]
16	14	No	144 ± 30 (14)
14	14	No	398 ± 49 (15)
12	14	No	411 ± 32 (11)
16	14	To mothers exposed to 14 h photoperiods during gestation	66 ± 38 (7)
14	14	To mothers exposed to 16 h photoperiods during gestation	282 ± 59 (7)

[a]Measured in mg at 34 days of age. Results are expressed as mean ± SEM. Numbers in parentheses indicate numbers of animals in each treatment group.

daylength varied according to the prenatal photoperiod, but was apparently not influenced by the foster mother during lactation (T. H. Horton, personal communication).

Recently, Horton's experiments were repeated in the Djungarian hamster. The results in this species were similar to those obtained with the montane vole. Hamsters were exposed to a 14 h daylength from the day of birth. When gestation occurred in 12 h or 14 h photoperiods, the testes were relatively large at 34 days of age compared to the testes of animals whose mothers were exposed to a 16 h photoperiod during gestation. The results of a cross-fostering experiment, with mothers exposed to 14 h or 16 h photoperiods during gestation, revealed that a photoperiodic message had been received by the fetus (Table 1).

The mechanism by which the fetal vole and hamster perceive photoperiodic information has not yet been elucidated. It seems likely that the mother transmits a photoperiodic message to the fetus, although it remains possible that light that penetrates the mother's body cavity is perceived directly by the fetus. In adult rodents, the circadian pattern of pineal melatonin secretion is essential for the regulation of photoperiodic responses (Goldman & Darrow 1983). As mentioned above, a maternally generated melatonin rhythm would be precisely reflected in the fetal circulation (Reppert 1982). Thus, it is tempting to speculate that the mother may transmit photoperiodic information to the fetus via her pattern of melatonin secretion.

Summary

The results of the studies reviewed in this paper suggest that the mother can transduce photic information into both entraining and photoperiodic sig-

nals for her young. Although the precise nature of these maternal signals has not yet been elucidated, it is clear that such information confers distinct adaptive advantages to the offspring.

REFERENCES

Aschoff J (ed) 1981 Handbook of behavioral neurology. Vol 4. Biological rhythms. Plenum, New York

Carter DS, Goldman BD 1983 Antigonadal effects of timed melatonin infusion in pinealectomized male Djungarian hamsters (*Phodopus sungorus sungorus*): duration is the critical parameter. Endocrinology 113:1261-1267

Davis FC, Gorski RA 1983 Entrainment of circadian rhythms in utero: role of the maternal suprachiasmatic nucleus. Soc Neurosci Abstr 9:625

Deguchi T 1975 Ontogenesis of a biological clock for serotonin:acetyl coenzyme A *N*-acetyltransferase in the pineal gland of rat. Proc Natl Acad Sci USA 72:2914-2920

Fuller CA, Sulzman FM, Moore-Ede MC 1978 Thermoregulation is impaired in an environment without circadian time cues. Science (Wash DC) 199:794-796

Goldman BD, Darrow JM 1983 Pineal gland and mammalian photoperiodism. Neuroendocrinology 37:386-396

Horton TH 1984 Growth and reproductive development of male *Microtus montanus* is affected by prenatal photoperiod. Biol Reprod 31:499-504

Jolly A 1972 Hour of birth in primates and man. Folia Primatol 18:108-121

Kaiser IH, Halberg F 1962 Circadian periodic aspects of birth. Ann NY Acad Sci 98:1056-1068

Lincoln DW, Porter DG 1976 Timing of the photoperiod and the hour of birth in rats. Nature (Lond) 260:780-781

Moore RY 1983 Organization and function of a central nervous system circadian oscillator: the suprachiasmatic hypothalamic nucleus. Fed Proc 42:2783-2789

Pratt BL 1981 Naturalistic studies of photoperiodism in Syrian and Djungarian hamsters. PhD Thesis, University of Connecticut

Reppert SM 1982 Maternal melatonin: a source of melatonin for the immature mammal. In: Klein DC (ed) Melatonin rhythm generating system. Karger, Basel, p 182-191

Reppert SM 1983 Time of birth in the rat is gated to the photoperiod by a circadian mechanism. Pediatr Res 17:154A

Reppert SM 1984 The developing circadian timing system: functional appearance of light-dark entrainment. Pediatr Res 18:144A

Reppert SM 1985 Maternal entrainment of the developing circadian system. Ann NY Acad Sci, in press

Reppert SM, Schwartz WJ 1983 Maternal coordination of the fetal biological clock in utero. Science (Wash DC) 220:969-971

Reppert SM, Schwartz WJ 1984a Ablation of the maternal suprachiasmatic nuclei disrupts the timing of the fetal circadian clock. Pediatr Res 18:144A

Reppert SM, Schwartz WJ 1984b The suprachiasmatic nuclei of the fetal rat: characterization of a functional circadian clock using ^{14}C-labeled deoxyglucose. J Neurosci 4:1677-1682

Reppert SM, Coleman RJ, Heath HW, Swedlow JR 1984 Pineal N-acetyltransferase activity in 10-day-old rats; a paradigm for studying the developing circadian system. Endocrinology 115:918-925

Rossendale PD, Short RV 1967 The time of foaling of thoroughbred mares. J Reprod Fertil 13:341-343

Schwartz WJ, Davidson LC, Smith CB 1980 In vivo metabolic activity of a putative circadian oscillator, the rat suprachiasmatic nucleus. J Comp Neurol 189:157-167

Sokoloff L, Reivich M, Kennedy C et al 1977 The [^{14}C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. J Neurochem 28:897-916

Stanfield B, Cowan WM 1976 Evidence for a change in the retinohypothalamic projection in the rat following early removal of one eye. Brain Res 104:129-133

Takahashi K, Deguchi T 1983 Entrainment of the circadian rhythms of blinded infant rats by nursing mothers. Physiol & Behav 31:373-378

Yellon SM, Goldman BD 1984 Photoperiod control of reproductive development in the male Djungarian hamster (*Phodopus sungorus*). Endocrinology 114:664-669

DISCUSSION

Follett: The natural breeding cycle in quail is quite similar to that of the Djungarian hamster, and therefore we were intrigued to find out what happened to birds born on the same daylength but in one case before the solstice and in the other case after the solstice. My colleague Trevor Nicholls carried out some experiments in which he used 13 h light:11 h dark (13L:11D) as the photoperiod on which to rear the young quail (unpublished work). He exposed the incubating eggs and the chicks to days of either 18 h or 8 h of light before moving all the chicks to 13L:11D at one week of age. The important point is that 13L:11D was read as a *short* day by chicks whose first week of life was spent in 18L:6D, but as a *long* day by those exposed for their first week to 8L:16D. Even more dramatic results were obtained when the eggs were hatched in 13L:11D and on the fourth day of life the chicks were given a single day of either 20 h or 8 h of light. They were then returned to 13 h days. This very long or very short day had as profound an influence upon how the chicks read 13L:11D as the photoperiodic regimen in the first experiment. Therefore, as with adult quail (Robinson & Follett 1982), the response to photoperiods such as 13L:11D is profoundly influenced by the photoperiodic history of the bird. Whether a daylength is read as 'short' or 'long' depends upon the bird's previous exposure. The ecological importance of this could be profound.

Zucker: In rodents the prenatal influence may very well dominate postnatal factors, at least temporarily. In Bruce Goldman's laboratory it has been shown that the prenatal signal influences development in *Phodopus* as late as 34 days of age, and Teresa Horton (1984) found that the photoperiodic signal that montane voles experienced prenatally determined their growth patterns up to 74 days of age. The prenatal maternal message that influences the animals' responses to the external environment is very long-lived. I find that surprising.

Menaker: That kind of thing is very well known in insects, and the mammals in which these observations have been made are mammals whose reproductive

strategies are very similar to those of insects. They are prepared so that as soon as the environmental resources become available the population explodes.

Wetterberg: What do you think about the possible role of the retina and the Harderian gland in the development of rhythms in rats and hamsters? Is there any evidence that these two endocrine tissues are involved?

Reppert: We know that enucleated pregnant animals still transfer signals to the fetuses, so the retina is not the source of the maternal signal. We have not looked at the Harderian gland in the mother.

Moore-Ede: You may get frustrated if you try to follow Occam's razor in these experiments. We all recognize that many subtle signals act as zeitgebers, and that sometimes a couple of subliminal signals add up to create one effective signal. While one cannot eviscerate the mother to remove all endocrine information, your single gland extirpation method may not be a feasible way of discovering the maternal entrainment signal. Trying to infuse an endocrine periodic signal into the mother to drive the fetal system may be more useful.

Reppert: That's a very good point, but removal of single organs seemed a reasonable initial strategy.

Klein: Silver (1977) has shown that in anophthalmic mice, which fail to develop a well-formed optic tract, the SCN are disorganized to an extent that varies from individual to individual in a manner correlated to the organization of the locomotor activity rhythm. This means that the optic tract passing by the SCN may in some way influence the organization of the nuclei.

Reiter: They may be totally independent.

Klein: That's possible, but there is a correlation between the development of the optic tract and the SCN in the animals and I suspect that there is a causal relationship there.

*Armstrong:*It would be interesting to see whether rat mothers are nocturnal in their feeding patterns and whether a nutritional signal is going from the mothers to the pups, in the form of circulating glucose or free fatty acids, rather than a hormonal signal. Perhaps the way to investigate this is to manipulate lighting and feeding schedules.

Reppert: It is conceivable that the signal used prenatally is different from that used postnatally, but food delivery systems may be important for either. We have used restricted feeding paradigms to look at the prenatal influence but have been unable to come up with anything very clear because the deoxyglucose probe may also be sensitive to nutritional manipulations. Postnatally, the pups show a maternally imposed rhythm, feeding mostly during the day. At weaning the pups undergo a 180° phase-shift so that they are now feeding mostly at night. This may be telling us that the developing circadian system is fairly resistant to changes in maternal behaviour postnatally.

Armstrong: Are you saying that the activity rhythm of the pups is dissociated from the feeding rhythm, i.e. that the pups are active at night but feed from the mother during the day?

Reppert: Yes. It's because the mother spends more time with the litter during the day since she is most active and is doing most of her feeding at night.

Armstrong: So the feeding rhythm in suckling pups is maternally enforced, i.e. exogenous?

Reppert: That's right.

*Illnerová:*You mentioned that postnatal entrainment by the mother can continue until about the fifth day after birth. The temperature in the nest may be important for this because it changes from day to night. During the night the mother leaves the nest more often. You could study this by keeping mother and pups together in one cage but with a second cage adjoining; Ader & Grota (1970) have found that mothers leave the nest much more frequently when they can go into another cage, so the entrainment might be stronger in this situation.

Reppert: That's a very good point. There is a vast discrepancy between laboratories in terms of how potent people think the postnatal influence is. The discrepancy may be due to strain differences or to differences in experimental design. The way to study this may well be to look at animals in a more naturalistic setting.

*Illnerová:*Even fetal entrainment might be a question of temperature; in lower vertebrates you can certainly get entrainment by a 24 h warm–cold schedule. Mothers might entrain their fetuses by their own rhythms in body temperature. Unfortunately this is very hard to study.

My other point concerns the synchronization of rhythms. When your rat mothers had SCN lesions, you concluded that rhythms might exist in new-born pups but they were difficult to find because they were not synchronized. We tried to look at this using chicken eggs to eliminate any maternal influence, and found exactly the same thing. We kept the eggs in constant darkness and two or three days before hatching we killed the chicks. We found a wide range of pineal *N*-acetyltransferase activities, corresponding to basal day-time values as well as to high night-time values, and no evidence of a rhythm, but this might be only because the rhythms in individual chicks were not synchronized.

Arendt: This work on maternal entrainment could have very important implications in clinical medicine. Do you know what happens to the development of circadian rhythms in humans when there is maternal separation postnatally? We have tried to look at postnatal development of pineal function in people, taking sequential urine samples from babies every 4 h during the first eight weeks of life. We looked at both melatonin and its major metabolite, but unfortunately there is so little produced that we cannot draw any firm conclusions. However, in some babies there seems to be some sort of rhythm at about six weeks of age, which is when the babies develop a sleep–wake cycle.

Reppert: Lou Sander has considered maternal influences on the development of human rhythms, and particularly the sleep–wake cycle (Sander et al 1972). He found that nursery-reared babies who were kept in constant environmental lighting conditions and who had an interaction with nurses every 4 h did

not appear to develop a strong diurnal variation in sleep–wake pattern, whereas infants who were reared by their mothers or foster mothers and who could demand an interaction when they wanted one developed a diurnal sleep–wake pattern by the end of the first postnatal week. He also did cross-fostering experiments which suggested that maternal influences are important. The problem in these studies is separating a passive, maternally driven response of the infant from an interaction that increases coupling strength between the circadian pacemaker and the other structures that to lead to the overt expression of a daily sleep–wake cycle.

I am intrigued by your melatonin work in babies and the utilization of the melatonin-generating system to look at the development of the human circadian system. One of the most important questions in terms of the developing circadian system is, when is the developing human sensitive to light at the level of the hypothalamus? I have always felt that studying the melatonin-generating system and its suppression by light may be an appropriate way to find out when that system is working, but I'm a bit disappointed that you are not able to show a rhythm earlier than six weeks.

REFERENCES

Ader R, Grota LJ 1970 Rhythmicity in the maternal behaviour of *Rattus norvegicus*. Anim Behav 18:144-150

Horton TH 1984 Growth and reproductive development of male *Microtus montanus* is affected by the prenatal photoperiod. Biol Reprod 31:499-504

Robinson JE, Follett BK 1982 Photoperiodism in Japanese quail: the termination of seasonal breeding by photorefractoriness. Proc R Soc Lond B Biol Sci 215:95-116

Sander LW, Julia HL, Stechler G, Burns P 1972 Continuous 24-hour interactional monitoring in infants reared in two caretaking environments. Psychosom Med 34:270-282

Silver J 1977 Abnormal development of the hypothalamus in a strain of genetically anophthalmic mice. J Comp Neurol 176:589-606

Photoperiodism, melatonin and the pineal
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Generation of melatonin rhythms

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Abstract. In mammals, information about the environmental photoperiod is relayed from the retina to the suprachiasmatic nuclei (SCN) in the anterior hypothalamus and via the sympathetic nervous system to the pineal gland where it influences the secretion of melatonin. Light plays a dual role: (1) to suppress the release of melatonin and (2) to entrain the circadian rhythm generators in the SCN, which govern the endogenous melatonin rhythm. Under normal daily light–dark cycles melatonin secretion is confined to the dark period. In most photoperiodic species the daily pattern of secretion changes in response to changes in daylength, and this acts as a physiological time cue in the brain for the control of seasonal cycles in reproduction, moulting and other processes.

To illustrate the underlying mechanisms that control the melatonin rhythm, results are presented from five experiments in which the blood plasma concentrations of melatonin were measured in Soay rams exposed to a variety of artificial changes in photoperiod including a switch from 16L:8D (16 h light:8 h dark) or 8L:16D to constant darkness, a switch from constant darkness to 1L:23D and a switch from 16L:8D to a 25 h or 23 h light–dark cycle. The results confirm that the melatonin rhythm is generated endogenously and will free-run under constant darkness with a period close to 24 h for at least 10 days. The rhythm can be entrained by exposure to IL:23D with the end of the light period acting as the 'melatonin-on' signal, and phase-shifts in the melatonin rhythm can be induced by phase-shifts in the light–dark cycle. The period for which melatonin concentrations are high each day (melatonin peak) also varies in duration under the different photoperiods, as a result of both the suppressive and the entraining effects of light. Two models explaining the control of melatonin peak duration are discussed.

1985 Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Foundation Symposium 117) p 129-148

The secretion of melatonin from the pineal gland shows a marked daily rhythm in all species of mammals investigated (Goldman 1983, Tamarkin et al 1985). Pineal activity is increased at night in both diurnal and nocturnal species as shown by studies of the pineal content of melatonin and associated enzymes in rodents, the concentrations of melatonin in peripheral blood and cerebrospinal fluid in sheep, horses, monkeys and humans and the levels of urinary metabolites of melatonin in rats and humans. The close link between the melatonin rhythm and the daily light–dark cycle is well established. However,

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when animals are shifted from a regular light–dark cycle to constant darkness the rhythm persists with a period close to 24 h (e.g. pineal enzymes in rats, Klein & Weller 1970; melatonin levels in the blood of sheep and horses, Rollag & Niswender 1976, Kilmer et al 1982; melatonin levels in the cerebrospinal fluid of macaques, Perlow et al 1981). The rhythm in melatonin is therefore circadian; it is generated endogenously but normally receives its temporal entrainment from the environmental light–dark cycle.

The neural mechanisms governing the secretory activity of the pineal gland have been determined largely from studies with the rat where the level of the pineal enzyme *N*-acetyltransferase (NAT) is often used as an index of melatonin production (Moore & Klein 1974, Klein & Moore 1979). Neurons in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus appear to act as the circadian pacemaker for rhythms in pineal NAT activity. Photic information reaches the SCN by way of the retinohypothalamic tract and the sympathetic nervous system serves as a relay between the SCN and the pineal gland. Noradrenaline released from the sympathetic nerve terminals, which are in intimate contact with the pineal cells, induces a cyclic AMP-mediated activation of NAT, which catalyses the conversion of serotonin to *N*-acetylserotonin, a precursor of melatonin. Activity in the sympathetic nerves innervating the pineal gland is assumed to increase at night leading to enhanced melatonin synthesis and release.

The neurons in the SCN show a circadian rhythm in electrical and metabolic activity (Inouye & Kawamura 1979, Schwartz et al 1980), which is influenced by the daily light–dark cycle. Electrical stimulation of the SCN strongly inhibits the electrical activity of the central sympathetic nervous system, as does the direct application of light to the retina or optic nerve stimulation (Nishino et al 1976). Thus, enhanced activity in the SCN or exposure to light leads to reduced pineal stimulation and decreased melatonin secretion. Carbachol, a cholinergic agonist, mimics the effect of light on the circadian rhythm in the pineal gland, indicating that acetylcholine is one of the neurotransmitters involved in photic effects on the SCN (Zatz & Brownstein 1979).

These observations on pineal function indicate that light has two effects on the daily rhythm of melatonin synthesis and secretion: (1) it suppresses the production of melatonin and (2) it entrains a circadian pacemaker in the SCN to set the phase of the rhythm relative to the light–dark cycle (Tamarkin et al 1985). The suppressive effect is rapid and intensity dependent and readily seen if animals are exposed to bright light at specific times during the dark period. In the rat, for example, a sudden exposure to light at night leads to a rapid decline in NAT activity in the pineal gland (4 min half-life); comparable effects are seen after the administration of β-adrenergic blockers, which prevent sympathetic nerve stimulation of the pineal gland (Vanecek & Illnerova 1979). Rollag & Niswender (1976) have shown that the peripheral blood

levels of melatonin decline rapidly in sheep when lights are switched on at night.

The entraining role of light in the generation of the melatonin rhythm is indicated by the way the daily melatonin rhythm is closely coupled to the light–dark cycle. However, the increased melatonin levels need not span the total period of darkness. In the rat and Syrian hamster pineal levels of melatonin begin to rise several hours after the onset of darkness and are elevated for 6–8 h irrespective of the duration of darkness (Goldman et al 1982). When sheep are exposed to a 48 h light–dark cycle of 8L:40D (8 h light:40 h dark) the 24 h rhythm in blood levels of melatonin persists since it can be entrained by the 8 h light period which occurs at the same circadian clock-time every other day; the rhythm is lost upon exposure to a 36 h cycle of 8L:28D since the circadian system fails to entrain to this period (Almeida & Lincoln 1982). Other studies in sheep have shown that after an 8 h advance in the time of lights-out produced by an abrupt switch from 16L:8D to 8L:16D there is a gradual phase-advancement of the onset of the daily melatonin peak leading to a coincidence between the onset of the peak and the onset of darkness in as few as two days but taking up to 14 days in some individuals (Bittman et al 1983). The most impressive studies on entrainment are those of Illnerova & Vanecek (1982) in which pineal NAT levels were measured in groups of rats exposed to a 1 min light pulse at different times during the night. They found that such light pulses led to phase-advances or delays of the rhythm depending upon when the light was given and that there appeared to be separate circadian oscillators governing the onset and decline of pineal activity.

Studies on the generation of the melatonin rhythm are still in their infancy but are important because they indicate how information about the external light–dark cycle is transduced into an endocrine signal leading to specific responses in the brain (see E. L. Bittman, this volume). We now present some recent observations recorded in sheep to illustrate further how the phase and duration of the melatonin rhythm are influenced by the light–dark cycle.

Materials and methods

The observations were made on groups of four to eight adult Soay rams housed in individual pens in light-proof sheds. Blood was sampled by means of a jugular catheter (Lincoln & Short 1980). Lighting of about 160 lux at the level of the rams' eyes was provided by white fluorescent strip lights. In addition a 15 W red bulb was used throughout to provide a minimal illumination during darkness to allow the animals to feed and to facilitate sampling. In five separate experiments, groups of rams were subjected to changes in the photoperiod (see results section) and hourly blood samples were collected

for 24–100 h at the beginning and end of the treatments for the measurement of melatonin.

The concentration of melatonin in the blood plasma was measured by radioimmunoassay by the method of Rollag & Niswender (1976) as modified by Almeida (1982). The lower limit of detection was 10 pg/ml and intra-assay coefficient of variation, based on duplicates of quality-control plasma pools, was 13.9%. All serial samples from individual animals in each experiment were assayed in the same or adjacent assays.

Analysis

The daily profiles of melatonin were analysed for peaks by a method developed with the help of Dr P. Warner (Ebling 1985). The procedure was as follows:

(1) all melatonin values for each ram in each sampling period were ranked and divided into 10–20 pg/ml classes to obtain a frequency distribution;

(2) this was used to assess any discontinuity between high and low values; if there was no discontinuity evident the median value was determined;

(3) a runs test (Seigel 1956) was performed on each profile to determine whether there was significant clustering of the melatonin values relative to the discontinuity/median;

(4) where significant clustering was observed, the melatonin peak was defined as the period with four or more consecutive values above the discontinuity/median; shorter runs separated by only a single low value were taken as an extension of the same peak.

The time of the onset of the melatonin peak was used as the phase reference point, and the period of the rhythm was taken as the time between the onset of successive peaks. The significance of differences in the duration of peaks was assessed by Student's *t* test. Some of the results of Experiment 1 have been published previously (Almeida & Lincoln 1984).

Results

Experiment 1: long days to constant darkness

Four rams held initially on long days (16L:8D) were exposed to constant darkness for 10 days and the hourly changes in the blood plasma concentrations of melatonin were measured on days 1–3 and day 10 (Fig. 1). Significant melatonin peaks occurred on day 1, with the onsets just before or coincident with the onset of darkness. Peaks were also evident on days 2, 3 and 10 in each ram, indicating that the melatonin rhythm persisted under constant darkness. The period of the rhythm was initially 24.3 ± 1.2 h (mean ± SEM,

based on peak onsets for days 1 and 3) and later 23.0 ± 0.4 h (based on days 3 and 10). The mean duration of the melatonin peak on day 1 (13.8 ± 1.9 h; long day into darkness) was not significantly different from that under constant darkness on day 10 (13.0 ± 1.2 h).

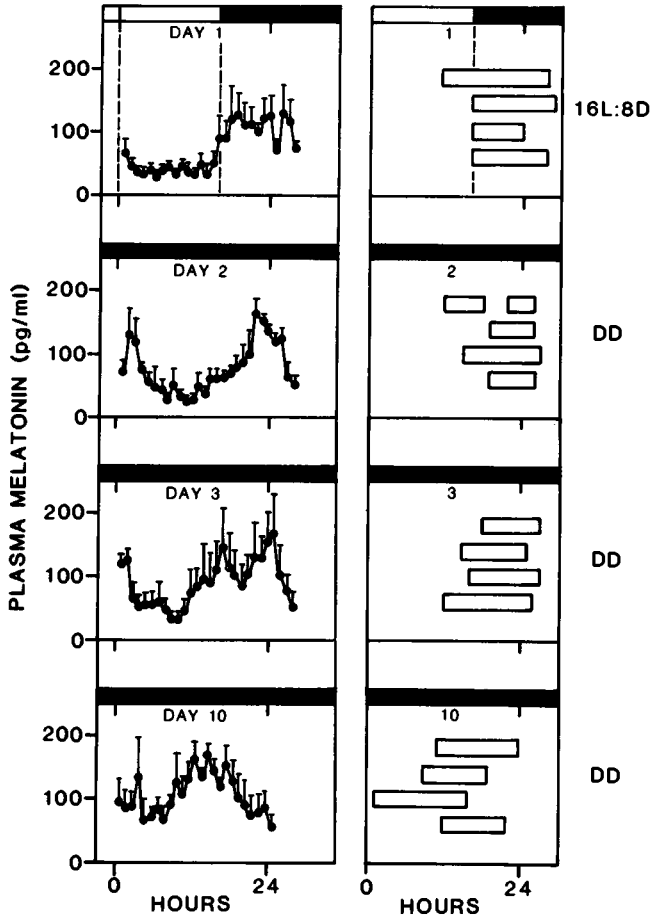

FIG. 1. Melatonin concentrations in blood plasma (mean ± SEM, $n = 4$) and timing of melatonin peaks (open bars for individual rams) in Soay rams transferred from 16L:8D to constant darkness. Samples were collected hourly for 24 h on days 1, 2, 3 and 10 (day 1 taken as last day under 16L:8D).

Experiment 2: constant darkness to a 1 h light pulse every 24 h

Seven rams housed under constant darkness for eight weeks were exposed to a 1 h light pulse every 24 h (1L:23D) for a further two weeks and blood

samples were collected for two days at the beginning and end of the 1L:23D treatment (Fig. 2). Significant melatonin peaks were evident in the plasma profiles of all the rams at the end of the eight weeks under constant darkness (day 1), though the peaks were randomly distributed throughout 24 h with no evidence of synchrony between individual rams. Exposure to the first 1 h light pulse was associated with a transitory decrease in the plasma levels of

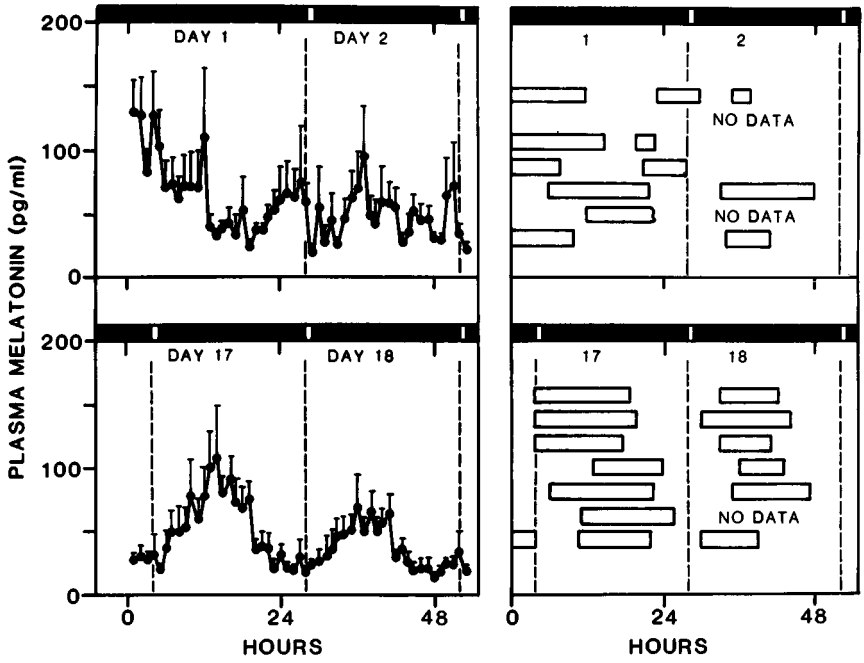

FIG. 2. Melatonin concentrations in blood plasma (mean ± SEM, $n = 7$) and timing of melatonin peaks (open bars for individual rams) in Soay rams exposed to a 1 h light pulse every 24 h (1L:23D) after an eight-week period under constant darkness. Samples were collected on days 1–2 and 17–18 (day 1 taken as the last day under constant darkness).

melatonin but did not cause an immediate synchronization of the rhythms (see mean melatonin levels, days 1 and 2, Fig. 2). However, after 16 days under 1L:23D the synchronizing effect of the light pulse was clearly evident; all the rams showed significant melatonin peaks within the first 8 h after the light pulse (mean time from end of light pulse to onset of peak: 2.9 ± 1.0 h) and the period between the peaks was close to 24 h (mean period 25.8 ± 1.6 h). The mean duration of the melatonin peaks under 1L:23D was 13.3 ± 0.9 h (Fig. 2, days 17 and 18).

Experiment 3: long days to a 1 h light pulse every 24 h with phase-shift

Eight rams preconditioned to long days (16L:8D) were exposed to 32 h darkness followed by a 1 h light pulse every 24 h (1L:23D) for two weeks. During this period the end of the light period was thus phase-advanced by 7 h. The plasma concentration of melatonin was measured in blood samples collected

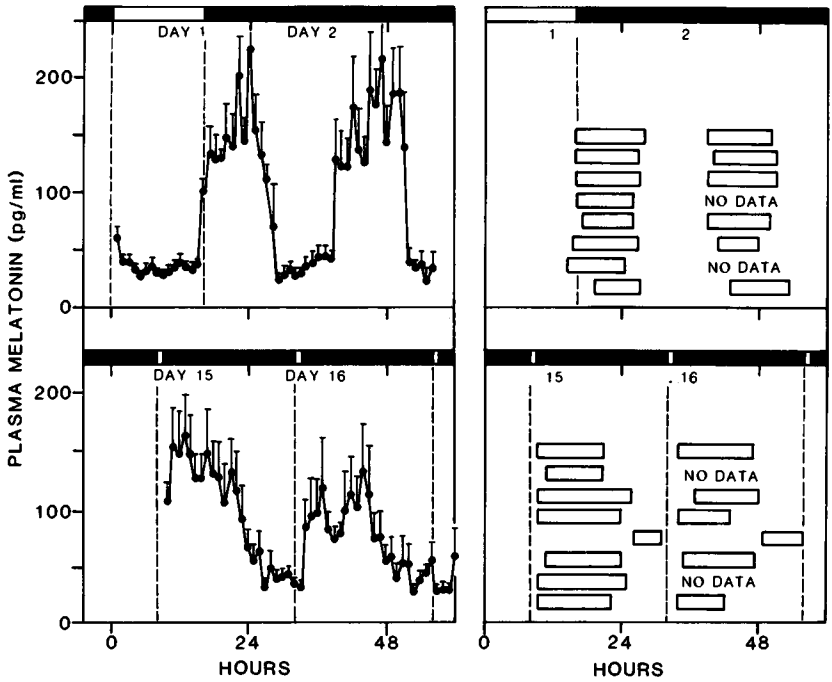

FIG. 3. Melatonin concentrations in blood plasma (mean ± SEM, $n = 8$) and timing of melatonin peaks (open bars for individual rams) in Soay rams transferred from 16L:8D to 32 h darkness and then to a 1 h light pulse every 24 h (1L:23D). The end of the light pulse was set 7 h in advance of the time of the end of the light period under 16L:8D. Samples were collected on days 1–2 and 15–16 (day 1 taken as the last day under 16L:8D).

at hourly intervals for two days during the change from long days to constant darkness and again after 13 days under the 1L:23D regimen (Fig. 3). Significant melatonin peaks were observed in all the rams on day 1 with the onset of the peaks closely synchronized to the onset of darkness. The peaks recurred on day 2 after a period close to 24 h (mean period 23.5 ± 0.4 h), indicating the persistence of a circadian rhythm of melatonin under constant darkness. The mean duration of the melatonin peak on day 1 (11.3 ± 0.5 h) was not

significantly changed by day 2 (12.0 ± 0.5 h). After 13 days under the 1L:23D regimen significant melatonin peaks were still evident in all the rams, but there was a phase-advance in the timing of the peaks; their onset now occurred within 4 h following the 1 h light pulse in seven out of eight rams. The mean period of the melatonin rhythm under 1L:23D was 25.0 ± 0.7 h. The mean duration of the melatonin peak was 12.7 ± 1.0 h, which was not significantly different from the values for days 1 and 2 (Fig. 3).

Experiment 4: short days to two 1 h light pulses every 24 h

Eight rams held initially under short days (8L:16D) were exposed to darkness for 32 h followed by a regimen with two 1 h light pulses every 24 h (1L:7D:1L:15D) for two weeks. The timing of the pulses was arranged so

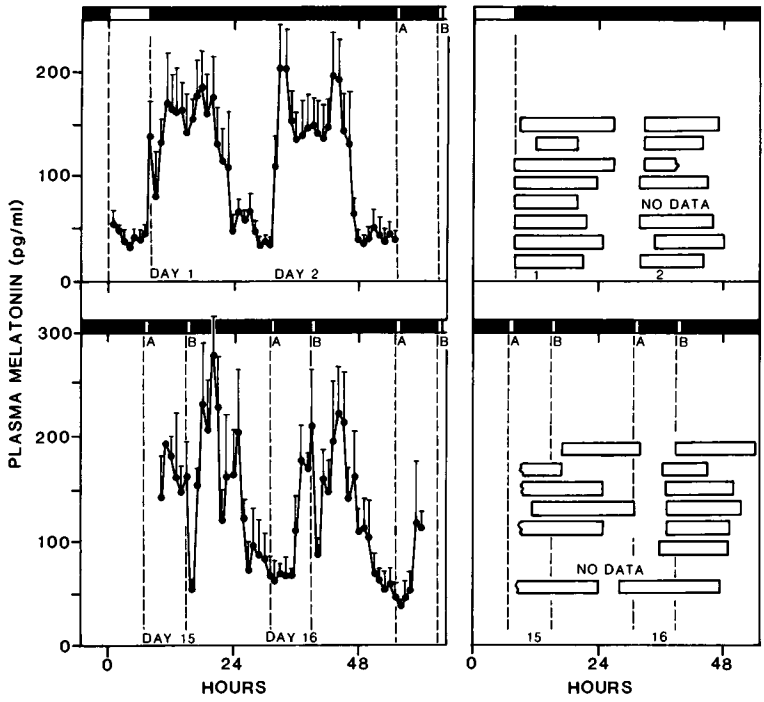

FIG. 4. Melatonin concentrations in blood plasma (means ± SEM, $n = 8$) and timing of melatonin peaks (open bars for individual rams) in Soay rams transferred from 8L:16D to 32 h darkness and then to two 1 h light pulses every 24 h (1L:7D:1L:15D). The end of light pulse A was set at the same time as the end of the light period under 8L:16D. Samples were collected on days 1–2 and 15–16 (day 1 taken as the last day under 8L:16D).

that the first pulse (pulse A) ended at the same time as the light period under the initial 8L:16D regimen, while the second pulse (pulse B) was given 8 h later. Blood samples were collected for two days during the change from short days to constant darkness and again after 13 days under 1L:7D:1L:15D (Fig. 4). On day 1 significant melatonin peaks were evident in all the rams with the onset of the peaks closely synchronized to the time of lights-out. Melatonin peaks were also evident on day 2, indicating that the circadian rhythm of melatonin secretion persisted under constant darkness (mean period 24.1 ± 0.7 h). There was no significant change in the mean duration of the melatonin peaks from day 1 (15.6 ± 1.3 h) to day 2 (13.8 ± 0.5 h). After 13 days of the 1L:7D:1L:15D regimen, significant daily melatonin peaks were still evident in all the rams. Analysis of the peaks was confounded by the fact that sampling was commenced during melatonin peaks in four of the animals. However, in six of the seven rams for which there were data, the onset of the melatonin peak was delayed compared to days 1 and 2; the onset occurred midway between the first and second light pulses (Fig. 4, day 16). The second pulse caused a transitory decline in plasma melatonin levels in all the animals (Fig. 4, days 15 and 16, pulse B). The mean duration of the melatonin peaks under 1L:7D:1L:15D was 13.9 ± 0.7 h, which was not significantly different from the values on days 1 and 2.

Experiment 5: long days to a 25 h or 23 h light–dark cycle

Six rams held initially under long days (16L:8D) were exposed to a 25 h light–dark cycle (16L:9D) for 13 days, returned to long days (16L:8D) for 13 days and then exposed to a 23 h cycle (16L:7D) for 13 days. The effect was first to phase-delay the onset of darkness by 1 h daily for 13 days and then gradually to phase-advance the onset of darkness back to its original time. The hourly changes in the plasma concentrations of melatonin were measured at the onset and end of the two treatments (Fig. 5). On day 1 under 16L:8D a significant melatonin peak was evident in all the rams, with the onset of the peaks closely synchronized to the end of the light period and a mean peak duration of 9.8 ± 0.3 h. After 13 days under the 25 h cycle the melatonin peak was shifted in clock time in four of the six animals to follow the change in the light–dark cycle; in these animals the onset of the melatonin peak preceded the end of the light period by 2–3 h. After a further 13 days under 16L:8D the timing of the melatonin peak had fully adjusted to the light cycle in five out of the six animals and the onset of the peak was now coincident with or occurred within 2 h following lights-out. At this stage the melatonin rhythm was clearly phase-shifted relative to day 1 (Fig. 5, day 37 vs. day 1). After 13 days of the 23 h cycle the timing of the melatonin peaks coincided with the dark

period in five of the six rams, indicating a phase-advance in the melatonin rhythm to follow the light–dark cycle.

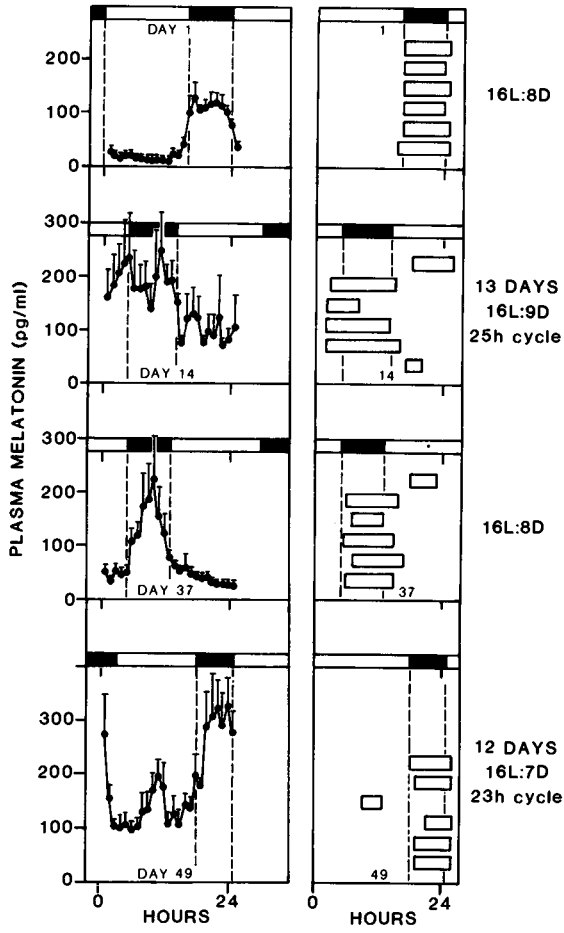

FIG. 5. Melatonin concentrations in blood plasma (mean ± SEM, $n = 6$) and timing of melatonin peaks (open bars for individual rams) in Soay rams transferred from 16L:8D to a 25 h cycle of 16L:9D for 13 days, switched back to 16L:8D for 13 days and then transferred to a 23 h cycle of 16L:7D for 12 days. Samples were collected hourly for 24 h at the beginning and end of the 25 h and 23 h cycle treatments.

Discussion

The results illustrate three aspects of the control of the daily rhythm in melatonin secretion: (1) the rhythm is generated endogenously and persists with

a period close to 24 h under constant darkness (e.g. Experiments 1, 3 and 4); (2) a light–dark cycle with a period of 24 h, as occurs naturally, or one close to 24 h will entrain the melatonin rhythm (e.g. Experiments 2, 3, 4 and 5); (3) light has a rapid suppressive effect on melatonin release at least during the subjective night (e.g. Experiment 4). By using sheep in these studies we have been able to monitor hourly changes in blood plasma levels of melatonin over many hours during changes in the photoperiod; this allowed the analysis of the hormone profiles of individual animals for changes in the phase of the melatonin rhythm and the duration of the daily melatonin peak. Most previous studies of the generation of the melatonin rhythm have involved killing groups of animals to measure the pineal content of melatonin or related enzymes; it was thus not possible to assess individual responses and the assumption was that glandular content parallels release.

Previous work on sheep kept under natural or artificial 24 h light–dark cycles has shown that the period for which levels of melatonin are high in peripheral blood closely reflects the period of darkness (Rollag & Niswender 1976, Arendt et al 1981, Bittman et al 1983). However, the extent to which this daily pattern is the result of the entraining effect of the light–dark cycle or of the more direct suppressive effect of light is not immediately apparent. In the present experiments constant darkness and light pulses of short duration were used to provide clues to the mechanism involved. When sheep were exposed to a 1 h light pulse every 24 h after a period under constant darkness, the melatonin rhythm became entrained such that the onset of the melatonin peak occurred shortly after the brief light period. This occurred in rams that previously had poorly defined melatonin rhythms due to prolonged exposure to constant darkness (Experiment 3) and in rams with well-synchronized rhythms due to recent exposure to cycles of 16L:8D (Experiment 3). These results indicate that the 24 h light cycle entrains the endogenous melatonin rhythm; the end of the light period provides the 'melatonin-on' signal.

The duration of the daily period of increased melatonin secretion is also affected by the photoperiod. It is longer under short days than under long days (e.g. 8L:16D vs. 16L:8D, Bittman et all 1983). Therefore, besides setting the phase of the melatonin rhythm the photoperiod modifies the duration of secretion. We found that the mean duration of the melatonin peak under constant darkness and under the 1L:23D regimen was 12–14 h (e.g. Experiments 1, 2 and 3), while the peak duration under long days was <10 h (Experiment 5). One explanation for this effect of daylength upon duration of the melatonin peak is that the end of the light period entrains the onset of the melatonin peak, while the beginning of the light period acts to suppress melatonin secretion directly. Under the 16L:8D regimen the duration of the night (8 h) is less than the duration of the endogenous melatonin peak of 12–14 h observed under constant dark; thus the suppressive effect of light at dawn

curtails melatonin secretion and leads to a short duration peak. Consistent with this is the observation that the duration of the melatonin peak is extended when dawn is delayed, for example on day 1 when rams were transferred from 16L:8D to constant darkness (Experiments 1 and 3). This model in which 'lights-off' acts to entrain the endogenous rhythm and 'lights-on' acts to suppress melatonin secretion is similar to that proposed for the golden hamster, although in that species the roles of dawn and dusk may differ since dawn appears to provide the principal entraining signal (Tamarkin et al 1980).

The work of Illnerova & Vanecek (1982) on the pineal NAT rhythm in rats provides an alternative model for the control of melatonin secretion by photoperiod. After exposing groups of rats to a 1 min light pulse at different times during the dark period they found that the suppressive effect of light upon pineal NAT levels varied according to when the treatment was given. Early in the dark period a brief light pulse caused only a transitory decline in NAT levels, but a similar light pulse given late in the night caused a much longer suppression of pineal activity. Further studies, which involved keeping animals in constant dark after the pulse treatments, revealed that the suppressive effects were associated with phase changes in the circadian rhythm governing NAT activity; phase-delays were induced by light pulses given early in the dark period whereas phase-advances were induced by pulses late in the night. The phase–response curve for the evening increase in NAT activity was different from that for the morning decrease in NAT activity and it was concluded that separate circadian oscillators control the onset and end of NAT activity (Illnerova & Vanecek 1982). These observations suggest that the photoperiod may influence the duration of the daily melatonin peak by dictating the relative phase of the underlying oscillators. Thus under long days the evening oscillator system might be delayed and the morning oscillator advanced, resulting in a melatonin peak of short duration, while the reverse changes might occur under short days, leading to a peak of longer duration. The advantage of this model over the one discussed initially is that it can explain how the period of melatonin secretion during light–dark cycles can be longer than the period of secretion observed under constant darkness when the control is endogenous; for example, under 8L:16D the peak duration may approach 16 h. With a multi-oscillator control it might by predicted that under prolonged darkness the melatonin rhythm would split into two components or become disorganized. Indeed, poorly defined and variable melatonin rhythms were observed in the rams held for eight weeks under constant darkness (Experiment 2).

One recent study on sheep has shown that a 1 h light pulse given in the dark phase under 8L:16D causes only a transitory suppression of melatonin levels when given early in the night but causes longer-term suppression when given later in the night (Brinklow et al 1984). The late-night interruption

regimen thus produces a short-duration melatonin peak, i.e. a long-day response. In our study the exposure of rams to two 1 h light pulses every 24 h failed to produce a short-duration melatonin peak; the onset of the melatonin peak occurred midway between the two pulses and the second pulse caused only a transitory suppression of melatonin secretion (Experiment 4). It remains to be established whether a two-pulse regimen with a longer interval between the pulses can be used as a skeleton photoperiod to control the duration of the melatonin peak.

In conclusion, we have presented results for the sheep illustrating that the daily melatonin rhythm is generated endogenously and light acts both to entrain the rhythm and to suppress melatonin secretion. This dual effect of light dictates the phase of the melatonin rhythm under a normal 24 h light–dark cycle and determines the duration of the daily melatonin peak.

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REFERENCES

Almeida OFX 1982 Melatonin and the control of seasonal breeding in the Soay ram. PhD Thesis, University of Edinburgh

Almeida OFX, Lincoln GA 1982 Photoperiodic regulation of reproductive activity in the ram: evidence for the involvement of circadian rhythms in melatonin and prolactin secretion. Biol Reprod 27:1062-1075

Almeida OFX, Lincoln GA 1984 Central mechanisms in the control of seasonal breeding. Acta Zool Fenn 171:151-156

Arendt J, Symons AM, Laud C 1981 Pineal function in the sheep: evidence for a possible mechanism mediating seasonal reproductive activity. Experientia 37:584-586

Bittman EL 1985 The role of rhythms in the response to melatonin. In: Photoperidoism, melatonin and the pineal. Pitman, London (Ciba Found Symp 117) p 149-169

Bittman EL, Dempsey J, Karsch FJ 1983 Pineal melatonin secretion drives the reproductive response to daylength in the ewe. Endocrinology 113:2276-2283

Brinklow BR, Forbes JM, Rodway RG 1984 Melatonin in the plasma of growing sheep subjected to short and skeleton long photoperiods. Experientia 40:758-760

Ebling FJP 1985 Central mechanisms in the control of seasonal breeding in the Soay ram. PhD Thesis, University of Edinburgh

Goldman BD 1983 The physiology of melatonin in mammals. Pineal Res Rev 1:145-182

Goldman BD, Carter DS, Hall VD, Roychoudhury R, Yellon SM 1982 Physiology of pineal melatonin in three hamster species. In: Klein DC (ed) Melatonin rhythm generating system. Karger, Basel, p 210-231

Illnerova H, Vanecek J 1982 Two-oscillator structure of the pacemaker controlling the circadian rhythm of *N*-acetyltransferase in the rat pineal gland. J Comp Physiol 145:539-548
Inouye ST, Kawamura H 1979 Persistence of circadian rhythmicity in a mammalian hypothalamic 'island' containing the suprachiasmatic nucleus. Proc Natl Acad Sci USA 76:5962-5966
Kilmer DM, Sharpe DC, Berglund LA, Grubaugh W, McDowell KJ, Peck LS 1982 Melatonin rhythms in pony mares and foals. J Reprod Fertil (suppl) 32:303-307
Klein DC, Moore RY 1979 Pineal *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase control by the retinohypothalamic tract and the suprachiasmatic nucleus. Brain Res 174:245-262
Klein DC, Weller JE 1970 Indole metabolism in the pineal gland. A circadian rhythm in *N*-acetyltransferase activity. Science (Wash DC) 169:1093-1095
Lincoln GA, Short RV 1980 Seasonal breeding: Nature's contraceptive. Recent Prog Horm Res 36:1-52
Moore RJ, Klein DC 1974 Visual pathways and the central neural control of a circadian rhythm in pineal serotonin *N*-acetyltransferase activity. Brain Res 71:17-33
Nishino H, Koizumi K, Brooks CM 1976 The role of the suprachiasmatic nuclei of the hypothalamus in the production of circadian rhythms. Brain Res 112:45-59
Perlow MJ, Reppert SM, Boyar RM, Klein DC 1981 Daily rhythms in cortisol and melatonin in primate CSF. Neuroendocrinology 32:193-196
Rollag MD, Niswender GD 1976 Radioimmunoassay of serum concentrations of melatonin in sheep exposed to different lighting regimens. Endocrinology 98:482-489
Schwartz WJ, Davidsen LC, Smith CB 1980 In vitro metabolic activity of a putative circadian oscillator, the rat supra-chiasmatic nucleus. J Comp Neurol 189:157-167
Seigel S 1956 Non-parametric statistics. McGraw-Hill, New York
Tamarkin L, Reppert SM, Klein DC, Pratt B, Goldman BD 1980 Studies on the daily pattern of pineal melatonin in the Syrian hamster. Endocrinology 107:1525-1529
Tamarkin L, Baird CJ, Almeida OFX 1985 Melatonin: a coordinating signal for mammalian reproduction? Science (Wash DC) 227:714-720
Vanecek J, Illnerova H 1979 Changes of a rhythm in rat pineal serotonin *N*-acetyltransferase following a one-minute light pulse at night. Prog Brain Res 52:245-248
Zatz M, Brownstein MJ 1979 Intra-ventricular carbachol mimics the effects of light on the circadian rhythm in the rat pineal gland. Science (Wash DC) 203:358-361

DISCUSSION

Armstrong: The free-running rhythms of individual animals seem to be very close, with a τ of about 24 h. I would expect them to be much further apart. Do you get social synchronization in sheep? Or does bleeding animals at the same time of day perhaps interfere with the free-running rhythm?

Lincoln: We certainly see examples of social synchronization in terms of overt behaviour in our rams, but we would not be able to assess such an effect for the melatonin rhythm. I don't think the timing of bleeding is very important especially where we sample for two complete 24 h cycles; however, we do find that melatonin levels tend to be elevated at the beginning of a serial bleed.

Turek: In Fig. 3 (p 135), one animal entrained in an unusual way. From the free-running pattern in constant darkness, it looks as though the average period of the sheep melatonin rhythm may be less than 24 h. The entrainment pattern

that you see in most of your sheep in 1L:23D is then what you would expect, because the light is occurring in the delay region of the phase–response curve. Perhaps the aberrant animal has a free-running period greater than 24 h, so light needs to be coincident with the advance region of the phase–response curve to produce entrainment. This raises the question of whether dusk or dawn is the entraining signal. I don't think we can say from your results that lights-off is the entraining signal just because the onset of the melatonin peak occurs after the lights go off. If you put your animals under T-cycles, you might find that you could easily get the melatonin peak to occur just *before* the light pulse.

Illnerová: In rats on long photoperiods the melatonin rhythm seems to be entrained by both lights-on and lights-off, but on short photoperiods the rhythm is entrained completely by lights-on (Illnerová & Vaněček 1985). On short days, the phase relationship between the evening rise in melatonin production and the morning decline may be already stable, and the rhythm is synchronized with the 24 h day by the morning onset of light.

Lewy: In Dr Lincoln's skeleton photoperiod study, dusk was apparently more important than dawn for entrainment, which is consistent with the τ being shorter than 24 h. This makes sense, and is consistent with the prediction that for animals with τ greater than 24 h dawn would be relatively more important than dusk for entrainment (Lewy 1983).

Moore-Ede: I was struck by the similarity between the timing of entrainment by a single light pulse and entrainment by two. The single light pulse came close to the middle of subjective day, and the second light pulse that you added fell near the middle of subjective night. But the second light pulse seemed to make no difference in that the melatonin rhythm still entrained to the first pulse. This made me question whether you were seeing entrainment by light or by something else. For example, were the animals being fed on a regular schedule?

Lincoln: These animals had a hopper system to provide pelleted food for up to five days at a time. They gradually work their way through this supply, so we avoid the problem of the sudden introduction of food once daily. In the experiment with two light pulses, we thought that the animals might act like quail and flip over to the second of the light pulses, interpreting that as the onset of night. But from our limited melatonin results for the 15 days, this didn't appear to happen.

Bittman: It could be a question of light intensity, which may influence the range of entrainment and the degree of phase-shifting. Any conclusions you make about the effect of a given light pulse presented at a particular time should be conditional upon exactly what light intensity is used, as well as upon the duration of the light pulse.

Lincoln: The lighting in our sheds is about 160 lux at the animals' eye level, provided by four white fluorescent strip lights (length 1.8 m) in a room approx-

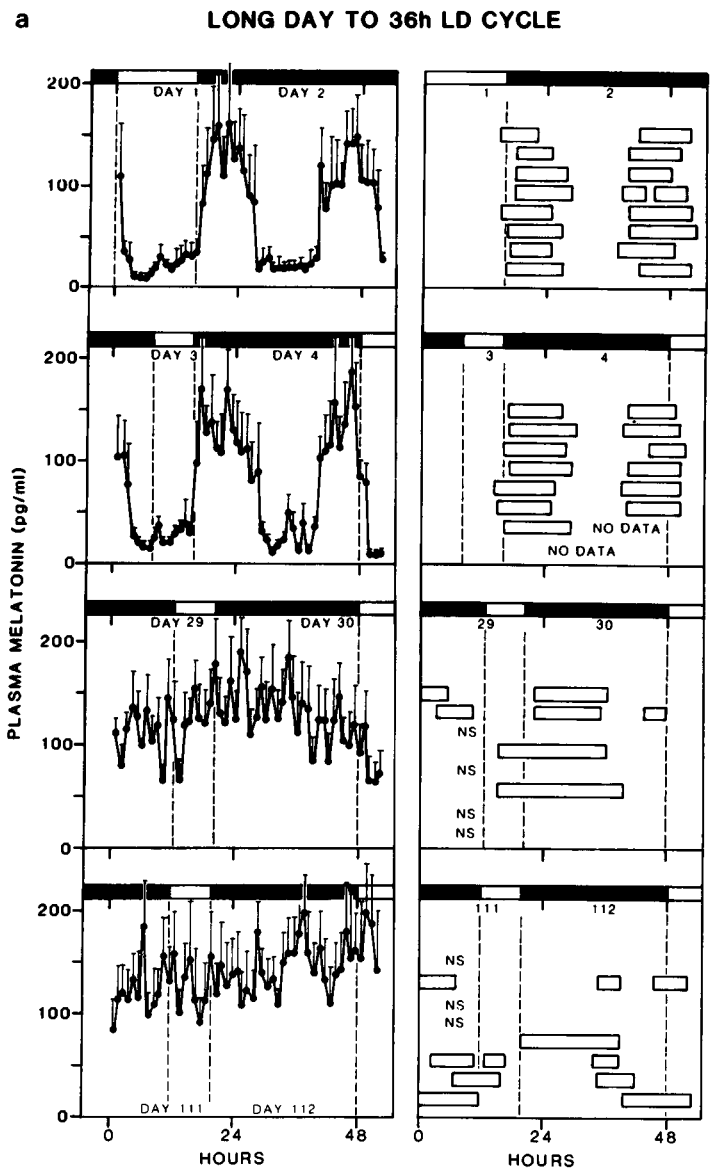
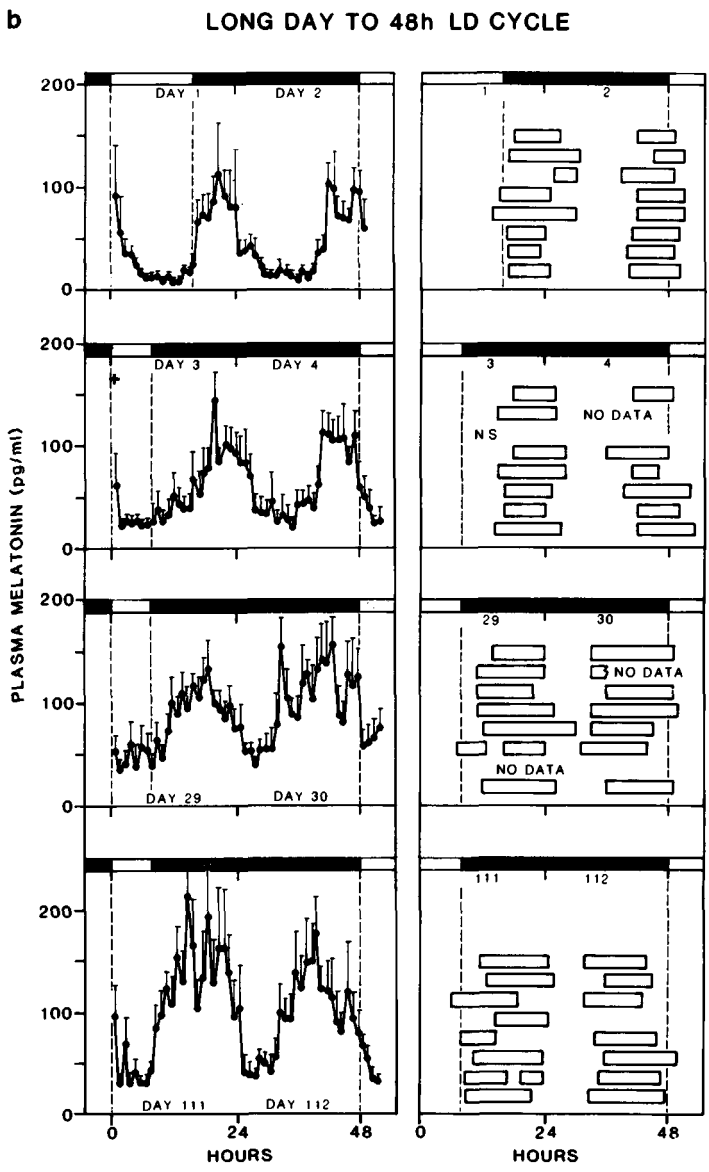


FIG. 1. (*Lincoln*) Melatonin concentrations in blood plasma (mean ± SEM, $n = 8$) and timing of melatonin peaks (open bars for individual rams) in Soay rams transferred from 16L:8D to either 8L:28D (a) or 8L:40D (b). Samples were collected hourly on days 1–4, 28–29 and 111–112 (day 1 taken as the last day under 16L:8D). Data from Almeida & Lincoln (1982) with melatonin peaks redefined as in this volume (p 132).



imately 4 × 5 m. I can't predict whether increasing the light intensity would alter the patterns of melatonin secretion in sheep, but one might expect more clearly defined changes from day to night.

Bittman: I have had the opportunity to measure melatonin in some ewes that were blinded for studies of extraretinal photoreception (Legan & Karsch

1983). Three years after blinding, these animals all had discrete and well-organized free-running melatonin patterns. How stable is the melatonin pattern of your Soay rams in constant dark? Does the organization of the rhythm or the duration of the peak vary with time after the animal is put into constant dark or blinded?

Lincoln: In our rams held in constant darkness the melatonin rhythm was poorly defined after eight weeks, but we have no information on the effect of blinding.

Klein: I am impressed by your observation that under 8L:28D animals become totally disorganized and show tonically high melatonin values (Almeida & Lincoln 1982 and Fig. 1 above). This is the first report of animals having continuously high melatonin. The concentrations are nowhere near normal day values; they are in the range of normal night values.

*Bittman:*I have seen patterns like that in a few animals with surgical insults, for example in some of Gary Jackson's ewes with frontal hypothalamic knife cuts. Steven Yellon has reported such patterns in ganglionectomized young ewes and in intact lambs before the melatonin rhythm fully matures (Yellon & Clayton 1983).

Lincoln: There are three situations where we get inter-peak melatonin levels that are higher than normal: (1) with the 8L:28D regimen mentioned by Dr Klein and illustrated in Fig. 1 above; (2) after constant darkness for a prolonged period; (3) when animals are exposed to a 23 h or 25 h regimen as shown in Fig. 5 (p 138). In the last case the melatonin peak is entrained to the dark period in most of the animals but the day-time melatonin levels are higher than normal. When the rams are given two weeks to readjust to a 24 h cycle, the full melatonin rhythm reappears with low day-time values.

Bittman: In these experiments where you see high inter-peak melatonin levels of between 100 and 200 pg/ml, the melatonin rhythm might be a bit better behaved if you tried the same light–dark schedule but with a higher light intensity.

Lewy: After long periods in constant dark, animals become desynchronized from each other. Under these conditions, do your animals, analysed individually, show consistently elevated melatonin concentrations?

Lincoln: Yes. The melatonin peak is no longer as well defined as it is under a normal 24 h light–dark cycle, i.e. the minimum values are increased. Perhaps at this point we should consider the problem of the melatonin assay. With the Rollag and Niswender method using the Rollag 1055 antibody we get nocturnal plasma melatonin levels around 200 pg/ml. I suspect that with Jo Arendt's assay for melatonin the peak levels would be less than 100 pg/ml, so our assay may be less specific.

Goldman: Mark Rollag provided us with an antibody that had not been used previously for radioimmunoassay and we compared this with the 1055 anti-

body. The two are very different. In plasma samples from sheep the new antibody indicated melatonin values which were only about one-half to one-third those found by Dr Steven Yellon using the 1055 antibody, although the patterns were generally the same. So it is possible that half of what is being measured with R1055 is not melatonin. Also, Steven Yellon sent us samples from a lamb (day-time) and a ganglionectomized sheep that showed unexpectedly high levels of melatonin with the 1055 antibody; both of these sera showed the expected low melatonin values when we assayed them using the new antibody. I'm not prepared to say which result is correct, but there is certainly a discrepancy.

Klein: Although it's possible that Gerald Lincoln's high values obtained with sheep under 8L:28D are just the consequence of an assay problem, they may be real. Normally, animals that free-run show a discrete melatonin rhythm. The animals under 8L:28D don't; their melatonin levels are constantly high, and this has never been seen before. It is remarkable because I don't think you can simulate this artificially by giving an animal drugs. The clock must be profoundly fouled up in some way, or the two parts are operating completely independently and are stretched out to cover a 24 h period.

Lincoln: We are looking at an event downstream from the clock function. My impression from the melatonin profiles is that the clock does not behave like a simple oscillator; the melatonin rhythm can become poorly defined and disrupted under certain experimental photoperiods, as mentioned earlier, which implies a multiple-oscillator system.

Klein: The 8L:28D schedule probably disturbs the normal relationship between two SCN 'clocks'. Instead of being out of phase by 6–12 h, they may be pulled apart so that the morning oscillator stimulates the pineal for 12 h or more and then the evening oscillator stimulates for a subsequent 12 h.

Illnerová: I don't think it is possible to pull the clocks apart far enough to cover the whole day. There may be a maximum phase relationship between them, and you cannot extend this.

Turek: You didn't say much about the importance of the melatonin pattern, but you have shown that when animals on a fixed light–dark cycle go into the refractory condition there is a change in the pattern. This suggests that the melatonin pattern indeed regulates reproductive condition. Have you any further information on that?

Lincoln: No.

REFERENCES

Almeida OFX, Lincoln GA 1982 Photoperiodic regulation of reproductive activity in the ram: evidence for the involvement of circadian rhythms in melatonin and prolactin secretion. Biol Reprod 27:1062-1075

Illnerová H, Vaněček J 1985 Entrainment of the circadian rhythm in rat pineal *N*-acetyltransferase activity under extremely long and short photoperiods. J Pineal Res 2:67-78

Legan SJ, Karsch FJ 1983 Importance of retinal photoreceptors to the photoperiodic control of seasonal breeding in the ewe. Biol Reprod 29:316-325

Lewy AJ 1983 Biochemistry and regulation of mammalian melatonin production. In: Relkin RM (ed) The pineal gland. Elsevier North-Holland, New York, p 77-128

Yellon SM, Clayton JA 1983 Evidence that the pineal times puberty in the lamb. Biol Reprod 28(suppl 1):27

Photoperiodism, melatonin and the pineal
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The role of rhythms in the response to melatonin

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Abstract. Rhythmicity of melatonin secretion is critical to the regulation of mammalian reproduction by daylength. In the ewe, photoperiod determines ovarian function by modulating the ability of oestradiol to suppress pituitary secretion of luteinizing hormone (LH). This influence of daylength depends in turn upon the pineal gland, which mediates photic control of the frequency at which the brain stimulates the pituitary to secrete gonadotropin. Photoperiod determines the pattern of melatonin secretion, most notably setting the duration of the nightly elevation in serum concentrations. Replacement of melatonin in pinealectomized ewes drives responsiveness to oestradiol negative feedback; LH levels are determined by the duration of the nightly melatonin infusion rather than by the photoperiod to which ovariectomized, oestradiol-implanted pinealectomized ewes are exposed. Refractoriness to stimulatory and inhibitory photoperiods may reflect circannual modulation of the responsiveness of neuroendocrine mechanisms to melatonin signals of a given duration.

1985 Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Foundation Symposium 117) p 149-169

Although as students of the pineal gland we have long been struck by the rhythmicity of melatonin synthesis, we have only recently begun to appreciate the functional importance of this feature. In none of the physiological effects of pineal secretion is the significance of rhythmicity so apparent as in the photoperiodic control of seasonal reproduction in mammals. Studies in both a long- and a short-day breeder now indicate not only that rhythmicity is critical, but also that a particular characteristic of this rhythm—the duration of nightly melatonin secretion—determines whether reproduction is induced or suppressed.

Important though circadian rhythmicity of pineal secretion is, it represents only one of several oscillations which underlie seasonal reproduction. In the ewe and perhaps other photoperiodic species, daylength determines gonadal condition by setting the frequency of ultradian rhythms of pituitary function. At the other extreme of frequencies, responsiveness to a particular melatonin signal is restricted to a specific phase of the annual reproductive cycle. In

the absence of changes in the melatonin signal or even when there is no pineal output, animals of some species alternate between phases of fertility and infertility whose combined durations result in nearly annual periodicity. In this review, I will consider not only the importance of temporal patterns of melatonin secretion, but also the nature of the rhythmic endocrine system that the pineal modulates and the limits of its responsiveness to melatonin.

Influences of daylength on the secretion of luteinizing hormone

It is well established that a variety of species utilize daylength as an environmental cue for restricting reproduction to particular times of year. That this confers a selective advantage is suggested by the differences in breeding seasons both within species, when populations are geographically separated, and between species, when gestation lengths differ. By responding to photoperiod these animals time birth to maximize the chances of survival of the young. The physiological basis of the phenomenon is most clearly studied by altering the timing or frequency of breeding seasons through manipulation of the photic environment. In such experiments, the eyes and the pineal gland of various mammals have been shown to participate in photoperiodic regulation of spermatogenesis, follicular maturation, gonadal steroidogenesis, sexual behaviour and implantation. The generalization that retinal photoreception controls seasonal breeding through pineal-mediated mechanisms in all mammals yet studied (Goldman 1983) is becoming progressively more meaningful; although much comparative work remains to be done, evidence for such a system has been found in representatives of six mammalian orders (Chiroptera, Rodentia, Perissodactyla, Artiodactyla, Carnivora and Marsupialia).

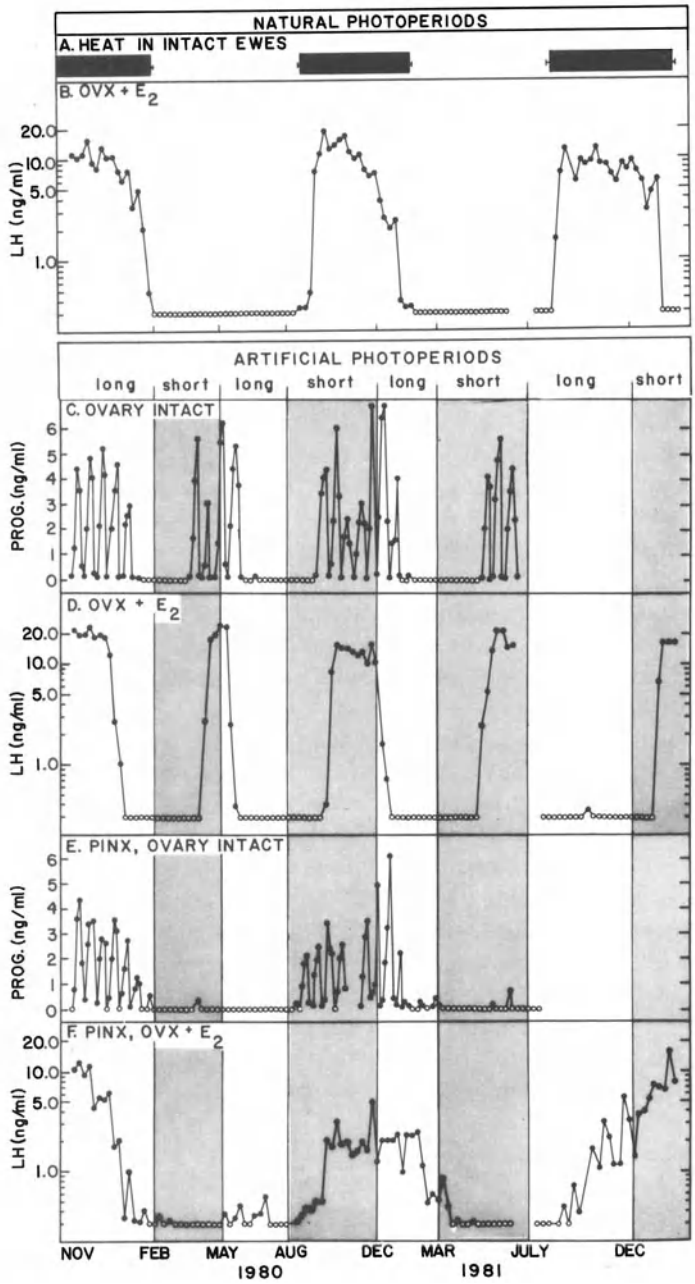
The relative sophistication of our understanding of gonadal–hypothalamo–hypophyseal relationships in sheep (Martin 1984, Karsch et al 1984) has suited the ewe particularly well to the analysis of the endocrine basis of the reproductive response to photoperiod. Sheep are short-day breeders; in Suffolk ewes oestrous cycles begin in late summer and continue almost until spring (Fig. 1A). As in other seasonal breeders, the responsiveness of luteinizing hormone (LH) secretion to the negative feedback influences of gonadal steroid hormones changes with season (Lincoln & Short 1980, Legan & Karsch 1980). In the ewe, the ability of oestradiol to suppress serum LH exhibits spectacular fluctuations whose timing coincides with transitions between the breeding season and anoestrus (Legan & Karsch 1980, Fig. 1B). In contrast, there is little seasonal change in the steroidogenic potency of LH, the positive feedback action of oestradiol or the behavioural efficacy of ovarian hormones (Goodman & Karsch 1980).

Experiments reveal that daylength regulates both ovarian cyclicity and this

critical negative feedback relationship. Doubling the frequency of the shifts in photoperiod results in two breeding seasons per year (Fig. 1C) and provokes a corresponding change in the pattern of LH secretion in ovariectomized ewes bearing subcutaneous capsules that maintain invariant physiological levels of oestradiol (Legan & Karsch 1980, Fig. 1D). Exposure to short days permits LH to 'escape' from negative feedback inhibition at any time of year, while long days heighten the potency of oestradiol negative feedback and thus suppress serum LH to undetectable values.

Surgical removal of the pineal gland disrupts these reproductive responses to photoperiod. Although the effects of this operation can be subtle in ewes in natural conditions, artificial photoperiodic challenges lose their ability to influence ovarian cyclicity (Fig. 1E, Bittman et al 1983b). Pinealectomy has corresponding effects on the responsiveness of the negative feedback axis to daylength: long days cease to suppress LH secretion and short days fail to drive a rise in serum gonadotropin concentrations in ovariectomized ewes implanted with oestradiol (Fig. 1F). In all respects, the effects of pinealectomy in ewes agree with those of pineal removal or denervation on testicular function and androgen feedback responsiveness in the ram (reviewed by Lincoln & Short 1980, Bittman 1984). These findings also correspond closely to the effects of similar surgical insults in long-day breeders. In the golden hamster, pineal removal or denervation eliminates photoperiodic regulation of testicular function, oestrous cyclicity and steroid feedback responsiveness (reviewed by Reiter 1980).

The influences of daylength on patterns of ovine gonadotropin secretion have been studied in detail. As in a variety of species, LH secretion occurs episodically in ewes and reflects pulsatile release of the neuropeptide gonadotropin-releasing hormone (GnRH) from the median eminence. In intact sheep the frequency of these pulses varies dramatically with season (Lincoln & Short 1980, Goodman et al 1982, Martin 1984). LH pulse amplitude tends to be reciprocally related to frequency. Although seasonal changes in endogenous GnRH pulse amplitude have not been ruled out, experiments involving pulsatile GnRH administration to ewes after section of the pituitary stalk indicate that changes in GnRH pulse frequency are the primary cause of seasonal alterations in the LH pulse pattern (Clarke et al 1984). During the breeding season of the ewe, LH pulses occur as frequently as twice per hour in the follicular phase but slow considerably under the influence of progesterone secreted after ovulation (Goodman et al 1982). During anoestrus, LH pulse frequency drops markedly despite the absence of progesterone. Although removal of the ovaries precipitates an increase in the LH secretory pattern during both seasons, the final pulse frequency during the breeding season is twice that of castrates in anoestrus. The effects of replacement of oestradiol on LH pulse patterns also depend upon time of year: during the winter, chronic


NATURAL PHOTOPERIODS
A. HEAT IN INTACT EWES
B. OVX + E2
LH (ng/ml)
20.0
10.0
5.0
1.0
ARTIFICIAL PHOTOPERIODS
long
short
long
short
long
short
long
short
C. OVARY INTACT
PROG. (ng/ml)
6
5
4
3
2
1
0
D. OVX + E2
LH (ng/ml)
E. PINX, OVARY INTACT
PROG. (ng/ml)
F. PINX, OVX + E2
LH (ng/ml)
NOV
FEB
MAY
AUG
DEC
MAR
JULY
DEC
1980
1981

castrates show a decrease in mean pulse amplitude without detectable alterations in LH pulse frequency. During the summer, however, LH pulse frequency drops in ovariectomized ewes within hours of oestradiol administration and may cease entirely as soon as three days after placement of the oestradiol implant.

The activity of the LH pulse generator is a critical determinant of the ewe's reproductive state, and fluctuations in pulse frequency are basic to seasonal breeding. Since frequent LH pulses are required to drive oestradiol to levels adequate to engage the positive feedback mechanism which triggers the LH surge, oestradiol cannot reduce LH pulse frequency if follicular maturation is to culminate in ovulation. This condition is satisfied during the breeding season, when only progesterone can slow LH pulses. During anoestrus, however, the oestradiol secreted by the follicle under the influence of LH reduces the frequency of further gonadotropic stimulation. The sustained rises of oestradiol required to activate the LH surge system do not occur and the oestrous cycle is suspended.

Seasonal changes in LH pulse amplitude and frequency can be driven by photoperiod (Fig. 2, Bittman et al 1985). Exposure of ovariectomized ewes to 8L:16D (8 h light:16 h dark) during the natural anoestrous season produces a pulsatile LH pattern quite similar to that of a castrate in the breeding season, while ewes kept in 16L:8D or exposed to natural long photoperiods exhibit low frequency, high amplitude LH pulses at this time of year. This response to daylength is pineal dependent: pinealectomized ewes fail to exhibit frequent LH pulses when exposed to short days during anoestrus, even when oestradiol is absent (Fig. 2). When oestradiol is present, the effects of pinealectomy are even more dramatic: whereas pineal-intact ewes exposed to 8L:16D during anoestrus exhibit frequent LH pulses, such pulses cease entirely in similarly treated pinealectomized ewes.

FIG. 1. Effects of season, photoperiod and pinealectomy on oestrous cyclicity and the potency of oestradiol negative feedback in Suffolk ewes. (A) Mean (±SEM) dates of onset and termination of breeding seasons, as determined by behavioural oestrus in a flock of intact ewes maintained outdoors. (B) Serum LH (mean of two weekly samples) in a representative ovariectomized, oestradiol-implanted (OVX + E_2) ewe maintained outdoors. Open circles indicate undetectable hormone levels. Note that the negative feedback effect of oestradiol waxes and wanes in synchrony with anoestrus and the breeding season. (C–F) Results from representative individuals exposed to artificial photoperiods. Open areas represent long photoperiods of 16 h light:8 h dark (16L:8D); stippling indicates short photoperiods (8L:16D). (C) Serum progesterone (PROG, determined twice weekly) in an intact ewe. (D) Serum LH in an ovariectomized, oestradiol-implanted ewe. Note that photoperiod regulates both oestrous cyclicity and oestradiol negative feedback on LH secretion. (E) Serum progesterone in a pinealectomized (PINX), ovary-intact ewe. (F) Serum LH in a pinealectomized, ovariectomized, oestradiol-implanted ewe. Note that pinealectomy eliminates photoperiodic regulation of oestrous cyclicity and the negative feedback potency of oestradiol, but that ewes continue to show approximately annual reproductive cycles.

These findings indicate that daylength acts through the pineal gland to determine whether LH is secreted at or above the frequency required to drive oestradiol to levels adequate to trigger a preovulatory LH surge. Changes

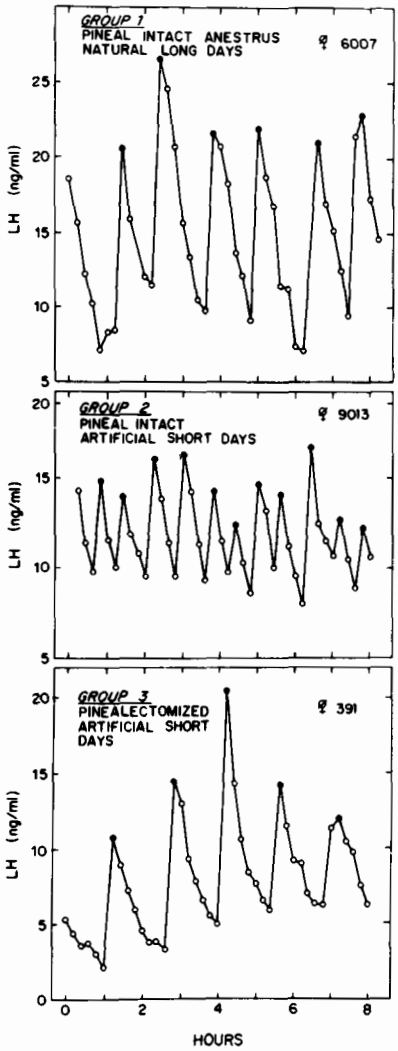


FIG. 2. Serum LH, sampled at 12 min intervals, in representative ovariectomized ewes. Closed circles represent statistically identified peaks of LH pulses. Results were obtained during July (middle of natural anoestrus). Ewes in groups 2 and 3 had been exposed to 8L:16D for 16 weeks before the day of sampling, and ewes in group 3 had been pinealectomized. Short photoperiods doubled LH pulse frequency by a pineal-dependent mechanism. Reprinted from Bittman et al (1985) with the permission of the publisher (Karger, Basel).

in oestradiol feedback potency are of the utmost physiological importance, for throughout anoestrus levels of oestradiol remain adequate to suppress LH and thus prevent completion of follicular maturation. Nevertheless, the effects of daylength in castrated ewes receiving no steroid treatment tell us a great deal about the nature of the pineal's action. Since each LH pulse reflects release of a bolus of GnRH from the brain, pineal-mediated changes in LH pulse frequency reflect modulation of a neural ultradian oscillator. The pineal regulates the frequency code that is the signature of the neurosecretory process which drives reproduction. This action of daylength, exhibited in the absence of steroids, may indeed be the primary mechanism whereby breeding seasonality is enforced. Attempts to explain the seasonal changes in steroid feedback potency on the basis of photoperiodic influences either on the number and affinity of neural and pituitary receptors for these hormones or on their metabolism by neural target tissues have generally failed.

Role of melatonin

The pineal-intact ewe shows a robust nocturnal rise in serum melatonin concentrations. Experiments in blind sheep (E.L. Bittman et al, unpublished work 1982) and in ewes held in constant darkness (Rollag & Niswender 1976) reveal free-running oscillations of serum melatonin, which indicate true circadian rhythmicity. The phase and amplitude of the night-time rise of serum melatonin remain to be explored systematically over a full range of photoperiods. Among ewes entrained to photoperiods between 8L:16D and 16L:8D, however, the inverse relationship between the duration of elevated serum titres and daylength is the most consistent regularity of the secretory pattern (Rollag & Niswender 1976, Arendt et al 1981, Bittman et al 1983a, Karsch et al 1985a). Melatonin levels rise shortly after the onset of darkness and remain elevated for most of the dark period, but do not remain constant throughout the night. In individual ewes, frequent sampling (every 5 or 12 min) reveals episodic fluctuations whose cause and function remain to be identified. Serum melatonin patterns respond quickly to abrupt changes in photoperiod (Bittman et al 1983b).

Removal of the pineal gland eliminates the night-time rise in serum melatonin; immunoreactive material remains at or below the normal day-time concentrations of <50 pg/ml (Fig. 3, small closed circles). In light of the elimination of reproductive photoperiodism by pinealectomy, we were curious to determine whether replacement of melatonin would drive the negative feedback potency of oestradiol in such ewes. This was accomplished by intravenous infusion using battery-operated syringe pumps which were turned on each night at dusk. Pumps can be loaded with an appropriate amount of

solution to sustain a rise in serum melatonin of the desired duration (Fig. 3). The infusions were designed to produce melatonin elevations which either restored the nocturnal pattern typical of the pineal-intact animal or gave a pattern that systematically differed from that normally seen under the prevailing photoperiod. Thus, pinealectomized, ovariectomized oestradiol-implanted

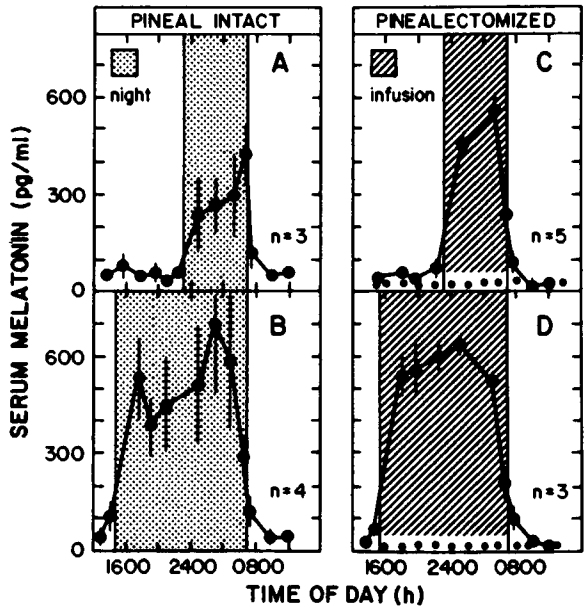


FIG. 3. Left: mean (±SEM) serum melatonin concentrations in pineal-intact ewes exposed to long (A) or short (B) days (stippled area indicates darkness). Long days were 16L:8D; short days were 8L:16D. Note that nightlength determines the duration of elevated serum melatonin concentrations. Right: mean (±SEM) concentrations of melatonin in serum of pinealectomized ewes infused with melatonin in the long-day (C) or short-day (D) pattern (cross-hatched area indicates period of infusions). Mean melatonin concentrations for 24 h in the same pinealectomized ewes, but in the absence of melatonin infusion, are indicated by the small points in the lower portion of panels C and D. Number of animals indicated by n. Reprinted with permission from Bittman & Karsch (1984).

ewes maintained in long or short days received either the long-day or the short-day melatonin pattern. Infusions were continued nightly for several months, and the consequences for steroid feedback responsiveness were assessed by measurement of serum LH.

Initial experiments revealed that infusion of the short-day (long duration) melatonin pattern freed LH from oestradiol feedback inhibition (Bittman et al 1983b, Fig. 4). The latency and the amplitude of this response were similar to those of pineal-intact ewes shifted to short days during anoestrus. After

90 nights of 16 h melatonin infusions, these ewes showed a pulsatile pattern of LH secretion similar to that of pineal-intact ewes, i.e. high frequency, low amplitude pulses prevailed (Bittman et al 1985).

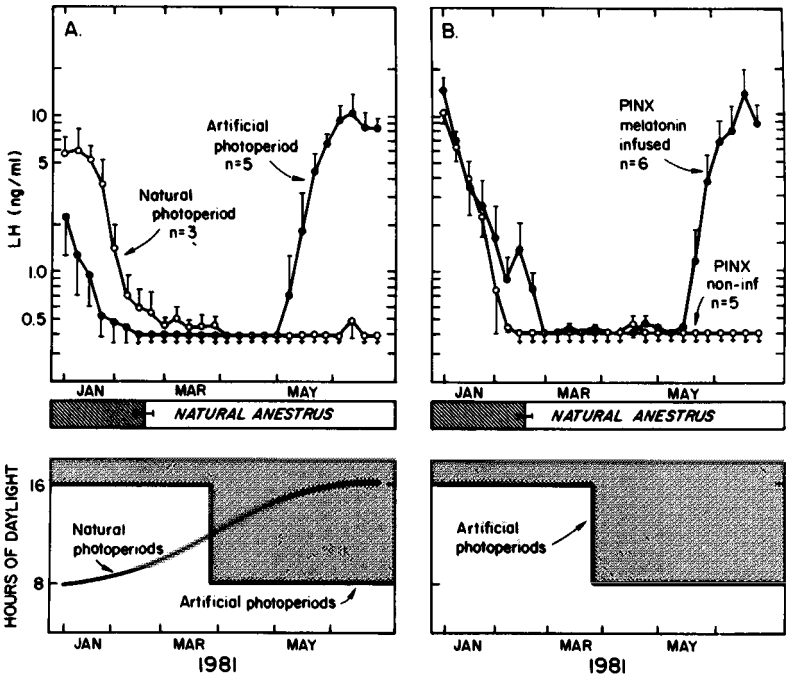


FIG. 4. Serum LH (mean ± SEM of two weekly samples) in ovariectomized, oestradiol-treated ewes. Left: results from pineal-intact ewes. Open circles, ewes maintained outdoors; closed circles, ewes exposed to artificial photoperiods. Photoperiodic history illustrated in the bottom panel; stippled area indicates darkness. Right: results from pinealectomized (PINX) ewes. Open circles, ewes not treated with melatonin (non-inf); closed circles, ewes infused nightly with melatonin (see Fig. 3 for serum melatonin patterns in ewes given corresponding treatments). Number of individuals indicated by n. Horizontal bar in middle of figure illustrates timing (mean date ± SEM) of onset of anoestrus in a flock of 10–15 intact ewes maintained outdoors over a five-year period; shading indicates breeding season. Reprinted with permission from Bittman et al (1983b).

We next determined whether short-duration melatonin infusions would mimic the inhibitory influence of long days (Bittman & Karsch 1984, Fig. 5). After long-term pinealectomized ewes had been primed with long-duration nightly infusions to induce high LH titres, the experimental group was transferred to 16L:8D and began to receive 8 h nightly infusions. A control group of pinealectomized ewes continued to receive the 16 h infusion and remained in short days. Within seven weeks, the LH titres of these two groups diverged markedly. While the short-day melatonin pattern maintained high LH levels

in the control ewes, the combination of transferring pinealectomized sheep to long days with switching to the long-day melatonin pattern potentiated oestradiol negative feedback. Serum LH fell to undetectable levels along a time course similar to that exhibited by pineal-intact animals subjected to the same photoperiodic treatments. LH levels did not drop in two pinealectomized ewes not infused with melatonin (Fig. 5).

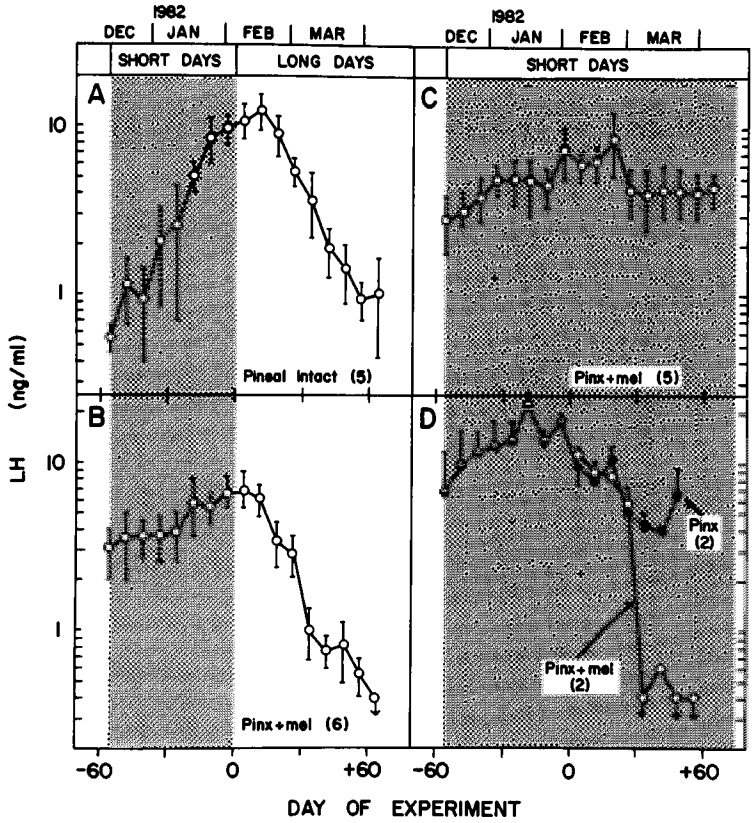


FIG. 5. Mean (± SEM) weekly serum LH concentrations in ovariectomized, oestradiol-implanted ewes. Shaded areas indicate short days (8L:16D), non-shaded areas long days (16L:8D). (A) Pineal-intact ewes transferred from short to long photoperiods at day 0. (B) Pinealectomized (Pinx) ewes housed in short days and infused with a short-day melatonin (mel) pattern before transfer on day 0 to long days and infusion of a long-day melatonin pattern. (C) Pinealectomized (Pinx) ewes maintained in short days throughout the experiment and given a short-day melatonin pattern. (D) Pinealectomized ewes maintained in short days and given either no melatonin infusions (closed circles) or a long-day pattern of melatonin beginning on day 0 (8 h infusion during the latter half of the 16 h night, open circles). Arrows below points indicate undetectable LH levels; number of ewes given in parentheses. See Fig. 3 for serum melatonin patterns in ewes subjected to these treatments. Reprinted with permission from Bittman & Karsch (1984).

These results are consistent with either of two interpretations. First, melatonin may act merely to permit daylength to regulate reproduction. A photoperiodic time-measurement system whose operation is independent of the pineal might require a daily melatonin signal only for the expression of its output. According to this model, effects of photoperiod on the secretory pattern of melatonin are only coincidental to the discrimination of daylength. Alternatively, melatonin patterns may drive feedback responsiveness. According to this hypothesis, photoperiod is irrelevant to reproductive function beyond its ability to determine patterns of melatonin secretion. Neither possibility was ruled out by the experiments described, because the patterns of infused melatonin corresponded to the prevailing daylengths and experimental photoperiods were changed simultaneously with alterations in the infusion regimen. To discriminate between the permissive and the driving role of melatonin, we deliberately presented pinealectomized ewes with melatonin patterns inappropriate to the daylengths in which they were housed.

The first such experiment involved sheep that were initially insensitive to oestradiol negative feedback. When such ewes were kept in short days but given the long-day melatonin pattern, LH levels fell much as in pineal-intact ewes exposed to long days (Bittman & Karsch 1984, Fig. 5D). In a second experiment, pinealectomized ewes initially in the anoestrous state were kept in short days. While LH levels rose in control ewes infused with melatonin for 16 h nightly, feedback responsiveness remained high in sheep receiving 8 h infusions despite exposure to the 'stimulatory' photoperiod. Finally, pinealectomized ewes held in long days received a short-day melatonin pattern (16 h nightly). Despite the 'inhibitory' daylengths, these infusions led to unambiguous reproductive induction during the anoestrous season (Yellon et al 1985). In all these experiments, the latency and amplitude of the LH response of melatonin-treated ewes were in good agreement with those of pineal-intact ewes exposed to the stimulatory or inhibitory photoperiods in which the pattern produced by the infusion would normally occur. The daylength to which the pinealectomized ewes were actually exposed had no detectable influence on the reproductive response. Furthermore, the duration of the nightly melatonin infusion determined the pattern of pulsatile LH secretion without changing pituitary sensitivity to GnRH (Bittman et al 1985). Thus we can rule out the possibility that melatonin has a merely permissive action. Instead, these results strongly indicate that melatonin acts on the brain to drive responses to both stimulatory and inhibitory daylengths.

The data also suggest that the duration of the nightly melatonin rise carries critical information about photoperiod. Although a melatonin infusion of fixed duration was not presented at different phases of the circadian cycle, driving exogenous melatonin patterns against daylength required distortion of the normal phase of the nightly rise in serum concentration. In various experiments

melatonin administration began 8 h before lights-off, or was delayed until 8 h after the night began. In no instance did such a departure from the normal phasing of the melatonin rise appear to affect the response. These data are in excellent agreement with an elegant series of experiments in a long-day breeder, the Djungarian hamster (Goldman 1983). In pubertal hamsters, the duration of the nocturnal rise in pineal melatonin content is closely related to nightlength. Pinealectomy eliminates testicular responses to both long and short days, and infusions of melatonin over durations which match those of the elevated pineal content provoke responses appropriate to the infusion pattern regardless of daylength. In these studies, the time of day of the melatonin infusion had little or no effect on the reproductive response as long as the duration was held constant.

It is not yet clear whether the duration of melatonin secretion codes daylength in other photoperiodic mammals. Indeed, it has been argued that the phase of melatonin secretion is important in Syrian hamsters (Watson-Whitmyre & Stetson 1983). Even within an animal, different photoperiodic functions may be driven by various codes, some of which may have pineal-independent components (Bartness & Wade 1984). Nevertheless, the evidence for this kind of message raises the question of how the duration of the melatonin signal is measured. Although it is possible that the melatonin target utilizes a circadian-based mechanism, it seems more likely at present that the detector operates as an hourglass. In hamsters, this system appears to be responsive to melatonin at a variety of circadian phases, to start timing the duration anew when the signal is interrupted and to survive lesions of the suprachiasmatic nucleus (Goldman 1983, Bittman et al 1979). The participation of circadian rhythms in photoperiodic time measurement may be accounted for solely at the level of generation of the pineal melatonin signal. A hybrid time-measurement system which utilizes a circadian-based melatonin signal and an interval-type duration timer may confer advantages in accuracy or temperature compensation, and may permit the adoption of different threshold daylengths for multiple responses to photoperiod (for further discussion see Silver & Bittman 1984).

How might ancestral receptor systems, acutely responsive to melatonin, have developed the capacity to measure the duration of the signal? Is the duration measurement accomplished within the melatonin-sensitive cells, or through reference to an anatomically distinct timer? How do the systems which measure melatonin duration overlap with those which control LH pulse frequency and oestradiol feedback responses? A parsimonious model would stipulate that cells within the LH pulse generator, or those which concentrate gonadal steroids, respond directly to melatonin. This is not necessarily the case, however, for melatonin may well influence various neurotransmitter systems, which in turn modulate the LH pulse generator.

Circannual rhythms, photorefractoriness and melatonin responsiveness

In many seasonal breeders, photoperiod enforces a particular reproductive condition only for a limited period of time. For example, Syrian hamsters become refractory to short days: their testes return to breeding condition within 30 weeks of introduction to inhibitory photoperiods (Reiter 1980). Thereafter, hamsters remain in reproductive condition indefinitely. In the ewe, short and long photoperiods lose their respective abilities to induce and suppress reproduction. When maintained in constant long, short or intermediate daylengths, sheep exhibit up to three annual reproductive cycles (reviewed by Karsch et al 1984). The physiological bases of photorefractoriness and circannual rhythmicity are not yet known. It has been suggested that fluctuations in pineal function or target responsiveness may underlie these phenomena.

Most evidence suggests that melatonin patterns convey a faithful representation of photoperiod over protracted periods of time. In golden hamsters, rhythms of pineal melatonin content do not change as the testes regress and spontaneously recrudesce during prolonged exposure to short days unless hibernation occurs (Rollag et al 1980, Vanecek et al 1984). Furthermore, regression and regrowth occur along a roughly similar time course when exogenous melatonin is administered daily (Bittman 1984). Injection of melatonin into hamsters whose gonads have spontaneously recrudesced cannot induce a second regression until refractoriness is broken by exposure to many weeks of long days (Bittman 1984). Taken together, these data indicate that the melatonin target loses its responsiveness and must somehow be regenerated by exposure to long days. Evidence suggests that this restorative signal is itself pineal mediated, and we may speculate that it consists of a long-day pattern of melatonin secretion sustained over a period of 10 weeks or longer.

The basis for photorefractoriness in sheep is more controversial. Almeida & Lincoln (1984) have noted circannual testicular cycles in Soay rams and reported that these animals lose their regular serum melatonin patterns as they become photorefractory. In the Suffolk ewe, we have observed refractoriness to both long and short days, using oestradiol feedback potency as an index of reproductive function (Karsch et al 1985b). We have not observed any alterations in serum melatonin patterns under either circumstance. Experiments in pinealectomized ewes also support the hypothesis that refractoriness results from target insensitivity to melatonin. Nightly infusions of melatonin in the short-day pattern have proved unable to maintain elevated serum LH indefinitely in ovariectomized, oestradiol-treated ewes housed in 8L:16D. Gonadotropin titres fall after eight weeks, much as in pineal-intact photorefractory sheep (Karsch et al 1985b).

Refractoriness to a particular melatonin signal, however, does not necessar-

ily mean that melatonin patterns are no longer detected by a duration-sensitive brain target. It seems more likely that in a 'refractory' sheep or hamster the effectors that consult the photoperiodic time-measurement system require an alternation between long and short photoperiods, and are readily apprised of a change in photoperiod by melatonin target tissues which remain sensitive. It is also not clear that refractoriness need be induced by a persistent melatonin signal for reproductively active and quiescent states to alternate. Although pinealectomized ewes no longer respond to photoperiod challenges, they continue to show nearly annual periodicity in ovarian cyclicity and oestradiol feedback responsiveness for at least three years (Bittman et al 1983a, Fig. 1E,F). Ewes were not totally isolated from fluctuations in temperature or other possible annual environmental signals in this study, so it is not clear whether this annual periodicity reflects persistence of endogenous rhythms in the absence of the pineal gland. This may be the case, however, because individuals tended to become less synchronous as the oscillations progressively damped. Such ewes showed less regularity in the timing of annual reproductive transitions than did pineal-intact ewes maintained outdoors; intervals between successive breeding-season onsets were 343 ± 35 days and 361 ± 8 days respectively (mean $\pm$ SEM). One significant role of the melatonin signal may be to serve as a zeitgeber for the entrainment of circannual rhythms by photoperiod.

Conclusion

Seasonal reproduction in the ewe results from the operation of at least three distinct endogenous rhythms. Photoperiodic information is encoded by the circadian system in the form of the duration of the melatonin signal. This signal is detected and measured by a poorly understood mechanism whose responsiveness or output may be gated by a circannual clock. The melatonin target in turn regulates the neural LH pulse generator. This ultradian oscillator uses a frequency code to establish the level of gonadal function appropriate to the season, possibly because its period determines its own sensitivity to steroid negative feedback.

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REFERENCES

Almeida OFX, Lincoln GA 1984 Reproductive photorefractoriness in rams and accompanying changes in the patterns of melatonin and prolactin secretion. Biol Reprod 30:143-158

Arendt J, Symons AM, Laud C 1981 Pineal function in sheep: evidence for a possible mechanism mediating seasonal reproductive activity. Experientia 37:584-586

Bartness TJ, Wade GN 1984 Photoperiodic control of body weight and energy metabolism in Syrian hamsters (*Mesocricetus auratus*): role of pineal gland, melatonin, gonads and diet. Endocrinology 114:492-498

Bittman EL 1984 Melatonin and photoperiodic time measurement: evidence from rodents and ruminants. In Reiter RJ (ed) The pineal gland. Raven Press, New York, p 155-192

Bittman EL, Karsch FJ 1984 Nightly duration of pineal melatonin secretion determines the reproductive response to inhibitory daylength in the ewe. Biol Reprod 30:585-593

Bittman EL, Goldman BD, Zucker I 1979 Testicular responses to melatonin are altered by lesions of the suprachiasmatic nuclei in golden hamsters. Biol Reprod 21:647-656

Bittman EL, Karsch FJ, Hopkins J 1983a Role of the pineal gland in ovine photoperiodism: regulation of seasonal breeding and negative feedback effects of estradiol upon LH secretion. Endocrinology 113:329-336

Bittman EL, Dempsey RJ, Karsch FJ 1983b Pineal melatonin secretion drives the reproductive response to daylength in the ewe. Endocrinology 113:2276-2283

Bittman EL, Kaynard AH, Olster DH, Robinson JE, Yellon SM, Karsch FJ 1985 Pineal melatonin mediates photoperiodic control of pulsatile luteinizing hormone secretion in the ewe. Neuroendocrinology 40:409-418

Clarke IJ, Cummins JT, Findlay JK, Burman KJ, Doughton BW 1984 Effects on plasma luteinizing hormone and follicle stimulating hormone of varying the frequency and amplitude of gonadotropin releasing hormone pulses in ovariectomized ewes with hypothalamo-pituitary disconnection. Neuroendocrinology 39:214-221

Goldman BD 1983 The physiology of melatonin in mammals. Pineal Res Rev 1:145-182

Goodman RL, Karsch FJ 1980 Control of seasonal breeding in the ewe: importance of changes in response to sex-steroid feedback. Prog Reprod Biol 5:134-154

Goodman RL, Bittman EL, Foster DL, Karsch FJ 1982 Alterations in the control of luteinizing hormone pulse frequency underlie the seasonal variation in estradiol negative feedback in the ewe. Biol Reprod 27:580-589

Karsch FJ, Bittman EL, Foster DL, Goodman RL, Legan SJ, Robinson JE 1984 Neuroendocrine basis of seasonal reproduction. Recent Prog Horm Res 40:185-232

Karsch FJ, Wayne NL, Bittman EL 1985a Importance of duration of the nocturnal increase in melatonin secretion in determining the reproductive response to photoperiod. In: Labrie F, Proulx L (eds) Endocrinology. Excerpta Medica, International Congress Series No. 655. Elsevier, Amsterdam, p 139-142

Karsch FJ, Bittman EL, Robinson JE et al 1985b Loss of response to an inductive melatonin pattern contributes to onset of anestrus in the ewe. Biol Reprod, in press

Legan SJ, Karsch FJ 1980 Photoperiodic control of seasonal breeding in the ewe: modulation of the negative feedback action of estradiol. Biol Reprod 23:1061-1068

Lincoln GA, Short RV 1980 Seasonal breeding: Nature's contraceptive. Recent Prog Horm Res 36:1-52

Martin GB 1984 Factors affecting the secretion of luteinizing hormone in the ewe. Biol Rev Camb Philos Soc 59:1-87

Reiter RJ 1980 The pineal and its hormones in the control of reproduction in mammals. Endocr Rev 1:109-131

Rollag MD, Niswender GD 1976 Radioimmunoassay of serum concentrations in sheep exposed to different lighting regimes. Endocrinology 98:482-489

Rollag MD, Panke ES, Reiter RJ 1980 Pineal melatonin content in male hamsters throughout the seasonal reproductive cycle. Proc Soc Exp Biol Med 165:330-334

Silver R, Bittman EL 1984 Reproductive mechanisms: interaction of circadian and interval timing. Ann NY Acad Sci 423:488-514

Vanecek J, Jansky L, Illnerova H, Hoffmann K 1984 Pineal melatonin in hibernating and aroused golden hamsters (*Mesocricetus auratus*). Comp Biochem Physiol A Comp Physiol 77:759-762

Watson-Whitmyre M, Stetson MH 1983 Simulation of peak pineal melatonin release restores sensitivity to evening melatonin injections in pinealectomized hamsters. Endocrinology 112:763-765

Yellon SM, Bittman EL, Lehman MN, Olster DH, Robinson JE, Karsch FJ 1985 Importance of duration of nocturnal melatonin secretion in determining the reproductive response to inductive photoperiod in the ewe. Biol Reprod 32:523-529

DISCUSSION

Goldman: Your experiments on refractoriness, and your earlier studies with Irv Zucker in which you showed that in the Syrian hamster the pineal is involved in the regaining of photosensitivity (Bittman & Zucker 1981) have important implications. If we could remove the pineal from sheep or hamsters and then put it back a week or two later, would there be an effect on the animal whatever the time of year? In other words, does the animal make use of pineal information all year long in both long days and short days?

Bittman: In the Syrian hamster we are a bit handicapped because it is sometimes difficult to see both long-day and short-day effects. In the sheep we can conveniently assay both, and the pineal is certainly involved in both.

Turek: Can you break the refractory period in sheep with a melatonin infusion paradigm?

Bittman: That has not been tried, but I expect it would work.

Follett: We are investigating photorefractoriness in birds and have begun to wonder if photoperiod operates at two different levels to induce refractoriness (e.g. Follett & Nicholls 1984). Do you think this might be true in sheep? For example, could you induce refractoriness in pinealectomized animals kept under long days and maintained on a short-day pattern of melatonin?

Bittman: I would predict that we would see exactly the same time course for the development of refractoriness as we do when the animals are kept in short days, but the experiment has not been done. We have also not tested whether we can get refractoriness to long days by infusing a long-day melatonin pattern in ewes under either long or short photoperiods.

Follett: There is usually a long lag before a photoperiodic response becomes visible in sheep, for example the seven weeks of short days before a rise occurs in LH secretion, and one wonders if the lag reflects an unwinding of refractoriness. In your animals given a short-day melatonin pattern but kept under long days, did the rise in LH occur at the normal time, i.e. after seven weeks?

Bittman: In none of our experiments have we seen any evidence that the ambient photoperiod affects the latency of the response of pinealectomized ewes to melatonin.

Lincoln: At what two levels does refractoriness operate in the bird, Professor Follett?

Follett: Our ideas at the moment are as follows (see Follett & Nicholls 1984). When a bird is exposed to long days two separate processes are begun. Firstly there is a positive drive on the system that increases gonadotropin secretion and so gonadal growth. At the same time, however, a second process is begun that some eight weeks later switches off the synthesis/secretion of luteinizing hormone-releasing hormone and causes refractoriness. We think that it might be possible to tease these processes apart in birds because one of them is dependent on the thyroid gland and the other is not.

Herbert: I'm a bit puzzled by the problem of steroid-dependent and steroid-independent feedback. Three questions arise from your results, Dr Bittman. First, you raised the possibility of constant-release implants of oestradiol being physiological, but from what we know of steroid hormones we must question whether this is true. For the ewe it would certainly not be true for progesterone; infusions of progesterone would be totally unphysiological because many responses to this hormone depend upon intermittent exposure.

Bittman: Some old experiments, which have been consistently replicated, show that the ewe makes a fair amount of oestradiol throughout anoestrus, as well as during both luteal and follicular phases in the breeding season (Robinson 1951, Smeaton & Robertson 1971, Yuthasastrakasol et al 1975).

Herbert: Yes, but the levels are not constant.

Bittman: No, they're not. Scaramuzzi & Baird have shown that the pulse frequency of oestradiol secretion varies quite dramatically between seasons and between phases of the oestrous cycle (Baird 1973, Scaramuzzi & Baird 1977).

Herbert: My second point is that one does not normally get castrated animals in the real world. We should realize that we may not be looking at a physiologically relevant system. Finally, although we usually separate steroid-dependent and steroid-independent effects, I'm not really sure this is sensible. It may well be that the neural nature of the GnRH pulse generator means that when it is operating during anoestrus it is necessarily more sensitive to oestrogen feedback because of the characteristics of that particular neuronal population. So when we get down to explaining the neural networks we may not actually be dealing with two systems.

Bittman: I agree. The relationship between changes in steroid feedback potency and pulse frequency in the castrate is a crucial question. With Lewis C. Krey of the Rockefeller University I have been studying responses in the hamster, but we have only negative results. We do not see a change in the

number or affinity of nuclear androgen receptors as a function of photoperiod, nor can we find changes in the pattern of metabolism of testosterone (Bittman & Krey 1984, Krey & Bittman 1985). But there could be a small subset of perhaps 10 cells that are changing their steroid feedback response, and we would never pick these up with our present methods.

Tamarkin: Which androgen receptors did you look at?

Bittman: Among neuroendocrine tissues, those in the preoptic area, the medio-basal hypothalamus and the pituitary. We used the seminal vesicle as a peripheral reference tissue. We also looked at steroid metabolism in these and other tissues. However, we did not see any effect of photoperiod on receptors or metabolism. The steroid receptor experiments were done in hamsters left in long days for seven weeks after castration and then implanted with silastic testosterone capsules which maintained serum testosterone at low, intermediate or high physiological levels. Half the hamsters were then moved to short days; the other half remained on long days. After an additional eight weeks we assayed occupied nuclear androgen receptors by exchange.

Turek: We have tried with a number of different experimental paradigms to separate out the steroid-dependent and steroid-independent systems. We have used a variety of melatonin regimens and lesions, but whenever we affect one system we affect the other. If there is a separation of the two, it is probably at the level of the GnRH neurons. It may be that when there is a change in the drive to the system there is also a change in the way those neurons respond to a steroid hormone. There certainly does not seem to be any separation at a 'gross' physiological level.

Bittman: Jane Robinson (1983) has shown that you can dissociate the time at which LH pulses slow in ewes that are becoming refractory to long days from the time at which castrated animals with implants of oestradiol show a dramatic fall in LH. I'm not sure what that means, but it's possible that, although pulse frequency changes smoothly in castrates without steroid replacement, there is a critical pulse frequency at which a discontinuity occurs in the steroid feedback response. I should also point out that it is not clear that melatonin or oestradiol is acting directly at the level of the pulse generator. These signals could be relayed to the pulse generator from other areas. In the rat preoptic area the oestradiol-concentrating cells are not the GnRH-producing cells (Shivers et al 1983). So several different cells may be involved: oestradiol-sensitive cells, GnRH-producing cells, which probably oscillate in the absence of steroids, and perhaps a third cell type on which melatonin acts. With that sort of system, the models become pretty complicated (see Fig. 5 in Bittman et al 1985).

Zucker: One point about steroid-dependent and steroid-independent systems is that the two sexes may differ. In the male golden-mantled ground squirrel there is negative feedback inhibition of LH release by the testes at every stage of the breeding cycle (Zucker & Licht 1983a). But in the female

there is only negative feedback inhibition by steroids during the breeding season; for the rest of the year the animal's LH levels are completely indifferent to gonadal steroids. If you ovariectomize one of these squirrels during the mating season you will see a rise in LH; the concentrations remain elevated for about one month but then decline, becoming undetectable and remaining that way until the next breeding season (Zucker & Licht 1983b).

Arendt: I'd like to come back to the question of whether the duration of melatonin secretion or the phase is important. Is there any good reason why melatonin itself should not entrain the photosensitive phase?

Bittman: If there is a circadian phase of melatonin sensitivity, it might be controlled by melatonin. But I don't see any compelling reason to include any circadian rhythmicity in our model of the system that measures the duration of melatonin secretion. Our evidence from ewes receiving melatonin patterns mismatched with photoperiod, and work done in Bruce Goldman's laboratory in which Djungarian hamsters received discontinuous presentations of melatonin or were treated at different phases of the day (reviewed by Goldman 1983) both argue against this. It is more parsimonious to think of a system that is a combination of a circadian rhythm, which generates the melatonin signal, and an interval timer, which is in the target system. Rae Silver and I have considered the possible adaptive advantages of such a system; perhaps it makes it easy for animals to vary photoperiodic responses according to temperature, nutrition and other environmental inputs (Silver & Bittman 1984). One can speculate about melatonin-sensitive phases but I don't think there is any evidence for them. It would be helpful if somebody could think up a way of presenting melatonin in some sort of resonance design to test for a circadian basis of melatonin sensitivity. I have tried to design such an experiment, but it is not clear to me how to combine ahemeral melatonin and photoperiod treatments.

Arendt: Do you think you could drive the cycle in the sheep against the natural photoperiod in natural light? In other words, if you advance oestrus once are you likely to be able to advance it again using, for example, melatonin implantation at intervals?

Bittman: To interpret seasonal reproduction in ewes kept outdoors, several experiments need to be done to investigate circannual rhythms. One approach would be to try to establish phase control with melatonin in pinealectomized animals and then to cut off the melatonin infusion to see whether the animals free-run from the phase at which the melatonin is stopped. That sort of experiment should be done under constant conditions because outdoors there are many influences that the animal might respond to. David Kennaway has looked at pinealectomized merino ewes kept outside for long periods (Kennaway et al 1984) and we have made some observations on Suffolk ewes kept outdoors for six months after pinealectomy (Bittman et al 1983). All of them

showed sporadic ovulation during anoestrus, but they would not cycle regularly until the onset of the normal breeding season. Perhaps they were responding to some other environmental signal; there is now evidence that the time of year at which you do the pinealectomy is important.

Zucker: To address the question of circannual rhythmicity one has to study animals under constant conditions. You mentioned that your pinealectomized animals are not responsive to photoperiod. Some species such as ground squirrels show 'true' circannual rhythmicity, going through reproductive cycles with a period of about eleven months when maintained under constant conditions from birth. They have a somewhat different form of organization. It is not pineal dependent, and in this species photoperiod has not been shown to be important for regulating the cycle. So we have to conclude either that there is no refractoriness in such animals or that there is refractoriness but it has nothing to do with the pineal gland.

Hoffmann: But a circannual cycle must be entrained to function properly under natural conditions. Is there solid evidence that neither the pineal nor photoperiod is involved in the synchronization of this cycle in squirrels?

Zucker: There is good evidence that the pineal gland is not involved because pinealectomized golden-mantled ground squirrels continue to show circannual cycles in both body mass and reproduction for several years.

Hoffmann: But that is not evidence that the pineal is not involved in *synchronization*.

Zucker: Nobody has yet been able to synchronize circannual cycles in these animals with any manipulation of the photoperiod.

Hoffmann: But the experiments of Pengelley et al (1976) provided no rigid evidence to exclude a role for photoperiod in synchronizing the cycles.

Zucker: I don't know what you would consider to be rigid evidence. Squirrels that are maintained in constant darkness or constant light or on any one of a number of different light–dark cycles all show free-running circannual rhythms. In none of these cases does one get a τ of 12 months. The more critical experiments of exposing animals to natural variations in photoperiod duration, i.e. keeping animals on a latitudinal timer and not varying temperature, we are in the process of doing now. It is too early to discern the outcome of these experiments.

REFERENCES

Baird DT 1973 Pulsatile secretion of LH and ovarian estradiol during the follicular phase of the sheep estrous cycle. Biol Reprod 18:359-364

Bittman EL, Krey LC 1984 Effects of daylength on nuclear androgen receptor occupancy in neuroendocrine tissues of the golden hamster. Neurosci Abstr 14:818 (No. 243.8)

Bittman EL, Zucker I 1981 Photoperiodic termination of hamster refractoriness: participation of the pineal gland. Biol Reprod 24:568-572

Bittman EL, Karsch FJ, Hopkins J 1983 Role of the pineal gland in ovine photoperiodism: regulation of seasonal breeding and negative feedback effects of estradiol upon luteinizing hormone secretion. Endocrinology 113:329-336

Bittman EL, Kaynard AH, Olster DH, Robinson JE, Yellon SM, Karsch FJ 1985 Pineal melatonin mediates photoperiodic control of pulsatile luteinizing hormone secretion in the ewe. Neuroendocrinology 40:391-400

Follett BK, Nicholls TJ 1984 Photorefractoriness in Japanese quail—possible involvement of the thyroid gland. J Exp Zool 232:573-580

Goldman BD 1983 The physiology of melatonin in mammals. Pineal Res Rev 1:145-182

Kennaway DJ, Dunstan EA, Gilmore TA, Seamark RF 1984 Effects of pinealectomy, oestradiol and melatonin on plasma prolactin and LH secretion in ovariectomized sheep. J Endocrinol 102:199-207

Krey LC, Bittman EL 1985 Does photoperiod influence metabolism of testosterone by neuroendocrine tissues of male golden hamsters? Biol Reprod 32(suppl 1):247

Pengelley ET, Asmundson SJ, Barnes B, Aloia RC 1976 Relationship of light intensity and photoperiod to circannual rhythmicity in the hibernating ground squirrel, *Citellus lateralis*. Comp Biochem Physiol A Comp Physiol 53:273-277

Robinson JE 1983 Daylength dictates the frequency of the LH-pulse generator in the ovariectomized ewe. Biol Reprod 28(suppl 1):62

Robinson TJ 1951 Reproduction in the ewe. Biol Rev Camb Philos Soc 26:121-157

Scaramuzzi RJ, Baird DT 1977 Pulsatile release of luteinizing hormone and the secretion of ovarian steroids in sheep during anestrus. Endocrinology 101:1806

Shivers B, Harlan R, Morrell JI, Pfaff DW 1983 Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurons. Nature (Lond) 304:345-347

Silver R, Bittman EL 1984 Reproductive mechanisms: interaction of circadian and interval timing. Ann NY Acad Sci 423:488-514

Smeaton TC, Robertson HA 1971 Studies on the growth and atresia of Graafian follicles in the ovary of the sheep. J Reprod Fertil 25:243-252

Yuthasastrakasol P, Palmer WM, Howland BE 1975 Luteinizing hormone, oestrogen, and progesterone levels in peripheral serum of anoestrous and cyclic ewes as determined by radioimmunoassay. J Reprod Fertil 43:57-65

Zucker I, Licht P 1983a Seasonal variations in plasma luteinizing hormone levels of gonadectomized male ground squirrels (*Spermophilus lateralis*). Biol Reprod 29:278-285

Zucker I, Licht P 1983b Circannual and seasonal variations in plasma luteinizing hormone levels of ovariectomized ground squirrels (*Spermophilus lateralis*). Biol Reprod 28:178-185

Photoperiodism, melatonin and the pineal
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Role of the pineal gland in the photoperiodic control of reproductive and non-reproductive functions in mink (*Mustela vison*)

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Abstract. Mink are long-lived mammals that exhibit seasonal changes in body weight, gonadal activity, pelage and plasma prolactin levels. Mating in February–March is followed by an obligatory delay in implantation during which the corpora lutea stay quiescent. All these events are controlled by annual variations in daylength. The role of the pineal gland and its product, melatonin, in conveying photic information to the target organs has been studied. Pineal denervation by bilateral ablation of the cervical superior ganglia rendered the mink unresponsive to artificial manipulations of the daily photoperiod: prolactin and progesterone secretion and the spring moult were no longer stimulated by long days or inhibited by short days in pregnant females; in the same way the increase in body weight in late summer and the autumn moult were no longer advanced by artificial shortening of the photoperiod. Pinealectomy seemed to desynchronize body weight, prolactin and moulting cycles from those in intact mink. Melatonin injections reproduced the effects of short days on hormonal secretion during the delayed implantation period. Melatonin capsules given to males during the phase of testicular activity delayed the decrease in body weight, testicular regression and onset of the spring moult exactly as did short days. In contrast, melatonin administered during the phase of testicular inactivity triggered an increase in body weight, the onset of the spring moult and testicular recrudescence in this short-day breeder.

These results support the hypothesis that in mink all photoperiodic signals are conveyed by the pineal gland. But although the pineal seems essential for the seasonal timing of the cycles, it does not modify the events themselves once they are initiated.

1985 Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Foundation Symposium 117) p 170-187

During the two past decades much information has been gathered about the role of the mammalian pineal gland in the control of seasonal cycles. Studies of the importance of the pineal in the transduction of photoperiodic signals

received by the retina have focused mainly on the regulation of reproduction (Reiter 1981). However there is also evidence that the pineal plays a role in the seasonal regulation of body weight (Plotka et al 1982), pelage changes (Lincoln et al 1980), antler growth (Plotka et al 1982), thyroid activity (Vriend 1983) and thermoregulation (Ralph et al 1979). Whether it is a question of reproductive function or not, indole amines, and melatonin in particular, always seem to contribute to the chemical messages released by the pineal gland to act on the neuroendocrine axis (Hoffmann 1973, Vriend 1983, Goldman 1983).

The mink seems to be a particularly convenient model for studying the role of the pineal gland as a neuroendocrine integrator and for testing the hypothesis that all the photoperiodic signals initiating seasonal cycles are conveyed by the pineal gland. Under natural environmental conditions male and female mink show marked cycles in body weight, gonadal activity, moulting periods, pelage composition and hormonal patterns, which are synchronized by annual changes in daylength. Furthermore, the duration of pregnancy depends on the length of the daily photoperiod.

Role of the pineal gland in the photoperiodic control of delayed implantation and spring moult

Mink breed from mid-February to late March; the later the mating, the shorter the pregnancy. This variable duration is due to an obligatory delay in implantation during which the corpora lutea are quiescent and blastocyst growth is inhibited (Enders 1952). The reactivation of the corpora lutea followed by implantation, which occurs by the vernal equinox (Allais & Martinet 1978), is triggered by increasing daylength (Holcomb et al 1962, Martinet et al 1981) as is the spring moult, which begins in mid-April (Duby & Travis 1972, Martinet et al 1983).

Pregnancy and spring moult were studied simultaneously because both depend on the same hormonal support, prolactin, which is luteotrophic in the mink and is involved in the control of moulting (Martinet et al 1984, Duncan & Goldman 1984).

Effects of superior cervical ganglionectomy

As the pineal gland receives an autonomic supply from the superior cervical ganglia, ablation of these ganglia is generally considered to have the same effect as pinealectomy. Thus bilateral ganglionectomy was performed in females two to four weeks before mating. When they were transferred on

the first day of pregnancy (March 6 ± 1 day) to a long (15 h light:9 h darkness) or short (8 h light:16 h darkness) photoperiod, a striking difference appeared between intact and ganglionectomized animals (Fig. 1). In the long-day group, the increase in plasma prolactin and progesterone concentrations and the

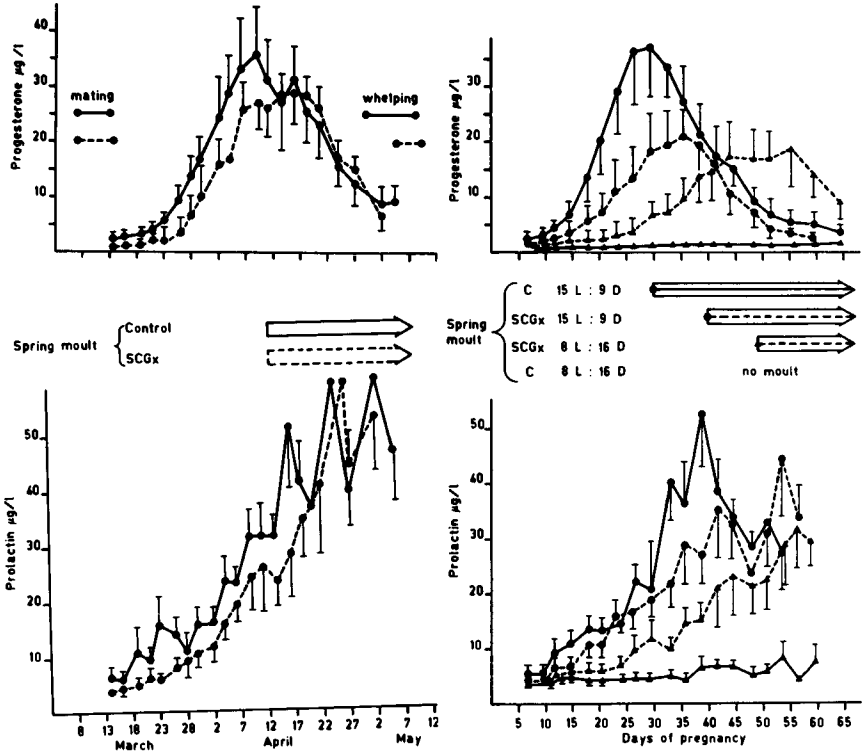

FIG. 1. Plasma prolactin and progesterone concentrations (mean ± SEM) in intact (C, solid lines) or ganglionectomized (SCGx, dashed lines) female mink maintained under natural daylight (left) or transferred (right) after mating to 15 h light:9 h darkness (15L:9D, circles) or 8 h light:16 h darkness (8L:16D, triangles). Horizontal bars show the onset and progression of the moult. From Martinet et al (1985) with the permission of the publisher.

onset of moult were significantly delayed by ganglionectomy. In contrast, ganglionectomy completely suppressed the inhibitory effect of short days on prolactin and progesterone secretion and the onset of moult. These results, which confirmed earlier observations in mink (Murphy & James 1972) and in a marsupial (Renfree et al 1981), clearly showed that removal of the autonomic supply of the pineal rendered the mink unresponsive to artificial manipulations of daylength.

But when the females were left under natural environmental conditions, the onsets of prolactin and progesterone secretion and of moult were not modified by ganglionectomy (Fig. 1). This difference in response between ganglionectomized animals left outside and those submitted to artificial manipulations of the daily photoperiod may be explained if the function of the pineal is only to synchronize endogenous rhythms with the natural daylength variation. In fact, ganglionectomy altered the timing of the recorded events, but did not change the events themselves; that is, the hormone profiles, the progression of the moult and the structure of the summer coat (Table 1) were unaffected.

The fact that no desynchronization of body weight and prolactin cycles was observed between intact and pinealectomized females for 12 to 14 months after the operation supports this hypothesis (Fig. 2). Such a delay in the effect of pinealectomy has been already reported in the timing of recurrent oestrous periods in ferrets (Herbert et al 1978) and may explain why pinealectomy was ineffective in reducing the period of embryonic diapause in spotted skunks maintained under natural daylight (Mead 1972).

Effects of melatonin

It is generally assumed that the daily rhythm of melatonin secretion conveys information about daylength to the neuroendocrine axis, and many effects of the daylight cycle have been reproduced by melatonin administered in different ways (Goldman 1983).

Injections of 100 μg melatonin were given daily one hour before lights-off from day 7 of pregnancy or pseudopregnancy to female mink transferred after mating to long (15 h light: 9 h darkness) or short (11 h light:13 h darkness) days. In those maintained under long days and given melatonin injections or maintained under short days without melatonin administration, the increase in plasma prolactin and progesterone levels was delayed or inhibited compared to that in females maintained under long days and given the vehicle only (Fig. 3). The appearance of blue pigmentation indicative of the initiation of hair follicle activity was also delayed (Fig. 4). Prolactin and progesterone secretion and moult were inhibited in all the females of the short-day group given melatonin (Figs. 3 & 4).

Daily injections of melatonin one hour before lights-off therefore mimicked and strengthened the inhibitory effect of short days on prolactin secretion and consequently on luteal cell and hair follicle activity. As melatonin injections have not been tested at other times in the light–dark cycle, and as nothing is known of the rhythm of melatonin secretion in mink, a comparison with results obtained in rodents or sheep would be premature. However, it seems

TABLE 1 Effects of manipulation of the photoperiod, melatonin and superior cervical ganglionectomy on the spring moult in mink (Allain et al 1981, Martinet et al 1985)

		Moulting period			*Hair follicles/bundle*	
Light and treatment	*Sex*	*Onset*	*Duration (days)*	*Gradient*	*Before moult*	*After moult*
Natural	♂	April 26	64	Caudad	20.3 ± 0.5	13.5 ± 0.6**
Natural + melatonin implants from January 25	♂	July 25	62	Caudad	20.8 ± 0.5	14.0 ± 1.3**
Natural + melatonin implants from March 7	♂	July 2	50	Caudad	18.7 ± 0.8	13.2 ± 0.4**
8L:16D from December 21	♂	July 30	67	Caudad		
Natural	♀	April 13	57	Caudad	14.5 ± 0.5	12.3 ± 0.9*
Natural + ganglionectomy	♀	April 14	66	Caudad	15.1 ± 0.7	11.9 ± 0.8*
15L:9D	♀	April 2	57	Caudad	15.1 ± 0.7	11.9 ± 0.7**
15L:9D + ganglionectomy	♀	April 12	73	Caudad	14.8 ± 0.3	13.1 ± 0.4*
8L:16D	♀	April to August	?	Incomplete	14.3 ± 0.5	?
8L:16D + ganglionectomy	♀	April 21	56	Caudad	16.0 ± 0.7	12.4 ± 0.7**

*$P < 0.05$, **$P < 0.01$ (Student's t test).
8L:16D, 8 h light:16 h darkness.

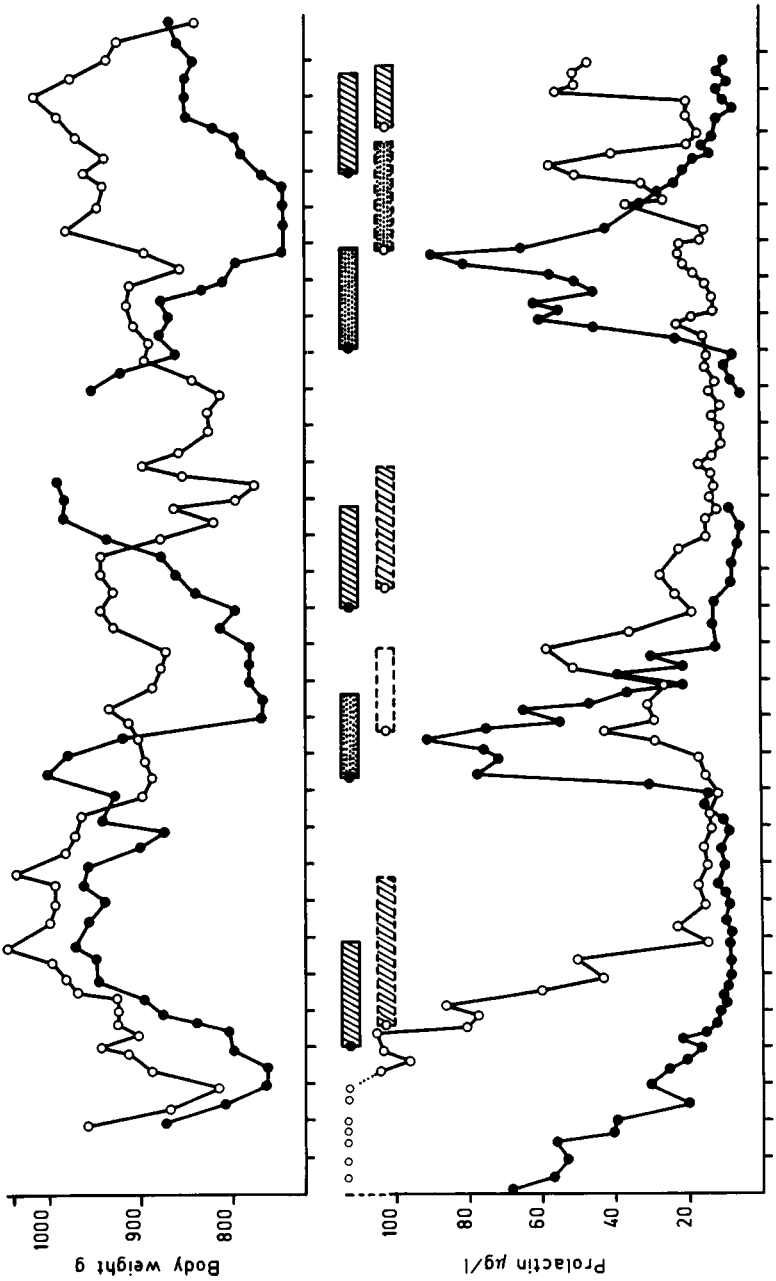

FIG. 2. Body weight, plasma prolactin concentrations and moulting periods in four intact female mink (black circles) and one female mink pinealectomized in February 1982 (open circles). Horizontal bars show the duration of spring (dotted) or autumn (striped) moult. The white bar represents an atypical moult in the pinealectomized female.

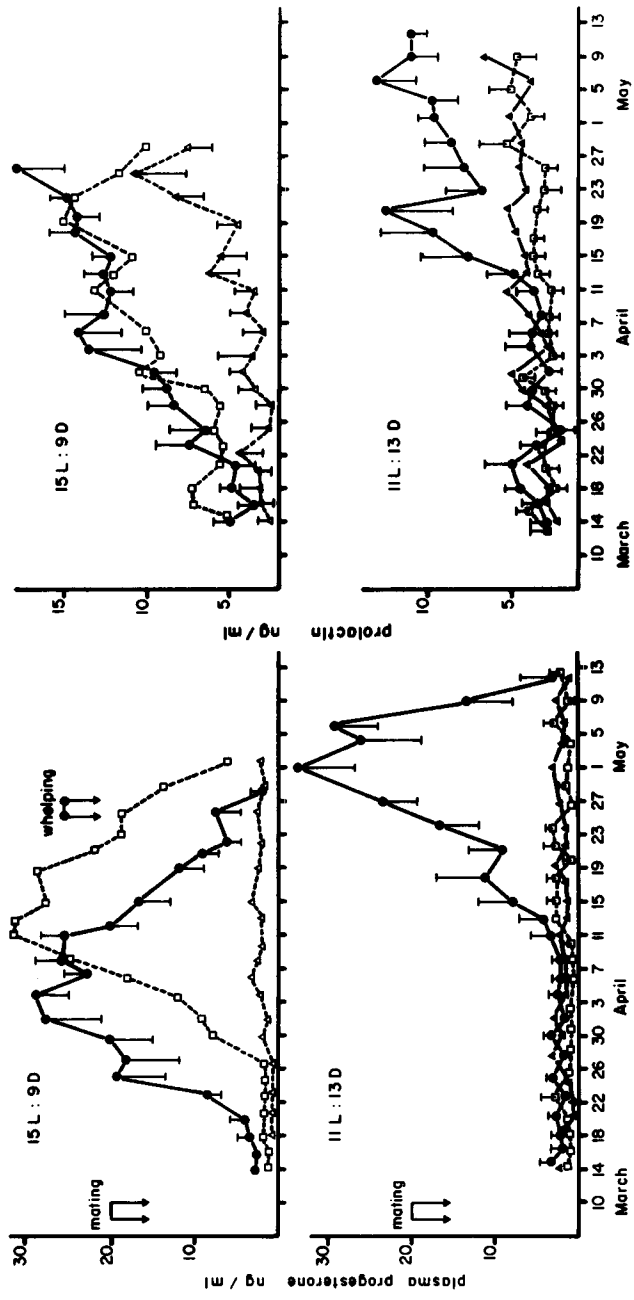

FIG. 3. Effects of melatonin and photoperiod on plasma prolactin and progesterone concentrations (mean ± SEM) in female mink. Some animals were transferred after mating to 15 h light:9 h darkness (15L:9D) and were given a daily afternoon injection of melatonin [dashed lines: squares, $n = 4$ (note the two different types of response)] or vehicle only (solid lines: $n = 2$; triangles, $n = 4$ (note the two different types of response)] or vehicle only (solid lines: $n = 5$). Others were transferred after mating to 11 h light:13 h darkness (11L:13D) and were given a daily afternoon injection of melatonin (dashed lines: $n = 6$) or vehicle only [solid lines: circles, $n = 4$; triangles, $n = 2$ (note the two different types of response)] (Martinet et al 1983).

likely that in mink, as in other mammals, melatonin conveys information about daylength from the pineal to the neuroendocrine axis controlling prolactin secretion and consequently luteal cell and hair follicle activity.

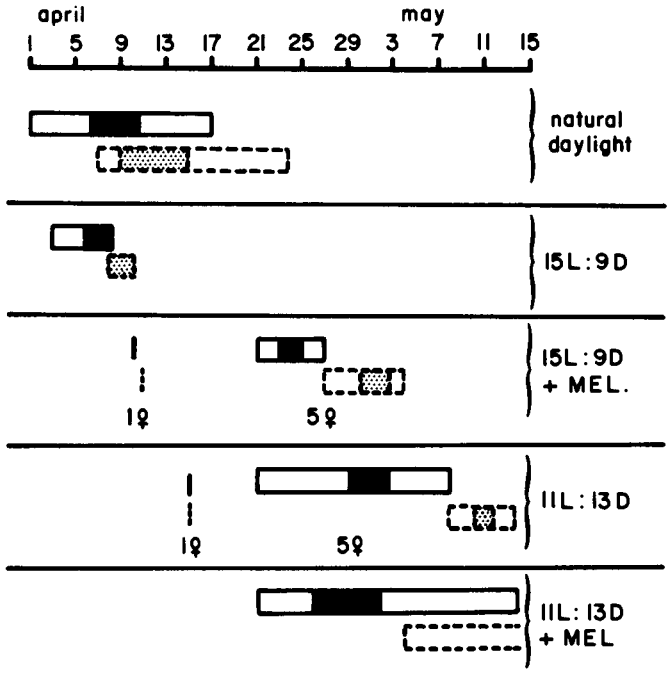

FIG. 4. Onset of moulting in female mink maintained under natural daylight or transferred after mating to 15 h light:9 h darkness (15L:9D) or 11 h light:13 h darkness (11L:13D) and given a daily afternoon injection of 100 μg melatonin (MEL) or vehicle only (Martinet et al 1983). Appearance of blue pigmented skin: on the head ■ (mean ± SEM), ▭ (range); on the flank ▩ (mean ± SEM), ⊏⊐ (range).

Seasonal cycles and their photoperiodic requirements

A marked annual variation in body weight is observed in males and females with a steady increase from August to January, followed by a dramatic decrease in the males just before mating (Fig. 5) and a lesser decrease in females during the breeding season (Fig. 2). When the daily photoperiod is artificially shortened from July, the increase in body weight in female mink is advanced (Fig. 6).

Testis volume begins to increase in December, reaches a peak in February and then decreases very rapidly and remains low from May to the next

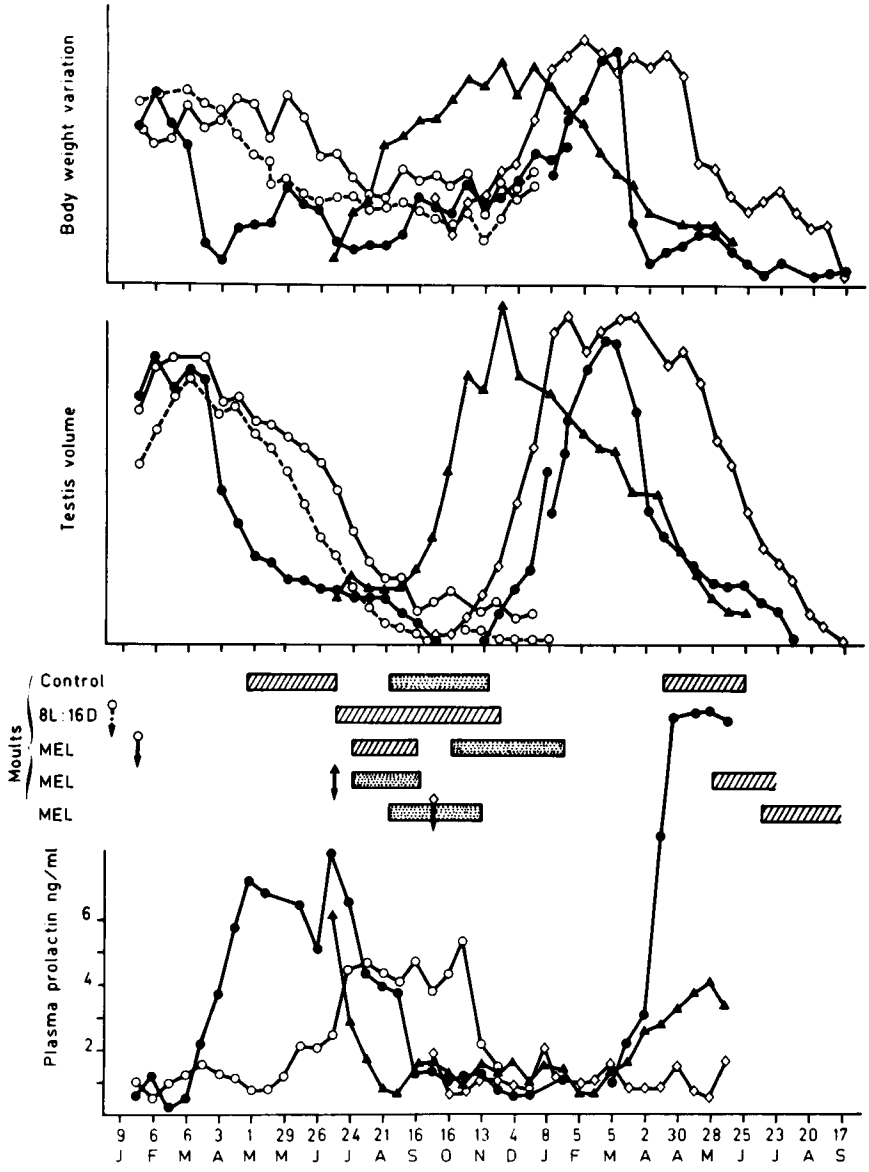

FIG. 5. Seasonal variations of body weight, testis volume and plasma prolactin concentrations in control males (black circles) and in males implanted with melatonin capsules in January (open circles and solid lines), July (triangles) or October (diamonds) or submitted to 8h light:16h darkness (8L:16D) from December (open circles and dashed lines). Horizontal bars show spring (striped) or autumn (dotted) moulting periods (redrawn from Allain et al 1981).

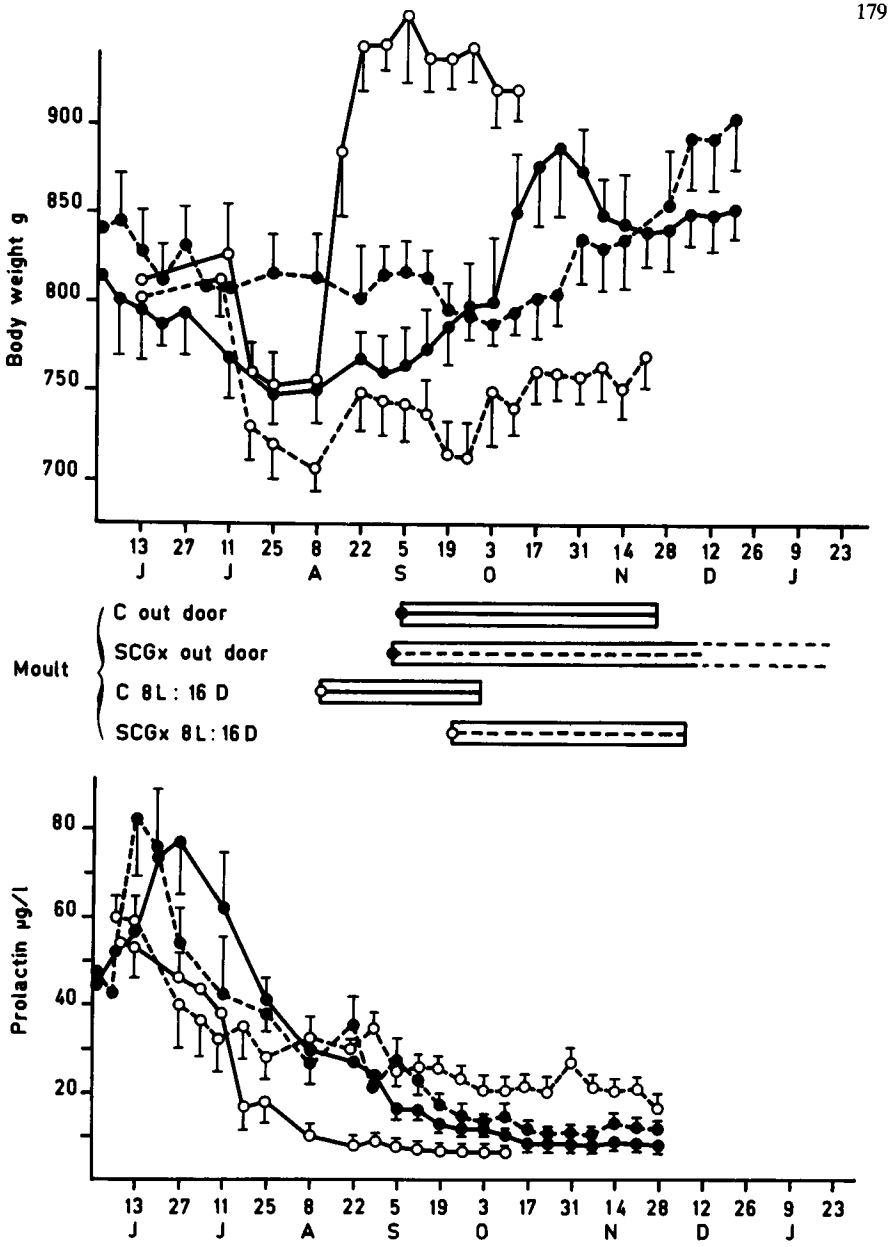

FIG. 6. Body weight, plasma prolactin concentrations (mean ± SEM) and moulting periods in intact female mink (solid lines) and in females ganglionectomized in February (SCGx, dashed lines) maintained either under natural daylight (black circles) or under 15 h light:9 h darkness (15L:9D) from March to July and then under 8 h light:16 h darkness (8L:16D) from July 10 (open circles) (Martinet et al 1985).

November (Fig. 5) (Boissin-Agasse & Boissin 1979). Testis recrudescence is induced by short days (Duby & Travis 1972, Boissin-Agasse et al 1982) and regression is advanced by long days (Duby & Travis 1972). There seems to be a period when testes are refractory to the stimulatory effect of short days since a spontaneous testicular regression was observed in males maintained under a regimen of 8 h light:16 h darkness for more than three months (Fig. 5). But testis recrudescence requires short days because it did not occur when the animals were maintained under long days (Boissin-Agasse et al 1982).

The spring and autumn moults are also controlled by increasing and decreasing daylengths (Duby & Travis 1972). However, in males maintained under 8 h light:16 h darkness from the winter solstice, the spring moult was delayed by about three months but was not suppressed (Fig. 5). Females maintained under 16 h light:8 h darkness showed a moult that had an autumnal gradient, but led to the growth of a thin summer coat, at nearly the same time as females under natural daylight (Table 2 and Martinet et al 1984). The annual moulting cycle therefore seems to be controlled by an endogenous rhythm synchronized by daylength variations.

The seasonal cycle of plasma prolactin concentrations closely parallels that of daylength (Martinet et al 1982).

Role of the pineal gland in the control of seasonal cycles

Effects of pinealectomy or ganglionectomy

The results presented in Fig. 2, although from only one animal, suggest that the pineal gland is necessary for the timing of body weight, prolactin and moult cycles in mink maintained under natural conditions. Mink may possess an endogenous pacemaker that drives these cycles.

In females ganglionectomized in February and then maintained under long days, the shift to a short-day regimen in July did not induce the fast increase in body weight, the decrease in plasma prolactin concentrations and the early autumn moult observed in intact mink (Fig. 6). So pineal denervation suppresses the response to photoperiodic manipulation of all the photoperiod-dependent variables recorded (body weight, time of moult, prolactin concentration).

Effects of melatonin

The observations of Rust & Meyer (1969), who initiated whitening of the fur in the short-tailed weasel by implanting melatonin capsules, raise the question of whether melatonin induces the winter pelage. The following experiment was designed to examine this and to determine when in the annual cycle

TABLE 2 Effects of manipulation of the photoperiod or melatonin administration on the autumn moult in mink (Allain et al 1981, Martinet et al 1984)

		Moulting period			*Hair follicles/bundle*	
Light and treatment	*Sex*	*Onset*	*End*	*Gradient*	*Before moult*	*After moult*
Natural	♂	August 25	Nov 20	Cephalad	13.5 ± 0.6	20.3 ± 0.5*
Natural + melatonin implants from July 2	♂	July 25	Sept 23	Cephalad	11.6 ± 1.2	19.8 ± 1.0*
Natural	♀	Sept 6	Dec 5	Cephalad	14.5 ± 0.4	17.2 ± 0.4*
8L:16D from June 25	♀	July 30	Sept 20	Cephalad	13.0 ± 0.3	16.3 ± 0.4*
16L:8D from June 25	♀	Sept 12	Dec 15	Incomplete	13.0 ± 0.7	10.9 ± 0.7*

*$P < 0.01$ (Student's t test).
8L:16D, 8 h light:16 h darkness.

winter fur growth can be induced in mink. Silastic capsules filled with melatonin were implanted subcutaneously in July, October, January or March in adult males maintained in natural conditions (Allain et al 1981). In those treated from July 2 the moult began after four weeks, leading to a mature winter coat in mid-September, two months earlier than in untreated animals (Fig. 5). The cephalad gradient and density increase were typical of an autumn moult (Table 2). The moult was accompanied by an increase in body weight and a decline in plasma prolactin levels. Testicular recrudescence began when the coat was complete. When given in October, melatonin did not modify the timing of the moult, which was already in progress, but slightly accelerated testis recrudescence (Fig. 5). After melatonin treatment the timing of events was the same as in mink submitted to a short-day regimen (Duby & Travis 1972).

When melatonin capsules were implanted in January or March, during the period of testis activity and before the spring moult, the decrease in body weight and testis volume, the increase in plasma prolactin levels and the onset of moult were delayed by about three months, exactly as in males maintained under a regimen of 8 h light:16 h darkness (Fig. 5 and Table 1). Though given melatonin, the males showed a typical spring moult with a caudad gradient, followed by a decrease in hair density (Table 1). Melatonin, like ganglionectomy, modified the timing of events but not the events themselves. The decrease in body weight and regression of the testes were closely parallel and always preceded the increase in plasma prolactin concentrations and the spring moult. Though it is not known if prolactin in mink is a modulator of gonadotropin secretion and testis activity, it could be the common denominator in the control of body weight, testis size and moulting period.

It is not easy to interpret these results because the melatonin continuously released from the capsules may have masked the probable rhythm in endogenous melatonin release. However, it should be noted that melatonin influences body weight, testis size and moulting periods only during photosensitive phases and is unable to prevent spontaneous testicular regression, which does not depend on photoperiodic stimuli. In the mink, as in other photoperiodic mammals, refractoriness to melatonin may coincide with photorefractoriness, Furthermore, the non-reproductive as well as the reproductive functions of mink become refractory to both photoperiod and melatonin administration (Herbert 1981, Almeida & Lincoln 1984).

Conclusions

These results support the hypothesis that in the mink all photoperiodic signals are transduced by the pineal gland into neuroendocrine messages since gang-

lionectomy suppressed the response of a variety of functions (reproduction, moult, body weight regulation) to a modification of the photoperiod. If the annual change in daylength is the only cue driving seasonal cycles in mink, the main role of the pineal may be to synchronize endogenous cycles with environmental light–dark cycles. Evidence supporting the notion of an endogenous component in the seasonal cycles is: (1) the recurrence of cycles of prolactin secretion, body weight and moulting period in a pinealectomized female; (2) the decrease in plasma prolactin concentrations and occurrence of autumn moult in females kept under an artificial summer solstice; (3) the delayed, but marked, decrease in body weight and testis volume and increase in plasma prolactin concentrations as well as the initiation of spring moult in males kept under constant artificial winter solstice or under conditions of constant melatonin release.

So many pieces are missing from the puzzle of how photoperiod controls seasonal cycles in mink and how the pineal gland is involved in this control that no valuable comparison can be made with other species such as the hamster or sheep. It would seem more interesting to pose a few questions about the mechanism by which the pineal gland controls or partly controls photoperiod-dependent functions. Do the non-reproductive effects of the pineal gland depend on changes in the endocrine reproductive axis, or are both the reproductive and the non-reproductive influences of the pineal gland secondary to its effects on body weight regulation? Testis activity seems to be partly responsible for the increase in body weight during late summer and autumn since castration suppressed body weight gain from December, that is, from the onset of testicular recrudescence. But the moulting periods were not modified either by castration or by testosterone capsules (Allain & Martinet 1984, 1985). On the other hand, the regulation of body weight could be the key to the series of events initiated by increasing or decreasing daylengths. Body weight gain in late summer and weight loss in late winter always precede the moulting periods and testis changes. In the mink, seasonal cycles in body weight are highly correlated with the level of food intake (Charlet-Léry et al 1984), but melatonin capsules that reproduce the effects of short days do not seem to increase food intake (D. Allain, unpublished work 1985). Another question thus arises. Is body weight regulation totally or partially (Syrian hamster: Bartness & Wade 1984) pineal dependent? Body weight regulation may also depend on the modulation of metabolic activity by the thyroid gland which, in turn, is controlled by the pineal gland (Vriend 1983); in mink plasma thyroxine concentrations show a biphasic seasonal change with increasing levels coinciding with periods of hair growth (Boissin-Agasse et al 1981).

Given the complexity of the interactions between endocrine glands and of the role of the pineal gland as the probable integrator of all photoperiodic

stimuli, no general conclusions can be drawn until these primary questions have been answered.

REFERENCES

Allain D, Martinet L 1984 Seasonal coat changes in the mink in relation to the castration in the male and the reproductive status in the female. In: Rougeot J (ed) Third Int Sci Cong Fur Anim Prod, 25–27 April 1984, Versailles, France. Les Colloques de l'I.N.R.A. (Institut National de la Recherche Agronomique), Paris, p 61-65

Allain D, Martinet L 1985 Role of the testis in the regulation of moulting periods and pelage changes in the mink (*Mustela vison*). In Boissin J et al (eds) Endocrine regulations as adaptive mechanisms to the environment. Colloques du Centre National de la Recherche Scientifique, Paris, in press

Allain D, Martinet L, Rougeot J 1981 Effect of melatonin implants on changes in the coat, plasma prolactin level and testis cycle in the mink (*Mustela vison*). In: Ortavant R et al (eds) Photoperiodism and reproduction. Les Colloques de l'I.N.R.A. (Institut National de la Recherche Agronomique), Paris, p 263-271

Allais C, Martinet L 1978 Relation between daylight ratio, plasma progesterone levels and timing of nidation in mink (*Mustela vison*). J Reprod Fertil 54:133-136

Almeida OFX, Lincoln GA 1984 Reproductive photorefractoriness in rams and accompanying changes in the pattern of melatonin and prolactin secretion. Biol Reprod 30:143-158

Bartness TJ, Wade GN 1984 Photoperiodic control of body weight and energy metabolism in Syrian hamsters (*Mesocricetus auratus*): role of the pineal gland, melatonin, gonads and diet. Endocrinology 114:492-498

Boissin-Agasse L, Boissin J 1979 Variations saisonnières du volume testiculaire et de la testostéronémie chez deux mustélidés: le furet (*Mustela furo L.*) et le vison (*Mustela vison S.*). J Physiol (Paris) 75:227-232

Boissin-Agasse L, Maurel D, Boissin J 1981 Seasonal variations in thyroxine and testosterone levels in relation to the moult in the adult male mink (*Mustela vison*). Can J Zool 59:1062-1066

Boissin-Agasse L, Boissin J, Ortavant R 1982 Circadian photosensitive phase and photoperiodic control of testis activity in the mink (*Mustela vison*), a short-day mammal. Biol Reprod 26:110-119

Charlet-Léry G, Fiszlewicz M, Morel MT, Rougeot J 1984 Variations au cours du cycle annuel de l'état nutritionnel du vison mâle adulte. I—Poids vif, niveau d'ingestion, digestibilité, rétention azotée. Ann Zootech (Paris) 33:73-98

Duby RT, Travis HF 1972 Photoperiodic control of fur growth and reproduction in the mink (*Mustela vison*). J Exp Zool 182:217-226

Duncan MJ, Goldman BD 1984 Hormonal regulation of the annual pelage color cycle in the Djungarian hamster, *Phodopus sungorus*. II Role of prolactin. J Exp Zool 230:97-103

Enders RK 1952 Reproduction in the mink (*Mustela vison*). Proc Am Philos Soc 96:691-755

Goldman BD 1983 The physiology of melatonin in mammals. Pineal Res Rev 1:145-182

Herbert J 1981 The pineal gland and photoperiodic control of the ferret's reproductive cycle. In: Follett BK, Follett DE (eds) Biological clocks in seasonal reproductive cycles. John Wright, Bristol, p 261-276

Herbert J, Stacy PM, Thorpe DH 1978 Recurrent breeding seasons in pinealectomized or optic-nerve sectioned ferrets. J Endocrinol 78:389-397

Hoffmann K 1973 The influence of photoperiod and melatonin on testis size, body weight and pelage colour in the Djungarian hamster (*Phodopus sungorus*). J Comp Physiol 85:267-282

Holcomb LC, Schaible PJ, Ringer RK 1962 The effects of varied lighting regimes on reproduction in mink. Q Bull Michigan Agric Exp Stn 44:666-678

Lincoln GA, Klandorf H, Anderson N 1980 Photoperiodic control of thyroid function and wool and horn growth in rams and the effect of cranial sympathectomy. Endocrinology 107:1543-1548

Martinet L, Allais C, Allain D 1981 The role of prolactin and LH in luteal function and blastocyst growth in mink (*Mustela vison*). J Reprod Fertil (suppl) 29:119-130

Martinet L, Ravault JP, Meunier M 1982 Seasonal variations in mink (*Mustela vison*) plasma prolactin measured by heterologous radioimmunoassay. Gen Comp Endocrinol 48:71-75

Martinet L, Allain D, Meunier M 1983 Regulation in pregnant mink (*Mustela vison*) of plasma progesterone and prolactin concentrations and regulation of onset of the spring moult by daylight ratio and melatonin injections. Can J Zool 61:1959-1963

Martinet L, Allain D, Weiner C 1984 Role of prolactin in the photoperiodic control of moulting in the mink (*Mustela vison*). J Endocrinol 103:9-15

Martinet L, Allain D, Chabi Y 1985 Pineal denervation by cervical sympathetic ganglionectomy suppresses the role of photoperiod on pregnancy or pseudopregnancy, body weight and moulting periods in the mink (*Mustela vison*). J Endocrinol, in press

Mead RA 1972 Pineal gland: its rôle in controlling delayed implantation in the spotted skunk. J Reprod Fertil 30:147-150

Murphy BD, James DA 1972 The effects of light and sympathetic innervation to the head on nidation in mink. J Exp Zool 187:267-276

Plotka ED, Seal US, Verme LJ 1982 Morphologic and metabolic consequences of pinealectomy in deer. In: Reiter RJ (ed) The pineal gland, vol III. Extra-reproductive effects. CRC Press, Boca Raton, Florida, p 153-168

Ralph CL, Firth BT, Gern WA, Owens DW 1979 The pineal complex and thermoregulation. Biol Rev Camb Philos Soc 54:41-72

Reiter RJ (ed) 1981 The pineal gland, vol II. Reproductive effects. CRC Press, Boca Raton, Florida

Renfree MB, Lincoln DW, Almeida OFX, Short RV 1981 Abolition of a seasonal embryonic diapause in a wallaby by pineal denervation. Nature (Lond) 293:138-139

Rust CC, Meyer RK 1969 Hair color, molt and testis size in male short-tailed weasels treated with melatonin. Science (Wash DC) 165:921-922

Vriend J 1983 Pineal-thyroid interactions. Pineal Res Rev 1:183-206

DISCUSSION

Turek: It's important not to think of the hamster and the sheep as the only photoperiodic species, and your results in the mink raise a number of interesting questions. You showed that pinealectomy affects the response of animals to an artificial photoperiodic challenge, but does not affect changes that occur in animals under a natural light–dark cycle. This reinforces the idea that the pineal gland is involved in photoperiod-induced changes in the reproductive system but not in those changes that occur on a circannual basis or that are controlled by another internal timing system, such as spontaneous testicular recrudescence in the golden hamster. The hamster does not show a circannual rhythm but it does have an internal timing system regulating spontaneous testicular recrudescence, which occurs a fixed number of weeks after short-day-induced testicular regression. It seems that melatonin interacts with such a timing system quite differently at different times of the year (Turek & Losee 1979), but we are really in the dark about what that internal timing system is.

Martinet: I think that the mink and the hamster are similar from this point of view, but we observe the inverse situation in the mink, perhaps because it is a short-day breeder. In this species short days or melatonin capsules delay the decrease in body weight and the testis regression that normally begin in March, but cannot prevent them. On the other hand, short days are needed for body weight increase and testicular recrudescence to occur. Testis growth does not seem to occur spontaneously. I do not know if an endogenous circannual rhythm in body weight and testis size exists in male mink, but circannual variations in body weight were observed for at least two years in a pinealectomized female.

Turek: In birds, there is tremendous species variation in the roles of the pineal gland and the retina in producing melatonin. In the mammalian system, species variation occurs at a different level, i.e. in how the animal's reproductive system responds to pinealectomy or melatonin, and I think this is clearly seen when we compare the hamster and the mink.

Goldman: I agree with your comment about species differences, but it would be useful if we could find some general principles. One generalization, as you mentioned, is that the pineal gland seems to be involved in synchronizing those cycles that have components under photoperiodic control. I would also like to suggest that, apart from the difference between short-day and long-day breeders, the major difference between species like the mink, the sheep and the hamster is that some but not others have a photoperiodic response superimposed on a circannual rhythm. I think that an explanation for seasonal rhythms which takes into account both these components may help to clear up some of the confusion.

Herbert: There are some striking parallels between Dr Martinet's results and our observations in ferrets. To produce an immediate difference between normal and pinealectomized ferrets you need to 'drive' the animals by exposing them to an artificial photoperiod; if you just leave the animals in a natural photoperiod the effects of pinealectomy are very much delayed. This is also true for the mink. However, we were never actually able to demonstrate anything we could call a circannual rhythm in ferrets, although we followed pinealectomized animals for up to five years. All we showed was that individual animals came in and out of oestrus at apparently random intervals. We built all kinds of complicated models with oscillators to try to explain this, but they were never satisfactory.

Zucker: In golden-mantled ground squirrels there is a clear persistence of circannual rhythmicity in individual animals studied for several years after removal of the pineal gland (Zucker 1985). This occurs in both males and females, but the results are most extensive for females maintained in a fixed photoperiod of 10 h light:14 h dark. Using two different phase reference points, we have observed a change in the period of the free-running circannual rhythm

after pinealectomy. It is 27 days shorter the first year and 58 days shorter the second year.

Martinet: Since in our experiment the pinealectomized or ganglionectomized females were kept outside, the overt rhythm observed might have been driven by external synchronizers other than the annual change in daylength. However, as the period of the rhythm seemed to be longer than 12 months, the existence of an endogenous rhythm in mink cannot be ruled out.

Zucker: In the squirrel the cycle is endogenous for the particular photoperiod I mentioned. We have also done the experiments with a long-day photoperiod and get similar results. But in the absence of a pineal, the animal does not respond to a fixed photoperiod as a normal animal would, so to be on safe ground we should probably repeat these experiments with animals kept in constant light or constant darkness.

Short: Although it has become a platitude, it is still worth thinking about the concept that it is not hormones that have evolved, but the uses to which they are put. This seems to be true for prolactin, so perhaps you could speculate about what the prolactin rhythm is doing in the mink, Dr Martinet? For example, do you think that prolactin is responsible for the weight loss in males? Every mammal that we know of has the same circannual prolactin rhythm, and yet different mammals use the prolactin to do different things. Wallabies, for example, use prolactin to *inhibit* the corpus luteum (Renfree 1981), which is exactly the opposite to mink, where prolactin is luteotrophic.

Martinet: I have no indication what the role of prolactin is in body weight regulation in the mink, but in this species prolactin is certainly necessary for luteal function and for the initiation of hair follicle activity in spring. Bromocriptine injections or short days, which lower plasma prolactin levels, inhibit the initiation of the spring moult, whereas prolactin injections or long days, which increase plasma prolactin levels, advance it. On the other hand, short days or bromocriptine injections can induce hair follicle activity and an early autumn moult (Martinet et al 1984).

REFERENCES

Martinet L, Allain D, Weiner C 1984 Role of prolactin in the photoperiodic control of moulting in the mink (*Mustela vison*). J Endocrinol 103:9-15

Renfree MB 1981 Marsupials: alternative mammals. Nature (Lond) 293:100-101

Turek FW, Losee SH 1979 Photoperiodic inhibition of the reproductive system: a prerequisite for the induction of the refractory period in hamsters. Biol Reprod 20:611-616

Zucker I 1985 Pineal gland influences period of circannual rhythms of ground squirrels. Am J Physiol 249:R111-R115

Photoperiodism, melatonin and the pineal
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Melatonin administration: effects on rodent circadian rhythms

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Abstract. Previous research has demonstrated a lack of effect of pinealectomy upon the generation of rodent circadian activity rhythms and only a subtle effect upon their re-entrainment after phase-shifts of the light–dark cycle. In contrast, our pharmacological studies on melatonin indicate that the pineal gland may be more important than hitherto believed. The main outcome of a preliminary pilot experiment on the effect of daily injections of melatonin, arginine vasotocin, melanocyte-stimulating hormone and a β-blocker on rat free-running activity rhythms in constant darkness was that melatonin appeared to have entrainment properties. This was clearly demonstrated in a second experiment although entrainment did not occur until the onset of the activity rhythm coincided with the daily injection. In contrast, when melatonin was administered *ad lib.* in the drinking water to six rats housed in constant dim light, there was apparently a lengthening of the free-running period. The effects of 17 days of melatonin injections given at four different times of day to different groups of rats on re-entrainment of activity rhythms after a 5 h phase-advance of darkness were assessed. Results were confounded by the response of some control rats. However, after an 8 h advance of darkness and daily injection at the time of day of the previous dark onset, melatonin-injected rats phase-advanced, whereas vehicle-injected and uninjected control rats phase-delayed. Thus melatonin can alter the direction, but not necessarily the rate, of re-entrainment. The relevance of some of these findings with pharmacological doses of melatonin to the function of endogenous melatonin is discussed.

1985 Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Foundation Symposium 117) p 188-207

On the basis of a limited number of pinealectomy (Px) studies in a few species, it is generally considered that the pineal gland is unimportant for the generation or maintenance of mammalian circadian activity rhythms (Richter 1967, Quay 1968). A role for the pineal gland in the synchronization of activity rhythms in rats and hamsters has been proposed. After a phase-shift in the light–dark (LD) cycle a faster rate of re-entrainment is found in Px animals than in sham-operated or intact controls. In rats and hamsters this increase in rate is modest, is restricted to the first few transient cycles after the lighting

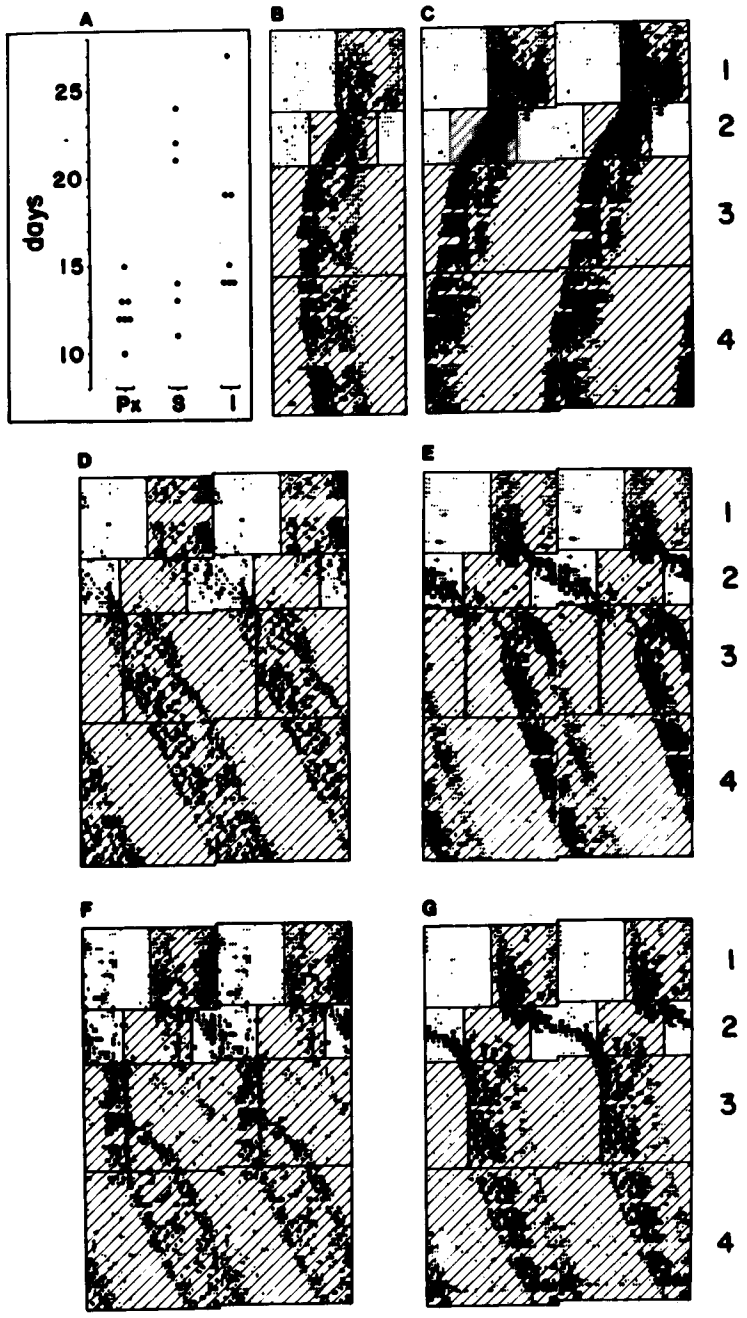
inversion and is age dependent (Kincl et al 1970, Quay 1972, Finkelstein et al 1978). These findings have been evaluated in detail by Rusak (1982). The consensus of opinion is that if the pineal gland plays any role in the circadian pacemaker system of mammals, it is a secondary or subsidiary role.

Such a conclusion may be considered premature, for several reasons. First, the number of mammalian species investigated is small. Rats and hamsters do not represent the placental mammals. Among the avian species there are considerable differences in the effects of Px (Ueck 1982). After Px, sparrows become arrhythmic in constant conditions (Gaston & Menaker 1968), starlings change their free-running period (Gwinner 1978) and the circadian activity rhythm of quail appears unaffected (Simpson & Follett 1981). These three results may reflect species differences in the strength of internal coupling between the sub-oscillators that compose the pacemaker for the circadian activity rhythm. Where the coupling is strong, for example in quail, Px has no effect, but where the coupling is weak, as in sparrows, Px results in loss of mutual entrainment between self-sustained oscillators of the circadian system (Gwinner 1978). Gwinner (1978) suggests that these species differences reflect quantitative rather than qualitative differences in the organization of the circadian system.

Second, Px alone may not be the most appropriate method for evaluating the importance of the pineal gland if there are extra-pineal sources of melatonin (Ebihara et al 1984). Third, in the re-entrainment experiments discussed, a 12 h phase-shift in the LD cycle is the least useful of all phase-shift paradigms, since the pacemaker is put in a situation of ambiguity for it may advance or delay. Indeed, when a 5 h phase-advance of darkness is utilized, which results in a very slow re-entrainment (see Fig. 4C), we have found that on average Px rats re-entrain 40% faster than sham-operated and intact controls (Fig. 1A). This finding clearly illustrates the need for implementing appropriate experimental paradigms to elucidate the role of the pineal gland within the mammalian circadian pacemaker system. However, in subsequent experiments involving the response to light pulses, entrainment to 8 h and 48 h days and changes to the free-running period after an abrupt switch from constant dark (DD) to constant light (LL), no obvious difference between Px, sham-operated and intact rats was noted.

Melatonin entrains free-running rhythms

As an alternative to Px, several years ago we began a pharmacological approach which was based upon the following rationale. In the rat, elevated nocturnal levels of pineal melatonin decrease rapidly when lights are switched on for a short time in the middle of the dark period. This decrease can also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be achieved by β-blockade of the pineal sympathetic innervation (Axelrod 1974). From this perspective, injection of a β-blocker mimics the effect of light on pineal melatonin levels. The rodent free-running activity rhythm can be phase-shifted by a single light pulse of 15 or 60 min duration while two light pulses given daily (skeleton photoperiods) entrain the rhythm (Pittendrigh & Daan 1976b). Therefore, as the pineal gland might be part of the circadian pacemaker system, it seemed reasonable to ask whether daily injections of a β-blocker (pindolol, 0.4 mg/kg) could entrain or phase-shift rat free-running activity rhythms. In other words, could a drug pulse mimic the effects of a light pulse? At the same time we were interested in ascertaining if daily, subcutaneous injections of melatonin (1 mg/kg), arginine vasotocin (AVT) (10 μg/kg) or melanocyte-stimulating hormone (MSH) (40 μg/kg) could influence free-running rhythms (four rats per drug group). As a control we used a solution containing 0.01 M-acetic acid, which acts as an antioxidant, and 2% alcohol.

In this pilot test a 7 h phase-advance of darkness (Stage 2) was implemented during a 12 h:12 h LD cycle (Stage 1) to bring dark onset to a more convenient time of day for injection. Most rats had not re-entrained entirely by the time injections commenced (Stage 3, DD) (Fig. 1B, D: control rats). Nevertheless, the following findings are worth noting. In one rat (Fig. 1C) pindolol appeared to exaggerate the trend to phase-advance. This was the only animal to keep advancing (or free-running with a period less than 24 h) in Stage 4 (DD, no injections). Injection of AVT to one rat (Fig. 1E) appeared to mask the onset of the free-running rhythm while in a second rat (Fig. 1F) it either temporarily entrained the rhythm or induced a burst of activity around the time of injection. The most important finding was that the melatonin-treated rats entrained in Stage 3 (Fig. 1G). The onset of activity early in Stage 4 coincided with the time of day of injection in Stage 3, showing that rats free-ran from this point in time. Three of the four MSH-injected rats phase-advanced during Stage 2 and therefore any possible entrainment properties of the hor-

FIG. 1. (A) Number of days taken to re-entrain activity rhythms after a 5 h advance in the LD cycle in pinealectomized (Px), sham-operated (S) and intact (I) rats that phase-advanced. (B) Single and (C–G) double-plotted rat running-wheel activity records. Hatched areas indicate darkness. Stage 1, 12:12 LD cycle; Stage 2, 7 h phase-advance of darkness; Stage 3, DD with subcutaneous injections of pindolol, AVT or melatonin given at time indicated by vertical arrows in D–G; Stage 4, DD. (B) Control rat that phase-advanced in Stage 2 and free-ran with a period less than 24 h in Stage 3 and eventually with a period greater than 24 h in Stage 4. (C) Rat treated with pindolol in Stage 3 which continued to free-run with a period less than 24 h in Stage 4. (D) Control rat that phase-delayed in Stage 2 and free-ran with a period greater than 24 h in Stages 3 and 4. (E, F) Rats injected with AVT during Stage 3. (G) Rat injected with melatonin in Stage 3.

mone could not be ascertained in Stage 3 (unpublished work with G. J. Coleman, K. Greenwood & S. McConnell 1981).

To demonstrate unequivocally that exogenous melatonin can entrain the rat circadian activity rhythm it is necessary first to let rats free-run (Stage 1), then to inject them daily with melatonin until entrainment is well demonstrated (Stage 2), and then to cease injection and determine the phase of the subsequent free-run (Stage 3). We did this with 10 experimental and 10 control rats and the results for eight rats are shown in Fig. 2 (Redman et al 1983). It is clear that entrainment does not take place until the onset of activity coincides with time of day of melatonin injection (Fig. 2A–D). There is apparently a very narrow window in time (activity onset) when the circadian pacemaker is susceptible to the entraining influence of exogenous melatonin. Melatonin injections did not phase-shift the activity rhythm. When injections were discontinued some rats free-ran almost immediately whereas in others considerable time elapsed from the termination of injection to the re-establishment of the original free-running period (Fig. 2A–D). Eight control rats were unaffected by the injection procedure (e.g. Fig. 2E, F), one became temporarily synchronized for a few days (Fig. 2G) and one became entrained (Fig. 2H). At present the importance of the retinae, dorsal raphe nuclei, suprachiasmatic nuclei and the locus coeruleus as neural targets for the melatonin entrainment effect are being investigated.

Hamsters, in contrast to rats, do not entrain to daily injections of melatonin given in the same dose and by the same route (Fig. 3A) (M. Menaker et al, unpublished work 1984), which confirms unpublished findings of Ellis & Turek (Turek et al 1982). The reason for this lack of effect in hamsters is unknown but the result emphasizes the importance of a comparative approach.

Melatonin may lengthen the free-running period

To try to circumvent the entrainment problem associated with control injections we decided to attempt to entrain rats to melatonin dissolved in the drinking water. Rats in our laboratory drink at least 80% of their total daily water intake in the dark portion of a 12:12 LD cycle, and this pattern continues under free-running conditions of DD with most drinking taking place in the active phase. Therefore, rats should start imbibing water at about the same time every day, the exact timing depending upon the length of the free-running period. Melatonin given orally is absorbed into the bloodstream at physiological levels in sheep and dogs (Kennaway & Seamark 1980, Sääf et al 1980). Six rats were maintained on tap water under very dim LL until the free-running period was stable. Then three rats were administered melatonin in the drinking water (0.05 mg/ml), and three were given the vehicle solution. Subsequently

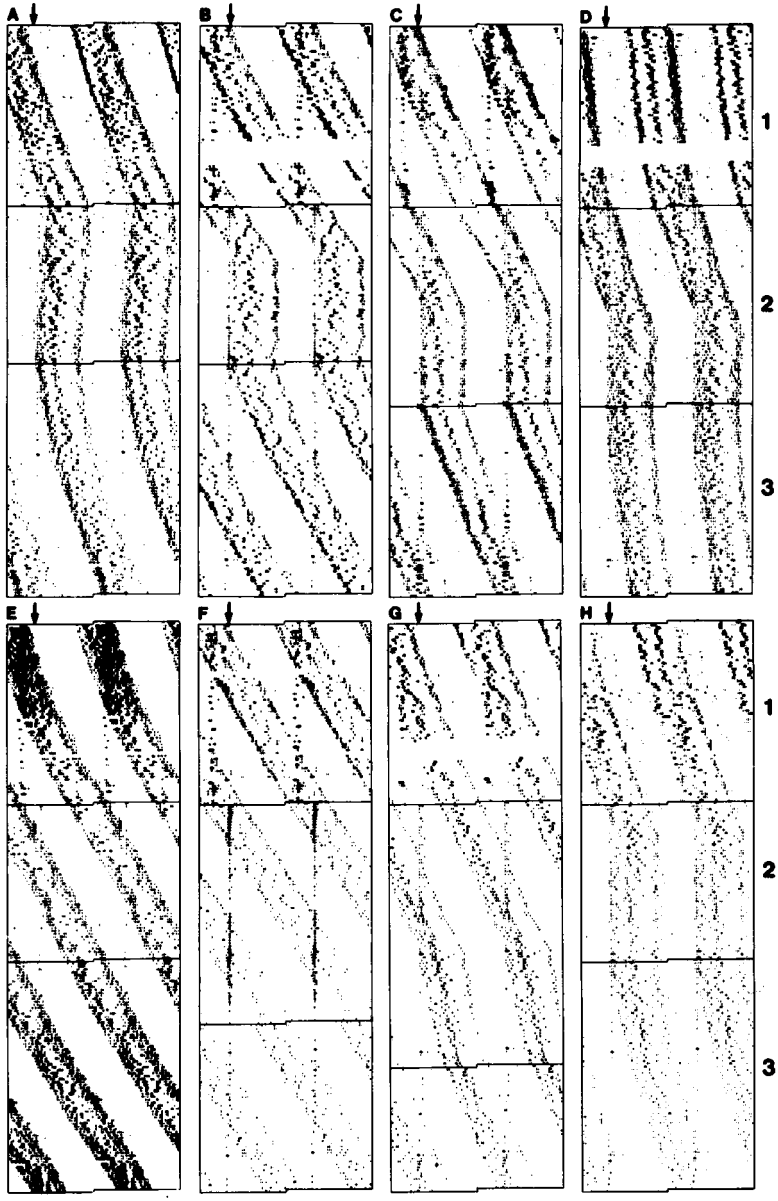

FIG. 2. Double-plotted running-wheel activity records of rats housed under DD and free-running with periods greater than 24 h. Stage 1, pre-injection; Stage 2, daily injection; Stage 3, post-injection. (A–D) Melatonin injections in Stage 2. (E–H) Control injections in Stage 2. Time of day of injection in Stage 2 is indicated by the arrows at the top.

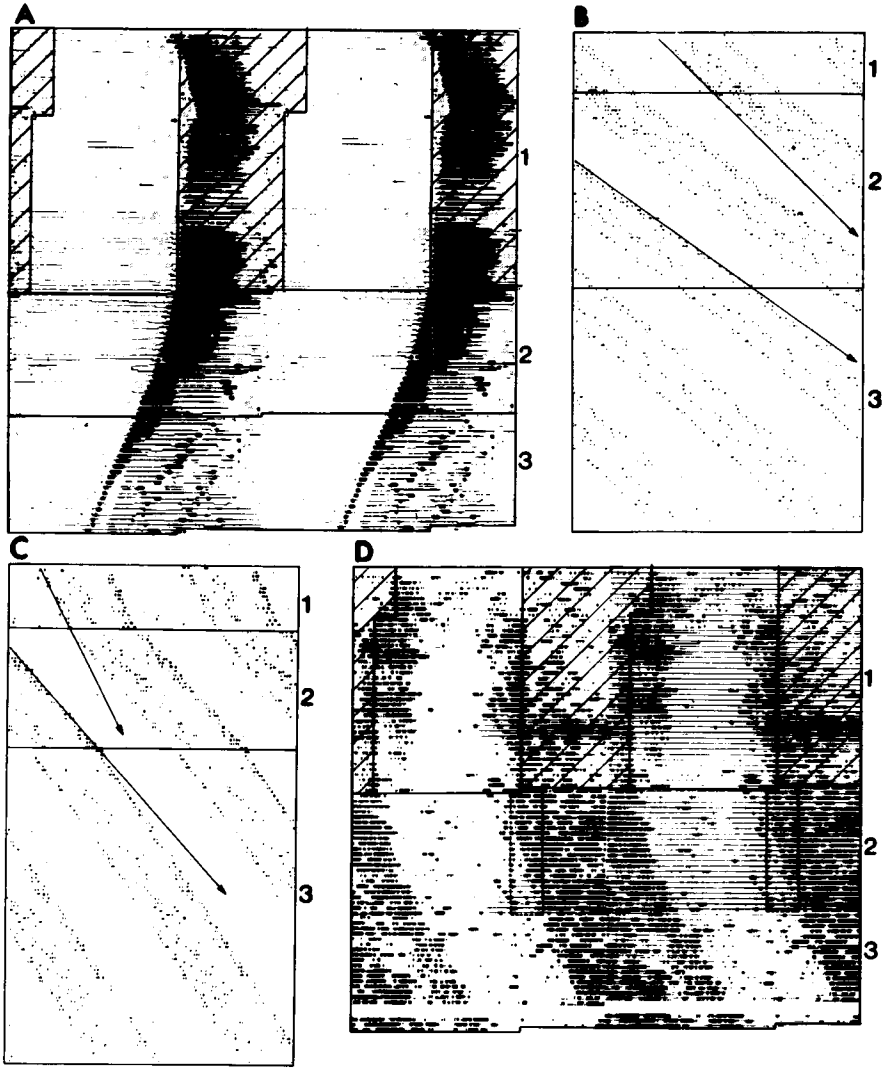

FIG. 3. (A) Double-plotted activity record of one hamster. Stage 1, 12:12 LD followed by 14:10 LD; Stage 2, dim LL and melatonin injections at time of previous dark onset; Stage 3, dim LL and no injections. (B, C) Double-plotted activity records of rats housed under dim LL and free-running with periods greater than 24 h. Stage 1, tap water; Stage 2, melatonin in drinking water; Stage 3, vehicle solution offered in drinking water *ad lib*. Arrows indicate melatonin-induced changes to the free-running period. (D) Double-plotted activity record of rat offered melatonin in drinking water for 3 h per day in Stage 2 at time indicated by vertical lines. Stage 1, 12:12 LD followed by 14:10 LD; Stages 2 and 3, dim LL.

the melatonin-treated rats were offered tap water again, two of the vehicle-treated rats were given the melatonin solution and the sixth rat remained on the vehicle solution.

No sign of entrainment to melatonin was found under these conditions but it appeared that the free-running period was lengthened (Fig. 3B, C). With only six rats this finding must be treated with caution since the free-running period normally tends to lengthen over many months under dim LL. Furthermore, if the period did lengthen in response to melatonin it did not always shorten when the melatonin solution was removed. Nevertheless, this experiment is certainly worth repeating with a larger experimental population.

In a second attempt to use the drinking water as a route of melatonin administration, a higher concentration (0.1 mg/ml) was offered for 3 h every 24 h, and tap water for the remaining 21 h. In this experiment rats ($n = 12$) were housed under an LD cycle and then switched to dim LL. On the day the laboratory was put onto LL, melatonin was presented for 3 h starting 1 h before the time of the previous dark onset. Melatonin was offered daily at this time for 24 days. No sign of entrainment and no obvious change in the free-running period were seen (Fig. 3D) (M. Menaker et al, unpublished work 1984).

Melatonin alters direction and speed of re-entrainment

We have carried out a series of experiments to examine the influence of subcutaneous melatonin injections (1 mg/kg) on rat re-entrainment patterns after phase-shifts in the LD cycle (J. Redman & S. M. Armstrong, unpublished work 1985). The initial series of experiments capitalized on the slow entrainment rate of our rats when exposed to a 5 h advance in darkness. In the first experiment we injected rats on the day we advanced darkness at the time of the new dark onset (Fig. 4A, Group 1). The major finding was that melatonin altered the direction of re-entrainment (Fig. 4D, cf. control in Fig. 4C). We had 15 vehicle-injected control rats and 15 melatonin-injected rats. As expected, most control rats slowly phase-advanced in response to the 5 h advance of darkness. Eight of the melatonin-treated rats initially phase-delayed, but subsequently two of these phase-advanced (Table 1). The fact that only six rats finally phase-delayed meant, presumably, that we were not injecting at the time of maximum sensitivity to melatonin. For instance, it is not known whether the circadian pacemaker in the central nervous system (CNS) changes immediately after the phase-shift to the new LD cycle (Pittendrigh & Daan 1976a) or whether this change takes many days (as reflected by the activity pattern). Hence, we did not know if we were injecting into the dead-zone of the pacemaker's phase–response curve or into the delay

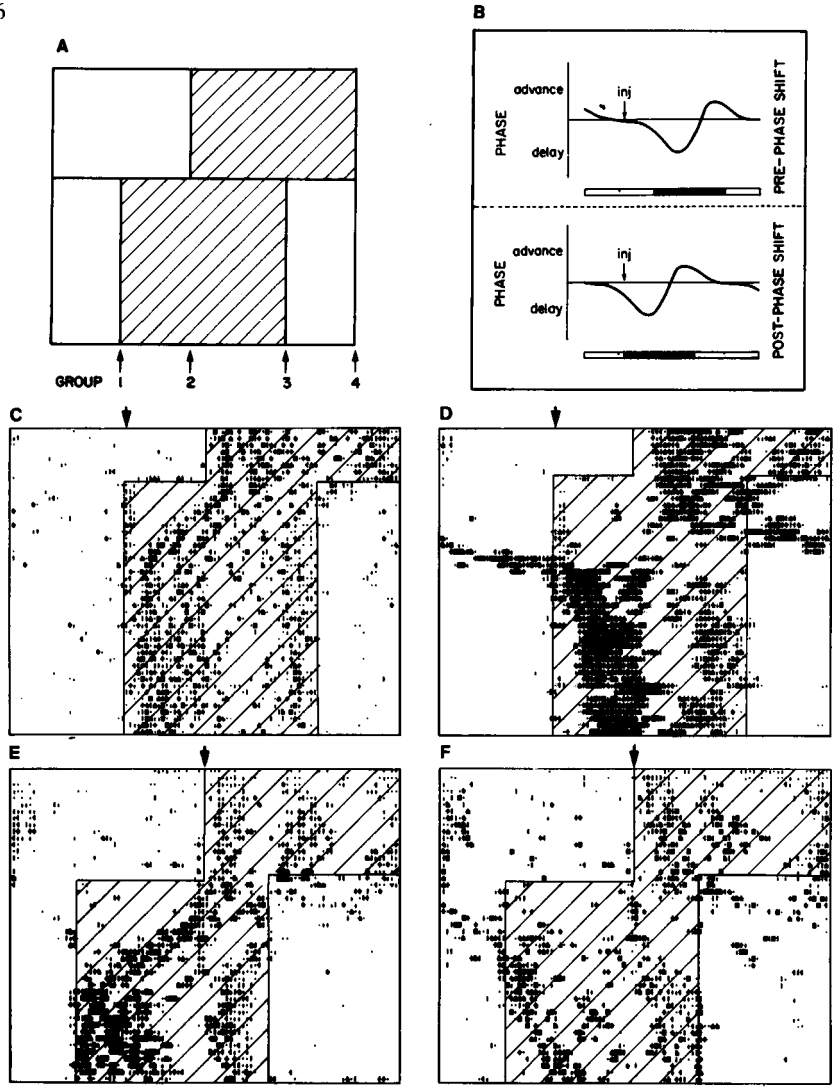

FIG. 4. (A) Diagram of 5 h phase-advance of darkness paradigm and time of day of injections for four groups of rats. (B) Diagram illustrating, for Group 1 rats, possible confounding of time of day of injection with change in phase of pacemaker after the 5 h phase-advance of darkness. (C) Activity plot for control (vehicle-injected) rat that phase-advanced in response to a 5 h phase-advance of darkness. (D) Activity plot for melatonin-injected rat that phase-delayed after a 5 h phase-advance of darkness. In (C) and (D) injections were given for 17 days, starting on the day of the phase-shift, and coincided with the time of the new dark onset (indicated by arrow). (E) Activity plot for melatonin-injected rat that phase-advanced after an 8 h phase-advance of darkness. (F) Activity plot for control rat that phase-delayed in response to an 8 h phase-advance of darkness. In (E) and (F) injections were given for 14 days, starting on the day of the phase-shift, and coincided with the time of dark onset before the phase-shift (indicated by arrow).

portion of the curve (see Fig. 4B), or even if this was an important determinant of melatonin's effect. We therefore chose three additional times of day at which to inject (Fig. 4A), expecting to find an optimum time when all melatonin-injected rats would phase-delay and all controls phase-advance. Group 2 rats showed no delay response to melatonin; all phase-advanced but re-entrained faster than controls, at least in the first seven days after the phase-shift.

TABLE 1 Influence of melatonin injections (1 mg/kg, s.c.) on direction of entrainment of rat activity rhythms after a 5 h (Groups 1 to 4) or 8 h (Group 5) phase-advance of darkness

	N	*Advance*	*Delay*
Group 1			
Control	15	14	1
Melatonin	15	9	6
Group 2			
Control	15	13	2
Melatonin	15	15	0
Group 3			
Control	15	2	13
Melatonin	15	4	11
Group 4			
Control	15	8	7
Melatonin	15	4	11
Group 5			
Uninjected	10	0	10
Control	10	0	10
Melatonin	10	10	0

Most Group 3 rats, controls as well as melatonin-injected, phase-delayed. A similar phenomenon occurred with Group 4 rats (Table 1). It appeared that at the dark–light transition all rats were susceptible to the stress of handling or to the injection or to the chemical content of the vehicle.

Melatonin-treated rats in Group 2 re-entrained faster than controls, indicating that melatonin administered at this time facilitates phase-advances. Rats in our laboratory consistently phase-delay when subjected to an 8 h phase-advance of darkness. Therefore, it would be striking if melatonin administered at Time 2 made rats phase-advance while control rats phase-delayed. The 8 h phase-advance procedure was applied to 10 melatonin-treated and 10 vehicle-treated rats. In addition we included 10 untreated rats to ascertain whether any aspect of the injection procedure had an affect on re-entrainment. Table 1 (Group 5) shows that all uninjected and vehicle-treated rats phase-delayed while the melatonin-treated rats phase-advanced (Fig. 4E, F).

This finding is important for several reasons. The paradigm offers a simple

behavioural test for investigating the effects of natural and synthetic pineal compounds on the circadian system. It will allow us to ascertain the minimum effective dose of melatonin, the best route of administration and the minimum number of days of injection required. In combination with CNS lesions or radioactive labelling techniques, it will allow us to trace which brain areas and neural systems are involved in the phase-advance effect. For instance, we already know from a Px study that the pineal gland itself is not necessary for the effect (unpublished work with M. Chesworth 1984).

Recently, it has been shown that a melatonin pellet implanted near the suprachiasmatic nuclei of intact and Px rats accelerates re-entrainment of the circadian rhythm in blood corticosterone (Murakami et al 1983). At present it is unclear how this finding relates to our own.

An even simpler behavioural test of melatonin's effect on the circadian pacemaker system has now been found. In our laboratory, rats under a 12:12 LD cycle often exhibit a negative phase-angle difference (the interval between dark onset and the onset of activity), which can be up to several hours long in some individuals. Melatonin injected 1 h before dark onset can reduce this negative phase-angle difference (Fig. 5A) providing the difference is not greater than 3 h (Fig. 5B) (unpublished work with M. Chesworth 1984). It is interesting to compare this result with that of clorgyline, a type A monoamine oxidase inhibitor (Wehr et al 1982). Clorgyline increases the negative phase-angle difference in hamsters, and lengthens the free-running period. We have shown that melatonin decreases the negative phase-angle difference in rats and lengthens the free-running period.

Throughout our experiments some control rats showed susceptibility to the injection procedure. This raises the possibility that our findings on melatonin could be simply an exaggerated stress response. To test this, six male C57BL mice were subjected to 30 min immobilization stress at the same time every day while under DD free-running conditions. Stress did not entrain the circadian activity rhythm (Fig. 5C), although masking of activity occurred in one animal as the onset of activity crossed the stress-time (Fig. 5D). The duration of the active phase altered in another mouse as the end of activity passed through the stress-time (Fig. 5E) (M. Menaker et al, unpublished work 1984). Since these results with mice are consistent with the effects of stressing wild and laboratory rats (Richter 1967), we conclude that our findings on melatonin cannot be attributed simply to a stress response.

Discussion and speculations

In summary, exogenous melatonin exerts a number of effects on the rat circadian pacemaker system, but at present it is impossible to integrate the results

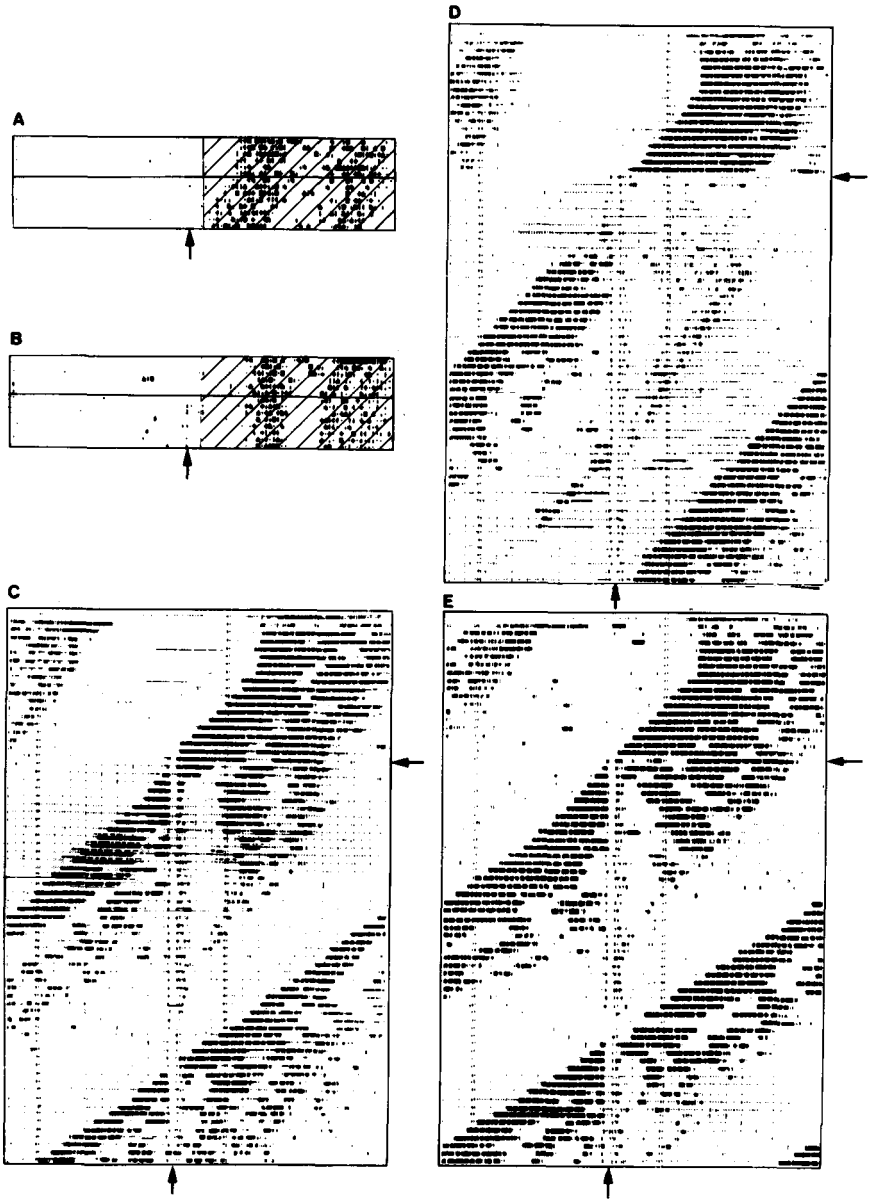

FIG. 5. (A) Rat activity plot showing reduction in negative phase-angle difference by melatonin injections given 1 h before dark onset (12:12 LD). (B) Rat activity plot showing failure of melatonin to reduce negative phase-angle difference because it is greater than 3 h. (C–E) Three male C57BL mice free-running with periods less than 24 h in DD and stressed by immobilization (30 min) at time of day indicated by vertical arrow, starting on day indicated by horizontal arrow.

into any global function for the mammalian pineal gland. Naturally, this raises the question of whether our findings are pharmacological or physiological. We cannot yet answer this question, but there is one observation that should be made about the entraining properties of melatonin. In the laboratory rat, whether under an LD cycle or free-running conditions, nocturnal activity co-varies with endogenous melatonin release. A similar phase relationship is found between the time of injection of exogenous melatonin and the free-running activity rhythm in that activity onset is entrained by the injection (Fig. 2A–D). In the diurnally active starling, endogenous melatonin release co-varies with the inactive, sleep and rest portion of the circadian cycle; therefore, activity and melatonin levels are 180° out of phase. Gwinner & Benzinger (1978) demonstrated that daily melatonin injections entrain the free-running activity rhythm of Px starlings; melatonin injections are followed by sleep and rest. Thus, in the rat the onset of activity is entrained by melatonin injections whereas in the starling it is the onset of inactivity/sleep that is entrained. These species differences suggest that exogenous melatonin does not simply have a gross pharmacological effect but may mimic the underlying physiological function of endogenous melatonin.

As the relationship between melatonin and activity rhythms varies between species, the interesting question arises of whether the effects of melatonin on the human circadian system will turn out to be 'rat-like' because we are mammals, or 'starling-like' because we are diurnally active vertebrates. The latter is most likely since melatonin induces sleep and tranquillity in humans (Armstrong et al 1982). This raises a very important point. In starlings melatonin injections can have immediate effects on the circadian system. Indeed, it was suggested that melatonin entrains activity rhythms in starlings by acting via the sleep mechanism; melatonin induces sleep and sleep alters the phase and entrains the circadian pacemaker (Gwinner & Benzinger 1978). Although at that time there was no supportive evidence for such a proposal it has recently been claimed that a portion of sleep, termed 'anchor sleep', can entrain human circadian rhythms (Minors & Waterhouse 1983). Therefore, melatonin could possibly entrain human circadian rhythms via its action on the sleep–wake mechanism; melatonin could be a potent chronobiotic (a chemical compound with the propensity to entrain circadian rhythms). It is not surprising, therefore, to find preliminary reports that melatonin can ameliorate jet lag (Short 1983, Short & Armstrong 1984). Jet lag is thought to reflect internal dissociation of the multitude of bodily rhythms. Different rhythms take different amounts of time to re-entrain to the new lighting regimen after the abrupt phase-shift imposed by intercontinental jet travel. If melatonin does prevent or cure jet lag, by implication it should prevent internal dissociation, thereby confirming a speculation that the pineal gland is a 'synchroniser of regulators' (Armstrong et al 1982).

Acknowledgements

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REFERENCES

Armstrong S, Ng KT, Coleman GJ 1982 Influence of the pineal gland on brain–behavior relationships. In: Reiter RJ (ed) The pineal gland. Vol III. Extra-reproductive effects. CRC Press Inc, Boca Raton, p 81–106

Axelrod J 1974 The pineal gland: a neurochemical transducer. Science (Wash DC) 184:1341-1348

Ebihara S, Uchiyama K, Oshima I 1984 Circadian organization in the pigeon, *Columba livia*: the role of the pineal organ and the eye. J Comp Physiol 154:59-69

Finkelstein JS, Baum FR, Campbell CS 1978 Entrainment of the female hamster to reversed photoperiod: role of the pineal. Physiol & Behav 21:105-111

Gaston S, Menaker M 1968 Pineal function: the biological clock in the sparrow? Science (Wash DC) 160:1125-1127

Gwinner E 1978 Effects of pinealectomy on circadian locomotor activity rhythms of European starlings, *Sturnus vulgaris*. J Comp Physiol 126:123-129

Gwinner E, Benzinger I 1978 Synchronization of a circadian rhythm in pinealectomized European starlings by daily injections of melatonin. J Comp Physiol 127:209-213

Kennaway DJ, Seamark RF 1980 Circulating levels of melatonin following its oral administration or subcutaneous injection in sheep and goats. Aust J Biol Sci 33:349-353

Kincl FA, Chang CC, Zbuzkova V 1970 Observation on the influence of changing photoperiod on spontaneous wheel-running activity of neonatally pinealectomized rats. Endocrinology 87:38-42

Minors DS, Waterhouse JM 1983 Does 'anchor sleep' entrain circadian rhythms? Evidence from constant routine studies. J Physiol (Lond) 345:451-467

Murakami N, Hayafugi C, Sasaki Y, Yamazaki J, Takahashi K 1983 Melatonin accelerates the re-entrainment of the circadian adrenocortical rhythm in inverted illumination cycle. Neuroendocrinology 36:385-391

Pittendrigh CS, Daan S 1976a A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. J Comp Physiol 106:223-252

Pittendrigh CS, Daan S 1976b A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. J Comp Physiol 106:291-331

Quay WB 1968 Individuation and lack of pineal effect in the rat's circadian locomotor rhythm. Physiol & Behav 3: 109-118

Quay WB 1972 Pineal homeostatic regulation of shifts in the circadian activity rhythm during maturation and aging. Trans NY Acad Sci 34:239-254

Redman J, Armstrong S, Ng KT 1983 Free-running activity rhythms in the rat: entrainment by melatonin. Science (Wash DC) 219:1089-1091

Richter CP 1967 Sleep and activity: their relation to the 24-hour clock. Proc Assoc Res Nerv Ment Dis 45:8-29

Rusak B 1982 Circadian organization in mammals and birds: role of the pineal gland. In; Reiter RJ (ed) The pineal gland. Vol III. Extra-reproductive effects. CRC Press Inc, Boca Raton, p27-51

Sääf J, Wetterberg L, Backström M, Sundwall A 1980 Melatonin administration to dogs. J Neural Transm 49:281-285

Short RV 1983 Method for minimizing disturbances in bodily performance and function following rapid time changes. Australian Provisional Patent Application PF 9418

Short RV, Armstrong S 1984 Method for minimizing disturbances in circadian rhythms of bodily performance and function. Australian Patent Application 27977/84

Simpson SM, Follett BK 1981 Pineal and hypothalamic pacemakers: their role in regulating circadian rhythmicity in Japanese quail. J Comp Physiol 144:381-389

Turek FW, Earnest DJ, Swann J 1982 Splitting of the circadian rhythm of activity in hamsters. In: Aschoff J et al (eds) Vertebrate circadian systems. Springer-Verlag, Berlin, p 203-214

Ueck M 1982 Morphology and physiology of the pineal organ in the evolution of vertebrates. Verh Dtsch Zool Ges 1982:61-80

Wehr TA, Lewy AJ, Wirz-Justice A, Craig C, Tamarkin L 1982 Antidepressants and a circadian rhythm phase-advance hypothesis of depression. In: Collu R et al (eds) Brain peptides and hormones. Raven Press, New York, p 263-276

DISCUSSION

Arendt: When you gave rats melatonin at the time when they would normally be making it, rather than at the beginning or at the end of the dark phase, you got faster entrainment, and that struck me as an important point. Have you ever tried giving rats a priming dose of melatonin before imposing a phase-shift?

Armstrong: No.

Turek: If endogenous melatonin is playing any role in the regulation of the circadian activity rhythm, you would expect to see an effect of pinealectomy on the free-running rhythm. And if melatonin is important for entrainment, you might also expect to see an effect of melatonin on the phase–response curve. There is no effect of pinealectomy on the phase–response curve or the free-running rhythm of hamsters (Aschoff et al 1982), but have you found any effect in rats?

Armstrong: After our initial study with the 5 h phase-advance paradigm, when pinealectomy seemed to help re-entrainment, we looked at the effects of light pulses in sham-operated, pinealectomized and control animals because we thought we might see a change in period. We also tried putting animals on 8 h days or 48 h days, and switching animals from constant darkness to constant light to look at the change in τ. But we saw no obvious differences between pinealectomized and sham-operated animals.

Zucker: In rats, Cheung & McCormack (1982) found no effects of pinealectomy on free-running τ in either constant light or constant dark, which is consistent with what you are saying. So your paradigm is useful as a way of manipulating the rate of phase-shifting of circadian rhythms, although it is not necessarily relevant to how animals usually entrain under normal light–dark cycles.

Arendt: Pinealectomy may not do much to circadian rhythms, but there is still retinal melatonin to think about.

Armstrong: One of our reasons for taking a pharmacological approach was that we hoped to be able to circumvent the problem of these other sources of melatonin. It has been shown that after pinealectomy there is still a melatonin rhythm in rats (Ozaki & Lynch 1976, Lynch et al 1975). It has something to do with the feeding pattern, because if you deprive the rats of food you get rid of that rhythm.

Moore-Ede: It's very tricky trying to study the effects of pharmacological agents, particularly by injection. David Borsook in our lab spent almost a year documenting a beautiful phase–response curve in the squirrel monkey that turned out to be an artifact. It did not matter whether he used the drug or the vehicle for intracranial and intraventricular microinjections; he got the same phase–response curve. A few of your control animals show apparent entrainment, so one wonders whether a component of the phase-shifting is caused by handling. We found with some of our injections that the amplitude of the phase–response curve was larger when the drug was present than when we just used vehicle, so there were additive effects, which were very confusing. The other important factor is the exact protocol one uses, but I presume that when you did control experiments you used exactly the same vehicle, with the alcohol and other components.

Armstrong: Yes.

Klein: We may be overlooking the most important fact—that we don't *need* melatonin to entrain the rhythm; apparently a perturbation can do the trick. This could be very useful for travellers with jet lag; they may not have to take a drug. But what is the mechanism? How is the rhythm entrained? Is a small amount of 'stress' sufficient? The stress of giving injections is not really very great; it is not nearly as bad as half an hour of immobilization stress, which is often used to exaggerate stress-related responses.

Reiter: It depends how you define stress.

Klein: The point is that the stress of exercise, for example running, jogging or walking, might be enough to re-entrain our rhythms. This may be a more profitable line to pursue than trying to manipulate rhythms with melatonin.

Hoffmann: Efforts have been made to overcome jet lag by taking melatonin, but rodents may not be very good models for investigating this. Have any studies been done in other species like the monkey and what sort of results have been obtained in humans?

Armstrong: Roger Short and I have been trying to get some data on humans and we have monitored seven subjects so far. Melatonin seems to help, and certainly in double-blind tests people could tell in retrospect which capsules contained melatonin. Whether in humans melatonin actually entrains circadian rhythms quicker than placebo does is another question. One of our results is

shown in Fig. 1, which gives records of core body temperature monitored with a PMS-8 Vitalog during a flight that Roger Short took round the world from Australia via the USA, Germany and England back to Melbourne. The first box shows four days of baseline in Australia, the shaded area indicating when Roger was asleep. There is quite a nice rhythm; body temperature was high during the day, fell usually just before sleep and rose again at about the time of awakening. After four days Roger flew to Los Angeles and then on to Dallas. In Dallas he took melatonin (5 mg) at 2345 local bedtime (1445 Melbourne time), went to sleep for four or five hours, woke up, took another capsule of melatonin and went back to sleep. There was a drop in the temperature after he took melatonin and on the subsequent days he got quite a good night's sleep. He took melatonin for four nights only, and after a few days his temperature started to drop before he went to sleep in the evening and picked up again when he woke in the morning. The records are a bit erratic, but it seems to me that after four days Roger's rhythms had already re-entrained. Of course we don't have a control for this, but re-entrainment seemed to be fairly fast compared to what one would expect from published reports of the effects of phase-shifts. Next, Roger flew off to Europe and took melatonin again. As before it decreased the body temperature. It's interesting that in sparrows pinealectomy wipes out the body temperature rhythm (Binkley et al 1971), and Martin Moore-Ede's group has shown that the duration of human sleep is dependent on the phase of the temperature rhythm (Czeisler et al 1980). So perhaps in humans melatonin changes the sleep–wake cycle via an effect on core temperature.

When Roger returned to Melbourne in Australia he did not take melatonin, but his rhythm was nevertheless quite well entrained. However, during sleep there was always a bump in the temperature record, and I think that this is the residual effect of the peak of activity he showed at that time in Europe and the USA. Our problem is one of analysis, in that we have to determine whether melatonin's effect on the shape of the temperature curve during sleep is exogenous or endogenous. It is very hard to build in good controls for these studies.

Lewy: I am impressed by the fact that administration of melatonin appears to hasten the return of the night-time temperature minimum to its pre-flight phase position. In my opinion, this part of the temperature curve is usually the least affected by 'masking'.

Hoffmann: Have there been no systematic studies in humans or in monkeys? For example, it would be interesting to put people in bunkers, subject them to phase-shifts and then give them melatonin.

Armstrong: Our problem in Australia is that although as scientists we can take melatonin we are not allowed to give it to anyone else. This has really held us up; we would have liked to have done these studies 18 months ago.

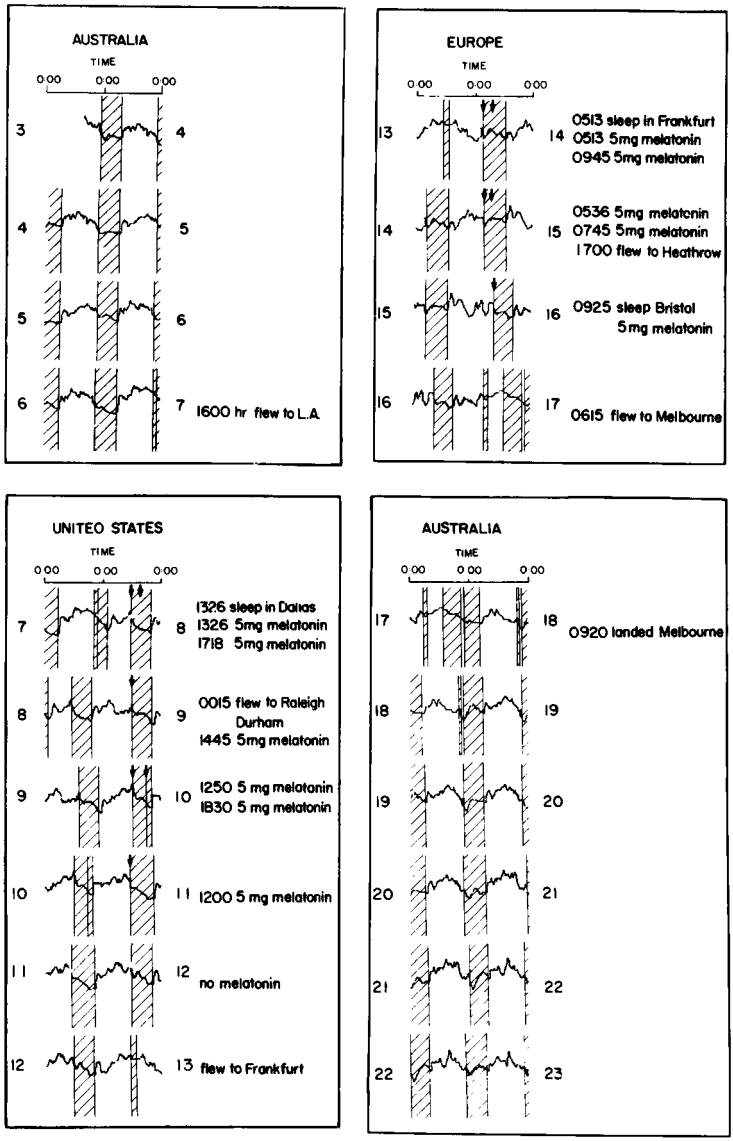

FIG. 1. (*Armstrong*) Daily body temperature rhythms of one male subject on an around-the-world flight from Melbourne (Australia) to Raleigh (Durham, USA) to Frankfurt and Bristol (Europe) and back to Melbourne. Temperature and activity were monitored every 12 min with a PMS-8 Vitalog microcomputer with extended memory. The data have been double-plotted (over 48 h) to facilitate inspection. 0.00 time is midnight in Melbourne. Hatched bars indicate time spent asleep. The vertical arrows above the sleep period on days 8–11 and 14–16 indicate oral consumption of one 5 mg capsule of melatonin (Sigma).

Arendt: We are trying to do some phase-shift studies with melatonin at the moment, and have some uncontrolled results from 10 people who all reported favourable effects on sleep after crossing several time-zones.

Moore-Ede: These experiments with melatonin in humans are very difficult. One needs an idea of how a control group would behave. Phillipa Gander has been collecting data from a study of international pilots whose body temperatures have been monitored continuously (unpublished work). She has also monitored her own temperature when flying back and forth between the USA and New Zealand. What emerges from the results is that changes in sleep, posture or darkness (although it's not clear which) have very strong masking effects. Certainly a combination of the three has a dramatic effect on temperature at any phase of the cycle. So it is very hard to tell what makes temperature drop faster or slower in any situation, because about 50% of the amplitude of the human temperature rhythm is probably contributed exogenously by masking elements. You therefore have to do a phase determination study where you suspend or control the exogenous variables over a period of at least 24 h. You need to get someone either to stand up for 36 h, or, more conveniently, to lie down for 36 h, and you must control meals, illumination etc. You can then get very clean patterns and precise determinations of phase. The trough of temperature is a very good phase marker and the peak of cortisol is another; these are really the things that you must show are shifting to prove that the circadian system is being reset. If you don't do this and you don't have appropriate control groups, you will never get the answer. You will always be interpreting fuzzy-looking patterns.

The other point is that in humans the system that times cortisol and temperature rhythms takes quite a long time to adapt. If you put someone onto night shifts they will take a couple of weeks to adapt fully. I think we are looking at long-term transients, and the problem is that when you are hopping from town to town and country to country the system does not stabilize.

Armstrong: Apparently the exogenous component (i.e. masking) of the temperature rhythm is more pronounced in older people, so perhaps we should use younger subjects in these studies (Phillipa Gander, personal communication).

Lewy: In addition to the masking effects that Martin Moore-Ede mentioned, you might also get a masking effect with melatonin *per se*. In Fig. 1, there is already a change in the temperature pattern on the first night after melatonin. What does an acute dose of melatonin do to body temperature in humans?

Armstrong: I don't know, but it has a hypothermic effect in rodents and birds (Ralph et al 1979).

Vollrath: One thing I noticed when I used an intranasal spray of melatonin was an immediate onset of shivering. This was fairly consistent, so I think that melatonin may affect temperature regulation.

REFERENCES

Aschoff J, Gerecke U, von Goetz Chr, Groos GA, Turek FW 1982 Phase responses and characteristics of free-running activity rhythms in the golden hamster: independence of the pineal gland. In: Aschoff J et al (eds) Vertebrate circadian systems: structure and physiology. Springer-Verlag, Berlin, p 129-140

Binkley S, Kluth E, Menaker M 1971 Pineal function in sparrows: circadian rhythms and body temperature. Science (Wash DC) 174:311-314

Cheung PW, McCormack CW 1982 Failure of pinealectomy or melatonin to alter circadian activity rhythm of the rat. Am J Physiol 242:R261-R264

Czeisler CA, Weitzman ED, Moore-Ede MC, Zimmerman JC_2 Knauer RS 1980 Human sleep: its duration and organization depend on its circadian phase Science (Wash DC) 210:1264-1267

Lynch HJ, Ozaki Y, Shakal D, Wurtman RJ 1975 Melatonin excretion of humans and rats: effects of time of day, sleep, pinealectomy and food consumption. Int J Biometeorol 19:267-279

Ozaki Y, Lynch HJ 1976 Presence of melatonin in plasma and urine of pinealectomized rats. Endocrinology 99:641-644

Ralph CL, Firth BT, Gern WA, Owens DW 1979 The pineal complex and thermoregulation. Biol Rev Camb Philos Soc 54:41-72

Photoperiodism, melatonin and the pineal
Editors: David Evered and Sarah Clark

The pineal and pubertal development

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Abstract. The pineal gland, through its major secretory product melatonin, influences seasonal breeding in species such as the hamster and the sheep. Recent studies from our laboratory have shown that melatonin also affects sexual development in the rat. A role for melatonin in humans has not yet been found.

The laboratory rat is sensitive to daily administration of melatonin at the beginning of sexual maturation. The male rat is most sensitive between day 20 and day 30 of life. Melatonin does not permanently inhibit sexual maturation, since normal but delayed sexual development occurs after 45 days of life whether melatonin administration is discontinued or maintained indefinitely. In female rats, daily injection of melatonin during the prepubertal period delays the vaginal opening and disrupts the normal cyclicity of the first oestrous cycles. In both male and female rats, the inhibitory action of melatonin is highly dependent upon the time of injection, with maximal effects when melatonin is given in the late photoperiod. The inhibitory action of melatonin is most likely exerted at the hypothalamic level, possibly through interference with the control of pulsatile secretion of gonadotropin-releasing hormone. In contrast to some published work, our experiments provide no evidence for modifications of diurnal or nocturnal melatonin secretion during puberty in humans. Our results with the rat indicate that melatonin may be an important factor for the timing of sexual maturation.

1985 Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Foundation Symposium 117) p 208-230

Numerous studies of the role of the pineal gland in many species have led to the concept that this small organ located in the brain is an active neuroendocrine gland with specific effects on reproductive biology. Two types of pineal secretory products have been described: indole amines and polypeptides (Reiter 1980). The concept has developed that the pineal gland is responsible for transducing environmental information such as light or season into new secretory signals (Wurtman et al 1964), which promote or repress reproductive behaviour (Reiter 1980). The pineal influence is mostly inhibitory and the signal is carried mainly by the indole amine melatonin (Cardinali 1981, Reiter 1980). However, it is likely that more than one pineal hormone is responsible for

the endocrine action(s) of the pineal gland. Although there is evidence that melatonin mediates most of the neuroendocrine functions attributed to the pineal gland, the antigonadotrophic activity of mammalian pineal extracts has also been attributed to the peptide arginine vasotocin ([Arg^8]oxytocin, AVT) (Vaughan et al 1974). This octapeptide was identified by Milcu et al (1963) and it has been suggested but not confirmed that melatonin is the releasing factor for AVT (Pavel 1978, Benson et al 1976). Clearly, more work is needed to establish the exact role, if any, of AVT in mammals.

Melatonin and sexual development

Melatonin, which was isolated and characterized by Lerner et al (1958), exhibits a nycthemeral rhythm, with peak production and secretion during the night (Lynch 1971). This hormone has been shown to mediate the antigonadotrophic effects of photoperiods in species with a seasonal reproductive cycle, such as the ewe and the hamster (Carter & Goldman 1983, Arendt et al 1983, Bittman et al 1983). Pinealectomy can prevent the gonadal regression that occurs in hamsters under non-stimulatory photoperiods (Hoffman 1979), indicating that the effects of darkness on gonadal development are mediated by the pineal. Similar observations have been made with other mammalian species. The observation in 1963 that daily melatonin injections in female rats could delay vaginal opening, reduce ovarian weight and decrease the incidence of vaginal oestrus (Wurtman et al 1963) opened a new field of research linking melatonin and reproduction. Today melatonin is known to be a possible inhibitor of sexual development in mammals (Tamarkin et al 1976, Goldman et al 1979). Pinealectomy induces premature pubertal development which can be counteracted by melatonin treatment (Relkin 1971, Kinson & Robinson 1970).

Melatonin and sexual development in the rat

The neuroendocrine reproductive system of the adult rat seems to be relatively unresponsive to pinealectomy or exogenous melatonin (Reiter 1980). However, under such conditions as neonatal steroid treatment, underfeeding or anosmia, the hypothalamo–pituitary–gonadal axis increases its sensitivity to melatonin (Blask & Nodelman 1979, Blask et al 1980). In the young rat, melatonin diminishes ovarian and uterine weight (Wurtman et al 1963) and retards testicular and accessory sex organ development (Debeljuk 1969, Kinson & Peat 1971). The physiological site and mechanism of action of melatonin are uncertain; glands and organs in both brain and periphery have been pro-

posed as targets. Before it became possible to measure plasma concentrations of pituitary and gonadal hormones, it was very difficult to assess the role of the pineal gland and of its secretory product, melatonin, in species that

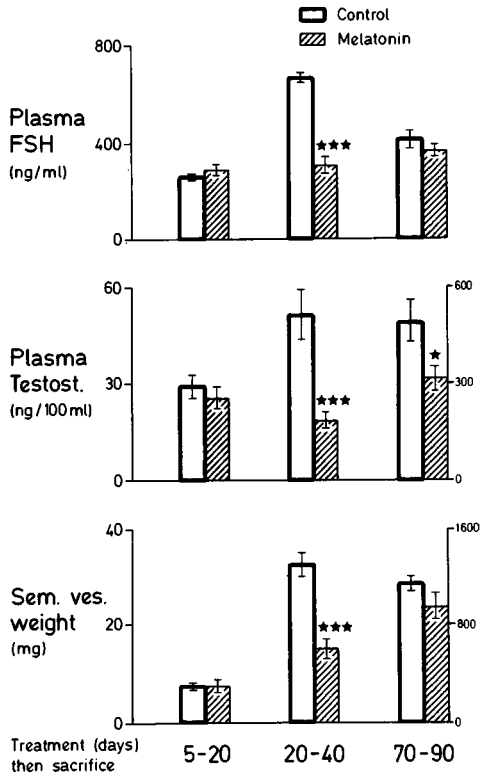

FIG. 1. Influence of melatonin on plasma FSH and testosterone concentrations and seminal vesicle (sem. ves.) weights of male rats during different stages of development. Groups of about 12 animals (▨) received daily injections of 100 μg melatonin during three different developmental periods: 5–20 days of age (prepubertal age), 20–40 days of age (pubertal age) and 70–90 days of age (adulthood). Control animals (□) received 100 μl physiological NaCl solution containing 10% ethanol at the same time. The rats were killed on the day after the last injection between 1000 and 1100 h. Plasma hormone levels and seminal vesicle weights are shown as means ± SE. $^{***}P < 0.001$, $^{*}P < 0.05$ (determined by Student's t test). Reproduced with permission from Lang et al (1983).

are insensitive to seasonal variations of the photoperiod like the laboratory rat. It is only recently that the effects of melatonin could be studied thoroughly in this species.

To investigate the role of melatonin in the male rat kept in a 12 h:12 h light–dark cycle, we administered melatonin daily during different periods of sexual development. The effects of these injections were tested on several variables

of the neuroendocrine reproductive axis: plasma concentrations of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and of testosterone, testicular weight, seminal vesicle weight and pituitary content

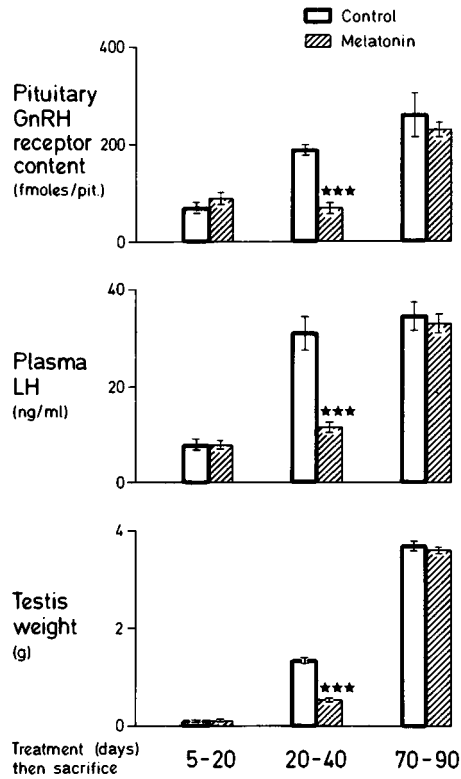

FIG. 2. Influence of melatonin on pituitary (pit.) GnRH receptor numbers, plasma LH concentrations and testes weights of male rats during different stages of development. Groups of about 12 rats were treated as described in Fig. 1. Significance is explained in Fig. 1. Reproduced with permission from Lang et al (1983).

of receptors for gonadotropin-releasing hormone (GnRH) (Aubert et al 1982, Lang et al 1983). All these variables were decreased in the male rat when 100 μg melatonin were administered daily at 1600–1700 h during the period of development from day 20 to day 40 of life (Figs. 1 & 2). Melatonin was administered subcutaneously in saline. In contrast, no change was observed when melatonin was injected either from day 5 to day 20, or from day 70 to day 90 of life (Lang et al 1983). This inhibiting effect of melatonin on sexual maturation in the male rat was found to be dose dependent in a dose range from 5 μg to 100 μg melatonin given daily (Lang et al 1983). Interestingly, chronic treatment of the rat with 10 μg melatonin per day did not

influence the rhythm of endogenous melatonin secretion. The nocturnal profiles of plasma melatonin for control and treated rats were parallel, with peaks of melatonin at 2400 h and 0300 h.

These results raised several questions which were further investigated in our laboratory. Is the time of injection during the light–dark cycle of importance? Does sexual maturation resume after cessation of melatonin administration? Does prolonged melatonin administration maintain the state of delayed sexual maturation indefinitely? Where does melatonin act?

The time of administration of melatonin within the day–night cycle is critical for melatonin action. Daily injections of 100 μg melatonin given to the male rat from day 20 to day 40 of life had their highest inhibiting activity when given during the late photophase (i.e. 9 h after the onset of light) and a smaller effect during the late scotophase (i.e. 9 h after the onset of darkness). The animals were kept in a 12 h:12 h light–dark cycle. Administration of melatonin during these two critical periods of the light–dark cycle reduced the weights of testes and seminal vesicles, lowered plasma levels of testosterone, LH and FSH and decreased the number of pituitary GnRH receptors (Figs. 3 & 4), changes that reflect delayed sexual maturation at 40 days (Lang et al 1984a).

A further study was designed to determine more precisely the age at which melatonin exerts its greatest inhibiting effect, to investigate whether spontaneous sexual maturation resumes after discontinuation of melatonin administration at 45 days of age and to evaluate rats treated daily with melatonin continuously until 115 days of age (Lang et al 1984b). Administration of melatonin to young male rats from day 20 to day 30 of life had the same inhibitory effect on sexual maturation at 40 days as melatonin injections given from day 20 to day 40. In contrast, administration of melatonin from day 30 to day 40 only slightly decreased the plasma testosterone concentration, weight of seminal vesicles and pituitary GnRH receptor content. Melatonin administration from day 38 to day 40 had no effect. Daily melatonin administration from day 20 to day 45 was followed by resumption of sexual maturation, as observed at 70 days. The recovery was complete by 80 days of age when all the variables studied reflected complete sexual maturation. Finally, in rats treated continuously with melatonin from day 20 to day 115, sexual maturation occurred but was delayed by about 20 to 30 days. The beginning of sexual maturation was observed at 60 days of age, and full development was attained only at 100 days (Fig. 5). These results demonstrate that the inhibitory action of melatonin (administered late in the light phase to male rats kept in a 12 h:12 h light–dark cycle) is strongest between day 20 and day 30 of life and is reversible whether or not melatonin administration is continued after day 45 (Lang et al 1984b).

The suppression of the pubertal peaks of pituitary GnRH receptor number, pituitary FSH content and plasma FSH concentration in melatonin-treated

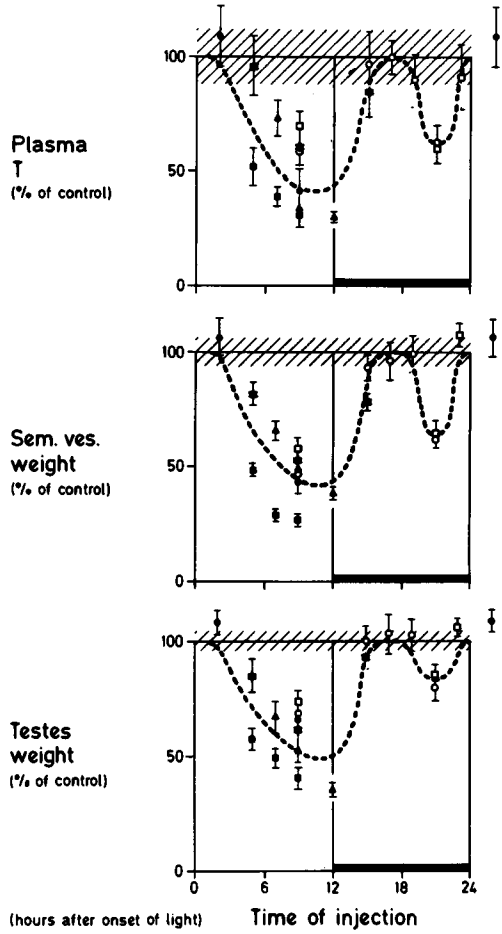

FIG. 3. Effects of daily melatonin injections given to male rats from day 20 to day 40 of life at different times during the day–night cycle. The weights of testes and seminal vesicles (sem. ves.) and plasma testosterone (T) concentrations were measured on day 41. Control animals received 100 μl of saline containing 10% ethanol 9 h after the onset of light. All results are expressed as percentages of the mean of control values, which is represented by the line on the top of each graph (± SEM: hatched area). The dotted lines represent suggested patterns of sensitivity to the inhibitory action of melatonin. The results are from six experiments, each including a control group of rats and groups treated at different times during the day (10–12 animals/group). The experiments were done at different times of the year and the mean values (± SEM) are represented as follows: ● = May, * = June, ▲ = August, ■ = December, ○ = February, □ = April. Closed symbols (●, *, ▲, ■) are for experiments with a light period from 0700 h to 1900 h; open symbols (○, □) are for experiments with a light period from 1200 h to 2400 h (○) and from 2400 h to 1200 h (□). All rats were maintained on a 12 h:12 h light–dark schedule as indicated at the bottom of the graphs. Reproduced with permission from Lang et al (1984a).

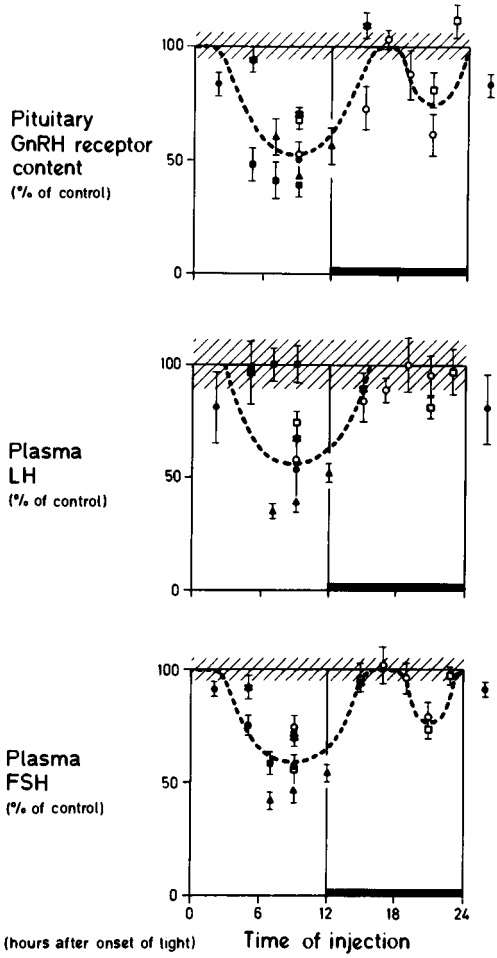

FIG. 4. Effects of daily melatonin injections given to male rats from day 20 to day 40 of life at different times during the day–night cycle. Pituitary GnRH receptor numbers and plasma levels of LH and FSH were measured. Experimental conditions are described in the legend to Fig. 3. Reproduced with permission from Lang et al (1984a).

rats strongly suggests that melatonin interferes with the pubertal increase in GnRH secretion.

Is melatonin the principal indole amine inhibiting sexual maturation in young male rats? The effect on sexual maturation of six different pineal indoles including melatonin and the metabolite 6-hydroxymelatonin was studied in animals after daily injections from day 20 to day 40 of life (Lang et al 1985b). Only 5-methoxytryptamine and 6-hydroxymelatonin, in addition to melatonin,

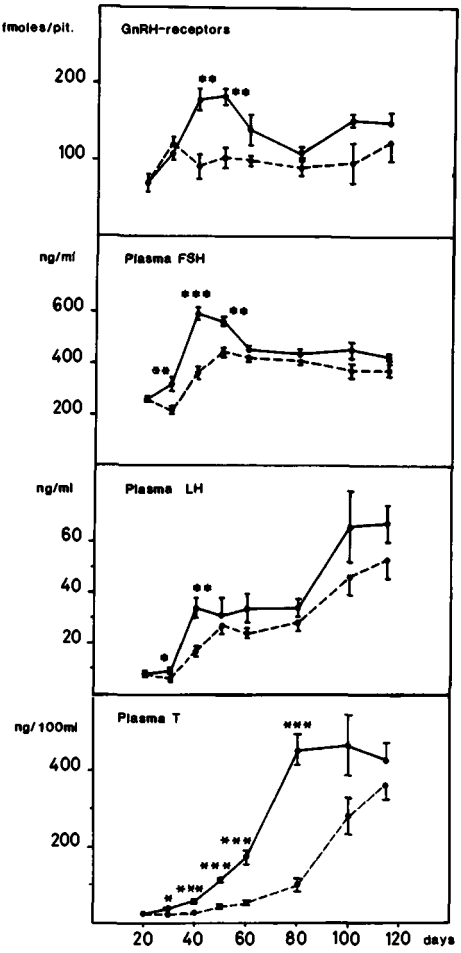

FIG. 5. Influence of prolonged melatonin administration during sexual development on pituitary GnRH receptor number and plasma FSH, LH and testosterone (T) concentrations. Twenty-day-old male Wistar rats (10 animals/group) received 100 μg melatonin daily (s.c.) for days of age 20–30, 20–40, 20–50, 20–60, 20–80, 20–100 or 20–115 (●--●). Control animals received 100 μl saline containing 10% ethanol at the same time (●—●). The rats were killed the day after the last injection between 1000 h and 1100 h. Values are shown as means ± SEM (vertical bars). Statistical significance for each result was determined by Student's *t* test with saline-treated rats as controls. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. Reproduced from Lang et al (1984b).

inhibited the neuroendocrine reproductive axis during sexual maturation. Their potencies when injected in the afternoon were about one-tenth that of melatonin. *N*-Acetylserotonin, serotonin, 5-hydroxytryptophol and 5-methoxytryptophol did not influence sexual maturation whether injected in the

morning or in the evening. Plasma extracts from rats injected daily with one of the three biologically active indoles and killed 10–20 min after the last injection were analysed chromatographically. No increase in plasma melatonin levels was observed after the administration of 6-hydroxymelatonin. In contrast, plasma melatonin levels in the 5-methoxytryptamine-treated animals

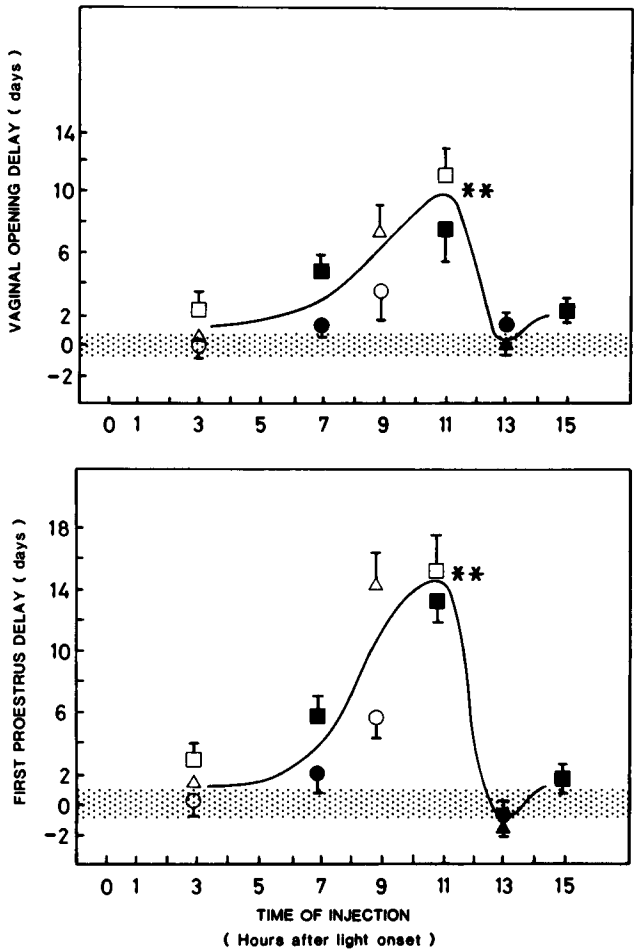

FIG. 6. Vaginal opening and first pro-oestrus in female rats after daily melatonin administration from day 15 of life. Each rat received melatonin at one of the different times of the day. Results are expressed as days of delay by comparison with the mean of the respective control groups. The dotted area represents the mean ± SE for the control groups. The open symbols represent results from three series of experiments with female rats housed in light–dark (LD) cycles of 12 h:12 h, the closed symbols from animals housed in LD 16 h:8 h. $^{**}P < 0.01$ vs. control values. Reproduced with permission from Rivest et al (1985).

were increased one hour after the 5-methoxytryptamine injection. These results suggest that 5-methoxytryptamine or part of it might be acetylated to melatonin (Lang et al 1985b), so inhibition of sexual maturation in this case might be mainly due to melatonin and not primarily due to 5-methoxytryptamine itself as suggested by Pevet (1983).

In the immature female rat housed in a 12 h:12 h light–dark cycle, daily administration of 100 μg melatonin 9–11 h after the onset of light (starting on day 15 of life) delayed vaginal opening by 10 days, dissociated vaginal opening from the first pro-oestrus (Fig. 6) and disrupted the initial oestrous cycles (Rivest et al 1985). Melatonin's action on sexual maturation was associated with a 30% lower pituitary GnRH receptor number in animals killed in the afternoon of pro-oestrus and dioestrus. Furthermore, although plasma levels of LH, FSH and oestradiol were similar to those of control animals in samples taken during the dioestrous phase, there was an enhanced pro-oestrous surge of these hormones under melatonin treatment. In the pituitary, FSH concentrations were higher than normal during dioestrus and lower during pro-oestrus. This hormonal pattern suggests a build-up phenomenon due to the low frequency of pro-oestrous surges in melatonin-treated rats (Rivest et al 1984, Rivest et al 1985).

In summary, daily administration of melatonin to male and female rats delays sexual maturation. Melatonin seems to act at the hypothalamic level, probably at the site controlling the secretion of GnRH, since we observed that the number of pituitary GnRH receptors is significantly decreased in both sexes with daily melatonin injections. It should be recalled that the number of pituitary GnRH receptors is directly related to the extent of prior stimulation by GnRH. The period of sensitivity to administration of melatonin is dependent on the time of the day, with maximal sensitivity 10–12 h after the onset of light; the time of appearance of this window of sensitivity was independent of the lighting regimen tested. Furthermore, melatonin acts mainly during the period of sexual maturation and in a reversible manner; treatment of adult animals has no visible effect in the male or the female. Finally, melatonin seems to be the principal indole amine responsible for the timing of sexual maturation in the rat.

Melatonin and human puberty

In humans, as in other animals, melatonin is secreted in a cyclic fashion. Melatonin concentrations are increased during the night in plasma, cerebrospinal fluid and urine (Arendt et al 1977, Lang et al 1981a, Waldhauser & Wurtman 1983, Vaughan 1984). Destructive tumours of the pineal region have been observed in association with precocious puberty (Kitay 1954), but

TABLE 1 Mean plasma melatonin concentrations (MT, pg/ml) during normal puberty

		Stages of puberty					
		P1	*P1*	*P2*	*P3*	*P4*	*P5*
Girls	*n*	11	11	14	13	14	16
	Mean age (years)	7.5	8.4	11.1	12.0	12.5	14.3
	Mean MT (± SEM)	9.5 ± 3.2	8.0 ± 2.0	9.8 ± 2.4	12.1 ± 2.1	9.5 ± 2.5	6.7 ± 1.3
Boys	*n*	11	13	15	14	15	15
	Mean age (years)	9.4	10.5	12.6	13.5	14.2	16.5
	Mean MT (± SEM)	11.4 ± 3.2	7.2 ± 1.1	6.3 ± 1.3	11.4 ± 1.8	11.4 ± 3.0	10.7 ± 2.0

Blood was taken between 0900 h and 1100 h from the girls and between 1300 h and 1500 h from the boys. P1 = prepubertal stage, P2 to P4 = progressive pubertal stages, P5 = adulthood.

it has been difficult, up to now, to assess the role of the pineal since most tumours are not pinealocytomas but dysgerminomas secreting human chorionic gonadotropin.

Similarly, during normal pubertal development, it has been difficult to analyse the possible changes in melatonin secretion because most observations on plasma melatonin in prepubertal and pubertal subjects have been made on plasma samples collected during day-time (Silman et al 1979, Tamarkin et al 1982, Ehrenkranz et al 1982, Lenko et al 1982). With one exception (Silman et al 1979), these studies found no difference in day-time melatonin concentrations between prepubertal children and adults, as observed in our study (Table 1). Studies that also measured nocturnal melatonin proved more fruitful. The day–night increment was studied in five children with early puberty and was found to be lower than in age-matched prepubertal children (Attanasio et al 1983). Similar observations were made in normal children, with a decline of the day–night increment in the serum melatonin concentration from the prepubertal stage to the early puberty stage (Gupta et al 1983). Recently, Waldhauser et al (1984) found a difference in midnight levels of melatonin between prepubertal children less than seven years old and prepubertal and pubertal subjects over seven years old. In two studies (Ehrenkranz et al 1982, Tamarkin et al 1982), the night-time melatonin levels of prepubertal children were comparable to those of adults. Melatonin concentrations tended to be higher before puberty, but the differences noted were not statistically significant.

Measurements of urinary melatonin can provide a better integrated picture of the total amount of melatonin secreted (Lang et al 1981a). Urinary excretion in 43 normal children and in 12 boys with delayed puberty was studied (Lang et al 1985a). There was no significant change in excretion in normal children during pubertal development (Table 2), as assessed from either 12 h night-time urine collections or 24 h urine collections, or in boys with delayed puberty

TABLE 2 Mean (± SEM) urinary melatonin excretion during normal and delayed puberty

		Urinary melatonin excretion (ng/m²)[a]		
Puberty	*Urine sample*	*P1*	*P2*	*P3–P5*
Normal	Night	28.8 ± 3.8 (15)	33.0 ± 1.8 (14)	23.3 ± 4.13 (14)
Normal	24 h	42.1 ± 6.5 (15)	49.3 ± 13.7 (14)	30.5 ± 6.6 (14)
Normal (obese patients)	Night	29.4 ± 3.8 (10)	30.4 ± 6.2 (6)	21.9 ± 2.7 (17)
Delayed	Night	38.6 ± 6.9 (12)	—	—

[a] Total for 12 h (night samples) or 24 h.
Night-time urine samples were collected from 2000 h to 0800 h, 24 h samples from 0800 h to 0800 h.
P1 = prepubertal stage, P2 to P4 = progressive pubertal stages, P5 = adulthood.
Figures in parentheses give numbers of subjects studied.

(Fig. 7) and in obese patients serving as control subjects (Table 2). Because similar values were obtained in girls and in boys, results are given for both sexes in Table 2 (Sizonenko et al 1982). In contrast, Penny (1982) reported increased excretion of melatonin in the urine during puberty.

The nocturnal pattern of melatonin was compared in prepubertal and post-pubertal subjects. Eight healthy boys were studied throughout the night: four (aged 11 to 12 years) were prepubertal (Stage P1) and four (aged 17.5 to 18.5 years) were at the end of the pubertal period (Stage P5). Blood samples

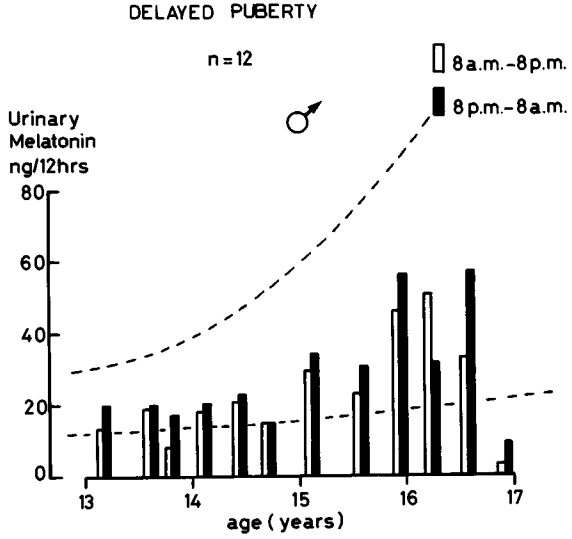

FIG. 7. Urinary melatonin excretion during night and day in 12 human males with delayed puberty. Dark columns represent 2000 h–0800 h urine collections, open columns 0800 h–2000 h collections. Interrupted lines represent 95% confidence limits for night-time urinary melatonin excretion in relation to age in 28 normal pubertal subjects.

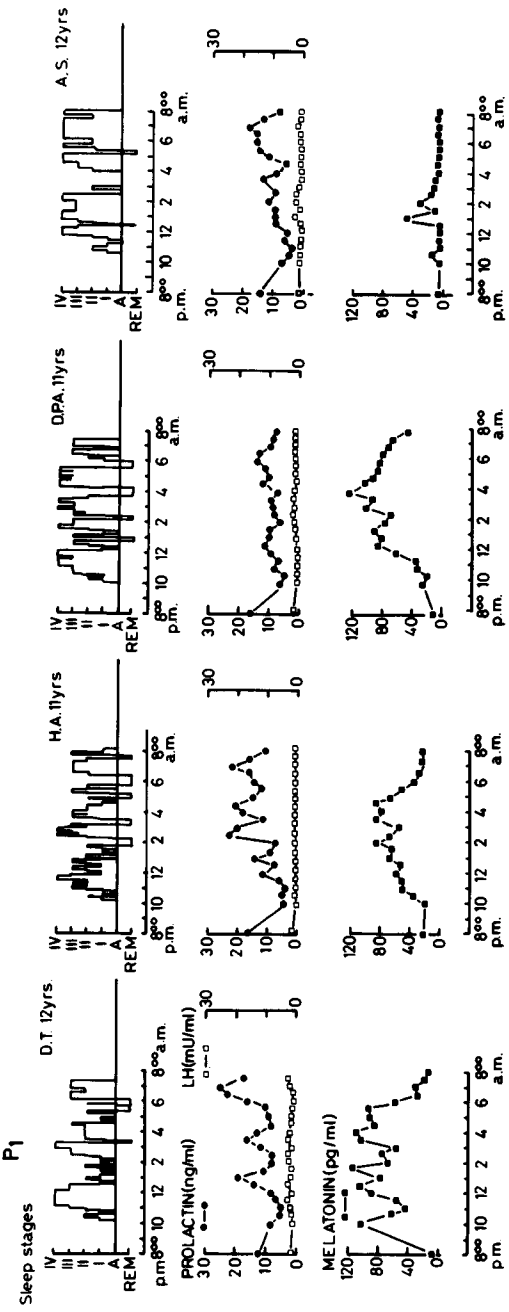

FIG. 8. Patterns of circulating melatonin ■-■, LH □-□ and prolactin ●-● in four prepubertal (Stage P1) boys over a 12 h period. An electroencephalograph recorded stages (I to IV) of sleep. REM, rapid eye movements; A, awake.

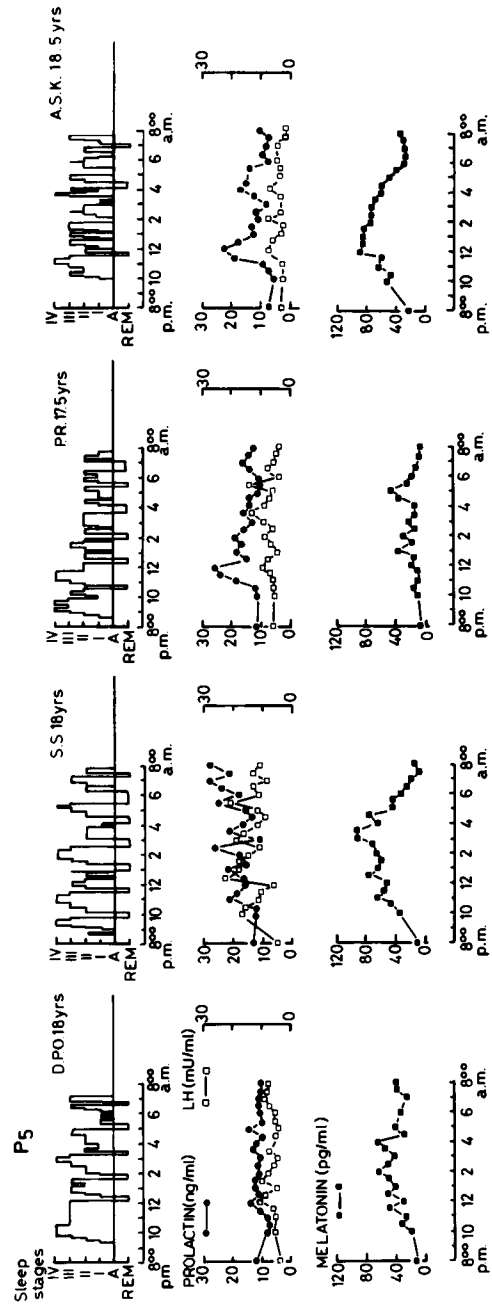

FIG. 9. Patterns of circulating melatonin ■-■, LH □-□ and prolactin ●-● in four pubertal (Stage P5, near adulthood) boys over a 12 h period. An electroencephalograph recorded stages of sleep. REM, rapid eye movements; A, awake.

were obtained every 30 min for determinations of plasma melatonin, LH and prolactin concentrations. Sleep electroencephalograms were also obtained. Results showed that the secretory pattern of melatonin does not differ in the two stages of puberty studied (Figs. 8 & 9). In contrast, nocturnal plasma concentrations of LH were higher at Stage P5 than at Stage P1.

It is difficult from the work presented and the studies cited to attribute a role to the pineal gland during human pubertal development. Measurements of urinary melatonin metabolites such as 6-hydroxymelatonin did not show a correlation between daily excretion rates and age or pubertal stage, with the exception of an increased excretion observed at the time of onset of breast development (Tetsuo et al 1982). In this study, no change in excretion was seen during puberty in boys. The significance of this difference between girls and boys is not known. Results obtained in studies of different disorders also offer no definite conclusion. In boys with delayed puberty, Cohen et al (1982) measured higher day-time melatonin levels than in a control population. Attanasio et al (1983) found that the day–night increment in subjects with delayed puberty was similar to that in prepubertal children. Tamarkin et al (1982) did not find abnormal changes in plasma melatonin in obese children and patients with Prader-Willi's syndrome. Similarly, Ehrenkranz et al (1982) reported a normal daily rhythm of plasma melatonin in precocious puberty.

These seemingly discordant findings do not completely rule out an action of melatonin during human pubertal development. It is not excluded that melatonin induces subtle rather than gross changes in gonadotropin secretion, which cannot be defined because the means of investigation are inadequate (for example, a change in the pulsatile pattern of LH secretion). In addition, in other animals melatonin is active only at a certain time during the day or the night. If this is also true for humans, it makes many studies either irrelevant or difficult. It is also possible that a rhythmic and life-long modulation of melatonin receptors or of target-tissue receptivity to melatonin action takes place. Melatonin receptors have been identified (Cardinali et al 1979, Cohen et al 1978, Lang et al 1981b), and the sensitivity of such receptors may change during pubertal development. Thus, in some cases, measurements of melatonin receptors on target tissues might yield more information than determinations of plasma levels of melatonin.

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REFERENCES

Arendt J, Wetterberg L, Heyden T, Sizonenko PC, Paunier L 1977 Radioimmunoassay of melatonin: human serum and cerebrospinal fluid. Horm Res (Basel) 8:65-75

Arendt J, Symons AM, Land CA, Pryde SJ 1983 Melatonin can induce early onset of the breeding season in ewes. J Endocrinol 97:395-400

Attanasio A, Borrelli P, Marini R, Cambiaso P, Cappa M, Gupta D 1983 Serum melatonin in children with early and delayed puberty. Neuroendocrinol Lett 5:387-392

Aubert ML, Lang U, Nawratil MF, Bradtke J, Sizonenko PC 1982 Ontogenesis of pituitary receptor sites for gonadotropin releasing hormone (GnRH) in male and female rats. In: Saez JM et al (eds) Ontogenesis of the endocrine system. Les Editions INSERM (Paris) 110:319-334

Benson B, Matthews MJ, Hurby VJ 1976 Characterization and effects of a bovine pineal antigonadotrophic peptide. Am Zool 16:17-19

Bittman EL, Dempsey RJ, Karsch FJ 1983 Pineal melatonin secretion drives reproductive response to daylight in the ewe. Endocrinology 113:2276-2283

Blask DE, Nodelman JL 1979 Antigonadotrophic and prolactin-inhibitory effects of melatonin in anosmic male rats. Neuroendocrinology 29:406-412

Blask DE, Nodelman JL, Leadem CA, Richardson BA 1980 Influence of exogenously administered melatonin on the reproductive system and prolactin, and levels in underfed male rats. Biol Reprod 22:507-512

Cardinali DP 1981 Melatonin: a mammalian pineal hormone. Endocr Rev 2:327-346

Cardinali DP, Vacas MI, Boyer EE 1979 Specific binding of melatonin in bovine brain. Endocrinology 105:437-441

Carter DS, Goldman BD 1983 Progonadal role of the pineal in the Djungarian hamster: mediation by melatonin. Endocrinology 113:1268

Cohen HN, Roselie D, Chabner B, Schmidt TJ, Lippmann 1978 Evidence for a cytoplasmic melatonin receptor. Nature (Lond) 274:894-895

Cohen HN, Hay JD, Annesley TM et al 1982 Serum immunoreactive melatonin in boys with delayed puberty. Clin Endocrinol 17:517-521

Debeljuk L 1969 Effect of melatonin on the gonadotrophic function of the male rat under constant illumination. Endocrinology 84:937-939

Ehrenkranz JR, Tamarkin L, Comite F et al 1982 Daily rhythm of plasma melatonin in normal and precocious puberty. J Clin Endocrinol & Metab 55:307-310

Goldman NB, Hall V, Hollister C, Roychoudhury P, Tamarkin L, Westrom W 1979 Effects of melatonin on the reproductive system in intact and pinealectomized male hamsters maintained under various photoperiods. Endocrinology 104:82-88

Gupta D, Riedel L, Frick HJ, Attanasio A, Ranke MB 1983 Circulating melatonin in children in relation to puberty, endocrine disorders, functional tests and racial origin. Neuroendocrinol Lett 5:63-78

Hoffman K 1979 Photoperiod, pineal melatonin and reproduction in hamsters. Prog Brain Res 52:397-415

Kinson GA, Peat F 1971 The influence of illumination, melatonin and pinealectomy on testicular function in the rat. Life Sci 10:259-269

Kinson GA, Robinson S 1970 Gonadal function of immature male rats subjected to light restriction, melatonin administration and removal of the pineal gland. J Endocrinol 47:391-392

Kitay JI 1954 Pineal lesions and precocious puberty: a review. J Clin Endocrinol & Metab 14:622-625

Lang U, Kornemark M, Aubert ML, Paunier L, Sizonenko PC 1981a Radioimmunological determination of urinary melatonin in humans: correlation with plasma levels and typical 24 hour rhythmicity. J Clin Endocrinol & Metab 53:645-650

Lang U, Aubert ML, Sizonenko PC 1981b Location of melatonin receptors. Pediatr Res 15:80 (abstr)

Lang U, Aubert ML, Conne BS, Bradtke JC, Sizonenko PC 1983 Influence of exogenous melatonin on melatonin secretion and on the neuroendocrine reproductive axis of intact male rats during sexual maturation. Endocrinology 112:1578-1584

Lang U, Rivest RW, Schlaepfer LV, Aubert ML, Sizonenko PC 1984a Diurnal rhythm of melatonin action on sexual maturation of the male rats. Neuroendocrinology 38:261-268

Lang U, Aubert ML, Rivest RW, Vinas-Bradtke JC, Sizonenko PC 1984b Daily afternoon administration of melatonin does not irreversibly inhibit sexual maturation in male rat. Endocrinology 115:2303-2310

Lang U, Lenko HL, Bradtke JC, Delavy B, Aubert ML, Sizonenko PC 1985a Pineal gland and puberty. In: Grumbach MM et al (eds) The control of the onset of puberty II (Stresa conference 1981). Williams & Wilkins, Baltimore, in press

Lang U, Aubert ML, Rivest RW, Vinas-Bradtke JC, Sizonenko PC 1985b Inhibitory action of exogenous melatonin, 5-methoxytryptamine and 6-hydroxymelatonin on sexual maturation of male rats: activity of 5-methoxytryptamine might be due to its conversion to melatonin. Biol Reprod, in press

Lenko HL, Lang U, Aubert ML, Paunier L, Sizonenko PC 1982 Hormonal changes in puberty. VII. Lack of variation of daytime plasma melatonin. J Clin Endocrinol & Metab 54:1056-1058

Lerner AB, Case JD, Lee TH, Takahashi Y, Mori W 1958 Isolation of melatonin, the pineal factor that lightens melanocytes. J Am Chem Soc 80:2587-2594

Lynch HJ 1971 Diurnal oscillations in pineal melatonin content. Life Sci 10:741-743

Milcu S, Pavel S, Neascu C 1963 Biological and chromatographic characterization of a polypeptide with pressor and oxytocic activities isolated from bovine pineal gland. Endocrinology 72:563-566

Pavel S 1978 Arginine vasotocin as a pineal hormone. J Neural Transm (suppl) 13:135-155

Penny R 1982 Melatonin excretion in normal males and females: increase during puberty. Metabolism 31:816-823

Pevet P 1983 Is 5-methoxytryptamine a pineal hormone? Psychoneuroendocrinology 8:61-73

Reiter RJ 1980 The pineal and its hormones in the control of reproduction in mammals. Endocr Rev 1:109-131

Relkin R 1971 Relative efficacy of pinealectomy, hypothalamic and amygdaloid lesions in advancing puberty. Endocrinology 88:415-418

Rivest RW, Lang U, Aubert ML, Nawratil MF, Scherrer A, Sizonenko PC 1984 The use of two different lighting regimens allows the dissociation of the mechanisms responsible for the action of melatonin on estrous cycles and those provoking the proestrous surge of gonadotropins. Annu Rev Chronopharmacol 1:211-214

Rivest RW, Lang U, Aubert ML, Sizonenko PC 1985 Daily administration of melatonin delays rat vaginal opening and disrupts the first estrus cycles: evidence that these effects are synchronized by the onset of light. Endocrinology 116:779-787

Silman RE, Leone RM, Hooper RJL, Preece MA 1979 Melatonin, the pineal gland and human puberty. Nature (Lond) 282:301-303

Sizonenko PC, Lang U, Aubert ML 1982 Neuro-endocrinologie de la puberté. Role de la mélatonine chez l'homme. Ann Endocrinol 43:453-464

Tamarkin L, Westrom WK, Hamill AI, Goldman BD 1976 Effect of melatonin on the reproductive systems of male and female Syrian hamsters: a diurnal rhythm in sensitivity to melatonin. Endocrinology 99:1534-1541

Tamarkin L, Abastillas P, Chen HC, McNemar A, Sidbury JB 1982 The daily profile of plasma melatonin in obese and Prader-Willi syndrome children. J Clin Endocrinol & Metab 55:491-495
Tetsuo M, Poth M, Markey SP 1982 Melatonin metabolite excretion during childhood and puberty. J Clin Endocrinol & Metab 55:311-313
Vaughan GM 1984 Melatonin in humans. Pineal Res Rev 2:141-201
Vaughan MK, Vaughan GM, Klein DC 1974 Arginine-vasotocin: effects on development of reproductive organs. Science (Wash DC) 186:938-939
Waldhauser F, Wurtman RJ 1983 The secretion and actions of melatonin. Biochem Actions Horm 10:187-225
Waldhauser F, Weiszenbacher G, Frisch H, Zeitlhuber U, Waldhauser M, Wurtman RJ 1984 Fall in nocturnal serum melatonin during prepuberty and pubescence. Lancet 1:362-365
Wurtman RJ, Axelrod J, Chu EW 1963 Melatonin a pineal substance: effect on the rat ovary. Science (Wash DC) 141:277-278
Wurtman RJ, Axelrod J, Fischer JE 1964 Melatonin synthesis in the pineal gland: effect of light mediated by the sympathetic nervous system. Science (Wash DC) 143:1328-1330

DISCUSSION

Bittman: Bulbectomy makes adult rats sensitive to photoperiod and to melatonin, so I am wondering whether the time of offset of melatonin sensitivity in your young rats might correspond to a change in the relationship of the innervation from the olfactory bulb to the forebrain of the animal. Is there any way of linking the effect of bulbectomy in adults with the transitory phase of sensitivity to melatonin in maturing rats?

Sizonenko: I do not know why young rats are sensitive to melatonin. In adult rats melatonin is most active if you do something to the animal, for example put it under stress, but I am not sure whether this is relevant to maturing animals.

Bittman: Since a lot of neural maturation goes on in young rats for many weeks after birth (Cotman & Nieto-Sampredo 1984), perhaps loss of sensitivity to melatonin at day 50 or so is correlated with a growth of projections from the olfactory bulb.

Reiter: Pierre Sizonenko's observations in young rats are certainly consistent with the effects of the so-called potentiating agents in adults. Olfactory bulbectomy, underfeeding and androgen sterilization will all severely exaggerate the response of the system to the pineal and to melatonin (Reiter & Sorrentino 1971). Adult rats recover from this exactly as Pierre's maturing animals do; there is a lot of parallelism. It's amazing that, although sexual maturation can be severely delayed by melatonin, something happens between 75 and 100 days of age so that, regardless of its severity, the inhibition is overcome.

Hoffmann: Young rats, even without any manipulation, are marginally photoperiodic. We put rats into 16 h light:8 h dark (16L:8D) or 8L:16D from birth and, after eight weeks, found differences in testis weight that were small but significant (Hoffmann 1981). Compared with the results of similar experiments in *Phodopus* there were differences in quantity not in quality. So I think

the phase dependence of melatonin sensitivity in young rats parallels what occurs in other photoperiodic species like hamsters.

Sizonenko: We have started studying the effects of photoperiod in our rats. If we compare animals kept in 8L:16D from days of life 0 to 42, 20 to 42, 0 to 50 or 20 to 50 to animals kept in 16L:8D, we see a marked difference between the two groups, testicular weight and seminal vesicle weight being smaller in the former group. With 12L:12D, testicular weight is slightly lower than in the group maintained on 16L:8D for 42 or 50 days, but the difference is not usually significant. We can therefore use the 12L:12D photoperiod schedule from birth in our experiments to study the specific effect of melatonin in non-pinealectomized animals without worrying about the effects of the photoperiod itself on the reproductive system.

Illnerová: I cannot quite agree with Dr Hoffmann that young rats are only marginally photoperiodic. I think that they are entirely photoperiodic after birth. Professor Sizonenko has found that the inhibitory action of melatonin applied in the late afternoon on the growth of reproductive organs is strongest between day 20 and day 30 of life. Dr Vaněček and I have found that rats are photoperiodic even earlier than this (Vaněček & Illnerová 1985). We transferred pregnant rats to either 18L:6D or 8L:16D regimens one week before parturition and we maintained litters with their mothers under the same lighting regimens. We measured *N*-acetyltransferase profiles, testis weights and brown fat weights and found that even eight-day-old rats with closed eyes responded to photoperiod. In short photoperiods the *N*-acetyltransferase peak was more prolonged than in long photoperiods. On day 15, when rats open their eyes, there was a great difference between the 18L:6D group and the 8L:16D group; the length of the *N*-acetyltransferase peak differed by about 4–5 h. What is more, in these 15-day-old rats, testis weight was significantly higher and brown fat weight lower under the long photoperiod than under the short one. So photoperiodic regulation may occur before rats open their eyelids. As we put our rats onto the different photoperiod regimens one week before birth, photoperiod might even have an effect on the fetus.

Zucker: I don't see any contradiction between what you, Pierre Sizonenko and Klaus Hoffmann are saying. We are all using the term 'rat', but there is no reason to believe that we are all dealing with the same preparation. Laboratory selection procedures are important. Results different from yours have been published, but I'm confident that both can be correct.

Reppert: Marilyn Duncan in my laboratory has studied the effects of long and short days (16L:8D and 8L:16D) on albino rats during gestation, putting the pups into 12L:12D after birth. We looked at the pups on postnatal day 40, 50 and 65 but saw no differences in the testicular and seminal vesicle weights (unpublished work). Irv Zucker's point is well taken; there may be strain differences, so what happens in Dr Sizonenko's rats may not happen in ours.

Turek: I agree. If we keep rats in our laboratory on long or short days we see no difference in testis size. Yet, if we give a small dose of testosterone via a silastic capsule the animals on short days show testicular regression and a decline in both serum LH and serum FSH levels (Wallen & Turek 1981). Animals on long days are not inhibited by this negative feedback hormone. The effect is pineal dependent because pinealectomized animals don't show this short-day response. The entire photoperiodic machinery is present in these rats, but for some reason, perhaps because of artificial selection pressures, the system may be shut off so the animals are no longer photoperiodic. There is no reason to expect that this will be the same in rats all over the world.

Reiter: I suspect that potentiating factors like olfactory bulbectomy simply work by increasing negative feedback sensitivity.

Goldman: I have no doubt that there are differences between strains of rats, but it is also true that no two of these experiments have been the same. We need to determine where the important differences lie.

Turek: That's not fair. Many people have put rats on long and short days; some people see an effect and others do not.

Goldman: I was referring to Dr Vaněček and Dr Illnerová's experiments. These were done in neonatal rats, but if you do the same experiments in adult rats you don't get effects, perhaps because these animals have lost the capacity to respond to melatonin. I do think that Dr Vaněček and Dr Illnerová's findings and Professor Sizonenko's results with melatonin injections in young rats may be very closely related, and it may well be the age factor that is responsible for differences between their results and those obtained in other laboratories, often with older animals.

Armstrong: We may be barking up the wrong tree with these experiments on neonatal rats. Rats are burrowing animals and therefore in the wild the young will not see a light–dark cycle until they are 16–19 days of age. The way we bring rats up in the laboratory is very artificial (Daly 1973).

Illnerová: Dr Reiter has mentioned that it is necessary to sensitize adults rats to short photoperiods if you want to get a photoperiodic response, and one way to do this is to restrict food. Many people wean rats at the age of 21 days, but this is very early because rats are naturally weaned at 28 days (Babický et al 1970). Early weaning means that the rats are hungry and can therefore be sensitized to short photoperiods. At what age did you wean your rats, Professor Sizonenko?

Sizonenko: At 21 days. But this was the same for controls, so we think that we can still compare control and melatonin-treated groups of animals.

Herbert: We have been comparing photoperiodic responses and pubertal responses and have made some interesting observations on endogenous opioids in puberty. N.D. Martensz (unpublished work) has been looking at β-endorphin in the hypothalamus of rats and sees a marked change through

puberty. Although the total amount of β-endorphin stays the same, the processing alters. The prepubertal rat has more or less pure β-endorphin (1–31), but as it goes through puberty the proportion of metabolites rises. That is consistent with the idea that opioid peptides are important in seasonal breeding. However, the big difference between prepubertal rats and non-breeding hamsters is in the response to naloxone. If you give a prepubertal rat naloxone you get an enormous LH surge, but in an adult female rat the effect of naloxone is much smaller. In breeding and non-breeding hamsters, it's the opposite.

Follett: Much has been made of the fact that melatonin secretion is affected by photoperiod. Has that been taken into account in the human studies, when samples may have been taken at different times of year?

Arendt: We have always taken it into account.

Sizonenko: In our experiments samples were taken throughout the year.

Tamarkin: In clinical work we, like you, have found tremendous variability amongst patients and normal subjects. You need to be careful how you select subjects in different situations. Sandy Markey has followed individuals through puberty, and it turns out by chance that a group of girls who showed high levels of 6-hydroxymelatonin at Tanner stage 2 continued to show high levels of 6-hydroxymelatonin throughout development (unpublished work). The levels did not change, so high melatonin levels observed in a small group of subjects may be just a function of population selection.

Turek: Is this the population that Wurtman studied?

Sizonenko: No. Waldhauser and Wurtman found a decrease in the night levels of melatonin in children aged seven, but this is well before pubertal development starts (Waldhauser et al 1984).

Tamarkin: Tetsuo et al (1982) measured 6-hydroxymelatonin in children as young as six, so they covered that critical period referred to by Waldhauser, but they did not find elevated levels, declining throughout puberty. Sandy Markey and I assayed peak plasma levels of melatonin in a number of patients and normal volunteers using Mark Rollag's radioimmunoassay (Markey et al 1985). We compared the results with measurements of 6-hydroxymelatonin in the 24 h urine samples, and the correlation was very good (about 0.7), so we believe that the 24 h profiles of melatonin in the plasma are accurately reflected in the urinary output of melatonin. However, in the two puberty studies we were involved with we did not see any change in melatonin levels during development in humans.

Sizonenko: We also get an excellent correlation between urine melatonin levels and plasma levels measured during the night.

Arendt: To complete the picture one could look at urinary 6-hydroxymelatonin sulphate. The levels are far higher than the levels of urinary melatonin itself, which only represents about 1% of the amount secreted.

Reiter: Does anybody who has measured blood levels of melatonin in hu-

mans feel that there is a change between one and 20 years of age, either in absolute levels or in the nocturnal rise?

Sizonenko: Unfortunately we don't have enough night samples to give a definite answer to that question.

Wetterberg: We have reported that at 2 a.m. the concentrations of melatonin in serum as well as in the first morning urine sample are two to three times higher in children than in adults (Wetterberg 1979). We have seen very high values, up to 250 pg/ml, in some subjects but there is no obvious difference between concentrations before and after puberty. In summary, the overall values for melatonin in serum and urine are higher in children.

Reiter: In the rat, pineal melatonin levels have been compared in 25-day-old, 40-day-old, 55-day-old and 70-day-old animals (Reiter et al 1985). The pineal glands produce the same amount of melatonin at the four different ages; if there is no change in the secretion or metabolism of melatonin during this time while the body size of the animals is changing tremendously, we would predict that the concentration of melatonin in the blood would decrease with age. One of the critical factors in pubertal development in the human is body size. If melatonin production remains constant there may be an effective decrease in levels with increasing body size and blood volume.

Klein: That is what happens with melatonin. Sandy Markey and his co-workers showed that the total amount of 6-hydroxymelatonin per individual does not change during development; the line is flat (Tetsuo et al 1982).

Tamarkin: In other words, as you grow your output of melatonin remains about the same at every stage of development.

Reiter: But this is output in the urine. The blood concentration of melatonin could be dropping during that time because the body is getting much larger.

Sizonenko: Our urine results indicate that secretion of melatonin increases with body size, so we correct our figures for body surface area to see whether there is a difference between individuals. When we do the correction our studies show that melatonin secretion in the urine does not decrease with age from four to 20 years.

Klein: You measure free melatonin. Perhaps 6-hydroxymelatonin has a different pattern.

Lewy: Using the gas chromatographic–negative chemical ionization mass spectrometric assay for 6-hydroxymelatonin (Tetsuo et al 1981), R. Sack and others in my laboraotry (personal communication) have found a very significant decline with age, but they only studied individuals between 22 and 94 years of age.

Klein: That is consistent with ageing studies in the rat.

Lewy: I have a question about when either plasma or urine values should be corrected for body surface area. Are you suggesting, Professor Reiter, that it would be appropriate to correct plasma values for surface area?

Reiter: No. All I am saying is that, if the pineal is secreting the same amount of melatonin throughout development, then as the blood volume increases the melatonin concentration would decrease.

Arendt: Does pineal size change relative to body size during development?

Reiter: The rat pineal gland does not change much after the 25th day of age, but I don't know about humans.

Klein: After day 21 in the rat the body gets bigger but the gland does not grow significantly in comparison. The potential for melatonin production, as estimated by hydroxyindole *O*-methyltransferase activity, is highest around that time if you correct for body weight (Klein & Lines 1969).

REFERENCES

Babický A, Ošťádalová I, Pařízek J, Kolář J, Bíbr B 1970 Use of radioisotope techniques for determining the weaning period in experimental animals. Physiol Bohemoslov 19:457-467

Cotman CW, Nieto-Sampredo M 1984 Cell biology of synaptic plasticity. Science (Wash DC) 225:1287-1294

Daly M 1973 Early stimulation of rodents: a critical review of present interpretations. Br J Psychol 64:435-460

Hoffmann K 1981 Photoperiodic function of the mammalian pineal organ. In: Oksche A, Pévet P (eds) The pineal organ: photobiology—biochronometry—endocrinology. Elsevier, Amsterdam

Klein DC, Lines SV 1969 Pineal hydroxyindole-O-methyltransferase activity in the growing rat. Endocrinology 84:1523-1525

Markey SP, Higa S, Shih M, Danforth DN Jr, Tamarkin L 1985 The correlation between human plasma melatonin levels and urinary 6-hydroxymelatonin excretion. Clin Chim Acta, in press

Reiter RJ, Sorrentino S Jr 1971 Factors influential in determining the gonad-inhibiting activity of the pineal gland. In: The pineal gland. Churchill Livingstone, Edinburgh & London (Ciba Found Symp) p 329-344

Reiter RJ, Esquifino AI, Champney TH, Craft CM, Vaughan MK 1985 Pineal melatonin production in relation to sexual development in the male rat. In: Gupta D et al (eds) Paediatric neuroendocrinology. Croom Helm, London, p 190-202

Tetsuo M, Markey SP, Colburn RW, Kopin IJ 1981 Quantitative analysis of 6-hydroxymelatonin in human urine by gas chromatography-negative chemical ionization mass spectrometry. Anal Biochem 110:208-215

Tetsuo M, Poth M, Markey SP 1982 Melatonin metabolite excretion during childhood and puberty. J Clin Endocrinol & Metab 55:311-313

Vaněček J, Illnerová H 1985 Effect of short and long photoperiods on pineal *N*-acetyltransferase rhythm and on growth of testes and brown adipose tissue in developing rats. Neuroendocrinology, in press

Waldhauser F, Weiszenbacher G, Frisch H, Zeithuber U, Waldhauser M, Wurtman RJ 1984 Fall in nocturnal serum melatonin during prepuberty and pubescence. Lancet 1:362-365

Wallen EP, Turek FW 1981 Photoperiodicity in the male albino laboratory rat. Nature (Lond) 289:402-404

Wetterberg L 1979 Clinical importance of melatonin. Prog Brain Res 52:539-547

Photoperiodism, melatonin and the pineal
Editors: David Evered and Sarah Clark

Melatonin, light and chronobiological disorders

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Abstract. Human plasma melatonin concentrations can be measured accurately and sensitively by gas chromatography–negative chemical ionization mass spectrometry. With this assay, we have shown that: (1) in rats and in humans, plasma melatonin is exclusively derived from the pineal gland; (2) propranolol and clonidine reduce melatonin levels in humans; (3) some blind people appear to have free-running melatonin secretory circadian rhythms; (4) bright light can acutely suppress human melatonin production according to a linear fluence–response relationship; (5) manic-depressive patients appear to be supersensitive to light, even when they are well; (6) melatonin levels are greater in manic patients than in depressed patients; (7) in experiments to test the clock–gate model and the hypothesized phase–response curve, two different effects of light appear to present in humans: an acute suppressant effect (mainly in the evening during long photoperiods) and an entrainment effect (particularly during the morning but also in the evening). When blood is sampled for measuring melatonin levels as a marker for circadian phase position, bright light should be avoided after 5 p.m. (the dim light melatonin onset). Bright-light exposure in the morning appears to advance circadian rhythms, whereas bright-light exposure in the evening appears to delay them. Once a patient has been 'phase typed' (phase-advanced vs. phase-delayed), predictions can be made about whether morning or evening light would be more effective in treating the sleep or mood disorder.

1985 Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Foundation Symposium 117) p 231-252

Melatonin has potential importance as a biological marker: the timing of its production for circadian phase position; the suppression of its synthesis by light for retinally mediated hypothalamic responses; and the area under the curve of plasma concentrations for adrenergic function. Previous studies in which plasma melatonin levels have been measured have provided evidence that patients with affective disorders may have abnormalities in circadian and seasonal rhythms, responses to light and adrenergic function.

Pineal origin of plasma melatonin

For plasma melatonin to be useful as a biological marker, it would be best if the circulating hormone was primarily derived from the pineal gland, as substantial extra-pineal contributions might confound interpretation of plasma level results. This has been a controversial area. More than one radioimmunoassay has shown that melatonin persists in the circulation following pinealectomy (Ozaki et al 1976, Yu et al 1981). Our question was limited to whether or not plasma melatonin was derived exclusively from the pineal gland or whether other sources made substantial contributions as well. (Another controversial question, not to be confused with this one, is whether or not extra-pineal production of melatonin occurs.)

We originally reported that pinealectomy reduces plasma melatonin to undetectable levels in the rat (Lewy et al 1980a). Of the plasma melatonin assays, the gas chromatography–negative chemical ionization mass spectrometry (neg CI GCMS) assay is among the most sensitive, if not the most sensitive (Lynch 1983) (the minimal detectable concentration of plasma melatonin in our assay is less than 1 pg/ml), and is probably the most specific (Arendt 1981, Rollag 1981). Using this assay, we found low, but measurable, day-time levels of melatonin in sham-operated rats (4 ± 1 pg/ml) and high levels in night-time samples (52 ± 7 pg/ml) and in day-time samples obtained after administration of the β-adrenergic agonist isoprenaline. In all three conditions no melatonin could be detected in the pinealectomized animals. Since that study, Dr Markey has used the neg CI GCMS assay he developed for urinary 6-hydroxymelatonin to demonstrate similar results in the rat (Markey & Buell 1982) and in the monkey (Tetsuo et al 1982). With Dr E. Neuwelt of the Department of Neurosurgery, Oregon Health Sciences University, we published a study showing that no melatonin could be detected in the plasma of a *human* after pinealectomy (Neuwelt & Lewy 1983). Consequently, we feel that this controversial question is gradually becoming resolved for most mammalian species, including humans, with the evidence favouring the pineal as the exclusive source of circulating melatonin.

Melatonin as a marker for adrenergic function

The use of plasma melatonin and urinary 6-hydroxymelatonin as biological markers for pineal adrenergic acitivity is particularly attractive because melatonin production can be affected by adrenergic drugs. We have preliminary evidence that propranolol reduces the amplitude of the night-time peak in melatonin production, apparently with a linear dose–response relationship (others, beginning with Hanssen et al (1977), have obtained similar results,

but a complete review of the literature cannot be given here). Furthermore, although we are not sure precisely where clonidine (an α-adrenergic agonist) acts, this drug does appear to reduce melatonin production (Lewy 1983) in a dose-dependent way; our preliminary data were based on four subjects but were statistically significant with $P < 0.01$. Tricyclic antidepressant medication causes a (twofold) increase in urinary 6-hydroxymelatonin production measured at one, two and three weeks after initiating treatment in depressed patients.

Melatonin as a marker for effects of light

A reassessment of the effects of light in humans has occurred since the publication of our finding that, whereas ordinary room light does not suppress melatonin production in humans, sunlight and bright artificial light are very effective (Lewy et al 1980b). Our data also suggested a linear relationship between the intensity of light and the degree of suppression of melatonin production.

After a pilot study involving self-experimentation (A.J.L.), two volunteers were tested; they slept during the dark between 3 and 11 a.m. for one week. We then measured their melatonin concentrations and documented a four-hour phase-delay. On the eighth day we exposed the volunteers to sunlight at 7 a.m.—their melatonin levels were immediately and profoundly reduced (Lewy et al 1980b).

Questioning what property of sunlight might be responsible for this effect, we decided to test intensity (Lewy et al 1980b). We found that 2500 lux light immediately and profoundly decreased melatonin levels between 2 and 4 a.m. when subjects were awakened at this time, whereas 500 lux had little effect. Furthermore, when the subjects were returned to sleeping in the dark, their plasma melatonin resumed high night-time levels within 20–40 minutes. We also tested 1500 lux in two of these volunteers and found that it caused a 50% suppression of melatonin production; concentrations again rebounded to night-time values when subjects were returned to darkness (Lewy et al 1980b).

Since then, we have demonstrated light suppression of melatonin production in patients (Lewy et al 1981, Lewy et al 1982, Lewy et al 1985a). Most recently, in association with Dr George Brainard and members of Dr Michael Menaker's group, we exposed six normal volunteers to six different intensities of monochromatic light [509 ± 10 (50% bandwidth) nm]; we were able to obtain more complete fluence–response curves. This study also demonstrated that waking subjects in the dark for one hour had no suppressant effect on melatonin production. With Dr Brainard, we also determined that the most effective wavelengths for light suppression of melatonin production in humans are

around 509 nm, corresponding approximately to those for rhodopsin activation.

The fluence–response relationship between light intensity and reduction of melatonin production may be useful in evaluating retinally mediated hypothalamic effects of light. Accordingly we have found that manic-depressive patients may be supersensitive to light; this characteristic may be a 'trait marker'. Originally, we studied four manic-depressive patients (two manic and two depressed, not taking medications) whose melatonin levels decreased 50% in response to a two-hour exposure to 500 lux light; six normal controls did not show suppressed melatonin production at this intensity (Lewy et al 1981). In a more recent study (Lewy et al 1985a), in 11 euthymic (currently well) patients with a history of manic-depressive illness who had not been taking medications for the previous two to four weeks, 500 lux suppressed melatonin production twice as much as in a group of 24 normal volunteers (Mann Whitney test: $U = 228.5$, $Z = 3.43$, $P = 0.003$).

Melatonin as a marker for biological rhythms

Our preliminary results suggested that the onset of nocturnal melatonin production (melatonin onset) is most advanced in mania and is also advanced in patients with endogenous depression compared to normal controls (Lewy et al 1979). When we compared the peak plasma melatonin levels, we found that more melatonin was produced during a manic episode (77 ± 19 pg/ml) than during a depressive episode (53 ± 14 pg/ml) ($P < 0.05$, Student's t test). Melatonin production in healthy control subjects appeared to be intermediate (60 ± 12 pg/ml). We compared patients to themselves and we controlled for many variables such as medication and time of year (Lewy 1983). Low melatonin production is probably state dependent, although some investigators (e.g. L. Wetterberg) consider it to be a trait marker for depression. Caution is needed here, because we have found that urinary 6-hydroxymelatonin production decreases with age.

Two totally blind individuals were studied once a week for four weeks, one of whom appeared to have a stable phase-delayed position (at about 120°) of his melatonin circadian secretory rhythm. (It is possible that he was free-running with a period so close to 24 hours that we were not able to detect any difference over the four weeks; this seems unlikely, however, given that 16 months earlier he was also in the same phase position.) The other subject appeared to be free-running, in that each week his melatonin circadian secretory rhythm was delayed by approximately four to six hours (Lewy & Newsome 1983). The temperature rhythm in the free-running subject also appeared to coincide with the melatonin rhythm in that the temperature minimum seemed to occur close to the melatonin maximum.

It is reasonable to infer from these preliminary results that at least three subgroups exist for totally blind people: (1) those with normal circadian rhythms, (2) those who have a 24-hour period but who have an abnormal phase position, and (3) those with periods different from 24 hours, who thus have a constantly changing phase position. (People in the last group may have symptoms or physiological abnormalities that are only present during days when they are maximally out of phase.) These data suggest that the light–dark cycle is important for entrainment of the melatonin circadian secretory rhythm, although our results also suggest that other time cues may be used for entrainment.

Treatment of winter depression using bright light exposure

The first published case of a manic-depressive with a seasonal mood cycle treated by bright artificial light (Lewy et al 1982) described a man with a 13-year history of depressive episodes that generally started before and lasted through much of the winter, and remitted in the late winter and early spring. We exposed him to 2000 lux fluorescent light for three hours between 6 and 9 a.m. and between 4 and 7 p.m. After four days he appeared to switch out of his depression.

Rosenthal et al (1984) studied nine more patients, using a double-blind cross-over design. The patients did not respond significantly to a lengthening of winter days with ordinary-intensity light; only bright artificial light appeared to switch them out of their depressive episodes. In a follow-up study (Rosenthal et al 1985), sleep was measured and sleep deprivation was rigidly controlled. The response did not appear to be mediated by sleep deprivation.

The phase–response curve and the clock–gate model

We recently completed a study (Lewy et al 1984) that assessed both the suppressant (tonic) and the entrainment (phasic) effects of light on the onset of night-time melatonin production (Fig. 1). On day 1 of the study, which occurred during the summer under the natural photoperiod ('dusk' was between 7:30 and 9 p.m. and 'dawn' was between 6 and 7:30 a.m.), we measured the onset of melatonin production in four healthy volunteers. Throughout the study, volunteers slept between 11 p.m. and 6 a.m. In the first week of the study (Figs. 1 & 2), we advanced dusk to 4 p.m.—the melatonin onset advanced 1.5 hours the first night (day 2), remained at that time for at least one more day (days 3 and 4), and then advanced an additional hour by the end of the week (day 8). We have concluded that, if we sample evening melatonin levels under dim light (the dim light melatonin onset), we can avoid the suppressant effect of light (without immediately affecting entrainment

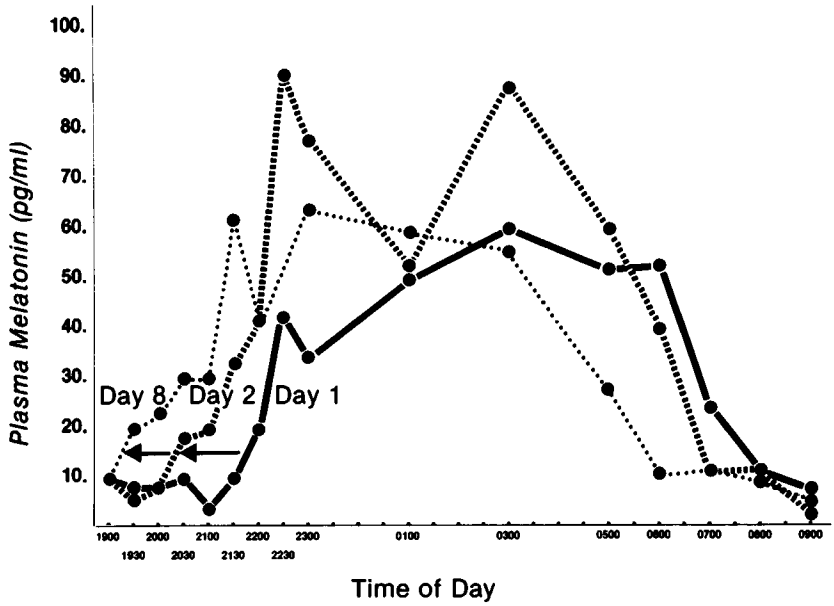

FIG. 1. Mean plasma melatonin concentrations in four healthy subjects. Standard errors have been deleted for clarity [they have been published in tabular form by Lewy et al (1985c)]. On day 1 'dusk' was between 7:30 and 9 p.m. and 'dawn' was between 6:00 and 7:30 a.m. On day 2 dusk was advanced to 4:00 p.m. The melatonin onset shifted to occur 1.5 hours earlier on day 2. By day 8 it had advanced by another hour as had the offset.

of the melatonin circadian secretory rhythm), thereby eliminating variability in the melatonin onset due to the changing time of sunset during the year. The onset of melatonin production may be the best part of the melatonin secretory curve to use for accurate assessment of circadian phase position, because the onset is probably much less affected than other parts of the curve by changes in β-adrenoceptor sensitivity (Zatz et al 1976) and perhaps by substrate availability, which could decrease melatonin levels during the night and thereby confound determination of the acrophase, maximum and offset. Use of the melatonin onset, which generally occurs between 7 p.m. and 11 p.m., has several other advantages over measuring the entire night-time secretory curve, including conservation of blood reserves, sleep and sample analysis time. Most significant is the fact that the melatonin onset is a discrete event that can be determined with high resolution.

The change in phase position between days 2 and 8 after dusk was advanced on day 2 (Fig. 1) suggests an entrainment effect of light in the evening; the rapid change in phase on day 2 itself suggests removal of an additional suppressant effect of light [although other explanations are plausible (Elliott & Tamarkin 1982, Illnerova & Vanecek 1982)]. In the second week of the study (Fig. 3),

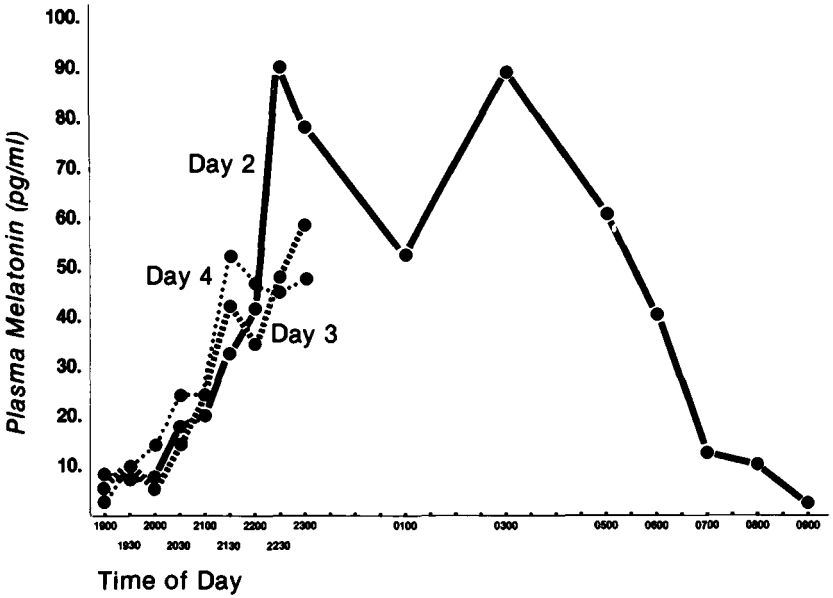

FIG. 2. Same study as Fig. 1. The melatonin onset appeared to remain unchanged between day 2 and day 3, but shifted to a slightly earlier time by day 4.

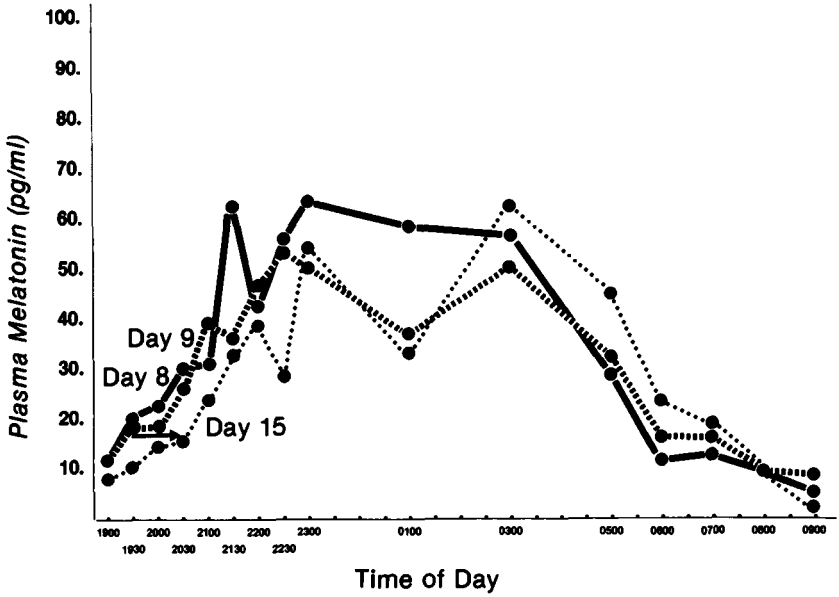

FIG. 3. Same study as Fig. 1. In the second week of this study, dawn was delayed to 9:00 a.m. No change in the melatonin onset occurred in the first few days (in addition to day 8, only day 9 and day 15, the first and last days of the week of delayed dawn, are shown in this figure). By the end of the week, the melatonin onset and offset had shifted to occur one hour later.

we delayed dawn to 9 a.m.—the melatonin onset did not change on the first night (day 9) but gradually delayed to occur one hour later by day 15 at the end of the week (this suggested an entrainment effect of light only).

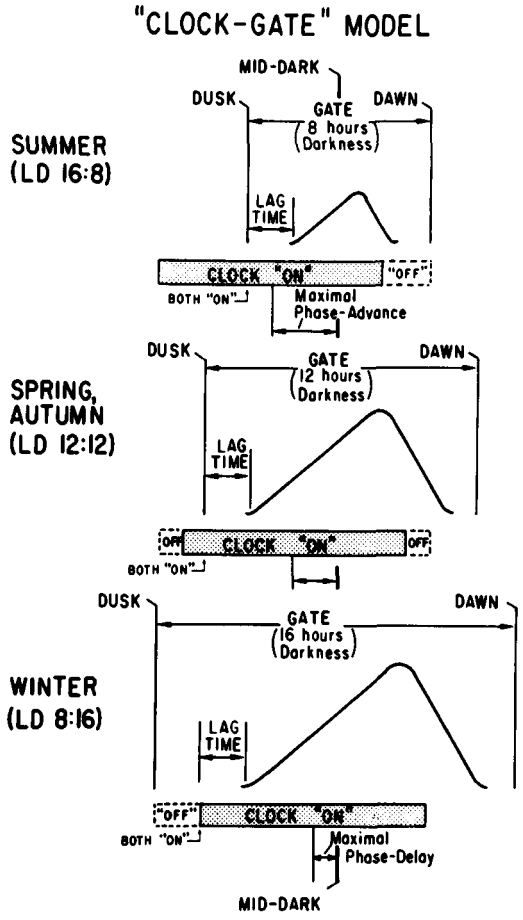

FIG. 4. The clock–gate model, showing patterns of melatonin production during different photoperiods. The clock–gate model assumes two effects of light: an acute suppressant effect and an entrainment effect. According to the phase–response curve shown in Fig. 5, dawn and, to some extent, dusk synchronize the endogenous circadian pacemaker to the 24-hour light–dark cycle. During long photoperiods, the scotoperiod (dark period) gates the circadian rhythm in melatonin production. [For this figure, the 'on' and 'off' signals of the 'clock' were assumed to be of equal duration throughout the year. It was also assumed that the endogenous (free-running) period would be greater than 24 hours (see Fig. 5).] According to this model, although the melatonin onset is later in the summer than in the winter, the clock could be shifted earlier (cueing more to dawn than to dusk). The study referred to in Figs. 1–3 provides evidence consistent with the clock–gate model. From Lewy (1983) by permission of Elsevier North-Holland.

This study provides evidence consistent with the 'clock–gate' model we have described elsewhere in detail (Lewy 1983). This model (Fig. 4) explains the seasonal onset–offset phase paradox (late onset and early offset of melatonin production in the summer compared to the winter). (In Fig. 4, it is assumed that the endogenous circadian 'on' and 'off' signals are of equal duration. This may or may not be true in humans.) The clock–gate model assumes that both dawn and dusk entrain the underlying pacemaker for melatonin production. According to this model, even if the 'clock' is 'on', the signal

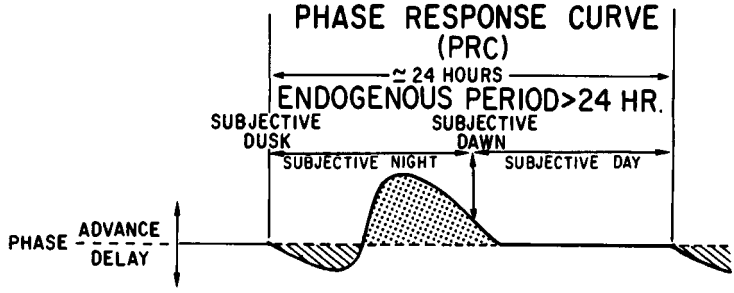

FIG. 5. Hypothesized phase–response curve (PRC) for animals (including humans) whose endogenous (free-running) periods are greater than 24 hours. Advance responses of increasing magnitude are plotted above the abscissa, delay responses below the abscissa. The abscissa itself is circadian time. Because most PRC studies are performed in constant darkness, the terms subjective day, subjective night etc. are used (these are based on the animal's activity–rest cycle). Light pulses during subjective day have minimal effects. Light pulses in the first half of subjective night cause phase-delays. Light pulses during the second half of subjective night cause phase-advances. The magnitude of these phase-shifts decreases near subjective dawn and dusk. There is an inflection point in the middle of the night at which the phase direction reverses. Near the margins of the photoperiod, the area under the advance portion of the curve is greater than that under the delay portion, because animals whose endogenous (intrinsic) periods are greater than 24 hours must use light to advance more than to delay their circadian rhythms; otherwise, their circadian rhythms would drift later and later each day. The study referred to in Figs. 1-3 provides evidence consistent with the hypothesized phase–response curve. From Lewy et al (1983).

for melatonin production cannot begin until after dusk. There is also a one- to two-hour lag time involving mRNA and *N*-acetyltransferase synthesis following 'clock on' or dusk (whichever is later) before the onset of night-time melatonin production (Romero et al 1975). On the basis of our knowledge of phase–response curves, we would expect dawn to be relatively more important than dusk for entrainment in species with intrinsic periods greater than 24 hours (Fig. 5). Dusk would then be more important for the suppressant effect on melatonin production. In the summer the underlying pacemaker is advanced relative to its position in the winter. This is seen in the earlier offset of melatonin production in the summer. As explained by the clock–gate

model, the melatonin onset is later in the summer because of the suppressant effect of light [also demonstrated by Tamarkin et al (1980)]. Thus, we can explain the seasonal onset–offset phase paradox with one endogenous pacemaker and two effects of light (suppression and entrainment). Whether or not there are separate pacemakers for dawn and dusk is not known, but one pacemaker can explain these data.

An alternate explanation for the seasonal onset–offset phase paradox can be based on the existence of two separate endogenous circadian pacemakers, one for the onset of melatonin production and one for the offset. Illnerova & Vanecek (1982) and Elliott & Tamarkin (1982) have proposed a two-oscillator timing mechanism, suggested by the 'splitting' phenomenon of the activity–rest cycle during constant bright-light conditions (Pittendrigh & Daan 1976). No study, however, has yet been designed that can conclusively prove whether one or two pacemakers govern the melatonin secretory rhythm. Furthermore, although the clock–gate model can explain much of the rodent data (Illnerova & Vanecek 1980), humans may be regulated differently from rodents. For example, brief exposure to light in the last half of the night results in suppression of melatonin production for the remainder of the night in rodents, whereas in humans melatonin production resumes with the return of darkness, if it is not too late in the night (Lewy et al 1980b).

'Phase typing' patients with chronobiological sleep or mood disorders

We originally treated winter depressive patients by exposing them to bright light between 6 and 9 a.m. and between 4 and 7 p.m. We have recently found that most of these patients respond much better to morning light alone than to evening light alone; the response to morning light alone is even greater than the response to light at both times (Lewy et al 1984, 1985b). (When bright light is administered in the morning, it is avoided in the evening, and vice versa.) These results are consistent with our observation that these patients have phase-delayed circadian rhythms (Lewy et al 1984, 1985b). This is in contrast to the phase-advanced circadian rhythms that characterize most other endogenous (melancholic) depressives, who have early morning awakening. The endogenous depressives appear to respond preferentially to evening light alone rather than to morning light (Lewy et al 1983, 1984, 1985b). At the very least, the early morning awakening is ameliorated in these patients. Relief for the remaining depressive symptoms may vary between individuals and is currently being evaluated. In preliminary studies, bright-light exposure also appears to be effective in the morning in patients with delayed sleep phase syndrome and in the evening in those with advanced sleep phase syndrome.

Consequently, we are currently recommending that patients suspected of having chronobiological sleep or mood disorders be 'phase typed' (Lewy et al 1984, 1985b, 1985c). Then a prediction can be made about whether or not bright-light exposure in the morning or evening would be clinically effective. Patients with phase-advanced circadian rhythms should respond to bright light in the evening, whereas patients with phase-delayed circadian rhythms should respond to bright light in the morning. Monitoring the melatonin onset and its response to light in patients suspected of having biological rhythm abnormalities and comparing these responses with those of normal control subjects may help elucidate how light regulates circadian and seasonal rhythms in humans. Such an evaluation may also lead to improved diagnosis and treatment of certain types of sleep and mood disorders, as well as aid in the understanding and treatment of 'jet lag' (Daan & Lewy 1984) and problems associated with shift-work.

Acknowledgements

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REFERENCES

Arendt J 1981 Current status of assay methods of melatonin. Adv Biosci 29:3-7

Daan S, Lewy AJ 1984 Scheduled exposure to daylight: a potential strategy to reduce 'jet lag' following transmeridian flight. Psychopharmacol Bull 20:566-568

Elliott JA, Tamarkin L 1982 Phase relationship of two circadian oscillators regulates pineal melatonin rhythm in Syrian hamsters. Endocrinology 110:A326

Hanssen T, Heyden T, Sundberg T, Wetterberg L 1977 Effect of propranolol on serum-melatonin. Lancet 2:309-310

Illnerova H, Vanecek J 1980 Pineal rhythm in *N*-acetyltransferase activity in rats under different artificial photoperiods and in natural daylight in the course of a year. Neuroendocrinology 31:321-326

Illnerova H, Vanecek J 1982 Two-oscillator structure of the pacemaker controlling the circadian rhythm of *N*-acetyltransferase in the rat pineal gland. J Comp Physiol 145:539-548

Lewy AJ 1983 Biochemistry and regulation of mammalian melatonin production. In: Relkin RM (ed) The pineal gland. Elsevier North-Holland, New York, p 77-128

Lewy AJ, Newsome DA 1983 Different types of melatonin rhythms in some blind subjects. J Clin Endocrinol & Metab 56:1103-1107

Lewy AJ, Wehr TA, Gold P, Goodwin FK 1979 Plasma melatonin in manic-depressive illness. In: Usdin E et al (eds) Catecholamines: basic and clinical frontiers. Pergamon Press, New York, vol 2:1173-1175

Lewy AJ, Tetsuo M, Markey SP, Goodwin FK, Kopin IJ 1980a Pinealectomy abolishes plasma melatonin in the rat. J Clin Endocrinol & Metab 50:204-205

Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Markey SP 1980b Light suppresses melatonin secretion in humans. Science (Wash DC) 210:1267-1269

Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Rosenthal NE 1981 Manic-depressive patients may be supersensitive to light. Lancet 1:383-384

Lewy AJ, Kern HE, Rosenthal NE, Wehr TA 1982 Bright artificial light treatment of a manic-depressive patient with a seasonal mood cycle. Am J Psychiatry 139:1496-1498

Lewy AJ, Sack RL, Fredrickson RH, Reaves M, Denney D, Zielske DR 1983 The use of bright light in the treatment of chronobiologic sleep and mood disorders: the phase-response curve. Psychopharmacol Bull 19:523-525

Lewy AJ, Sack RL, Singer CL 1984 Assessment and treatment of chronobiologic disorders using plasma melatonin levels and bright light exposure: the clock-gate model and the phase response curve. Psychopharmacol Bull 20:561-565

Lewy AJ, Nurnberger JI, Wehr TA et al 1985a Supersensitivity to light—possible trait marker for manic-depressive illness. Am J Psychiatry 142:725-727

Lewy AJ, Sack RL, Singer CM 1985b Treating phase typed chronobiologic sleep and mood disorders using appropriately timed bright artificial light. Psychopharmacol Bull 21:368-372

Lewy AJ, Sack RL, Singer CM 1985c Immediate and delayed effects of bright light on human melatonin production: shifting 'dawn' and 'dusk' shifts the dim light melatonin onset (DLMO). Ann NY Acad Sci 453:253-259

Lynch HJ 1983 Assay methodology. In: Relkin RM (ed) The pineal gland. Elsevier North-Holland, New York, p 129-150

Markey SP, Buell PE 1982 Pinealectomy abolishes 6-hydroxymelatonin excretion by male rats. Endocrinology 111:425-426

Neuwelt EA, Lewy AJ 1983 Disappearance of plasma melatonin after removal of a neoplastic pineal gland. New Engl J Med 308:1132-1135

Ozaki Y, Lynch HJ, Wurtman RJ 1976 Melatonin in rat pineal, plasma and urine: 24-hour rhythmicity and effect of chlorpromazine. Endocrinology 98:1418-1424

Pittendrigh CA, Daan S 1976 A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: a clock for all seasons. J Comp Physiol 106:333-355

Rollag MD 1981 Methods for measuring pineal hormones. In: Reiter RJ (ed) The pineal gland, anatomy and biochemistry. CRC Press, Boca Raton, vol 1:273-302

Romero JA, Zatz M, Axelrod J 1975 Beta-adrenergic stimulation of pineal *N*-acetyltransferase: adenosine 3′,5′-cyclic monophosphate stimulates both RNA and protein synthesis. Proc Natl Acad Sci USA 72:2107-2111

Rosenthal NE, Sack DA, Gillin JC et al 1984 Seasonal affective disorder. Arch Gen Psychiatry 41:72-80

Rosenthal NE, Sack DA, Carpenter CD, Parry BL, Mendelson WB, Wehr TA 1985 Antidepressant effect of light in seasonal depression. Am J Psychiatry 142:163-170

Tamarkin L, Reppert SM, Klein DC, Pratt B, Goldman BD 1980 Studies on the daily pattern of pineal melatonin in the Syrian hamster. Endocrinology 107:1525-1529

Tetsuo M, Perlow MJ, Mishkin M, Markey SP 1982 Light exposure reduces and pinealectomy virtually stops urinary excretion of 6-hydroxymelatonin by Rhesus monkeys. Endocrinology 110:997-1003

Yu HS, Pang SF, Tang PL, Brown GM 1981 Persistence of circadian rhythms of melatonin and *N*-acetylserotonin in the serum of rats after pinealectomy. Neuroendocrinology 32:262-265

Zatz M, Kebabian JW, Romero JA, Lefkowitz RJ, Axelrod J 1976 Pineal adrenergic receptor: correlation of binding of ^{3}H-alprenolol with stimulation of adenylate cyclase. J Pharmacol Exp Ther 196:714-722

DISCUSSION

Tamarkin: Have you looked at the same individual for a couple of days to see how reproducible the melatonin rhythm is?

Lewy: We have just begun to address this issue.

Tamarkin: Our results indicate that in many individuals the nocturnal increase in melatonin levels is not predictable. We cannot say that it will always start at, for example, 10 p.m.

Lewy: To use melatonin as a marker for circadian phase position, we recommend the use of DLMO (dim light melatonin onset) (Lewy et al 1984). On the night of the blood drawing, subjects are kept away from bright light after 5 p.m. Melatonin levels are sampled every 30 min between 6 and 11 p.m. This reduces variability in the timing of the onset due to the changing time of dusk throughout the year. We think that bright light in the evening suppresses the signal that results in stimulation of melatonin production—a suppressant effect of light separate from its phase-controlling effects. We determine a DLMO on admission to the hospital and again after a week of standardized light–dark and sleep–wake schedules.

Tamarkin: You have just said that it is best to use dim light to study the onset of nocturnal melatonin production and yet you argue that room light of perhaps 500 lux would be insufficient to suppress melatonin secretion. So why do you need to use dim light? Is the intensity of light necessary to suppress melatonin production the same as the intensity needed to entrain the melatonin rhythm? What intensity of light did you use in your study on the clock–gate model when you shifted the melatonin rhythm?

Lewy: We used light greater than 2000 lux to shift rhythms in our circadian study. Although we suspect, from data on bright-light suppression of melatonin production (Lewy et al 1980), that humans also require bright light for entrainment, we do not know the precise range of intensities that might affect entrainment. It is possible that 500 lux light may be effective in some individuals, particularly in the morning. Furthermore, patients may be more sensitive to light than normal subjects (Lewy et al 1981, 1985). Consequently, we are being cautious when we keep our subjects in dim light (50–200 lux) to measure the melatonin onset (Lewy et al 1984).

*Menaker:*This question of entrainment versus suppression is important. Are there no results from bunker experiments indicating how much light is needed to entrain the human circadian system in general?

Moore-Ede: Entrainment of the human system does not occur when you allow subjects to provide themselves with a reading light during the night. But if you place someone in an absolute light–dark cycle, switching the lights on and off on a fixed schedule, and use about 600 lux as a reasonable level of light in the room you do get entrainment. When we applied a light–dark cycle to subjects

with free-running rhythms we showed entrainment not only of the sleep–wake cycle, but also of temperature and cortisol rhythms (Czeisler et al 1981). We also demonstrated phase control. The subjects didn't think that the light cycles made any sense; they did not perceive them as providing time cues in the intellectual sense, but the cycles certainly entrained the rhythms.

Lewy: Light may have a social cue effect as well as the direct neuronal effect on the hypothalamus. The social cue effect may be stronger under an absolute light–dark cycle (because subjects are forced to lie in their beds and sleep during total darkness), whereas zeitgeber strength for the neuronal effect is probably related to light intensity (Lewy 1983).

Menaker: We find that in the hamster we need 2.5 orders of magnitude more light to shift the circadian system than to suppress melatonin production (D.J. Hudson & M. Menaker, unpublished work). All the 'bright' and 'dim' lights that you are talking about differ in intensity by less than an order of magnitude. It's important to recognize that; the difference between 600 lux and 2500 lux is really very small.

Lewy: I agree with you with regard to hamsters. But for humans, there may be a difference between 600 and 2500 lux.

Tamarkin: In your first study of light suppression one subject was asked to sleep later than normal. She went to bed at about 3 a.m. and woke up 7 h later, and you found that her melatonin rhythm shifted (Lewy et al 1980). Did this shift occur in normal ward light?

Lewy: No. That patient slept in the dark between 3 a.m. and 11 a.m. and we saw a 4 h phase-delay in the melatonin rhythm.

Tamarkin: So you didn't have to expose the subject to bright light to shift the rhythm.

Lewy: Yes we did; bright light at dawn was delayed by 4 h. We (Lewy et al 1984) as well as Wetterberg (1978) have shown that holding the activity–rest cycle constant and delaying dawn causes a delay in the melatonin rhythm.

Bittman: Wever (1985) has been exploring the limits of entrainment using very bright light, and he has gone up to 32 h without problems. This raises the possibility that the shape of the phase–response curve is entirely dependent upon how bright the light pulses are. You need to consider this when you are constructing hypothetical phase–response curves, and I think it may force a re-interpretation of some of your experiments with morning and evening bright light.

Lewy: I agree that the brighter the light the greater will be the amplitude of the phase–response curve. However, light intensity should not affect the direction of the phase-shifts (delay responses in the evening, advance responses in the morning).

Goldman: Can anything be said about the effect of short light pulses on the human sleep–wake cycle or temperature rhythm?

Moore-Ede: Only a few people have been studied in constant darkness with minimal light exposure, and I don't believe any of those were subjected to short light pulses.

Armstrong: Do the patients with phase-advanced rhythms have a short τ, or a long τ with a big advance portion in the phase–response curve?

Lewy: I can't tell you. We would like to analyse this but we do not have a temporal isolation unit. Ideally, we would study these patients in Portland, Oregon, and then send them to the appropriate laboratory to see what their τ values are when the rhythms free-run.

Armstrong: What percentage of the human population have a τ shorter than 24 h?

Moore-Ede: It is extremely rare to have a τ shorter than 24 h for the internally synchronized circadian system. Mills et al (1977) have reported this in a couple of schizophrenics, and some people have a τ close to 24 h, for example 24.1 h. But most τ values are 25 h $\pm$ 0.5 h, and the distribution tails off very rapidly once you get close to 24 h.

Armstrong: Does that suggest that most people with a short τ are in the clinical population?

Moore-Ede: Mills' two subjects with τ values of less than 24 h were certainly schizophrenic, so I think that if you get a free-running period under 24 h you should look for a clinical problem.

Lewy: But you must distinguish between a τ shorter than 24 h and a 'short' τ. You can certainly get a phase-advance with a τ greater than (but close to) 24 h, whereas with a τ of 25 h you may get a relatively delayed phase position.

Reppert: One of the blind individuals you studied appeared to be entrained to the outside world and had a stable phase position like that of a sighted person. How do you define your blind individuals? Is there no way they could perceive light, perhaps through a residual portion of functional retina? Did you find out whether light would suppress melatonin production in these people?

Lewy: No, but we exposed them to bright light and asked them whether or not they could detect it.

Reppert: But that would be irrelevant in terms of the hypothalamus. It's conceivable that someone who has no conscious perception of light might well have hypothalamic light perception.

Menaker: You cannot evoke a cortical response in RD mice with degenerate retinas (Drager & Hubel 1978), and yet these mice are no different in their phase-shifting behaviour from normal litter mates with perfectly good retinas. So you really cannot tell from the subjective impression of the patient whether light has affected one or another part of the brain.

Marks: I am wondering whether your effects of light on mood are necessarily mediated by the eyes and by melatonin. There is a nice rhythm in calcium in humans, with plasma concentrations going up in the summer and down in the

winter; and occasionally people become ill because of hypercalcaemia during the summer. The effects are due to bright light, acting not through the eyes but through the skin, probably changing the amount of vitamin D produced. You can certainly get abnormalities of mood in disorders of calcium metabolism, so perhaps in your patients depression is mediated by calcium and not melatonin.

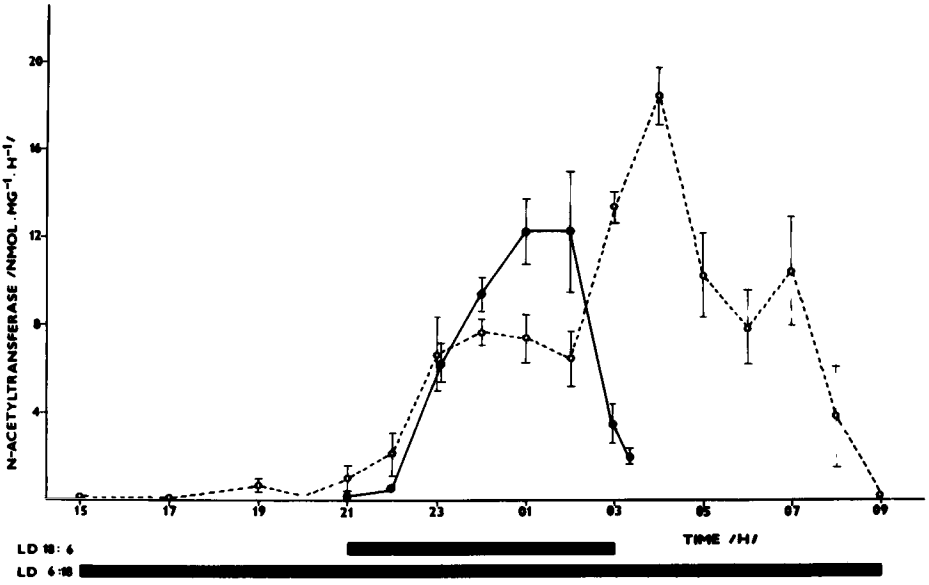

FIG. 1. (*Illnerová*) The rhythm in pineal *N*-acetyltransferase activity in rats maintained under 18L:6D (filled circles) or under 6L:18D (open circles) for at least 14 days prior to the experiment. Data are expressed as means ± SEM for four animals. Lines under the abscissa indicate periods of darkness. From Illnerová & Vaněček (1985) with the permission of the publishers.

Lewy: We have no conclusive evidence that melatonin is involved in winter depression and its treatment with bright light exposure. However, melatonin remains a highly useful marker for biological rhythms and the effects of light. Animal studies (Klein et al 1971) indicate that the eyes mediate these effects.

Illnerová: I would like to comment on your observation that morning light is more effective than evening light in patients who are phase-delayed. My contribution will deal with the importance of the evening lights-off and of the morning lights-on in the entrainment of the *N*-acetyltransferase (NAT) rhythm in the rat pineal gland, which drives the rhythm in melatonin production. Under a long photoperiod of 18 h light:6 h dark (18L:6D) the evening NAT rise occurs within 1.5 h after lights-off and the morning decline just before lights-on (Fig. 1). Under a very short photoperiod of 6L:18D the pattern of the rhythm is shifted towards the morning hours. The evening NAT rise only occurs about 7 h

after lights-off; however, the morning decline occurs about 1 h before lights-on (Fig. 1). Under artificial lighting regimens, the timing of the evening NAT rise depends on the duration of the dark period (Fig. 2). As the dark period lengthens, the interval between lights-off and the NAT rise increases steadily, from 1.4 h under 20L:4D to 7.2 h under 6L:18D. There is no such correlation

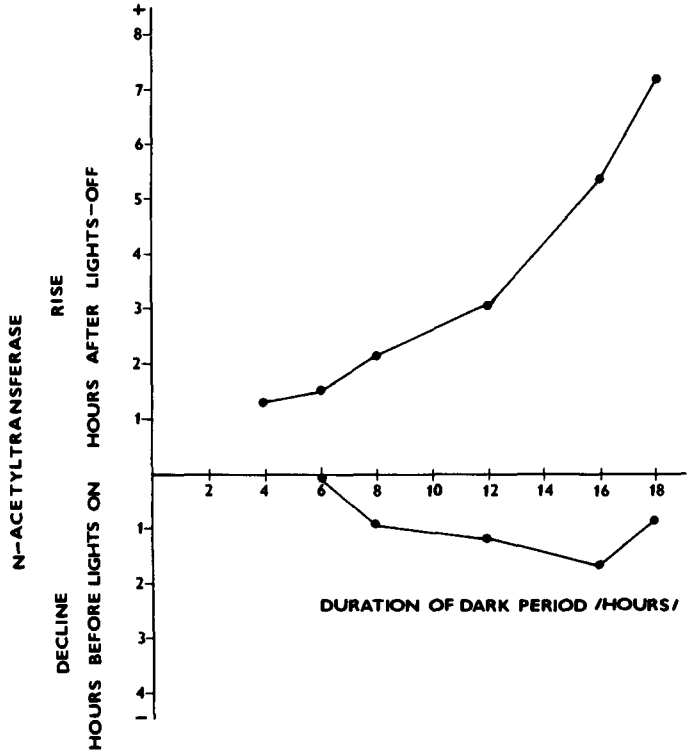

FIG. 2. (*Illnerová*) Time intervals between the evening lights-off and the rise in pineal *N*-acetyltransferase activity (upper part) and between the morning lights-on and the decline in *N*-acetyltransferase activity (lower part). Intervals after lights-off are shown by the sign +, intervals before lights-on by the sign −. The times of the rise and of the decline were arbitrarily chosen as the times when *N*-acetyltransferase activity attained the value of 3 nmol mg^{-1} during its evening rise and morning decline respectively. Intervals were read from figures in Illnerová & Vaněček (1980, 1982, 1983, 1985).

between the timing of the morning NAT decline and duration of the dark period. Apart from under the regimen 18L:6D, the interval between the decline in NAT activity and lights-on is about 1 h. Hence it seems that, at least in rats, the position of the NAT rhythm is locked to the morning lights-on and not to the evening lights-off.

The relative importance of evening and morning light in the entrainment of the NAT rhythm under long and short photoperiods may be deduced from phase–response curves (PRCs) showing shifts of the evening NAT rise and of the morning NAT decline one day after the presentation of 1 min light pulses (Fig. 3). Under 18L:6D as well as under 6L:18D, a 1 min light pulse applied

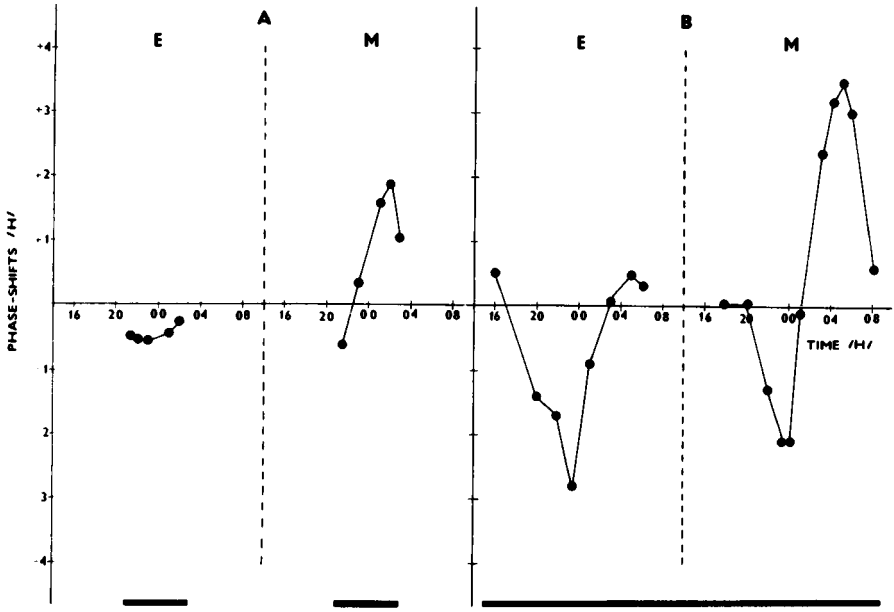

FIG. 3. (*Illnerová*) Phase–response curves representing phase-shifts of the evening *N*-acetyltransferase rise (E) and of the morning *N*-acetyltransferase decline (M) one day after application of 1 min light pulses to rats maintained for at least 14 days under 18L:6D (A) or under 6L:18D (B). Rats were either exposed to a light pulse at a particular time of night or left unpulsed; they were then released into constant darkness and the next night the evening rise in pineal *N*-acetyltransferase activity and the morning decline were determined. Values of phase-shifts were read from graphic illustrations as the time intervals between the NAT rises in pulsed and unpulsed rats, and as the time intervals between the NAT declines in pulsed and unpulsed animals. Abscissa denotes time of pulse presentation. Phase-delays are shown by the sign −, phase-advances by the sign +. From Illnerová & Vaněček (1985) with the permission of the publishers.

within 1 h before the expected lights-on phase-advances the morning NAT decline the next morning. A 1 min light pulse applied within 1 h after lights-off phase-delays the evening NAT rise only in rats maintained under 18L:6D and not in those maintained under 6L:18D. Hence, at the end of the dark period, the pacemaker controlling the NAT rhythm may still be reset by light under long as well as under short photoperiods, while at the beginning of the dark period the pacemaker is sensitive to light only under long photoperiods. From

these observations it may be inferred that under long photoperiods the NAT rhythm may be entrained by the evening as well as by the morning light, while under short photoperiods the rhythm may be synchronized only by the morning light. PRCs representing phase-shifts of the evening NAT rise under both photoperiods show only phase-delays, whereas PRCs representing shifts of the NAT decline have phase-delays and notable phase-advances. The existence of different PRCs for the NAT rise and the NAT decline may be consistent with a hypothesis of a two-oscillator pacemaker controlling the NAT rhythm (Illnerová & Vaněček 1982), proposed originally by Pittendrigh & Daan (1976) for the locomotor activity rhythm in nocturnal rodents, though other explanations cannot be excluded. The NAT rise may be controlled by the evening oscillator E, the NAT decline by the morning oscillator M. Under long photoperiods, the phase-relationship between E and M may be compressed, and synchronization of the rhythm may proceed by entrainment of both E and M by lights-off and lights-on. Under short photoperiods, the rhythm may be synchronized only by lights-on through entrainment of M, and the phase relationship between E and M may be stable.

What do melatonin rhythms look like in humans under long and short photoperiods? We hoped that we would see an extension of the peak in winter, as in rats (Illnerová & Vaněček 1980), but unfortunately this did not happen (Fig. 4), perhaps because our subjects as urbanized persons spent a lot of time at home and did not experience much more bright light during summer than during winter. Nevertheless, during winter, the rhythm in the plasma melatonin concentration was phase-delayed compared with the summer pattern. The shift might be due to the phase-shift of the rest–activity cycle caused by the introduction of 'summer-time' in summer. However, a more plausible explanation for the phase-shift is the change in daylength. We may conjecture that, since in winter humans do not experience light of sufficiently strong intensity until later in the morning than in summer, the rhythm may extend more towards the morning hours, and it may be entrained only by the morning light, as in rats.

Sizonenko: We have measured plasma melatonin concentrations in immature rats and find that in long photoperiods the melatonin peak occurs about 4 h after darkness and in short photoperiods about 8 h after darkness. When we keep our animals in 12L:12D we see two peaks, one at about 4 h and one at about 8 h. Do you see the same phenomenon? And which peak do you think is the most important—the first or the second?

Illnerová: When we maintained rats under 12L:12D we observed two peaks in night-time NAT activity only in five- and eight-day-old rats and not in 15-day-old or adult rats (Vaněček & Illnerová 1985). However, with an analysis of variance test we were not able to prove the bimodality of NAT rhythms in neonatal rats statistically.

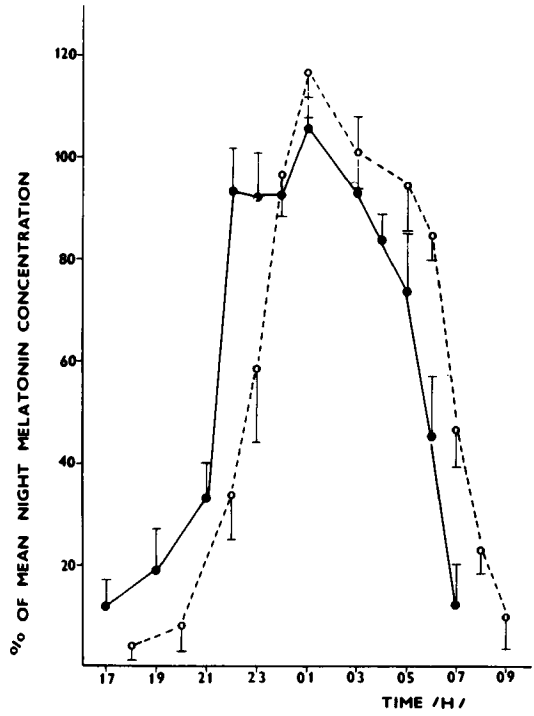

FIG. 4. (*Illnerová*) Rhythms in plasma melatonin concentrations of urbanized humans in July (filled circles) and January (open circles). Five healthy volunteers were sampled between 4 July and 5 July, and these five people plus two other volunteers were sampled between 13 January and 14 January. For each individual, the mean night-time melatonin concentration was calculated from the five highest values and was used as the 100% value. Individual patterns of melatonin rhythms were expressed as percentages of the mean night-time values. Points are means for five or seven people ± SEM. Values were calculated from individual rhythm patterns determined by Illnerová et al (1985).

Sizonenko: But my animals are 45 days old.

*Illnerová:*We have not seen two peaks at that age, but we have frequently observed fluctuations of the night-time NAT activity in adult rats.

Armstrong: Given that the suprachiasmatic nuclei (SCN) drive the pineal and that Pickard & Turek (1983) have shown that there are two oscillators in these nuclei, is it possible that NAT levels are giving us a picture of what is happening in the SCN? Is that where you think your evening and morning oscillators are?

*Illnerová:*Of course we would like to know, but it would be hard to do the appropriate lesion experiments.

Turek: One needs to be extremely cautious in trying to determine from results of this sort how many oscillators underlie a particular circadian rhythm. We know nothing about transients in this situation; we know nothing about coupling between the master pacemaker(s) in the SCN and the melatonin rhythm. I'm quite sure all the phenomena described could be predicted on the basis of a single pacemaker and certain coupling properties between the pacemaker and the driven rhythm.

REFERENCES

Czeisler CA, Richardson GS, Zimmerman JC, Moore-Ede MC, Weitzman ED 1981 Entrainment of human circadian rhythms by light-dark cycles: a reassessment. Photochem Photobiol 34:239-247

Drager UC, Hubel DH 1978 Studies of visual function and its decay in mice with hereditary retinal degeneration. J Comp Neurol 180:85-114

Illnerová H, Vaněček J 1980 Pineal rhythms in *N*-acetyltransferase activity in rats under different artificial photoperiods and in natural daylight in the course of a year. Neuroendocrinology 31:321-326

Illnerová H, Vaněček J 1982 Two-oscillator structure of the pacemaker controlling the circadian rhythm of *N*-acetyltransferase in the rat pineal gland. J Comp Physiol 145:539-548

Illnerová H, Vaněček J 1983 The evening rise in the rat pineal *N*-acetyltransferase activity under various photoperiods. Neurosci Lett 36:279-284

Illnerová H, Vaněček J 1985 Entrainment of the circadian rhythm in rat pineal *N*-acetyltransferase activity under extremely long and short photoperiods. J Pineal Res 2:67-78

Illnerová H, Zvolský P, Vaněček J 1985 The circadian rhythm in plasma melatonin concentration of the urbanized man: the effect of summer and winter time. Brain Res 328:186-189

Klein DC, Reiter RJ, Weller JL 1971 Pineal *N*-acetyltransferase activity in blinded and anosmic male rats. Endocrinology 89:1020-1023

Lewy AJ 1983 Effects of light on melatonin secretion and the circadian system of man. In: Wehr TA, Goodwin FK (eds) Circadian rhythms in psychiatry. The Boxwood Press, Pacific Grove, California, p 203-219

Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Markey SP 1980 Light suppresses melatonin secretion in humans. Science (Wash DC) 210:1267-1269

Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Rosenthal NE 1981 Manic-depressive patients may be supersensitive to light. Lancet 1:383-384

Lewy AJ, Sack RL, Singer CM 1984 Assessment and treatment of chronobiologic disorders using plasma melatonin levels and bright light exposure: the clock-gate model and the phase response curve. Psychopharmacol Bull 20:561-565

Lewy AJ, Nurnberger JI, Wehr TA et al 1985 Supersensitivity to light—possible trait marker for manic-depressive illness. Am J Psychiatry 142:725-727

Mills JN, Morgan R, Minors DS, Waterhouse JM 1977 The free-running circadian rhythms of two schizophrenics. Chronobiologia 4:353-360

Pickard GE, Turek FW 1983 The suprachiasmatic nuclei: two circadian clocks? Brain Res 268:201-210

Pittendrigh CS, Daan S 1976 A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: a clock for all seasons. J Comp Physiol 106:333-335

Vaněček J, Illnerová H 1985 Effect of short and long photoperiods on pineal *N*-acetyltransferase rhythm and on growth of testes and brown adipose tissue in developing rats. Neuroendocrinology 41:186-191

Wetterberg L 1978 Melatonin in humans: physiological and clinical studies. J Neural Transm (Suppl) 13:289-310

Wever RA 1985 Ann NY Acad Sci, in press

Photoperiodism, melatonin and the pineal
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Melatonin and affective disorders

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Abstract. The pineal hormone melatonin has a clear 24 h rhythm with a nocturnal peak. Serum melatonin concentrations have been reported to be decreased in subgroups of patients with affective disorders. When clusters of clinical items were correlated with the maximal nocturnal melatonin levels, significant negative regressions were found for items interpreted as retardation symptoms, especially those related to emotional or conative functions. These results point to the possibility of a 'low melatonin syndrome' in depression, characterized by low nocturnal serum levels of melatonin, an abnormal dexamethasone suppression test, a disturbed 24 h rhythm in cortisol levels and a less pronounced daily and annual cyclic variation in depressive symptomatology. Healthy persons show a rebound increase of nocturnal serum melatonin levels following evening suppression by bright light. One hour of the same light exposure did not alter the nocturnal melatonin levels in patients with major depressive disorders. This indicates a possible alteration in the pineal response to environmental lighting in depressed patients. The studies reported support the hypothesis of a decreased pineal function in some types of affective disorders.

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Freud, in his paper 'Mourning and Melancholia' in 1917, stated that some subtypes of depression appeared to have somatic origins. This paper was published long before the neurotransmitters noradrenaline, acetylcholine and serotonin were discovered. Twenty years ago Schildkraut (1965) published the catecholamine hypothesis of affective disorders. According to this hypothesis and others developed over the last two decades depression is associated with decreased levels of catecholamines such as noradrenaline and dopamine. Another major hypothesis explaining the biochemistry of affective disorders concerns the indole amine pathway and proposes that depression is associated with decreases in brain serotonin activity. Both these hypotheses have been rewarding since they have provided a basis for the development of many effective antidepressant drugs.

In this paper I focus on the aetiology of depression from the biochemical point of view and present some data on melatonin in affective disorders.

The genetic model of depression

This is one of the oldest models, and is again drawing interest from the scientific community. Progress in molecular biology has helped towards the understanding of the inheritance of affective disorders. However, the genetic models have been complex and limited to diagnostic systems for depressive syndromes, without any specific biological markers to prove the presence of any one genetic subtype. Many investigators have therefore set out to search for biological markers in depression and many laboratories are investigating the biochemical genetics of the biogenic amine-metabolizing system.

It has been proposed that there are at least two biochemical subtypes of depression that could be separated by biological markers and pharmacological methods. According to this concept one subtype is associated with brain noradrenaline depletion and the other with brain serotonin depletion. The drugs to treat depression have been developed to increase the net effect of noradrenaline (e.g. imipramine) or serotonin (e.g. amitriptyline).

Biological markers of affective disorders

Several markers have been claimed to be potentially helpful as trait markers, to indicate a constitutional (genetic) vulnerability to affective disorders, or as state markers to show the severity of the disease state. Two of the most used tests for state markers are the dexamethasone suppression test and the thyroid-stimulating hormone response to thyrotropin-releasing hormone. Other biological markers used in research on depression are the urinary level of 3-methoxy-4-hydroxyphenylglycol, amine metabolite levels in cerebrospinal fluid, catechol *O*-methyltransferase activity in erythrocytes, monoamine oxidase in platelets, dopamine β-hydroxylase in plasma, choline uptake by erythrocytes and, recently, the affinity of imipramine for binding sites on platelets.

Melatonin as a possible marker in affective disorders

New diagnostic trends emphasize the importance of early biological markers for the different subtypes of depression to enable one to choose the right drug for the individual patient, or at least to predict better the response to pharmacotherapy. It is in this search for additional and/or more specific biological markers that interest in melatonin in patients with affective disorders should be focused. Studies of animal populations have provided information about biochemistry and biological effects much faster than work on humans. However, since there is no animal model for affective disorders, the hypothetical links between catecholamines, indole amines and depression must be studied in humans.

The basis for our interest in melatonin in affective disorders is an observation made by Wetterberg et al (1979). In short, one patient with depression had low levels of melatonin even during night-time. Her cortisol levels were higher during depression than during recovery, and the peak melatonin concentration was reached earlier during the disease state than during recovery.

The potential use of melatonin as a marker in depression was obvious. Not only is melatonin dependent on both the noradrenergic and serotonergic neuronal systems for its regulation, but melatonin also seems to be related to the hypothalamic–pituitary–adrenal axis, which has been shown to be affected in depressive states. Additionally, melatonin is useful in indicating the phase and the amplitude of the biological clock mechanism. The variation in melatonin concentrations over the 24 h period has allowed scientists to study the hypothesis of free-running rhythm failure in subtypes of depression and to test the phase-advance theory of affective illness introduced in 1968 by Halberg. This theory is based on an internal as well as external desynchronization of physiological functions. Temporal external desynchronization (jet lag) can occur during flights over time-zones, and rapid time-zone changes may even precipitate affective illness in predisposed persons as Weller & Jauhar (1981) showed in London in a study at Heathrow Airport.

Melatonin and seasonal variation

Seasonal variations in the incidence of depression and suicide and in the use of electroconvulsive therapy as well as other treatment modalities in affective disorders are well documented. Circannual rhythms in pineal function in animals have been reported over the last two decades, and a seasonal variation in pineal gland weights in human autopsy material has also been described (Wetterberg 1978). Seasonal variations in melatonin production measured as 24 h serum levels have also been shown (Beck-Friis et al 1984).

From these introductory remarks it seems clear that different forms of depression could be biochemically linked to a disturbance in melatonin production, secretion or function. The close relation between the pituitary, adrenal, thyroid and gonadal systems and the pineal gland, and especially melatonin, may be reflected in corresponding neuropsychoendocrine dysfunctions of clinical importance. Serum and plasma melatonin determinations may thus be of interest in relation to different disease states and diagnostic subgroups.

Reports of melatonin in depression

In 1979 three research groups independently reported lowered night-time melatonin concentrations in some depressed patients (Wetterberg et al,

Mendlewicz et al, Wirz-Justice & Arendt). The same year Lewy et al (1979) reported melatonin levels to be higher in manic patients than in controls.

To follow up early findings of a possible relationship between the pineal gland and adrenal glands, Wetterberg et al (1981) reported that low melatonin was related to high cortisol and an abnormal dexamethasone suppression test and suggested that low melatonin levels in depression allowed for production of high levels of corticotropin-releasing factor (CRF). It seemed possible that melatonin or another pineal substance acted as a CRF-inhibiting factor under physiological conditions. Several studies involving extensive neuroendocrine testing have been reported over the last five years, e.g. an investigation of melatonin and cortisol secretion by Branchey et al (1982). A summary of the pharmacoendocrine studies of growth hormone, prolactin and melatonin in patients with affective disorders has been presented by Checkley & Arendt (1984).

The current view of melatonin as a tool in the diagnosis of mental diseases in general has recently been reviewed by Wetterberg (1985). Specific findings relating serum melatonin to clinical variables in patients with depression have been reported by Wetterberg et al (1984) and by Beck-Friis et al (1984, 1985a,b). The conclusions are based on the examination of 87 people, including acutely ill patients with major depression according to the research diagnostic criteria of Spitzer et al (1978) and patients in clinical remission as well as healthy controls. The melatonin measurements were made on 10 blood samples taken over 24 h with two-hour intervals between samples from 2000 h to 0800 h.

Melatonin determination by radioimmunoassay

The melatonin samples were analysed by a radioimmunoassay that is commercially available (KALAB, P.O. Box 634, Danville, CA 94526, USA). The lower limit of detection was 0.01 nmol/l. The interassay coefficient of variation was 4.8% for melatonin levels > 0.15 nmol/l ($n = 60$). The interassay variation for samples containing > 0.10 nmol/l was 12.3%, for samples with > 0.15 nmol/l 7.4% and four samples with < 0.10 nmol/l 28%, calculated from 10 independent 24 h profiles (i.e. 100 serum samples).

Several factors influence melatonin levels in serum

It is clear that factors other than psychiatric diagnoses are of importance for melatonin levels. It has been reported that light (Wetterberg 1978, Lewy et al 1980), age (Touitou et al 1981, Iguchi et al 1982), body weight (Arendt

et al 1982, Ferrier et al 1982), body height (Beck-Friis et al 1984), the use of glasses (Erikson et al 1983), drugs (Hanssen et al 1977) and genetic variation (Wetterberg et al 1983) are among factors which in different degrees and under different circumstances may influence melatonin levels in humans. Even when all these factors are taken into account, there are still some reports indicating a correlation between some subtypes of depression and melatonin concentrations.

A low melatonin syndrome in depression

For the sake of simplicity the results of Beck-Friis et al (1984, 1985a,b) are discussed here. For details the reader is referred to the original papers. The findings from our group point to the possibility of a special clinical syndrome termed the 'low melatonin syndrome'. In a subgroup of depressed patients the following features constitute the syndrome: low nocturnal melatonin levels, an abnormal dexamethasone suppression test, a disturbed 24 h rhythm in cortisol concentrations and a less pronounced daily and annual cyclic variation in depressive symptomatology. The syndrome may furthermore be characterized by conative and emotional retardation symptoms, i.e. sadness, lassitude and inability to feel emotions. On the functional level the syndrome may be interpreted by a loss of rhythm amplitude, which could be a sign of dysfunction between the pineal gland and the suprachiasmatic nuclei in the hypothalamus.

Melatonin levels could serve as a marker for noradrenergic tone in the brain. If low melatonin concentrations reflect a deficiency of noradrenaline at receptor sites, the possibility that the low melatonin syndrome might respond to noradrenergic antidepressive pharmacotherapy should be tested. It is clear that a dysfunction in the serotonergic system could theoretically also lead to a low melatonin syndrome.

From a clinical point of view it is interesting that patients with low melatonin syndrome have longer depressive periods than patients with higher melatonin levels at night, which may indicate that those in the low melatonin group receive less adequate therapy.

The hypothalamic–pituitary–adrenal axis and melatonin

A link between the pineal gland and the hypothalamic–pituitary–adrenal axis has been discussed in several reports. A reciprocal relationship may exist between the pineal and the adrenal glands, at least at certain times of day. The low melatonin levels seen in the low melatonin syndrome are also present

during remission, so such melatonin concentrations seem to be a trait marker for vulnerability to depression. We have hypothesized that low melatonin could be a permissive factor for lower CRF-inhibiting activity during the depressive state, causing an increased release of CRF. Such an increase of CRF might cause a down-regulation of the receptors responsible for production of adrenocorticotropic hormone (ACTH) at the pituitary level. In our patients with low melatonin syndrome, morning levels of ACTH before the dexamethasone suppression test were not increased (Beck-Friis et al 1985a).

Studies from other laboratories support the notion that there is a subgroup of depressed patients with low melatonin secretion during the night. Claustrat et al (1984) studied 11 patients of whom 10 were female. Eight male controls were also studied over a 24 h period. The depressed patients showed a significant melatonin rhythm but with lower amplitude and lower mean values than the controls. The melatonin rhythm was not significantly phase-advanced compared to the controls. In nine of the 11 patients nocturnal melatonin secretion was less marked and frequently associated with high cortisol levels in plasma.

6-Sulphatoxy melatonin in depression

Boyce (1985) from Australia studied about eight persons, of whom seven were female, who had been admitted to hospital and fulfilled the criteria for melancholia according to DSM-III. Three of the patients lacked a marked nocturnal increase in the melatonin metabolite 6-sulphatoxy melatonin. The data of Boyce may be interpreted as supporting the previous results since it is likely that the 6-sulphatoxy metabolite in urine reflects melatonin production in the pineal gland to a major extent.

Cluster headache and melatonin

Patients with cluster headache are in many respects similar to patients with affective disorders, with characteristic diurnal and seasonal clusters of disease symptoms. The circadian secretion of melatonin was thus investigated in 24 patients with cluster headache and nine healthy controls (Waldenlind et al 1984). Eight of the patients were examined twice, both in active cluster period and in clinical remission. The nocturnal secretion of melatonin in humans is abolished by β-adrenoceptor blocking drugs, indicating a sympathetic innervation of the pineal gland. The finding of lower serum melatonin concentrations during cluster periods may indicate a change in vegetative tone in a 'hypo-sympathetic' direction, as may the presence of the vegetative symptoms associated with cluster headache attacks. These results, together with

the cyclic nature of the cluster headache periods and attacks, are in agreement with a hypothesis of hypothalamic dysfunction in cluster headache. A continued comparison between depression and cluster headache in relation to melatonin regulation seems warranted.

Rebound in nocturnal melatonin release after exposure to light is lacking in depression

Beck-Friis et al (1985c) reported that light exposure in the evening lowered the nocturnal rise of melatonin concentrations in healthy controls. Thirty minutes to one hour of light was sufficient to suppress the melatonin increase. Furthermore, some patients with major depression did not show the rebound in nocturnal melatonin release that was reported to occur in controls after such evening suppression by bright light. This finding may be interpreted as a deficiency in building up transmitter substance in depressed patients or at least as a failure to sensitize the receptor mechanism responsible for the rebound increase in the controls. If these preliminary results are confirmed, exposure of depressed patients during an acute episode to a half-hour period of light may help to test their central nervous system receptor sensitivity. Such a test may prove rewarding in predicting the response to treatment, whether this is psychopharmacological therapy, electroconvulsive therapy, light or psychotherapy.

In summary, the studies reported indicate that there may be a subgroup of patients with affective disorders who have decreased pineal function measurable as low nocturnal melatonin levels in serum.

REFERENCES

Arendt K, Hampton S, English J, Kwasowski P, Marks V 1982 24-hour profiles of melatonin, cortisol, insulin, C-peptide and gip following a meal and subsequent fasting. Clin Endocrinol 16:89-95

Beck-Friis J, von Rosen D, Kjellman BF, Ljunggren JG, Wetterberg L 1984 Melatonin in relation to body measures, sex, age, season and the use of drugs in patients with major affective disorders and healthy subjects. Psychoneuroendocrinology 9:261-277

Beck-Friis J, Ljunggren JG, Thorén M, von Rosen D, Kjellman BF, Wetterberg L 1985a Melatonin, cortisol and ACTH in patients with major depressive disorder and healthy humans with special reference to the outcome of the dexamethasone suppression test. Psychoneuroendocrinology 10:173–186

Beck-Friis J, Kjellman BF, Aperia B, Undén F, von Rosen D, Ljunggren JG, Wetterberg L 1985b Serum melatonin in relation to clinical variables in patients with major depressive disorders and a hypothesis of a low melatonin syndrome. Acta Psychiatr Scand 71:319-330

Beck-Friis J, Borg G, Wetterberg L 1985c Rebound increase of nocturnal serum melatonin levels following evening suppression by bright light exposure in healthy man: relation to cortisol levels and morning exposure. Conference on medical and biological effects of light, Oct 31–Nov 2 1984. The New York Academy of Sciences, in press

Boyce PM 1985 6-Sulphatoxy melatonin in melancholia. Am J Psychiatry 142:125-127

Branchey L, Weinberg U, Branchey M, Linkowski P, Mendlewicz J 1982 Simultaneous study of 24-hour patterns of melatonin and cortisol secretion in depressed patients. Neuropsychobiology 8:225-232

Checkley S, Arendt J 1984 Pharmacoendocrine studies of GH, PRL, and melatonin in patients with affective illness. In: Brown GM et al (eds) Neuroendocrinology and psychiatric disorder. Raven Press, New York, p 165-190

Claustrat B, Chazot G, Brun J, Jordan D, Sassolas G 1984 A chronobiological study of melatonin and cortisol secretion in depressed subjects; plasma melatonin, a biochemical marker in major depression. Biol Psychiatry 142:125-127

Erikson C, Küller R, Wetterberg L 1983 Non visual effects of light. Neuroendocrinol Lett 5:412

Ferrier IN, Arendt J, Johnstone EC, Crow TJ 1982 Reduced nocturnal melatonin secretion in chronic schizophrenia: relationship to body weight. Clin Endocrinol 17:181-187

Halberg F 1968 Physiologic considerations underlying rhythmometry with special reference to emotional illness. In: de Ajusiaguerra J (ed) Cycles biologiques et psychiatrie. Masion et Cie, Paris, p 73

Hanssen T, Heyden T, Sundberg I, Wetterberg L 1977 Effect of propranolol on serum melatonin. Lancet 2:309

Iguchi H, Kato KI, Ibayashi H 1982 Age-dependent reduction in serum melatonin concentrations in healthy human subjects. J Clin Endocrinol & Metab 55:27-29

Lewy AJ, Wehr TA, Gold PW, Goodwin FK 1979 Plasma melatonin in manic-depressive illness. In: Usdin E et al (eds) Catecholamines: basic and clinical frontiers. Pergamon Press, Oxford, p 1173-1175

Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Markey SP 1980 Light suppresses melatonin secretion in humans. Science (Wash DC) 210:1267-1269

Mendlewicz J, Linkowski P, Branchey L, Weinberg U, Weitzman ED, Branchey M 1979 Abnormal 24 hour pattern of melatonin secretion in depression. Lancet 2:1362

Schildkraut JJ 1965 The catecholamine hypothesis of affective disorders: a review of supporting evidence. Am J Psychiatry 122:509-522

Spitzer RL, Endicott J, Robins E 1978 Research diagnostic criteria. Rationale and reliability. Arch Gen Psychiatry 35:773-782

Touitou Y, Fèvre M, Lagoguey M et al 1981 Age- and mental health-related circadian rhythms of plasma levels of melatonin, prolactin, luteinizing hormone and follicle-stimulating hormone in man. J Endocrinol 91:467-475

Waldenlind E, Ekbom K, Friberg Y, Sääf J, Wetterberg L 1984 Decreased nocturnal serum melatonin levels during active cluster headache periods. Opusc Med 29:109-112

Weller MPJ, Jauhar P 1981 Travel induces disturbances in circadian rhythms as precipitants of affective illness. In: Perris C et al (eds) Biological psychiatry. Elsevier/North-Holland Biomedical Press, Amsterdam, p 1253-1256

Wetterberg L 1978 Melatonin in humans. Physiological and clinical studies. J Neural Transm (suppl) 13: 289-310

Wetterberg L 1985 The human pineal gland: melatonin as a tool in the diagnostics of endocrine and mental diseases. In: Mess B et al (eds) The pineal gland. Current state of pineal research. Akadémiai Kiadó, Budapest, p 329-339

Wetterberg L, Beck-Friis J, Aperia B, Petterson U 1979 Melatonin/cortisol ratio in depression. Lancet 2:1361

Wetterberg L, Aperia B, Beck-Friis J et al 1981 Pineal-hypothalamic-pituitary function in patients with depressive illness. In: Fuxe K et al (eds) Steroid hormone regulation of the brain. Pergamon Press, Oxford, p 397-403

Wetterberg L, Iselius L, Lindsten J 1983 Genetic regulation of melatonin excretion in urine. A preliminary report. Clin Genet 24:403-406

Wetterberg L, Beck-Friis J, Kjellman BF, Ljunggren JG 1984 Circadian rhythms in melatonin and cortisol secretion in depression. In: Usdin E et al (eds) Frontiers in biochemical and pharmacological research in depression. Raven Press, New York, p 197-205

Wirz-Justice A, Arendt J 1979 Diurnal, menstrual cycle, and seasonal indole rhythms in man and their modification in affective disorders. In: Obiols J et al (eds) Biological psychiatry today. Elsevier/North-Holland, Biomedical Press, Amsterdam, p 294-302

DISCUSSION

Turek: You say that some depressed patients have low melatonin compared with normal controls, but there is probably a subgroup of normal humans with low melatonin, and it may be more appropriate to compare your subgroup of depressed patients with these normal people who have a similar melatonin pattern.

Wetterberg: Yes. We have reported on 87 subjects, and in the control group of 33 healthy individuals we find some with serum melatonin concentrations as low as those found in depressed patients. If low melatonin was a trait marker for all types of depression you would expect to find a frequency as high as 25% in the general population. From a clinical point of view, we would like to test the hypothesis that low melatonin may be a trait marker for depression. If the finding is confirmed, it may help, particularly in families with depression, to prevent the onset of depression and perhaps give useful information about treatment.

Turek: If the percentage of depressed patients with low melatonin is the same as the percentage of people in a normal population who have low melatonin, what use is melatonin as a marker?

Wetterberg: Low melatonin may be a trait marker for a subtype of depression. Not all depressed patients, however, show the same biochemical profile. I would predict, for example, that patients with 'winter depression' would not be dexamethasone positive and would have normal or even high melatonin levels. In the general population, low melatonin may be a marker for people belonging to specific families of a subtype at risk for depression. Since the patients may require specific therapy, their recognition may be of clinical relevance.

Menaker: But if you have people with low melatonin and normal affect and people with low melatonin and depression, what is the evidence that low melatonin *is* a trait marker for depression?

Wetterberg: We are testing the hypothesis that low melatonin is a trait marker for one subtype of depression. In this syndrome diurnal and seasonal variation

are less pronounced than in patients with high melatonin levels. The disease periods are also longer. This may tell us that patients with low melatonin are less effectively treated. Several different symptoms differentiate these patients from others with depression, and that is why we hypothesize that low melatonin may be a trait marker for one subgroup.

Lewy: Can you identify patients with low melatonin levels on the basis of their clinical histories?

Wetterberg: No. Confirmatory studies are needed. We will now study parents and children in families with depression and measure melatonin profiles to test the low melatonin hypothesis further.

Short: If there are a very small number of genes regulating melatonin synthesis we would expect there to be enormous polymorphism in the population with respect to melatonin profiles. Until we have done some endocrine epidemiology to define the extent of this pleomorphism in the normal population, it's going to be difficult to determine the precise significance of the few individuals with affective disorders who have upset melatonin patterns.

Lewy: Although many of us agree that some depressed patients have low melatonin levels, there is disagreement about whether this feature is a state marker or a trait marker. I have only studied manic-depressive patients, but I think that melatonin is probably a state marker. Melatonin levels may be simply mirroring adrenergic tone, which may be increased in mania and decreased in depression (Lewy 1983). But you feel that melatonin is a trait marker because when your patients get well, their melatonin levels remain low.

Wetterberg: Yes. We have examined both patients and healthy controls at up to five time points in the year and in different clinical states. The pattern and levels of melatonin are stable for any particular patient from one time point to the next. This is in contrast to cluster headache and alcoholism in which diagnostic melatonin levels differ between clinical states, possibly reflecting a difference in noradrenergic receptor function at different times.

Armstrong: Is it theoretically possible to have a normal serum melatonin curve but to have tissue resistance to melatonin so that as far as the brain is concerned there is no melatonin there?

Klein: Of course.

Lewy: But if low melatonin levels are just a marker for depression, depressed patients are not necessarily going to respond to exogenous melatonin.

Illnerová: Although in some depressed patients you see low melatonin concentrations, this may not be the most important thing because you can also get low melatonin in normal people. The important difference is the 'rebound' phenomenon that you have shown in healthy controls but not in depressed patients, i.e. the increase in the peak melatonin concentration after exposure to light at night. In how many people have you seen this rebound phenomenon and is it never seen in depressed patients? Is there a difference in the rate of rise

of the melatonin concentration after exposure to light or is the difference only in the height of the peak?

Wetterberg: Only six controls and six patients have been studied so far, so we don't yet have enough results to answer your question.

Klein: You have reported that in long winter nights in Sweden the period of melatonin secretion is prolonged by one hour (Beck-Friis et al 1984), but Helena Illnerová has seen only a *shift* of a little more than one hour in Czechoslovakia. Can this difference be explained simply on the basis of distance from the poles? Are nights that much longer in Sweden?

Wetterberg: Yes, in winter the dark periods are significantly longer and in summer they are shorter in Sweden than in Czechoslovakia. We have recently introduced 'summer time' in Sweden, which means that we get up one hour earlier during the summer months. We do not have data from the period before 'summer time' was introduced in Sweden.

Lewy: We prefer to keep people in dim light in the evening when we want to determine the onset of melatonin production (Lewy et al 1984). If you are interested in the seasonal pattern, you should do your studies under natural conditions, but if you want to use melatonin as a marker for circadian phase position (to tell whether a person is phase-advanced or phase-delayed), you should standardize dusk throughout the year, because late evening light in the summer may suppress the rise in melatonin. This may be happening in summer in Lennart's study in Sweden.

Illnerová: It's really a question of how much strong light these people experience.

Tamarkin: In your studies of kindreds, where the genetic trait of low melatonin is exhibited in healthy subjects, have you found a perturbation of dexamethasone suppression?

Wetterberg: We haven't looked at that yet.

Tamarkin: Is it possible that some sort of link exists between the response to the provocative dexamethasone suppression test and low melatonin levels?

Wetterberg: The pineal may affect the pituitary directly or at the hypothalamic level via the control of corticotropin-releasing factor (CRF), which influences ACTH and cortisol levels. We hypothesize that the activity of a CRF-inhibiting factor (CRF-IF) is closely related to melatonin levels. We find that in depression ACTH levels are low, although there is presumably an increase in CRF, and this may be a result of down-regulation of CRF receptors. We do not know if melatonin itself or some closely related substance may function as CRF-IF.

Short: If you think that melatonin is really a trait marker, do you see any advantages to be gained from therapeutic administration of melatonin?

Wetterberg: If we assume that there are at least two subgroups of depressed patients—those with low melatonin and those with high melatonin— adminis-

tration of melatonin may help one group but not the other. This is another reason why we want to identify which patients have low and which have high melatonin levels. Of course the dose of melatonin, the time of day melatonin is administered, the route of administration etc. may also affect patients differently in the two groups. Clinical trials taking these and other variables into consideration are needed to answer your question.

Short: Has anyone given large doses of tryptophan by mouth to see whether it alters melatonin production?

Klein: We have done this in sheep. They are very resistant to tryptophan but you can produce an enormous increase in melatonin levels if you give them hydroxytryptophan. We want to determine if this response could be used as a clinical test to assess the capacity of patients to convert hydroxytryptophan to melatonin during the day. Our results have not been analysed yet, but if this works in humans it might be a useful way of screening patients.

Reiter: We gave high tryptophan loads to rats and saw markedly increased pineal serotonin levels and *N*-acetyltransferase activity, but no appreciable increase in the pineal melatonin content. Unfortunately we did not look at the activity of hydroxyindole *O*-methyltransferase.

Klein: Were blood melatonin levels affected?

Reiter: We didn't measure them.

Klein: We measured melatonin in the pineal and in the blood of sheep and found no changes after high doses of tryptophan (Namboodiri et al 1983).

Rollag: We injected tryptophan (up to 10 mg/kg) into hamsters at different times of day and found a modest increase of about 50% in the pineal melatonin content (M.D. Rollag & M.H. Stetson, unpublished work). The increase was certainly no more than twofold and never as great as 10-fold or 100-fold. This is consistent with earlier studies in rats (Quay 1963, Snyder et al 1967, Young & Anderson 1982).

Illnerová: When Snyder et al (1967) administered tryptophan to rats at different times of day and killed them 1 h later, the pineal serotonin content of the animals increased to the same steady-state level by day as at night.

Arendt: Is there any evidence that the quantities of tryptophan we get in the diet influence pineal serotonin and melatonin synthesis?

Rollag: In that injections of 10 mg/kg, a massive amount of tryptophan, do not markedly change pineal melatonin content, experimental evidence to date suggests that dietary tryptophan does not play an important regulatory role in pineal melatonin synthesis.

Herbert: We are always told that the pineal is outside the blood–brain barrier. This is true for the rat but is probably not true for the primate. Tryptophan is taken up in the brain by a carrier-mediated system and you might therefore expect marked differences between rats and humans in tryptophan access to the pineal.

REFERENCES

Beck-Friis J, von Rosen D, Kjellman BF, Ljunggren JG, Wetterberg L 1984 Melatonin in relation to body measures, sex, age, season and the use of drugs in patients with major affective disorders and healthy subjects. Psychoneuroendocrinology 9:261-277

Lewy AJ 1983 Biochemistry and regulation of mammalian melatonin production. In: Relkin RM (ed) The pineal gland. Elsevier North-Holland, New York, p 77-128

Lewy AJ, Sack RL, Singer CM 1984 Assessment and treatment of chronobiologic disorders using plasma melatonin levels and bright light exposure: the clock-gate model and the phase response curve. Psychopharmacol Bull 20:561-565

Namboodiri MAA, Sugden K, Klein DC, Mefford IN 1983 5-Hydroxytryptophan elevates serum melatonin. Science (Wash DC) 221:659-661

Quay WB 1963 Effect of dietary phenylalanine and tryptophan on pineal and hypothalamic serotonin levels. Proc Soc Exp Biol Med 114:718-721

Snyder SH, Axelrod J, Zweig M 1967 Circadian rhythm in the serotonin content of the rat pineal gland: regulating factors. J Pharmacol Exp Ther 158:206-213

Young SN, Anderson GM 1982 Factors influencing melatonin, 5-hydroxytryptophol, 5-hydroxyindoleacetic acid, 5-hydroxytryptamine and tryptophan in rat pineal glands. Neuroendocrinology 35:464-468

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Some effects of melatonin and the control of its secretion in humans

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Abstract. Whether or not the pineal gland has a significant physiological role in humans is not known. There has nevertheless been speculation about the potential therapeutic use of melatonin (in view of its hypnotic and possible zeitgeber properties) in conditions such as insomnia and jet lag, and in shift-workers. Our work concerns the effects of melatonin administration in humans and the interactions between melatonin and other circadian variables.

Chronic (one month), timed (1700 h), low-dose (2 mg daily) melatonin administration to normal subjects without environmental control consistently increased evening fatigue and slightly modified the 24 h prolactin rhythm without effect on cortisol, growth hormone, luteinizing hormone, thyroxine, testosterone or self-rated mood. In five out of 11 subjects the endogenous melatonin rhythm was advanced by one to three hours. During fractional desynchronization of circadian rhythms by increasing imposed 'day' length (26–29 h, 24 days, 500 lux), 5 mg melatonin *per os* at lights-out in two subjects resulted in better entrainment of the fatigue rhythm to the zeitgeber than in five out of six control subjects, without major consistent effects on other measured circadian variables.

Using a new radioimmunoassay for 6-hydroxymelatonin sulphate (aMT6s), the major melatonin metabolite, we have shown that the urinary aMT6s rhythm is closely correlated to that of melatonin in plasma and is completely suppressed by an acute dose of atenolol (100 mg *per os*), a peripheral β-adrenergic antagonist. During fractional desynchronization by increasing imposed 'day' length in one subject and decreasing imposed 'day' length in two subjects, the urinary aMT6s rhythm behaved similarly to that of core temperature.

The results suggest that fatigue (or alertness) may be entrained by melatonin, but whether critical performance rhythms can be suitably manipulated remains to be clarified. It is likely that melatonin production is linked to the so-called 'strong' circadian oscillator.

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Melatonin has clear and dramatic effects in photoperiodic seasonal breeders: if suitably administered it is equipotent with light in driving the seasonal repro-

ductive cycle and other seasonal functions (E. L. Bittman, G. A. Lincoln et al, L. Martinet & D. Allain, this volume, Kennaway et al 1982, Arendt et al 1983). The pineal gland itself is an essential component of the response to changing photoperiod (Reiter 1980, Bittman et al 1983, M. H. Hastings et al, this volume).

Human photoperiodism has not aroused much interest until recently. Seasonal rhythms have been demonstrated in a variety of factors including birth-rate, the incidence of depression and other diseases, hormone concentrations in plasma and the duration of sleep. Whether or not these rhythms depend on the length of exposure to natural light is an open question. Recently Rosenthal et al (1984) have described the occurrence of Seasonal Affective Disease or winter depression, its treatment by application of very bright light of a skeleton spring photoperiod and possibly the partial dependence of remission on suppression of plasma melatonin. These experiments suggest that some populations are acutely sensitive to the length of the natural photoperiod and that melatonin is an important factor in their response.

In animals photoperiodic responses are accompanied by changes in circadian rhythms (e.g. Symons et al 1983). In rats melatonin will entrain the rest–activity cycle (Redman et al 1983). This and other evidence suggests that the pineal is involved in circadian rhythm generation or entrainment in many mammals, possibly including humans.

Importance of the pineal and melatonin in human physiology

There is no good evidence for a major physiological role of the human pineal gland.

Most information concerning the pineal in humans is derived from measurement of physiological and pathological variations in the concentrations of melatonin and its principal metabolites. Peripheral melatonin is largely derived from the pineal (see Arendt 1985 for references) and it is considered to be a good index of pineal function.

In our opinion this has not led to any major insights, although the possible changes in melatonin secretion during puberty, in old age and in treated and untreated affective disease (see Arendt 1985 for references) may well be of importance. It is very pertinent that lack of melatonin, at least in adults, is not necessarily associated with any major abnormality. For example, we have observed that three of our normal volunteers have very low plasma concentrations of melatonin (< 10 pg/ml) during both the day and the night with no lack of well-being, with the possible exception of sub-clinical sleep disturbance in one case. Cortisol, growth hormone (GH), prolactin (PRL), luteinizing hormone (LH), thyroxine (T4) and testosterone were measured in this last subject and in one of the other two, and showed no apparent

abnormality. It has been claimed that melatonin and cortisol secretion are closely linked; however, the rhythms are readily dissociated (e.g. Fèvre-Montange et al 1981). In diabetic autonomic neuropathy no night-time rise of melatonin is found but cortisol levels and rhythmicity appear to be entirely normal (O'Brien et al 1984). It may well be, of course, that melatonin is reaching target areas (hypothalamus?) in appropriate amounts in such subjects, and peripheral observations are not pertinent.

Notable in some photoperiodic seasonal breeders (sheep) is the rather small effect of pinealectomy compared with the dramatic effects of melatonin administration. An innervated and intact pineal is essential for sheep to respond to changing artificial photoperiod (Bittman et al 1983), but pinealectomy does not abolish synchronized seasonal cycling (Lincoln & Forbes 1984). Melatonin administration to pinealectomized ewes and ganglionectomized rams on the other hand will drive the seasonal reproductive cycle irrespective of the ambient photoperiod. Administration of melatonin to human volunteers may well be more informative than observations of variations in endogenous melatonin concentrations. Melatonin may not be necessary in normal physiology; however, it may be sufficient to manipulate neuroendocrine function.

There has been speculation about the potential therapeutic use of melatonin, in view of its hypnotic (Cramer et al 1974) and possible zeitgeber properties (Redman et al 1983), in conditions such as insomnia, jet lag and depression, and in shift-workers. Before melatonin can be used clinically it is essential to ensure that it has no undesirable side-effects during long-term administration. Its close association with reproductive function in seasonal breeders indicates that possible effects on sex hormones, in particular, must be carefully investigated. Sugden (1980) has shown a very low toxicity of melatonin, and numerous studies have reported little effect of high-dose melatonin during the day, with the exception of exacerbation of depression (Carman et al 1976) and sleepiness (Lerner & Nordlund 1978).

Although others have studied the effects of melatonin in humans by giving very large oral doses (Lerner & Nordlund, 1978) or smaller amounts intranasally (Vollrath et al 1981) or intravenously (Lerner & Nordlund 1978), no one has previously considered the effects of chronic, timed feeding of low doses of melatonin. This was clearly a necessary approach in humans in that all the major physiological effects of melatonin are observed after either chronic timed administration or implantation.

Administration of melatonin to healthy volunteers without environmental control

In two placebo double-blind cross-over studies subjects took either 2 mg melatonin (as an 0.04% solution in corn oil containing 2% ethanol) in 50 ml milk,

or placebo (vehicle only) at 1700 h daily for one month (spring) or three weeks (autumn). In spring 12 subjects (10 M, 2 F) took part and in autumn 11 subjects, identical to the spring cohort but minus one man, were studied. In spring, blood samples were taken hourly for 24 h on the day after the last dose of melatonin or placebo. In autumn, a one week washout period was interposed between each section of the experiment, and blood samples were taken hourly for 24 h on the day of the last dose. Mood and fatigue were self-rated on 10 cm visual analogue scales four times daily (0800, 1200, 1900, 2200 h) in spring and five times daily in autumn (0800, 1200, 1900, 2030, 2200 h) and daily sleep logs were kept [time of sleep onset and offset, sleep latency (minutes), night awakenings and sleep quality (10 cm visual analogue scale)]. Plasma samples from the spring experiment were analysed for melatonin, cortisol, GH, PRL, LH, testosterone and T4. Melatonin, cortisol and PRL were assayed in the autumn study. A preliminary report of this study has appeared (Arendt et al 1984).

Measurement of melatonin in plasma from spring and autumn experiments permitted an assessment of 24 h profiles in the presence and absence of exogenous hormone (Fig. 1). In the presence of exogenous melatonin in autumn, five of the 11 volunteers showed a clear 1–3 h phase-advance in the rhythm of endogenous melatonin. This was not related to the order of administration of melatonin and placebo and is highly unlikely to have been an artifact produced by secondary absorption peaks, as shown by our previous pharmacokinetic experiments. In the remaining subjects profiles for endogenous melatonin were either indistinguishable in the presence and absence of exogenous melatonin, or possibly absent. In one woman there was a delayed clearance of melatonin; she had previously also shown a delayed clearance of synthetic steroids. In spring only two out of 12 subjects showed a phase-advance in the rhythm of endogenous melatonin one day after the last dose of exogenous melatonin. In all other cases the profiles after melatonin or placebo were indistinguishable. Although in the group as a whole no significant phase-shifts were seen on either occasion, these observations nevertheless suggest that, at least in some individuals, melatonin is able to modify the timing of its own secretion, possibly by a direct effect on central rhythm-generating mechanisms.

In both autumn and spring there was a highly significant increase in evening fatigue and in some cases actual evening sleep during melatonin feeding (Fig. 2). The onset of the effect was slow, requiring five days for a significant effect in the group as a whole. This would be consistent with an effect on the timing mechanisms of fatigue (or its converse, alertness) rather than a pharmacological hypnotic effect, although the latter cannot be excluded. An actual phase-shift in the fatigue rhythm was not demonstrable because no significant difference was found in the 0800 h readings, although there was

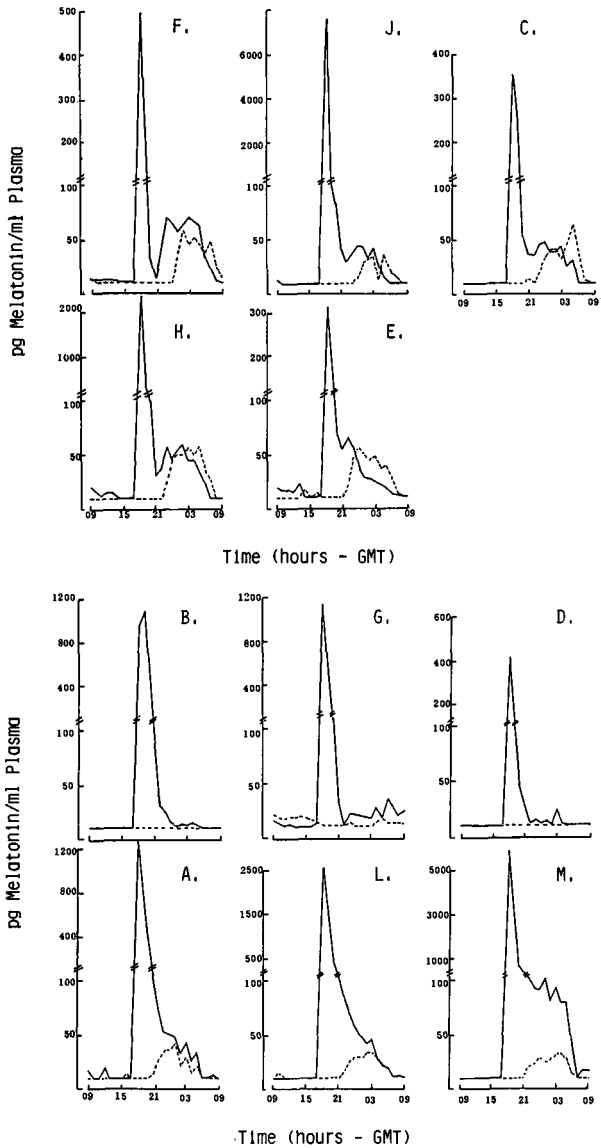


FIG. 1. Twenty-four-hour profiles of plasma melatonin concentrations in 11 subjects after either three weeks taking 2 mg melatonin daily (—) or three weeks of placebo treatment (-----) in autumn. Note that (i) in five subjects (C,E,F,H,J) there is a clear phase-advance of the endogenous melatonin peak; (ii) individual peak levels of exogenous melatonin vary from 320 to 7500 pg/ml; (iii) three subjects have very low or undetectable endogenous melatonin (B,D,G); (iv) two subjects (A,L) show no change in the endogenous rhythm; (v) one subject (M) shows impaired clearance of melatonin; (vi) in general the shape of the 24 h profile for endogenous melatonin is similar whether or not exogenous melatonin has been taken; (vii) there is a change of scale for the exogenous melatonin peak.

a tendency for volunteers to awake early ($P < 0.1$) when taking melatonin. In a minority of individuals (4/12) a marked intermittence of evening fatigue was noted, similar to the 'relative coordination' effects of weak zeitgebers described by Wever (1979). No other variables of sleep or mood showed significant changes in volunteers taking melatonin.

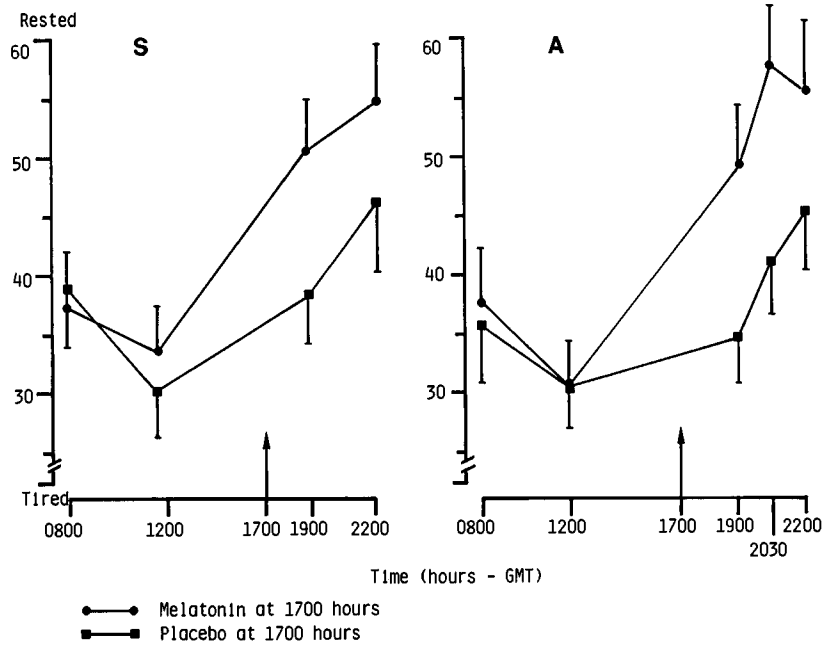


FIG. 2. Fatigue ratings at different times of day in volunteers taking 2 mg melatonin (●—●) or placebo (■—■) daily at 1700 h (arrows) for one month in spring (S, 12 subjects, 10M, 2F) or autumn (A, 11 subjects, 9M, 2F). Fatigue ratings are mean values for one month; bars show SE. Analysis of variance indicated a highly significant increase in early evening fatigue ($P < 0.02$) in volunteers taking melatonin.

The work of N. E. Rosenthal et al on Seasonal Affective Disease, or winter depression, in which light-induced remission is partially reversed by melatonin administration (personal communication), suggests that a raised plasma concentration of melatonin in early evening is 'depressogenic'. Such was not the case in our study and it seems likely that different populations are susceptible to raised melatonin levels to different extents.

Melatonin had no effect on the levels or 24 h rhythm of GH, LH, testosterone or T4 (measured in spring only). In both spring and autumn a slightly early (1 h) decline in the night-time PRL rhythm was seen, but the cortisol rhythm and concentration were unchanged.

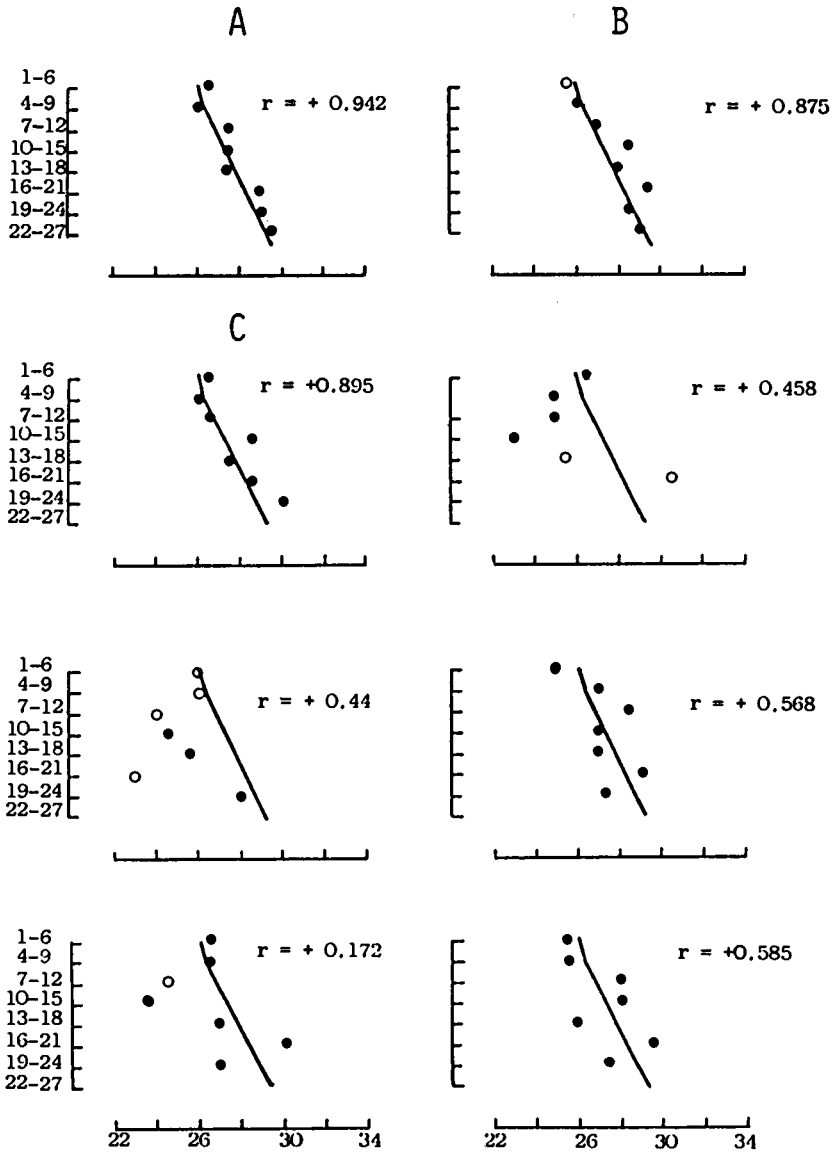
Thus, chronic low-dose melatonin treatment is well tolerated, will modify the timing of fatigue and has no undesirable effects on other major endocrine variables. These experiments reinforced our opinion that melatonin may be of use therapeutically, if only for its effects on fatigue.

Administration of melatonin to healthy volunteers in environmental isolation

To determine whether melatonin has important zeitgeber properties in human circadian systems it is necessary to perform experiments in environmental isolation, with extensive monitoring and analytical facilities such as are available at the Max-Planck Institut, Andechs, West Germany.

We have used the fractional desynchronization protocol described by Wever (1979), whereby in environmental isolation the light–dark zeitgeber period is extended from 26 h to 29 h by 10 min per day. In these conditions, with 500 lux, circadian components linked to the so-called 'strong' oscillator, such as core body temperature and cortisol, lose entrainment to the zeitgeber at a zeitgeber period length of around 27 h, whereas the sleep–wake cycle (linked to the 'weak' oscillator) remains entrained. If a light–dark zeitgeber with bright light (2000–3000 lux) is applied, most measured components remain entrained up to 29 h (Wever et al 1983). This intensity of light exceeds the threshold for suppressing human melatonin at night (Lewy et al 1980), so it was reasonable to speculate that some of the entraining effects of bright light relate to melatonin production and entrainment. We have attempted to reinforce the entraining strength of the 500 lux light–dark cycle under fractional desynchronization conditions by feeding 5 mg melatonin as a gelatine capsule to two subjects at lights-off for the duration of the experiment. In these preliminary experiments the circadian rhythm of self-rated fatigue (10 cm visual analogue scale) or alertness, recorded seven times per cycle according to previous protocols (Wever 1979), remained entrained to the zeitgeber throughout the study in volunteers taking melatonin, whereas in five out of six control subjects poor entrainment was observed (Fig. 3).

FIG. 3. Entrainment of the fatigue rhythm (self-rated seven times daily, 10 cm visual analogue scale) during fractional desynchronization achieved by increasing imposed daylength (26–29 h, 500 lux light–dark cycle) in environmental isolation. Data were averaged over six-day moving windows (vertical axis) and are presented as the best-fitting periods. For technical and analytical procedures see Wever (1979). Reliable best-fitting periods ($P < 0.001$) are shown as filled circles, unreliable ones as open circles. The period length of the zeitgeber is shown as a diagonal line. The correlation between changes in the zeitgeber period and changes in the best-fitting period for the fatigue rhythm is also shown separately for each subject. The critical r ($\alpha = 0.05$, two-tailed) is 0.754. Subjects A and B took 5 mg melatonin at lights-off daily. All other subjects were untreated. Note that reliable entrainment to the zeitgeber period is seen in subjects A and B and control subject C. All other controls showed evidence of desynchronization and/or arrhythmicity.


A
B
C
1-6
4-9
7-12
10-15
13-18
16-21
19-24
22-27
r = + 0.942
r = + 0.875
r = +0.895
r = + 0.458
r = + 0.44
r = + 0.568
r = + 0.172
r = +0.585
22
26
30
34
Period (hours)

No major consistent effects of melatonin on other measured circadian variables (core body temperature, cortisol, urine volume, Na^+, K^+, Ca^{2+}, verbal reasoning, low and high memory-loaded performance tasks, mood) were observed.

Our observations suggest that melatonin may have zeitgeber properties with respect to fatigue and altertness, but its effects are by no means comparable to those of bright light where most overt rhythms remain coupled to the zeitgeber. From a practical point of view it would be of interest and importance to assess the ability of melatonin to increase the rate of resynchronization of fatigue and alertness, together with body temperature and performance rhythms, after phase-shifts in real time, such as those seen during rapid travel across time-zones.

Melatonin metabolites

Observations of human pineal function have generally been restricted by methodology. Melatonin assays have always been difficult and observations consis-

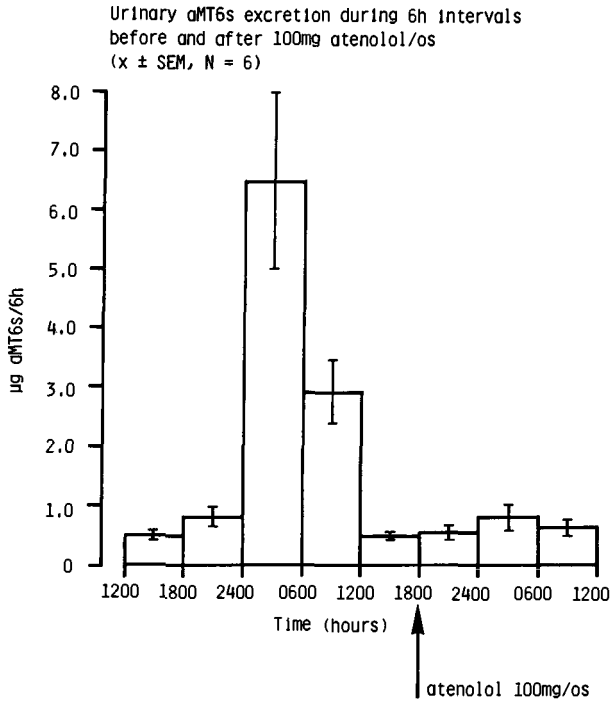


FIG. 4. Amount of aMT6s in six-hour sequential urine samples before and after a dose of 100 mg atenolol (a peripheral β_1-adrenergic antagonist) in six normal healthy volunteers. Atenolol completely abolished the night-time rise in aMT6s excretion.

tent between laboratories are still rare. Moreover it is necessary to sample blood over at least 24 h to obtain a complete secretory profile; this is a difficult clinical undertaking. Various important aspects of human pineal function are only amenable to study by urine measurements, for example development of melatonin rhythms in infancy, long-term monitoring of single individuals, studies on the circadian oscillatory control of the melatonin rhythm in isolation, and extensive screening of different pathological states.

Urinary melatonin is unreliable as a measure in that it only represents a small percentage of the secreted product. An assay for a major excreted metabolite would overcome these problems. Such a metabolite in humans is 6-hydroxymelatonin sulphate (aMT6s) (Jones et al 1969), for which we have developed a simple, direct radioimmunoassay (RIA) (Arendt et al 1985). The assay will measure plasma aMT6s but, more importantly, is sufficiently sensitive to assay urine diluted by a factor of 50. Both plasma aMT6s (area under the 24 h curve) and urinary aMT6s (total 24 h excretion) correlate well with plasma melatonin. Comparison of the RIA for aMT6s with the estimation of total conjugated metabolites of melatonin in urine by gas chromatography–mass spectrometry (Tetsuo et al 1980) gave a correlation of $r = 0.93$ ($N = 100$) (S. P. Markey & J. Arendt, unpublished work 1985) with, as expected, lower values for the RIA, which only measures one metabolite. The metabolite aMT6s is extremely stable in urine for at least 24 h at 4 °C and for at least six months at −20 °C (C. Bojkowski, unpublished work 1985).

Studies on the control of melatonin secretion in humans using a novel assay for aMT6s

Current evidence suggests that peripheral β-adrenergic receptors mediate the stimulation of pineal melatonin production in humans, but very little is known of the central mechanisms governing human melatonin secretion. Acute administration of 100 mg atenolol, a peripheral β_1-adrenergic antagonist, at 1800 h completely suppressed the urinary aMT6s rhythm in six normal healthy volunteers (Fig. 4), confirming the importance of β_1-adrenoceptors in the control of the human pineal.

In rodents and primates the suprachiasmatic nucleus (SCN) is considered to generate an endogenous melatonin rhythm which is entrained, via the retinohypothalamic projection, to the light–dark cycle. Moore-Ede et al (1983), reviewing the circadian time-keeping system, have pointed out that the weak oscillator appears to reside in the SCN but that the strong oscillator is anatomically distinct from it (at least in the ground squirrel). We have begun experiments to investigate the circadian oscillatory control of melatonin secretion in humans and to observe how it varies in the unusual photoperiodic conditions of Antarctica.

In three normal healthy volunteers living in an Antarctic base (Halley), aMT6s, measured in sequential 6 h urine samples over 24 h in March, June, October and January, showed a marked 24 h rhythm on all occasions with high values during the night (P. Griffiths et al, unpublished work 1984). During the Antarctic winter, subjects saw only artificial light (max 500 lux) for 68 days, so the bright light needed to suppress melatonin acutely at night is not necessary for entrainment to a 24 h day.

Using the aMT6s assay we have made preliminary observations of the circadian behaviour of aMT6s in one volunteer subjected to fractional desynchronization by increasing zeitgeber period (consecutive 10 min increases in cycle length, 26–29 h, ≈ 500 lux, Andechs) and two volunteers subjected to fractional desynchronization by decreasing zeitgeber period (24–22.8 h, ≈ 500 lux, 12 min consecutive decreases in cycle length followed by 16 cycles at 22.8 h, Manchester; see Folkard et al 1984 for procedures). Core body temperature was continuously monitored (10 times per second, Andechs; every 2 min, Manchester) by an indwelling rectal thermometer. The concentration of aMT6s was measured by direct RIA in sequential urine samples from both studies and results were expressed in mass per unit time after correction for urine volume. Periodogram analysis (Wever 1979) (Fig. 5) revealed that the aMT6s rhythm remained entrained to the zeitgeber for the first half of the cycle-lengthening experiment and then showed a free-running rhythm of 24.4 h. Temperature behaved in a somewhat similar fashion, showing a free-running rhythm of 25.3 h in the second half of the study, but also had an entrained component (Fig. 5). The two subjects under decreased cycle length were dissimilar. In one both temperature and aMT6s overtly desynchronized on day 16. In the other temperature remained entrained up to day 16 when no further readings were obtained, whereas aMT6s showed only weak zeitgeber coupling from day 14.

Clearly aMT6s (and, by implication, melatonin) does not behave like a weak oscillator variable. If it is more strongly related to strong oscillator components, and if the anatomical site of the strong oscillator is indeed distinct from the SCN, the anatomical basis for melatonin rhythm generation in humans may be different from that in rodents and sub-human primates.

Conclusions

The absence of an overt melatonin rhythm in humans may not have important physiological consequences. Administered melatonin nevertheless has a marked effect on fatigue (at least when given in the early evening) but does not significantly modify a number of mood and endocrine variables with the exception of prolactin. In five out of 12 subjects it phase-advanced the rhythm

of its own secretion, suggesting an effect on circadian rhythm-generating systems. It may extend the range of entrainment of the fatigue (alertness) rhythm in environmental isolation but insufficient data are available to draw conclusions. Under fractional desynchronization protocols melatonin behaves more

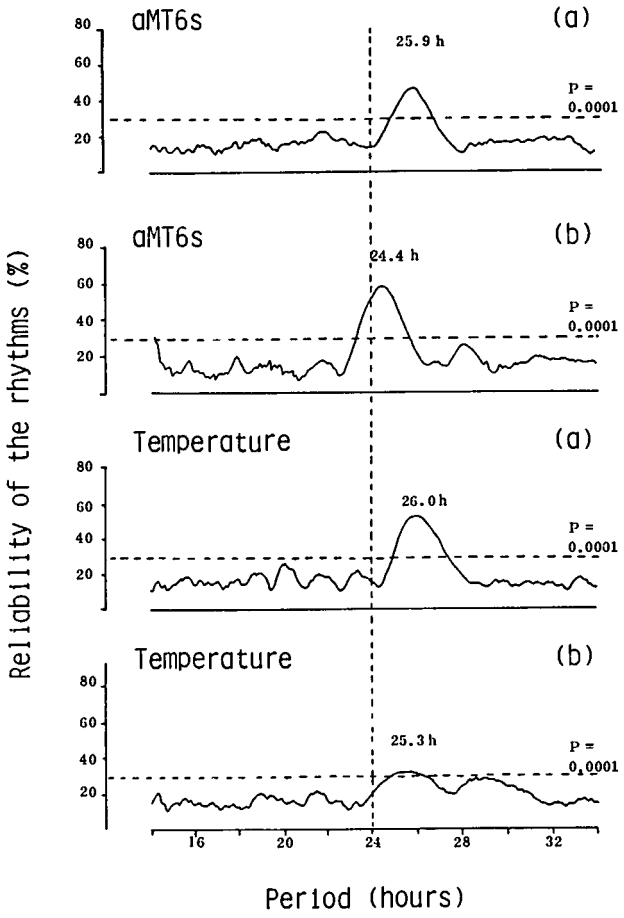


FIG. 5. Periodogram analysis (see Wever 1979 for details) of the urinary aMT6s rhythm and core body temperature rhythm in one volunteer in environmental isolation subjected to increasing imposed daylength (26–29 h, 500 lux light–dark cycle). Cycle lengths up to 27 h (a) and from 27 h to 29 h (b) are grouped separately. Note that the average period of the aMT6s rhythm (25.9 h) in (a) indicates entrainment to the zeitgeber (26–27 h imposed) and that the average period of the rhythm (24.4 h) in (b) indicates desynchronization from the zeitgeber (27–29 h imposed). The temperature rhythm behaved similarly, but in addition to the free-running period of 25.3 h an entrained component is seen in the 27–29 h section. Reliability of the rhythm > 30% is equivalent to $P < 0.0001$ (Wever 1979).

like a strong oscillator variable than a weak oscillator variable. These experimental approaches are ongoing with the objectives of determining (1) the time-dependency of melatonin effects in humans, (2) details of the control of human pineal function and (3) whether melatonin has therapeutic potential in disturbances of biological rhythm such as occur in insomnia, jet lag, shift-work, depression and old age.

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REFERENCES

Arendt J 1985 Mammalian pineal rhythms. Pineal Res Rev 3:161-214

Arendt J, Symons AM, Laud CA, Pryde SJ 1983 Melatonin can induce early onset of the breeding season in ewes. J Endocrinol 97:395-400

Arendt J, Borbely AA, Franey C, Wright J 1984 The effect of chronic, small doses of melatonin given in the late afternoon on fatigue in man: a preliminary study. Neurosci Lett 45:317-321

Arendt J, Bojkowski C, Franey C, Wright J, Marks V 1985 Immunoassay of 6-hydroxymelatonin sulfate in human plasma and urine: abolition of the urinary 24-hour rhythm with atenolol. J Clin Endocrinol & Metab 60:1166-1173

Bittman EL 1985 The role of rhythms in the response to melatonin. In: Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Found Symp 117) p 149-169

Bittman EL, Karsch FJ, Hopkins JW 1983 Role of the pineal gland in ovine photoperiodism: regulation of seasonal breeding and negative feedback effects of estradiol upon luteinising hormone secretion. Endocrinology 113:329-336

Carman JS, Post RM, Buswell R, Goodwin FK 1976 Negative effects of melatonin on depression. Am J Psychiatry 133:1181-1186

Cramer H, Rudolph J, Consbruch V 1974 On the effects of melatonin on sleep and behaviour in man. Adv Biochem Psychopharmacol 11:187-191

Fèvre-Montange M, Van Cauter E, Refetoff S, Désir D, Tourniaire J, Copinschi G 1981 Effects of 'jet lag' on hormonal patterns. II. Adaptation of melatonin circadian periodicity. J Clin Endocrinol & Metab 52:642-649

Folkard S, Minors DS, Waterhouse JM 1984 Is there more than one circadian clock in humans? Evidence from fractional desynchronisation studies. J Physiol (Lond) 357:341-356

Fraser S, Cowen P, Franklin M, Franey C, Arendt J 1983 Direct radioimmunoassay for melatonin in plasma. Clin Chem 29:396-397

Hastings MH, Herbert J, Martensz ND, Roberts AC 1985 Melatonin and the brain in photoperiodic mammals. In: Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Found Symp 117) p 57-77

Jones RL, McGeer PL, Greiner AC 1969 Metabolism of exogenous melatonin in schizophrenic and non-schizophrenic volunteers. Clin Chim Acta 26:281-285

Kennaway DJ, Gilmore TA, Seamark RF 1982 Effect of melatonin feeding on serum prolactin and gonadotrophin levels and the onset of seasonal estrous cyclicity in sheep. Endocrinology 110:1766-1722

Lerner AB, Nordlund JJ 1978 Melatonin: clinical pharmacology. J Neural Transm (Suppl) 13:339-347

Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Markey SP 1980 Light suppresses melatonin secretion in humans. Science (Wash DC) 210:1267-1269

Lincoln GA, Forbes JM 1984 Seasonal physiology of pinealectomised rams. Soc Study Fertil Abstr No 33

Lincoln GA, Ebling FJP, Almeida OFX 1985 Generation of melatonin rhythms. In: Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Found Symp 117) p 129-148

Martinet L, Allain D 1985 Role of the pineal in the photoperiodic control of reproductive and non-reproductive functions in mink (*Mustela vison*). In: Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Found Symp 117) p 170-187

Moore-Ede MC, Czeisler CA, Richardson GS 1983 Circadian timekeeping in health and disease. Part 1. Basic properties of circadian pacemakers. N Engl J Med 309:469-476

O'Brien IAD, Lewin IG, O'Hare JP, Wright J, Arendt J, Corrall RJM 1984 Abnormal circadian rhythm of melatonin in diabetic autonomic neuropathy. Clin Sci (Lond) 66:38P

Redman J, Armstrong S, Ng KT 1983 Free-running activity rhythms in the rat: entrainment by melatonin. Science (Wash DC) 219:1080-1081

Reiter RJ 1980 The pineal and its hormones in the control of reproduction in mammals. Endocr Rev 1:109-131

Rosenthal NE, Sack DA, Gillin JC et al 1984 Seasonal affective disorder. A description of the syndrome and preliminary findings with light therapy. Arch Gen Psychiatry 41:72-79

Sugden D 1980 Actions of melatonin on the central nervous system. PhD Thesis, CNAA, p 66

Symons AM, Arendt J, Laud CA 1983 Melatonin feeding decreases prolactin levels in the ewe. J Endocrinol 99:41-46

Tetsuo M, Markey SP, Kopin IJ 1980 Measurement of 6-hydroxy-melatonin in human urine and its diurnal variations. Life Sci 27:105

Vollrath L, Semm P, Gammel G 1981 Sleep induction by intranasal application of melatonin. Adv Biosci 29:327-329

Wever RA 1979 The circadian system of man: results of experiments under temporal isolation. Springer-Verlag, New York

Wever RA, Polasek J, Wildgruber CM 1983 Bright light affects human circadian rhythms. Pfluegers Arch Eur J Physiol 396:85-87

DISCUSSION

Klein: Were your subjects with very low plasma melatonin concentrations depressed?

Arendt: No. It's a fairly common observation that a small percentage of normal people have absolutely no detectable melatonin. In our assay this means less than 10 pg/ml throughout 24 h. We thought that one of our subjects with no detectable melatonin might have a sleep disturbance, but I'm not sure about that. We've looked at a whole series of anterior pituitary hormones in these people with very low levels of melatonin and they are perfectly normal.

Klein: This underlines the fact that there is great variation in the population, so melatonin should only be used as a marker for depression with great caution.

Short: Let's remind ourselves that pleomorphism is the substrate for natural selection. If a species has tremendous pleomorphism in a certain characteristic, and Jo Arendt has convincingly shown that there are quite a few normal people walking around with no detectable melatonin, that characteristic is unlikely to have enormous adaptive significance. I would guess, however, that there are no sheep walking around without melatonin; I don't think they would survive.

Arendt: We have certainly never found intact sheep with no detectable melatonin and we have looked at about 50 or 60.

Moore-Ede: Isolation experiments are difficult to do, but it is important when you are studying melatonin as a zeitgeber (a potential entraining agent) to establish that you are not inducing a masking effect instead of entrainment. Have you had a chance to look at the effects on phase of taking people off melatonin and putting them onto placebo?

Arendt: Not in isolation. But we tested placebo in our feeding study and the phase-advance was not seen when we gave placebo immediately after the melatonin trial. We don't think there was any masking in our isolation experiments because when Simon Folkard analysed the fatigue rhythms from that study he found that fatigue clearly increased *before* melatonin was given.

Lewy: Do you get an effect the first time you give the melatonin? Doesn't melatonin acutely increase sedation and fatigue?

Arendt: Not in the doses we used. We noticed this in our initial feeding study; none of our subjects reported a feeling of immediate fatigue.

Tamarkin: Norman Rosenthal has claimed that some of his patients taking 200 μg of melatonin show some degree of sleepiness (unpublished work).

Arendt: But he gives melatonin differently, in three spaced doses.

Tamarkin: But the plasma levels achieved are no higher than those you report.

Arendt: We certainly saw no immediate fatigue in normal subjects. Perhaps our ratings were insufficiently sophisticated. We didn't know what to expect. There are many reports of pharmacological hypnotic effects of melatonin, but none were obtained with the sort of oral dose we are using, and all the reported effects were acute.

Turek: The only rhythm that was affected in your desynchronization experiments was the fatigue rhythm, but if this rhythm is linked to the strong oscillator which regulates other rhythms, would you not expect melatonin to affect other rhythms also?

Arendt: Obviously we cannot look directly at the sleep–wake cycle with this particular experimental protocol, but in the two subjects there was certainly no consistent entrainment of rhythms in temperature, cortisol, electrolytes and various performance measures.

Turek: Melatonin does not sound like a very good zeitgeber.

Arendt: I agree.

Turek: In your other experiments you showed that in five of 11 subjects the endogenous melatonin rhythm was phase-advanced by an hour or so after you administered melatonin. Do you think the exogenous melatonin affects the underlying endogenous rhythm? If so, you should see a phase-advance in the endogenous rhythm the next day. Or do you think exogenous melatonin acts in a pharmacological way, stimulating the pineal gland to such an extent that the gland no longer responds to internal signals immediately after the exogenous melatonin is administered?

Arendt: I think the effect is probably pharmacological. The results I showed with the phase-advance in the endogenous rhythm were for blood samples taken on the day of the last dose of melatonin. In the spring experiment we took samples the day *after* the last dose, for precisely the reasons that you mentioned, and we looked at the endogenous profiles. Only two out of 12 subjects showed a phase-advance; all the rest were indistinguishable from placebo-treated controls and there were no phase-delays. So it does not look as though melatonin is affecting a clock system for melatonin rhythm generation, but how it works I don't know. Has anybody looked at the direct effect of melatonin on the pineal *in vitro*?

Klein: We have looked, but we have never seen any feedback. Of course that doesn't mean that it doesn't occur.

Illnerová: Melatonin and *N*-acetylserotonin inhibit rat liver *N*-acetyltransferase and bovine pineal *N*-acetyltransferase, but not the night-active rat pineal *N*-acetyltransferase (Howd et al 1976).

Lewy: Gerald Lincoln's and Helena Illnerová's data and Jo Arendt's results all show changes in the circadian phase position of the melatonin rhythm under different photoperiods, and these changes fit with the clock–gate model I have described (see p 238). If τ is greater than 24 h you would expect a delay in phase in the winter compared to the summer, but if τ is shorter than 24 h you would expect an advance, and many of the results presented are consistent with this.

Klein: It's difficult to make judgements about the clock on the basis of measurements of melatonin, because melatonin is an output. In the rat there is a lag before the pineal responds with an increase in *N*-acetyltransferase activity and melatonin production after stimulation, and it has been shown that this lag can vary according to the past treatment of the animal (Illnerová & Vaněček 1982). The timing of the peaks in melatonin and *N*-acetyltransferase is not fixed; the increase in *N*-acetyltransferase activity does not always occur a given number of hours after you start drug administration. It depends on dose and prior treatment. Therefore, one cannot rely only on melatonin measurements to make predictions about what is happening in the clock. You need to test, for example, that the pineal gland always responds to a catecholamine challenge in the same way. If it does, then perhaps melatonin rhythms do reflect the activity

of an endogenous oscillator. However, there may be some elasticity in the linkage between the two, especially in timing. A more direct measure of the SCN would be better.

*Arendt:*If we assume that melatonin *is* a good reflection of its own rhythm-generating system and that the melatonin rhythm is generated within the SCN, how can we explain our results indicating that melatonin rhythms are associated with strong oscillator components when the strong oscillator is apparently located *outside* the SCN?

Moore-Ede: This is a question that is not resolved. I don't think it is clear that the melatonin rhythm is definitely associated with the 'strong' oscillator. Your data are certainly suggestive in that melatonin does not seem to follow the sleep–wake cycle. However, in rhesus monkeys SCN lesions more readily cause disruption of the melatonin rhythm than the cortisol rhythm (Reppert et al 1981), and you could argue from this alone that the melatonin rhythm is more strongly driven by the SCN than by components outside the SCN. So I don't know the answer, but the results presented at this symposium do suggest that the melatonin rhythm is in some way coordinated by the SCN.

Klein: It's possible that the SCN do act as a strong oscillator and that when you destroy these nuclei a second oscillator, which the SCN normally control, then takes over. However, we found that when we destroyed the SCN in the rhesus monkey the melatonin rhythm was severely disrupted (Reppert et al 1981). In fact, there was no well-defined rhythm in these animals. If a weak oscillator was operational it was not impressive. I can't see how our results provide convincing support for the weak oscillator story. The only thing that is clear to me is that when you destroy the SCN pineal rhythmicity dramatically deteriorates.

Reppert: It's worth adding that, even though we disrupted the major rhythmic component in these monkeys, time series analysis showed that minor components remained, with 24 h or 25 h cycle lengths (Reppert et al 1981). These rhythms were unexplained, but they may represent extra components in the melatonin rhythm-generating system in primates. However, the number of animals that we studied was very small so we can't draw definite conclusions.

Turek: Everyone here seems afraid to say much about the multi-oscillator concept and multiple master pacemakers. Yet many people believe that it has been proved that in mammals there are two master pacemakers in the brain that regulate the entire circadian system. If we actually look at the data things are not so clear. The results of SCN lesion studies in primates are perfectly compatible with the idea that some rhythms are entrained by other zeitgebers such as social cues and presentation of food. In my opinion, there is really very little hard data to support the multiple master pacemaker concept. Wever & Aschoff's desynchronization experiments can also be interpreted in several ways. In fact J. Zulley (personal communication) has recently reanalysed the data and has shown that there is not that much desynchronization between the

body temperature rhythm and the sleep–wake cycle; if you take 'naps' into consideration the correlation is much closer than has previously been assumed. We need to be careful because people start building hypotheses, for example about the role of circadian disorganization in mental illness, on what they assume is a factual basis. But that 'factual' basis, i.e. proof of a multiple master pacemaker system, does not exist.

Moore-Ede: Having personally been responsible for gathering much of the information on temperature, cortisol and other rhythms in humans in free-running situations, I do not find it quite so easy to dismiss the idea that there are two pacemaker systems. Furthermore Ziad Boulos's results showing that rhythms in food anticipation persist after SCN lesions (Boulos et al 1980) and our data from squirrel monkeys on persisting temperature rhythms provide pretty strong evidence that sites outside the SCN can generate rhythmicity (Fuller et al 1981).

Turek: But the question is whether sites outside the SCN act as master pacemakers in synchrony with the SCN. I don't think anyone would deny that there are oscillators outside the SCN.

Zucker: There is no question that some of the rhythms Martin Moore-Ede mentioned, especially the feeding rhythm, are usually subservient to the SCN, even though they may persist in the absence of the SCN.

Moore-Ede: The SCN obviously play a very important role in the interaction between the environment and other oscillators and in the entrainment of the system. I don't think there is any dispute about that. The point is, does a capability for stable rhythm generation persist after you take out the SCN? The idea has arisen from certain rodent experiments that the SCN alone can drive everything, but this is a matter for debate. There are strong believers on both sides.

REFERENCES

Boulos Z, Rosenwasser AM, Terman M 1980 Feeding schedules and the circadian organization of behavior in the rat. Behav Brain Res 1:39-65

Fuller CA, Lydic R, Sulzman FM, Albers HE, Tepper B, Moore-Ede MC 1981 Circadian rhythm of body temperature persists after suprachiasmatic lesions in the squirrel monkey. Am J Physiol 241(10):R385-R391

Howd RA, Seo KS, Wurtman RJ 1976 Rat liver *N*-acetyltransferase: inhibition by melatonin. Biochem Pharmacol 25:977-978

Illnerová H, Vaněček J 1982 Complex control of the circadian rhythm in N-acetyltransferase activity in the rat pineal gland. In: Aschoff et al (eds) Circadian systems: structure and function. Springer-Verlag, Heidelberg, p 285-296

Reppert SM, Perlow MJ, Ungerlieder LG, Mishkin M, Tamarkin L, Orloff DG, Hoffman HJ, Klein DC 1981 Effects of damage to the suprachiasmatic areas of the anterior hypothalamus on the daily melatonin and cortisol rhythms in the Rhesus monkey. J Neurosci 1:1414-1425

Photoperiodism, melatonin and the pineal
Editors: David Evered and Sarah Clark

Melatonin and malignant disease

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Abstract. A possible role for the pineal and melatonin in malignant disease is suggested by the studies of hormone-dependent tumours presented here. The most dramatic observation of the effect of the pineal and melatonin in malignancy is that melatonin protects against, while pinealectomy enhances, 7,12-dimethylbenz[*a*]anthracene-induced mammary tumours in rats. Melatonin may protect against tumours through a suppressive effect on prolactin secretion or an action on oestrogen receptors. Melatonin can change the concentration of oestrogen receptors in hamsters, both juvenile and adults, and in a human breast cancer cell line. The long-term effect of melatonin in the ovariectomized adult hamster and in human breast cancer cells is to inhibit oestrogen-stimulated growth. A possible relationship between melatonin and oestrogen receptors was examined in women with breast cancer. An inverse correlation was observed between the oestrogen receptor concentration in each patient's tumour and her peak plasma melatonin level, suggesting that the more hormone dependent the breast cancer the more blunted the daily melatonin rhythm. Thus, the more robust the daily melatonin rhythm the greater the protective effect on hormone-dependent breast cancer. Unfortunately, this hypothesis is not easily tested as shown in a study of women at high risk for developing breast cancer. These women had daily melatonin profiles that did not differ from those of a normal population. Thus, the use of plasma melatonin as a screening tool for breast cancer risk is not reasonable; however the basic and clinical data argue that the role of melatonin in endocrine-related cancers requires more extensive clinical investigation.

1985 Photoperiodism, melatonin and the pineal. Pitman, London (Ciba Foundation Symposium 117) p 284-299

Is there a role for the pineal gland and melatonin in malignant disease? Numerous studies have reported an effect of the pineal in the induction or the growth of experimentally induced malignant tumours. Some studies have shown that the pineal and melatonin enhance tumorigenesis, while some have shown the converse and others have reported no effect at all. Evaluation of these studies is hampered by the diversity of the experimental approaches. Various tumour models have been used with a variety of treatment paradigms. In effect, comparison of these studies is nearly impossible.

We have recently reviewed published work in an attempt to gain some historical perspective of the role of the pineal and melatonin in malignant disease. To our knowledge the first association between the pineal and malignancy was made by Georgiou in 1929. In his study pinealectomy inhibited tumorigenesis and thus he concluded that the pineal actually stimulated cancer growth.

Most results suggest just the opposite. The extensive work of Ebels, Lapin and Das Gupta (cf. Lapin 1979) has pointed toward an inhibitory effect of the pineal on tumorigenesis. Unfortunately, many of these studies were phenomenological in approach; that is, a specific tumour model was employed and the effect of pineal removal or replacement then assessed. What was not considered in these studies was why an association between the pineal and a specific tumour might exist and how the pineal and its hormone(s) could affect tumorigenesis.

Our interest in this field was prompted by the studies of the effect of melatonin on seasonal breeding in the Syrian hamster. The dramatic change in reproductive function during melatonin treatment suggested that melatonin might have an inhibitory effect in rats treated with a carcinogen whose tumour-inducing effect was dependent upon pituitary and ovarian hormones. The tumour model chosen for study was the rat treated with 7,12-dimethylbenz[*a*]anthracene (DMBA).

Tumour development in DMBA-treated rats

Female rats administered DMBA once in adulthood (50 days of age) develop mammary carcinoma 20 to 80 days later (sometimes the first appearance of a tumour is even later). The induction of tumours is dependent upon the pituitary and the ovary; removal of either prevents tumour growth (Dao 1962). Specifically, tumorigenesis in these DMBA-treated animals is dependent on prolactin and oestrogen action (Hollander & Diamond 1978), which led us to ask whether the pineal and melatonin might be involved. Subsequently, we looked at these hormone systems to assess how melatonin might affect tumorigenesis in the DMBA-treated rat.

Our first experiment tested the effect of daily afternoon treatment with melatonin (500 μg/animal) on rats administered DMBA (Tamarkin et al 1981). During the 90 days of treatment after DMBA administration none of the melatonin-treated rats developed mammary tumours, while 50% of the vehicle-treated animals developed tumours (Fig. 1). Animals were observed for 50 more days and during this period without melatonin treatment five of 24 animals originally treated with melatonin did develop tumours, while more than 85% of the vehicle-treated animals had mammary tumours

at the end of the experiment. These results suggested that a single daily dose of melatonin might inhibit tumorigenesis. However, what was the relationship between this single daily dose of melatonin and the pineal gland?

The pineal gland produces and secretes melatonin primarily at night, creating a daily rhythm in the levels of this hormone in the circulation. One possible explanation for the protective effect of melatonin in DMBA-treated rats is

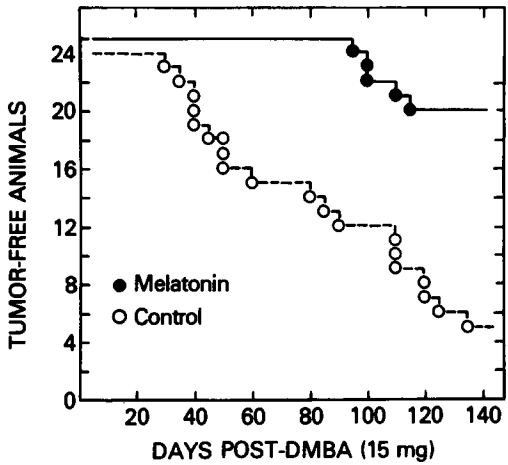

FIG. 1. Incidence of mammary tumours in rats given 15 mg of DMBA. From 0 to 90 days, half the animals received daily s.c. injections of melatonin (500 μg/animal) and the remaining animals received vehicle (control) at 1600 h (lights on from 0600 to 2000 h). All animals were examined twice weekly for tumours until 140 days after DMBA treatment.

that the daily pineal melatonin signal was enhanced by daily afternoon melatonin injections. One question we asked was whether, if we removed the daily melatonin signal by pinealectomy, the incidence of tumorigenesis in DMBA-treated rats would change.

From previous observations we knew that few rats develop mammary tumours when given a low dose of DMBA (Dao 1962). Our next experiment examined the effect of pinealectomy or sham pinealectomy on the incidence of tumours in rats treated with a low dose of DMBA (Tamarkin et al 1981). As previously observed, few intact animals (25%) developed mammary tumours; however, 84% of the pinealectomized animals did develop tumours with this dose (Fig. 2). These results suggest that the endogenous melatonin rhythm plays a role in the aetiology of DMBA-induced mammary tumours.

A conclusive demonstration that melatonin can prevent DMBA-induced tumours is still wanting, since a single melatonin injection in pinealectomized rats treated with DMBA did not fully protect the majority of these animals from developing tumours. Perhaps multiple daily injections of melatonin

would have been more effective in inhibiting tumorigenesis. (A multiple injection paradigm was effective in inducing gonadal quiescence in the hamster.) However, these data and similar observations from other laboratories (Aubert et al 1980, Blask et al 1984, Shah et al 1984) do suggest that enhancement of the daily melatonin signal inhibits tumour development, while elimination of this daily hormonal rhythm promotes tumorigenesis.

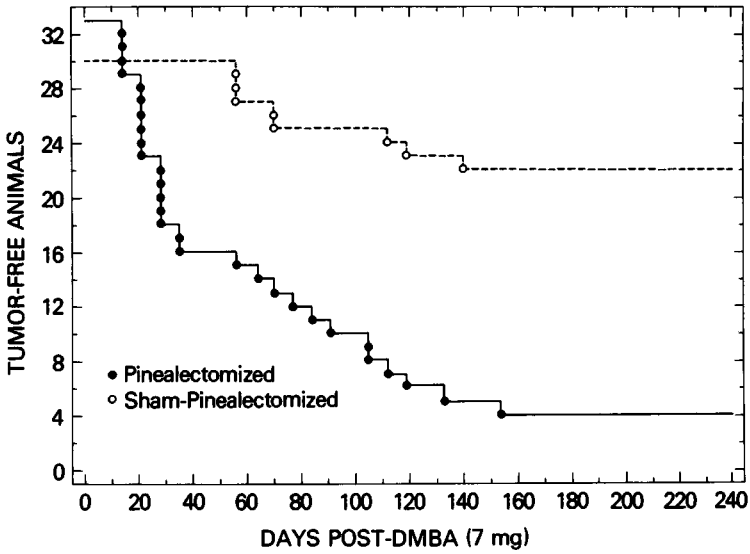

FIG. 2. Incidence of mammary tumours in rats pinealectomized or sham-pinealectomized 30 days before being given a low dose (7 mg) of DMBA. All animals were examined twice weekly for tumours until 240 days after DMBA treatment.

Interactions of melatonin with other hormone systems

The protective role of melatonin in DMBA-induced mammary cancer may be related to a suppressive effect of melatonin on prolactin secretion. DMBA-treated animals injected daily with melatonin had significantly lower plasma prolactin concentrations than did vehicle-injected DMBA-treated animals. This lowered level of prolactin may at least partially account for the inhibition of tumorigenesis by melatonin.

Melatonin could also have influenced tumorigenesis in DMBA-treated rats through an effect on oestrogen action. DMBA-treated rats with or without melatonin injections had essentially the same levels of oestradiol, so our next task was to examine the oestrogen receptor. We chose for that model system the juvenile Syrian hamster, which is devoid of endogenous oestrogens. In

the absence of endogenous oestrogens, our method of choice to determine oestrogen receptor concentrations in uteri was a dextran-coated charcoal assay of cytosol. Cytosol was prepared from homogenized uteri from five or six animals, so that sufficient protein could be added to five or six different concentrations of [^{3}H]oestradiol with or without a 200-fold excess of diethylstilboestrol. The resultant saturation curves were analysed by Scatchard analysis for the estimation of the equilibrium dissociation constant (K_d) and the receptor concentration.

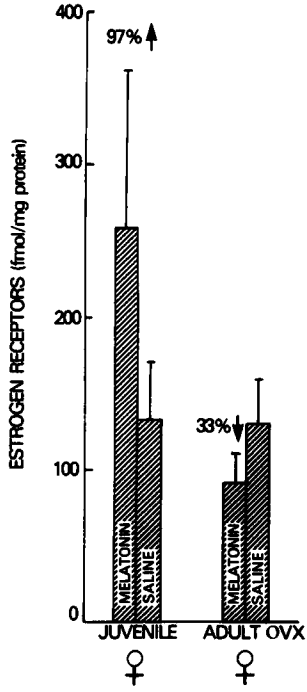

FIG. 3. Oestrogen receptor concentrations in uteri from juvenile and ovariectomized (OVX) adult hamsters treated with 25 μg melatonin or saline 45 min before being killed. The bars represent the mean ± SE of oestrogen receptor concentrations from 11 juvenile hamster experiments and 14 adult OVX hamster experiments. For each experiment four to six uteri were used to generate a five- or six-point Scatchard plot.

Injection of 2.5 μg or 25 μg of melatonin 1 h before the hamsters were killed caused approximately a 30% increase in oestrogen-specific binding in juvenile animals (Danforth et al 1983a). This increase was transient and a time course study revealed that within 20 min of a single 2.5 μg injection of melatonin oestrogen-specific binding rose more than 30% and remained elevated for 90 min. By the second hour after treatment oestrogen receptor concentrations

were essentially identical to control. The K_d for the oestradiol-specific binding was unchanged by any dose of melatonin or any time during the time course examined.

One obvious question that needed to be addressed was what effect melatonin had on adult oestrogen receptors. To our surprise the response of adults was exactly opposite to that of juvenile animals (Almeida & Tamarkin 1984). In paired experiments, 45 min after melatonin treatment of hamsters the concentration of uterine oestrogen receptors had declined by 33% in ovariectomized adults, but had increased by 97% in juvenile animals (Fig. 3).

This differential response to melatonin during development was also demonstrated by assessing specific uterine uptake of [^{3}H]oestradiol *in vivo*. In this experiment juvenile or ovariectomized adult hamsters were treated with or without melatonin and/or diethylstilboestrol 40 min before [^{3}H]oestradiol injection. One hour later all animals were killed, and their uteri were excised and solubilized overnight. Specific oestradiol uptake was determined by the subtraction of [^{3}H]oestradiol uptake in the presence of diethylstilboestrol from [^{3}H]oestradiol uptake alone. The results support the oestrogen receptor study: melatonin treatment caused a 27% increase in specific oestradiol uptake in juvenile animals, while the same dose of melatonin at the same time of day caused a 36% decrease in oestradiol uptake in ovariectomized adults (Almeida & Tamarkin 1984).

This antagonism of oestrogen action by melatonin in adults was also observed in ovariectomized adults treated with 40 μg of oestradiol at 0800 h. Daily afternoon injection of 25 μg of melatonin for four days resulted in 57% less uterine growth than in animals treated with oestradiol alone (Almeida & Tamarkin 1984). These studies suggest that melatonin's action is affected by changes that occur during development, and may be very different in juvenile and adult tissue.

Effects on the human breast cancer cell line MCF-7

As oft-times suggested, malignant tissue more closely resembles juvenile tissue than adult. With this in mind we examined the effect of melatonin on the human breast cancer cell line MCF-7. This cell line was derived from the malignant pleural effusion of a patient with hormone-dependent breast cancer. Since these cells are oestrogen responsive we first determined the effect of melatonin on their receptors.

One method we used for looking at specific oestradiol uptake in these cells was the very simple one of measuring the ability of intact cells to take up [^{3}H]oestradiol specifically after melatonin treatment (Danforth et al 1983b). After cell disruption to terminate the incubation period, oestradiol was

extracted overnight in ethanol. This procedure is less traumatic than determining the concentration of oestrogen receptors in cell cytosol, and, we hoped, subject to fewer methodological problems.

In MCF-7 cells, as in the juvenile hamster, a 40 min incubation in 1 nM-melatonin induced a transient twofold increase in oestradiol-specific binding (Danforth et al 1983b). The dissociation equilibrium constant was the same for vehicle-treated and melatonin-treated cells. A 1 h pretreatment of these cells with 20 μM-cycloheximide completely prevented the melatonin-induced increase in specific oestradiol binding, suggesting that the response was mediated by a protein synthesized *de novo*.

This acute effect of melatonin on these cells is contrary to our expectations of an inhibitory effect of melatonin on tumorigenesis. The physiological significance of this transient increase in oestrogen receptor numbers is also puzzling.

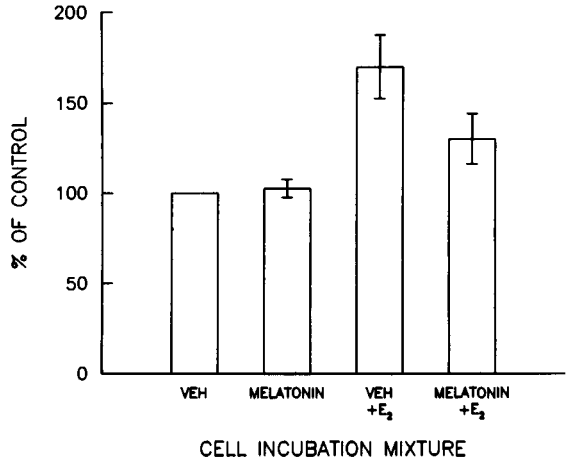

FIG. 4. Effects of melatonin on oestradiol-stimulated growth of MCF-7 cells cultured in IMEM medium with 5% fetal calf serum (twice charcoal stripped). Cells were incubated in melatonin (1 nM) or vehicle (VEH) for 24 h. Some cultures were then supplemented with oestradiol (E_2, 10^{-11} M). After a total of seven days, growth was assessed by cell counts and is expressed as percentage of control (mean ± SE). Results are from three experiments; for each experiment six tissue culture wells were used for each treatment.

Thus, we determined the long-term effect of melatonin treatment on oestrogen-stimulated MCF-7 cells.

Incubation of MCF-7 cells with 1 nM-melatonin for 24 h and then with melatonin and 10^{-11} M-oestradiol for six more days resulted in a 25% decrease in oestradiol-stimulated growth (Fig. 4). Melatonin alone had neither a stimulatory nor an inhibitory effect on cell growth during the seven days of study (Danforth et al 1984). To date, we have not been able to reconcile the acute

effect of melatonin on oestrogen receptors with its long-term effect on cell growth.

Melatonin in patients with breast cancer

On the basis of the very dramatic inhibitory effect of melatonin on DMBA-induced mammary tumours in rats and our other results suggesting a negative effect of melatonin on long term oestrogen action, we undertook a clinical study of patients participating in the National Cancer Institute Breast Cancer Protocol. Randomly selected patients with clinical stage I or II primary breast cancer were recruited for a 24 h blood study just before or just after surgical removal of their tumours. No patients had evidence of other malignancy or major illness and all were drug free during the study. The concentrations of melatonin and gonadal hormones in the plasma were determined and compared with those found in age-matched normal women. Additionally, oestrogen, progesterone and glucocorticoid receptor concentrations were determined in the tumours removed from the breast cancer patients.

In our initial (Tamarkin et al 1982) and subsequent (Danforth et al 1985) studies we observed that some patients had little or no nocturnal increase in plasma melatonin levels. We hypothesized that this might be related to the hormone dependency of the individual's tumour, and indeed, determination of oestrogen receptor concentrations in the tumours of these patients revealed that those patients with the oestrogen receptor-positive tumours had the lower levels of melatonin at night (Fig. 5) (note: measuring oestrogen receptor concentrations is one way in way in which the hormone dependency of breast tumours is currently assessed). Comparison of oestrogen receptor concentrations in each patient's tumour with her peak plasma melatonin level revealed a highly significant inverse correlation, such that those patients with the highest oestrogen receptor concentrations in their tumours had the lowest peak levels of melatonin (Fig. 6). Further analysis revealed that this inverse correlation also applied to the progesterone receptor, but did not hold for the glucocorticoid receptor. Additionally, no relationship was found between the day–night melatonin difference and plasma concentrations of oestrone, oestradiol, progesterone, luteinizing hormone or follicle-stimulating hormone. These data and the data of Bartsch et al (1981) suggest that the absence of a clear daily rhythm in melatonin concentrations might be related to the presence of hormone-dependent breast cancer, and perhaps plays a role in the aetiology of this disease.

We attempted to examine this latter question by recruiting 23 women at high risk for breast cancer from 10 kindreds with familial breast cancer (Danforth et al 1985). The relative risk for each individual having breast cancer

was calculated by means of additive and multiplicative models for combining separate risk estimates derived from age, pedigree type (mother, sister or second-degree relative), age of menarche, first full-term pregnancy, parity

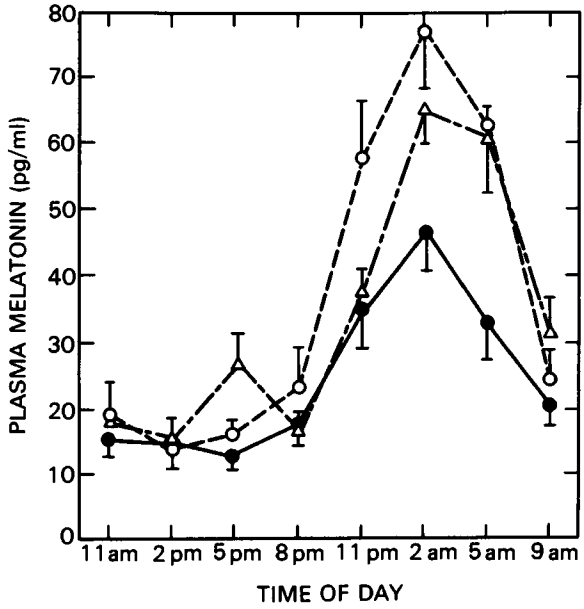

FIG. 5. Twenty-four hour profiles of plasma melatonin concentrations (means ± SE) in women with oestrogen receptor-positive (●, $N = 10$) or oestrogen receptor-negative (○, $N = 10$) breast cancer and in normal women (△, $N = 8$).

and history of fibrocystic disease. At the time of study all subjects were disease free and evaluation of plasma melatonin profiles revealed that some women did have a reduced nocturnal increase in melatonin concentrations (Fig. 6). However, this lowered day–night melatonin difference did not correlate with degree of risk. Thus, we have no *a priori* way of determining if lowered melatonin levels do indeed indicate increased risk of developing hormone-dependent breast cancer.

In conclusion, although melatonin may play a subtle role in the regulation of oestrogen-responsive tissues, and may be useful as a predictor of clinical breast cancer and as a clinical screening tool, the variation in plasma melatonin concentrations seen in a normal population far exceeds our ability ro resolve this question. Perhaps a more promising approach to the question of melatonin's role in cancer is suggested by the observation of Burns (1973), who

administered melatonin intramuscularly to women with breast carcinoma and found a reduction in urinary oestrogen concentrations which may be significant in the remission of the disease. We are currently examining a simple means

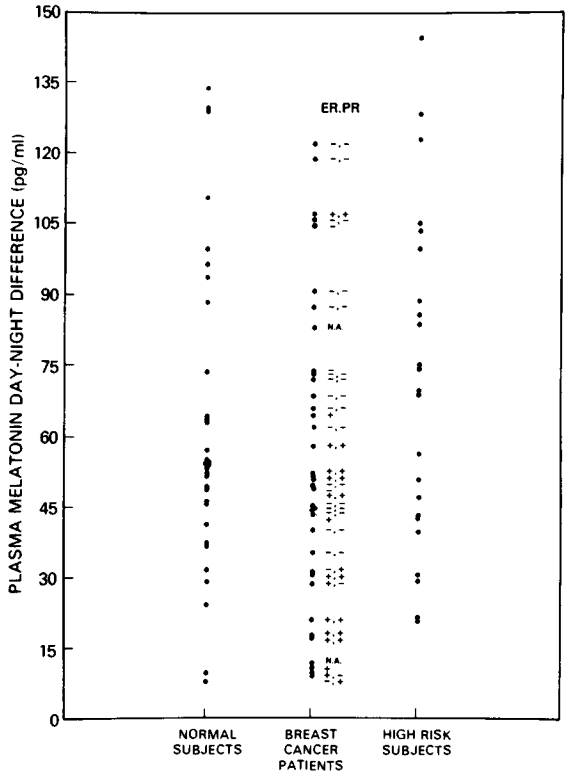

FIG. 6. Day–night difference in plasma melatonin concentrations in normal subjects, women with breast cancer and women at high risk for breast cancers. Each point represents the melatonin day–night difference (peak night-time concentration minus mean day-time concentration) for one subject. Oestrogen receptor (ER) and progesterone receptor (PR) concentrations in each breast cancer patient's tumour are designated as positive (+), negative (−) or not available (NA).

of creating a daily melatonin signal in humans, by determining the pharmacokinetics of orally administered melatonin. If melatonin administration does arrest breast cancer, it will have significant advantages over traditional therapies, all of which are accompanied by a host of physiological and psychological problems. It is only from clinical trials with melatonin that the role of the pineal in endocrine-related cancers will become clear.

REFERENCES

Almeida OFX, Tamarkin L 1984 Differential response of uterine estrogen receptors to melatonin in adult and juvenile Syrian hamsters. 7th Int Congr Endocrinol Abstr 192

Aubert C, Janiaud P, Lecalvez J 1980 Effect of pinealectomy and melatonin on mammary tumor growth in Sprague-Dawley rats under different conditions of lighting. J Neural Transm 47:121-130

Bartsch C, Bartsch H, Jain AK, Laumas KR, Wetterberg L 1981 Urinary melatonin levels in human breast cancer patients. J Neural Transm 52:281-294

Blask D, Hill S, Orstead M, Massa J 1984 Interaction between melatonin (MEL) and underfeeding in suppressing 7,12-dimethylbenzanthracene (DMBA)-induced mammary tumorigenesis. 7th Int Congr Endocrinol Abstr 324

Burns JK 1973 Administration of melatonin to non-human primates and to women with breast carcinoma. J Physiol (Lond) 229:38P-39P

Danforth DN Jr, Tamarkin L, Do R, Lippman ME 1983a Melatonin-induced increase in cytoplasmic estrogen receptor activity in hamster uteri. Endocrinology 113:81-85

Danforth DN Jr, Tamarkin L, Lippman ME 1983b Melatonin increases estrogen receptor binding activity of human breast cancer cells. Nature (Lond) 305:323-325

Danforth DN Jr, Tamarkin L, Lippman ME 1984 Melatonin-induction of estrogen receptor hormone binding activity is associated with inhibition of E_2-stimulated growth of MCF-7 human breast cancer cells. 7th Int Congr Endocrinol Abstr 494

Danforth DN Jr, Tamarkin L, Mulvihill JJ, Bagley CS, Lippman ME 1985 Plasma melatonin and the hormone-dependency of human breast cancer. J Clin Oncol 3:941-948

Dao TL 1962 The role of ovarian hormones in initiating the induction of mammary cancer in rats by polynuclear hydrocarbons. Cancer Res 22:973-981

Georgiou E 1929 Uber die Natur und die Pathogenese der Krebstumoren. Z Krebsforsch 28:562-572

Hollander VP, Diamond EJ 1978 Hormonal control in animal breast cancer. In: Sharma RK, Criss WE (eds) Endocrine control in neoplasia. Raven Press, New York, p 93-119

Lapin V 1979 Pineal influence on tumor. Prog Brain Res 42:523-533

Shah PN, Mhatre MC, Kothari LS 1984 Effect of melatonin on mammary carcinogenesis in intact and pinealectomized rats in varying photoperiods. Cancer Res 44:3403-3407

Tamarkin L, Cohen M, Roselle D, Reichert C, Lippman M, Chabner B 1981 Melatonin inhibition and pinealectomy enhancement of 7,12-dimethylbenz(a)anthracene-induced mammary tumors in the rat. Cancer Res 41:4432-4436

Tamarkin L, Danforth D, Lichter A et al 1982 Decreased nocturnal plasma melatonin peak in patients with estrogen receptor positive breast cancer. Science (Wash DC) 216:1003-1005

DISCUSSION

Short: Could you very briefly review what is known about circadian rhythms in organ-specific cell mitotic rates?

Tamarkin: I don't think there is consensus of opinion on that, but of course melatonin may well affect some aspects of cellular regulatory mechanisms. We have seen a direct effect of melatonin on uterine oestrogen receptors, and Virginia Fisk has found that melatonin affects ovarian function, but I can't predict what the impact would be on cell cycles. It's surprising in a way that

melatonin acts on the uterus and ovary, because many people believe that the site of action of melatonin should be in the brain.

Reiter: Cell cycles in individual organs generally vary tremendously in terms of peaks and troughs. Rubin et al (1984) have done an interesting study in hamsters where the cell cycles are very uniform. They looked at tongue, heart and a number of other tissues and found that pinealectomy did not modify cell cycle rhythms. This doesn't mean, however, that melatonin does not affect tissues where cell division is dependent upon hormones. In fact, hormone-dependent tumours may be the ones that are most influenced by the pineal; many experiments on melatonin and tumour growth are contradictory, but results are certainly more uniform for hormone-dependent tumours.

Tamarkin: We have seen a diurnal rhythm in oestrogen receptor numbers, but pinealectomy doesn't affect it. So it seems that at least this rhythm is not driven by the pineal gland.

Reiter: The results of your experiments on melatonin effects on the uteri of ovariectomized adult hamsters confirm Roger Hoffman's observations (Hoffman 1978).

Zucker: The difference between juvenile and adult hamsters in responses to melatonin is very interesting and it's possible that an ovarian history-dependent factor accounts for this. Have you investigated whether a single oestrous cycle is enough to discriminate between animals that respond to melatonin with an increase in oestrogen binding and those that respond with a decrease? I predict that a single injection of oestradiol to a juvenile animal would give you the adult pattern.

Tamarkin: You are probably right in that the tissue may not be the same once it sees oestradiol. Our choice of the immature animal was unfortunate in retrospect because it started us off on the wrong track.

Zucker: You used 40 μg of oestradiol to get a uterine growth response in ovariectomized adult hamsters, and I'm sure you appreciate that this is not a physiological dose and that your treatment was essentially a pharmacological manipulation. I cannot explain why you needed such high levels, but did you determine what dose of oestradiol would give you negative feedback inhibition of luteinizing hormone and follicle-stimulating hormone secretion in that preparation?

Tamarkin: No. We were simply looking at uterine growth. We just did not see consistent growth in animals treated with what I consider to be a reasonable dose of oestradiol, i.e. 1 μg.

Bittman: The actions of oestradiol on prolactin levels would also be interesting to investigate, to determine whether oestrogen effects are in fact a reflection of prolactin release. Do you think that if you treated animals with prolactin or gave them ectopic pituitaries you could reverse some of melatonin's effects, particularly those in the adult animal?

Tamarkin: It's very possible.

Bittman: In immature or adult hamsters or in the MCF-7 cells could the effect of melatonin be on the translocation of oestrogen receptors?

Tamarkin: We looked at both nuclear and cytosolic receptors in MCF-7 cells and the effects of melatonin were the same on both, so I don't think translocation is affected.

Bittman: And was the amount of oestradiol bound to nuclear receptors in hamster uteri, which you found required large doses of oestrogen, comparable to what one sees in the rat? In other words, could it just be that there is less efficient translocation in the hamster, so that levels of nuclear receptor occupation (or amounts of a particular class of nuclear oestradiol receptor occupied) are comparable to those found in the uteri of rats given lower doses of oestradiol?

Tamarkin: I don't know. We did not look at oestrogen receptor characteristics in hamster uteri.

Sizonenko: Have you looked for melatonin receptors in cells in the uterus? Do you think there are binding sites for melatonin, or does it act in some other way?

Tamarkin: We've tried to find melatonin binding sites but it is not easy. If you use a high dose of melatonin that you think will be saturating you will get melatonin uptake, but the whole thing falls apart when you try to generate a Scatchard plot. The other problem is that non-specific binding is inexplicably high.

Sizonenko: I'm a little confused by the interpretation of some of your results. You showed that melatonin acutely increased oestrogen binding to MCF-7 tumour cells, but in the long term it decreased oestrogen-stimulated growth of these cells. How can you reconcile these two effects?

Tamarkin: It's a problem. It could be that the short-term effect on oestrogen receptors is a red herring.

Sizonenko: Do you think that the modification of growth is a post-receptor effect then?

Tamarkin: I don't know. The only information I have that may be relevant is on protein kinases. It's been suggested that MCF-7 cells are very dependent on cyclic nucleotides for their growth. Cho-Chung has shown that dibutyryl cyclic AMP will inhibit cell growth, and she has a hypothesis that cyclic AMP-dependent protein kinases are involved in the activation of oestrogen receptors in this cell line (Cho-Chung et al 1981). We looked at the effects of melatonin on cyclic AMP in MCF-7 cells and did not see anything. However, we also investigated the binding of ^{32}P-labelled cyclic AMP to the cells by photoaffinity labelling using an azido derivative and UV light to get specific binding to protein kinases. On a sodium dodecyl sulphate gel we saw labelling of protein kinase I of M_r 48000 (48K), some protein kinase 2 (56K) and its breakdown

product (36K). We did not see binding in the presence of a 1000-fold excess of cyclic AMP, so the bands represent specific binding. In cells treated with melatonin there was a change in this specific binding of cyclic AMP to protein kinases, so perhaps studying oestrogen receptors was the wrong way to look at the problem; it could be that activation of an intracellular event associated with phosphorylation is the most important response to melatonin.

Rollag: I have also been focusing upon the intracellular mechanism of action for melatonin, but in the frog dermal melanophore, where melatonin causes pigment aggregation. This melatonin-induced pigment aggregation can be prevented with pertussis toxin, which chemically modifies the inhibitory nucleotide- binding protein, N_i, in the membrane, and also modifies transducin if it is present. If melatonin acts via N_i, as my pertussis toxin experiments suggest, it probably inhibits the generation of cyclic AMP by adenylate cyclase. Melatonin's actions would then only be evident if adenylate cyclase was in a stimulated state; you would not see any effect on basal cyclic AMP production. In your MCF-7 experiments, an action of melatonin on N_i might explain why you saw effects on oestradiol-stimulated growth but not on basal growth.

Zucker: You have found a correlation between oestrogen receptor concentrations and melatonin. The issue of the relation between hormone levels and other parameters that we measure is an important one. Results from a different system suggest that one should be sceptical about precise correlations between hormone levels and other variables. The study (Damassa et al 1977) was designed to discover whether or not reproductive behaviour in male rats is related to the plasma levels of testosterone. Although copulatory behaviour in rats is highly testosterone dependent, plasma levels of testosterone and sex behaviour of intact animals did not correlate well. Among animals that copulated there was a substantial range in plasma testosterone levels; the lowest concentration of testosterone detected in intact rats was an order of magnitude greater than the minimal testosterone level necessary to activate the behaviour. It is not surprising, therefore, that there was no correlation between the level of the hormone in the blood and the behaviour studied. I wonder whether this is relevant in the present context; perhaps the levels of melatonin that one sees are in every case much greater than those required to mediate any particular function.

Tamarkin: It's not necessarily just absolute concentrations that should be considered. The relative change in levels may be what the system is monitoring. Bruce Goldman and Eric Bittman have shown that for melatonin the *relative* increase in concentration at night is important, and perhaps also the duration of the peak. It seems that the system must 'see' not only high levels but also low levels. Certainly, one cannot expect to get a bigger or faster effect simply by increasing concentrations. In experiments I did in female hamsters with Bruce Goldman, the closer the injections of melatonin were to the onset of the dark

period the more rapid was the response, and it was certainly quicker than in any animal exposed to short days.

Arendt: Are there known effects of photoperiod on tumour growth?

Tamarkin: In a DMBA model, yes.

Reiter: Dave Blask (1984) has shown that rats with exaggerated sensitivity to melatonin, induced by a potentiating factor like anosmia or underfeeding, show reduced growth of DMBA-induced mammary tumours. If these animals are put in short photoperiods or deprived of light there is a further marked inhibition of tumour growth. Hormone-dependent tumours, especially breast tumours, seem to be particularly sensitive.

Sizonenko: In your female rats treated with the DMBA, Dr Tamarkin, what happened to ovarian cycles? We know that there is some sort of imbalance between oestrogen and progesterone in melatonin-treated female rats, and it is possible to decrease the number of oestrous cycles with melatonin.

Tamarkin: We didn't study ovarian cycles; all we did was look for the presence of mammary tumours.

Short: For human breast cancer you suggested that risk was based on family history.

Tamarkin: Yes, to some extent. The parameters analysed were age, number of first-degree relatives with the disease, menarche, parity and history of fibrocystic disease.

Short: So you have looked at reproductive history as well as family history. My point is that recent studies of the incidence of breast cancer in monozygotic and dizygotic twin pairs have shown no difference in concordance rates (Holm et al 1980), which for me rules out the significance of family history. I don't think we can really accept that as a major risk factor.

Tamarkin: In the kindreds that have been followed, it does seem to be a substantial risk factor. But perhaps only a subset of breast cancers are related to family history. It's curious that people in the high risk group are not more likely than members of the normal population to develop hormone-dependent disease.

REFERENCES

Blask DE 1984 The pineal: an oncostatic gland. In: Reiter RJ (ed) The pineal gland. Raven Press, New York, p 253-284

Cho-Chung YS, Clair T, Bodwin JS, Berghoffer B 1981 Growth arrest and morphological change of human breast cancer cells by dibutyryl cyclic AMP and L-arginine. Science (Wash DC) 214:77-79

Damassa DA, Smith ER, Tennent B, Davidson JM 1977 The relationship between circulating testosterone levels and male sexual behavior in rats. Horm Behav 8:275-286

Hoffman RA 1978 Influence of melatonin and the pineal gland on uterine sensitivity to estrogen in hamsters. J Neural Transm (Suppl) 13:367-368

Holm NV, Hauge M, Harvald B 1980 Etiologic factors of breast cancer elucidated by a study of unselected twins. J Natl Cancer Inst 65:285-298

Rubin NH, Reiter RJ, Hokanson JA 1984 Apparent absence of influence of melatonin on the circadian rhythm of cell division. Annu Rev Chronopharmacol 1:215-218

General discussion II

Melatonin assay technology (summarized by Dr J. Arendt)

Arendt: Melatonin has always been a problem to assay so we were pleased to find an alternative in the 6-hydroxymelatonin sulphate assay, at least in humans. The kind of validation procedures we have employed for melatonin radioimmunoassay (RIA) have been chromatography (TLC), comparison with other methods (originally bioassay, but later, via Sheila Fraser in Oxford and Al Lewy, gas chromatography–mass spectrometry [GCMS]) and checking the effects of pinealectomy. In sheep, humans, monkeys and rats pinealectomy reduces circulating dark-phase melatonin to low or undetectable levels; hence, if a given assay system can show this, it is at least measuring something to do with pineal function. We are currently using a direct, unextracted melatonin RIA for work in sheep and humans. It also works well in wallabies. It was largely developed by Sheila Fraser in Oxford, using one of our antibodies. Of course we worry about what else we might be measuring, but extracted melatonin assays also have this problem. We have recently done a comparison between this direct assay and Rollag's assay in sheep with Karsch's group in Michigan—the 24 h profiles were identical, but our absolute values were somewhat lower.

In principle, if assay recoveries are good, then the lower the measured values the better. The noise level of an assay becomes important if, for example, people report variations in day-time levels and attribute significance to them when the noise level is very high. RIA is by far the most extensively used procedure for melatonin assay and is the only practical method for the large number of samples generated by sheep and human circadian and seasonal studies. In fact there has been a great deal of consistency between laboratories in sheep studies. We all agree that the duration of melatonin secretion follows the length of the dark phase even if our absolute levels vary. There is, however, an apparent discrepancy between Lincoln's group and that of Karsch in measurements of melatonin patterns in photorefractory sheep. Likewise, in constant light, not everyone finds that melatonin is always undetectable. Is it possible that exotic photoperiods are generating cross-reactivity?

As you know, Lennart Wetterberg organized an international comparison of melatonin RIAs in human plasma some years ago. Coded samples were assayed blind, in some cases spiked with melatonin, by a number of different

laboratories. It was a very useful exercise—those of us who got it right were given considerable confidence in our technology. If enough people are interested in doing this for sheep or other species, we would be prepared to set it up.

Rollag: I agree with you in many ways. Currently, I am content with the state of the art as regards the melatonin assay. I would include *N*-acetyltransferase (NAT) activity as a good validation tool; it is a good predictor of pineal melatonin content.

I don't think one can ever be absolutely certain that RIA measures melatonin in all samples. One gains confidence with experience, but there is always a chance that in some experimental paradigms, some species, some fluids or some tissues unexpected interference will arise. Thus, there is good reason to have a crisis of confidence when unpredicted responses are found in a new area. The best way to gain confidence in RIA techniques is to demonstrate that independent methods give similar results. One can use other antibodies or, even better, a different technique such as GCMS, bioassay or NAT assay.

A second point I would like to make is that, although RIA methods are usually described in terms of the antibodies used, for an extracted hormone like melatonin it is the extraction methodology that is important. Using the same antibody one can change the purification system to solve most problems that arise. Some separation procedures may be extremely expensive but nevertheless solve the problem; high performance liquid chromatography (HPLC) and gas chromatography are such possibilities. The strength of the melatonin assay is that such purification procedures are easily applied.

Goldman: I agree very much with your comments. Our laboratory is not set up to do HPLC, although I agree that it is needed. From experience with other hormones we consider it useful to compare two antibodies to see if they give the same results. This does not, of course, prove that they are both correct, but if they do not agree then at least one is wrong. We have used this approach in our attempts to achieve a suitable melatonin RIA for serum.

We tried Mark Rollag's 1055 antibody in rodents and, like everyone else, got values in the 100–200 pg/ml range during the day, which no one believes. We then tried another Rollag antibody, 1056, which I believe has not been used before for RIA. With the same extraction procedure, this gave much lower values than the 1055, but our results were somewhat inconsistent from assay to assay. Finally, we set upon using a simple dichloromethane extraction that gave us good, repeatable results. The 1056 antibody always gives much lower values in rodent serum (< 10 pg/ml day-time) and a very clear day/night difference.

We have now attempted to use these two antibodies to compare plasma melatonin values in different species, including several rodents. We find that the greatest discrepancies appear in rodent blood, but there are also discrepancies in most of the other species we have looked at, especially sheep. In

monkeys, and surprisingly in a large series of horse plasmas obtained from Dan Sharp, we have good agreement between results obtained with the R1055 and R1056 antibodies. To illustrate the sort of discrepancy one can find we compared three antibodies: 1055, 1056 and one obtained from David Kennaway. Using pooled day-time blood from Syrian hamsters, extracted with dichloromethane and split up between the three assays, we obtained values of less than 10 pg/ml with 1056, 100–200 pg/ml with 1055 and more than 800 pg/ml with Kennaway's antibody. I am not confident that simply by changing the extraction procedure one can get rid of that kind of error.

Rollag: That is a rather unfair assessment of Kennaway's assay. If I recall correctly, you do not do lipidex purification as he does. The assay is more than the antibody; it is also the purification. It is important that the specified purification procedures are employed when different RIAs are compared.

Arendt: I agree, and would add that assays are very dependent on the operator. It is clear from the literature that the same assay can give beautiful results in one pair of hands and not in another.

Sizonenko: Unfortunately many people using these assays are not critical enough and report values during the day that other people do not find. For example, reports have appeared of 100–200 pg/ml in day-time in humans, but we find a maximum of 5,10 or 20 pg/ml. This is very frustrating in human work. To my knowledge there are only two ways of stimulating human melatonin production during the day. One is by exercise and the other, reported by Desir et al (1983), is with a β_2-agonist. We have repeated this work. We have tested three different types of exercise with trained and untrained subjects on treadmills, and have used marathon and half-marathon runners. We cannot find any change in melatonin concentrations during the day using an RIA which gives a very nice nocturnal rise, and which in pinealectomized patients gives undetectable levels. Dr F. Beguin in the Department of Obstetrics and Gynaecology in Geneva has infused the β_2-agonist into 10 women for us and again we have found no change in melatonin levels during the day-time. This is a real problem because no journal is going to accept these negative results, and I think that those that are published are probably wrong. We should emphasize that people must be very critical about their values.

Arendt: What would you think about an international quality control scheme for melatonin RIA?

Sizonenko: The problem is that only people who are aware of the difficulties will participate in a control scheme.

Marks: Journals may not accept papers where people do not participate in quality control schemes. This is becoming increasingly common in clinical chemistry. If you cannot show that your analytical data are worthwhile, reputable journals are very concerned about publishing it.

Reppert: I am not sure that it has to be formalized. I was involved with the

studies on exercise mentioned by Pierre Sizonenko. The problem could easily be resolved by sending half of the sample to him, but there is still the possibility that experimental differences (protocol, type of exercise, duration etc.) are the root of the problem. I think we must do more work comparing values between ourselves to settle these issues.

Pévet: Dr B. Viven-Roels, in our group, has measured the melatonin concentration in the plasma of a sea-water fish (*Gadus morhua*) using two different antibodies, that of Rollag and that of Claustrat (Kopp et al 1980). With Rollag's antibody she found very high concentrations of melatonin (more than 2000 pg/ml) without clear changes during the day/night cycle. With the antibody of Claustrat, on the contrary, with the same plasma and with the same extraction she found clear day/night variations, with low values (5–200 pg/ml) during day-time and high values (2000 pg/ml) during darkness. Clearly, with the antibody of Rollag, at least during day-time, she was able to detect something that was probably not melatonin.

Wetterberg: We have noted interference by plasticizer in melatonin RIA. Plasticizers have numerous methoxy groups, and it is a matter for speculation whether they have biological actions similar to those of melatonin (Wetterberg et al 1984).

Lewy: We use the negative chemical ionization–GCMS with a deuterated standard to assay melatonin (Lewy & Markey 1978). Each sample is corrected for recovery, so recovery is effectively corrected back to 100%. We measure two fragment ions at a specific retention time and employ all the optimum principles for mass spectrometry. We can usually identify interference by the shape of recorded peaks, using a chart recorder running fast. I frequently run other people's samples for validation purposes and I am happy to do it blind for anyone provided the number of samples is kept down.

Tamarkin: I have some experience of mass spectrometry using a quadrupole system and I don't think you should give the impression that there is one unflawed system that can act as a standard. Even with great care mass spectrometry can be wrong; for instance, mass resolution can be affected by the geometry of the lenses in a quadrupole mass spectrometer, and, more importantly, by the residue on them. Moreover the chemical derivatization employed in GCMS changes not only melatonin but also other molecules. RIAs are very objective when one is analysing a large number of samples, as the sheer numbers reduce bias.

Lewy: RIAs, particularly the recent ones, are very good. Extreme specificity is probably not necessary in some circumstances and assays should be chosen with this in mind. Nevertheless none of the classical RIA validation processes rules out the possibility of occasional interference, so the best method is probably a technique like mass spectrometry that both identifies and measures a substance at the same time. By the way, chemical derivatization improves the

specificity of GCMS. There are several good reviews of the melatonin assays (Arendt 1978, Rollag 1981, Lynch 1982).

Menaker: Does anyone know a good antibody for immunohistochemical localization of melatonin?

[No one was able to answer this question.]

Summary

Melatonin RIAs can be very powerful tools. They require extensive validation by classical techniques and comparison with others, preferably with non-RIA methodology. Both GCMS and HPLC are useful validation techniques, but no one technique should be considered the standard for all other methods. Even GCMS, whilst theoretically the most specific method, can have problems. With a suitable antibody, sample purification should give the necessary specificity, although with highly specific antibodies direct RIA is possible without prior purification. In general, it is important to compare values obtained with as many methods as possible and to swap samples between groups, especially if conflicting results are obtained. In the end, the duplication of results in independent laboratories is the final seal on the validity of an experimental technique.

How important is the duration of the melatonin signal?

Turek: If we want to approach the question of where melatonin acts in the brain and how it acts at a physiological level, we need to identify the characteristics of the melatonin signal that are important for its effects. Bruce Goldman and Eric Bittman have shown that one critical characteristic of the melatonin signal is its duration. For years I fought that idea intellectually, and tried to look instead at the complex relationship between the phase of the melatonin rhythm and a possible internal circadian oscillator. I now feel that Eric and Bruce are probably right, but I would like to see whether we can find some holes in the hypothesis that duration is the only critical factor. Are there data that do not fit this hypothesis? For example, there is some question about whether signal duration is important in the golden hamster, and Stetson I believe has recent data indicating that duration is not the whole story (M. Stetson, personal communication).

Reiter: I think we should entertain several options. Certainly the duration hypothesis is very attractive because it fits with many of the results, but it does not refute other ideas like the coincidence model. It's odd that humans seem to have been overlooked as far as models are concerned; we are worried about

whether levels of melatonin are high or low at night in humans, but for some reason are not concerned about whether a coincidence model or a duration model applies. There are papers relating depression to aberrant melatonin rhythms, but the changes emphasized are alterations in rhythm magnitude rather than duration or coincidence. The emphasis is clearly different for animal models and humans. At the moment I agree with Fred Turek that duration is important, but I don't think we should consider it exclusively.

Fred mentioned the question of where in the brain melatonin works, but I think we must be cautious here. Most of us agree that melatonin has effects in the central nervous system, but there are probably many sites of action and some of the tissues may show rhythms that determine whether they ignore or respond to melatonin at any time. For example, if melatonin administered at one time has an action on the neuroendocrine axis, it doesn't mean that it will have the same effect at another time. I suspect that melatonin's widespread effects on the organism as a whole are not all mediated by a single site within the central nervous system.

Turek: And presumably not all the responses are affected by the duration of the melatonin signal; some may vary with phase or amplitude.

Goldman: History is sometimes a better predictor than science, and on that basis I think we should be very careful about simply accepting the idea that duration is the most important characteristic of the melatonin signal. Eric Bittman and I, and our respective collaborators, probably agree more closely than other people in the field with respect to making predictions based on the idea that the duration of the melatonin peak is critical. I think the reason for our agreement stems from our experiences with melatonin infusion experiments. Most of us have done studies with implants and injections of melatonin, but we frequently disagree over interpretation of the results because, by their nature, such experiments do not allow us to test the hypothesis that duration is critical. Milt Stetson (personal communication) has recently obtained results from the Syrian hamster that may challenge the duration concept, and indicate that phase is more important. However, I believe that when his results are considered in context with the results of the many other injection experiments, the picture is still very confusing for the Syrian hamster. The only way really to answer the question is through an experiment in which it is possible to make a fair comparison between the effects of phase, duration, amplitude and total amount of melatonin, and the only way in which this has been done to date is in studies employing programmed infusions of melatonin.

Hoffmann: Are there any indications from different species that something other than duration is important?

*Goldman:*Milt Stetson (personal communication) has tested the effects of injections at every hour of the day in pineal-intact Djungarian hamsters and the results are very similar to those for Syrian hamsters. So the injection

experiments in Syrian and Djungarian hamsters are comparable, but we only have comparable infusion experiments for Djungarian hamsters and sheep, not for Syrian hamsters.

Bittman: I think we should thank the people who have worked on devising good melatonin assays for giving us a tool for determining when we are in a physiological range. One of the problems in interpreting the old injection experiments is that the melatonin concentrations were often not physiological, and so the results have to be taken with a pinch of salt. Watson-Whitmyre & Stetson (1983), however, have found differences in the effects of the same dose given to Syrian hamsters at different times of day, which leads them to conclusions discrepant from ours based on ewe experiments. So, to determine whether the phase or the duration of the melatonin signal is important, we must ask further questions. Could changes in the rate of degradation of melatonin with the time of day explain some of the discrepancies? What is the relevance of the content of melatonin in the pineal to the secretion of melatonin, in particular in rodents?

I also want to make the point that animals may have pineal-independent means for measuring photoperiodic time. Bartness & Wade (1984) have found effects of photoperiod on the body weight of pinealectomized Syrian hamsters. The neural control mechanisms mediating photoperiodic control of both body weight and reproduction in this species are dependent on the paraventricular nuclei and are driven by the circadian system (Bartness et al 1985). Clearly, if you can get a response in the absence of the pineal, then monitoring the duration of the melatonin signal is not the only way the animal has of measuring photoperiod. Perhaps daylength-responsive, sympathetically innervated structures other than the pineal (e.g. brown adipose tissue) also measure the duration of a circadian-based autonomic input.

My final comment relates to refractoriness to photoperiod, which by extrapolation we could say occurs when the animal becomes insensitive to a melatonin signal of a particular duration. Brian Follett keeps asking me whether the photoperiod influences the rate at which this happens, and I don't think we can answer on the basis of the model which we think can explain reproductive induction or inhibition. We have proposed the existence of a photoperiod-*independent* duration or interval timer, which monitors the pattern of melatonin secretion that is determined by the pineal through the circadian system. That is not necessarily the case for the induction of refractoriness; perhaps there *is* a clock driven by the circadian system, and daylength *does* make a difference at the level of the melatonin target. If we look at one specific case like reproductive induction or inhibition we can draw conclusions, but they do not necessarily extend to other photoperiodic responses.

Lincoln: I would like to try to focus on the way in which the duration signal is read in the hypothalamus. Is there a critical duration effect, with a long-

duration or continuous melatonin signal (for example under short days, from a melatonin implant or from melatonin in the drinking water) producing a short-day response, and a short-duration signal or no melatonin producing a long-day response. How short can the 'short' duration peak of melatonin be each day to induce a long-day response; if this short signal can be distinguished from the absence of melatonin then it may be acting merely as a time cue in the brain, synchronizing other rhythms. Has anyone tested how short the melatonin peak can be to produce a long-day response?

Goldman: In the Djungarian hamster there certainly is a difference between a short-duration signal and no melatonin. If the Djungarian hamster is raised from birth on short days so that its gonads are inhibited, and it is then pinealectomized at 23 days of age, the gonads remain small for a few weeks but can be stimulated by short-duration infusions of melatonin for 4–6 h a day.

Lincoln: But could you give an injection of melatonin and get the same effect?

Goldman: Tim Bartness is doing that now and he seems to be getting positive results (unpublished work). He is using adult Djungarian hamsters that have been pinealectomized after being maintained on short days and that therefore have regressed testes. He can stimulate gonadal recrudescence with small doses of melatonin (1 μg or less) given by injection, but we do not know how long melatonin persists in the circulation after these injections.

Zucker: I think it's possible that in the same animal some end-points may be coded by duration and others by phase. The only evidence that we have is from studies of meadow voles in which we have monitored four different variables. There is a very clear effect of photoperiod: short days will decrease body mass, decrease food intake, increase nest-building activity and cause regression of the reproductive apparatus (Dark et al 1983). If we put animals on long days and give them a melatonin capsule, which provides a relatively constant long-duration melatonin signal, we can simulate some but not all of these responses to short days. Admittedly this is a non-physiological treatment, but the results do raise the possibility that both duration and phase may play a role in the same animal.

Goldman: Tim Bartness has recently had a very similar experience with melatonin implants in Djungarian hamsters (unpublished work), but I do not believe that such results necessarily tell us anything about the relative importance of phase vs. duration of the melatonin peak. I certainly agree that if you give a pharmacological treatment in the form of a constant-release implant all 'photoperiodic' variables do not necessarily respond in the same way in a given animal.

Herbert: Before we accept the duration hypothesis, we must ask ourselves what duration we are talking about. We are all thinking in terms of a neat square wave going up and down, but of course we don't know that the brain

reads it like that. We don't know what the brain interprets as an increased melatonin signal, whether absolute levels or levels relative to the day-time determine the response, or whether indeed some of the variations that we have all seen during the night-time are important. So before we reduce the argument to a simple dichotomy between a time-of-day effect and a duration effect, we must learn a lot more about what 'duration' means. It is certainly important to remember that we are often looking at different kinds of responses. Lise Martinet, for example, has shown very clearly that in the mink one can separate a prolactin response from a gonadotropin response, and it may be that this is simply because the part of the brain responsible for controlling these two systems can read the same melatonin signal in two different ways.

Marks: Can I throw a spanner in the works? Growth hormone, which has not been mentioned, is also secreted episodically during the night, but nobody pretends that it is not working during the rest of the day—we know very well that it is, although we don't really know why or how. In a disease like acromegaly you need not necessarily ever have absolutely high growth hormone levels; it is just that the concentration doesn't drop normally during the day. It's possible that something similar can occur with melatonin. The effect of melatonin may actually persist for very much longer than we think; although we may not be able to detect it in plasma during the day, it may still be above the critical concentration at which it actually exerts its effects. At the moment our assays are not sensitive enough, and when we say that there is no melatonin there we may be wrong.

Turek: I agree. The troughs may be as important as the peaks. Also, we have not discussed the pulsatile nature of melatonin secretion. Is it possible that pulse frequency rather than signal duration is the critical variable?

Klein: Before we accept any theory we should be convinced that the particular variable fits an important criterion: it should reflect an alteration in photoperiod from, for example, 11 h light:13 h dark (11L:13D) to 10½L:12½D because in sheep the gonads are known to respond to such small changes.

Turek: Stetson has measured pineal melatonin in Syrian hamsters exposed to daylengths near the photoperiodic threshold, and there is a difference between the effects of stimulatory and non-stimulatory photoperiods (M. Stetson, personal communication).

Moore-Ede: But that's not the problem. The question is, is the change in the duration of the melatonin signal responsible for the alteration we see in the end-point we are measuring?

Goldman: The closest we have come to answering that is in our infusion experiments in Djungarian hamsters. A 6 h infusion of melatonin does not give any inhibition of testicular growth but an 8 h infusion produces a full effect. So there is a critical difference between 6 h and 8 h signals. With a 7 h infusion we see an intermediate response, so I suspect that the switch-over from no response to full inhibition is very precisely regulated.

Lewy: I would like to go back to the question of what melatonin does. Originally, the amplitude of the melatonin rhythm was the main focus of interest, but now that focus has shifted to the phase and duration of the melatonin signal. With the possible exception of vasopressin, melatonin production is unique in the way it is regulated by the light–dark cycle and in its conserved phase relationship to the light–dark cycle in both diurnal and nocturnal species. I think that somewhere in this unique property of melatonin lies the key to its function.

Closing remarks

Short: It's impossible to summarize the whole of this meeting in a few minutes, so I would like to revert to the words of Sir Francis Bacon (1620); he said, 'The human mind is often so awkward and ill-regulated in the career of invention that it is at first diffident, and then despises itself. For it appears at first incredible that any such discovery should be made, and when it has been made, it appears incredible that it should so long have escaped men's research.' He must surely have been writing about the interrelationship between photoperiodism, melatonin and the pineal gland.

REFERENCES

Arendt J 1978 Melatonin assays in body fluids. J Neural Transm (Suppl) 13:265-278

Bacon F 1620 Aphorism CX, from *Novum organum*. Bonhamum Nortonium & Ioannem Billium, London

Bartness TJ, Wade GN 1984 Photoperiodic control of body weight and energy metabolism in Syrian hamsters (*Mesocricetus auratus*): role of pineal gland, melatonin, gonads, and diet. Endocrinology 114:492-498

Bartness TJ, Bittman EL, Wade GN 1985 Paraventricular nucleus lesions exaggerate dietary obesity but block photoperiod-induced weight gains and suspension of estrous cyclicity in Syrian hamsters. Brain Res Bull, in press

Dark J, Zucker I, Wade GN 1983 Photoperiodic regulation of body mass, food intake and reproduction in meadow voles. Am J Physiol 245:R334-R338

Desir D, Kirkpatrick C, Fevre-Montange M, Tourniaire J 1983 Ritodrine increases plasma melatonin in women. Lancet 1:184-185

Kopp N, Claustrat B, Tappaz M 1980 Evidence for the presence of melatonin in the human brain. Neurosci Lett 19:237-242

Lewy AJ, Markey SP 1978 Analysis of melatonin in human plasma by gas chromatography negative chemical ionization mass spectrometry. Science (Wash DC) 201:741-743

Lynch HJ 1982 Assay methodology. In: Relkin R (ed) The pineal gland: current endocrinology—basic and clinical aspects. Elsevier Biomedical, New York, p 129-150

Rollag MD 1981 Methods for measuring pineal hormones. In: Reiter RJ (ed) The pineal gland: anatomy and biochemistry, vol 1. CRC Press, Boca Raton, p 273-302

Watson-Whitmyre M, Stetson MH 1983 Simulation of peak pineal melatonin release restores sensitivity to evening melatonin injections in pinealectomized hamsters. Endocrinology 112:763-765

Wetterberg L, Sääf J, Norén B, Waldenlind E, Friberg Y 1984 Interference with the radioimmunoassay of melatonin by dimethyl phthalate. J Steroid Biochem 20(6B):1475

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