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THE PINEAL GLAND AND MELATONIN: Molecular and Pharmacologic Regulation

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ABSTRACT

The pineal gland expresses a group of proteins essential for rhythmic melatonin production. This pineal-specific phenotype is the consequence of a temporally and spacially controlled program of gene expression. Understanding of pineal circadian biology has been greatly facilitated in recent years by a number of molecular studies, including the cloning of N-acetyltransferase, the determination of the in vivo involvement of the cAMP-inducible early repressor in the regulation of N-acetyltransferase, and the identification of a pineal transcriptional regulatory element and its interaction with the cone-rod homeobox protein. Likewise, appreciation the physiological roles of melatonin has increased dramatically with the cloning and targeted knockout of melatonin receptors. With these molecular tools in hand, we can now address more specific questions about how and why melatonin is made in the pineal at night and about how it influences the rest of the body.

INTRODUCTION

Because of its remarkable anatomical features—an unpaired spherical organ seemingly located in the center of the brain—the pineal gland has historically

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been regarded as having almost mystical significance. Rigorous scientific analysis of pineal gland function has, however, markedly lagged behind other glandular structures. Descartes made one of the most enduring philosophical statements in pineal biology in the seventeenth century by designating it the seat of the soul. More recently, two observations at the turn of the twentieth century led to many of our more recent scientific insights. One was the discovery that pineal gland extracts lighten the skin of amphibians. The other was that destructive tumors of the pineal gland lead to precocious puberty, which stimulated the work by Kitay & Altschule (1), who demonstrated that pineal gland extracts inhibit ovarian function. In 1958, Lerner and colleagues (2) isolated melatonin, N-acetyl-5-methoxytryptamine, and identified it as the active skin-lightening ingredient of the pineal gland. Axelrod and associates then showed that the biosynthesis of melatonin involves N-acetylation (3) of serotonin by serotonin N-acetyltransferase (NAT) followed by methylation (4) of the 5-hydroxy moiety by hydroxyindole-O-methyl-transferase (HIOMT) (Figure 1). Wurtman and

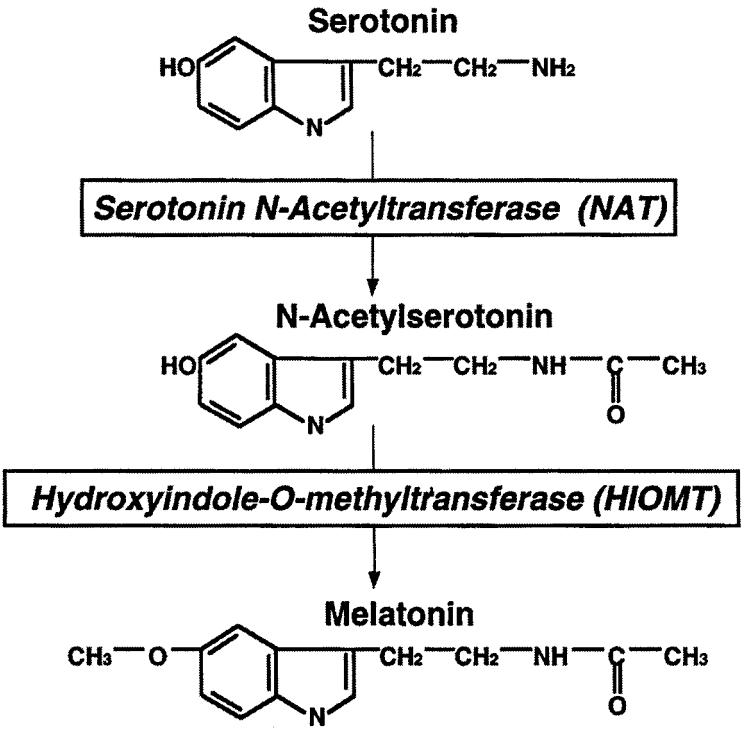

Figure 1 Melatonin synthesis. Serotonin is acetylated by serotonin N-acetyltransferase (NAT) to produce N-acetylserotonin. N-acetylserotonin is then methylated by hydroxyindole-O-methyltransferase (HIOMT) to form melatonin.

colleagues (5) then demonstrated that melatonin is the active ovary-suppressing ingredient of the pineal.

The next series of breakthroughs concerned circadian rhythms in pineal gland function. Quay (6) demonstrated a dramatic rhythm in serotonin concentration in the pineal gland, with levels at noon being up to 10 times higher than values at midnight. Quay also noted that pineal serotonin concentrations are by far the greatest in the body, with noontime concentrations about 100 times higher than those in the brain. HIOMT displays negligible diurnal rhythmicity, and investigators turned to NAT to account for these dramatic fluctuations in serotonin. Klein & Weller (7) discovered a dramatic rhythm in NAT, with nighttime peaks 50–100 times daytime troughs, and reasoned that NAT activity at night accounts for the decreased levels of serotonin that are consumed in melatonin synthesis.

In humans and other primates as well as in rodents, melatonin secretion peaks at night, which is surprising because rodents are nocturnal whereas primates are active during the day. Peak plasma melatonin levels are about 0.3 nM. Thus, to be physiologically relevant, putative melatonin receptors should have subnanomolar dissociation constants, and to be biologically relevant, doses of exogenous melatonin should elicit effects at low nanomolar concentrations. Circulating melatonin is metabolized by the liver P-450 enzymes, which hydroxylate melatonin at the 6-carbon position followed by conjugation with sulfuric or glucuronic acid, creating the principal urinary metabolite, 6-sulfatoxymelatonin. Because its release is maximal at night, melatonin levels in the first morning-urine collection provide a good index of nocturnal secretion.

MOLECULAR CLONING OF NAT

Though the diurnal variations in NAT activity were first reported in 1970, cloning of the NAT cDNA did not take place until 1995, in large part because of the small size of the pineal gland, which precluded obtaining sufficient tissue for enzyme purification. Successful cloning of NAT by two independent laboratories required novel strategies. Coon et al (8) employed expression cloning with a nighttime sheep pineal cDNA library. Borjigin et al (9) utilized a subtractive hybridization technique based on the polymerase chain reaction (10), isolating pineal gland messages that are expressed differentially between night and day. In rats, the time course for expression of NAT catalytic activity and mRNA are essentially the same, with an abrupt increase in expression between midnight and 0200 and a similarly abrupt decline from peak levels at 0600 to undetectable values at 0800, 1 h after the lights are turned on in the morning. The perfect coincidence in rats in the magnitude and timing of NAT mRNA, catalytic values, and protein (J Borjigin, SH Snyder, unpublished data) indicates that the NAT rhythm is driven primarily by transcriptional alterations. By contrast, posttranscriptional control may be much more important in sheep,

in which NAT activity has a sevenfold variation whereas mRNA values vary less than twofold. Similarly, in monkeys, nighttime elevations in NAT activity greatly exceed the modest increases in mRNA (11). Light exposure during subjective night elicits a precipitous decline of rat NAT protein and catalytic activity. This decrease appears to be mediated by proteasomal degradation of NAT, as several proteasomal inhibitors block the decline (12).

The chicken pineal gland provides additional insights into NAT regulation, as—unlike mammalian pineals—it has an endogenous clock, with NAT rhythms persisting in organ culture (13, 14). In addition, chicken pineal gland is photoreceptive, so exposure of the pineal to light alters the NAT rhythm (15). In chick pineal cultures, protein synthesis inhibitors increase NAT mRNA throughout the day and night, which suggests that a protein with rapid turnover suppresses NAT mRNA (16).

The dissociation between transcriptional and translational control of NAT is more dramatic in fish, another species whose pineals are photosensitive and contain endogenous clocks. In trout, NAT mRNA is the same during both day and night, despite a robust rhythm of NAT (17). Pike behave very much like chickens in that the pineal gland possesses an endogenous clock and has photosensitive pinealocytes (18). NAT mRNA varies diurnally in pike pineal gland and is maintained under constant lighting conditions. Zebrafish NAT is regulated in a similar fashion (17).

PINEAL SPECIFICITY OF MELATONIN FORMATION

Tissue-specific expression of proteins is often regulated by transcription factors that are more-or-less unique for individual tissues (19). Well characterized examples are the olfactory-specific transcription factor Olf-1 (20) and myo-D, which is selective for muscle tissue (21). Such transcription factors act on consensus regulatory elements present in the promoters of tissue-specific proteins. Tissue-specific proteins in the pineal include NAT and HIOMT. Additionally, in the night subtractive pineal cDNA library used to discover NAT, we identified an alternatively spliced form of ATP7B, a copper transporter disrupted in Wilson's disease, a disorder of copper metabolism. This night-specific protein, designated pineal night-specific ATPase (PINA), is expressed a hundred times more at night than during the day (J Borjigin, SH Snyder, unpublished data). A twelve-nucleotide sequence in the upstream promoter region of PINA binds factors found exclusively in pineal and retina nuclear extracts (22). Similar sequences that bind the pineal- and retina-specific nuclear factor occur in the promoter regions of NAT and HIOMT and in other areas of the PINA gene. These sequences have been designated the pineal regulatory element (PIRE) (22). PINA possesses seven PIRE sequences, whereas the NAT promoter has three and the A and B promoters for HIOMT have four and three, respectively.

Independently, researchers searching for retinal-specific transcription factors identified a novel member of the homeobox gene family that is selectively expressed in the retina and the pineal gland and that has been designated CRX (23–25). CRX accounts for at least part of the PIRE binding activity of pineal and retina extracts (22). CRX mRNA is abundant in the pineal during the day and increases by threefold at night, with a nocturnal peak that precedes that of NAT by 1–2 h. The high daytime expression and the modest diurnal rhythm of CRX transcripts suggest that CRX primarily directs tissue-specific expression rather than nighttime specificity.

Besides sharing exclusive expression of CRX, the retina and pineal gland manifest a number of other similarities. For example, pineal proteins such as NAT, PINA (J Borjigin, SH Snyder, unpublished data), and HIOMT are also expressed in the retina. In addition, a number of retina-specific proteins also occur in the pineal gland. In situ hybridization studies reveal very high levels of retina-specific markers in neonatal rat pineal gland (26), including rod and cone phosphodiesterases, interphotoreceptor retinoid binding protein, rod cyclic nucleotide channels, visual pigments, transducin, and arrestin. Critical cone-related elements are sometimes expressed in the pineal at levels exceeding those of the retina.

NIGHT SPECIFICITY OF MELATONIN SYNTHESIS

Circadian rhythms in NAT activity are observed in all species examined and form the biochemical basis of the melatonin rhythm. NAT and melatonin rhythms are regulated by a suprachiasmatic nucleus (SCN) clock and light, in the form of adrenergic innervation of the pineal. Norepinephrine acts at beta-adrenergic receptors to stimulate cAMP levels in the pineal. Removal of the superior cervical ganglia interferes with the regulation of NAT by light. cAMP activates cAMP-dependent protein kinase, which phosphorylates CRE binding protein (CREB), which in turn binds to CRE sites to activate transcription. Feedback regulation of this system at the transcriptional level determines the abrupt rises and falls in pineal NAT.

Besides CREB, CRE is also regulated by another family of leucine zipper transcription factors designated cAMP response element modulators (CREM). Some members of the CREM family are also activators, but others, derived by alternative splicing, are repressors (27). One of these is the inducible cAMP early repressor (ICER) (28), a small protein, which contains only the DNA-binding element and functions as a dominant repressor of cAMP-induced transcription (29). ICER lacks domains for activation and phosphorylation; thus, unlike CREB, it is not influenced by phosphorylation. Interestingly, ICER mRNA displays a pronounced diurnal variation in the pineal gland, with a peak during the second part of the night just preceding the decline of melatonin synthesis. By

contrast, CREB is a constitutive protein regulated by norepinephrine-induced phosphorylation in the pineal gland (30).

The promoter region of NAT contains CRE elements that bind ICER as well as CREB. The physiologic role of the interaction between ICER and CRE has been established in mice with targeted deletion of the CREM gene (31). In CREM knockout mice, NAT expression retains its diurnal pattern of expression, but at substantially higher levels at all times compared with controls. The enhancement of NAT expression in CREM knockouts indicates that ICER normally down-regulates NAT expression. In vitro studies utilizing a reporter construct attached to the NAT promoter indeed demonstrate that ICER inhibits NAT expression (31). Not all diurnal genes are CREM sensitive; the fos-related antigen (Fra-2), a transcription factor that also varies diurnally in the pineal gland (32), does not change in CREM knockout mice.

These findings indicate that the diurnal regulation of NAT depends on a delicate interplay of the various transcription factors that bind to CRE. Adrenergically stimulated phosphorylation of CREB at night turns on NAT transcription. The extent of this transcription is itself determined by the balance between CREB phosphorylation by protein kinase and dephosphorylation. Dephosphorylation of CREB leads to a decline of the unstable ICER mRNA. Inhibition of NAT transcription occurs through ICER, which is subject to transcriptional activation by CREB and feedback inhibition by itself. Though ICER mRNA has a marked diurnal rhythm, ICER protein levels are relatively stable. Thus, it appears that throughout the 24-h cycle, ICER can bind to the CRE element in NAT and modulate the rate and magnitude of NAT induction (33).

ROLE OF THE PINEAL AND MELATONIN IN MAMMALS

A key function of the pineal gland is to transform information about environmental lighting into biological rhythms, which has led to its designation in some species as a "third eye." In mammals, light information reaches the pineal gland via a circuitous route. The mammalian pineal consists of the large, cone-shaped, superficial pineal connected by a stalk to the deep pineal that is intimately associated with the habenula from which it may derive partial innervation. However, the principal innervation of the pineal emanates from the peripheral sympathetic nervous system, which conveys the influence of light (Figure 2). Light-dark information detected by the retina is relayed by the retinohypothalamic pathway to the SCN of the hypothalamus (34), which has been established as the principal biological clock in mammals. Cells from the SCN project to the paraventricular hypothalamic nucleus (35). Fibers from this nucleus descend to synapse in the intermediolateral column of the spinal

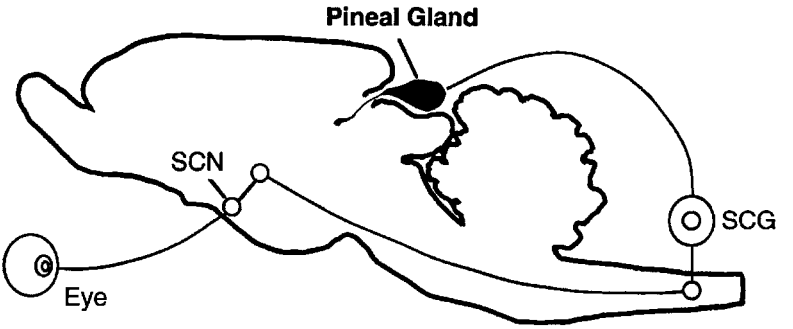

Figure 2 Neuronal pathway regulating pineal melatonin synthesis in rats. Light-dark information is transmitted to the pineal through the suprachiasmatic nucleus (SCN) and superior cervical ganglion (SCG).

cord (36). Preganglionic sympathatic neurons from this region then project to the superior cervical ganglia (37) from which postganglionic neurons ascend along the internal carotid artery to enter the pineal gland (38). The circadian release of norepinephrine at night (39) then determines the NAT and melatonin rhythms in the pineal.

Melatonin, the only known hormonal output of the pineal, has various biological effects ranging from light-dark entrainment of behavioral and physiological response to regulation of seasonal reproductive activity (40). How does melatonin exert these effects? The best insights into actions of melatonin at target organs come from studies of melatonin receptors. These have been made possible by the development of iodo-melatonin as a radioligand (41). 2-[^{125}I]iodomelatonin is about 10 times more potent than melatonin itself in receptor interactions, and 2-[^{125}I]iodomelatonin has been a valuable ligand for labeling receptors. Autoradiographic investigations reveal high-affinity binding sites in the SCN, pars tuberalis, and retina. The affinity of 2-[^{125}I]iodomelatonin for this site is in the picomolar range, and the receptor is coupled to pertussus toxin–sensitive guanine nucleotide binding proteins (42). Fairly substantial levels of receptor binding also occur in the paraventricular nucleus of the thalamus, anterior hypothalamus, and many other parts of the brain. Receptors in the anterior hypothalamus may be responsible for actions of melatonin on reproductive behavior (43), especially in photoperiodic rodents. The pituitary receptors probably mediate photoperiodic regulation of prolactin secretion (44). Nakazawa et al (45) showed that melatonin potently inhibits luteinizing hormone release from the median eminence. Melatonin receptors in the inner plexiform layer of the retina may be responsible for melatonin's effects on retinal physiology (42). High-affinity melatonin receptors have also been reported

in various blood vessels and may mediate the hypothermic actions of melatonin (46).

Of all the target tissues of melatonin, the SCN has attracted the most attention because of its central role in circadian rhythms. Neuronal firing of SCN neurons recorded in vitro exhibit a circadian rhythm that peaks during the light phase and is minimal during the dark phase of the circadian cycle (47). Melatonin can inhibit SCN firing in vitro in organ culture experiments (48–50). The circadian peak in SCN neuronal activity can be phase-shifted by melatonin in a dose- and time-dependent manner in vitro (47, 51). Melatonin can entrain mammalian circadian rhythms (52, 53) and attenuate the phase-delaying effects of light pulses applied during subjective night (54).

Molecular cloning of melatonin receptors has greatly enhanced our insight into melatonin-SCN interactions. Expression cloning of the receptors was based on the ability of melatonin to induce aggregation of melanin in amphibian melanophores. Utilizing an immortalized cell line of *Xenopus laevis* dermal melanophores for the expression cloning strategy, Ebisawa et al (55) cloned a high-affinity receptor designated Mel1A, which was then shown to be present in all mammalian species (56). Subsequently, a separate Mel1B receptor was cloned from mammalian species and shown to be about 60% identical at the amino acid level to Mel1A (57). The receptors differ somewhat in expression localization, with Mel1A being most concentrated in the SCN and other brain regions, whereas Mel1B appears to be more prominent in the retina. Targeted deletion of Mel1A receptors has revealed surprising differential actions of melatonin within SCN (58). 2-[^{125}I]iodomelatonin binding to receptors throughout the brain, detected by autoradiography, is essentially abolished in mutant animals, fitting with earlier evidence that quantitatively, Mel1A receptors are much more prominent than are Mel1B sites. In SCN organ cultures, the inhibitory effects of melatonin on firing are abolished in the Mel1A knockouts. By contrast, the phase-shifting effect of melatonin on SCN firing persists in knockout mice. Conceivably the phase shifting is mediated by the low levels of Mel1B receptors detected by PCR analysis in SCN. These data suggest that the predominant Mel1A receptor mediates the inhibitory action of melatonin on the SCN, and the low-level Mel1B receptor may be involved in the phase-shifting response of melatonin (58). Drugs specific to each of the melatonin receptor subtypes may shed light on the differential actions of melatonin in the central nervous system.

PHARMACOLOGIC AND POTENTIAL THERAPEUTIC ACTIONS OF MELATONIN

There is limited evidence that physiologic secretion of melatonin normally regulates the sleep cycle. In one study, serum melatonin levels were significantly

lower in elderly insomniacs than in age-matched non-insomniac individuals (59). In another study, electrophysiologic recordings provided evidence that the steepest increase in nocturnal sleepiness correlates with the rise in urinary 6-sulfatoxymelatonin excretion (60).

Many studies have examined the hypnotic actions of melatonin (61). Although consistent hypnotic effects have been observed, especially with daytime administration (62, 63), these are not as prominent as those obtained with conventional sleeping medications such as benzodiazepines. Many of the studies have employed relatively high doses of melatonin, about 50 mg, which produce micromolar plasma levels that could well interact not only with melatonin receptors, but with a variety of serotonin receptor subtypes. To approximate physiologic levels of melatonin, a study by Wurtman and colleagues (64, 65) evaluated doses as low as 0.1–1.0 mg, which produce serum levels of about 0.3 nM, comparable to nocturnal peaks. In a 30-min sleep test conducted at midday, doses as low as 0.1 mg consistently decreased sleep-onset latency by about 10 min and increased sleep duration by about 10 min. In this study, as in many others, low doses of melatonin also decreased body temperature. Whether there is any link between the hypothermic effects of melatonin and its sedative actions is unclear.

A number of studies have investigated the role of melatonin in phase-shifting of the sleep-wake cycle (66). The therapeutic potential of these studies includes the use of melatonin in treating jet lag (67) and in entraining blind subjects to the 24-h rhythm (68, 69). There is solid evidence in animal studies supporting a differential sensitivity of melatonin targets, such as SCN, to the hormone depending on the time of day (47, 70). In one study of subjects traveling eastward across many time zones, 5 mg of melatonin given at 6:00 PM before departure and at bedtime after arrival hastened adaptation to sleep and alleviated jet-lag symptoms (71). Variable effects of melatonin on jet lag have been obtained in other studies (69). Whether these actions reflect a direct hypnotic effect or a resynchronization of the circadian rhythm is unclear.

Melatonin is a free radical scavenger and, thus, has been promoted as an antioxidant agent (72) with potential roles in treating cancer and modulating aging and other conditions. In one study, melatonin appeared to be more effective in protecting against oxidative damage than other antioxidants, including vitamin E, glutathione, and mannitol (73). The antioxidant effects of melatonin occur at concentrations thousands of times higher than physiologic levels. Whether or not melatonin proves to be an effective antioxidant drug in vivo is unclear (74), but it certainly does not function physiologically as an antioxidant, except conceivably within the pineal gland itself.

Melatonin has also been reported to influence immune responses. There are reports of melatonin receptors with a dissociation constant of about 0.3 nM in human CD4 lymphocytes, but not in B lymphocytes (75), and Mel1a mRNA

was detected in lymphocytes from rat thymus and spleen (76). Melatonin enhances the production of interleukin-4 in bone marrow T-helper cells and of granulocyte-macrophage colony-stimulating factor in stromal cells (77). It also can protect bone marrow cells from apoptosis (78). Because these studies were also performed using concentrations of melatonin much higher than physiologic levels, it remains to be seen whether at physiological concentrations the hormone can produce these effects (79).

SUMMARY

Based on molecular cloning of NAT, identification of the mechanisms for selective nighttime and pineal specific regulation of pineal gene expression, characterization and cloning of melatonin receptors, and the use of melatonin receptor knockout mice, understanding of pineal physiology has increased dramatically in the last few years. It is now clear that the pineal gland is crucial in communicating the effects of light to a variety of biologic rhythms. The 100-fold diurnal variation in NAT expression and its exquisite regulation by the cAMP response element system underlie the ability of the pineal gland to carry out its entraining role. The prominent regulation of NAT at transcriptional, translational, and other levels may serve as a paradigm for characterizing other regulatory proteins.

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