

43 Circadian Rhythms in Indole Metabolism in the Rat Pineal Gland

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ABSTRACT There are circadian rhythms in the concentration of serotonin, N-acetylserotonin, and melatonin in the rat pineal gland. These rhythms are regulated by the rhythm in the activity of the enzyme that converts serotonin to N-acetylserotonin, N-acetyltransferase. The rhythmic changes in the activity of this enzyme are controlled transsynaptically by norepinephrine. The effects of norepinephrine are mediated intracellularly by a cyclic AMP mechanism requiring protein synthesis. The endogenous neural signals that drive this system appear to originate in the CNS, possibly in the suprachiasmatic nucleus. The generation or the transmission of these signals can be blocked by environmental lighting acting via the eye.

INDOLE METABOLISM in the rat pineal gland is remarkably dynamic during the course of a typical day. Under conditions that provide alternating periods of light and darkness of about 12 hr, daily rhythms occur in the activities of the enzymes and the concentration of the compounds in the pathway that converts serotonin to melatonin. Most of these rhythms have been found to be truly circadian: They persist when animals are in constant darkness or are blinded. These rhythms and the questions of how these rhythms are generated and integrated are discussed in this chapter.

Rhythms in the serotonin-melatonin pathway in the rat pineal gland

The conversion of serotonin (5-hydroxytryptamine) to melatonin (N-acetyl 5-methoxytryptamine) involves two enzymes and the intermediate, N-acetylserotonin (N-acetyl 5-hydroxytryptamine). The enzyme that converts serotonin to N-acetylserotonin is serotonin N-acetyltransferase (E.C.2.3.1.5); the acetyl group comes from acetyl coenzyme A (Weissbach et al., 1960; 1961). The enzyme that converts N-acetylserotonin to melatonin is hydroxyindole-O-methyltransferase (E.C.2.1.1.4); the methyl group donor is S-adenosyl methionine (Axelrod and Weissbach, 1961). Details of this pathway appear in Figure 1.

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During the hours of daylight, the concentration of serotonin in the rat pineal gland is about 0.5 mM (Quay, 1963; Snyder et al., 1965; Illnerová, 1971). At night the concentration gradually falls to about one-half this value. Concurrently, the activity of serotonin N-acetyltransferase increases and reaches values that are 15- to 70-fold higher than the day values (Klein and Weller, 1970a; Ellison et al., 1972; Deguchi and Axelrod, 1972a). This increase in enzyme activity is accompanied by an increase in the concentration of N-acetylserotonin to values that are 10- to 30-fold greater than day values (Klein and Weller, 1973a). The concentration of melatonin also increases at night and reaches values that are about 7- to 10-fold greater than the day values (Quay, 1964; Lynch, 1971). The activity of hydroxyindole-O-methyltransferase has been reported to increase at night (Axelrod et al., 1965; Klein and Lines, 1969). This increase is much smaller than that of N-acetyltransferase, N-acetylserotonin, or melatonin and has been difficult to observe consistently (Quay, 1967; Lynch and Ralph, 1970; Reiter and Klein, 1971).

When the dark-light transition takes place there is a rapid decrease (halving time \cong 3 min) in the activity of N-acetyltransferase (Klein and Weller, 1972; Deguchi and Axelrod, 1972a) and a rapid increase in the concentration of serotonin to daytime values within 14 min (Illnerová, 1971). A rapid decrease in the concentration of N-acetylserotonin also occurs (Klein and Weller, 1973a). The melatonin content of the pineal gland also decreases at this time (Lynch, 1971), but the rate of this change has not been reported.

The data describing these rhythms have been abstracted from the original reports referenced above or unpublished data from this laboratory as detailed below and are presented in Figure 1. The lighting cycles were not precisely the same in the original reports. For the sake of comparison, we have altered the length of the light and dark periods (no more than 2 hr) to normalize the lighting cycles used in the original studies. The serotonin graph is based on the reports of Snyder et al. (1965), and Illnerová (1971). The N-acetyltransferase graph is based on several

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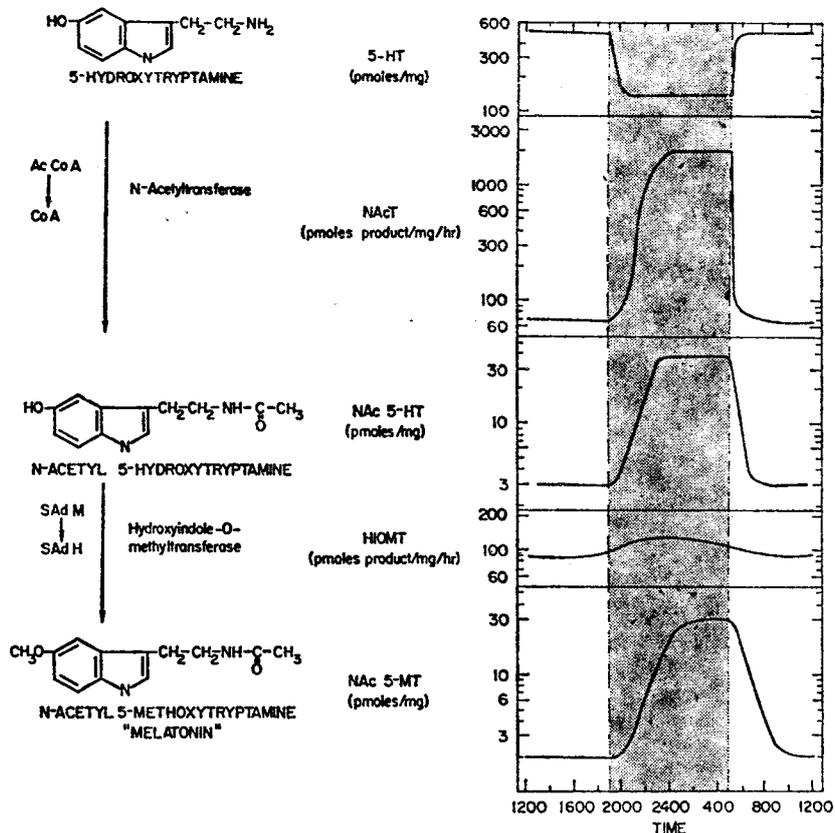


FIGURE 1 Rhythms in indole metabolism in the rat pineal gland. The metabolic pathway from 5-hydroxytryptamine to melatonin is on the left. The daily variations in the concentrations of metabolites and activities of enzymes are on the right. The shaded portion indicates the dark period of the lighting cycle. The data have been abstracted from reports in the liter-

ature as detailed in the text. AcCoA, acetyl coenzyme A; CoA, coenzyme A; S AdM, S-adenosyl methionine; S AdH, S-adenosyl homocysteine; 5-HT, 5-hydroxytryptamine, serotonin; NAcT, N-acetyltransferase; HIOMT, hydroxyindole-O-methyltransferase; NAc 5-MT, N-acetyl 5-methoxytryptamine, melatonin.

reports from this laboratory (Klein and Weller, 1970a; Klein et al., 1971b; Klein and Weller, 1972). The N-acetylserotonin graph is based on the report of Klein and Weller (1973a). The hydroxyindole-O-methyltransferase graph is based on the reports of Klein and Lines (1969) and Reiter and Klein (1971) and on unpublished reports from this laboratory. The melatonin graph is based on the report of Lynch (1971).

The following hypothesis has been proposed to explain the integrated regulation of the rhythms in indole metabolism (Klein et al., 1971a). The initial perturbation in the system is the increase in the activity of N-acetyltransferase. This causes a 20- to 70-fold increase in the rate of conversion of serotonin to N-acetylserotonin and results in the 50% decrease in the steady state levels of serotonin and the 10- to 30-fold increase in the concentration of N-acetylserotonin. The latter effect causes an increase in the production of melatonin by mass action: With more substrate available more product is produced,

because the amount of N-acetylserotonin in the pineal gland, assuming uniform distribution, is never at concentrations that saturate hydroxyindole-O-methyltransferase (Klein and Weller, 1973a). The increased production of melatonin causes a 7- to 10-fold increase in the gland content of melatonin and the increased release of melatonin. It is probable that large tonic changes in hydroxyindole-O-methyltransferase activity produced by constant light or darkness (Axelrod et al., 1965) will modify the effects of large changes in N-acetyltransferase activity on melatonin production.

The rapid changes in indole metabolism also appear to be due primarily to the change in the activity of N-acetyltransferase. When it drops, the production of N-acetylserotonin is apparently cut off, which results in a decrease in the concentration of N-acetylserotonin and melatonin. The content of serotonin increases apparently because serotonin is not being used in the N-acetylation pathway.

THE CIRCADIAN VERSUS DAILY NATURE OF THE RHYTHMS IN INDOLE METABOLISM IN THE PINEAL GLAND The term *circadian* is used by the student of biological periodicities to describe a rhythm that has two characteristics: a complete cycle of about a day's length, and the persistence of the rhythm in the absence of lighting cues under conditions of either constant darkness or constant lighting. This sets the endogenously generated circadian rhythms apart from the exogenously generated daily rhythms that are totally dependent upon environmental cues of lighting transitions (light→dark; dark→light) and disappear in the absence of lighting cues in both constant darkness and constant lighting. The rhythms in the concentration of serotonin, N-acetylserotonin, melatonin, and the activity of N-acetyltransferase are all circadian; they persist in sighted animals in the dark or in blinded animals in constant lighting (Snyder et al., 1965; Klein and Weller, 1970a; Ralph et al., 1971; Klein et al., 1971b; Reiter et al., 1971). In contrast, the rhythm in hydroxyindole-O-methyltransferase is a daily rhythm; it is absent in conditions of constant lighting and constant darkness (Axelrod et al., 1965).

THE EFFECT OF LIGHT ON THE CIRCADIAN RHYTHMS IN PINEAL INDOLE METABOLISM Although the circadian rhythms in indole metabolism in the pineal gland do not depend upon lighting for their generation, all are known to be influenced by light. The changes that are associated with the light→dark transition do not occur if lighting is maintained, and the circadian rhythms are never detected when animals are kept in constant light conditions (Snyder et al., 1965; Klein and Weller, 1970a; Reiter and Klein, 1971; Ralph et al., 1971; Klein and Weller, 1973a). In all cases light has been shown to act via the eye. In the next section the mechanism through which light appears to act on the generation of the circadian rhythms is discussed.

GENERATION OF THE CIRCADIAN RHYTHMS IN PINEAL INDOLE METABOLISM AND INTEGRATION OF LIGHTING SIGNALS The circadian rhythms in pineal serotonin, N-acetylserotonin, and N-acetyltransferase activity depend upon neural input (Fiske, 1964; Klein and Weller, 1973a; Klein et al., 1971a). All the neural input to the pineal gland comes from the superior cervical ganglia (Ariens-Kappers, 1960). Removal of these ganglia, which leads to the disappearance of pineal nerve fibers, or decentralization of the ganglia, which leaves the post-ganglionic connections to the pineal gland intact but cuts input to the ganglia, abolishes the rhythms in serotonin and N-acetyltransferase activity (Snyder et al., 1965; Klein et al., 1971a). The N-acetylserotonin rhythm is also abolished by removal of the ganglia (Klein and Weller,

1973a). Electrical stimulation of the sympathetic nerve chain leading to the ganglia increases the activity of N-acetyltransferase (Volkman and Heller, 1971). These observations indicate that the rhythms are not generated in the pineal gland or in the superior cervical ganglia but rather that they are driven by a generator located distal to the superior cervical ganglia.

The superior cervical ganglia receive inputs from cell bodies in the upper thoracic spinal segment, which are in turn controlled by nerve fibers originating in central structures. In studies on the central structures involved in the regulation of the N-acetyltransferase rhythms that are detailed by Moore elsewhere in this volume, it has been found that lesions in the medial forebrain bundle, which transmits impulses to the superior cervical ganglia, block the rhythm in pineal N-acetyltransferase. This indicates that the endogenous driving mechanism is probably in the brain. It has also been shown that lesions of the supra-chiasmatic nuclei, a pair of cell groupings immediately above the optic chiasm in the anterior hypothalamus, also block the pineal N-acetyltransferase rhythm. When all the input to this region appears to be obliterated, the rhythm in N-acetyltransferase activity persists, indicating that the supra-chiasmatic nuclei may be the originating site of the circadian rhythm generator driving the N-acetyltransferase rhythm.

As we have discussed above, constant light can block the rhythm in N-acetyltransferase activity. Moore et al., in this volume, have demonstrated the existence of a unique neural pathway from the retina to the supra-chiasmatic nuclei. This retinohypothalamic projection consists of unmyelinated fibers that course with the primary optic nerves and branch off at the level of the optic chiasm. Since this projection is the only apparent direct input from the retina to the supra-chiasmatic nucleus, it is possible that it is by these fibers that light can act to either block the generation of the circadian rhythm signals or their transmission. Present collaborative investigations by this laboratory and Moore's are directed at testing the hypothesis that the retinohypothalamic projection mediates the effects of light on the N-acetyltransferase rhythm and that the supra-chiasmatic nucleus is the site of the endogenous circadian rhythm generator.

TRANSSYNAPTIC GENERATION OF INDOLE RHYTHMS As mentioned above, the rhythms in N-acetyltransferase activity, serotonin concentration, and N-acetylserotonin concentration are blocked when neural input to the pineal gland is blocked by either decentralization or removal of the superior cervical ganglia. The nerve fibers that leave the superior cervical ganglia and terminate in the pineal gland contain norepinephrine (Pellegrino de Iraldi and Zieher, 1966; Bondareff and Gordon, 1966; Wolfe et al.,

1962). The possibility that norepinephrine mediates the neural regulation of indole metabolism by being released from nerve fibers in the pineal gland and stimulating pinealocytes has been investigated with organ culture of the pineal gland. This technique makes it possible to maintain a pineal gland under defined conditions (Shein et al., 1967; Klein and Weller, 1970b). When cultured pineal glands are treated with L-norepinephrine there is an increase in the activity of N-acetyltransferase (Klein et al., 1970a; Klein and Weller, 1973b), an increase in the N-acetylserotonin in pineal gland (Klein and Weller, 1973a), and a decrease in the amount of serotonin in pineal glands (Klein et al., 1973). L-Norepinephrine treatment also causes an increase in the conversion of radiolabeled tryptophan to radiolabeled N-acetylserotonin (Klein and Berg, 1970) and melatonin (Axelrod et al., 1969; Klein and Berg, 1970) in the culture medium and an increase in the total amount of N-acetylserotonin in the culture medium (Klein and Weller, 1973a). These effects are mediated by a highly specific receptor that is not responsive to indoleamines and is in general less responsive to closely related catecholamines and to the D-isomeric form of norepinephrine. In addition, all these effects of norepinephrine are blocked by a beta-adrenergic receptor blocking agent, propranolol, indicating that this is a beta-receptor (Wurtman et al., 1971; Klein et al., 1973b; Klein and Weller, 1973a, b). Deguchi and Axelrod (1972a, b) have also presented in vivo evidence indicating that the activity of N-acetyltransferase is regulated by a beta-adrenergic receptor. From these in vivo and in vitro findings, it seems highly probable that norepinephrine is the neurochemical involved in the transsynaptic transmission of neural information regulating the activity of N-acetyltransferase, which in turn causes the changes in the concentration of serotonin and the production and concentration of N-acetylserotonin and melatonin to occur. The adrenergically regulated changes that occur in organ culture are similar to those that occur at night in the dark. This means that during the night in the dark the release of norepinephrine occurs. Conversely, light must block this release.

Recent studies on the regulation of the rapid decrease in N-acetyltransferase activity, which appears to be responsible for the rapid changes in serotonin and N-acetylserotonin in the pineal gland, indicate this decrease may be under adrenergic control. A single injection of a beta-adrenergic blocking agent to an animal in the dark results in a rapid decrease in enzyme activity (Deguchi and Axelrod, 1972a). Thus it would appear that simple displacement of the norepinephrine by the blocking agent is sufficient to cause a rapid reversal of the norepinephrine effects. However, in unpublished studies, we attempted

similar experiments in organ culture using compounds known to compete with norepinephrine at postsynaptic sites. All were used at 10- or 100-fold higher concentrations than was norepinephrine. Treatment with these compounds did not reverse the effects of a 6 hr treatment with norepinephrine. This failure may only be a result of the artificial nature of organ culture. It is also possible that these findings are an indication that the displacement and removal of norepinephrine alone may not be sufficient to reverse the effects of norepinephrine and that another active mechanism is involved in the reversal of the stimulatory effects of norepinephrine.

THE MECHANISM OF ACTION OF NOREPINEPHRINE The transsynaptic regulation by norepinephrine of the dark induced changes in indole metabolism appears on an intracellular level to involve adenosine 3',5'-monophosphate (cyclic AMP). Norepinephrine, acting via a beta-adrenergic receptor, stimulates the activity of adenylyl cyclase in broken cell preparations (Weiss and Costa, 1967; 1968) and increases the concentration of cyclic AMP in cultured pineal glands (Strada et al., 1972). The effects of norepinephrine on indole metabolism are mimicked by ⁶N, 2'O-dibutyryl cyclic AMP (dibutyryl cyclic AMP), an analog of cyclic AMP that can inhibit the breakdown of cyclic AMP by pineal phosphodiesterase, and by theophylline (Klein and Berg, 1970; Berg and Klein, 1971). The following effects of norepinephrine on indole metabolism are mimicked by dibutyryl cyclic AMP: stimulation of the conversion of radiolabeled tryptophan and serotonin to radiolabeled N-acetylserotonin and melatonin (Shein and Wurtman, 1969; Klein et al., 1970b; Berg and Klein, 1971; Klein and Weller, 1973b), increased amount of N-acetylserotonin in the glands and medium (Klein and Weller, 1973a), increased activity of N-acetyltransferase (Klein et al., 1970a; Klein and Weller, 1973b), and decreased amount of serotonin in the gland (Klein et al., 1973).

The mechanism of action of cyclic AMP is not clear. The effect of both norepinephrine and dibutyryl cyclic AMP are blocked by cycloheximide but not by actinomycin D (Klein and Berg, 1970), which indicates that new synthesis of RNA is not required but that new synthesis of protein is necessary. This may mean that new molecules of active N-acetyltransferase are made. However, it is also possible that inactive N-acetyltransferase molecules are made continually and that the effect of norepinephrine is to stabilize the newly formed enzyme. It is also possible that inactive N-acetyltransferase molecules are always available and that there is new synthesis of an activating enzyme stimulated by norepinephrine. The cyclic AMP activated protein

kinase in the pineal gland (Fontana and Lovenberg, 1971) may be involved, but this has not been proven yet.

As indicated above, the regulation of the rapid decrease in N-acetyltransferase activity stimulated by light appears to involve the adrenergic receptor (Deguchi and Axelrod, 1972a). The intracellular mechanism involved is not known; it may involve the spontaneous degradation of N-acetyltransferase. We have found (Binkley et al., 1973) that when homogenates of pineal glands obtained at night are incubated at 37°C, there is a rapid disappearance of enzyme activity. This disappearance can be prevented by the cosubstrate of the enzyme, acetylcoenzyme A, and a small fragment of acetylcoenzyme A, cysteamine. Perhaps the rapid decrease in N-acetyltransferase is regulated via this stabilizing mechanism. Light may act by blocking the release of norepinephrine and allowing reuptake into nerve fibers to occur, and perhaps by stimulating a second mechanism that blocks the stabilization of the enzyme, by a cysteamine-like structure.

POSSIBLE FUNCTIONS OF THE RHYTHMS IN PINEAL INDOLE METABOLISM. The large changes in indole metabolism that occur in the pineal gland during the course of the day provide a means of turning environmental light signals into biochemical messages that are measurements of the *duration* of the dark and light periods. Long dark periods would allow longer periods of high melatonin and N-acetylserotonin production to occur, and these would presumably result in periods during which blood levels of these compounds would be elevated. In this role of translating environmental lighting information into chemical information, the pineal gland has been termed a neuroendocrine transducer (Wurtman and Anton-Tay, 1969). The rapid effects of light on N-acetyltransferase resulting in rapid effects on the production of N-acetylserotonin and the short blood half-life of melatonin (Kopin et al., 1961) could greatly enhance the precision of this system and allow small differences in the duration of the dark period to be transduced as significant differences in the duration of the high blood level of melatonin. The pineal gland has been implicated in the gonadal atrophy that occurs when hamsters are deprived of light (Reiter and Fraschini, 1969). When these animals are switched from a lighting cycle, which provides 14 hr of light and 10 hr of darkness, to one that provides 14 hr of darkness and 10 hr of light, gonadal atrophy is initiated. Large differences in gonadal weight are detected in about 6 to 10 weeks. It is possible that the effect of extended darkness on gonadal weight is mediated in part by changes in the production and release of N-acetylserotonin and melatonin.

General implications

The experiments discussed here indicate that, in the pineal gland, norepinephrine can regulate the metabolism of serotonin by a cyclic AMP mechanism requiring protein synthesis, and that this regulation involves the increased formation of N-acetylated derivatives, which is accompanied by a decrease in the steady-state levels of serotonin. This mechanism appears to be the basis of the circadian rhythms in indole metabolism in the pineal gland. Reis et al. (1969) have detected rhythms in the concentration of serotonin in many areas of the cat brain. These rhythms are not synchronized with each other. The N-acetyltransferase activity has been detected in several areas of the brain (Ellison et al., 1972), and this enzyme might be involved in regulating serotonin rhythms in some of these areas. The N-acetylation mechanism could function to modulate the amount of serotonin available for release as a neurotransmitter. Alternatively, the N-acetylated derivatives of serotonin could function as transmitters for local hormones.

Jouvet has implicated serotonin in the mechanism underlying sleep (see Jouvet, this volume). Disturbances in serotonin metabolism in the raphe nuclei, which contain all the cell bodies of the serotonergic neurons in the brain, result in disturbances in sleep. The raphe nuclei also receive adrenergic input. These areas are similar therefore to the pineal gland in two respects: both have serotonin containing cell bodies and both receive adrenergic input. However, it is not known if the cells in the raphe nuclei are similar to the pineal gland in the manner in which the concentration of serotonin is regulated by norepinephrine via the N-acetylation mechanism. Perhaps they are.

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