

DEVELOPMENT OF A CIRCADIAN RHYTHM IN THE ACTIVITY OF PINEAL SEROTONIN *N*-ACETYLTRANSFERASE

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Abstract—Pineal serotonin *N*-acetyltransferase (EC 2.3.1.5) is a neurally regulated enzyme. It is detectable in the rat as early as 4 days prior to birth. A circadian rhythm in enzyme activity appears on the fourth day after birth. It develops most rapidly during the second week and achieves an adult magnitude by the end of the third week at which time nocturnal values are more than 30-fold greater than daytime values. Norepinephrine, which appears to be the neurotransmitter regulating this enzyme, can cause a 2- to 3-fold stimulation of *N*-acetyltransferase in organ cultures of pineal glands from 4-day-old animals and a 17-fold increase in the activity of glands from 15-day-old animals. Apparently the norepinephrine-sensitive system controlling pineal *N*-acetyltransferase activity also develops most rapidly during the first few weeks of life. The circadian rhythm in the activity of serotonin *N*-acetyltransferase develops in the pineal glands of both male and female rats at the same rate. A similar rhythm for the enzyme was not observed in twelve other tissues of the rat.

SEROTONIN *N*-ACETYLTRANSFERASE (acetyl CoA : arylamine *N*-acetyltransferase; EC 2.3.1.5) catalyses the conversion of serotonin to *N*-acetylserotonin (WEISSBACH, REDFIELD and AXELROD, 1960, 1961). In the pineal gland of the adult male rat there is a circadian rhythm in the activity of this enzyme with nocturnal values 15- to 30-fold greater than daytime values (KLEIN and WELLER, 1970a). The nocturnal increase can be prevented by light acting via the eye (KLEIN and WELLER, 1970a). The central neural regulation of the rhythm reaches the pineal gland via postsynaptic fibres from the superior cervical ganglia (KLEIN, WELLER and MOORE, 1971). The increase in enzyme activity appears to be regulated via the release of norepinephrine (NE) from sympathetic terminals in the pineal gland (KLEIN *et al.*, 1971). NE acts apparently by stimulating adenylyl cyclase (WEISS and COSTA, 1967), which promotes an increase in the intracellular concentration of adenosine-3',5'-monophosphate (cyclic AMP) (STRADA, KLEIN, WELLER and WEISS, 1972). Studies with N⁶, 2'-O-dibutyryl cyclic AMP indicate that the increase in *N*-acetyltransferase activity is most probably mediated by cyclic AMP acting via a mechanism which depends upon protein synthesis (KLEIN and BERG, 1970). The circadian rhythm in pineal *N*-acetyltransferase appears to cause the large diurnal fluctuations in the concentration of serotonin (QUAY, 1963; SNYDER, ZWEIG, AXELROD and FISCHER, 1965) and melatonin (*N*-acetyl 5-methoxytryptamine) (QUAY, 1964; LYNCH, 1971) in the pineal gland (KLEIN and WELLER, 1970a).

In the present report we describe the ontogenetic development of the circadian rhythm in *N*-acetyltransferase activity in male and female rats and compare this pattern of development to the established pattern of appearance in the pineal gland of NE-containing neurons (HAKANSON, LOMBARD DES GOUTTES and OWMAN, 1967).

Abbreviations used: NE, norepinephrine; Cyclic AMP, adenosine-3', 5'-monophosphate.

the NE-sensitive adenylyl cyclase system (WEISS, 1971), the circadian serotonin rhythm (ZWEIG, SNYDER and AXELROD, 1966), and pineal hydroxyindole-*O*-methyltransferase activity (ZWEIG and SNYDER, 1968; KLEIN and LINES, 1969).

MATERIALS AND METHODS

Studies in vivo. Osborne-Mendel rats (NIH strain) were obtained from the NIH colony. Animals were weaned at 21 days and then were fed Purina Lab Chow and water *ad libitum*. The ages given are correct to within ± 1 day. Animals were shipped from the NIH colony to our laboratory facilities at least 4 days before being killed. The source of light in the NIH colony was General Electric Daylight Fluorescent Lamps, and in our laboratory facilities it was General Electric incandescent bulbs. In both facilities the intensity of light in the cages was 10 to 30 ft-candles with the lighting cycle consisting of 14 h of light and 10 h of darkness (lights were turned on at 0500 h).

Pineal glands were obtained between 1300 and 1400 h for the determination of the daytime activity of *N*-acetyltransferase and between 2215 and 2300 h for the nocturnal determination. All animals were decapitated; those older than 21 days were first stunned by a blow to the head. Glands were rapidly removed and stored in 0.15 M NaCl at room temperature for not more than 5 min. Fetal pineal glands were removed with the aid of a dissecting microscope.

N-acetyltransferase activity was measured by the method of KLEIN and WELLER (1970a). Each gland was rapidly homogenized in 20 μ l of 0.1 M sodium phosphate buffer (pH 6.8), containing 20 nmoles of [3 - 14 C] serotonin (28 μ Ci/ μ mole) and 40 nmol of acetyl coenzyme A. The reaction was terminated by the addition of 20 μ l of an ethanol-HCl solution (1 : 1, v/v) containing 20 nmol of *N*-acetyl serotonin and melatonin. The samples were centrifuged at 18,000–20,000 g for 2 min and stored at -20°C . The [14 C] *N*-acetyl serotonin and [14 C]melatonin were isolated following two-dimensional thin-layer chromatography on precoated silica gel plates (initial development with chloroform : methanol : acetic acid; 90 : 10 : 1 by vol.; followed by development with ethyl acetate in the second dimension).

In one study *N*-acetyltransferase activity was measured in 13 tissues from 6 rats. The entire pineal gland was assayed. Other tissues were homogenized in 20 vol. of 0.1 M sodium phosphate buffer (pH 6.8); 10 μ l of homogenate were added to an assay tube containing 10 μ l of buffer, 20 nmoles of [3 - 14 C] serotonin and 40 nmol of acetyl coenzyme A. Assays were run as already described.

Organ culture studies. Animals used for these studies were delivered to our laboratory on the morning of the experiment. Pineal glands were cultured for 6 h by the method of KLEIN and WELLER (1970b). At the end of the experiment pineal glands were frozen on dry ice, stored at -20°C , and subsequently homogenized in 30 μ l of 0.1 M sodium phosphate buffer (pH 6.8); 10 μ l of homogenate was assayed for enzyme activity as already described.

Materials. [3 - 14 C] Serotonin (56 μ Ci/ μ mole) was obtained from Amersham Searle (Chicago, Ill.); NE, serotonin, *N*-acetylserotonin, and melatonin were from Regis Chemical Co. (Chicago, Ill.); and acetyl coenzyme A was from Mann Research Co. (Orangeburg, New York).

Statistics. A statistical analysis was performed by Student's '*t*' test. Data are given as the means \pm S.E.M.

RESULTS

To determine whether there was a circadian rhythm in female rats and whether there were differences in the activity of *N*-acetyltransferase between males and females, 12- to 70-day-old rats of both sexes were tested for enzyme activity (Table 1). A rhythm was observed in females and there were no consistent differences in the values for pineal *N*-acetyltransferase during the night or day between males and females.

The embryonic rat exhibited measurable pineal *N*-acetyltransferase activity at 4 days prior to birth (Fig. 1). Only daytime values were determined for these animals. Daytime and nocturnal values were about equal at 1 day after birth. At 4 days of postnatal age, a statistically significant night-day difference was first detectable. At about 7 days of postnatal age the daytime values decreased sharply to about one-half to one-third of the daytime values at birth. The average nocturnal values continued to increase rapidly until the third postnatal week at which time adult values of enzyme activity were approximated. Between 21 and 70 days of age the ratio of nocturnal to diurnal activity of the enzyme was 30–70.

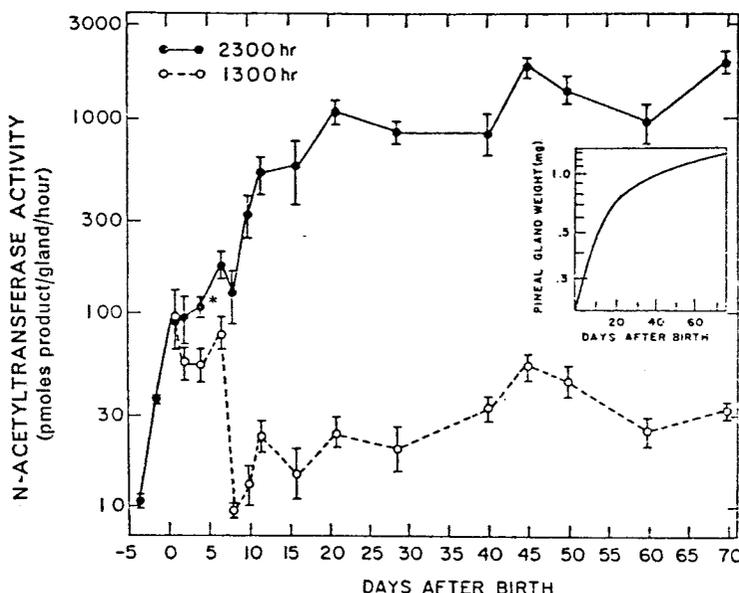


FIG. 1.—Development of pineal *N*-acetyltransferase activity. Animals younger than 12 days of postnatal age were not segregated by sex. The values at 21, 50, and 60 days after birth are based on groups of males. The remaining values are the pooled male and female values presented in Table 1. Each plotted point is based on three to eight determinations, with the s.e.m.'s shown. The insert is based on data from previous studies in this laboratory. Nocturnal values for enzymatic activity are significantly higher ($P < 0.01$) than daytime values for rats older than 4 days of postnatal age (*).

In a series of organ culture experiments we examined the effects of 0.01 M NE on the activity of *N*-acetyltransferase in pineal glands from rats of different ages (Table 2). Pineal glands were incubated for 6 h. In two experiments in which pineal glands from 3-day-old animals were tested, a 2.2- to 3.5-fold increase in enzyme activity was observed. This response increased rapidly as a function of postnatal age of the animals.

TABLE 1.—DAYTIME AND NOCTURNAL ACTIVITY OF PINEAL *N*-ACETYLTRANSFERASE ACTIVITY IN MALE AND FEMALE RATS

| Postnatal age (days) | <i>N</i> -acetyltransferase activity (pmol of product/h/per gland) | | | |
|----------------------|--|---------------|-------------|--------------|
| | Males | | Females | |
| | Daytime | Nocturnal | Daytime | Nocturnal |
| 12 | 21.2 ± 4.3 | 357.8 ± 46.7 | 17.1 ± 10.8 | 376.8 ± 56.3 |
| 16 | 16.5 ± 5.5 | 482.0 ± 263.0 | 15.9 ± 6.5 | 654.0 ± 33.3 |
| 29 | 30.2 ± 6.4 | 958.0 ± 117 | 9.9 ± 1.7 | 722 ± 146 |
| 40 | 19.5 ± 2.0 | 1073 ± 144 | 41.8 ± 7.1 | 819 ± 353 |
| 45 | 60.6 ± 13.5 | 1965 ± 100.9 | 48.3 ± 5.9 | 1710 ± 350.8 |
| 70 | 33.9 ± 2.5 | 2246 ± 415 | 31.2 ± 5.1 | 1552 ± 209 |

Values represent means ± s.e.m. for 3 or 4 observations. See text for details of tissue sampling and assay.

TABLE 2.—STIMULATION BY NOREPINEPHRINE OF THE ACTIVITY OF *N*-ACETYLTRANSFERASE IN CULTURED PINEAL GLANDS

| Postnatal age (days) | <i>N</i> -acetyltransferase activity Percent of unincubated controls | | |
|----------------------|---|-------------|--------------|
| | Control | NE | (NE/Control) |
| 3 | 95 ± 18.6 | 329 ± 55.2 | 3.5 |
| 3 | 113 ± 23.6 | 245 ± 36.5 | 2.2 |
| 6 | 103 ± 23.6 | 365 ± 31.5 | 3.5 |
| 12 | 105 ± 32.9 | 703 ± 152.3 | 6.6 |
| 15 | 32 ± 7.3 | 516 ± 6.1 | 17 |

Individual pineal glands were cultured for 6 h under control conditions or in the presence of 0.01 M norepinephrine (NE). Each group comprised 4–6 glands. Treatment with NE caused a significantly higher activity of the enzyme over that in unincubated controls ($P < 0.01$ at all ages). Values represent means ± S.E.M.

A 17-fold higher value for enzyme activity was detected in NE-treated glands from 15-day-old animals.

To determine whether rhythms of the magnitude seen for pineal *N*-acetyltransferase develop in other tissues of the rat, *N*-acetyltransferase activity was measured during the night and the day in 13 tissues (Table 3). Enzyme activity was detectable in all tissues examined. In this study, the pineal gland exhibited a 62-fold increase in

TABLE 3.—SURVEY OF TISSUES FOR CIRCADIAN RHYTHM IN THE ACTIVITY OF SEROTONIN *N*-ACETYLTRANSFERASE

| Tissue | Daytime enzyme activity (pmol/mg of tissue) | Ratio Night/Day |
|--------------------|--|--------------------|
| Pineal | 44 | 62.0 |
| Thyroid | 100 | 2.0 |
| Cerebrum | 140 | 1.5 |
| Cerebellum | 150 | 1.4 |
| Olfactory lobe | 170 | 1.3 |
| Spleen | 180 | 0.9 |
| Pituitary | 200 | 1.6 |
| Heart | 270 | 1.1 |
| Kidney | 280 | 1.4 |
| Intestine | 290 | 1.0 |
| Adrenal | 300 | 1.0 |
| Submaxillary gland | 390 | 1.2 |
| Liver | 840 | 1.5 |

Tissues were obtained at 1300 h and at 2300 h. Each value for the daytime enzyme activity is the mean of three determinations. The night/day ratio is the ratio of the mean of three nocturnal determinations to the mean of three diurnal determinations of enzyme activity. The S.E.M. for nocturnal and diurnal means was less than 35 per cent in each case. Tissues were obtained from 8-week-old male rats. There was a significant ($P < 0.05$) night/day difference only in the case of the pineal gland.

enzyme activity during the dark phase of the lighting cycle. A significant ($P > 0.05$) circadian rhythm was not observed in other tissues.

DISCUSSION

Our investigation has revealed that the circadian rhythm in the activity of pineal *N*-acetyltransferase develops most rapidly between the seventh and fifteenth postnatal day of life. We thought it of interest to compare the ontogenetic development of this rhythm to that of mechanisms that are known to be necessary for the rhythm in adults. Intact innervation to the pineal gland is known to be necessary for the rhythm in adults (KLEIN *et al.*, 1971). The development of sympathetic innervation of the pineal gland has been described using fluorescent histochemistry (HAKANSON *et al.*, 1967). At 0–4 days after birth, catecholamine-containing nerve fibres are apparent only at the surface of the gland. At 5–6 days, only a few delicate nerve fibres are visible in the parenchyma. Adult levels of parenchymal catecholamine fluorescence are reached at 3–4 weeks after birth, with the most rapid development occurring during the second week. In view of the known dependence of the rhythm in *N*-acetyltransferase on neural input, it is not surprising that the circadian rhythm in *N*-acetyltransferase develops most rapidly during the second week after birth (when innervation is developing) and not before.

The postsynaptic mechanisms which regulate *N*-acetyltransferase activity appear to include the stimulation by NE of adenylyl cyclase (KLEIN, BERG and WELLER, 1970). WEISS (1971) has described the development of the activity of NE-sensitive adenylyl cyclase in the pineal gland. Sensitivity of adenylyl cyclase to NE cannot be detected at birth but develops during the first 2 postnatal-weeks and achieves adult levels at 15 days of postnatal age. At 2 and 4 days of postnatal age there is a measurable response of pineal adenylyl cyclase to norepinephrine. Our observations here that there is a rapid increase during the period between the third and fifteenth day after birth in the response of *N*-acetyltransferase in cultured pineal glands to NE is consistent with the hypothesis that the development of the NE-sensitive adenylyl cyclase is necessary for the development of the *N*-acetyltransferase rhythm.

Both the innervation of the pineal gland from the superior cervical ganglia and the sensitivity of adenylyl cyclase to NE appear to develop at about the same time that a rhythm in *N*-acetyltransferase develops. Can the rhythm in pineal *N*-acetyltransferase be explained solely by these two mechanisms? We think it improbable on the basis of a comparison of the submaxillary gland with the pineal gland. Like the pineal gland, the submaxillary gland receives innervation from the superior cervical ganglia, contains a NE-sensitive adenylyl cyclase (WOLFE, MUENZER and GORDON, 1969, and personal communications), exhibits a circadian rhythm for NE (WURTMAN and AXELROD, 1966; WURTMAN, AXELROD, SEDVALL and MOORE, 1967), and responds to environmental lighting with changes in NE content (MOORE and SMITH, 1971). However, unlike the pineal gland, the submaxillary gland does not appear to have a circadian rhythm for *N*-acetyltransferase. Apparently other specific mechanisms are present in the pineal gland that allow the *N*-acetyltransferase rhythm to develop.

One interesting aspect of the development of the *N*-acetyltransferase rhythm is that at the beginning of the second week of postnatal life there is a decrease in daytime levels of the enzyme. This decrease might arise because innervation of the gland introduces an inhibitory influence on the gland. Alternatively, differentiation might

result in the appearance of mechanisms in the pineal gland which suppress the activity of the cyclic AMP-*N*-acetyltransferase system. It is possible that the development of sensitivity of pineal adenylyl cyclase to norepinephrine may be a consequence of the development of a specific receptor protein that will be associated with adenylyl cyclase. Perhaps in the absence of norepinephrine, the receptor protein inhibits adenylyl cyclase activity, and this inhibition is removed when norepinephrine is released and interacts with the receptor.

Another possibility is that norepinephrine metabolism may change markedly at about 7 days after birth. Consistent with this idea is the observation by MOORE and SMITH (1971) that the total amount of norepinephrine in the pineal gland of rats raised in constant lighting is about 3-fold greater at 6 days than at 10 or 85 days of age. Perhaps there is greater daytime release of norepinephrine at 6 days of postnatal age in normal animals than in older animals.

We have compared the factors regulating the concentration of pineal serotonin and the activity of *N*-acetyltransferase and have concluded that in adult rats the circadian rhythm in serotonin is regulated by the inverse circadian rhythm in *N*-acetyltransferase (KLEIN and WELLER, 1970a). The observation by ZWEIG *et al.* (1966) of rhythmic changes in the concentration of serotonin in the pineal gland at 6 days of postnatal age and our finding of a rhythm in the activity of *N*-acetyltransferase at this age both support the hypothesis that *N*-acetyltransferase regulates serotonin in the pineal gland of neonatal rats. The possibility that serotonin regulates *N*-acetyltransferase activity is doubtful in view of our finding that serotonin cannot stimulate *N*-acetyltransferase activity in cultured pineal glands (KLEIN and WELLER, 1970a).

The ontogenetic studies on serotonin *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase activity (ZWEIG and SNYDER, 1968; KLEIN and LINES, 1969) indicate that the appearance of the latter enzyme probably determines the onset of melatonin production. Up to 10 days of postnatal age, pineal glands can produce *N*-acetylserotonin but do not have the ability to convert *N*-acetylserotonin to melatonin. This latter ability increases rapidly during the next few weeks. This sequential ontogenetic relationship indicates that it is possible that *N*-acetylserotonin may trigger the development of hydroxyindole-*O*-methyltransferase.

The findings that both male and female rats exhibit a similar rhythm in *N*-acetyltransferase and that male and female pineal hydroxyindole-*O*-methyltransferase activity (KLEIN and LINES, 1969) is similar indicate that both sexes possess essentially the same enzymatic capacity for converting serotonin to melatonin.

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