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Physiology

Ontogenesis of a biological clock for serotonin:acetyl coenzyme A *N*-acetyltransferase in pineal gland of rat

(circadian/neural regulation/melatonin)

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ABSTRACT Serotonin:acetyl coenzyme A *N*-acetyltransferase (EC 2.3.1.5) activity in pineal gland was assayed in rats which were born and raised under diurnal lighting conditions, in continuous darkness, or in constant light. *N*-Acetyltransferase activity in the pups raised under diurnal lighting showed a rhythmic pattern, with high enzyme activity during dark period. The pups raised in continuous darkness also showed a rhythmic pattern, the phase of which was delayed by 8 hr in 7-week-old pups; the rhythmic phase of *N*-acetyltransferase began in the evening in 12-day-old pups and was regularly delayed by 1 hr every week. The pups raised in constant illumination also showed a rhythmic pattern; the rhythmic phase was delayed by 3 hr every week. When the mother rats were coupled in darkness and maintained in darkness during pregnancy and after the pups were born, their pups again showed a rhythmic pattern. These observations indicate that the biological clock for *N*-acetyltransferase is generated independently of environmental lighting. When mothers were coupled under reversed lighting conditions and transferred into darkness or light, the rhythmic pattern in their pups was inverted 180° from that of pups born of diurnal mothers. When the pups were raised in darkness, the rhythmic phase of *N*-acetyltransferase in the pups was similar to that of their mothers. It is suggested that in the absence of light-darkness cycle, the mother rat sets the rhythm of the pups to synchronize with her own rhythm. When pups were reared by a foster mother with a different rhythmic pattern from that of their original mother, the rhythmic phase in the pups was closer to that of the original mother, suggesting that the original mother plays the predominant role in setting the rhythm of the pups.

The circadian rhythms in indole metabolism in rat pineal gland have been extensively studied during the past decade (1) since Quay found the circadian change in serotonin content (2). Serotonin:acetyl coenzyme A *N*-acetyltransferase (EC 2.3.1.5) activity, that acetylates serotonin to *N*-acetylserotonin (3), has been shown to play the major role in generating the rhythmic changes in indole metabolism in the pineal gland (4). The neuronal pathway that regulates the circadian change in serotonin *N*-acetyltransferase activity has been established (5–7). The signal for *N*-acetyltransferase rhythm presumably arises in suprachiasmatic nuclei in hypothalamus and is transmitted through superior cervical ganglion to pineal gland.

N-Acetyltransferase activity shows a 20- to 50-fold increase at night in darkness (4, 6). When the rats are maintained in continuous darkness or they are blinded, the rhythmic change in *N*-acetyltransferase activity or serotonin content persists, indicating that in the absence of light, *N*-acetyltransferase activity is driven by the endogenous biological clock located in the hypothalamus (4, 6–8). The circadian rhythms in serotonin content and *N*-acetyltransferase activity appear as early as 6 days after birth when the pups are maintained under diurnal lighting conditions (9, 10). The present study was undertaken to investigate whether the biological clock that controls *N*-acetyltransferase rhythm is set inborn, independent of environmental lighting, or is set after the newborns are exposed to light-darkness stimulus.

Abbreviation: *N*-acetyltransferase, serotonin:acetyl coenzyme A *N*-acetyltransferase.

MATERIALS AND METHODS

Wistar rats weighing 100–110 g were supplied from Shizuoka Laboratory Animal Agricultural Cooperative Assoc. Five rats were housed in a clear plastic cage (30 × 35 × 18 cm) with a screen cover at 25–27°. The rats were maintained under diurnal lighting conditions with light on from 5 a.m. to 7 p.m. (diurnal rat) or under reversed lighting conditions with illumination from 5 p.m. to 7 a.m. (reversed rat). An overhead fluorescent lamp provided 70–110 footcandles (750–1200 lux) of illumination at the level of cages. Food and water were given *ad libitum*. They were kept in our animal rooms for at least 1.5 months before they were coupled. Two or 3 days before parturition, the pregnant rat was housed separately in a plastic cage. They were transferred under the following lighting conditions: (*i*) diurnal lighting, (*ii*) continuous illumination [70–120 footcandles (760–1300 lux) at the level of cages], or (*iii*) dark room equipped with double-door light baffles. Litters were used for experiment only when there were 9 to 12 pups. The infant rats were weaned from their mother 4 weeks after birth. A dim red light (15 W) was used when the rats were manipulated or killed in darkness. Four or five rats of mixed sexes were randomly chosen from different littermates at each time point. There was no difference in *N*-acetyltransferase activity between males and females. After decapitation, the pineal gland was quickly removed and chilled in ice. *N*-Acetyltransferase activity was assayed by the method of Deguchi and Axelrod (11).

RESULTS

Development of Circadian Rhythm under Diurnal Lighting Conditions. When infant rats were raised under diurnal lighting conditions for 7 weeks after birth, *N*-acetyltransferase activity increased between 8 p.m. and 11 p.m. reaching a 50-fold higher level compared to the daytime level (Fig. 1A). When rats were born and raised in continuous darkness for 7 weeks, *N*-acetyltransferase activity also showed a rhythmic pattern (Fig. 1B). The rats killed between 5 a.m. and 12 a.m. showed consistently high enzyme activity, whereas the rats killed between 2 p.m. and 2 a.m.

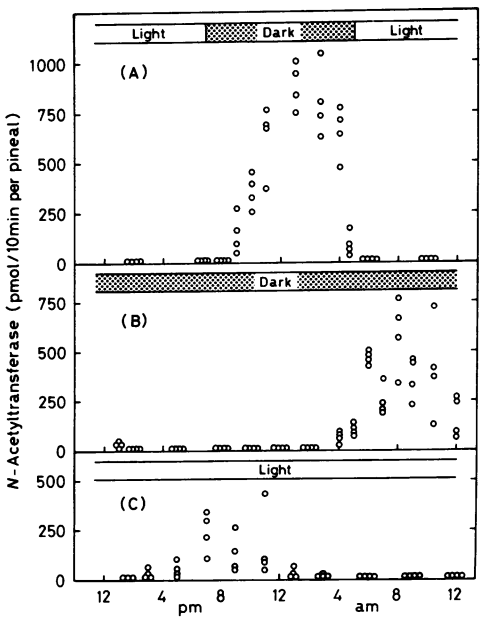

FIG. 1. Rhythm in *N*-acetyltransferase activity in 7-week-old rats. The pregnant rats, coupled under diurnal lighting conditions, were transferred under various lighting conditions before parturition. The pups were raised there for 7 weeks, and enzyme activity was assayed. (A) Diurnal lighting; (B) continuous darkness; (C) constant light.

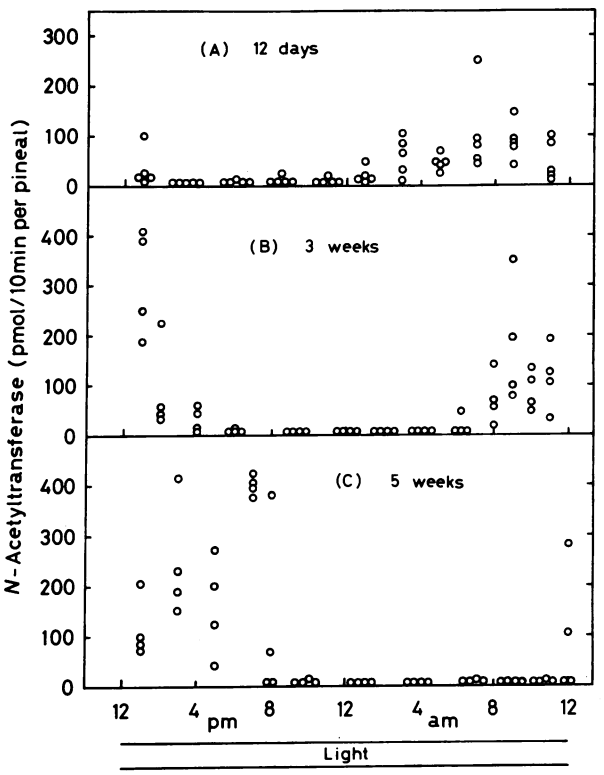

FIG. 2. Rhythm of *N*-acetyltransferase activity in the pups raised in continuous light. The mother rats, coupled under diurnal lighting, were transferred into continuous illumination before parturition. Enzyme activity was assayed in 12-day-old pups (A), 3-week-old pups (B), or 5-week-old pups (C).

had low enzyme activity. The rhythmic phase was delayed by about 8 hr compared to that of the diurnal rats (Fig. 1A). High level of enzyme activity showed a larger variation compared to the night-time level of the diurnal rats. The high level of enzyme activity was approximately two-thirds of that in the diurnal rats. When rats were born and raised in continuous illumination for 7 weeks, *N*-acetyltransferase activity was far lower than that of the diurnal rats or the rats raised in darkness. *N*-Acetyltransferase activity again showed a rhythmic pattern, with high enzyme activity between 5 p.m. and 1 a.m. and low activity between 3 a.m. and 2 p.m. (Fig. 1C). The rhythmic phase was advanced by 4 hr or was delayed by about 20 hr compared to the diurnal rats.

To study the phase shift in rhythmic pattern, *N*-acetyltransferase activity was measured 12 days, 3 weeks, or 5 weeks after birth. When the pups were raised under diurnal lighting conditions, *N*-acetyltransferase activity began to increase 1 hr after the onset of darkness and decreased in the morning when the light was turned on (data not shown). The night level of *N*-acetyltransferase activity was between 150 and 300 pmol/10 min per gland in 12-day-old pups, between 300 and 600 pmol in 3-week-old pups, and between 500 and 900 pmol in 5-week-old infants.

Development of Circadian Rhythm in Continuous Light. The rats raised in continuous light also showed a rhythmic change at all ages examined (Fig. 2). There was much larger variation in enzyme activity compared to the diurnal rats or the rats raised in darkness (see Fig. 4). The rhythm began at 1 a.m. in 12-day-old pups, at 6 a.m. in 3-week-old pups, and at 12 a.m. in 5-week-old rats. The rhythmic phase was delayed by about 3 hr every week. When extrapolated, the rhythmic phase seems to begin between 8 p.m. and 9 p.m. shortly after birth. The high level of enzyme activity was about one-third of that in the diurnal rats.

When adult rats were maintained under reversed lighting conditions for 4 weeks, the rhythm in *N*-acetyltransferase activity was inverted, with a high enzyme activity between 10 a.m. and 5 p.m. (data not shown). The rats were coupled and transferred into constant light. *N*-Acetyltransferase activity in 3-week-old pups showed a rhythmic pattern, with high enzyme activity between 7 p.m. and 5 a.m. (Fig. 3). The rhythmic phase was inverted 180° compared to that of the pups born of diurnal mothers (Fig. 2B). The results indicate that the rhythmic pattern of *N*-acetyltransferase activity in the pups raised in continuous light is presumably determined by the lighting conditions where their mother lived during pregnancy.

When 5-week-old rats raised in continuous light were transferred under diurnal lighting, *N*-acetyltransferase activity rapidly synchronized with darkness. Six days after

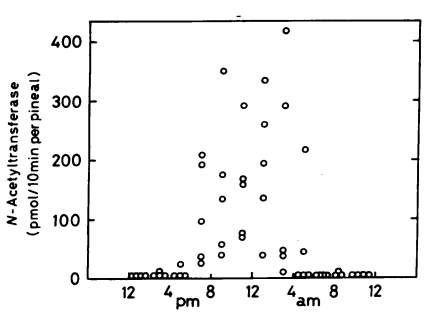

FIG. 3. Rhythm of *N*-acetyltransferase activity in continuous light in the pups born of reversed mother. The mother rats maintained under reversed lighting conditions were coupled and transferred into constant light before parturition. Enzyme activity in the pups was assayed 3 weeks after birth.

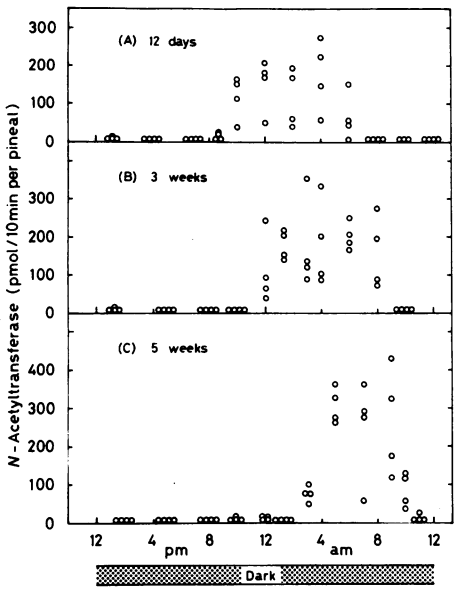

FIG. 4. Rhythm of *N*-acetyltransferase activity in the pups raised in continuous darkness. The mother rats were coupled under diurnal lighting conditions and transferred into darkness before parturition. Enzyme activity was assayed in 12-day-old pups (A), 3-week-old pups (B), or 5-week-old rats (C).

they were moved into diurnal lighting, all rats exhibited a marked nocturnal increase, with the same level of *N*-acetyltransferase activity as those of rats raised under diurnal lighting conditions (data not shown).

Development of Circadian Rhythm in Continuous Darkness. The pups raised in continuous darkness showed a rhythmic pattern at all ages examined (Fig. 4). The rhythm began at 10 p.m. in 12-day-old pups, at 12 p.m. in 3-week-old pups, and at 3 a.m. in 5-week-old rats. The rhythmic phase was delayed by about 1 hr every week. The high level of enzyme activity was approximately two-thirds of that in the diurnal pups. Shortly after they were born, the rhythm in *N*-acetyltransferase activity seems to begin in the evening, a similar observation obtained with the pups raised in continuous light (Fig. 2). The question will be raised why the pups that have never been exposed to diurnal lighting set their biological clock in the evening. In these studies their mothers were maintained under diurnal lighting conditions before the pups were born. It might be possible that the fetus can perceive the environmental lighting and set their biological clock as to synchronize with environmental darkness where their mother lived.

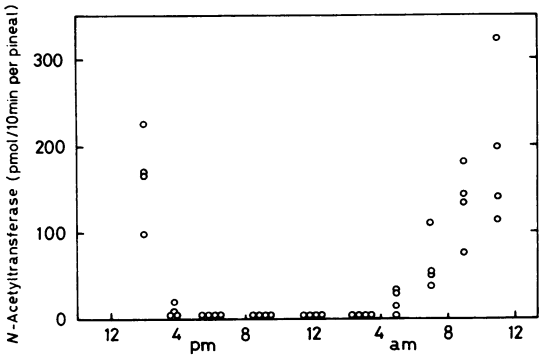

FIG. 5. Rhythm in *N*-acetyltransferase activity in the pups born of mothers coupled in darkness. Male and female rats were transferred from diurnal lighting conditions into darkness 10 days before they were coupled. The rats were maintained in darkness during pregnancy and after the pups were born. Enzyme activity in the pups was assayed 23 days after birth.

To study this possibility, mother rats were coupled in darkness and kept there during pregnancy and after the pups were born. *N*-Acetyltransferase activity in the pups was assayed 23 days after birth (Fig. 5). *N*-Acetyltransferase activity in the pups again showed a rhythmic pattern, with high enzyme activity between 5 a.m. and 2 p.m. There was about 8 hr delay in the rhythmic phase compared to the diurnal pups. This delay could be accounted for as follows: When adult rats were maintained in continuous darkness, the rhythmic phase of *N*-acetyltransferase activity was regularly delayed by about 1 hr every week (Deguchi, submit-

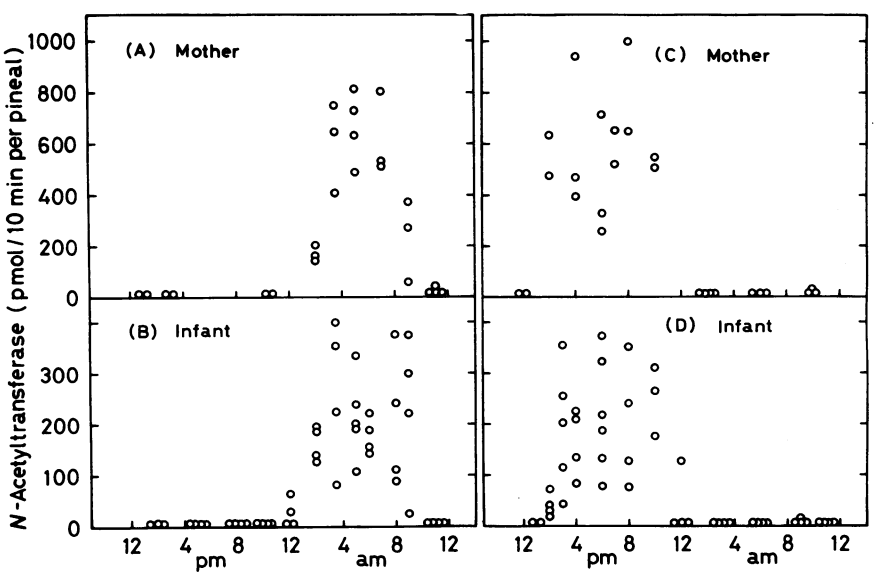

FIG. 6. Relation between the rhythmic phases of pups and mothers. Mother rats were coupled under diurnal lighting conditions and transferred into darkness before parturition. *N*-Acetyltransferase activity was assayed 3 weeks after birth in the mothers (A) and in the pups (B). Another group of female rats was coupled under reversed lighting conditions and transferred into darkness before parturition. Enzyme activity was assayed 3 weeks after birth in the mothers (C) and in the pups (D).

ted), which was a similar shift to that observed in the pups raised in continuous darkness (Fig. 4). In the above experiment, the mother rats were maintained in darkness for 31 days before the pups were born. Meanwhile, the rhythm of *N*-acetyltransferase activity in the mothers must have been delayed by about 5 hr. After birth the rhythm in the pups was presumably delayed by about 3 hr. Eight hours' delay in the rhythmic phase in the pups could be the sum of these delays. The above observations suggest that the pups born of a mother whose rhythm has been shifted would show a larger shift in the rhythmic phase.

To study the relation of rhythmic patterns between mothers and pups, mother rats were coupled under diurnal lighting and transferred into darkness. *N*-Acetyltransferase activity in both mothers and pups was measured 3 weeks after birth. The rhythmic phase of the pups (Fig. 6B) was similar to that of their mothers (Fig. 6A). When mother rats were coupled under reversed lighting conditions and transferred into darkness, the rhythmic phase of *N*-acetyltransferase activity in the pups (Fig. 6D) was again simiar to that of their mothers (Fig. 6C). The rhythmic phase in the pups born of reversed mothers (Fig. 6D) was 180° out of phase to that of the pups born of diurnal mothers (Fig. 6B). The results suggest that the rhythmic pattern of *N*-acetyltransferase activity in the pups raised in the absence of light was set to synchronize with that of their mother.

The question will be raised when do mother rats set the biological clock in their pups, during pregnancy or after birth? To answer the question, one group of mother rats was coupled under diurnal lighting and another under reversed lighting conditions at the same time. They were transferred into darkness 2 days before the pups were born. Within 24 hr after the pups were born, their mothers were exchanged. The pups born of a diurnal mother were reared by a reversed mother (Fig. 7A). The pups born of reversed mother were reared by diurnal mother (Fig. 7B). *N*-Acetyltransferase activity in the pups was assayed 3 weeks after birth. Both groups of pups showed a rhythmic pattern, the phase of which was 180° out of phase with each other. The pups born of a diurnal mother and reared by a reversed mother showed a rhythmic pattern that was closer to that of their original mother (Fig. 6A) than to their foster mother (Fig. 6C). The pups born of a reversed mother and reared by a diurnal mother showed a closer relation to their original mother (Fig. 6C) than to their foster mother (Fig. 6A). There was, however, about a 3-hr delay in the rhythmic phase of the pups compared to that of original mother, suggesting that both original mother and foster mother can affect the rhythmic pattern of *N*-acetyltransferase activity in the pups. The original mother, however, seems to have a predominant role in determining the rhythmic phase in the pups.

Relation Between Environmental Light-Darkness and Maternal Effect. The above observations would indicate that in the absence of environmental light-darkness, the mother acts as a synchronizer for the biological clock of *N*-acetyltransferase activity in their pups. Which is a predominant "Zeitgeber" for the pups, maternal effect or environmental lighting? To study the question, the pregnant rats were blinded on the seventh day of pregnancy and were maintained under diurnal lighting conditions. *N*-Acetyltransferase activity was assayed in the pups and in their mother 10 days after birth. The rhythm in the mothers was delayed, with low enzyme activity at 11 p.m. and high level at 3 a.m. (Table 1) because they were blinded 24 days before enzyme assay. *N*-Acetyltransferase activity in the pups, on the other hand, was low during the light period and increased after the light was turned off, reaching the maximal level at 11 p.m. Thus, the rhythmic pattern in the pups did not coincide with the rhythm of their mothers, but was synchronized with the onset of darkness.

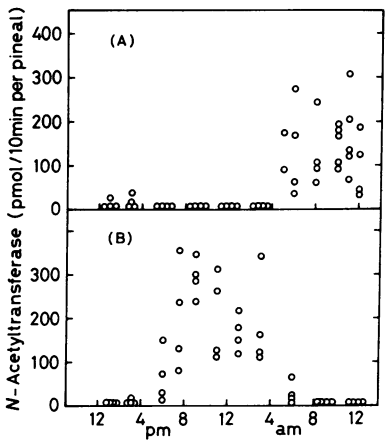

FIG. 7. Rhythm in *N*-acetyltransferase activity in the pups reared by foster mother. Mother rats were coupled under diurnal lighting conditions or under reversed lighting conditions and were transferred into darkness 2 days before the pups were born. Within 24 hr after the pups were born, their mother was exchanged. *N*-Acetyltransferase activity was assayed in the pups 3 weeks after birth. (A) *N*-Acetyltransferase activity in the pups born of diurnal mother and reared by reversed mother. (B) *N*-Acetyltransferase activity in the pups born of reversed mother and reared by diurnal mother.

DISCUSSION

The present study demonstrated that the circadian rhythm of *N*-acetyltransferase activity in rat pineal gland develops even when the infant rats were born and raised in continuous darkness or in constant illumination. *N*-Acetyltransferase activity also showed a rhythmic pattern in the pups when their mother was coupled in darkness and maintained in darkness during pregnancy and after the pups were born. These results indicate that the biological clock that regulates the carcadian rhythm in *N*-acetyltransferase activity is generated in the central nervous system, independent of environmental lighting.

Table 1. Dissociation of rhythmic phase in pups and mother rats

Time killed	Lighting condition	*N*-Acetyltransferase (pmol/10 min per pineal)	
		Pup	Mother
11 a.m.	Light	15 ± 4	—
7 p.m.	Light off	13 ± 4	—
8:30 p.m.	Dark	66 ± 7	—
11 p.m.	Dark	115 ± 12	0 ± 0
3 a.m.	Dark	96 ± 17	382 ± 42

Mother rats, coupled under diurnal lighting conditions, were bilaterally enucleated on the seventh day of pregnancy under ether anesthesia. They were maintained under diurnal lighting conditions. *N*-Acetyltransferase activity in the pups and mothers was assayed 10 days after birth. There were four rats in each group. The results are expressed as mean ± standard errors.

In the rats raised in constant illumination, *N*-acetyltransferase activity also showed a rhythmic pattern. *N*-Acetyltransferase activity in their mothers (37 rats) was also assayed at various times of a day after 3 or 5 weeks in continuous light. None of them showed significant increase in *N*-acetyltransferase activity (data not shown). When adult rats or 3-week-old pups raised under diurnal lighting conditions were transferred into continuous lighting, *N*-acetyltransferase activity did not increase at all (data not shown). These results indicate that light completely suppresses *N*-acetyltransferase activity in the rats raised under diurnal lighting, whereas in the rats born and raised in continuous light a mechanism that partially prevents the suppressive effect of light develops.

The rhythmic phase of *N*-acetyltransferase activity in the rats raised in continuous darkness or in constant illumination was regularly delayed as they grew. The circadian rhythm of *N*-acetyltransferase activity shifted in a regular manner among individual rats so that *N*-acetyltransferase activity still showed a rhythmic pattern 7 weeks after birth. A larger variation in *N*-acetyltransferase activity in the rats raised in darkness or under illumination might suggest a partial desynchronization of the rhythm among individual rats. The delay in the rhythmic phase was approximately 3 hr every week in light and 1 hr every week in darkness. The rhythmic phase of adult rats was also delayed by about 1 hr every week in darkness or in the blinded rats (Deguchi, submitted). Thus, the period of free-running rhythm of *N*-acetyltransferase activity in rat pineal gland is longer than 24 hr both in darkness and under illumination.

When the pups were born of mothers who had been maintained under diurnal lighting conditions (dark: from 7 p.m. to 5 a.m.) during pregnancy, the *N*-acetyltransferase rhythm in the pups seems to begin at 8 p.m. or 9 p.m. shortly after birth. In contrast, the pups born of mothers who had been maintained under reversed lighting conditions showed a rhythmic pattern that was 180° out of phase to that of pups born of diurnal mothers. When *N*-acetyltransferase activity was assayed 3 weeks after birth in darkness, the rhythmic phase of *N*-acetyltransferase in the pups was identical or similar to that of their mothers. It might be possible that the pups were born with a rhythmic pattern identical or similar to that of their mothers. In continuous darkness, the rhythmic phase was delayed at a similar rate in both pups and mothers, resulting in a similar pattern 3 weeks after birth. In constant light, however, the rhythm of *N*-acetyltransferase activity in mothers was completely suppressed, whereas the rhythm in the pups partially persisted and was regularly delayed. The circadian rhythm in *N*-acetyltransferase activity appears 6 days after birth (9, 10), at the time when sympathetic innervation to the pineal gland begins (12). The biological clock for *N*-acetyltransferase rhythm, however, might have been set in the hypothalamus and have started to run before sympathetic nerves from the superior cervical ganglion innervate the pineal gland. Although it was suggested that the original mother could have a predominant role in setting the circadian clock in the pups, the exact mechanism of how the original and foster mothers interact to set the biological clock of the pups is still unclear.

When the circadian rhythm of the mother rat was different from environmental light-darkness, the rhythmic phase of *N*-acetyltransferase activity in the pups was determined by the onset of darkness. Thus, among two "Zeitgeber" so far as we know, the environmental lighting is a more predominant determinant than the mother. It is also interesting that 10 days after birth, when the eyelids of the pups are still closed, *N*-acetyltransferase activity synchronizes with environmental darkness, but not with the rhythm of their mother. A nonretinal pathway to pineal gland would be involved in setting the circadian rhythm in the newborn rats, as has been indicated by Zweig *et al.* (9).

N-Acetyltransferase in rat pineal gland offers an ideal model for studies of the circadian nature of mammalians. In the present study the factors that affect the ontogenesis of a biological clock were analyzed by following the development of circadian rhythm of *N*-acetyltransferase activity under various environmental conditions.

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