Diurnal Fluctuation in the Enzyme Activity and the Messenger RNA Level of Pinea
Serotonin N-Acetyltransferase in Normal and Hereditary Microphthalmic Rats

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The enzyme activity and the messenger RNA level of pineal serotonin N-acetyltransferase were more than 20- and 50-fold higher, respectively, in the dark period than in the light period in normal rats. In hereditary microphthalmic rats, however, the serotonin N-acetyltransferase activity and its mRNA level did not undergo a great diurnal change through the light and dark periods. These results indicate that the diurnal rhythms of the activity and the mRNA level of serotonin N-acetyltransferase are not detected in the pineal gland of hereditary blind rats, suggesting free-running rhythms in individual animals due to desynchronization of their circadian rhythms by a lack of their optic nerve.

Key words: circadian rhythm; serotonin N-acetyltransferase mRNA; hereditary blind rat; pineal

Melatonin synthesis in the pineal gland is regulated by the activity of the enzyme, serotonin N-acetyltransferase [arylalkylamine N-acetyltransferase; EC 2.3.1.87], which converts serotonin to N-acetylserotonin. Like the diurnal rhythm of melatonin concentration in the pineal and serum, the pineal serotonin N-acetyltransferase activity exhibits intensive diurnal fluctuation, in which the activity is more than 15 to 30 times greater at night than the day value. 1-3 It appeared that large rhythmic changes in serotonin N-acetyltransferase activity under various physiological conditions are affected by both transcriptional and/or posttranscriptional events. More recently, the cloning and tissue-specific expression of serotonin N-acetyltransferase gene in sheep, rat, and human were reported. 5-10 Roseboom et al. 11 demonstrated that, in contrast to the small rhythm in ovine pineal serotonin N-acetyltransferase mRNA, there is a more than 150-fold night/day rhythm in rats' mRNA, suggesting that transcriptional mechanisms contribute heavily to the rhythm in serotonin N-acetyltransferase activity.

Serotonin N-acetyltransferase rhythm has the characteristics of a true circadian rhythm since it becomes free-running in the absence of visual cues under conditions of constant darkness or blindness. 11 The diurnal rhythm of this enzyme activity in rats blinded by surgically bilateral orbital enucleation has been examined by many workers. 4-10 There are some disagreements in the literature about the rhythmic changes in the pineal activity of these blind animals; Deguchi showed a persisting rhythm but Klein et al. reported the disappearance of a rhythmic pattern. We have maintained hereditary blind microphthalmic rats that completely lack the optic nerve. In hereditary blind animals, the congenital absence of visual input to the circadian clock might affect the development of daily rhythms of biochemical and physiological activities. Therefore, we paid attention to diurnal rhythms in serotonin N-acetyltransferase and its mRNA level in hereditary blind rats and attempted to see if they only have constant enzyme activity and its mRNA level of serotonin N-acetyltransferase throughout the day or have some kind of diurnal fluctuation.

The hereditary microphthalmic rats from the Donryu strain were produced through brother-sister mating in our laboratory, characteristics of those rats have been reported by Sugita et al. 13 Normal male Donryu rats weighing about 250 g were obtained from SEASCO, Saitama, Japan. The rats were housed individually in stainless wire-mesh cages kept in a room maintained at 22 ± 2°C and under a 12 h-12 h LD cycle (lights on 05:00 h) for two weeks before the experiments. Quay 14 reported that a similar circadian rhythm in rat pineal serotonin contents is found in males and females. Moreover, a circadian rhythm in the activity of serotonin N-acetyltransferase develops in the pineal glands of both male and female rats at the same rate, and it reaches an adult magnitude by the end of the third week after birth. 12 Therefore, hereditary blind rats used here were 13-14 weeks-old and both males and females, weighing about 220 g, and similar results for the enzyme activity in males and females were obtained. Twenty-three hereditary microphthalmic male and female rats from the Donryu strain were used, and 16 normal male rats of the same strain for controls. The animals were fed on laboratory chow (crude protein: 24%, Type MF, Oriental Yeast Co., Tokyo) and water ad libitum. Rats were sacrificed at 14:00, 20:00, 02:00, and 08:00 h by decapitation, during the dark period at 02:00 and 20:00 h animals were killed under dim red light.

The brains were rapidly removed and frozen on dry ice. Serotonin N-acetyltransferase activity in rat pineal gland was assayed by the method of Deguchi and Axelrod 15 with a slight modification. A rat pineal was homogenized with 70 μ1 of a reaction mixture containing 2.5 μmol of potassium phosphate (pH 6.5), 0.1 μmol of tryptamine, and 3.4 nmol (7.5 kBq) of acetyl-l-[14C]coenzyme A (Du Pont/NEN Research products, purchased from the Japan Radioisotope Association, Tokyo) in a glass microhomogenizer (Radinot). The reaction mixture was incubated at 37°C for 10 min, and the reaction was stopped by adding 0.5 ml of 0.05 M borate buffer (pH 10). The reaction mixture was extracted with 6 ml of toluene-isooamyl alcohol (97:3), and the radioactivity in the organic phase was measured in an NT (Nonion-toluene) scintillation solution containing 4 g of DPO per liter of 3 parts of nonylphenoxo polyethoxy ethanol and 7 parts of toluene. 16 Enzyme activity was calculated for product formed per gland per 10 min.

Eighteen hereditary microphthalmic female rats and 11 normal female rats from the Donryu strain were fed on the laboratory chow and water ad libitum. Rats were killed by decapitation at 11:00 h for the measurement of the daytime levels of serotonin N-acetyltransferase mRNA and at 23:00 h for the nocturnal measurement, at 23:00 h animals were killed in dim red light as described above.

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Fig.  Enzyme Activity and Its Messenger RNA Level of Serotonin N-Acetyltransferase in the Pineal Glands of Normal and Hereditary Blind Rats.

Normal rats (A) and hereditary blind rats (B) for the enzyme activity were maintained under regular lighting conditions (a 12h:12h LD cycle, light on 05:00-17:00), and killed at 14:00, 20:00, 02:00, and 08:00. Normal rats (C) and hereditary blind rats (D) for the mRNA level were maintained under regular lighting conditions (a 12h:12h LD cycle, light on 05:00-17:00), and killed at 11:00 in the light period and 23:00 in the dark period. Data for the mRNA level in the pineal gland are expressed as percentage of the maximum value in the an experiment. Horizontal lines are mean values.

Serotonin N-acetyltransferase mRNA was analyzed by the lysate RNase protection assay that we modified for efficient measurement of mRNA levels in small tissues. Briefly, complementary DNA corresponding to nucleotide 291–690 base pairs of rat serotonin N-acetyltransferase (arylamidylamine N-acetyltransferase) cDNA (Gene Bank accession No. U40803) was obtained by reverse transcriptase-PCR using total RNA from rat pineal gland as the template. Each pineal gland was transferred to a micro-homogenizer containing 50 μl lysis buffer (50 mM guanidine thiocyanate, 0.1 M EDTA, 0.1 M Tris-HCl, pH 7.0, 1% 2-mercaptoethanol) and homogenized. Homogenates were centrifuged at 15,000 rpm for 5 min at room temperature. Forty-five microfilters of the supernatant were mixed with 5 μl of lysis buffer containing 250,000 cpm of antisense RNA probe. Digestion buffer containing RNase A and RNase T1 was added and excess probes were digested at 37°C. After treatment with protease K, protected probes were precipitated and then separated on a denaturing polyacrylamide gel. The values for serotonin N-acetyltransferase mRNA levels in the pineal gland are given as a percentage of the maximum value in an experiment.

The activities of serotonin N-acetyltransferase in normal rat pineal glands were low at 8:00 and 14:00 h during the light period with a mean value of 22.9 ± 0.9 (mean ± SE) picomoles of product formed per pineal gland per 10 min (Fig. (A)). The enzyme activities in normal rat pineal were increased 3 h after the start of darkness, and the maximum activity was obtained at 02:00 h during the period of darkness, where the mean values for the enzyme activities at 20:00 and 02:00 were 347 ± 58 and 606 ± 102, respectively, picomoles of product per gland per 10 min, showing about 15- to 30-fold increase from the value for the light period. Thus, normal rats in this experiment show the typical appearance of the serotonin N-acetyltransferase rhythm in the pineal gland maintained in a normal diurnal lighting schedule.

Under similar lighting conditions, however, hereditary blind rats did not show any rhythmic pattern of serotonin N-acetyltransferase activity in their pineal glands during a 24-h period (Fig. (B)). Hereditary blind rats continuously maintained in a 12h:12h LD cycle for 13 weeks after birth were killed at 4 times of the day. There were no significant differences in the enzyme activities among the mean values at each time point of 14:00, 20:00, 02:00, and 08:00. Of the 23 measurements made in hereditary blind rats, some individuals showed increased activities of serotonin N-acetyltransferase, more than 200 pmol of product per gland per 10 min even in the light period, and some had less than 50 pmol of product during the darkness, and thus the values at each time point were extensively varied. This would be explained by the hypothesis that, in hereditary blind rats, each animal maintains its circadian rhythm in pineal serotonin N-acetyltransferase activity, which persists with various free-running times, but the group rhythm disappears because they become desynchronized from each other. We are now examining diurnal rhythmic changes in self-sustained locomotor activity and metabolic activity in hereditary blind rats.

Pineal serotonin N-acetyltransferase mRNA levels have been analyzed mainly by Northern blot analyses using total RNA preparations obtained from pooled pineal glands. In this experiment, however, we have used a microscale lysate RNase protection assay system that enabled us to analyze mRNA content of a single pineal gland and observed great individual to individual fluctuation (Fig. (C, D)). In normal rat pineal glands, the serotonin N-acetyltransferase messenger RNA levels were nearly undetectable at 11:00 h in the light period (1.6 ± 0.9% of the maximum value of the experiment), but increased about 50-fold at 23:00 h in the dark period (81.6 ± 9.2%). Some hereditary blind rats showed very abundant serotonin N-acetyltransferase mRNA, but the others showed nearly undetectable amounts of the mRNA at both the clock times in the light period and in the dark period (Fig. (D)). Thus the levels of serotonin N-acetyltransferase mRNA varied extensively among the individual pineal glands of hereditary blind rats, in accordance with the case with enzyme activities described above. These results suggest that each blind rat has its own fluctuation rhythm of mRNA content and activity of serotonin N-acetyltransferase. Alternatively, some rats may have consistently low levels and the others always have high levels. The latter is, however, less plausible, since if this is the case, more rats should exhibit 'intermediate' values between the high (nighttime value of normal rats) and the low (daytime value of normal rats) values.

In summary, we have shown that pineal serotonin N-acetyltransferase activity in hereditary blind rats fluctuates independent of the light cycle and the fluctuation is attributable to changes in its mRNA level.

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