Circadian Rhythm of Serotonin *N*-Acetyltransferase Activity in Organ Culture of Chicken Pineal Gland

Abstract. *When chicken pineal glands were organ-cultured in darkness, serotonin* N-*acetyltransferase activity was low during daytime, increased at midnight, and decreased on the next morning. The autonomous increase of* N-*acetyltransferase activity was suppressed by illumination of the glands. When pineal glands were cultured under a light-dark cycle (LD 12:12), the change of* N-*acetyltransferase activity continued to oscillate in phase with the light-dark cycle for 3 days.*

Circadian rhythm of melatonin synthesis in the pineal gland is controlled by a change of serotonin *N*-acetyltransferase activity. The neuronal pathway that regulates the circadian rhythm of *N*-acetyltransferase activity in rat pineal has been well established (*1*, *2*).

N-Acetyltransferase activity in chicken pineal gland exhibits a circadian change with a high enzyme activity at night in darkness and a low level during daytime (*3*). In contrast to that in the rat pineal, the *N*-acetyltransferase rhythm in chicken pineal is not affected by bilateral denervation of superior cervical ganglion or by injection of catecholamines (*4*, *5*). A difference in melatonin synthesis between mammalian and avian pineal glands was reported with hydroxyindole *O*-methyltransferase activity (*6*). To elucidate the regulation mechanism of the *N*-acetyltransferase rhythm, I used organ culture of chicken pineal glands. When chicken pineals were cultured for a long period, *N*-acetyltransferase activity autonomously increased four- to tenfold at night and returned to a low level on the next morning. I now report that chicken pineal gland contains an endogenous oscillator or a time-keeping system for *N*-acetyltransferase rhythmicity.

Chickens (*7*) (10 to 12 days old) were decapitated, and pineals were quickly removed and placed in culture medium on ice. The pineals were placed in organ culture by a modification (*8*) of the method used for rat pineal (*9*). After various periods of incubation in darkness, the culture dishes were removed in darkness and chilled on ice. *N*-Acetyltransferase activity was assayed immediately by a modification (*10*) of the method described for rat pineal (*11*).

In 10-day-old chickens raised under a light-dark cycle (LD 12:12), pineal *N*-acetyltransferase activity was low during daytime and increased 8- to 11-fold at night in darkness (Fig. 1A). When chickens were killed at 6 p.m. and the pineals were cultured in continuous darkness, *N*-acetyltransferase activity was low during the subsequent 2 to 3 hours and started to increase at 10 p.m., reaching a maximum at midnight (Fig. 1B). The activity decreased to the daytime level on the next morning at 10 a.m. The maximum enzyme activity was the same as that of the in vivo rhythm (Fig. 1A). When chickens were killed at either 2 p.m. or 11 a.m., *N*-acetyltransferase activity was low during daytime and increased at around 10 p.m. reaching a maximum at midnight (Fig. 1, C and D). The maximum enzyme activities were 70 and 40 percent of the in vivo rhythm, respectively. The phase of the increase and decrease of *N*-acetyltransferase activity was similar whenever chickens were killed at 11 a.m., 2 p.m., or 6 p.m. The phase of the change in activity was delayed 2 to 3 hours compared to that of the rhythm in vivo of chickens maintained under an LD cycle (Fig. 1A). This phase delay could be in part due to manipulation of the pineals on ice for 30 to 60 minutes prior to culture. When pineals were kept at 0°C for 5 hours prior to culture, the increase of *N*-acetyltransferase activity was delayed 5 hours, an indication that the time-keeping system in chicken pineal gland stops at 0°C. When a small number of chickens were killed at one time and the pineals were subjected to culture rapidly, the phase of the increase of *N*-acetyltransferase activity became very close to that of the in vivo rhythm (*12*). Cycloheximide (3 μg/ml) or actinomycin D (10 μg/ml) completely prevented the nocturnal rise of *N*-acetyltransferase activity in cultured

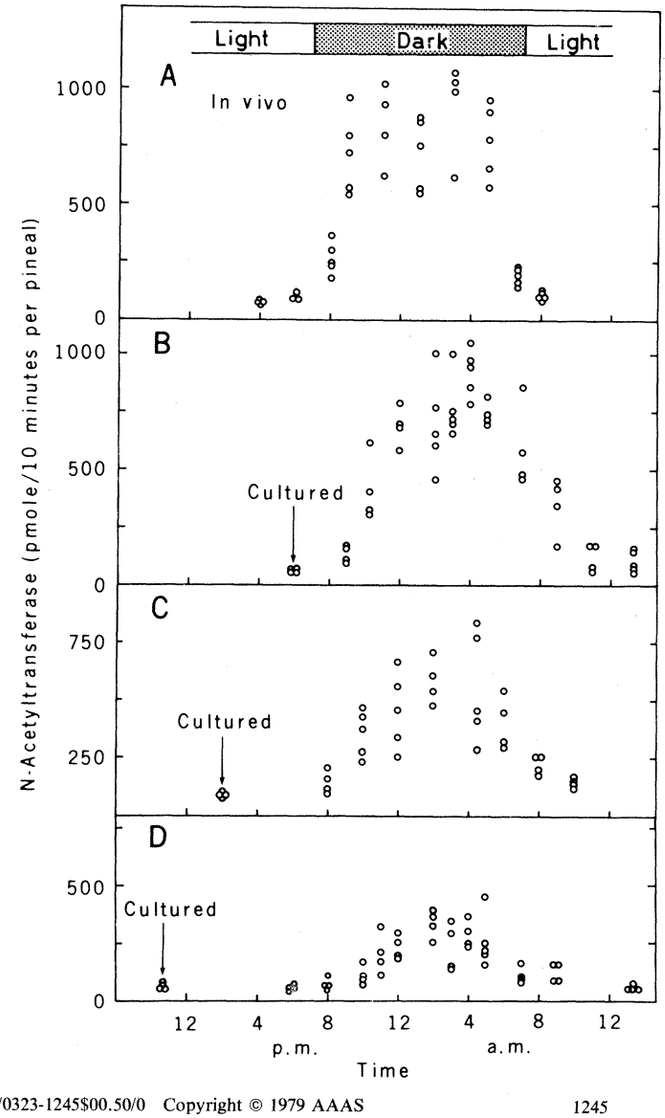

Fig. 1. Circadian change of pineal *N*-acetyltransferase activity in vivo and in organ culture. Chickens were raised under the light-dark schedule, as shown on the top, for 10 days after hatching, and *N*-acetyltransferase activity was measured (A). The chickens were killed at 6 p.m. (B), at 2 p.m. (C), or at 11 a.m. (D); the pineals were then placed in culture in continuous darkness. Each point represents the enzyme activity of individual pineal.

0036-8075/79/0323-1245$00.50/0

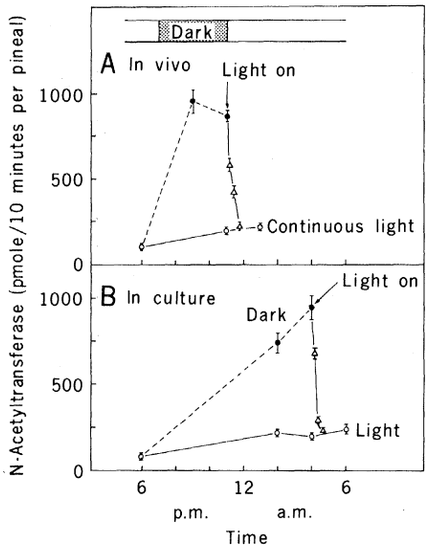

Fig. 2 (left). Effect of illumination on the nocturnal increase of *N*-acetyltransferase activity in vivo (A) and in cultural pineal glands (B). There were five samples in each group. The vertical bars indicate the standard error of the mean.

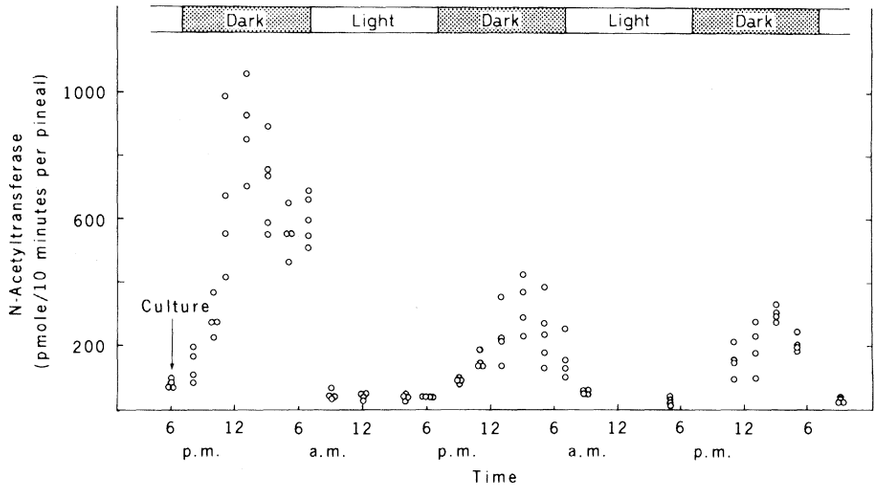

Fig. 3 (right). Circadian change of *N*-acetyltransferase activity of pineals cultured under a light-dark cycle with lights on from 7 a.m. to 7 p.m. Each point represents the enzyme activity of individual pineal.

pineals indicating an involvement of new synthesis of protein and messenger RNA in this process (*12*).

When the chickens were exposed to light until midnight, *N*-acetyltransferase rhythm was suppressed with only a twofold increase at midnight (Fig. 2A). When the light was turned on at midnight after *N*-acetyltransferase activity had reached its maximum, the activity rapidly decreased during subsequent 20 to 30 minutes, in agreement with reports on rat and chicken pineals (*2, 5, 13*). When pineals were cultured under continuous illumination, the autonomous rise of *N*-acetyltransferase activity was suppressed (Fig. 2B). There was only a 2- to 2.5-fold increase of the enzyme activity. When the pineals cultured in darkness were exposed to light after *N*-acetyltransferase activity had reached maximum, the activity declined to one-quarter of the initial level within 20 minutes.

When chicken pineals were cultured under an LD 12:12 cycle, the change of *N*-acetyltransferase activity continued to oscillate in phase with the environmental lighting schedule (Fig. 3). *N*-Acetyltransferase activity increased in the dark-time and decreased during the light period. The maximum enzyme activity on the first night was comparable to that of the in vivo rhythm. On the second and third nights the maximum enzyme activity was one-third of the night level of the in vivo rhythm. The daytime levels were also reduced to half or less on days 2 and 3 of culture. This reduction of the enzyme activity could be due to a necrosis of pinealcytes inside the glands or due to less than optimum culture conditions, or both. When pineals were cultured in continuous darkness for 3 days, the circadian change of *N*-acetyltransferase activity was not evident on the second and third nights of culture (data not shown).

The above study demonstrated that serotonin *N*-acetyltransferase activity in cultured pineal glands of chickens exhibits a circadian change comparable to that of the rhythm in vivo. When cultured in darkness, *N*-acetyltransferase activity autonomously increased at midnight and decreased during daytime. The pattern of the increase and decrease of the enzyme activity was similar, regardless of the time when chickens were killed. The time schedule of the change in *N*-acetyltransferase activity in cultured pineals was presumably determined by the environmental lighting schedule to which the chickens had been exposed. These observations indicate that chicken pineal gland contains a timekeeping system or an endogenous oscillator for *N*-acetyltransferase rhythmicity. Although the circadian oscillation of the enzyme activity in culture persisted for one cycle in continuous darkness, I failed to detect the circadian increase of *N*-acetyltransferase activity on the second and third expected nights in continuous darkness. In contrast, the rhythmic change of the enzyme activity was clearly observed for at least 3 days under an LD cycle. These observations would indicate either that the chicken pineal gland can keep the time schedule only one cycle in the absence of light-dark schedule, or that the circadian oscillation in chicken pineal could easily desynchronize between individual pineal glands or between individual pineal cells in a gland in the absence of an LD cycle. Although I have no conclusive evidence at present, the latter possibility would be likely. The lack of the persistent rhythmicity in continuous darkness could also be due to some inadequacy of the culture conditions, which might result in a desynchronization of the individual rhythms. Binkley and Geller reported (*14*) that *N*-acetyltransferase activity in chicken pineal persisted for at least two nights when they were kept in continuous darkness. However, the nighttime levels of the enzyme activity on the second expected night was damped, an observation differing from those reported for rat pineals in which *N*-acetyltransferase rhythm persisted for 10 weeks in continuous darkness or for blinded rats with the same amplitude (*15*).

The *N*-acetyltransferase rhythm in cultured chicken pineals was suppressed by illumination of the glands. Turning on the light at midnight resulted in a rapid decline of the enzyme activity. This observation suggests that chicken pineal gland contains a photoreceptor, the stimulation of which is somehow transduced to the synthesis and degradation of *N*-acetyltransferase molecules.

Preliminary data on chicken pineal glands have been reported (*12*). Several other groups of investigators found a circadian change of *N*-acetyltransferase activity in organ culture of chicken pineal glands (*16*). Thus the control mechanism of circadian rhythm of serotonin *N*-acetyltransferase activity is not the same in mammalian and avian pineal glands. In rat the endogenous oscillator is located in hypothalamus and regulates *N*-acetyltransferase rhythm through sympathetic nerves originating in superior cervical

ganglion (*1*, *2*), while in the chicken the pineal gland contains an endogenous oscillator and a photoreceptor for the *N*-acetyltransferase rhythmicity.

Menaker and his associates have shown that the free-running circadian rhythms of locomotor activity and body temperature in the house sparrow in constant darkness were abolished by pinealectomy and restored by transplantation of pineal gland (*17*). They further showed that the phase of the rhythm of locomotor activity in the recipient sparrow is determined by the phase of the rhythm of the pineal-donor sparrow. On the basis of these observations, they proposed that the avian pineal gland is an endogenous driving oscillator for the locomotor activity rhythm. Hendel and Turek have reported that melatonin controls the running activity rhythm in sparrow (*18*). These observations are consistent with the results reported here that the activity of serotonin *N*-acetyltransferase that regulates the synthesis of melatonin exhibits a circadian rhythm in isolated chicken pineal glands.

TAKEO DEGUCHI
Department of Medical Chemistry, Tokyo Metropolitan Institute for Neurosciences, Tokyo 183, Japan

References and Notes

1. J. Axelrod, *Science* **184**, 1341 (1974); D. C. Klein and J. L. Weller, *ibid.* **169**, 1093 (1970); D. C. Klein, G. R. Berg, J. Weller, W. Glinsmann, *ibid.* **167**, 1738 (1970); R. J. Wurtman, H. M. Shein, F. Larin, *J. Neurochem.* **18**, 1683 (1971); D. C. Klein, J. L. Weller, R. Y. Moore, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 3107 (1971); T. Deguchi and J. Axelrod, *ibid.* **69**, 2208 (1972); T. Deguchi, *Mol. Pharmacol.* **9**, 184 (1973); R. Y. Moore and D. C. Klein, *Brain Res.* **71**, 17 (1974).
2. T. Deguchi and J. Axelrod, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2547 (1972).
3. S. Binkley, S. E. MacBride, D. C. Klein, C. L. Ralph, *Science* **181**, 273 (1973); S. Binkley, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 2347 (1976).
4. C. L. Ralph, S. Binkley, S. E. MacBride, D. C. Klein, *Endocrinology* **97**, 1373 (1975).
5. S. Binkley, S. E. MacBride, D. C. Klein, C. L. Ralph, *ibid.* **96**, 848 (1975).
6. J. K. Lauber, J. E. Boyd, J. Axelrod, *Science* **161**, 489 (1968).
7. Chickens (White Leghorn cockerels) were supplied from Tokyo Metropolitan Agricultural Laboratory 1 day after hatching. They were maintained in our facility with lights on from 7 a.m. to 7 p.m. An overhead fluorescent lamp provided 480 to 840 lux at the level of cages. Food and water were freely available. When chickens were killed at night in darkness, a dim red light (7.5 W) was used.
8. Four or five pineals were placed on a nylon mesh in a Falcon plastic dish (5 cm in diameter) containing 4.5 ml of culture medium. The culture medium consisted of a Fitton-Jackson modification of BGJb medium (Gibco) supplemented with 10 percent fetal calf serum, 2 m*M* L-glutamine, ascorbic acid (0.3 mg/ml), streptomycin (100 μg/ml), and penicillin (100 U/ml). Organ culture was carried out at 40°C in an atmosphere of 95 percent O_2 and 5 percent CO_2 in a transparent incubator box placed in a CO_2 incubator. The inside of the incubator was kept dark. When indicated, a fluorescent lamp (30 W) provided light through a glass door incubator. The intensity of the illumination was 900 to 1100 lux at the level of dishes.
9. D. C. Klein and J. Weller, *In Vitro* **6**, 197 (1970); R. W. Holz, T. Deguchi, J. Axelrod, *J. Neurochem.* **22**, 205 (1974).
10. 5-Methoxytryptamine was used as a substrate instead of tryptamine, because it and serotonin were slightly (15 to 20 percent) better substrates for *N*-acetyltransferase of chicken pineal. A pineal gland was homogenized with 70 μl of a reaction mixture containing 2.5 μmole of potassium phosphate (*p*H 6.5), 20 nmole of 5-methoxytryptamine, and 12 nmole of [1-^{14}C]acetyl coenzyme A (5 mCi/mmole; Radiochemical Centre, Amersham, England) in a small glass homogenizer. The reaction was carried out at 37°C for 10 minutes and stopped by the addition of 0.5 ml of 0.5*M* borate buffer (*p*H 10). The radioactive melatonin produced was extracted into 5 ml of a mixture of toluene and isoamyl alcohol (97:3), and the radioactivity was measured.
11. T. Deguchi and J. Axelrod, *Anal. Biochem.* **50**, 174 (1972).
12. T. Deguchi, in *Integrative Control Functions of Brain* M. Ito, Ed. (Kodansha-Elsevier, New York, 1978), vol. 1, p. 345; in preparation.
13. D. C. Klein and J. L. Weller, *Science* **177**, 532 (1972).
14. S. Binkley and E. B. Geller, *J. Comp. Physiol.* **99**, 67 (1975).
15. T. Deguchi, *J. Neurochem.* **25**, 91 (1975).
16. C. A. Kasal, M. Menaker, J. R. Perez-Polo, *Science* **203**, 656 (1979); S. A. Binkley, J. B. Riebman, K. B. Reilly, *ibid.* **202**, 1198 (1978).
17. S. Gaston and M. Menaker, *ibid.* **160**, 1125 (1968); S. Binkley, E. Kluth, M. Menaker, *ibid.* **174**, 311 (1971); N. H. Zimmerman and M. Menaker, *ibid.* **190**, 477 (1975); M. Menaker, *J. Neural Transm.* (Suppl.), in press.
18. R. C. Hendel and F. W. Turek, *Physiol. Behav.* **21**, 275 (1978).
19. I thank E. Amano for technical assistance and Drs. Kiyohisa Takahashi and Masaki Nakane for criticism in preparation of the manuscript. Supported in part by grants from The Naito Foundation (76-182) and from the Ministry of Education of Japan.

6 August 1978; revised 28 November 1978

Mitochondrial DNA Analyses and the Origin and Relative Age of Parthenogenetic Lizards (Genus *Cnemidophorus*)

Abstract. *Morphological, karyological, and allozyme analyses indicate that the parthenogenetic lizards* Cnemidophorus neomexicanus *and diploid* C. tesselatus *are hybrids formed, respectively, by crosses involving the bisexual species* C. tigris *and* C. inornatus, *and* C. tigris *and* C. septemvittatus. *Mitochondrial DNA, which is inherited maternally, was obtained from each of these species. Analyses of the mitochondrial DNA's and their restriction endonuclease digestion products by electron microscopy and agarose gel electrophoresis support the hybridization hypothesis by indicating that* C. tigris *(specifically the subspecies* marmoratus*) was the maternal parent species for both* C. neomexicanus *and* C. tesselatus. *Furthermore, these data imply that these two parthenogenetic species are younger than some races of* C. tigris.

Approximately 13 species of whiptail lizards (*Cnemidophorus*) are known to reproduce parthenogenetically (*1*). Ten of these species, including *C. neomexicanus* and *C. tesselatus*, occur in the southwestern United States and northern Mexico. Analyses of karyology and morphology suggested that *C. neomexicanus* and diploid *C. tesselatus* originated, respectively, by hybridization between the bisexual species *C. tigris* and *C. inornatus* (*2*), and between *C. tigris* and *C. septemvittatus* (*3*). Subsequent analyses of allozymes (*4*) added support to this hypothesis by demonstrating that both parthenogenotes are heterozygous for alleles unique to one or the other of the postulated parental spe-

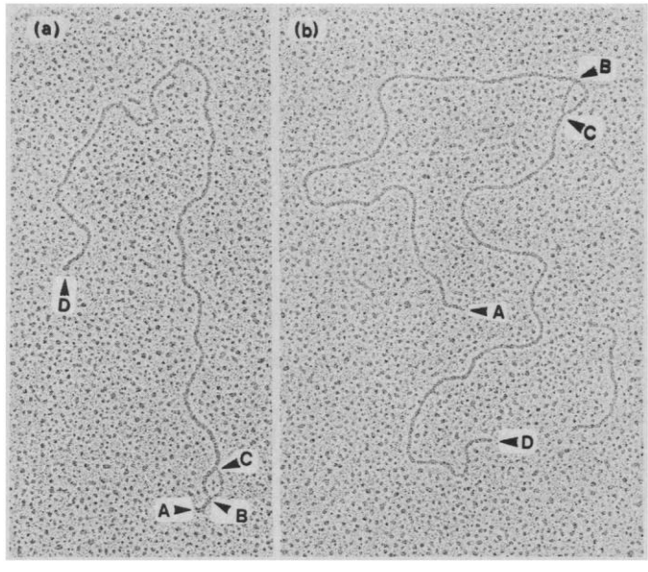

Fig. 1. Electron micrographs of fragments of mitochondrial DNA that contain the respective origins of DNA replication from (a) *Cnemidophorus inornatus* and (b) *C. tigris mundus*. The lengths AB and CD define the position of the D-loop, BC, on the fragment (*8*). During replication, D-loops expand unidirectionally, thus decreasing either AB or CD. The origin, defined as the fixed fork of the D-loop, and the direction of replication are determined by measurements of fragments that contain expanding D-loops (*8*). For the two examples above, the origin is at C, and the direction is toward A. Points A and D indicate the positions of the Eco R1 recognition sites 1 and 3 in *C. inornatus* and sites 3 and 5 in *C. t. mundus* (Fig. 3) (Table 1).

0036-8075/79/0323-1247$00.50/0