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The Localization of the Site of Arylalkylamine *N*-Acetyltransferase Circadian Expression in the Photoreceptor Cells of Mammalian Retina

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To investigate the molecular mechanism of the melatonin rhythm in the mammalian retina, we examined the temporal mRNA expression pattern of arylalkylamine (serotonin) *N*-acetyltransferase (AA-NAT), the rate-limiting enzyme in melatonin synthesis in the rat retina. Rat AA-NAT mRNA was detected exclusively in the retinal photoreceptors in the outer nuclear layer—low during the day and increased more than threefold at night. The nocturnal AA-NAT expression in rat retina was also confirmed by RNase protection and the AA-NAT enzymatic activity. This is the first report to localize the site of AA-NAT mRNA circadian expression in mammalian photoreceptor cells.

Key Words: **circadian rhythm; arylalkylamine *N*-acetyltransferase; photoreceptors; retina; rat; mammals; serotonin; melatonin.**

Melatonin is made from serotonin, and serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AA-NAT) is the rate-limiting enzyme in melatonin synthesis (1). Therefore nocturnal rhythmic melatonin production is due primarily to changes in AA-NAT activity.

Circadian melatonin production in the pineal has been observed in most vertebrate species, and is considered to play an important role in circadian activity and seasonal changes in physiology (1,2). In amphibians and avians, retinal photoreceptors have been shown to have a circadian clock that regulates rhythmic melatonin synthesis in the retina (3), and retinal melatonin production is thought to act as a local modulator (4). In mammals, however the suprachiasmatic nucleus(SCN) have been considered to be a only site of circadian clock (5). But there are several indications mammalian retina also have a subclock independent of the SCN. For examples, the rhythm of the photoreceptor disk shedding is persisted in the SCN lesioned rat (6,7) or the persistence of circadian rhythmic expression of melatonin and AA-NAT in the pinealectimized rat (8). Recently circadian rhythms of melatonin synthesis were clearly shown in cultured neural retinas of the golden hamster (9), suggesting that the mammalian retina contains an endogenous clock, regulating the rhythmic synthesis of melatonin. In previous report, we showed the circadian rhythmic expression of AA-NAT mRNA in rat retina (10).

In this study, to investigate the molecular and cellular mechanism of the rhythmic synthesis of melatonin in the mammalian retina, we examined the temporal expression pattern of serotonin AA-NAT gene by RNase protection assay and determined the distribution of AA-NAT transcript in the rat retina by *in situ* hybridization. Retinal AA-NAT enzymatic activity was also determined. Our findings suggest that the retinal AA-NAT mRNA circadian expression might be regulated by an endogenous circadian clock in photoreceptor cells even in mammals.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (4 weeks old; 60-80 g) were obtained from CLEA JAPAN, INC. (Tokyo) and were housed in a 12 h light-12 h dark cycle [LD 12:12; lights on zeitgeber time (ZT) 0-12]

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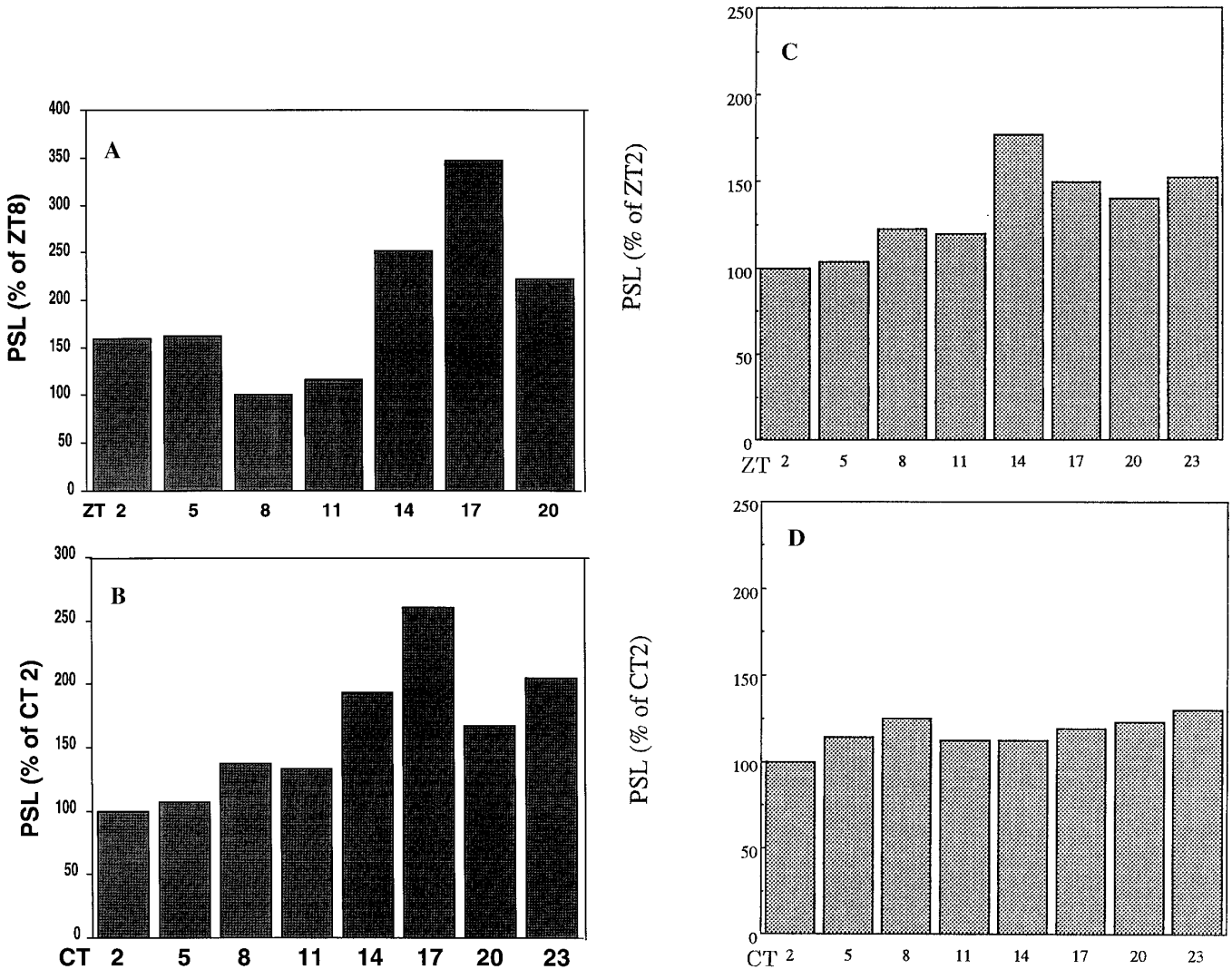

FIG. 1. Temporal expression pattern of AA-NAT mRNA in the retina. (A) Quantification of AA-NAT mRNA levels in LD 12:12 from the RNase protection. The abundance of AA-NAT mRNA was normalized to actin mRNA. The minimum value at ZT 8 was expressed as 100 %. Rats were housed in LD 12:12 (lights-on at ZT 0) and were killed at each time point indicated. (B) Quantification of AA-NAT mRNA levels in DD from the RNase protection. Rats were housed in LD 12:12 and transferred to DD. They were killed on the 3rd day after the LD/DD transition at each time point indicated below. The minimum value at CT 2 was expressed as 100 %. (C) Quantification of Fra-2 mRNA levels in LD 12:12 from the RNase protection. The abundance of AA-NAT mRNA was normalized to actin mRNA. The minimum value at ZT 2 was expressed as 100 %.(D) Quantification of Fra-2 mRNA levels in DD from the RNase protection. Rats were housed in LD 12:12 and transferred to DD. They were killed on the 3rd day after the LD/DD transition at each time point indicated below. The minimum value at CT2 was expressed as 100 %.

for at least 1 week before the day of the experiment. A white fluorescent lamp was used as a source of light during the day (150-200 lux at the level of the cages). We recorded the drinking rhythm of rats in constant darkness (DD) by using the chronobiology kit (Stanford Software System, USA) to determine circadian time.

Animals were decapitated, and tissues were dissected, quick-frozen and kept in liquid nitrogen until used. In darkness, dissections were carried out under dim red light. Animals used for *in situ* analysis were anesthetized with barbiturate and were perfused from the left ventricle with 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Tissues were prepared and treated as described later.

RNase protection assay. Rats were decapitated at the a specified Zeitgeber Time (ZT) on the day or Circadian Time (CT), the brain was opened and the optical nerves were cut cerebrospinally. The eye was removed from the brain, put in ice-cold Krebs - Ringer (composition in mM: 129 NaCl, 1.3 $MgSO_4$, 22.4 $NaHCO_3$, 1.2 $KH2PO_4$, 4.2 KCl, 10.0 Glucose, 1.5 $CaCl_2$, pH 7.4) for 0.5 min. Eyes from 10 rats were pooled, frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

Total RNA was extracted by the acid guanidium phenol chloroform procedure(11) from the eye of rat. The KpnI-NaeI 280 bp fragment of rat AA-NAT cDNA [bases 1-861; GenBank accession number U18913; The full-length cDNA fragment of rat AA-NAT was kindly provided by Dr. Solomon H. Snyder (12)] was subcloned into the plasmid pBluescriptII -KS(+). The EcoRI - HindIII 939 bp fragment of rat fos related antigen 2(Fra 2) RT-PCR product was subcloned into the plasmid pBluescriptII -KS(+), and this plasmid was cut by SalI and removed 5′ half of Fra2 and selfligated using Sal I site. A complete description of RNase protection is found in the instruction

manual for the RPAII kit(Ambion). Hybridizations were performed as follows: 5μg total RNA for eye with AA-NAT probe, Fra2 probe, β-actin probe,100 000 cpm probe, 43 °C for 12-15 hr. RNase digestion: RNAseA/T1 dilution, 1:100; 37 °C 30 min.100ml ethanol was added to the reaction after addition of the digestion stop buffer to facilitate precipitation of the protected fragments. The size of protected the AA-NAT RNA and the Fra2 RNA transcript was 280 bp and 500 bp, respectively and the size of protected the β-actin cDNA transcript(control) was 126 bp. After precipitation for 30 min at -20 °C, the protected fragments were pelleted by a TOMY Eppendorf centrifuge at 12000 rpm for 20 min., dissolved in the buffer provided by Ambion and separated by 5% urea gel. After electrophoresis, the gel was dried onto Watmann 3MM paper and exposed to an imaging plate (Fuji Film) and quantified using a Fujix Bio-imaging analyzer BAS 2000 (Fuji Film).

AA-NAT enzyme assay. AA-NAT enzymatic activity in rat retinas was measured as described (13). Retinas were dissected from eyes and cytoplasmic fractions were extracted using inhibitor cocktail (1 mM DTT, 0.5 mM PMSF, 0.5 mg/ml leupeptin, 1 mg/ml pepstatin A, 70 mg/ml TPCK, 10 mg/ml trypsin inhibitor, 5 mg/ml aprotinin), as previously described (14).

The AA-NAT activity in the retinal cytoplasmic extracts was determined by the method described previously using *p*-phenetidine as a substrate (15). 10 μl of enzyme preparation was incubated with 40 μl of 20 mM phosphate buffer (pH 6.5), containing 0.22 mM ^{14}C-acetyl CoA (5.2 mCi/mole, Amersham Japan) and amino substrate at 37°C for 30 min. The product *N*-[^{14}C] acetylamine was extracted with toluene and isoamylalcohol (97:3, v/v), and the radioactivity was measured. The amount of protein was also measured by the dye-binding method of Bradford with bovine serum albumin as a standard.

In situ hybridization. Detailed methods followed as described (16,17). Rats were killed as described above and eyes were fixed with 4% paraformaldehyde in phosphate buffered saline (pH 7.4) for 1 h at room temperature. Then tissues were embedded in Tissue-Tek OCT compound (Miles). Eight μm cryosections were cut.

Antisense and sense cRNA probes (digoxigenin-labeled) were generated using a DIG RNA labeling kit (Boehringer Mannheim). The template for transcription was a 1,367 bp cDNA fragment of rat AA-NAT (bases 1-1,367). The whole cDNA fragment was subcloned into the pBluescript II SK+ vector. As a control, digoxigenin-labeled RNA probe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used.

Sections were immersed in 50 μl of hybridization solution containing 50 % formamide, 10 % dextran sulfate, 20 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 5 mM EDTA, 10 mM vanadyl ribonucleoside complex, 1× Denhardt's solution, 1 mg/ml yeast tRNA, 0.25 % SDS, and a digoxigenin-labeled RNA probe (50-100 ng/ml). The sections were covered with a coverslip and incubated in a moist chamber at 50°C for 15-16 h. Then they were washed in 2× SSC/50 % formamide at 50°C for 2 h and incubated in 20 mg/ml RNase at 37°C for 30 min followed by 2× SSC for 1 h and in 0.1× SSC for 20 min at room temperature for washing.

Digoxigenin-labeled RNA probes were detected, after hybridization to target mRNA, by enzyme-linked immunoassay using a DIG nucleic acid detection kit (Boehringer Mannheim). The digoxigenin probe was visualized with anti-digoxigenin antibody conjugated to alkaline phosphatase, and stained with Nitroblue Tetrazolium (NBT) and X-phosphate (5-brome-4-chloro-3-indolyl phosphate).

RESULTS

Circadian expression of retinal AA-NAT mRNA. The temporal pattern of AA-NAT mRNA level in the retina was examined by RNase protection assay (Figs. 1A, B). In LD 12:12 (Fig. 1A), AA-NAT mRNA exhibited a daily rhythm with a peak at ZT 17 (5 h after light-off). AA-NAT mRNA level was low during the day and increased at night to levels more than threefold higher than the late afternoon value (ZT 11). The rhythmic expression of retinal AA-NAT mRNA persisted in constant darkness (DD; Fig. 1B). The night-to-day ratio of AA-NAT mRNA was 2.5 on the 3rd day after transferring from LD 12:12 to DD. These results indicate that the AA-NAT mRNA level in the rat retina is regulated by a circadian clock.

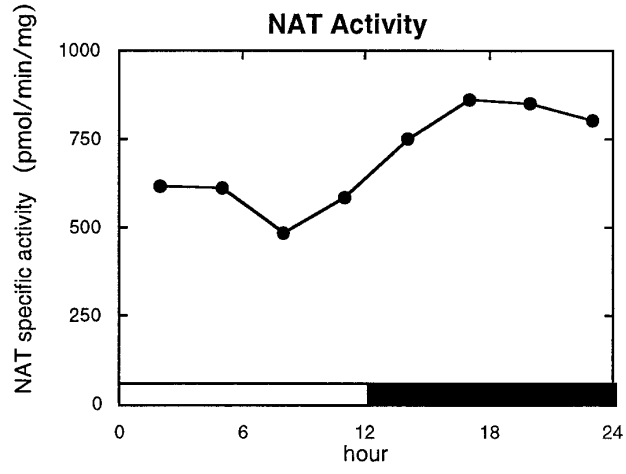

FIG. 2. Daily rhythm of AA-NAT enzymatic activity in the retina. Rats were housed in LD 12:12 (lights on at 0), and retinas were obtained at each time point indicated in Fig.1A. AA-NAT enzyme activity in rat retinas was measured using *p*-phenetidine as a substrate as previously described (13).

To investigate Fra-2 is involved in the AA-NAT circadian expression in rat retina as reported in the rat pineal(18,19), we also carried out RNase protection assay of the same source of RNA samples by Fra-2 probe(Figs. 1C, D) The results indicate that Fra-2 did not show significant circadian expression as observed in pineal.

Daily rhythm of retinal AA-NAT activity. To determine the relationship between AA-NAT mRNA and enzymatic activity, the temporal pattern of AA-NAT activity in the retina was measured with rats housed in LD 12:12. AA-NAT activity was low during the day and increased at night (Fig. 2). Compared with the rhythm of retinal AA-NAT mRNA, AA-NAT activity rhythm showed a broader peak and a smaller amplitude. In addition, AA-NAT activity began to increase at ZT 8 (4 h before light-off), while AA-NAT mRNA decreased during the day (Fig. 2). These findings suggest that the daily rhythm of AA-NAT activity is regulated, at least in part, at the post-transcriptional level.

Distribution of AA-NAT transcript in the retina. To assign the distribution of AA-NAT mRNA in the rat retina, we carried out a modified *in situ* hybridization (see method) using digoxigenin-labeled antisense cRNA probes (Fig. 3). The sense strand-specific hybridization

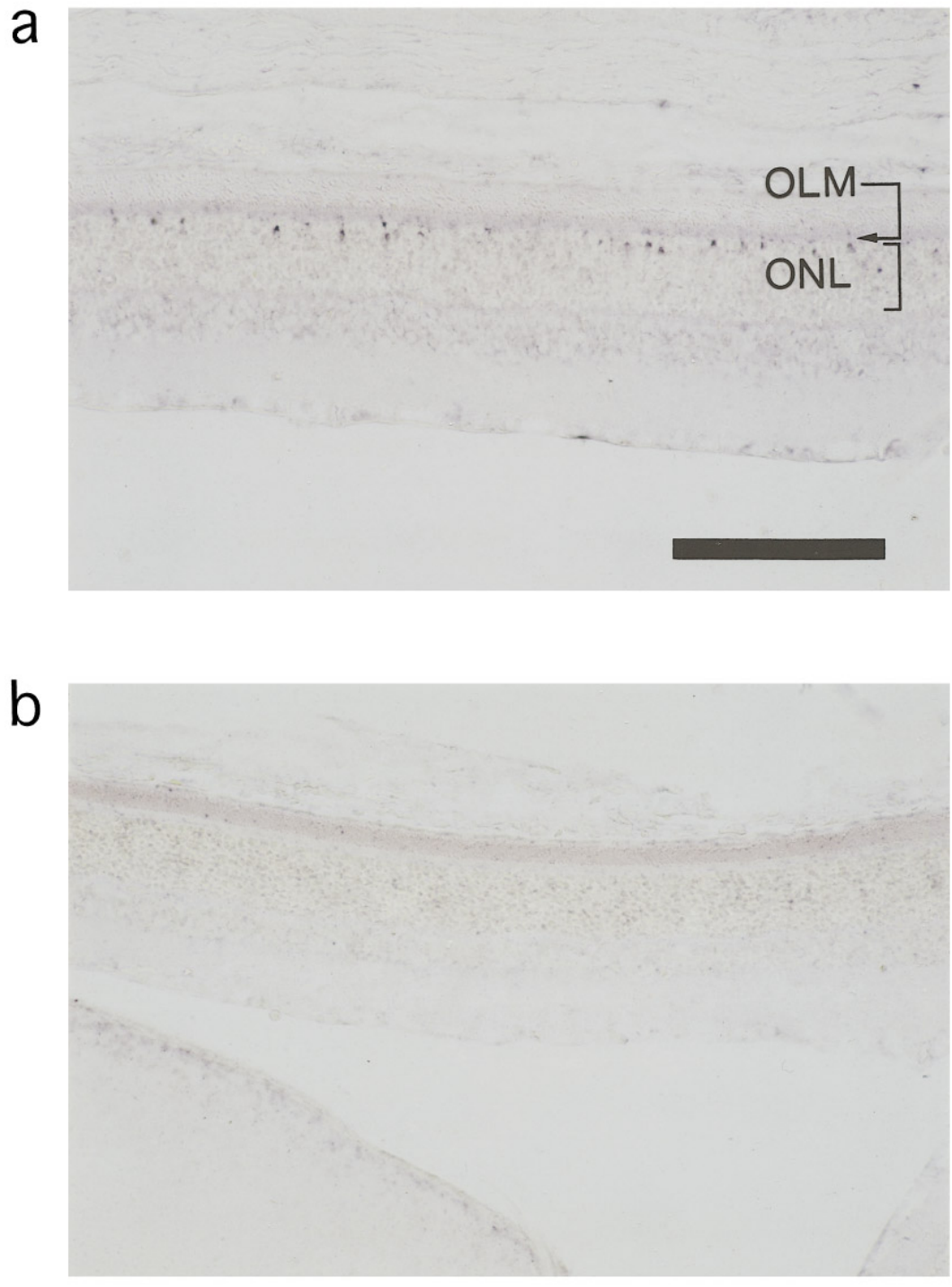

FIG. 3. Distribution of AA-NAT transcript in the retina determined by *in situ* hybridization. Rats were housed in LD 12:12 (lights-on ZT 0-12), and retinas were obtained at ZT 17 (A) or ZT 5 (B). AA-NAT mRNA was detected using a digoxigenin-labeled antisense cRNA probe. The strand-specific hybridization signal for AA-NAT transcript was observed in the outer nuclear layer (exclusively in the marginal region near the outer limiting membrane), and the signal was stronger at night (ZT 17, A) than in the day (ZT 5, B) in LD 12:12. Scale bar, 200 μm. ONL, outer nuclear layer; OLM, outer limiting membrane.

signal for rat AA-NAT transcript was detected in the outer nuclear layer (exclusively in the marginal region near the outer limiting membrane), and the signal was stronger at night (ZT 17; Fig. 3A) than at day (ZT 5; Fig. 3B) in LD 12:12 (lights-on ZT 0-12). In contrast, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA did not show significant daily changes (data not shown). The result strongly suggest that the site of AA-NAT mRNA circadian expression is in photoreceptor cells even in mammals.

DISCUSSION

Here we report the circadian expression of AA-NAT mRNA in the rat retinal photoreceptors. Retinal AA-NAT mRNA exhibited a daily rhythm with peak levels at night in LD 12:12 (examined by RNase protection assay and *in situ* hybridization), and the rhythm persisted in DD. AA-NAT activity in the retina also showed a daily rhythm with peaks at night in LD 12:12. In previous report(10), temporal Northern blot analysis data is very consistent with the data obtained in this report. These findings indicate that the AA-NAT mRNA circadian expression in the rat retina is regulated by an endogenous circadian clock.

In amphibians and avians, retinal photoreceptors have been shown to have a circadian clock that regulates rhythmic melatonin synthesis in the retina (1,3,20,21). *In situ* hybridization analysis in this study revealed that rat AA-NAT transcript was also detectable in the outer nuclear layer that contains photoreceptor cells. As the number of cone cells is known to be less than that of rod cells in rat retina, the specific signal of AA-NAT mRNA seemed to be detect in cone cells. Thus, mammalian photoreceptor cells might also be important in the generation of retinal AA-NAT rhythm as observed in amphibians and avians. This is the first report to localize the site of AA-NAT mRNA circadian expression in mammalian photoreceptor cells.

In mammals, circadian melatonin production in the pineal is controlled by the circadian oscillator in the SCN of hypothalamus (5). However, there are increasing the number of reports suggesting that mammalian photoreceptor cells are driven by a circadian oscillator in the retina independent of the SCN (6-10). Therefore, the rhythm of rat photoreceptor AA-NAT mRNA may be regulated by an endogenous circadian clock in the retina. To examine this possibility in mammals, experiments using SCN-lesioned animals is underway in our laboratory.

Several reports have demonstrated that the relationship between AA-NAT mRNA and activity differs from species to species (1,12). In the rat pineal, transcriptional events play a more important role in regulating AA-NAT activity than post-transcriptional events do (1,10,22). But AA-NAT activity in the retina may also be regulated, at least in part, at the post-transcriptional level, because AA-NAT activity began to increase earlier (at ZT 8) than the AA-NAT mRNA level do in LD condition(Figs. 1 and 2).

The promoter sequence analysis of the rat AA-NAT gene (18) demonstrates the existence of cAMP responsive element [CRE, (23,24)] and activator protein-1 [AP-1, (25)] sites in the 5′ upstream region. Therefore, cAMP responsive element-binding protein [CREB, (14,26)], inducible cAMP early repressor [ICER, (27)] and Fos-related antigen-2 [Fra-2, (18,19)] may be involved in the circadian transcription of AA-NAT gene in the rat retina. In this study, we first tested temporal Fra-2 mRNA expression pattern in compared with the same source of RNA used in the circadian AA-NAT mRNA expression under LD and DD conditions. In contrast to pineal(18,19), Fra-2 product did not seem to be involved in the circadian expression of AA-NAT mRNA in the rat retina(Fig. 1). The data suggest that AP-1 is not so important in the circadian expression of AA-NAT in the retina than that in the pineal(18,19).

The elucidation of the mRNA rhythm of the AA-NAT gene in the rat retina will provide an important clue in solving the molecular mechanism of a circadian clock in the mammalian retina.

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