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Transcripts Encoding Two Melatonin Synthesis Enzymes in the Teleost Pineal Organ: Circadian Regulation in Pike and Zebrafish, But Not in Trout*

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ABSTRACT

In this report the photosensitive teleost pineal organ was studied in three teleosts, in which melatonin production is known to exhibit a daily rhythm with higher levels at night; in pike and zebrafish this increase is driven by a pineal clock, whereas in trout it occurs exclusively in response to darkness. Here we investigated the regulation of messenger RNA (mRNA) encoding serotonin *N*-acetyltransferase (AA-NAT), the penultimate enzyme in melatonin synthesis, which is thought to be primarily responsible for changes in melatonin production. AA-NAT mRNA was found in the pineal organ of all three species and in the zebrafish retina. A rhythm in AA-NAT mRNA occurs *in vivo* in the pike pineal organ in a light/dark (L/D) lighting environment, in constant lighting (L/L), or in constant darkness (D/D) and *in vitro* in the zebrafish pineal organ in L/D and L/L, indicating that these transcripts are regulated by a circadian clock. In contrast, trout pineal AA-NAT mRNA levels are stable *in vivo* and *in vitro* in L/D, L/L, and D/D. Analysis of mRNA encoding the first enzyme in melatonin synthesis, tryptophan hydroxylase, reveals that the *in vivo* abundance of this transcript changes on a circadian basis in pike, but not in trout. A parsimonious hypothesis to explain the absence of circadian rhythms in both AA-NAT and tryptophan hydroxylase mRNAs in the trout pineal is that one circadian system regulates the expression of both genes and that this system has been disrupted by a single mutation in this species. (*Endocrinology* **139:** 905–912, 1998)

MELATONIN is an internal "Zeitgeber" involved in the timing and control of a number of rhythmic functions and behaviors in vertebrates (1–3). Circulating melatonin levels are higher during the night than during the day and reflect changes in the rate of melatonin synthesis in the pineal gland (4–6).

Systems responsible for the rhythmic production of melatonin by the pineal gland typically include a circadian clock, which drives the rhythm, and a photodetector, which mediates effects of light. However, the anatomical organization of these components differs markedly among vertebrates. In mammals, light acts through photodetectors in the retina to synchronize the activity of a circadian clock located in the hypothalamic suprachiasmatic nuclei. Rhythmic signals from the suprachiasmatic nuclei course through central and peripheral nerves to the pineal organ to drive rhythmic production of melatonin (4, 7, 8). In contrast, the entire system (the photodetector, circadian clock, and melatonin synthesis enzymes) is contained within the pineal gland of many nonmammalian vertebrates (9–16). Because of this, melatonin production by these organs continues to follow a circadian pattern *in vitro* in the absence of external cues, and light acts directly on these structures to reset this clock and turn off melatonin production. It is of interest to add that melatonin production in the retina of a number of vertebrates is also controlled by this type of self-contained system (16–20). These autonomous oscillatory systems are expected to play an important role in the discovery of the molecular structure of vertebrate clocks.

Teleost pineal organs are of further interest as circadian clock models for two reasons. First, the zebrafish has emerged as an excellent vehicle for genetic manipulation (21–23), which could further our understanding of clock function. Second, the trout represents a genetic variant among teleosts because the increase in melatonin at night in the trout pineal is not driven by a clock, whereas it is clock driven in other teleosts (24–27). Identification of the basis of this difference would provide new insight into vertebrate clock mechanisms.

Analysis of the regulation of the rhythm in melatonin synthesis in vertebrates, including fish, has revealed that it is driven primarily by a rhythm in the activity of the penultimate enzyme in melatonin synthesis, serotonin *N*-acetyltransferase [arylalkylamine *N*-acetyltransferase (AA-NAT); EC 2.3.1.87] (4, 26, 28). The molecular basis of the increase in AA-NAT activity in some, but not all, vertebrate pineal organs is due in part to an increase in AA-NAT messenger RNA (mRNA) (29). This is the case in the chicken pineal organ (30, 31), suggesting that the clock and this gene are linked, as is also true for tryptophan hydroxylase (TPH; EC 1.14.16) in

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several nonmammalian pineal glands and retinae (32–37). TPH is the first enzyme in melatonin synthesis.

Although the regulation of pineal AA-NAT activity and melatonin production has been investigated in teleosts (26, 28), the regulation of the abundance of pineal AA-NAT mRNA has not. To pursue this, AA-NAT mRNA was studied in the trout, pike, and zebrafish. An independent measure of clock function was obtained by studying TPH mRNA. The results of these studies are presented here.

Materials and Methods

Animals

Trout. Trout (*Oncorhynchus mykiss*; ~300 g; female) were commercially obtained. Fish were maintained in the laboratory in oxygenated recycled filtered pond water under conditions resembling (unless otherwise specified) the temperature and photoperiod (L = 1000 lux intensity at the water surface) of their natural habitat.

Pike. Pike (*Esox lucius*; 300-1500 g; male and female) were collected (mid-December to mid-March) from ponds of the Poitou-Charentes (France) and held in a commercial hatchery in small ponds supplied with river water under natural conditions.

For *in vivo* experiments, trout and pike were adapted for at least 24 h to the experimental photoperiod (dim red light was substituted for the dark period) and then were killed by decapitation at selected times during 24-h cycles of white light/red light (L/R), constant white light (L/L), or constant red light (R/R) conditions. For *in vitro* experiments, fish were killed upon delivery (~1200 h), and pineal organs were immediately cultured as described below.

Zebrafish. Adult zebrafish (*Danio rerio*; male and female) of the AB line were bred in the laboratory and raised in a 14-h light, 10-h dark (14:10 L/D) cycle to adulthood (2–6 months) before use in the experiments. Pineal organs were removed during the afternoon and cultured as described below.

Tissue

To remove pineal organs, the skull cap was removed, and pineal organs were quickly detached from the meninges and the roof of the diencephalon and frozen in liquid nitrogen for subsequent analysis or immersed in culture medium (see below). Other tissues were obtained by rapid dissection and were frozen in liquid nitrogen.

Organ culture

Trout. The culture medium used in all experiments was RPMI 1640 supplemented with bicarbonate (24 mmol/liter), HEPES (15 mmol/liter), and FCS (10%; Life Technologies, SARL, Cergy Pontoise, France) along with penicillin (100 IU/ml), streptomycin (100 μg/ml), glutamine (2 mmol/liter), and ascorbic acid (5.68 mmol/liter; Sigma Chemical Co., La Verpillière, France).

Pineal organs were cultured in 24-well culture plates (two pineals per well) containing 500 μl culture medium, renewed every 24 h. The temperature was maintained constant at 15 C (26). The pineal organs were cultured for 3 days under L/D, constant light (L/L), or constant darkness (D/D). At selected intervals of the third 24-h cycle, organs were frozen in liquid nitrogen, under room light for the midday samples or under dim red light for the midnight samples, and then stored at −80 C. Other conditions are indicated in *Results* and the figure legends.

Zebrafish. Pineal organs were cultured for 3 days at 21 C as described previously (16). They were then collected at different times of the L/D cycle. For the L/L experiments, continuous light was started at the normal time of light onset, then pineal organs were harvested after 30 and 42 h in constant light and processed immediately for total RNA as described below. Other experimental details are indicated in the figure legends.

Northern blot analysis

RNA preparation. Total RNA was extracted using a guanidine HCl/phenol method (Trizol, Life Technologies, Gaithersburg, MD) as described by the manufacturer. Polyadenylated [poly(A)$^+$] RNA were purified using oligo(deoxythymidine) latex beads (Oligotex, Qiagen, Chatsworth, CA) from total RNA as described by the manufacturer. RNA was then fractionated on a 1.5% agarose-0.66 M formaldehyde gel. RNA was transferred to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL) by capillary blotting and cross-linked to the membrane using UV.

Probes. A fragment of trout AA-NAT was amplified from trout pineal complementary DNA (cDNA) using degenerate primers designed against regions highly conserved among available AA-NAT sequences. These primers correspond to amino acids 49–55 and 117–123 of the sheep AA-NAT (GenBank accession no. U29663) and amplified a 184-bp fragment, excluding primers (Fig. 1). This fragment was cloned into pCRII (Invitrogen, Carlsbad, CA), and three independent clones were sequenced in both directions. The cloned fragment (clone T3) was excised using *Eco*RI and used to generate a ^{32}P-labeled probe by random priming.

A fragment of chicken TPH was amplified from chicken pineal cDNA, yielding a 1337-bp fragment corresponding to bases 83–1420 of the chicken TPH cDNA (GenBank accession no. U26428). This fragment was ^{32}P labeled directly by random priming.

Hybridization. Northern blots from all three fish species were probed for AA-NAT as previously described (38) for 1.5 h at 68 C in hybridizing solution (QuikHyb, Stratagene, La Jolla, CA) containing probe at 10^6 cpm/ml. After a final wash for 15 min at 60 C in 0.1 × SSC-0.1% SDS, blots were exposed overnight to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). In addition, some blots were stripped and probed using the chicken TPH probe under the same conditions as those described for AA-NAT, except they were hybridized at 60 C and the final wash was performed at 50 C.

After being probed for either AA-NAT or TPH, blots were stripped and probed using a ^{32}P-labeled, random primed, human β-actin probe (Clontech, Palo Alto, CA) for normalization. Blots were hybridized under the same conditions as those described for AA-NAT.

Quantitation and normalization

Band intensities for AA-NAT, TPH, and β-actin were measured by integrating the volume under the peaks of interest (ImageQuant soft-

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          56                                                          116
Sheep     FiSVS GnCPLnLDEv qhFLtlCPEL SLGWFvEGrL VAFIIGSlWd eeRLtQesla lHrPrg
Chicken   FiSVS GdCPLhLDEi rhFLtlCPEL SLGWFeEGrL VAFIIGSlWd qdRLsQaalt lHnPrg
Trout     FvSVS GeCPLtLDEv lnFLsqCPEL SLGWFeEGqL VAFIIGSgWg keRLeQeamt qHiPet
Consensus F-SVS G-CPL-LDEv --FL--CPEL SLGWFeEG-L VAFIIGS-W- -eRL-Qea-t -H-P--
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FIG. 1. Sequence analysis of the trout AA-NAT probe (clone T3; GenBank accession no. AF033500). A fragment of trout AA-NAT was generated by PCR from trout pineal cDNA. The deduced amino acid sequence of this fragment (excluding primers) is compared with relevant regions of the sheep and chicken AA-NAT amino acid sequence (GenBank accession no. U29663 and U46502, respectively). *Uppercase letters* designate residues that conform to the consensus of all three sequences. *Lowercase letters* in the consensus designate trout residues that match either sheep or chicken. The *underlined* residues are 100% conserved among previously sequenced AA-NATs.

ware, Molecular Dynamics). Multiple transcripts were summed and normalized to the β-actin signal. Images in the figures are reproduced from autoradiographic film, except in some cases where the image was generated from the PhosphorImager, as indicated.

Results

Clone T3 corresponds to AA-NAT

Trout pineal clone T3 encodes a region of the open reading frame of AA-NAT that includes the putative arylalkylamine-binding domain (Fig. 1); this region contains three stretches of amino acids that are 100% conserved among all known AA-NAT sequences (29). These three stretches are also highly conserved in clone T3; the overall nucleic acid sequence of clone T3 is 72% and 71% identical to sheep and chicken, respectively, whereas the amino acid sequence is 69% identical to both sheep and chicken. A search of the available databases found significant similarity only with known AA-NATs, indicating that clone T3 is derived from trout AA-NAT.

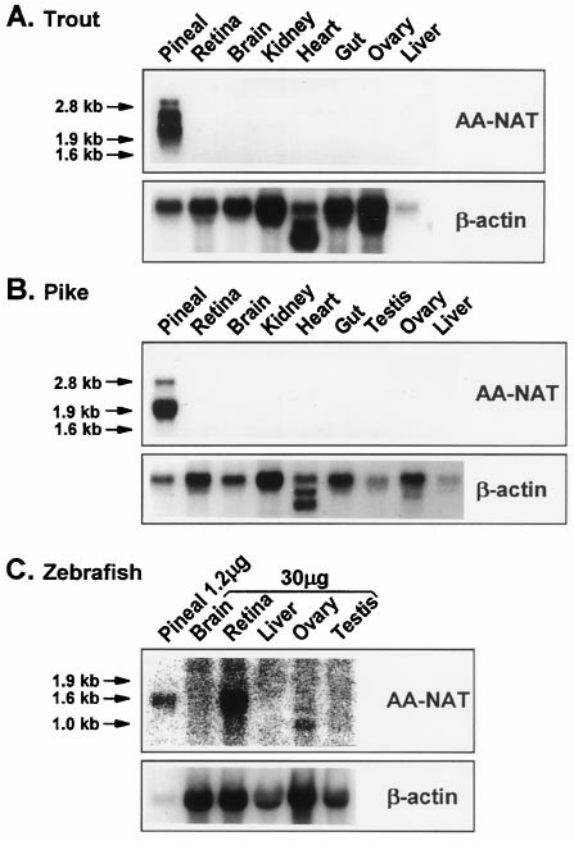

FIG. 2. Tissue-specific expression of AA-NAT mRNA in trout (A), pike (B), and zebrafish (C). Northern blot analysis was performed on total RNA obtained from selected tissues removed at midnight. RNA preparation and Northern blot analysis were performed as described in *Materials and Methods*. Each lane contains 20 μg (trout), 10 μg (pike), or 30 μg (zebrafish; 1.25 μg for pineal) total RNA. After detection of AA-NAT mRNA, the blots were stripped and probed for β-actin mRNA. Mol wt standards are shown on the *left*. The image of zebrafish AA-NAT transcripts was generated by a PhosphorImager.

AA-NAT mRNA is expressed at high levels in the teleost pineal gland, but not in other tissues

Blots containing total RNA from selected tissues from trout, pike, and zebrafish collected at midnight were probed using clone T3 (Fig. 2). In trout and pike, AA-NAT transcripts were only detectable in the pineal organ and not in retina, brain, kidney, gut, heart, testis, ovaries, or liver (Fig. 2, A and B). AA-NAT transcripts were also not detected in Northern blots of specific brain regions (data not shown). In zebrafish, AA-NAT transcripts were detectable in the retina as well as in the pineal organ; a transcript was not detectable in other tissues, except for a weak low mol wt signal in the ovaries (Fig. 2C).

Multiple forms of pineal AA-NAT mRNA exist in the pike and trout, but not in zebrafish

The population of pineal AA-NAT mRNA transcripts in two of the teleosts examined was different from that in all other vertebrates examined. In most species, AA-NAT mRNA is present as a single 1.0- to 1.6-kb band; however, multiple bands are present in trout and pike. In the former, there are three major forms [2.2, 2.6, and 3.0 kilobases (kb)] and an additional minor form (1.8 kb; Fig. 2A). In the latter, pineal AA-NAT mRNA is present as two prominent bands (2.0 and 2.8 kb; Fig. 2B). In zebrafish, however, only a single 1.7-kb band is present (Fig. 2C).

Northern blot analysis of poly(A)$^+$ RNA from pike and

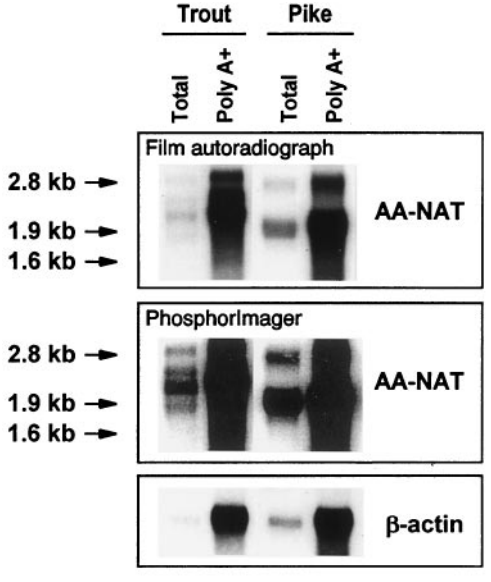

FIG. 3. Polyadenylated RNA in trout and pike pineal organs. RNA preparation, poly(A)$^+$ RNA purification, and Northern blot analysis were performed as described in *Materials and Methods*. Each lane contains 2 μg (trout) or 3 μg (pike) total RNA or poly(A)$^+$ RNA. RNA was purified from pooled pineal organs collected throughout a 24-h cycle. The *top* and *bottom panels* were generated by overnight exposure to autoradiography film; the *middle panel* is a darker image generated by the PhosphorImager of the same blot and is presented with the intention of demonstrating the presence of the very weak 1.8-kb band in the trout total RNA; this weak band has been found to be consistently detectable by visual inspection of the original images. After detection of AA-NAT mRNA, the blots were stripped and probed for β-actin mRNA. Mol wt standards are shown to the *left*.

trout pineal organs generated images similar to those obtained with total RNA, indicating that these multiple forms of AA-NAT mRNA represent mature species (Fig. 3). However it was observed that the minor 1.8-kb band in total RNA fractions of the trout pineal organ was not present in the poly(A)$^+$-selected fraction, suggesting that it is a nonpolyadenylated transcript of AA-NAT mRNA. The significance of multiple AA-NAT mRNA transcripts in teleosts is not clear.

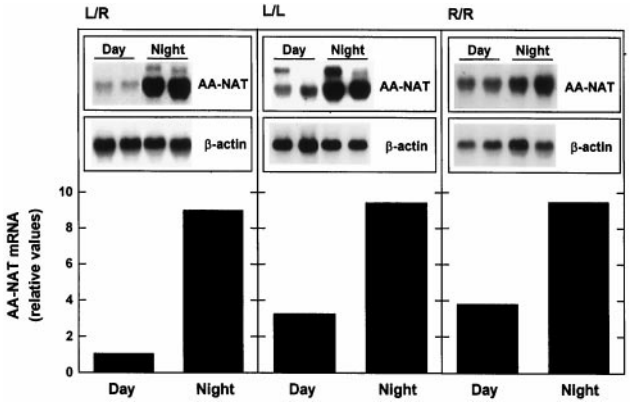

FIG. 4. Pike pineal AA-NAT mRNA exhibits a circadian rhythm *in vivo*. Pike were maintained in aquaria as described in *Materials and Methods* for at least 24 h under an L/R cycle (11:13; lights on at 0800 h), in constant white light (L/L), or in constant red light (R/R) before being killed. Pineals were removed during the day (1200 h) and during the night (2400 h). RNA preparation and Northern blot analysis were performed as described in *Materials and Methods*. Each lane contains 10 μg total RNA obtained from a pool of two or three pineal organs. After detection of AA-NAT mRNA, the blots were stripped and probed for β-actin mRNA. The values of AA-NAT mRNA are the mean of duplicates for one experiment. Results were confirmed in an independent experiment.

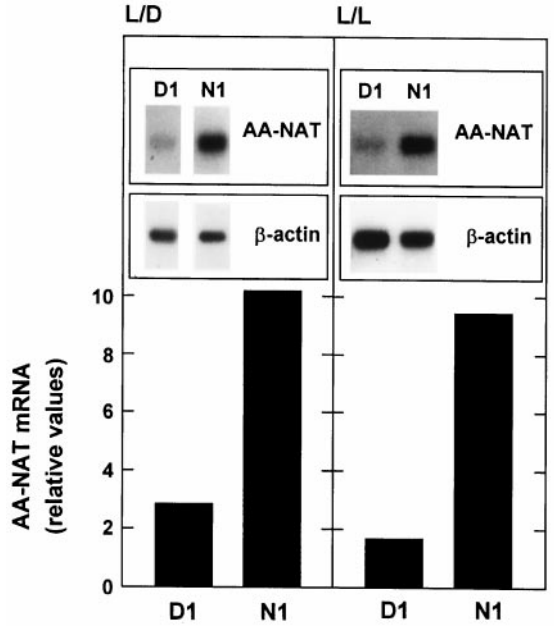

FIG. 5. Zebrafish pineal AA-NAT mRNA exhibits a circadian rhythm driven by a pineal clock *in vitro*. Zebrafish pineal organs were cultured for 2 days either under an L/D cycle (14:10; lights on at 0600 h) or under constant light (L/L) before sampling. The pineal organs were collected at midday (1300 h; D1) or midnight (0100 h; N1). Each lane contains 1.5 μg total RNA obtained from a pool of 40–50 pineal organs. After detection of AA-NAT mRNA, the blots were stripped and probed for β-actin mRNA. Results were confirmed in an independent experiment.

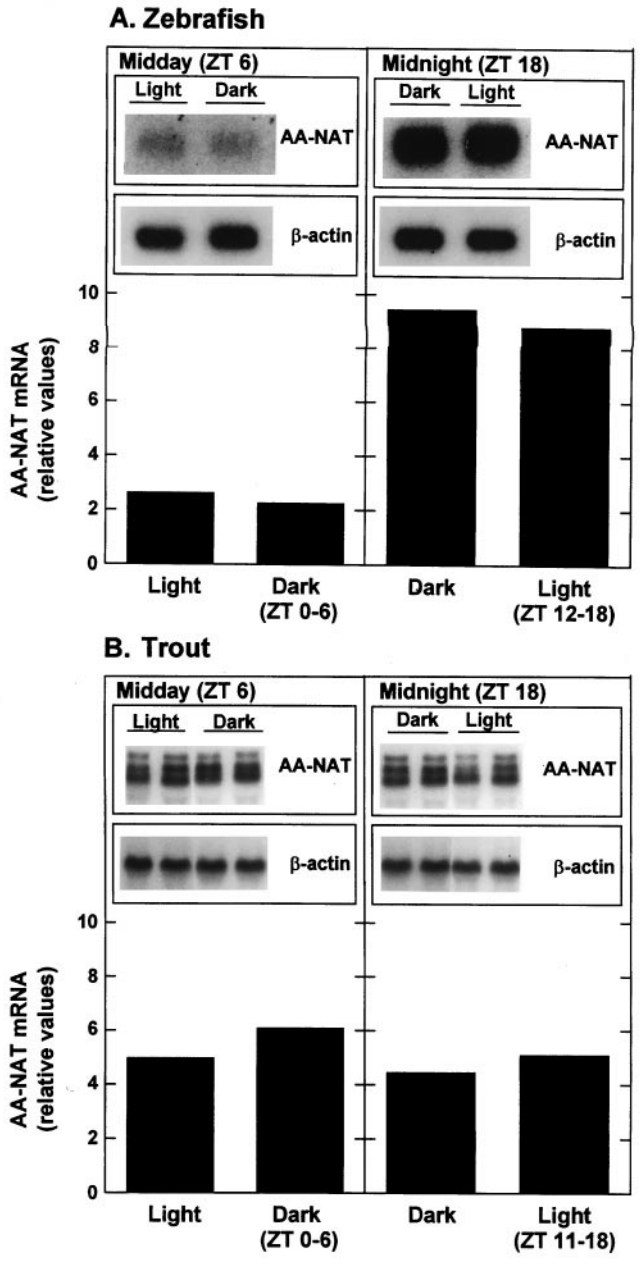

FIG. 6. Effects of extended photoperiods on zebrafish (A) and trout (B) AA-NAT mRNA *in vitro*. Zebrafish and trout pineal organs were cultured in controlled lighting for 2 days under an L/D cycle (12:12; lights on at 0800 h). On the third day, some pineal organs were exposed to unexpected dark for 6 h (lights were not turned on at 0800 h). On the third night, some pineal organs were exposed to unexpected light for 6 h (lights were not turned off at 2000 h). RNA preparation and Northern blot analysis were performed as described in *Materials and Methods*. For trout, each lane contains 20 μg total RNA obtained from a pool of 5 pineal organs. For zebrafish, each lane contains 1 μg total RNA obtained from a pool of 40–50 pineal organs. After detection of AA-NAT mRNA, the blots were stripped and probed for β-actin mRNA. The values of AA-NAT mRNA are the mean of duplicates for one experiment. Results were confirmed in an independent experiment.

A circadian rhythm in the abundance of pineal AA-NAT mRNA is apparent in pike and zebrafish, but not in trout

Pike. Pike pineal AA-NAT mRNA levels in the intact animal maintained in a 11:13 L/D cycle (L/R) were 8-fold higher at 2400 h than at 1200 h. This rhythm persisted in constant light (L/L) or constant darkness (R/R), although the apparent amplitude was 4- and 2.5-fold, respectively (Fig. 4). The reduction in the average amplitude may reflect asynchrony among individuals, which can develop in constant lighting conditions.

Zebrafish. An *in vitro* rhythm in the abundance of AA-NAT mRNA was detected in zebrafish after 3 days in L/D; the 1.7-kb transcript was 5-fold more abundant at midnight than at midday (Fig. 5). This pattern was maintained when organs were cultured in L/L after the first day in L/D (Fig. 5) and also when either the dark or the light period of the normal L/D cycle was extended by 6 h (Fig 6A).

Trout. Trout pineal AA-NAT mRNA did not exhibit a marked night/day difference under any lighting condition tested in *in vivo* studies (L/R, L/L, or R/R; Fig. 7). Similarly, a rhythm was not detected *in vitro* under L/D, L/L, or D/D (Fig. 8). Exposure of cultured pineal organs to darkness during the first 6 h of the normal light period of the day or to light during the first 6 h of the normal dark period of the night did not alter AA-NAT mRNA levels (Fig. 6B).

A circadian rhythm in the abundance of pineal TPH mRNA is apparent in vivo in pike, but not in trout

The abundance of TPH mRNA in pike and trout pineal organs was studied using a chicken TPH probe; major transcripts of 2.4 and 2.8 kb were detected in pike and trout, respectively (Figs. 9 and 10). The pattern of TPH mRNA abundance was similar to that obtained with the AA-NAT probe. In pike, TPH mRNA levels were higher during subjective night than during subjective day in L/L or R/R (2.7- and 2-fold, respectively; Fig. 9). Under a light/dark cycle (L/R), a modest increase in TPH mRNA appeared to occur at night (Fig. 9). In trout, the abundance of the TPH mRNA

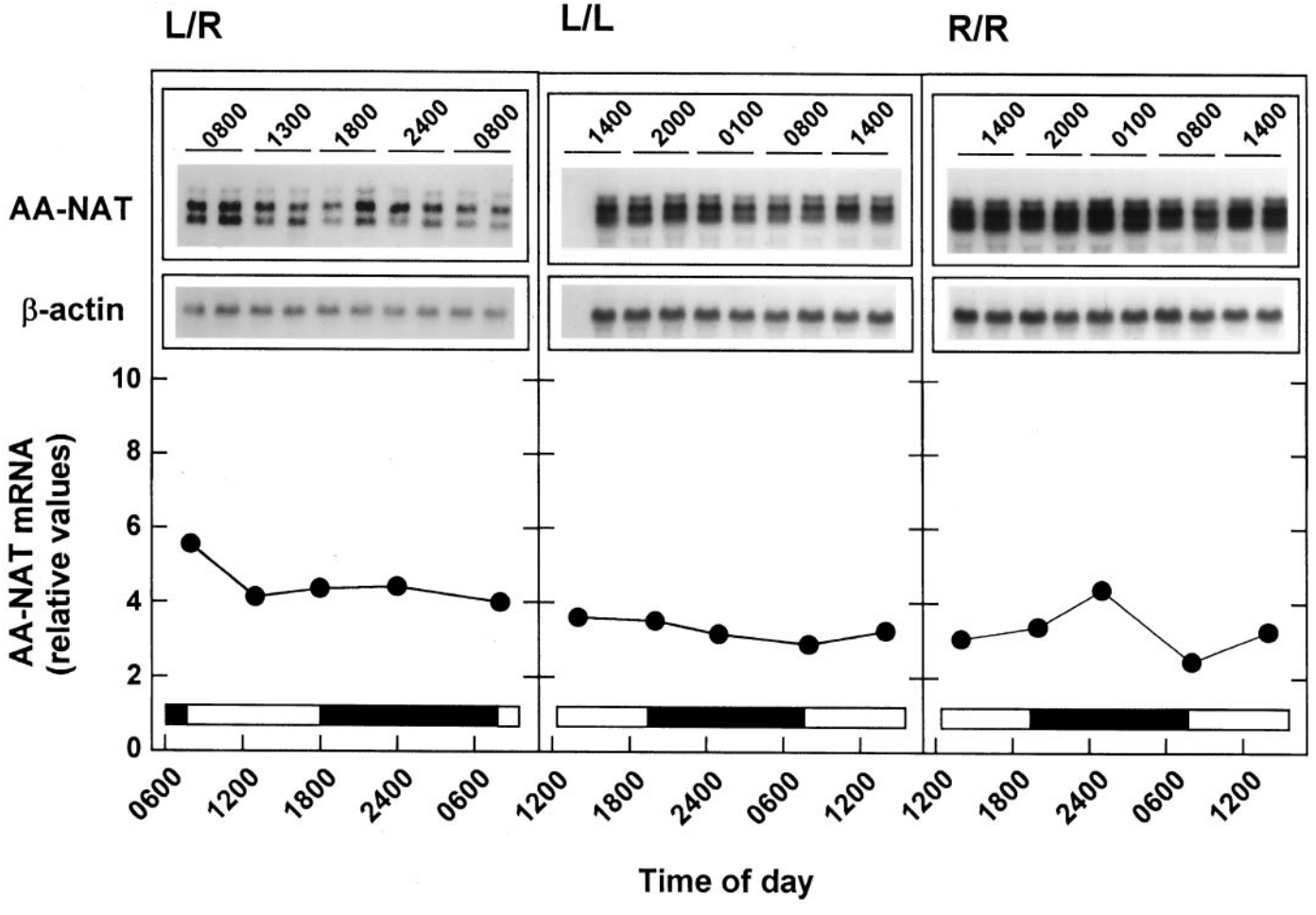

FIG. 7. Light does not alter trout pineal AA-NAT mRNA abundance *in vivo*. Trout were maintained in aquaria, as described in *Materials and Methods*, for at least 24 h either under an L/R cycle (10:14; lights on at 0800 h) or in constant white light (L/L) or in constant red light (R/R), before being killed. The light/dark bars shown for L/L and R/R designate the subjective photoperiod. Pineal organs were removed throughout the 24-h cycles at the indicated times. RNA preparation and Northern blot analysis were performed as described in *Materials and Methods*. Each lane contains 20 μg total RNA obtained from a pool of five pineal organs. After detection of AA-NAT mRNA, the blots were stripped and probed for β-actin mRNA. The values of AA-NAT mRNA are the mean of duplicates for one experiment. Results were confirmed in an independent experiment.

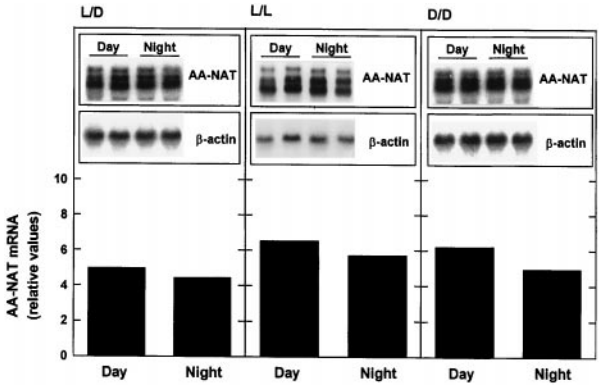

FIG. 8. Light does not alter trout pineal AA-NAT mRNA abundance *in vitro*. Trout pineal organs were cultured in controlled lighting for 3 days either under an L/D cycle (12:12; lights on at 0800 h) or in constant light (L/L) or in constant darkness (D/D) as described in *Materials and Methods*. The light/dark bars shown for L/L and D/D designate the subjective photoperiod. Pineal organs were collected during the day (1400 h) or during the night (0200 h). RNA preparation and Northern blot analysis were performed as described in *Materials and Methods*. Each lane contains 20 μg total RNA obtained from a pool of five pineal organs. After detection of AA-NAT mRNA, the blots were stripped and probed for β-actin mRNA. The values of AA-NAT mRNA are the mean of duplicates for one experiment. Results were confirmed in an independent experiment.

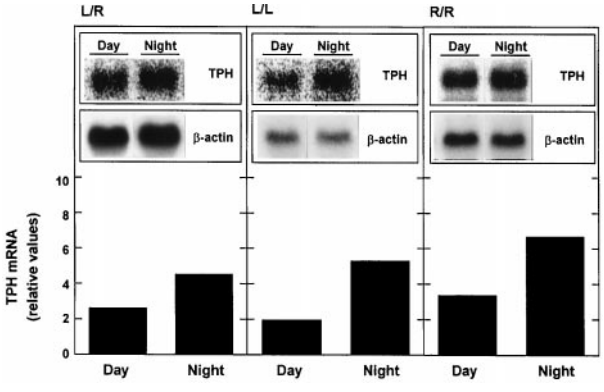

FIG. 9. TPH mRNA exhibits a circadian rhythm in pike pineal organs *in vivo*. The blots shown in Fig. 4 were stripped and probed for TPH. The values of TPH mRNA are the mean of duplicates for one experiment. Results were confirmed in an independent experiment. The images of pike TPH transcripts were generated by a PhosphorImager.

did not change under any lighting conditions tested (L/R, L/L, and R/R; Fig. 10).

Discussion

The results of this study are of special importance because they demonstrate that there is a circadian rhythm in the abundance of AA-NAT mRNA in the pineal organ of two teleosts, but not in a third. The evidence that this rhythm persists under constant lighting conditions *in vivo* in pike and *in vitro* in zebrafish indicates that the clock driving this rhythm is located within the pineal organ, and that it is not driven by changes in lighting. An endogenous pineal clock probably drives the AA-NAT mRNA rhythm in the pike, as melatonin production exhibits a circadian rhythm in constant lighting conditions even in cultured pike pineal photoreceptors (15).

The failure to detect a rhythm in AA-NAT mRNA in the trout pineal organ is especially interesting because this may reflect differences at one or more points in the regulatory mechanism. This includes the clock and output pathways that link the clock and the AA-NAT gene and in the AA-NAT gene itself. Our observation that the circadian rhythm in TPH mRNA seen in the pike is not present in the trout suggests that there is a difference upstream of both genes, perhaps in the clock or in an output pathway. A parsimonious hypothetical explanation is that a single circadian system regulates the expression of both TPH and AA-NAT in pike and other teleosts and that the system has been disrupted in trout by a single mutation. It is also possible that multiple mutations have occurred that disrupt each rhythm independently in this species.

The results of this study have raised another interesting question regarding retinal AA-NAT mRNA. We were unable to detect AA-NAT mRNA transcripts in the pike and trout retina by Northern blot, as is the case in the rat (38), but not in chicken, sheep, or human (30, 39, 40). The inability to detect retinal AA-NAT mRNA in these species is surprising because previous studies have clearly indicated that AA-NAT activity is present in the pike and trout retina at levels generally similar to those in the pineal organ (41) (Falcón, J., unpublished observation). This apparent discrepancy may reflect conditions within the fish retina that make it possible for large amounts of AA-NAT protein to accumulate, although AA-NAT mRNA is not abundant. Another hypothetical explanation is that a second AA-NAT gene exists, that this is expressed in the retina, and that mRNA encoded by this gene is not detected by the AA-NAT probe used in this study.

The final point of interest is related to how melatonin production is regulated. It would appear that the rhythm in melatonin in many teleosts reflects a rhythm in AA-NAT activity. In the case of pike and zebrafish, our studies indicate that the endogenous clock in the pineal gland contributes to the rhythmic component of melatonin production by driving a rhythm in AA-NAT mRNA, as is the case in the chicken pineal gland (30, 31). In contrast, clock-driven changes in AA-NAT mRNA do not play a role in melatonin production in the trout pineal gland, where light appears to turn activity off without changing mRNA levels. The available data on regulation of AA-NAT activity by light during the dark in rats and chickens indicate that large and rapid changes in activity occur without similar changes in mRNA (30, 38). Preliminary studies using anti-AA-NAT sera suggest that these changes in activity are closely linked to changes in protein (29). Accordingly, it is reasonable to suspect that a similar mechanism functions in the teleost pineal gland, and moreover, that this is either the dominant or the only regulatory mechanism operating in the trout pineal. Regulation of AA-NAT activity in the trout pineal appears similar to that in the mammalian pineal gland regarding the clock, because in neither system is a clock involved, and cAMP activates the system. In mammals, pineal cAMP is elevated by adrenergic activation, and in the trout, pineal cAMP is elevated by exposure to darkness, probably through a phosphodiesterase mechanism (4, 26, 42). In both, cAMP may control AA-NAT activity through regulation of protein. However, it is impor-

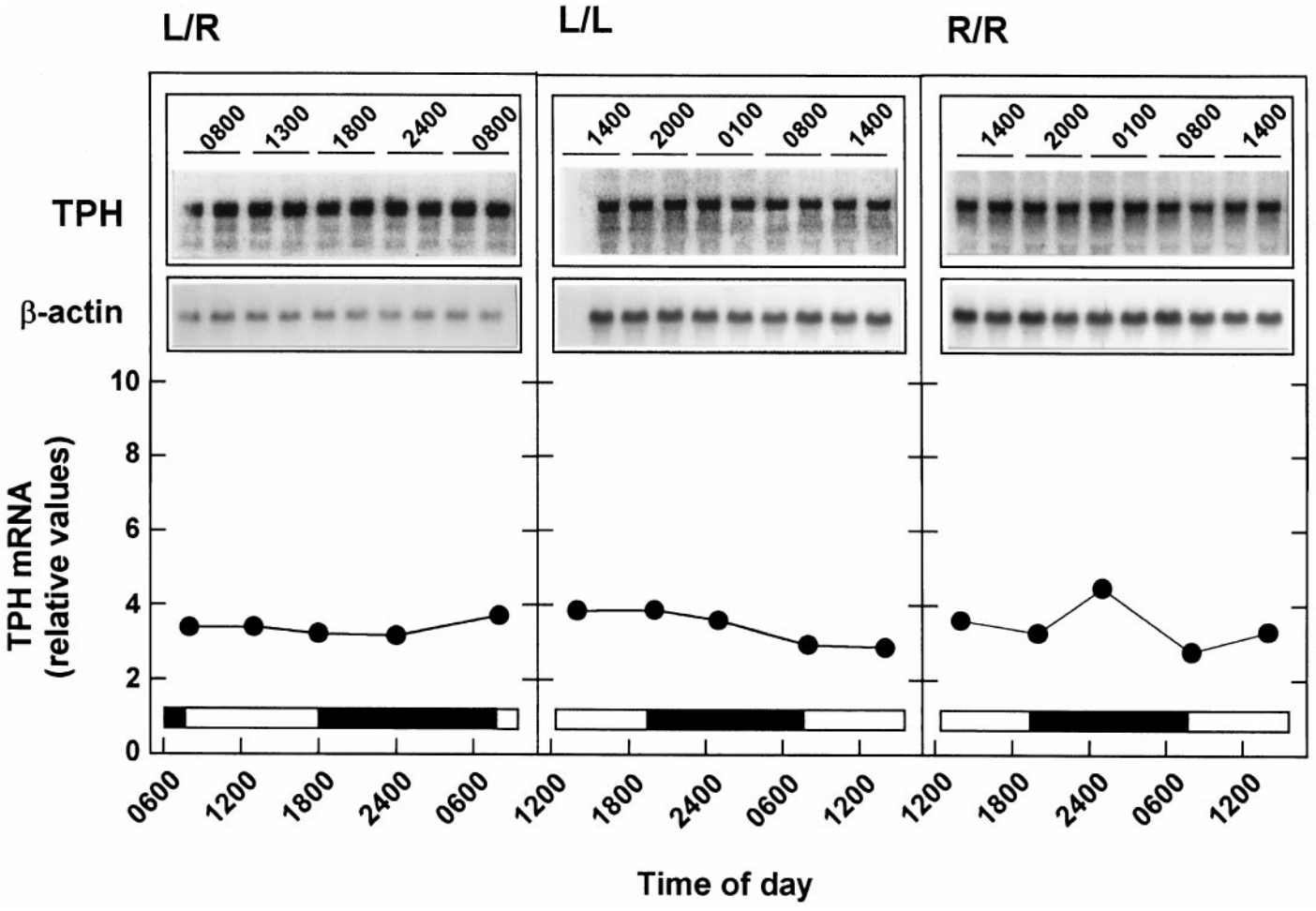

FIG. 10. Light does not alter trout pineal TPH mRNA abundance *in vivo*. The blots shown in Fig. 7 were stripped and probed for TPH. The light/dark bars shown for L/L and R/R designate the subjective photoperiod. The values of TPH mRNA are the mean of duplicates for one experiment. Results were confirmed in an independent experiment. The images of trout TPH transcripts were generated by a PhosphorImager.

tant to note that cAMP also appears to act in some pineal glands to alter the expression of the AA-NAT gene (29, 38).

These studies have raised a number of interesting issues regarding clock regulation of genes in the fish pineal organ and the use of this model to study the molecular basis of clock function. Continued study of this system might provide important insights into the molecular basis of vertebrate clock function.

Acknowledgments

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