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Regulation of Arylalkylamine *N*-Acetyltransferase-2 (AANAT2, EC 2.3.1.87) in the Fish Pineal Organ: Evidence for a Role of Proteasomal Proteolysis

JACK FALCÓN*, KRISTINA M. GALARNEAU, JOAN L. WELLER, BENNY RON, GALIT CHEN, STEVEN L. COON, AND DAVID C. KLEIN

Section on Neuroendocrinology (J.F., K.M.G., J.L.W., S.L.C., D.C.K.), Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-4480; Laboratoire de Neurobiologie Cellulaire (J.F.), Département des Neurosciences, CNRS-UMR 6558, F-86022 Poitiers Cédex, France; and Fish Physiology Department (B.R., G.C.), National Center for Mariculture, Oceanographic & Limnological Research Institute, Eilat 88112, Israel

ABSTRACT

In fish, individual photoreceptor cells in the pineal organ and retina contain complete melatonin rhythm generating systems. In the pike and seabream, this includes a photodetector, circadian clock, and melatonin synthesis machinery; the trout lacks a functional clock. The melatonin rhythm is due in part to a nocturnal increase in the activity of the arylalkylamine *N*-acetyltransferase (AANAT) which is inhibited by light. Two AANATs have been identified in fish: AANAT1, more closely related to AANATs found in higher vertebrates, is specifically expressed in the retina; AANAT2 is specifically expressed in the pineal organ. We show that there is a physiological day/night rhythm in pineal AANAT2 protein in the pike, and that light exposure at midnight decreases the abundance of AANAT2 protein and activity. In culture, this decrease is blocked by inhibitors of the proteasomal degradation pathway. If glands are maintained under light at night, treatment with these inhibitors increases AANAT2 activity and protein. Organ culture studies with the trout and seabream also indicate that the light-induced decrease of AANAT2 activity is prevented when proteasomal proteolysis is blocked. A cAMP-dependent pathway protects AANAT2 protein from degradation. These results provide a clue to understanding how light regulates the daily rhythm in melatonin secretion in fish photoreceptor cells and provides evidence that proteasomal proteolysis is a conserved element in the regulation of AANAT in vertebrates. (*Endocrinology* **142:** 1804–1813, 2001)

MELATONIN is an important component of the vertebrate circadian system; it is made in two sites involved in phototransduction, the pineal gland and retina. Pineal-derived melatonin controls the daily rhythm in circulating melatonin and provides a hormonal signal of night time to the organism. This signal plays a role in the timing and control of a number of biological rhythms (1, 2). Retinal melatonin has a local paracrine function related to photic adaptation (3).

In all vertebrates, melatonin production follows a circadian pattern that is controlled by melatonin rhythm generating systems that are typically composed of three basic elements, a photodetector, an endogenous clock and melatonin synthesizing machinery. In mammals, the melatonin rhythm generating system includes the retina (photodetector), suprachiasmatic nucleus (clock), and pineal gland (melatonin synthesis) (2). In contrast, in birds and fish, complete melatonin rhythm generating systems are located within individual photoreceptor cells in the pineal gland and retina (4). In most cases, this includes a photodetector, circadian clock, and melatonin synthesis machinery; however exceptions exist. For example, trout melatonin rhythm generating systems appear to lack a functional circadian clock. In species with cellular melatonin rhythm generating systems, melatonin production persists on a circadian basis when the pineal glands are placed in culture (5, 6).

Large changes in melatonin production are typically associated with similar changes in the activity of the penultimate enzyme in melatonin synthesis, arylalkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87), which controls the rate at which serotonin is converted to *N*-acetylserotonin (7, 8). In most vertebrates, only a single AANAT gene exists. However, in fish there are two AANAT genes, termed AANAT1 and AANAT2 (9–11). AANAT2 is the dominant form expressed in the pineal organ. AANAT1 is expressed predominantly in the retina, and is more similar to the AANAT in other vertebrate classes. AANAT1 acetylates indole- and phenyl-ethylamines equally well; AANAT2 strongly prefers indole-ethylamines.

The mechanisms controlling AANAT protein vary among vertebrates. For example, in some—but not all—cases changes in AANAT messenger RNA (mRNA) occur; in these species, low levels of AANAT mRNA determine when AANAT activity can increase. In most cases, mechanisms appear to exist at a post transcriptional level to control levels of AANAT protein (8, 12, 13).

In the pike pineal organ, the endogenous clock regulates

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Address all correspondence and requests for reprints to: David C. Klein, NIH 49–6A82, Bethesda, Maryland 20892-4480. E-mail: klein@helix.nih.gov.

* Current address: Laboratoire Aragó, Observatoire Oceanologique de Banyuls, UMR CNRS 7628, Université de Paris VI, 66651 Banyuls/Mer-Cedex, France.

changes in AANAT2 activity and AANAT2 mRNA (6, 10). Rhythmic changes in AANAT2 activity drive the rhythm in melatonin production. The translation of endogenously driven changes in AANAT2 mRNA into changes in AANAT2 activity is influenced by an exogenous signal –light. Light acts through two distinct pathways: one adjusts and resets the endogenous circadian clock and, as a result, the rhythm in AANAT2 mRNA and activity (6); this effect is of a gradual nature and is likely to involve shifting the rhythmic expression of clock genes (14). Light also acts rapidly, independently of the clock to suppress AANAT2 activity. This suppressive influence is in part responsible for low day levels of AANAT2 activity and for the light-induced suppression of enzyme activity that occurs when pike are exposed to a pulse of light at night. The importance of this mechanism is most obvious in trout, which, as indicated above, lack a pineal circadian clock and express the AANAT2 gene constitutively. In this case, light appears to be the dominant regulatory mechanism controlling day/night changes in AANAT activity and melatonin production (6).

The precise molecular details mediating the immediate effects of light on AANAT2 activity are poorly known. Studies in the rat, sheep, and chicken pineal organ indicate that AANAT activity and protein change in parallel on a daily basis. Studies of the rat and chicken pineal in culture indicate that AANAT protein is influenced by a cAMP-dependent mechanism that blocks destruction of AANAT protein *via* proteasomal proteolysis (12, 13). The high degree of homology between the different vertebrates AANATs, especially in the putative N-terminal region flanking the catalytic core of the molecule, suggests a similar mechanism might also operate in fish to control AANAT2 protein. However, regulation of fish AANAT2 protein has not been examined and it is not clear that mechanisms exist to control AANAT2 protein. In addition, second messenger control of fish pineal AANAT2 is still a matter of debate (6, 15). And, limited observations suggest that proteasomal proteolysis plays a minor role in controlling fish AANAT2 activity (15).

The present study focused on the question of whether AANAT2 protein in the fish pineal organ is regulated in a manner consistent with a role in regulating AANAT activity, as is the case in birds and mammals. Three fish species, pike, seabream and trout, were studied. Pike is a well studied nondomesticated fresh water species; the pike pineal gland contains a complete melatonin rhythm generating system, as is the case in the chicken (16). The seabream pineal organ also contains an endogenous clock; this species is of interest because it is of marine origin and is domesticated (17). In addition, it is abundantly available for analysis. A better understanding of melatonin production in this species might improve commercial breeding. Trout were used because AANAT2 activity is regulated by light without the mediation of an endogenous clock (18).

The results of the study indicate that there is a daily rhythm in the pike pineal gland in AANAT2 protein and that AANAT2 protein and activity change in a parallel manner *in vivo* and *in vitro*. Results from studies of all three species make apparent that regulatory systems are present in the fish pineal organ through which AANAT2 protein can be increased by elevation of cAMP or by inhibition of proteasomal proteolysis.

Materials and Methods

Animals

Pike (*Esox lucius* L., teleost; 250–500 g) of both sexes were obtained during the winter from naturally breeding populations in ponds at Poitou-Charentes ("Pisciculture Ichtyos," Vienne, France). For the *in vivo* experiments, fish were maintained for 24 h in oxygenated and filtered mixture of tap water and pond water, under conditions resembling their natural habitat with respect to temperature and illumination (natural photoperiod was 11 L (0730–1830)/13D; L = 1000 lux intensity at the water surface). They were killed by decapitation at selected time intervals of the LD cycle, and tissues were sampled and frozen in dry ice. Light samples were collected under fluorescent light, and dark samples under dim red light. For *in vitro* organ culture studies, animals were killed soon after their arrival to the laboratory, *i.e.* between 1700 and 1730 h; the pineal organs were removed and immersed in culture medium, and maintained at 4 C for no more than 1 h before the initiation of organ culture.

Trout (*Oncorhynchus mykiss,* teleost; 300 to 1,000 g) of both sexes were obtained from a commercial breeder ("Pisciculture du Moulin de Pied de Lance," Vienne, France). Animals were killed soon after their arrival at the laboratory (1100 and 1130 h); their pineal organs were removed and placed in culture medium; all other conditions were as above.

Seabream (*Sparus aurata,* teleost; 200–300 g) of both sexes were obtained from the National Center for Mariculture at Eilat (Israel). The photoperiod was 13.75 L (0545–1930)/10.25D. They were killed early in the afternoon, and the pineal organs were collected and prepared as above.

Organ culture

The culture medium was RPMI 1640 supplemented as indicate before (19). The pineal organs were cultured in 24-well culture plates containing 0.5 ml culture medium. Temperature was maintained constant at 22 C (seabream), 20 C (pike) or 12 C (trout). It has been shown that these temperatures are optimal for the culture of these organs (6). After removal, the pineal organs were cultured for 24 h under photoperiodic conditions similar to the natural ones (L = 1000 lux intensity). Experimental treatments were initiated thereafter as detailed in Fig. 1.

The organs were transferred to wells in a 96-well culture plate containing 300 μl of culture medium, and the drugs indicated or vehicle (controls). The final concentration of vehicle (dimethylsulfoxide) never exceeded 1%.

Treatments of trout pineal organs were always initiated at 1200 h because in this species, exposure to darkness at any time of the light/dark (LD) cycle produces similar increases in pineal AANAT2 activity and melatonin secretion (20, 21). Treatments of pike and seabream pineal organs were initiated at the onset of darkness, because the circadian oscillator that governs AANAT2 activity and melatonin secretion prevents a dark-induced rise in AANAT2 activity during the day and second half of the night (22, 23). Following the indicated treatments, organs were collected under fluorescent white light (light samples) or dim red light (dark samples) and frozen on dry ice. Treatments are described in the text and legends.

Assay of AANAT1 and AANAT2 activities

AANAT1 and AANAT2 activities were determined following modification (9, 11) of a published radiochemical procedure (24). To measure AANAT2 each pineal organ was sonicated in 50 μl of assay buffer (0.2 M, pH 6 phosphate buffer), and the homogenate was added to a 50 μl buffer solution containing tryptamine (10 mM), acetyl-CoA (0.5 mM) and [^{3}H]-acetyl-CoA (4 mCi/mmol). Incubation was for 60 min at 12 C (trout), or 20/22 C (pike, seabream). *N*-[^{3}H]-Acetyltryptamine was extracted into chloroform and radioactivity was determined. For the estimation of retinal AANAT1 activity phenylethylamine was used as the substrate, and the homogenates were incubated for 20 min at 37 C (9).

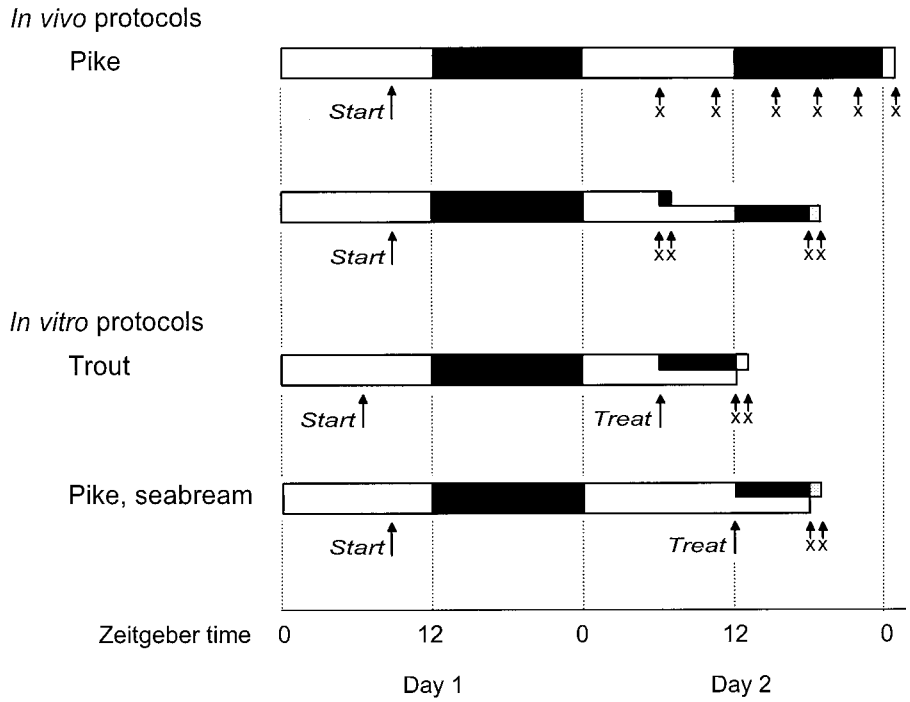

FIG. 1. Schematic representation of experimental design. The lighting cycle is represented by the *white (light)* and *black (dark) bars*. For the *in vivo* experiments "*Start*" indicates when animals were placed into laboratory holding tanks and "x" represents when pineal glands were removed and placed on dry ice. For the *in vitro* studies, "*Start*" indicates when pineal glands were removed and placed into organ culture, "*Treat*" indicates glands were treated with drugs and "x" represents when glands were removed from culture and placed on dry ice.

Production of anti-AANAT2 antisera (3345)

pAANAT2$_{4-29}$ [Accession No. AF034082] was conjugated to cationized BSA for use as an immunogen. Polyclonal anti-pAANAT2$_{4-29}$ serum was raised in a rabbit [#3345] (Covance Laboratories, Inc., Vienna, VA). Antiserum was immunopurified by exposure to sections of blots containing the immunizing peptide (pAANAT2$_{4-29}$); the adsorbed antibodies were eluted with 0.1 N acetic acid, pH 2.85, containing BSA (25).

Production of recombinant proteins

Recombinant glutathione S-transferase (GST) pike (p)AANAT2 (Accession No. AF034082, 10) and pAANAT1 (Accession No. AF034084) fusion proteins were obtained after transformation and expression in *Escherichia coli* strain BL21(DE3) pLysS. The methods used have been described (26), except that cells were grown overnight at 20 C after addition of isopropylthiogalactopyranoside. After centrifugation, cells (from 1 liter) were resuspended in 50 ml lysis buffer (2× PBS, 10 mM dithiothreitol, 1 mM EDTA) and stored at −80 C. Upon thawing, cells were lysed by sonication at 4 C followed by centrifugation at 48,000 × *g* for 45 min. A 5 ml sample of glutathione Sepharose 4B, previously equilibrated in 1× PBS, was added to the supernatant, and the slurry was mixed at 4 C for 1 h. The resin was then poured into a 100 ml column (Bio-Rad Laboratories, Inc.) and washed with 10 column volumes of buffer 2 (1× PBS, 0.5 M NaCl, 10 mM DTT, 1 mM EDTA) followed by 10 column volumes of buffer 3 (20 mM Tris [pH 7.5], 0.5 M NaCl, 10 mM DTT, 1 mM EDTA, 10% [wt/vol] glycerol). The protein was eluted at room temperature using 10 mM glutathione in buffer 3 (pH adjusted to ~8). Fractions containing the fusion protein (judged by absorption at 280 nm) were combined and dialyzed against buffer 3 containing 1 mM DTT. This and subsequent steps were carried out at 4 C in buffers sparged with helium. The GST-fusion protein was cleaved by adding 5 U thrombin per mg protein, and the reaction was monitored by SDS-PAGE. After cleavage, free GST and thrombin were removed by passage over a second column consisting of 2.5 ml glutathione Sepharose 4B and 0.5 ml benzamidine Sepharose 4B equilibrated in dialysis buffer. The recovered protein (>90% pure) was dialyzed against 50 mM sodium citrate (pH 6.5), 0.1 M NaCl, 10 mM DTT, 1 mM EDTA, 10% (wt/vol) glycerol. The final yield from 1 liter cells was approximately 4 mg protein.

Western blots

Pineal proteins were resolved on pre-formed 14% Tris/glycine (1 mm) gels using the standard Novex polyacrylamine gel electrophoresis protocol (Novex, Invitrogen, Carlsbad, CA). The gels were run at 125 V for 2 h. Rainbow standards (Amersham Pharmacia Biotech, Piscataway, NJ) were used to determine the molecular weight of the proteins.

Before electroblotting, gels were equilibrated (5 min) in 10 mM [3-cyclohexylamino]-1-propanesulfonic acid buffer, pH 11, containing 20% methanol and 0.1% SDS. The proteins were transferred in this buffer to Immobilon-P membrane (0.45 μm; Millipore Corp., Bedford, MA) with a semidry blotting following the manufacturers protocol system (Investigator Graphite Electroblotter system; Genomic Solutions; Chelmsford, MA). The transfer currents were 400 μA/cm^2 (20 min), then 800 μA/cm^2 (20 min) and finally 1200 μA/cm^2 (45 min).

After transfer, the membranes were air dried and then blocked for 2 h in phosphate buffer saline (PBS, pH 7.4) containing 10% nonfat dry milk, 0.2% Tween-20 and 0.05% thimerosal (TPBS). They were then incubated overnight (18 h at room temperature) in the primary antibody solution (1/250 dilution in PBS containing 1 mg BSA fraction V, and 0.05% thimerosal). The membranes were then washed successively in TPBS (2 × 5 min) and PBS (2× 5 min) before a 1 h exposure to horseradish peroxidase conjugated goat antirabbit IgG (0.0083 μg/ml in TPBS containing 0.1% normal goat serum). The membranes were then washed successively in TPBS (3 × 10 min) and PBS (3 × 5 min) and immunodetection was performed using Luminglo. Immunopositive reactions on the blots were finally exposed to BIOMAX MR or Kodak XAR 5 films. The immunopositive bands were quantified using the ImageQuant program (Molecular Dynamics, Inc., Sunnyvale, CA) after the films were scanned.

Chemicals

Acetyl-CoA was from Roche Molecular Biochemicals (Ingelheim, Germany). [^{3}H]-acetyl-CoA, was from NEN Life Science Products (Boston, MA). Clasto-lactacystine (β-lactone), lactacystine, MG132, proteasome inhibitor I, calpain inhibitor II were from Calbiochem (La Jolla, CA). Synthetic pAANAT2$_{4-29}$ was made by Research Genetics, Inc. (Huntsville, AL). RPMI 1640 was from Life Technologies, Inc. (Rockville, MD). Nonfat dry milk and glutathione Sepharose 4B were from Bio-Rad Laboratories, Inc. (Hercules, CA). Goat antirabbit IgG conjugated to horseradish peroxidase and Luminglo were from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Normal goat serum was from Pierce Chemical Co. (Rockford, IL). All other products were from Sigma (St. Louis, MO).

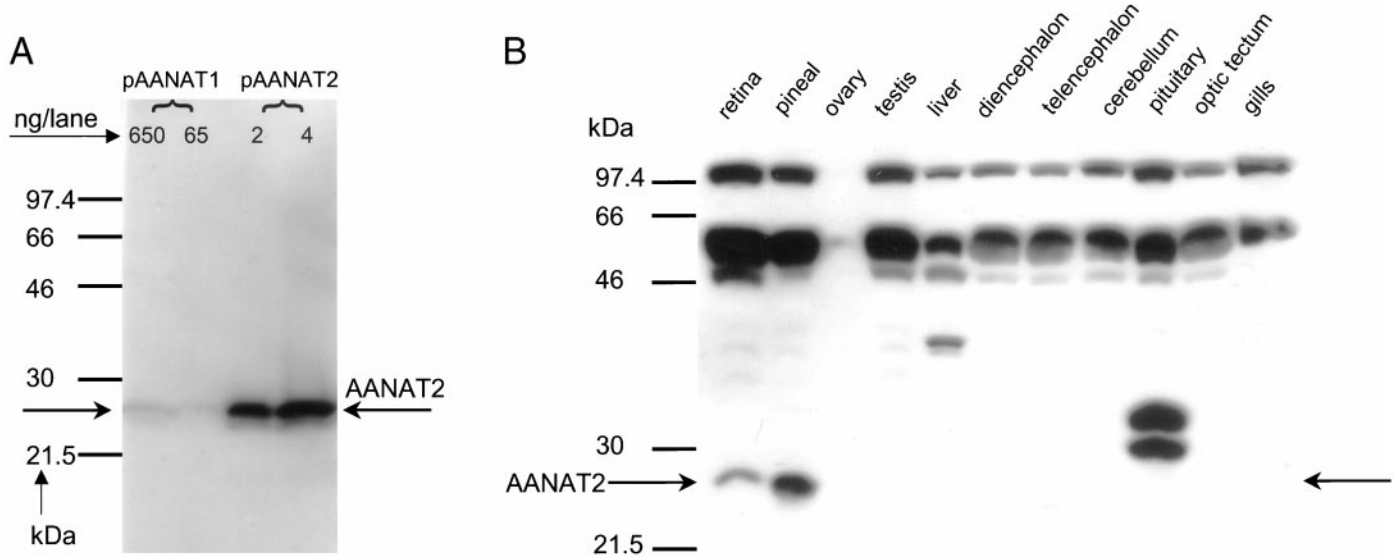

FIG. 2. Immunodetection of pike AANAT2 protein on Western blots using serum 3345. The utility of immunopurified anti-pAANAT2$_{4-29}$ serum 3345 in Western blot analysis of pAANAT2 was evaluated using expressed protein and tissue homogenates. A, *left panel*, Immunopurified serum 3345 detects a major band (26 kDa) in Western blots loaded with recombinant pAANAT2 protein (*two right lanes*). A faint signal was seen in lanes loaded with pAANAT1 (Accession No. AF034084) (*two left lanes*). Position of the molecular weight standards is indicated on the *left*, and the amount of protein loaded is indicated on the top of the blot. B, *right panel*, Pike tissues were obtained in the middle of the night (0100) of a light/dark cycle and prepared for Western blot analysis as indicated in *Materials and Methods*. The retinal lane was loaded with 140 μg of protein and the pineal lane with 50 μg of protein containing 2.36 (pAANAT1) and 0.27 (pAANAT2) nmol/h of AANAT activity, respectively (pAANAT1 and pAANAT2 activities were estimated as described in *Materials and Methods*). The optic tectum lane was loaded with 72 μg of protein; other lanes were loaded with 92 μg of protein. Bands at 26 kDa were only detected in the pineal organ and retina, indicating that this 26 kDa signal is a reliable indicator of AANAT2 protein. Position of the molecular weight standards is indicated on the *left*. For further details, see *Materials and Methods*.

Statistics

All experiments were run in duplicate or triplicate. Variability did not exceed 10%. Values were compared using the Student's *t* test, or by ANOVA followed by Tukey's comparison of means (Prism software, GraphPad Software, Inc., San Diego, CA).

Results

Physiological daily variations in pineal AANAT2 protein and AANAT2 activity are similar

Western blot analysis was used to determine if AANAT2 protein in the teleost pineal organ exhibited a daily rhythm, as is true of AANAT2 activity (27). *In vivo* studies were done in the pike using an antiserum (3345) raised against pAANAT2$_{4-29}$. This antiserum reacted with an approximately 26-kDa band of pineal protein (Fig. 2); the signal generated by the recombinant pAANAT2 protein had the same mass (Fig. 2A). An approximately 26-kDa band was also detected in the retina, albeit at a distinctly less intense level (Fig. 2B). Bands of this mass were not detected in other tissues, although nonspecific immunoreactive bands of higher mass were apparent, including two strong at approximately 30–35 kDa in the pituitary (Fig. 2B). Based on this pattern of expression and the predicted mass of pike AANAT2 protein (24 kDa), the approximately 26 kDa band of protein was used as an indicator of the amount of pAANAT2 protein. The observation that there is a difference between the predicted and apparent masses of both the expressed and recombinant pAANAT2 protein suggests that this is due to an inherent feature of the protein.

The pAANAT2 band was more intense in samples obtained at night, compared with those obtained during day (Fig. 3A). In addition, exposure to 1 h of light at night decreased the strength of the signal (Fig. 3B); exposure to darkness at midday failed to elevate AANAT2 activity and protein to night levels (Fig. 3B), consistent with the known involvement of a pineal clock in the regulation of pAANAT2 mRNA and activity as discussed (27, 19). In all cases, changes in AANAT2 activity were paralleled by similar changes in AANAT2 protein, as has been seen in other species (8, 12).

AANAT2 protein and activity increase during subjective night and following exposure to cAMP protagonists in the cultured pike pineal organ

To determine if light acted directly on the pineal photoreceptor to control AANAT2 protein and if the pineal clock regulated day/night changes in AANAT2 protein, the *in vivo* study in the pike was extended using organ culture. Baseline reference values for this study were established by incubating pineal organs from the pike during day, or during night, or in the light during subjective night. This confirmed (19), that pike AANAT2 activity was higher in glands obtained from organ culture in the dark at night compared with those obtained during the day (Fig. 4).

There was an approximately 7-fold increase of pike AANAT2 activity and protein levels at midnight compared with day values, or values obtained at mid-subjective night in constant light (Fig. 4). In addition, midnight illumination of dark-adapted organs induced a dramatic decrease of both pAANAT2 activity and pAANAT2 protein levels. As shown

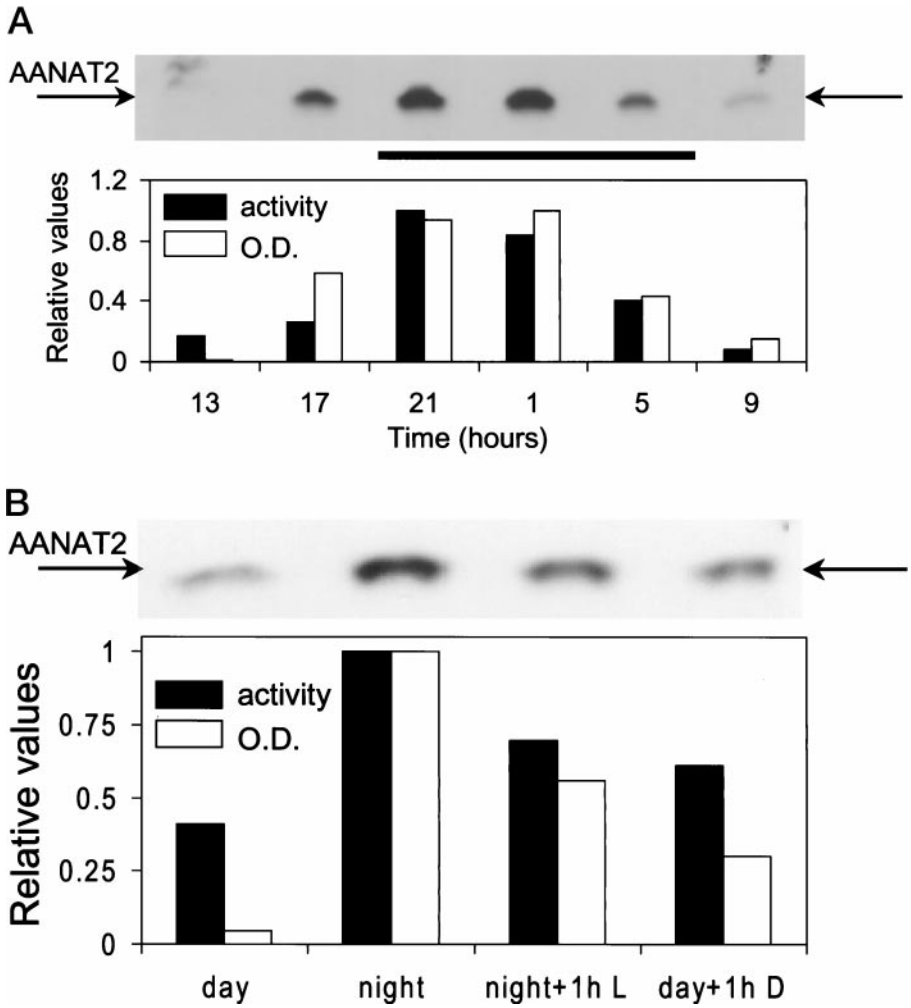

FIG. 3. *In vivo* studies: pike pineal pAANAT2 protein and activity change in parallel during a 24 h period. Samples of 5 pineal organs were collected at the times indicated (see Fig. 1 for a schematic representation of the experimental design). Glands were pooled and homogenized. Each lane was loaded with 80 μg of proteins. Only the band at 26 kDa is shown (see Fig. 2). Pike AANAT2 protein content (*upper panel*) was immunodetected in a sample of homogenate using antiserum 3345; and the signal was measured by determination of optical density (OD) (*lower panel*). Pike AANAT2 activity (*lower panel*) was measured in a another sample of the same homogenate. OD and AANAT2 activity are normalized to the maximum values detected. For the latter, 1 in the ordinate was 0.4 (3A, *top panel*) and 0.18 (3B, *lower panel*) nmol/h/80 μg prot. A, Organs were sampled at the indicated times during a light/dark cycle; *black bar* represents the night. Statistical analysis was done by comparing the three samples collected during the day *vs.* the three collected during the night. Mean day and night pAANAT2 activities were, respectively, 0.069 $\pm$ 0.023 and 0.3 $\pm$ 0.07 nmol/h/80 μg prot. ($P < 0.025$); mean day and night OD values were, respectively, 0.29 $\pm$ 0.023 and 0.79 $\pm$ 0.18 ($P < 0.03$); Student's *t* test (means $\pm$ SEM, n = 3). In an independent duplicate experiment the mean day and night pAANAT2 activities were, respectively, 0.036 $\pm$ 0.009 and 0.452 $\pm$ 0.032 nmol/h/80 μg prot. ($P < 0.001$); mean day and night OD values were, respectively, 0.165 $\pm$ 0.023 and 0.694 $\pm$ 0.12 ($P < 0.002$); Student's *t* test (means $\pm$ SEM, n = 4). B, Organs were sampled at mid-day (day) and midnight (night). In addition, a set of glands was obtained from animals that had been exposed to an hour of dark during the day period (day+1 h D); and, a set of glands was obtained from animals that had been exposed to an hour of light at night (night + 1 h L) (L = 1000 lux intensity). Identical results were obtained in another independent experiment conducted under similar conditions.

previously (6), agents that increase intracellular cAMP levels, such as the adenylate cyclase activator forskolin, increase pAANAT2 activity. Here we found that *in vitro* treatments with forskolin during the day increased pAANAT2 protein, and that activity and protein changed to a similar degree (Fig. 4). Similar results were obtained by treatment with isobutylmethylxanthine, an inhibitor of cAMP phosphodiesterase (not shown).

To test the hypothesis that AANAT2 protein is destroyed by proteasomal proteolysis in the fish pineal gland, we first examined the effects of an inhibitor of proteasomal degradation, MG132. Treatment with MG132 increased both pAANAT2 protein and activity. The effects of MG132 appeared to be larger than those due to forskolin treatment (Fig. 4). This was most evident when drug treatments were done under illumination. In dark-adapted pineal organs, treatment with both MG132 and forskolin totally prevented the suppressive effects of light on pAANAT2 activity and protein. It is notable that forskolin and/or MG132 had similar effects on AANAT2 protein and AANAT2 activity in all three different lighting conditions, providing convincing evidence that either agent enhances AANAT protein levels, and that this does not appear to be an effect that is dependent upon environmental lighting (Fig. 4).

Inhibitors of proteasomal proteolysis increase AANAT2 activity in cultured pike, trout and seabream pineal organs

The above effects of MG132 on AANAT2 protein provide direct evidence that the fish pinealocyte has the intracellular machinery required to destroy AANAT2 protein by proteasomal proteolysis. In addition, these studies indicated that the inhibition of proteasomal proteolysis results in an increase in AANAT2 activity. This indication was examined further *in vitro* using other proteasomal proteolysis inhibitors and pike pineal organs, and pineal organs from two other species, trout and seabream. Treatment with the indicated inhibitors of proteasomal proteolysis elevated AANAT2 activity 1.5- to 4-fold (Table 1; Fig. 6), suggesting that low levels of AANAT2 activity during the day appear to reflect in part continuous degradation by proteasomal proteolysis.

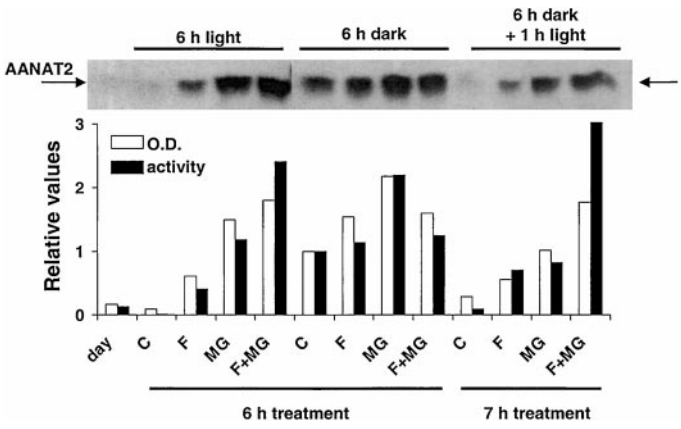

FIG. 4. *In vitro* studies: effects of MG132 and forskolin on pike AANAT2 protein and activity. Pike pineal organs were removed and cultured for 24 h under photoperiodic conditions similar to the natural ones. A schematic representation of the experimental design appears in Fig. 1. After 24 h of culture, proteasomal proteolysis inhibitors (10^{-4} M) and/or forskolin (F; 10^{-5} M), or vehicle were added to the culture medium as indicated in Table 1. The glands were cultured for 6 h either in the dark or light and were placed on solid CO_2. In some cases, glands that were in the dark for 6 h were exposed to an additional hour of light. The lighting conditions are indicated at the top of the figure. Samples of 5 pineal organs were collected at the times indicated, pooled, and homogenized (see *Materials and Methods*). AANAT2 activity and protein were measured in two samples of the same homogenate. AANAT2 activity and OD reported in the lower graph is the mean of independent measurements of two preparations, and are normalized to the value measured at 6 h after the onset of darkness (1 = 0.36 nmol/h/80 μg prot. for AANAT2 activity). The AANAT activity values and OD values from each experiment were within 30% of the mean, except for OD values for the three lowest samples ("day", "C 6 h light" and "C 6 h dark +1 h light"), for which each value was ≤ the mean value. Day, Pineal organs sampled during day (before the experiment started); C, control for each lighting condition; F, forskolin (10^{-5} M); MG, MG132 (10^{-4} M). Similar results were obtained when forskolin was replaced by the nonspecific phosphodiesterase inhibitor isobutylmethylxanthine (IBMX; not shown). For further details see *Materials and Methods*. Similar effects on AANAT2 activity were obtained in the experiment in Fig. 6.

The effects of inhibitors of proteasomal proteolysis were also examined in the dark, when AANAT2 activity is already elevated (Table 2; Fig. 6). Treatment for 6 h at this time, with some, but not all, inhibitors, significantly increased AANAT2 activity in the trout and pike; in other experiments with trout, stimulatory effects of proteasomal proteolysis inhibitors were apparent within 3 h of treatment (not shown). Effects of proteasomal proteolysis inhibitors on AANAT in seabream pineal glands during the night were not consistently statistically significant (*e.g.* Table 2 *vs.* Fig. 6), reflecting uncontrolled variability.

The significant effects of inhibitors at night in trout and pike clearly indicate that proteasomal proteolysis of pineal AANAT is ongoing and contributes to the steady-state levels in the dark. However, it appears that proteolysis is reduced at night, compared with that during the day, because the effect of the inhibitors is reduced in pike and trout, and not statistically significant in seabream.

As indicated above, light exposure at night causes a decrease in AANAT2 activity. This was confirmed here: a 30- to 60-min exposure to light suppressed *in vitro* activity 50–80% (Fig. 5). This light-induced decrease in AANAT2 activity was prevented or reduced by treatment with proteasomal proteolysis inhibitors (Fig. 5), which resulted in levels of AANAT2 activity that were equal to, or greater, than nocturnal values (Figs. 5 and 6).

The magnitude of the effects of lactacystin (10^{-4} M) or MG132 (10^{-4} M) were similar to those of forskolin (10^{-5} M) in the dark or under illumination (Fig. 6). Treatment with forskolin and a proteasomal proteolysis inhibitor in the light, produced a larger response than that produced by either drug alone, there was, however, no evidence of marked potentiation (Fig. 6, seabream and pike). In dark-adapted pineal organs, no further increase was observed. One hour of light after 6 h of dark resulted in a reduction in AANAT2

TABLE 1. *In vitro* studies: effects of proteasomal proteolysis inhibitors on AANAT2 activity of illuminated fish pineal organs

	6 h dark	6 h light		
	Vehicle	Vehicle	MG132	Calpain Inhibitor II
Trout	100 ± 12.8 (7)	38.9 ± 3.3 (8)^{a}	n.d.	57.8 ± 9.3 (6)^{a,b}
Pike	100 ± 23.8 (6)	17.2 ± 2.8 (6)^{a}	62.0 ± 13.8 (7)	n.d.
Seabream^{c}	100 ± 16.7 (3)	47.8 ± 6.5 (4)^{a}	120.3 ± 22.4 (6)^{b}	92 ± 9.3 (5)^{b}

Following removal from animals, the pineal organs were cultured for 24 h under photoperiodic conditions similar to the natural ones (L = 1000 lux intensity). A schematic representation of the experimental design is provided in Fig. 1. Experimental treatments were initiated at 19:30 (pike, seabream) or 12:00 (trout); the organs were then cultured for 6 h in the presence of the drug (10^{-4} M) or vehicle, under the light conditions as indicated. Values are expressed as a percent of the nocturnal control in the dark. The 100% values are 116 (trout), 467(pike) and 768 (seabream) pmoles/h/pineal organ. The mean ± SEM (n is the number *in parentheses*) from one of two (pike, seabream) or three (trout) experiments is presented; similar results were obtained in other experiments. One-way ANOVA indicated significant variations in all groups at $P < 0.008$: ^{b} indicates values significantly different from the 6 h light group at $P < 0.05$ or below; ^{a} indicates values significantly different from the 6 h dark group at $P < 0.05$. n.d., Not determined. ^{c} Seabream pineal AANAT activity was also elevated by treatment with 10^{-4} M lactacystin [147.3 ± 13.4 (5)^{a,b}], proteasome inhibitor I [192.1 ± 25.2 (5)^{a,b}], or β-Lactone [114.7 ± 18.9 (4)^{b}]. For further detail, see *Materials and Methods.*

TABLE 2. *In vitro* studies: effects of proteolysis inhibitors on AANAT2 activity of fish pineal organs cultured in the dark

	6 h dark	
	Vehicle	MG132[a]
Trout	100 ± 12.8 (7)	174.1 ± 43.1 (6)
Pike	100 ± 16.1 (10)	245.0 ± 72.1 (5)[b]
Seabream	100 ± 18.9 (8)	148.5 ± 27.4 (9)

After removal, the pineal organs were cultured for 24 h under photoperiodic conditions similar to the natural ones (L = 1000 lux intensity). A schematic representation of the experimental design is provided in Fig. 1. Experimental treatments were initiated at 19:30 (pike, seabream) or 12:00 (trout); the organs were then cultured for 6 h in the dark and in the presence of either the drug (10^{-4} M) or vehicle. Values are expressed as a percent of the nocturnal control in the dark, to which they are compared. The 100% values are 116 (trout), 348 (pike) and 428 (seabream) pmol/h/pineal organ. Mean ± SEM (n is the number *in parentheses*) from one representative experiment out of 2 (pike, seabream) or 3 (trout). [a] In similar experiments, it was found that other inhibitors of proteasomal proteolysis significantly elevated AANAT activity: proteasome inhibitor I elevated AANAT activity in the pineal gland of the trout [285.7 ± 57.2 (5)] and pike [217.0 ± 38 (5)] ($P < 0.005$); β-lactone elevated AANAT activity in the pike pineal gland [207.7 ± 17.4 (5)] at (***, $P < 0.001$). [b] Student's *t* test, $P < 0.05$. For further details, see *Materials and Methods.*

activity that was prevented by the cotreatment with forskolin and MG132 but not either drug alone (Fig. 6, pike).

Discussion

This study has advanced understanding of the regulation of AANAT2 in the fish pineal gland in three ways. First, *in vivo* and *in vitro* evidence from the pike indicates that previously described day/night and photically regulated changes in AANAT2 activity are associated with similar changes in AANAT2 protein. Second, *in vitro* evidence is presented that indicates that regulatory pathways exist through which cAMP can alter AANAT protein. Third, results of this study indicate that fish pineal AANAT2 protein and activity are influenced by proteasomal proteolysis. These points will be discussed sequentially below.

Daily and photic regulation of AANAT2 protein. The indication that day/night and photically regulated changes in AANAT2 activity are associated with changes in AANAT2 protein comes from *in vivo* and *in vitro* studies in pike. AANAT2 protein was more abundant during the night than during day. These changes in AANAT2 protein closely parallel changes in AANAT activity reported here and in the literature (12, 13).

The link between protein and activity was also found to persist in the pike following two lighting manipulations, *i.e.* when animals were exposed to an hour of light during the night, or to an hour of darkness during the day. In both cases there were similar changes in both activity and protein. It should be added that exposure to light during the night did not return pAANAT2 activity or protein levels to those seen during the day; this may reflect a high level of ongoing AANAT2 synthesis, because AANAT2 mRNA is maximally elevated during the night (10). In addition, the failure of exposure to darkness during the day to elevate either AANAT2 protein or activity may reflect low levels of AANAT2 mRNA at this time, which would limit AANAT2 protein synthesis. The daily rhythm in AANAT2 mRNA is regulated by the endogenous pineal clock; and, it is possible that this clock might influence AANAT2 protein through other mechanisms. For example, it might act through a second messenger to regulate AANAT2 protein stability.

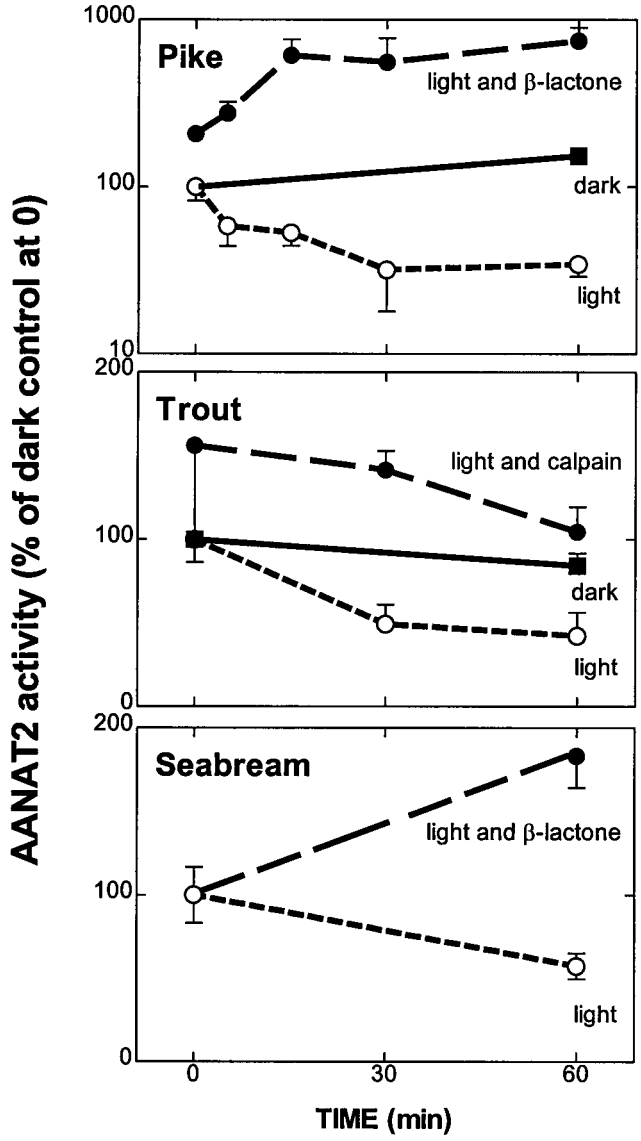

FIG. 5. *In vitro* studies: effects of proteasomal proteolysis inhibitors on the light-induced decrease in fish pineal AANAT2 activity. Pineal glands were removed and placed into culture (trout, 12:00; pike, seabream, 19:30). A schematic representation of the experimental design appears in Fig. 1. After overnight culture, proteasomal proteolysis inhibitors (5×10^{-5} M, *solid circles*) or vehicle (*open circles*) were added and the culture was continued in the dark. A set of glands from each experimental group was removed 6 h later (0 in the abscissa). The remaining glands were cultured in the light (1000 lux) or darkness (*solid squares*). Glands were removed and place on solid CO_2 at the times indicated. For further details, see *Materials and Methods*. Values are expressed as a percent of the nocturnal controls at 0; mean ± SEM (n = 5 glands; one experiment). Similar results were obtained in two (pike; seabream) or three (trout) other experiments. In the pike, ordinates are on a logarithmic scale; similar results were obtained in the pike with MG132, proteasome inhibitor I, and lactacystin; in all cases the two way ANOVA analysis indicated that the effects of time ($P < 0.02$) and drug ($P < 0.006$) were significant.

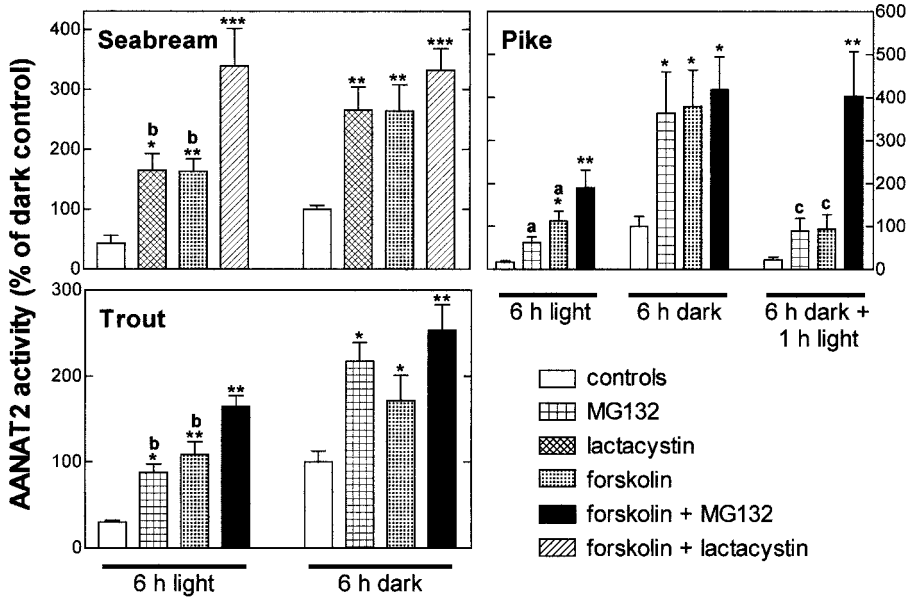

FIG. 6. *In vitro* studies: effects of proteolysis inhibitors and forskolin on AANAT2 activity. See Fig. 1 for a schematic representation of the experimental design. Following overnight culture, inhibitors (10^{-4} M) and/or forskolin (FSK; 10^{-5} M), or vehicle were added at 12:00 (trout) or 19:30 (pike, seabream) of a light-dark cycle. Glands were cultured for 6 h either in the dark, or under light (1000 lux) and then placed on solid CO_2. In some cases, glands that had been in the dark for 6 h were exposed to light for 1 h; they were then placed on solid CO_2. Values are expressed as a percent of the 6 h dark control; mean ± SEM (n = 6 to 8) from one representative experiment out of two. Student's *t* test; within a given experimental group, values are compared with those obtained 1) in the absence of drug: *, $P < 0.01$, **$P < 0.001$; 2) in the presence of the two tested drugs: [a], $P < 0.01$; [b], $P < 0.001$; [c], $P < 0.0001$. Two-way ANOVA indicated, in all cases, significant effects of drug ($P < 0.0001$) and of light regimen ($P < 0.02$). A significant interaction between the effects of both factors was also seen in the case of pike ($P < 0.03$).

The *in vivo* demonstration that there is a close link between AANAT2 protein and activity was supported by the results of *in vitro* studies, which consistently found that AANAT2 activity and protein changed in parallel. Thus it appears that an important element regulating AANAT2 activity is the abundance of AANAT protein. However, it should be noted that changes in AANAT2 activity reported here and in the literature typically are not as dramatic as those seen in melatonin synthesis (27). This difference is of special importance because it might reflect regulation of melatonin synthesis at other steps in the conversion of tryptophan to melatonin or in melatonin destruction. For example, tryptophan hydroxylase and melatonin deacetylation have been identified as a potential regulatory point in melatonin synthesis (28, 29). It is also possible that AANAT2 activity as measured in homogenates is not an entirely reliable indicator of the activity of AANAT within the cell, which might be subject to regulation—either inhibition or activation—that is not apparent following homogenization (30). It has also been pointed out that melatonin synthesis may be regulated by negative feedback mechanisms (31, 32).

cAMP regulation of AANAT2. The organ culture studies presented here make it clear that regulatory pathways exist in the fish pineal gland through which cAMP can alter AANAT2 protein. This is consistent with the highly conserved nature of PKA sites in the AANAT family and evidence from mammalian and avian pineal studies, which indicate that AANAT protein is subject to cAMP regulation (12, 13). The mechanism through which cAMP is thought to act involves inhibition of proteasomal proteolysis (13). It seems reasonable to suspect that this process is also functional in the fish pineal, as discussed in the following section. Accordingly, it is reasonable to consider that cAMP can regulate AANAT protein and activity by inhibiting proteasomal proteolysis.

An important issue to address is whether this regulatory pathway is involved in the physiological control of AANAT2. Reliable *in vivo* physiological data on pineal cAMP from any fish species is not available. Analysis of cAMP in superfused pineal organs and static cultures of fish photoreceptor cells have revealed changes in cAMP consistent with a role in regulating AANAT2 activity and protein (6). However, similar changes are not seen in static organ cultures (15, 33) in which AANAT2 activity or melatonin synthesis did change. This failure to detect changes in cAMP may indicate that other second messenger systems, including calcium, participate in regulating AANAT2 activity and melatonin synthesis. It is also possible that dramatic compartmented changes in cAMP occur in the fish pinealocyte, which are undetectable when the gland is homogenized. Such highly compartmentalized changes, which could occur in discrete regions in which AANAT2 destruction occurs, are not unrealistic to propose, when one considers the organization of the fish pinealocyte and the heterogeneous nature of cells in the pineal organ. The fish pinealocyte is similar to a retinal photoreceptor, and has distinct outer and inner segments, cell body, and pedicles. The fish pineal organ contains photoreceptors, glia, and neurons. Accordingly, cAMP levels in nonmelatonin producing cells might mask dramatic compartmentalized subcellular changes in melatonin-producing cells.

Proteasomal proteolysis of AANAT2. The conclusion that pineal AANAT2 is subject to proteasomal proteolysis in the fish pineal organ is supported by the results of pharmacological studies of three species presented here. In these studies, inhibitors of the proteasomal degradation pathway (34, 35) increased the abundance of pineal AANAT2 activity and protein under a variety of conditions in which AANAT2 protein and activity are otherwise at low levels. For example, inhibition of proteasomal degradation increases AANAT2 activity (pike and seabream) and protein (pike) during subjective night in the light. In addition, treatment with inhibitors of proteasomal degradation at night in culture, inhibits the light-induced decrease in AANAT2 protein and activity. In the case of trout, inhibitors of proteasomal degradation increase AANAT2 activity during the day or subjective day in the dark. This finding indicates that proteasomal proteolysis contributes to the low steady-state levels of AANAT2 protein and activity seen during the day, and that this route of metabolism appears to play a role in the light-induced decrease in these parameters.

Under conditions in which AANAT2 protein and activity are already elevated, including darkness at night, proteasome inhibitors were found to either have no effect in the dark, or to enhance the dark-induced rise in AANAT2 activity. In the case of enhancement, it would appear that proteasomal proteolysis of AANAT2 is ongoing at night, albeit at levels relatively reduced from day levels; in the case were no observed effect of these inhibitors was seen, it would appear that proteasomal proteolysis was maximally inhibited.

We suspect that proteolysis at night is most probably of physiological importance and modulates the amplitude of the AANAT2 peak in the dark, and the shape of the AANAT2 oscillations throughout the LD cycle. Indeed, in species displaying AANAT2 mRNA oscillations (*e.g.* pike), a basal level of proteolysis at night would cause AANAT2 activity to closely follow changes in AANAT2 mRNA that occur before the dark to light transition (10). Light-activated proteolysis during the day would also prevent AANAT2 activity from increasing dramatically before night onset, when AANAT2 mRNA abundance is increasing. In the case of the trout, where the abundance of AANAT2 mRNA remains constant throughout the LD cycle (18), it would appear that the photically regulated difference in proteasomal proteolysis is the major mechanism driving the daily rhythm in AANAT2 activity and melatonin production (20, 21).

It is relevant to comment on a report that failed to observe an effect of proteasomal inhibitors on AANAT2 activity in the trout pineal organ (15), in contrast to studies presented here, where proteasomal inhibitors were found to statistically increase AANAT2 protein and activity under a variety of conditions. Although the effects of proteasomal inhibitors were seen in all three species examined, their effectiveness varied somewhat on a species-to-species basis. Small effects of the proteasome inhibitors in this species may have occurred, but have been masked by the large variation in AANAT2 activity. Similarly, it is possible that this characteristic of the trout pineal gland might explain the failure of others to observe statistically significant effects of proteasomal proteolysis inhibitors. Other technical differences, including culture conditions (9, 11, 32, 36) and duration of treatment, might also contribute to the differences observed. As discussed above, it is possible that large changes in melatonin production might be due to large changes in the activity of AANAT2 in the intact cell that are not seen when the enzyme is homogenized and AANAT2 is assayed under ideal conditions. For example, protein might be inhibited in the cell during the day, whereas this inhibition is not apparent in broken cells assays. This would explain the small differences between daytime and nighttime AANAT2 values or between daytime values of organs treated with or without proteasome inhibitors.

Conclusions

Three major findings emerge from this study. First, the daily variations in AANAT2 activity result from variations in the amount of AANAT2 protein. Second the acute inhibitory effect of light on enzyme activity results from direct proteolysis of the protein, through proteasomal proteolysis, independent of the presence or not of a circadian clock. This receptor-mediated process provides the photoreceptor cells with a rapid switch to turn off AANAT2. The magnitude of the turn-on depends on the amount of mRNA present at a given time of the LD cycle. In clock-controlled pineal organs, the dark-induced rise in activity will occur only when high mRNA levels coincide with low proteolysis; but in the absence of clock-controlled mRNA oscillations AANAT2 activity will increase any time of the 24 h cycle darkness is applied. Third, a cAMP-dependent pathway protects AANAT2 protein from degradation; this may involve phosphorylation of AANAT through highly conserved N- and C-terminal cAMP-dependent protein kinase phosphorylation sites. Proteasomal proteolysis appears as a conserved mechanism between vertebrate species as divergent as the rat and the fish are, although the mechanisms through which the process is triggered are different.

References

1. **Zachmann A, Ali MA, Falcón J** 1992 Melatonin in fish: rhythmic production by the pineal and effects; an overview. In: Ali MA (ed) NATO Advanced Study on Rhythms in Fish. Plenum Press, New York, pp 149–166
2. **Arendt J** 1995 Melatonin and the mammalian pineal gland. Chapman and Hall, London
3. **Cahill GM, Besharse JC** 1995 Circadian rhythmicity in vertebrate retinas: regulation by photoreceptor oscillations. Prog Retin Eye Res 14:267–291
4. **Bolliet V, Ali MA, Lapointe FJ, Falcón J** 1996 Rhythmic melatonin secretion in different teleost species: an *in vitro* study. J Comp Physiol B 165:677–683
5. **Zatz M** 1996 Melatonin rhythm: trekking toward the heart of darkness in the chick pineal. Cell Dev Biol 7:811–820
6. **Falcón J** 1999 Cellular circadian clocks in the pineal. Prog Neurobiol 58:121–162
7. **Klein DC** 1985 Photoneural regulation of the mammalian pineal gland. Ciba Found Symp 117:38–56
8. **Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Bégay V, Falcón J, Cahill GM, Cassone VM, Baler R** 1997 The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. Recent Prog Horm Res 52:307–357
9. **Falcón J, Bolliet V, Collin JP** 1996 Partial characterization of serotonin *N*-acetyltransferases from northern pike (*Esox lucius*, L.) pineal organ and retina. Effects of temperature. Pflügers Arch 342:386–396
10. **Coon S, Bégay V, Deurloo D, Falcón J, Klein DC** 1999 Two arylalkylamine *N*-acetyltransferase genes mediate melatonin synthesis in fish. J Biol Chem 274:9076–9082
11. **Benyassi A, Schwartz C, Coon SL, Klein DC, Falcón J** 2000 Melatonin synthesis: arylalkylamine N-acetyltransferases in trout retina and pineal organ are different. Neuroreport 11:255–258
12. **Gastel JA, Roseboom PH, Rinaldi PA, Weller JL, Klein DC** 1998 Melatonin

production: proteasomal proteolysis in serotonin *N*-acetyltransferase regulation. Science 279:1358–1360
13. **Zatz M, Gastel J, Heath III JR, Klein DC** 2000 Chicken pineal melatonin synthesis: light and cAMP control abundance of serotonin *N*-acetyltransferase (AANAT, EC 2.3.1.87) protein. J Neurochem 74:2315–2321
14. **Hall JC** 1995 Tripping along the trail to the molecular mechanisms of biological clocks. Trends Neurosci 18:230–240
15. **Kroeber S, Meissl H, Maronde E, Korf HW** 2000 Analyses of signal transduction cascades reveal an essential role of calcium ions for regulation of melatonin biosynthesis in the light-sensitive pineal organ of the rainbow trout (*Oncorhynchus mykiss*). J Neurochem 74:2478–2489
16. **Takahashi JS, Murakami N, Nikaido SS, Pratt BL, Robertson LM** 1989 The avian pineal, a vertebrate model system of the circadian oscillator—cellular-regulation of circadian-rhythms by light, 2nd messengers, and macromolecular-synthesis. Recent Prog Horm Res 45:279–352
17. **Raloff J** 2000 Downtown fisheries? Advances may make fish farming a healthy prospect, even for inner cities. Science News 157:305–320
18. **Bégay V, Falcón J, Cahill G, Klein DC, Coon LC** 1998 Transcripts encoding two melatonin synthesis enzymes in the teleost pineal organ: circadian regulation in pike and zebrafish but not in trout. Endocrinology 139:905–912
19. **Falcón J, Brun-Marmillon J, Claustrat B, Collin JP** 1989 Regulation of melatonin secretion in a photoreceptive pineal organ: an *in vitro* study in the pike. J Neurosci 9:1943–1950.
20. **Gern WA, Greenhouse SS** 1988 Examination of *in vitro* melatonin secretion from superfused trout (*Salmo gairdneri*) pineal organs maintained under diel illumination or continuous darkness. Gen Comp Endocrinol 71:163–174
21. **Thibault C, Falcón J, Greenhouse SS, Lowery CA, Gern WA, Collin JP** 1993 Regulation of melatonin production by pineal photoreceptor cells: role of cyclic nucleotides in the trout. J Neurochem 61:332–339
22. **Bolliet V, Bégay V, Taragnat C, Ravault JP, Collin JP, Falcón J** 1996 Photoreceptor cells of the pike pineal organ as cellular circadian oscillators. Eur J Neurosci 9:643–653
23. **Molina-Borja M, Falcón J, Urquiola E, Ravault JP, Collin JP** 1996 Production of melatonin by the seabream pineal: an *in vivo* and *in vitro* study. Fish Physiol Biochem 15:413–419
24. **Sugden D, Namboodiri MAA, Weller JL, Klein DC** 1983 Melatonin synthesizing enzymes: serotonin *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase. In: Parvez H, Parvez S, Nagatsu T (eds) Methods in Biogenic Amine Research. Elsevier/North Holland Biomedical Press, Amsterdam, The Netherlands, pp 567–572
25. **Smith DE, Fisher PA** 1984 Identification, developmental regulation, and response to heat-shock of antigenically related forms of a major nuclear-envelope protein in drosophila embryos - application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. J Cell Biol 99:20–28
26. **De Angelis J, Gastel J, Klein DC, Cole PA** 1998 Kinetic analysis of the catalytic mechanism of serotonin *N*-acetyltransferase (EC 2.3.1.87) J Biol Chem 273:3045–3050
27. **Falcón J, Guerlotté J, Voisin P, Collin JP** 1987 Rhythmic melatonin biosynthesis in a photoreceptive pineal organ: a study in the pike. Neuroendocrinology 45:479–486
28. **Falcón J, Balemans MGM, Vanbenthem J, Collin JP** 1985 *In vitro* uptake and metabolism of [^{3}H]-indole compounds in the pineal organ of the pike.1. A radiochromatographic study. J Pineal Res 2:341–356
29. **Grace MS, Besharse JC** 1994 Melatonin deacetylase activity in the pineal gland and brain of the lizards *Anolis-carolinensis* and *Sceloporus-jarrovi*. Neuroscience 62:615–623
30. **Namboodiri MAA, Sugden D, Klein DC, Grady R, Mefford IN** 1985 Rapid nocturnal increase in ovine pineal *N*-acetyltransferase activity and melatonin synthesis - effects of cycloheximide. J Neurochem 45:832–835
31. **Bégay V, Falcón J, Thibault C, Ravault JP, Collin JP** 1992 Pineal photoreceptor cells - photoperiodic control of melatonin production after cell-dissociation and culture. J Neuroendocrinol 4:337–345
32. **Yañez J, Meissl H** 1995 Secretion of methoxyindoles from trout pineal organs in-vitro - indication for a paracrine melatonin feedback. Neurochem Int 27:195–200
33. **Falcón J, Thibault C, Blazquez JL, Vaudry H, Ling N, Collin JP** 1990 Atrial natriuretic factor increases cyclic GMP and cyclic AMP levels in a directly photosensitive pineal organ. Pflügers Arch 417:243–245
34. **Mykles DL** 1998 Intracellular proteinases of invertebrates: calcium-dependent and proteasome/ubiquitin-dependent systems. Int Rev Cytol 184:157–289
35. **Sheaff RJ, Singer JD, Swanger J, Smitherman M, Roberts JM, Clurman BE** 2000 Proteasomal turnover of p21(Cip1) does not require p21(Cip1) ubiquitination. Mol Cell 5:403–410
36. **Max M, Menaker M** 1992 Regulation of melatonin production by light, darkness, and temperature in the trout pineal. J Comp Physiol A 170:479–489