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Regulation of Indoleamine N-Acetyltransferase Activity in the Retina: Effects of Light and Dark, Protein Synthesis Inhibitors and Cyclic Nucleotide Analogs

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The regulation of indoleamine N-acetyltransferase (NAT) in the posterior eye was investigated in vivo, and in vitro in cultured eye cups. Surgical separation of neural retina from the retinal pigment epithelium–choroid complex indicated that NAT was localized to neural retina. The activity of retinal NAT fluctuated in vivo in a rhythmic fashion, with peak activity in the dark phase of the light–dark cycle. The rhythm of NAT activity persisted for up to 3 days in constant darkness, with a rhythmic period of approximately 25 h. The rhythm was suppressed by constant light, and could be phase-shifted by exposure to a new light–dark cycle. These observations indicate that retinal NAT activity occurs as a circadian rhythm that is entrained by light and dark. Retinas also responded to light and dark in vitro with changes of NAT activity. A significant increase in retinal NAT activity occurred in eye cups cultured in darkness during the dark phase of the light–dark cycle. This increase was completely suppressed in eye cups cultured at the same time of day in light. The dark-induced increase in NAT was completely blocked by protein synthesis inhibitors, and mimicked in light by cyclic AMP analogs.

The similarity of the regulation of NAT activity in retina to that in pineal, and the possible relationship of the retinal NAT rhythm to cyclic metabolism in photoreceptors are discussed.

INTRODUCTION

Melatonin is a hormone that is synthesized and released from pineal glands in a circadian fashion (reviewed by Reiter[42]). The rhythmic synthesis of pineal melatonin appears to be regulated by an induction of indoleamine N-acetyltransferase (NAT; E.C. 2.3.1.5)[30], the enzyme that catalyzes the conversion of serotonin to N-acetylserotonin. Recent evidence indicates that melatonin may also be synthesized in the retina. Melatonin-like immunoreactivity (MLI) has been localized to retina[14,29,39], and the MLI content of retina did not decrease after pinealectomy[43]. The enzymes for the synthesis of melatonin from serotonin, NAT[12,29,36] and hydroxyindole-O-methyltransferase (E.C.2.1.1.4)[3,16,41], have been identified in retina, and the synthesis of radiolabeled melatonin from radiolabeled serotonin in retinal explants has been demonstrated[17,27]. Furthermore, recent demonstration of circadian NAT activity in retinas from chickens[12,13,29] suggests a broad analogy between retinal and pineal indoleamine metabolism. Although the significance of such a system in retina remains undetermined, it has been widely suggested that local indole metabolism may influence other circadian aspects of retinal metabolism, particularly within the photoreceptor pigment epithelial complex[6,26]. This view is supported by the recent demonstration of the induction of photoreceptor disc shedding, a circadian process, with melatonin and other methoxyindoles[8].

In order to gain insight into the regulation of melatonin synthesis in retina, we have studied the regulation of NAT in retinas of the African clawed frog (*Xenopus laevis*). This species was chosen for the study because the concentrations of serotonin and melatonin and the activities of the melatonin-synthesizing enzymes are high in its retina[3,4,25], and because it has been used extensively in previous studies of photoperiodic regulation of photoreceptor membrane turnover[9–11]. In this paper, we report that retinal NAT activity cycles in vivo as a circadian rhythm with peak activity at night, and that a dark-induced increase in retinal NAT occurs in vitro in cultured eye cups. In vitro the dark-induced increase in activity is prevented by protein synthesis inhibitors, and is mimicked in light by cyclic AMP analogs.

MATERIALS AND METHODS

Animals

Post-metamorphic *X. laevis* (3.5–6.0 cm body length) obtained from Nasco, Fort Atkinson, WI, were maintained at 24–26 °C under a 12 h light–dark cycle and were fed commercial frog pellets (Nasco) 3 times weekly. Animals were maintained on this schedule for a minimum of 1 month before use.

In vivo experiments

To study the effects of light and dark on retinal NAT activity frogs were kept in constant-temperature incubators (26 °C) with a light intensity of 2×10^{-3} W/cm^2 incident at the level of animal containers. At various times during the light–dark cycle, or during constant light or dark treatment, frogs were killed by decapitation and pithing. Eyes were removed and retinas were isolated free of pigment epithelium, in phosphate-buffered saline (PBS; 0.14 M NaCl, 0.1 M sodium phosphate, pH 7.2) under a dissecting microscope. The retinas were immediately frozen on dry-ice and were stored (<30 h) at —80 °C until assayed for NAT activity. Sacrificing, dissection and freezing of retinas in 'dark' were performed under dim red light (Kodak Wratten filter No. 2).

In vitro experiments

To study the effects of drugs, light, and dark on retinal NAT activity in vitro, animals were sacrificed as described above and eye cups were prepared by surgical removal of cornea, iris and lens and cultured using procedures described previously[10]. Eye cups were cultured at a pH of 7.5 under an atmosphere of 95% O_2–5% CO_2 in amphibian tissue culture medium (Grand Island Biologicals) supplemented with sodium bicarbonate to a final bicarbonate concentration of 30 mM. Eye cups were kept in plastic culture dishes on a rotary shaker (60 rpm) in either light (2×10^{-3} W/cm^2 incident at culture dishes) or darkness for periods of 4–10 h. At the end of the incubation period, retinas were removed from eye cups in PBS and frozen on dry-ice. Cyclic nucleotide analogs and protein synthesis inhibitors used in these experiments were obtained from Sigma Chemicals, St. Louis, MO.

N-Acetyltransferase assay

Retinal NAT activity was assayed by measuring the transfer of a [^{14}C]acetyl group from acetyl-coenzyme A to tryptamine[23,29]. Each retina was sonicated in 100 μl of ice-cold 0.5 M potassium phosphate (pH 6.5) containing 0.68 mM acetyl-coenzyme A (Sigma). Aliquots (25 μl) of homogenate were mixed with 10 μl of reaction mixture containing 0.1 μmol of tryptamine (Sigma) and 1.91 nmol (0.1 μCi) of [acetyl-1-^{14}C]acetyl coenzyme A (New England Nuclear, Boston, MA; 52.3 mCi/mmol), and incubated at 25 °C for 15 min. The final concentrations of acetyl-coenzyme A and tryptamine were 0.54 mM and 2.9 mM, respectively. The reaction was stopped by addition of 1 ml of water-saturated chloroform. The reaction product was extracted and prepared for liquid scintillation counting as described by Parfitt et al.[40].

The protein concentration of the homogenate was determined by the method of Lowry et al.[35].

Data analysis

Data are expressed as group mean ± standard error of the mean. Each experimental treatment consisted of analysis of 4–10 retinas, each from a different animal. All key experiments were repeated at least twice. Data were analyzed for statistical significance by one-way analysis of variance. Comparison of experimental groups to a single control group was made by Dunnet's *t*-test, and multiple comparisons among groups by Newman-Keuls test.

RESULTS

Effects of light and dark on retinal NAT activity in vivo

The activity of NAT in the posterior eye was investigated. The gross localization of NAT was studied by separating the neural retina from the pigment epithelium (PE)–choroid. When examined in the light- and dark-phases of the 12 h light–dark cycle, activity in the retina was considerably greater in the dark than in the light. Significant NAT activity could not be measured in PE–choroid in light or dark, indicating that NAT activity in the posterior eye was localized to neural retina.

Retinal NAT activity, measured at regular intervals during the 12 h light–dark cycle, showed statisti-

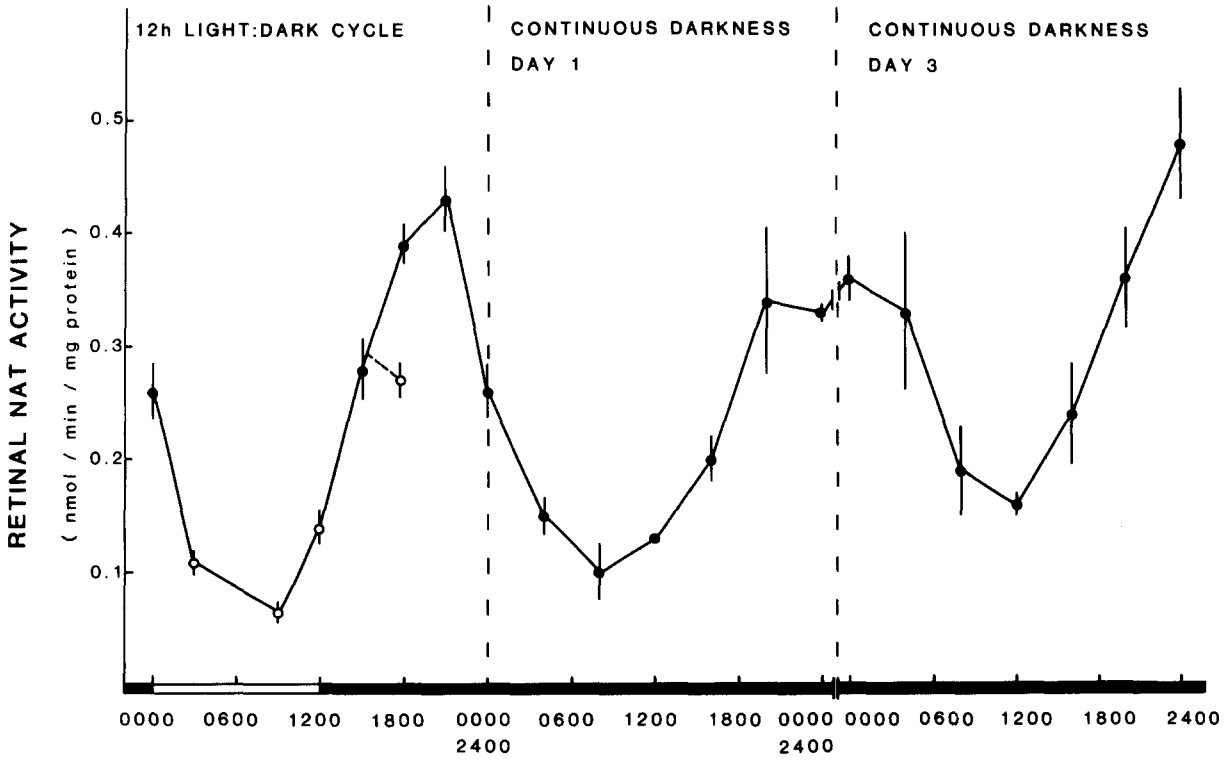

Fig. 1. Retinal NAT activity during the light–dark cycle and continuous exposure to darkness. Animals were kept on the 12 h L:12 h D light–dark cycle for at least 1 month prior to the experiment. Lights were on from 00.00–12.00 h. White bars along the x-axis and open circles (○) represent light; dark bars and solid circles (●) represent darkness. The open circle during the dark-phase of the light–dark cycle represents animals exposed to light for 2.5 h (15.15–17.45 h). n = 4–6/group.

cally significant fluctuations ($P < 0.001$, Fig. 1). NAT activity decreased significantly during the first hours of light and reached its lowest point following approximately 9 h of light exposure. The activity of the enzyme began to rise before the onset of darkness ($P < 0.05$), and continued to rise during the dark phase of the light–dark cycle ($P < 0.01$), reaching a maximum following 6–9 h of exposure to darkness. NAT activity during the 9th hour of darkness was 6–7-fold higher than that during the 9th hour of light. Enzyme activity began to decline during the last 3 h of darkness ($P < 0.01$). Thus, retinal NAT activity occurs in a rhythmic fashion with peak activity during the latter part of the dark phase of the light–dark cycle.

Exposure to light for 2.5 h during the first half of the dark phase, when NAT activity is normally increasing, prevents the continuation of the rise in enzyme activity (Fig. 1), suggesting that light has an inhibitory effect on the induction of retinal NAT. Although this effect is striking during the dark-period, it should be emphasized that a significant rise in NAT activity is detected during the last 3 h prior to light offset. Thus, the dark increase in NAT activity may not simply reflect a release from light inhibition.

This is further emphasized by analyses of the effects of constant darkness and constant light. During the first and third day of treatment with constant darkness, retinal NAT activity continued to cycle ($P < 0.01$) with activity decreasing during the subjective light phase and increasing during the subjective dark phase (Fig. 1). However, the peak of the rhythm progressively shifted to a later time in the subjective dark phase. The shift of the rhythm in constant darkness is characteristic of 'free-running' circadian rhythms, with rhythmic periods of slightly greater than 24 h[1]. Our data suggest that retinal NAT activity is regulated by a free-running rhythm of approximately 25 h.

The conclusion that the dark rise in retinal NAT activity is not simply the result of release from inhibition by light is also supported by the effects of con-

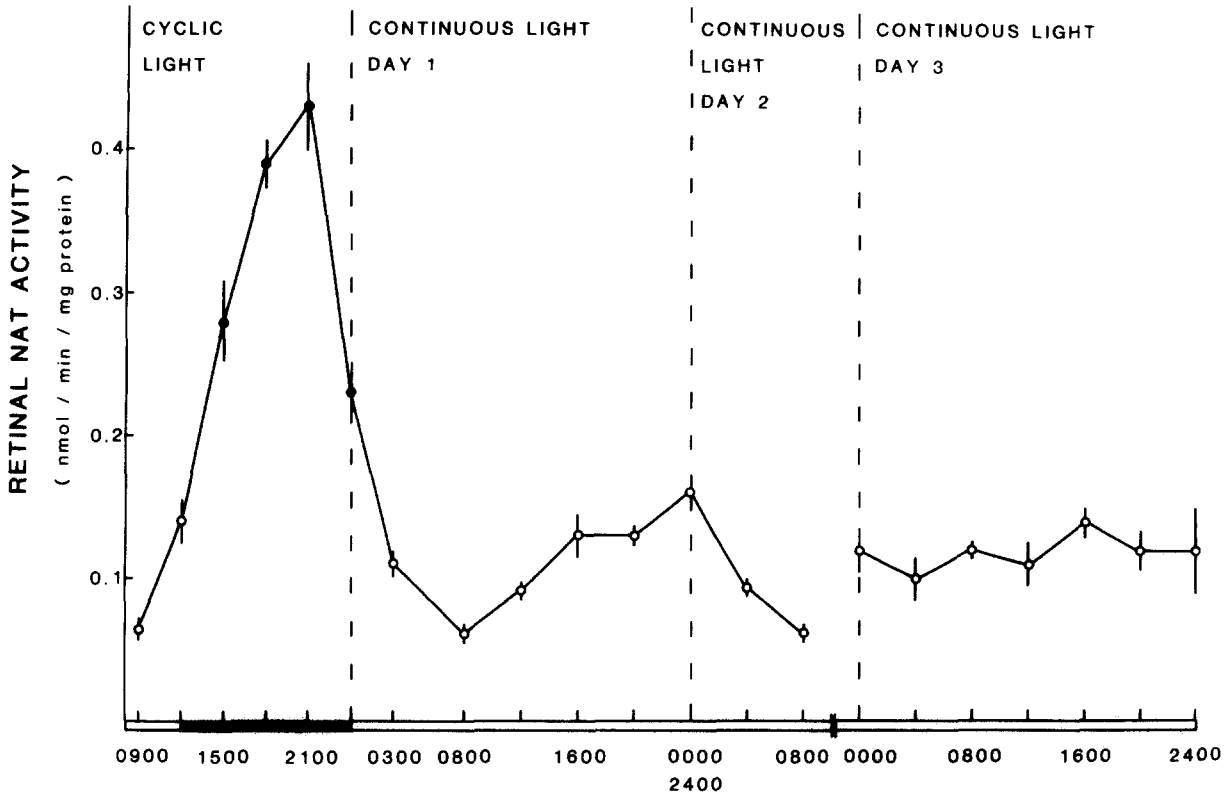

Fig. 2. Effect of constant light exposure on the rhythm of retinal NAT activity. Data for cyclic light are the same as those in Fig. 1. See legend to Fig. 1 for details.

stant-light treatment (Fig. 2). A small, but significant ($P < 0.01$) rise in retinal NAT activity occurred during the subjective dark phase of the first day of continuous light, and a significant ($P < 0.01$) decline in activity occurred during the subjective light phase of the second day of treatment. Thus, even in the absence of darkness a low amplitude rhythm in NAT activity persists for a short period. The rhythm is totally eliminated by the third day of continuous light (Fig. 2). The observed activity during the third day of continuous light was greater than that at the nadir of the rhythm during the 12 h light–dark cycle ($P < 0.01$), a finding that was confirmed in an independent experiment (constant light 120 ± 14 pmol/min/mg vs cyclic light 65 ± 8 pmol/min/mg, $P < 0.01$). This result suggests that the rhythm in retinas exposed to a 12 h light–dark cycle is regulated not only by positive, or inducing factors, but also by negative, or repressing factors.

To determine if darkness might act as a stimulus for the inducing component of the rhythm, the effects of 3 h of dark treatment following 48 h of continuous light was examined (Table I). Dark treatment significantly increased NAT activity in these retinas, whose rhythm had been blocked by continuous light.

All of these results suggest that retinal NAT is regulated by a free-running biological rhythm or clock

TABLE I

Dark-induced increase in retinal NAT activity following 48 h of continuous light

Animals were kept in constant temperature (26 °C) incubators in light or dark as indicated. Light exposure began at the normal time of light onset of the light–dark cycle. Dark exposure began after the 48 h of constant light. NAT activity was determined as described in Methods.

Treatment	*n*	*NAT activity (pmol/min/mg protein)*
48 h light	6	120 ± 15
48 h light + 3 h dark	6	180 ± 10*
52 h light	4	100 ± 15

* $P < 0.01$.

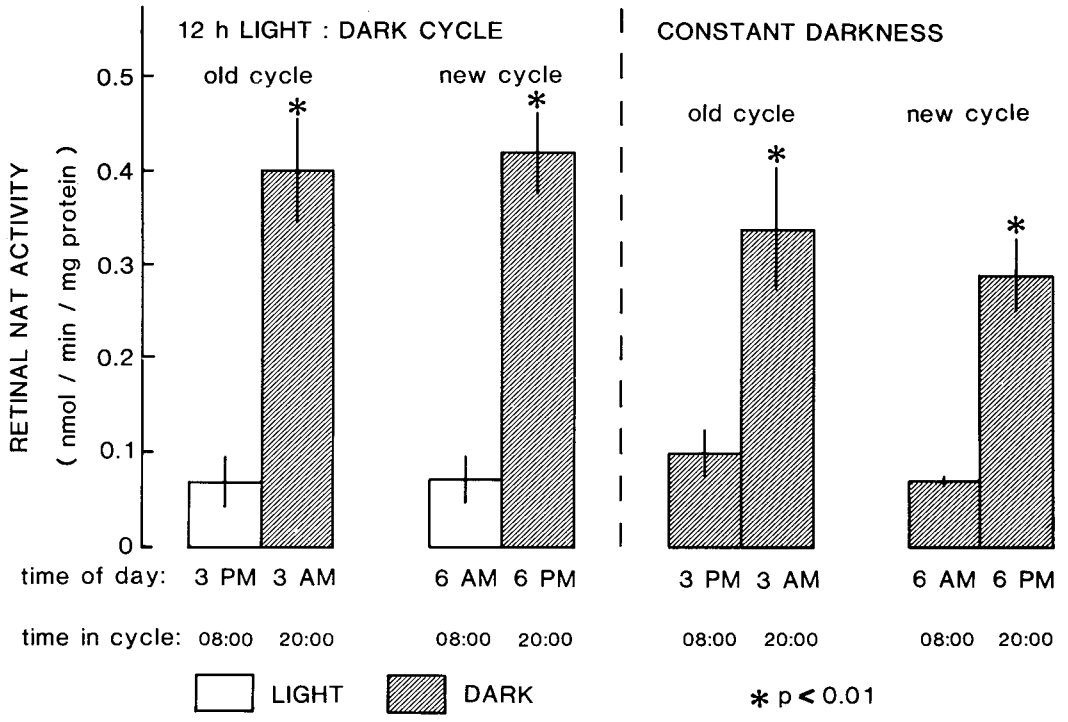

Fig. 3. Phase-shift in the rhythm of retinal NAT activity. Animals were entrained to a 12 h L:12 h D cycle ('old cycle', lights on at 07.00 h) and re-entrained to the 'new cycle' (lights on at 23.00 h) for 4–6 weeks. They were sacrificed and NAT activity determined during the 8th hour of the subjective light or the 8th hour of subjective darkness for each cycle, respectively. Constant dark-treated animals were sacrificed during the second day of constant darkness. n = 4.

that is entrained by the light–dark cycle. Additional experiments were conducted to determine if this rhythm, once established, was permanent, or if it displayed plasticity and could be re-entrained to a new light–dark cycle (Fig. 3). Frogs were exposed for 1 month to a new 12 h light–dark cycle with lights going out 9 h earlier than the normal cycle. Measurements of retinal NAT activity during subjective light and subjective dark phases during the 12 h light–dark cycle and during the second day of continuous darkness indicated that the rhythm had phase-shifted and was re-entrained to the new cycle (Fig. 3).

Effects of light and dark on retinal NAT activity in vitro

The effects of light and dark on retinal NAT activity in cultured eye cups was investigated to determine if the inhibitory effect of light or the inducing effect of darkness was mediated within the eye. Eye cups were prepared just prior to the time of light offset (12.00 h) and cultured in darkness beginning at normal light offset for various periods of time. NAT activity increased in a time-dependent manner during these incubations (Fig. 4), to a level approximately half that seen in vivo. However, if eye cups prepared at the same time were cultured under bright light instead of darkness, NAT activity failed to increase (data not shown).

Effect of protein synthesis inhibitors on the dark-induced increase in NAT activity in vitro

When eye cups were cultured in darkness in the presence of puromycin (25 μg/ml) or cycloheximide (25 μg/ml), retinal NAT activity failed to increase. In fact, the protein synthesis inhibitors caused a significant decline in activity (Table II). The results suggest that dark induction involves new enzyme synthesis and that the turnover rate of the enzyme is rapid.

Effects of cyclic nucleotide analogs

The induction of NAT in the pineal gland is regulated by a cyclic AMP-dependent mechanism[2,21,22,24]. In order to investigate the possibility that cyclic nucleotides are involved in the regulation of the enzyme

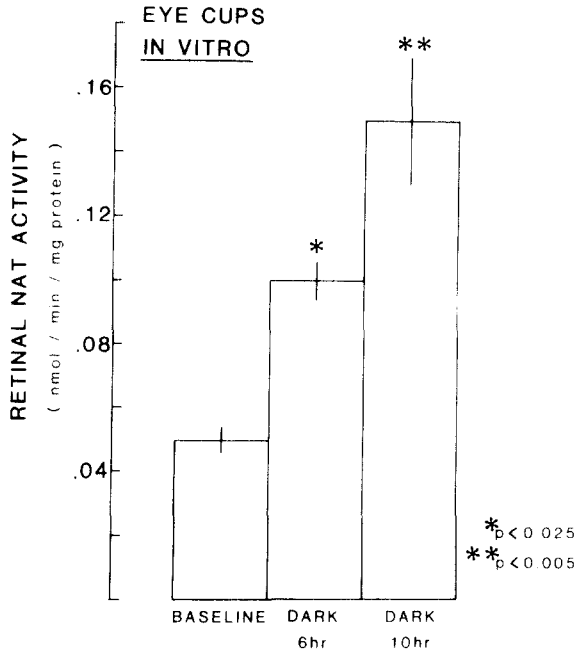

Fig. 4. Dark-induced increase in NAT activity in retinas of cultured eye cups. Eye cups were prepared just prior to the time of light offset (12.00 h) of the light–dark cycle and were cultured in darkness as described in Methods. At the end of the incubation period retinas were removed from the eye cups, frozen, and assayed for NAT activity. n = 5/group.

in retina, we examined the effects of cyclic AMP- and cyclic GMP-analogs on retinal NAT activity. As described above, when eye cups prepared just prior to 12.00 h (the time of normal light offset) were cultured in light instead of in darkness, NAT activity did not increase (Table III). However, if dibutyryl-cyclic

TABLE II

Effect of protein synthesis inhibitors on the increase in retinal NAT activity in vitro

Eye cups were prepared just prior to the time of light offset (12.00 h) of the light–dark cycle and were cultured in darkness with or without protein synthesis inhibitors at 25 μg/ml.

Treatment	*n*	*NAT activity (pmol/min/mg protein)*
Pre-culture control	10	66 ± 6.5
6 h culture	10	120 ± 8.7*
6 h culture		
+ Cycloheximide	5	30 ± 0.6**
+ Puromycin	5	33 ± 2.3**

* Greater than pre-culture control, $P < 0.01$.
** Less than pre-culture and cultured controls, $P < 0.01$.

TABLE III

Effects of cyclic AMP analogs on retinal NAT activity of eye cups cultured in light during the subjective dark-phase of the light–dark cycle

Eye cups were prepared just prior to the normal time of light offset (12.00 h) and were cultured in light to block the nocturnal rise in NAT activity. Cyclic AMP analogs were added to the culture medium at 2 mM.

Condition	*n*	*NAT activity (pmol/min/mg protein)*
Pre-culture control	4	97 ± 1
4 h culture	5	80 ± 6
4 h culture		
+ dibutyryl-cyclic AMP	5	190 ± 18*
+ 8-bromo-cyclic AMP	5	140 ± 16*

* $P < 0.01$.

AMP or 8-bromo-cyclic AMP (2 mM) were included in the culture medium, significant increases in NAT activity occurred even in light (Table III).

The above experiment demonstrates that cyclic

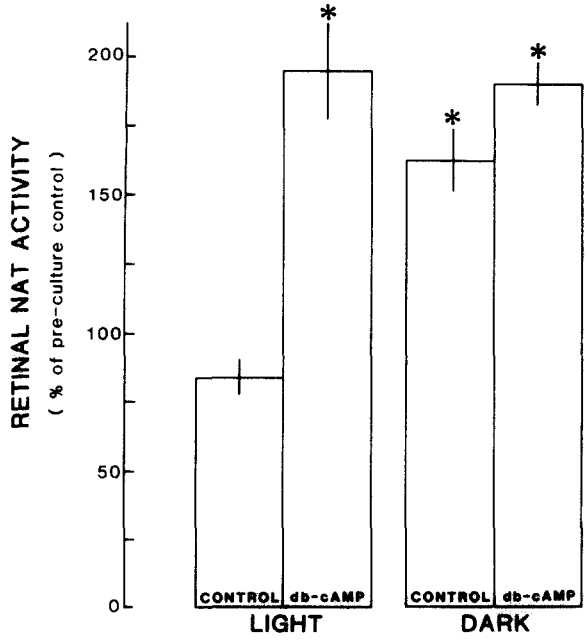

Fig. 5. Effect of dibutyryl-cyclic AMP on NAT activity of retinas cultured in light or dark. Eye cups were prepared just prior to light offset (12.00 h), and were subsequently cultured for 4 h in light or dark, with (db-cAMP) or without (control) 2.0 mM dibutyryl-cyclic AMP. Data are expressed as percent of pre-culture control, which was the activity in eye cups prepared at the time of light offset but not subsequently cultured: 75 ± 8 pmol/min/mg protein. n = 5/group, $P < 0.01$ compared to the pre-culture control.

TABLE IV

Effects of dibutyryl-cyclic AMP on retinal NAT activity of eye cups cultured during the light phase of the light–dark cycle

Eye cups were cultured in light from 04.00 h to 08.00 h as described in Methods. The concentration of dibutyryl-cyclic AMP was 2.0 mM.

Condition	*n*	*NAT activity (pmol/min/mg protein)*
Pre-culture control	5	64 ± 8
4 h culture	4	48 ± 4
4 h culture + dibutyryl-cyclic AMP	5	56 ± 9

AMP-analogs can substitute for darkness as a stimulus to increase NAT activity, and suggests that the effects of darkness on retinal NAT may be mediated by a cyclic AMP-dependent mechanism. To test this hypothesis an experiment was conducted to determine if the effects of darkness and dibutyryl-cyclic AMP were additive or non-additive (Fig. 5). Dibutyryl-cyclic AMP increased retinal NAT activity in eye cups cultured in light, but had no significant effect on that in eye cups where enzyme activity was already increased by culturing in darkness. Thus, the effects of dibutyryl-cyclic AMP and darkness were not additive. The activating effect of the cyclic nucleotide analogs was apparently specific for the adenine nucleotide because addition of dibutyryl-cyclic GMP (2 mM) to the culture medium had no significant effect on NAT activity in light or darkness (data not shown).

The effect of dibutyryl-cyclic AMP on NAT activity depends on the time within the light–dark cycle that the analog is administered. The stimulatory effect of dibutyryl-cyclic AMP described above was observed during the early portion of the normal dark phase. Dibutyryl-cyclic AMP added to the medium of eye cups cultured in the middle of the light phase (04.00–08.00 h) had no significant effect on retinal NAT activity (Table IV).

DISCUSSION

Considerable evidence suggests that melatonin is synthesized in the retina, as well as in the pineal gland, and that locally synthesized melatonin plays a role in cyclic metabolism in retina. First, melatonin-like immunoreactivity has been detected in retina of mammalian and non-mammalian vertebrates using highly specific antibodies[14,29,39]. This melatonin-like substance does not disappear following pinealectomy[43]. Second, the synthesis of radiolabeled melatonin from its precursor, serotonin, and the synthesis of radiolabeled serotonin from its precursor, tryptophan, have been demonstrated in retina[17,27,37]. Thirdly, melatonin and a synthetic analog, 6-chloromelatonin, have been shown to influence the rhythmic turnover of outer segment membranes of rod photoreceptors[8], a cyclic process that is not blocked by pinealectomy[5,20,34]. It is therefore important to gain insight into the mechanisms that control this humoral system in retina.

In the pineal gland, melatonin synthesis is regulated by induction of NAT[30]. In retina of chickens and rats, NAT activity and melatonin levels both occur in a rhythmic fashion, with peak detectability at night[12,13,15,29,36,43], suggesting that induction of NAT may also play a role in retinal melatonin biosynthesis. In this paper we have described a circadian rhythm in retinal NAT activity of the frog, *X. laevis*, and using an in vitro eye cup preparation have demonstrated that the increase in NAT activity that occurs in darkness is blocked by protein synthesis inhibitors and mimicked by cyclic AMP analogs.

Retinal NAT activity occurred as a circadian rhythm, with activity peaking during the dark phase of the light–dark cycle. In vitro, the dark-induced increase in activity was completely blocked by protein synthesis inhibitors, suggesting that the rhythm of activity represents a rhythm of protein synthesis (enzyme induction). The effects of darkness could be mimicked by cyclic AMP analogs. Thus, the regulation of NAT activity in retina appears to be similar to that in the pineal, where NAT activity occurs as a circadian rhythm that is blocked by protein synthesis inhibitors and is stimulated by cyclic AMP (reviewed by Axelrod[2]).

The circadian rhythm of retinal NAT was entrained by the cycle of light and dark, and showed plasticity in that it would phase-shift in response to a change in the light–dark cycle. The detailed studies on the regulation of the NAT rhythm in retina suggest that it is controlled by both positive (inducing) and negative (repressing) influences. When the rhythm is blocked by exposure to constant light for

two days, exposure to dark for 3 h causes an increase in NAT activity, suggesting that darkness may serve as a positive stimulus in the regulation of the rhythm. This effect of darkness may be mediated by cyclic AMP, beause cyclic AMP-analogs can mimic the effects of darkness during the early hours of the subjective dark phase. In contrast, the expression of NAT activity appears to be repressed and unresponsive to addition of cyclic AMP analogs during the light phase of the light–dark cycle.

Locally synthesized melatonin may have several functions within the eye. Based on observations that melatonin influences the uptake and release of neurotransmitters in hypothalamus[18,44], melatonin may modulate synaptic transmission in retinal neurons. Melatonin may also be involved in dark-adaptive melanin pigment aggregation in retinal pigment epithelium[19,31,38]. In addition, there are many similarities between the regulation of photoreceptor membrane turnover and that of NAT activity. For example, the shedding of outer segment discs[9,11,32] and expression of retinal NAT activity both have circadian components which persist for at least 3 days in constant darkness[9,32]. In addition, the 'free-running' rhythms of disc shedding and NAT activity have a period slightly greater than 24 h[33], and are blocked by constant light treatment for several days. Following constant light, a 3 h exposure to darkness will increase both shedding[9,20,28] and NAT activity. These correlations, taken together with the observations that disc shedding can be stimulated in vitro by melatonin[8], suggest that the retinal melatonin system may play an important role in the regulation of photoreceptor membrane turnover.

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