Technical Brief

Diurnal regulation of arylalkylamine N-acetyltransferase activity in chicken retinal cells in vitro: Analysis of culture conditions

Rashidul Haque, Angel L. Alonso-Gómez, Shyam S. Chaurasia, P. Michael Iuvone

Department of Pharmacology, Emory University School of Medicine, Atlanta, GA

Purpose: Arylalkylamine N-acetyltransferase (AANAT) is a key regulatory enzyme in the synthesis of melatonin, which displays daily fluctuations in chicken retinal photoreceptors in vivo. The purpose of the present study was to determine if cultures of embryonic neural retina cells express diurnal rhythms of AANAT activity.

Methods: Cell cultures were prepared from chick embryonic day 6 neural retina and incubated for 4 to 8 days in vitro (DIV). Cells were incubated under a daily light-dark (LD) cycle and were harvested day and night. Culture conditions were modified to test the effects of cell density, serum concentration, incubation temperature, S-(4-nitrobenzyl)-6-thioinosine (NBTI), and taurine on AANAT activity. AANAT activity was assayed in cell homogenates by measuring the catalytic formation of N-acetyltryptamine from tryptamine and acetyl coenzyme A.

Results: Cells cultured in medium containing 10% fetal bovine serum (FBS) failed to show any diurnal fluctuation in AANAT activity on DIV 5 and 6. However, if the culture medium was replaced on DIV 4 with one containing 1% FBS, and 5 µM NBTI or 5 mM taurine, the cells expressed significant diurnal rhythms of enzyme activity. NBTI was more potent and effective than taurine. Culture conditions were optimized with respect to cell density, serum concentration, incubation temperature, and NBTI concentration. Under optimized conditions, overall cell survival and the density of photoreceptor cells were increased relative to that with the other culture conditions tested.

Conclusions: The results indicate that diurnal rhythms of AANAT activity are expressed in embryonic retinal cells incubated under particular culture conditions. The results show that the mechanisms regulating melatonin synthesis in chicken retinal cells are established during early embryonic life. This culture preparation will be useful in elucidating the photic control mechanisms involved in regulation of melatonin biosynthesis in photoreceptor cells.

Melatonin is a neurohormone synthesized in the pineal gland [1] and the retina [2]. Retinal melatonin is made primarily in photoreceptor cells and appears to act as a local neuromodulator [3-6]. Melatonin synthesis is organized as a daily rhythm, which reflects the rhythmic expression of arylalkylamine N-acetyltransferase (AANAT) [7-9], the penultimate enzyme in the melatonin biosynthetic pathway. The rhythmic synthesis of melatonin in vertebrates, including birds, is regulated by two conserved mechanisms: one is the regulation by endogenous circadian clocks located in photoreceptors [10-13] and the other is the regulation by light, which negatively modulates AANAT expression and promotes degradation of the enzyme by a proteasomal mechanism [14-16].

Cultured cells from embryonic chick neural retina have been used extensively to study mechanisms of melatonin production in photoreceptors [17-21]. However, the cells used in these studies were not light sensitive, so the inductive effects of darkness on melatonin synthesis were mimicked using depolarizing concentrations of extracellular K+ or cAMP antagonists. The aim of the present study is to determine if dispersed cultures of embryonic neural retina cells can be induced to express diurnal rhythms of AANAT activity that are entrained to an imposed daily light-dark cycle.

METHODS

Cell cultures: Retinal cell cultures were prepared from six-day-old chick embryo (Gallus domesticus) as described by Adler et al. [22] with slight modifications [17,18]. Retinas were dissociated by brief trypsinization and cells were seeded at a density of 9x10^6 cells/60 mm polyaniline-coated culture dishes, unless noted otherwise. Cells were incubated for 4-8 days in vitro (DIV) in 6 ml of medium 199 containing 10% fetal bovine serum (FBS), 20 mM HEPES, linoleic acid-BSA (110 µg/ml), 2 mM glutamine, and penicillin G (100 units/ml). When the medium was changed during the culture period, serum concentration was reduced to 1%, except when serum concentration was studied. It should be noted that multiple lots of serum were screened to find sera that yielded cultures with a high proportion of cells expressing the photoreceptor phenotype, relative to other cell types, and high levels of AANAT activity. After the initial experiment to determine
optimum concentration of S-(4-nitrobenzyl)-6-thioinosine (NBTI), media were always supplemented with 5 µM NBTI before and after replacement. After seeding, cells were typically incubated at 37±0.5 °C under an atmosphere of 5% CO₂ in air for one day and thereafter at 40±0.5 °C for the rest of the experiment. Cells were exposed to a daily lighting regimen of 12 h light and 12 h darkness, with lights on at zeitgeber time (ZT) 0. Illumination was provided by an 8 W cool white fluorescent lamp (General Electric, Ohio, USA). Irradiance at the level of the culture dishes was 30-60 µW/cm². When indicated in the figure legends, medium from the dishes was removed and replaced with 3 or 6 ml of fresh medium. To collect cells for AANA T activity measurements, culture dishes were washed with Ca²⁺/Mg²⁺-free Hank’s balanced salt solution and scraped into 150 µl of 0.25 M potassium buffer (pH 6.5) containing 1.33 mM acetyl CoA. Samples were frozen on dry ice and stored at -80 °C until assayed.

AANAT activity assay: AANAT activity was determined by incubating cell homogenates at 37 °C for 15 min with 1 mM acetyl coenzyme A and 2 mM tryptamine as substrates and quantification of the reaction product, N-acetyltryptamine, by HPLC with electrochemical or fluorometric detection [23]. Homogenate protein content was determined by the method of Lowry et al. [24], using bovine serum albumin as standard.

Photomicroscopy and cell counting: To assess relative appearance and cell density, cultures were examined using a Zeiss Axiovert microscope (Carl Zeiss, Thornwood, NY) equipped with a video camera and Scion Image and Adobe Photoshop software. Random fields (3 to 4) from each dish were photographed and photoreceptor cells, identified by their refractile lipid droplet in the inner segment and polarized cell body [22], were counted. Although the cultures contained other cell types, including glia and neurons, the clumping of cells grown at the density used in this study made it impossible to quantify these cell types.

Data analysis: Data are shown as means with the standard errors of the mean (SEM). Comparisons among the group means were analyzed for statistical significance by ANOVA and the Student-Newman-Keuls multiple-comparison test.

Materials: Fertilized eggs were purchased from HyLine International (Covington, GA, USA); linoleic acid/bovine serum albumin, penicillin G were from Sigma (St. Louis, MO, USA); Medium 199, Hank’s balanced salt solution were from Mediatech, Inc (Herndon, VA); HEPES, L-glutamine, and trypsin were from Life Technologies (Grand Island, NY, USA); fetal bovine serum was from Atlanta Biologicals (Lot 5022F; Norcross, GA, USA) or HyClone (Lot AGJ7101; Logan, UT, USA); S-(4-nitrobenzyl)-6-thioinosine (NBTI) was obtained from Research Biochemicals International (Natick, MA).
RESULTS

Effect of media change and serum concentration on AANAT activity: Cells prepared from embryonic day 6 neural retinas were incubated in a medium containing 10% fetal bovine serum. The cells were subjected to a 12 h light:12 h dark cycle (LD) from DIV 1. Under these conditions, AANAT activity was low and showed no day-night difference on DIV 5 and 6 (Figure 1). When 50% of the medium was replaced on DIV 4 with a medium containing 1% serum, AANAT activity was higher on DIV 5 and 6 compared to cells with no fresh medium, but no evidence of a diurnal fluctuation of AANAT activity was observed. In contrast, when the medium was completely replaced with one containing 1% serum, significant day-night differences of AANAT activity were observed on DIV 5 and 6. Activity was low during the daytime and high at night (p<0.02), as it is in vivo [25,26]. These findings suggest that some component of the medium is exhausted after 5 days of incubation in LD, that some metabolite accumulates in the medium under these conditions which inhibits the diurnal expression of AANAT activity, and/or that serum inhibits the diurnal expression of the enzyme. In all subsequent experiments, medium was completely exchanged on DIV 4.

To assess the role of serum, cultures were subjected to medium replacement on DIV 4 with media containing 0, 1, or 5% serum, and AANAT activity was measured on DIV 6. All groups had higher AANAT activity (Figure 2) compared to cells with no change of medium. A day-night fluctuation in AANAT activity was observed, but neither serum-free nor 5% serum supported this effect. Thus, serum appears to be required for diurnal expression of AANAT activity, but high concentrations of serum may have an inhibitory effect. In all subsequent experiments, the medium was changed on DIV 4 to one containing 1% serum.

Effect of incubation temperature on AANAT activity: The body temperature of resting chickens is 39 °C, but increases as high as 45 °C during periods of high activity [27]. Temperature has been identified as an important variable regulating rhythmic melatonin secretion from cultured pineal glands of 2-4 week old chicks [28]. Therefore, we examined the effects of different incubation temperatures of 39 °C and above. Cells incubated at 39 °C exhibited significant diurnal fluctuations of AANAT activity (p<0.01; Figure 3) on each day from DIV 4-7. Cells incubated at 40 °C had comparable daytime AANAT activity to those incubated at 39 °C, but nighttime levels were significantly higher at 40 °C than at 39 °C on DIV 4 (p<0.001), DIV 5 (p=0.006) and DIV 6 (p=0.047; Figure 3). In contrast, AANAT activity of cells incubated at 42 °C was arrhythmic (data not shown). Consequently, 40 °C was chosen as the optimal incubation temperature.

Figure 3. Daily rhythm of AANAT activity in chicken retinal cell cultures. Cultures were prepared from embryonic day 6 retinas and incubated as described in the legend to Figure 1. After incubation for 1 day at 37 °C, cells were cultured under LD at either 39 °C or 40 °C for six more days. Cells were harvested on days 4, 5, 6, and 7 in vitro (DIV 4 to 7) at ZT 6 and ZT 18. The horizontal bars on the bottom of the graph represent the periods of light (open bar) and darkness (filled bar). Data are shown as means with the error bars representing the SEM (N=3). Significant diurnal rhythms of AANAT activity were observed on each day from DIV 4-7 in cells incubated at 39 °C (p<0.01) and 40 °C (p<0.001). Daytime values were not significantly different at the two temperatures, but nighttime activity was significantly higher at 40 °C than at 39 °C on DIV 4 (p<0.001), DIV 5 (p=0.006), and DIV 6 (p=0.047).

Figure 4. Concentration-response analysis of the effect of NBTI on daily rhythm of AANAT activity. Cultures were prepared from embryonic day 6 retinas and incubated as described in the legend to Figure 1, with complete replacement of the culture medium on DIV 4, except that the concentration of NBTI added on DIV 1 and 4 was varied. Cells were harvested on the night of DIV 6 at ZT 18 and on DIV 7 at ZT 10 and ZT 18. Data are shown as means with the error bars representing the SEM (N=3-4). Significant diurnal fluctuations of AANAT activity were observed in cells incubated with 5 μM NBTI (p=0.030) and 20 μM NBTI (p=0.036), but not with 1 μM NBTI (p=0.661).
Effects of S-(4-nitrobenzyl)-6-thioinosine (NBTI) and taurine on AANAT activity: In preliminary experiments (data not shown), we found that changing the medium dramatically reduced the survival of photoreceptor cells and neurons. Paes-de-Carvalho and Maia [29] reported that adenosine and the adenosine uptake inhibitor NBTI had a neuroprotective effect on cultured chick retinal cells. We, therefore, examined the effect of NBTI concentration on the diurnal regulation of AANAT activity in cultured cells (Figure 4). NBTI was present in the culture medium from DIV 1. AANAT activity was measured the night of DIV 6 and both day and night on DIV 7. NBTI produced a concentration-dependent increase of AANAT activity under all illumination conditions. A significant diurnal variation of AANAT activity was observed at 5 and 20 µM concentrations of NBTI (1 µM, P=0.661; 5 µM, P=0.042; and 20 µM, P=0.037). The magnitude of the rhythm appeared slightly larger with 5 µM NBTI compared to the other concentrations, and this concentration was used in all subsequent studies.

We also investigated whether taurine affects the diurnal variation of AANAT activity of cultured cells (Figure 5). Cells incubated in the presence of 5 mM taurine showed a significant light-dark difference in AANAT activity (p<0.001). However, activity at night in cells treated with NBTI was significantly higher than that in cells treated with taurine (p=0.038). Taurine was not included in subsequent studies.

Cell density and rhythm of AANAT activity: We examined the daily rhythm of AANAT activity in chicken retinal cells seeded at relatively low (4.5x10^6 cells/dish) and high (9x10^6 cells/dish) densities. The results are shown in Figure 6. There was no AANAT rhythm observed in cells cultured in low density (P=0.155), but a significant difference of AANAT activity was observed over a period of two days in cells seeded at high density (P<0.001).

Microscopic appearance and photoreceptor cell density under different culture conditions: To assess the overall appearance of cells cultured under different conditions, cultures were examined on DIV 6 by phase-contrast photomicroscopy. Representative pictures are shown in Figure 7A-F. The control condition represents the culture condition that produced the optimal AANAT rhythms, which includes seeding at a density of 9x10^6 cells/60 mm dish and replacement of the culture medium on DIV 4 with a medium containing 1% fetal bovine serum and 5 µM NBTI. Under this condition, dishes were nearly covered with a monolayer of neuron-like cells, possibly growing on a glial cell layer (Figure 7A). Many of these neuron-like cells expressed a photoreceptor phenotype, with the refractile lipid droplet and polarized cell body characteristic of chicken cone cells [22]. Other cell types were present as well. The apparent cell density of cultures not subjected to medium replacement (no MR) on DIV 4 was lower.
than that of controls (Figure 7B), as observed also for cells exposed on DIV 4 to fresh medium containing 0% or 5% fetal bovine serum (Figure 7C,D). In 0% serum and to a lesser extent in 5% serum, cells appeared to organize in clusters connected by neurites. Cells treated with taurine instead of NBTI (Figure 7E) had many polarized cells in monolayer and some cell clusters. In cultures seeded at a density of 4.5x10^6 cells/dish but otherwise treated like controls, cell density was very low, and multiple cell types could be discerned, including photoreceptors, multipolar neurons, and apparently undifferentiated round cells (Figure 7F).

Due to the high-density and clustering of cells, it was not possible to count multipolar neurons and round cells. However, we were able to estimate the number of cells with an apparent photoreceptor phenotype by counting polarized cells with a refractile lipid droplet. By this measure, the apparent density of photoreceptor cells was highest in the controls and lowest in the cultures seeded at low density (Figure 8). The apparent photoreceptor density under all other conditions was intermediate relative to these conditions.

**DISCUSSION**

The experiments reported here demonstrate that retinal cell cultures prepared from six-day-old chick embryos are capable of expressing a light-driven diurnal fluctuation of AANAT activity. Our observations demonstrate that four factors are important to establish the rhythm of AANAT activity in cell culture: (1) serum concentration, (2) addition of NBTI to the media, (3) media replacement during culture period, and (4) density of cells seeded per dish.

E6 chick retinal cells are prepared and incubated in a medium containing 10% fetal bovine serum. Under these conditions, the majority of cells differentiate as photoreceptor cells [17,30]. However, cells incubated in 10% serum under a daily light-dark cycle failed to exhibit a diurnal rhythm of AANAT activity (Figure 1). In contrast, if the medium was changed to a medium containing 1% serum after 4 days in vitro, when photoreceptor differentiation is largely complete [30], a prominent day-night rhythm was observed. Interestingly, the serum concentration appeared to be critical. Neither serum-free medium nor one containing 5% serum was as effective as 1%
serum in supporting the expression of the AANAT activity rhythm. Besides providing an optimal serum concentration, the addition of fresh medium may have other beneficial effects. Cells that received fresh medium on DIV 4 had higher AANAT activity regardless of serum concentration compared to cells incubated in 10% serum without addition of fresh medium. It is probable that certain components of the medium become depleted due to cellular utilization and/or degraded, possibly to toxic metabolites, by light exposure.

NBTI promotes survival of neurons and photoreceptor cells following media change [29]. NBTI is an adenosine transport inhibitor [31], which increases the concentration of extracellular adenosine available to act on G protein-coupled receptors located on photoreceptor cells [32]. Adenosine is taken up by and released from cultured chick photoreceptor cells [33]. Adenosine is implicated in extracellular signaling [34] and, in chick embryo retina, adenosine promotes accumulation of cAMP via activation of A1 receptors as well as inhibition of dopamine-dependent accumulation of cAMP via A2A receptors [35,36]. Replacing the culture medium with fresh, serum-containing medium causes extensive death of chick retinal neurons and photoreceptor cells, an effect not observed when cultures are preincubated with adenosine or NBTI [29]. Possible mechanisms of how NBTI promotes photoreceptor survival are: (1) accumulation of extracellular adenosine, with consequent activation of A1 receptors and intracellular accumulation of cAMP in retinal neurons, which promotes the synthesis and release of trophic or neuroprotective factors by retinal cells in culture [37,38], (2) a decrease in the accumulation of cAMP caused by down regulation of A2A receptors and adenylyl cyclase [39], or (3) a cAMP-independent mechanism. We found that NBTI increases AANAT activity in a concentration-dependent manner. As AANAT in chick retina is expressed primarily in photoreceptor cells [14], this result may reflect photoreceptor survival and/or a direct effect of endogenous adenosine on AANAT expression. Cell counts indicated that cultures maintained in the presence of NBTI had a higher number of cells expressing an apparent photoreceptor phenotype than did cells incubated without NBTI, but in the presence of taurine.

A 5 µM concentration of NBTI supports a robust diurnal rhythm of AANAT activity in cultured cells. A diurnal rhythm of AANAT was also observed in cells incubated in a medium containing taurine; however, the amplitude of the rhythm was smaller than that observed in the presence of NBTI. Taurine is a regulator of rod photoreceptor development and is required for survival of photoreceptor cells in vivo [40,41]. Chick retina accumulates taurine in photoreceptors, in cells from the inner nuclear layer, and in processes of the inner plexiform layer [42]. Taurine has no effect on opsin expression in cell cultures prepared from embryonic day 8 retina, though it stimulates opsin expression in rat retinal cell culture [43].

Varying the seeding density between 4.5 million and 9 million cells per 60 mm dish significantly affects the diurnal expression of AANAT activity. There was no detectable diurnal rhythm of AANAT activity in cells seeded at the lower density (Figure 6). However, in the same experiment, cells seeded at the higher density demonstrated significant diurnal rhythms of enzyme activity. The reason for this density-dependent effect is unclear. One possibility is that at the higher density, the cultures contained Müller glial cells while those seeded at the lower density were virtually devoid of glia. Glia may contribute retinoids needed for differentiation and survival of cone cells as well as for phototransduction by the photoreceptors [44,45]. It is also possible that photoreceptor-glia interactions or photoreceptor-neuron interactions may contribute to the effects of the all of the treatments tested.

It is interesting to note that other aspects of photoreceptor metabolism in chick cell cultures may not be as sensitive to culture conditions as is regulation of AANAT activity. For example, Stenkamp and colleagues [46,47] found that chick embryo photoreceptor cells in culture undergo light-evoked photomechanical movements that are mediated by dopamine. This effect occurs in cultures seeded at low density and incubated in the presence of 10% fetal bovine serum without media replacement. The reason for this discrepancy is not apparent. The light-sensitive elements in these cultures have not been identified. Perhaps light-evoked dopamine release and the consequent photomechanical movement occur by a mechanism distinct from light-regulated AANAT activity. Other possible explanations include differences in the embryonic age of the cultures, the atmosphere in which the cells are incubated, and the relative need for retinoids in the two responses. The photomechanical movements were studied in cultures seeded on embryonic day 8 while the present study used cultures generated on embryonic day 6. Stenkamp and colleagues [46,47] incubated the cells under an atmosphere of 9% O2, 5% CO2, and 86% N2, while the cells in the current study were incubated under 5% CO2. The lower O2 content used to study photomechanical movement may have resulted in less light-evoked generation of reactive oxygen species, thus making media replacement unnecessary. Retinoid metabolism may be another variable. Exogenous retinoic acid increases AANAT activity in chick retinal cell cultures (T. Ivanova and P.M. Iuvone, unpublished observation) and the expression of another melanin synthesizing enzyme, hydroxyindole Omethyltransferase, in retinoblastoma cells [48]. The higher cell density and presence of glial cells that metabolize retinoids may be important for AANAT expression and melanin synthesis but not photomechanical movement. Additional studies are needed to assess the relative importance of these variables.

The use of NBTI to improve cell survival following media replacement may have other applications. Most in vitro transfection protocols require media replacement with a serum-free or low-serum medium during the transfection and return to serum-containing medium during the post-transfection incubation period. In a recent study, Toy et al. [49] reported that survival of cultured chick retinal cells in the post-transfection period was much better if the cells were refed with the original, conditioned medium rather than with fresh medium. The enhanced cell survival afforded by NBTI in cells refed with fresh medium [29; present results], suggests that the adenosine transport inhibitor may be a useful addition to transfection protocols.
In conclusion, the current study establishes conditions for expression of diurnal rhythms of AANAT activity in cultured retinal cells. This culture preparation will be useful in elucidating the photic control mechanisms involved in regulation of melatonin biosynthesis in photoreceptor cells.

ACKNOWLEDGEMENTS
The authors wish to thank James H. Wessel III and Brenda Bondesen for assistance with this study. This research was supported by NIH grant R01-EY04864.

REFERENCES