Circadian clockwork machinery in neural retina: Evidence for the presence of functional clock components in photoreceptor-enriched chick retinal cell cultures

Shyam S. Chaurasia, 1 Nikita Pozdeyev, 1 Rashidul Haque, 1 Amy Visser, 1 Tamara N. Ivanova, 1 P. Michael Iuvone 1,2

1 Department of Pharmacology, 2 Department of Ophthalmology, Emory University School of Medicine, Atlanta, GA

Purpose: Circadian clocks in retinas regulate a variety of biochemical and physiological processes. Retinal neurons, particularly photoreceptor cells, are thought to contain autonomous circadian clocks that control iodopsin expression, cFos expression, cAMP levels, and melatonin synthesis. Photoreceptor-enriched cell cultures prepared from chick embryo retina and entrained to a daily light-dark (LD) cycle exhibit circadian rhythms of cAMP levels and the activity of arylalkylamine N-acetyltransferase (AANAT), a key regulatory enzyme in melatonin synthesis. The present study was conducted to investigate the expression of circadian clockwork machinery comprised of clock genes; a clock-controlled gene, Aanat; and a clock output, melatonin, in the photoreceptor-enriched cultured retinal cells.

Methods: Photoreceptor-enriched cell cultures were prepared from E6 neural retinas and incubated under 14 h:10 h light-dark cycle (LD) of illumination for 8 days and then transferred to constant (24 h/day) darkness (DD). Cells were collected every 4 h in LD and DD, and RNA was isolated. cDNA was prepared from each sample and transcripts of clock genes and Aanat were measured using real-time polymerase chain reaction (PCR). Melatonin release into the culture medium was assayed by HPLC with fluorescence detection at intervals of 3 h in LD and DD.

Results: Cultured neural retina cells exposed to a light-dark cycle showed rhythmic expression of clock genes. Bmal1 and Npas2 (also known as Mop4) peaked late in the day in LD and in DD. Clock mRNA was high at night in LD, but arrhythmic in DD. Cry1 and Per2 transcripts increased rapidly in the early morning and were low at night. The rhythm of Per2 was reduced in amplitude in constant darkness (DD). Levels of Cry1 and Per2 transcripts were stimulated by light exposure at night. Melatonin release and Aanat mRNA were low during the day and high at night. Rhythmic expression of clock genes and Aanat was not observed in cultures not exposed to a LD cycle but treated otherwise identically to cultures described above.

Conclusions: Photoreceptor-enriched cell cultures derived from chick embryo neural retina contain a complete circadian clockwork system that is entrained by the light-dark cycle, and has a core timekeeping mechanism and circadian output in the form of melatonin synthesis.

Circadian clocks are self-sustaining genetically based molecular machines that impose approximately 24 h rhythmicity on physiology and behavior, synchronizing these functions with the solar day-night cycle [1]. These clocks provide a selective advantage to organisms by allowing them to anticipate temporal changes in their environment. Circadian clocks contain three main components: an input or entrainment mechanism, a self-sustaining timing device, and an output mechanism. The current model for the molecular basis of the timing device in vertebrates consists of interlocking transcriptional-translational feedback loops involving a highly conserved set of “clock genes” [1-3]. These feedback loops consist of positive (CLOCK, BMAL1, and MOP4, also known as NPAS2) and negative components (PER1 and 2; CRY1 and 2). The temporal delay between transcription and translation of the clock components and some poorly understood phosphorylation events controlling nuclear import/export and protein degradation result in a daily rhythm in the transcripts and protein products of the clock genes.

The retina is characterized by the presence of autonomous circadian clocks and a photoentrainment mechanism [3-7]. Retinal circadian clocks regulate a variety of cellular, biochemical, and physiological processes, including expression of immediate early genes, activities of the enzymes in signal transduction pathways, rod outer segment disc shedding, phagocytosis by retinal pigment epithelium (RPE), and the release of the neurohormone melatonin [3,8]. Circadian clocks in the retina also play a role in the daily modulation of visual sensitivity and electroretinography (ERG) responses to anticipate the changes in ambient lighting and to adjust visual sensitivity for the bright conditions of day and the darkness of night [9,10].

Several studies have demonstrated that melatonin is rhythmically synthesized by the retinal photoreceptors, with markedly higher levels at night than during the day, reflecting the actions of light and the circadian clock [4,11-14]. Arylalkylamine N-acetyltransferase (AANAT; EC 2.3.1.87) has been identified as a key regulatory enzyme in the melatonin synthetic pathway [15]. In chickens, the enzyme is ex-
pressed primarily in retinal photoreceptors and the pineal gland [16,17]. The levels of chicken retinal AANAT activity and melatonin biosynthesis exhibit circadian rhythms, peaking at night [11]. Additionally, AANAT activity in chicken retina is under the control of light, which dramatically suppresses enzyme activity and promotes degradation of the enzyme by a proteasomal mechanism [17]. Recently, we reported that photoreceptor-enriched chicken retinal cell cultures, entrained to a daily light-dark (LD) cycle for eight days, exhibit circadian and photic regulation of AANAT activity that recapitulates regulation in vivo (6). It was also shown that cAMP levels are controlled in a circadian fashion, which in turn, regulates the circadian and photic control of AANAT activity [7].

In search of a suitable model system to investigate circadian clock organization in the retina, we have utilized a photoreceptor-enriched chick retinal cell culture preparation that shows rhythms of cGMP-gated channel activity [18], iodopsin expression [5], levels of cAMP [7], and the activity of AANAT [6]. The purpose of this study was to determine if these cultured cells, entrained to a daily cycle of light and darkness, rhythmically express clock genes. In addition, output from the circadian clock was assessed by determining circadian rhythms of Aanat mRNA and melatonin release in these cells.

METHODS

Cell preparation and culture: Monolayer cultures of retinal cells were prepared from neural retinas of 6-day-old chicken embryos (E6) as described by Adler [19] with modifications [6,20]. Neural retinas were dissociated in 0.25% trypsin, and cells were seeded at a density of about 3.6×10^6 cells on polyornithine-coated 35 mm Primaria six-well culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) in 3 ml medium 199 containing 20 mM HEPES, linoleic acid-BSA (110 µg/ml), 2 mM glutamine, penicillin-streptomycin (100 U/ml) and 10% fetal bovine serum. Cells were maintained at 39.5°C ± 0.4°C under a humidified atmosphere of 5% CO_2 in air. Illumination was provided by an 8 W cool white fluorescent lamp (General Electric, Cleveland, OH) and the irradiance at the level of the culture dishes was 30-60 µW/cm². Days in vitro (DIV) are numbered successively from the day of dissection (DIV 0). On DIV 1, S-(p-nitrobenzyl)-6-thioinosine (NBTI) was added in a final concentration of 5 µM. Medium was replaced on DIV 4 and 7 with medium 199, 1% fetal bovine serum, 1% equine serum, 5 nM insulin-like growth factor-1, 5 µM 9-cis-retinoic acid, and the above given concentrations of HEPES, glutamine, linoleic acid-BSA, NBTI and penicillin-streptomycin. Fertilized eggs and cultured cells were exposed to a daily lighting regime of 14 h light (L) and 10 h dark (D) from day 1 of incubation, with light onset at Zeitgeber time (ZT) 0. DIV9, cells were transferred to constant darkness (DD). All measurements were made on DIV 8-10 at the times indicated in the figures.

To assess the proportion of cells expressing a photoreceptor phenotype in these cultures, cells were fixed on DIV 9 in 3.7% formaldehyde, and were stained with oil red O, which labels lipid droplets [21] that are characteristic of avian cone photoreceptors in vivo and in vitro [22,23], and with DAPI, to label the nuclei of all cells in the cultures. A total of 3,064 cells were counted from randomly selected microscope fields from 12 culture dishes. In this sample, 78±2% of the DAPI labeled cells stained with oil red O. This estimate of the percentage of photoreceptors is consistent with previous estimates made on cultures generated from E6 chick retina and grown at lower density [24,25].

**RNA isolation and first strand cDNA synthesis:** Cells were washed twice with Hanks’ Balanced Salt Solution (HBSS) and collected in 150 µl of Buffer RLT, and processed for RNA isolation by a silica-based filter-binding RNeasy mini kit (Qiagen Inc., Valencia, CA). Samples were treated with RNase-free DNase I following the manufacturer’s instructions (Qiagen Inc.). First strand cDNA synthesis was performed as described [26]. Briefly, total RNA (2 µg) was reverse transcribed in the literature a 20 µl reaction using oligo-dT primer (Invitrogen, Carlsbad, CA), RNase inhibitor and Superscript III reverse transcriptase (Invitrogen). The reaction proceeded for 1 h at 50°C, followed by 15 min at 72°C to inactivate the enzyme.

**Quantitative real time PCR:** Real time PCR amplification of cDNA was performed with SYBR Green master mix (Bio-Rad, Hercules, CA) in a Bio-Rad iCycler (Bio-Rad) as described in previous reports [26,27]. Briefly, the reaction mixture included 2 µl of cDNA, 1X SYBR Green mix, and 300 nM gene specific forward and reverse primers. PCR reaction includes initial denaturation at 95°C for 3 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Each sample was assayed in triplicate and normalized to the expression of a housekeeping gene, hypoxanthine phosphoribosyl transferase (Hprt). cDNA fragments of Bmal1 (GenBank accession number U46502) and Aanat (GenBank accession number EF205219) were used as references for the normalization of the data. Areal time PCR primers were designed using PrimerQuest (Integrated DNA Technologies, Caraville, IA) on the indicated GenBank sequences. Each primer pair yielded a single product, as determined by agarose gel electrophoresis, melt curve analysis, and DNA sequencing.

### TABLE 1. PRIMERS

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Accession number</th>
</tr>
</thead>
</table>
| Bmal1       | F: TGAGGAGTCTGCTTGTCAGTTCCTCA  
R: ACGeTGCATCATGTAGTGAGAA | AF205219 |
|            | R: ACGCTGTCCATGCTATGTGGAGAA | AF205219 |
| NPAS2       | F: CCAGGGCAATTTGCACTCCACAA  
R: AGGATGTGGCCTCATAGGCTCAA | AF396828 |
| Clock       | F: ACGGTCAAGGACTGAGATGCTCT  
R: CTGCAAAAGGCTGTGCAGATCAT | AF246959 |
| Per2        | F: TGGTACAGGTCAGACACTTCACAA  
R: TTTCCGGAGTCTGCCAGCTGATTA | AF246956 |
| Cry1        | F: AGAGATGTCCTCGAAGGCTGCAA  
R: ACTGTGCAAGGACACCGAATCT | AY034432 |
| Aanat       | F: ACGGACCACTCTTACACACGCAAGA  
R: CTGCTTTCACGCAAAAACCAAGGCAAT | U46502  

©2006 Molecular Vision
ber AF205219), *Npas2/Mop4* (GenBank accession number AF396828), *Clock* (GenBank accession number AF246959), *Cry1* (GenBank accession number AY034432), *Per2* (GenBank accession number AF246956), *Hprt* (GenBank accession number AJ132697), and *Aanat* (GenBank accession number U46502) transcripts were generated by PCR, gel purified, quantitated, and used as standards in the real-time PCR assays. The primers used in the experiment for the expression of *Aanat* and clock genes (Table 1) were designed using the software PrimerQuest (Integrated DNA Technologies, Coralville, IA) and validated for the single PCR product as verified by melting curve analysis, agarose gel electrophoresis and DNA sequencing. Moreover, each primer set was designed spanning intron-exon borders and/or bracketing one or more introns.

**Estimation of melatonin:** At ZT 0 on day 8 in vitro, medium was replaced with 1 ml of fresh medium using the same formulation described for medium changes on DIV 4 and 7. Thereafter, 1 ml of media was collected from the culture dishes and replaced every 3 h on DIV 8 in LD and on DIV 9 in DD. Levels of melatonin in culture media were determined by reversed-phase high performance liquid chromatography (HPLC). To 100 µl of culture media 20 µl of 1 N HClO₄ was added to precipitate proteins. After centrifugation at 15,000 g for 10 min, a 100 µl aliquot of supernatant was injected into the HPLC system. The separation was performed on an Ultrasphere ODS 250x4.6 mm column, 5 mm (Beckman Coulter, Fullerton, CA) with 40% methanol in water as the mobile phase. Melatonin was detected by fluorescence with excitation and emission wavelengths set at 283 and 352 nm, respectively. External standards of melatonin with concentrations ranging from 50 to 2000 pg/injection were used to build a calibration curve.

**Statistical analysis:** Data are expressed as mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keul’s multiple comparison test.

**RESULTS**

*Circadian clock components in chicken photoreceptor-enriched cultured retinal cells:* Real-time PCR analysis showed robust rhythms in the expression of *Bmal1* and *Npas2* tran-

---

**Figure 1.** Temporal expression of positive modulators of the circadian clockwork system. Relative mRNA levels of *Bmal1* (A) and *Npas2* (B) in photoreceptor-enriched retinal cell cultures collected at the indicated zeitgeber times (ZT) in light-dark (LD) and dark-dark (DD). Each data point represents clock gene transcripts normalized to *Hprt* mRNA and expressed relative to the lowest values in LD. The open horizontal bar at the X-axis represents times of light exposure; the black bars represent times of darkness. Analysis of variance (ANOVA) indicated significant rhythms of *Bmal1* and *Npas2* transcripts in LD and DD (p<0.001), with highest levels in the late day and early night; n=4-6 cultures per time. C: *Clock* mRNA showed significantly higher values during the night (ZT 16) than during the day in LD (p<0.001); transcript levels increased on the first day of DD (ZT 0 compared to ZT 20; p<0.05) but there were no significant rhythms on DD1 or DD2. There were 5-6 cultures for each time.
scripts in cultured photoreceptor cells. In LD, Bmal1 mRNA levels peak at about ZT 12 (p<0.001) and continue to display circadian rhythms for two days in DD (p<0.001; Figure 1A). The circadian oscillatory pattern of Npas2 mRNA is similar to that of Bmal1 transcript with high levels at ZT 8-12 (p<0.001), which persist in constant darkness (p<0.001; Figure 1B). The timing of the peak levels of Bmal1 and Npas2 is similar to that seen in 2 week posthatch chicken retina in vivo [22]. The expression of Clock mRNA showed a diurnal rhythm with highest levels at night in LD (p<0.001; Figure 1C). Clock mRNA levels increased during the first day in DD (p<0.05) and became arrhythmic (Figure 1C).

Figure 2. Temporal expression of negative modulators of the circadian clockwork system. Circadian profiles of Cry1 (A) and Per2 (B) transcripts in the photoreceptor-enriched retinal cell cultures collected at the indicated zeitgeber time (ZT) in light-dark (LD) and dark-dark (DD). Each data point represents clock gene transcripts normalized to Hprt mRNA, expressed relative to the lowest values in LD. ANOVA indicated significant differences (p<0.001) with high levels of both transcripts in the first half of the day in LD and DD; There were 6 cultures for each time. Acute light exposure at night induces Cry1 (C) and Per2 (D) mRNA expression. On day in vitro 9, cells were kept in constant darkness until ZT 18, when one group of cells was collected. Another group of cells remained in darkness for an additional 2 h (solid symbol), while a third group of cells was exposed to light for 2 h prior to cell harvesting (open symbol). Exposure to light significantly increased Cry1 and Per2 transcript levels (p<0.001; n=5-6 culture dishes per group).
In LD and DD, levels of Cry1 increased during the subjective day with highest levels from ZT 4-12 (p<0.001); they decreased during the subjective night to show a trough in the late night (Figure 2A). In LD, Per2 mRNA levels showed a large increase after light onset, reaching a peak at about ZT 4, and then declined to a minimum at about ZT 16 (p<0.001; Figure 2B). In DD, a similar, statistically significant pattern was observed (p<0.01), but with greatly reduced amplitude (Figure 2B). Light exposure for 2 h at night (ZT 18) significantly increased the levels of Cry1 and Per2 transcripts (p<0.001; Figure 2C,D). The magnitude of light induction was greater for Per2 than for Cry1. Induction by a light pulse and persistent rhythms in DD are indicative of both photic and circadian control.

Circadian output of the photoreceptor-enriched cell cultures: Cultured photoreceptor cells showed circadian fluctuations of Aanat mRNA peaking at about ZT 20 in LD (p<0.001). In DD, Aanat transcript levels continued to be robustly rhythmic (p<0.001; Figure 3), with broader peaks at night (ZT 12-20).

Melatonin release into the culture medium was significantly higher during the night at ZT 17-20 compared to that seen in the day (p<0.001; Figure 4), which corresponds to the levels of Aanat mRNA expression in these cells. In constant darkness, rhythmic melatonin release persisted (p<0.001), but with lower amplitude (Figure 4).

Photoentrainment of circadian gene expression: To determine if the rhythms of clock gene and Aanat expression are entrained by the light cycle or caused by culture conditions, such as medium exchange, cells were prepared and seeded into culture dishes and then incubated in either darkness or in LD for eight days. All cells were subjected to the same culture conditions and schedule of medium changes, which were performed under dim room light. In cells incubated in LD, robust day-night rhythms of Bmal1, Npas2, and Aanat transcripts were observed (ZT 12 compared to ZT 20, p<0.001; Figure 5A), consistent with previous results (Figure 1, Figure 3). In contrast, cells that were not cultured under an LD cycle showed no day-night differences in the levels of these transcripts when sampled at the same two times of the subjective day (with respect to medium changes; Figure 5B), indicating that photoentrainment, rather than culture conditions, sets the circadian oscillations in the photoreceptor cultures.

DISCUSSION

In this study, we have demonstrated that photoreceptor-enriched cell cultures derived from neural retina exhibit an autonomous circadian clock that is entrained to the daily LD cycle. They express transcripts encoding several positive and negative clock components, and a clock output in the form of melatonin secretion. The rhythmic expression of circadian clock genes in LD and DD demonstrates the presence of a circadian pacemaker.

The presence of an autonomous circadian clock in the retina of vertebrates was first conclusively demonstrated in cultured eyecups of the African clawed frog Xenopus laevis, where the activity of the AANAT was shown to be regulated by a circadian clock [28]. Moreover, the circadian rhythm of this enzyme could be phase-shifted by light in vitro, thus demonstrating that the photoreceptor responsible for the entrain-
ment resided within the eyecup. Subsequently, Cahill and Besharse [13] showed that the circadian pacemaker controlling this rhythm was located in photoreceptors. The presence of circadian clocks in the retinas of many vertebrates, including fishes, reptiles, birds, and mammals, has now been firmly established [5,27,29-31].

The avian retina rhythmically expresses several clock genes in vivo. Bmal1 and Npas2 are highly rhythmic in LD and DD, with highest levels during the late day and early night [27]. Clock mRNA is also expressed in chick retina, but with a low amplitude rhythm [32]. Cry1 and Per2 transcripts peak during the beginning of the subjective day [33,34]. Cry2 mRNA is also expressed in chick retina, but its temporal expression pattern is unknown [35]. The clock in the chicken retina also controls the expression of Aanat in photoreceptors [16,17]. Multiple cell types in the avian retina may contain functional circadian clocks. There is evidence of circadian rhythms in photoreceptors [4,5,18], cells in the inner nuclear layer [36], and retinal ganglion cells [37].

Cultures of embryonic chick retinal cells have been used extensively in studies of retinal development [19]; regulatory mechanisms involved in retinal melatonin biosynthesis, phototransduction, and receptor-mediated signaling [5,24,38-40]; and photoreceptor retinomotor movements [41,42]. Photoreceptor-enriched cell cultures express circadian rhythms of cAMP level and AANAT activity following entrainment to LD cycle of illumination [6,7]. Light exposure for 2 h at night dramatically decreases cAMP and AANAT activity to the levels observed during the day [6,7]. From earlier studies, we know that cAMP protagonists stimulate Aanat mRNA and activity in these cells [24,38,39]. Thus, circadian control of cAMP may play an important role in generating circadian rhythms of Aanat transcription and melatonin synthesis. Although the previous studies mentioned above suggest the possibility that retinal photoreceptors contain a circadian clock that responds to light, these data are not sufficient to identify the nature of the circadian pacemaker in retinal photoreceptors. Moreover, these studies examined the regulation of clock-controlled genes; none of them showed the actual clock components.

In the present study, we found that cultured chick retinal cells rhythmically express Aanat and clock genes known to be essential components of the core molecular clockwork in the mammalian brain. Aanat mRNA levels show robust circadian regulation, with high levels at night in LD and DD. Bmal1 and Npas2 are rhythmically transcribed with high levels at about ZT 12, as seen in vivo [27], approximately 8 h prior to the peak of Aanat mRNA rhythm. Clock transcript levels peak later at night in LD, but were arrhythmic in constant darkness. Cry1 and Per2 mRNAs showed highest levels during the day,

![Figure 5. Photoentrainment of clock gene and Aanat transcript rhythms.](http://www.molvis.org/molvis/v12/a24/)

Cell cultures were prepared as described in Methods culture dishes from the same batch of cells were divided into two groups: one kept in incubators without illumination and the other in incubators with a daily light-dark (LD) cycle. Other than exposure to LD, the culture conditions and schedule of medium changes were identical for all cultures. Cultures were sampled on day in vitro (DIV) 8 at ZT 12 and ZT 20 for LD cultures and on DIV 8 at the same times of day for cultures kept in the incubators without illumination. Relative levels of Bmal1, Npas2, and Aanat transcripts were measured. A: Cells cultured under a daily cycle of light and darkness from DIV 0-8 displayed significant differences between zeitgeber times (ZT) 12 and ZT 20 for all three transcripts, with Bmal1 and Npas2 higher at ZT 12 and Aanat higher at ZT 20 (asterisk indicates p<0.01; n=6). B: Cells incubated without a daily cycle of illumination, but treated identically otherwise, displayed no significant difference between the two times of day for any of the transcripts measured; n=5-6.
about 8-12 h out of phase with the Aanat transcript rhythm. The chicken Aanat promoter contains a circadian E-box enhancer element where the circadian clock components BMAL1 and CLOCK/NPAS2 bind and enhance transcription [43]. The temporal expression patterns of the positive and negative clock components are consistent with a model in which BMAL1/CLOCK or BMAL1/NPAS2 drive circadian Aanat expression at night, with expression being suppressed during the day by PER and CRY, which inhibit E-box driven transcription by BMAL1/CLOCK-NPAS2. This E-box mediated control, coupled with the circadian rhythm of cAMP, may cooperatively generate the circadian rhythms of Aanat transcription and melatonin synthesis.

For the most part, the rhythmic expression of clock genes in retinal cell culture recapitulates the temporal expression patterns seen in the chicken retina in vivo. One notable exception is Cry1, which damps quickly in DD in vivo [34], but not in vitro. However, the in vivo observation of Cry1 mRNA arrhythmicity in DD was made on whole retina and Cry1 is expressed in all retinal layers, including the photoreceptor layer [34]. Thus, Cry1 rhythmicity in photoreceptors in DD may have been masked by damping in other retinal cell types.

In the present study, we observed circadian rhythms of melatonin release in LD and DD. However, the rhythm in DD had greatly reduced amplitude compared to that in LD. This may have resulted from cellular damage caused by repeated (every 3 h) medium changes, or may reflect the influence of post-translational photic control of AANAT in DD. The rhythms of Aanat mRNA remained relatively robust in DD in the cell cultures, as in vivo [34]. However, AANAT activity rhythms in vivo and in vitro show reduced amplitude in DD [6,17]. The reduced amplitude in AANAT activity appears to reflect the absence of photic control mechanisms in DD. Light promotes the degradation of AANAT protein in chick retina by proteasomal proteolysis [17]. Thus, in LD, the high amplitude of AANAT activity rhythms reflects a combination of the circadian clock-control of Aanat transcription and the light-mediated degradation of AANAT protein during the day; the latter effect is lost in DD.

Previous studies have shown that some cultured cell types and peripheral tissues can be induced to rhythmically express clock genes by serum shock or medium exchange [44,45]. Thus, an important consideration in the present study is whether the rhythms of clock gene expression and Aanat expression are a function of light-dark cycles or culture conditions, such as medium exchange. Several lines of evidence indicate that the rhythms are photoentrained. In the present study, rhythms of Clock mRNA were observed in LD but not in DD. In LD, Per2 levels were rapidly induced after light onset at dawn, resulting in a high amplitude rhythm; in DD, the large increase in Per2 expression was not observed at subjective dawn but a lower amplitude circadian rhythm of expression persisted. In addition, Per2 mRNA levels were strongly induced by light exposure at night. The induction of Per2 by light probably plays a key role in the photo-entrainment of circadian clocks in the retinal cell cultures, as in other circadian clock systems [33,46-49]. More definitively, retinal cell cultures never exposed to LD cycles, but subjected to the same culture conditions and media changes as cultures exposed to LD, failed to display day-night changes of clock gene and Aanat transcript expression.

The light sensitivity of clock gene expression and the circadian control of Aanat in retinal cell cultures suggest that circadian clocks are expressed by the photoreceptor cells in these cultures. However, the cultures are not pure, with only about 80% of the cells expressing a photoreceptor phenotype. Thus, we cannot exclude the possibility that nonphotoreceptor cells in the cultures express the clocks that regulate circadian Aanat expression in photoreceptors via neurohumoral communication or gap junctions. With respect to the latter possibility, it should be noted that gap junction uncoupling agents disrupt circadian expression of Aanat and iodopsin in explant cultures of chick retina [50].

In conclusion, photoreceptor-enriched retinal cell cultures express photoentrained circadian clocks and clock outputs. These cell cultures may serve as an excellent model to facilitate the biochemical and genetic dissection of the retinal circadian clockwork.

ACKNOWLEDGEMENTS

This work was supported by grant EY04864 from the National Institutes of Health. Preliminary reports of some of these data were presented at the 2005 meeting of the Association for Research in Vision and Ophthalmology (Ft. Lauderdale, FL) and the Xth Congress of the European Pineal and Biological Rhythm Society (Frankfurt, Germany; 2005).

REFERENCES


