Temporal coupling of cyclic AMP and Ca2+/CaM-stimulated adenylyl cyclase to the circadian clock in chick retinal photoreceptor cells

Shyam S. Chaurasia, *Emory University*
Rashidul Haque, *Emory University*
Nikita Pozdeyev, *Emory University*
Chad R. Jackson, *Emory University*
P Michael Iuvone, *Emory University*

Journal Title: Journal of Neurochemistry

Volume: Volume 99, Number 4

Publisher: Wiley: 12 months | 2006-11, Pages 1142-1150

Type of Work: Article | Post-print: After Peer Review

Publisher DOI: 10.1111/j.1471-4159.2006.04154.x

Permanent URL: http://pid.emory.edu/ark:/25593/fjd1x

Final published version:
http://onlinelibrary.wiley.com/doi/10.1111/j.1471-4159.2006.04154.x/abstract

Copyright information:

Published in final edited form as:
J Neurochem. 2006 November ; 99(4): 1142–1150. doi:10.1111/j.1471-4159.2006.04154.x.

Temporal coupling of cyclic AMP and Ca^{2+}/CaM-stimulated adenylyl cyclase to the circadian clock in chick retinal photoreceptor cells

Shyam S. Chaurasia[1], **Rashidul Haque**[1], **Nikita Pozdeyev**[1], **Chad R. Jackson**[1], and **P. Michael Iuvone**[1,2,*]

[1]Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322

[2]Department of Ophthalmology, Emory University School of Medicine, Atlanta, GA 30322

Abstract

Cyclic AMP signaling pathways play crucial roles in photoreceptor cells and other retinal cell types. Previous studies demonstrated a circadian rhythm of cyclic AMP level in chick photoreceptor cell cultures that drives the rhythm of activity of the melatonin synthesizing enzyme arylalkylamine N-acetyltransferase (Ivanova and Iuvone, 2003a) and the rhythm of affinity of the cyclic nucleotide-gated channel for cyclic GMP (Ko et al., 2004). Here we report that the photoreceptor circadian clock generates a rhythm in Ca^{2+}/calmodulin-stimulated adenylyl cyclase activity, which accounts for the temporal changes in the cyclic AMP levels in the photoreceptors. The circadian rhythm of cyclic AMP in photoreceptor cell cultures is abolished by treatment with the L-type Ca^{2+} channel antagonist nitrendipine, while the Ca^{2+} channel agonist, Bay K 8644, increased cyclic AMP levels with continued circadian rhythmicity in constant darkness. These results indicate that the circadian rhythm of cyclic AMP is dependent, in part, on Ca^{2+} influx. Photoreceptor cell cultures exhibit a circadian rhythm in Ca^{2+}/calmodulin-stimulated adenylyl cyclase enzyme activity with high levels at night and low levels during the day, correlating with the temporal changes of cyclic AMP in these cells. Both of the Ca^{2+}/calmodulin-stimulated adenylyl cyclase genes, type 1 and type 8 (*Adcy1* and *Adcy8*), displayed significant daily rhythms of mRNA expression under a light-dark cycle, but only the *Adcy1* transcript rhythm persisted in constant darkness. Similar rhythms of *Adcy1* mRNA level and Ca^{2+}/calmodulin-stimulated adenylyl cyclase activity were observed in retinas of 2 week old chickens. These results indicate that a circadian clock controls the expression of *Adcy1* mRNA and Ca^{2+}/calmodulin-stimulated adenylyl cyclase activity; and calcium influx into these cells gates the circadian rhythm of cyclic AMP, a key component in the regulation of photoreceptor function.

Keywords

circadian clock; cyclic AMP; adenylyl cyclase; photoreceptors; calmodulin; retina

Introduction

Circadian clocks are complex biological machines in which a set of programmed processes generate ~24 h rhythms in physiology and behavior (Reppert and Weaver 2002; Hastings et al. 2003; Iuvone et al. 2005). Vertebrate retinal neurons, including photoreceptor cells, are characterized by the presence of self-sustaining autonomous circadian clocks (Cahill and

*Address for Correspondence: P. Michael Iuvone Department of Pharmacology Emory University School of Medicine 1510 Clifton Road, Atlanta, GA 30322 Phone: 404−727−5859 Fax: 404−727−0365 Email: miuvone@pharm.emory.edu.

Besharse 1993; Pierce et al. 1993; Tosini and Menaker 1996; Ivanova and Iuovne 2003a,b; Chaurasia et al. 2005). Circadian clocks have been implicated in several retinal functions including visual sensitivity; expression of immediate early genes and visual pigment genes; affinity of the cone cyclic nucleotide-gated channel (CNGC) for cyclic GMP; and the synthesis of the neuromodulators dopamine and melatonin (for reviews see Tosini and Fukuhara 2002; Iuvone et al. 2005). The chick retina is an excellent model system for studying circadian organization because of its robust rhythmic expression of clock genes and clock controlled genes, as well the availability of a photoreceptor cell culture preparation that retains photosensitivity and circadian clock function (reviewed in Iuvone et al., 2005). The level of melatonin in chicken retina is high at night and low during the day (Hamm and Menaker, 1980). This rhythm of melatonin is derived from circadian rhythms of tryptophan hydroxylase and arylalkylamine *N*-acetyltransferase (AANAT), the first and penultimate enzymes in the biosynthetic pathway of melatonin from tryptophan (Bernard et al. 1997; Chong et al., 1998; Iuvone et al., 1999). In chicken retina, AANAT is expressed primarily in photoreceptor cells and is intricately controlled by light and by the circadian clock. Cyclic AMP contributes to the transcriptional and posttranslational control of AANAT activity in retina (reviewed in Iuvone et al., 2005). Cyclic AMP has also been implicated in the circadian control of the cone photoreceptor CNGC (Ko et al., 2004), and may serve as a coordinating clock signal for many circadian rhythms in photoreceptor cells (reviewed in Iuvone et al., 2005). Recently, chick photoreceptor cell cultures were shown to express circadian rhythms of cyclic AMP (Ivanova and Iuvone, 2003a). Levels of the second messenger are high at night and low during the day in cells exposed to a light-dark (LD) cycle and, with slightly reduced amplitude, in constant (24 h / day) darkness (DD). These temporal changes in cyclic AMP couple the circadian oscillator to the rhythms of AANAT activity (Ivanova and Iuvone, 2003a) and CNGC affinity for cyclic GMP (Ko et al., 2004).

K^+-induced depolarization of chick retinal photoreceptors stimulates cyclic AMP formation and AANAT activity (Avendano et al., 1990; Iuvone et al., 1991; Gan et al., 1995). These effects of depolarization require Ca^{2+} influx through L-type voltage-gated channels and are blocked by inhibitors of calmodulin (CaM) and adenylyl cyclase (Gan et al., 1995; Alonso-Gomez and Iuvone, 1995), suggesting the involvement of Ca^{2+}/CaM-stimulated adenylyl cyclases. The type 1 (ADCY1) and type 8 (ADCY8) adenylyl cyclases are the principal isoforms of the enzyme stimulated by Ca^{2+} and CaM (reviewed by Wang and Storm, 2003). Transcripts encoding the neural-specific ADCY1 are prominently expressed in retinal photoreceptor cells and ganglion cells of mammalian retina (Xia et al., 1993; Fukuhara et al., 2004), and *Adcy1* mRNA expression is circadian in rat retina (Fukuhara et al., 2004). To our knowledge, expression of *Adcy8* mRNA in photoreceptors has not been examined. The present study was undertaken to examine the temporal expression patterns of *Adcy1* and *Adcy8* in chick photoreceptor cells and to determine if a circadian rhythm of Ca^{2+}/CaM-stimulated adenylyl cyclase activity contributes to the circadian rhythm of cyclic AMP in these cells.

Materials and methods

Animals and tissue collection

Male White Leghorn chickens (*Gallus gallus domesticus*) were acquired (Hyline International, Convington, GA) on the day of hatching. Animals were maintained for a minimum of two weeks in heated brooders on a 12h light : 12h dark (LD) cycle with lights on from zeitgeber time (ZT) 0 to ZT12. Following this, the animals were subjected to constant (24h/day) darkness (DD). All experiments under conditions of darkness were performed under dim red light (No. 92 filter; Eastman Kodak, Rochester, NY). Chickens were killed by decapitation and retinas were rapidly dissected and frozen on dry ice. The experimental protocols were approved by the Emory University Institutional Animal Use and Care Committee and conform to the

guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Cell preparation, culture conditions and drug treatments

Fertilized eggs were purchased from Hyline International (Covington, GA) and incubated for six days in a 14h light-10h dark cycle. Monolayer cultures of retinal cells are prepared from neural retinas of 6-day-old chicken embryos (E6) as described by Adler et al. (1984) with modifications (Ivanova and Iuvone, 2003b; Chaurasia et al., 2006). Neural retinas were dissociated in 0.25% trypsin and cells were seeded at a density of ~3.6×10^6 cells on polyornithine-coated 35 mm Primaria six-well culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in 3 ml medium 199 supplemented with 20 mM HEPES, linoleic acid-BSA (110 μg/ml), 2mM glutamine, pencillin-streptomycin (100 U/ml) and 10% fetal bovine serum. Cells were maintained at 39.5±0.4°C under a humidified atmosphere of 5% CO_2 in air. Illumination was provided by an 8W cool white fluorescent lamp (General Electric, Ohio, USA); the irradiance at the level of the culture dishes was 30−60 μW/cm^2. Days in vitro (DIV) are numbered successively from the day of dissection (DIV 0). On the 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 HEPES, glutamine, linoleic acid-BSA, NBTI and penicillin-streptomycin at the concentrations described above. 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. On DIV 9, cells were transferred to DD and maintained in darkness for the remainder of the experiment. The cells were sampled on DIV 8−10 during the hours indicated in the figures. Approximately 80% of the cells at this stage express a photoreceptor phenotype (Chaurasia et al., 2006).

Cyclic AMP Assay

Cultured cells were collected on DIV 8 in LD and DIV 9 in DD and homogenized in 6% trichloroacetic acid (TCA). To determine the recovery of cyclic AMP during extraction, ~2,000 dpm of [^{3}H]cyclic AMP marker (Perkin-Elmer Life and Analytical Sciences, Inc. Shelton, CT, USA) was added to each sample. Homogenates were centrifuged at 2,500 × *g* at 4°C for 15 min, and the supernatant fluid removed and extracted three times with 5 ml of ethyl ether saturated with water. The extracted aqueous phase was evaporated and the residue dissolved in sodium acetate buffer, pH 6.2, which was used directly in the immunoassay. Cyclic AMP levels were measured by radioimmunoassay according to manufacturer's instructions (Perkin-Elmer Life and Analytical Sciences, Inc.). The protein in the pellet was solubilized in 0.1 N sodium hydroxide and quantified (Lowry et al. 1951) using bovine serum albumin as standard. The cyclic AMP levels were normalized to the protein concentration and expressed as pmol of cyclic AMP per mg of protein.

Preparation of membranes and measurement of adenylyl cyclase activity

Cultured cells were collected at ZT 5 and ZT 17 on DIV 8 in LD and DIV 9 in DD. Cell from six dishes were pooled in a microfuge tubes for each sample and 5−6 samples were taken in each group. Cells were washed twice with Ca^{2+},Mg^{2+}-free Hanks balanced salt solution, harvested and homogenized in 150 μl of buffer TME, which contains 200 mM Tris-maleate, pH 7.5, 1 mM $MgSO_4$, 1.2 mM EGTA, 0.5 mM dithiothreitol, and Protease Inhibitor Cocktail III (5μl/ml; EMD Biosciences, La Jolla, CA, USA). Neural retinas were dissected from 2 week old chickens at ZT 4 and ZT 16 in DD and homogenized in the solution described above. Partially purified membranes were prepared by centrifugation at 20,000 × *g* for 20 min. Supernatant fraction was discarded, and membranes were resuspended in 300 μl of buffer TME and washed twice. The pellet was resuspended in 100 μl of buffer TME and stored at −80°C.

Measurement of adenylyl cyclase activity involves assessing the conversion of [α-^{32}P]ATP to [^{32}P]cyclic AMP using the method of Salomon et al. (1974) with modifications. The incubation mixture contained 80 mM Tris-HCl, pH 7.5, 10 μM GTP, 1 mM DTT, 5 mM MgSO4, 0.5 mM cyclic AMP, 5 mM phosphocreatine, 0.1 mg/ml creatine phosphokinase, 4 units/ml adenosine deaminase, 0.2 mM EGTA, 50 μM ATP, [α^{32}P]ATP (0.5 μCi/tube) in the presence or absence of 195 μM $CaCl_2$, 120 nM calmodulin and 10 μM forskolin. Free [Ca^{2+}], with 195 μM $CaCl_2$ added, was calculated to be 2μM using WEBMAXCLITE v1.15 (http://www.stanford.edu/%7Ecpatton/webmaxc/webmaxclite115.htm). The reaction was initiated by the addition of 20−30 μg membrane protein in a final reaction volume of 250 μl and incubated at 37°C for 10 min. The reaction was stopped by addition of 750 μl of 10% TCA containing [^{3}H]cyclic AMP (~2000 dpm). After centrifugation, cyclic AMP was extracted by employing sequential chromatography on columns of Dowex AG50-W4 cation exchange resin (200−400 mesh) and neutral alumina. ^{32}P and ^{3}H in the resulting elute were quantified by liquid scintillation counting.

Laser Capture Microdissection (LCM)

The retinal photoreceptor layer was isolated by LCM as described previously (Haque et al. 2002; Chaurasia et al. 2005). Briefly, the eye cups with retinas were embedded in Tissue-Tek OCT Compound, frozen on dry ice and stored at −80°C. Frozen tissues were cut at 8−10 μm using a cryostat (CN 1850, Leica Microsystems, Deerfield, IL, USA) and mounted on uncharged glass slides (VWR International, West Chester, PA, USA). Sections were fixed in nuclease-free 75% ethanol, rehydrated in nuclease-free distilled water for 30 s, stained for 20 s (HistoGene, Arcturus Engineering, Mountain View, CA, USA) and rinsed in nuclease-free water for 30 s. They were then dehydrated by sequential immersion into 75, 95 and 100% ethanol for 30 s each and cleared in xylene for 5 min. After air-drying for 30 min, the slides were kept in a vacuum dessicator for 45 min-2 h. Laser capture was performed by lifting the photoreceptor cell layer (outer nuclear layer plus inner segments) of retina onto HS-CapSure non-contact LCM film (Arcturus Engineering). The PixCell IIe LCM system (Arcturus Engineering) was set to the following parameters: 7.5 μm laser spot size, 100 mW power, 1−2 ms duration. One photoreceptor layer, including central and peripheral retina, was obtained from each animal. Microdissected cells were immediately processed using the PicoPure RNA isolation kit (Arcturus Engineering). To eliminate possible genomic DNA contamination, RNA samples were treated with RNase-free DNase I (Qiagen, Inc., Valencia, CA) as described by the manufacturer. The total extracted RNA was used for first strand cDNA synthesis and real-time PCR was performed as described below.

RNA isolation and first strand cDNA synthesis

Retinal tissue and photoreceptor-enriched cultured neural retinal cells were extracted in buffer RLT™ (Qiagen Inc.) and processed for RNA isolation by a silica-based filter-binding RNeasy™ mini kit (Qiagen Inc.). Samples were treated with RNase-free DNase I following the manufacturer's instructions (Qiagen Inc.). First strand cDNA synthesis was performed as described earlier (Chaurasia et al. 2005). Briefly, total RNA (2μg) was reverse transcribed in a 20 μl reaction using oligo-dT primer (Invitrogen, Carlsbad, CA), RNase inhibitor, T4 gene 32 protein (Ambion, Austin, TX, USA), and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reaction proceeded for 1 hr 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, USA) in a Bio-Rad iCycler (Bio-Rad) as described in previous reports (Chong et al. 2003; Chaurasia et al. 2005). Briefly, the reaction mixture included 2μl of cDNA,

1X SYBR Green mix and 300nM 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 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Each sample was assayed in triplicate and normalized to the expression of a housekeeping gene, hypoxanthine phosphoribosyl transferase *(Hprt)*. cDNA fragments of *Adcy1*, *Adcy*, and *Hprt* transcripts were generated by PCR, gel purified, quantified by spectrophotometer, and used as standards in the real-time PCR assays. The primers used for generation of standard cDNAs and expression analysis of *Adcy1*, *Adcy8*, and *Hprt* (Table 1) were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA) and validated for a single PCR product by melting curve analysis and agarose gel electrophoresis.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Comparison between two groups was accomplished with Student's t-test. Comparisons of multiple groups were made by analysis of variance (ANOVA) followed by Student-Newman-Keul's multiple comparison test. All data were subjected to normality and equal variance tests; if either test failed, data were analyzed by Mann Whitney Rank Sum test (2 groups) or Kruskal-Wallis ANOVA on Ranks with Dunn's multiple comparison test.

Results

Calcium influx is required for cyclic AMP circadian rhythm generation in photoreceptor cell cultures

Cells incubated under LD displayed a statistically significant rhythm in cyclic AMP level (Fig. 1), peaking at night (ZT 20 vs. ZT 10, $p<0.001$). This rhythm in cyclic AMP level persisted on the first day of constant darkness (DD; ZT 20 vs. ZT 10, $p<0.001$). Treatment of cells with nitrendipine (3 μM) completely abolished the cyclic AMP rhythms in LD and DD, reducing cyclic AMP levels at night to daytime values. In contrast, Bay K 8644 (3 μM) significantly enhanced the cyclic AMP levels in these cells at all times of the day tested with a sustained rhythm in LD and DD ($p<0.05$, ZT 20 vs. ZT 10 on both days) (Fig. 1). These findings indicate that the circadian rhythm of cyclic AMP is dependent, at least in part, on Ca^{2+} influx.

Ca^{2+}/CaM-stimulated adenylyl cyclase activity in photoreceptor cell cultures

The most parsimonious interpretation of the results described above is that Ca^{2+} influx results in stimulation of one or more Ca^{2+}/CaM-stimulated adenylyl cyclases and that the circadian rhythm of cyclic AMP arises from a rhythm in the abundance of the cyclic AMP synthesizing enzymes. To test this hypothesis, adenylyl cylase activity was measured in crude membrane preparations of homogenized cells collected during the daytime (ZT 5) and nighttime (ZT 17) in LD on DIV 8 and in DD on DIV 9. There was no significant difference in basal adenylyl cyclase activity between daytime and nighttime samples in either LD or DD (Fig. 2). Adenylyl cyclase activity was significantly stimulated by addition of Ca^{2+} and CaM. Moreover, stimulation of cyclase activity by Ca^{2+}/CaM in nighttime samples (9 fold) was significantly greater than that in daytime samples (5 fold) in LD ($p\leq0.001$; Fig. 2). The difference in Ca^{2+}/CaM-stimulated adenylyl cyclase activity persisted in DD, with stimulation in nighttime samples approximately twice as large as that in daytime samples. The stimulation of adenylyl cyclase by Ca^{2+}/CaM was potentiated by forskolin, and the night/day difference in stimulation was maintained in the presence of the adenylyl cyclase activator in both LD ($p\leq0.001$) and DD ($p<0.05$; Fig. 2).

Daily rhythms of *Adcy1* and *Adcy8* transcript levels in photoreceptor cell cultures

To evaluate whether the circadian rhythm of cyclic AMP levels, which is dependent on Ca^{2+} influx into the cultured photoreceptor cells, is derived from the rhythmic transcription of adenylyl cyclase genes, we investigated the mRNA levels of two Ca^{2+}/CaM-stimulated adenylyl cyclases- *Adcy1* and *Adcy8* (Fig 3). The cultures cells expressed transcripts for both *Adcy1* and *Adcy8*, with higher levels of *Adcy1* mRNA. Photoreceptor cell cultures incubated under an LD cycle displayed prominent daily fluctuations of *Adcy1* transcript, peaking at ZT16 on DIV 8 ($p<0.001$). The rhythmic expression of the *Adcy1* transcript persisted for two days in constant darkness (DD) on DIV 9 ($p<0.001$) and DIV 10 ($p=0.022$), indicative of circadian control (Fig. 3a). In contrast, the expression of *Adcy8* mRNA was rhythmic in LD with high levels during the early morning hours of the day and low levels during the night on DIV 8 in cultured photoreceptor cells (Fig. 3b, $p<0.001$). On day 1 of DD, there was a trend towards continued rhythmicity of *Adcy8* transcript levels, but it was not statistically significant ($p=0.112$). On the second day of DD, *Adcy8* mRNA levels increased from ZT0 to ZT8 ($p=0.018$), and remained at that level for the remainder of the day.

Adcy1 and *Adcy8* are expressed in chicken photoreceptor cells *in vivo*

The photoreceptors cell cultures used in this study are from embryonic neural retina and are not completely pure, with approximately 80% of the cells expressing the photoreceptor phenotype (Chaurasia et al., 2006). To determine if photoreceptors express *Adcy1* and *Adcy8 in vivo* in posthatch chickens, the photoreceptor layer was isolated by laser capture microdissection of frozen sections of retina obtained from 2 week old chickens killed during the daytime (ZT 4) and night (ZT 16) in LD (Fig. 4). Both transcripts were readily detectable in the isolated photoreceptor layers, with significantly higher expression at ZT16 compared to ZT4 for *Adcy1* ($p\leq0.005$) and at ZT4 compared to ZT16 for *Adcy8* ($p\leq0.005$). The relative levels of *Adcy1* transcript were higher than those of *Adcy8* mRNA at both times of day.

Adcy1 transcript and adenylyl cyclase activity in neural retina is under the control of an endogenous circadian clock *in vivo*

Adcy1 mRNA expression was examined in neural retina of chickens entrained to LD for 2 weeks and then released into DD for two days. A significant daily rhythm of *Adcy1* mRNA expression was observed in LD (Fig. 5; $p=0.008$), with highest levels of transcript at night (ZT 16) and lowest levels during early hours of the daytime (ZT0−4). In DD, we found a significantly daily variation in the expression of *Adcy1* transcript in neural retina for at least 2 days ($p\leq0.001$ for both days in DD). *Adcy8* transcript levels displayed significant variation only in LD ($p<0.001$), with highest levels during the daytime; this pattern was not maintained on either day in DD (data not shown).

Membranes prepared from neural retina in DD also displayed a circadian fluctuation in Ca^{2+}/CaM-stimulated adenylyl cyclase activity (Fig. 6), similar to that observed in photoreceptor cell culture, with higher activity at night ($p<0.001$). As a control, membranes were treated with nitrendipine (3 μM), which had no effect on the circadian rhythm of adenylyl cyclase activity. This result indicates that the effect of the calcium channel blocker on the circadian rhythm of cyclic AMP is not due to a direct effect on adenylyl cyclase.

Discussion

Our data provide the first experimental evidence for a requirement of Ca^{2+} influx in the generation of the circadian rhythm of cyclic AMP level in photoreceptor cells. They also demonstrate for the first time the existence of daily rhythms of Ca^{2+}/CaM-stimulated adenylyl cyclase activity in retinal cells, and have identified regulation of *Adcy1*, and possibly *Adcy8*, transcript levels as factors in the regulation of these rhythms.

The levels of cyclic AMP in photoreceptor cells, as well as the activity of AANAT, are subject to dual control by a circadian clock and light. The levels of the cyclic nucleotide and AANAT activity in photoreceptor cell cultures oscillate in constant darkness with peaks during the subjective night (Ivanova and Iuvone, 2003a,b). Light exposure at night lowers cyclic AMP and AANAT to daytime levels. In the present study, we demonstrated that the circadian rhythm of cyclic AMP in photoreceptor cell cultures is abolished by nitrendipine, a dihydropyridine L-type Ca^{2+} channel blocker, with low levels observed both day and night. In contrast, the L-type Ca^{2+} channel agonist, Bay K 8644, elevated cyclic AMP levels during both subjective day and subjective night. The plasma membrane of vertebrate retinal photoreceptor cells are relatively depolarized in darkness, and hyperpolarized in a graded fashion in response to light of increasing intensity (Hagins et al., 1970). Photoreceptors have dihydropyridine-sensitive Ca^{2+} channels (Taylor and Morgans, 1998; Nachman-Clewner et al., 1999; Krizaj, 2005), which open upon depolarization (Uchida and Iuvone, 1999) and mediate the Ca^{2+} influx required for the synthesis of cyclic AMP (Iuvone et al., 1991). Moreover, the induction of AANAT activity requires sustained Ca^{2+} influx in photoreceptors and the enzyme activity decreases upon closure or inhibition of channel activity (Iuvone and Besharse, 1986; Avendano et al., 1990). Collectively, these observations indicate that the suppression of cyclic AMP level and AANAT activity by light is mediated by hyperpolarization of the plasma membrane and closure of the Ca^{2+} channels. Thus, differential Ca^{2+} influx can explain the light-dark difference in cyclic AMP level, but alone does not account for the sustained oscillation of the cyclic nucleotide in constant darkness, when photoreceptors would be depolarized and voltage-gated Ca^{2+} channels activated both day and night.

Two possible mechanisms for the sustained oscillation of cyclic AMP are circadian control of intracellular Ca^{2+} and circadian gating of the availability of Ca^{2+}/CaM-stimulated adenylyl cyclases. The latter mechanism was explored in this study. We show here that Ca^{2+}/CaM-stimulated adenylyl cyclase activity is higher in membranes prepared from photoreceptor cell cultures during the subjective night than in membranes prepared during subjective day, in both LD and constant darkness. Thus, in the presence of a fixed Ca^{2+} concentration *in vitro*, cyclic AMP production is higher at night than during the day, indicative of a rhythm of cyclase availability. This observation does not preclude a possible contribution of circadian control of intracellular Ca^{2+}, which has yet to be explored.

Of the 10 adenylyl cyclases known in vertebrates, ADCY1 and ADCY8 are directly stimulated by Ca^{2+}/CaM *in vitro* and *in vivo* (Choi et al., 1992; Cali et al., 1994). ADCY1 is synergistically stimulated by intracellular Ca^{2+} and G protein-coupled receptor activation *in vivo* (Wayman et al., 1994), whereas ADCY8 is not. We found that *Adcy1* and *Adcy8* transcripts are present in the chicken photoreceptor cell cultures and in neural retina. Using laser capture microdissection (LCM), we also showed that *Adcy1* and *Adcy8* are expressed in photoreceptors of two week old chickens *in vivo*. Previous studies in mammalian retina have shown the expression of *Adcy1* mRNA in photoreceptors (Xia et al., 1993; Fukuhara et al., 2004). Photoreceptor cells cultured under LD showed rhythmic patterns of *Adcy1* and *Adcy8* expression. Similar results were observed in whole neural retina and in LCM-isolated photoreceptors of two week old chickens *in vivo*. Rhythms of *Adcy1* mRNA, which peaked during the subjective night, persisted in constant darkness *in vitro* and *in vivo*, indicative of circadian control. In contrast, *Adcy8* mRNA levels were higher during the daytime and arrhythmic in constant darkness. The amount of *Adcyl* transcript was ~10 fold higher in cultured photoreceptor cells and ~5 fold higher in LCM dissected photoreceptors compared to *Adcy8* transcripts. These results clearly suggest that the circadian rhythm of Ca^{2+}/CaM-stimulated adenylyl cyclase activity and cyclic AMP level is generated, at least in part, by a rhythm in the abundance of ADCY1 protein. Unfortunately, we were unable to find an antibody that specifically recognizes ADCY1 in chicken retina to directly test this hypothesis. A recent study has also shown the circadian expression of *Adcy1* mRNA in the rat retina, and provided

evidence for a role of ADCY1 in the circadian gating of melatonin synthesis (Fukuhara et al. 2004).

In addition to regulation of melatonin biosynthesis and CNGC affinity for cyclic GMP, other circadian processes in photoreceptor cells may be gated or driven by the circadian rhythm of cyclic AMP. For example, induction of c-Fos mRNA and protein in photoreceptors is regulated in a circadian fashion, with peak levels during the subjective night (Yoshida et al., 1993; Humphries and Carter, 2004). Similar to cyclic AMP, light exposure at night suppresses c-Fos transcript levels in photoreceptors (Yoshida et al., 1993). In many cell types, c-Fos expression is induced by a cyclic AMP-dependent mechanism (Greenberg et al., 1985; Webster and Kedes, 1990; Buckmaster et al., 1991). Induction of nocturin, an RNA deadenylase (Baggs and Green, 2003), in *Xenopus* photoreceptors is stimulated by phospho-CREB (Liu and Green, 2002), which undergoes a circadian rhythm with peak levels at night. It must be noted, however, that CREB can be phosphorylated and *c-fos* transcription can be activated by multiple protein kinases and a definitive link between the rhythm of cyclic AMP and those of phospho-CREB and c-Fos have yet to be established.

In summary, we have demonstrated the circadian expression of Ca^{2+}/calmodulin-stimulated adenylyl cyclase activity in chicken retinal photoreceptors. The clock-controlled expression of *Adcy1* appears to contribute to the light-sensitive, Ca^{2+}-dependent rhythm of cyclic AMP, which provides a circadian clock output capable of regulating multiple aspects of cellular physiology. Coupled with the diurnal expression of *Adcy8*, this regulatory system may be responsible for the many light-driven and circadian rhythms associated with photoreceptor cell biology.

Acknowledgements

The authors thank Hong Zhou and Amy Visser for excellent technical assistance. This work was supported by grant R01EY004864 and P30EY006360 from the National Institute of Health. Preliminary reports of some of these data were presented at the 2004 meeting of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, FL, and the X^{th} Congress of the European Pineal and Biological Rhythm Society, Frankfurt, Germany, 2005.

Abbreviations

AANAT, arylalkylamine N-acetyltransferase
Adcy1, adenylyl cyclase type 1
Adcy8, adenylyl cyclase type 8
BSA, bovine serum albumin
CaM, calmodulin
CNGC, cyclic nucleotide-gated channel
DD, constant darkness
DIV, days in vitro
DTT, Dithiothreitol
EGTA, ethylene glycol tetraacetic acid
GCL, ganglion cell layer
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hprt, hypoxanthine guanine phosphoribosyltransferase
INL, inner nuclear layer
LCM, laser capture microdissection
LD, light-dark
ONL, outer nuclear layer
PCR, polymerase chain reaction
PhR, photoreceptors
TCA, trichloroacetic acid

TME, tris-maleate EDTA
ZT, zeitgeber time

REFERENCES

Adler R, Lindsey JD, Elsner CL. Expression of cone-like properties by chick embryo neural retina cells in glial-free monolayer cultures. J. Cell Biol 1984;99:1173–1178. [PubMed: 6470040]

Alonso-Gómez AL, Iuvone PM. Role of calmodulin in the regulation of serotonin N-acetyltransferase activity in cultured chick retinal photoreceptor cells. Invest.Ophthalmol.Vis.Sci 1995;36:S512.

Avendano G, Butler BJ, Iuvone PM. K^+-evoked depolarization induces serotonin N-acetyltransferase activity in photoreceptor-enriched retinal cell cultures. Involvement of calcium influx through L-type channels. Neurochem. Int 1990;17:117–126.

Baggs JE, Green CB. Nocturnin, a deadenylase in Xenopus laevis retina: a mechanism for posttranscriptional control of circadian-related mRNA. Curr Biol 2003;13:189–98. [PubMed: 12573214]

Bernard M, Iuvone PM, Cassone VM, Roseboom PH, Coon SL, Klein DC. Avian melatonin synthesis: photic and circadian regulation of serotonin N-acetyltransferase mRNA in the chicken pineal gland and retina. J. Neurochem 1997;68:213–224. [PubMed: 8978728]

Buckmaster A, Nobes CD, Edwards SN, Tolkovsky AM. Nerve Growth Factor is Required for Induction of c-Fos Immunoreactivity by Serum, Depolarization, Cyclic AMP or Trauma in Cultured Rat Sympathetic Neurons. Eur J Neurosci 1991;3:698–707. [PubMed: 12106477]

Cahill GM, Besharse JC. Circadian clock functions localized in *Xenopus* retinal photoreceptors. Neuron 1993;10:573–577. [PubMed: 8476609]

Cali JJ, Zwaagstra JC, Mons N, Cooper DM, Krupinski J. Type VIII adenylyl cyclase. A Ca2+/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. J Biol Chem 1994;269:12190–5. [PubMed: 8163524]

Chaurasia SS, Rollag MD, Jiang G, Hayes WP, Haque R, Natesan A, Zatz M, Tosini G, Liu C, Korf HW, Iuvone PM, Provencio I. Molecular cloning, localization and circadian expression of chicken melanopsin (Opn4): differential regulation of expression in pineal and retinal cell types. J. Neurochem 2005;92:158–170. [PubMed: 15606905]

Chaurasia SS, Pozdeyev N, Haque R, Visser A, Ivanova TN, Iuvone PM. Circadian clockwork machinery in neural retina :Evidence for the presence of circadian clock in photoreceptor-enriched chick retinal cell cultures. Mol. Vis 2006;12:215–223. [PubMed: 16604054]

Choi EJ, Wong ST, Hinds TR, Storm DR. Calcium and muscarinic agonist stimulation of type I adenylylcyclase in whole cells. J Biol Chem 1992;267:12440–2. [PubMed: 1319996]

Chong NW, Cassone VM, Bernard M, Klein DC, Iuvone PM. Circadian expression of tryptophan hydroxylase mRNA in the chicken retina. Mol. Brain Res 1998;61:243–50. [PubMed: 9795235]

Chong NW, Chaursaia SS, Haque R, Klein DC, Iuvone PM. Temporal-spatial characterization of chicken clock genes: circadian expression in retina, pineal gland and peripheral tissues. J. Neurochem 2003;85:851–860. [PubMed: 12716417]

Fukuhara C, Ivanova TN, Chan GC, Storm DR, Iuvone PM, Tosini G. Gating of the cAMP signaling cascade and melatonin synthesis by the circadian clock in mammalian retina. J. Neurosci 2004;24:1803–11. [PubMed: 14985420]

Gan J, Alonso-Gomez AL, Avendano G, Johnson B, Iuvone PM. Melatonin biosynthesis in photoreceptor-enriched retinal cell cultures: role of cyclic AMP and K+-evoked, Ca^{2+}-dependent induction of serotonin *N*-acetyltransferase activity. Neurochem. Int 1995;27:147–155. [PubMed: 7580870]

Greenberg ME, Greene LA, Ziff EB. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J Biol. Chem 1985;260:14101–10. [PubMed: 3877054]

Hagins WA, Penn RD, Yoshikama S. Dark current and photocurrent in retinal rods. Biophys. J 1970;10:380–412. [PubMed: 5439318]

Hamm HE, Menaker M. Retinal rhythms in chicks: circadian variation in melantonin and serotonin *N*-acetyltransferase activity. Proc. Natl. Acad. Sci. U S A 1980;77:4998–5002. [PubMed: 6933543]

Haque R, Chaurasia SS, Wessel JH III, Iuvone PM. Dual regulation of cryptorchrome 1 mRNA expression in chicken retina by light and circadian oscillators. Neuroreport 2002;13:2247–2251. [PubMed: 12488805]

Hastings MH, Reddy AB, Maywood ES. A clockwork web:circadian timing in brain and periphery, in health and disease. Nat. Rev. Neurosci 2003;4:649–661. [PubMed: 12894240]

Humphries A, Carter DA. Circadian dependency of nocturnal immediate-early protein induction in rat retina. Biochem. Biophys. Res. Commun 2004;320:551–556. [PubMed: 15219864]

Iuvone PM, Besharse JC. Cyclic AMP stimulates serotonin N-acetyltransferase activity in Xenopus retina in vitro. J. Neurochem 1986;46:33–39. [PubMed: 2415681]

Iuvone PM, Gan J, Avendano G. K(+)-evoked depolarization stimulates cyclic AMP accumulation in photoreceptor-enriched retinal cell cultures: role of calcium influx through dihydropyridine-sensitive calcium channels. J. Neurochem 1991;57:615–621. [PubMed: 1712831]

Iuvone PM, Cong NW, Bernard M, Brown AD, Thomas KB, Klein DC. Melatonin biosynthesis in chicken retina. Regulation of tryptophan hydroxylase and arylalkylamine N-acetyltransferase. Adv. Exp. Med. Biol 1999;460:31–41. [PubMed: 10810497]

Iuvone PM, Tosini G, Pozdeyev N, Haque R, Klein DC, Chaurasia SS. Circadian clocks, clock networks, arylalkylamine N-acetyltransferase, and melatonin in the retina. Prog Ret Eye Res 2005;24:433–456.

Ivanova TN, Iuvone PM. Melatonin synthesis in retina: circadian regulation of arylalkylamine *N*-aceyltransferase activity in cultured photoreceptor cells of embryonic chick retina. Brain Res 2003a; 973:56–63. [PubMed: 12729953]

Ivanova TN, Iuvone PM. Circadian rhythm and photic control of cAMP level in chick retinal cell cultures: a mechanism for coupling of circadian oscillator to the melatonin-synthesizing enzyme, arylalkylamine *N*-aceyltransferase, in photoreceptor cells. Brain Res 2003b;991:96–103. [PubMed: 14575881]

Ko GY, Ko ML, Dryer SE. Circadian regulation of cGMP-gated channels of vertebrate cone photoreceptors: role of cAMP and Ras. J. Neurosci 2004;24:1296–304. [PubMed: 14960600]

Krizaj D. Compartmentalization of calcium entry pathways in mouse rods. Eur. J. Neurosci 2005;22:3292–3296. [PubMed: 16367794]

Liu X, Green CB. Circadian regulation of nocturnin transcription by phosphorylated CREB in Xenopus retinal photoreceptor cells. Mol. Cell. Biol 2002;22:7501–7511. [PubMed: 12370297]

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem 1951;193:265–275. [PubMed: 14907713]

Nachman-Clewner M, St Jules R, Townes-Anderson E. L-type calcium channels in the photoreceptor ribbon synapse: localization and role in plasticity. J. Comp. Neurol 1999;415:1–16. [PubMed: 10540354]

Pierce ME, Sheshberadaran H, Zhang Z, Fox LE, Applebury ML, Takahashi JS. Circadian regulation of iodopsin gene expression in embryonic photoreceptors in retinal cell culture. Neuron 1993;10:579–584. [PubMed: 8476610]

Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature 2002;418:935–941. [PubMed: 12198538]

Salomon Y, Londos C, Rodbell M. A highly sensitive adenylyl cyclase assay. Anal. Biochem 1974;58:541–548. [PubMed: 4827395]

Taylor WR, Morgans C. Localization and properties of voltage-gated calcium channels in cone photoreceptors of Tupaia belangeri. Vis. Neurosci 1998;15:541–52. [PubMed: 9685206]

Tosini G, Menaker M. Circadian rhythms in cultured mammalian retina. Science 1996;272:419–421. [PubMed: 8602533]

Tosini G, Fukuhara C. The mammalian retina as a clock. Cell Tissue Res 2002;309:119–126. [PubMed: 12111542]

Uchida, K.; Iuvone, PM. Intracellular Ca2+ concentrations in cultured chicken photoreceptor cells: sustained elevation in depolarized cells and the role of dihydropyridine-sensitive Ca2+ channels.; Mol. Vis. 1999. p. 1http://www.molvis.org/,molvis/v5/p1

Wang H, Storm DR. Calmodulin-regulated adenylyl cyclases: cross-talk and plasticity in the central nervous system. Mol. Pharmacol 2003;63:463–468. [PubMed: 12606751]

Wayman GA, Impey S, Wu Z, Kindsvogel W, Prichard L, Storm DR. Synergistic activation of the type I adenylyl cyclase by Ca2+ and Gs-coupled receptors in vivo. J. Biol. Chem 1994;269:25400–25405. [PubMed: 7929237]

Webster KA, Kedes L. The c-fos cyclic AMP-responsive element conveys constitutive expression to a tissue-specific promoter. Mol. Cell. Biol 1990;10:2402–2406. [PubMed: 2157971]

Xia Z, Choi EJ, Wang F, Blazynski C, Storm DR. Type I calmodulin-sensitive adenylyl cyclase is neural specific. J. Neurochem 1993;0:305–311. [PubMed: 8417150]

Yosdida K, Kawamura K, Imaki J. Differential expression of c-fos mRNA in rat retinal cells: regulation by light/dark cycle. Neuron 1993;10:1049–1054. [PubMed: 8318229]

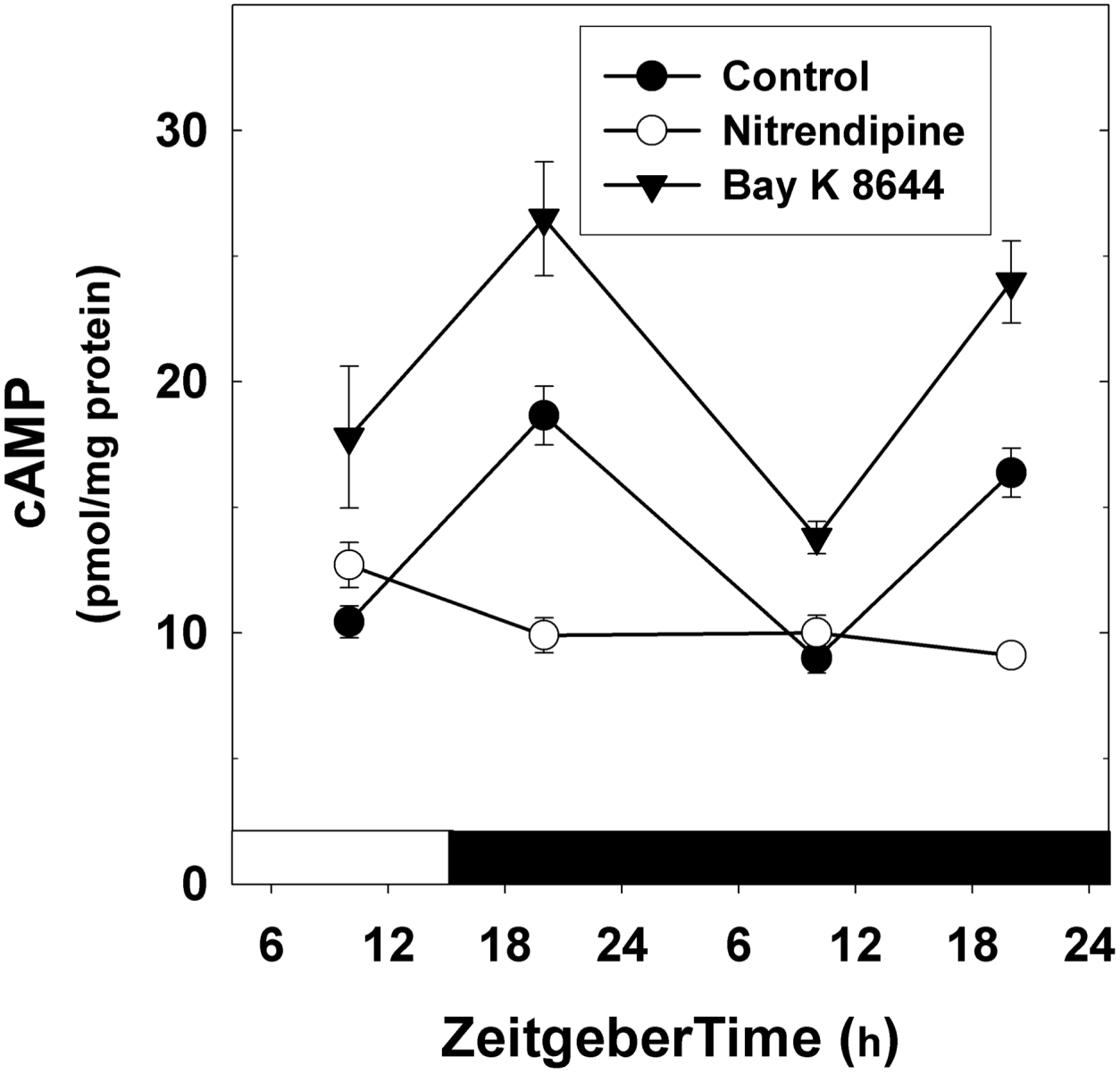

Figure 1. Ca^{2+} influx is required for cyclic AMP circadian rhythm generation

Cells were prepared from E6 retinas and incubated for 8 days under a 14 h light and 10 h dark (LD) cycle and 1 day in constant darkness (DD) as described in Materials and methods. On DIV7, culture medium was replaced with fresh media containing the L-type Ca^{2+} channel blocker, nitrendipine (3μM), the Ca^{2+} channel agonist, Bay K 8644 (3 μM), or vehicle. Cyclic AMP was determined in the cells harvested on DIV 8 in LD and DIV 9 in DD. Cells treated with vehicle exhibited a circadian rhythm of cyclic AMP level. Levels of the cyclic nucleotide were significantly lower during the day (ZT10) than at night (ZT 20) in LD ($P<0.001$; n=10−12) and in DD ($P<0.001$; n=12). Treatment of cells with nitrendipine totally suppressed the nocturnal increase in cyclic AMP concentration and abolished the circadian rhythm of cyclic AMP in LD and DD (n=5−6). In contrast, the circadian rhythm of cyclic AMP level persisted in the presence of Bay K 8644 ($p<0.05$; n=11−12), with higher levels of cyclic AMP compared to vehicle controls. The horizontal white and black bars above the x-axis represent times of light and darkness, respectively. Data are presented as mean ± SEM.

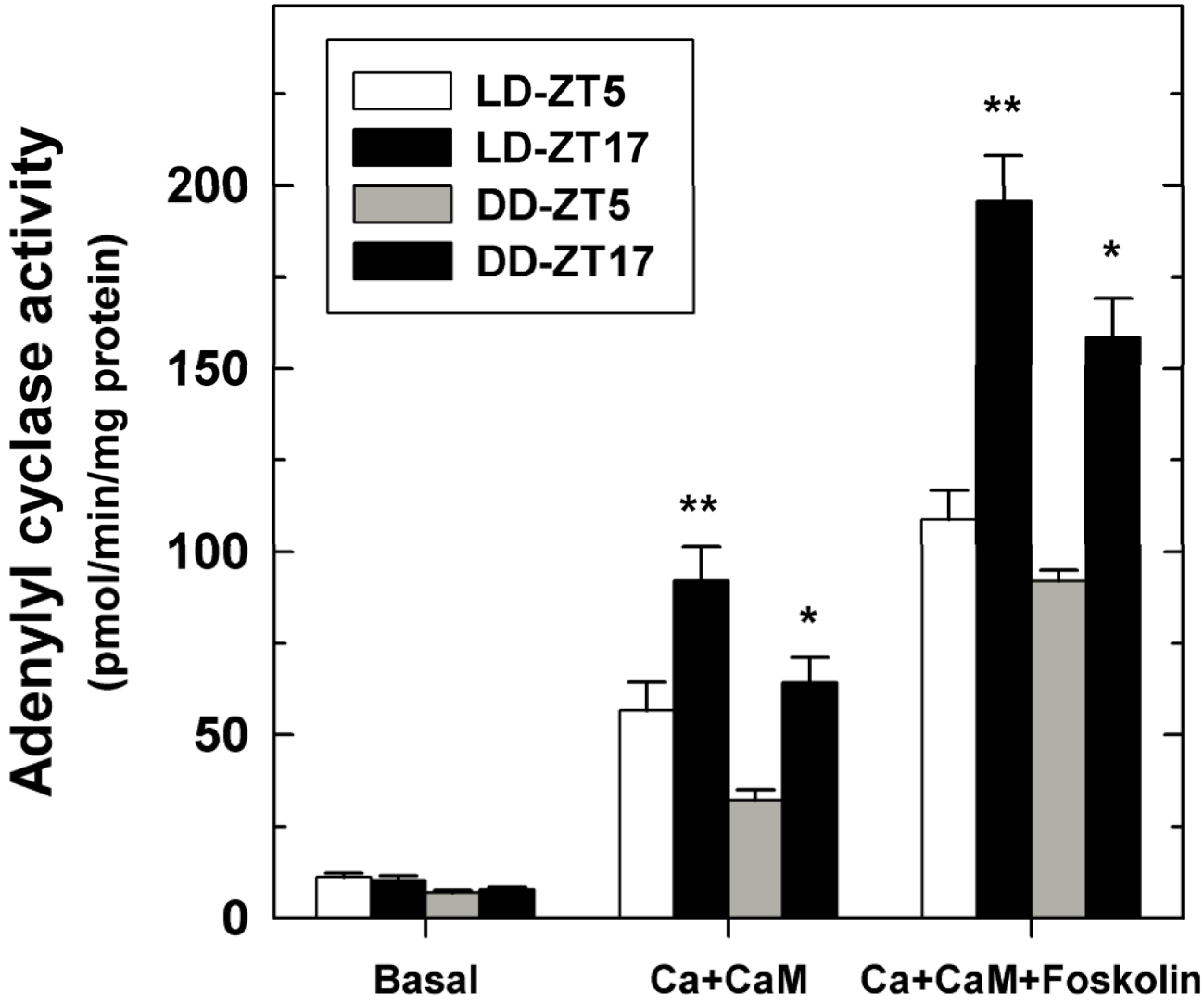

Figure 2. Adenylyl cyclase activity in photoreceptor cell cultures

Cells were prepared from E6 retinas and incubated as described in Materials and methods. Cells were harvested at ZT 5 and ZT 17 on DIV 8 in LD and DIV 9 in DD. Adenylyl cyclase activity was determined in crude membrane preparations by measuring the formation of radiolabelled cyclic AMP from [α^{32}P]ATP, as described in Materials and methods. Cells from 6 dishes were pooled together to prepare membranes for each sample, which was assayed for activity under three conditions: basal, Ca^{2+} (2μM) + CaM (120 nM), and Ca^{2+} + CaM + forskolin (10μM). Each time point is the mean ± SEM from 5−6 membrane preparations. No significant differences of basal activity were observed between ZT 5 and ZT 17 in either LD or DD. A circadian fluctuation in Ca^{2+}/CaM-stimulated adenylyl cyclase activity was observed in LD (**$p \leq 0.001$) and DD (*$p < 0.05$), with activity higher at night (ZT 17) than during the daytime (ZT 5). A similar circadian fluctuation was observed in activity measured in the presence of Ca^{2+}, CaM, and forskolin (LD, **, $p \leq 0.001$; DD, *,$p < 0.05$).

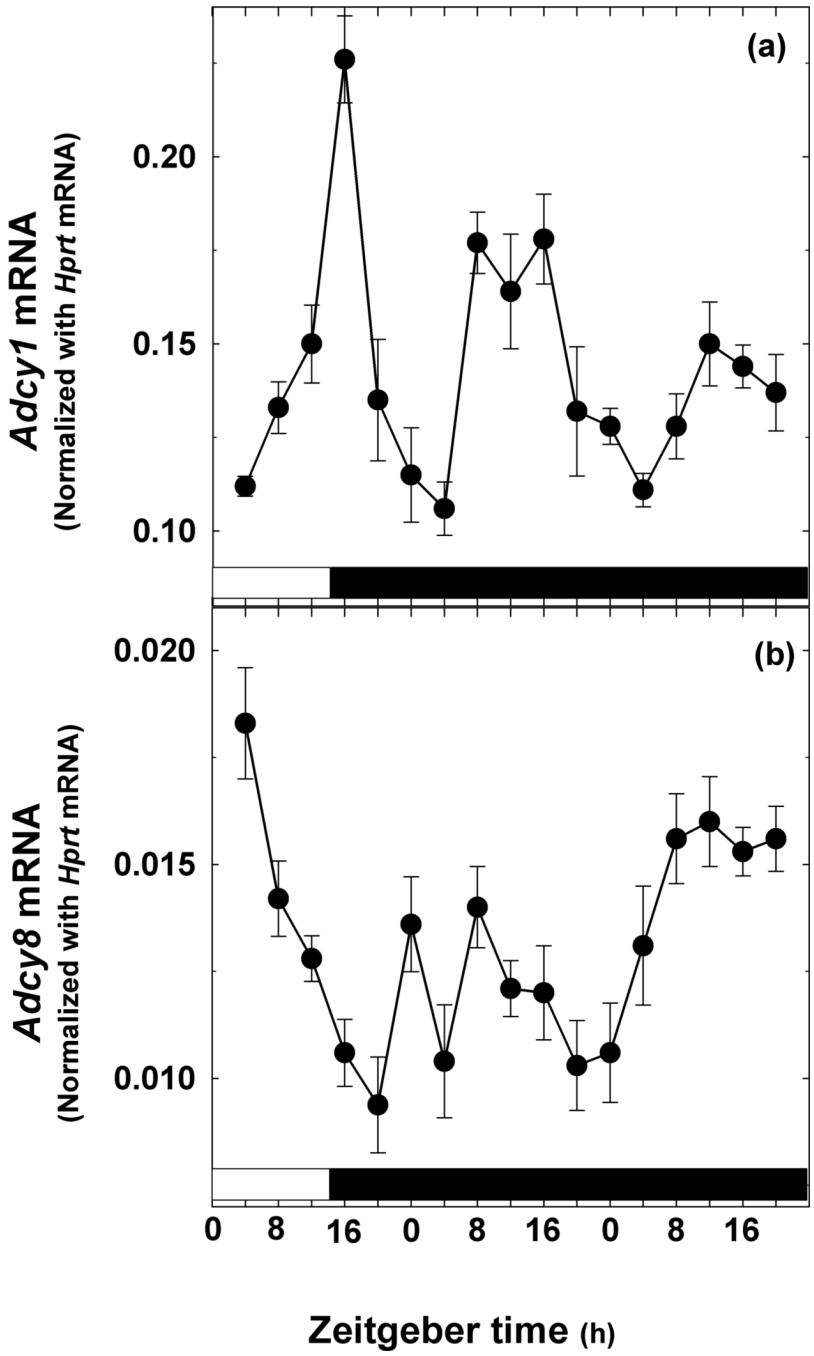

Figure 3. *Adcy1* and *Adcy8* mRNA rhythm in photoreceptor cell cultures

Cells were prepared from E6 retinas and incubated as described in Materials and methods. Cells were harvested at the times indicated in the figure in LD on DIV 8 and DD on DIV9 and 10. Relative mRNA levels for *Adcy1* and *Adcy8* were quantified by real-time RT-PCR. Transcript levels were normalized with *Hprt* mRNA. (a) *Adcy1* mRNA expression was rhythmic in LD ($p<0.001$, n = 5−6 dishes / time point) and in constant darkness ($p<0.001$ for DD1, n=6; $p=0.022$ for DD2, n=6) with highest levels during the late afternoon hours. (b) In LD, *Adcy8* transcript level displayed a daily rhythm with high values during the early hours of the day ($p<0.001$, n=6) relative to those at night. On day 1 of DD, there was a trend towards continued rhythmicity of *Adcy8* transcript levels, but it was not statistically significant

(p=0.112). On the second day of DD, *Adcy8* mRNA levels increased from ZT0 to ZT8 (p=0.018), and remained at that level for the remainder of the day.
The horizontal white and black bars above the x-axis represent times of light and darkness, respectively.

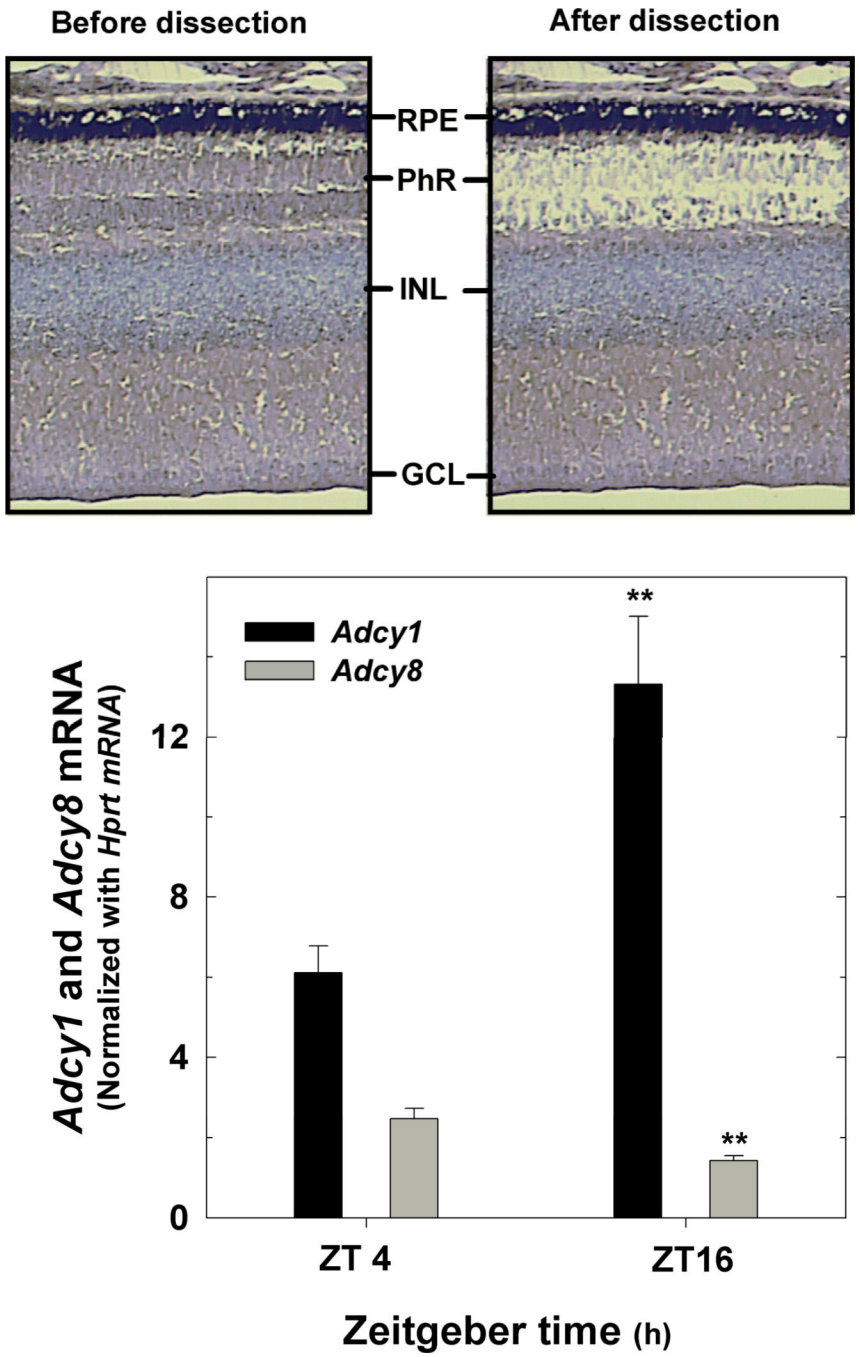

Figure 4. Expression of *Adcy1* mRNA in retinal photoreceptors dissected by laser capture microdissection (LCM)

One-day old chicks were housed in a 12-h light: 12-h dark (LD) cycle with lights on at ZT 0 for 2 weeks. Eyes were collected in LD at the times indicated in the figure and prepared for LCM as described in Materials and methods. (a) Photomicrograph of representative retinal section prior to dissection. (b) Photomicrograph of a retinal after removal of the photoreceptor layer by LCM. (c) Relative mRNA levels in the photoreceptor layers were quantified by real-time RT-PCR. *Adcy1* and *Adcy8* transcript levels were normalized with *Hprt* mRNA. *Adcy1* mRNA expression was significantly higher at ZT 16 than at ZT4 (**$p \leq 0.005$, n=7) whereas

Adcy8 transcript showed higher levels at ZT4 (**p≤0.005, n=7). RPE, retinal pigment epithelium; PhR, photoreceptors; INL, inner nuclear layer; GCL, ganlion cell layer.

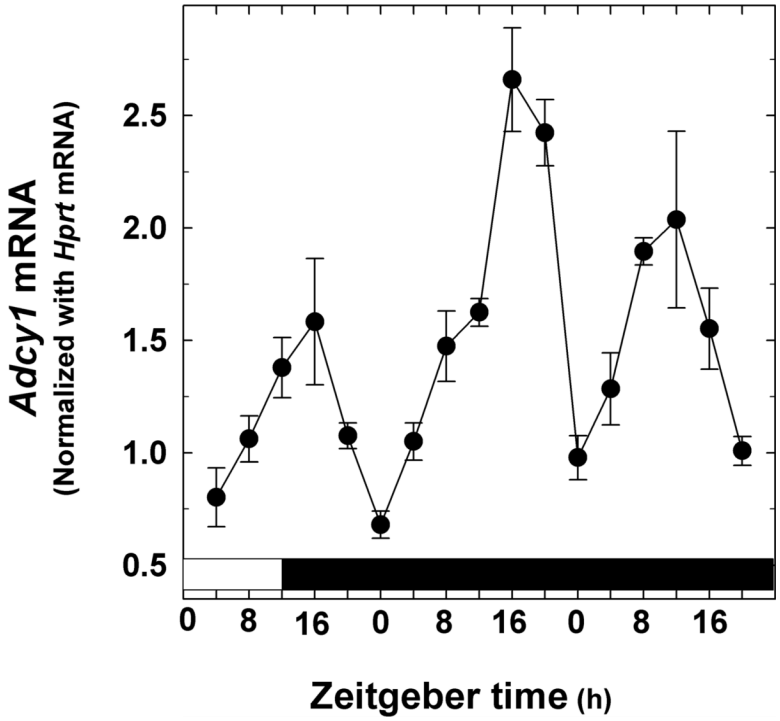

Figure 5. *Adcy1* transcript in neural retina is regulated by a circadian clock *in vivo*

One-day old chicks were housed in a 12-h light: 12-h dark (LD) cycle with lights on at ZT 0 for 2 weeks and later kept under constant darkness (DD) for 2 days. Neural retina was collected in LD, DD1 and DD2 at the times indicated in the figure. Relative mRNA levels were quantified by real-time RT-PCR. Each data point represents *Adcy1* transcript from the six individual chickens. *Adcy1* transcript levels are normalized with *Hprt* mRNA. *Adcy1* mRNA expression was rhythmic in LD ($p=0.008$, $n=6$) and in constant darkness ($p<0.001$ for DD1, $n=6$; $p=0.001$ for DD2, $n=6$) with lowest levels in the early morning. The horizontal white and black bars above the x-axis represent times of light and darkness, respectively.

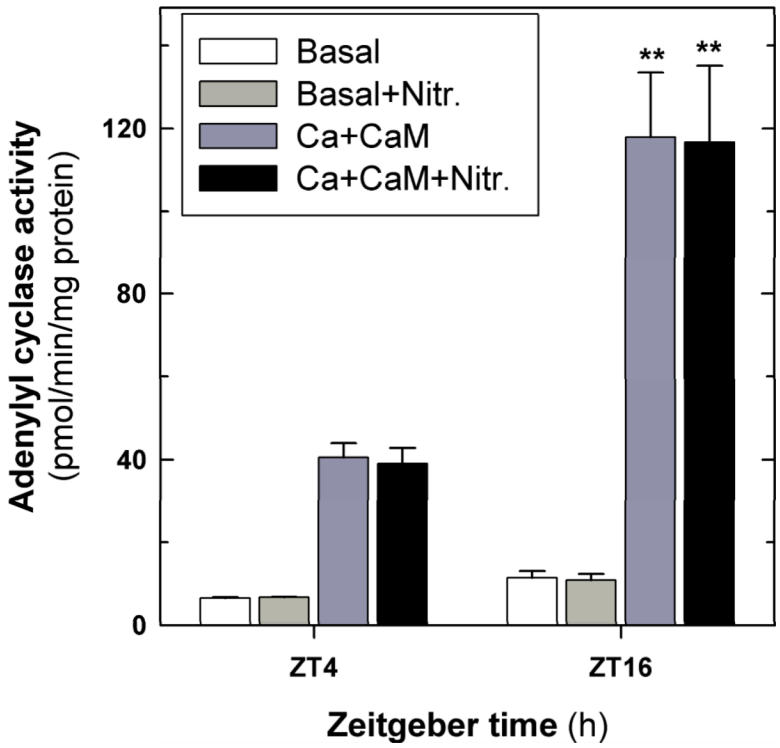

Figure 6. Circadian Regulation of Ca^{2+}/CaM-stimulated adenylyl cyclase activity in neural retina

One-day old chicks were housed in a 12-h light: 12-h dark (LD) cycle with lights on at ZT 0 for 2 weeks and then transferred to DD. Neural retina was collected on DD1 at the times indicated in the figure. Adenylyl cyclase activity was determined in crude membrane preparations as described in Materials and methods. Retinal membranes were prepared and assayed for activity under four conditions: basal, basal + nitrendipine (3 μM), Ca^{2+} (2μM) + CaM (120 nM), and Ca^{2+} + CaM + nitrendipine. Each time point is the mean ± SEM from 5 membrane preparations. No significant differences of basal or basal + nitrendipine activity were observed between ZT 4 and ZT 16 in DD. A circadian fluctuation in Ca^{2+}/CaM-stimulated adenylyl cyclase activity was observed in DD (**, $p<0.001$), with activity higher at night (ZT 16) than during the daytime (ZT 4). Nitrendipine added to the retinal membranes in vitro did not affect the circadian fluctuation observed in activity measured in the presence of Ca^{2+} and CaM (**, $p<0.001$ at ZT16 compared to ZT4).

Table 1

PCR primers

Primers were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA) using the GenBank accession numbers listed. Primers were designed to generate PCR products that span exon-intron boundaries. Each primer pair yielded a single product of the correct size as determined by agarose gel electrophoresis and melt curve analyses. Amplicons from the "Standard" primers were gel purified and served as standard cDNAs for the "Real-time" PCR assays.

Name	Sequence (5‘−3’)	GenBank Accession Number	Size (bp)
Standard:			
Adcy1	F: AGGTGACTGTTACTATTGTGTATCAGGC	XM_418882	328
	R: GCCATATCCTGGTTCTACTTCATAGTC		
Adcy8	F: GGAGAAACAGACTTCCTGGGTACAA	XM_418437	292
	R: AAGCAAACATCACTCCAACCGCATC		
Hprt	F: CCGCTCCATGGCGACTCACA	AJ132697	590
	R: AGTCGAGGGCGTATCCAACAACAA		
Real-time:			
Adcy1	F: AGGTGACTGTTACTATTGTGTATCAGGC	XM_418882	179
	R: CTAGGACACCACAAAGCACCCTTC		
Adcy8	F: GGAGAAACAGACTTCCTGGGTACAA	XM_418437	175
	R: CATTGTGCTCCCTTAACTCCTTCATTTC		
Hprt	F: CATGGACAGGACAGAGAGACTG	AJ132697	186
	R: CTCTTCAACCTAATGAAGTCCACAG		