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Dopamine D₄ receptor activation controls circadian timing of the adenylyl cyclase 1/cyclic AMP signaling system in mouse retina

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Abstract

In the mammalian retina, dopamine binding to the dopamine D₄ receptor affects a light-sensitive pool of cyclic AMP by negatively coupling to the type 1 adenylyl cyclase. The type 1 adenylyl cyclase is the primary enzyme controlling cyclic AMP production in dark-adapted photoreceptors. A previous study demonstrated that expression of the gene encoding the type 1 adenylyl cyclase, *Adcy1*, is down regulated in mice lacking *Drd4*, the gene encoding the dopamine D₄ receptor. The present investigation provides evidence that dopamine D₄ receptor activation entrains the circadian rhythm of *Adcy1* mRNA expression. Diurnal and circadian rhythms of *Drd4* and *Adcy1* mRNA levels were observed in wild type mouse retina. Also, rhythms in the Ca²⁺-stimulated adenylyl cyclase activity and cyclic AMP levels were observed. However, these rhythmic activities were damped or undetectable in mice lacking the dopamine D₄ receptor. Pharmacologically activating the dopamine D₄ receptor 4 hrs before its normal stimulation at light onset in the morning advances the phase of the *Adcy1* mRNA expression pattern. These data demonstrate that stimulating the dopamine D₄ receptor is essential in maintaining the normal rhythmic production of the type 1 adenylyl cyclase from transcript to enzyme activity. Thus, dopamine/dopamine D₄ receptor signaling is a novel zeitgeber that entrains the rhythm of *Adcy1* expression and, consequently, modulates the rhythmic synthesis of cyclic AMP in mouse retina.

Keywords

calmodulin; neuromodulator; entrainment; light; gene expression

Introduction

Retinal dopamine is synthesized and released from a subset of amacrine and interplexiform cells. Dopamine synthesis and release are stimulated by light. Released dopamine diffuses throughout the retina to activate D₁-like and D₂-like receptors, each contributing to unique biological processes (reviewed by Witkovsky, 2004). The dopaminergic signaling system is key in retinal processing controlling melatonin production in photoreceptor cells (Tosini *et al.*, 2008), Na/K ATPase activity (Shulman & Fox, 1996), rod-cone coupling (Ribelayga *et al.*, 2008), horizontal cell and AII amacrine cell coupling (Baldridge *et al.*, 1998), and light adaptation (Nir *et al.*, 2002).

Several lines of evidence suggest a functional relationship between dopamine and cyclic AMP in the regulation of circadian physiology in the retina. Dopamine D₄ receptor (D₄R)

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activation suppresses a light-sensitive pool of cyclic AMP in retina (Cohen & Blazynski, 1990; Cohen *et al.*, 1992) by negatively coupling to the type 1 adenylyl cyclase (AC1), the primary cyclase controlling cyclic AMP production in the dark-adapted mouse retina (Jackson *et al.* 2009). In chickens and rats, cyclic AMP production in retina fluctuates in a circadian pattern (Ivanova & Iuvone, 2003; Fukuhara *et al.*, 2004). Cyclic AMP can reset or entrain circadian rhythms in the rat suprachiasmatic nucleus (Prosser & Gillette, 1989) and Aplysia eye (Eskin, 1982). In mouse retina, dopaminergic amacrine cells co-express the six core circadian clock genes (Gustincich *et al.*, 2004; Ruan *et al.*, 2006; Dorenbos *et al.*, 2007), suggesting this cell contains a functional oscillator. Activation of dopamine D₂-like receptors, probably D₄Rs, on *Xenopus* photoreceptors phase shifts the circadian rhythm of melatonin release (Cahill and Besharse, 1991a). In addition, D₁ receptor agonists affect the phase of circadian Period 2-driven luciferase (Per2::Luc) rhythms in the inner retina of the mouse (Ruan *et al.*, 2008), and targeted deletion of the D₂ receptor gene in mouse decreases light-evoked induction of *Per1* throughout the retina and dampens the daily rhythm of *Per1* mRNA levels (Yujnovsky *et al.*, 2006). Thus, dopamine appears to function as a coordinator of circadian retinal physiology.

Dopamine release is stimulated by light in retinas of most vertebrate species (Witkovsky, 2004). In some species, dopamine release is also regulated in a circadian fashion with highest levels during the subjective daytime; however, circadian regulation of dopamine is strain-dependent in mice and related to melatonin synthesis (Nir *et al.*, 2000; Doyle *et al.*, 2002b; Pozdeyev *et al.*, 2008). Mouse strains that make melatonin, such as the C3H/f+/, show circadian rhythms of dopamine metabolism that persist in constant darkness, while those that have mutations in the genes that encode enzymes in the melatonin biosynthetic pathway, such as the C57Bl/6, do not (Doyle *et al.*, 2002b; Pozdeyev *et al.*, 2008). Daily injections of melatonin can induce circadian rhythms of dopamine metabolism in C57Bl/6 mice (Doyle *et al.*, 2002b). Diurnal and circadian rhythms of *Drd4* mRNA levels have been observed in mouse and rat retina (Storch *et al.*, 2007; Bai *et al.*, 2008; Klitten *et al.*, 2008). Circadian rhythms of *Adcy1* mRNA, the transcript that encodes AC1, have been observed in retinas of several vertebrate species (Fukuhara *et al.*, 2004; Chaurasia *et al.*, 2006; Storch *et al.*, 2007). Thus, the present study was conducted to investigate a functional role for dopamine and the D₄R in the circadian regulation of AC1 mRNA expression and enzyme activity in mouse retina, which ultimately controls circadian rhythms of cyclic AMP synthesis.

Materials and Methods

Animals

All animal experimental procedures conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Emory University's Institutional Animal Care and Use Committee. Mice lacking dopamine D₄ receptors (*Drd4*^{-/-}, Rubinstein *et al.*, 1997) and wild-type mice (WT), all on a C57Bl/6 background, were used for this investigation. The *Drd4*^{-/-} mice were produced by targeted deletion of exon 2. The splicing of exons 1 and 3 caused a frame shift resulting in a premature stop codon. These mice develop normally, have normal retinal morphology and dark-adapted electroretinograms, but have reduced photopic electroretinogram amplitudes (Nir *et al.*, 2002). Mice were approximately 3 months of age. Mice were genotyped by polymerase chain reaction analysis of genomic DNA. Mice were provided food and water *ad libitum* and were housed on a 12-hour light/dark (LD) cycle with lights on at zeitgeber time (ZT) 0 and lights off at ZT12, unless a lighting schedule change was necessary for specific experimental protocols. Retinal samples were collected at designated ZT or circadian time (CT) points determined by the experimental protocol, immediately frozen on dry ice, and stored at -80°C. Circadian rhythms were investigated in mice entrained to the

LD cycle and subsequently kept in constant (24 h/day) darkness (DD - first day of constant darkness; DD2 - second day of constant darkness) for sampling. CT rather than ZT was used to designate subjective time of day in constant darkness. All manipulations of mice during “dark” conditions were performed under dim red light.

RNA isolation, quantification, and quantitative real-time PCR

Total RNA was extracted from whole retina using the RNeasy kit and protocol (Qiagen Inc., Valencia, CA, USA), and was quantified by fluorescence using the Quant-iT RNA Assay Kit (Invitrogen/Molecular Probes, Eugene, Oregon, USA). Reverse transcription was performed on total RNA (at least 250 ng) preparations using QuantiTect Reverse Transcription Kit (Qiagen Inc. Valencia, CA, USA) containing oligo-dTs and random primers, thus, enabling measurement of ribosomal RNA as well as mRNA. RT-PCR reactions were performed in 25 μ L total volume with 2 μ L cDNA, 1X QuantiFast Syber Green PCR Kit (Qiagen Inc., Valencia, CA, USA) and 300 nM intron-spanning gene specific forward and reverse primers in a Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA). The quantification of transcript level was performed by comparing the threshold cycle for amplification of the unknown to those of six concentrations of standard cDNAs for each respective transcript. Each sample was assayed in duplicate. Two RNAs were used for normalization, either hypoxanthine guanine phosphoribosyltransferase (*Hprt*) mRNA or 18S ribosomal RNA, with comparable results. Results are expressed relative to the lowest mean value in each experiment.

Laser capture microdissection (LCM)

Whole mouse eyes, frozen and embedded in OCT (Tissue-Tek, Torrance, CA, USA), were cut at 10 μ m thickness and mounted on polyethylene naphthalate membrane glass slides (Applied Biosystems, Foster City, CA, USA). In a sterile environment, frozen sections were thawed for 30 s and fixed in 75% ethanol for 30 s followed by a wash in RNase-free water for 30 s. The sections were stained with HistoGene (Applied Biosystems, Foster City, CA, USA) staining solutions for approximately 15 s followed by a wash with RNase-free water for 30 s. The sections were dehydrated in graded ethanol solutions (75%, 30 s; 95%, 30 s; 100%, 30 s), cleared in xylene (>5 min), and air-dried for 5 min.

LCM was performed to capture the outer nuclear layer and inner segments of photoreceptor cells onto HS-CapSure non-contact LCM films using ArcturusXT system (Applied Biosystems, Foster City, CA, USA). A low-energy ultraviolet laser was used to cut around the border of the desired tissue area, followed by low-energy infrared laser pulses were used to attach the tissue to LCM film. The outer nuclear layer and inner segments were lifted away from the tissue section, leaving all unwanted cells behind and providing a pure population for cell-specific molecular analysis. When dissecting the photoreceptor cell layer (inner segments and outer nuclear layer), the row of nuclei abutting the outer plexiform layer were untouched to avoid cross contamination with the inner retina. Additionally, outer segments were not captured, because mRNA levels are highest in the photoreceptor soma and inner segments, and to avoid contamination from the adjacent retinal pigment epithelium. The PicoPure RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) was used to purify total RNA from captured tissue. Total RNA was reverse transcribed using random primers and subjected to real-time PCR analysis as described above.

In vivo agonist and antagonist experiments

Mice, maintained in LD, were injected subcutaneously for 6 six days with a selective D₄R antagonist, L-745,870 (Patel *et al.*, 1997) at 1 mg/kg, 30 min before light onset (ZT 23.5). The drug and control were prepared in 0.3% carboxymethylcellulose (Sigma-Aldrich, St. Louis, MO, USA). Retinas were dissected during the subjective day and subjective night of

DD1 following the 6th day of injections at CT 5, CT 10, CT 15, and CT 20. Since the rhythmic expression of *Adcy1* has been extensively studied, we chose this truncated time scale to determine the effect of D₄R antagonism on the peak of *Adcy1* retinal expression. RNA was isolated and assayed for *Adcy1* expression.

WT mice were injected intraperitoneally with PD 168,077 (1 mg/kg, Tocris, Ellisville, MO, USA), a selective D₄R agonist, or vehicle 4 hrs before the onset of light (ZT 20) for 2 days during a 12 hour light/dark cycle. The injections were performed under dim red light. On the third day mice were kept in constant darkness to eliminate any light response. They were injected with PD 168,077 or vehicle 4 hrs before subjective light onset, and retinas were removed at ZT 21, CT 0, CT 4, and CT 8. Retinal RNA was isolated and assayed for *Adcy1* mRNA. An additional group of animals, on a 12 hr LD cycle, were treated with a single injection of PD 168,077 administered at ZT 20, to investigate an acute effect of the drug on *Adcy1* mRNA expression. PD 168,077 was dissolved in dimethyl sulfoxide, and then diluted with sterile phosphate buffered saline to a final dimethyl sulfoxide concentration of approximately 1%.

Retinal cyclic AMP accumulation

WT and *Drd4*^{-/-} mice were kept in DD and dissected at various times of subjective day or subjective night (CT 2, 8, 14, 20). Retinas were placed in cold, oxygenated Earle's salt solution (115 mM NaCl, 3 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 0.9 mM NaH₂PO₄, 26 mM NaHCO₃, 5.5 mM glucose) at pH 7.4, equilibrated with 95% O₂/5% CO₂. Individual retinas were transferred to cell culture plates containing Earle's salt solution supplemented with 1 mM 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO, USA). The retinas were incubated in darkness at 37°C in an atmosphere of 95% O₂/5% CO₂ for 15 min in a water bath. Retinas were immediately frozen on dry ice and stored at -80°C until cyclic AMP levels were measured by radioimmunoassay (RIA, Perkin Elmer, Waltham, MA, USA).

Adenylyl cyclase assay

Retinas were dissected from WT and *Drd4*^{-/-} mice during the day and night (ZT4 and ZT16, respectively), and were immediately frozen and stored at -80°C. Basal and Ca²⁺/calmodulin-stimulated adenylyl cyclase activities were measured by assessing the conversion of [α -³²P] ATP to [³²P]cyclic AMP by partially purified retinal membranes, as described previously (Jackson *et al.*, 2009).

Statistical analysis

Analysis of multiple group data was performed using one-, two-, and three-way analysis of variance (ANOVA, SigmaStat) with Student-Newman-Keuls (SNK) multiple comparison test where applicable. Comparisons of two groups were made with two-tailed Student's t-test. Error bars represent standard error of the mean (SEM).

Results

Daily rhythms of *Drd4* transcript levels in mouse retina

Diurnal (LD) and circadian rhythms (DD2) of *Drd4* mRNA were evaluated by measuring the transcript level at 4 hr intervals over a 24 hr period. *Drd4* mRNA levels fluctuated as daily rhythms in mice exposed to LD or DD2 (Fig. 1). In LD, transcript levels were high at the time of light onset, decreased dramatically by ZT4, and remained depressed until the lights went out (Fig. 1A; 2-way ANOVA, factor time $F_{6,81} = 24.486$, $P < 0.001$). In the dark phase of the LD cycle, *Drd4* mRNA levels increased by ZT16 and remained elevated through the remainder of the night. During DD2, relative transcript levels were higher overall compared to those in LD (Fig. 1; 2-way ANOVA, factor – lighting condition LD vs.

DD2, $F_{1,81} = 157.86$, $P < 0.001$). A notably dampened but significant rhythm persisted in the second day of constant darkness with a trough at CT 8 and a peak at CT 20 (Fig. 1B; SNK LD vs. DD2, $q_2 = 17.769$, $P < 0.001$).

Dopamine D4 receptors and circadian cyclic AMP synthesis in retina

Cyclic AMP accumulation in mouse retina, measured in DD, occurred as a circadian rhythm with levels being lower during the subjective day and rising significantly during the early subjective night (Fig. 2; 2-way ANOVA, factor – time, $F_{3,38} = 9.163$, $P < 0.001$), peaking at ~ CT 14. Interestingly, cyclic AMP accumulation in retinas of *Drd4*^{-/-} mice was significantly lower at all times of subjective day and subjective night and displayed no detectable rhythm (Fig. 2; factor – genotype $F_{1,38} = 108.64$, $P < 0.001$, genotype $\times$ time $F_{3,38} = 11.657$, $P < 0.001$). These data suggest that D₄R expression is necessary to maintain normal cyclic AMP levels as well as the circadian rhythm of its synthesis.

Knowing that the circadian rhythm of cyclic AMP is influenced by D₄R, we examined daily expression pattern of *Adcy1* in wild type and *Drd4*^{-/-} mice. On DD2, *Adcy1* transcript levels in WT control retina displayed a significant rhythm with highest levels in the middle of the subjective day (Fig. 3; 2-way ANOVA, factor – genotype $F_{1,40} = 30.77$, $P < 0.001$; factor – time $F_{3,40} = 5.225$, $P = 0.004$; genotype $\times$ time, $F_{3,40} = 4.979$, $P = 0.005$). In contrast, *Adcy1* expression in *Drd4*^{-/-} retinas showed no statistically significant rhythm and transcript levels in the knock out were significantly lower at CT 8 (SNK, $q_2 = 8.101$, $P < 0.001$) and CT 20 ($q_2 = 4.563$, $P = 0.003$) compared to that in WT retinas (Fig. 3). To determine if these changes were occurring in photoreceptor cells, the photoreceptor layer was isolated from retinal frozen sections of WT and *Drd4*^{-/-} mice at mid-day and mid-night in LD and subjected to qRT-PCR. As shown in Figure 4, *Adcy1* transcript level was significantly higher at ZT 6 than at ZT 18 in photoreceptors of WT mice (2-way ANOVA, genotype $F_{1,17} = 6.169$, $P = 0.024$; time $F_{1,17} = 3.95$, $P = 0.063$; time $\times$ genotype, $F_{1,17} = 0.026$; SNK WT ZT 6 vs. ZT18, $q_2 = 4.517$, $P = 0.005$). In contrast, no day-night difference of *Adcy1* mRNA was observed in photoreceptors of *Drd4*^{-/-} mice ($q_2 = 0.434$, $P = 0.763$).

To further test the role of D₄R in rhythmic *Adcy1* expression, WT mice were treated for 6 days with the D₄R antagonist, L745,870 (1 mg/kg, i.p.), administered at ~ZT23.5, approximately 30 min prior to the time of light onset (ZT 0) of the daily light-dark cycle. The rationale for administering the drug at this time of day was to block the D₄R before the light-evoked increase of dopamine release upon light onset. When assessed in constant darkness (DD1) following the sixth day of drug treatment, a significant rhythm in *Adcy1* mRNA levels was observed in the vehicle-treated mice but not in those treated with L745,870 (Fig. 5; 2-way ANOVA, factor – time $F_{3,34} = 10.557$, $P < 0.001$; factor – treatment [vehicle vs. L745,870] $F_{1,34} = 41.195$, $P < 0.001$; genotype $\times$ treatment $F_{3,34} = 22.482$, $P < 0.001$).

Adenylyl cyclase activity was assayed in retinal membranes to determine if there is a rhythm in Ca²⁺/CaM-dependent enzyme activity in WT mouse retina and, if so, if the rhythm is disrupted in retinas of *Drd4*^{-/-} mice (Fig. 6). In WT samples, Ca²⁺/CaM increased adenylyl cyclase activity at both ZT 4 and ZT 16, but the increase at ZT 16 was significantly larger than that at ZT 4; no significant difference in basal activity was observed between the two time points (3-way ANOVA, time $F_{1,40} = 9.317$, $P = 0.004$; assay condition [basal vs. Ca²⁺/CaM] $F_{1,40} = 217.70$, $P < 0.001$; genotype $F_{1,40} = 326.39$, $P < 0.001$; time $\times$ genotype $F_{1,40} = 17.239$, $P < 0.001$; assay condition $\times$ genotype, $F_{1,40} = 126.34$, $P < 0.001$; time $\times$ assay condition $\times$ genotype, $F_{1,40} = 10.501$, $P = 0.002$). In retinal membranes from *Drd4*^{-/-} mice, basal activity was lower than that in WT samples at both times of day, indicating an overall reduction of adenylyl cyclase. Ca²⁺/CaM-stimulated activity was nearly abolished in

Drd4^{-/-} samples compared to WT controls. Moreover, there was no difference in basal or Ca²⁺/CaM-stimulated activity between ZT 4 and ZT 16 in the *Drd4*^{-/-} samples.

Collectively these data indicate that the D₄R and its activation plays a critical role in regulating the rhythmic expression of AC1 at the transcript and enzyme activity levels in the retina, thereby maintaining the circadian rhythm of cyclic AMP synthesis.

Dopamine D₄ receptor activation mediates rhythmic behavior of *Adcy1* expression

Based on the results showing retinal *Adcy1* mRNA expression is rhythmic and controlled by the D₄R, we next hypothesized that activation of the D₄R at an atypical time of day could change its normal expression pattern, thereby causing a phase shift in its rhythm. To test this hypothesis we injected mice with PD 168,077, a selective D₄R agonist, 4 hrs before the normal surge in dopamine release at light onset (ZT 0). WT mice were injected with drug or vehicle for 2 days under a normal light/dark cycle and on a 3rd day in constant darkness. PD 168,077 treatment caused *Adcy1* mRNA levels to increase earlier in the day than in the vehicle treated controls (Fig. 7; 2-way ANOVA: treatment [vehicle vs. PD 168,077] $F_{1,30} = 4.556$, $P=0.04$; time $F_{3,30} = 3.810$, $P=0.02$; treatment $\times$ time $F_{3,30} = 3.296$, $P=0.03$). Mice injected with PD 168,077 (1mg/kg) showed a steady increase in *Adcy1* levels at CT 0 and CT 4 – when compared to the ZT 21 time point. Conversely, the vehicle-treated animals did not show a significant increase in expression until the CT 8 time point, providing evidence that D₄R activation in the late night can phase shift the *Adcy1* rhythm. As a control for a possible direct stimulatory effect of D₄R activation on *Adcy1* expression, mice were injected with a single dose of PD 168,077 (1mg/kg) administered 4 h before subjective dawn, with *Adcy1* levels quantified 4 h later (CT 0). Under these conditions, PD 168,077 did not cause a significant increase in *Adcy1* gene expression (*Adcy1* mRNA/18S rRNA: Vehicle = $[18.9 \pm 1.3] \times 10^{-3}$; PD 168,077 = $[18.5 \pm 0.9] \times 10^{-3}$; $N = 6/\text{group}$), indicating that the advance of the *Adcy1* mRNA expression pattern observed in Figure 6 is not due to acute induction of *Adcy1* by the D₄R.

DISCUSSION

Our results indicate that the D₄R is critical for maintaining normal circadian expression of AC1 and cyclic AMP synthesis in mouse retina. Our major findings are as follows: AC1 mRNA and enzyme activity are rhythmically controlled by D₄R; the phase of *Adcy1* mRNA expression rhythm is shifted by activation of the D₄R at night in darkness, when dopamine levels are low. Overall, the data suggest that D₄R activation acts as an entrainment stimulus synchronizing activity rhythms of AC1, thereby modulating the circadian synthesis of cyclic AMP in the mouse retina.

The retina uses endogenous circadian oscillators to regulate retinal physiology under light-adapted conditions (Storch *et al.* 2007; Cameron *et al.*, 2008). This allows for fine-tuning of the retinal signals across a large range of light intensities as a function of time-of-day. The mechanisms involved are not well understood and there are many different processes contributing to light- and dark-adaptation, including electrical coupling between photoreceptors and retinal neurons (Vardi & Smith, 1996). Ribelayga *et al.* (2008) have shown that the retinal clock controls rod-cone coupling by a mechanism that involves dopamine. This study's focus was to define novel circadian signaling processes that may contribute to circadian regulation of retinal function through activation of the D₄R. Klitten *et al.* (2008) demonstrated that *Drd4* mRNA shows rhythmic expression in rat retina and pineal gland, thus setting up a possible site for circadian regulation. This modulation may control the circadian fine-tuning of retinal signaling, perhaps through connexin phosphorylation at rod-cone gap junctions or other cyclic AMP-dependent metabolic and synaptic actions (Urschel *et al.*, 2006; Michaelides *et al.*, 2010).

D₄R control of the rhythmic expression of *Adcy1*

The classical circadian feedback loop, consisting of the core clock genes *Bmal1*, *Clock*, *Period1/2*, and *Cryptochrome1/2*, has served as the defining standard for identifying cells or a population of cells with circadian oscillators (Ruan *et al.*, 2006). In the retinal circadian research field there have been contrasting views concerning where the circadian pacemaker(s) are located. Using single-cell RT-PCR, Ruan *et al.*, (2006) assayed mouse rod, horizontal, bipolar, amacrine and dopaminergic interplexiform cells for the presence of six core clock genes. All cell types, except rods, demonstrated expression of all six of the core clock genes. Also, this study demonstrates that *rd* mice, whose photoreceptor layer endogenously degenerates, maintains robust circadian rhythms of *Period2*-driven luciferase activity in the inner retina, further suggesting oscillators required for rhythmic *Period 2* expression in the inner nuclear layer are not contained within photoreceptor cells. However, these experiments do not address photoreceptor circadian phenomena, such as outer segment disc shedding (LaVail, 1976), rod-cone coupling (Ribelayga *et al.*, 2008), Na/K⁺ ATPase activity (Shulman & Fox, 1996), photoreceptor protein phosphorylation (Pozdeyev *et al.*, 2008), and melatonin synthesis (Tosini & Menaker, 1996) that are observed in mammalian retina. Moreover, another study showed expression of the six core clock genes in the microdissected photoreceptor layer of the rat retina and demonstrated that rhythmic *Period1*-driven luciferase and melatonin synthesis persists in isolated photoreceptor layers (Tosini *et al.*, 2007). The D₄R is the sole dopamine receptor subtype located on photoreceptor cells (Cohen *et al.*, 1992) and has been shown to be involved in many of the aforementioned physiological functions (Witkovsky, 2004). Additionally, Klitten *et al.* (2008) demonstrated by *in situ* hybridization that the *Drd4* transcript shows rhythmic expression only in photoreceptors of rat retina, although some hybridization was also observed in the inner retina. The common link between D₄R and rhythmic physiological functions suggests a possible site for circadian regulation in photoreceptor cells.

Storch *et al.* (2007) demonstrated that *Drd4* expression in mouse eye was rhythmic in LD even when the core clock mechanism was disrupted by elimination of *Bmal1*. This observation suggested that the rhythm of *Drd4* mRNA was light-driven and not regulated directly by circadian clocks. However, our results indicate that *Drd4* mRNA expression in mouse retina is circadian, being rhythmic in both LD and DD, although the amplitude of the rhythm was reduced in constant darkness compared to that in cyclic light. These findings indicate that rhythmic *Drd4* expression is regulated by both light and retinal circadian oscillators. Previous studies have shown that dopamine metabolism in retinas of wild type C57Bl/6 mice is light driven and not circadian (Doyle *et al.*, 2002b; Pozdeyev *et al.*, 2008). The larger amplitude of the *Drd4* transcript rhythm observed in LD compared to DD was due to lower relative expression levels in light phase of the LD cycle when dopamine is released, suggesting that endogenous dopamine may down regulate *Drd4* expression.

Our lab has recently reported that *Adcy1* expression is modulated by D₄R activation (Jackson *et al.*, 2009). Currently, we show that the rhythm in *Adcy1* is damped or undetectable in *Drd4*^{-/-} mice or in mice treated for six days with a D₄R antagonist. These findings provide strong evidence that the D₄R plays a critical role in regulating the circadian expression of *Adcy1*. Currently, the mechanism for how D₄R activation controls *Adcy1* mRNA expression is not known; however, there are studies that show D₄R activation not only affects cAMP levels, but also activates the MAPK signaling pathways (Oldenhof *et al.*, 1998; Oak *et al.*, 2001).

D₄R control of rhythmic adenylyl cyclase enzyme activity

The D₄R regulates cyclic AMP production in retina primarily by controlling the activity of the type 1 adenylyl cyclase at the protein level (Jackson *et al.*, 2009). Three adenylyl cyclase

isozymes are known to interact with Ca^{2+} and CaM: AC1, AC3, and AC8 (Patel *et al.*, 2001), with AC1 being primarily responsible for cyclic AMP accumulation in dark-adapted mouse photoreceptors (Jackson *et al.*, 2009). While it was previously reported that *Adcy1* mRNA is expressed in a circadian rhythm in mouse retina (Storch *et al.*, 2007), it was unknown if this resulted in daily changes in adenylyl cyclase activity. In the current study we demonstrated rhythmic activity of Ca^{2+} /CaM-stimulated adenylyl cyclase in retinal membranes from wild type mice with higher activity at night (Fig. 6). In contrast, Ca^{2+} /CaM-stimulated enzyme activity was greatly reduced and arrhythmic in retinal membranes of *Drd4*^{-/-} mice. These results suggest that there is less AC1 available for stimulation by Ca^{2+} /CaM activity and that the amount of AC1 protein does not fluctuate rhythmically in *Drd4*^{-/-} retina, which is consistent with the reduced levels and damped rhythms of *Adcy1* in *Drd4*^{-/-} retinas. However, we cannot exclude the possibility that post-translational modifications contribute to these differences. We conclude that the rhythmic changes seen in enzyme activity are directly related to the dopamine/D₄R signaling system in mammalian retina.

Dopamine/D₄R signaling system controls cyclic AMP in mouse retina

Cyclic AMP is a key second messenger that translates external cues into intracellular responses (Sands & Palmer, 2008). One way to regulate cyclic AMP is through the D₄R signaling pathway, which is negatively coupled to AC1 in retina (Jackson *et al.*, 2009). Therefore, disrupting this receptor's function should lead to abnormalities in cyclic AMP regulation, as seen by Nir *et al.* (2002) and Jackson *et al.* (2009). In chick and rat, retinal rhythms of cyclic AMP are observed (Ivanova & Iuvone, 2003; Fukuhara *et al.*, 2004); to date this had not been established in mouse retina. The present results definitively show that cyclic AMP accumulation is circadian in retinas of WT mice. However, cyclic AMP levels in *Drd4*^{-/-} retinas are constitutively low and display no circadian rhythm. This result is striking because other dopamine receptors beside the D₄R, most notably the D1 receptor, regulate cyclic AMP accumulation in retina (Witkovsky, 2004). In contrast to the D₄R, dopamine D1 receptors activate adenylyl cyclase. Thus, it may seem surprising that removal of the D₄R did not reveal a D1-receptor mediated increase of cyclic AMP accumulation. Deletion of the D₄R does not affect the expression of *Drd1* (Jackson *et al.*, 2009), the gene that encodes the D1 receptor. Light stimulates cyclic AMP accumulation in the *Drd4*^{-/-} retina except under conditions where synaptic transmission from photoreceptors to inner retinal neurons is blocked by exogenous glutamate (Nir *et al.*, 2002). This stimulatory effect of light is eliminated by blocking D1 receptors (Nir *et al.*, 2002). The constitutively low levels of cyclic AMP in the current study can be attributed to the reduced and constitutive expression of *Adcy1* in the *Drd4*^{-/-} retina and to the experiment having been conducted in constant darkness. These observations have several important implications. They support the generally held view that D1 receptors are expressed only in the inner retina. They suggest that D1 receptors may couple to adenylyl cyclase isoforms other than AC1, since *Adcy1* and Ca^{2+} /CaM-stimulated adenylyl cyclase are dramatically reduced in retinas of *Drd4*^{-/-} mice (Jackson *et al.*, 2009; present results). The observations also suggest that the circadian rhythm of retinal cyclic AMP accumulation may derive primarily from photoreceptors, although additional experimentation is needed to conclusively establish this point. Lastly, these data indicate that the circadian nature of cyclic AMP synthesis in mammalian retina is controlled primarily by the dopamine/D₄R signaling system.

Is the dopamine/D₄R signaling system an entrainment mechanism in retina?

The current study shows that *Adcy1* expression rhythms are damped or lost in the absence of D₄R signaling. Conceivably, *Adcy1* expression rhythms could be driven by dopamine. Alternatively, dopamine released in the morning at light onset may serve to entrain or resynchronize circadian clocks in mouse retina that control *Adcy1* rhythms. The experiments

in this study were performed with mice on a C57Bl/6 background, which show light-driven but not circadian control of dopamine metabolism (Doyle *et al.*, 2002a; Pozdeyev *et al.*, 2008). This suggests that dopamine does not drive the *Adcy1* rhythm, since the transcript rhythm persists in constant darkness but dopamine rhythms do not. This conclusion is further supported by our observation that acute administration of a D₄R agonist did not induce *Adcy1* mRNA expression. In contrast, injections of the D₄R agonist 4 hours prior to subjective dawn for three days caused *Adcy1* mRNA levels to increase earlier in the day compared to vehicle injected controls, supporting the hypothesis that dopamine entrains the rhythm of *Adcy1* expression. In *Xenopus* retina, quinpirole, a D₂/D₄ dopamine receptor agonist, can phase shift the circadian rhythm of melatonin release from photoreceptors (Cahill & Besharse, 1991a). Similarly, dopamine synchronizes multiple circadian oscillators regulating rhodopsin promoter activity in zebrafish photoreceptors (Yu *et al.*, 2007).

Another possible explanation for the apparent phase shift of the *Adcy1* transcript rhythm following three days of PD 168,077 administration is that the agonist may have changed the pattern of D₄R availability at the plasma membrane. If repeated treatment with the agonist resulted in up-regulation of the receptor late in the evening, there might be a direct stimulatory effect of D₄R activation that could have been missed following a single injection of PD 168,077. This mechanism, however, seems unlikely since agonist treatment usually down-regulates receptors (Duman and Nestler, 1995). In addition, the half-life of PD 168,077 in the rat central nervous system is relatively short (~1 hr; Moreland *et al.*, 2005) and changes in receptor up- or down-regulation usually occur in response to tonic receptor occupancy (Duman and Nestler, 1995).

In apparent contradiction to our results, Ruan *et al.* (2008) found that tonic exposure to a dopamine D1 receptor agonist, but not a D2/D4 agonist, altered the phase of Per2::Luc rhythms in isolated mouse retina. However, the Per2::Luc signal emanates primarily from the inner retina and persists in retinas lacking photoreceptors (Ruan *et al.*, 2006). In contrast, our data show that the D₄R affects the *Adcy1* rhythm in photoreceptors. Coupled with the observation of reduced amplitude of the *Per 1* mRNA rhythms throughout the retinas of *Drd2* null mice (Yujnovsky *et al.*, 2006), these observations illustrate a major role for dopamine, acting through multiple receptor subtypes, in the circadian organization of the mammalian retina.

In conclusion, the dopamine/D₄R signaling system plays an important role in retinal circadian regulation, particularly in photoreceptors. The rhythm in D₄R may control subordinate rhythms that are driven by its timely activation by dopamine, thereby, controlling the phase of rhythmic gene expression (*Adcy1*), adenylyl cyclase enzyme activity, and cyclic AMP production. Our results, in conjunction with studies performed on cold-blooded vertebrates, support the hypothesis that dopamine, acting through D₂-like (D₂ or D₄) receptors, serves as an entrainment stimulus to synchronize circadian clocks regulating retinal gene expression and function.

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Abbreviations

AC	Adenylyl Cyclase
<i>Adcy1</i>	Gene encoding the type 1 adenylyl cyclase
ANOVA	Analysis of Variance
CaM	Calmodulin
CT	Circadian Time
D₄R	Dopamine D ₄ Receptor
DD	Constant Darkness
<i>Drd4</i>	Gene encoding the dopamine D ₄ receptor
<i>Drd4</i>^{-/-}	Mice lacking the dopamine D ₄ receptor
<i>Hprt</i>	Gene encoding hypoxanthine guanine phosphoribosyltransferase
LD	Light/Dark
SEM	Standard Error of the Mean
SNK	Student-Newmann-Keuls test
WT	Wild-type
ZT	Zeitgeber Time

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Fig. 1.

Diurnal and circadian rhythms of *Drd4* mRNA expression in mouse retina. *Drd4* mRNA levels were determined by real-time RT-PCR as described in Materials and methods under light/dark (LD; panel A) and constant dark (DD2; panel B) conditions. *Drd4* transcript levels show significant rhythms (* $P \leq 0.01$ vs Time 0; ** $P < 0.001$ vs Time 0). In DD2 the *Drd4* mRNA levels were significantly higher than in LD ($P < 0.001$, LD vs DD2) and the rhythm had a lower amplitude. Data expressed as means $\pm$ SEM, $n = 5-7$ mice per group. White bars on the X-axis represent daytime in light; gray bars represent subjective daytime in constant darkness; black bars represent night or subjective night in darkness.

Fig. 2. Cyclic AMP accumulation is circadian in retina of WT mice ($P < 0.001$) but not in retina of *Drd4*^{-/-} mice. Cyclic AMP accumulation was measured in constant darkness (DD) as described in Materials and methods. *** $P < 0.001$ vs all other groups in WT and *Drd4*^{-/-} mice; WT vs *Drd4*^{-/-} at the same time-of-day, ** $P < 0.001$ at CT 2 and 8, * $P < 0.05$ at CT20. Data expressed as means $\pm$ SEM, $n = 4-6$ per group.

Fig. 3.

Comparison of rhythmic expression of *Adcy1* mRNA in *Drd4*^{-/-} retinas compared to WT controls. *Adcy1* mRNA levels in retinas dissected on DD2 were determined by real-time RT-PCR as described in Materials and methods. Closed circles represent WT samples exhibiting a daily rhythm ($p < 0.001$); open circles represent *Drd4*^{-/-} samples, which show no detectable rhythm. * $P = 0.003$, ** $P < 0.001$ vs *Drd4*^{-/-} at the same time of day. Data expressed as mean $\pm$ SEM, $n = 6$ per group.

Fig. 4.

The day-night rhythm of *Adcy1* transcript expression in photoreceptor cells is absent in *Drd4*^{-/-} mice. Eyes were dissected from WT and *Drd4*^{-/-} mice at mid-day and mid-night in LD. The photoreceptor layer was isolated by LCM as described in Materials and methods and subjected to qRT-PCR analysis. **P=0.005 vs. WT ZT18. Data are expressed as mean ± SEM, n=5–6 per condition.

Fig. 5. Effect of treatment with the D₄R antagonist, L-745,870, on the *Adcy1* transcript rhythm. Mice, maintained in LD, were injected at ZT 23.5 for six days with 1 mg/kg L-745,870 or vehicle as described in Materials and methods. After the last injection, retinas were dissected during the following subjective day or subjective night in DD1 at the times indicated in the figure. Mice injected with vehicle showed a statistically significant rhythm in *Adcy1* mRNA levels ($P < 0.001$), while those injected with L-745,870 did not. * $P < 0.01$, ** $P < 0.001$ vs Vehicle at the same time of day.

Fig. 6.

Adenylyl cyclase activity in *Drd4*^{-/-} mouse retina at ZT 4 and ZT 16 compared with WT controls. Basal and Ca²⁺/CaM-stimulated adenylyl cyclase activities were determined in retinal membranes as described in Materials and methods. ZT 4 groups: Ca²⁺/CaM-stimulated adenylyl cyclase activity was significantly increased in WT samples (*P < 0.001), however when comparing stimulated groups, *Drd4*^{-/-} samples failed to reach comparable WT levels of enzyme activity (**P < 0.001). Basal activity was significantly lower in *Drd4*^{-/-} samples compared to WT controls, but a significant increase is also observed between the basal and Ca²⁺/CaM-stimulated in the *Drd4*^{-/-} samples (***P < 0.05). ZT 16 group: Ca²⁺/CaM-stimulated activity in WT samples showed a significant increase compared to basal levels (*P < 0.001). Ca²⁺/CaM-stimulated activity in *Drd4*^{-/-} was significantly lower than those in WT controls (**P < 0.001). A decrease in basal activity between WT and *Drd4*^{-/-} is also observed (***P < 0.05). ZT4 and ZT16 comparison: Ca²⁺/CaM-stimulated adenylyl cyclase activity in membranes from WT mice is significantly higher at ZT 16 than at ZT 4 (#P < 0.001), demonstrating a rhythm in cyclase activity. Conversely, there is no detectable difference in activity between the two time points in the *Drd4*^{-/-} samples. All statistics were determined by three-way ANOVA and post-hoc Student-Newman-Keuls method, n = 6 per group.

Fig. 7.

Effect of pre-dawn activation of D₄R on the temporal expression pattern of *Adcy1* mRNA in mouse retina. Mice were injected at ZT 20 for three days with 1 mg/kg PD 168,077 or vehicle as described in Materials and methods. After the third injection, retinas were dissected during the following subjective night or subjective day in DD at the times indicated in the figure. In the PD 168,077 treatment group (open circles) a significant increase is observed from CT 21 to CT 0 (*P = 0.017) and to CT 4 (**P = 0.002). The treatment group peaks at CT 4 with values that are significantly higher than those of vehicle controls at that time (P = 0.004).