Essential roles of dopamine D4 receptors and the type 1 adenylyl cyclase in photic control of cyclic AMP in photoreceptor cells

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Abstract

Light and dopamine regulate many physiological functions in the vertebrate retina. Light exposure decreases cyclic AMP formation in photoreceptor cells. Dopamine D4 receptor (D4R) activation promotes light adaptation and suppresses the light-sensitive pool of cyclic AMP in photoreceptor cells. The key signaling pathways involved in regulating cyclic AMP in photoreceptor cells have not been identified. In the present study, we show that the light- and D4R-signaling pathways converge on the type 1 Ca^{2+}/calmodulin-stimulated adenylyl cyclase (AC1) to regulate cyclic AMP synthesis in photoreceptor cells. In addition, we present evidence that D4R activation tonically regulates the expression of AC1 in photoreceptors. In retinas of mice with targeted deletion of the gene (*Adcy1*) encoding AC1, cyclic AMP levels and Ca^{2+}/calmodulin-stimulated adenylyl cyclase activity are markedly reduced, and cyclic AMP accumulation is unaffected by either light or D4R activation. Similarly, in mice with disruption of the gene (*Drd4*) encoding D4R, cyclic AMP levels in the dark-adapted retina are significantly lower compared to wild-type retina and are unresponsive to light. These changes in *Drd4*^{-/-} mice were accompanied by significantly lower *Adcy1* mRNA levels in photoreceptor cells and lower Ca^{2+}/calmodulin-stimulated adenylyl cyclase activity in retinal membranes compared with wild-type controls. Reduced levels of *Adcy1* mRNA were also observed in retinas of wild-type mice treated chronically with a D4R antagonist, L-745,870. Thus, activation of D4R is required for normal expression of AC1 and for the regulation of its catalytic activity by light. These observations illustrate a novel mechanism for cross-talk between dopamine and photic signaling pathways regulating cyclic AMP in photoreceptor cells.

Keywords

dopamine; photoreceptors; adenylyl cyclase; cyclic AMP; retina; calmodulin

Introduction

Dopamine is a neuromodulator that influences many physiological functions in the retina (reviewed by (Witkovsky 2004). It enhances cone input signals and decreases rod input signals, uncouples horizontal cell and AII amacrine cell gap junction networks, enhances contrast

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sensitivity, and modulates light- and dark-adaptation. Also, it has trophic actions within the retina, affecting circadian rhythms, retinal development, and ocular growth (Witkovsky 2004). Activation of dopamine receptors, particularly the dopamine D4 receptor (D4R) subtype from the D2 family of receptors, suppresses the light-sensitive pool of cyclic AMP in dark-adapted mouse photoreceptors (Cohen and Blazynski 1990;Cohen *et al.* 1992). Cyclic AMP levels in photoreceptor cells are highest in darkness and reduced by light exposure. Although it is known that light and D4R activation regulate the same pool of cyclic AMP, they appear to do so by different mechanisms. Nir et al. (2002) showed that the effect of light is not directly dependent on D4R activation; however, in mice lacking D4Rs (*Drd4*^{-/-} mice), cyclic AMP levels were significantly lower in darkness and unresponsive to light when compared with wild-type controls. These observations suggested that D4R activation may exert a trophic regulatory influence on components of the cyclic AMP signaling system.

Cyclic AMP is synthesized by a large family of adenylyl cyclase isoforms (Hanoune and Defer 2001). In chicken cone photoreceptor cells, membrane potential regulates cyclic AMP synthesis in a Ca^{2+}-dependent manner (Iuvone *et al.* 1991). Light exposure and dopamine receptor activation both decrease intracellular Ca^{2+} and cyclic AMP levels in photoreceptor cells (Krizaj and Copenhagen 2002;Thoreson *et al.* 2002;Ivanova *et al.* 2008). The type 1 adenylyl cyclase, AC1, is activated by Ca^{2+} through the Ca^{2+} binding protein calmodulin (CaM) and is uniquely expressed in neural tissues, including the retina (Xia *et al.* 1993). Therefore, we examined the potential role of AC1 in mediating the light- and D4R-evoked reductions in cAMP, and investigated which components of the cyclic AMP signaling system are aberrant when D4R signaling is disrupted.

Materials and methods

Experimental Procedures

Animals—All 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. *Drd4*^{-/-} mice, lacking dopamine D4 receptors (Rubinstein *et al.* 1997), *Adcy1*^{-/-} mice, lacking the type 1 adenylyl cyclase (Wu *et al.* 1995), and normal control [wild-type (WT)] mice, all on a C57BL/6J background, were studied. Mice were genotyped by polymerase chain reaction (PCR) analysis of genomic DNA. Mice were housed on a 12 h light/12 h dark cycle with lights provided by cool white fluorescent tubes. Mice were sacrificed by cervical dislocation. All samples were collected in the morning, unless otherwise specified, immediately frozen on dry ice, and stored at -80°C.

Ex Vivo Retinal Incubations—Mice were dark adapted overnight. Retinas were dissected and placed in cold, oxygenated Earle's salt solution (115 mM NaCl, 3 mM KCl, 1.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 26 mM $NaHCO_3$, 5.5 mM glucose) at pH 7.4. To assess the effects of light on cyclic AMP accumulation, individual retinas where transferred to 6-well plates containing Earle's salt solution supplemented with 1 mM 3-isobutyl-1-methylxanthine (Sigma), 10 mM glutamate, and test drug or vehicle. Exogenous glutamate inhibits synaptic transmission to second order neurons in the retina, isolating the photoreceptor response to light in this assay (Cohen et al., 1992). The retinas were incubated in darkness at 37 °C in an atmosphere of 95% O_2/5% CO_2 for 5 minutes in a water bath. Subsequently, retinas were incubated in darkness or light for 10 additional minutes. To access the effects of D4R activation, retinas were incubated with or without PD168,077, a D4R-specific agonist (Glase *et al.* 1997), for 15 minutes in darkness. Retinas were immediately frozen on dry ice and stored at -80 °C until assayed. Cyclic AMP levels (pmol/mg protein) were measured by radioimmunoassay (RIA, Perkin Elmer, Waltham, Massachusetts 02451, USA).

Adenylyl Cyclase Assay—Partially purified membranes were prepared by homogenizing whole mouse retina in TME buffer containing 10 mM Tris-maleate, pH 7.5, 1 mM $MgSO_4$, 1.2 mM EGTA, 0.5 mM DTT and Protease Inhibitor Cocktail III (EMD Biosciences, La Jolla, CA, USA). Homogenates were centrifuged at $20,000 \times g$ for 20 min at 4°C. The resulting pellet was washed three times and resuspended in TME buffer. The protein content of the crude membrane preparation was determined (Lowry *et al.* 1951), using bovine serum albumin as a standard. Adenylyl cyclase activity was assayed by assessing the conversion of $[\alpha\text{-}^{32}P]$ ATP to $[^{32}P]$cyclic AMP using the method of (Salomon *et al.* 1974) with minor modifications. The final reaction mixture contained 80 mM Tris maleate, pH 7.5, 10 mM GTP, 1 mM DTT, 5 mM $MgSO_4$, 0.5 mM cyclic AMP, 0.2 mM EGTA, 5 mM phosphocreatine, 0.1 mg/ml creatine phosphokinase, 4 units/ml adenosine deaminase, 50 μM ATP, $[\alpha^{32}P]$ATP (0.5 μCi/tube), with or without 195 μM $CaCl_2$, and 120 nM CaM as indicated. Free $[Ca^{2+}]$, with 195 μM $CaCl_2$ added, and was calculated to be 2 μM using WEBMAXCLITE v1.15 (http://www.stanford.edu/%7Ecpatton/webmaxc/webmaxclite115.htm). The reaction was initiated by the addition of membranes (20-30 μg protein) in a final reaction volume of 250 μl and samples were incubated at 37°C for 10 min. The reaction was stopped by addition of 750 μl of 10% TCA containing $[^3H]$cyclic AMP (~4000 dpm) for recovery. After centrifugation, cyclic AMP was extracted by employing sequential chromatography on columns of Dowex AG50-W4 cation exchange resin and neutral alumina. The resulting elute (2.5 ml) was mixed with scintillation fluid (14 ml) and counted in a liquid scintillation counter using dual channel counting, one channel measuring $[^3H]$ for recovery of internal standard and the other channel measuring $[^{32}P]$-labeled product.

Acute and Sub-chronic Drug Treatment—A selective dopamine D4 receptor antagonist, L-745,870 (Patel *et al.* 1997), was administered for 1 or 6 days by subcutaneous injection (1mg/kg, s.c.) thirty minutes before light onset in the morning (ZT 23.5). Control animals were injected with a vehicle, 0.3% carboxymethylcellulose (Sigma-Aldrich, St. Louis, MO, USA). The rationale for injecting drug before light onset was to block D4Rs prior to the burst of dopamine release that occurs at light onset (Nir *et al.* 2000). On the first or sixth day, retinas were dissected and processed for analysis of *Adcy1* mRNA expression. In a separate experiment, mice were injected with L-745,870 or vehicle for six days as described above, but retinas were not dissected until the morning of the seventh day to allow for drug elimination.

Western Blotting—Retinas were homogenized in 50 mM Tris-HCl, 50 mM NaCl, 0.1 mM PMSF, 0.5% Triton X-100, pH 7.4 and were incubated at 4°C for 30 min with mixing. Homogenates were centrifuged at 20,000 X g for 30 min at 4°C. Pilot studies demonstrated that this procedure solubilized all detectable CaM protein (data not shown). 50 μg of supernatant protein was denatured by boiling for 5 min and was separated on a 10% Bis-Tris Criterion XT precast gel (BioRad Laboratories). The proteins on the gel were transferred to PVDF membrane using a semi-dry method. CaM was detected using a mouse monoclonal IgG1 antibody (05-173, Upstate, Lake Placid, NY, 1:500) plus horseradish peroxidase-labeled goat anti-mouse IgG (1:5,000, sc-2005, Santa Cruz Biotechnology, Santa Cruz, CA) with detection by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). The α-CaM antibody recognizes a C-terminal amino acid sequence that is 100 % conserved in CaM 1, 2 and 3 isoforms.

RNA Isolation and Quantitative Real-time PCR—Total RNA was extracted by a silica filter-binding method using the RNeasy kit (Qiagen Inc., Valencia, CA). Reverse transcription (RT) was performed on total RNA (2 μg) preparations using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo-dT or random primers (Invitrogen); oligo-dT was used in assays where mRNA expression was normalized with hypoxanthine

phosphoribosyltransferase (*Hprt*) mRNA or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA and random primers were used for normalization to 18S rRNA.

Reactions were performed in 25 μl total volume with 2μl cDNA, 1X SYBR Green PCR Master mix (Bio-Rad, Hercules, CA, USA) and 300 nM intron-spanning gene specific forward and reverse primers in a Bio-Rad iCycler (Bio-Rad). The PCR was heated at 95°C for 2 min, followed by 40 cycles of denaturing, annealing and extension at 95°C, 53° - 57°C, and 72°C for 30 seconds each, respectively. 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 gene. Each sample was assayed in duplicate.

Laser Capture Microdissection—Whole mouse eyes were embedded in OCT (Tissue-Tek, Torrance, CA), frozen on dry ice, and stored at -80°C. Frozen tissues were cut at 8 μm thickness and mounted on uncharged glass slides (VWR Scientific). The frozen sections were thawed for 30 sec and fixed in 75% ethanol for 30 sec followed by a wash in RNase-free water for 30 sec. The sections were stained with HistoGene (Arcturus Engineering, Mountain View, CA) staining solutions for 15 seconds followed by a wash with RNase-free water for 30 sec. The sections were dehydrated in graded ethanol solutions (75%, 30 sec; 95%, 30 sec; 100%, 30 sec) and cleared in xylene (5 min). After air-drying for 5 min, the slides were kept in a vacuum desiccator for a minimum of 30 min.

Laser capture microdissection (LCM) was performed by lifting the outer nuclear layer and inner segments of photoreceptor cells, the inner nuclear layer, and the ganglion cell layer individually onto HS-CapSure non-contact LCM films using a PixCell IIe LCM system (Arcturus Engineering). In this technique, a thermoplastic polymer membrane is placed above the tissue section. The cells of interest are visually located by light microscopy and a focused, low energy infrared laser is used to soften the film, allowing it to bond to the cells on the surface of the tissue section. The film absorbs the infrared energy, preventing damage to DNA, RNA, or protein in the underlying cells (Bonner *et al.* 1997;Parlato *et al.* 2002). Removal of the film lifts the desired cells from the tissue section without damaging other cells. When dissecting the photoreceptor cell layer (inner segments and outer nuclear layer) or the inner nuclear layer, we leave untouched the cells abutting the outer plexiform layer to avoid cross contamination. Outer segments are 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 specificity of this approach has been validated using cell type specific markers (Liu *et al.* 2004).

Total RNA was extracted from the captured cells using the PicoPure RNA Isolation Kit (Arcturus Engineering). Samples were reverse transcribed using random primers and subjected to real-time PCR analysis as described above.

Statistical analysis—Statistical comparison of multiple group data was performed using one-way or two-way analysis of variance (ANOVA) with Student-Newman-Keuls test where applicable. Comparisons of two groups were made with Students t-test.

Results

Identification of the adenylyl cyclase isoform regulated by light and D4R activation

Light exposure and D4R activation decrease the same pool of cyclic AMP in mouse photoreceptor cells (Cohen and Blazynski 1990). Previous studies suggest that cyclic AMP in photoreceptor cells is regulated by a Ca^{2+}-stimulated adenylyl cyclase (see Introduction). The primary Ca^{2+}-stimulated isoforms are the type-1 and type-8 adenylyl cyclases, AC1 and AC8 respectively (Wang and Storm 2003). Therefore, we investigated the expression of these

isoforms in mouse retina. Using LCM to isolate retinal cell layers, we determined that *Adcy1* mRNA, which encodes AC1, was expressed in the photoreceptor layer, inner nuclear layer, and the ganglion cell layer (Fig.1). In contrast, *Adcy8* mRNA was expressed in the inner nuclear and ganglion cell layers, but was undetectable in the photoreceptor layer. Therefore, in subsequent experiments, we focused our attention on AC1 as a putative cyclase isoform regulated by light and D4R in photoreceptor cells.

To assess the contribution of AC1 to retinal adenylyl cyclase activity, basal and Ca^{2+}/calmodulin-stimulated enzyme activities were assessed in retinal membranes prepared from wild type and *Adcy1$^{-/-}$* mice. In membranes prepared from WT retinas, Ca^{2+}/CaM increased adenylyl cyclase activity ~ 5 fold ($p<0.001$) compared to basal conditions without Ca^{2+} and CaM in the reaction (Fig.2A). Both basal activity and Ca^{2+}/CaM-stimulated activity were markedly reduced in retinal membranes from *Adcy1$^{-/-}$* mice, indicating that AC1 is a major adenylyl cyclase isoform controlling cyclic AMP synthesis in the mouse retina. A small amount of Ca^{2+}/CaM-stimulated activity was observed in *Adcy1$^{-/-}$* retinal membranes ($p=0.015$), which probably reflects a contribution of AC8 from the inner retina. In order to investigate the role of AC1 in the photic regulation of cyclic AMP, the effect of light exposure on cyclic AMP accumulation was examined in retinas of wild type and *Adcy1$^{-/-}$* mice incubated *in vitro*. The level of cyclic AMP accumulation was high in dark-adapted retinas from wild type mice and was significantly reduced by light exposure ($p<0.001$; Fig. 2B). In retinas of *Adcy1$^{-/-}$* mice, cyclic AMP accumulation in darkness was markedly lower compared to wild type retinas ($p<0.001$) and light exposure had no significant effect ($p = 0.714$). A control experiment found no compensatory changes in the levels of transcripts encoding the related type 8 adenylyl cyclase isoform in retinas of mice deficient in AC1 (WT: 1.39 ± 0.03; *Adcy1*-/-: 1.26 ± 0.06 *Adcy8* mRNA / *Hprt* mRNA$*10^{-2}$; N=6).

Further studies investigated whether AC1 is coupled to the D4R, measuring cyclic AMP accumulation in vehicle and D4R agonist (PD 168,077) treated retinas from WT, *Adcy1$^{-/-}$*, and *Drd4$^{-/-}$* mice. Incubation of dark-adapted WT retinas with PD 168,077 resulted in a concentration-dependent decrease of cyclic AMP accumulation, with an EC_{50} of 30nM (Fig. 3A). The effect of PD 168,077 was absent in *Drd4$^{-/-}$* mouse retinas, confirming involvement of D4Rs (data not shown). Similarly, treatment with a supra-maximal concentration (3μM) of PD 168,077 in dark-adapted retinas of *Adcy1$^{-/-}$* mice failed to decrease cyclic AMP accumulation (Fig. 3B; $p = 0.92$). In parallel with the previous light experiment (Fig. 2), the basal level of cyclic AMP accumulation in the *Adcy1$^{-/-}$* retinas is significantly lower ($p<0.001$) compared with WT controls (Fig. 3B). *Drd4* mRNA levels were unaffected in retinas of *Adcy1$^{-/-}$* mice (WT: 5.89 ± 0.81; *Adcy1$^{-/-}$*: 6.86 ± 1.23 *Drd4* mRNA / *Hprt* mRNA$*10^{-2}$; N=6). These data indicate that light and D4R activation control cyclic AMP production in mouse retina via AC1.

Expression of AC1 in Drd4$^{-/-}$ mouse retina

A previous study reported that cyclic AMP levels in dark-adapted retinas of *Drd4$^{-/-}$* mice were low compared to WT controls and unresponsive to light (Nir et al., 2002). The present study confirms these observations; cyclic AMP accumulation in dark-adapted WT retinas was approximately twice that of dark-adapted retinas from *Drd4$^{-/-}$* mice ($p<0.001$; Fig. 4) and, while light exposure significantly reduced cyclic AMP accumulation in WT retinas ($p<0.001$), it failed to do so in retinas of mice lacking D4Rs (Fig. 4). These observations led us to hypothesize that the expression of component(s) of the cyclic AMP signaling pathway regulated by light and by D4R was dysfunctional in retinas of *Drd4$^{-/-}$* mice. To test this hypothesis, the activity of Ca^{2+}/CaM-stimulated adenylyl cyclase, the transcript level of *Adcy1*, and the protein and transcript levels of calmodulin were examined.

There was no significant difference in basal adenylyl cyclase activity between membranes prepared from wild-type and *Drd4*^{-/-} mice (Fig. 5A; p=0.705). However, adenylyl cyclase activity in the presence of Ca^{2+}/CaM was significantly lower in retinal membranes prepared from *Drd4*^{-/-} mice compared to WT controls (p<0.001). In addition, quantitative real-time PCR revealed markedly lower levels of *Adcy1* transcripts in *Drd4*^{-/-} retinas than in WT samples (Fig. 5B; p<0.001).

The aforementioned measurements were based on mRNA from whole retina. To determine if a difference in *Adcy1* expression occurred in photoreceptors of the two genotypes, LCM was employed to isolate the photoreceptor layer. Figures 6A and B show a typical dissection of the photoreceptor inner segments and outer nuclear layer. RT-PCR analysis of the captured photoreceptors revealed that *Adcy1* transcript levels were markedly lower in samples from the *Drd4*^{-/-} mice compared to the corresponding WT samples (Fig. 6C; p=0.006).

To further explore the mechanisms responsible for the decrease in cyclic AMP accumulation in retinas of D4R deficient mice, we studied the expression of CaM transcripts and protein. Four transcripts encode highly conserved CaM isoforms in mouse: *Calm1, Calm2, Calm3, and Calm4* (Skinner *et al.* 1994;Hwang and Morasso 2003). *Calm1-3* transcripts were found to be abundantly expressed in mouse retina (Fig. 7A). *Calm4* mRNA was detectable, but relative abundance was <1% of *Calm1* mRNA level. No differences in *Calm1-4* transcript levels were observed between WT and *Drd4*^{-/-} mouse retinas (Fig. 7A). *Calm* transcript expression was further explored in LCM isolated retinal layers, but no significant differences were observed between samples from WT and *Drd4*^{-/-} mice (data not shown). Similarly, Western blot analysis of retinal homogenates, using an antibody that recognizes an amino acid sequence that is 100 % conserved in CaM 1, 2, and 3, revealed no difference between the WT and *Drd4*^{-/-} mice (Fig. 7B). Thus, the dysfunction of Ca^{2+}/CaM-stimulated adenylyl cyclase activity in *Drd4*^{-/-} mouse retina appears to be due primarily to low levels of expression of the AC1 and not to changes in CaM expression.

As a further control for compensatory changes, we examined the relative expression of transcripts encoding the five dopamine receptor subtypes in retinas of WT, *Adcy1*^{-/-}, and *Drd4*^{-/-} mice (Supplemental Fig. S1). *Drd1, Drd2, Drd4, and Drd5* transcripts were observed in mouse retina; *Drd3* mRNA expression was not detectable. *Drd4* was the most highly expressed subtype. No significant differences were observed among genotypes, except for the absence of *Drd4* mRNA in retinas of *Drd4*^{-/-} mice.

Effect of D4R antagonism on Adcy1 expression

To investigate the role of D4R activation in the expression of *Adcy1*^{-/-}, the effects of a D4R-selective antagonist, L-745,870 (Patel *et al.* 1997), were examined in WT mice. L-745,870 was administered 30 minutes prior to light onset in the morning to antagonize the surge of dopaminergic activity that occurs when dark-adapted retinas are first exposed to light in the morning (Nir *et al.* 2000). Following a single injection, L-745,870 had no significant effect on *Adcy1* mRNA level measured 1.5 or 5 h after light onset (Fig. 8A). In contrast, retinas of mice treated with L-745,870 for 6 days had significantly lower levels of *Adcy1* transcript compared to vehicle-treated controls (Fig. 8B; p<0.001). Recovery of *Adcy1* mRNA levels following drug elimination for >29 h was examined in mice treated with L-745,870 for 6 days. In mice exposed to light in the morning for 4-5 hours to stimulate dopamine release, no difference was observed in *Adcy1* transcript levels between vehicle and antagonist treated samples (Fig. 8C), indicative of recovery. However, in mice that were kept in darkness for the same period to prevent the morning increase of dopaminergic activity, *Adcy1* mRNA levels remained lower in the L-745,870-treated mice than in the vehicle controls (Fig. 8C; p<0.05). These observations suggest that D4R activation tonically supports *Adcy1* expression.

Discussion

Dopamine D4 receptors are highly expressed in retina relative to other central nervous system structures (Oak *et al.* 2000) and appear to be the only dopamine receptor subtype expressed by photoreceptors (Cohen *et al.* 1992). Activation of these receptors decreases a light-sensitive pool of cyclic AMP in mouse photoreceptors (Cohen and Blazynski 1990;Cohen *et al.* 1992;Nir *et al.* 2002). The absence of D4Rs has physiological consequences that manifest as impaired light adaptation and a decreased rate of dark adaptation following light exposure (Nir *et al.* 2002). Effects are observed in both the a-wave and b-wave of the electroretinogram, consistent with effects on synaptic transmission from the photoreceptors to inner retinal neurons. It is unknown at present if the dysfunctional cyclic AMP signaling is responsible for these deficits of adaptation in *Drd4*$^{-/-}$ mice, but recent studies have shown that the absence of D4Rs compromises light-evoked dephosphorylation of the PKA site of phosducin (Pozdeyev *et al.* 2008), a regulatory phosphoprotein that binds to transducin-$\beta\gamma$ subunits (Lee *et al.* 1987).

A number of other photoreceptor functions are influenced by dopamine and/or cyclic AMP and could contribute to the physiological abnormalities observed in *Drd4*$^{-/-}$ mice. Dopamine, acting through D4Rs, inhibits Na^+/K^+-ATPase in rat rod outer segments (Shulman and Fox 1996). Dopamine increases the rate of rhodopsin dephosphorylation in frog retina (Udovichenko *et al.* 1998) and PKA has been shown to phosphorylate the γ-subunit of cGMP phosphodiesterase purified from bovine rod outer segments (Xu *et al.* 1998). Also, D4R activation regulates melatonin synthesis in photoreceptors by decreasing cyclic AMP formation (Iuvone 1986;Iuvone and Besharse 1986;Tosini and Dirden 2000;Zawilska and Nowak 1994;Zawilska *et al.* 1995). Dopamine and cyclic AMP affect rod and cone photoreceptor Ca^{2+} currents in salamander (Stella, Jr. and Thoreson 2000), photoreceptor retinomotor movements in Xenopus and fish (Besharse *et al.* 1982;Pierce and Besharse 1985;Hillman *et al.* 1995), and reset the phase of the circadian clock in *Xenopus* photoreceptors (Cahill and Besharse 1991;Hasegawa and Cahill 1999).

Previous observations that light-regulated cyclic AMP signaling is dysfunctional in retinas of *Drd4*$^{-/-}$ mice (Nir *et al.* 2002), confirmed in the present study, suggest that D4R activation regulates the expression of components of the cyclic AMP signaling system in photoreceptor cells. The major goal of this study was to identify which adenylyl cyclase(s) are regulated by light and the D4R, and to determine if expression of the cyclase or related signaling components is aberrant when D4R activation is disrupted. The results provide compelling support for the conclusions that light and dopamine acutely regulate the catalytic activity of AC1 in photoreceptor cells and that D4Rs tonically regulate the expression of the cyclase.

Cyclic AMP accumulation in dark-adapted retinas of *Adcy1*$^{-/-}$ mice is dramatically lower than that in wild type mice, indicating that AC1 contributes significantly to synthesis of the cyclic nucleotide under these conditions. In addition, the failure of either light or dopamine receptor activation to further reduce cyclic AMP accumulation in *Adcy1*$^{-/-}$ retinas indicates that the response to both stimuli is mediated by a reduction in AC1 activity. While we can not exclude the possibility that other adenylyl cyclases contribute to the light- and dopamine-regulated pool of cyclic AMP in photoreceptors, their contribution would seem to be small relative to that of AC1. The possibility must be considered that disruption of *Adcy1* results in down-regulation of other cyclase genes, but this was not seen, at least for *Adcy8*. The inhibition of AC1 catalytic activity by light and dopamine likely involves the reduction in intracellular Ca^{2+} in photoreceptors elicited by both stimuli (Krizaj and Copenhagen 2002;Thoreson *et al.* 2002;Ivanova *et al.* 2008), decreasing CaM stimulation of the cyclase. The reduction of Ca^{2+} in response to light occurs secondary to decreased cGMP-gated currents, hyperpolarization of the plasma membrane, and closure of voltage-gated Ca^{2+} channels. The

decrease in Ca^{2+} subsequent to D4R activation is less well understood, but may involve a G_i/G_0-dependent inhibition of voltage-gated channels (Mei *et al.* 1995), dopamine receptor-mediated interactions of Ca^{2+} and Cl^- channels (Thoreson *et al.* 2002), or modulation of Ca^{2+} channels by cyclic AMP-dependent phosphorylation (Stella, Jr. and Thoreson 2000). Inhibition of AC1 activity by D4R may also involve direct effects of G_i, G_o, or transducin (Oak *et al.* 2000;Yamaguchi *et al.* 1997).

Disruption of *Drd4* or subchronic antagonism of D4Rs with L-745,870 in wild type mice decreased the expression of *Adcy1* mRNA. The reduced expression of *Adcy1* in retinas of *Drd4*$^{-/-}$ mice was accompanied by lower Ca^{2+}/CaM-stimulated adenylyl cyclase activity and a lower level of cyclic AMP accumulation in dark-adapted retinas compared to wild type controls. In addition, the photic control of cyclic AMP accumulation was absent in photoreceptors of mice lacking D4Rs, presumably due to the reduced expression of AC1. These results emphasize the importance of AC1 in the light-evoked reduction of cyclic AMP and suggest that D4R activation plays a significant role in regulating the expression of the cyclase.

The decreased expression of *Adcy1* mRNA following L-745,870 treatment suggests that D4R receptor activation is required for the normal expression of the cyclase in photoreceptors. The observation that repeated but not acute treatment with the antagonist also suggests that receptor activation has a trophic influence on the expression of the cyclase. Moreover, *Adcy1* expression level recovered 1 day after the last of the six injections of antagonist in mice exposed to light to stimulate dopamine release, but not in mice kept in darkness.

The mechanism whereby D4Rs regulate expression of the Ca^{2+}/CaM-stimulated adenylyl cyclases is presently unknown. In pinealocytes, increasing cyclic AMP levels inhibits *Adcy1* gene expression (Chan *et al.* 2001). The decrease of cyclic AMP levels in response to D4R activation in photoreceptors may elicit an increase in expression of the cyclase gene. However, this mechanism appears contradictory to the observation that both cAMP accumulation and *Adcy1* expression are low in *Drd4*$^{-/-}$ retina. The D4R also contains SH3 binding domains (Oldenhof *et al.* 1998) and D4R stimulation activates Akt/nuclear factor kappa-B signaling, increases ERK1/2 phosphorylation, and induces cFOS (Zhen *et al.* 2001;Bitner *et al.* 2006). Thus, D4R activation in retina may recruit signaling cascades that regulate *Adcy1* gene expression.

In summary, the present study demonstrates that the photic- and dopamine-signaling pathways in photoreceptor cells converge on AC1 to regulate cyclic AMP formation. D4R activation not only acutely inhibits cyclic AMP formation in retina, but also exerts a trophic influence on the expression of the type 1 Ca^{2+}/CaM-stimulated adenylyl cyclase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations

AC1, type 1 adenylyl cyclase; AC8, type 8 adenylyl cyclase; CaM, calmodulin; D4R, dopamine D4 receptor; WT, wild type.

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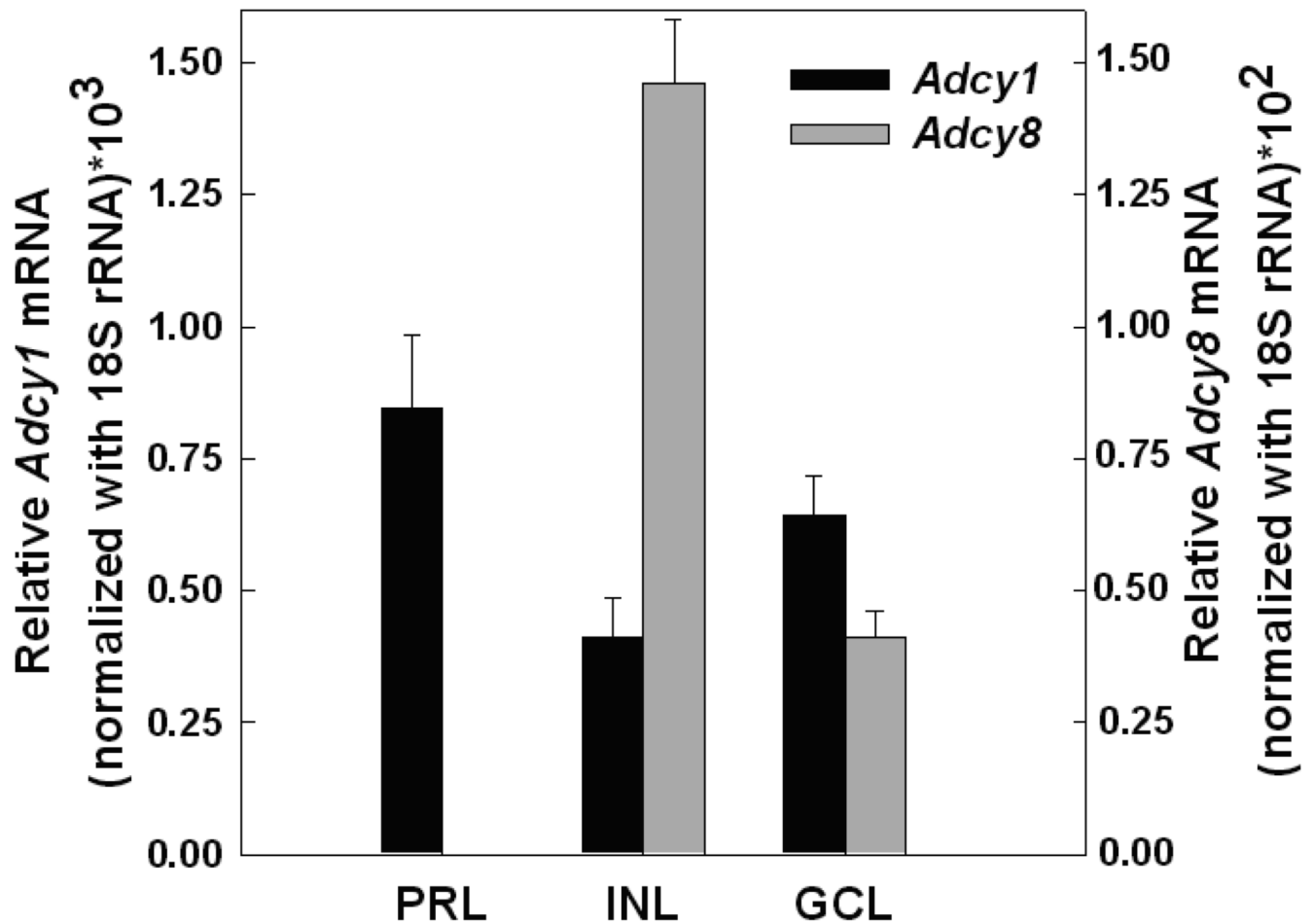
Figure 1. Expression of *Adcy1* and *Adcy8* transcripts in retinal layers isolated by laser capture microdissection (LCM)
The ganglion cell layer (GCL), inner nuclear layer (INL), and photoreceptor layer (PRL; inner segments and outer nuclear layer) were captured from frozen sections of retina as described in *Material and methods*. *Adcy1* and *Adcy8* mRNA levels, normalized to 18S rRNA, where determined by real-time RT-PCR. N= 4 retinas per group, except for *Adcy8* GCL, where N= 3.
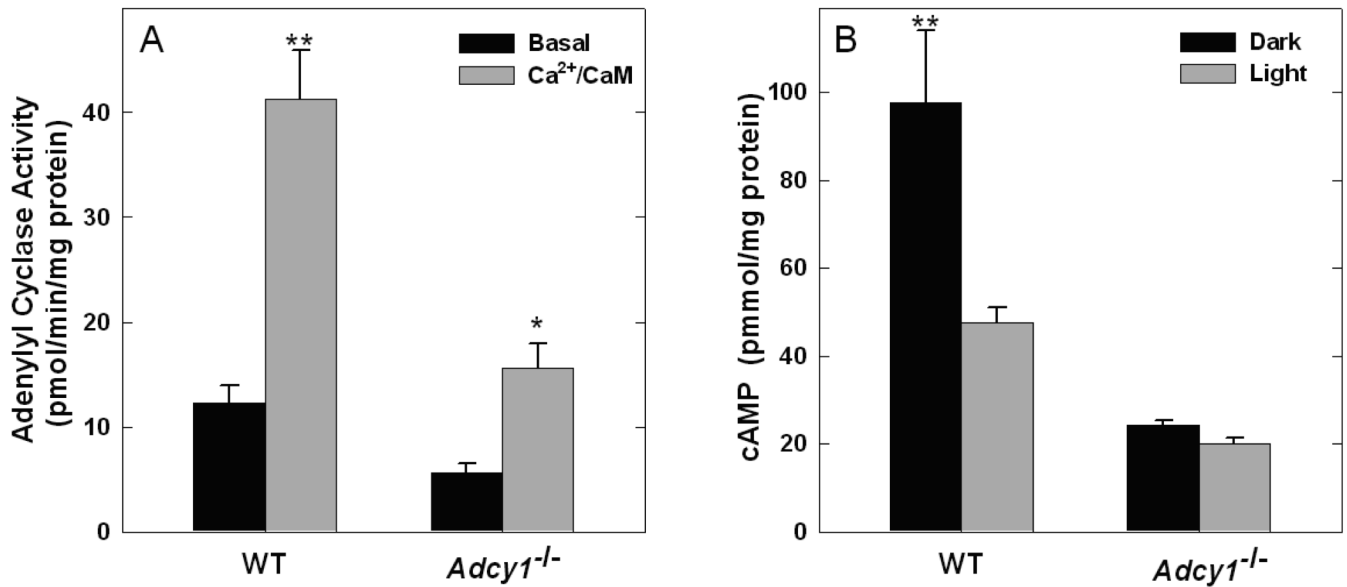
Figure 2. Adenylyl cyclase activity and cyclic AMP accumulation is low and unresponsive to light in mouse retinas lacking the type 1 adenylyl cyclase
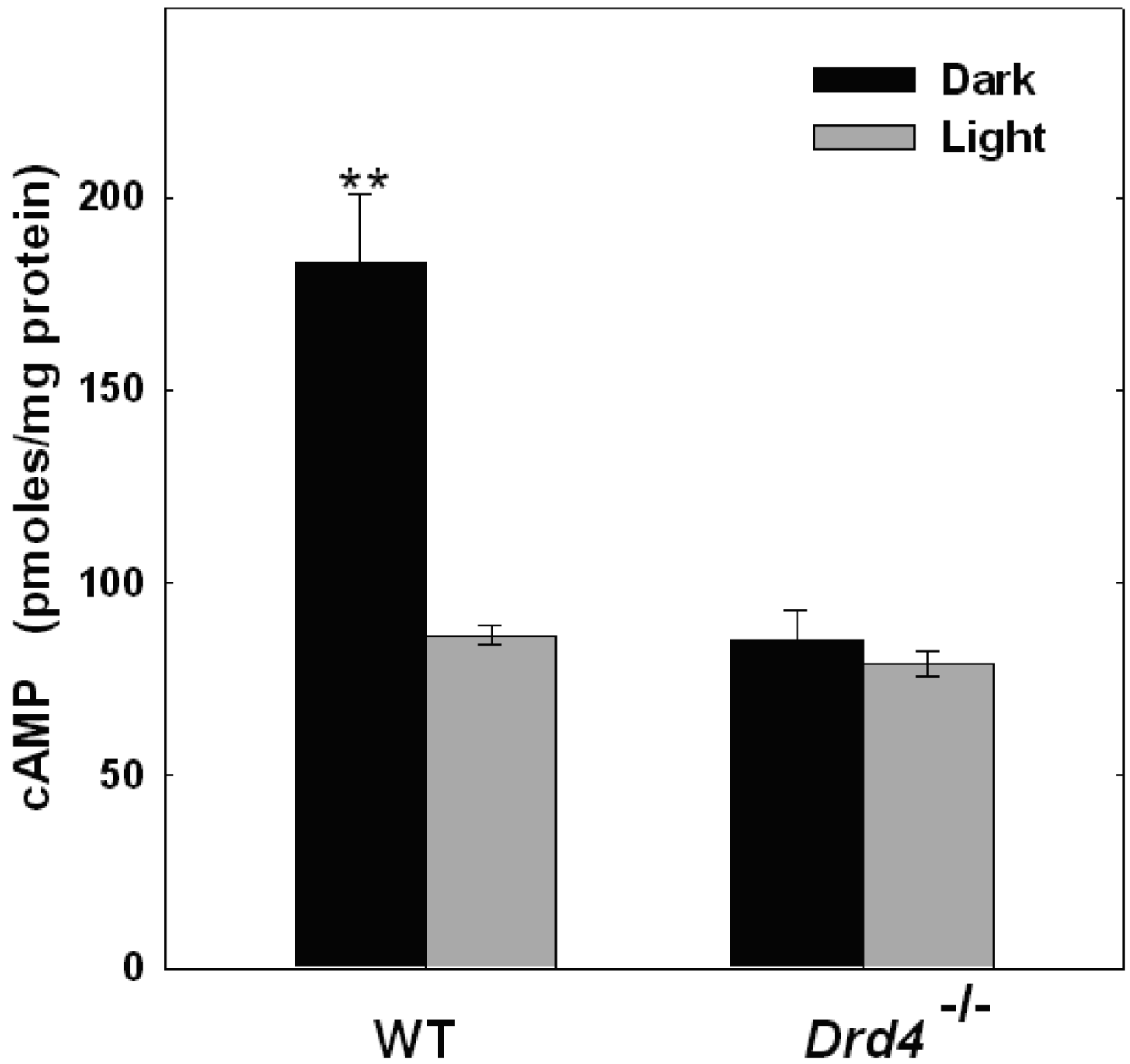
A. Adenylyl cyclase activity was measured in retinal membranes prepared from WT and *Adcy1*$^{-/-}$ mice in the presence or absence of Ca^{2+} and CaM, as described in *Materials and methods*. 2-factor ANOVA revealed significant effects of genotype ($p < 0.001$), Ca^{2+}/CaM ($p < 0.001$), and a significant interaction of genotype and Ca^{2+}/CaM ($p = 0.004$). **$p < 0.001$ *vs.* WT Basal, *Adcy1*$^{-/-}$ Basal, and *Adcy1*$^{-/-}$ Ca^{2+}/CaM; *$p = 0.015$ *vs. Adcy1*$^{-/-}$ Basal . B. Cyclic AMP accumulation was measured in retinal explants incubated in darkness for 15 min or in darkness (5 min) followed by light exposure (10 min), as described in *Materials and methods*. N= 6 per group; 2-factor ANOVA revealed significant effects of genotype ($p < 0.001$, light conditions ($p = 0.004$), and interaction of genotype and light conditions. **$p < 0.001$ *vs.* all other groups.
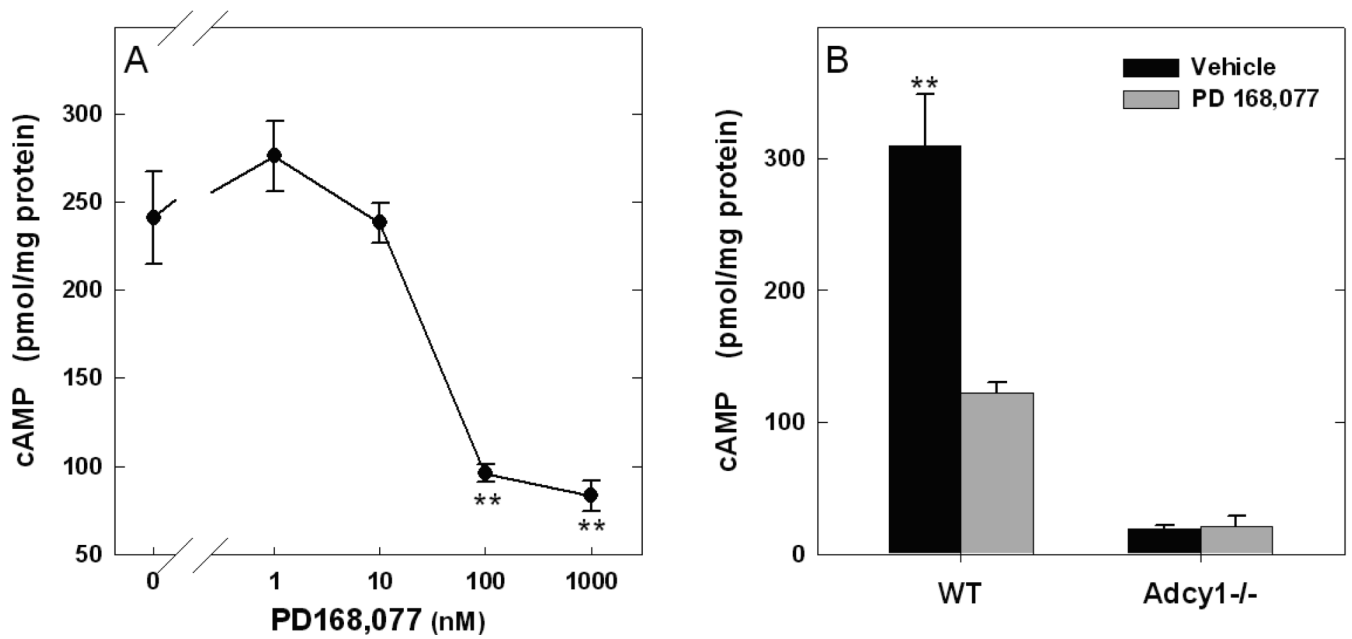
Figure 3. PD 168,077, a D4R agonist, inhibits cyclic AMP accumulation in WT but not *Adcy1*^{-/-} retinas

A. PD 168,077 inhibits cyclic AMP accumulation during a 15 min incubation with an EC_{50} value of 30 nM. **p<0.001 *vs.* control. B. In the presence of PD168077 (3μM) there is a significant reduction in cyclic AMP accumulation in WT, but not in *Adcy1*^{-/-} retinas. N=4 / group; **p<0.001 *vs.* all other groups.

Figure 4. Light decreases cyclic AMP accumulation in retinas of WT but not *Drd4*^{-/-} mice

Retinas were incubated in dark or light as described in Fig. 2B. N= 4-6; **p<0.001 *vs.* all other groups.
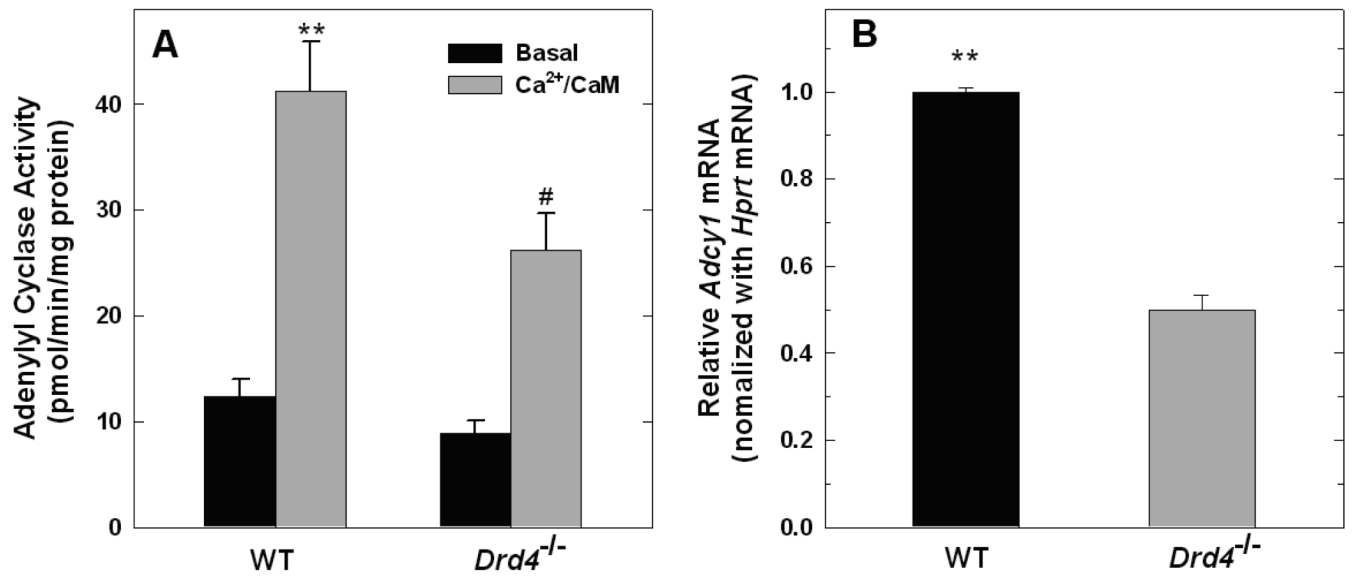
Figure 5. Ca^{2+}/CaM-stimulated adenylyl cyclase activity and *Adcy1* mRNA levels in retinas of WT and *Drd4*$^{-/-}$ mice

A. Ca^{2+}/CaM stimulated adenylyl cyclase activity is significantly lower in the *Drd4*$^{-/-}$ retinal membranes compared to WT controls. N= 9 / group; **p<0.001 *vs.* all other groups.; #p<0.01 *vs. Drd4*$^{-/-}$ Basal. B. *Adcy1* mRNA is significantly lower in retinas of *Drd4*$^{-/-}$ mice compared to WT controls. *Adcy1* mRNA level was normalized to *Hprt* mRNA and expressed relative to WT. N= 6 / group; **p<0.001 *vs. Drd4*$^{-/-}$.
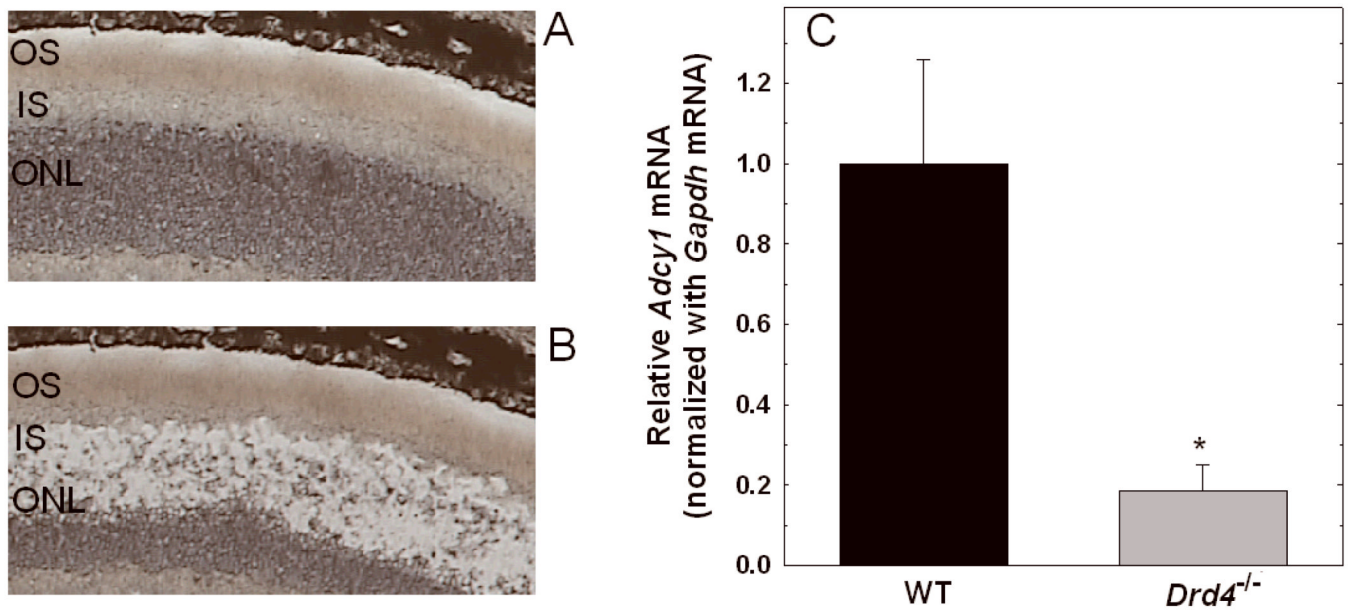
Figure 6. Decreased expression of *Adcy1* mRNA in photoreceptors of *Drd4*^{-/-} mouse retina

Photoreceptor inner segments and cell bodies were isolated by laser capture microdissection (LCM) as described in Methods. A. Representative retinal section before dissection. B. Retinal section after capture of inner segments and cell bodies. OS, outer segments; IS, inner segments, ONL, outer nuclear layer. C. *Adcy1* mRNA was measured in captured IS/ONL from WT and *Drd4*^{-/-} mice, normalized to *Gapdh* mRNA, and expressed relative to WT. N= 4 (WT) and 6 (*Drd4*^{-/-}); *p=0.006 *vs.* WT mice.
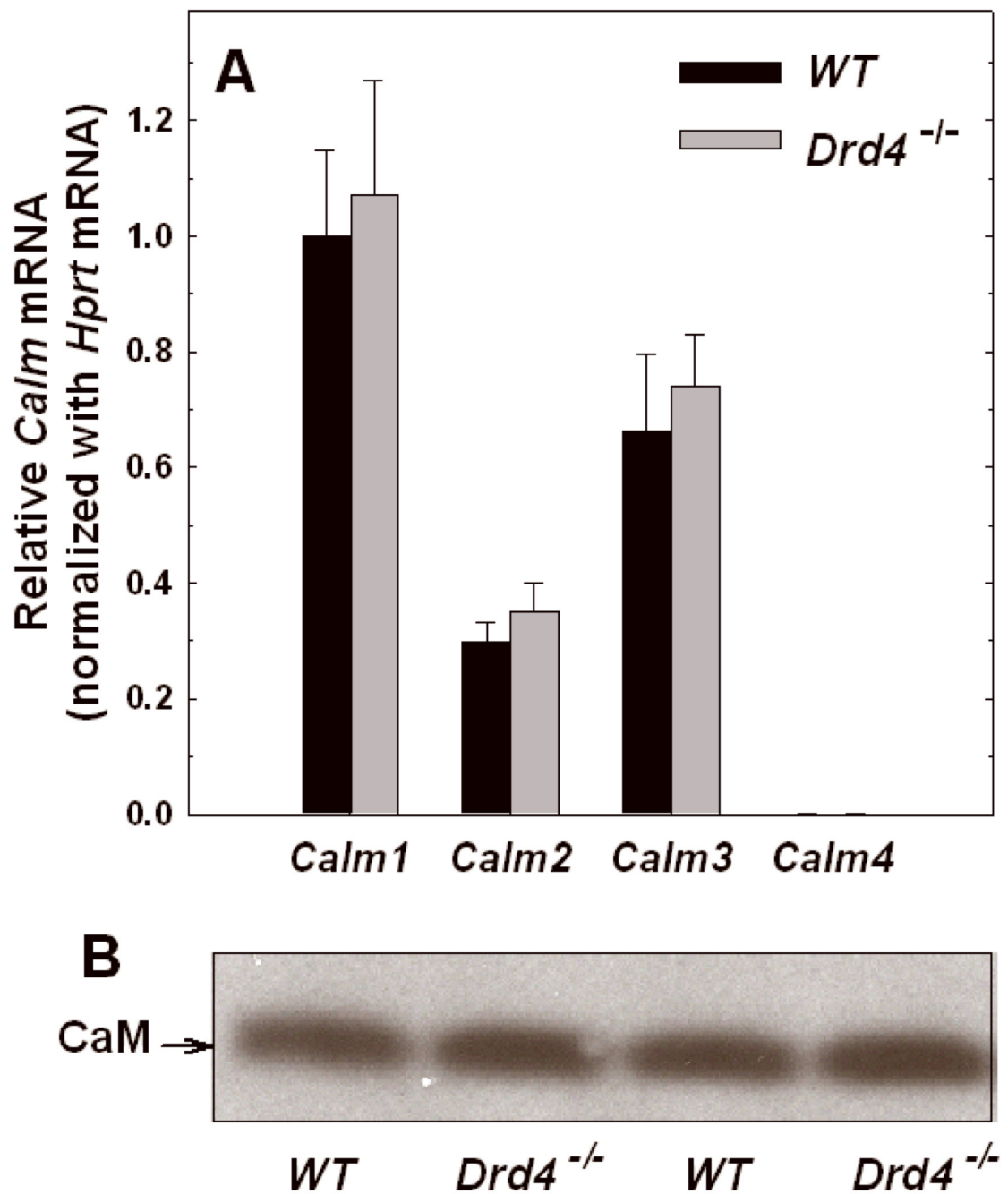
Figure 7. Calmodulin expression in wild-type and *Drd4*^{-/-} mouse retina
A. *Calm1-4* transcripts normalized to *Hprt* mRNA and expressed relative to WT *Calm1* mRNA. The values are means ± SEM (N=6). B. Western blot analysis of calmodulin protein from the wild-type and *Drd4*^{-/-} mouse retina. The blot shown is representative of an N of 6 from two separate experiments.
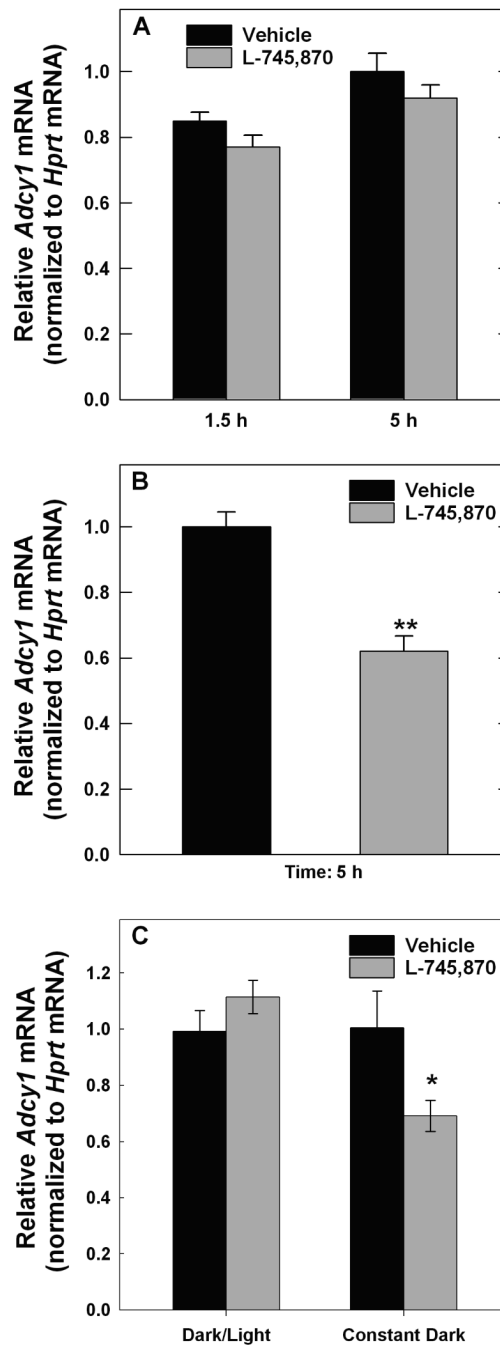
Figure 8. Effect of L-745,870 on *Adcy1* transcript expression in WT mouse retina
Mice were injected subcutaneously with L-745,870 (1 mg/kg body weight) or vehicle ~30 min before light onset in the morning. **A**. A single injection failed to significantly affect *Adcy1* mRNA, measured 1.5 or 5 h after light onset. N= 6. **B**. Mice were injected with L745,870 or vehicle daily for 6 days and retinas dissected 5 h after light onset following the last injection. *Adcy1* mRNA was significantly lower in L-745,879 treated mice compared to vehicle controls. *Adcy1* mRNA was normalized to *Hprt* mRNA and expressed relative to Vehicle values from samples collected 5 h after light onset. N=8; **p<0.001 *vs.* Vehicle. **C**. Mice were injected for 6 days as in Figure 8B, but retinas were not dissected until the following day to allow for drug elimination. One set of subjects (Dark/Light) were exposed to light in the morning for 4-5 h

to stimulate dopamine release, while the other set (Constant Darkness) remained in the dark for the same period of time. Light exposed samples showed recovery of *Adcy1* mRNA levels, while those kept in darkness did not; N=6, *P=0.014 *vs.* Vehicle.

Table 1

Real time PCR primers.

Primer Name	Sequence	Accession Number
Adcy1	F:5'-CACAGCAGGAACCAAGGCTAAGAA-3' R:5'-TGCCAACTCGGAGAACAAAGTCGT-3'	AF053980
Adcy8	F:5'-TCCGCTTGGAGACAGAGAACCAAA-3' R:5'-GAATACTGACGTTCTCATAGCGATGG-3'	NM_009623
Calm1	F:5'-AGTGTGATGGTTCATTGCTGTGCG-3' R:5'-TGATGGTGTGCTCAAGTCCACAGA-3'	NM_009790
Calm2	F:5'-TGGAGTTGGTCCAATGAGGGAACA-3' R:5'-ACGCAGAGTTACAGCTCCACACTT-3'	NM_007589
Calm3	F:5'-AAACCATCTGGCTGGCTTTCTGAG-3' R:5'-TTTACAGAACAGCGGGACATGGCT-3'	NM_007590
Calm4	F:5'-TCCTTGACCAGAATGGTGATGGCT-3' R:5'-TTCAATGTGGAGGCGCACAAACTC-3'	NM_020036
Hprt	F:5'-TGGACAGGACTGAAAGACTTGCTC-3' R:5'- GCAGGTCAGCAAAGAACTTATAGCCC-3'	BC004686
18S rRNA	F:5'-TAGAGTGTTCAAAGCAGGCCCGA-3' R:5'-ATGCTTTCGCTCTGGTCCGTCTT-3'	X00686
Gapdh	F:5'-CAGTATGACTCCACTCACGGCAAATTCAA R:5'-GTGATGGGTGTGAACACGAGAA	NM_008084