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Chad R. Jackson Date

Dopamine and dopamine D_4 receptor activation: Signaling components, gene regulation, endogenous circadian systems, and visual function in mouse retina

By

Chad R. Jackson
Doctor of Philosophy, 2010

Graduate Division of Biological and Biomedical Sciences
Molecular and Systems Pharmacology

_____
Advisor: P. Michael Iuvone, Ph.D.

_____
Committee Member: Gary Miller, Ph.D.

_____
Committee Member: Gianluca Tosini, Ph.D.

_____
Committee Member: Randy Hall, Ph.D.

_____
Committee Member: Yue Feng, Ph.D.

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

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Chad Robert Jackson

B.A., Earlham College, 2002

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A dissertation submitted to the Faculty of the
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in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in
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Molecular and Systems Pharmacology, 2010

Abstract

Dopamine and dopamine D_4 receptor activation: Signaling components, gene regulation, endogenous circadian systems, and visual function in mouse retina

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The retina is the primary neural structure responsible for image formation. It functions over a wide range of light intensities, in part due to its endogenous rhythmic behavior. Dopamine, a rhythmic neuromodulator, promotes light-adaptive effects in the retina. Dopamine, produced by a small subset of amacrine and interplexiform cells, mediates photoreceptor input signals, gap junction connectivity, contrast sensitivity, and Na^+/K^+-ATPase activity in the mouse retina. Also, it is involved in trophic actions, such as circadian rhythms, retinal development, and ocular growth. In the mammalian photoreceptor layer only dopamine D_2-like receptors are expressed. In this layer, dopamine reduces a light-sensitive pool of cyclic AMP, which is related to photoreceptor Ca^{2+} influx. Mice lacking the D_4R are unresponsive to light and dopaminergic agonists and display deficits in basal cAMP levels. In other vertebrate retinas cAMP levels are rhythmic, connecting this second messenger to the retina's endogenous circadian clock(s). Thus, I sought to investigate if the D_4R is coupled to Ca^{2+}-sensitive adenylyl cyclase(s) controlling retinal cAMP levels and determine if D_4R activation affects retinal gene expression and circadian rhythms. Using molecular and biochemical assays it was determined that the type 1 adenylyl cyclase is the primary enzyme responsible for cAMP production in mouse retina. Additionally, we found that D_4R modulates the expression of the *Adcy1* gene (encoding the type 1 adenylyl cyclase). Knowing cAMP is rhythmic in vertebrate retina we assayed the *Adcy1* transcript, cAMP, and Ca^{2+}/CaM-stimulated adenylyl cyclase activity and found each demonstrates a significant rhythm. However, this rhythm was not detected in mice lacking the D_4R. We show that antagonizing or abnormal stimulation of the D_4R significantly affects the normal *Adcy1* retinal rhythm. Microarray analysis revealed that ~4.8% of the measured retinal transcriptome is affected by the D_4R. Visual contrast sensitivity tests show mice lacking the D_4R have an impaired ability to detect changes in contrast compared with WT controls. These data demonstrate that the D_4R is essential for modulating rhythmic cAMP, influencing basal and circadian retinal gene expression, and visual function regulating visual contrast sensitivity. This investigation shows that the DA/D_4R signaling system plays a necessary role in retinal function.

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List of Abbreviations

3-isobutyl-1-methylxanthine.....IBMX

Adenosine triphosphate.....ATP

Adenylyl cyclase.....AC

Age-related macular degeneration.....AMD

Arylalkylamine N-acetyltransferase.....AANAT

Brain and Muscle ARNT-like protein.....*Bmal1*/BMAL1

Calmodulin.....CaM

Central nervous system.....CNS

Circadian locomoter output cycles protein kaput.....*Clock*/CLOCK

Circadian time.....CT

Clock-controlled genes.....CCG

Coimmunoprecipatation.....Co-IP

Constant Darkness 1st day.....DD

Constant Darkness 2nd day.....DD2

Cryptochrome.....*Cry*/CRY

cyclic adenosine monophosphate.....cAMP

ΔCycle time.....ΔCT

Dimethyl sulfoxide.....DMSO

Dopamine.....DA

Dopamine D_1 receptor.....D_1R

Dopamine D_2 receptor.....D_2R

Dopamine D_3 receptor.....D_3R

Dopamine D_4 receptor.....*Drd4*/D_4R

Dopamine D_5 receptor.....D_5R

Electroretinogram.....ERG

False discovery rate.....FDR

Free-running period.....FRP

Ganglion cell layer.....GCL

Glyceraldehyde-3-phosphate dehydrogenase.....*Gapdh*

Hyopxanthine phosphoribosyltransferase.....*Hprt*

Ingenuity pathway analysis.....IPA

Inner nuclear layer.....INL

Inner plexiform layer.....IPL

Inner segments.....IS

Intracellular loop IC

Laser capture microdissection.....LCM

L-dihydroxyphenylalanine.....L-DOPA

Light/Dark cycle.....LD

Melatonin 1 receptor.....MT_1

Melatonin 2 receptor.....MT_2

Optimal cutting temperature compound .....OCT

Optokinetic tracking.....OKT

Outer nuclear layer.....ONL

Outer plexiform layer.....OPL

Outer segments.....OS

Period.....*Per*/PER

Photoreceptor layer.....PRL

Protein Kinase A.....PKA

Quantitative real-time polymerase chain reaction.....qRT-PCR

Quantitative trait loci.....QTL

Retinal ganglion cell layer.....RGC

Retinal pigment epithelium.....RPE

Retinohypothalamic tract.....RHT

Retinoic acid-related orphan nuclear receptor alpha.....RORα

Suprachiasmatic nucleus.....SCN

Type 1 adenylyl cyclase.....*Adcy1*/AC1

Type 3 adenylyl cyclase.....AC3

Type 8 adenylyl cyclase.....AC8

Vesicular monoamine transporter.....VMAT

Wild-type.....WT

Zeitgeber time.....ZT

Chapter I: Introduction

1.1 Project Overview. Vision is a unique neurological pathway that connects humans, as well as other animal species with their immediate environment. This system helps us locate food, alerts us of present dangers, and enables us to emotionally connects with others. Also, vision is responsible for controlling and setting circadian biological rhythms, such as the sleep/wake cycle, blood pressure, and cortisol levels. These rhythms enable animals to predict temporal changes in the day, preparing them for daily stressors (*e.g.*, physical and emotional stress), and optimizes their biology to meet these challenges. Malfunctions in the visual system dramatically changes the quality of life, making this area an important research topic.

Vision is controlled primarily by the retina. In mammals, the retina is located in the posterior portion of the mammalian eye (Figure 1-1.1). Light is focused onto the retina via the lens. Light strikes the retina sending signals to the central nervous system (CNS) via an elaborate neuronal network. This delicate process is under the control of many neuromodulators and cell communication systems ensuring this biological process is maintained and conserved in most species.

Elucidation of the visual process has been the topic of many studies, including this project which sought to define how dopamine (DA)and dopamine D_4 receptor (D_4R) activities affect retinal functioning at the cellular and network levels. The research strategies were focused on delineating key components of the D_4R signaling pathway, determining if D_4R activation modulates intrinsic retinal rhythms, and exploring the molecular systems controlled by its activation, thereby affecting vision. Overall, the central hypothesis is that DA, released at dawn by dopaminergic amacrine and interplexiform cells, works via volume transmission activating D_4Rs throughout the retinal layers. This activation reduces cyclic adenosine monophosphate

(cAMP) levels by inhibiting adenylyl cyclase(s), thereby affecting retinal gene expression, and resets the retinal circadian clock at dawn.

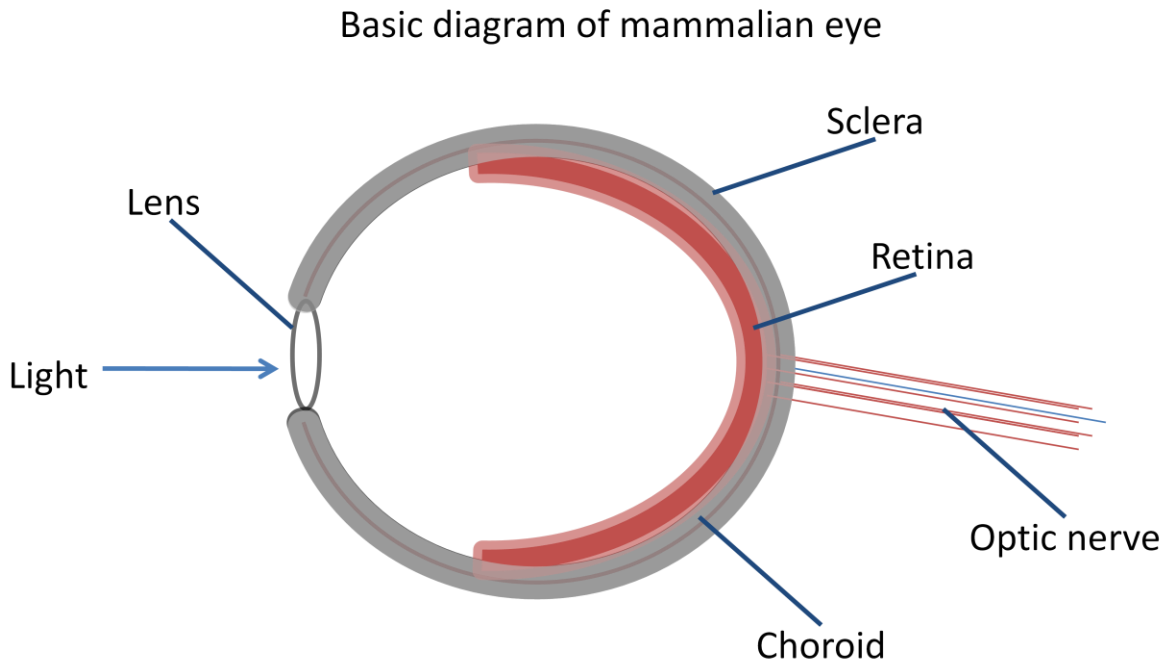


Figure 1-1.1. Transection of a mammalian eye, displaying basic structure and components. The retina is located in the posterior part of the eye just below the choroid.

1.2 Vision

1.2.1 Neural retina: cellular structure and function. The neural retina (Fig. 1-1.1), located just below the choriod, contains several cellular layers primarily responsible for detecting light and transmitting the stimuli to the CNS. From front to back, the mammalian retinal layers are as follows: 1) ganglion cell layer (GCL), 2) inner plexiform layer (IPL), 3) inner nuclear layer (INL), 4) outer plexiform layer (OPL), 5) outer nuclear layer (ONL), 6) inner and outer segments (IS and OS), and 7) retinal pigment epithelium (RPE) (Fig. 2-1.2).

The photoreceptor layer (PRL), containing the rod and cone photoreceptors, is the primary cellular layer regulating image formation (Fig. 2-1.2). Rod photoreceptors are rod-shaped neurons that mediate visual sensitivity, and night vision, absorbing at a peak wavelength of 498 nm. The rod photoreceptor is composed of 4 basic parts; 1) the spherule (synapse), 2) nucleus, 3) inner segment (contains mitochondria for energy production), and 4) outer segment (contains rhodopsin for light detection). On the other hand, cones have cone-shaped outer segments, mediating color and daytime visions. Humans have three types of cone photoreceptors that are sensitive to red, green, and blue light (S-cones: ~437 nm, M-cones: ~533 nm, L-cones: ~564 nm, respectively, (Ebrey and Koutalos 2001)). Both photoreceptor cell types translate light stimuli into electrical signals through opsins.

Opsins are seven transmembrane spanning integral proteins that enclose a binding pocket for 11-*cis* retinal (Palczewski, Kumasaka et al. 2000). 11-*cis* retinal is the key for the visual process. It is a derivative of retinol (vitamin A) which is obtained from the diet due to lack of endogenous biological synthesis. Exclusion of this compound from the diet can lead to a

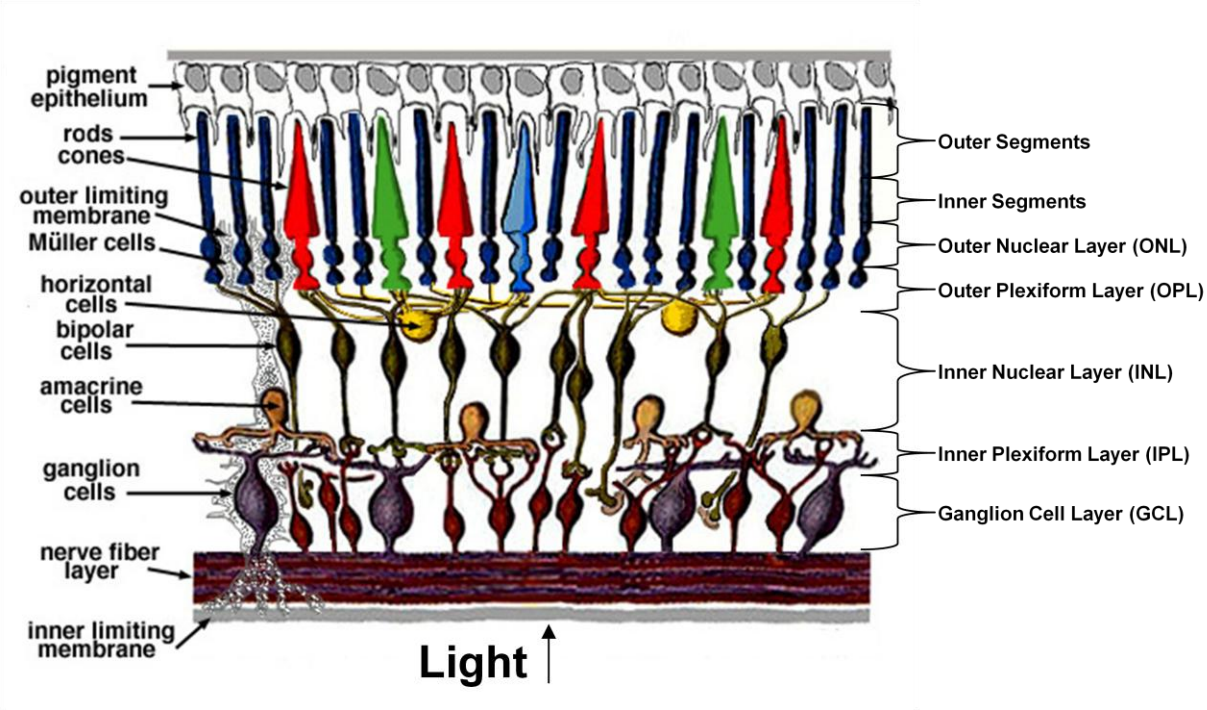


Figure 2-1.2. Diagram of a typical mammalian retina showing its multiple cellular layers. From top to bottom, the retinal layers are as follows; 1) Retinal pigment epithelium (RPE) - mainly responsible for the visual pigment cycle, 2) Photoreceptor layer (PRL, rods and cones), made up of the outer segments (opsin filled discs), inner segments (mitochondria), outer nuclear layer (soma), and outer plexiform layer (synapse) - responsible for light detection, 3) Inner nuclear layer (INL, contains bipolar, horizontal, and amacrine cells) - organize signals from photoreceptor layers. 4) Inner plexiform layer (IPL) - chemical synapse transferring INL information. 5) Ganglion cell layer (GCL) - coordinates signals from INL and involved in central circadian regulation and pupillary light reflex (Picture adapted from http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=webvision.TOC).

condition called night blindness (Dowling and Wald 1959). Once 11-*cis* retinal is isomerized into all-*trans* retinal by light, photoreceptors undergo hyperpolarization, reducing the tonic release of the neurotransmitter glutamate onto the 2nd order neurons in the INL. Photoreceptors synapse with these INL cells in the OPL, transferring the information to the next cellular level for processing.

The bipolar, horizontal and amacrine cells, located in the INL of mammalian retina, are largely responsible for organizing the photoreceptor responses to light into coordinated signals before the information is transferred to the GCL. Bipolar cells, as the name implies, are neurons that have two synapses located at opposing poles of the cell (Fig. 2-1.2). There are several different forms of bipolar cells (9-11 types), each of which carry specific information to the IPL where ganglion cells are awaiting the processed information.

Horizontal cells are mainly responsible for modulating light responses of photoreceptors. They receive input from multiple photoreceptors and synapse directly with cones. This sharpens the photoreceptor's receptive fields by inhibiting adjacent photoreceptors surrounding a particular area. This process is termed lateral inhibition.

Amacrine cells receive the majority of information from cone bipolar cells. Although, bipolar cells form few synaptic connections with amacrine cells, there are synapses formed within the amacrine layer (Freed and Sterling 1988; Calkins, Schein et al. 1994; Jacoby, Stafford et al. 1996). Conversely, amacrine cells also form synaptic connections onto bipolar cells modulating the information sent to the GCL, overall influencing the ganglion cell response to incoming stimuli (Meister, Lagnado et al. 1995). There are many different types of amacrine cells, some producing neuromodulators (e.g., dopamine [DA], acetylcholine, GABA) that work

distally and locally to control cellular function. For example, starburst amacrine cells form excitatory cholinergic synapses (O'Malley, Sandell et al. 1992) within the INL and seem to be important in directional visual sensitivity (He and Masland 1997; Yoshida, Watanabe et al. 2001). Additionally, there are dopaminergic amacrine and interplexiform cells who adjust the retinal network, adapting the mammalian retina to increases in light intensity. The importance of retinal dopamine will be discussed thoroughly in latter sections.

The refined light-response signal reaches the retinal ganglion cell (RGC) layer, which is responsible for transmitting the information to the brain. Like the aforementioned cells, RGCs have receptive fields with on- and off-centers. There are two main types of RGCs (M-cells and P-cells), together these neurons maintain the retina's ability to detect changes in illumination contrasts, movement, color, and acuity.

Even though not part of the neural retina, the RPE is an important cellular layer to the vision process (Fig. 2-1.2). A primary function of the RPE is to phagocytize the photoreceptor discs as they are being shed. Also, the RPE contains the cellular retinoid binding protein (CRBP) (Saari, Bredberg et al. 1982) which delivers retinol to the first visual cycle enzyme in the RPE, lecithin retinol acyl transferase (LRAT) (Saari and Bredberg 1989). After numerous enzymatic steps, the newly generated 11-*cis* retinal enters the photoreceptors where it binds to an opsin completing the visual cycle. When this process breaks down these cells become damaged, which can lead to serious disease states, such as age-related macular degeneration (AMD).

In conclusion, each of these retinal layers is important to the visual process. They communicate with each other through direct synaptic interactions and by release of

neuromodulators. The culmination of the cellular processes works to translate a light stimulus into a coordinated signal, ultimately forming an image.

1.3 **Retinal Dopamine**

1.3.1 Importance of retinal dopamine. DA is one of the key neuromodulators in the central nervous system (CNS), controlling functions such as cognition, emotional feelings, and movement. Abnormalities in this system can manifest into disease states, such as Parkinson's disease (Barbeau 1968), schizophrenia, and mania (Kopin, Kaufman et al. 1976). In retina, DA functions to maintain normal physiological processes and mediates overall retinal light adaptation (Witkovsky 2004). DA regulates numerous photoreceptor layer functions, including: 1) outer segment disc shedding (Besharse et al. 1988), 2) Na^+/K^+-ATPase activity (Shulman and Fox 1996), 3) the balance of rod/cone inputs to horizontal cells (Witkovsky, Stone et al. 1989), and 4) gap junction connectivity between rods and cones (Krizaj, Gabriel et al. 1998). DA also serves as a potent modulator of photoreceptor calcium currents (Thoreson, Stella et al. 2002). These phenomena are DA receptor specific and are presumed to be coordinated by a D2-like receptor (i.e., mouse D_4R) (Cohen, Todd et al. 1992). This section serves to define DA's known roles in mammalian retina from production to utilization.

1.3.2 Retinal dopamine production and release. DA production begins with the amino acid L-tyrosine, which is hydroxylated by tyrosine hydroxylase to form L-dihydroxyphenylalanine (L-DOPA, Fig. 3-1.3). This secondary compound interacts with DOPA decarboxylase to form the active compound of interest, DA (Fig. 3-1.3). Once produced, DA is stored in vesicles by the vesicular monoamine transporter (VMAT) proteins (Witkovsky, Arango-Gonzalez et al. 2005; Edwards 2007).

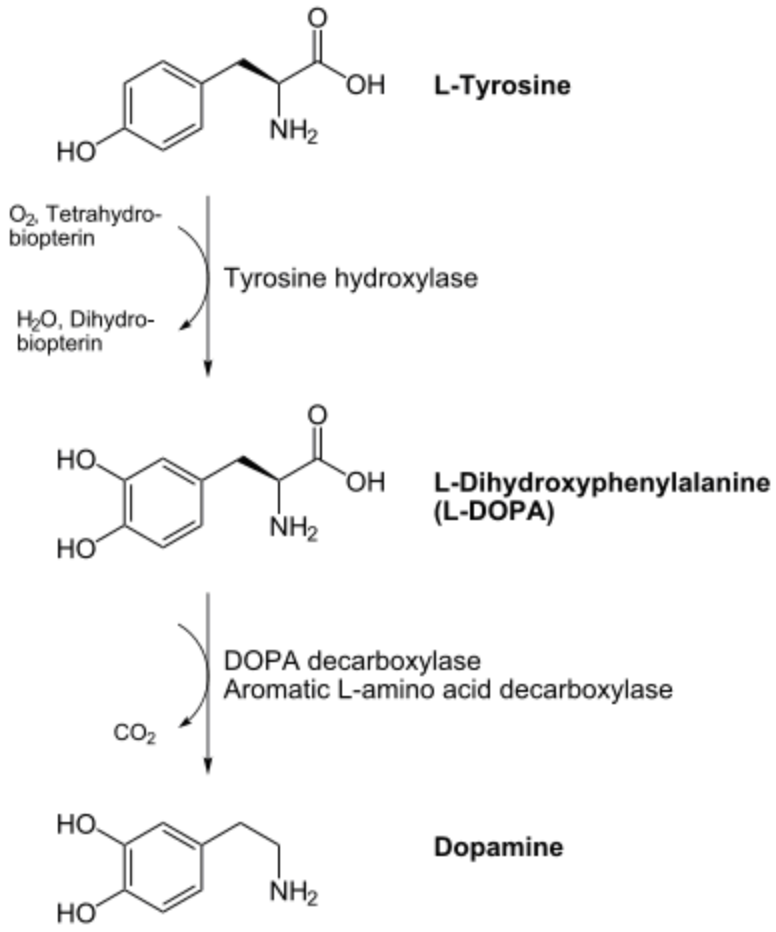


Figure 3-1.3. Dopamine biosynthesis (image adapted from chem4513.pbworks.com)

It remains there until the cell receives a stimulus, enabling the vesicles to fuse with the plasma membrane and release the monoamine from the neuron.

Retinal DA is mainly produced by a small subset of dopaminergic amacrine cells located at the interface between INL and the IPL in the mammalian retina (Fig. 4A/B-1.3). Studies have implicated the rod photoreceptor signaling system in controlling the release of DA from the aforementioned cells (Boatright, Hoel et al. 1989; Besharse and Iuvone 1992). As shown in Figure 4B-1.3, tyrosine hydroxylase (the rate-limiting enzyme in DA synthesis) staining implies DA production occurs in the neuronal soma, as well as throughout the dendritic and axonal arbors. Other references show that RPE cells, located posterior to the neural retina, may produce and secrete DA (Ming, Li et al. 2009). However, it is generally accepted that functional retinal DA is produced and released primarily from amacrine and interplexiform cells.

DA release is under rhythmic control (Nir, Haque et al. 2000), with a burst of release upon light onset. This rhythm is generated in part by melatonin's actions on dopaminergic cells. These rhythmically produced neuromodulators have an antagonistic relationship in retinal physiology, inhibiting the production and release of one another in their respective cell types. Melatonin is mainly produced by photoreceptor cells from the amino acid tryptophan. Its rhythmic expression is coordinated by its penultimate enzyme arylalkylamine N-acetyltransferase (AANAT), which is directly regulated by the core molecular clock (Chen and Baler 2000; Fukuhara, Liu et al. 2004). Melatonin binds to its receptors (MT_1 and MT_2) located throughout the retinal network to promote dark-adaptive effects, and in particular on amacrine and interplexiform cells, where it inhibits DA release (Boatright, Rubim et al. 1994). On the other hand, DA, released via volume transmission at the onset of light promotes light-adaptive effects and inhibits the synthesis and release of melatonin from photoreceptor cells by activating

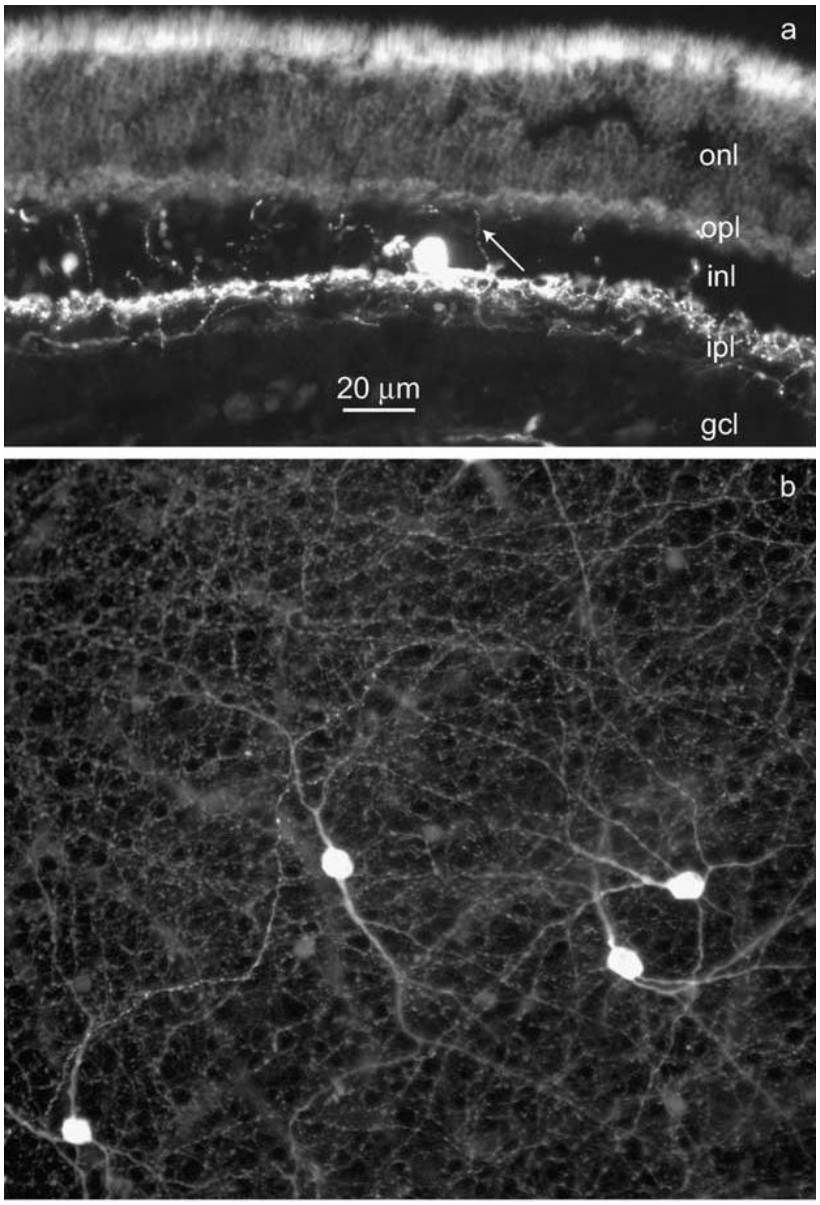


Figure 4A/B -1.3. Tyrosine hydroxylase staining of dopaminergic retinal cells. This diagram shows that the dopaminergic processes seem to be of two kinds: thick and abnormally shaped dendrites with diameter of about 0.5 µm, and many smaller axons extending several mm from the cell body (Dacey 1990; Witkovsky 2004). Most of these processes extend along the horizontal plane of the distal IPL (Fig. 4A-1.3), near the OFF bipolar cell termination region (Famiglietti and Kolb 1976). Image adapted from Witkovsky 2004.

D_2-like receptors (Zawilska and Iuvone 1992; Nguyen-Legros, Chanut et al. 1996; Tosini and Dirden 2000). These findings imply that at least two cell types in the retina contain circadian oscillators (i.e., photoreceptors and dopaminergic cells). However, the issue of circadian oscillator localization has been greatly debated in the literature, which will be discussed in detail later in this chapter.

1.3.3 Dopamine receptor subtypes in the retina. Once released, DA binds to dopamine receptors located throughout the retinal network facilitating biochemical responses (e.g., increases/decreases in cAMP). There are five known dopamine receptor subtypes; dopamine D_1, D_2, D_3, D_4, and D_5 receptors, all of which are G-protein coupled receptors. They are divided into two subclasses: the D1-like receptors (D_1R and D_5R), that when activated increase the intracellular cellular levels of cAMP by stimulating adenylyl cyclases (AC), and the D_2-like receptors (D_2R, D_3R, D_4R). Activation of D_2-like receptors decreases the amount of cAMP in the cell by inhibiting AC, except D_3R, in the case where its heterotrimeric g-protein alpha subunit is of the G_z subtype that increases cAMP (Dearry, Gingrich et al. 1990; Dearry, Falardeau et al. 1991). Each subtype has been identified in mammalian retinas, except for the D_3R (Appendix I).

Numerous studies, using autoradiography and immunocytochemistry, have sought to define the location of the receptor subtypes. D_1Rs are largely present in the INL and GCL (Veruki and Wassle 1996; Nguyen-Legros, Simon et al. 1997). Dopaminergic amacrine and interplexiform cells also express D_2-like receptors (Veruki 1997). Using flourescent ligands Muresan et al. (1997) demonstrated that photoreceptors in amphibians solely express D_2-like receptors. Others have shown that mammalian photoreceptors solely express the D_4R subtype (Cohen, Todd et al. 1992) and these receptors control a light-sensitive pool of cAMP in

photoreceptors (Nir, Harrison et al. 2002). It is noted that D_4Rs are found throughout each retinal layer, but the photoreceptors only express this subtype (Cohen, Todd et al. 1992). Studies localizing D_5R show they are expressed mainly in the RPE (Versaux-Botteri, Gibert et al. 1997). Each subtype has unique pharamcodynamics and kinetics producing distinct biological responses to DA stimulation. In the future, more sensitive and selective techniques will enable better detection and localization of dopamine receptor subtypes in the mammalian retina, further detailing DA's complete function.

There are few dopaminergic cells located in the mammalian retina, making direct synaptic connections with most retinal cells numerically difficult. Therefore, most actions of DA are mediated by volume transmission (Witkovsky 2004), reaching distant retinal cells that are many microns away from its site of release. With the help of extensive processes, dopaminergic cells provide DA to the most distal portions of the OPL even though a defined synapse is not formed (Dacey 1990; Kolb, Cuenca et al. 1990; Savy, Moussafi et al. 1995). Therefore, DA interacts with the retinal layers, solely through a DA receptor mediated system, ultimately helping the retina adjust to light onset.

1.3.3.1 Dopamine D_4R signaling. One focus of this thesis is to elucidate additional unknown key players in the D_4R signaling pathway as they are related to retinal function. However, there is a breath of information that has previously defined many aspects to this signaling system. It is noted that most discoveries involving the D_4R signaling come from heterologous expression systems (e.g. HEK293T, CHO, etc.), therefore some results may conflict with *in vivo* investigations.

In humans, the D_4R is polymorphic, most extensively at the 3rd intracellular loop (IC3). The IC3 contains a region of variable tandem repeats (VNTR), in which a 48-bp sequence exists

as a 2- to 11-fold repeat named D4.2 to D4.11 (Rondou, Haegeman et al. 2010). These VNTRs could possibly give rise to differential regulation, thereby activating alternative signaling pathways. As mentioned previously, the D_4R is in the D2-like family of dopaminergic receptors and these receptors are generally $G_{i/o}$-coupled, which reduce cellular cAMP levels by inhibiting AC. Studies have shown that the VNTRs can play a role in the potency of the polymorphic D_4R variants (Asghari, Sanyal et al. 1995) in coupling to AC. A goal of this work is to define which adenylyl cyclase is coupled to the D_4R in mouse retina, providing additional information into how mammalian retinas use DA to function.

cAMP, created from adenosine triphosphste (ATP) by AC, is a paramount second messenger which propagates external stimulus information into a useable intracellular signal. It influences the activity states of such factors as protein kinase A (PKA) and DARPP-32 (Hemmings, Greengard et al. 1984). Each of these factors modulates downstream factors that lead to regulation of transcription. For example, D_4R signaling has been shown to activate NF-κB (transcription factor, (Zhen, Zhang et al. 2001)), Kruppel-like factor-2 (quiescence regulator, (Sarkar, Das et al. 2006)), and AP-1 (transcription factor, (Bitner, Nikkel et al. 2006)), all activating gene transcription. In the PRL it is known that D_4R activation leads to a decrease in a light-sensitive pool of cAMP (Nir, Harrison et al. 2002), that most likely affects the retinal transcriptome upon activation. Moreover, in rat retina and chick retinal cells (Ivanova and Iuvone 2003; Iuvone, Tosini et al. 2005) it has been shown that cAMP levels are rhythmic, providing a potential avenue for a rhythmic gene regulation system.

In the retina the D_4R signaling system controls many aspects of retinal physiology, of which some were stated in the introduction to this section (e.g., outer segment disc shedding and rod/cone coupling, Ch. 1.3.1). Many of these biological processes are rhythmic and have a direct

relationship with DA. It is clear that DA binds to different dopamine receptors throughout the retinal layers; however, it is unclear what exact physiological effect DA has on the different cell types in the retina (e.g. Does DA function to modulate circadian rhythms?). Knowing more about this system will help us understand how the retina adapts to changes in illumination.

1.4 Circadian Rhythms

1.4.1 Biological Clock. Circadian rhythms are endogenous biological fluctuations of physiology, driven by a molecular mechanism, paced near a 24 hour cycle. These biological clocks, which are present in a range of species from bacteria to humans, provide an adaptive advantage, ensuring that an organism's internal biochemistry, physiological processes, and behavior are optimal for their immediate environment. The first reported account of the existence of a biological clock was Jean-Jacques d'Ortous deMairan's (1729) description of the relationship between internal physiology and the solar cycle. However, it was not until 1935 that Erwin Bunning published data showing evidence of the first biological clock demonstrating that period length is heritable in bean plants. These observations and many other experiments have led to the discovery of a transcriptional/post-translational feedback loop system that drives ~24 hour biological rhythms in fungi , plants, insects, and mammals (Fig. 5-1.4).

There are two known types of daily biological rhythms - diurnal and circadian. Both rhythms oscillate with a *period* ~ 24 hours long and are entrained by external cues, such as light exposure, temperature change, or feeding. These stimuli are categorized as zeitgebers (German for *time- giver*), that reset the output rhythm according to its own onset of action. A main difference between diurnal and circadian rhythms is that diurnal rhythms only persist as long as

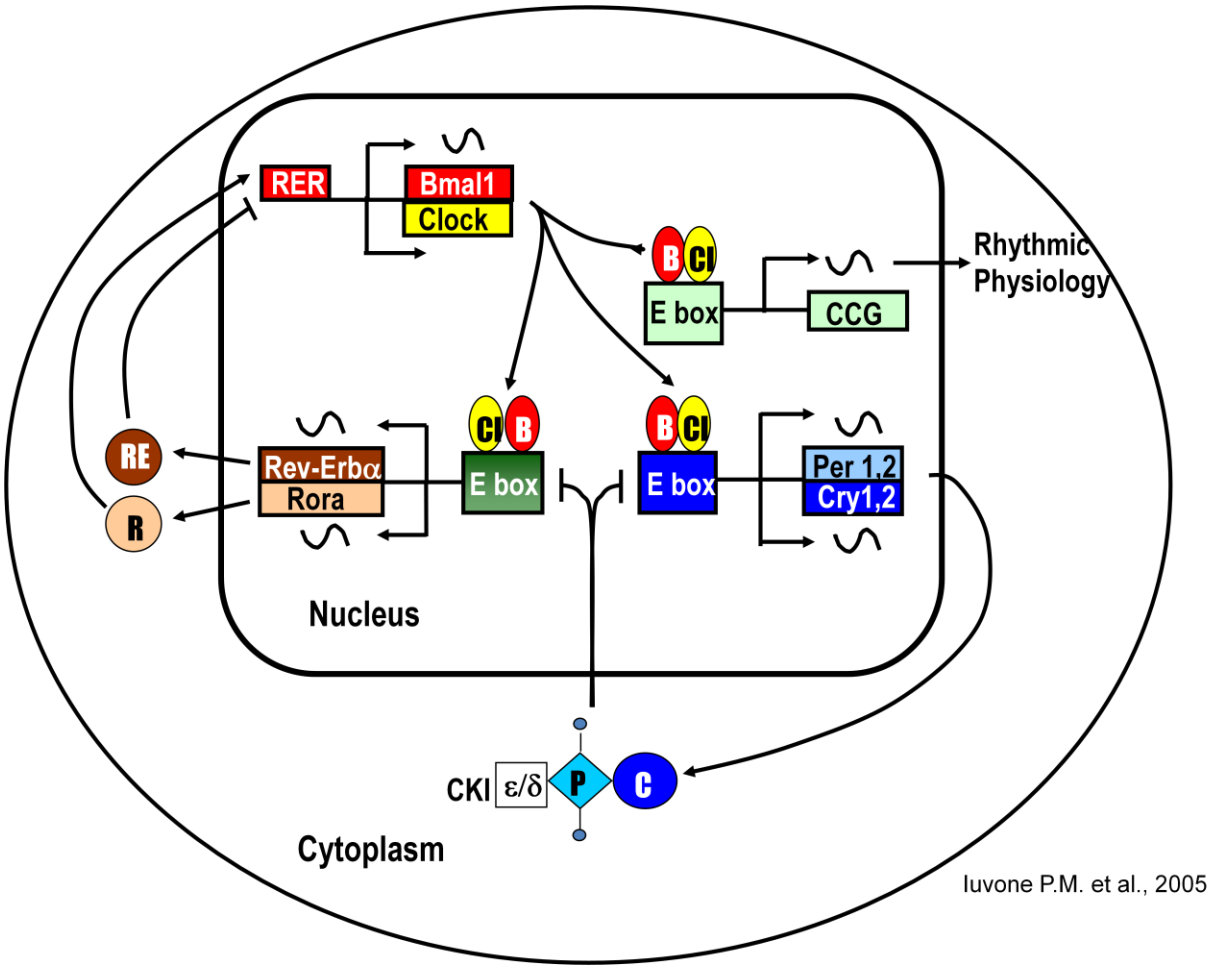


Figure 5-1.4. Molecular circadian clock. Core circadian clock-two loop translation/transcriptional feedback system. This figure depicts the basic molecular clock mechanism that drives all biological circadian rhythms in mammals.

they are synchronized to the zeitgeber. When the external stimulus is removed, the rhythm is abolished or becomes arrhythmic. In order for a biological process to be termed "circadian", its overt observable rhythmic output (e.g., opening/closing of flower petals, fluctuations in gene expression, etc.) must persist in absence of the zeitgeber, such as constant darkness (referred to as DD) or absence of feeding cues. In constant conditions, the time needed for one circadian oscillation to occur is known as the free-running period (FRP). The majority of these types of studies are based on measuring an animal's activity (i.e., wheel-running activity or sleep onset (Zulley, Wever et al. 1981)). However, activity is not the only output response used to observe circadian clocks. Currently, gene expression, protein levels, phosphorylation states, and cell growth are just a few parameters that can be evaluated to test circadian oscillators.

In mammals, light is the overbearing zeitgeber driving circadian biology. The suprachiasmatic nucleus (SCN), located in the anterior hypothalamus, is termed *master clock* because it mainly functions to transmit retinal environmental light timing cues to the peripheral oscillators in mammalian tissues via polysynaptic and humoral pathways. Light information is relayed from the retina to the SCN via the retinohypothalamic tract (RHT), and serves to coordinate and synchronize the rhythmic timing of this tissue. Individual SCN neurons are capable of generating self-sustained oscillations (Welsh, Logothetis et al. 1995); however, it is the SCN neuron conglomerate that encodes luminance history and generates rhythmic output signals organizing physiology (*e.g.*, sleep/wake cycle, mating, etc.).

1.4.2 Molecular clock. The sleep/wake cycle, body temperature, and mating are all examples of observable circadian outputs that are ultimately driven by a cellular biological clock. In 1971 there was a leap in the circadian field when a genetic basis for rhythms generation was determined by looking for the persistence of circadian behaviors (*i.e.*, pupal eclosion and

locomotor activity) in mutagenized *Drosophila melanogaster* (Konopka and Benzer 1971). This study determined that the mutant phenotypes presented abnormal period lengths due to a single locus, known now as the *Period* gene. Similar studies in many other models, including mammals, have determined that circadian physiology is modulated by two interlocking transcription-translation feedback loops (Fig. 5-1.4).

Circadian locomotor output cycles protein kaput (CLOCK) and Brain and Muscle ARNT-like protein (BMAL1) are the positive effectors of the first loop of the mammalian clock. Both are basic helix-loop-helix (bHLH)-PAS domain-containing transcription factors that form heterodimers in the cytoplasm, enter the nucleus (Kwon, Lee et al. 2006), and bind to E-box sequences of the promoters of genes. When these transcription factors bind to E-box sequences (canonical sequence of CACGTG) they drive expression of clock-controlled genes (CCG), such as AANAT mRNA, and the negative effectors of the first loop: Period (in mice, *Per1*, *Per2*, and *Per3*) and Cryptochrome (*Cry1* and *Cry2*) genes (Kume, Zylka et al. 1999; Iuvone, Tosini et al. 2005). *Per* and *Cry* transcripts, once translated into protein in the cytoplasm, heterodimerize and are transported into the nucleus where they inhibit the activity of the CLOCK/BMAL1 heterodimer (Fig. 5-1.4), thereby reducing the amount of *Per* and *Cry* genes being transcribed. Further decline of this signal is mediated by phosphorylation of the *PER/CRY* protein complex by Casein Kinase 1δ and Casein Kinase 1ε (Loudon, Meng et al. 2007). This event triggers proteasomal degradation of the *Per1/2* and *Cry1/2* complex, enabling the CLOCK/BMAL1 complex to drive subsequent gene expression.

The second loop of the molecular clock regulates the expression of the *Bmal1* gene. The CLOCK/BMAL1 complex drives the expression of secondary regulatory factors, REV-ERBα and RORα (retinoic acid-related orphan nuclear receptors), that compete for the ROR elements

in the *Bmal1* promoter (Sato, Panda et al. 2004). The ROR family of proteins activate the rhythmic expression of *Bmal1*, while the REV-ERB family acts as repressors. This view of the molecular clock is an oversimplified model; however, it serves to demonstrate the most important and basic components of the system. Post-translational events, such as proteasomal degradation, phosphorylation, acetylation, ubiquitination, and sumoylation are also key components in the generation and timing of circadian rhythms (Gatfield and Schibler 2007; Sehgal 2008).

The culmination of these transcription factors' activities regulates circadian biology from molecular mechanisms to overall behavior. Deregulation of the molecular clock system causes severe disease phenotypes, such as depression (Soria, Martinez-Amoros et al. 2010), sleep disorders (Ebisawa 2007), and even cancer (Stevens 2009). In mammals, numerous studies have shown that peripheral tissues, outside the CNS, contain circadian clocks. For example, liver (Lamia, Storch et al. 2008), muscle (McDearmon, Patel et al. 2006), and fibroblast (Murphy, Vick et al. 2006) all have been shown to contain circadian oscillators. Not to be excluded, the retina also contains endogenous circadian rhythms that can be observed from the molecular expression to functional electroretinogram (ERG) levels (Cameron and Lucas 2009). Recent studies have shown that disruption of the endogenous retinal clocks causes abnormalities in cellular responses to light stimulation (Storch, Paz et al. 2007).

1.4.3 Retinal Circadian Rhythms. The retina is a heterogenous mixture of cell types, all with specialized functions. Many of these functions are maintained by the circadian clock and themselves show rhythmic activity, for example outer segment disc shedding and cone-ERGs (Goldman, Teirstein et al. 1980; Cameron and Lucas 2009). Therefore, it is to be expected that each cell type is unique in regulation of its transcriptome, as well as its circadian clock gene

expression. Defining which cell type(s) contain the required circadian elements to produce functional circadian oscillators has been the subject of many investigations. It is believed these oscillators are responsible for coordinating and maintaining circadian retinal physiology.

While most biological rhythms are modulated by the SCN it seems that the retina controls its own rhythmic physiology by light and endogenous neuromodulators. One of the first demonstrations that a clock exists in the retina was the finding that rod outer segment disc shedding has a relationship with cyclic lighting conditions (LaVail 1976). Additionally, many experiments performed in frog *Xenopus laevis* and chicken retinas has provided evidence of a circadian clock (Cahill and Besharse 1993; Pierce, Sheshberadaran et al. 1993; Thomas, Tigges et al. 1993), with possible localization in the photoreceptor cells. However, it was not until 1996 that cultured mammalian retinas were reported to maintain circadian release of melatonin, providing solid evidence that an endogenous clock is present in the mammalian retina (Tosini and Menaker 1996).

In early research, retinal degenerative animal models (e.g., *rd* mouse and Royal College Surgeons [RCS] rat) were studied to localize which cell types contain circadian oscillators. Early in the lives of these animals their photoreceptors degenerate, leaving melatonin production/release arrhythmic; however, daily rhythms of DA and its metabolites levels are maintained in the absence of photoreceptors (Tosini and Menaker 1998; Doyle, McIvor et al. 2002). Initially, it was concluded that photoreceptors were necessary for maintaining rhythmic melatonin production, but were not required for maintaining DA rhythms. However, it was subsequently noted that there is photoreceptor-loss compensation; AANAT mRNA is upregulated in the INL and remains rhythmic (Sakamoto, Liu et al. 2004), which possibly could

explain the persistence of the DA rhythm. These data suggest that at least three retinal cell types can contain circadian clocks and that the clock is an integral part of retinal physiology.

A standard indication of a functional circadian clock is the presence of the core clock genes in a given cell type. There have been many creative approaches assaying for core clock genes (*i.e., Bmal1, Clock, Per1&2, Cry1&2*) as direct evidence of the location of these circadian oscillators in retinal cell types. Using *in situ* hybridization, laser capture microdissection (LCM), and RT-PCR with rodent models, several investigators have provided evidence that clock genes are located in the photoreceptor and inner nuclear cells (Namihira, Honma et al. 1999; Namihira, Honma et al. 2001; Tosini, Davidson et al. 2007). However, localizing circadian oscillator(s) in mouse retina has been more difficult with conflicting results obtained in different studies. First, it has been well established that INL cells of mouse contain a circadian oscillator. Ruan et al. (2006) used single cell RT-PCR to detect robust expression of the core clock genes in inner retinal neurons and dopaminergic cells. Similar findings were observed in Gustincich et al. (2004) in genetically labeled dopaminergic neurons. Next, photoreceptors have been the site for the most controversy because initially it was reported that, in mice, *Per1*, *Clock*, and *Bmal1* mRNAs were present in photoreceptors cells (Gekakis, Staknis et al. 1998). Subsequent studies confirmed (Yujnovsky, Hirayama et al. 2006), while others failed to confirm that *Per1* was expressed in mouse photoreceptors (Witkovsky, Veisenberger et al. 2003; Storch, Paz et al. 2007). Ruan et al. (2006) found that photoreceptors expressed the core clock genes; however, no single neuron expressed detectable levels of all six of the core clock genes, implying that these cells do not contain the necessary components to create a functional oscillator. Clock gene expression levels are unique to each cell type; therefore not detecting each clock component may be due to low transcript levels and the limits of the detection system.

These data do not parallel or explain the occurrence of the circadian phenomena (e.g., outer segment disc shedding, rod/cone coupling, cone-mediated ERGs, etc.) that persist without a "functional" clock in the photoreceptor layer. One thought is that the rhythmic DA binding to the D_4R on photoreceptors cells affects or coordinates the rhythmic behavior of the PRL, as well as the whole retina. Also, it has been shown that the D_4R mRNA transcript (*Drd4*) is rhythmic in PRL of the rat retina (Klitten, Rath et al. 2008). This data provides evidence that the D_4R exist rhythmically in the mammalian retina. If this is true then this receptor expression pattern and DA stimulation could serve as a regulatory site for intercellular circadian signaling in photoreceptors.

1.4.4 Overall hypothesis. It is known that DA modulates retinal light adaptive mechanisms and affects circadian rhythms. Also, it affects photoreceptor signals, uncouples horizontal and amacrine cells gap junctions, and it is important for overall retinal development (Witkovsky 2004). In the photoreceptor layer DA activates D_4Rs, independently reducing a light sensitive pool of cAMP (Nir, Harrison et al. 2002). Investigation of mice lacking the D_4R (*Drd4*$^{-/-}$) shows that cAMP in these retinas is unresponsive to light and have lower basal cAMP levels, suggesting an irregularity in the cAMP signaling system. Therefore, I hypothesize that in the *Drd4*$^{-/-}$ mouse retina there is a deregulation in expression of adenylyl cyclase(s). Several retinal processes (e.g., rod/cone coupling, photoreceptor cAMP level, etc.), that are modulated by dopamine, display rhythmic behavior. Previous findings have lead to the formation of the hypothesis that if DA released at dawn, by amacrine and interplexiform cells, binds to D_4Rs inhibiting adenylyl cyclase(s) reducing cAMP; thereby, affecting the retinal transcriptome and resetting the retinal clock at dawn (Fig. 6-1.4).

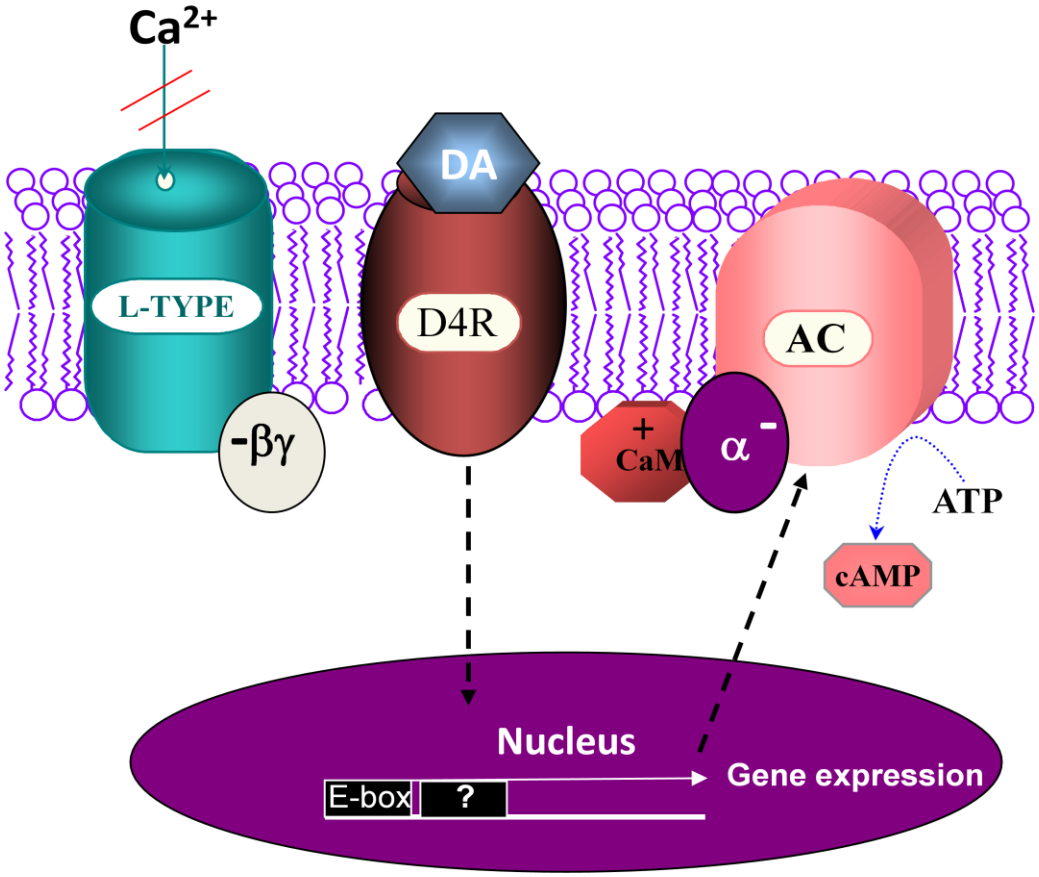


Figure 6-1.4. Central hypothesis. A burst of dopamine is released at the onset of daily light. Dopamine, via volume transmission, activates dopamine receptors throughout the retinal network, but in the PRL it only activates the D_4R, thereby inhibiting L-type Ca^{2+} channels and reducing cAMP by inhibiting adenylyl cyclase(s). Once activated it affects the retinal transcriptome, including the transcript(s) encoding adenylyl (AC), and resets the retinal circadian clock.

Chapter II:

Essential roles of dopamine D_4 receptors and the type 1 adenylyl cyclase in photic control of cyclic AMP in photoreceptor cells[1]

[1] This chapter has been published. Jackson, C.R., Chaurasia, S.S., Zhou, H., Haque, R., Storm, D.R., Iuvone, P.M. Essential roles of dopamine D4 receptors and the type 1 adenylyl cyclase in photic control of cyclic AMP in photoreceptor cells (2009). J. Neurochem. 109, 148-157

2.1 Summary. Light and dopamine regulate many physiological functions in the vertebrate retina. Light exposure decreases cyclic AMP formation in photoreceptor cells. Dopamine D_4 receptor (D_4R) 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 D_4R-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 D_4R activation. Similarly, in mice with disruption of the gene (*Drd4*) encoding D_4R, 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 D_4R antagonist, L-745,870. Thus, activation of D_4R 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.

2.2 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 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 D_4 receptor (D_4R) subtype from the D_2 family of receptors, suppresses the light-sensitive pool of cyclic AMP in dark-adapted mouse photoreceptors(Cohen and Blazynski 1990; Cohen, Todd 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 D_4R 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 D_4R activation; however, in mice lacking D_4Rs (*Drd4*$^{-/-}$ mice), cyclic AMP levels were significantly lower in darkness and unresponsive to light when compared with wild-type controls. These observations suggested that D_4R 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, Gan 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, Stella et al. 2002; Ivanova, Alonso-Gomez 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, Choi et al. 1993). Therefore, we examined the potential role of AC1 in mediating the light- and D_4R-evoked reductions in cAMP, and investigated which components of the cyclic AMP signaling system are aberrant when D_4R signaling is disrupted.

2.3 Materials and Methods

2.3.1 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 D_4 receptors (Rubinstein, Phillips et al. 1997), *Adcy1*$^{-/-}$ mice, lacking the type 1 adenylyl cyclase (Wu, Thomas et al. 1995), and normal control [wild-type (WT)] mice, all on a C57BL/6J background, were studied. Mice were genotyped by 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 killed by cervical dislocation. All samples were collected in the morning, unless otherwise specified, immediately frozen on dry ice, and stored at −80°C.

2.3.2 *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 $CaCl_2$, 0.8 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, St. Louis, MO, USA), 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, Todd et al. 1992). The retinas were incubated in darkness at 37°C in an atmosphere of 95% O_2/5% CO_2 for 5 min in a water bath. Subsequently, retinas were incubated in darkness or light for 10 additional minutes. To access the effects of D_4R activation, retinas were incubated with or without PD 168077, a D_4R-specific agonist (Glase, Akunne et al. 1997), for 15 min 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, MA, USA).

2.3.3 Adenylyl cyclase assay. Partially purified membranes were prepared by homogenizing whole mouse retina in 10 mM Tris-maleate, pH 7.5, 1 mM $MgSO_4$, 1.2 mM EGTA, 0.5 mM dithiothreitol and Protease Inhibitor Cocktail III (EMD Biosciences, La Jolla, CA, USA) henceforth referred to as TME buffer. Homogenates were centrifuged at 20,000 X 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, Rosebrough et al. 1951), using bovine serum albumin as a standard. Adenylyl cyclase activity was assayed by assessing the conversion of [α-^{32}P] ATP to [^{32}P]cyclic AMP (Salomon, Londos et al. 1974) with minor modifications. The final reaction mixture contained 80 mM Tris maleate, pH 7.5, 10 mM GTP, 1 mM dithiothreitol, 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, [α-^{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% trichloroacetic acid containing [^{3}H]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 [^{3}H] for recovery of internal standard and the other channel measuring [^{32}P]-labeled product.

2.3.4 Acute and sub-chronic drug treatment. A selective dopamine D_4 receptor antagonist, L-745,870 (Patel, Freedman et al. 1997), was administered for 1 or 6 days by subcutaneous injection (1 mg/kg, s.c.) 30 min before light onset in the morning. Control animals were injected with vehicle, 0.3% carboxymethylcellulose (Sigma-Aldrich). The rationale for injecting drug before light onset was to block D_4Rs prior to the burst of dopamine release that occurs at light onset (Nir, Haque 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.

2.3.5 Western blotting. Retinas were homogenized in 50 mM Tris-HCl, 50 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 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 (Bio-Rad Laboratories, Hercules, CA, USA). The proteins on the gel were transferred to polyvinylidene difluoride membrane using a semi-dry method. CaM was detected using a mouse monoclonal IgG1 antibody (05–173, Upstate, Lake Placid, NY, USA, 1 : 500) plus horseradish peroxidase-labeled goat anti-mouse IgG (1 : 5000, sc-2005, Santa Cruz Biotechnology, Santa Cruz, CA, USA) 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.

2.3.6 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, USA). RT was performed on total RNA (2 μg) preparations using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) 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 2 μL cDNA, 1X SYBR Green PCR Master mix (Bio-Rad) and 300 nM intron-spanning gene specific forward and reverse primers in a Bio-Rad iCycler (Bio-Rad). Primer sequences for real-time PCR are provided in Table 1. 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 s 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.

2.3.7 Laser capture microdissection. Whole mouse eyes were embedded in OCT (Tissue-Tek, Torrance, CA, USA), 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 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 (Arcturus Engineering, Mountain View, CA, USA) staining solutions for 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) and cleared in xylene

Table 1-2.3 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

(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, Emmert-Buck et al. 1997; Parlato, Rosica 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, Fukuhara et al. 2004).

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

2.3.8 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 Student's t-test.

2.4 Results

2.4.1 Identification of the adenylyl cyclase isoform regulated by light and D_4R activation. Light exposure and D_4R 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 ~ fivefold ($p < 0.001$) 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.

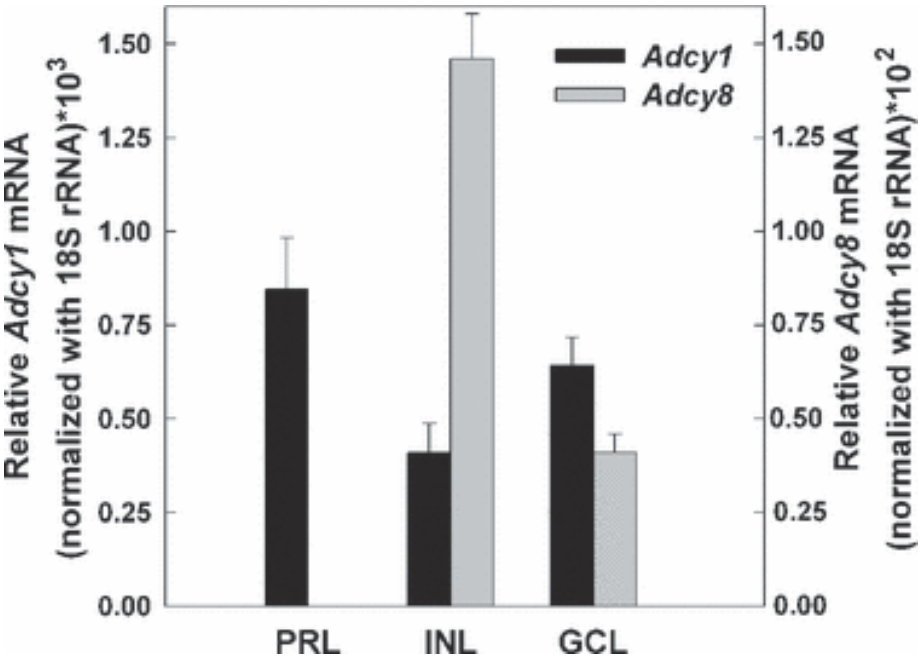
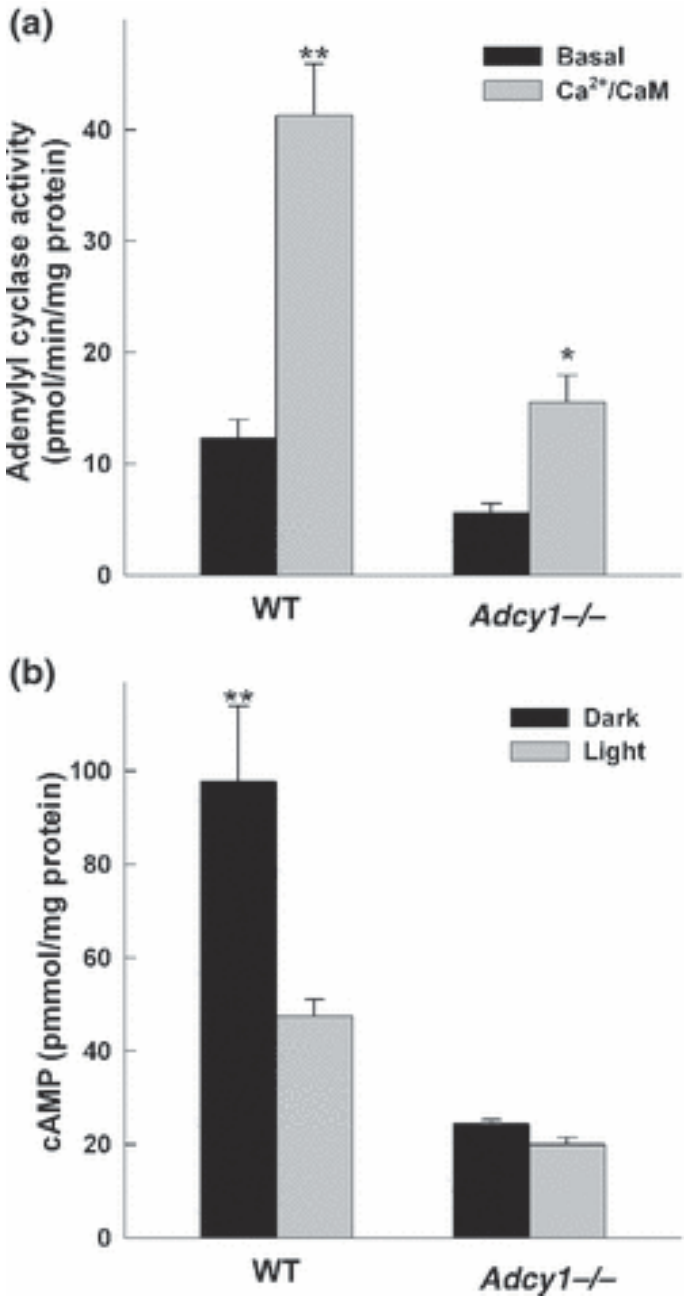


Fig. 1-2.4. Expression of *Adcy1* and *Adcy8* transcripts in retinal layers isolated by laser capture microdissection (LCM). The ganglion cell layer (GCL), inner nuclear layer (INL), and photoreceptor layer (PRL; inner segments and outer nuclear layer) were captured from frozen sections of retina as described in Material and methods. *Adcy1* and *Adcy8* mRNA levels, normalized to 18S rRNA, were determined by real-time RT-PCR. n = 4 retinas per group, except for *Adcy8* GCL, where n = 3.

Fig. 2-2.4. 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 Material 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 Material 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.


(a)
Adenylyl cyclase activity (pmol/min/mg protein)
0
10
20
30
40
**
*
Basal
Ca2+/CaM
WT
Adcy1–/–
(b)
cAMP (pmmol/mg protein)
0
20
40
60
80
100
**
Dark
Light
WT
Adcy1–/–

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 D_4R, measuring cyclic AMP accumulation in vehicle and D_4R agonist (PD 168077) treated retinas from WT, *Adcy1*$^{-/-}$, and *Drd4*$^{-/-}$ mice. Incubation of dark-adapted WT retinas with PD 168077 resulted in a concentration-dependent decrease of cyclic AMP accumulation, with an EC_{50} of 30 nM (Fig. 3a). The effect of PD 168077 was absent in *Drd4*$^{-/-}$ mouse retinas, confirming involvement of D_4Rs (data not shown). Similarly, treatment with a supra-maximal concentration (3 μM) of PD 168077 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 D_4R activation control cyclic AMP production in mouse retina via AC1.

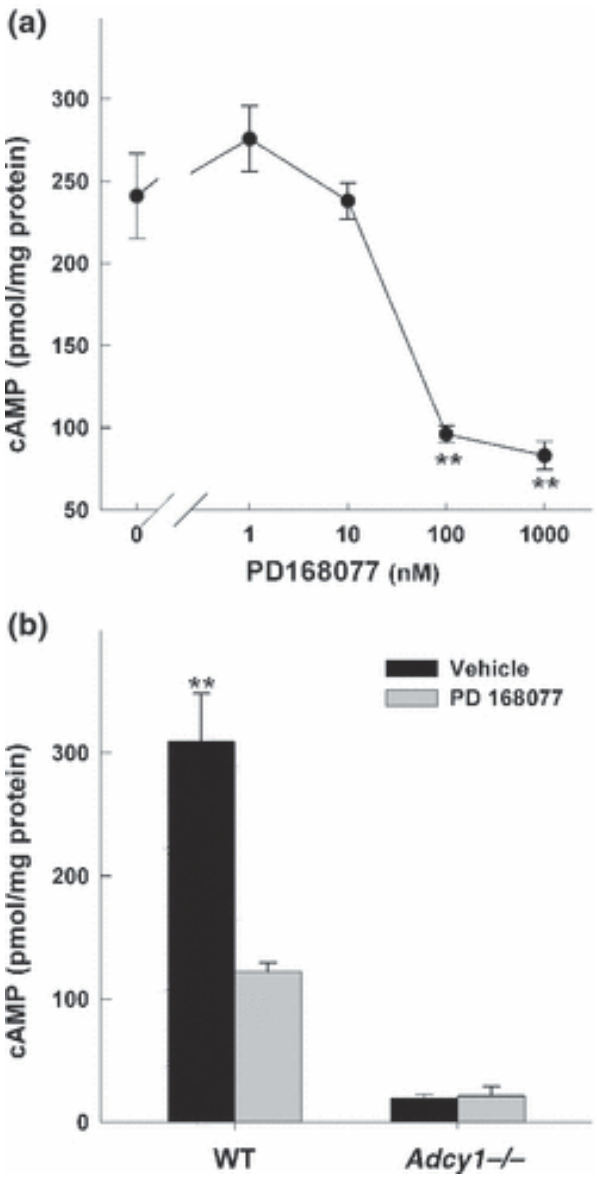


Fig. 3-2.4. PD 168077, a D_4R agonist, inhibits cyclic AMP accumulation in WT but not *Adcy1*$^{-/-}$ retinas. (a) PD 168077 inhibits cyclic AMP accumulation during a 15 min incubation with an EC_{50} value of 30 nM. $^{**}p < 0.001$ vs. control. (b) In the presence of PD 168077 (3 μM) there is a significant reduction in cyclic AMP accumulation in WT, but not in *Adcy1*$^{-/-}$ retinas. $n = 4$ per group; $^{**}p < 0.001$ vs. all other groups.

2.4.2 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, Harrison 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 D_4Rs (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 D_4R 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. Figure 6 shows 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; $p = 0.006$).

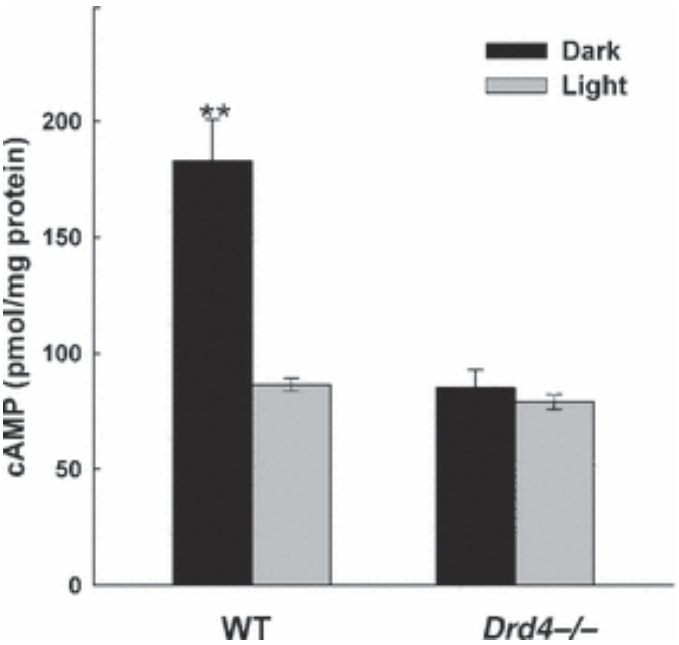


Fig. 4-2.4. Light decreases cyclic AMP accumulation in retinas of WT but not *Drd4*$^{-/-}$ mice. Retinas were incubated in dark or light as described in Fig. 2b. $n = 4–6$; $^{**}p < 0.001$ vs. all other groups.

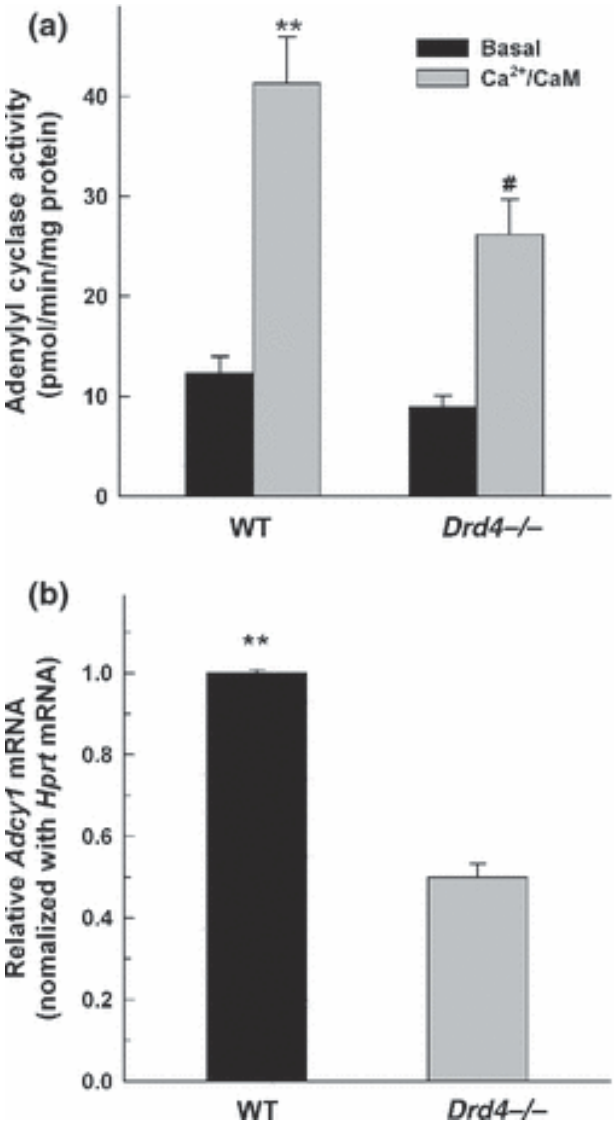


Fig. 5-2.4. Ca^{2+}/CaM-stimulated adenylyl cyclase activity and *Adcy1* mRNA levels in retinas of WT and *Drd4*$^{-/-}$ mice. (a) Ca^{2+}/CaM stimulated adenylyl cyclase activity is significantly lower in the *Drd4*$^{-/-}$ retinal membranes compared to WT controls. $n = 9$ per group; $^{**}p < 0.001$ 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. $n = 6$ per group; $^{**}p < 0.001$ vs. *Drd4*$^{-/-}$.

To further explore the mechanisms responsible for the decrease in cyclic AMP accumulation in retinas of D_4R 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, Kerns 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 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. S1-2.4). *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.

2.4.3 Effect of D_4R antagonism on *Adcy1* expression. To investigate the role of D_4R activation in the expression of *Adcy1*$^{-/-}$ retinas, the effects of a D_4R-selective antagonist, L-745870 (Patel, Freedman et al. 1997), were examined in WT mice. L-745870 was administered

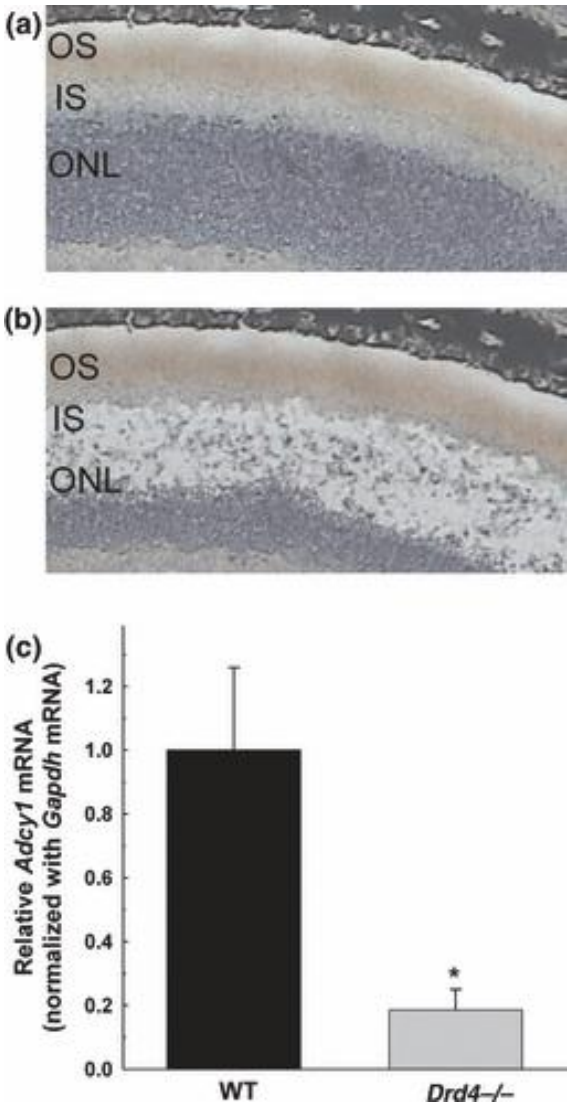


Fig. 6-2.4. Decreased expression of *Adcy1* mRNA in photoreceptors of *Drd4*$^{-/-}$ mouse retina. Photoreceptor inner segments and cell bodies were isolated by laser capture microdissection (LCM) as described in Methods. (a) Representative retinal section before dissection. (b) Retinal section after capture of photoreceptor 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.

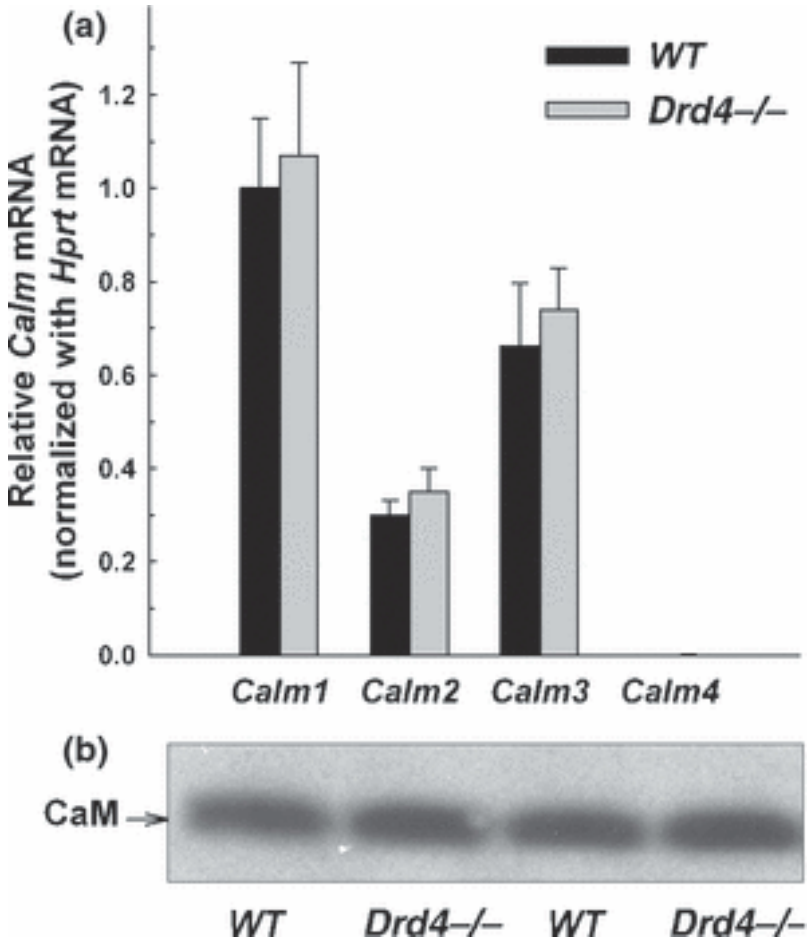


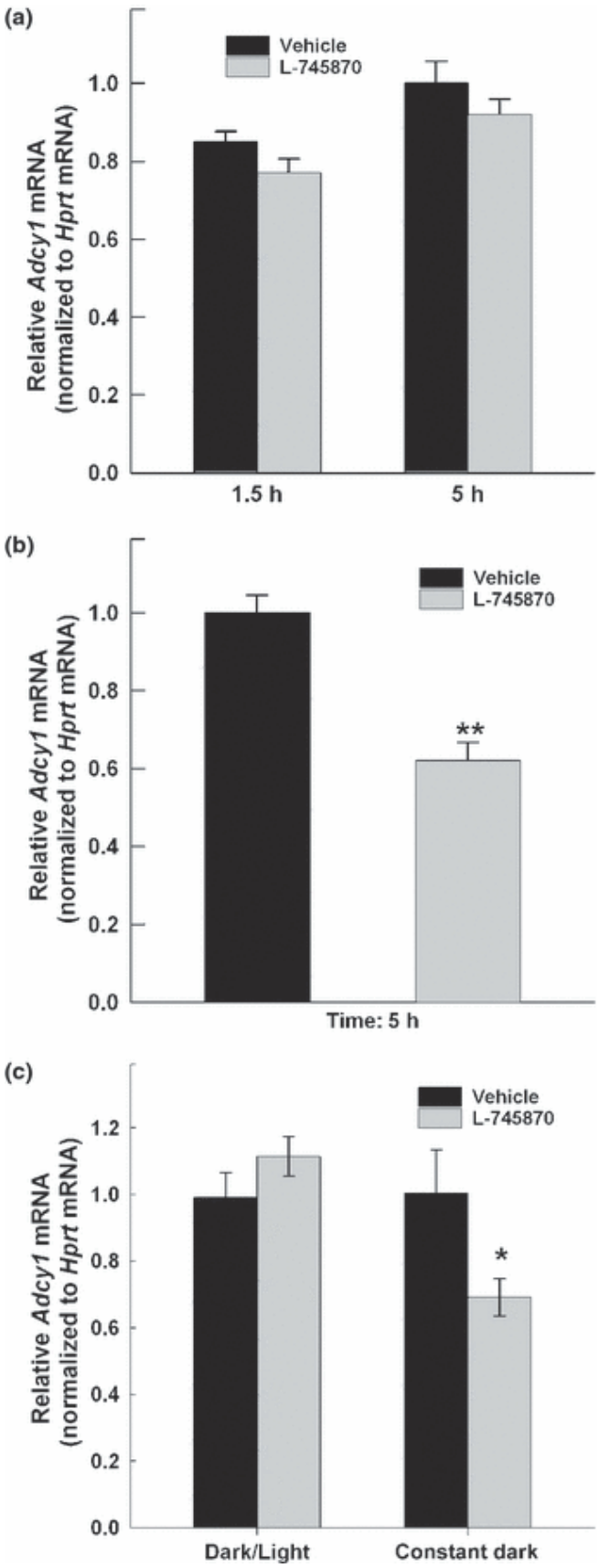
Fig. 7-2.4. Calmodulin expression in wild-type and *Drd4*$^{-/-}$ mouse retina. (a) *Calm1-4* transcripts normalized to *Hprt* mRNA and expressed relative to WT *Calm1* mRNA. The values are means ± SEM (n = 6). (b) Western blot analysis of calmodulin protein from the wild-type and *Drd4*$^{-/-}$ mouse retina. The blot shown is representative of an n of 6 from two separate experiments.

30 min 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, Haque et al. 2000). Following a single injection, L-745870 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-745870 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-745870 for 6 days. In mice exposed to light in the morning for 4–5 h 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-745870-treated mice than in the vehicle controls (Fig. 8c; $p < 0.05$). These observations suggest that D_4R activation tonically supports *Adcy1* expression.

2.5 Discussion

Dopamine D_4 receptors are highly expressed in retina relative to other central nervous system structures (Oak, Oldenhof et al. 2000) and appear to be the only dopamine receptor subtype expressed by photoreceptors (Cohen, Todd et al. 1992). Activation of these receptors decreases a light-sensitive pool of cyclic AMP in mouse photoreceptors (Cohen and Blazynski 1990; Cohen, Todd et al. 1992; Nir, Harrison et al. 2002). The absence of D_4Rs has physiological consequences that manifest as impaired light adaptation and a decreased rate of dark adaptation following light exposure (Nir, Harrison 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

Fig. 8-2.4. Effect of L-745870 on *Adcy1* transcript expression in WT mouse retina. Mice were injected subcutaneously with L-745870 (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 L-745870 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-745879 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 (b), 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.


(a)
Vehicle
L-745870
Relative Adcy1 mRNA
(normalized to Hprt mRNA)
1.0
0.8
0.6
0.4
0.2
0.0
1.5 h
5 h
(b)
Vehicle
L-745870
Relative Adcy1 mRNA
(normalized to Hprt mRNA)
**
Time: 5 h
(c)
Vehicle
L-745870
Relative Adcy1 mRNA
(normalized to Hprt mRNA)
1.2
*
Dark/Light
Constant dark

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 D_4Rs compromises light-evoked dephosphorylation of the protein kinase A site of phosducin (Pozdeyev, Tosini et al. 2008), a regulatory phosphoprotein that binds to transducin-βγ subunits (Lee, Lieberman 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 D_4Rs, inhibits Na^+/K^+-ATPase in rat rod photoreceptors (Shulman and Fox 1996). Dopamine increases the rate of rhodopsin dephosphorylation in frog retina (Udovichenko, Newton et al. 1998) and PKA has been shown to phosphorylate the γ-subunit of cGMP phosphodiesterase purified from bovine rod outer segments (Xu, Tanaka et al. 1998). Also, D_4R activation regulates melatonin synthesis in photoreceptors by decreasing cyclic AMP formation (Iuvone 1986; Iuvone and Besharse 1986; Zawilska and Nowak 1994; Zawilska, Derbiszewska et al. 1995). Dopamine and cyclic AMP affect rod and cone photoreceptor Ca^{2+} currents in salamander (Stella and Thoreson 2000), photoreceptor retinomotor movements in *Xenopus* and fish (Besharse, Dunis et al. 1982; Pierce and Besharse 1985; Hillman, Lin 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, Harrison et al. 2002), confirmed in the present study, suggest that D_4R 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 D_4R, and to determine if expression of the cyclase or related signaling

components is aberrant when D_4R 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 D_4Rs 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 cannot 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, Stella et al. 2002; Ivanova, Alonso-Gomez 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 D_4R activation is less well understood, but may involve a G_i/G_0-dependent inhibition of voltage-gated channels (Mei, Griffon et al. 1995), dopamine receptor-mediated interactions of Ca^{2+} and Cl^- channels (Thoreson, Stella et al. 2002), or modulation of Ca^{2+} channels by cyclic AMP-dependent phosphorylation (Stella and Thoreson 2000). Inhibition of AC1 activity by D_4R may also involve direct effects of G_i, G_o, or transducin (Yamaguchi, Harmon et al. 1997; Oak, Oldenhof et al. 2000).

Disruption of *Drd4* or subchronic antagonism of D_4Rs with L-745870 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 D_4Rs, presumably because of the reduced expression of AC1. These results emphasize the importance of AC1 in the light-evoked reduction of cyclic AMP and suggest that D_4R activation plays a significant role in regulating the expression of the cyclase.

The decreased expression of *Adcy1* mRNA following L-745870 treatment suggests that D_4R 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 D_4Rs regulate expression of the Ca^{2+}/CaM-stimulated adenylyl cyclases is presently unknown. In pinealocytes, increasing cyclic AMP levels inhibits *Adcy1* gene expression (Chan, Lernmark et al. 2001). The decrease of cyclic AMP levels in response to D_4R 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 D_4R also contains Src homology 3 binding domains (Oldenhof, Vickery et al. 1998) and D_4R stimulation activates Akt/nuclear factor kappa-B signaling, increases extracellular signal-regulated kinases 1/2 phosphorylation, and induces

cFOS (Zhen, Zhang et al. 2001; Bitner, Nikkel et al. 2006). Thus, D_4R 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. D_4R 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.

Chapter III:

Dopamine D_4 receptor activation controls circadian timing of the adenylyl cyclase 1 / cyclic AMP signaling system in mouse retina

3.1 Summary. In the mammalian retina, dopamine binding to the dopamine D_4 receptor (D_4R) affects a light-sensitive pool of cyclic AMP by negatively coupling to the type 1 adenylyl cyclase (AC1). AC1 is the primary cyclase controlling cyclic AMP production in dark-adapted photoreceptors. Previous studies from our laboratory demonstrated that expression of the gene encoding AC1, *Adcy1*, is down-regulated in mice lacking *Drd4*, the gene encoding the D_4R. The present investigation provides evidence that D_4R 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 Ca^{2+}-stimulated adenylyl cyclase activity and cyclic AMP levels were observed. However, these rhythmic activities were dampened or undetectable in mice lacking the D_4R (*Drd4*$^{-/-}$). Pharmacologically activating the D_4R 4 hrs before its normal stimulation at light onset in the morning advances the phase of *Adcy1*'s expression pattern. These data demonstrate that stimulating the D_4R is essential in maintaining the normal rhythmic production of AC1 from transcript to enzyme activity. Thus, dopamine/D_4R signaling is a novel zeitgeber that entrains the rhythm of *Adcy1* expression and, consequently, modulates the rhythmic synthesis of cyclic AMP in mouse retina.

3.2 Introduction. The dopaminergic signaling system is a key feature in retinal processing controlling melatonin production in photoreceptor cells (Cahill, Grace et al. 1991), Na/K ATPase activity (Shulman and Fox 1996), rod-cone coupling (Ribelayga, Cao et al. 2008), horizontal cell and AII amacrine cell coupling (Baldridge, Vaney et al. 1998), and light adaptation (Nir, Harrison et al. 2002). Dopamine (DA) is synthesized and released from a small set of amacrine and/or interplexiform cells. Its synthesis and release are stimulated by light and extracellular DA diffuses throughout the retina to activate D_1-like (D_1 and D_5) receptors and D_2-like (D_2 and D_4) receptors, each contributing to unique biological processes (reviewed by Witkovsky, 2004).

Dopamine may play important roles in regulating circadian physiology in the retina. Dopaminergic amacrine cells in mouse retina express circadian clock genes (Gustincich, Contini et al. 2004; Ruan, Zhang et al. 2006; Dorenbos, Contini et al. 2007). Dopamine D_4 receptor (D_4R) activation suppresses a light-sensitive pool of cyclic AMP in retina (Cohen and Blazynski 1990; Cohen, Todd et al. 1992) by negatively coupling to the type 1 adenylyl cyclase (AC1), the primary cyclase controlling cyclic AMP production in mouse retina (Jackson et al. 2009). In chickens and rats, cyclic AMP production in retina fluctuates in a circadian pattern (Ivanova and Iuvone 2003; Fukuhara, Liu et al. 2004). Cyclic AMP can reset or entrain circadian rhythms in the rat suprachiasmatic nucleus (Prosser and Gillette, 1989) and Aplysia eye (Eskin et al., 1982; Eskin and Takahashi, 2003). Activation of dopamine D2-like receptors, probably D_4Rs, on *Xenopus* photoreceptors phase shifts the circadian rhythm of melatonin release (Cahill and Besharse, 1991). Activation of D_1 receptors sets the phase of a circadian clock in the inner retina of the mouse (Ruan et al., 2008), and targeted deletion of the D_2 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). These observations lead us to hypothesize that dopamine, acting in part through cyclic AMP, coordinates circadian rhythms in retinal physiology.

Dopamine metabolism and 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. In mice, the control of dopamine by circadian clocks is strain-dependent (Nir et al., 2000; Doyle et al., 2002; Pozdeyev et al., 2008). Diurnal and circadian rhythms of *Drd4* mRNA levels have been observed in mouse and rat retina (Storch, Paz et al. 2007; Bai, Zimmer et al. 2008; Klitten, Rath et al. 2008). Recently, our lab discovered dual roles for D_4R activation in retina: inhibition of cyclic AMP formation by AC1 and regulation of the transcript level of *Adcy1*, which encodes AC1 (Jackson, Chaurasia et al. 2009). Circadian rhythms of *Adcy1* mRNA expression in retina have been observed in several vertebrate species (Fukuhara et al., 2004; Chaurasia et al., 2006), including mouse (Storch et al., 2007). Thus, the present study was conducted to investigate a possible role for dopamine and the D_4R in the circadian regulation of AC1 expression and activity in mouse retina.

3.3 Materials and Methods

3.3.1 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 D_4 receptors (*Drd4*$^{-/-}$, Rubinstein et al. 1997) and wild-type mice, all on a C57Bl/6 background, were used for this investigation. Mice were genotyped by PCR 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. Mice were approximately 3 months of age. 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.

3.3.2 RNA isolation, quantification, and quantitative real-time PCR. Total RNA was extracted from whole retina using the Qiagen 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). 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 μL total volume with 2 μL 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). 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.

3.3.3 *In vivo* agonist and antagonist experiments. Mice were injected subcutaneously for 6 six days with a selective D_4R antagonist, L-745,870 (Patel et al. 1997) at 1 mg/kg, 30 min before light onset. The drug and control were prepared in 0.3% carboxymethylcellulose (Sigma-

Aldrich, St. Louis, MO). Retinas were dissected following the 6th day of injections at ZT 5, ZT 10, ZT 15, and ZT 20. RNA was isolated and assayed for *Adcy1* expression.

WT mice were injected intraperitoneally with PD 168,077 (1mg/kg, Tocris, Ellisville, MO), a selective D_4R 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 (DD) 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 light/dark cycle, were treated with a single injection of PD 168,077 administered at ZT 20, to determine if there is an acute effect of the drug. PD 168,077 was dissolved in dimethyl sulfoxide (DMSO), and then diluted with sterile phosphate buffered saline to a final DMSO concentration of approximately 1%.

3.3.4 Retinal cyclic AMP accumulation. WT and *Drd4*$^{-/-}$ mice were kept in DD and dissected at various times of subjective day or subjective night. Retinas were placed in cold, oxygenated Earle's salt solution (115 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 26 mM $NaHCO_3$, 5.5 mM glucose) at pH 7.4, equilibrated with 95% O_2/5% CO_2. Individual retinas where transferred to cell culture plates containing Earle's salt solution supplemented with 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma, St. Louis, MO, USA). The retinas were incubated in darkness at 37°C in an atmosphere of 95% O_2/5% CO_2 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).

3.3.5 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 Ca^{2+}/calmodulin-stimulated adenylyl cyclase activities were measured by assessing the conversion of [α-^{32}P] ATP to [^{32}P]cyclic AMP by partially purified retinal membranes, as described previously (Jackson, Chaurasia et al. 2009).

3.3.6 Statistical analysis. Analysis of multiple group data was performed using one-, two-, and three-way analysis of variance (ANOVA, SigmaStat) with Student–Newman–Keuls 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).

3.4 Results

3.4.1 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; $P < 0.001$). 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, transcript levels were higher overall compared to those in LD (Fig. 1; $P < 0.001$ LD vs. DD2). 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; $P < 0.001$).

3.4.2 Dopamine D_4 receptors and circadian cyclic AMP synthesis in retina. Cyclic AMP accumulation in mouse retina, measured in DD, occurs as a circadian rhythm with levels being lower during the subjective day and rising significantly during the early subjective night (Fig. 2;

P <0.001), peaking at ~ CT 14. In mouse photoreceptors, cyclic AMP levels are controlled by light, dopamine and D_4R (Cohen, Todd et al. 1992; Nir, Harrison et al. 2002). Interestingly, cyclic AMP accumulation in retinas of *Drd4*$^{-/-}$ mice was significantly lower at all times of subjective day and subjective night ($p<0.05$) and displayed no detectable rhythm (Fig. 2). These data suggest that D_4R expression is necessary to maintain normal cyclic AMP levels as well as the circadian rhythm of its synthesis.

Previous studies from our lab showed that D_4Rs signal through AC1 and that AC1 is the primary cyclase controlling cyclic AMP production by mammalian retina in darkness (Jackson, Chaurasia et al. 2009). In addition, the level of *Adcy1* mRNA, which encodes AC1, fluctuates as a circadian rhythm in retinas of chick, rat, and mouse with peak expression during the subjective day (Fukuhara, Liu et al. 2004; Chaurasia, Haque et al. 2006; Storch, Paz et al. 2007). Hence, 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 ($p<0.001$; Fig. 3). In contrast, *Adcy1* expression in *Drd4*$^{-/-}$ retinas showed no statistically significant rhythm and transcript levels in the knock out were significantly lower at ZT 8 ($p<0.001$) and ZT 20 ($p<0.002$) compared to that in WT retinas (Fig. 3).

To further test the role of D_4Rs in rhythmic *Adcy1* expression, WT mice were treated for 6 days with the D_4R antagonist, L745,870 (1 mg/kg, i.p.), administered approximately 30 min prior to the time of light onset of the daily light-dark cycle. The rationale for administering the drug at this time of day was to block the D_4Rs before the light-evoked increase of dopamine release upon light onset. When assessed in constant darkness on the sixth day of drug treatment,

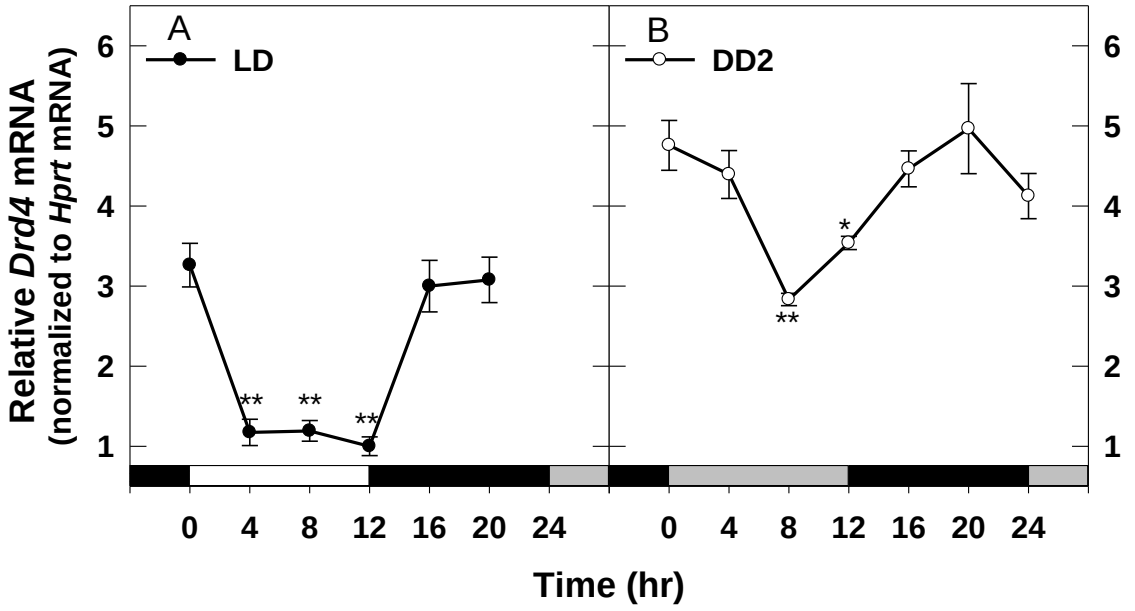


Fig. 1-3.4. Diurnal and circadian rhythms of *Drd4* mRNA expression in mouse retina. *Drd4* mRNA levels were determined by real-time RT-PCR as described in Materials and methods under light/dark (LD; panel A) and constant dark (DD2; panel B) conditions. *Drd4* transcript levels show significant rhythms (*P≤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. Two-way ANOVA indicated significant effects of *time* (P<0.001) and *lighting conditions* (P<0.001), and a significant interaction of *time* and *lighting conditions* (P<0.001). Data expressed as means ± SEM, n = 5-7 mice per group. White bars on the X-axis represent daytime in light; gray bars represent subjective daytime in darkness; black bars represent night or subjective night in darkness.

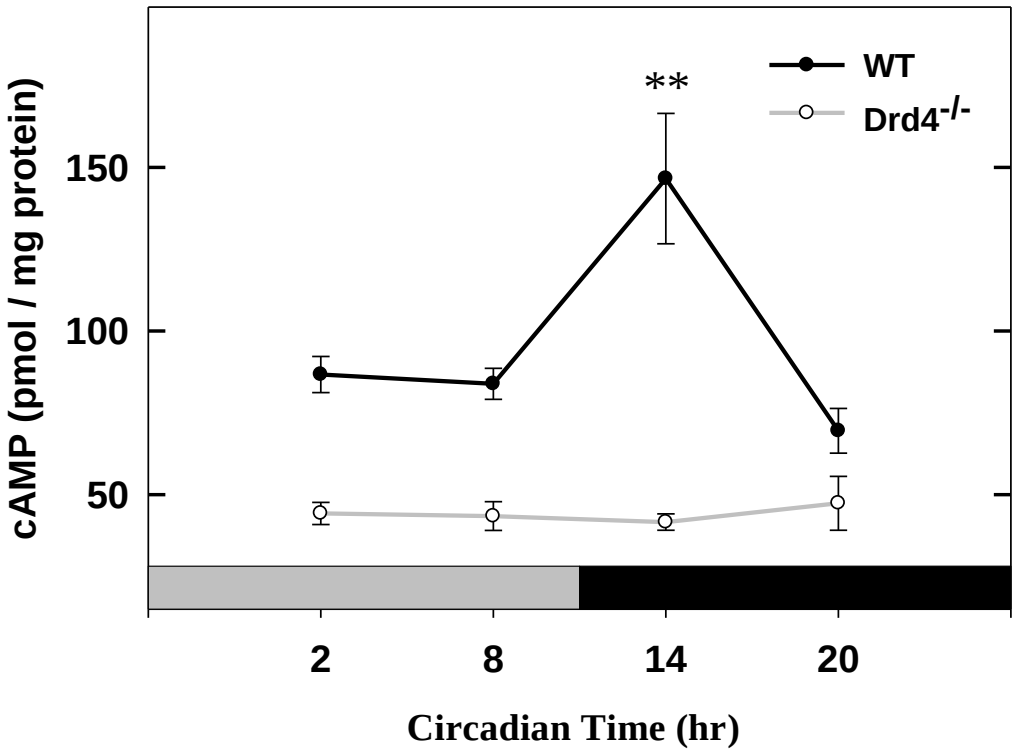


Fig. 2-3.4. Cyclic AMP accumulation is circadian in retina of WT mice (P<0.001) but not in retina of *Drd4*$^{-/-}$ mice. Cyclic AMP accumulation was measured in constant darkness (DD) as described in Materials and methods. Two-way ANOVA showed significant effects of *time* (P<0.001), and *genotype* (P<0.001), and a statistically significant interaction of *time* and *genotype* (P<0.001). *P<0.001 *vs* all other groups in WT and *Drd4*$^{-/-}$ mice; $^{\Delta}$P < 0.05 *vs Drd4*$^{-/-}$ at the same time-of-day. Data expressed as means ± SEM, n = 4-6 per group.

a significant rhythm in *Adcy1* mRNA levels was observed in the vehicle-treated mice ($p<0.001$) but not in those treated with L745,870 (Fig. 4). AC1 is a Ca^{2+}/calmodulin (CaM)-stimulated adenylyl cyclase. Adenylyl cyclase activity was assayed in retinal membranes to determine if there is a rhythm in Ca^{2+}/CaM-dependent enzyme activity in WT mouse retina and, if so, if the rhythm is disrupted in retinas of *Drd4*$^{-/-}$ mice. In WT samples, Ca^{2+}/CaM increased cyclase activity at both ZT 4 and ZT 16 (Fig. 5, $p<0.001$), but the increase at ZT 16 was significantly larger than that at ZT 4 ($p<0.001$); no significant difference in basal activity was observed between the two time points. 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. Ca^{2+}/CaM-stimulated activity was nearly abolished in *Drd4*$^{-/-}$ samples compared to WT controls. Moreover, there was no difference in basal or Ca^{2+}/CaM-stimulated activity between ZT 4 and ZT 16 in the *Drd4*$^{-/-}$ samples.

Collectively these data indicate that the D_4R plays a critical role in regulating the rhythmic expression of AC1 at the transcript and protein levels, thereby maintaining normal levels and circadian synthesis of cyclic AMP.

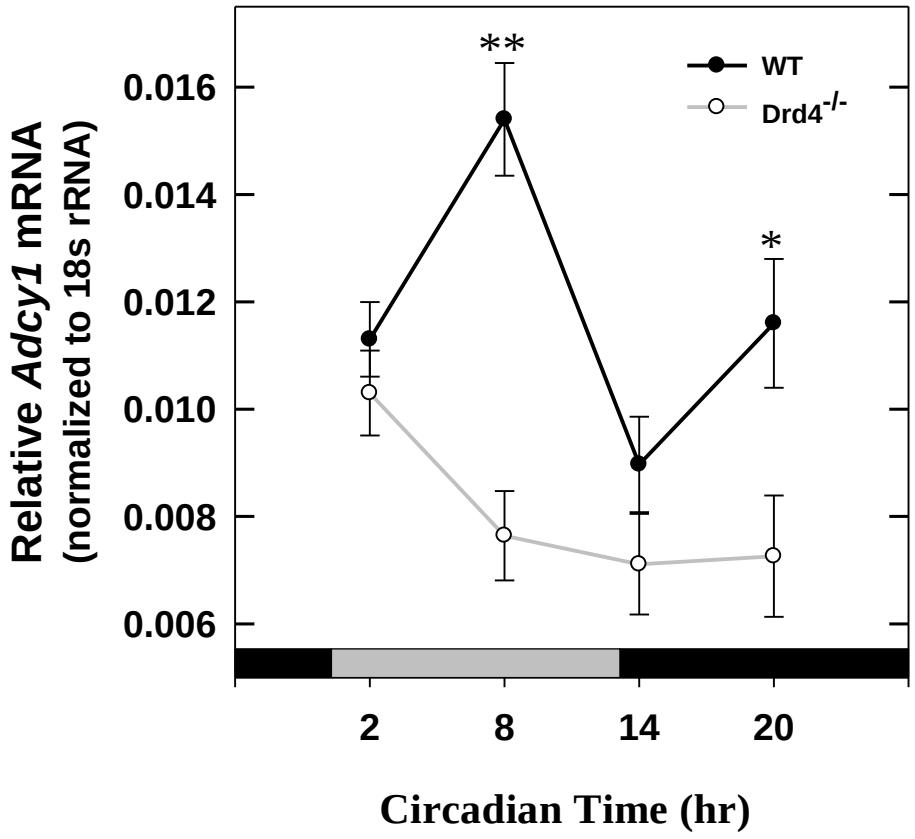


Fig. 3-3.4. Comparison of rhythmic expression of *Adcy1* mRNA in *Drd4*$^{-/-}$ retinas compared to WT controls. *Drd4*$^{-/-}$ mRNA levels in retinas dissected in DD were determined by real-time RT-PCR as described in Materials and methods. Two-way ANOVA showed significant effects of *time* (P=0.004), and *genotype* (P<0.001), and a statistically significant interaction of *time* and *genotype* (P=0.005). 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 ± SEM, n = 6 per group.

Fig. 4-3.4. Effect of treatment with the D_4R antagonist, L-745,870, on the *Adcy1* transcript rhythm. Mice 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 DD 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. Two-way ANOVA showed significant effects of *time* ($P<0.001$), *treatment* ($P<0.001$), and a statistically significant interaction of *time* and *treatment* ($P<0.001$); n= 5-6 per group at each time. *$P<0.01$, **$P<0.001$ *vs* Vehicle at the same time of day.

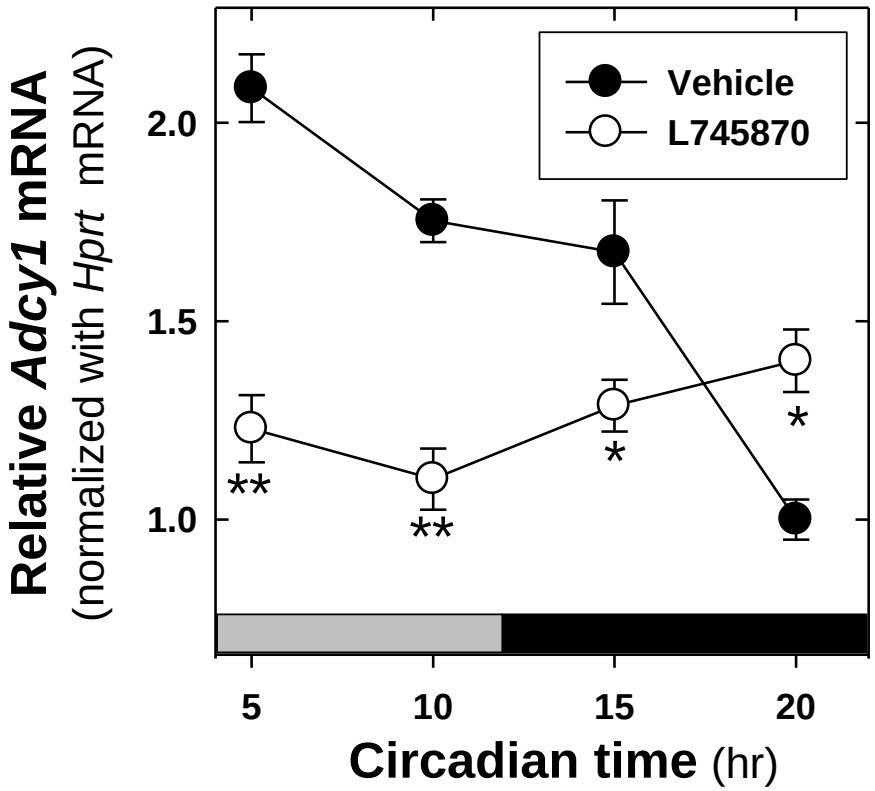
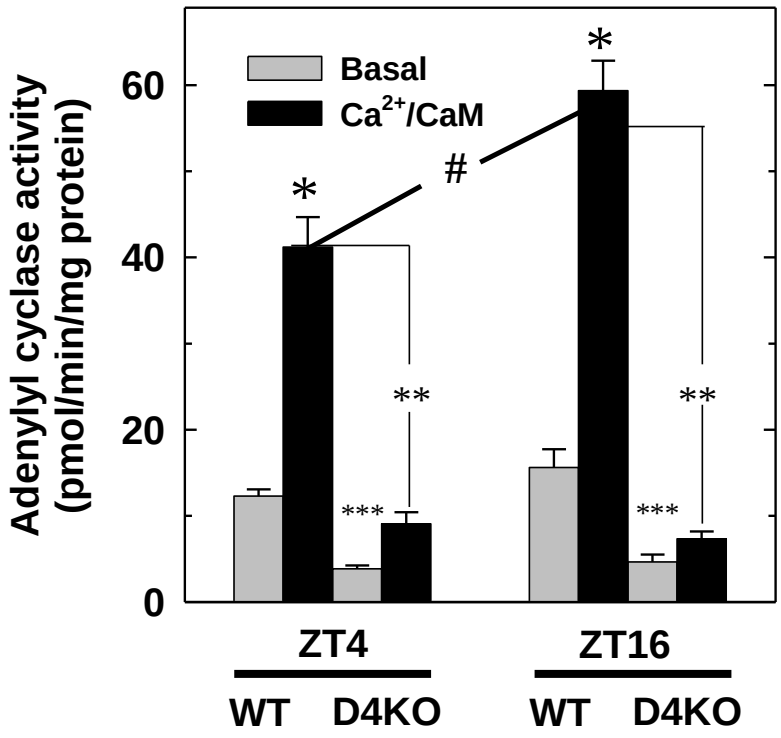

Relative Adcy1 mRNA
(normalized with Hprt mRNA)
2.0
1.5
1.0
Vehicle
L745870
**
**
*
*
5
10
15
20
Circadian time (hr)

Fig. 5-3.4. Adenylyl cyclase activity in *Drd4*$^{-/-}$ mouse retina at ZT 4 and ZT 16 compared with WT controls. Basal and Ca^{2+}/CaM-stimulated adenylyl cyclase were determined in retinal membranes as described in Materials and methods. **ZT 4 groups:** Ca^{2+}/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 stimulated in the *Drd4*$^{-/-}$ samples (***$P < 0.05$). **ZT 16 group**; Ca^{2+}/CaM-stimulated activity in WT samples showed a significant increase compared to basal levels (*$P < 0.001$). Ca^{2+}/CaM-stimulated activity in *Drd4*$^{-/-}$ were 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**: Ca^{2+}/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 determined by three-way ANOVA and post-hoc Student-Newman-Keuls method, n= 6 per time and group.


Adenylyl cyclase activity
(pmol/min/mg protein)
60
40
20
0
Basal
Ca2+/CaM
*
*
#
**
**
***
***
ZT4
ZT16
WT
D4KO
WT
D4KO

3.4.3 Dopamine D_4 receptor activation modulates rhythmic behavior of *Adcy1* expression.

Based on the results showing *Adcy1* mRNA expression is rhythmic, controlled by the D_4R in mouse retina, and attenuated in mice lacking D_4Rs, we asked whether activation of the D_4R at an atypical time of day could change its normal expression pattern, thereby causing a phase shift in its rhythm. Previous studies have shown that dopamine can affect *Period* gene expression in retina (Steenhard and Besharse 2000; Ruan, Zhang et al. 2006; Yujnovsky, Hirayama et al. 2006). Also, there is a large increase of retinal dopamine synthesis and release at the onset of light in the morning (Nir et al. 2000), suggesting that dopamine may act as an entrainment stimulus re-setting retinal clocks at dawn each day. To test this hypothesis we injected mice with PD 168,077 4 hrs before the normal spike in dopamine release. 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. 6). 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 CT 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 D_4R activation can phase shift the *Adcy1* rhythm. As a control, 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. Acute stimulation of D_4R did not cause a significant increase in *Adcy1* gene expression (Fig. 7).

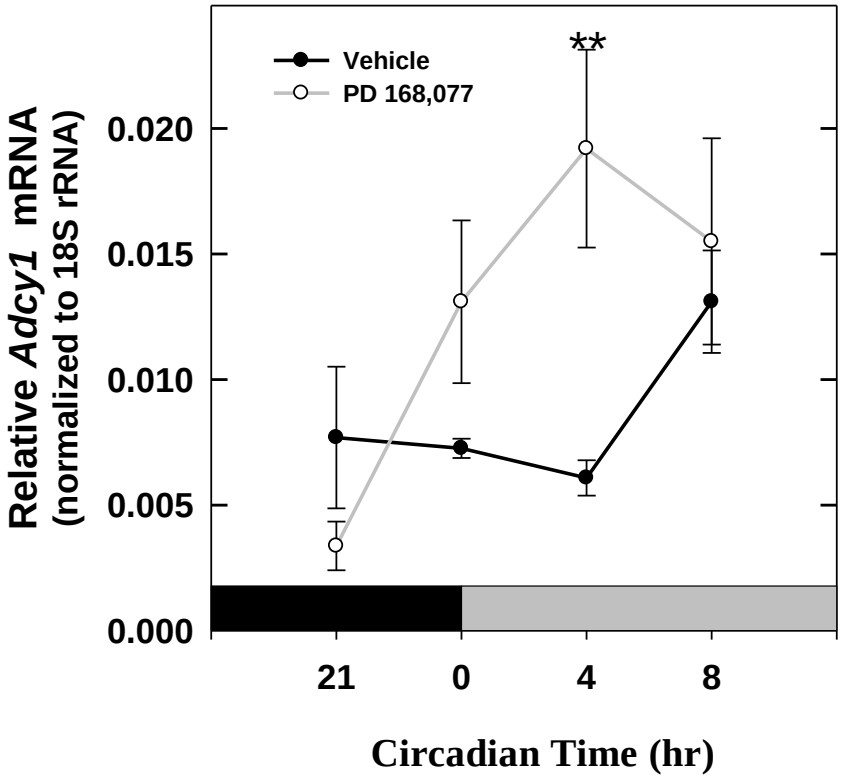


Fig. 6-3.4. Effect of pre-dawn activation of D_4R on the temporal expression pattern of *Adcy1* mRNA in mouse retina. Mice were injected at ZT 20 for three days with 1 mg/kg PD 168,077 or vehicle as described in Materials and methods. After the third injection, retinas were dissected during the following subjective day or subjective night in DD at the times indicated in the figure. Two-way ANOVA showed significant effects of *time* (P=0.02), *treatment* (P<0.04), and a statistically significant interaction of *time* and *treatmen*t (P<0.03); n=4-5 per group. 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).

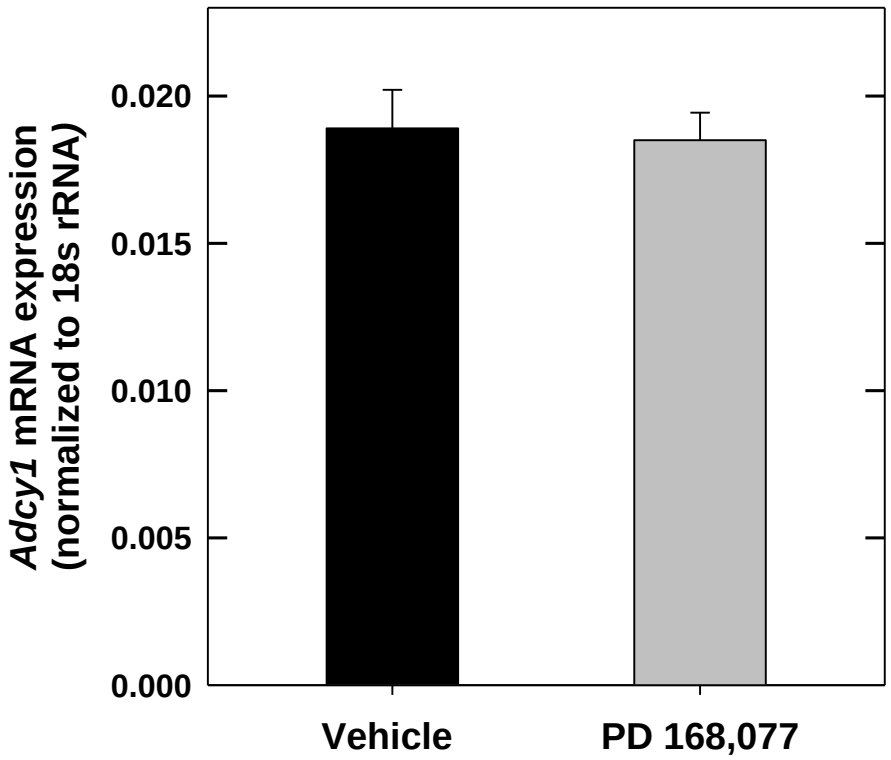


Fig. 7-3.4. Effect of acute pre-dawn stimulation of the D_4R with PD 168,077 does not cause an increase in *Adcy1* mRNA expression in mouse retina. Mice, injected with a dopaminergic agonist 4 hours before the normal release of retinal dopamine did not affect *Adcy1* transcript levels when assayed. Two-tailed student t-test was used to determine significance.

3.5 Discussion

The retina uses endogenous circadian oscillators to adapt to changes in illumination (Storch et al. 2007). This allows for fine tuning of the retinal signals across a large range of light intensities. Even though there are many different processes aiding in light/dark adaptation, such as electrical coupling between photoreceptors and retinal neurons (Vardi and Smith 1996). Ribelayga et al., (2008) shows that the retinal clock, not environmental light, controls rod-cone coupling mediated by a novel circadian signaling processes that may aid in light adaptation through activation of the D_4R. The focus of the study was to define novel circadian signaling processes that may aid in light adaptation through activation of the D_4R. 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 study shows that the D_4R is critical for maintaining the normal rhythmic 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 D_4R. The phase of *Adcy1* mRNA expression rhythm is altered by activation of the D_4R at an abnormal time. Overall, the data suggest that D_4R activation acts as an entrainment stimulus synchronizing activity rhythms of AC1, thereby modulating the rhythmic synthesis of cyclic AMP in the mouse retina.

3.5.1 D_4R control of the rhythmic expression of *Adcy1*. The classical circadian feedback loop mechanism, 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. 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 all 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 *Per2*-driven luciferase activity in the inner retina, further suggesting oscillators required for rhythmic *Per 2* expression in the INL 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, Cao et al. 2008), Na/K^+ ATPase activity (Shulman and Fox 1996), photoreceptor protein phosphorylation (Pozdeyev, Tosini et al. 2008), and melatonin synthesis (Tosini, Doyle et al. 2000) that are observed in mammalian retina. Moreover, another study found transcripts of all six clock genes in the microdissected photoreceptor layer of the rat retina and demonstrated that rhythmic *Per1*-driven luciferase and melatonin synthesis persists in isolated photoreceptor layers (Tosini, Davidson et al. 2007). The D_4R is the sole dopamine receptor subtype located on photoreceptor cells (Cohen, Todd et al. 1992) and has been shown to be involved in many of the aforementioned physiological functions (Witkovsky 2004). The common thread between D_4R and rhythmic physiological functions suggests a possible site for circadian regulation in photoreceptor cells.

Storch et al. (2007) showed by microarray and RT-PCR analyses that *Adcy1* mRNA was rhythmically expressed in mouse retina. In addition, they reported that *Drd4* expression in mice exposed to LD was rhythmic even when the core clock mechanism is disrupted by elimination of *Bmal1*. This latter observation suggested that the rhythm of *Drd4* mRNA was light-driven and not regulated directly by circadian clocks. However, our results demonstrated rhythmic *Drd4* mRNA expression 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 circadian oscillators. Previous studies have shown that dopamine metabolism in retinas of wild type C57Bl6 mice is light driven and not circadian (Doyle, McIvor et al. 2002; Pozdeyev, Tosini et al. 2008). The larger rhythmic amplitude of the *Drd4* transcript in LD compared to DD was due to lower 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 D_4R activation (Jackson, Chaurasia et al. 2009). Currently, we show that the rhythm in *Adcy1* is dampened or undetectable in *Drd4*$^{-/-}$ mice or in mice treated for six days with a D_4R antagonist. These finding provide strong evidence that D_4R is active in regulating the circadian expression of AC1.

3.5.2 D_4R control of rhythmic adenylyl cyclase enzyme activity. Our results provide evidence that the D_4R regulates cyclic AMP production by controlling the activity of the type 1 adenylyl cyclase at the enzymatic activity level. Three adenylyl cyclase isozymes are known to interact with Ca^{2+} and CaM: AC1, AC3, and AC8 (Patel, Du et al. 2001), with AC1 being primarily responsible for cyclic AMP accumulation in dark-adapted mouse retina (Jackson et al., 2009). 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. 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 DA/D_4R signaling system in mammalian retina.

3.5.3 Dopamine/ D_4R signaling system controls cyclic AMP in mouse retina. Cyclic AMP is a key second messenger that translates external cues into intracellular responses. One way to regulate cyclic AMP is through the D_4R signaling pathway, which is negatively coupled to AC1 in retina (Jackson, Chaurasia 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 and Iuvone 2003; Fukuhara, Liu et al. 2004); to date this had not been established in mouse retina. This investigation definitively shows that cyclic AMP accumulation is rhythmic in retinas of WT mice. However, cAMP levels in *Drd4*$^{-/-}$ retinas display no circadian phenotype. These data lead us to believe that the rhythmic nature of cAMP synthesis in mammalian retina is controlled by the DA/D_4R signaling system.

3.5.4 Is the DA/D_4R signaling system an entrainment mechanism in retina? The current study shows that *Adcy1* expression rhythms are damped or lost in the absence of D_4R signaling. Conceivably, *Adcy1* expression rhythms could be driven by dopamine. Alternatively, DA 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/6J background, which show light-driven but not circadian control of dopamine metabolism (Doyle, Grace et al. 2002; Pozdeyev, Tosini 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 D4 agonist does not induce *Adcy1* mRNA expression. In contrast,

the observation that injections of the D4 receptor agonist 4 hours prior to subjective dawn for three days causes *Adcy1* mRNA levels to increase earlier in the day compared to vehicle injected controls supports the hypothesis that dopamine entrains the rhythm of expression. In *Xenopus* retina, quinpirole, a D2/D4 dopamine receptor agonist, can phase shift the circadian rhythm of melatonin release from photoreceptors (Cahill and Besharse 1991). Similarly, dopamine synchronizes multiple circadian oscillators regulating rhodopsin promoter activity in zebrafish photoreceptors (Yu, Gao et al. 2007).

In conclusion, the DA/D_4R signaling system seems to play an important role in retinal circadian regulation. The rhythm in D_4R may control subordinate rhythms that are driven by its timely activation by DA, thereby, controlling the phase of rhythmic gene expression (*Adcy1*), adenylyl cyclase activity, and cyclic AMP production. Our results, in conjunction with studies performed on cold-blooded vertebrates, support the hypothesis that dopamine, acting through D_2 or D_4 receptors, serves as an entrainment stimulus to synchronize circadian clocks regulating retinal gene expression and function.

Chapter IV:

Dopamine D_4 receptor signaling in mouse retina controls gene regulation and visual function

4.1 Summary. In mammalian retina, dopamine and dopamine D_4 receptor (D_4R) activity controls a multitude of physiological responses, such as outer segment disc shedding, melatonin synthesis, Na^+/K^+-ATPase activity, and the signaling balance between rods and cones. The D_4R is located throughout the retinal layers, but with respect to the photoreceptor layer it is the only dopamine receptor subtype expressed, suggesting dopamine's actions on this layer is solely mediated through this receptor. An activated D_4R is negatively coupled to the type 1 adenylyl cyclase (AC1); the main cyclase controlling rhythmic cAMP production in the mouse retina. Although it is known that D_4R activation mediates rhythmic retinal behaviors such as melatonin synthesis and controls rhythmic AC1 gene expression, little is known of what other genes it might regulate to drive the physiological responses of the retina. The core of these processes is controlled at the transcriptional level, making it an important site for investigation. Therefore, we used Affymetrix microarrays to determine the global changes in gene expression of retinas obtained from mice lacking D_4R (*Drd4*$^{-/-}$). We found approximately 475 genes that were differentially expressed in the knockout mouse. Using gene ontology and pathway analysis we found a trend in the top 50 genes with known functions, showing that 16% have enzymatic activity, 44% are cell signaling molecules, 12% are chromatin remodeling molecules, and 14% have protein interaction properties, with the remaining transcripts having no known function. Furthermore, we accessed visual performance in *Drd4*$^{-/-}$ mice and found that while maintaining normal visual acuity, these mice have diminished levels of contrast sensitivity and have trouble adapting to light changes. D_4R signaling controls a variety of genes in the mouse retina, and serves to coordinate retinal functioning and preserve visual performance.

4.2 Introduction. Previous investigations of dopamine D_4 receptor (D_4R) signaling have focused on the reduction of cellular cyclic AMP (cAMP) levels by inhibiting adenylyl cyclases, through its heterotrimeric $G_{\alpha i}$ protein subunit (Cohen, Todd et al. 1992; Chio, Drong et al. 1994; Tang, Todd et al. 1994; McAllister, Knowles et al. 1995). The D_4R is a focal point for controlling many retinal functions such as: outer segment disc shedding (Besharse and Spratt 1988); inhibition of melatonin synthesis (Iuvone and Besharse 1986; Tosini and Dirden 2000); rod photoreceptor Na^+/K^+ ATPase activity (Shulman and Fox 1996); regulation of a light-sensitive pool of cAMP and light adaptation (Cohen and Blazynski 1990; Cohen, Todd et al. 1992; Nir, Harrison et al. 2002); regulation of phosducin phosphorylation (Pozdeyev, Tosini et al. 2008); and control of AC1 levels in mouse retina (Jackson, Chaurasia et al. 2009). While these physiological functions are well established, it is not known what specific retinal genes are affected by DA/D_4R signaling.

Moreover, several of these physiological responses are under rhythmic regulation in the mouse retina, making this receptor a possible steward for retinal circadian rhythms. It is known that the *Drd4* transcript is rhythmic in the rat photoreceptor layer (Klitten, Rath et al. 2008) and the D_4R is imperative for normal light adaptation (Nir, Harrison et al. 2002). Circadian rhythms are an intrinsic part of retinal physiology controlling functions such as: rod/cone coupling (Ribelayga, Cao et al. 2008) and visual sensitivity (Storch, Paz et al. 2007; Cameron and Lucas 2009). Both are paramount in maintaining normal vision and enables the tissue to function at the wide range of light intensities occurring over a 24 hr period. Previous investigations have sought to define where the central circadian oscillator(s) are located, driving these rhythmic phenomena. The dopaminergic amacrine cells located in the retina contain the canonical core clock genes (i.e., *Bmal1*, *Clock*, *Period 1* and *2*, *Cryptochrome 1* and *2*) that make up the basic feedback

network of the clock mechanism (Gustincich, Contini et al. 2004). Results of single cell RT-PCR analysis demonstrated that other inner retinal neurons (INL) and ganglion cells (GCL) also contain all six core clock genes (Ruan, Zhang et al. 2006); however, no one photoreceptor contained detectable levels of all six core clock genes. Moreover, dopamine and GABA influence clock function as determined by mPER2::LUC rhythms in cultured mouse retina (Ruan, Allen et al. 2008). These data provide evidence that the circadian clocks are located in the INL and the GCL, though this does not explain the basis for the photoreceptor circadian phenomena of outer segment disc shedding or melatonin synthesis.

We and others have shown that *Drd4* mRNA is expressed in a circadian manner in mouse retina (Storch, Paz et al. 2007), possibly linking it to the intrinsic retinal clock. In rat retina, a similar rhythm of *Drd4* mRNA occurs in photoreceptor cells (Klitten, Rath et al. 2008) Also, it is well established that DA utilization demonstrates rhythmic activity in mouse retina upon the stimulation of light (Nir, Haque et al. 2000). We have shown that the rhythms of *Adcy1* and cAMP are lost in the *Drd4*$^{-/-}$ retinas (Chapter 3). These observations led us to investigate broader aspects of the D_4R signaling system by looking at global gene expression changes in *Drd4*$^{-/-}$ mice via microarray analysis. It is known that *Drd4*$^{-/-}$ mice have deficits in adapting to changes in light (Nir, Harrison et al. 2002), which could be investigated by looking at gene expression changes. Therefore, we also sought to determine if *Drd4*$^{-/-}$ mice had any additional visual abnormalities by measuring visual acuity and contrast sensitivity.

4.3 Materials and methods

4.3.1 Animal Care/Usage and Tissue Collection. Microarray experiments were preformed on retinas from 2 month old *Drd4*$^{-/-}$ (n = 5, (Rubinstein, Phillips et al. 1997)) mice on a C57BL/6J

background and wildtype (WT) littermate controls (n = 5). Mice were entrained to a 12 hr light/12 hr dark cycle (LD) for at least 2 weeks. Food and water were provided *ad libitum* and mice were genotyped by PCR analysis of genomic DNA. Four hours after light onset mice were killed by cervical dislocation, retinas were dissected from the eyes (both retinas pooled), frozen on dry ice, and stored at -80°C until RNA processing. Rhythmic gene expression analysis and optokinetic tracking were preformed on male mice approximately 2 months of age that were also maintained on a 12 hr:12 hr LD cycle. This investigation complied to the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and was approved by Emory University's Institutional Animal Care and Use Committee.

4.3.2 Microarray analysis. WT and *Drd4*$^{-/-}$ retinal samples were hybridized to Affymetrix GeneChip Mouse Exon 1.0 ST Arrays (Fremont, CA) at the National Institutes of Health Neuroscience Microarray Consortium. These arrays provide comprehensive coverage of the mouse transcriptome with approximately four probes per exon and 40 probes per gene enabling analysis at the gene expression and alternative splicing levels. The Aroma.affymetrix package was used to build up transcripts from the exon-level probes in order to simulate a measure at the single transcript level (Bengtsson, Irizarry et al. 2008). Differential gene expression levels were determined by R (2.10.1) and BioConductor 2.5 statistical software, noting significant changes by P-value ($P < 0.05$) and fold change (~1.5 fold change, www.r-project.org). Four levels of processing were used in the analysis which allowed a false discovery rate (FDR) of 5%, meaning that each gene identified as differentially expressed has a 95% chance of being meaningfully different. First, *pre-processing quality controls* were performed to check for consistency of intensity and distribution of the non-normalized signal. Next, *normalization* was used to reduce chip effects. Also, *post-normalization quality checks* were performed to determine if the

samples have similar distribution. Finally, *post-normalization* was employed by using the R package Limma to produce a linear model for analyzing the results of the microarray hybridizations (Smyth, Michaud et al. 2005).

4.3.3 GeneNetwork analysis. Approximately 600 genes were analyzed using GeneNetwork (Gene Network.org, an open source website for the analysis of changes in transcriptome and phenotypes in the mouse). The ultimate goal of this analysis was to identify genetic networks modulated by the D_4R. Upstream modifiers were identified and used to generate pathway networks. Ultimately, this program was used to locate modifier genes responsible for possible downstream aberrations in gene expression that define genetic changes that underlie functional retinal phenotypes. The software package enables us to refine the search by choosing the species (e.g. mouse), group (e.g. strain of mouse), type (tissue of interest, e.g. retina), and database. A summary of the analytical tools is presented in (Geisert, Lu et al. 2009).

4.3.4 Ingenuity Pathway Analysis. Ingenuity Pathway Analysis (IPA, www.ingenuity.com) is a multifaceted software that enables researchers to quickly identify putative relationships, mechanisms, function, and pathways of relevance from data produced by a particular platform (e.g., microarrays, western blots, etc.). The software extracts information from published scientific data concerning genes, proteins, drugs, biomarkers, chemicals, and cellular and disease processes. The 430 differentially regulated genes (Up:336 transcripts and Down:94 transcripts) identified in the microarray study, by statistical analysis and which have known functions, were entered into the IPA software and multiple network pathways with possible functional significance were produced.

4.3.5 Rhythmic gene expression. Retinas from WT and *Drd4*$^{-/-}$ were isolated from mouse eyes at circadian time (CT) 2, 8, 14, and 20 to access rhythmic expression patterns of target genes found through microarray analysis and differential core clock gene expression. Total RNA was isolated using a Qiagen RNeasy kit (Qiagen Inc. Valencia, CA, USA, for microarray and gene expression analysis). Reverse transcription was performed on total RNA (at least 250 ng) preparations using QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, CA, USA) containing oligo-dT and random primers for production of cDNA. Then qRT-PCR reactions were performed with 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). The quantification of transcript levels was performed by comparing the Δ cycle time (ΔCT) of the target transcript to the ΔCT of ribosomal RNA 18s ($^{\Delta\Delta}$CT method, http://pathmicro.med.sc.edu/pcr/realtime-home.htm).

4.3.6 Optokinetic tracking (OKT). 2 month old male *Drd4*$^{-/-}$ and *Adcy1*$^{-/-}$ mice were tested for levels of visual acuity and contrast sensitivity. Each strain was tested using Optomotry to identify optical kinetic tracking; Optomotry is a virtual-reality system that quantifies visuomotor behavior (Optomotry, Cerebral Mechanics Inc.). The results were compared with WT controls, as described by Douglas et al. (2005). The tracking device produces a three-dimensional sine wave grating, creating a virtual cylinder that the mouse tracks by responding with movements of the head and neck indicative of vision. All tests were done in the middle of the day to eliminate any chance for changes in visual sensitivity due to circadian effects.

Visual threshold acuity was measured at 100% contrast, 12.0 degrees/sec (d/s) drift speed, 0.4 Hz temporal frequency with varying spatial frequency. The contrast threshold was measured using mouse presets spatial frequencies (0.031, 0.064, 0.092, 0.103, 0.192, 0.272

cycles/degree) with drift speed of 12.0 d/s, temporal frequency of 0.4 Hz and varying contrast (Prusky, Alam et al. 2004). Contrast sensitivity was calculated for each spatial frequency as a Michelson contrast using the equation published in Prusky et al. (2006).

4.4 Results

4.4.1 Microarray analysis of gene expression changes: WT vs. *Drd4*$^{-/-}$. While much is known about the physiological processes affected by the DA/D_4R signaling system, there is limited information about the genes that are regulated by D_4R activation. In this study we used Affymetrix GeneChip mouse exon 1.0 ST microarray to discern the contribution of the D_4R in regulating the retinal transcriptome. Using R (2.10.1) and BioConductor 2.5 we determined that 4.8% (555 transcripts, 475 with known function) of the total transcripts represented on the chip were differentially expressed in *Drd4*$^{-/-}$ retinas when taking fold-change and p-value of at least 0.05 as our significance criteria. After application of the required normalization methods were employed, there were a total of 11,563 transcripts that were identified through chip analysis. Table 1 shows the top 50 differentially expressed genes and there known molecular functions. The complete list of transcripts is presented in Table S1 (Appendix I). The most common physiological theme in Table 1 is represented by transcripts encoding for proteins involved in cell signaling/signal transduction (44%, e.g., *Trpc1*). This category was followed by transcripts representing proteins involved in regulation of enzymatic activities (16%, e.g., *Gucalb*) and protein-protein interacting transcripts (14%, e.g., *Lrfn2*). Also, transcripts encoding chromatin remodeling proteins (12%, e.g., *Hdac9*) were highly differentially expressed in the *Drd4*$^{-/-}$ retinas. These findings imply that this receptor plays a major role in transmitting information to the cell, driving a large portion of the retinal transcriptome.

4.4.2 qRT-PCR confirmation analysis. mRNA expression profiles of *Aqp1*, *Trpc1*, *Irf7*, *Rgs20*, *Gnaz*, *Hdac9*, *Mbnl2*, *Dpf3*, *Gucalb*, retinal associated genes, were selected to confirm the microarray data (Fig. 1). Retinal samples were collected at CT 4, the time point corresponding to the microarray study. From the chosen transcripts, all confirmed the microarray results showing the same increases or decreases observed in the expression profiles.

4.4.3 Rhythmic gene regulation. In general, retinal associated transcripts that were differentially expressed in the microarray and qRT-PCR experiments showed rhythmic expression profiles when assayed in WT retinas (Fig. 2). *Aqp1, Trpc1, Guca1b, Mbnl2, Hdac9, and Irf7* were assayed at CT 2, CT 8, CT 14, and CT 20 in WT and *Drd4*$^{-/-}$ retinas determining circadian expression of each transcript. *Aqp1* demonstrated a strong rhythmic expression pattern in WT samples ($P < 0.001$), peaking around CT 14 (Fig. 4a, $^{**}P < 0.001$). Expression patterns were significantly altered in the *Drd4*$^{-/-}$ samples at all circadian time points (Fig. 2a, $P < 0.001$); however, these samples maintained rhythmic behavior (Fig. 2a, $P < 0.011$) in the knockout samples, but did not correlate temporally with the WT samples. When assayed, *Trpc1* did not show any significant rhythmic behavior in the WT or *Drd4*$^{-/-}$ retinas (Fig. 4b). This discrepancy could be due to different sampling conditions (LD vs. DD). It was noted that when visually assessed the expression pattern of *Trpc1* follow the same trend of expression of other rhythmic transcripts analyzed (e.g., *Hdac9*). Using two-way ANOVA and post-hoc Student-Newman-Keuls statistical tests revealed that *Guca1b*, *Mbnl2*, and *Hdac9* have similar rhythmic expression profiles. All of these transcripts showed rhythmic expression in WT samples ($P<0.001$, each)but not in the *Drd4*$^{-/-}$ samples (Fig. 2c-e). Each transcript peaked around CT 2 and troughs around CT 14 to CT 20. Significant differences in gene regulation were seen at multiple time points for each transcript (Fig. 2c *Guca1b* - CT2, $^{**}P < 0.001$; Fig. 2d

Table 1-4.4. Top 50 differentially expressed gene transcripts from microarray analysis. Significance levels were determined by first accessing P-value (≤ 0.05), followed by fold-change. Transcripts are labeled by their respective gene symbol and in the second column a (+) or a (–) symbol denotes the genes' expression pattern relative to WT samples(41 transcript decreased and 9 increased). There are a variety of cellular functions represented in this list, however, a few standout: 1) 8 transcripts - enzymatic activity, 2) 22 trancripts - cell signaling (receptors, GPCRs, channels, etc.), 3) 7 transcripts - protein function/interaction, 4) 6 trancripts - chromatin remodeling, and 5) 4 transcripts - unknown function.

Table 1-4.4: Top 50 differentially expressed genes in *Drd4*$^{-/-}$ mouse retina from the microarray analysis

Gene Symbol	*Drd4*$^{-/-}$ relative to the WT samples	Known Function
Acadm	-	Medium chain acyl-CoA dehydrogenase (MCAD) mitochondrial beta-oxidation enzymes
Guca1b	-	Regulator of guanylyl cyclase activity
Med13l	-	Transcription regulator
Mina	-	Myc induced nuclear antigen
Ppp2r2b	-	Serine/threonine protein phosphatase 2A, Signal transduction mechanisms
Aqp1	-	Water channel
2610034M16Rik	-	PDZ signaling
Pcmtd2	-	AdoMet-MTases are enzymes that use S-adenosyl-L-methionine (SAM or AdoMet) as a substrate for methyltransfer
Trpc1	-	Transient receptor potential cation channel
Adcy1	-	cAMP production
Irf7	+	IRF transcription factors - regulation of interferons
Mylip	-	Sterol regulation of cholesterol uptake
Plekhf2	-	Targets proteins to membrane lipids
Hdac9	-	Histone modification
Ankrd33b	-	Ankyrin repeats mediate protein-protein interactions
Glmn	-	Hepatocyte growth factor
St8sia1	-	Roles in cell proliferation and motility
Frmpd1	+	PDZ signaling
Smarcad1	-	ATP-dependent RNA or DNA unwinding
Cpt1a	-	enzyme
Rgs20	-	Regulator of G protein signaling domain
Rab30	-	Interacts with GTPase activating proteins
Dpf3	-	Chromatin remodeling
Mbnl2	-	Zine finger protein - mediates transcription
Stk39	-	Serine/Threonine protein kinase
Scn4a	+	Sodium channel
Clip4	-	Mediate protein-protein interactions
Hpcal1	-	Calcium binding/sensor
Scap	+	Regulator of fatty acid synthesis
Eps8l1	-	SH3 domain
Lrfn2	+	Participates in protein-protein interactions
2410042D21Rik	-	Unknown function
Emid2	+	EMI domain containing 2 precursor
Rps6ka3	-	Ribosomal Kinase
Racgap1	+	GTPase-activator protein
Fastkd2	-	FAST kinase-like protein
Zfp238	-	DNA binding
Bmpr1a	-	morphogenetic protein receptor
Mapkap1	-	Map kinase interacting protein
Rpap2	-	RNA polymerase associated protein
Usp16	-	Peptidase
Wdr47	-	adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly
Adamts3	-	Zinc-dependent metalloprotease
Klhl36	-	Unknown function
Pcp4l1	+	Unknown function
Pde6b	-	rod cGMP-specific 3',5'-cyclic phosphodiesterase subunit beta
Tmem221	+	Unknown function
Rnf170	-	Zine binding motif
Nipal1	-	Ion transport
Sik2	+	salt inducible kinase - Serine/Threonine protein kinase

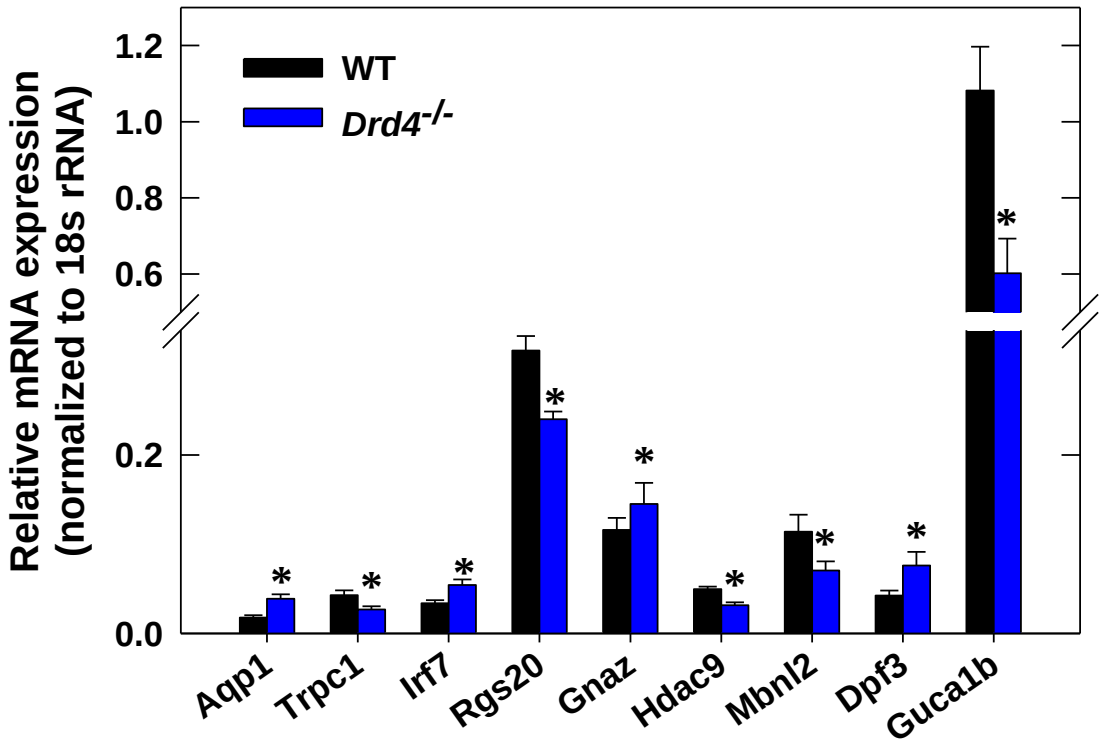


Figure 1-4.4. qRT-PCR results from nine randomly chosen transcripts verified microarray data. RNA isolated from mouse retinas at ZT 4 (corresponding to the microarray study) was converted to cDNA and accessed by qRT-PCR analysis to confirm the changes seen in *Drd4*$^{-/-}$ in the microarray study. *P < 0.05, n = 6 mice per strain, mean ± SEM.

Mbnl2 - CT 2 and CT 14,*P<0.05 and **P <0.001, respectively; Fig. 2e *Hdac9* - CT 2, **P <0.001). Lastly, *Irf7* demonstrated rhythmic behavior in WT samples (Fig. 2f, P <0.001), but similar regulation was not seen in the *Drd4*$^{-/-}$ retinas assayed at the same time points with significant differences at CT 2, CT 8, and CT 14 (Fig. 2f, **P<0.001). These data provided evidence that the D_4R is not only important for the regulation of genes expression, but also supports their rhythmic expression patterns.

If these genes are truly under circadian regulation, then we expected changes in the expression patterns of the core clock gene (i.e., *Cry1*, *Cry2*, *Bmal1*, and *Per2*) in the *Drd4*$^{-/-}$ retinas. Using the same methods and samples, we assayed the expression profiles for the core clock genes. *Cry1* was significantly reduced in *Drd4*$^{-/-}$ samples (Fig. 3a, P <0.025), with the biggest change in expression at the CT 20 time point. Conversely, *Cry2* showed no difference between genotypes (Fig. 3b, P = 0.47). Each *Cry* transcript displayed a significant difference in levels as a function of time, which indicated a potential rhythm in expression (Fig. 3b, P <0.042 and P <0.01). *Bmal1*, encoding a positive component of the circadian feedback system (Hastings, Reddy et al. 2003), only displayed a significant difference in the *Drd4*$^{-/-}$ mice (Fig. 3c, P <0.002). At CT 20 there is a significant change in the expression pattern of *Bmal1* in the knockout mouse compared with WT controls (*P <0.05). This reduction may be due to *Drd4*$^{-/-}$ rhythmic profile or gene expression reduction over the time points assayed (Fig. 3c, P <0.002). *Per2*, demonstrated a rhythmic expression profile in both strains of mice (Fig. 3d, P <0.001), but also had no significant changes in expression between the two genotypes (Fig. 3d, P = 0.21). Overall, core clock gene expression was altered in *Drd4*$^{-/-}$ mice, but the changes seem minor compared to the aberrations seen in the differentially expressed microarray genes (Fig. 2).

Figure 2-4.4. Circadian expression of select genes differentially expressed in *Drd4*$^{-/-}$ mouse retinas. qRT-PCR was utilized to analyze retinal mRNA levels of *Aqp1*, *Trpc1*, *Gucalb*, *Mbnl2*, *Hdac9*, and *Irf7* in the 1st day of constant darkness (DD) at four time points. (a) *Aqp1* shows a strong rhythmic gene expression pattern peaking around CT 14 ($P < 0.001$) in WT samples, and a dampened rhythmic expression pattern in the Drd4$^{-/-}$ samples ($P < 0.011$). There is a significant difference between the two strains of mice at CT 2 (*$P < 0.05$), CT 8 (**$P < 0.001$), and CT 14(**$P < 0.001$), indicating a difference in gene expression patterns. (b) *Drd4*$^{-/-}$ and the controls samples did not show a circadian expression pattern in *Trpc1* gene expression. Additionally, there was no significant difference between mice in expression patterns. (c) WT samples showed a rhythmic pattern of expression of *Gucalb* ($P < 0.001$), which peaked at CT 2; the time point that is significantly higher than the knockout model (**$P < 0.001$). There was no rhythm detected in the *Drd4*$^{-/-}$ samples. (d) *Mbnl2* demonstrated rhythmic expression patterns in WT samples ($P < 0.001$), conversely, the *Drd4*$^{-/-}$ sampled showed no rhythmic pattern. The two strains of mice differ in expression at CT 2 (*$P < 0.05$) and CT 14 (**$P < 0.001$) where the WT samples zenith and nadir. (e) *Hdac9* showed a circadian expression pattern, which was highest at CT 2 and lowest at CT 14 ($P < 0.001$) in WT samples. This expression pattern was not observed in the *Drd4*$^{-/-}$ samples. Also, *Hdac9* showed significant differential expression in *Drd4*$^{-/-}$ samples at CT 2 (**$P < 0.001$) when compared to WT controls. (f) *Irf7* showed a significant difference in expression patterns between WT and *Drd4*$^{-/-}$ samples at CT 2, CT 8, and CT 14 (**$P < 0.001$, respectively). While a rhythm was not detected in the *Drd4*$^{-/-}$ samples, WT retinas demonstrated a circadian phenotype ($P < 0.001$). Significant differences were determined by Two-Way ANOVA, using Student-Newman-Keuls where applicable for pairwise multiple

comparisons. Closed circles (●) represent WT samples, with open circles (○) representing *Drd4*$^{-/-}$ samples. Points represented are means ± SEM (n = 6 mice per group).

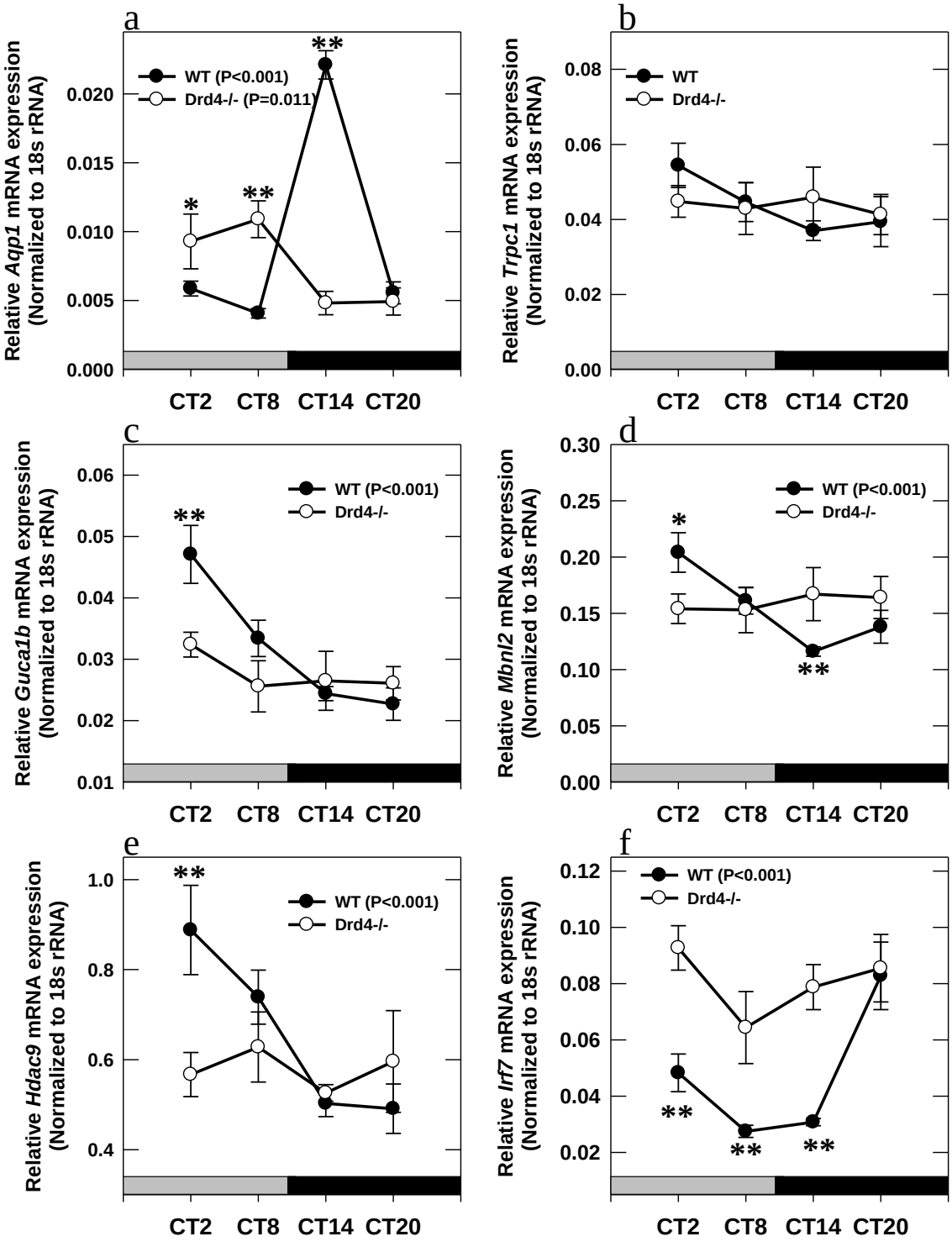
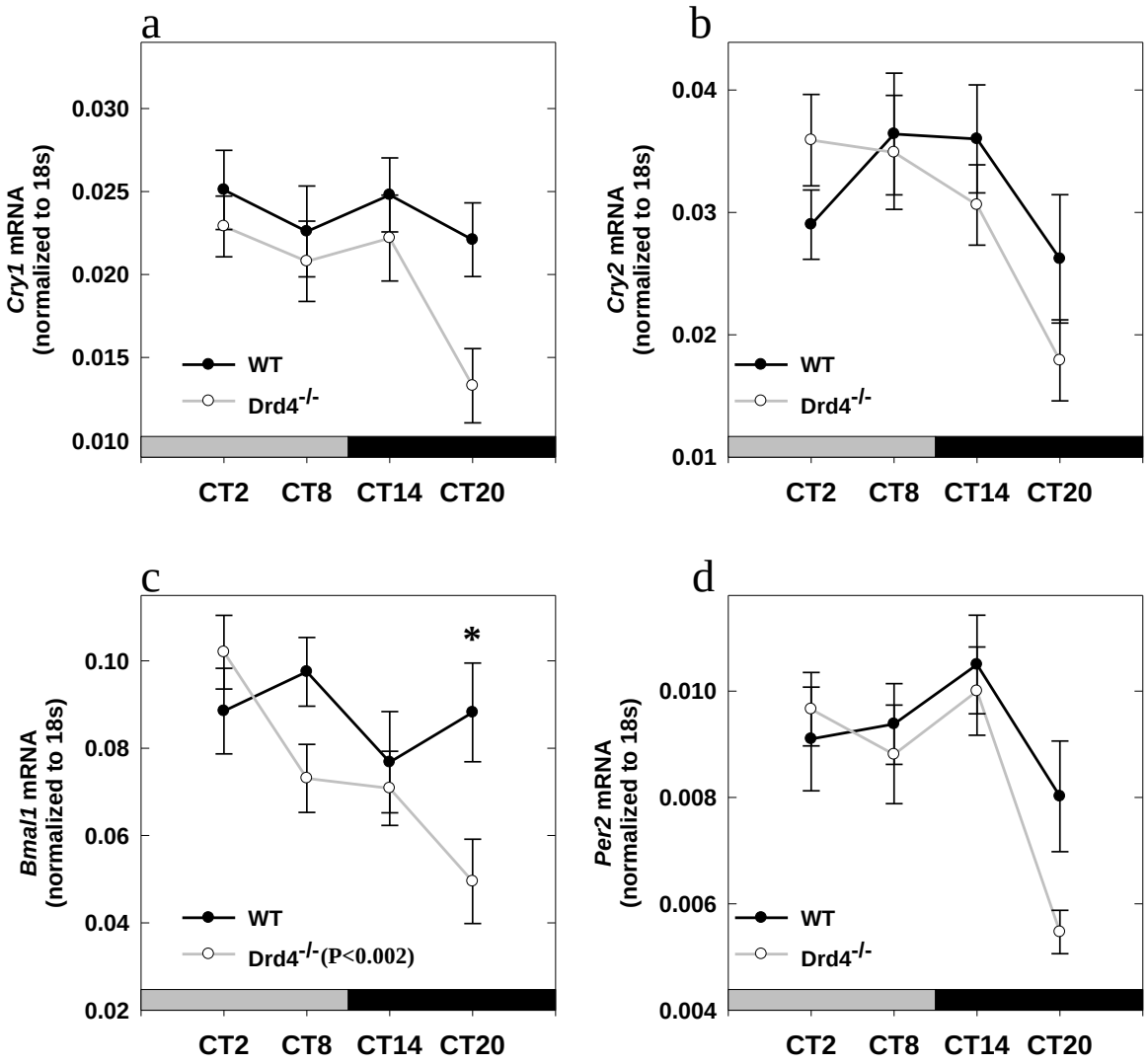

a
Relative Aqp1 mRNA expression (Normalized to 18s rRNA)
WT (P<0.001)
Drd4-/- (P=0.011)
0.000
0.005
0.010
0.015
0.020
CT2 CT8 CT14 CT20
b
Relative Trpc1 mRNA expression (Normalized to 18s rRNA)
WT
Drd4-/-
0.00
0.02
0.04
0.06
0.08
CT2 CT8 CT14 CT20
c
Relative Guca1b mRNA expression (Normalized to 18s rRNA)
WT (P<0.001)
Drd4-/-
0.01
0.02
0.03
0.04
0.05
0.06
CT2 CT8 CT14 CT20
d
Relative Mbnl2 mRNA expression (Normalized to 18s rRNA)
WT (P<0.001)
Drd4-/-
0.00
0.05
0.10
0.15
0.20
0.25
0.30
CT2 CT8 CT14 CT20
e
Relative Hdac9 mRNA expression (Normalized to 18s rRNA)
WT (P<0.001)
Drd4-/-
0.4
0.6
0.8
1.0
CT2 CT8 CT14 CT20
f
Relative Irf7 mRNA expression (Normalized to 18s rRNA)
WT (P<0.001)
Drd4-/-
0.02
0.04
0.06
0.08
0.10
0.12
CT2 CT8 CT14 CT20

Figure 3-4.4. Core clock gene expression, *Cry1*, *Cry2*, *Bmal1*, and *Per2*, between WT and *Drd4*$^{-/-}$ mouse retinas. Mouse retinas were removed in DD1 at CT 2, CT8, CT 14 and CT 20 to access rhythmic patterns of core clock gene expression. Significance levels were determined by Two-Way ANOVA, using Student-Neuman-Keuls post hoc test measuring all-pairwise multiple comparisons. Closed circles (●) denotes WT samples and open circles (○) denote *Drd4*$^{-/-}$ samples. (a) The ANOVA determined that genotype does affect the *Cry1* circadian expression pattern ($P < 0.025$). (b) Overall, there is no difference in *Cry2* expression between the WT and *Drd4*$^{-/-}$ samples ($P = 0.47$), however a Tukey test determined that at CT 20 there was a significant change in expression suggesting a possible rhythm ($P < 0.01$). (c) There is an effect of genotype on *Bmal1* expression in the *Drd4*$^{-/-}$ samples ($P < 0.045$). There is a rhythm of expression in the *Drd4*$^{-/-}$ samples (*$P = 0.002$), which peaks at CT 2 and troughed at CT 20. (d) *Per2* demonstrated rhythmic expression in both strains of mice ($P < 0.001$); however, there was no significant difference between the mice ($P = 0.21$).


a
Cry1 mRNA (normalized to 18s)
0.030
0.025
0.020
0.015
0.010
WT
Drd4-/-
CT2 CT8 CT14 CT20
b
Cry2 mRNA (normalized to 18s)
0.04
0.03
0.02
0.01
WT
Drd4-/-
CT2 CT8 CT14 CT20
c
Bmal1 mRNA (normalized to 18s)
0.10
0.08
0.06
0.04
0.02
*
WT
Drd4-/-(P<0.002)
CT2 CT8 CT14 CT20
d
Per2 mRNA (normalized to 18s)
0.010
0.008
0.006
0.004
WT
Drd4-/-
CT2 CT8 CT14 CT20

4.4.4 GeneNetwork analysis. Next, we used Gene Network to locate potential loci that may be driving the expression of our differentially-expressed transcripts. We loaded the top 600 differentially expressed genes (not all significantly different) into the online software package (www.genenetwork.org) and generated Quantitative Trait Loci (QTL) heatmaps (Fig. S1, Appendix II). We included some transcripts just outside of our significance criteria to give us the best chance to identify potential regulatory loci. From a crude visual observation of the heatmaps, we concluded that the genomic loci modulating gene expression were located in chromosomes 12 and15. GeneNetwork enabled us to look at pathway analysis to see which transcripts are closely related. The microarray analysis gave us the opportunity to choose transcripts that were highly differential (i.e., *Drd4, Aqp1, Guca1b*), intermediate (i.e., *Pde6a, Rhot1, Rgs9bp*), and marginally expressed (i.e., *Nr1, Ttc14*). The correlation with the D_4R pathway is shown in Figure. 4. This analysis allowed us to force in the other dopamine receptor subtype transcripts into a relationship with *Drd4* to perform a correlation. Additionally, we included *Rho* (rhodopsin) to insure a network generated displayed a photoreceptor specific signature. Figure 4 shows that *Aqp1, Rgs9bp, Nr1, Rho, Rhot1, Guca1b, and Pde6a* are tightly correlated with *Drd4*. These interactions are specific to the *Drd4,* for when other dopamine receptor transcripts are analyzed no specific connections with the differentially expressed transcripts relate to this network. These results provide further support that the D_4R is functionally active in regulating retinal gene transcription, most likely in the photoreceptor layer.

4.4.5 Ingenuity Pathway Analysis. Microarray data is limited in the usable information that it provides, telling us only if transcript levels change due to specific conditions (e.g., altered genotypes). What is not determined from microarray analysis is if the proteins encoded by the

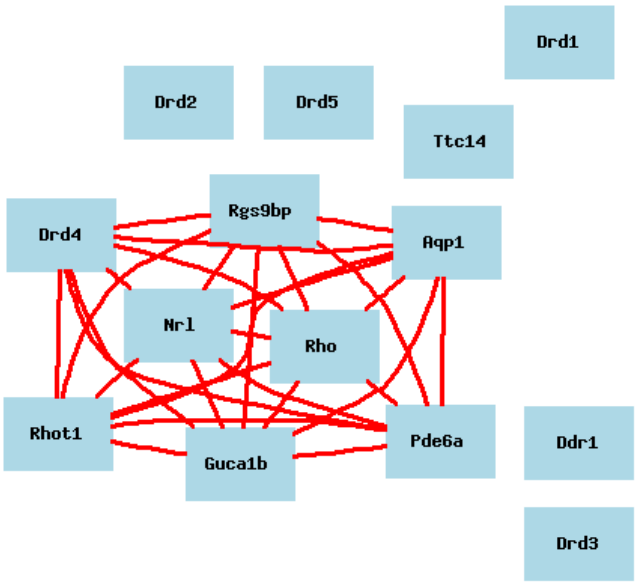


Figure 4-4.4. Genetic pathway analysis of *Guca1b* and *Rho*, relating to *Drd4* using GeneNetwork. These three transcripts were put into GeneNetwork's pathway analysis tool along with the other transcripts shown in the figure. The red lines demonstrate a correlation of $r > 0.7$ or higher between the connected genes. These genes were differentially expressed in the *Drd4*$^{-/-}$ retinas in relation to the *Drd4* gene. The other dopamine receptor subtypes were forced into the analysis with D_4R. Notice that there was no correlation between these genes and the D_4R network.

anomalous transcripts interact with each other, form signaling networks or directly interact (stimulating or inhibiting) other proteins. Ingenuity Pathway Analysis software was used to generate network pathways from a list of the Up-regulated genes (350 transcripts, higher expression in WT samples relative to the *Drd4*$^{-/-}$ samples) and Down-regulated genes (125 transcripts, lower expression in WT samples relative to the *Drd4*$^{-/-}$ samples) from the normalized microarray data (IPA, www.ingenuity.com, Redwood City, California) . This analysis generated 25 network pathways (Fig. S2, Appendix II; 20 up-regulated and 5 down-regulated) and a lists of genes that are most highly correlated with one another. Figure 5 shows the most highly correlated network among the up-regulated transcripts (out of 20 networks), which determined that these proteins are important in cellular process such as: 1) Nervous System Development and Function, 2) Cell Morphology, and 3) Cellular Assembly and Organization. Of the 5 pathways generated for the down-regulated gene list Figure 6 displays the most significant relationships. The proteins represented relate to biological functions such as gene expression, small molecule biochemistry, and cellular development. Firm conclusions cannot be drawn from this information without further biological investigation. This information is important for generating new hypotheses and provides insights for novel signaling networks. From the generated networks it appears that the putative *Drd4*-induced gene expression may be mediated by the SRC pathway. Nine of 20 pathways (Up-regulated group, WT vs. *Drd4*$^{-/-}$) have *Drd4* integrated into their pathway through a *SRC* link (Fig. S2, Appendix II). In the case of the "down" genes there are only 5 large networks and there is no apparent bias as to how *Drd4* integrates into the pathways. However, network 1 (Fig. 6) of the down pathways integrates *Drd4* through *KCNJ7*, a G-protein-regulated inward rectifier K^+ channel gene, which has been implicated in

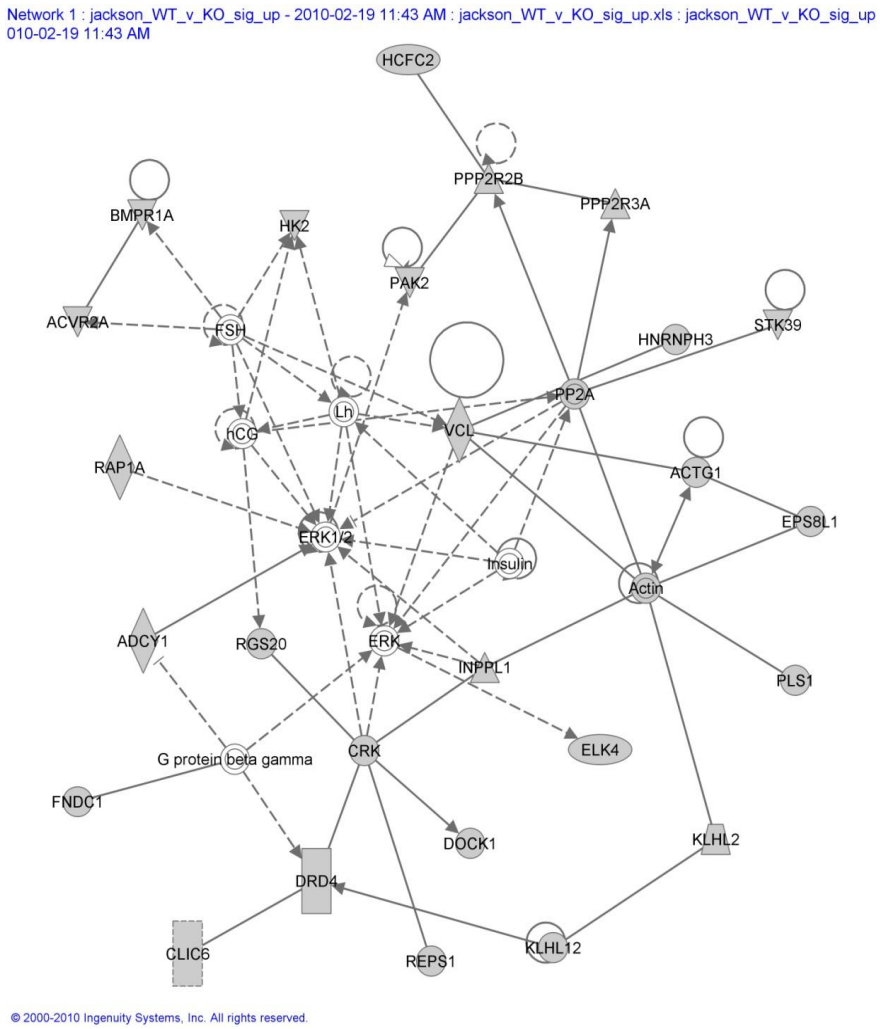


Figure 5-4.4. Pathway analysis of top up-regulated genes via Ingenuity pathway analysis software.

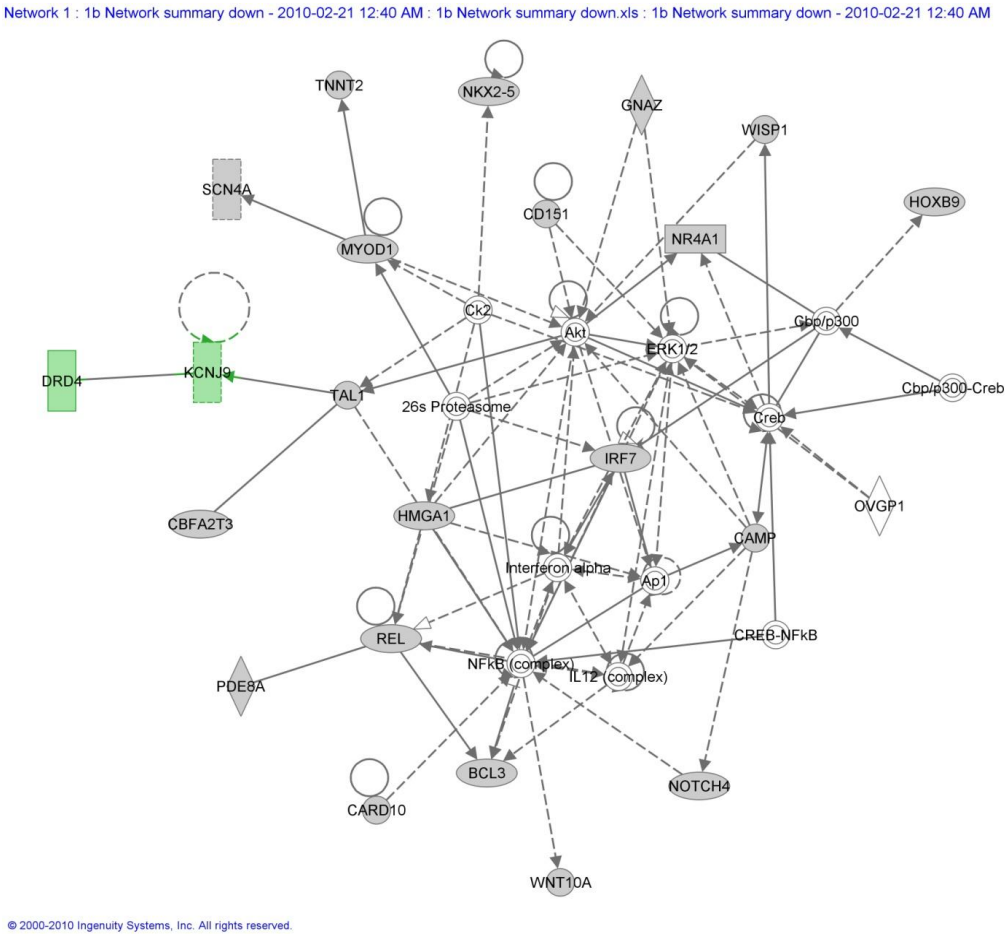


Figure 6-4.4. Pathway analysis of the top down-regulated genes via Ingenuity pathway analysis software.

disease phenotypes such as Parkinson's disease . For a complete account of the UP- and Down - regulated gene network pathways refer to Appendix II data sets.

4.5 Retinal function in *Drd4*$^{-/-}$ mice

4.5.1 Visual Acuity and Contrast Sensitivity Assessment. To examine visual retinal functioning we tested and compared visual acuity and contrast sensitivity in our *Drd4*$^{-/-}$, *Adcy1*$^{-/-}$, and WT mouse models. This was accomplished by placing the mice in a virtual-reality cylindrical drum that created a sine-wave gradient (black and white bars) on four LCD monitors surrounding a central stage where the animal was sitting. The mice have an innate-behavioral response to the moving gradient, displayed as movement of their head and neck.

When compared with WT controls both *Drd4*$^{-/-}$ and *Adcy1*$^{-/-}$ showed significant reductions in contrast sensitivity (Fig. 7a/b, **P <0.001 and *P <0.05), while maintaining normal visual acuity (Fig. 8). In each case the mice were tested over a range of spatial frequencies (distance between the black and white bars of the gradient), because previous research has shown that when tested for contrast sensitivity mice displayed a inverted U-shaped response curve (Prusky and Douglas 2004). *Drd4*$^{-/-}$ mice had a reduced contrast sensitivity at each spatial frequency measured (**P <0.001, *P <0.05, Fig. 7a). *Adcy1*$^{-/-}$ mice were tested under the same conditions to see if the cAMP signaling system played a role in contrast regulation. It is important to note that expression of the *Adcy1* transcript, which encodes the type 1 adenylyl cyclase, is controlled by the D_4R (Jackson, Chaurasia et al. 2009) and could potentially contribute to contrast regulation. These mice also demonstrated reduced contrast sensitivities at

most of the spatial frequencies, excluding 0.192 c/d and 0.272 c/d (**P <0.001, Fig. 7b). These data indicate that the D_4R and AC1 play an important role in contrast sensitivity

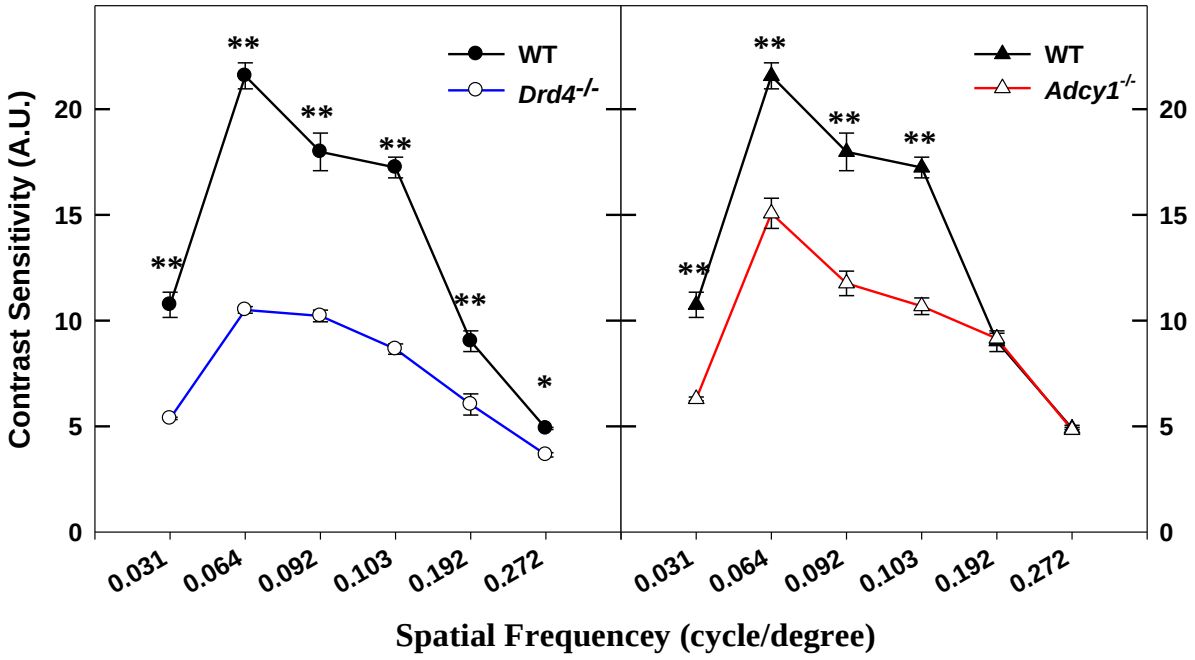


Figure 7-4.5. Contrast sensitivity in *Drd4*$^{-/-}$ (open circles) and *Adcy1*$^{-/-}$ (open triangles) mice. Animals were housed in a 12 hr light/dark cycle for at least 2 weeks. Contrast sensitivities were measured over multiple spatial frequencies to access the differences over the range of visual performance ensuring changes were not seen at only a single measurement. All animals were tested approximately during the mid-day time point (ZT 4 - ZT 8), to eliminate circadian effects. (a) *Drd4*$^{-/-}$ mice demonstrate significant reductions (*P <0.05 and **P <0.001, mean ± SEM: n = 4) in contrast sensitivity at all spatial frequencies when compared with WT controls (n = 3, black-filled circles). (b) *Adcy1*$^{-/-}$ mice show a reduction in contrast sensitivity in four of the six spatial frequencies tested (**P < 0.001, mean ± SEM: n = 3, Two-Way ANOVA and post-hoc Student-Newman-Keuls) when compared with WT controls (closed triangles).

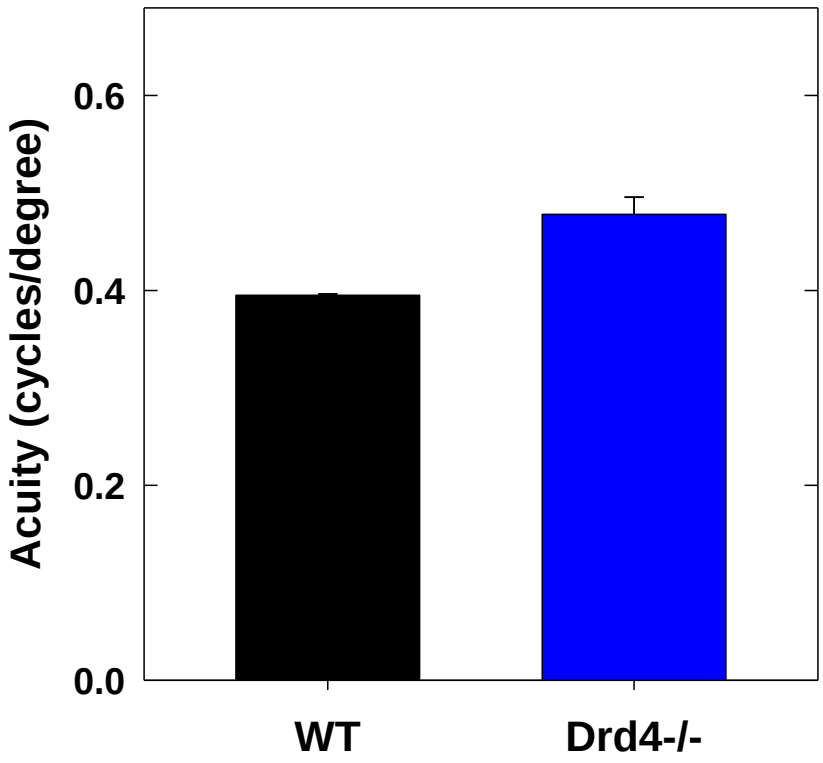


Fig. 8-4.5. Mice lacking the D_4R demonstrate normal visual acuity as determined by student t-test statistical analysis.

regulation in mouse retina. However, it is possible that some of the aberrations seen could be due to abnormalities in central visual cortex in processing, although visual cortex lesions do not impair OKT measurements of acuity (Douglas, Alam et al. 2005).

4.6 Discussion. The results of this investigation indicate that the DA/D_4R signaling system regulates the expression of hundreds of genes (~500 genes) in the retinal transcriptome. To our knowledge, this is the most comprehensive investigation of D_4R signaling in relation to gene expression. Some differentially expressed genes exhibit rhythmic behavior in WT samples, while similar patterns of rhythms were not detected in the *Drd4*$^{-/-}$ mouse retina. Also, our results leads us to believe that the D_4R signaling system is very important in maintaining normal visual contrast sensitivity in mammals. The differentially expressed genes found in the microarray analysis are associated with a variety of functions, such as cell signaling, chromatin remodeling, and protein-protein interactions. These findings provide key points of interest to investigate new molecular processes and signaling pathways that maybe associated with dopamine's overall role in transitioning the retina from a rod photoreceptor dominant system to a cone photoreceptor dominant system (Witkovsky 2004).

We identified 475 genes, with known functions that are differentially expressed in mice lacking the D_4R. These genes represent a breadth of biological functions, however, solely examining the changes in gene expression levels defines little in terms of overall function. Recent published data from our lab shows that D_4R activation controls the expression of *Adcy1* (transcript for AC1), which is the primary cyclase controlling cAMP production in mouse retina (Jackson, Chaurasia et al. 2009), and that rhythmic cAMP in mammalian retina is abolished in

Drd4$^{-/-}$ retinas. The culmination of these findings leads us to believe that the lowered levels of AC1 can be attributed to the lack of D_4R signaling, plus this defect causes abnormal cAMP signaling potentially affecting downstream targets. Moreover, we suspect other abnormally expressed transcripts will encode for proteins are that important for other cellular processes, such as light adaptation.

Gene ontology and pathway analyses revealed that the D_4R influences photoreceptor-signaling pathways (e.g., *Guca1b* and *Pde6a*). The D_4R may signal through the SRC system, potentially controlling these transcripts' expression. GeneNetwork analysis revealed a correlation between the highly differentially expressed *Guca1b* and *Rho*. The results also show that the correlation is D_4R specific because when the other dopamine receptor subtypes are forced into the analysis they show no network connectivity (Fig. 3).

The study of D_4R signaling is evolving, especially in its regulation of cAMP production (Rondou, Haegeman et al. 2010). Our IPA pathway analysis shows that this receptor may signal through Src homology domains implicating the mitogen-activated protein kinase (MAPK) pathway as an important step.

The pathway analysis results also identified the *Kcnj7* (G protein-activated inward rectifier potassium channel 2 isoform Girk2-1) as a potential signaling target of the D_4R pathway. Interestingly, a previous study has shown that this channel maybe involved in Parkinson's disease (Bandmann, Davis et al. 1996), in which the dopamine neurons in the substatia nigra are destroyed.

The retina is a highly rhythmic tissue, coordinating much of its physiology to the 24 hr day/night cycle. The INL and GCL both exhibit the core clock components indicative of a

functional circadian clock (Gustincich, Contini et al. 2004; Ruan, Zhang et al. 2006), while a single photoreceptor does not possess all of the core components at detectable levels. Conversely, melatonin production, outer segment disc shedding, Na^+/K^+ ATPase activity, and rod/cone horizontal cell coupling are just a few of the circadian phenotypes displayed in the PRL of mammalian retina. It seems that DA plays a major role in regulating each of these processes. To date, only one study has investigated whether or not D_4R activation controls aspects of the circadian clock. Ruan et al. (2008) showed that dopamine mediates circadian function through D_1R and could not detect significant changes in PER2::LUC expression via D_2R-like stimulation. However, they used a transgenic mouse where the luciferase reporter gene is driven by the *Per2* promoter and luciferase is expressed primarily in the inner retina. Our results showed that there are slight changes in core clock gene expression in our *Drd4*$^{-/-}$ model when assaying endogenous circadian transcripts in whole retinal extracts. Also, our analysis identified several gene transcripts that show rhythmic expression in WT retina, but not in retinas of *Drd4*$^{-/-}$ mice. While the current data does not support the conclusion that the rhythmic differentially expressed genes are regulated directly by the circadian clock, we can firmly say that the D_4R is important for regulating gene transcripts in mammalian retina and that some of these transcripts are rhythmic.

The retina is an intrinsically rhythmic tissue, not only at the gene regulation level, but also in functionality. It is clear that the DA/D_4R signaling system controls a portion of the retinal transcriptome, as well as supports light adaptation mechanisms and contrast sensitivity. These data demonstrate that this signaling system supports indispensable components retinal function.

Chapter V:

Conclusions and Future directions

5.1 Conclusion summary.

Chapters 2, 3, and 4, provide new evidence that the DA/D_4R signaling pathway in the retina is critical in gene regulation, as well as modulating circadian phenomena, overall, maintaining normal retinal functioning.

The data presented show that the D_4R is coupled to AC1 and controls its activity levels by modulation of its mRNA transcript. Also, we demonstrate that rhythmic retinal cAMP is primarily controlled by the rhythmic activity of AC1. This entire system is dependent on the expression and activation of the D_4R, seeing that the normal rhythm of *Adcy1* (transcript encoding for AC1) can be altered by stimulating the D_4R at an abnormal time point. Lack of this receptor not only significantly reduces basal levels of retinal cAMP, but also eliminates the rhythmic cAMP levels. To my knowledge this is the first complete characterization of how the D_4R signaling system regulates cAMP levels in mammalian retina (Chapters II).

cAMP is a potent regulator of gene expression (Hagiwara, Shimomura et al. 1996; Iuvone, Tosini et al. 2005). Knowing this and that *Drd4*$^{-/-}$ retinas have significantly lower cAMP levels led to the finding that ~4.8% of the retinal transcriptome is affected by the absence of the D_4R. In addition, we found that this receptor is important in regulating contrast sensitivity in mice (Chapter IV). These data enables us to form gene and protein networks, potentially locating novel chromosomal loci that are critical for this signaling pathway affecting visual function.

5.1.1 Aim 1 : Novel findings and future studies. The first aim focuses on identifying which adenylyl cyclase(s) couple to the D_4R when stimulated by DA. Upon photoreceptor hyperpolarization there is a drop in Ca^{2+} levels that is followed by a drop in the cAMP levels. The literature shows that Ca^{2+} drives cAMP production in the vertebrate photoreceptors

implicating a Ca^{2+}-modulated adenylyl cyclase (Iuvone, Gan et al. 1991; Ivanova and Iuvone 2003; Chaurasia, Haque et al. 2006). There are at least three adenylyl cyclases that are affected by Ca^{2+}; AC1 and AC8 are stimulated, while AC3 is inhibited (Choi, Xia et al. 1993). Also, the neural specific AC1 is highly expressed in photoreceptors (Xia, Choi et al. 1993) and may account for the increase in Ca^{2+}/calmodulin stimulation seen in photoreceptor-enriched chick retinal cell cultures (Iuvone, P.M. et al. 1996).

It was found that *Drd4*$^{-/-}$ mouse retinas show approximately 50% reduction in *Adcy1* transcript levels; plus cAMP and Ca^{2+}-stimulated cyclase activity are significantly reduced in these mice when compared to WT controls (Chapter II). Using the *Adcy1*$^{-/-}$ mouse model enabled us to determine that AC1 is the primary cyclase inhibited by D_4R activation in mammalian retina. Also, the investigation determined that D_4R activation controls *Adcy1* mRNA expression in mouse retina, adding an additional layer to retinal cAMP regulation. These novel findings help further define elements of the D_4R signaling pathway(s).

The cAMP signaling pathway and MAP kinase signaling pathway both have been shown to be affected by D_4R activation (Rondou, Haegeman et al. 2010). Identifying AC1 as a main target of D_4R implies that this specific regulation is important for maintaining normal physiology. There is an ~90% reduction in cAMP when AC1 is absent; AC8 and AC3 were assayed and showed no compensation (Chapter II, Jackson et al. 2009). Using the same techniques and knockout strategies could verify or eliminate the possibility of these cyclases contribution to retinal cAMP regulation.

AC1's main known function is the conversion of ATP into cAMP, however, it is possible other proteins (e.g., signaling proteins, etc.) bind to ACs adding a new avenue to the D_4R

signaling pathway. AC1 is a large protein complex (100 kD) and to date there are no reliable primary antibodies for measuring its protein level, making techniques like co-immunoprecipatation (Co-IP) nearly impossible to perform to identify novel binding partners. Identification of novel binding partners could provide new insight to alternative signaling pathways involved in D_4R activation.

Future studies should focus on identifying the exact signaling pathway(s) of the D_4R signaling system. Showing that activation of this receptor controls gene regulation is only part of the story. Investigating what signaling pathways, DNA targets of transcription factors, and post-translation mechanism will provide a clearer picture in order to better understand the role of DA in retinal functioning.

5.1.2 Aim 2 : Novel findings and future studies. Aim 2 focused on elucidating if D_4R activation influences mammalian retinal circadian clocks. The accepted paradigm for a cell to have a functional circadian clock is that it must contain the canonical core clock gene elements (Chapter 1, Fig. 5-1.4). Using this as a benchmark, the mammalian retina has been extensively studied showing that there are retinal clocks contained in the INL and GCL. The photoreceptor layer also displays several circadian phenomena (Chapter I, 1.4.3); however, Ruan et al. (2006) analyzed individual photoreceptors but did not detect all the alleged components of the circadian clock. The lack of detection could be due to the sensitivity limits of the technique, seeing that single cells were being analyzed. Another rhythm detected in the rodent retina (rat photoreceptor layer) is the *Drd4* transcript (Storch, Paz et al. 2007; Klitten, Rath et al. 2008). In the case of photoreceptor cells the D_4R is the only dopamine receptor subtype expressed (Cohen, Todd et al. 1992). DA, released in rhythmic manner (Nir, Haque et al. 2000), mediates circadian physiology in the retina, such as rod/cone coupling (Ribelayga and Mangel 2010). These data

imply that the DA/D_4R signaling pathway potentially mediates circadian clocks in the mammalian retina.

In Chapter 2 the data shows the D_4R controls *Adcy1*expression in mouse retina. In Chapter 3 we and others (Storch, Paz et al. 2007) show that *Adcy1*, a clock controlled gene, is rhythmic in WT mouse retina, but in *Drd4*$^{-/-}$ retinas there is no detectable rhythm of this transcript. Agonist stimulation or antagonist blockade of the D_4R, at different times during the day, manipulates the normal rhythmic pattern of *Adcy1*, proving that receptor activation has an effect on certain circadian rhythms in mammalian retina. To our knowledge this is the first evidence that the DA/D_4R signaling system acts as a zeitgeber coordinating a portion of retinal circadian function. However, this type of investigation has some limitations; 1) testing in whole retina, 2) no direct testing of core clock components, and 3) multiple oscillators in multiple cell types. In circadian biology it is thought that in order to have a functional clock there must be core clock genes present (Bunger, Wilsbacher et al. 2000). In our studies we looked at *Adcy1* as an observable output of circadian clock functioning, however, direct evidence of changes in the core clock mechanism, in specific cell types, will provide a better demonstration of the role of this DA/D_4R signaling system in clock regulation.

Previous studies show that multiple circadian oscillators exist in the mammalian retina (Ruan, Zhang et al. 2006; Dorenbos, Contini et al. 2007; Tosini, Davidson et al. 2007; Ruan, Allen et al. 2008) that occur in different cell types. One main focus of their studies was to demonstrate where the primary oscillator(s) are located in the retina. A general consensus is that oscillators are located in the INL and GCL, with less defining evidence of a clock located in the photoreceptor layer. In this investigation we did not pinpoint the exact location of the D_4R signaling regulation of the *Adcy1* transcript. Future studies should serve to directly measure the

location of *Adcy1* circadian regulation. A luciferase expression system that is driven by the *Adcy1* promoter (*Adcy1*::LUC transgenic mouse), along with primary retinal cell culture, where individual cell types are assayed for rhythmic expression could serve to answer this outstanding question. Individual cell types could show how they endogenously regulate this rhythm, plus agents (e.g., dopamine, GABA, etc.) could be added to the system to determine what retinal neuromodulators affect circadian rhythmicity.

Knowing how the DA/D_4R signaling system is involved in regulating the circadian clock is important because it has been shown that the retinal circadian clock is paramount in retinal function (Storch, Paz et al. 2007). Recent published data shows that a D2-like mechanism is directly involved in circadian regulation of rod-cone signal flow in mammals (Ribelayga and Mangel 2010).

5.1.3 Aim 3 : Novel findings and future studies. In aim 3, hundreds of genes were identified as being differentially expressed in mice lacking the D_4R compared to WT mice. This investigation broadened the data showing that *Adcy1* is lower in *Drd4*$^{-/-}$ retinas (Chapter I) by looking at changes in global retinal gene expression. Also, it shows that while *Drd4*$^{-/-}$ mice have normal visual acuity, the have deficits detecting changes in contrast. Moreover, when looking at cone photoreceptor function these knockout mice have difficulty adapting to light after dark adaptation (Nir, Harrison et al. 2002). The culmination of these data shows that the DA/D_4R signaling system not only is important for gene regulation but also has a direct effect on retinal function.

The D_4R is highly expressed in retina, making this tissue an ideal site to study this signaling system. 475 genes, with known functions, show differential expression patterns in the

knockout mouse. These genes encode for proteins that control multiple cellular functions, such as, cell signaling, histone modification, protein-protein interaction, and enzymatic processes. Gene and protein network software (IPA and GeneNetwork) identified potential new targets (i.e., SRC), which may be key players in this signaling system regulating the differentially-expressed genes seen in the retinas of mice lacking the D_4R. It is still unclear if any of the changes in gene expression specifically affect certain aspects of retinal function; this type of ongoing research will spearhead D_4R signaling research.

5.2 Final conclusion. This investigation has given new insights into the DA/D_4R signaling system in mouse retina. The totality of the data demonstrates that this signaling system is vital for normal mammalian retinal function. This research on retinal doapminergic signaling mechanisms provides novel evidence of additional signaling component(s) (AC1), evidence of rhythmic gene regulation, and data on visual contrast sensitivity. This research will provide a better foundation for visual research in humans with dysfunctional dopaminergic systems.

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