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NPAS2 and CLOCK in the mammalian retina: their localization and roles in clock-controlled gene regulation and visual function

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B.A., Dartmouth College, 2004

Advisor: P. Michael Iuvone, Ph.D.

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Abstract

NPAS2 and CLOCK in the mammalian retina: their localization and roles in clock-controlled gene regulation and visual function

By Christopher Kwan Hwang

Circadian rhythms are biological rhythms that occur on a daily basis. These rhythms are thought to be controlled by an autonomous transcriptional-translational feedback loop called the circadian clock. Traditionally, the clock has been thought to be comprised of two positive-regulator proteins (CLOCK and BMAL1) and four negative-regulator proteins. However, in certain parts of the brain, recent studies have demonstrated that NPAS2 has an overlapping role with CLOCK and led to my investigation of NPAS2's role in the retinal circadian clock. I determined that NPAS2 is expressed rhythmically in a subset of retinal ganglion cells and regulates the expression of a clock-controlled gene, adenylyl cyclase type 1 (*Adcy1*), selectively in the ganglion cell layer (GCL). I also discovered that *Npas2*$^{-/-}$ mice show strikingly similar reductions in the contrast sensitivity rhythm to that of *Adcy1*$^{-/-}$ and *Drd4*$^{-/-}$ mice in which contrast sensitivity has previously been shown to be disrupted. In *Drd4*$^{-/-}$ mice, I discovered that the expression of *Npas2* and *Adcy1* transcripts in the GCL are arrhythmic, suggesting that contrast sensitivity is modulated through a dopamine-NPAS2-adenylyl cyclase pathway in the GCL. Next I explored the role of CLOCK in the contrast sensitivity regulation and determined that contrast sensitivity in *Clock*$^{-/-}$ mice contrast sensitivity is arrhythmic. Using a luciferase reporter assay, I determined that the *Adcy1* promoter is selectively activated by the NPAS2/BMAL1 heterodimer but not the CLOCK/BMAL1 heterodimer in neuronal NG108-15 cells and that the transcript expression of *Npas2* and *Adcy1* in the GCL of *Clock*$^{-/-}$ mice are arrhythmic, suggesting that CLOCK regulates the contrast sensitivity rhythm in part by regulating the expression of NPAS2. In addition to contrast sensitivity, I also investigated the roles of NPAS2 and CLOCK in the regulation of electroretinogram (ERG) responses. In *Clock*$^{-/-}$ mice, the light-adapted ERG rhythm and dark-adapted ERG responses are significantly disrupted. In contrast, both light-adapted and dark-adapted ERG responses in *Npas2*$^{-/-}$ mice are not disrupted, suggesting that the functional role of NPAS2 outside of the GCL is limited. In summary, these studies demonstrate that CLOCK and NPAS2 have differential roles in retinal circadian clocks and in visual function.

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celebrate with me. Because of my wife, I went through the rigorous training period in science without losing sight of the bigger purpose that God has for me in my life. Pauline is my best friend whom I cherish and love dearly, and my words just cannot express the level of appreciation for her. Additionally, I would like to thank my parents, brother, sister-in-law, parents-in-law, and friends for their love and support.

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Chapter 1: Introduction

1.1 What are circadian rhythms?

Circadian rhythms are biological rhythms that occur over a roughly 24-hour period and on a daily basis. Common examples include sleep-wake cycles, locomotor activity, hormone secretion, blood pressure, and body temperature. The term “circadian” has Latin roots and literally means “around a day.” *Circa* in Latin means “around,” and *diem* or *dies* means “a day.” Collectively circadian rhythms refer to biological rhythms with a period of around a day. These physiological rhythms confer an adaptive advantage to organisms by allowing them to anticipate daily fluctuations in the environment and optimize their biochemical, physiological, and behavioral properties accordingly. Consequences of disruptions in circadian rhythms are significant and numerous, and they include metabolic disorders (e.g., obesity, diabetes, metabolic syndrome) (Marcheva et al., 2009; Marcheva et al., 2010), mood disorders (e.g., bipolar, major depressive, and seasonal affective disorders) (Hampp and Albrecht, 2008; Hampp et al., 2008; Albrecht, 2010), and cancers (Fu and Lee, 2003; Collis et al., 2007; Sahar and Sassone-Corsi, 2009).

Traditionally three prerequisites must be met for a 24-hour rhythm to be called a circadian rhythm: 1) the rhythm must phase-shift in accordance to an entrainment stimulus referred to as a zeitgeber (German word for “time giver”) like the sun light; 2) the rhythm must persist in the absence of a zeitgeber (the period referred to as the free-running period); and 3) the rhythm has to be temperature-

compensated (i.e., the period has to be similar at different temperatures). Due to the predictable variation in the environmental light intensity between the day and night, the sun light is a potent zeitgeber to entrain circadian rhythms in organisms. In a laboratory setting, the room light is used to model the natural light cycle to entrain circadian rhythms. In a typical laboratory light cycle, the light is on for 12 hours and off for the next 12 hours. The time at which the light turns on is designated zeitgeber time 0 (ZT0), and the zeitgeber time ranges from ZT0 to ZT24 in a 24-hour cycle. In the free-running period (i.e. in constant darkness), the time designation changes from zeitgeber time to circadian time (CT) to denote the absence of the zeitgeber. For example, in a 12-hr light-12-hr dark cycle in which light turns on at 7 am and off at 7 pm, then 7 am would be designated as ZT0 and 7 pm as ZT12. In constant darkness when organisms are free-running, 7 am would be labeled as CT0 and 7pm as CT12.

1.2 Molecular mechanism of circadian rhythms

Circadian rhythms in organisms have been observed for centuries, but the mechanism for circadian rhythms has not been elucidated until recently. In the 1960's, studies using RNA and/or protein synthesis inhibitors showed that the mechanism may involve gene regulation (Karakashian and Hastings, 1962; Feldman, 1967). Several years later in 1971, Konopka and Benzer first identified a gene that was linked to altered circadian rhythm periods of locomotor activity and eclosion in *Drosophila melanogaster* (Konopka and Benzer, 1971). In this

study, three different mutations in the same gene resulted in three different phenotypes: one with a shorter circadian period, another with a longer circadian period, and one with arrhythmic circadian period. Now we know the gene as the period gene. However, it was not until 20 years ago that a clock gene in mammals was identified. In 1994, the work from the Takahashi lab resulted in the identification of the first circadian gene in mammals, which was suitably named the circadian locomotor output cycles kaput gene, abbreviated as *Clock* (Vitaterna et al., 1994). Using a forward genetic screen, they identified mice that have an antimorphic, dominant negative mutation in the *Clock* gene, which results in the absence of locomotor activity rhythms. In the ensuing five years, additional genes in the circadian rhythm mechanism such as brain and muscle ARNT-like protein 1 (*Bmal1*) (Hogenesch et al., 1997; Hogenesch et al., 1998), period 1 and 2 (*Per1* and *2*) (Albrecht et al., 1997; Sun et al., 1997), and cryptochrome 1 and 2 genes (*Cry1* and *2*) (Kobayashi et al., 1998; Kume et al., 1999; van der Horst et al., 1999) were identified, and the period and cryptochrome genes were shown to play different roles in a negative feedback loop that is at the core of the circadian oscillator.

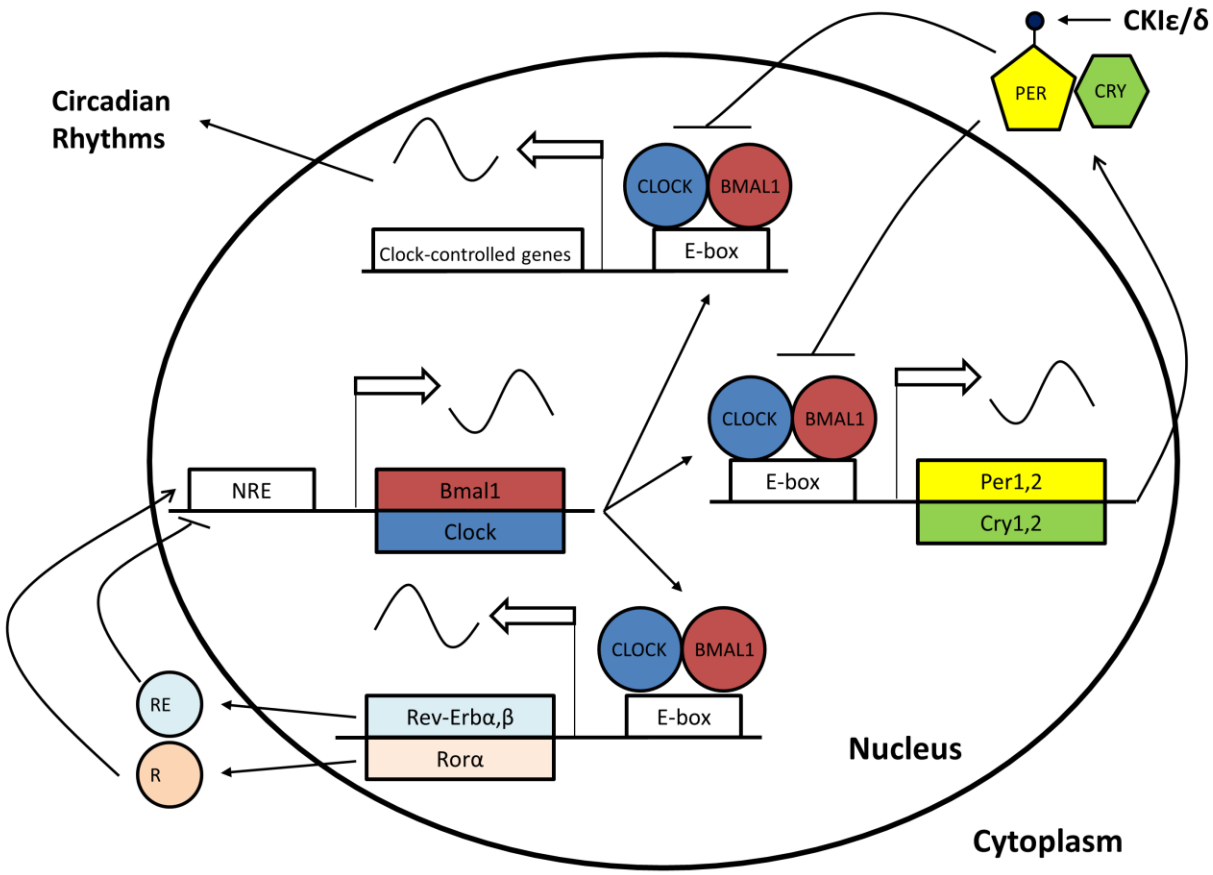

Figure 1-1 The molecular mechanism of the circadian clock.

The clock is comprised of six core clock proteins, two of which are CLOCK and BMAL1. CLOCK and BMAL1 are bHLH transcription factors that heterodimerize and drive the expression of many clock-controlled genes that regulate circadian physiology. They also drive the expression of the four other clock proteins called PERIOD1/2 and CRYPTOCHROME1/2, which function to inhibit the activity of CLOCK and BMAL1, and RORα and REV- ERBα,β, which antagonistically regulate the expression of CLOCK and BMAL1. Phosphorylation of the PERIOD protein by casein kinase Iδ and Iε contributes to the time delay of the clock.

Circadian rhythms are thought to be driven by an autonomous transcriptional-translational feedback loop called the circadian clock (Figure 1-1). CLOCK and BMAL1 are the positive regulators of the clock. They are basic-helix-loop-helix (bHLH) transcription factors that heterodimerize, bind to a six-nucleotide element in the promoter called the circadian E-box, and drive the expression of their negative regulators, PERIOD 1 and 2 and CRYPTOCHROME 1 and 2 (Gekakis et al., 1998; Reppert and Weaver, 2002; Ko and Takahashi, 2006), as well as clock-controlled genes, which are output genes that are thought to drive different physiologic rhythms (Akhtar et al., 2002; Duffield et al., 2002; Panda et al., 2002; Storch et al., 2002). Following the translation of the PERIOD and CRYPTOCHROME proteins in the cytoplasm, the PERIOD proteins bind to the CRYPTOCHROME proteins, translocate into the nucleus, and inhibit the activity of the CLOCK/BMAL1 heterodimer, thereby inhibiting the transcription of the genes that encode them. Post-translational processes like the phosphorylation of PERIOD proteins by casein kinase Iδ and Iε (CKIδ and CKIε) have been shown to contribute to the time delay needed for a 24-hour clock (Lowrey and Takahashi, 2000; Lee et al., 2001; Lee et al., 2004).

In addition, CLOCK and BMAL1 drive the expression of orphan nuclear receptors, RORα and REV-ERBα and β. These proteins comprise another loop within the circadian clock machinery, and their effects are antagonistic. RORα functions as a positive regulator, and REV-ERBα and β as repressors. They compete to bind to a six-nucleotide element called the nuclear receptor element

(NRE) in the promoters of *Bmal1* (Preitner et al., 2002; Sato et al., 2004; Cho et al., 2012), *Clock* (Crumbley and Burris, 2011), and *Npas2* genes (Crumbley et al., 2010) to regulate their expression.

Recently a new CLOCK homolog protein called neuronal PAS-domain protein 2 (NPAS2) was identified. Like CLOCK, NPAS2 is a bHLH transcription factor that exhibits its functions after heterodimerizing with BMAL1 (King et al., 1997; Zhou et al., 1997; Hogenesch et al., 1998). NPAS2 has been shown to have an integral role in the clock machinery in the forebrain (Reick et al., 2001) and suprachiasmatic nuclei (SCN) (Debruyne et al., 2006; DeBruyne et al., 2007b). However, in other tissues, the role of NPAS2 as a clock protein is not as clear. For example, NPAS2 is able to substitute for CLOCK in preserving the *Per2*-luc rhythm in the SCN of *Clock*$^{-/-}$ mice (Debruyne et al., 2006; DeBruyne et al., 2007b), but in the liver of *Clock*$^{-/-}$ mice, NPAS2 cannot substitute for CLOCK, and *Per2*-luc rhythm is abolished even though *Npas2* expression in the liver is undisrupted (DeBruyne et al., 2007a). In peripheral oscillators, NPAS2 appears to function as an output of the clock. Although NPAS2 cannot substitute for CLOCK to preserve the *Per2*-luc rhythm in the liver (DeBruyne et al., 2007a), NPAS2 substitutes for CLOCK in regulating the expression of a circadian gene for the Factor VII protein (Bertolucci et al., 2008).

1.3 Circadian rhythms in the retina

1.3.1 The structure of the retina

The retina is a light-sensitive neural tissue that lines the posterior part of the eye (Figure 1-2). From the optic disc, the retina extends anteriorly to the ora serrata, which is a junction between the ciliary body and retina. The retina has three layers containing neural cell bodies (ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL)) and two layers containing synaptic junctions (inner plexiform layer (IPL) and outer plexiform layer (OPL). The ONL contains the cell bodies of rod and cone photoreceptors. The ONL plus inner and outer segments of photoreceptors, where the cytoplasm and opsins are located, respectively, are collectively termed the photoreceptor layer (PRL). The OPL contains synapses between photoreceptors and cells in the INL, which include bipolar, horizontal, and amacrine cells. The GCL contains retinal ganglion cells (RGCs) and displaced amacrine cells. The IPL houses synapses between cells of INL and GCL.

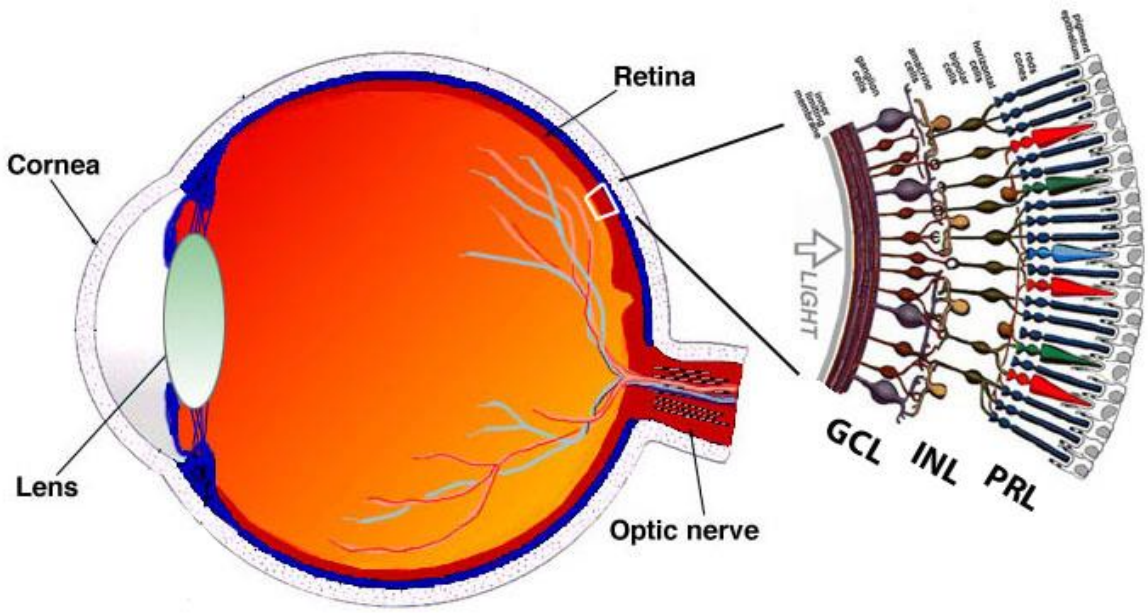

Figure 1-2 The structure of the neural retina.

In the back of the eye, there is a light-sensitive neural tissue called the retina. The retina detects light, converts it to complex neural signals, and transmits them to the brain via the optic nerve. The retina is comprised of heterogeneous cell types, and a nuclear staining of a retinal section reveals three distinct retinal layers. Photoreceptors, in the photoreceptor layer, first detect light and transmit information to bipolar cells in the inner nuclear layer. Bipolar cells transmit information to retinal ganglion cells in the ganglion cell layer. In the inner nuclear layer, there are horizontal and amacrine cells which function to modulate the responses in the vertical visual pathway. (Image adapted from http://webvision.med.utah.edu/)

Light enters the eye through the clear external membrane of the eye called the cornea and becomes focused through the lens, which is a transparent body located inside the eye behind the cornea. It traverses through the clear vitreous and reaches the retina. Light must pass through the GCL, IPL, INL, OPL, and ONL before it reaches the outer segments of photoreceptors, where discs that contain light-sensitive opsin proteins are located. Opsin protein is a multipass transmembrane protein that contains a dietary vitamin A-derived compound called 11-*cis* retinal. In the dark, photoreceptors stay depolarized through the opening of cGMP-mediated sodium/calcium channels. Upon exposure to light, 11-*cis* retinal converts to 11-*trans* retinal, which results in the activation of the transducin and phosphodiesterase proteins and lowers the intracellular level of cGMP (Hall, 2011). Consequently, cGMP-mediated sodium/calcium channels close, and photoreceptors become hyperpolarized. 11-*trans* retinal converts to 11-*trans* retinol before being transported into the retinal pigment epithelium (RPE) through a cellular retinol binding protein (CRBP). In the RPE, 11-*trans* retinol is recycled back to 11-*cis* retinal, and CRBP transports the retinal back to a photoreceptor (Purves, 2012).

All retinal signaling from photoreceptors to RGCs occur through electrotonic conduction (Hall, 2011). This means that, instead of action potentials, direct current transmits information from one retinal neuron to another. Therefore, the signal is not all or none as in action potentials, but direct current allows a graded response, which is important to detect the level of illumination. Between

photoreceptors and RGCs, there are several other retinal neurons in the INL, which include horizontal, amacrine, and bipolar cells. Horizontal cells connect laterally between photoreceptors and bipolar cells, and their outputs are always inhibitory. Because of the lateral connection and inhibitory output, the horizontal cell circuitry mediates a process called lateral inhibition that contributes to contrast detection (Hall, 2011). A focal light on the retina will stimulate the area, but the surrounding area will be inhibited through lateral inhibition. Amacrine cell is another cell-type in the INL. There are several different types of amacrine cells, and some of them are dopaminergic, GABAergic, and cholinergic amacrine cells, which synthesize and release neuromodulators to mediate their functions in the retina. A special type of amacrine cells called AII amacrine cells function as the primary conduit to transmit neural input from rod bipolar cells to cone bipolar cells. Lastly, cone bipolar cells transmit neural signals to RGCs through electrotonic conduction.

Unlike the rest of retinal neurons, RGCs transmit visual information to the brain through action potentials (Hall, 2011). There are several types of RGCs. A vast majority of RGCs are involved in image-forming functions. One of the critical functions of RGCs is contrast detection. Through a center surround antagonism, RGCs allow organisms to detect contrast in their environment (Purves, 2012), which is discussed in greater detail in Chapter 2. Additionally, some RGCs are motion-sensitive and differentially respond to the direction of a visual stimulus. These RGCs are collectively known as the direction-selective ganglion cells

(DSGCs) and are comprised of three subtypes: On DSGCs, Off DSGCs, and On-Off DSGCs [reviewed in (Vaney et al., 2012)]. Each DSGC subtype responds to global motion in at least one direction. In addition to visual functions, RGCs mediate non-visual functions such as the entrainment of circadian rhythms. Circadian rhythms are thought to be regulated by a molecular feedback loop called the circadian clock. The master clock of mammals resides in the SCN of the anterior hypothalamus (Reppert and Weaver, 2002). Because light is the main entrainment stimulus to entrain circadian clocks (Reppert and Weaver, 2002), light must first be converted into a neural signal in the retina and transmitted to the SCN through the retino-hypothalamic tract for entrainment. The SCN subsequently synchronizes the phases of multiple peripheral oscillators in the body (Yamazaki et al., 2000), and the peripheral oscillators are thought to mediate their local circadian rhythms.

In the retina, a small subset of RGCs were recently discovered to be intrinsically photosensitive RGCs (ipRGCs). An ortholog of a novel opsin called melanopsin in melanophores in Xenopus was localized in RGCs of mice (Provencio et al., 1998; Provencio et al., 2000). These cells are responsible for relaying the light entrainment input to the SCN as several studies have shown that these cells project their axons directly to the SCN (Gooley et al., 2001; Hannibal, 2002) and that melanopsin confers photosensitivity to ipRGCs (Berson et al., 2002; Hattar et al., 2002).

1.3.2 Circadian rhythms in the retina

Many studies investigating retinal physiology in vertebrates using *Xenopus* or avian models have uncovered circadian rhythms in the retina at several levels, including retinomotor movements (Pierce and Besharse, 1985, 1988), electroretinogram (Manglapus et al., 1999), melatonin synthesis (Cahill and Besharse, 1990; Ivanova and Iuvone, 2003), and phototransduction events (Pierce et al., 1993; Ko et al., 2001, 2003). In addition to physiologic rhythms, circadian rhythms in gene regulation, protein synthesis, and post-translational events like phosphorylation have been described. Similar circadian rhythms have been observed in mammals, but the two physiologic processes implicated in the circadian biology that are central to this project are electroretinogram (ERG) responses and contrast sensitivity, which are discussed in detail in Chapter 2.

In the mammalian eye, one of the first circadian rhythms detected was photoreceptor outer segment disc shedding in rats (LaVail, 1976). This study showed that the outer segment disc shedding occurred rhythmically in constant darkness for at least three days and peaked just after light onset in LD and during the early subjective day in DD. However, it was not clear whether the disc shedding rhythm was regulated by the master clock in the SCN or a local clock in the retina, because it was not known whether the retina contained a local clock, that is, until a breakthrough study in 1983.

1.3.3 An autonomous clock in the retina

In 1983, Besharse and Iuvone demonstrated the presence of an automatous clock in the *Xenopus* retina (Besharse and Iuvone, 1983). Using primary retinal cultures, the authors demonstrated that the enzymatic activity of retinal serotonin N-acetyl-transferase (NAT; also known as AANAT for arylalkylamine N-acetyl-transferase) is rhythmic, and that the rhythm was sustained for several days in DD, and was phase-entrained by light. A subsequent study in the Besharse lab showed that photoreceptor cells were sufficient for the melatonin rhythm in the *Xenopus* retina to persist, suggesting that a circadian clock is localized in the photoreceptor layer (Cahill and Besharse, 1993).

Thirteen years after the discovery of an autonomous clock in the *Xenopus* retina in 1996, the presence of a circadian clock in the mammalian retina was shown in a study from the Menaker lab. Using cultured retinas of the golden hamsters, Tosini and Menaker showed that the melatonin rhythm persists in constant darkness and that the melatonin rhythm can be entrained using different light cycles (Tosini and Menaker, 1996). Additionally, they showed that the retinas from hamsters with a “tau” mutation (later identified as a mutation in the CKIε protein (Lowrey et al., 2000)), which show a shortened period of the locomotor activity rhythm, exhibit a shortened period of the melatonin rhythm, suggesting that a similar clock in the SCN that regulates the locomotor rhythm is present in the mammalian retina. However, the localization of a circadian clock in the mammalian retina has not been conclusive.

1.3.4 Localization of a clock in the retina

Tosini and Menaker attempted to determine the localization of a clock in mammals by using mice with the *rd* mutation (Tosini and Menaker, 1998), which causes a degeneration of rod phtoreceptors followed by a degeneration of cone photoreceptors (Carter-Dawson et al., 1978; Jimenez et al., 1996). They showed that the melatonin rhythm disappears when the photoreceptor degeneration is complete, although the melatonin synthesis persists at all ages of mice, suggesting that a circadian clock in mammals could also be localized in the photoreceptor layer and that AANAT, the rate-limiting enzyme for the melatonin synthesis, is expressed outside the photoreceptor layer. However, subsequent studies in rats with photoreceptor degeneration demonstrated that photoreceptors are not necessary for the circadian rhythms in the retina to persist. One study showed that the circadian rhythms of dopamine synthesis and metabolism are preserved in rat retinas with photoreceptor degeneration (Doyle et al., 2002). Another study showed that the circadian rhythms of *Aanat* transcript (*in vivo*) and melatonin release (*in vitro*) persist in rat retinas with photoreceptor degeneration (Sakamoto et al., 2004). The latter studies demonstrate that a circadian clock outside of the PRL exists and that circadian rhythms can persist without SCN or photoreceptors at least in mammals.

The localization of a clock in mammalian photoreceptors has been elusive. It is thought that all six core clock genes (*Clock, Bmal1, Per1, Per2, Cry1, Cry2*) have to be expressed for a circadian clock machinery to be functional (Ruan et al.,

2006). Although a limited expression of clock gene transcripts in the mammalian PRL has been previously reported (Gekakis et al., 1998; Ruan et al., 2006), several studies have shown that some of the core clock gene transcripts are not detected in mammalian photoreceptors. One study using *Per1*-GFP transgenic mice showed using immunohistochemistry that GFP did not localize in the PRL (Witkovsky et al., 2003). Another study using in situ hybridization did not detect *Cry1* or *Cry2* transcripts in the PRL in contrast to their detection in the GCL and INL of WT mice (Miyamoto and Sancar, 1998). A study from the McMahon lab performed a single cell PCR analysis on individual mouse rod and cone photoreceptors and found that none of them expressed detectable levels of all six core clock gene transcripts, while the cells in the INL and RGCs did (Ruan et al., 2006). Moreover, a study from the Weitz lab similarly showed that the protein expressions of CLOCK, PER1, and PER2 are limited to GCL and INL and are not detectable in the PRL (Storch et al., 2007). In contrast, Tosini and coworkers demonstrated the expression of all six clock genes in the rat PRL isolated by laser capture microdissection (LCM) and robust circadian rhythms of *Per1*-luc and melatonin synthesis in PRL isolated from *Per1*-luc transgenic rats (Tosini et al., 2007).

Considering the numerous circadian rhythms in mammalian photoreceptors, these results are surprising. There are at least three explanations for this apparent paradox. One explanation is that there are species differences in the localization of retinal clocks. Also, the circadian rhythms in photoreceptors can

be modulated remotely by a retinal clock located outside the PRL. Dopaminergic amacrine cells, which are thought to contain a circadian clock (Gustincich et al., 2004; Ruan et al., 2006; Ruan et al., 2008), could regulate circadian rhythms of photoreceptors humorally through the circadian dopamine release. Because dopamine D4 receptors (D4Rs), whose expression is also circadian, are the only dopamine receptor subtype expressed in the PRL (Cohen et al., 1992), it is possible that the circadian rhythms of photoreceptors could be entrained through the circadian dopamine release and D4Rs in the photoreceptors. Dopamine has been shown to regulate circadian rhythms of protein phosphorylation in photoreceptor cells (Pozdeyev et al., 2008). Another explanation is simply that only a select few photoreceptors could be clock cells in the PRL, making their detection extremely difficult. This latter possibility is supported by the localization of clock protein immunoreactivity in cone photoreceptors but not rods in the mouse (Liu et al., 2012).

1.3.5 The implication of the dopamine pathway in the retinal clock mechanism

In the retina, dopamine synthesis occurs in dopaminergic amacrine cells. The rate-limiting enzyme of the dopamine synthesis pathway is tyrosine hydroxylase (TH), which converts tyrosine to dihydroxyphenylalanine (DOPA), which is then converted immediately to dopamine by DOPA decarboxylase (Figure 1-3). The newly synthesized dopamine is stored in vesicles until being released upon stimulation [reviewed in (Edwards, 2007)]. Light regulates many aspects of dopaminergic amacrine cells. The spike rate of dopaminergic amacrine cells

significantly increases just after the light onset (Zhang et al., 2007) and is thought to cause a spike-dependent release of dopamine into the extracellular space (Puopolo et al., 2001). Moreover, the activity of TH is maximal just 15 minutes after light onset (Iuvone et al., 1978) and is correlated temporally with a burst in dopamine synthesis and utilization (Nir et al., 2000). The effect of dopamine is thought to be mediated through a volume transmission in which dopamine is released into the extracellular space and activates dopamine receptors on retinal neurons [reviewed in (Witkovsky, 2004)].

There are five different subtypes of dopamine receptors (D1R, D2R, D3R, D4R, and D5R). An *in situ* hybridization and qRT-PCR analyses showed that all of the dopamine receptor subtypes except for D3R are expressed in the retina (Derouiche and Asan, 1999; Jackson et al., 2009). Although dopamine receptors are expressed in most retinal neurons, the only subtype expressed in photoreceptors is the D4R subtype (Cohen et al., 1992). This finding is significant, as there are many circadian rhythms in the photoreceptors that have been shown to be regulated by dopamine, including the electrical coupling rhythm between cone and rod photoreceptors (Ribelayga et al., 2008), modification of the balance of rod and cone photoreceptor input to horizontal cells (Witkovsky et al., 1988), and Na/K ATPase activity in photoreceptors (Shulman and Fox, 1996). In addition to the circadian rhythms in the PRL, dopamine also regulates the circadian rhythm of light-adapted ERG responses (Jackson et al., 2012), gap junction permeability among AII amacrine cells

(Hampson et al., 1992), and receptor field properties of ON-center and OFF-center RGCs (Jensen and Daw, 1984, 1986). The extensive involvement of dopamine in many circadian rhythms in the retina suggests that dopamine may be involved in the retinal circadian clock.

Indeed, dopamine has been shown to regulate the expression of a clock-controlled gene and to modulate the retinal clock. In *Drd4*$^{-/-}$ mice, the retinal transcript expression of a clock-controlled gene, adenylyl cyclase type 1 (*Adcy1*), is arrhythmic and low (Jackson et al., 2011). This study showed that the administration of D4R antagonists in WT mice can abolish the retinal *Adcy1* rhythm and that the administration of D4R agonists can phase-shift the *Adcy1* transcript rhythm in WT mice. Dopamine has also been implicated in modulating the circadian clock in the whole retina of mammals and in photoreceptors of non-mammalian vertebrates. In the retina of *Drd2*$^{-/-}$ mice, the amplitude of the *Per1* transcript rhythm is reduced (Yujnovsky et al., 2006). Dopamine, via D1 receptors, entrains the clock regulating *Per2*-luc reporter expression in the inner retina (Ruan et al., 2008). In *Xenopus* photoreceptors, the administration of D2/D4 receptor agonist, quinpirole, can shift the circadian rhythm of melatonin release (Cahill and Besharse, 1991). In photoreceptor cells of zebrafish, dopamine functions to synchronize different circadian oscillators to regulate rhodopsin promoter activity (Yu et al., 2007). Although it is not entirely clear how dopamine is involved in the retinal clock machinery, the preponderance of data

linking dopamine to the retinal clock shows that dopamine is indispensable for the retinal oscillator.

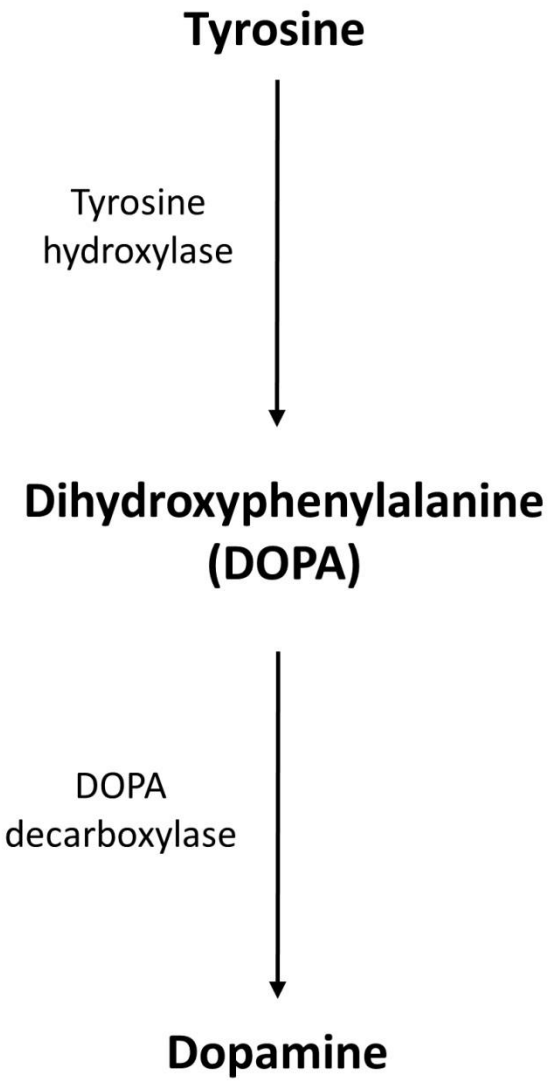

Figure 1-3 The biosynthesis of dopamine.

Through the rate-limiting enzyme, tyrosine hydroxylase, tyrosine is converted to dihydroxyphenylalanine (DOPA), which is quickly converted to dopamine through DOPA decarboxylase.

1.4 Project Objectives

The two overall objectives of my dissertation research were first to characterize the localization and role of NPAS2 in the mouse retina and second to determine whether CLOCK colocalizes with NPAS2-expressing and to elucidate the differential roles of CLOCK and NPAS2 in mediating different dimensions of vision.

In Chapter 2 of the dissertation, I show the justification for the euthanasia method utilized for my research and the specificity of the laser capture microdissection procedure. First using high performance liquid chromatography, I measured steady-state levels of dopamine and its major metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), in mice euthanized via cervical dislocation or CO_2 overdose and demonstrated that the CO2 overdose method significantly decreases the steady-state level of retinal dopamine while that of DOPAC is unaffected. As a result, all experimental mice sacrificed for this dissertation were euthanized through cervical dislocation. Second, I validate my LCM method through gene expression analyses on each retinal layer dissected via LCM, using qPCR with gene primers specific for the GCL, INL, or PRL.

In Chapter 3 of the dissertation, I determined the localization of NPAS2, its role in regulating a clock-controlled gene, *Adcy1*, and its involvement in the dopamine-adenylyl cyclase pathway to modulate contrast sensitivity. I localized NPAS2 in a

subset of RGCs in the GCL using immunohistochemistry, enzymatic assay, and LCM. I determined that *Adcy1* transcripts are rhythmic and dually regulated by both NPAS2 and D4Rs in the GCL. Using a luciferase reporter assay, I determined that cotransfecting the luciferase reporter vector with expression vectors for NPAS2 and BMAL1 results in a substantial induction in the luciferase levels, demonstrating that the NPAS2/BMAL1 heterodimer directly activates the *Adcy1* promoter. Next, I showed through qPCR analyses of GCL samples from WT and *Drd4*$^{-/-}$ mice that the deficiency in D4Rs leads to abolishment of *Npas2* transcript rhythm. Using optokinetic tracking, I determined that day-time contrast sensitivity is similarly diminished in *Npas2*$^{-/-}$, *Drd4*$^{-/-}$, and *Adcy1*$^{-/-}$ mice, leading to my conclusion that the dopamine-NPAS2-adenylyl cyclase pathway modulates day-time contrast sensitivity.

In Chapter 4 of the dissertation, I elucidated the differential roles of NPAS2 and CLOCK in regulating different visual functions. I showed that CLOCK is co-localized in NPAS2-expressing RGCs. Although CLOCK does not appear to directly activate the *Adcy1* promoter in the luciferase reporter assay, the *Adcy1* transcript rhythm in the GCL of *Clock*$^{-/-}$ mice is abolished. I performed LCM and determined that *Npas2* transcripts in the GCL of *Clock*$^{-/-}$ mice are similarly arrhythmic and at low levels, but the *Clock* transcript level in WT and *Npas2*$^{-/-}$ mice are not different, suggesting that CLOCK is upstream of NPAS2 and indirectly regulates *Adcy1* transcript expression in the GCL. Using the optokinetic tracking, I showed that the contrast sensitivity rhythm in *Clock*$^{-/-}$ mice is

completely abolished, while the contrast rhythm is reduced in *Npas2*$^{-/-}$ mice. Moreover, the deficiency of CLOCK nearly abolishes the light-adapted ERG response rhythm and substantially affects dark-adapted ERG responses. In accordance with the retinal expression pattern of NPAS2, I determined that NPAS2 is not necessary for normal light-adapted and dark-adapted ERG responses.

In conclusion, the findings support a model of a clock hierarchy in which NPAS2 is downstream of CLOCK and acts as an output of the clock. NPAS2 is expressed in the retina in a subset of RGCs, modulates the contrast sensitivity rhythm by regulating the rhythmic expression of a clock-controlled gene, *Adcy1*. CLOCK is co-expressed with NPAS2 in the GCL, regulates the rhythmic expression of *Npas2* transcript, and appears to exhibit a dominant role over NPAS2 in regulating different dimensions of vision.

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Chapter 2: Description, justification, and validation of essential experimental procedures

Section 2.1 has been published:

Hwang CK, Iuvone PM. A comparison of two methods of euthanasia on retinal dopamine levels. *Molecular Vision*. 2013 May 29; 19: 1122-4.

2.1 A comparison of two methods of euthanasia on retinal dopamine levels

2.1.1 Descriptive Abstract

Mice are commonly used in biomedical research, and euthanasia is an important part of mouse husbandry. Approved, humane methods of euthanasia are designed to minimize the potential for pain or discomfort, but may also influence the measurement of experimental variables. Here, we have compared the effects of two approved methods of mouse euthanasia on the levels of retinal dopamine. We examined the level of retinal dopamine, a commonly studied neuromodulator, following euthanasia by CO_2-induced asphyxiation or by cervical dislocation. We found that the level of retinal dopamine in mice euthanized through CO_2 overdose substantially differs from that in mice euthanized through cervical dislocation.

2.1.2 Introduction

Mice are commonly used in biomedical research today as the genetic manipulation in mice is becoming more feasible and affordable. An important part of mouse husbandry is euthanasia, which should be performed as painlessly and efficiently as possible. There are several ways in which laboratory mice are euthanized, two of which include carbon dioxide (CO_2)-induced asphyxia and cervical dislocation. CO_2 overdose has been favored for euthanasia, because the lowering of pH in the cerebrospinal fluid (CSF) that occurs secondary to CO_2 overdose is associated with anesthetic depth and insensibility to pain in humans (Meyer et al., 1961). Nevertheless, there are many labs that regularly utilize cervical dislocation to euthanize laboratory mice. Although humane treatment of laboratory mice is of critical importance, the method of euthanasia may have a substantial effect on postmortem measurements of experimental variables and could significantly affect experimental results. Hence, in this study, we tested the hypothesis that CO_2 overdose alters the retinal level of dopamine, an important regulator of visual function (Jackson et al., 2012).

2.1.3 Methods

Animals

All animal experimental procedures were approved by Emory University's Institutional Animal Care and Use Committee (IACUC) and conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Five-months-old adult male 129/Sv mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used for this study. Animals were kept in a 12-hour light:12-hour dark cycle. Lights were on from 7 am to 7 pm, and food and water were provided ad libitum. At approximately 1 pm, all animals were euthanized by either carbon dioxide (CO_2) overdose or cervical dislocation at the same time and in the same room. The room light intensity was ~700 lux. CO_2 euthanasia consisted of gradual exposure to CO_2 for 5 minutes as recommended by Pritchett et al. (Pritchett et al., 2005) for adult mice. Eyes were enucleated, and retinas collected and stored immediately in -80˚ C.

Analysis of dopamine and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), by high-performance liquid chromatography (HPLC)

Levels of retinal dopamine and its major metabolite, DOPAC, were determined by ion-pair reverse-phase HPLC with coulometric detection (guard cell at 0.6 V and coulometric analytical cell at 0.3 V) using a modification of the method as described by Pozdeyev et al. (Pozdeyev et al., 2008). Two retinas from each mouse were homogenized in 200 µl of 0.2N HClO4 solution containing 0.01% of sodium meta-bisulfite and 25 ng/mL 3,4-dihydroxybenzylamine hydrobromide

(internal standard). Samples were subsequently centrifuged at 15,000 x g for 10 minutes at 4˚ C, and 50 µl of the supernatant was used for HPLC analysis using an Ultrasphere ODS 5 µm 250 x 4.6 mm column (Beckman Coulter, Fullerton, CA, USA) with a mobile phase containing 0.1 M sodium phosphate, 0.1 mM EDTA, 0.35 mM sodium octyl-sulfate, and 5.5% acetonitrile, pH 2.7. Standards of dopamine and DOPAC ranging from 2 to 20 ng/ml were analyzed with the samples. The precipitate obtained by centrifuging the homogenized retinas was resuspended in 100 µl of 1 N NaOH by sonication. An aliquot of 5 µl was used to estimate the amount of protein in each sample of retinas (Lowry et al., 1951).

Statistics

Comparisons of two groups were made with Student's t-test using SigmaPlot 12 (Systat Software Inc., San Jose, CA). Error bars represent standard error of the mean (SEM), and $p < 0.05$ were considered significant.

2.1.4 Results

The level of retinal dopamine was significantly different in mice that were euthanized by CO_2 overdose compared to those in mice euthanized by cervical dislocation (Figure 2-1A; $p = 0.003$). The normalized mean level of retinal dopamine of mice euthanized by the cervical dislocation method was 1.07 ng per mg of retinal protein, while the normalized mean level of retinal dopamine of mice euthanized by the CO_2 overdose method was 0.78 ng per mg of retinal protein. However, the level of retinal DOPAC, dopamine's major metabolite, was not statistically different between the two groups of mice (Figure 2-1B; $p > 0.05$). The normalized mean level of retinal DOPAC of mice euthanized by the cervical dislocation method was 0.59 ng per mg of retinal protein, while the normalized mean level of retinal DOPAC of mice euthanized by the CO_2 overdose method was 0.50 ng per mg of retinal protein.

2.1.5 Discussion

The current study compared two methods of euthanasia approved by the American Veterinary Association on the level of retinal dopamine. We found that changing euthanasia method from cervical dislocation to CO_2 overdose was a major change in experimental condition that significantly altered the level of retinal dopamine.

Although it is not clear why dopamine levels are diminished in mice that were euthanized through CO_2 overdose, we can conjecture that the mechanism involves factors of time, hypoxia, and respiratory acidosis that results from having excess CO_2 in the blood. Dopamine turnover in mouse retina exposed to light is rapid (Nir et al., 2000). CO_2-induced asphyxiation requires several minutes and changes in oxygenation and pH could affect dopamine synthesis and metabolism within that period of time. In contrast, cervical dislocation is rapid and retinas are typically dissected and frozen within 1-2 minutes. The rate-limiting step in dopamine biosynthesis is catalyzed by tyrosine hydroxylase, which requires molecular O_2 for activity (Shiman et al., 1971). Thus, hypoxia associated with breathing concentrated CO_2 could inhibit tyrosine hydroxylation and dopamine biosynthesis. Normally blood has a buffering capacity for CO_2 in which water combines with CO_2 to produce hydrogen and bicarbonate ions to keep the CO_2 levels tightly regulated. However, when the capacity of the buffering system in the blood is exceeded, the excess CO_2 leads to acidosis. Acidosis alters dopamine reuptake by affecting the activity of dopamine transporters (Pastuszko

et al., 1982; Barrier et al., 2003). As a result of hypoxia, extracellular dopamine could be favored to be metabolized by catechol-*O*-methyl transferase (COMT) rather than monoamine oxidase (MAO), which requires oxygen to metabolize dopamine. These factors could explain why the level of retinal dopamine was affected while the level of retinal DOPAC was preserved.

In conclusion, we show that mice euthanized by CO_2 overdose have significantly different retinal dopamine levels compared to mice euthanized by cervical dislocation. Thus, the use of CO_2 as a method of euthanasia could result in experimental artifact that could compromise results when studying labile biological processes.

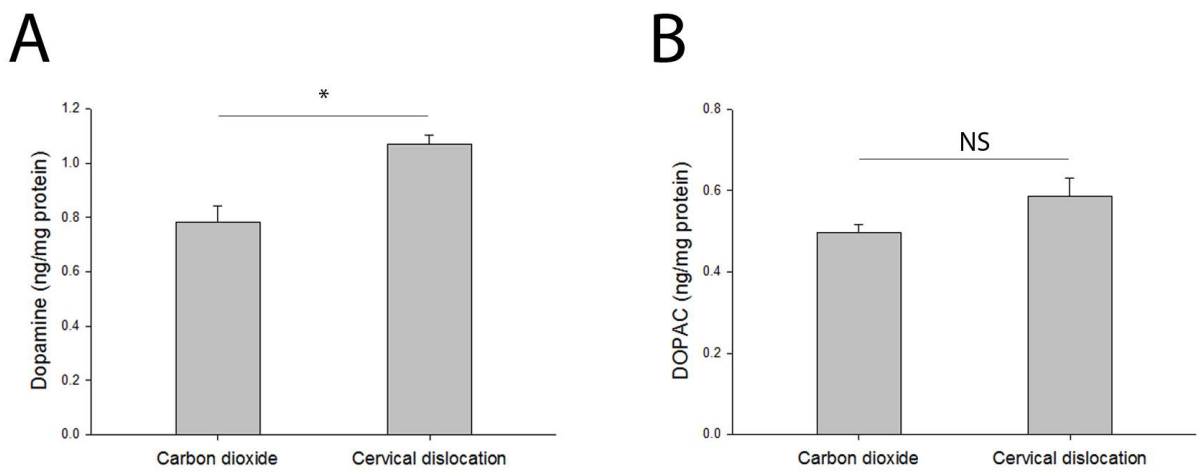

Figure 2-1 Retinal dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) levels in euthanized mice.

A, Retinal dopamine levels in mice that were euthanized through carbon dioxide overdose were significantly different compared to those in mice euthanized through cervical dislocation (t-test, $p = 0.003$, $n = 5$ for each group). B, Retinal DOPAC levels between mice euthanized through carbon dioxide and those through cervical dislocation were similar (t-test, $p = 0.119$, $n = 5$ for each group). The amount of retinal dopamine or DOPAC (ng) was normalized to the amount of retinal protein (mg) for each sample. Data expressed as mean ± SEM.

2.2 Validation of the Specificity of Laser Capture Microdissection

2.2.1 Introduction

Traditionally retinal transcripts have been studied using the whole retina. However, the retina is comprised of heterogeneous cell types, and in some instances, the transcript expression of a gene in different retinal layers could be substantially different from each other. To overcome this shortcoming, laser capture microdissection (LCM) has been utilized in different studies. Through LCM, a specific retinal layer can be dissected and analyzed for expressions of different transcripts using qRT-PCR. In this study, different retinal layers were dissected using LCM, and qRT-PCR was performed using gene primers specific for different retinal layers to demonstrate the specificity of the LCM technique.

2.2.2 Methods

Laser capture microdissection (LCM)

Whole rat eyes, embedded in optimal cutting temperature compound (OCT; Sakura Finetek, Torrance, CA) and frozen in -80°C, were used for LCM. Rat eye sections were made at 12 µm thickness and mounted on polyethylene naphthalate membrane glass slides (Applied Biosystems, Foster City, CA). The sections were stained with HistoGene staining solution (Applied Biosystems), dehydrated in graded ethanol solutions (75%, 95%, 100% ethanol), and cleared in the xylene solution. LCM was performed to microdissect the GCL, INL, and PRL (which included both the outer nuclear layer and inner segments of photoreceptors) onto HS CapSure non-contact LCM films using the ArcturusXT

system (Applied Biosystems). RNAqueous-Micro Kit (Life Technologies) was subsequently used to purify both large and small RNA species from the captured tissue.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Retinal layer-specific transcripts (*Brn3a* (GCL), *mGluR6* (INL), and *Rho* (PRL)) were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) after the total RNA was reverse-transcribed to cDNA using QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA). With 2 µL cDNA from each sample, qRT-PCR was performed in Bio-Rad iCycler (Bio-Rad Laboratories Inc., Hercules, CA) with a 25 µL total volume containing cDNA, QuantiFast SYBR Green PCR Master mix (Qiagen), and 1 µM forward and reverse primers for the gene of interest, and the fluorescence threshold value was calculated using MyiQ cycler software. The transcript level for each gene was normalized to the level of the housekeeping gene 18S rRNA and quantified according to the delta-delta Ct method(Livak and Schmittgen, 2001).

2.2.3 Results

qRT-PCR testing with specific retinal layer markers showed that LCM is specific. Retinal ganglion cell marker, *Brn3a*, was selectively expressed only in the GCL samples (Figure 2-2A). A marker for rod and ON-type cone bipolar cells, *mGluR6*, was selectively expressed only in the INL samples (Figure 2-2B). Rod photoreceptor marker, *Rho*, was primarily expressed in the PRL samples (Figure

2-2C). Although there was a small expression of *Rho* in the INL samples, this level was 500-fold lower than the one in the PRL samples.

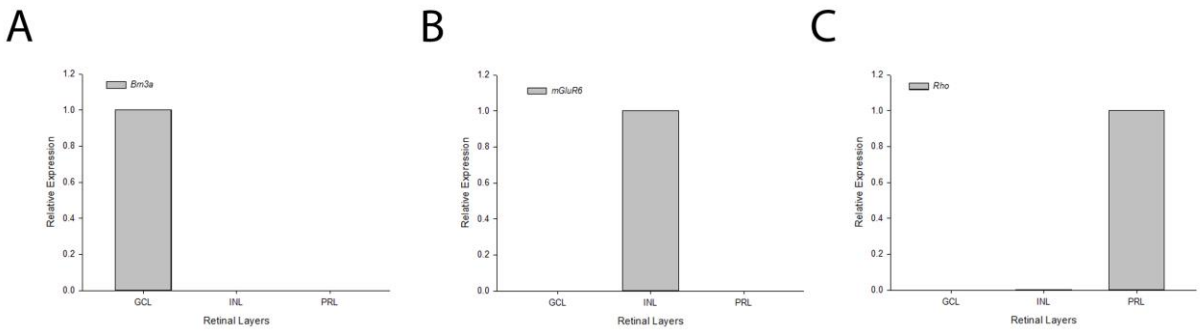

Figure 2-2 The specificity of laser capture microdissection (LCM) is demonstrated.

The selective expression of the retinal layer-specific markers *Brn3a* (ganglion cell layer (GCL)), *mGluR6* (inner nuclear layer (INL)), and *Rho* (photoreceptor layer (PRL)) demonstrates the specificity of LCM. (A) *Brn3a*, which is a marker for retinal ganglion cells, was expressed only in the GCL-microdissected tissues. (B) *mGluR6*, which is a marker for rod and ON-type cone bipolar cells, was expressed only in the INL-microdissected tissues. (C) *Rho*, which is a marker for rod photoreceptors, was expressed 500-fold higher in the PRL-microdissected tissues than in the INL-microdissected tissues and was not expressed in the GCL-microdissected tissues.

2.3 Contrast sensitivity and optokinetic tracking (OKT) test

2.3.1 Contrast sensitivity

Contrast sensitivity is the ability of an organism to detect a difference in luminance, and it gives organisms a selective advantage by allowing them to distinguish an object from its background. In the retina, contrast information is processed by RGCs. Each RGC forms a circuit with multiple photoreceptors (except for the RGCs in the fovea that receives input from a single cone photoreceptor [reviewed in (Purves et al., 2012)], which contribute to a receptive field of each RGC. A receptive field of an RGC is thought to be comprised of a center-surround mechanism. In this mechanism, RGCs exhibit three different responses to a small flash of light depending on the area of the receptive field stimulated. In an excitatory center and inhibitory surround (ON-center) receptive field, a small flash of light confined to the center area will evoke an excitatory response. If confined to the immediate surrounding area, then an inhibitory response will be evoked. If both center and surround areas are stimulated, then the response will be minimal to none. In an inhibitory center and excitatory surround (OFF-center) receptive field, the opposite will occur (Barlow, 1953; Kuffler, 1953). ON-center RGCs form dendritic processes at the lower half of the IPL and comprise approximately 50% of RGCs, while OFF-center RGCs form dendritic processes at the upper half of the IPL [reviewed in (Wandell, 1995)]. Therefore, contrast is detected when the center and surround areas receive different levels of luminance.

In the laboratory setting, contrast sensitivity is measured using a greyscale, sinusoidal grating. The grating is sinusoidal, because it allows the mean luminance level to be constant when the contrast is lowered. For example, the contrast decreases as the black bars become lighter shades of grey and white bars darker shades of grey, but the mean luminance stays constant. In this context, contrast (C) is defined mathematically as

$$C = (Lmax – Lmin)/(Lmax + Lmin).$$

C ranges from 0 to 1 (equivalent to 0% to 100%). Lmax is the luminance of the brighter bar and Lmin of the dimmer bar. Lmin is 0 when the bar is black, and Lmax is 100 when the bar is white. At 100% contrast, the grating presents alternating black and white bars. In a sinusoidal grating, the contrast decreases as a result of an equal level of change in both Lmin and Lmax.

The size of the bars is termed spatial frequency and is expressed as cycles per degree, where a cycle is defined as one sinusoidal period, which is comprised of one brighter bar and one dimmer bar. Contrast sensitivity is measured across a range of spatial frequencies. A graphical representation of contrast sensitivity across numerous spatial frequencies is called the contrast sensitivity function (CSF).

The shape of the CSF is that of a normal distribution curve [reviewed in (Wandell, 1995; Kalloniatis and Luu, 2011)]. There is a peak at one of the spatial frequencies, and the graph decreases to both the right and left of the peak. It is thought that the peak represents the width of a bar that corresponds to the diameter of the center area of the receptive field. Therefore, at a lower spatial frequency (i.e., wider bars), the width of the bar extends beyond the diameter of the center area into the opposing surrounding area, and consequently, the neural response is weaker. Similarly, at a higher spatial frequency (i.e., narrower bars), bars of dimmer and brighter luminance can both stimulate the center area, and the resulting neural response will be weaker. The extent of the drop-off in lower spatial frequencies is thought to correspond to the strength of the opposing surround area.

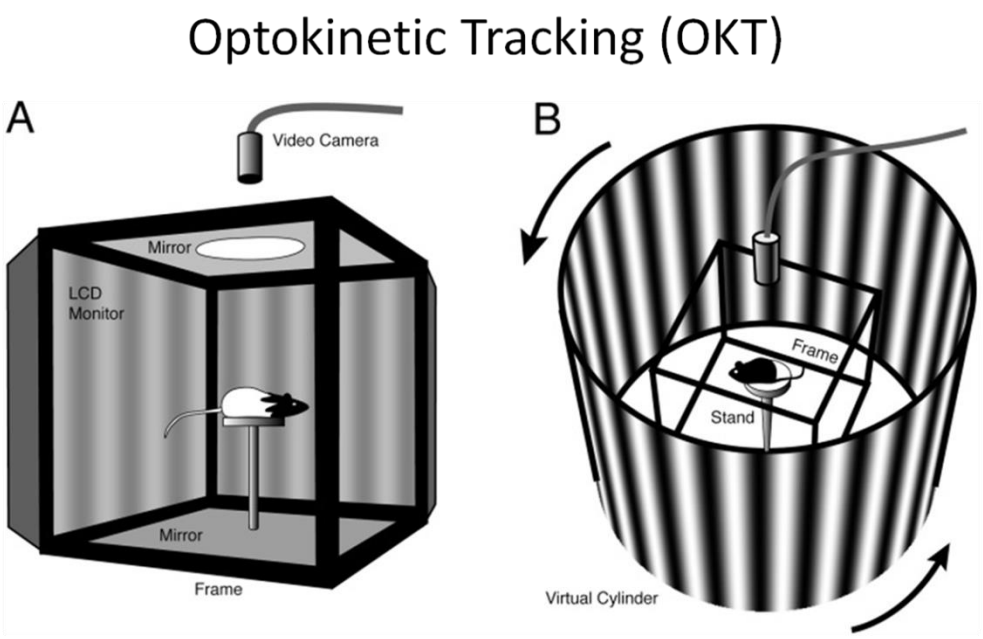

Figure 2-3 The optokinetic tracking test.

In this setup, a mouse is placed on a raised platform surrounded by four LCD monitors, which project an image of a virtual cylinder of a sinusoidal gradient. In case of contrast sensitivity measurement, the contrast is lowered until the mouse can no longer detect contrast and stops tracking, which is called the threshold. The threshold is measured across a range of spatial frequencies. In case of visual acuity (i.e., spatial frequency threshold), the contrast is held constant at 100%, and the spatial frequency of the gradient is lowered until the mouse stops tracking. (Image adapted from www.cerebralmechanics.com).

2.3.2 Optomotor Response/Optokinetic Tracking:

One of the commonly used tests to measure contrast sensitivity is called the optokinetic tracking (OKT) test (Figure A2-1). An animal (e.g., mouse) is placed on a raised platform in an arena comprised of four LCD monitors, which projects an image of a virtual cylinder comprised of a greyscale, sinusoidal gradient. The cylinder will rotate, and the animal will track if contrast is detected. The contrast is gradually lowered until the threshold, at which contrast is no longer detected, is reached (i.e., the animal stop tracking). The threshold is measured across a range of spatial frequencies, and contrast sensitivity (reciprocal of the threshold) at multiple spatial frequencies are plotted to form CSF.

The exact term to describe the reflexive head movement of a mouse when tracking the drum is disputed. Optokinetic reflex refers to the movement of the eye as it tracks a moving stimulus. Optomotor response refers to the movement of the head as it tracks a moving stimulus, but this term has primarily been used to describe the movement in insects and fish. Prusky and coworkers determined that the underlying mechanism for the head tracking movement in mice is similar to optokinetic reflex mechanism in mammals (Prusky et al., 2008). Therefore, they started describing the movement as optokinetic tracking, although they initially described the movement as optomotor response in their earlier manuscript (Prusky et al., 2004).

The exact mechanism still needs to be elucidated. However, the movement is generated from a combination of sensory and motor components that begin with a detection of the movement of the virtual drum and ending with a head tracking movement. Another study from the Prusky lab determined that the visual cortex is not involved in the optokinetic tracking (Douglas et al., 2005). They created a large lesion in the visual cortex, and the visual acuity and contrast sensitivity of mice were still normal. They concluded that the pathway is subcortical, possibly through the midbrain (olivary nucleus). They hypothesized that contrast detection when the distance to the moving stimulus changes involves the cortex (e.g. water maze test), whereas contrast detection when the distance is fixed does not involve the cortex. Moreover, because the OKT test specifically tests posterior-to-anterior motion, and it is possible that the contrast sensitivity function is at least partially influenced by DSGCs.

2.4 The electroretinogram and circadian rhythm

2.4.1 What is an ERG?

ERG graphically represents field potential changes across the corneal surface elicited by a light stimulus. It is non-invasive and simple to setup, and thus ERG is used widely for both animal and human clinical studies. An anesthetized animal is placed on a raised platform facing a ganzfeld dome, and two electrodes (recording and reference electrodes) are strategically placed on the body. After dilating the pupil, a recording electrode is placed directly on or closely adjacent to the cornea, and a reference electrode is placed elsewhere (typically on the ipsilateral cheek). These electrodes record field potential changes elicited by a visual stimulus.

There are several types of ERGs, but the most common type is the flash ERG. As the name suggests, the flash ERG records retinal responses to a flash of light. In the retina, there are different cell types, which can be categorized into one of the following three groups: first order neurons, second order neurons, and third order neurons. The first order neurons are rod and cone photoreceptors and are the first retinal neurons to detect light. The responses from photoreceptors are represented by the initial negative deflection of the ERG waveform (Figure 1-3), which is called the a-wave (Brown, 1968). Among different second order neurons in the retina, ON bipolar cells are thought to produce the ensuing positive deflection of the ERG waveform (Stockton and

Slaughter, 1989), which is called the b-wave. Horizontal cells and OFF bipolar cells, which are other second order neurons in the retina, contribute to the repolarization of the b-wave (Bush and Sieving, 1994). In addition to the b-wave, there are oscillatory potentials (OP), which are the small wavelets that appear superimposed on the positive b-wave. The exact origin of OP is still debated, but they are thought to originate from third-order neurons like amacrine and/or retinal ganglion cells (Heynen et al., 1985; Yu and Peachey, 2007).

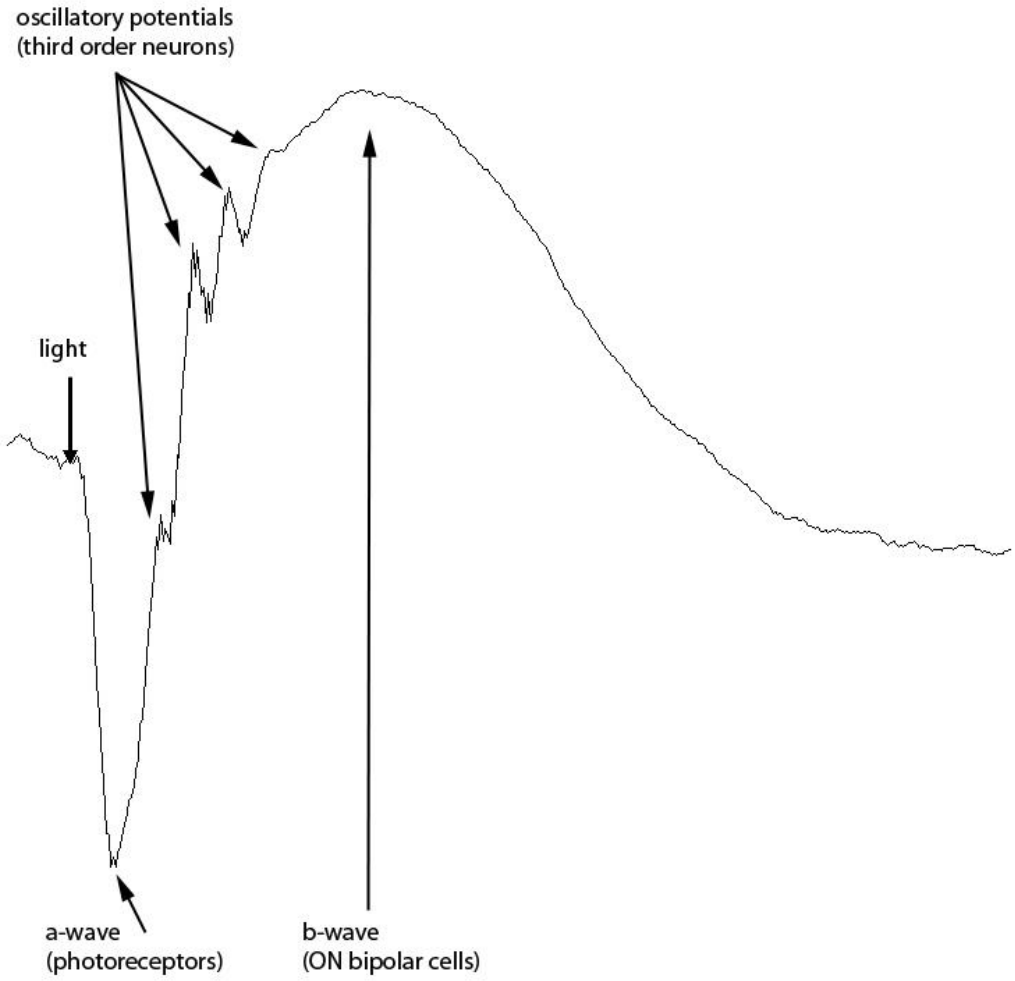

Figure 2-4 A representative image of a flash electroretinogram (ERG) waveform.

Upon exposure to a flash of light, field potential changes are depicted by a waveform on an ERG recording. An ERG waveform is characterized by an a-wave (which represent the activity of photoreceptors), b-wave (which represent the activity of ON-bipolar cells), and oscillatory potentials (which represent the activity of one or more third-order neurons).

2.4.2 Analyzing ERG responses

A- and b-waves are analyzed by measuring their amplitudes and implicit times (also known as latency). The amplitude is simply the height from the pre-flash baseline to the peak of the a- or b-wave, while the latency is the elapsed time from the flash of light until the peak of the a- or b-wave. The general shape of ERG waveforms is retained in different conditions. However, the magnitude of the amplitude and latency of the individual elements can significantly be altered depending on the experimental conditions, such as the flash intensity and light adaptation. There is a strong correlation between the flash intensity and amplitude as well as between the flash intensity and latency [reviewed in (Cameron et al., 2008a)]. For example, a low flash intensity correlates with low amplitude and long latency, while a high flash intensity correlates with high amplitude and short latency. Moreover, the length of time the animal is exposed to a background light, known as the light adaptation time, correlates with the amplitude and latency (Cameron et al., 2008b). For example, the longer the light adaptation time, the greater the amplitude and shorter the latency are. Conversely, the shorter the light adaptation time, the smaller the amplitude and longer the latency are.

2.4.3 Studying cone or rod-specific ERG responses

In dark-adapted retinas, there is a 1000-fold difference in light sensitivity between rod and cone photoreceptors (Nikonov et al., 2006). Therefore the rod pathway

can be isolated in dark-adapted retinas by using dim flash intensities that are below the threshold at which cone photoreceptors are activated, and this condition is termed scotopic. Above the threshold in dark-adapted retinas, both rod and cone photoreceptors are activated, and this condition is called mesopic. In order to isolate the cone pathway, retinas are light-adapted by first saturating ("bleaching") rod photoreceptors with a constant background light and then recording cone responses elicited by flashes of light bright enough to activate cone photoreceptors. This condition is termed photopic. However, in some species, the number of cone photoreceptors is too low to generate a visible a-wave on the waveform. For example, in mice cone photoreceptors constitute only 3% of retinal photoreceptors, while rod photoreceptors constitute the rest (Carter-Dawson and LaVail, 1979; Jeon et al., 1998). In contrast, in other species like humans, cone photoreceptors are much more numerous and can generate a large cone a-wave (Hood and Birch, 1993). Therefore, in species with a low percentage of cone photoreceptors, the photopic ERG waveform does not resemble scotopic or mesopic ERG waveforms.

2.4.4 Cone ERG responses are circadian

In mammals, a circadian rhythm in the light sensitivity of dark-adapted ERG b-wave has previously been shown in rabbits (Brandenburg et al., 1983; White and Hock, 1992) rats (Sandberg et al., 1986). In mice, the amplitude and latency of light-adapted ERG b-waves exhibit a circadian rhythm (Barnard et al., 2006; Cameron et al., 2008b). However, it is not clear whether dark-adapted ERG

responses are circadian. One study showed a small variation between the subjective day and subjective night dark-adapted ERG responses (Cameron et al., 2008b). While the a- and b-wave amplitudes among the different time points tested were constant at scotopic flash intensities, there was a small difference at mesopic flash intensities. The latency remained constant across the time points tested at all flash intensities. The authors concluded that the minor variation in dark-adapted ERG a- and b-waves under mesopic conditions represents the contribution from the cone pathway. Another study measured dark-adapted a- and b-wave amplitudes across a similar range of flash intensities and did not detect a circadian rhythm (Jackson et al., 2012). The differences between the results from these two studies could be due to strain differences and/or insufficient number of time points in the latter study. Nevertheless, the circadian regulation of ERG responses appears to be significantly more apparent in light-adapted conditions.

The role of circadian clocks in regulating ERG responses has recently been investigated. In mice with retina-specific deletion of the *Bmal1* gene, light-adapted ERG b-waves are arrhythmic and of low amplitude, similar to those of WT mice at the midnight time point (Storch et al., 2007). This suggests that local clocks in the retina are necessary to regulate the circadian rhythm of light-adapted ERG responses. However, the animals tested at the subjective-night time point were kept in constant light conditions. Constant light has a strong inhibitory effect on light-adapted ERG responses (Cameron et al., 2008b).

Consequently, it is debatable whether the light-adapted ERG rhythm in WT mice in this study is an artifact from continuous light exposure or attributable to circadian regulation. Moreover, the authors did not test the effect of the light adaptation time, as the light-adapted ERG responses were recorded with flashes of light and rod-saturating background light simultaneously turned on. Therefore, the role of the circadian clock in the regulation of light-adapted ERG responses was not clear.

In *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ mice, light-adapted ERG b-waves measured in animals free-running in constant dark conditions are arrhythmic, suggesting that a functional clock is necessary for the circadian light-adapted ERG b-wave amplitudes. However, in contrast to *Bmal1*$^{-/-}$ mice, the amplitudes in *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ mice are large and even larger than those of WT mice at the midday time point (Cameron et al., 2008b). The authors hypothesized that the difference may be due to the fact that *Bmal1* and *Cry1/2* deficiencies stop the clock at different phases of the circadian oscillation. Nonetheless, this study showed that light-adapted ERG responses are regulated by a circadian clock.

In 2006, a surprising discovery was made in mice deficient in melanopsin (*Opn4*$^{-/-}$); these mice failed to exhibit the circadian rhythm of light-adapted ERG responses (Barnard et al., 2006). With the data showing that a retinal circadian clock is involved in regulating the circadian light-adapted ERG responses, this finding suggests that ipRGCs are involved regulating a local clock in the retina

and has important implications. These cells could function to synchronize local clocks in the eye in addition to the central clock in the SCN, and thus, in the absence of melanopsin, local clocks become unsynchronized, leading to arrhythmic light-adapted ERG responses. This model is consistent with findings showing that ipRGCs make synaptic connections with dopaminergic amacrine cells and modulate their responses (Zhang et al., 2008; Zhang et al., 2012). Alternatively, retinal clock cells could be upstream of ipRGCs and could use ipRGCs as an output to regulate the light-adapted ERG rhythm. Nevertheless, this study was performed under similar testing conditions as those in the *Bmal1*$^{-/-}$ mice study (Storch et al., 2007) and thus has similar limitations. Moreover, these two studies have findings that are contradictory. The implicit times in WT mice kept in constant light were rhythmic in one study (Storch et al., 2007) but arrhythmic in another (Barnard et al., 2006). The reason of this discrepancy is unclear, but emphasizes the point that using animals kept in constant light conditions to study the circadian regulation of light-adapted ERG responses is disfavored. Consequently the role of ipRGCs in the regulation of light-adapted ERGs is not entirely clear.

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Chapter 3: Circadian rhythm of contrast sensitivity is regulated by a dopamine-NPAS2-adenylyl cyclase 1 signaling pathway in retinal ganglion cells

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3.1 Abstract

Spatial variation in light intensity, called spatial contrast, comprises much of the visual information perceived by mammals, and the relative ability to detect contrast is referred to as contrast sensitivity (Purves et al., 2012). Recently, retinal dopamine D4 receptors (D4Rs) have been implicated in modulating contrast sensitivity (Jackson et al., 2012); however, the cellular and molecular mechanisms have not been elucidated. Our study demonstrates a circadian rhythm of contrast sensitivity that peaks during the daytime and that its regulation involves interactions of D4Rs, the clock gene *Npas2*, and the clock-controlled gene adenylyl cyclase 1 (*Adcy1)* in a subset of retinal ganglion cells (RGCs). Targeted disruption of the gene encoding D4Rs reduces the amplitude of the contrast sensitivity rhythm by reducing daytime sensitivity and abolishes the rhythmic expression of *Npas2* and *Adcy1* mRNA in the ganglion cell layer (GCL) of the retina. *Npas2*$^{-/-}$ and *Adcy1*$^{-/-}$ mice show strikingly similar reductions in the contrast sensitivity rhythm to that in mice lacking D4Rs. Moreover, *Adcy1* transcript rhythms were abolished in the GCL of *Npas2*$^{-/-}$ mice. Luciferase reporter assays demonstrated that the *Adcy1* promoter is selectively activated by NPAS2/BMAL1. Our results indicate that the contrast sensitivity rhythm is modulated by D4Rs via a signaling pathway that involves NPAS2-mediated circadian regulation of *Adcy1*. Hence, we have identified a circadian clock mechanism in a subset of RGCs that modulates an important aspect of retinal physiology and visual processing.

3.2 Introduction

Contrast sensitivity refers to the level of spatial variation in light intensity an organism is able to detect (2012). Dopamine, through dopamine receptor subtype 4 (D4R), modulates contrast sensitivity, and dopamine deficiency in the retina alone is sufficient to cause contrast sensitivity deficits (Jackson et al., 2012). However, the underlying cellular and molecular mechanisms through which retinal dopamine modulates contrast sensitivity have not been elucidated.

Dopamine secretion, as well as synthesis and metabolism, is controlled by light and modulated by the circadian clock (Iuvone et al., 1978; Doyle et al., 2002; Ribelayga et al., 2004). The circadian clock is an autonomous oscillator responsible for regulating physiology and behavior, thereby conferring adaptive significance to living organisms by allowing them to anticipate regular changes in their environment. The oscillator is driven by a transcriptional-translational feedback loop in which CLOCK/BMAL1 heterodimers drive the rhythmic expression of Period and Cryptochrome clock genes and of clock-controlled genes (Gekakis et al., 1998; Hogenesch et al., 1998; Reick et al., 2001), which are thought to mediate the biological effects of the circadian clock. The Period and Cryptochrome proteins form oligomers and repress the action of CLOCK/BMAL1 (van der Horst et al., 1999; Zheng et al., 2001).

In the mammalian retina, the rhythmic transcript expression of the *Drd4* gene, which encodes the dopamine D4 receptor (D4R), persists in constant darkness

(Jackson et al., 2011), and there is evidence that dopamine itself modulates retinal clocks (Ruan et al., 2008). Hence, we tested the hypothesis that contrast sensitivity is rhythmic and modulated by clock components. In this study, we investigated the retinal CLOCK homolog protein neuronal PAS-domain protein 2 (NPAS2) and found it localized in mouse retina primarily (if not exclusively) in retinal ganglion cells (RGCs), many of which detect motion and contrast in the visual field (Barlow et al., 1964; Weng et al., 2005; Sun et al., 2006). Traditionally, CLOCK and BMAL1 proteins were thought to be the sole positive regulators in the circadian clock feedback-loop system. However, NPAS2 (Zhou et al., 1997), which shares extensive sequence identity with CLOCK (King et al., 1997; Hogenesch et al., 2000), was identified as another positive regulator in the circadian clock (Reick et al., 2001; DeBruyne et al., 2007). Like CLOCK, NPAS2 forms a heterodimer with BMAL1 that binds to the circadian E-box to drive the expression of clock-controlled genes (Hogenesch et al., 1998; Kume et al., 1999; Reick et al., 2001).

We investigated the role of NPAS2 in the dopamine pathway that modulates contrast sensitivity. We discovered that contrast sensitivity is circadian and is modulated by NPAS2-dependent regulation of adenylyl cyclase type 1 (AC1), the protein product of the clock-controlled gene *Adcy1*, in RGCs. Additionally, we determined that D4Rs are required for the rhythmic expression of *Npas2* transcripts in the ganglion cell layer (GCL) and to regulate the contrast sensitivity rhythm.

3.3 Materials and Methods

3.3.1 Animals

All animal experimental procedures were approved by Emory University's Institutional Animal Care and Use Committee (IACUC) and conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. *Adcy1*$^{-/-}$ mice, lacking the type 1 adenylyl cyclase (Wu et al., 1995), *Npas2*$^{-/-}$ mice, producing the non-functional NPAS2-β-galactosidase fusion protein (Garcia et al., 2000), *Drd4*$^{-/-}$ mice, lacking dopamine D4 receptors (D4Rs) (Rubinstein et al., 1997), and wild-type mice (WT, C57BL/6) were used for this investigation. Mice were housed on a 12-hour light /dark (LD) cycle in which lights were on at zeitgeber time (ZT) 0 and off at ZT12 with food and water provided ad libitum. Mice were of either sex, 9-12 weeks old, and genotyped by polymerase chain reaction of tail genomic DNA. Mice were entrained to LD and/or subsequently kept in constant darkness (DD2=second day of constant darkness) to study circadian function. Circadian time (CT) rather than ZT was used to designate subjective time of day in DD2. All experimental procedures in ‘‘dark’’ conditions were performed under dim red light.

3.3.2 LacZ Histochemistry

LacZ histochemistry was performed as described previously (Kerrison et al., 2005). Briefly, following enucleation, eyes were fixed for 5 minutes in 0.5% glutaraldehyde, embedded in optimal cutting temperature compound (OCT; Sakura Finetek, Torrance, CA), and immediately frozen on dry ice. Sections were

cut using a cryostat (Leica Microsystems, Buffalo Grove, IL) at 10-µm thickness and placed on charged glass slides (Fisher Scientific, Pittsburgh, PA). Sections were incubated for an additional 5 minutes in 0.5% glutaraldehyde, rinsed with PBS, and stained in X-gal solution at room temperature for 72 hours. The stained sections were mounted and viewed under the microscope (Eclipse E300, Nikon Instruments Inc., Melville, NY).

3.3.3 Immunofluorescence imaging

Mouse eyes were enucleated and fixed in 4% paraformaldehyde at 4^{o}C overnight. After removing the lens, the eyes were cryoprotected in 30% sucrose/PBS solution at 4^{o}C for one hour, frozen in OCT and stored at -80^{o}C. Sixteen micron thick retinal sections were made using a cryostat and mounted on charged glass slides (Fisher Scientific). All sections were permeabilized for 20 minutes at RT with 0.02% Triton-X100/PBS and blocked for 1 hour at RT in 10% normal goat serum (NGS) in PBS. Chicken Anti-β-galactosidase antibody (1:1000, Abcam, Cambridge, MA), rabbit anti-CLOCK (1:1000, Millipore), or rabbit anti-Brn3a antibody (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS, 0.1% Tween 20, and 10% NGS was incubated at 4°C overnight. Sections were washed three times in 0.1% Tween 20/PBS for 10 minutes each, and respective secondary antibodies—Alexa 568 goat anti-chicken IgG (Invitrogen, Life Technologies, Grand Island, NY) and Alexa 488 goat anti-rabbit IgG (Invitrogen)—were added to the sections for 1 hour at RT. Following three rinses in 0.1% Tween 20/PBS for 10 minutes each, slides were mounted with

VectaShield Hardset with DAPI (H-1500; Vector Laboratories). Imaging was performed with a confocal microscope (Nikon C1 confocal imaging system, Nikon Instruments Inc., Melville, NY). Cells labeled with β-galactosidase and Brn3a were counted across a length of 1 mm in 9 retinal sections from 3 different *Npas2*$^{-/-}$ mice (3 retinal sections per mouse).

3.3.4 Laser capture microdissection (LCM)

LCM was performed using the whole mouse eyes, embedded in OCT and frozen at -80°C using a modification of a method described previously(Jackson et al., 2011). Briefly, the tissue sections were cut at 10µm thickness and mounted on polyethylene naphthalate membrane glass slides (Applied Biosystems, Foster City, CA). In a sterile environment, frozen sections were placed in the 75% ethanol solution for 30 seconds and subsequently placed in RNase-free water for 30 seconds. The sections were stained with the HistoGene (Applied Biosystems) staining solution for approximately 15 seconds followed by a wash with RNase-free water for 30 seconds. The sections were dehydrated in graded ethanol solutions (75%, 30 s; 95%, 30 s; 100%, 30 seconds), cleared in xylene (> 5 minutes), and air-dried for 5 minutes. Laser capture microdissection was performed to capture the GCL, inner nuclear layer (INL), and photoreceptor layer (PRL), which included both outer nuclear layer and inner segments of photoreceptor cells, onto HS CapSure non-contact LCM films using ArcturusXT system (Applied Biosystems). The desired tissue area was lifted away from the tissue section, leaving all unwanted cells behind and providing a pure population

for cell-specific molecular analysis. In the PRL, outer segments were not captured to avoid contamination from the adjacent retinal pigment epithelium. The PicoPure RNA Isolation kit (Applied Biosystems) was used to purify total RNA from captured tissue. Subsequently, total RNA was reverse-transcribed to cDNA using QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA).

3.3.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

Adcy1, *Npas2, and Drd4* transcripts were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) using 2 µL cDNA from each sample. qRT-PCR was performed in Bio-Rad iCycler (Bio-Rad Laboratories Inc., Hercules, CA) with a 25 µL total volume containing cDNA, QuantiFast SYBR Green PCR Master mix (Qiagen), and 1 µM forward and reverse primers for the gene of interest. Each sample was assayed in duplicates, and the fluorescence threshold value was calculated using MyiQ cycler software. The levels of *Adcy1*, *Npas2,* and *Drd4* transcripts were normalized to the expression levels of the housekeeping gene 18S rRNA and quantified according to the delta-delta Ct method (Livak and Schmittgen, 2001).

3.3.6 Contrast sensitivity and visual acuity

An optomotor response test (optokinetc head tracking) was used to measure contrast sensitivity and visual acuity, as defined by spatial frequency threshold, using the OptoMotry instrument (CerebralMechanics, Inc.) as described previously (Prusky et al., 2004). Briefly, mice were placed one at a time on the

raised platform inside the device, where a sine wave grating projected by four interfacing LCD monitors moved in one direction, and were assessed for tracking behavior. Contrast sensitivity testing began with grating of 100% contrast and was gradually reduced until the contrast threshold was reached at each of six spatial frequencies (0.031, 0.064, 0.092, 0.103, 0.192, 0.272 cycles per degree). The reciprocal of the threshold was used as the contrast sensitivity value at each spatial frequency. Staircase procedure was utilized to determine the spatial frequency threshold for visual acuity in which a black and white sinusoidal grating at 100% contrast was increased in cycles per degree until tracking ceased. Testing occurred during mid-day (ZT6) and mid-night (ZT18) hours in LD and after two days in constant darkness during CT6 and CT18 time points in DD2. All measurements were made under photopic conditions (mean intensity at 100% contrast, 0.3 c/d, 12 d/s was ~300 cd/m^2; light intensity of the light bars and dark bars ranged from ~700 and ~20 cd/m^2 at 90% contrast and ~200 and ~140 cd/m^2 at 10% contrast. To minimize the effect of light adaptation when measuring CS in dark-adapted mice, the order of spatial frequency tested for each mouse was randomly chosen, and the testing period for each mouse was no longer than 20 minutes.

3.3.7 Luciferase Reporter Assays and Transfections

Fragments of the murine adenylyl cyclase type 1 (*mAdcy1*) promoter were cloned into the pGL3-basic vector (Promega, Madison, WI), which contains the firefly luciferase reporter gene (Chan et al., 2001). Reporters included a 610 bp

sequence (*mAdcy1* 610-luc), which contained a circadian clock E-box (CACGTG), and a 280 bp sequence (*mAdcy1* 280-luc), which lacks the E-box. Two additional reporter constructs were generated using the 280-luc reporter plasmid: one with a circadian clock E-box insert and the other with a mutated E-box (TATGTG) insert at the 5' end to produce b280-luc and m280-luc reporter constructs, respectively (Chan et al., 2001). Murine cDNAs encoding for Npas2 (BC_109166) and Bmal1 (BC_011080) were cloned into pSCT1 (Hampp et al., 2008). The mammalian cell line used for this study was NG108-15, which is a murine neuroblastoma-rat glioma hydridoma line. Cells were grown in standard Dulbecco's modified Eagle medium (DMEM) and supplemented with 10% fetal bovine serum, 0.1 mM sodium hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine, 100 units of penicillin, and 100 µg of streptomycin. The cells were transfected with DNA plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The amount of firefly luciferase reporter plasmid added to each well was 0.2 µg, and the amounts of expression vectors were as follows: 0.8 µg for NPAS2 and BMAL1, unless noted otherwise. The total amount of expression vectors in each well was adjusted to 1.6 µg with an empty expression plasmid. Twenty-four hours after transfection, luciferase activity was measured in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). Firefly luciferase activity was normalized to Renilla luciferase activity of the co-transfected Renilla luciferase reporter plasmid (Invitrogen). Dual-Luciferase Assay System kit (Promega) was used to determine the luciferase activity levels. All experiments were repeated at least three times.

3.3.8 Statistical analysis

Analyses of two groups were made with Student's t-test. Analyses of more than two groups were performed using one-, two-, or three-way analysis of variance (ANOVA) with Student's-Newman-Keuls (SNK) multiple comparison test where applicable using SigmaPlot 12 (Systat Software Inc., San Jose, CA). Error bars represent standard error of the mean (SEM) and $p<0.05$ were considered significant.

3.4 Results

3.4.1 Contrast sensitivity exhibits circadian rhythmicity

To determine whether visual function is regulated in a circadian manner, an optomotor response test (optokinetic head tracking, OKT) was used to measure contrast sensitivity and visual acuity (Douglas et al., 2004), the latter defined by spatial frequency threshold (Prusky et al., 2004), at mid-day and mid-night in the 12 h light: 12 h dark cycle (LD) and on the second day in constant darkness (DD2). A circadian rhythm of contrast sensitivity was observed in WT mice. Mice exhibited significantly higher contrast sensitivity during the daytime than at night in both LD (Figure 3-1A; $p < 0.001$) and DD2 (Figure 3-1B; $p < 0.001$). Although night-time contrast sensitivity remained similar in LD and DD2, daytime sensitivity was reduced in the DD2 cycle compared to that in the LD cycle. However, visual acuity, as defined by spatial frequency threshold, did not fluctuate between mid-day (ZT6) and mid-night (ZT18) time points in LD (Figure 3-1C). Additional visual acuity measurements at ZT2, ZT10, and ZT14 were not different from those at the mid-day and mid-night time points (data not shown).

3.4.2 *Npas2* shows circadian expression in retinal ganglion cells

To localize *Npas2* expression in the mouse retina, we used an established β-galactosidase reporter driven by the NPAS2 promoter (Garcia et al., 2000). β-galactosidase immunofluorescence and lacZ histochemistry analyses were performed. NPAS2-β-galactosidase was localized to a subset of cells in the GCL by immunofluorescence (Figure 3-2A). NPAS2-β-galactosidase was not detected

in either the INL or PRL. Likewise, LacZ histochemistry revealed staining only in the GCL (Figure 3-2C). Because a significant proportion of cells in the GCL are displaced amacrine cells (Jeon et al., 1998), we performed a colocalization study with antibodies for β-galactosidase and Brn3a, a marker for retinal ganglion cells (Nadal-Nicolas et al., 2009). Virtually all of the cells expressing NPAS2-β-galactosidase were Brn3a-positive RGCs, but not all Brn3a-positive RGCs expressed NPAS2- β-galactosidase (Figure 3-2B). Out of 403 beta-gal-positive cells counted, 380 cells were also Brn3a positive (94.3%).

To determine whether the *Npas2* transcript is rhythmically expressed in the GCL, the GCL was isolated from retinal sections using laser capture microdissection (LCM) and subjected to qRT-PCR. Eyes were collected at 6-hour intervals over a 24-hour period. *Npas2* transcript levels were robustly rhythmic (Figure 3-2D; $p = 0.007$). Transcript levels were high near the time of light onset, decreased significantly by ZT6, and remained low until the onset of light the next day.

The selective expression of NPAS2-β-galactosidase in a subpopulation of RGCs raised the possibility that these cells may be involved in the daily rhythm of contrast sensitivity. We previously determined that optimal daytime contrast sensitivity requires dopamine and dopamine D4 receptors (D4Rs) (Jackson et al., 2012). Therefore, we examined the expression of *Npas2* mRNA in the GCL of mice lacking D4Rs (*Drd4*$^{-/-}$). We found the expression of the clock gene transcript to be low and arrhythmic in the GCL of *Drd4*$^{-/-}$ mice (Figure 3-2D).

We also attempted to determine if the rhythm of *Drd4* expression was altered in *Npas2*$^{-/-}$ mice. However, the expression level of *Drd4* in the GCL was too low to reliably quantify in either WT or *Npas2*$^{-/-}$ mice.

3.4.3 Contrast sensitivity is similarly reduced in *Drd4*$^{-/-}$, *Npas2*$^{-/-}$, and *Adcy1*$^{-/-}$ mice

Contrast sensitivity of *Drd4*$^{-/-}$ mice was measured at mid-day and mid-night in LD and DD2. Compared to WT controls, the amplitude of the circadian rhythm of contrast sensitivity was significantly reduced in *Drd4*$^{-/-}$ mice (Figure 3-3A; $p < 0.001$). Day-time contrast sensitivity measurements in the LD cycle were significantly reduced at multiple spatial frequencies in these mice compared to control while night-time contrast sensitivity measurements were preserved in *Drd4*$^{-/-}$ mice. Likewise, in DD2, a dampened circadian rhythm of contrast sensitivity was observed in *Drd4*$^{-/-}$ mice compared to controls (Figure 3-4A) with significantly reduced contrast sensitivity in the subjective day ($p < 0.001$).

To assess the role of *Npas2* in contrast sensitivity, OKT was performed on *Npas2*$^{-/-}$ mice in mid-day and mid-night time points in LD and DD2. A dampened circadian rhythm of contrast sensitivity was observed in *Npas2*$^{-/-}$ mice in LD (Figure 3-3B; $p < 0.001$). Day-time contrast sensitivity measurements were significantly reduced while night-time contrast sensitivity was preserved. In DD2, a dampened circadian rhythm of contrast sensitivity was also observed in *Npas2*$^{-}$

$^{/-}$ mice (Figure 3-4B; $p < 0.001$) with significantly reduced day-time contrast sensitivity and no effect at night.

We hypothesized that the clock-controlled gene, *Adcy1,* could play a role in the dopaminergic pathway modulating day-time contrast sensitivity. *Adcy1* is an effector gene involved in the synthesis of cyclic AMP and is under circadian control (Fukuhara et al., 2004). D4Rs, which are expressed in all three retinal layers (Cohen et al., 1992), regulate circadian *Adcy1* transcript expression (Jackson et al., 2011), and its protein product, adenylyl cyclase 1 (AC1), is the primary enzyme through which D4Rs and light regulate the rhythmic production of cAMP in the retina (Jackson et al., 2009). We measured contrast sensitivity of *Adcy1*$^{-/-}$ mice in mid-day and mid-night in LD and DD2. Strikingly, contrast sensitivity deficits observed in *Adcy1*$^{-/-}$, *Npas2*$^{-/-}$, and *Drd4*$^{-/-}$ mice were all remarkably similar. Compared to WT controls, the amplitude of the circadian rhythm of contrast sensitivity was significantly reduced in *Adcy1*$^{-/-}$ mice (Figure 3-3C; $p < 0.001$). Day-time contrast sensitivity in LD was significantly reduced while night-time contrast sensitivity was preserved. A dampened circadian rhythm of contrast sensitivity was also observed in *Adcy1*$^{-/-}$ mice in DD2 (Figure 3-4C) with significantly reduced day-time contrast sensitivity (*$p < 0.001$).

There were no significant differences in visual acuity, as defined by spatial frequency threshold, among WT, *Adcy1*$^{-/-}$, *Drd4*$^{-/-}$, and *Npas2*$^{-/-}$ mice at either mid-day or mid-night (Figure 3-3D).

3.4.4 Differential regulation of *Adcy1* expression in *Npas2*$^{-/-}$ and *Drd4*$^{-/-}$ mice

To determine if NPAS2 selectively regulates the expression of the clock-controlled gene, *Adcy1*, in RGCs, LCM was used to isolate the GCL, INL, and PRL from the retina across five different time points in a day. In WT mice, there was a robust rhythm in transcript expression over the 24-hour period in the GCL (Figure 3-5A; $p = 0.004$) and PRL (Figure 3-5A; $p < 0.001$), but not in the INL (Figure 3-5A). Transcript levels were low near the time of light onset, peaked at ~ZT12, and decreased to basal levels at the onset of light the following morning. In *Npas2*$^{-/-}$ mice, the rhythmic expression of the *Adcy1* transcript was completely abolished in the GCL (Figure 3-5B), but was preserved in the PRL (Figure 3-5C; $p = 0.015$), consistent with the localization of NPAS2-β-galactosidase to RGCs. Given the layer-specific control of the *Adcy1* gene expression, the rhythmic expression of *Adcy1* mRNA in the whole retina was slightly, but not significantly diminished in *Npas2*$^{-/-}$ mice compared to WT mice (Figure 3-5D). In *Drd4*$^{-/-}$ mice, the rhythmic expression of *Adcy1* transcript was similarly abolished in the GCL, as observed in *Npas2*$^{-/-}$ mice (Figure 3-5B). However, the rhythmic expression of *Adcy1* was also abolished in the PRL, unlike in *Npas2*$^{-/-}$ mice (Figure 3-5C).

3.4.5 Activation of the *Adcy1* promoter by NPAS2/BMAL1 in NG108-15 cells

We examined the ability of NPAS2/BMAL1 to activate *mAdcy1*-luciferase reporters in NG108-15 cells. Co-transfection with NPAS2/BMAL1 activated the *mAdcy1* 610-luc reporter construct (Figure 3-6A), which contains the circadian E-

box (Chan et al., 2001). The *mAdcy1* 280-luc reporter, which lacks the circadian E-box, showed little or no activation by NPAS2/BMAL1 (Figure 3-6A), suggesting that the E-box is necessary to confer the regulatory effect of NPAS2/BMAL1. In order to test whether the presence of the circadian E-box was sufficient to mediate the regulatory effects of NPAS2/BMAL1, a 45 bp insert containing the circadian E-box (b280-luc) or a mutated circadian E-box (m280-luc) was cloned at the 5' end of the 280 bp promoter fragment. Remarkably, the insertion of the wild type E-box at the 5' end of 280-luc vector conferred a comparable activation as seen with the 610-luc vector that was dependent on NPAS2/BMAL1 (Figure 3-6B). In contrast, the mutated E-box at the same location in m280-luc did not confer NPAS2/BMAL1-dependent activity (Figure 3-6B). The activation by NPAS2/BMAL1 was dose-dependent (Figure 3-6C).

3.5 Discussion

In this study, we elucidated a molecular mechanism through which day-time contrast sensitivity is modulated (Figure 3-7). WT mice exhibit a robust rhythm in contrast sensitivity both in LD and DD2 at most spatial frequencies with much higher sensitivity during the daytime. A circadian rhythm of contrast sensitivity has not been reported previously. We discovered that contrast sensitivity is dependent on a dopamine D4 receptor-NPAS2-adenylyl cyclase signaling pathway in RGCs.

Npas2 transcript is rhythmically expressed in the GCL, and NPAS2 is required for the rhythmic expression of *Adcy1* transcripts in the GCL but not in the PRL. Although CLOCK is expressed in the murine GCL (Storch et al., 2007), NPAS2 deficiency alone was sufficient to abolish the rhythmic expression of *Adcy1* mRNA and to dampen the contrast sensitivity rhythm. The *Adcy1* promoter contains an E-box enhancer element (Chan et al., 2001) and we found that it is directly activated by NPAS2/BMAL1 in NG108-15 cells. This result is consistent with another study demonstrating that the circadian expression of the *Aanat* gene in chicken retinal cells involves NPAS2 binding to a circadian E-box (Haque et al., 2010). Similarly, cotransfecting NPAS2/BMAL1 with promoter constructs of the gene encoding monoamine oxidase A in NG108-15 cells activated transcription (Hampp et al., 2008); however, cotransfection with CLOCK/BMAL1 was without effect (Hampp et al., 2008). The ability of NPAS2 or CLOCK to activate *Adcy1* appears to be cell-type specific. A previous study found that

CLOCK/BMAL1 increased *Adcy1*promoter activity in transiently transfected rat photoreceptor cells (Fukuhara et al., 2004). Additionally, NG108-15 cells appear to possess a cell-type specific mechanism that confers specificity to NPAS2/BMAL1 over CLOCK/BMAL1 (Hampp et al., 2008). A similar cell-type specific mechanism in RGCs could potentially explain why CLOCK could not substitute for NPAS2 in maintaining the rhythmic expression of *Adcy1*.

Our observations indicate that D4Rs are involved in regulating clock gene expression specifically in a subset of RGCs. Dopamine has been implicated in modulating the circadian clock in the whole retina of mammals and in photoreceptor cells of non-mammalian vertebrates. In the retina of *Drd2*$^{-/-}$ mice, the amplitude of the *Per1* transcript rhythm is reduced (Yujnovsky et al., 2006). Dopamine, via D1 receptors, entrains the clock regulating Per2::LUC reporter expression in the inner retina (Ruan et al., 2008). In photoreceptor cells of *Xenopus*, the administration of D2/D4 receptor agonist, quinpirole, can shift the circadian rhythm of melatonin release (Cahill and Besharse, 1991). In photoreceptor cells of zebrafish, dopamine functions to synchronize different circadian oscillators to regulate rhodopsin promoter activity (Yu et al., 2007).

Based on the observation that *Npas2* mRNA expression is arrhythmic in the GCL of *Drd4*$^{-/-}$ mice, we postulate that D4Rs are upstream of NPAS2. However, *Drd4* expression is rhythmic, with high levels at night and low levels during the daytime. The *Drd4* rhythm is regulated by a circadian clock and light (Jackson et

al., 2011), with light playing a dominant role (Storch et al., 2007). Light-evoked dopamine release and activation of D4Rs during the daytime may result in a compensatory decrease in *Drd4* expression, driving a circadian clock-independent rhythm of *Drd4*. The circadian clock-controlled rhythm of *Drd4* raises the possibility that *Drd4* expression is regulated by clock genes, perhaps by NPAS2/BMAL1. Melanopsin ganglion cells appear to influence retinal circadian clocks as well as the light-evoked regulation of dopamine (Barnard et al., 2006; Zhang et al., 2008; Dkhissi-Benyahya et al., 2013). Thus, there may be reciprocal interactions between D4R and NPAS2, with NPAS2-containing ganglion cells affecting dopamine neurons and dopamine affecting rhythmic regulation of D4Rs.

In addition to dopamine, cyclic AMP is also implicated in the regulation of the circadian pacemaker. Cyclic AMP is necessary to maintain the rhythmic expression of PER2-luciferase in the suprachiasmatic nucleus of the brain (O'Neill et al., 2008). The effects of cyclic AMP are thought to be regulated through both CREB-dependent signaling and MAP kinase pathways. In mice overexpressing *Adcy1*, the levels of pERK and pCREB in the forebrain were both higher than in controls, showing that *Adcy1* affects both pathways (Wang et al., 2004). Dopamine D2 receptor-mediated activation of MAP kinase pathway induces *Per1* (Yujnovsky et al., 2006). D4Rs contain Src homology 3 binding motifs and could activate the MAPK signaling pathway and affect circadian clock

function by interacting with the Src-homology 2—Src-homology 3 adapter protein, Grb2 (Oldenhof et al., 1998; Oak et al., 2001).

Previous studies have shown that visual sensitivity, as defined by visual thresholds, is under circadian control in zebrafish (Li and Dowling, 1998), larval *Xenopus* (Solessio et al., 2004), mouse (Balkema et al., 2001), and human (Bassi and Powers, 1986; Tassi et al., 2000). Together with our data on circadian regulation of contrast sensitivity, this indicates that multiple aspects of visual function are under circadian control. However, it is unlikely that the circadian rhythm of visual sensitivity threshold is directly related to the observed rhythm of contrast sensitivity as the latter was measured under photopic conditions.

The exact mechanism through which a subset of RGCs expressing NPAS2-β-galactosidase modulate contrast sensitivity is not clear, but there are at least two possible explanations. First, the subset of RGCs expressing NPAS2- β-galactosidase could be direction-selective ganglion cells (DSGCs), which comprise a minority of RGCs [reviewed in (Vaney et al., 2012)]. The optomotor response test that was utilized in this study preferentially evaluates the response to posterior to anterior motion (Douglas et al., 2005). Therefore, it is possible that NPAS2 may play an essential role in the function of a subset of DSGCs that detect posterior to anterior motion. Second, the expression of NPAS2-β-galactosidase may be below the level of detection in other RGCs as a recent

study found NPAS2-like immunoreactivity in over 90% of the cells in the GCL (Liu et al., 2012).

Over 94% of the NPAS2-β-galactosidase positive cells were immunoreactive for the ganglion cell marker, Brn3a. A small number of cells in the GCL were β-galactosidase positive yet were not Brn3a positive. Although Brn3a is an excellent marker for retinal ganglion cells, Brn3a does not detect 100% of RGCs. In a recent study, a Brn3a antibody labeled 92% of RGCs identified by a retrograde labeling with Fluorogold, which detects over 98% of RGCs in rodents (Nadal-Nicolas et al., 2009; Salinas-Navarro et al., 2009). Therefore, these cells are likely a small minority of retinal ganglion cells that are not Brn3a-positive. Alternatively they could be displaced amacrine cells in the GCL; but this is unlikely as the β-galactosidase antibody did not label any cell in the INL except for one, which was a displaced retinal ganglion cell (Brn3a-positive).

Contrast sensitivity was similarly reduced in *Drd4*$^{-/-}$, *Npas2*$^{-/-}$, and *Adcy1*$^{-/-}$ mice, although *Adcy1* transcript rhythm was preserved in the PRL of *Npas2*$^{-/-}$mice. Our data suggest that *Adcy1* in the photoreceptor layer may not play a significant role in modulating contrast sensitivity. However, while we found NPAS2-β-galactosidase to be localized exclusively to RGCs, a recent study found NPAS2-like immunoreactivity in cone photoreceptors as well as in some cells in the INL (Liu et al., 2012). Therefore, it is possible that NPAS2 regulates additional factors in cone photoreceptors that could also contribute to modulating contrast

sensitivity and could explain why the contrast sensitivity rhythms were similarly reduced. Nevertheless, the observation that circadian expression of *Adcy1* is abolished in the GCL but not the PRL of *Npas2*$^{-/-}$ mice indicates that NPAS2 plays a major functional role in retinal ganglion cells to regulate contrast sensitivity.

The time course of *Npas2* expression in the GCL differs significantly from that of *Adcy1* expression. However, a time delay in the activation of clock and clock-controlled genes is not uncommon. For example, in the mouse retina, there is approximately an 8-hour delay from the time at which *Bmal1* transcript level peaks to the time that *Per1* and *Per2* levels peak (Ruan et al., 2006; Storch et al., 2007). Additionally, in the chicken retina, the *Aanat* gene, which is another clock-controlled gene regulated by NPAS2, peaks over 6 hours after the peak of *Npas2* transcript level (Haque et al., 2010). Although the *Adcy1* transcript level trends toward a peak at ZT12 in WT mice, the Adcy1 transcript levels at ZT6 and ZT12 are not statistically different. Therefore, the actual peak of Adcy1 transcripts in the murine GCL may occur sometime between ZT6 and ZT12, and the time delay we observed in our study is consistent with the ones previously reported in the literature.

We used NG108-15 cells to investigate if NPAS2/BMAL1 could activate the *Adcy1* gene promoter by NPAS2/BMAL1. These cells are used frequently in molecular studies to investigate gene activation using luciferase assays

(Takeuchi et al., 2000; Wan et al., 2000; Hampp et al., 2008; Liu et al., 2011). Like RGCs, NG108-15 cells are derived from a neuronal lineage. However, as with any cell line, NG108-15 cells are immortalized cells and may not accurately model primary cells.

In conclusion, we show for the first time that contrast sensitivity is circadian and is influenced by specific clock components. Dopamine, through a D4 receptor pathway, regulates the rhythmic expression of *Npas2* in the GCL. NPAS2 in RGCs regulates the rhythmic expression of *Adcy1*, which presumably generates a rhythm of cAMP that ultimately modulates day-time contrast sensitivity. Disruption of D4Rs, NPAS2, or AC1 reduces the amplitude of the rhythm but does not abolish it, suggesting that CLOCK may also contribute to the rhythm. The mechanism(s) whereby cyclic AMP in RGCs modulates the rhythm of contrast sensitivity is unknown, but may involve regulation of hyperpolarization-activated cyclic nucleotide-gated channels (Stradleigh et al., 2011), glutamate receptors (Knapp and Dowling, 1987; Liman et al., 1989; Greengard et al., 1991), GABA receptors (Feigenspan and Bormann, 1994; Veruki and Yeh, 1994), and/or gap junctions (Mills et al., 2007). Elucidation of these mechanisms may provide novel insights into ways to improve vision at night (e.g., night vision for shift workers, pilots and surgeons).

3.6 Figures and Figure Legends

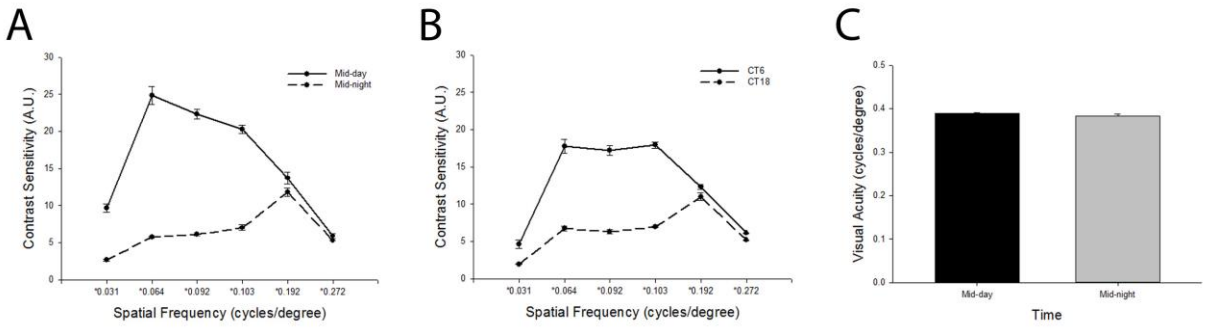

Figure 3-1 WT mice displayed circadian rhythm in contrast sensitivity but not in visual acuity (as defined by spatial frequency threshold).

(A) In LD, contrast sensitivity was significantly rhythmic in WT mice (two-way ANOVA, spatial frequency x time interaction, $F_{5,37} = 45.194$, $p < 0.001$; *$p < 0.001$, Student-Newman-Keuls *post hoc* test, n = 4-6 mice). (B) In DD2, contrast sensitivity was significantly rhythmic in WT mice (two-way ANOVA, spatial frequency x time interaction, $F_{5,34} = 67.866$, $p < 0.001$; *$p < 0.001$, Student-Newman-Keuls *post hoc* test, n = 4-6 mice). (C) Visual acuity measurements were not different between mid-day and mid-night time points (n = 9-10 mice). Data expressed as mean ± SEM.

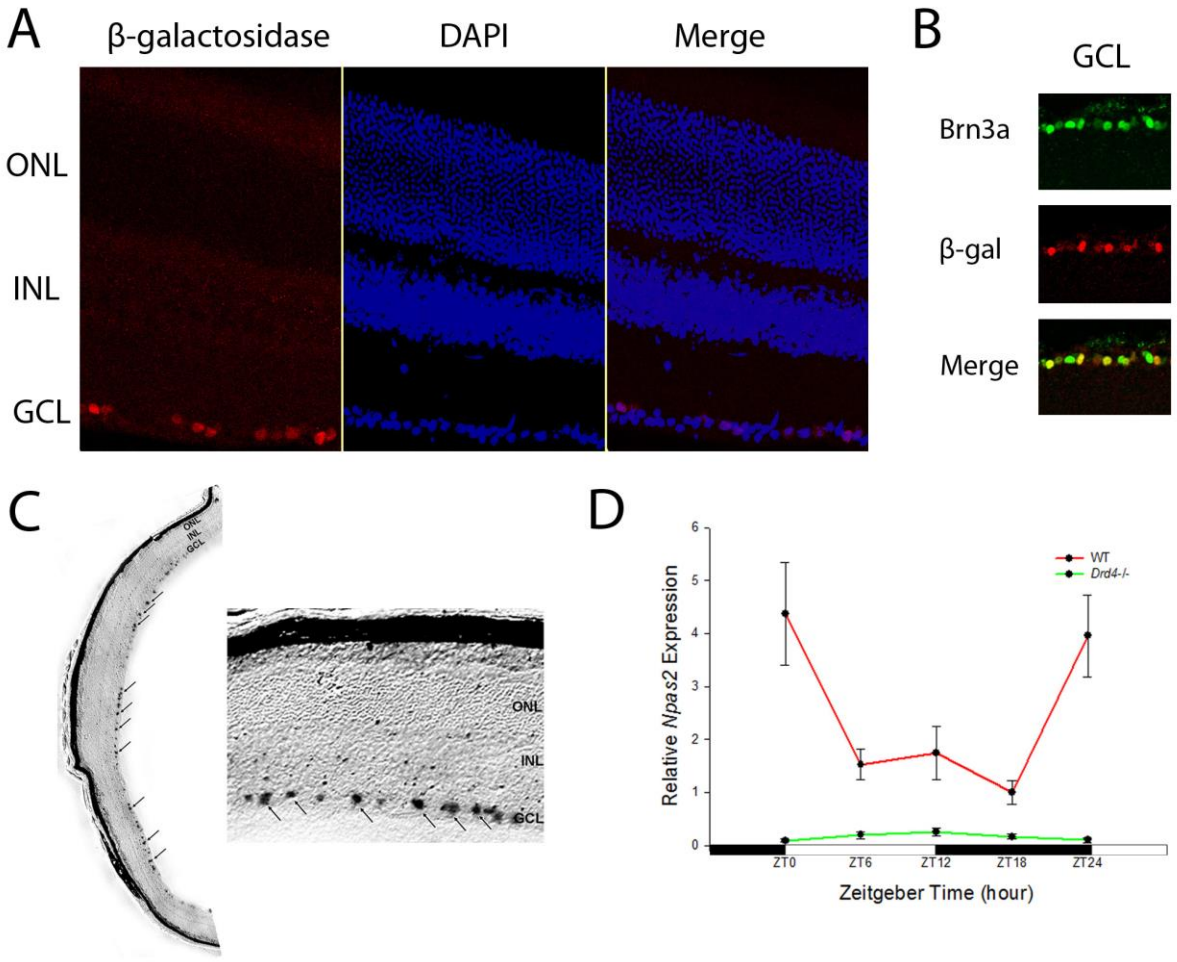

Figure 3-2 NPAS2 is rhythmically expressed in retinal ganglion cells.

Figure 3-2 NPAS2 is rhythmically expressed in retinal ganglion cells.

(A) Immunofluorescence staining shows NPAS2-β-galactosidase (red) localized to the GCL. (B) Immunofluorescence staining shows NPAS2-β-galactosidase (red) co-localizing with retinal ganglion cell marker, Brn3a (green). (C) LacZ histochemical staining is localized to the GCL. (D) *Npas2* transcript in the GCL of WT mice (red) is robustly rhythmic (one-way ANOVA, $F_{4,25} = 4.541$, $p = 0.007$), but in *Drd4*$^{-/-}$ mice (green), it was arrhythmic and barely detectable (one-way ANOVA, $F_{4,18} = 0.1.584$, $p = 0.222$; two-way ANOVA, D4 receptor deficiency x time interaction, $F_{4,41} = 5.802$, $p < 0.001$). Data expressed as mean ± SEM, n = 5-6 mice per time point.

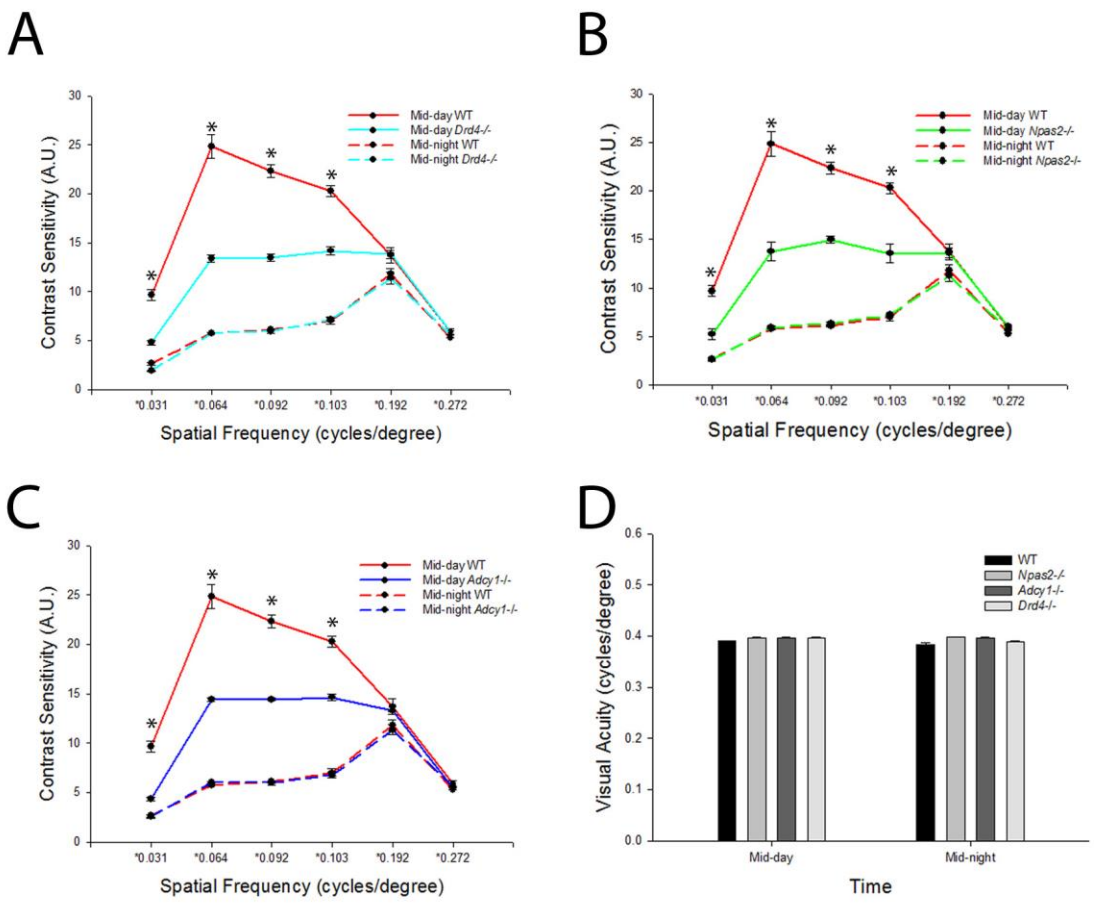

Figure 3.3 *Drd4*$^{-/-}$, *Npas2*$^{-/-}$, and *Adcy1*$^{-/-}$ mice display similar deficiencies in visual parameters in LD.

Figure 3-3 *Drd4*$^{-/-}$, *Npas2*$^{-/-}$, and *Adcy1*$^{-/-}$ mice display similar deficiencies in visual parameters in LD.

Contrast sensitivity is rhythmic but reduced in (A) *Drd4*-/- mice (two-way ANOVA, D4R deficiency x time interaction, $F_{5,25} = 5.802$, $p < 0.001$; *$p < 0.001$, Student-Newman-Keuls *post hoc* test, n=4-6 mice), (B) *Npas2*$^{-/-}$ mice (two-way ANOVA, NPAS2 deficiency x spatial frequency interaction, $F_{5,25} = 19.276$, $p < 0.001$; *$p < 0.001$, Student-Newman-Keuls *post hoc* test, n=4-6 mice), and (C) *Adcy1*$^{-/-}$ mice (two-way ANOVA, AC1 deficiency x spatial frequency interaction, $F_{5,25} = 45.067$, $p < 0.001$; *$p < 0.001$, Student-Newman-Keuls *post hoc* test, n=4-6 mice). There was a significant reduction in mid-day contrast sensitivity at multiple spatial frequencies in *Drd4*$^{-/-}$, *Npas2*$^{-/-}$, and *Adcy1*$^{-/-}$ mice (*$p < 0.001$, Student-Newman-Keuls *post hoc* test, n=4-6 mice) while the mid-night contrast sensitivity was preserved. (D) Like in WT mice, visual acuity measurements (as defined by spatial frequency thresholds) were normal and arrhythmic in *Npas2*$^{-/-}$, *Adcy1*$^{-/-}$, and *Drd4*$^{-/-}$ mice (n=6-8 mice) in LD. Data expressed as mean ± SEM.

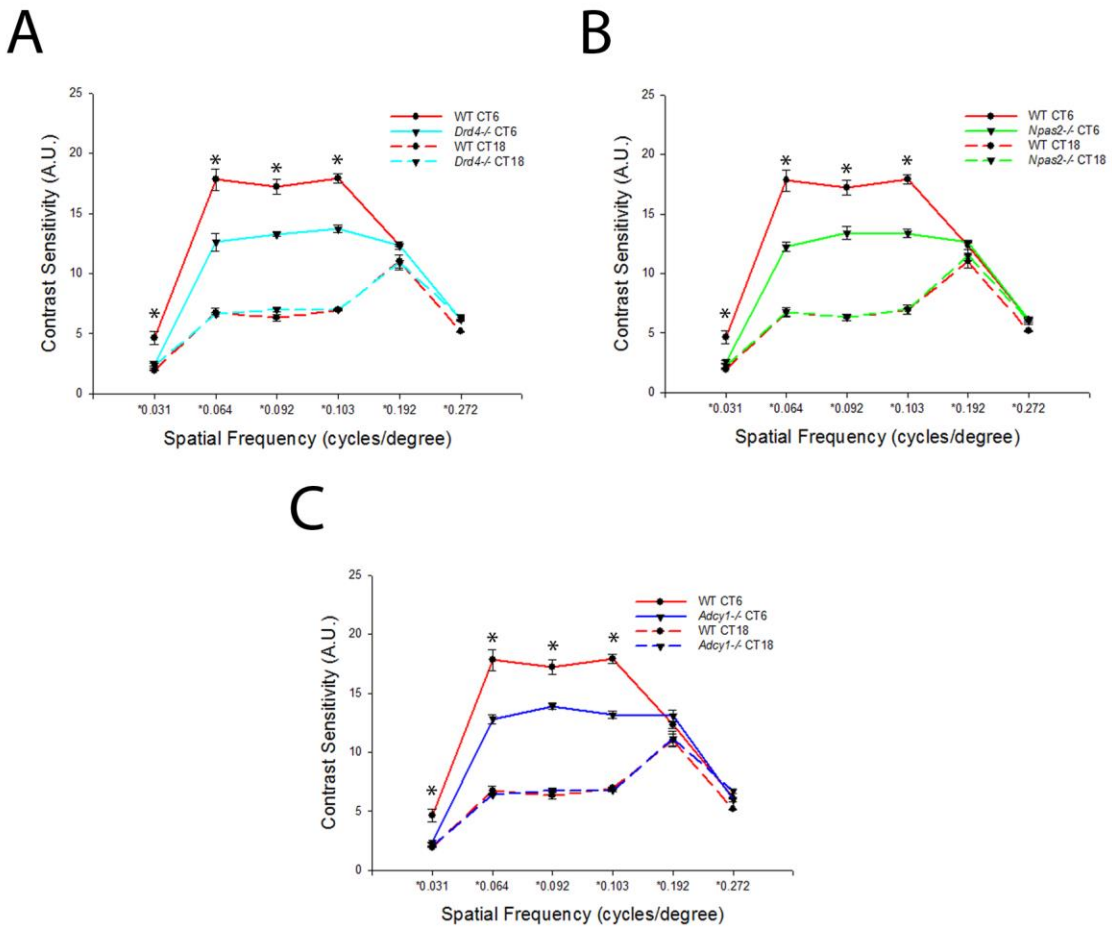

Figure 3-4 *Drd4*$^{-/-}$, *Npas2*$^{-/-}$, and *Adcy1*$^{-/-}$ mice display similar deficits in mid-day contrast sensitivity in DD2.

Figure 3-4 *Drd4*$^{-/-}$, *Npas2*$^{-/-}$, and *Adcy1*$^{-/-}$ mice display similar deficits in mid-day contrast sensitivity in DD2.

Compared to controls, contrast sensitivity is rhythmic but reduced (A) in *Drd4*$^{-/-}$ mice (two-way ANOVA, D4R deficiency x spatial frequency interaction, $F_{5,25}$ = 45.067, $p < 0.001$; *$p < 0.001$ at 0.03, 0.064, 0.092, and 0.103 cycles/degree, Student-Newman-Keuls *post hoc* test, n=3-6 mice), (B) in *Npas2*$^{-/-}$ mice (two-way ANOVA, NPAS2 deficiency x spatial frequency interaction, $F_{5,34}$ = 46.215, $p < 0.001$; *$p < 0.001$ at 0.03, 0.064, 0.092, and 0.103 cycles/degree, Student-Newman-Keuls *post hoc* test, n=3-6 mice), and (C) in *Adcy1*$^{-/-}$ mice (two-way ANOVA, AC1 deficiency x spatial frequency interaction, $F_{5,24}$ = 94.952, $p < 0.001$; *$p < 0.001$ at 0.03, 0.064, 0.092, and 0.103 cycles/degree, Student-Newman-Keuls *post hoc* test, n=3-6 mice). There was a significant reduction in mid-day contrast sensitivity at multiple spatial frequencies in *Npas2*$^{-/-}$, *Adcy1*$^{-/-}$, and *Drd4*$^{-/-}$ mice (*$p < 0.001$ at 0.03, 0.064, 0.092, and 0.103 cycles/degree, Student-Newman-Keuls *post hoc* test, n=4-6 mice) while the mid-night contrast sensitivity was preserved (for both *Adcy1*-/- and *Drd4*-/- mice, *$p < 0.05$ only at 0.272 cycles/degree, Student-Newman-Keuls *post hoc* test, n=4-6 mice). Data expressed as mean ± SEM.

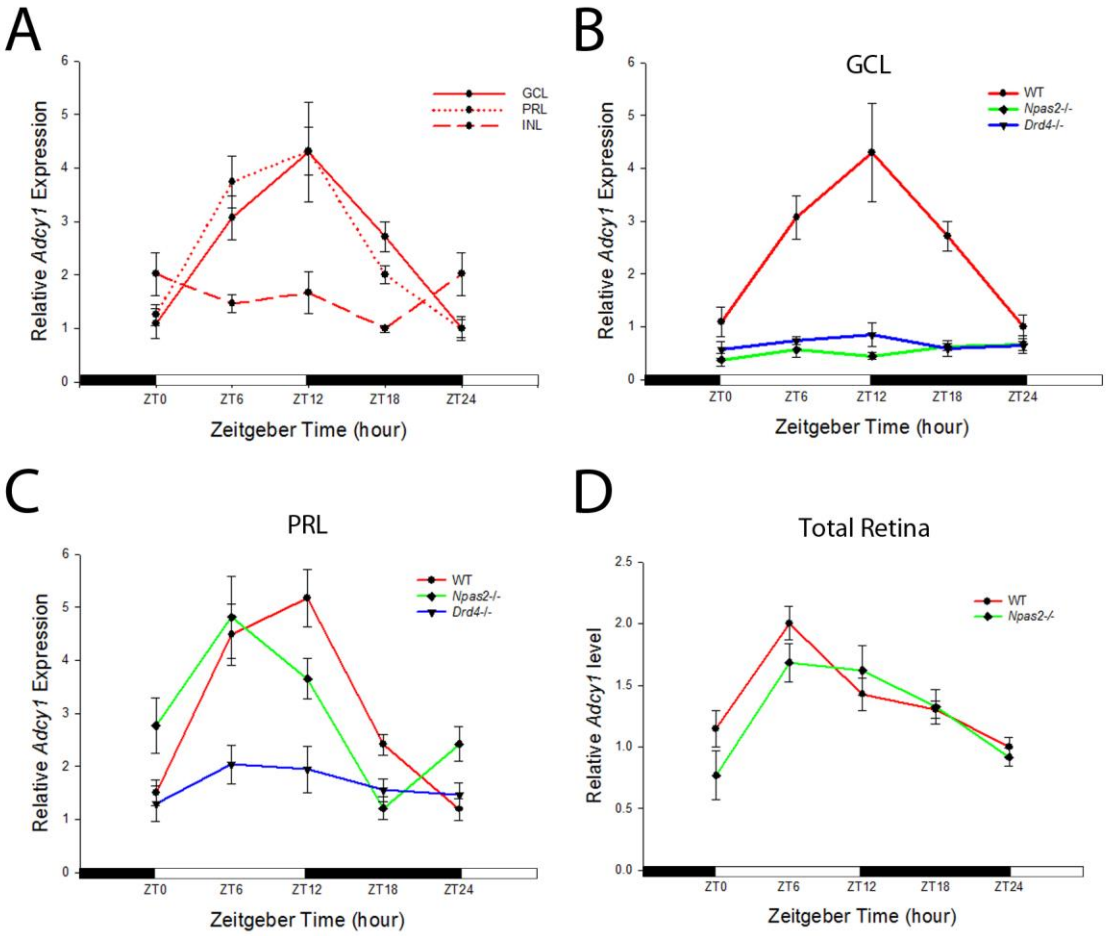

Figure 3-5 *Adcy1* transcript rhythms in different retinal layers and whole retinas of WT, *Npas2*$^{-/-}$, and *Drd4*$^{-/-}$ mice.

Figure 3-5 *Adcy1* transcript rhythms in different retinal layers and whole retinas of WT, *Npas2*$^{-/-}$, and *Drd4*$^{-/-}$ mice.

(A) In WT mice, *Adcy1* transcripts are robustly rhythmic the in GCL (solid red, one-way ANOVA on ranks, $H_4 = 15.548$, $p = 0.004$) and PRL (short-dashed red, one-way ANOVA, $F_{4,23} = 18.627$, $p < 0.001$), but not in the INL (long-dashed red). (B) *Adcy1* transcripts are arrhythmic and stay at low levels in the GCL of *Npas2*$^{-/-}$ (green, one-way ANOVA, $F_{4,20} = 0.975$, $p = 0.443$; two-way ANOVA, NPAS2 deficiency x time interaction, $F_{4,40} = 6.809$, $p < 0.001$) and *Drd4*$^{-/-}$ mice (blue, one-way ANOVA, $F_{4,18} = 0.644$, $p = 0.638$; two-way ANOVA, D4 receptor deficiency x time interaction, $F_{4,38} = 5.082$, $p = 0.002$). (C) In the PRL of *Npas2*$^{-/-}$ mice, the *Adcy1* transcript expression (green) is rhythmic and not significantly different from WT (red) (one-way ANOVA, $F_{4,19} = 4.092$, $p = 0.015$; two-way ANOVA, NPAS2 deficiency x time interaction, $F_{4,42} = 2.760$, $p = 0.055$). In the PRL of *Drd4*$^{-/-}$ mice (blue, one-way ANOVA, $F_{4,18} = 0.896$, $p = 0.487$; two-way ANOVA, D4R deficiency x time interaction, $F_{4,41} = 7.270$, $p < 0.001$), the *Adcy1* transcript expression is arrhythmic and stays at low levels. (D) The rhythmic expression of *Adcy1* transcripts in the whole retina is not significantly different between WT and *Npas2*$^{-/-}$ mice ($p = 0.407$, two-way ANOVA). Data expressed as mean ± SEM, n=5-6 mice per time point.

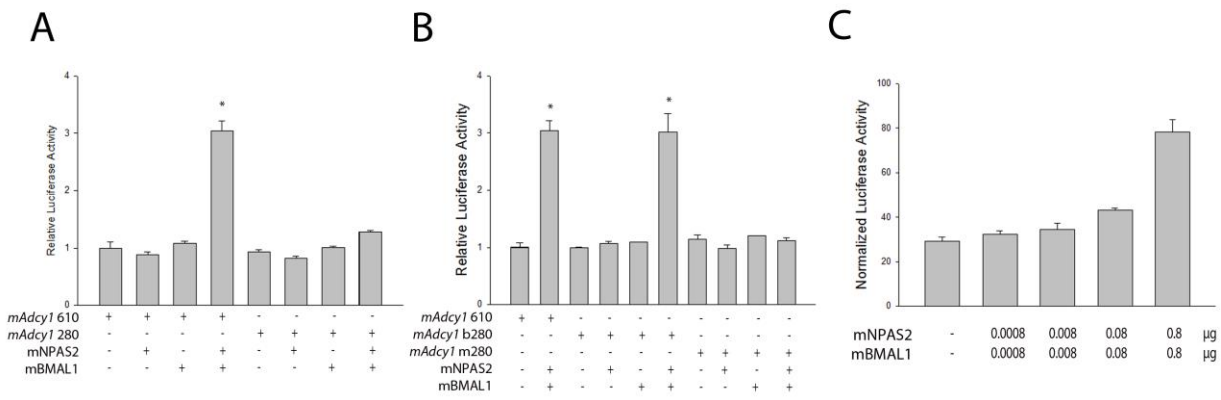

Figure 3-6 Regulation of *mAdcy1* promoter by NPAS2/BMAL1.

(A) Luciferase reporter plasmids containing either a 610 bp m*Adcy1* promoter (m*Adcy1* 610), which contains a circadian E-box, or a circadian E-box-lacking 280 bp m*Adcy1* promoter (m*Adcy1* 280) was used for the luciferase activity assays. NPAS2/BMAL1 activated m*Adcy1* promoter (*p < 0.001 vs. empty plasmid transfected control group), and the deletion of the circadian E-box region substantially reduced the luminescence level. (B) Insertion of the circadian E-box into the m*Adcy1* 280-luc vector (m*Adcy1* b280) confers high luciferase activity with NPAS2/BMAL1 (*p < 0.001 vs. empty plasmid transfected control group) while insertion of a mutated circadian E-box into the m*Adcy1* 280-luc vector (m*Adcy1* m280) had no effect. (C) Adcy1 promoter activation by NPAS2/BMAL1 was dose-dependent. The activation of 610 bp *Adcy1* promoter increased with higher levels of expression vectors (in µg) of NPAS2 and BMAL1. Presence (+) or absence (-) of the reporter and expression plasmids are shown. All values are the mean ± SE (n=3). Representative data of at least three independent experiments.

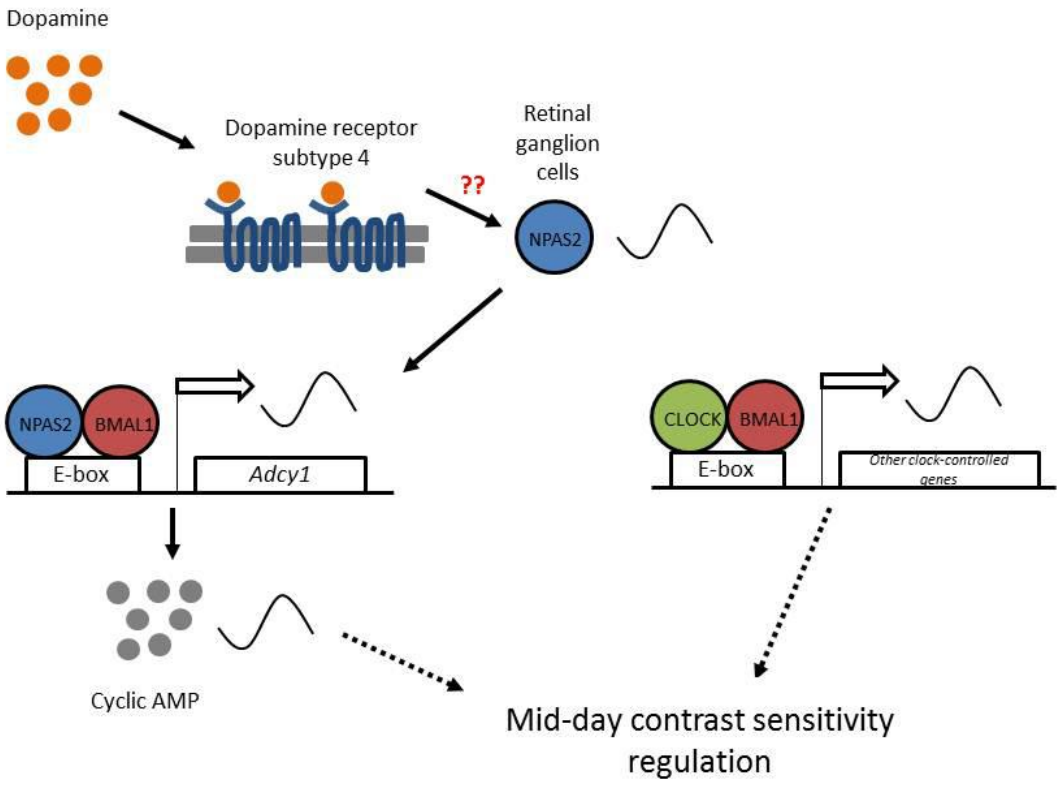

Figure 3-7 A model for mid-day contrast sensitivity regulation.

Dopamine, through a D4 receptor pathway, regulates the rhythmic expression of *Npas2* in the GCL. NPAS2 in the retinal ganglion cells regulates the rhythmic expression of the *Adcy1* gene in the GCL, which ultimately modulates day-time contrast sensitivity.

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Chapter 4: Differential roles of retinal CLOCK and NPAS2 in mediating different dimensions of vision

4.1 Abstract

Contrast sensitivity and light-adapted electroretinogram responses are two important physiologic functions in the retina that are driven by retinal circadian clocks. CLOCK and NPAS2 are homolog proteins that operate in the positive arm of the circadian clock machinery. In the retina, NPAS2 is expressed rhythmically in a subset of retinal ganglion cells and modulates the contrast sensitivity rhythm by regulating the rhythmic expression of *Adcy1* in the ganglion cell layer (GCL). Although CLOCK is also expressed in the GCL, it is not known whether CLOCK plays a role in modulating the contrast sensitivity rhythm. In this study, we show that CLOCK colocalizes with NPAS2 in NPAS2-expressing RGCs and that CLOCK appears to be upstream of NPAS2 to regulate the rhythmic expression of both *Npas2* and *Adcy1*. In *Clock*$^{-/-}$ mice, the circadian rhythm of contrast sensitivity is abolished. Additionally, the light-adapted ERG rhythm is severely disrupted, and dark-adapted ERG responses across all flash intensities are also substantially reduced in *Clock*$^{-/-}$ mice. In contrast, both light-adapted and dark-adapted ERG responses in *Npas2*$^{-/-}$ mice are indistinguishable from those in WT mice.

4.2 Introduction

Retinal circadian clocks drive many important physiologic functions in the retina. Recently, retinal clocks have been shown to an important role in regulating the circadian rhythm of contrast sensitivity (Hwang et al., 2013) and light-adapted electroretinogram (ERG) responses (Storch et al., 2007; Cameron et al., 2008). Although a clock protein, neuronal PAS-domain protein 2 (NPAS2), has been shown to modulate the circadian rhythm of contrast sensitivity (Hwang et al., 2013), the role of the NPAS2-homolog protein, CLOCK, in the contrast sensitivity regulation is not known. Furthermore, while a retinal circadian oscillator is implicated in the regulation of light-adapted ERG responses (Storch et al., 2007; Cameron et al., 2008), it is not clear whether CLOCK or NPAS2 is involved.

The circadian clock is driven by a transcriptional-translational feedback loop comprised of the positive regulators, CLOCK and BMAL1, which heterodimerize and drive the expression of their negative regulators, PERIOD 1 and 2 and CRYPTOCHROME 1 and 2 (Gekakis et al., 1998; Reppert and Weaver, 2002; Ko and Takahashi, 2006). These clock proteins also regulate the expression of clock-controlled genes, which are output genes that are thought to drive different physiologic rhythms (Akhtar et al., 2002; Duffield et al., 2002; Panda et al., 2002; Storch et al., 2002). NPAS2 is a CLOCK homolog protein that has been shown to be integral in the circadian clock machinery in the forebrain (Reick et al., 2001) and suprachiasmatic nucleus (SCN) (Debruyne et al., 2006; DeBruyne et al., 2007b). In the mammalian retina, NPAS2 appears to be localized primarily, if not

exclusively, in retinal ganglion cells (RGCs) (Hwang et al., 2013). Retinal NPAS2 modulates the contrast sensitivity rhythm by regulating the expression of a clock-controlled gene, *Adcy1*, in the ganglion cell layer (GCL) (Hwang et al., 2013). Although CLOCK is expressed in the GCL (Gekakis et al., 1998; Ruan et al., 2006; Storch et al., 2007), it is not clear whether CLOCK plays a role in regulating the *Adcy1* expression in the GCL or the circadian rhythm of contrast sensitivity.

BMAL1 appears to be required for the circadian rhythm of light-adapted ERG responses (Storch et al., 2007), However, it is unknown if the BMAL1 binding partner underlying the ERG rhythm is CLOCK or NPAS2. CLOCK is widely distributed in the retina (Gekakis et al., 1998; Storch et al., 2007), and a recent study showed an NPAS2-like immunoactivity in the inner nuclear layer and cone photoreceptors, as well as in RGCs (Liu et al., 2012). Thus, either CLOCK or NPAS2 could play a role in mediating the effects of circadian clock on light-adapted ERG responses.

In this study, we determined that CLOCK colocalizes with NPAS2 in NPAS2-expressing RGCs and that CLOCK appears to be upstream of NPAS2 to regulate the rhythmic expression of both *Npas2* and *Adcy1*. In *Clock*$^{-/-}$ mice, the contrast sensitivity rhythm is ablated. Additionally, their light-adapted ERG rhythm is severely disrupted, and their dark-adapted ERG responses across all flash

intensities are also substantially reduced. In contrast, both light-adapted and dark-adapted ERG responses in *Npas2*$^{-/-}$ mice are normal.

4.3 Methods

4.3.1 Animals

Emory University's Institutional Animal Care and Use Committee (IACUC) approved all animal experimental procedures, all of which conform to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For this investigation, *Npas2*$^{-/-}$ mice, producing the non-functional β-galactosidase-NPAS2 fusion protein (Garcia et al., 2000), *Clock*$^{-/-}$ mice, lacking CLOCK protein (Debruyne et al., 2006), and wild-type mice (WT, C57BL/6J) of either sex were used and genotyped by polymerase chain reaction analysis of tail genomic DNA. Mice were 9-12 weeks old except for the mice used for toluidine staining, which were 2.5-3 months ("young" group) and 9-10 months old ("old" group). Food and water were provided ad libitum. Mice were housed on a 12-hour light /dark (LD) cycle. Lights were switched on at zeitgeber time (ZT) 0 and off at ZT12. Mice were entrained to the LD cycle and/or subsequently kept in constant darkness (DD=first day of constant darkness; DD2=second day of constant darkness), and circadian rhythms were studied thereafter. All experimental procedures in ''dark'' conditions were performed under dim red light. CT rather than ZT was used to designate subjective time of day in DD or DD2.

4.3.2 Light Microscopy of Retinas

Eyes of each genotype were fixed (2.5% glutaraldehyde in 0.1 M cacodylate buffer) for four days at 4˚C. Retinal sections including the optic nerve were subsequently obtained, stained in toluidine blue, and viewed under the microscope (Eclipse E300, Nikon Instruments Inc., Melville, NY). Quantitative cell count in each retinal layer (mid-periphery) was performed by counting nuclei in the following retinal segments: ONL and INL-per 20 µm segment of retina; GCL-per 100 µm segment of retina.

4.3.3 Laser capture microdissection (LCM)

Whole mouse eyes, embedded in OCT and stored at -80°C, were used for LCM using the method described previously (Hwang et al., 2013). Briefly, 12 µm-thick tissue sections were made and mounted on polyethylene naphthalate membrane glass slides (Applied Biosystems, Foster City, CA). Subsequently, the slides were placed in 75% ethanol for 30 seconds followed by a wash in RNase-free water for 30 seconds. HistoGene (Applied Biosystems) staining solutions were used to stain the tissue sections for approximately 15 seconds followed by a wash with RNase-free water for 30 seconds, followed by dehydration steps in graded ethanol solutions (75%, 30 s; 95%, 30 s; 100%, 30 seconds) and clearing step in xylene (> 5 minutes). The slides were then air-dried for 5 minutes. Laser capture microdissection was performed to isolate the ganglion cell layer (GCL) onto HS CapSure non-contact LCM films using ArcturusXT system (Applied Biosystems). Only the desired GCL and PRL areas were captured from the

tissue section with all unwanted cells left behind, generating a pure cell population for downstream molecular analysis. To purify total RNA from captured tissue, the PicoPure RNA Isolation kit (Applied Biosystems) was utilized, and the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) was used to reverse-transcribe the total RNA to cDNA.

4.3.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to quantify *Adcy1*, *Npas2,* and *Clock* transcripts, using 2 µL cDNA from each sample. qRT-PCR was performed with a 25 µL total volume containing cDNA, QuantiFast SYBR Green PCR Master mix (Qiagen), and 1 µM forward and reverse primers for the gene of interest in Bio-Rad iCycler (Bio-Rad Laboratories Inc., Hercules, CA). The fluorescence threshold value was calculated using MyiQ cycler software. The expression levels of *Adcy1*, *Npas2,* and *Clock* transcripts were normalized to the levels of the housekeeping gene 18S rRNA and quantified according to the delta-delta Ct method (Livak and Schmittgen, 2001). Each sample was assayed in duplicate.

4.3.5 Luciferase Reporter Assays and Transfections

Murine adenylyl cyclase type 1 (*mAdcy1*) promoter fragments were cloned into the pGL3-basic vector (Promega, Madison, WI), which contains the firefly luciferase reporter gene (Chan et al., 2001). The promoter fragments included a 610 bp sequence (*mAdcy1* 610-luc), which contains a circadian E-box

(CACGTG), and a 280 bp sequence (*mAdcy1* 280-luc), which lacks the E-box (Chan et al., 2001). Into the pSCT1 vector, murine cDNAs encoding for Npas2 (BC_109166), Clock (AF_000998), and Bmal1 (BC_011080) were cloned (Hampp et al., 2008). NG108-15, a murine neuroblastoma-rat glioma hydridoma cell line, was used for this study. Cells were grown in standard Dulbecco's modified Eagle medium (DMEM) and supplemented with 10% fetal bovine serum, 0.1 mM sodium hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine, 100 units of penicillin, and 100 µg of streptomycin in 12 well (3.8 cm^2) cell culture plates. DNA plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The amounts of expression vectors were as follows: 0.8 µg for NPAS2, CLOCK, and BMAL1, unless noted otherwise; 0.2 µg of firefly luciferase reporter plasmid and 0.02 µg of Renilla luciferase reporter plasmid (Invitrogen) per ml of medium. An empty expression plasmid was used to adjust the total amount of expression vectors in each well to 1.6 µg. Luciferase activity was measured in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA) 24 hours after transfection. Firefly luciferase activity was normalized to Renilla luciferase activity. The luciferase activity levels were determined using the Dual-Luciferase Assay System kit (Promega). Each experiment was done in triplicate, and all experiments were repeated at least three times.

4.3.6 Contrast sensitivity and visual acuity

To measure contrast sensitivity and visual acuity, as defined by spatial frequency threshold, an optomotor response test (optokinetc head tracking) was utilized, using the OptoMotry instrument (CerebralMechanics, Inc.) as previously described (Prusky et al., 2004). Briefly, a mouse was placed on the raised platform inside the device, where a sine wave grating, projected by four interfacing LCD monitors, moved either in clock-wise or counter-clockwise direction. Subsequently, the mouse was assessed for tracking behavior. Contrast sensitivity testing began with a 100% contrast grating, which was gradually reduced until contrast threshold was reached when the mouse stopped tracking at each of six spatial frequencies (0.031, 0.064, 0.092, 0.103, 0.192, 0.272 cycles per degree). At each spatial frequency, the reciprocal of the threshold was used as the contrast sensitivity value. To determine the spatial frequency threshold for visual acuity, the staircase procedure was utilized in which a black and white sinusoidal grating at 100% contrast was increased in cycles per degree until tracking ceased. Testing occurred during mid-day (ZT6) and mid-night (ZT18) hours in LD and during subjective day (CT6) and night (CT18) time points in DD2. All measurements were made under photopic conditions as characterized previously (Hwang et al., 2013). The order of spatial frequency tested for each mouse was randomly chosen, and the testing period for each mouse was no longer than 20 minutes to minimize the effect of light adaptation when measuring CS in dark-adapted mice.

4.3.7 Retinal Function Test with Electroretinogram (ERG)

Mice were dark-adapted overnight or for up to 2 days and then prepared for ERG under dim red illumination as described previously (Jackson et al., 2012). Briefly, mice were anesthetized with ketamine (70 mg/kg) and xylazine (7 mg/kg). Pupils were dilated with 1% tropicamide and 1% cyclopentolate, and corneal surfaces moistened with 10% methylcellulose eye drops. The DTL fiber electrode was placed over the cornea for ERG recordings. For the dark-adapted ERG, responses were recorded to flash stimuli presented in order of increasing luminance (UTAS BigShot; LKC Technologies, Gaithersburg, MD). ERG stimuli consisted of a 12-step series (−3.4 to 2.1 log cd·s/m^2) to isolate rod-dominant and rod-cone mixed responses. With 20-µs flashes presenting in order of increasing intensity, dark-adapted condition was maintained by increasing the interstimulus interval from 4 to 75 s. For the light-adapted ERG, 0.90 log cd·s/m^2 bright light flashes were presented at 0.75 Hz over a 20 min period on a steady background light (40 cd/m^2) to saturate rod photoreceptors. Data were collected in 2.6 min averaged bins for a total of eight bins. The a-wave and b-wave amplitudes were analyzed off-line. A low pass 60 Hz filter was used to remove the influence of oscillatory potentials from ERG waveforms. After the ERG recording, yohimbine (2.1 mg/kg) was administered to the mouse to reverse the effects of xylazine and to prevent corneal ulcers (Guillet et al., 1988). The core body temperature was monitored and maintained using a rectal probe and heating pad, respectively.

4.3.8 Statistical analysis

Student's t-test was used for analyses of two groups. One-, two-, or three-way analysis of variance (ANOVA) with Student's-Newman-Keuls (SNK) multiple comparison test where applicable were used for analyses of more than two groups. The qRT-PCR data from the ZT0 and ZT24 time points were combined as one time point for statistical analyses. All analyses were performed using SigmaPlot 12 (Systat Software Inc., San Jose, CA). Error bars represent standard error of the mean (SEM), and statistical significance level was $p<0.05$.

4.4 Results

4.4.1 Retinal morphology and structure preserved in young and old *Clock*$^{-/-}$ and *Npas2*$^{-/-}$ mice.

We used light microscopy to check for morphological and structural defects in the retinas of *Clock*$^{-/-}$ and *Npas2*$^{-/-}$ mice. As observed in *Bmal1*$^{-/-}$ mice (Storch et al., 2007), there was no observable difference in the retinal cell morphology or number or retinal structure among the three genotypes in the 3-month-old group (ONL, one-way ANOVA, $F = 4.200$, $p = 0.072$; INL, one-way ANOVA, $F = 0.706$, $p = 0.531$; GCL, one-way ANOVA on ranks, $H = 2.000$, $p = 0.511$) or in the 10-month-old group (ONL, one-way ANOVA, $F = 0.380$, $p = 0.699$; INL, one-way ANOVA, $F = 0.067$, $p = 0.936$; GCL, one-way ANOVA, $F = 0.143$, $p = 0.870$) (Figure 4-1).

4.4.2 CLOCK regulates the expression of *Adcy1* and *Npas2* transcripts in the ganglion cell layer

We examined whether CLOCK played a role in regulating the *Adcy1* expression in the GCL or whether the control of Adcy1 expression was restricted to NPAS2. To assess the effect of CLOCK on the expression of the *Adcy1* mRNA, LCM was performed to assess the expression of *Adcy1* mRNA in the GCL isolated from retinas of *Clock*$^{-/-}$ mice. Interestingly, *Adcy1* transcript levels in GCL of *Clock*$^{-/-}$ mice are low and arrhythmic, very similar to what we have previously observed in the GCL of *Npas2*$^{-/-}$ (Figure 4-2A; one-way ANOVA on ranks, $H_3 = 1.059$, $p = 0.787$; two-way ANOVA, CLOCK deficiency x time interaction, $F_{3,47} = 6.091$, $p < 0.001$), suggesting that CLOCK may regulate the expression of *Npas2*. Indeed, *Npas2* transcripts in *Clock*$^{-/-}$ mice are barely detectable and are not rhythmic (Figure 4-2B; one-way ANOVA on ranks, $H_3 = 8.490$, $p = 0.075$; two-way ANOVA, CLOCK deficiency x time interaction, $F_{3,47} = 4.846$, $p = 0.003$). In contrast, *Clock* transcripts in *Npas2*$^{-/-}$ mice are not altered compared to WT mice (Figure 4-2C; two-way ANOVA, factor – time $F_{3,47} = 0.628$, $P = 0.601$; factor – genotype $F_{1,47} = 3$

Because CLOCK is expressed in the PRL in addition to GCL, we wanted to investigate whether CLOCK played a role in regulating the Adcy1 expression in the PRL in addition to GCL. To assess the expression of *Adcy1* mRNA in the PRL, we used LCM to isolate the PRL from retinas of *Clock*$^{-/-}$ mice. Intriguingly, in contrast to what we have previously observed in the PRL of *Npas2*$^{-/-}$ mice, *Adcy1* transcript levels in the PRL of *Clock*$^{-/-}$ mice are low and arrhythmic (Figure 4-2B;

one-way ANOVA, F = 2.269, p = 0.115; two-way ANOVA, factor – time F = 3.228, P = 0.033; factor – genotype F = 10.441, P = 0.003)

.484, P = 0.069 time x spatial frequency interaction, $F_{3,47}$ = 0.672, p = 0.574).

4.4.3 Selective Activation of the *Adcy1* promoter by NPAS2/BMAL1 but not CLOCK/BMAL1 in NG108-15 cells

We wanted to determine whether CLOCK co-localizes with NPAS2 in the subset of RGCs, because NPAS2 should theoretically be able to substitute for CLOCK in regulating *Adcy1* if co-expressed. We performed immunofluorescence analysis and determined that CLOCK is expressed in all of the retinal ganglion cells expressing NPAS2-β-galactosidase (Figure 4-3A). Interestingly, although CLOCK co-localizes with NPAS2 in the subset of RGCs expressing NPAS2 in mice, CLOCK deficiency alone was sufficient to abolish the rhythmic expression of *Adcy1* mRNA (Hwang et al., 2013).

We therefore examined the relative ability of NPAS2/BMAL1 and CLOCK/BMAL1 to activate *mAdcy1*-luciferase reporters in NG108-15 cells. As reported previously (Hwang et al., 2013), co-transfection with NPAS2/BMAL1 activated the *mAdcy1* 610-luc reporter construct (Figure 4-3B), which contains the circadian E-box. In contrast, co-transfection with CLOCK/BMAL1 failed to activate the *mAdcy1* 610-luc reporter (Figure 4-3B). The *mAdcy1* 280-luc reporter, which lacks the circadian E-box, showed little or no activation by NPAS2/BMAL1 or CLOCK/BMAL1 (Figure 4-3B).

4.4.4 CLOCK is required for the circadian rhythm of contrast sensitivity.

With the *Adcy1* and *Npas2* transcript levels being arrhythmic and low in the GCL of *Clock*$^{-/-}$ mice, we hypothesized that contrast sensitivity would be altered in *Clock*$^{-/-}$ mice and assessed the role of CLOCK in contrast sensitivity by performing OKT at mid-day and mid-night time points in LD. Remarkably, the contrast sensitivity rhythm was completely abolished in *Clock*$^{-/-}$ mice (Figure 4-4A; two-way ANOVA, time $F = 0.0077$, $P = 0.931$; spatial frequency $F = 74.644$, $P < 0.001$; time x spatial frequency interaction, $F = 1.560$, $p = 0.205$, n=4-8 mice), with day-time contrast sensitivity measurements reduced to night-time contrast sensitivity measurements. In contrast, the rhythm was only dampened in *Npas2*$^{-/-}$ mice (Figure 4-4B) as previously reported (Hwang et al., 2013).

Unlike WT or *Npas2*$^{-/-}$ mice, *Clock*$^{-/-}$ mice do not track at the 0.031 cycles/degree spatial frequency, which is the lowest spatial frequency tested in this study, even at 100% contrast. Additionally, the spatial frequency threshold in *Clock*$^{-/-}$ mice was slightly higher than that of WT mice (Figure 4-4C; t-test, $p < 0.001$). The mean spatial frequency threshold for Clock-/- mice was 0.407±0.001, and that of WT mice was 0.390±0.002 cycles/degree.

4.4.5 CLOCK regulates the circadian control of light-adapted ERG responses.

Light-adapted ERG b-wave amplitudes are regulated by the circadian clock (Storch et al., 2007; Cameron et al., 2008). Although the localization of β-

galactosidase-NPAS2 protein in *Npas2*$^{-/-}$ mice was virtually exclusive to RGCs (Hwang et al., 2013), a recent study reported NPAS2-like immunoactivity in the INL and cone photoreceptors in addition to the GCL (Liu et al., 2012). Therefore, we studied both *Clock*$^{-/-}$ and *Npas2*$^{-/-}$ mice to determine their roles in the circadian regulation of light-adapted ERG responses. In agreement with previous results (Cameron et al., 2008; Jackson et al., 2012), the light-adapted ERG showed circadian rhythm in WT mice (Figure 4-5A; two-way ANOVA, factor – CT $F = 114.768$, $P < 0.001$; factor – light adaptation $F = 4.520$, $P < 0.001$). However, in *Clock*$^{-/-}$ mice, the circadian rhythm of the light-adapted ERG was nearly abolished with both CT6 and CT18 levels close to the CT18 levels of WT mice (Figure 4-5B; three-way ANOVA, genoptype X CT interaction $F = 51.236$, $P < 0.001$; genoptype X light adaptation interaction $F = 5.320$, $P < 0.001$; time X light adaptation interaction $F = 0.494$, $P = 0.812$; genotype X CT X light adaptation interaction $F = 0.611$, $P = 0.721$). Moreover, the light-adapted ERG revealed that *Clock*$^{-/-}$ mice cannot adapt to light in the subjective day; b-wave amplitudes did not increase during the 20 min of background illumumination (one-way ANOVA, $F = 0.578$, $p = 0.744$). In *Npas2*$^{-/-}$ mice, light-adapted ERG b-wave amplitudes were rhythmic and not different from those of WT mice (Figure 4-5C; three-way ANOVA, factor – genotype $F = 0.365$, $P = 0.547$; factor – CT $F = 164.833$, $P < 0.001$; factor – light adaptation $F = 7.620$, $P < 0.001$).

4.4.6 CLOCK modulates dark-adapted ERG responses.

Dark-adapted ERG responses are not regulated as circadian rhythms under scotopic conditions and are mildly rhythmic at the brightest mesopic flash intensities (Cameron et al., 2008). Dark-adapted ERG intensity-response curves were measured at subjective day and subjective night time points on the first day of constant darkness. As previously observed (Jackson et al., 2012), there was no evidence of circadian regulation of dark-adapted ERGs in *Clock*$^{-/-}$, *Npas2*$^{-/-}$, or WT mice at the two time points tested. However, in *Clock*$^{-/-}$ mice compared to WT mice, b-wave amplitudes were substantially reduced across all flash intensities (Figure 4-6A; three-way ANOVA, genotype X flash intensity interaction $F = 2.504$, $P = 0.010$; genotype X CT interaction $F = 9.549$, $P = 0.002$; flash intensity X CT interaction $F = 0.512$, $P = 0.865$; genotype X CT X flash intensity interaction $F = 0.175$, $P = 0.996$), while a-wave amplitudes were moderately reduced at the brightest flash intensities (Figure 4-6A; three-way ANOVA, factor – genotype $F = 22.032$, $P < 0.001$; factor – flash intensity $F = 151.025$, $P < 0.001$; factor – CT $F = 1.369$, $P = 0.244$). In contrast, in *Npas2*$^{-/-}$ mice, dark-adapted ERG responses at subjective day and night time points were indistinguishable from those in WT mice with respect to b-wave amplitudes (Figure 4-6B; three-way ANOVA, factor – genotype $F = 2.179$, $P = 0.141$; factor – flash intensity $F = 116.472$, $P < 0.001$; factor – CT $F = 1.538$, $P = 0.216$) and a-wave amplitudes (Figure 4-6B; three-way ANOVA, factor – genotype $F = 3.616$, $P = 0.060$; factor – flash intensity $F = 240.579$, $P < 0.001$; factor – CT $F = 0.0430$, $P = 0.836$).

4.5 Discussion

In this study, we compared the roles of CLOCK and NPAS2 in regulating different visual functions. In *Clock*$^{-/-}$ mice, contrast sensitivity is arrhythmic with mid-day values completely reduced to mid-night values, while in *Npas2*$^{-/-}$ mice the rhythm is preserved but substantially reduced. Additionally, we determined that CLOCK but not NPAS2 regulates light-adapted ERG responses and modulates dark-adapted ERG responses.

We previously showed that NPAS2 is required for the rhythmic expression of *Adcy1* transcripts in the GCL and the *Adcy1*-mediated modulation of day-time contrast sensitivity (Hwang et al., 2013). Interestingly, CLOCK is also required for the *Adcy1* transcript rhythm to persist in the GCL. Although NPAS2 colocalizes with CLOCK in the GCL, NPAS2 cannot substitute for CLOCK in *Clock*$^{-/-}$ mice, and CLOCK deficiency alone is sufficient to abolish the rhythmic expression of *Adcy1* transcripts in the GCL. This result is consistent with another study demonstrating that the circadian expression of the *Aanat* gene in chicken retinal cells requires both NPAS2 and CLOCK (Haque et al., 2010). The ability of CLOCK or NPAS2 to activate gene transcription appears to be cell-type specific, as a previous study found that CLOCK/BMAL1 increased *mAdcy1* promoter activity in transiently transfected rat photoreceptor cells (Fukuhara et al., 2004), and similarly, CLOCK/BMAL1-dependent mechanism appears to regulate the *Adcy1* expression in mouse photoreceptor cells, as the *Adcy1* rhythm is abolished in the PRL of *Clock*$^{-/-}$ mice, which is in sharp contrast to the robust *Adcy1* rhythm in the PRL of *Npas2*$^{-/-}$ mice, previously been shown to be

indistinguishable from that of WT mice (Hwang et al., 2013). Another study showed that NPAS2/BMAL1, but not CLOCK/BMAL1, cotransfected with the promoter constructs of monoamine oxidase A (*Maoa*) gene in NG-108-15 cells activated transcription; but in COS-7 cells, both NPAS2/BMAL1 and CLOCK/BMAL1 activated the *Maoa* promoter (Hampp et al., 2008). Moreover, the role of NPAS2 as a core clock protein appears to be cell-type dependent as well, and NPAS2 appears to be an output of the clock in peripheral oscillators like the retina and liver. For example, NPAS2 plays an integral part of the clock machinery in the forebrain and in the suprachiasmatic nuclei (SCN), as the *Per2* expression is disrupted in the forebrain in *Npas2*$^{-/-}$ mice (Reick et al., 2001) and NPAS2 is able to substitute for CLOCK to preserve clock oscillations in the SCN (Debruyne et al., 2006; DeBruyne et al., 2007b). In the liver, NPAS2 is unable to substitute for CLOCK to maintain the integrity of the clock (DeBruyne et al., 2007a), but in contrast, NPAS2 is able to substitutes for CLOCK in regulating the expression of the gene for a circadian protein called Factor VII (Bertolucci et al., 2008), suggesting that NPAS2 is involved in gene regulation downstream of the clock, but not the clock itself. Similar cell-type specific and/or gene-specific mechanisms in RGCs could potentially explain why both CLOCK and NPAS2 are required for the rhythmic expression of *Adcy1*.

We found that CLOCK is necessary for the rhythmic expression of *Npas2* in the GCL, but the deficiency of NPAS2 did not alter the expression of *Clock* in the GCL. This result is consistent with the findings from chicken photoreceptor

primary culture experiments (Haque et al., 2010). In that study, knocking down *Clock* led to a significant reduction in the expression levels of *Clock* and *Npas2*, but when *Npas2* was knocked down, only the expression level of *Npas2* decreased substantially. CLOCK-dependent regulation of *Npas2* appears to be cell-type specific. In the murine SCN and liver, *Npas2* transcript rhythm is unaffected in *Clock*$^{-/-}$ mice (Debruyne et al., 2006; DeBruyne et al., 2007a), suggesting that the regulation of the *Npas2* expression is not CLOCK-dependent. Nevertheless, in the murine retinal ganglion cells, our data suggest that CLOCK hierarchically regulates NPAS2 and that NPAS2 but not CLOCK directly regulates the expression of the *Adcy1* gene.

The light-adapted ERG rhythm is nearly absent in *Clock*$^{-/-}$ mice but normal in *Npas2*$^{-/-}$ mice and, thus, the circadian regulation of light adapted ERG rhythm can virtually be accounted for by CLOCK/BMAL1 complex alone. Our finding is similar to previous findings in mice deficient in other clock proteins, BMAL1 and Cry1 and 2 (Storch et al., 2007; Cameron et al., 2008). In addition to clock proteins, deficiencies in melanopsin or retina tissue-specific tyrosine hydroxylase lead to an abolishment of the circadian light-adapted ERG rhythm (Barnard et al., 2006; Jackson et al., 2012). Intrinsically photosensitive RGCs (ipRGCs) may provide an excitatory input in a retrograde signaling pathway to dopaminergic amacrine cells (Zhang et al., 2008; Zhang et al., 2012) and, therefore, disruption of melanopsin-expressing RGCs may lead to circadian clock desynchrony and a consequent loss of the light-adapted ERG rhythm.

In addition to disruptions in light-adapted ERG responses, dark-adapted ERG responses are also substantially reduced in *Clock*$^{-/-}$ mice across all flash intensities tested. A reduction in dark-adapted ERG b-wave amplitude was observed previously in *Bmal1*$^{-/-}$ mice (Storch et al., 2007). However, because the ERG recording was performed at one flash intensity just above the lower threshold for mesopic condition (Toda et al., 1999) at which both cone and rod photoreceptors are activated, it was not clear whether a similar reduction would be observed at scotopic flash intensities at which only rods are activated. Our data show that even at the dimmest scotopic flash intensities, dark-adapted b-wave amplitudes were substantially reduced in *Clock*$^{-/-}$ mice. In addition to the reduction in b-wave amplitudes, we observed a noticeable reduction in a-wave amplitudes in dark-adapted *Clock*$^{-/-}$ mice at brighter mesopic flash intensities. A modest circadian rhythm in dark-adapted ERG a-wave amplitudes in mesopic conditions has been previously reported (Cameron et al., 2008), and it is possible that in *Clock*$^{-/-}$ mice, dark-adapted ERG a-wave amplitudes are arrhythmic and persistently low. Although a reduction in a-wave was not observed in the previous study with *Bmal1*$^{-/-}$ mice (Storch et al., 2007), the difference is likely due to the fact that the difference in a-wave amplitudes do not become noticeable until brighter flash intensities. In contrast to our results, dark-adapted ERG responses were largely normal in *Cry1*$^{-/-}$; *Cry2*$^{-/-}$ mice except for a mild increase in dark-adapted ERG b-wave amplitudes limited to mesopic flash intensities (Cameron et al., 2008). The reason for this discrepancy is not clear, but our

findings may be unique to a constitutively repressed clock. Nevertheless, our findings in *Clock*$^{-/-}$ mice suggest that the circadian clock is not dispensable in general retinal function. This conclusion is consistent with the findings of other studies that provide robust evidence that circadian clocks exert a strong influence on mammalian retinal function (Green and Besharse, 2004), which include the regulation of the outer segment disc shedding (LaVail and Ward, 1978; Grace et al., 1999), release of melatonin (Tosini and Menaker, 1996) and dopamine (Doyle et al., 2002), opsin transcript expression (Pierce et al., 1993; von Schantz et al., 1999), and cGMP-gated channel ligand affinity in cone photoreceptors (Ko et al., 2001, 2003).

In conclusion, we determined that CLOCK regulates NPAS2 expression in the GCL and that CLOCK is required for many essential physiologic functions in the retina. In the absence of CLOCK, the rhythms of contrast sensitivity and light-adapted ERG b-wave amplitudes are abolished. Surprisingly, CLOCK also plays a role in regulating the dark-adapted ERG, which previously was not thought to be regulated by circadian clocks. A deficiency of NPAS2 did not alter light-adapted or dark-adapted ERG responses from those of WT mice.

4.6 Figures and Figure Legends

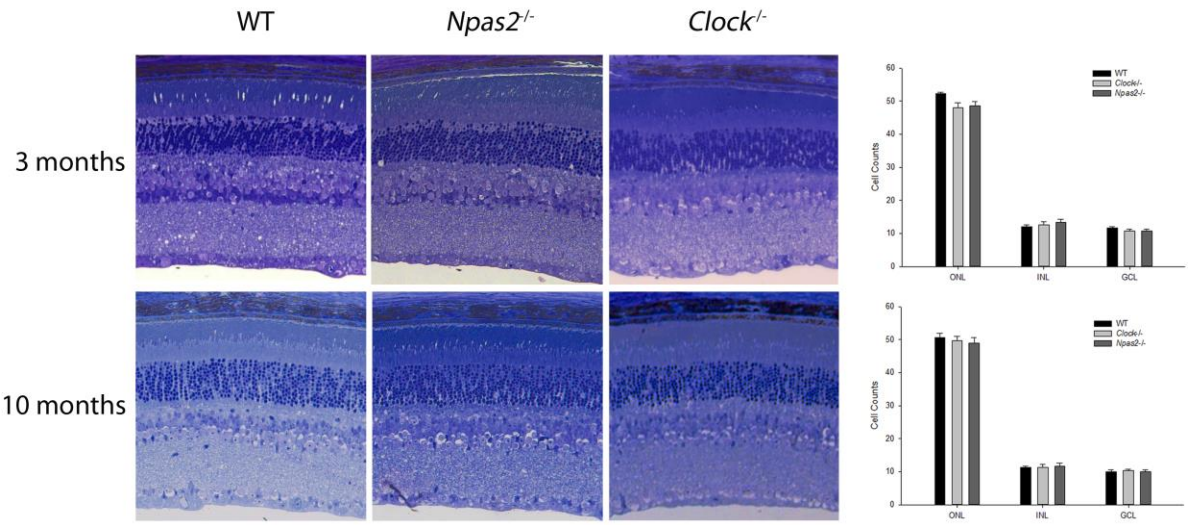

Figure 4-1 Toluidine blue staining of retinal sections.

Toluidine blue staining of retinal sections of 3-month-old (top row) and 10-month-old (bottom row) WT, *Npas2*$^{-/-}$, and *Clock*$^{-/-}$ mice. There is no difference in the retinal cell morphology, structure, or number among the three genotypes in the 3-month-old (ONL, one-way ANOVA, $F = 4.200$, $p = 0.072$; INL, one-way ANOVA, $F = 0.706$, $p = 0.531$; GCL, one-way ANOVA on ranks, $H = 2.000$, $p = 0.511$) and 10-month-old groups (ONL, one-way ANOVA, $F = 0.380$, $p = 0.699$; INL, one-way ANOVA, $F = 0.067$, $p = 0.936$; GCL, one-way ANOVA, $F = 0.143$, $p = 0.870$). Cell counts are reported as mean ± SEM, $n = 3$ for each group.

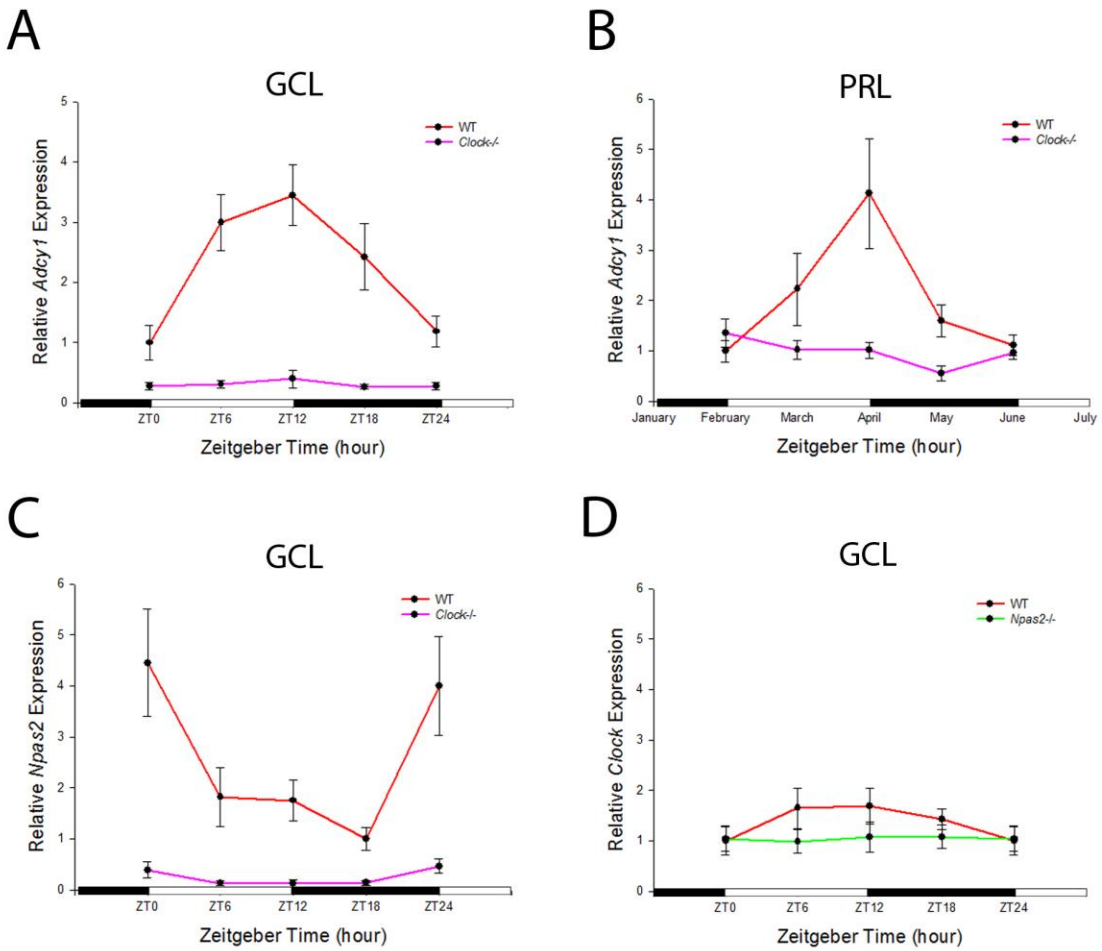

Figure 4-2 CLOCK is required for the rhythmic expression of *Adcy1* and *Npas2*.

Figure 4-2 CLOCK is required for the rhythmic expression of *Adcy1* and *Npas2*.

(A) Like in the GCL of *Npas2*$^{-/-}$ mice, the *Adcy1* transcript expression is arrhythmic and stayed at low levels in the GCL of *Clock*$^{-/-}$ mice (pink, one-way ANOVA on ranks, H = 1.059, p = 0.787; two-way ANOVA, CLOCK deficiency x time interaction, F = 6.091, p <0.001). (B) In the PRL of *Clock*$^{-/-}$ mice, the *Adcy1* transcript expression is arrhythmic and stayed at low levels (pink, one-way ANOVA, F = 2.269, p = 0.115; two-way ANOVA, factor – time F = 3.228, P = 0.033; factor – genotype F = 10.441, P = 0.003). (C) The *Npas2* transcript expression in the GCL of *Clock*$^{-/-}$ mice (pink) is arrhythmic and barely detectable (one-way ANOVA on ranks, H = 8.490, p = 0.075; two-way ANOVA, CLOCK deficiency x time interaction, F = 4.846, p = 0.003). (D) *Clock* transcript expressions in WT and *Npas2*$^{-/-}$ mice (green) are not significantly different (two-way ANOVA, factor – time F = 0.628, P = 0.601; factor – genotype F = 3.484, P = 0.069; time x spatial frequency interaction, F = 0.672, p = 0.574). Data expressed as mean ± SEM, n = 5-6 for each time point.

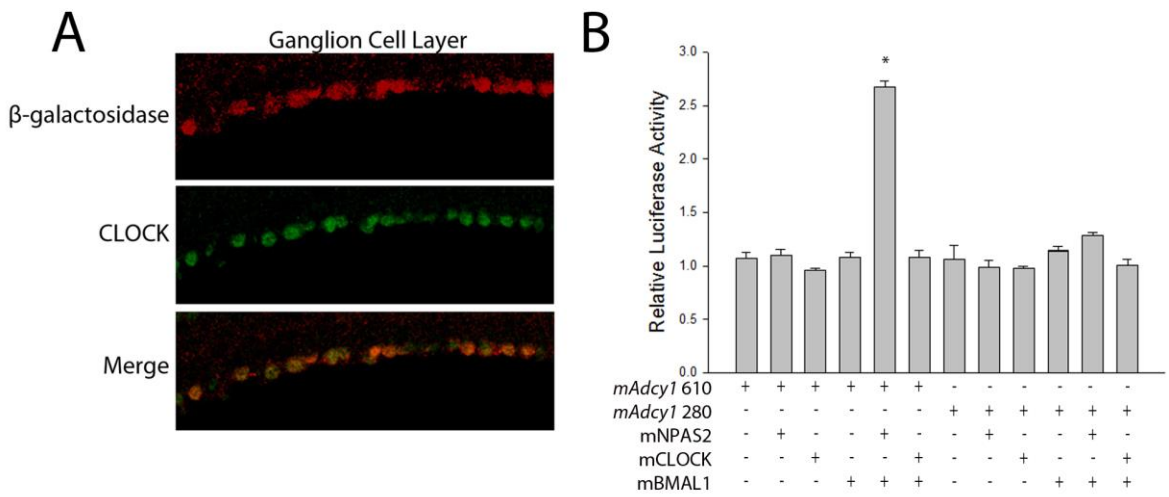

Figure 4-3 Selective regulation of *mAdcy1* promoter by NPAS2/BMAL1 but not by CLOCK/BMAL1.

(A) Immunofluorescence staining shows NPAS2-β-galactosidase (red) colocalizing with CLOCK (green) in the GCL. (B) Luciferase reporter plasmids containing either a 610 bp m*Adcy1* promoter (m*Adcy1* 610), which contains a circadian E-box, or a circadian E-box-lacking 280 bp m*Adcy1* promoter (m*Adcy1* 280) was used for the luciferase activity assays. Only NPAS2/BMAL1 activated m*Adcy1* promoter (*$p < 0.001$ vs. empty plasmid transfected control group), and the deletion of the circadian E-box region substantially reduced the luminescence level. Presence (+) or absence (-) of the reporter and expression plasmids are shown. All values are the mean ± SE (n = 3) and representative of at least three independent experiments.

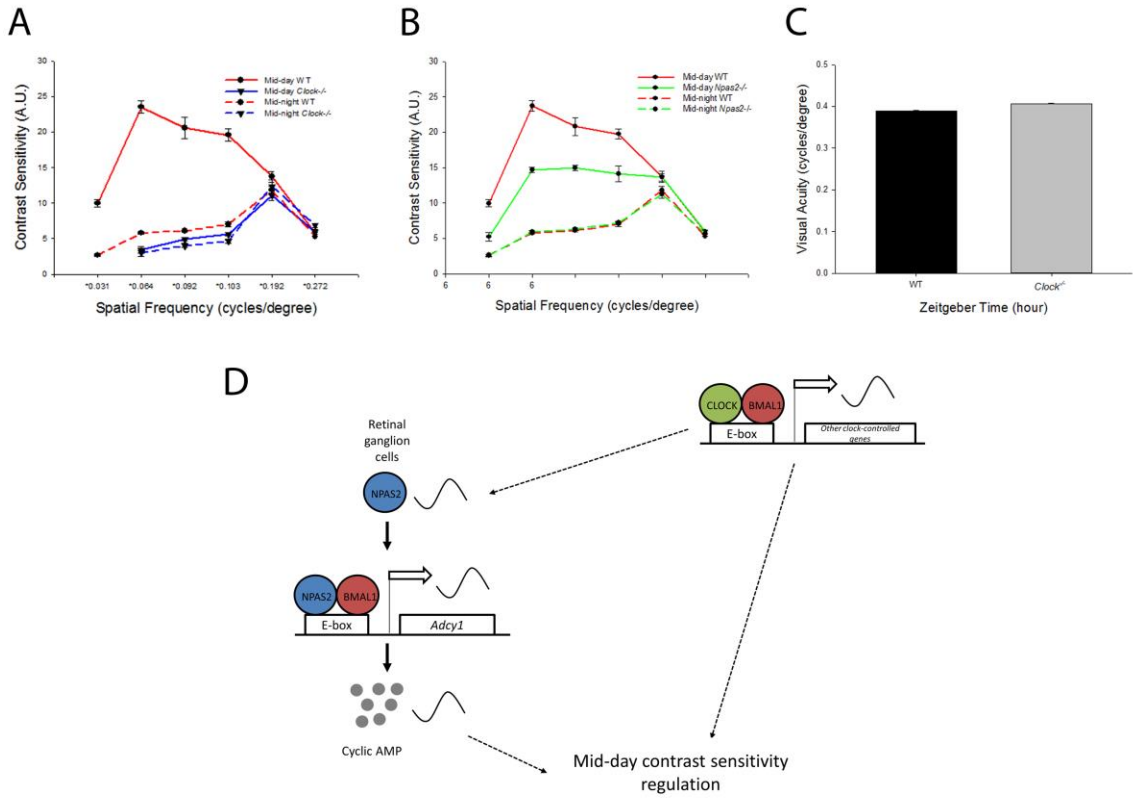

Figure 4-4 Contrast sensitivity of WT, *Npas2*$^{-/-}$, and *Clock*$^{-/-}$ mice.

Figure 4-4 Contrast sensitivity of WT, *Npas2*$^{-/-}$, and *Clock*$^{-/-}$ mice.

(A) In LD, contrast sensitivity is arrhythmic in *Clock*$^{-/-}$ mice (two-way ANOVA, factor – time F = 0.0077, P = 0.931; factor – spatial frequency F = 74.644, P < 0.001; time x spatial frequency interaction, F = 1.560, p = 0.205, n=4-8 mice). While mid-night contrast sensitivity remains low, mid-day contrast sensitivity level is reduced completely to mid-night contrast sensitivity level. (B) Contrast sensitivity is rhythmic but reduced in *Npas2*$^{-/-}$ mice (two-way ANOVA, NPAS2 deficiency x spatial frequency interaction, F = 19.276, p < 0.001; *p < 0.001, Student-Newman-Keuls *post hoc* test, n=4-6 mice). There is a significant reduction in mid-day contrast sensitivity at multiple spatial frequencies in *Npas2*$^{-/-}$ mice (*p < 0.001, Student-Newman-Keuls post hoc test, n=4-6 mice) while the mid-night contrast sensitivity was preserved. (C) Spatial frequency thresholds were slightly higher in *Clock*$^{-/-}$ mice than those in WT mice (n = 6-8 mice). (D) A model for the contrast sensitivity regulation. Data expressed as mean ± SEM.

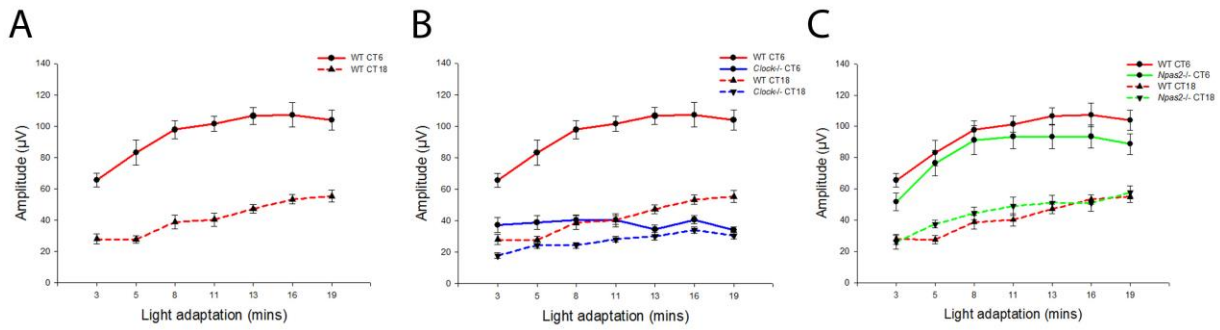

Figure 4-5 CLOCK regulates the circadian rhythm of photopic b-wave amplitude.

(A) Light-adapted ERG b-wave amplitudes are rhythmic and increase over time in WT mice (two-way ANOVA, factor – CT $F_{1,63} = 114.768$, $P < 0.001$; factor – light adaptation $F_{6,63} = 4.520$, $P < 0.001$). (B) Light-adapted ERG b-waves are low in *Clock*$^{-/-}$ mice (blue), which do not adapt to light in CT6 (solid blue, one-way ANOVA, $F_{6.34} = 0.578$, $p = 0.744$), but modestly adapt to light in CT18 (dotted blue, one-way ANOVA, $F_{6,34} = 0.4.718$, $p = 0.002$). Three-way ANOVA revealed significant interactions between genoptype and CT as well as between genotype and light adaptation (three-way ANOVA, genoptype X CT interaction $F_{1,119} = 51.236$, $P < 0.001$; genoptype X light adaptation interaction $F_{6,119} = 5.320$, $P < 0.001$; time X light adaptation interaction $F_{6,119} = 0.494$, $P = 0.812$; genotype X CT X light adaptation interaction $F_{6,119} = 0.611$, $P = 0.721$) (C) B-wave amplitudes in *Npas2*$^{-/-}$ mice (green) were not different from those of WT mice (red) in DD2 (three-way ANOVA, factor – genotype $F_{1,101} = 0.365$, $P = 0.547$; factor – CT $F_{1,101} = 164.833$, $P < 0.001$; factor – light adaptation $F_{1,101} = 7.620$, $P < 0.001$). N=4-7 mice for each group. Data expressed as mean ± SEM.

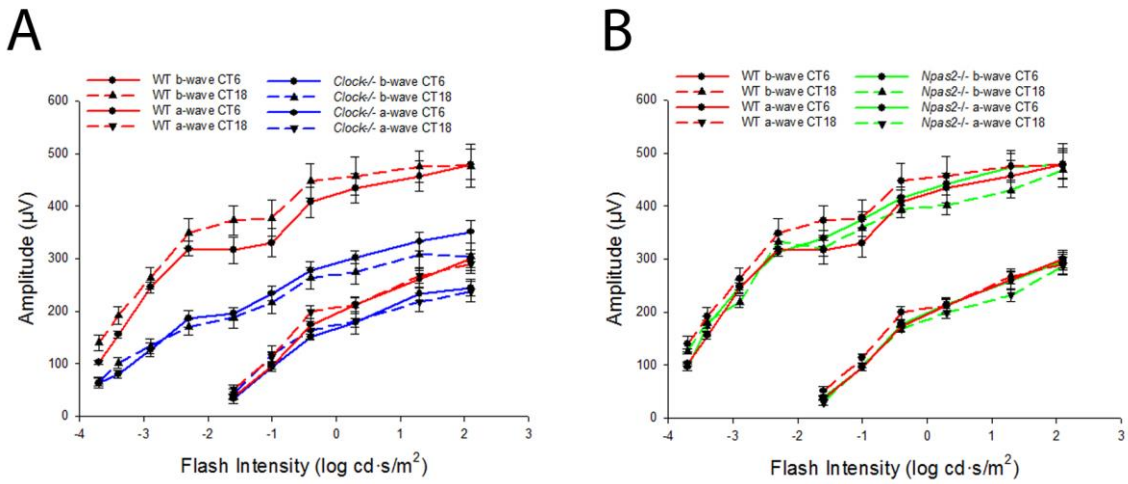

Figure 4-6 CLOCK modulates non-circadian dark-adapted ERG responses. (A) In *Clock*$^{-/-}$ mice, both dark-adapted ERG b-wave (three-way ANOVA, genotype X flash intensity interaction $F_{9,210} = 2.504$, $P = 0.010$; genotype X CT interaction $F_{1,210} = 9.549$, $P = 0.002$; flash intensity X CT interaction $F_{9,210} = 0.512$, $P = 0.865$; genotype X CT X flash intensity interaction $F_{9,210} = 0.175$, $P = 0.996$) and a-wave amplitudes were significantly reduced compared to WT mice (three-way ANOVA, factor – genotype $F_{1,124} = 22.032$, $P < 0.001$; factor – flash intensity $F_{5,124} = 151.025$, $P < 0.001$; factor – CT $F_{1,101} = 1.369$, $P = 0.244$). (B) In *Npas2*$^{-/-}$ mice, dark-adapted ERG b-wave (three-way ANOVA, factor – genotype $F_{1,201} = 2.179$, $P = 0.141$; factor – flash intensity $F_{9,201} = 116.472$, $P < 0.001$; factor – CT $F_{1,201} = 1.538$, $P = 0.216$) and a-wave amplitudes (three-way ANOVA, factor – genotype $F_{1,119} = 3.616$, $P = 0.060$; factor – flash intensity $F_{5,119} = 240.579$, $P < 0.001$; factor – CT $F_{1,119} = 0.0430$, $P = 0.836$) were not different from those of WT mice. Data expressed as mean ± SEM.

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Chapter 5: Summary and Future Directions

5.1 Summary

5.1.1 Retinal clocks regulate contrast sensitivity.

I determined that retinal clocks regulate the circadian rhythm of contrast sensitivity. In Chapter 3, I elucidated a dopamine-NPAS2-adenylyl pathway in a subset of retinal ganglion cells that modulates day-time contrast sensitivity. In Chapter 4, I showed that CLOCK and NPAS2 are co-expressed in RGCs and that CLOCK exhibits its effects partially by regulating the expression of *Npas2* transcripts in the GCL. The deficiency of CLOCK alone is sufficient to completely ablate the contrast sensitivity rhythm, implicating additional clock-controlled genes (in addition to *Adcy1*) in the regulation of contrast sensitivity.

The role of a dopamine-NPAS2-adenylyl cyclase pathway in contrast sensitivity

In certain parts of the mammalian brain, NPAS2 has an overlapping role as CLOCK and exhibits oscillatory functions. However, its role in the mammalian retinal clock has never been explored. The initial goal of my project was to determine the expression pattern of NPAS2 in the retina and its role in regulating clock-controlled genes. Using LCM, immunohistochemistry, and enzymatic assays, I determined that NPAS2 is expressed in a subset of RGCs. With that finding, I wanted to find out whether NPAS2 plays a role in regulating a clock-controlled gene. Among clock-controlled genes, I chose *Adcy1*, because it had been well characterized in the literature and studied in our laboratory for several years. Retinal *Adcy1* transcripts were known to be circadian and expressed in all three retinal layers, but the *Adcy1* transcript rhythm in the GCL has never been

analyzed. Therefore, I utilized LCM to dissect each retinal layer to perform qRT-PCR analyses on the *Adcy1* transcript expression across five time points.

In WT mice, I found the *Adcy1* transcript expression to be robustly rhythmic in the GCL and PRL, but not in the INL. In contrast, I discovered that the *Adcy1* transcript expression in the GCL of *Npas2*$^{-/-}$ mice is arrhythmic and low, but in the PRL of *Npas2*$^{-/-}$ mice, the *Adcy1* transcript rhythm persists similar to that in WT mice. With these results, I wanted to determine whether the NPAS2/BMAL1 could directly activate the *Adcy1* promoter using a luciferase reporter assay. Indeed, when the luciferase vector is cotransfected with both NPAS2 and BMAL1, there is a significant induction in the luciferase signal, showing that the NPAS2/BMAL1 heterodimer directly activates the Adcy1 promoter.

Chad Jackson, a previous graduate student in the laboratory, had determined that *Adcy1*$^{-/-}$ mice have reduced contrast sensitivity, and because RGCs are well-known detectors of contrast, I wanted to explore whether NPAS2 is involved in the contrast sensitivity regulation. Because *Adcy1* is a clock-controlled gene, I first decided to test whether contrast sensitivity itself is circadian. I discovered that there is a robust rhythm in contrast sensitivity in WT mice, which persists after two days in constant darkness. Upon testing *Npas2*$^{-/-}$ and *Adcy1* $^{-/-}$ mice, I found that their day-time contrast sensitivity functions are similarly reduced while night-time contrast sensitivity remains unchanged, suggesting that Adcy1 transcript rhythm in the GCL modulates the contrast sensitivity rhythm.

In order to confirm my hypothesis, I performed similar experiments on *Drd4*$^{-/-}$ mice. These mice have been previously characterized with reduced contrast sensitivity and arrhythmic retinal *Adcy1* transcript, but the *Adcy1* expression in the GCL of *Drd4*$^{-/-}$ mice had never been analyzed. I used LCM to determine the *Adcy1* transcript expression in the GCL of *Drd4*$^{-/-}$ mice and discovered that it is similarly arrhythmic and low as that in the GCL of *Npas2*$^{-/-}$ mice. I then measured contrast sensitivity in these mice and determined that day-time contrast sensitivity is similarly reduced in *Drd4*$^{-/-}$ mice as those in *Npas2*$^{-/-}$ and *Adcy1*$^{-/-}$ mice, while night-time contrast sensitivity remained similar to that of WT mice. Using LCM, I found out that *Npas2* transcript expression is arrhythmic in the GCL of *Drd4*$^{-/-}$ mice, suggesting that D4Rs are necessary for the *Npas2* transcript rhythm.

The role of CLOCK in contrast sensitivity

Because CLOCK is expressed in the GCL (Storch et al., 2007), I wanted to determine why CLOCK could not substitute for NPAS2 to regulate the expression of *Adcy1* transcripts. Using immunohistochemistry, I discovered that CLOCK is coexpressed in NPAS2-expressing retinal ganglion cells. I then performed LCM to determine the transcript expression level of *Adcy1* transcripts in *Clock*$^{-/-}$ mice and found out that the *Adcy1* transcript expression is arrhythmic and low, as in *Npas2*$^{-/-}$ mice.

I hypothesized that CLOCK could be upstream of NPAS2 and has a role in regulating the expression of *Npas2* transcripts. I tested this hypothesis by performing LCM and gene expression analyses for *Npas2* and *Clock* transcripts in WT and *Clock*$^{-/-}$ mice and WT and *Npas2*$^{-/-}$ mice, respectively. Indeed, qPCR analyses showed that the *Npas2* transcript rhythm in *Clock*$^{-/-}$ mice is completely abolished, while *Clock* transcripts are normal in *Npas2*$^{-/-}$ mice. With these results, I then hypothesized that the *Adcy1* promoter would confer specificity to the NPAS2/BMAL1 heterodimer over the CLOCK/BMAL1 heterodimer and repeated the luciferase reporter assay described above. Remarkably, I discovered that the luciferase level was induced only when *Adcy1*-luc was cotransfected with expression vectors for NPAS2/BMAL1, but not with those for CLOCK/BMAL1.

If CLOCK regulates the expression of *Npas2* in the GCL, and if NPAS2 in the GCL modulates day-time contrast sensitivity, then it would make sense to think that *Clock*$^{-/-}$ mice would also exhibit deficiencies in day-time contrast sensitivity as well. Therefore, I performed optokinetic tracking test on *Clock*$^{-/-}$ mice, and impressively, I saw that the contrast sensitivity rhythm is completely abolished in *Clock*$^{-/-}$ mice. Both day and night-time levels of contrast sensitivity are low and indistinguishable.

These results led to my conclusion that CLOCK regulates the circadian rhythm of contrast sensitivity in part by controlling the rhythmic expression of *Npas2* in the GCL.

5.1.2 Retinal clocks modulate ERG responses.

In addition to contrast sensitivity, I also explored the role of the retinal clock in ERG responses. BMAL1 is required for the circadian rhythm of light-adapted ERG responses, but it was not known whether CLOCK or NPAS2 mediates this effect. Therefore, I conducted light-adapted ERG tests in WT, *Clock*$^{-/-}$, and *Npas2*$^{-/-}$ mice after two days in constant darkness across a 20-minute light-adaptation period. Additionally, I assessed dark-adapted ERG responses in these animals across multiple scotopic and mesopic flash intensities.

The light-adapted ERG rhythm is robustly rhythmic in WT mice. However, in *Clock*$^{-/-}$mice, I determined that this rhythm is almost completely absent. Furthermore, the deficiency of CLOCK significantly disrupts the retina from properly adapting to light at day and night time-points, suggesting that CLOCK plays a critical role in mediating retinal light adaptation and light responses. In contrast to CLOCK, I discovered that the deficiency of NPAS2 does not affect the light-adapted ERG rhythm, suggesting that the CLOCK/BMAL1 heterodimer alone is involved in the regulation of the light-adapted ERG rhythm.

It was previously thought that the role of retinal clock is limited to the regulation of cone pathway-mediated ERG responses. Therefore, although dark-adapted ERG b-wave amplitudes at mesopic flash intensities are reduced in *Bmal1*$^{-/-}$ mice, it was assumed that b-wave amplitudes at scotopic flash intensities would not be

affected. However, my results show that this is not the case. I discovered that the deficiency of CLOCK substantially reduces dark-adapted ERG b-wave amplitudes not only at mesopic flash intensities, but also across all scotopic flash intensities tested. In addition, I noticed a noticeable reduction in a-wave amplitudes at the brightest mesopic flash intensities. In contrast, in *Npas2*$^{-/-}$ mice, all aspects of dark-adapted ERG responses are normal.

These results show that CLOCK heterodimerizes with BMAL1 to regulate the light-adapted ERG rhythm and modulate dark-adapted ERG responses at both scotopic and mesopic flash intensities. The functional role of NPAS2 outside of the GCL appears to be limited.

5.2 Future Directions

The results from my dissertation research have elucidated many important mechanistic details about the differential roles of NPAS2 and CLOCK in retinal clocks and in regulating different dimensions of vision. My findings have led to several additional scientific questions, and there are other questions that remain unanswered. I plan to address some of these questions in the future using the experimental details below.

5.2.1 The role of D4Rs in the regulation of transcript rhythms of clock genes in the PRL

As discussed in Chapter 1, the localization of a functional clock in the mammalian PRL has been elusive. Ruan and coworkers postulated in their earlier study that all six clock genes have to be expressed for a cell to have a functional clock (Ruan et al., 2006). In that study, they showed using single-cell qPCR that photoreceptors (mainly rod photoreceptors with a limited number of cone photoreceptors) express at most three clock genes, but do not express all six core clock genes. However, the authors stated that one of the limitations of single-cell qPCR is the relatively low sensitivity despite high specificity (Ruan et al., 2006). Therefore, it is possible that all six core clock genes are expressed in photoreceptors, but at lower levels than in other cell types, making their simultaneous detection in one cell difficult. Indeed, a recent study showed using LCM that all six clock genes are expressed in a circadian manner in the PRL of WT mice (Dkhissi-Benyahya et al., 2013). Furthermore, the study demonstrated that the phase of the rhythm observed in the PRL is different from that in the inner retina, suggesting that there could be a clock in the PRL and that its regulation may be different from that of the inner retina.

Dopamine may be an entrainment cue for not only the circadian physiology in photoreceptors, but also the clock gene rhythms in the PRL through D4Rs. Ruan and coworkers showed that dopamine regulates the phase of *Per2*-luc rhythms in retinal explants (Ruan et al., 2008). Although this action appears to be mediated through D1Rs in the study, *Per2*-luc rhythms in retinal explants are primarily driven by the cells in the inner retina (Ruan et al., 2008). Therefore, their results

are representative of an inner retinal clock and do not exclude the possibility of another dopamine receptor subtype (i.e. D4Rs) regulating clocks in the PRL. The PRL of WT and *Drd4*$^{-/-}$ mice can be collected using LCM across 5 different time points in LD and DD2, and gene expression analyses can be performed for transcript rhythms of *Clock, Npas2, Bmal1, Per1, Per2, Cry1*, and *Cry2* in the PRL. I anticipate that clock gene expression in the PRL of WT and *Drd4*$^{-/-}$ mice will be significantly different.

Additionally, I showed in Chapter 4 that the deficiency in CLOCK does not reduce the retinal *Drd4* transcript rhythm, which is primarily driven by *Drd4* transcripts in the PRL (Cohen et al., 1992), in either LD or DD2. These results show that the rhythmic expression of *Drd4* transcripts in the PRL occurs independently of CLOCK and suggest that another clock protein may substitute for CLOCK to preserve the *Drd4* transcript rhythm. NPAS2 could substitute for CLOCK in the PRL, as a recent study found an NPAS2-like immunoactivity in cone photoreceptors (Liu et al., 2012). For this investigation, *Npas2*$^{-/-}$ mice can be crossed with *Clock*$^{-/-}$ mice to generate *Npas2*$^{-/-}$x *Clock*$^{-/-}$ double knockout mice, and gene expression analysis can be performed for *Drd4* transcripts in the whole retina and clock gene expression in the PRL in LD and DD2. I anticipate that *Drd4* transcript rhythm as well as clock gene transcript rhythms will be severely disrupted in these mice.

5.2.2 The role of NPAS2 in the retinal dopamine level and metabolism

β-galactosidase-NPAS2 expression in *Npas2*$^{-/-}$ mice is limited to a subset of RGCs in the GCL. However, it is possible that the expression pattern of NPAS2 is different in WT mice compared to *Npas2*$^{-/-}$ mice, and a recent study found an NPAS2-like immunoactivity in the INL and cone photoreceptor cells, as well as in RGCs (Liu et al., 2012). Therefore, NPAS2 could be expressed and have a functional role in other retinal layers in WT mice. Indeed, I have preliminary data to show that *Th* transcript expression in the whole retina is rhythmic in WT mice and that the rhythm is significantly dampened in *Npas2*$^{-/-}$ mice. Moreover, the steady-state levels of dopamine measured using HPLC in *Npas2*$^{-/-}$ mice at ZT6 and ZT18 time points were significantly lower than those in WT mice. There are at least two explanations for these results. One is that NPAS2 expressed in ipRGCs could play a role in modulating the *Th* transcript rhythm in dopaminergic amacrine cells. Alternatively, NPAS2 expressed in dopaminergic amacrine cells or other cell types connected to the dopaminergic amacrine cells in the INL could directly mediate this effect. First, the retinal *Th* transcript expression and steady-state level of retinal DA could be assessed in *Opn4*$^{aDTA/aDTA}$ mice, which express diphtheria toxin A under the control of the melanopsin promoter and are thought to be deficient of ipRGCs. If these levels are not affected in *Opn4* $^{aDTA/aDTA}$ mice, then it is not likely that NPAS2 would mediate its effect through ipRGCs. Second, the INL in WT retinas can be dissected using LCM and assessed for the *Npas2* transcript expression. If *Npas2* transcripts are rhythmically expressed in the INL, but *Th* transcript or DA levels are not affected in *Opn4* $^{aDTA/aDTA}$ mice, then it would suggest that NPAS2 in the INL would mediate these effects.

5.2.3 The role of a dysfunctional clock in retinal diseases

Recently the clock has been implicated in angiogenesis [reviewed in (Jensen and Cao, 2013)]. The hypoxia-induced factor-1 protein (HIF-1α) is another heterodimeric partner of BMAL1 (Hogenesch et al., 1998), and in hypoxic conditions, HIF1α induces the expression of vascular endothelial growth factor (VEGF) (Forsythe et al., 1996), which then induces angiogenesis. Two clock genes, *Per2* and *Cry1*, were shown to inhibit the hypoxia-induced expression of VEGF in a tumor cell line (Koyanagi et al., 2003). Furthermore a mutation in the *Per2* gene disrupts endothelial progenitor cell function and increases vascular senescence (Wang et al., 2008). These results suggest that the clock could be critical in regulating neovascularization in hypoxic conditions and also in vascular repair and normal angiogenesis. Clinically, these results suggest that the clock could be involved in various retinal diseases in which hypoxia-induced neovascularization is involved, such as retinal tumors (e.g., uveal melanoma, retinoblastoma), age-related macular degeneration (AMD), diabetic retinopathy (DR), and retinopathy of prematurity (ROP), in which normal angiogenesis is also impaired.

A cell culture study can be performed using a vascular endothelial cell line. The clocks in these cells would first need to be synchronized through a “serum shock,” in which the cells are exposed to high level of serum for a short period of time. The clock synchrony could be verified by a rhythmic clock gene expression

(e.g., Per2) over a 24-hour time course. Next, the clock could be disrupted by knocking down *Per1/Per2* or *Cry1/Cry2*, and compare the levels of *Hif-1α* transcripts and proteins and of VEGF transcripts and proteins between the control and experimental groups. Additionally, there are several mouse models readily available to study neovascularization for retinal tumors (e.g., uveal melanoma) and AMD. The progression of the retinal tumor and/or AMD-like retinal changes can be assessed in WT mice and in mice with disrupted clocks (e.g. *Per1*$^{-/-}$; *Per2*$^{-/-}$ mice, *Cry1*$^{-/-}$; *Cry2*$^{-/-}$, *Bmal1*$^{-/-}$ mice). If this hypothesis is correct, then cells with disrupted clocks would express substantially higher levels of HIF-1α and VEGF compared to control cells and mice with disrupted clock would exhibit more severe forms of tumor (e.g., size, growth rate, metastasis) and neovascularization compared to control mice.

5.2.4 Visual defects in night-shift workers

My research shows that contrast sensitivity has a circadian rhythm, and a disruption in retinal clocks leads to a diminished or no rhythm. A translational research study could be conducted to determine whether humans with disrupted clock would exhibit deficits in contrast sensitivity. Night-shift workers are thought to have disrupted circadian rhythms due to irregular exposure to light [reviewed in (Kuhn, 2001)], and it is possible that their contrast sensitivity is also disrupted as a result. Two groups of people can be enrolled to measure their contrast sensitivity: one group with regular sleep-wake cycles and another group with irregular sleep-wake cycles like night-shift workers who are exposed to long

durations of light at night time. The state of their clocks could be monitored through salivary levels of melatonin, which is rhythmic in people with non-disrupted clocks (McIntyre et al., 1987), and people in either group could be excluded accordingly. For example night-shift workers with no apparent defect in salivary melatonin rhythms (i.e., normal rhythms) could be excluded, as would the people with regular sleep-wake cycles who exhibit abnormal salivary melatonin levels. I anticipate that the group with irregular sleep-wake cycles would exhibit deficiencies in contrast sensitivity.

5.3 References

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Appendix

List of mouse qRT-PCR primer sequences

The primer sequences (listed below) for qRT-PCR reactions were designed using Primer3 and ordered from Eurofins MWG Operon.

Adcy1 forward 5'- gggacgaattcaggtgactg -3'

Adcy1 reverse 5'- gcctctcccataaggacaca -3'

Npas2 forward 5'- ccctctgcagtgaaggaaaa -3'

Npas2 reverse 5'- tgttctcagccatcagcttg -3'

Clock forward 5'- tgaagccaaaatccaccact -3'

Clock reverse 5'- accagggaaccttgtcctct -3'

Drd4 forward 5'- gcaaggcaatgagagtcctg -3'

Drd4 reverse 5'- tggtgtagatgatggggttg -3'

18S forward 5'- cccgaagcgtttactttgaa -3'

18S reverse 5'- ccctcttaatcatggcctca -3'