AN AUTONOMOUS CIRCADIAN CLOCK IN THE INNER MOUSE RETINA REGULATED BY DOPAMINE AND GABA

By

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CHAPTER I

INTRODUCTION

Circadian rhythms are recurring changes in biological processes that repeat on a roughly 24 hr basis and thus potentially provide a selective advantage to organisms by allowing them to anticipate temporal changes in their environment. Circadian rhythms are driven by endogenous circadian clocks, which are self-sustaining molecular/cellular oscillators. Environmental cues, of which light is most important and powerful, synchronize the circadian clock to local solar day-night cycle and adjust the period of the internal clock to exactly 24 hrs. This phenomenon is called entrainment.

The change in the levels of illumination between day and night is enormous (more than 6-log unit change). As a sensory organ for vision and a self-sustained clock tissue, the retina anticipates the daily changes of environmental light and exhibits a circadian sensitivity rhythm compensating for the external day/night changes, to protect the sensitive eyes against possible damage by bright day light, and to enable visual sensitivity during dim night light. Besides driving the circadian rhythmicity of visual sensitivity, the retinal clock influences many other aspects of retinal physiology and function, including neurotransmitter synthesis and release (Tosini and Menaker, 1996; Doyle et al., 2002b; Doyle et al., 2002a); cellular events such as rod disk shedding (LaVail, 1976; Grace et al., 1996), intracellular signaling pathways (Gabriel et al., 2001), and gene expression

(Storch et al., 2007); as well as trophic processes such as photoreceptor survival (Ogilvie and Speck, 2002).

Whereas the existence of a self-sustained clock in photoreceptor is well established in amphibian and avian retinas based on molecular and physiological data (Cahill and Besharse, 1993; Pierce et al., 1993; Hayasaka et al., 2002; Ivanova and Iuvone, 2003a), the cellular location of the retinal clock in the mammalian retina remains to be elucidated at this point. *In situ* hybridization studies of the regional distribution of core circadian clock genes in the mammalian retina found them to be expressed predominantly in the inner nuclear and ganglion cell layers (Gekakis et al., 1998; Miyamoto and Sancar, 1998; Namihira et al., 2001; Thompson et al., 2003; Witkovsky et al., 2003), suggesting the potential for an alternate circadian organization.

Retinal physiology and function depend on a complement of chemical neurotransmitters, and previous studies have indicated that dopamine, melatonin, and γ-aminobutyric acid (GABA) are potent modulators of retinal physiology (Green and Besharse, 2004). Dopamine and melatonin mediate multiple light- and dark-adaptive responses in the retina, respectively. GABA suppresses dopamine synthesis and release (Marshburn and Iuvone, 1981; Puopolo et al., 2001), and increases melatonin production (Jaliffa et al., 1999). However, it is unclear whether these three neurotransmitters are involved in the regulation of the mammalian retinal circadian clock mechanism.

In this chapter, first, circadian rhythms in mammals and organization of the mammalian circadian system are defined. Next, cellular structure of the mammalian

retina and influence of the mammalian retinal clock are presented. Then, molecular nature of the master clock in the suprachiasmatic nucleus (SCN) and the retinal clock is illustrated. After that, locations of the potential circadian clock in non-mammalian vertebrate retinas and mammalian retinas are compared. Then, the roles of several retinal neurotransmitters in regulating retinal physiology are described. Following that, challenges to study the retinal circadian clock is discussed. Finally, objectives of this dissertation study are presented.

The Mammalian Circadian Clock

Circadian Rhythms in Mammals

In mammals, a broad spectrum of physiological parameters fluctuate in an orderly fashion with a period of approximate 24 hours, including the sleep–wake cycle, locomotor activity, hormone secretion, heartbeat, blood pressure, renal plasma flow, body temperature, pain tolerance, and even sensory perception. These roughly-24-hour cycles are known as circadian rhythms. The term “circadian” comes from the Latin *circa*, “around”, and *diem* or *dies*, “day”, meaning literally “about a day”. A circadian rhythm should satisfy three fundamental criteria: (1) The rhythm persists in constant conditions (e.g. constant darkness) with a period of ca. 24 hours; (2) The rhythm has similar periods over the physiological temperature range (i.e., it is temperature-compensated); (3) The rhythm can be reset by exposure to an external stimulus.

Organization of the Mammalian Circadian System

Circadian rhythms are controlled by an internal circadian clock. A circadian clock system generally consists of three parts: an input pathway that links the internal clock to external environmental cues, a self-sustained oscillator that generates daily oscillation, and an output pathway that converts oscillations into physiological rhythms. In the mammalian circadian system, the daily light/dark cycle is the most important and powerful entrainment cue, referred to as a *zeitgeber*. Therefore, in mammals the main input pathway to the circadian clock is the light input pathway (Reppert and Weaver, 2002). To entrain the central biological clock to the solar day, light signals are first captured by the retina and converted into chemical signals there, then transmitted as electrical signals through the retino-hypothalamic tract (RHT) to the SCN of the hypothalamus, the master circadian clock in mammals (Reppert and Weaver, 2002). The entrained SCN in turn coordinates the phase of multiple self-sustained peripheral oscillators throughout the body, like a conductor leading an orchestra (Yamazaki et al., 2000; Abe et al., 2002; Yoo et al., 2004). The peripheral oscillators further control a wide variety of local circadian output rhythms, which are the output pathway of the circadian clock system.

The retina is an important component in the mammalian circadian timing system, as it is the sole site for circadian photoreceptors (Foster, 1998; Barinaga, 2002), and light input from the retina modifies the entrained phase and developmental organization of the master SCN clock (Reppert and Weaver, 2002; Ohta et al., 2006). In addition to its role as

a light information sensor, the retina may play other roles in the circadian system. For example, enucleated hamsters have been reported to exhibit more variable free-running periods (*tau*) than do intact hamsters when the animals are maintained in constant darkness (Yamazaki et al., 2002), indicating the retina interacts continuously with the SCN to determine the *tau* of the entire system. Enucleated mice have also been found to show disrupted rhythms of p44/p42 mitogen-activated protein kinase (MAPK) phosphorylation in the SCN in constant darkness (Lee et al., 2003).

Cellular Structure of the Mammalian Retina

The mammalian retina is a thin and delicate tissue that lines the back of the eyeball. As shown in Figure 1-1, the mammalian retina, like other vertebrate retinas, is composed of three layers of nerve cell bodies and two layers of synapses. The outer nuclear layer (ONL) contains cell bodies of rod photoreceptors and cone photoreceptors; the inner nuclear layer (INL) contains cell bodies of horizontal, bipolar, and amacrine cells; and the ganglion cell layer (GCL) contains cell bodies of ganglion cells and displaced amacrine cells (Figure 1-1A). Separating the three nuclear layers are the synaptic layers, the outer plexiform layer (OPL) and the inner plexiform layer (IPL) where synaptic contacts occur (Figure 1-1A). During light transduction in the retina, light coming from the pupil and vitreous first strikes the ganglion cell layer of the neural retina and then passes through the entire retina to be received and transduced by photoreceptors. Light information is then vertically passed from photoreceptors to bipolar cells and to ganglion cells, which

finally transmit light information to the brain via their axons (Figure 1-1B). Besides this vertical light pathway, there are lateral signaling pathways which involve two types of interneurons, horizontal cells and amacrine cells, and which modify vertical light signal processing (Figure 1-1B).

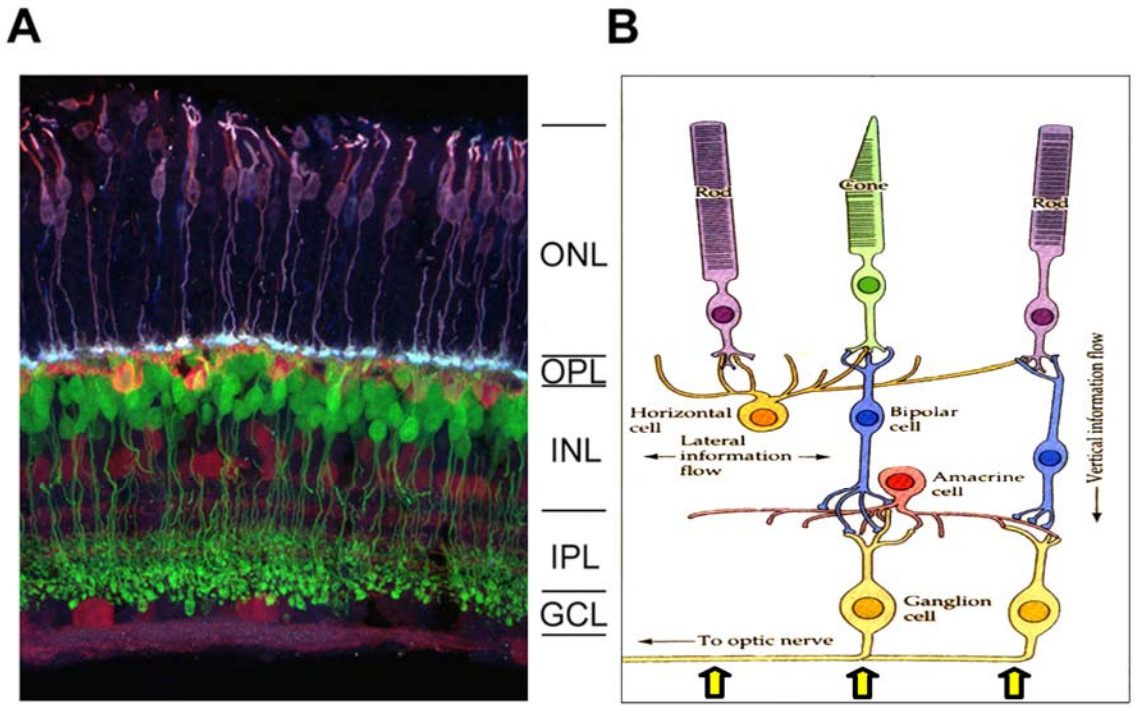

Figure 1-1: Cellular structure of the mammalian retina.
(A) Immunostaining of a *mGluR6*::GFP transgenic mouse retina showing different layers of cell bodies and synaptic terminals (Modified from Webvison). Photoreceptors are immunolabeled with anti-cone arrestin in bluish-purple; Bipolar cells are immunolabeled for *mGluR6* promoter driven GFP; Horizontal, amacrine and ganglion cells are immunstained for calbindin in red.
(B) Schematic diagram of the mammalian retina, showing the major retinal cell types as well as the vertical and lateral light flow pathways (Modified from Purves et al., 2000).
ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Circadian Rhythms in the Mammalian Retina

The mammalian retina is not only an important component in the light input pathway, but also an oscillator controlling multiple local rhythms at the physiological, cellular, and molecular levels (Table 1-1). One important example of retinal rhythms is visual sensitivity to light. Circadian rhythms in the ability to detect dim lights have been reported in human beings (Bassi and Powers, 1986) and in rats (Rosenwasser et al., 1979; Walker and Olton, 1979). Circadian sensitivity rhythms may compensate for external light intensity changes, to enable high visual acuity and protect the sensitive retinas against possible damage by bright light in the day (photopic vision is dominated by cones which have high temporal and spatial resolution but low sensitivity), and to allow visual function during dim night light (scotopic vision is dominated by sensitive rods). Indeed, circadian rhythms of visual sensitivity seem to prevail across vertebrate retinas, as the threshold light intensity required to evoke an escape response of zebrafish to a threatening object has also been found to show circadian rhythms (Li and Dowling, 1998).

Whereas there is absence of evidence supporting the influence of the master SCN clock on the retinal clock, several studies suggest local circadian control. Following lesions of the master SCN clock, circadian rhythms of visual detectability and rod outer-segment disk shedding persist (Teirstein et al., 1980; Reme et al., 1991). In addition, melatonin release rhythms in cultured hamster retinas can be reset by light (Tosini and Menaker, 1996), indicating that some retinal rhythms can be directly entrained by the

ambient light cycle. It has been reported that entrainment and phase-shifting of photoreceptor disk-shedding rhythms to new light-dark cycles are disrupted in optic nerve severed rats (Teirstein et al., 1980), however, it is not clear whether this is due to disruption of signals sent from the SCN (or other parts in the brain) to reset the photoreceptor disk-shedding rhythm, or due to ablation of melanopsin-expressing ganglion cells upon optic nerve section, which might serve as a synchronizer of the retinal clock (Zhang et al., unpublished data).

The retinal circadian clock and its two major neuromodulatory outputs, dopamine and melatonin, intimately influence pathological processes in the retina and eye. It is well documented that continuous high intensity illumination causes degeneration of photoreceptor and synaptic degeneration in the outer plexiform layer (Pecci Saavedra and Pellegrino de Iraldi, 1976). Rats with intravitreally injection of melatonin have been reported to develop more severe impairment of retinal structures compared to control animals (Bubenik and Purtill, 1980; Wiechmann and O'Steen, 1992). In contrast, rats treated with bromocriptine, a potent stimulator of dopamine receptors, have been found to develop damage to a much lesser extent in comparison to non-treated animals (Bubenik and Purtill, 1980). Consistently, intravitreally injection of the melatonin receptor antagonist luzindole has been shown to protect rat retinal photoreceptors from light damage (Sugawara et al., 1998).

Retinal dopamine and its rhythmicity may also be critical for regulating refractive error. Local administration of the dopamine agonist apomorphine to the occluded monkey

eye has been shown to retard excessive axial elongation and the concomitant development of myopia associated with visual deprivation (Iuvone et al., 1991).

Table 1-1: Circadian rhythms in the mammalian retina.

Category	Species
Photoreceptor disc shedding	Rat (LaVail, 1976, 1980), Hamster (Grace et al., 1996), Mice (Grace et al., 1999)
Visual sensitivity	Rat (Rosenwasser et al., 1979; Walker and Olton, 1979), Human (Bassi and Powers, 1986)
ERG b-wave	Rabbit (Brandenburg et al., 1983), Mouse (Barnard et al., 2006; Storch et al., 2007)
Intraocular pressure	Rabbit (Rowland et al., 1981), Human (Boyd and McLeod, 1964)
Extracellular pH	Rabbit (Dmitriev and Mangel, 2001)
Dopamine (DOPAC, HVA) content	Rat (Wirz-Justice et al., 1984; Pozdeyev and Lavrikova, 2000; Doyle et al., 2002a), Mouse (Doyle et al., 2002b)
Melatonin release	Rat (Sakamoto et al., 2004), Hamster (Tosini and Menaker, 1996), Mouse (Tosini and Menaker, 1998)
GABA turnover rate and release	Hamster (Jaliffa et al., 2001)
PKC level	Rat (Gabriel et al., 2001)

*In a recent microarray study, the Weitz lab identified 277 genes with rhythmic expression in constant darkness (Storch et al., 2007).

Molecular Nature of Mammalian Circadian Clocks

Molecular Nature of the Master SCN Clock

The molecular nature of the master SCN clock has been extensively studied. Within individual SCN clock neurons, a defined set of "clock genes" comprise two interlocking transcription/translation feedback loops (Figure 1-2; Reppert and Weaver, 2002). The first feedback loop (negative loop) is essential and determines the period and the amplitude of circadian oscillations, while the second feedback loop (positive loop) may be important to fine-tune and stabilize these oscillations (Emery and Reppert, 2004). In the first loop, two positive elements CLOCK and BMAL1 heterodimerize and activate transcription of their own repressor genes, namely *Period* (*Per*) 1 and 2, and *Cryptochrome* (*Cry*) 1 and 2, through E-box enhancers in their promoter region. Over the course of the day, the PER proteins accumulate in the cytoplasm and form complexes with the CRY proteins and casein kinase I ε and δ (CKIε and CKIδ), which phosphorylate the PER and CRY proteins. The resultant complex translocates back into the nucleus where the PER/CRY heterodimers interact with the CLOCK/BMAL1 heterodimers to repress the transcription of *Per* and *Cry* genes, leading to reduced night-time expression.

In the second feedback loop, the CLOCK/BMAL1 heterodimers activate transcription of two orphan nuclear receptor genes, *Rora* and *Rev-erbα*, also through E-box enhancers. The resultant proteins show opposing activities, with RORa activating *Bmal1* expression and REV-ERBα repressing *Bmal1* expression. They functionally and physically compete

for binding to the REV-ERB/ROR element (RRE) in the *Bmal1* promoter to regulate circadian expression of *Bmal1*.

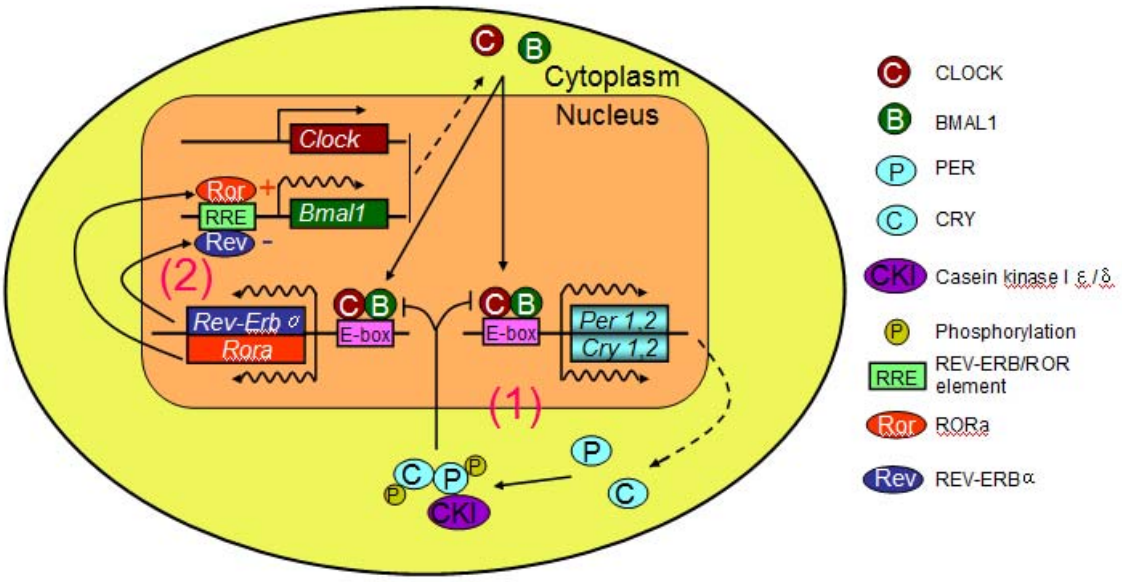

Figure 1-2: Mammalian circadian clockwork model.

The Mammalian Retinal Clock Operates in A Way Similar to the SCN Clock

It is generally believed that the mammalian retinal clock operates in the same manner as the SCN clock at the molecular level. The core clock genes in the SCN are also expressed in the mammalian retina (Gekakis et al., 1998; Miyamoto and Sancar, 1998; Namihira et al., 2001; Thompson et al., 2003; Witkovsky et al., 2003; Sakamoto et al., 2004). Additional important evidence supporting the notion that the mammalian retina uses the same basic molecular mechanism as the SCN clock comes from the studies of *tau* mutant hamsters, which show ca. 20 hr locomotor activity rhythms due to a point mutation in the *CkIε* gene (Lowrey et al., 2000). The *tau* mutant hamsters express disk shedding rhythms with a period of ca. 20 hr in constant darkness (Grace et al., 1996), and retinas from these mutant animals show a significantly shortened period (ranging from 20.0 to 22.1 hours) of melatonin release rhythms (Tosini and Menaker, 1996).

Circadian Rhythms of Clock Gene Expression in the Mammalian Retina

Quite a few studies have investigated the patterns of clock gene expressions in the rat retina under constant darkness, but the results are controversial. Using quantitative real-time RT-PCR (*i*Cycler), Tosini and colleagues found that *Per1* was the only core clock gene which showed circadian oscillation in the rat retina (Tosini and Fukuhara, 2002). However, in an *in situ* hybridization study, *Per2* but not *Per1* was found to exhibit circadian oscillation (Namihira et al., 2001). Moreover, in a Northern Blot study, *Per2* and *Bmal1* were found to show antiphase circadian expression in the rat retina, a pattern

similar to the SCN clock (Oishi et al., 1998). However, another article using *in situ* hybridization reported that a small but significant circadian variation were detected in *Clock* expression, but not in *Bmal1* expression (Namihira et al., 1999).

This controversy could be due to several possibilities. One possibility is that the amplitude of individual rat retinal oscillators is quite low which makes retinal ensemble rhythmicity of clock gene expression undetectable by some experimental strategies. Another possibility is that multiple circadian oscillators exist in the rat retina and show different phases (e.g. oscillators in the photoreceptors and oscillators in the inner retina are in antiphase). Theoretically there is another possibility that the retinal clockwork uses some retina-specific components instead of those defined components of the SCN clockwork. However, no evidence has been found to support the existence of such components to date.

Compared to the rat retina, the pattern of clock gene expression in the mouse retina is relatively less studied. Our lab has previously generated *Per1*::GFP mice in which a GFP reporter is driven by the mouse *Per1* promoter (Kuhlman et al., 2000). In this transgenic model, GFP immunoreactivity (i.e. *Per1* expression) has been found to show circadian oscillation in the whole retina as well as in dopaminergic amacrine cells (Witkovsky et al., 2003), suggesting that dopaminergic amacrine cells are putative clock neurons. In addition, *Cry1* and *Cry2* expression in the mouse retina has been reported to lack circadian variation by an *in situ* hybridization study (Miyamoto and Sancar, 1998).

Localization of Circadian Clock(s) in the Vertebrate Retina

Localization of Circadian Oscillators in Non-mammalian Vertebrate Retinas

Several lines of evidence indicate that in non-mammalian vertebrates, including amphibians (*Xenopus*) and avians (chicken), photoreceptors are the primary location for retinal rhythms generation. First, photoreceptors in non-mammalian vertebrate retinas express high levels of clock genes. In the *Xenopus* retina, *Per2* (Besharse et al., 2004), 3 *Cry* homologues (Zhu and Green, 2001), and *Clock* (Zhu et al., 2000) are expressed predominantly in photoreceptors; *Per1* is expressed at roughly equal levels in the three nuclear layers (Besharse et al., 2004). Higher expression levels of *Cry1* (Haque et al., 2002) and *Cry2* ((Bailey et al., 2002) in photoreceptors have also been reported in the chicken retina. Second, isolated *Xenopus* photoreceptor layers have been demonstrated to exhibit circadian rhythms of melatonin release (Cahill and Besharse, 1993). Similarly, in photoreceptor-enriched cultures prepared from embryonic chick retinas, iodopsin mRNA level has been found to show circadian oscillation (Pierce et al., 1993). Last but not least, disruption of the *Clock* gene specifically in *Xenopus* photoreceptors abolishes circadian melatonin release rhythmicity without affecting its production levels, further strengthening the conclusion that the circadian clock controlling *Xenopus* retinal melatonin release rhythmicity is located in its photoreceptors (Hayasaka et al., 2002).

Localization of Circadian Oscillators in the Mammalian Retina

While the existence of circadian oscillators in photoreceptors is well established in non-mammalian vertebrates, the location(s) of the circadian clock(s) in the mammalian retina is controversial. Similar to non-mammalian vertebrates, mammalian photoreceptors show some circadian rhythms, such as outer segment disc shedding (LaVail, 1976) and Fos expression (Yoshida et al., 1993; Humphries and Carter, 2004). In addition, isolated mammalian retinas also exhibit circadian release of melatonin, which is synthesized primarily in photoreceptors (Tosini and Menaker, 1996; Niki et al., 1998b; Tosini and Menaker, 1998). In contrast to the *Xenopus* and chicken retina, however, using *in situ* hybridization, *Per1* and *Clock* transcripts have been detected predominantly in the inner nuclear and ganglion cell layers of the mammalian retina with a lower level in photoreceptors; *Bmal1* transcripts have been found in the inner nuclear layer, ganglion cell layer, and photoreceptor layer at similar levels (Gekakis et al., 1998), whereas *Per2*, *Cry1* and *Cry2* have been detected exclusively in the inner nuclear and ganglion cell layers (Miyamoto and Sancar, 1998; Namihira et al., 2001). We recently found that *Clock* and at least one of the *Cry* genes were necessary for self-sustained retinal molecular rhythms (Ruan and McMahon, unpublished data). Thus, the mammalian inner nuclear and ganglion cell layers express all the core circadian clock genes known necessary for self-sustained rhythms generation, but the photoreceptors show limited expression of clock genes. Several other studies also suggest that circadian organization of mammalian retinas might be different from the current prevailing retinal circadian organization model

based primarily on data from the *Xenopus* retina. For example, retinas from the Royal College of Surgeons (RCS) rats lacking photoreceptors have been shown to preserve circadian rhythms in dopamine content and turnover, as well as melatonin release and the transcription of arylalkylamine *N*-acetyltransferase (AANAT), the rate-limiting enzyme in melatonin synthesis (Doyle et al., 2002a; Sakamoto et al., 2004), suggesting the persistence of circadian function in the absence of photoreceptors.

Whereas there is absence of evidence supporting an oscillator in the mammalian photoreceptors, several recent studies indicate that dopaminergic amacrine cells of the inner nuclear layer may contain an endogenous oscillator that controls dopamine metabolism and release rhythms. Using the transgenic *Per1*::GFP mouse model, *Per1* transcription has been shown to occur in most GABAergic amacrine cells, and rhythmically in the dopaminergic and NOS-positive subtypes of GABAergic amacrine cells (Witkovsky et al., 2003; Zhang et al., 2005). A recent microarray study has detected the presence of *Cry1* transcripts in the mRNA pool extracted from dopaminergic amacrine cells (Gustincich et al., 2004). Immunoreactive PER1, CRY1, CRY2, CLOCK, and BMAL1 have been detected in dopaminergic amacrine cells as well (Gustincich et al., 2004). Taken together, these results suggest a molecular basis for circadian regulation of dopamine transmission in dopaminergic amacrine cells. Indeed, the circadian rhythms observed in the mammalian photoreceptors might be driven by the rhythmic retinal dopamine level, as dopamine has been shown to inhibit photoreceptor disc shedding as well as melatonin synthesis and release by binding to D2-like receptors (Cahill and

Besharse, 1991; Dahl, 1992; Tosini and Dirden, 2000).

Regulation of the Mammalian Retinal Circadian Clock

Neurotransmitters in the Mammalian Retina

The mammalian retina contains all the major neurotransmitter systems and to a large extent, retinal function is mediated by neurotransmission. The important retinal neurotransmitters include two excitatory faster neurotransmitters, glutamate and acetylcholine (ACh), two inhibitory faster neurotransmitters, GABA and glycine, and two globally modulatory neurotransmitters, dopamine and melatonin (Figure 1-3). Glutamatergic transmission is used in vertical light flow pathways, between photoreceptors and their bipolar and horizontal cell targets, and between bipolar cells and their amacrine and ganglion cell targets (Pourcho, 1996; Linden et al., 2005). In lateral signaling pathways, horizontal and amacrine cells primarily use GABAergic and glycinergic signaling, with specialized circuits employing AChergic signaling (Pourcho, 1996; Linden et al., 2005). The neuromodulator dopamine is synthesized and released from dopaminergic amacrine cells and acts on target cells either by synaptic transmission or by non-synaptic diffusion (Witkovsky, 2004). Melatonin is produced in photoreceptors and acts on target melatonin receptors mainly through non-synaptic diffusion (Wiechmann and Smith, 2001). Previous studies have suggested that dopamine, melatonin, and GABA play important roles in regulating the retinal clock. They may

either function as a day/light signal (dopamine) or as a night/dark signal (melatonin and GABA) to regulate retinal physiology.

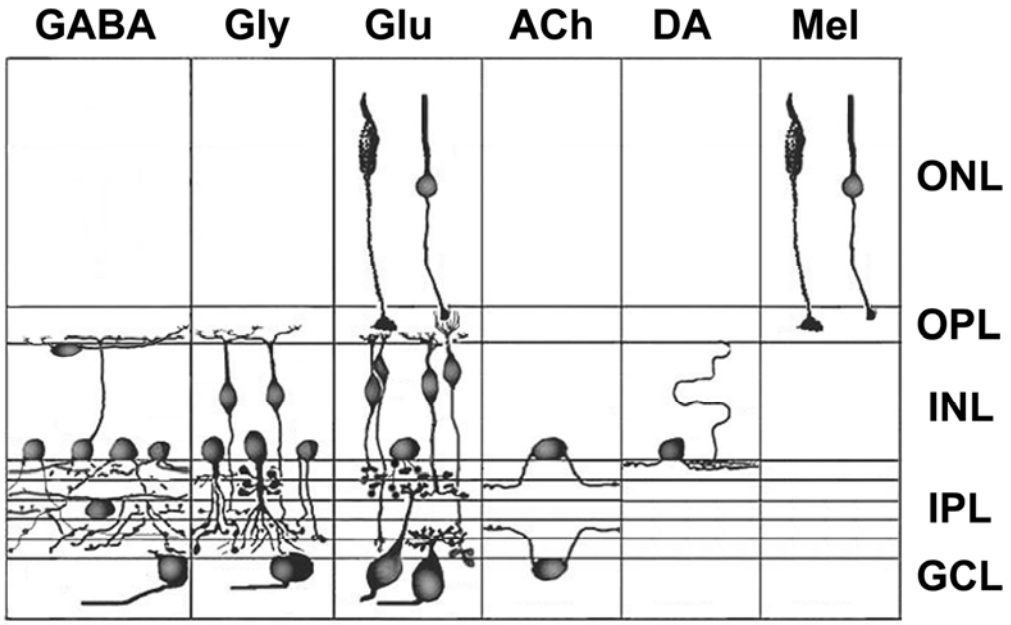

Figure 1-3: Diagram of the organization of neurotransmitters in the mammalian retina (Modified from Webvision).

Note that: (1) the inner plexiform layer is divided into five stratums where bipolar cells, amacrine cells, and ganglion cells interact each other; (2) there are multiple subtypes of bipolar cells, amacrine cells, and ganglion cells which have different morphology, function, and connection with other cells; (3) acetylcholine (ACh) is produced by a mirror symmetric pair of amacrine cells, with one of the mirror pair occurring in the inner layer and the other of the pair displacing in the ganglion cell layer.

Gly, glycine; Glu, glutamate; DA, dopamine; Mel, melatonin; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Dopamine Is A Light Signal in the Mammalian Retina

Dopamine is synthesized and released by the ca. 500 dopaminergic amacrine cells in each mammalian retina (Nguyen-Legros et al., 1981; Versaux-Botteri et al., 1986). Convergent evidence from three types of experiments substantiates the notion that retinal dopamine functions as a chemical signal for light.

First, this conclusion is grounded on the fact that retinal dopamine content and turnover show daily rhythms, with levels higher during the day than at night, in several mammalian species, including rat (Melamed et al., 1984; Wirz-Justice et al., 1984; Gibson, 1988; Pozdeyev and Lavrikova, 2000), mouse (Nir and Iuvone, 1994; Nir et al., 2000), and rabbit (Nowak and Zurawska, 1989).

The second type of evidence comes with the observation that light quickly induces dopamine synthesis and release in the mammalian retina. Light increases the activity of retinal tyrosine hydroxylase, the key enzyme in dopamine synthesis, to its highest level within 15 min of light onset (Iuvone et al., 1978), thus evoking a burst of dopamine synthesis and utilization within 30 min of light onset (Nir et al., 2000). In addition, light increases the spike rate of dopaminergic amacrine cells and evokes spike-mediated dopamine release (Puopolo et al., 2001).

The third line of evidence is that dopamine regulates retinal physiology to bias function toward “day” states through modulating a number of light-adaptive responses in the mammalian retina, i.e. outer segment disk shedding and phagocytosis (Reme et al., 1991), light-sensitive pool of cyclic AMP in photoreceptors (Cohen and Blazynski, 1990),

Na/K ATPase activity in rod photoreceptors (Shulman and Fox, 1996), coupling in horitontal cells (He et al., 2000), depolarization of horizontal cells (Hankins and Ikeda, 1994), dark-adapted electroretinogram b-wave (Skrandies and Wassle, 1988; Naarendorp and Sieving, 1991), electrical coupling among AII amacrine cells (Hampson et al., 1992), $GABA_A$ currents in amacrine cells (Feigenspan and Bormann, 1994), acetylcholine release by mirror symmetrical starburst amacrine cells (Yeh et al., 1984), discharge of ON-center and OFF-center cells (Jensen and Daw, 1984, 1986), and light sensitivity of ganglion cells (Jensen and Daw, 1986).

Melatonin Is A Dark Signal in the Mammalian Retina

In the mammalian retina, melatonin is primary synthesized and released from photoreceptors. Melatonin serves as a chemical signal for darkness. Melatonin release in the mammalian retina shows both light-influenced and circadian rhythms, with levels higher at night than during the day (Tosini and Menaker, 1996, 1998; Sakamoto et al., 2004). Indeed, melatonin release has served as the principal experimental measure of the retinal circadian clock. Light exposure at night results in rapid inactivation and degradation of arylalkylamine *N*-acetyltransferase (AANAT), the key enzyme in melatonin synthesis (Fukuhara et al., 2001). In contrast to the light-adaptive role of dopamine, melatonin modulates several dark-adaptive responses, including dark-adaptive pigment migration (Pang and Yew, 1979), cyclic GMP level (Faillace et al., 1996b), acetylcholine release (Mitchell and Redburn, 1991), and glutamate release (Faillace et al.,

1996a).

The Roles of Melatonin and Dopamine in the Mammalian Retinal Clock

As described above, dopamine and melatonin are important neurochemical messengers that act at multiple sites within the retinal circuitry to shape retinal function into "day" and "night" states. More interestingly, dopamine and melatonin have been demonstrated to be able to inhibit each other's release (Dubocovich, 1983; Tosini and Dirden, 2000). Because of the widespread influence of dopamine and melatonin on mammalian retinal physiology, and the fact that their receptors are located on most cell types in the mammalian retina (Scher et al., 2002; Witkovsky, 2004; Iuvone et al., 2005), it has been postulated that dopamine and melatonin are located at the core of the retinal circadian timing system, and that they either work alone or work together to drive multiple retinal rhythms (Green and Besharse, 2004; Iuvone et al., 2005).

Some recent evidence suggests that melatonin might be the predominant circadian signal in the mammalian retina as under constant darkness condition, circadian rhythms in dopamine level persist in C3H mice, which produce melatonin, but not in C57BL/6J mice, which do not produce melatonin (Doyle et al., 2002b). In addition, C57BL/6J mice receiving ten daily injections of melatonin exhibit a robust circadian rhythm of retinal dopamine content (Doyle et al., 2002b). These results are consistent with the notion that endogenous melatonin is involved in the regulation of circadian rhythms of retinal dopamine content. In addition, continuous activation or blockade of melatonin receptor

has been reported to abolish the circadian rhythms of dopamine release in the goldfish retina (Ribelayga et al., 2004). However, these results do not necessarily indicate that dopamine is required for the retinal circadian clock, since it is not clear whether overall retinal rhythmicity is abnormal in the absence of dopamine rhythmicity. Even if dopamine is not necessary for retinal rhythms generation, it may function in other aspects. It is acknowledged that in the *Xenopus* retina dopamine shifts circadian rhythm of melatonin release in a manner similar to light (Cahill and Besharse, 1991; Steenhard and Besharse, 2000). Thus, dopamine transmission is likely to be a mediator of light entrainment. In the mouse retina, the dopamine D2 receptor has been demonstrated to mediate light-induced transcriptional activation of *Per1* through an E-box dependent mechanism (Yujnovsky et al., 2006).

Although melatonin seems to be important for dopamine rhythms, it is not required for all retinal circadian rhythms. For instance, by comparing C57BL/6J and C3H mice, Grace et al. (Grace et al., 1999) have demonstrated that melatonin is not required for circadian rhythm of photoreceptor disk shedding in constant darkness.

GABA: Another Dark Signal in the Mammalian Retina?

In the retina, GABA is synthesized and released by horizontal cells and GABAergic amacrine cells, of which dopaminergic amacrine neurons are a subset (Young, 1994). GABA functions as a principal inhibitory neurotransmitter in modulating visual signal processing in the lateral pathway. GABA receptors are classified as ionotropic,

chloride-conducting $GABA_A$ and $GABA_C$ receptors, or as metabotropic $GABA_B$ receptors. All three types of GABA receptors are present in the mammalian retina (Yang, 2004). Our lab has recently identified *Per1* expression within GABAergic retinal neurons (Zhang et al., 2005). Consistently, GABA turnover rate and release in the golden hamster retina showed daily variations under both light/dark and constant darkness conditions, with levels higher at night than during the day (Jaliffa et al., 2001). In addition, GABA is known to suppress tyrosine hydroxylase activity (Marshburn and Iuvone, 1981), abolish the bursting activity of retinal dopaminergic neurons (Gustincich et al., 1997; Zhang et al., 2007) and inhibit dopamine release (Puopolo et al., 2001). Therefore, GABA could act indirectly through modulation of retinal dopamine. GABA also increases melatonin content in a dose-dependent manner in the hamster retina through a $GABA_A$ receptor-mediated mechanism (Jaliffa et al., 1999). Taken together, these data indicate that GABA may serve as another analog for "darkness" in the mammalian retina.

Challenges to the Study of the Retinal Circadian Clock

The mammalian retina is the most accessible portion of the central nervous system, and its structure and circuitry are well characterized. These features make the retina an appealing model to study the general principles of circadian pacemaking. However, examination of the mammalian retinal clock has been limited by the challenge of long-term culture of adult mammalian retina (Tosini and Menaker, 1996, 1998), although some other studies have succeeded in culturing retinas from neonatal mammals (Ogilvie

and Speck, 2002; Xin et al., 2007). Circadian rhythmicity of melatonin release are not detectable in mouse retinas cultured at 37^{o}C or 32^{o}C (Tosini and Menaker, 1998). When mouse retinas are cultured at 27^{o}C, melatonin release rhythms persist up to 5 cycles and the resulting free-running period (ranges from 24.3 to 27.3 h) is markedly longer than that of the mouse SCN pacemaker. Since the metabolism of mammalian photoreceptors consumes high energy (Ames et al., 1992), it is possible that photoreceptor survival at higher temperatures requires much higher level of oxygen (Tosini and Menaker, 1998). Another concern to use melatonin release as a measure for molecular clock motion is the fact that melatonin is an output but not a component of retinal circadian clock, which makes it difficult to determine whether melatonin modulates the retinal molecular clock mechanism itself and what role it may play in the organization of the retinal clock. Experiments using time-point sampling of retinal melatonin, dopamine or clock gene expression from animal populations do not require adult retinal culture, but can not address the independence of the retinal circadian clock from the master SCN clock or other clocks in the body, and moreover, the results are influenced by synchrony in the animal population.

Objectives of This Study

Despite the widespread influence of retinal clock on retinal physiology, the cellular location(s) and neural regulation of the mammalian retinal circadian clock remain unclear, which has greatly hindered the progress in studying the retinal circadian organization and

the mechanisms by which retinal circadian clock controls multiple retinal circadian output rhythms. In addition, although it is believed that dopamine, melatonin, and GABA play important roles in regulating retinal circadian physiological rhythms, it has not been directly demonstrated whether these neurotransmitters regulate the molecular clockwork in the mammalian retina at this point.

The overall objective of this study was to determine circadian organization of the mammalian retina, and specifically to examine the cellular location and explore the neural regulation of the mouse retinal circadian clock. I have taken advantage of several transgenic mouse models as well as used genetic, molecular, and pharmacological strategies to address these questions.

In Chapter II of this dissertation, I examined the circadian organization of the mouse retina using cell morphology, as well as genetic and retrograde labeling to identify individual retinal neurons, and single-cell real-time reverse transcription (RT)-PCR, to investigate the cell-type specific expression of core circadian clock genes; using PERIOD2::LUCIFERASE (PER2::LUC) fusion protein real-time reporting of molecular circadian rhythms (Yoo et al., 2004) and quantitative real-time RT-PCR to study clock gene expression rhythms following photoreceptor degeneration. The results indicate that there are neurons with the requisite molecular components to be potential circadian pacemakers among all the major classes of retinal neurons, with the exception of photoreceptors, and that the ability to generate endogenous circadian rhythms resides in neurons of the inner nuclear and ganglion cell layers.

In Chapter III, I developed an *in vitro* explant culture protocol for **intact** *mPer2*^{Luc} mouse retinas to monitor circadian rhythms in PER2::LUC expression. Using this protocol, I studied the properties of retinal molecular rhythms *in vitro*, the location within the retina of rhythms generation and the influence of melatonin and dopamine on retinal molecular rhythms. The findings reveal an inner nuclear layer retinal circadian clock that generates endogenous molecular rhythms independent of melatonin and which is regulated in its phase by dopamine.

In Chapter IV of this dissertation, using the protocol we developed in Chapter III, I further investigated the role of GABA and other fast neurotransmitters in the mouse retinal circadian clock. The results indicate that GABA, acting through $GABA_A$ and $GABA_C$ receptors, linked to membrane hyperpolarization and casein kinases, plays an important role in regulating the amplitude of the mammalian retinal circadian clock, and suggest that GABA and casein kinase act at the molecular level of PER proteins to reinforce the autonomous generation of the retinal "night" state.

Overall, the findings support a model of retinal circadian organization, in which multiple circadian oscillators are located in the inner nuclear layer of the mouse retina, and rhythms generation of these oscillators is independent of major forms of neural communication; however, dopamine, GABA and casein kinases play critical roles in setting the phase, amplitude and period of these autonomous oscillators.

CHAPTER II

CIRCADIAN ORGANIZATION OF THE MAMMALIAN RETINA

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Introduction

The mammalian retinal circadian clock exerts extensive control over retinal physiology and function, regulating a wide variety of retinal circadian rhythms, including photoreceptor disc shedding (LaVail, 1976, 1980; Grace et al., 1996; Grace et al., 1999), visual sensitivity (Rosenwasser et al., 1979; Bassi and Powers, 1986; Reme et al., 1991), rod-cone balance(Wang and Mangel, 1996; Manglapus et al., 1998), electroretinogram (ERG) b-wave amplitude (Brandenburg et al., 1983), extracellular pH (Dmitriev and Mangel, 2001), melatonin release (Tosini and Menaker, 1996; Niki et al., 1998a; Tosini and Menaker, 1998), dopamine synthesis (Doyle et al., 2002b; Doyle et al., 2002a), gamma-aminobutyric acid (GABA) turnover rate and release (Jaliffa et al., 2001), and intraocular pressure (Boyd and McLeod, 1964; Rowland et al., 1981).. The retinal circadian clock, and its dopamine and melatonin signaling molecules, also influence pathological processes in the eye including the susceptibility of photoreceptors to degeneration from light damage (Sugawara et al., 1998; Organisciak et al., 2000), photoreceptor survival in animal models of retinal degeneration (Ogilvie and Speck,

2002), and the degree of refractive errors in primate models of myopia (Iuvone et al., 1991). Despite its widespread influence, the cellular origin and organization of the circadian clock in the mammalian retina remains unclear.

Neural circadian clocks generate endogenous circadian rhythms through cell-autonomous autoregulatory transcription-translation feedback loops comprised of a defined set of "clock genes" in subsets of circadian pacemaker neurons. Gene targeting has demonstrated that in mammals the *Period* genes (*Per*) *1* and *2*, the *Cryptochrome* genes (*Cry*) *1* and *2*, as well as *Clock* and *Bmal1* are core molecular components of circadian clocks and that their expression is necessary for circadian rhythmicity (Reppert and Weaver, 2002). The prevailing model for circadian organization of vertebrate retinas, based on molecular and physiological data from amphibian and avian retinas, holds that photoreceptors contain self-sustained circadian clocks that rhythmically secrete melatonin as an output signal (Cahill and Besharse, 1993; Pierce et al., 1993; Hayasaka et al., 2002; Ivanova and Iuvone, 2003b). Although mammalian retinas also exhibit circadian melatonin rhythms (Tosini and Menaker, 1996; Niki et al., 1998a; Tosini and Menaker, 1998), *in situ* hybridization studies of the regional distribution of clock genes in the mammalian retina found them to be expressed predominantly in the inner nuclear and ganglion cell layers (Gekakis et al., 1998; Miyamoto and Sancar, 1998; Namihira et al., 2001; Thompson et al., 2003; Witkovsky et al., 2003) suggesting the potential for an alternate circadian organization.

In the current study we have examined the circadian organization of the mouse retina

using cell morphology, as well as genetic and retrograde labeling to identify individual retinal neurons, single-cell real-time reverse transcription (RT)-PCR, PER2::LUC fusion protein real-time reporting of molecular circadian rhythms (Yoo et al., 2004), and quantitative real-time RT-PCR (qPCR) to investigate the cell-type specific expression and endogenous rhythmicity of core circadian clock genes. Our results indicate that there are neurons with the requisite molecular components to be circadian pacemakers among all the major classes of retinal neurons, with the exception of photoreceptors, and that the ability to generate endogenous circadian rhythms resides in neurons of the inner nuclear and ganglion cell layers, independent of photoreceptors and of the central biological clock of the brain.

Materials and Methods

Harvesting of Individual Retinal Neurons for RT-PCR

Individual photoreceptors, rod bipolar cells, dopaminergic (DA) amacrine cells, type 2 catecholamine (CA) cells, and ganglion cells were collected from acute retinal cell cultures. Ganglion cell labeling was performed as previously described (Witkovsky et al., 2003). 2-4 month old C57BL/6J mice, hemizygous transgenic B6C3H mice harboring a *TH*::RFP reporter (Zhang et al., 2004), and DiI injected C57BL/6J mice were euthanized by cervical dislocation at Zeitgeber time (ZT) 6. Retinas were isolated in Leibovitz's L-15 medium (GIBCO), digested with papain (20 units/ml; Worthington), gently triturated in L-15 medium, and finally distributed into culture dishes at low density.

For collection of individual retinal neuron contents for RT-PCR, culture dishes were kept at room temperature and cells were observed with an inverted microscope (Nikon, Lewisville, TX). A modified perfusion system was used to avoid medium contamination of the RT-PCR samples (Figure 2-1). The inlet tube perfused the target cell with Ame's medium (Sigma; supplemented with 10 mM HEPES and 10 mM glucose, pH 7.4). The outlet speed was a little faster than the inlet speed. When there was very little medium left near the target cell, the tip of the glass pipette, filled with 10 μl of diethylpyrocarbonate (DEPC) treated intracellular solution (containing 140 mM KCl, 5mM EGTA, 3 mM $MgCl_2$ and 5 mM HEPES, pH 7.4), was quickly moved to the target cell. During this process, positive pressure was continuously applied to the pipette. A seal

was formed on the target cell and cellular contents were aspirated into the pipette by gently applying negative pressure. The tip of the pipette was then quickly lifted from the culture dish, positioned in front of a perfusion tube, washed with fresh medium for 1 min, and finally broken into an Eppendorf tube containing 13.5 µl of RT mixture 1 (containing 10 units of RNase inhibitor, 0.5 µl of 100 mM DTT, 1 µl of 10 mM dNTPs, 1 µl of 3 µg/µl random hexamers, 2.5 µl of 20 µg/µl bovine serum albumin). The tube was kept on dry ice until the end of the harvesting procedure, which lasted from 30 min to 90 min from the time of plating.

Individual horizontal cells were harvested from whole-mount *rd* mouse retinas in which the photoreceptors had degenerated allowing direct physical access to the horizontal cells. 3-4 month old C3H *rd* mice were sacrificed at ZT 6. The retinas were isolated and transferred to a perfusion chamber, stabilized using a nylon mesh with ganglion cell layer down, mounted on an upright microscope (Zeiss, Thornwood, NY), and perfused for 1 hr with 95% O_2-5% CO_2 bubbled Ame's medium (supplemented with 25 mM $NaHCO_3$ and 10 mM glucose) before harvesting. Superficial horizontal cells with relatively large cell bodies were harvested.

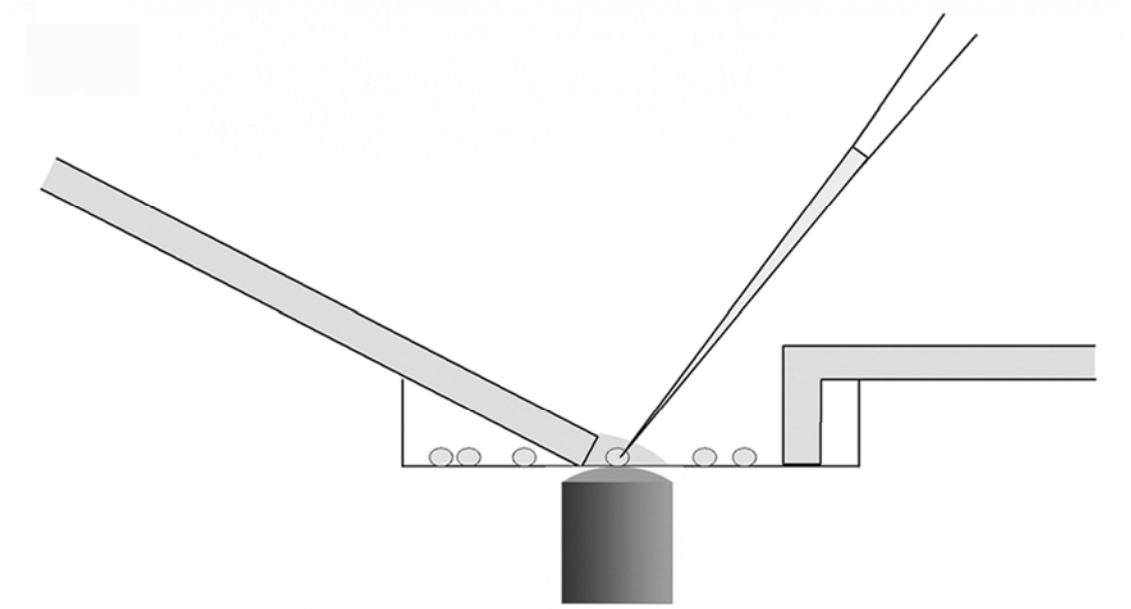

Figure 2-1: The cell harvesting method is shown.
A perfusion system was used to avoid medium contamination of the PCR samples. The outlet speed was faster than the inlet flow so that the depth of medium over the target cell was minimized during harvesting.

Animal Sampling for Whole Retina qPCR

Sixty 10-14 week old male C3H *rd* mice were purchased from Jackson Laboratories (Bar Harbour, ME). The mice were either maintained in light/dark (LD) cycles or, before being used, transferred into constant darkness (DD) at the light-dark transition and kept there for 36-48 hr. Five animals were sampled for each time point examined at ZT/ circadian time (CT) 2, 6, 10, 14, 18 and 22. For dissections during the dark phase, night-vision goggles (B.E. Meyers, Redmond, WA) were used under infrared illumination so that no visible light was necessary for the retina isolation procedure. The two retinas from each mouse were pooled as one sample and immediately homogenized in TRIZOL reagent (Invitrogen) under dim red light, frozen on dry ice and stored at -80°C until RNA extraction.

cDNA Preparation

For single-cell real-time PCR, single-cell samples were first incubated at 70°C for 10 min. After adding 6 µl of RT mixture 2 (containing 10 units of RNase inhibitor, 1.5 µl of 100 mM DTT, and 4 µl of 5X first-strand buffer) to each tube, the samples were kept at room temperature for 2 min. Single-cell cDNAs were then synthesized with 100 units of Superscript II (Invitrogen) by incubation at 42°C for 70 min. 20 µl of DEPC H_2O were added to each sample, bringing the final volume to 40 µl.

For whole retina qPCR, total RNA was isolated from whole retinal homogenates following the manufacturer's instructions (Invitrogen) and resuspended in 100 µl of

DEPC H_2O. RNA was quantified using OD at 260 nm. 1 μg total RNA was reverse transcribed into first-strand cDNA with 200 units of Superscript II and 3 μg random hexamers by incubation at 42°C for 70 min. 80 μl of DEPC H_2O was added to each sample, bringing the final volume to 100 μl.

TaqMan Real-time PCR

The following primers and probes were designed using ABI PrimerExpress software and ordered from ABI:

Per1 forward 5'-GAAAGAAACCTCTGGCTGTTCCTA-3',

Per1 reverse 5'-TGGTTGTACTGGGAATGTTGCA-3',

Per1 probe 5'-6FAM-CATCCTCAGGTATTTGGA-MGBNFQ-3';

Per2 forward 5'-TGCTGGCAGAGAGGGTACACT-3',

Per2 reverse 5'-GGTTGTTGTGAAGATCCTCTTCTCA-3',

Per2 probe 5'-VIC-CTATGAAGCGCCTAGAAT-MGBNFQ-3';

Cry1 forward 5'-CACCATCC GCTGCGTCTATA-3',

Cry1 reverse 5'-CTCAAGACACTGAAGCAAAAATCG-3',

Cry1 probe 5'-6FA M-CCTCGACCCCTGGTT-MGBNFQ-3';

Cry2 forward 5'-CGTGGAGGTGGTGACTGAGA-3',

Cry2 reverse 5'-CTGCCCATTCAGTTCGATGA-3',

Cry2 probe 5'-VIC-CACCCTCTATGACCTAGACAG-MGBNFQ-3';

Clock forward 5'-ACGGCGAGAACTTGGCATT-3',

Clock reverse 5'-TGATACGATTGTCAGACCCAGAA-3',

Clock probe 5'-6FAM-CCTGAGACAGCTGCTGACAAAAGCCA-MGBNFQ-3';

Bmal1 forward 5'-GAAGGTTAGAATATGCAGAACACCAA-3',

Bmal1 reverse 5'-TCCCGACG CCTCTTTTCA-3',

Bmal1 probe 5'-VIC-TGGGCCTCCCTTGCAT-MGBNFQ-3';
GABA-δ forward 5'-TTATCCGCCTACAGCCTGATG-3',
GABA-δ reverse 5'-TTGGCGAGGTCCATGTCA-3',
GABA-δ probe 5'-VIC-CATCCGCATCACCTC-MGBNFQ-3'.

PCR primers were designed to span introns. The amplicon lengths varied from 70 to 94 bp and PCR products for each reaction were further confirmed to be from cDNA, not genomic DNA, by agarose gel electrophoresis. All probes except the *Cry1* probe crossed exon junctions. The two primers of *Cry1* were on two exons separated by a 27119 bp intron. The primer and probe concentrations were optimized following the manufacturer's directions (ABI). The amplification efficiency (*E*) was determined on a total retinal cDNA dilution series. The *E* values of clock genes varied from 1.92 to 1.99.

Immediately after first-strand cDNA synthesis, real-time PCR was performed using an ABI Prism 7000. The reaction was first incubated at 50°C for 2 min, then at 95 °C for 10 min, followed by 55 cycles (single-cell) or 40 cycles (whole retina) of 95°C for 15 s and 60°C for 1 min. Samples were assayed in duplicate for single-cell PCR and in triplicate for whole retina qPCR. Each particular transcript was regarded as present in individual single-cell samples when its delta Rn exceeded a threshold value of 0.2 in less than 50 cycles of real-time PCR.

Retina Explant Cultures

mPer2^{Luc} knockin mice, which initially were on a 129SvEv X C57BL/6J genetic background (Yoo et al., 2004; a gift from J. Takahashi to S. Yamazaki), were maintained

as a continuous backcross to C57BL/6J for 10 generations. The resulting *mPer2*^{Luc} mice were crossed with C3H *rd* mice twice to produce *mPer2*^{Luc} knockin mice that are homozygous for *rd* gene. Approximately 30 min before lights off, *mPer2*^{Luc} knockin *rd* mice were euthanized by cervical dislocation. Eyes were enucleated and placed in Hank's balanced salt solution (HBSS; Invitrogen). Retinas were isolated from the rest of the eyecup with as little disruption and manipulation as possible and then gently placed ganglion cell layer up on Millicell culture membranes (Millipore, Billerica, MA) in 35 mm culture dishes with 1.0 ml Medium 199 (without L-glutamine, sodium bicarbonate, and phenol red; Sigma), supplemented with 0.7 mM L-glutamine, 4 mM sodium bicarbonate, 10 mM HEPES, 2% B27, 0.1 mM beetle luciferin (Promega), 25 units/ml penicillin and 25 μg/ml streptomycin (Invitrogen). Bioluminescence was measured with a LumiCycle (Actimetrics, Wilmette, IL). Cultures were maintained in a light-tight incubator at 36.8°C. Data were analyzed using LumiCycle data analysis software (Actimetrics) and ClockLab software (Actimetrics). The timing of the peak of the circadian oscillation was determined during the interval of 12 and 36 hours in culture.

Data Analysis

All statistical analyses were conducted with SPSS V12.0. For single-cell PCR, a χ^2 test was applied to compare percentages of clock gene expression between two paired cell types, as well as expression percentages between clock gene pairs. For sample sizes less than 5, Fisher's exact test was performed instead of χ^2 test. For whole retina qPCR,

the normalization method used was based on Dijk et al. (Dijk et al., 2004) with some modifications. In brief, the cycle threshold (*C*t) was converted to absolute amount of transcript (E^{-Ct}, E is amplification efficiency) and presented as $E^{-Ct}/E^{-Ct}_{(Max)}$. $E^{-Ct}_{(Max)}$ corresponds to the maximum E^{-Ct} in 6 ZTs and 6 CTs. Data were analyzed using one-way ANOVA for time differences and two-way ANOVA for light cycle differences.

Results

Coordinate Expression of Core Clock Genes Occurs Preferentially in Neurons of the Inner Nuclear and Ganglion Cell Layers

In order to localize potential clock neurons within the mouse retina, we used single-cell real-time RT-PCR to simultaneously detect all 6 core clock genes within single, identified retinal neurons. Six specific retinal neuron types were identified for collection either from acutely dissociated retinal cell cultures or from retinal whole mounts. Rods and rod bipolar cells were distinguished from other cells following dissociation based on their unique morphological characteristics (Figures 2-2A and 2B). Horizontal cells were harvested from whole-mount retinas of *rd* mice, in which the degeneration of photoreceptors permits direct access to horizontal cell somata (Figure 2-2C; Zhang and McMahon, unpublished data). Two types of amacrine cells, dopaminergic (DA) amacrines and type 2 catecholamine (CA) amacrines, were collected by dissociating retinas from mice harboring a tyrosine hydroxylase (TH) promoter-driven red fluorescent protein (RFP) transgene and visualized by RFP fluorescence (Zhang et al., 2004). Both DA and type 2 CA cells express the *TH*::RFP reporter, but are readily distinguished by soma size (Zhang et al., 2004), with DA cells having the relatively larger somas (Figures 2-2D and 2E). Ganglion cells were retrogradely labeled with DiI by injection into the lateral geniculate nuclei (LGN) and were visualized by fluorescence following retinal dissociation (Figure 2-2F).

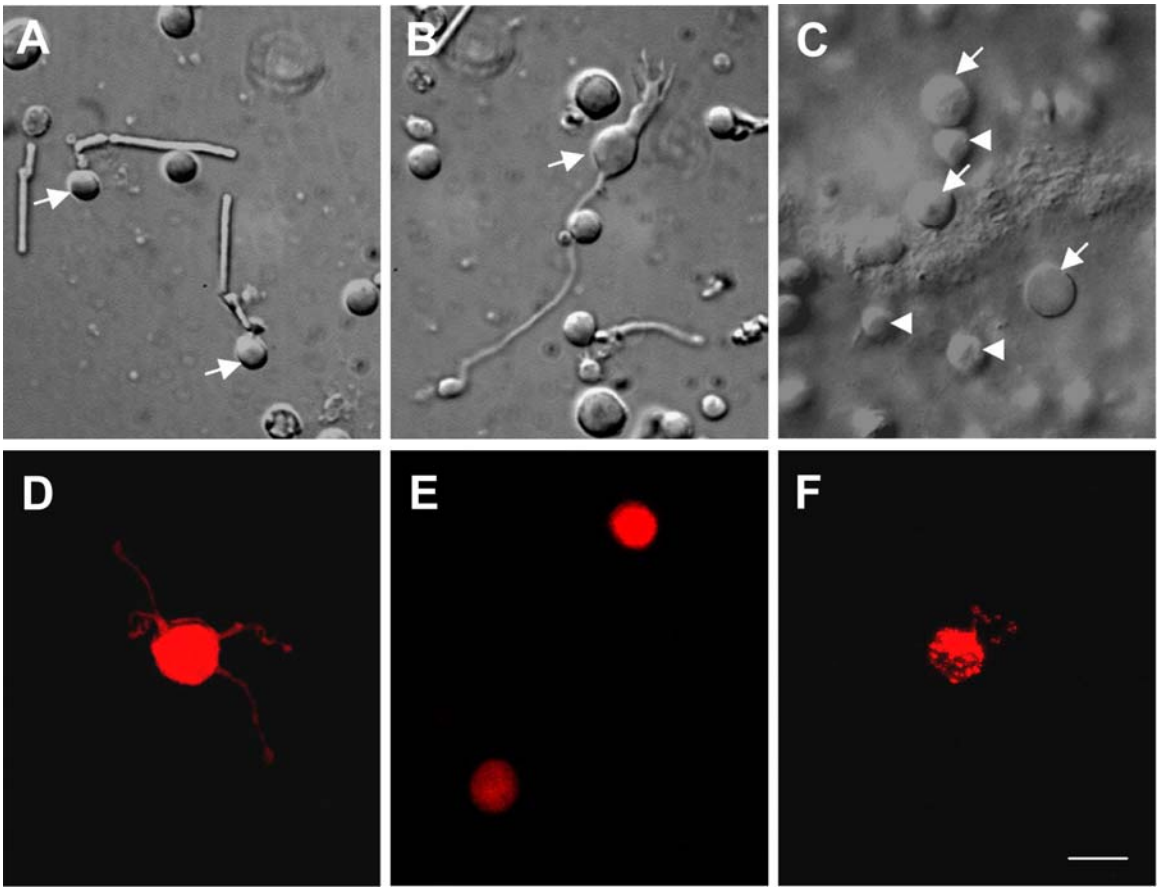

Figure 2-2: 6 types of mouse retinal cells were identified and harvested for RT-PCR.
(A and B) Rods (A; arrows) and rod bipolar cells (B; arrow) were identified in dispersed cultures based on morphology.
(C) Horizontal cells (arrows) were identified and harvested from whole-mount rd mouse retinas. The relatively small surface cells (arrow heads) are either bipolar cells or remaining photoreceptors.
(D and E) DA cells (D) and type 2 CA cells (E) were identified in dispersed cultures of *TH*::RFP transgenic mouse retinas by RFP fluorescence.
(F) Ganglion cells were identified in dispersed cultures by DiI fluorescence following injection and retrograde transport from the LGN. Scale bar = 10 μm.

Cellular contents of identified cells were collected by suction with a patch-type pipette (Figure 2-1), and subjected to RT-PCR for the 6 core clock genes. After harvesting each cell, we also collected medium near the target cell and processed it in parallel to the harvested cell. These medium controls were negative for all 6 core clock genes in all cells tested. Figure 2-3A shows the real-time PCR amplification plot for a representative DA cell. Whereas all clock gene transcripts were detected in this cell, the transcript for the *GABA-δ* receptor subunit, known to be absent in DA cells (Gustincich et al., 1999), was not detected. We initially examined core clock gene expression in 31 rods, 25 horizontal cells, 21 rod bipolar cells, 30 DA cells, 22 type 2 CA cells and 22 ganglion cells. Simultaneous detection of the 6 core clock genes in individual identified neurons yielded 0 rods (0%), 6 horizontal cells (24%), 2 rod bipolar cells (10%), 9 DA cells (30%), 2 type 2 CA cells (9%) and 4 ganglion cells (18%) that expressed all 6 clock genes (Figure 2-3B). The remaining cells of each type expressed 1 to 5 clock genes, except for 10 rods (32%) and 2 horizontal cells (8%) that did not express any core clock genes. In addition, 8 cells identified as cone-like by morphology were collected and the PCR results for them were similar to those obtained for rods. The percentage expression of each clock gene in each neuron type is shown in Figure 2-4. A number of steps were taken to minimize false positives and negatives in the PCR (see Methods).

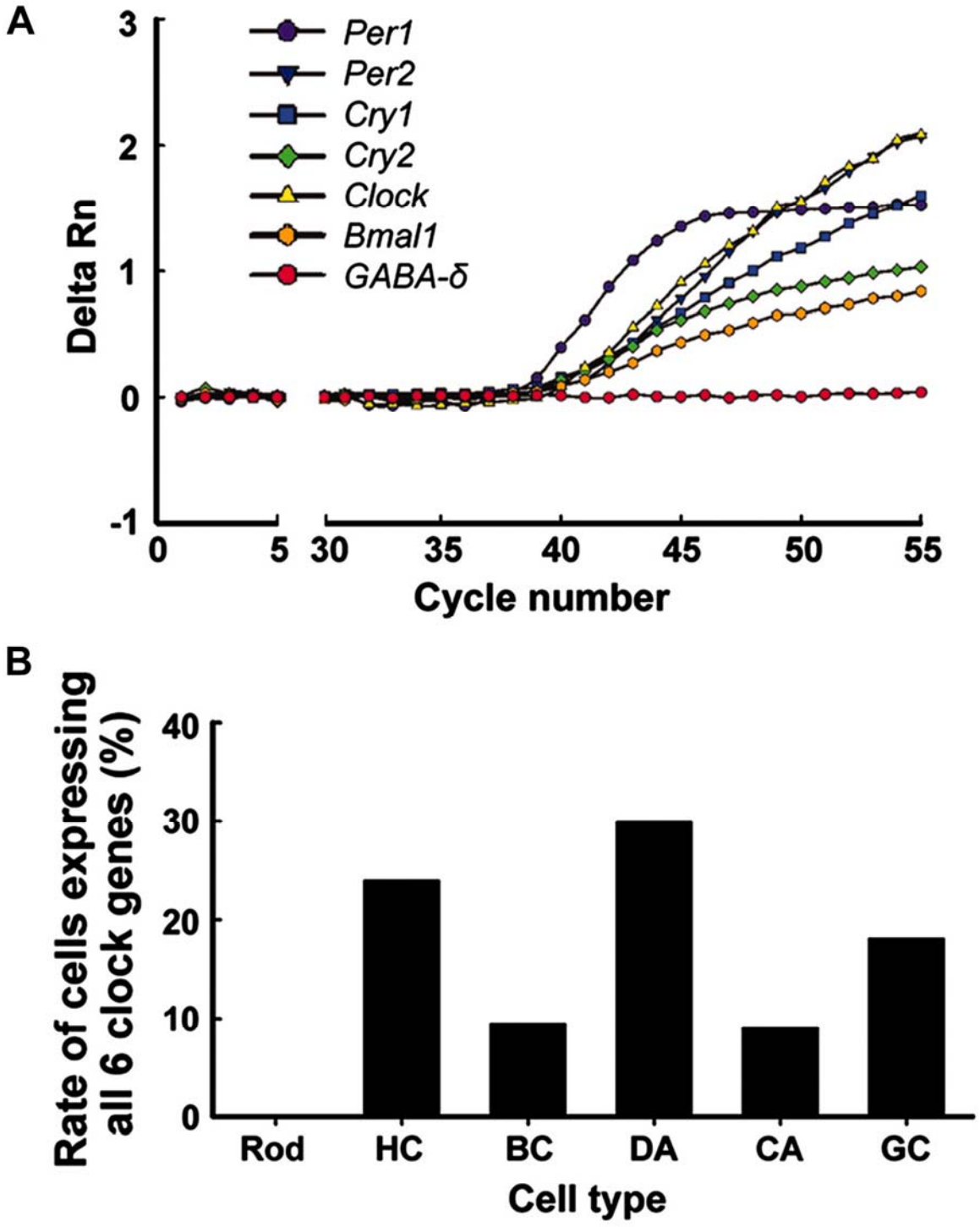

Figure 2-3: Simultaneous detection of all 6 core clock genes in individual retinal neurons.

Figure 2-3: Simultaneous detection of all 6 core clock genes in individual retinal neurons.
(A) Real-time PCR amplification plot of a representative DA cell in which all 6 clock gene transcripts, but not the *GABA-δ* receptor subunit transcript (a negative control gene in DA cells), were detected. Data is presented as the average of duplicate amplifications for each gene. Detection of specific PCR products was monitored as the relative fluorescence (delta Rn) in the intensity of reporter dye.
(B) The percentage of cells expressing all 6 core clock genes in each cell type is shown. Rod, rods; HC, horizontal cells; BC, rod bipolar cells; DA, dopaminergic amacrine cells; CA, type 2 catecholamine amacrine cells; GC, ganglion cells.

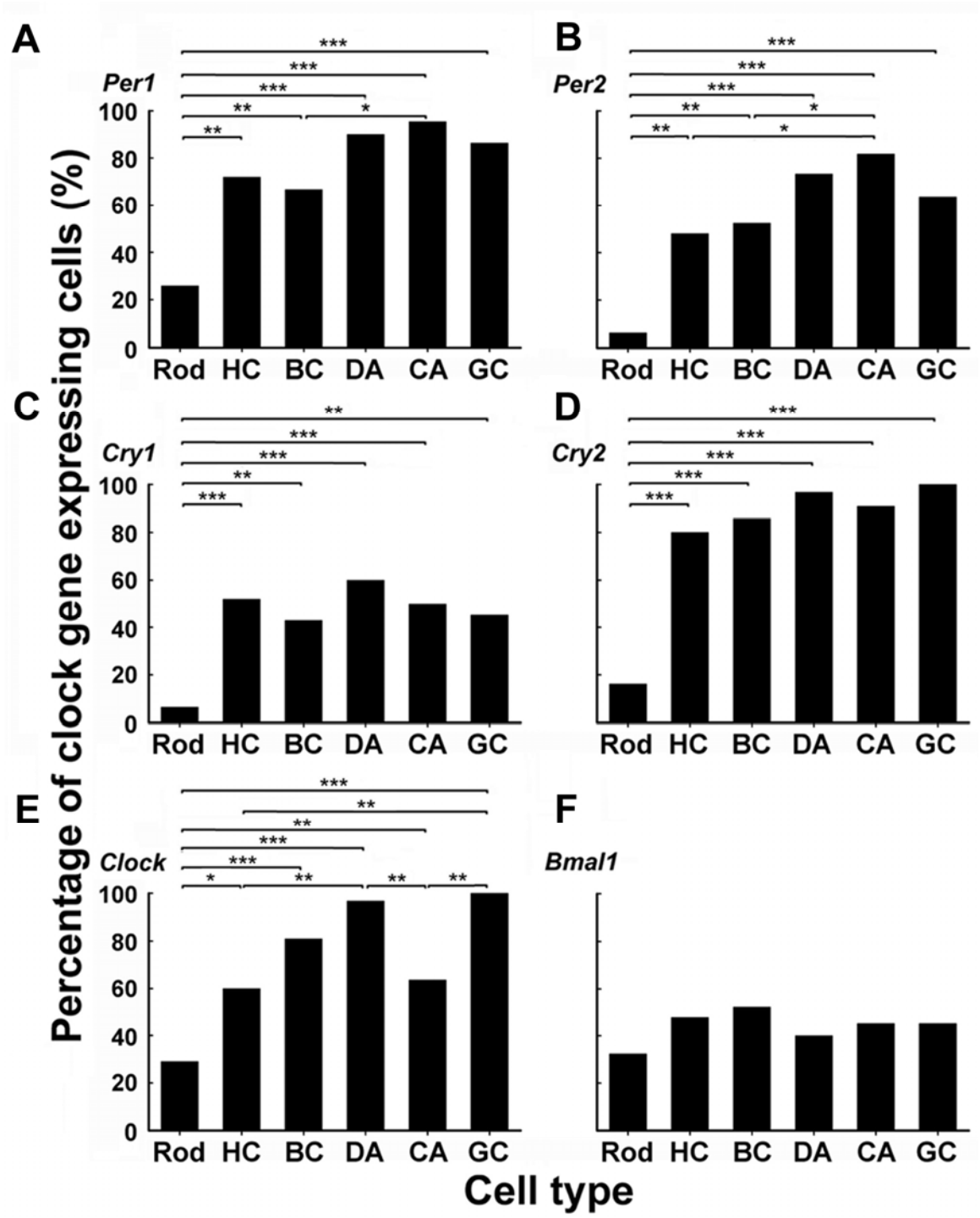

Figure 2-4: Expression rates of 6 core clock genes in 6 types of retinal cells.

Figure 2-4: Expression rates of 6 core clock genes in 6 types of retinal cells.
(A) *Per1* was expressed in > 67% of each retinal neuron type (horizontal, bipolar, amacrine and ganglion) but only in 26% of rods ($P < 0.01$, χ^2 test).
(B-E) Similar as *Per1*, *Per2*, *Cry1*, *Cry2* and *Clock* also showed high rates of expression in other retinal neurons (> 44%) and significantly lower rates of expression (< 28%) in rods ($P < 0.01$).
(F) In contrast to all other clock genes, there was no significant difference between rods and other retinal neurons in the percentage of cells positive for *Bmal1* expression, which ranged from 32-52% across all the cell types tested.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The cells reported in Figures 2-3 and 2-4 were collected at approximately ZT 7-8 during the day phase (4-5 hr before lights off in the mouse colony). To test if time of day has an effect on the detection rate of clock genes, an additional 31 rods and 16 DA cells were individually harvested at ZT 19-20 during the night and core clock gene expression was examined as described above. At this point during the night phase, the *Per* and *Cry* gene transcripts are expressed at lower levels in the whole retina, whereas *Bmal1* is expressed at higher levels (Ruan and McMahon, unpublished data). The detection rates of the core clock genes at ZT 19-20 were similar to those at ZT 7-8 in both neuron types with 0% of rods and 25% of DA neurons exhibiting expression of all 6 core clock genes (Figure 2-5). Thus, the circadian phase does not significantly affect the detection rates of clock genes in retinal neurons using our methods. Cell-to-cell variability in absolute levels of the transcripts precluded using this single-cell RT-PCR approach to quantify potential clock gene rhythms in specific neuron populations. Taken together, our single-cell real-time PCR data demonstrate the presence of potential circadian oscillator neurons among all the major cell types of the inner nuclear and ganglion cell layers of the retina.

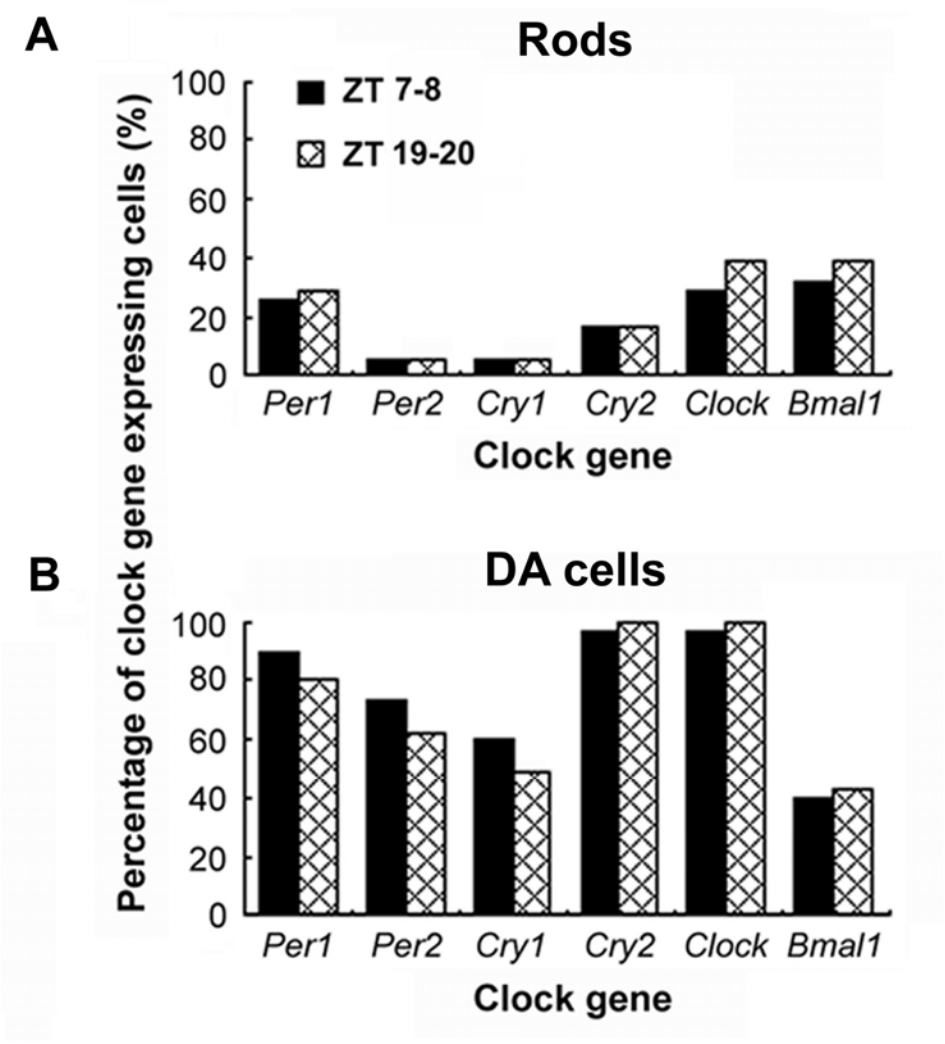

Figure 2-5: Detection of core clock genes in rods and DA cells during the day (ZT 7-8) and night (ZT 19-20).
No significant difference was found in the rates of clock gene expression between ZT 7-8 (■) and ZT 19-20 (⊠) in either rods (*a*) or DA cells (*b*).

rd Mouse Retinas Exhibit Robust Circadian Clock Gene Rhythms

To test whether the clock gene-expressing neurons of the inner nuclear and ganglion cell layers can generate endogenous circadian rhythms, we isolated and cultured retinas from *mPer2*^{Luc} knockin mice, which had been crossed with photoreceptor degenerate C3H mice, to introduce the *rd* photoreceptor degeneration gene. We used retinas from *mPer2*^{Luc} mice which were homozygous for *rd* and were 85 ± 2 days old. At this age, virtually all photoreceptors have degenerated in the retinas of mice homozygous for *rd* (Carter-Dawson et al., 1978; Jimenez et al., 1996; LaVail et al., 1997; Tosini and Menaker, 1998). Retinas were explanted onto membranes and luminescence from the PER2::LUC fusion protein was continuously measured in real-time in constant darkness at 36.8°C. PER2::LUC expression *in vitro* was robustly rhythmic with the peak of PER2::LUC expression occurring at hour 14.28 ± 0.56 of projected Zeitgeber time from the previous LD cycles (Figure 2-6A, n=6 retinas from 3 mice), phase delayed by 2 hr relative to the SCN (Yoo et al., 2004). The period of the retinal rhythms was 22.70 ± 0.20 hr, which is shorter than the period previously reported for the SCN (23.5 hr; Yoo et al., 2004), but is slightly longer than that of the cornea (22.2 hr; Yoo et al., 2004). Most explants were only maintained for 4-5 days, but one culture studied over a longer term exhibited robust circadian rhythms for > 25 days (Figure 2-6B). As with the cultured SCN (Yoo et al., 2004), fresh medium with fresh luciferin substrate, exchanged every 6-7 days, tended to increase the amplitude of the ongoing luminescence rhythms (Figure 2-6B). In contrast to the results with photoreceptor degenerate retinas, PER2::LUC expression in retinas

cultured from *mPer2*^{Luc} knockin mice that were heterozygous for *rd* (i.e. that retained their photoreceptors) only exhibited 1-2 circadian cycles and then damped out (data now shown). This might be due to a suppressive effect of the photoreceptors on gene expression in inner retinal neurons, as has been found in the Royal College of Surgeons (RCS) rat (Sakamoto et al., 2004), or it might be due to non-optimal culture conditions for photoreceptor-retaining retinas.

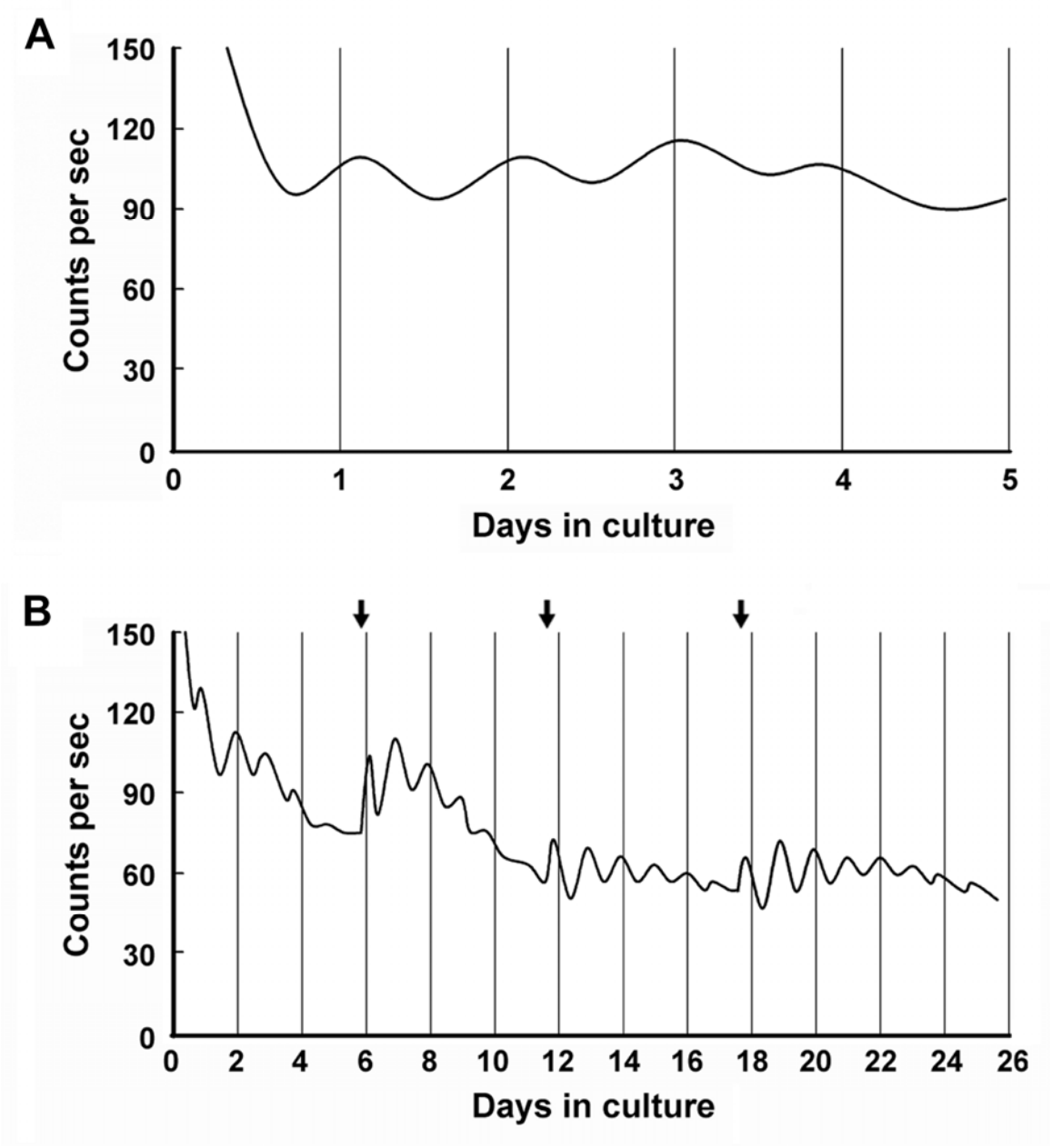

Figure 2-6: Real-time monitoring of PER2::LUC expression in isolated *rd* mouse retinas.
(A) Representative record showing retinal rhythms during the initial 5 days of culture. Retinas were dissected and explanted just before lights off (ZT 12). Vertical lines indicate the projected time of lights off on each day in DD (projected ZT 12). Photon counts per second are plotted against days in culture.
(B) Long-term culture of an *rd* mouse retina showing persistent circadian rhythms in PER2::LUC expression. Arrows indicate times of medium changes.

To further define the temporal expression patterns of all the core clock genes in *rd* mouse retinas, we quantified the rhythmic expression of core clock genes in whole retina RNA using qPCR. Mice 84-112 days of age were maintained in 12:12 LD cycles or in DD and the temporal pattern of clock gene expression in their retinas was sampled at 4 hr intervals. All core clock genes except *Clock* exhibited statistically significant variations in expression level over the 24 hr sampling period consistent with ongoing circadian rhythms in both LD and DD conditions (Figure 2-7). It is noteworthy that these retinal clock gene profiles were statistically significant, but were generally lower in amplitude than those previously reported in the SCN and liver (Yamamoto et al., 2004; Ueda et al., 2005), similar to retinal clock gene rhythms reported for the rat (Namihira et al., 2001; Kamphuis et al., 2005). Peak-trough amplitudes ranged from 2.95-fold for *Per2* in DD to 1.26-fold for *Bmal1* in DD (Figure 2-7 legend). *Per* and *Cry* genes peaked in their expression at or near ZT/CT 10, whereas *Bmal1* peaked in antiphase at or near ZT/CT 22. Peak *Per2* transcript level occurred about 4 hr earlier than the peak PER2 protein level (Figure 2-6, projected ZT 14). We also compared mean clock gene transcript levels between LD and DD conditions. Surprisingly, in *rd* retinas the expression of *Per1* was significantly higher in DD vs. LD ($F = 52.5$; $P < 0.001$; Two-way ANOVA) (Figure 2-7A), indicating a suppressive effect of light cycles on the overall retinal expression of *Per1*, even when the outer retinal photoreceptors had degenerated. Taken together, our whole retina qPCR data further demonstrate the existence of the molecular circadian clockworks in the inner nuclear and ganglion cell layers.

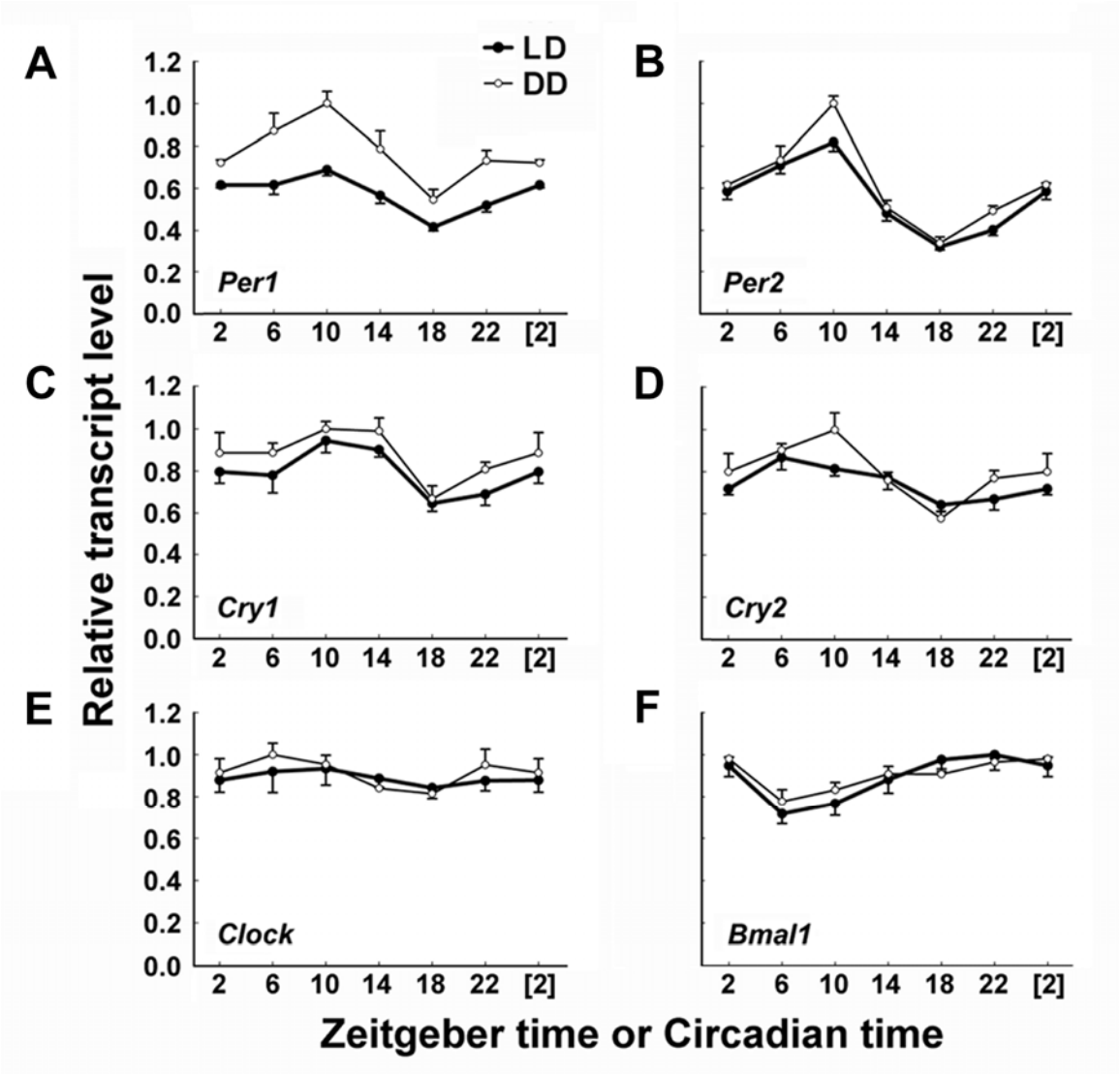

Figure 2-7: Temporal expression profiles of *Per1* (A), *Per2* (B), *Cry1* (C), *Cry2* (D), *Clock* (E), and *Bmal1* (F) in *rd* mouse retinas under LD (●) and DD (○) conditions. Data are presented as means ± SEM from 5 mice and expressed as a percentage of the maximum mean expression. Points at ZT/CT [2] are duplicates of ZT/CT 2 replotted to show 24 hour trends. One-way ANOVA revealed $P < 0.001$ for *Per1* and *Per2* under both LD and DD conditions, $P < 0.01$ for *Cry1* and *Bmal1* under both LD and DD conditions, $P < 0.05$ for *Cry2*/LD and $P < 0.001$ for *Cry2*/DD. For *Per1*, *Per2*, *Cry1*, *Cry2* and *Bmal1* expression, the peak-trough amplitudes under LD conditions were 1.65-, 2.54-, 1.46-, 1.35- and 1.39-fold, respectively, while under DD conditions they were 1.84-, 2.95-, 1.50-, 1.73- and 1.26-fold, respectively.

Discussion

Our examination of the circadian organization of the mouse retina using cell-type specific and temporal gene expression profiling of the core circadian clock genes, as well as real-time gene expression reporting, resulted in two main findings. First, coordinate expression of the 6 core circadian clock genes occurs in individual neurons from all the major cell types of the inner nuclear and ganglion cell layers of the retina, but not in photoreceptors, and second, these clock gene-expressing neurons of the inner retinal layers comprise a self-sustained circadian clock that can generate rhythmicity independent of the master SCN clock and of photoreceptors. These results support a novel paradigm for circadian organization of the retina in which retinal neurons other than rod or cone photoreceptors, are a primary locus of endogenous circadian rhythms generation.

Circadian Clocks in the Inner Nuclear and Ganglion Cell Layers

Individual horizontal, rod bipolar, DA amacrine, CA amacrine, and ganglion cells were found to coordinately express the core clock genes at varying rates according to cell-type. Thus, retinal neurons harboring the molecular genetic basis for endogenous rhythms generation are widely distributed among the inner nuclear and ganglion cell layers of the mammalian retina. Of note, is that the proportion of putative clock neurons expressing all 6 core clock genes in each of the retinal neuron populations tested was a minority, ranging from 9% in CA amacrines to 30% in DA amacrines. However, we

consider these to be minimum estimates of the proportion of putative clock neurons. The combinatorial nature of our criteria (i.e. simultaneous detection of 6 genes) means that false negatives have a multiplicative effect and even the low false negative rates we have demonstrated will significantly reduce the apparent number of putative clock neurons. Thus, it is likely that the actual proportion of retinal cells expressing sufficient molecular elements to support circadian rhythms generation is somewhat higher than our current results indicate, although the expression rate for *Bmal1* is limiting and effectively caps the maximum possible proportion of putative clock neurons in all cell types in a range of 40-60% (see Figure 2-4). It should be noted that although we have examined selected subpopulations of all the major retinal neuron types, we do not have morphological or genetic markers for all subtypes and thus, there may be additional neuronal subpopulations in the inner nuclear and ganglion cell layers that have circadian pacemaker capability. In addition, it must be recognized that at this point coordinate clock gene expression in horizontal cells has only been demonstrated in *rd* mouse retinas wherein there is the potential for functional reorganization.

Can a minority of pacemaker cells be sufficient to generate rhythms within retinal neuron populations? Recent findings suggest that the SCN circadian clock and the retinal circadian clock may be organized in a similar manner, with minority populations of pacemaker neurons driving high amplitude circadian rhythms throughout the entire neural ensemble. Selected subpopulations of SCN neurons do not express endogenous rhythms in clock genes (Silver and Schwartz, 2005) and disruption of neuropeptide

communication between SCN neurons blunts rhythmicity in many cells, revealing a pacemaker neuron population of 25-30% of SCN neurons in which self-sustained rhythmicity persists (Aton et al., 2005). Thus, by this criterion, the proportion of putative pacemaker neurons in the master SCN clock is similar to the proportions of DA cells, horizontal cells, and ganglion cells which we have identified as putative pacemaker neurons by molecular means. It is likely that these specific subpopulations engender endogenous rhythmicity to the inner retinal layers.

Real-time bioluminescence gene expression reporting in isolated *mPer2*^{Luc} knockin *rd* mouse retinas demonstrated that the neurons of the inner nuclear and ganglion cell layers comprise a functional circadian clock in isolation from the SCN master clock and in the absence of virtually all rods and cones. These results also demonstrate that inner retinal circadian molecular rhythms persist in the absence of melatonin rhythms since *rd* mouse retinas exhibit low, arrhythmic melatonin levels (Tosini and Menaker, 1998). Because a few cones may have remained in the *rd* retinas we tested (Carter-Dawson et al., 1978; Jimenez et al., 1996; LaVail et al., 1997), we cannot rigorously conclude, based on this experiment alone, that the observed PER2 expression rhythms were completely endogenous to the inner nuclear and ganglion cell layers. However, previous *in situ* hybridization experiments have demonstrated low levels of clock gene expression in the mammalian photoreceptor layer (Gekakis et al., 1998; Miyamoto and Sancar, 1998; Namihira et al., 2001; Thompson et al., 2003; Witkovsky et al., 2003), and our single-cell RT-PCR studies failed to find a single photoreceptor cell out of 70 tested, which

expressed the full complement of core clock genes. Thus, mammalian photoreceptors show a relative lack of molecular clock elements and they are, on this basis, unlikely to be a locus of endogenous rhythms generation. In this regard, a recent study found temporal variations in the expression level of mRNA for retinal arylalkylamine *N*-acetyltransferase (AANAT), a key enzyme in melatonin synthesis, consistent with ongoing circadian rhythms following lesion of the inner retina, suggesting the possibility of photoreceptor clock function (Sakamoto et al., 2006). However, neural mechanisms for SCN influence of mammalian retinal rhythms have been described (Brandenburg et al., 1981) and these *in vivo* sampling studies, limited to one 24 hr interval, did not address whether the AANAT variations were indeed endogenous rhythms that persist independent of the influence of the master SCN clock. Our data do not completely exclude the possibility of photoreceptor clock function, as it is possible that there is small number of photoreceptors expressing the full complement of clock genes that escaped our detection. However, when taken together, the lack of demonstrated coordinate expression of clock genes in photoreceptors, the lack of evidence for endogenous photoreceptor rhythmicity independent of both the inner retina and the SCN, along with the coordinate clock gene expression in more inner retinal neurons, as well as the persistent *in vitro* circadian rhythmicity from inner retinal neurons in *rd* retinas form convergent lines of evidence suggesting that neurons of the inner nuclear and ganglion cell layers constitute an endogenous circadian pacemaker within the mammalian retina.

Clock Genes in Photoreceptors

Our results do not provide support for the proposition that mammalian photoreceptors contain endogenous circadian clocks, but they do demonstrate a limited expression of clock genes in these cells. Each of the 6 clock genes was indeed detected within the population of photoreceptor cells we sampled and the genes exhibited a range in detection rate with *Per2* detected in about 10% of photoreceptors and *Bmal1* detected in about 35% of photoreceptors (Figure 2-4). Despite the demonstrated presence of clock genes in photoreceptors, consistent with previous *in situ* hybridization studies (Gekakis et al., 1998; Miyamoto and Sancar, 1998; Namihira et al., 2001; Thompson et al., 2003; Witkovsky et al., 2003), coordinate expression of all six core clock genes was never detected. The great majority of photoreceptor cells sampled was morphologically identifiable as rods, but based on a more limited sample, cones, like rods, showed similar low frequency expression of clock genes and none showed coordinate expression of all six core clock genes. We must be more cautious in interpreting the cone results since the morphological identification of mouse cones is less certain. It is likely that the clock genes expressed in photoreceptors serve non-clock functions. For example, *Clock* and *Bmal1*, which are expressed together in about 10% of rods, encode CLOCK and BMAL1 transcription factors that form heterodimers for activating the transcription of E-box containing genes, such as AANAT. However, since the full complement of required negative elements of the circadian clockworks (*Per* and *Cry* genes) are missing from these cells, it is unlikely that they can form a viable, cell autonomous circadian feedback

loop. Thus, our data are consistent with the notion that clock genes expressed in mouse photoreceptors may play a role in regulation of gene expression, but that circadian rhythmicity in these cells is likely to be driven by exogenous signals.

Circadian Organization of Vertebrate Retinas

Our results support a model for circadian organization of mammalian retinas distinct from the current prevailing paradigm based primarily upon data from amphibian and avian retinas. In amphibian and avian retinas, the photoreceptors are endogenous circadian oscillators that mediate rhythmic melatonin release (Cahill and Besharse, 1993; Pierce et al., 1993; Hayasaka et al., 2002; Ivanova and Iuvone, 2003b) and express high levels of the core clock genes (Zhu et al., 2000; Zhuang et al., 2000; Zhu and Green, 2001; Bailey et al., 2002; Haque et al., 2002), whereas in the mouse retina, clock genes and clock function have become more localized to the inner retinal layers, but melatonin secreted by the photoreceptors may still serve as an important circadian output signal. While a previous study demonstrated a lack of circadian rhythmicity of melatonin synthesis in isolated C3H *rd* mouse retinas (Tosini and Menaker, 1998), our results show that endogenous molecular rhythms generation in retinal neurons is intact after photoreceptor degeneration. In addition, cultured RCS rat retinas with photoreceptor degeneration exhibit circadian rhythms of melatonin release mediated by the inner retina (Sakamoto et al., 2004), and dopamine content varies between subjective night and subjective day even in retinas sampled from populations of photoreceptor degenerate

RCS rats (Doyle et al., 2002a) and melatonin-less mice (Nir et al., 2000), which are consistent with a photoreceptor-independent retinal circadian oscillator in mammals.

There is extensive influence upon photoreceptor function and gene expression of diffusible signals from inner retinal neurons. Basic photoreceptor functions that are under circadian control, such as disk shedding, melatonin secretion and AANAT gene expression, as well as Na^+/K^+ ATPase function, are regulated by the inner retinal circadian signal dopamine (LaVail, 1976; Nguyen-Legros et al., 1996; Shulman and Fox, 1996; Tosini and Menaker, 1996), acting on mouse cones through D4 dopamine receptors (Cohen et al., 1992). Dopamine even influences photoreceptor survival in *rd* mouse retinas (Ogilvie and Speck, 2002). Dopamine is secreted rhythmically in the retina and dopaminergic amacrine neurons, as a population, have previously been shown to exhibit circadian rhythms in the transcription of the clock gene *Per1* (Witkovsky et al., 2003), as well as to contain the clock gene products PER1, CRY1, CRY2, CLOCK and BMAL1 (Gustincich et al., 2004). Our results specifically co-localize the core components of the molecular circadian clockworks within individual DA neurons, defining their potential for endogenous circadian oscillation and further strengthening the inference that they are clock neurons. In addition, nitric oxide (NO) and GABA are potential circadian neurotransmitters within the retina, based on clock gene rhythmicity in inner retinal neuron subpopulations. Nitric oxide synthase expressing amacrine cells rhythmically express the clock gene *Per1* under LD and DD conditions (Zhang et al., 2005), and thus are also candidate circadian pacemaker neurons. Both DA and NO amacrines are also

GABAergic. Whether NO and GABA, like dopamine and melatonin, are rhythmically secreted in the retina remains to be determined.

Summary

In summary, putative circadian clock neurons that express the core clock genes necessary for circadian rhythms generation are found in all the major neuron populations of the mammalian retina, except in photoreceptors. In addition, following photoreceptor degeneration, retinal neuron populations continue to express endogenously generated molecular circadian rhythms. These findings provide an expanded basis for the study of mammalian retinal rhythms by localizing putative pacemaker neurons to the inner nuclear and ganglion cell neuronal layers, suggesting that there are likely many local pacemaker neurons embedded within the full spectrum of retinal circuits that could influence retinal physiology and function, photoreceptor survival and eye growth, through endogenous rhythms generation and neurotransmitter secretion. Cell communication is important for rhythms production in the neural circadian pacemakers of the mammalian and fly brains (Nitabach et al., 2002; Aton et al., 2005). The highly ordered and well characterized nature of retinal circuitry will likely facilitate elucidation of the general principles of neuronal communication in circadian pacemaking.

CHAPTER III

A CIRCADIAN CLOCK IN THE INNER RETINA REGULATED BY DOPAMINE

Introduction

The vertebrate retina is both a sensory organ and an endogenous circadian clock. As the locus of visual phototransduction, the retina initiates many organismal responses to light, and as a circadian clock, the retina gates the sensitivity of visual behaviors and physiology. The retinal clock, and its neuromodulatory outputs melatonin and dopamine, bias retinal function toward night and day states respectively (Manglapus et al., 1999; Green and Besharse, 2004; Iuvone et al., 2005), and drive endogenous rhythms in photoreceptor disc shedding (LaVail, 1976; Grace et al., 1996), melatonin release (Besharse and Iuvone, 1983; Tosini and Menaker, 1996, 1998), dopamine synthesis (Doyle et al., 2002b; Doyle et al., 2002a), ERG b-wave amplitude (Brandenburg et al., 1983), extracellular pH (Dmitriev and Mangel, 2001), visual sensitivity (Li and Dowling, 1998), rod-cone balance (Wang and Mangel, 1996; Manglapus et al., 1999), and intraocular pressure (Rowland et al., 1981). In mammals, the retina is the sole site for circadian phototransduction, and its output modifies the rhythmicity, period, and developmental organization of the central biological clock, the suprachiasmatic nucleus (SCN) of the hypothalamus (Yamazaki et al., 2002; Lee et al., 2003; Ohta et al., 2006). In addition, the mammalian retinal clock and its outputs influence trophic processes in the

eye including the susceptibility of photoreceptors to degeneration from light damage (Organisciak et al., 2000), photoreceptor survival in animal models of retinal degeneration (Ogilvie and Speck, 2002), and the degree of refractive errors in primate models of myopia (Iuvone et al., 1991). Despite the widespread influence of the mammalian retinal circadian clock on retinal, visual and circadian function, the cellular elements and mechanisms comprising the retinal pacemaker remain to be elucidated. Mammalian photoreceptor degenerate retinas and isolated photoreceptor layers can express circadian rhythms in gene expression (Ruan et al., 2006; Tosini et al., 2007b). Thus, it is important to identify in intact mammalian retinal circuits the loci of endogenous circadian rhythms generation.

Melatonin and dopamine are mutually inhibitory neurochemical outputs of the retinal clock, which are rhythmically released by photoreceptors and dopaminergic amacrine cells respectively (Witkovsky and Schutte, 1991; Cahill and Besharse, 1993; Doyle et al., 2002b). Melatonin and dopamine rhythms have served as the principal experimental measures of the retinal circadian clock, and previous models of retinal circadian organization have postulated that communication through these transmitters is critical for overall retinal rhythmicity (Tosini and Menaker, 1996; Doyle et al., 2002b; Doyle et al., 2002a; Green and Besharse, 2004; Iuvone et al., 2005). In fish, avian, and mammalian retinas, rhythmic melatonin levels are necessary to sustain retinal circadian rhythms of dopamine synthesis and metabolism, but not vice versa (Doyle et al., 2002b; Ribelayga et al., 2004; Megaw et al., 2006). However, these neurochemical rhythms assays of clock

output leave open the questions of whether dopamine or melatonin modulate the retinal molecular clock mechanism itself and what roles they may play in organization of the retinal clockworks.

Circadian clocks in mammalian tissues generate molecular circadian rhythms through coupled transcription/translation feedback loops, in which the positive gene elements *Clock* and *Bmal1* interact with the negative gene elements *Period* (*Per*) *1* and *2*, and *Cryptochrome* (*Cry*) *1* and *2* (Reppert and Weaver, 2002; Green and Besharse, 2004). In the current study, we developed an *in vitro* retinal explant culture protocol to monitor circadian rhythms in clock gene expression in intact retinas of adult mice, which harbor a PERIOD2::LUCIFERASE (PER2::LUC) fusion protein transgene as a real-time reporter of circadian gene dynamics (Yoo et al., 2004). Using this protocol, we studied the properties of retinal molecular rhythms *in vitro*, the location within the retina of rhythms generation and the influence of melatonin and dopamine on retinal molecular rhythms. Our findings reveal an inner nuclear layer (INL) retinal circadian clock that generates endogenous molecular rhythms independent of melatonin and which is regulated in its phase by dopamine.

Materials and Methods

Animals

mPer2^{Luc} knockin mice, which initially were on a 129SvEv X C57BL/6J genetic background (Yoo et al., 2004), were maintained as a continuous backcross to C57BL/6J for 13 generations. The resulting *mPer2*^{Luc} mice were crossed with C3H *rd* mice (The Jackson Laboratory, Bar Harbor, ME) to produce *mPer2*^{Luc} mice that are heterozygous for *rd* gene and genetically capable of producing melatonin. All animal use was conducted in accordance with the guidelines of the Vanderbilt University Animal Care Division and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal Explant Cultures

mPer2^{Luc} mice 28-60 days of age were killed at approximately Zeitgeber Time (ZT) 4 during the day phase (4 hr after lights on in the mouse colony). Eyes were enucleated and placed in Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA). Retinas were isolated and cut into two pieces. Each piece of retina was placed with the ganglion cell layer up on Millicell culture membrane (Millipore, Billerica, MA) and gently flattened with two end-blunted glass pipettes. The membrane was first transferred to 1.0 ml neurobasal medium (Invitrogen) supplemented with 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 2% B27 (Invitrogen), 25 units/ml penicillin and 25 μg/ml streptomycin

(Invitrogen), incubated in 5% CO_2 incubator at 37°C for 24 hr, and then transferred to 1.0 ml medium 199 (Sigma-Aldrich) supplemented with 0.7 mM L-glutamine, 4 mM sodium bicarbonate (Sigma-Aldrich), 10 mM Hepes (Sigma-Aldrich), 20 mM D-glucose (Sigma-Aldrich), 2% B27, 0.1 mM beetle luciferin (Promega, Madison, WI), 25 units/ml penicillin and 25 μg/ml streptomycin. Bioluminescence was measured with a LumiCycle (Actimetrics, Wilmette, IL). Cultures were maintained at 37°C. ClockLab software (Actimetrics) was used to determine the peak time (maximum phase) of PER2::LUC rhythms from the raw data, which was used as our phase marker.

Immunocytochemistry

Retinal explant wholemounts were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA), and blocked with 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in 0.1 M PBS for 2 hr, and then treated with primary antibody1:500 sheep anti-tyrosine hydroxylase (Chemicon, Temecula, CA) for 48 hr. After rinsing, a secondary incubation was performed for 2 hr with 1:500 Alexa Fluor 488 donkey anti-sheep IgG (Molecular Probes, Eugene, OR). Samples were mounted and cover slipped using Vectashield (Vector Laboratories, Burlingame, CA) and then visualized using Zeiss LSM5 PASCAL confocal microscopy (Carl Zeiss, Thornwood, NY).

Vertical Retinal Slice Imaging

Freshly isolated retinas were attached tightly to Millipore membrane filters, with the ganglion cell layer facing filters, sliced into 150-μm-thick sections with a tissue slicer (Stoelting, Wood Dale, IL), transferred onto Millicell membrane inserts and cultured with 1.0 ml neurobasal medium, and then incubated in 5% CO_2 incubator at 37°C for 1-2 days. Immediately before imaging, 0.1 mM beetle luciferin was added to the culture medium. Images of 30 min exposure duration were collected using a 20x objective (NA 0.35) coupled directly to a PIXIS camera system (Princeton Instruments, Trenton, NJ) cooled to -70°C. Signal-to-noise ratio was improved by 2x2 binning of pixels. Data were analyzed with Metamorph software (Molecular Devices, Sunnyvale, CA).

Results

PER2::LUC Rhythms from Intact Mouse Retinas

To study the retinal circadian clock in the intact retina, *mPer2*^{Luc} mice congenic on the C57BL/6J background, in which melatonin levels are greatly reduced due to point mutations in synthetic enzymes for melatonin (Ebihara et al., 1986; Doyle et al., 2002b), were crossed to C3H *rd*/*rd* mice to obtain F1 hybrid B6C3 mice. These mice have one wild type allele at the melatonin and *rd* loci, synthesize melatonin and do not undergo photoreceptor degeneration. When retinal explants from B6C3 F1 mice were cultured under standard conditions (medium 199 or DMEM, air, 37°C), they exhibited only low amplitude PER2::LUC oscillations (Figure 3-1). However, when retinal explants were first cultured in neurobasal medium in 5% CO_2 at 37°C for 24 hr *in vitro*, and subsequently transferred to medium 199 as before, PER2::LUC expression was robustly rhythmic for numerous circadian cycles (Figure 3-2A). Peak-to-trough amplitude typically increased on the second cycle in medium 199, after which the amplitude gradually decreased over 9-10 cycles (Figure 3-2A) with a damping rate of 2.5-4 cycles/e-fold decrease in amplitude. The first peak of PER2::LUC expression occurred at hr 14.81 ± 0.29 of projected Zeitgeber Time (i.e. 2.81 hrs after the time of lights off occurred on the light/dark cycle of the mouse colony; mean ± SEM; n = 11). This was essentially identical to the phase of PER2::LUC rhythms in photoreceptor degenerate retinas (Ruan et al., 2006), and phase delayed by 2 hr relative to the peak times of SCN

PER2::LUC rhythms (Yoo et al., 2004). As with the cultured SCN (Liu et al., 2007), a media change partially restored the amplitude of luminescence rhythms (Figure 3-2C). With media changes every 10 days, retinal rhythms could be readily sustained for a month or more (Figure 3-2C).

To control for whether bioluminescence light emission produced by the PER2::LUC fusion protein influenced retinal rhythms, we performed assays in which luciferin substrate was absent from the medium, and thus, there was no retinal light emission for the first two circadian cycles *in vitro*. Luciferin (0.1 mM) was then added to the medium of experimental explants, and an equal volume of vehicle was added to the medium of control explants at the beginning of the rising phase of the 3rd cycle *in vitro*. The delayed addition of luciferin revealed rhythms of PER2::LUC luminescence in the experimental explants that were similar in amplitude and peak time to the ongoing rhythms of control explants (n = 4; Figure 3-2B), indicating that bioluminescence light emitted by the PER2::LUC reporter in these retinas did not influence rhythms generation in our *in vitro* assay.

Histological examination of vertical slices from cultured retinas confirmed that all retinal layers were intact in these preparations, including the photoreceptor cell bodies and outer segments (Figure 3-2D). In addition, immunocytochemistry (ICC) with an antibody to tyrosine hydroxylase, the rate-limiting enzyme of dopamine synthesis, confirmed that cultured retinal explants retained a normal complement of dopamine neurons (Figure 3-2E).

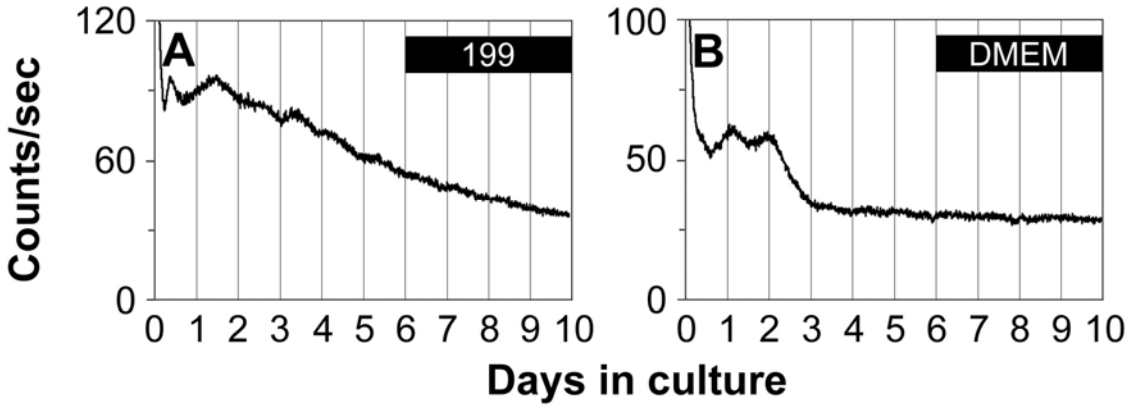

Figure 3-1: Mouse retinas show low amplitude PER2::LUC oscillations when directly cultured in medium 199 (A) or DMEM (B).

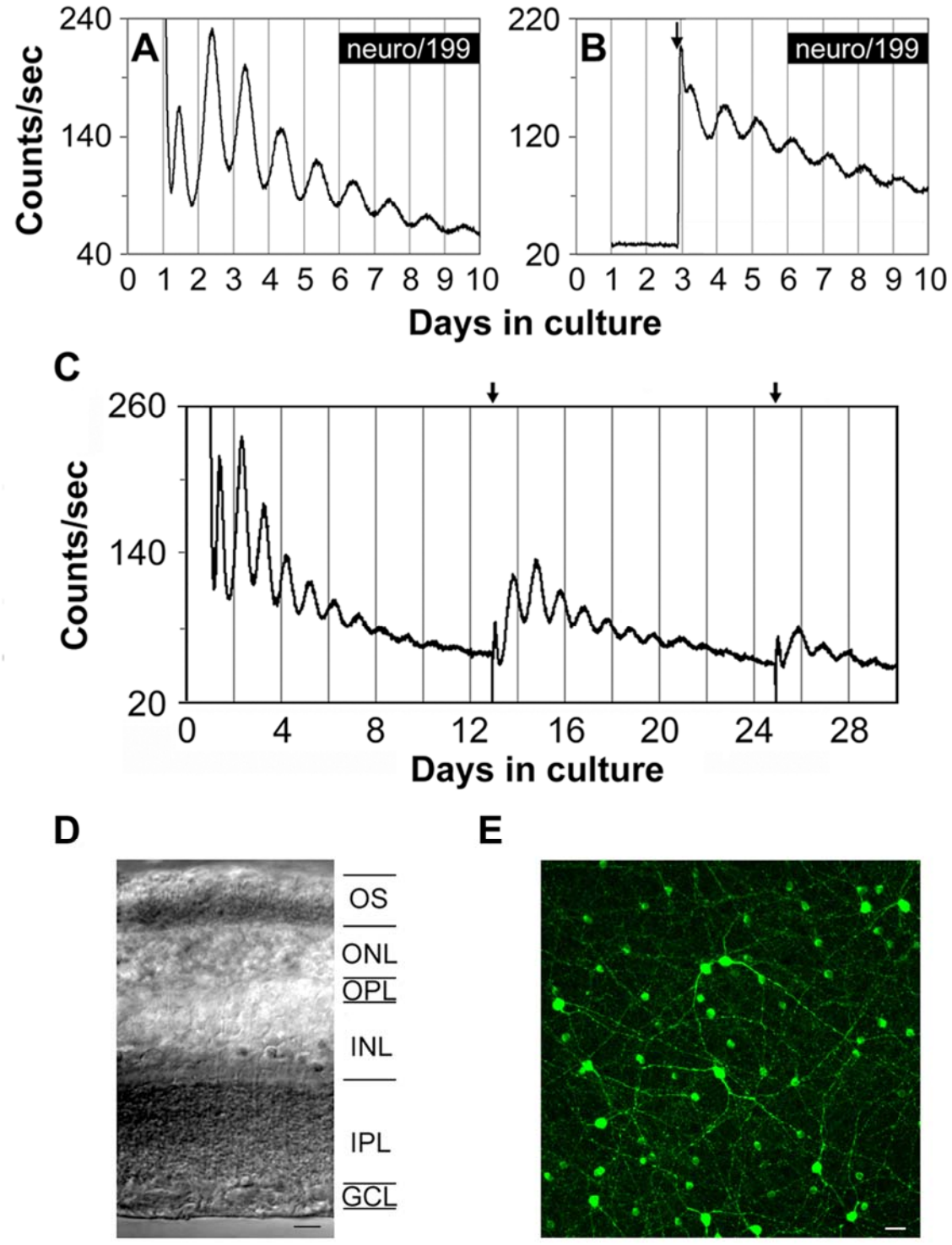

Figure 3-2: Bioluminescence rhythms from *mPer2*^{Luc} mouse retinal explants.

Figure 3-2: Bioluminescence rhythms from *mPer2*^{Luc} mouse retinal explants.
(*A*) A representative PER2::LUC bioluminescence trace recorded from a B6C3 *mPer2*^{Luc} mouse retinal explant.
(B) A representative PER2::LUC bioluminescence trace recorded from a mouse retinal explant in which luciferin substrate was absent from the medium in the first two cycles. Arrow indicates addition of luciferin.
(C) Long-term culture of an intact mouse retinal explant showing persistent circadian rhythms in PER2::LUC expression. Arrows indicate times of media changes.
(D) Shown is a representative DIC image of vertical retinal sections. Retinal explants were cultured for 9 days *in vitro* (DIV), and vertical retinal slices were cut with a tissue slicer at 150 μm. OS, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 20 μm.
(E) Flat-mount view showing tyrosine hydroxylase immunoreactivity in retinal explants that were cultured for 9 DIV. The immunoreactive cells with relatively large somata and 2 to 3 thick primary processes arose from the cell body are dopaminergic amacrine cells, whereas the immunoreactive cells with relatively small cell body and very few processes are type 2 catecholamine amacrine cells. Scale bar, 20 μm.

Circadian Oscillation of PER2::LUC Bioluminescence in the Inner Nuclear Layer

Multiple approaches, including *in situ* hybridization, ICC and single-cell RT-PCR, have established that the *Per2* clock gene is expressed, with varying levels and frequencies, in all the major subtypes of retinal neurons (Namihira et al., 2001; Ruan et al., 2006; Storch et al., 2007). Thus all retinal cell subtypes could potentially contribute to the tissue-level PER2::LUC luminescence rhythms recorded in our assays. To localize the PER2::LUC signal within the retina, we imaged vertical retinal slices for both bright-field and luminescence with a cooled CCD camera. The bioluminescence signal was concentrated in the mid-retina, in the inner nuclear layer which contains the nuclei of the horizontal, bipolar and amacrine cells, with low levels of luminescence detected in the photoreceptor and the ganglion cell layer (Figure 3-3A). To confirm that inner retinal PER2::LUC is rhythmic, populations of retinal slices maintained in constant culture conditions were sampled by imaging at 6 hr intervals (n = 2 independent runs; Figure 3-3B). PER2::LUC bioluminescence in the inner nuclear layer exhibited a circadian variation with a peak at projected ZT 8-14, close to the peak time observed in whole retinal explant preparations. In addition, individual retinal slices subject to time-lapse imaging for 48 hrs showed similar circadian rhythms in PER2::LUC bioluminescence emanating from the inner nuclear layer (n = 3 independent runs; Figure 3-3C). Similar inner nuclear layer localization of PER2::LUC expression was observed in retinas from C57BL/6J mice as well (data not shown). This indicates that the primary source of rhythmic PER2::LUC signals in our retinal rhythms assay is the inner nuclear

layer, with minor contributions from the other retinal layers.

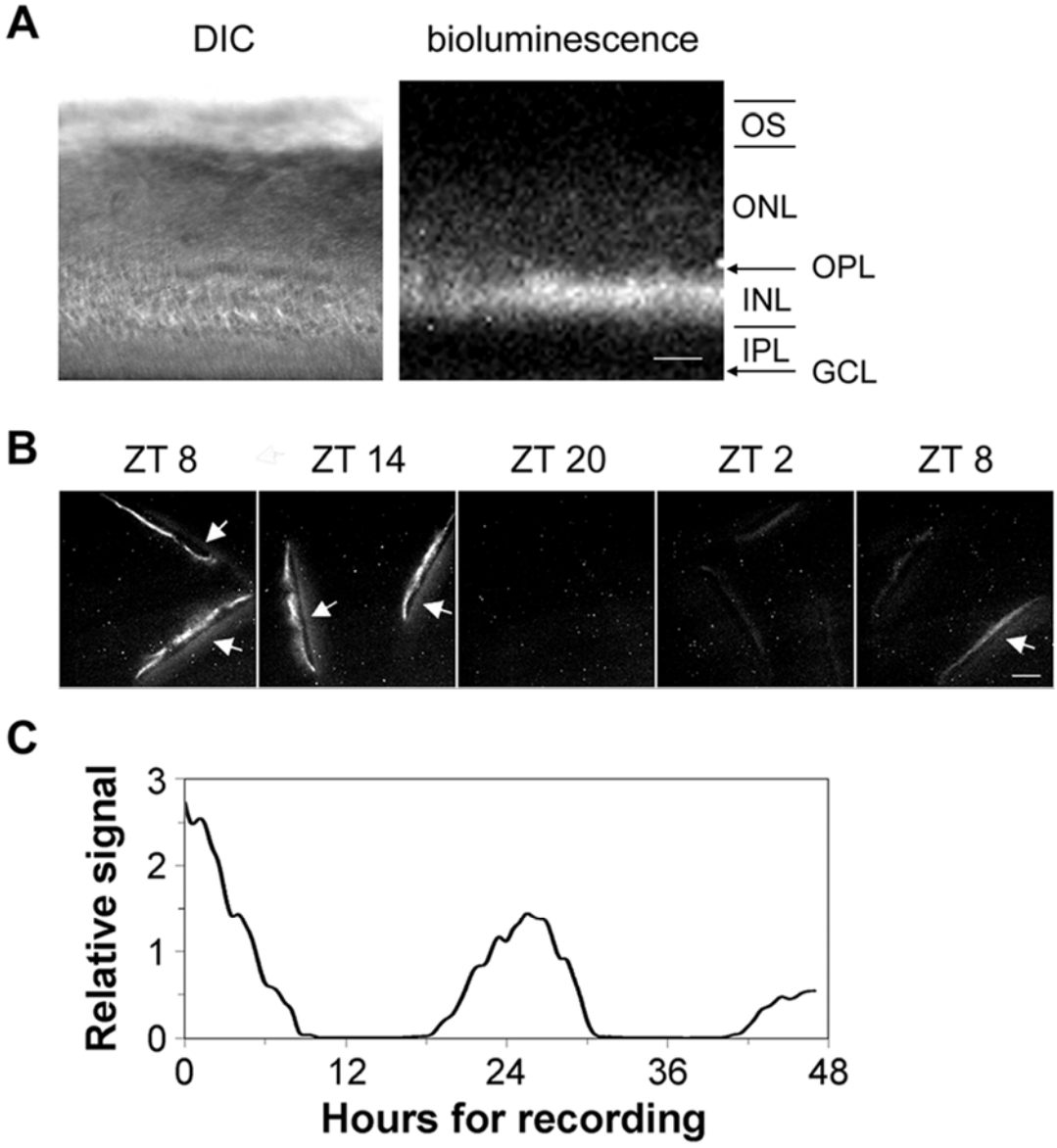

Figure 3-3: Circadian oscillation of PER2::LUC bioluminescence in the inner nuclear layer.

Figure 3-3: Circadian oscillation of PER2::LUC bioluminescence in the inner nuclear layer.

(A) Shown are DIC image (left panel) and luminescence image (right panel) of a vertical retinal slice. Retinal slices were prepared around ZT 4, incubated in CO_2 incubator for 1 day and then imaged. Note the bioluminescence signals were primarily located in the inner nuclear layer. OS, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 50 μm.

(B) PER2::LUC bioluminescence in the inner nuclear layer of cultured vertical retinal slices showed a circadian variation with a peak at projected ZT 8-14. Shown are representative images acquired by 30-min exposures at 6 hr intervals. Arrows point to membrane filters that became visible when signals in the inner nuclear layer were high. Scale bar, 100 μm.

(C) Continuous recording of PER2::LUC bioluminescence in a representative cultured vertical retinal slice. In this experiment, retinal slices were prepared around ZT 4, incubated in CO_2 incubator for 2 days, and then images (30 min exposure) were continuously collected for 47 hrs, starting at projected ZT 9.

Retinal Rhythms Generation Is Independent of Melatonin

Melatonin is a key circadian neurotransmitter in the retina and throughout the vertebrate circadian system (Cassone, 1990). To determine whether melatonin signaling is required to maintain retinal rhythmicity in clock gene expression, we assayed luminescence rhythms from retinal explants derived from *mPer2*^{Luc} C57BL/6J mice in which melatonin synthesis is genetically blunted (Ebihara et al., 1986; Doyle et al., 2002b). Circadian rhythms of PER2::LUC expression in C57BL/6J retinas were similar in their sustained nature to those of B6C3 F1 retinas, which have wild-type alleles for melatonin synthetic enzymes (n = 6; Figures 3-4A and 4B). Similarly, B6C3 retinas, treated with continuous application of 10 nM melatonin or continuous blockade of melatonin MT1 receptors with 5 μM luzindole, also exhibited robust circadian gene expression rhythms (n = 6 for each; Figures 3-4C and 4D). These results, taken together with previous results in which retinal molecular rhythms persist in photoreceptor degenerate retinas (Ruan et al., 2006), indicate that the mouse retina requires neither normal levels of melatonin nor melatonin rhythmicity to generate and sustain molecular circadian rhythms.

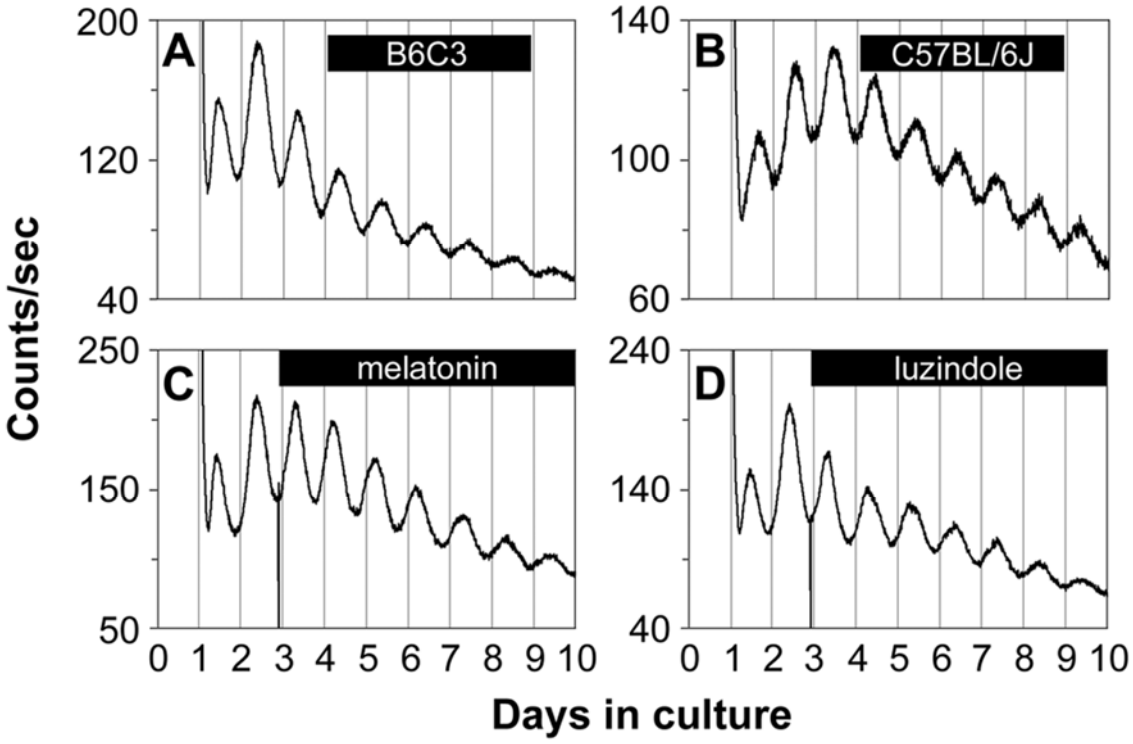

Figure 3-4: Generation of retinal PER2::LUC rhythms is independent of melatonin.
(A) *mPer2*^{Luc} retinal explants cultured from B6C3 mice exhibited sustained circadian rhythms in PER2::LUC expression.
(B) *mPer2*^{Luc} retinal explants cultured from C57BL/6J mice also showed robust circadian oscillation of PER2::LUC expression.
(C-D) Circadian PER2::LUC expression rhythms persisted upon continuous application of melatonin (10 nM; C) or the melatonin MT1 receptor antagonist luzindole (5 μM; D). Bars indicate the duration of treatment.

Dopamine Regulates the Phase of Retinal Rhythms

Next, we examined whether dopamine influences molecular rhythms of the mouse retinal clock. Manipulation of dopaminergic neurotransmission produced perturbations in the waveform of retinal PER2::LUC rhythms at the onset of treatments with dopaminergic agonists, but did not damp or disrupt rhythms generation (Figures 3-5A and 5B). To test the effects of dopamine on retinal phase, we applied dopaminergic reagents in a step protocol beginning at different circadian times on the 2nd cycle, using the peak time as a phase reference point of approximately Circadian Time 15 (CT 15). We then compared the transient change in period (phase shift) of PER2::LUC rhythms in reagent-treated explants relative to those of vehicle-treated explants on the cycle ensueing initiation of treatment (See Figure 3-5C legend for detail). Application of the non-selective dopamine receptor agonist (±)-2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene [ADTN; 100 μM; in vehicle containing 100 μM L-ascorbic acid (L-AA, an antioxidant)], or the D1 dopamine agonist SKF 38393 (50 μM) beginning in the early retinal subjective day (CT 3), phase advanced retinal rhythms by approximately 1.5 hrs (Figure 3-5C). In contrast, application of the D2/D4 receptor agonist quinpirole (50 μM) at CT 3, did not reset retinal phase (Figure 3-5C), whereas co-application of the two agonists together produced phase advances similar in amplitude to D1 agonist alone (Figure 3-5C). Application of D1 agonist beginning in the retinal subjective night (CT 18), induced phase delays of approximately 1 hr (Figure 3-5C), whereas application of the D2 agonist at that phase again did not affect retinal phase (Figure 3-5C). Phase

advances and delays induced by dopamine agonists were stable and persistent for multiple cycles. Although dopaminergic agonists were applied as a bolus and thus were potentially active for multiple circadian cycles, the periods of molecular rhythms of treated and vehicle control retinas were similar following the initial phase shift, indicating that these reagents likely have a limited effective half-life in our culture system. Acute pulse application experiments, performed in an attempt to define more precisely the phase-dependence of the resetting action of dopamine, did not yield useful data due to artifactual phase shifts induced by the repeated media changes themselves.

Manipulation of melatonin transmission did not have noticeable effects on the waveform of retinal PER2::LUC rhythms (Figures 3-4C and 4D) and did not significantly affect the phase of retinal molecular rhythms. Neither application of melatonin (10 nM), nor the melatonin receptor antagonist luzindole (5 μM) significantly altered the phase of retinal PER2::LUC rhythms (Figure 3-6). Melatonin and luzindole were each applied beginning at two different phases of the retinal rhythm, at approximately CT 3 or CT 18, but none of these treatments resulted in a statistically significant change in retinal phase (Figure 3-6). In addition, application of a higher dose of melatonin (10 μM) to B6C3 retinas, or application of 10 nM melatonin to C57BL/6J retinas also failed to alter retinal phase (Figure 3-6).

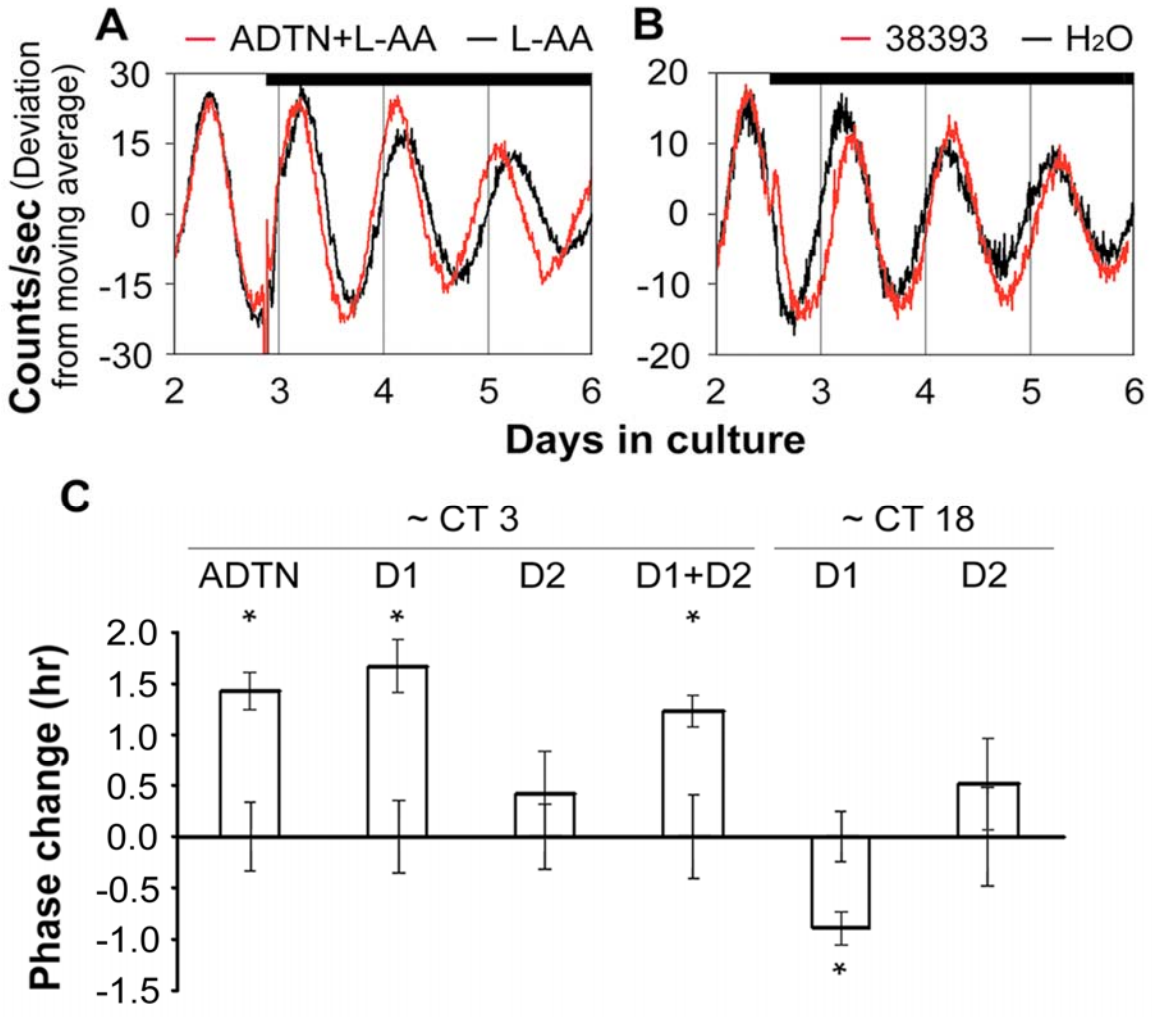

Figure 3-5: Dopamine regulates the phase of retinal PER2::LUC rhythms.

(A) Application of ADTN (100 µM) along with L-AA (100 µM) beginning at CT 3 advanced PER2::LUC rhythms compared to L-AA application alone.

(B) Application of the D1 receptor agonist SKF 38393 (50 µM) beginning at CT 18 delayed PER2::LUC rhythms compared to vehicle (H_2O). Data shown in (A) and (B) were baseline corrected by calculating a 24 hr moving average of the raw data, and then the deviation from the moving average was plotted as a function of days in culture. Bars indicate the duration of treatment.

(C) Phase change following application of dopamine agonists. Briefly, the peak times (as determined by ClockLab software) on the 3rd cycle (after treatment) were subtracted from the peak times on the 2nd cycle (before treatment) for both drug and vehicle, and then used to calculate the phase change of drug vs. vehicle controls. Bars show drug phase changes (means ± SEM), error bars from x axis show ± SEM for vehicle controls in each group. Drug concentrations were as follows: ADTN, 100 µM; the D1 agonist SKF 38393, 50 µM; the D2 agonist quinpirole, 50 µM. "*", $P < 0.01$; Student's t-test. N = at least 4 for each drug treatment and for vehicle controls.

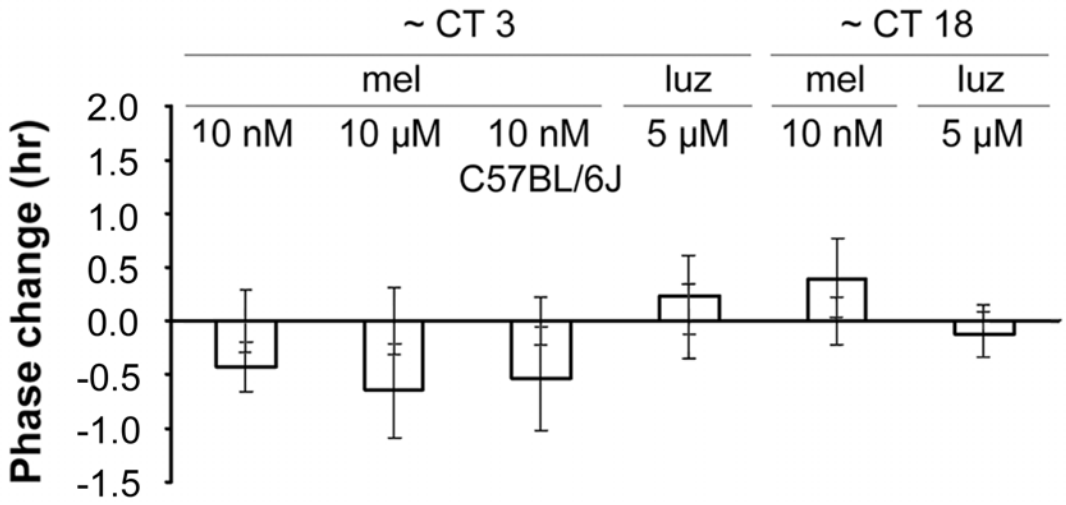

Figure 3-6: Melatonin does not regulate the phase of retinal PER2::LUC rhythms.
Phase change following application of melatonin reagents was calculated as in Figure 4. Drug concentrations are indicated above columns. Vehicles used for 10 nM melatonin (mel), 10 µM melatonin and 5 µM luzindole (luz) were 1 µl 0.01% ethanol, 1 µl 10% ethanol, and 1 µl DMSO, respectively. N = at least 4 for each drug treatment and for vehicle controls.

Discussion

In the present study we developed a retinal explant culture protocol that enables the assay of circadian clock gene expression rhythms in real time from isolated, intact PER2::LUC mouse retinas. Using this protocol, we characterized retinal gene expression rhythms, localized their source within the retinal layers, and examined the influence of the retinal neuromodulators dopamine and melatonin. We found that isolated mouse retinas exhibited sustained, endogenous circadian rhythms in gene expression localized primarily to the inner nuclear layer of the retina. Generation of these retinal molecular rhythms is independent of melatonin; however, their phase is regulated by dopamine.

Culture of Mouse Retina

Information regarding the mechanisms of the mammalian retinal clock has been limited by the challenge of long-term culture of the mammalian retina and the interpretational caveats imposed by using melatonin or dopamine neurochemical output rhythms as proxies for observing the clock mechanism itself. The protocol we have developed here, a modification of the explant culture method for SCN luminescence recording (Yamazaki and Takahashi, 2005), allows continuous readout of the rhythmic abundance of a putative molecular component of the circadian clock mechanism, PER2 protein, as bioluminescence intensity emitted by isolated, intact mouse retinal explants. Within our preparations, all layers of the retina were anatomically intact and the retinas

exhibited robust circadian rhythms of PER2::LUC expression. The precise mechanism by which the transition through bicarbonate-based media preserves retinal rhythmicity is unknown, however bicarbonate balance has wide-ranging effects on retinal physiology and increased bicarbonate concentration has previously been shown to increase the amplitude of circadian rhythms in photoreceptor disc-shedding in amphibian retinas (Flannery and Fisher, 1984).

An Inner Nuclear Layer Retinal Clock

Imaging of PER2::LUC luminescence signal from retinal vertical slice cultures of both B6C3 and C57BL/6J mice showed that PER2::LUC rhythms were predominantly localized to the inner nuclear layer of the retina, which contains the nuclei of retinal horizontal, bipolar, and amacrine cells. The pattern of PER2::LUC expression in the current study is consistent with previous single-cell RT-PCR and *in situ* hybridization studies demonstrating concentration of clock gene transcripts in the inner nuclear layer of the mouse (Gekakis et al., 1998; Witkovsky et al., 2003; Ruan et al., 2006), and immunocytochemistry studies showing predominant localization of PER1, PER2 and CLOCK protein in the inner nuclear layer as well (Witkovsky et al., 2003; Storch et al., 2007). Localization of rhythmic PER2::LUC expression to the inner nuclear layer is consistent with the persistence of PER2::LUC rhythms in photoreceptor degenerate mouse retinas (Ruan et al., 2006), and with the persistence of *Per2* rhythms in photoreceptor degenerate RCS retinas (Tosini et al., 2007a). The wide distribution of

PER2::LUC expression across the inner nuclear layer indicates that multiple inner nuclear layer cell types are sources for the rhythmic PER2::LUC signal and are likely participants in the inner retinal clock mechanism. Cell-type specific mapping of coordinate expression of the core circadian clock genes has demonstrated that significant proportions of horizontal, rod bipolar and dopaminergic amacrine neurons of the inner nuclear layer express all six core clock genes and thus are candidate cell types for self-sustained circadian rhythms generation (Ruan et al., 2006). In addition, transcription of the *Per1* clock gene has been shown to occur in most GABAergic amacrine cells, and rhythmically in the dopaminergic and NOS-positive subtypes of GABAergic amacrine cells (Witkovsky et al., 2003; Zhang et al., 2005), indicating the potential for these classes of retinal neurons to participate in the inner nuclear layer retinal clock. Additional experiments are needed to fully elucidate the specific roles of these inner nuclear layer retinal neuron subtypes and their neurotransmitters in the retinal clock network.

Although, our results suggest that the inner nuclear layer of the intact mouse retina contains a circadian clock, our study does not preclude the potential for circadian rhythms generation in the photoreceptor or ganglion cell layers. Tosini et al. have recently reported gene rhythms from photoreceptor layers isolated from rat retinas that carry a transcriptional transgenic reporter for *Per1*, rather than the knockin fusion protein reporter for PER2 used here (Tosini et al., 2007b).

Regulation of the Inner Retinal Circadian Clock by Dopamine

It is well established that dopamine and melatonin are important neurochemical messengers of the retinal circadian clock that act at multiple sites within the retinal circuitry to shape retinal function into "day" and "night" states (Morgan and Boelen, 1996; Manglapus et al., 1999; Green and Besharse, 2004; Iuvone et al., 2005). Our results distinguish the roles of these neurotransmitters in retinal circadian organization indicating that whereas dopamine has the additional role of regulating the phase of the core clock mechanism, melatonin's role is primarily that of an output messenger of the clock. Our finding that PER2::LUC rhythms persist in C57BL/6J mouse retinas with genetically blunted melatonin production and following pharmacological manipulation of melatonin signaling is consistent with previous studies showing that melatonin is not required for circadian photoreceptor disk shedding in constant darkness (Grace et al., 1999), and that photoreceptors, the primary source of retinal melatonin, are not required for molecular circadian rhythms generation (Ruan et al., 2006). In addition, in the present study neither manipulation of melatonin, nor blockade of MT1 receptors, significantly altered the phase of retinal PER2::LUC rhythms. Taken together, these results suggest that melatonin is an output messenger of the clock, which can influence dopamine release and other aspects of retinal physiology, but which does not directly affect or feed back on the molecular clock mechanism.

In contrast, dopamine is a key regulator of the endogenous retinal clock mechanism. Stimulation of dopamine D1 receptors reset the phase of retinal molecular rhythms,

producing phase advances during treatments initiated in the early subjective day and phase delays during treatments initiated in the subjective night. These effects on circadian phase demonstrate that retinal dopamine influences the core clock mechanism of mammalian retinas. The finding that D1 dopamine receptors are key to the action of dopamine on the retinal circadian pacemaker, suggests that dopamine targets retinal cell types in the inner nuclear layer clock to reset the retinal circadian oscillator. These potential targets include multiple cell types which are the primary sites of D1 receptor expression in the retina: horizontal, AII amacrine and bipolar cells (Witkovsky, 2004). The lack of phase setting of the inner retinal oscillator by the D2/D4 agonist quinpirole and melatonin suggests that photoreceptors and melatonin do not participate in the dopaminergic resetting process, as D4 receptors are expressed predominantly in retinal photoreceptors where they influence melatonin synthesis (Witkovsky, 2004). This D1-dependent resetting mechanism, that targets neurons of the inner nuclear layer, is distinct from dopamine-mediated light resetting of amphibian retinal melatonin rhythms which involves D2-like receptors on photoreceptor cells (Cahill and Besharse, 1991; Steenhard and Besharse, 2000).

Based on our results, we propose that endogenous retinal dopamine serves to synchronize the inner retinal circadian clock to the external light/dark cycle. *In vivo,* retinal dopamine release is rhythmic, exhibiting a peak near dawn each day, due to both circadian and light-driven regulation of retinal dopamine (Kramer, 1971). Retinal dopaminergic amacrine cells are light-responsive and receive excitatory light input from

M-cones via ON bipolar cells and from an additional, as yet unidentified, excitatory pathway (Zhang et al., 2007). Light increases the activity of retinal tyrosine hydroxylase, the key enzyme in dopamine synthesis, within 15 min of light onset (Iuvone et al., 1978), and evokes a sharp burst of dopamine synthesis and utilization within 30 min of light onset (Nir et al., 2000). On a molecular level, dopamine mediates acute light induction of the *Per1* gene in the mouse retina (Yujnovsky et al., 2006) and resets the *Xenopus* photoreceptor clock through induction of *xPer2* (Cahill and Besharse, 1991; Steenhard and Besharse, 2000). The phase dependence of dopamine-induced phase shifts we have observed corresponds with the likely phase resetting response to light, with phase advances induced in the early subjective day and phase delays in the early subjective night. The circuitry of mammalian retinal dopaminergic amacrine cells (Zhang et al., 2007), the molecular action of dopamine on retinal clock genes (Yujnovsky et al., 2006), and our direct demonstration of dopamine effects on clock phase, all indicate that dopamine transmission likely mediates light entrainment of the inner retinal mammalian circadian clock through its action on *Per* gene levels and rhythms.

In addition to setting the phase of the retinal clock, dopamine is a key output of the retinal clock inducing many of the functional changes in retinal neurons and circuits that define the "day" state of retinal function, such as cone-dominated processing in visual circuits and ERG amplitude (Manglapus et al., 1999; Ribelayga et al., 2002; Storch et al., 2007), and mediating circadian rhythms in behaviorally measured visual sensitivity (Li and Dowling, 2000). Thus, retinal dopamine apparently plays dual roles in the circadian

organization of the retina, serving as an output signal that mediates many of the physiological, morphological, and trophic rhythms in the retina, and serving as an input signal to regulate the phase of the molecular clock mechanism in relation to the external light/dark cycle. As both a key input to and output from the mammalian retinal clock, dopamine plays a central role in retinal circadian organization and is an important point for mechanistic intervention in ocular processes and pathologies regulated by the circadian clock.

Vertebrate Retinal Circadian Clock Organization

By characterizing key aspects of mammalian retinal circadian organization, our results provide additional insight into the principles of circadian organization of vertebrate retinas. First, within the retina there is colocalization of clock function with high levels of clock gene expression. Whereas photoreceptor clocks are widely expressed in non-mammalian retinas (Cahill and Besharse, 1993; Pierce et al., 1993) and photoreceptor rhythms have been described in mammalian preparations (Tosini et al., 2007b), the results of the present study clearly establish a distinct inner nuclear layer clock in the mammalian retina that is robust, sustained and resettable. This differential localization of circadian clock function to the photoreceptor and the inner nuclear layers follows the overall patterns of clock gene expression, which are weighted toward higher levels of expression in the photoreceptors of non-mammalian retinas and the inner nuclear layer of mammalian retinas (Gekakis et al., 1998; Namihira et al., 2001; Zhu and

Green, 2001; Haque et al., 2002; Thompson et al., 2003; Witkovsky et al., 2003; Ruan et al., 2006). Thus, while the current data does not exclude inner retinal oscillators in non-mammalian retinas, nor photoreceptor oscillators in mammalian retinas, it does indicate that the retinal layers and cell-types expressing retinal circadian rhythms differ across vertebrate retinas. Second, melatonin's role is primarily that of an effector of the retinal clock. Our results show that genetic and pharmacological manipulation of melatonin has no effect on the expression or phase of retinal molecular circadian rhythms, and the lack of effect of manipulation of retinal melatonin levels on the phase of amphibian retinal melatonin rhythms (Cahill and Besharse, 1991), suggests that this may be the case across vertebrate retinas. Third, dopamine mediates entrainment of retinal clocks, but the target cells and receptors for this mechanism vary across vertebrate retinas. Whereas the phase of the mouse retinal clock is reset by dopamine acting on D1 receptors localized primarily on the inner nuclear layer neurons, the amphibian retinal melatonin rhythm is phase shifted by dopamine acting on D2-like receptors on photoreceptors (Cahill and Besharse, 1991; Steenhard and Besharse, 2000). Knockout of D2 receptors in the mouse, which function as inhibitory autoreceptors on dopaminergic amacrine cells (Witkovsky, 2004), disrupts acute light-induction of *mPer1* (Yujnovsky et al., 2006), likely through dysregulation of dopaminergic signaling.

Summary

In summary, we have developed a real-time clock gene expression assay for the isolated mammalian retina and used it to characterize and localize the expression of retinal molecular circadian rhythms, as well as to examine the roles of dopamine and melatonin in the retinal clock. Our main findings are that there is a self-sustained endogenous retinal circadian clock in the inner nuclear layer that functions independently of melatonin and that the phase of this inner retinal circadian clock is regulated by dopamine. We propose that dopamine plays a key role in mammalian retinal circadian organization by mediating molecular-level entrainment of the retinal clock, as well as serving as a rhythmic output signal by which the retinal clock establishes a functional "day" state in retinal circuits. Future studies using this *in vitro* retinal explant clock model can further elucidate the cellular and molecular mechanisms of retinal circadian clocks and their influence on visual function as well as reveal additional principles regarding the functional organization of neural circadian oscillators.

CHAPTER IV

GABAERGIC REGULATION OF THE RETINAL CIRCADIAN CLOCK

Introduction

The vertebrate retina is both a sensory organ and an endogenous circadian clock. As the locus of visual phototransduction, the retina initiates many organismal responses to light; and as a circadian clock, the retina comprises many physiological or functional circadian rhythms, including photoreceptor disc shedding (LaVail, 1976, 1980; Grace et al., 1996; Grace et al., 1999), visual sensitivity (Rosenwasser et al., 1979; Bassi and Powers, 1986; Reme et al., 1991; Li and Dowling, 1998), rod-cone balance(Wang and Mangel, 1996; Manglapus et al., 1998), electroretinogram (ERG) b-wave amplitude (Brandenburg et al., 1983; Barnard et al., 2006; Storch et al., 2007), extracellular pH (Dmitriev and Mangel, 2001), melatonin release (Besharse and Iuvone, 1983; Tosini and Menaker, 1996; Niki et al., 1998a; Tosini and Menaker, 1998), dopamine synthesis (Doyle et al., 2002b; Doyle et al., 2002a), gamma-aminobutyric acid (GABA) turnover rate and release (Jaliffa et al., 2001), PKC level (Gabriel et al., 2001), and intraocular pressure (Boyd and McLeod, 1964; Rowland et al., 1981). In a recent microarray study, 277 genes representing a wide range of functions were found to show a circadian rhythm of expression in the mouse retina under constant darkness condition (Storch et al., 2007). In mammals, the retina is the sole site for circadian phototransduction, and its output

modifies the rhythmicity, period, and developmental organization of the central biological clock, the suprachiasmatic nucleus (SCN) of the hypothalamus (Yamazaki et al., 2002; Lee et al., 2003; Ohta et al., 2006). Despite the widespread influence of the retinal clock on visual and circadian function, the key neural mechanisms regulating this clock remain elusive.

GABA is a principal inhibitory neurotransmitter of the retina. GABA is synthesized and released by horizontal cells and GABAergic amacrine cells, and functions to modulate visual signal processing in these lateral cellular pathways (Pourcho, 1996). GABA is also closely related to circadian clock function. We have previously identified circadian clock gene expression within GABAergic horizontal and amacrine cells (Zhang et al., 2005; Ruan et al., 2006). In addition, the circadian oscillator neurons of the master SCN clock are also GABAergic (van den Pol, 1993) and GABA plays a key role in intercellular communication within this neural clock (Albus et al., 2005).

In the current study we examined the role of GABA and other fast neurotransmitters in the retinal circadian clock using *in vitro* retinal explants in which molecular circadian rhythms were monitored by the expression of a PERIOD2::LUCIFERASE (PER2::LUC) transgene (Yoo et al., 2004; Chapter III). Our findings indicate that retinal GABA, acting through $GABA_A$ and $GABA_C$ receptors, as well as membrane hyperpolarization and casein kinases, negatively regulates the amplitude of *Per2* expression rhythms.

Materials and Methods

Animals

mPer2^{Luc} knockin mice, which initially were on a 129SvEv X C57BL/6J genetic background (Yoo et al., 2004), were maintained as a continuous backcross to C57BL/6J for 13 generations. The resulting *mPer2*^{Luc} mice were crossed with C3H *rd* mice (The Jackson Laboratory, Bar Harbor, ME) to produce *mPer2*^{Luc} mice that are heterozygous for *rd* gene and genetically capable of producing melatonin. All animal use was conducted in accordance with the guidelines of the Vanderbilt University Animal Care Division and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals.

Retinal Explant Culture

Retinal explant culture was performed as described in Chapter III. Briefly, *mPer2*^{Luc} mice 30-60 days of age were killed by cervical dislocation at approximately Zeitgeber time (ZT) 4. Eyes were enucleated and placed in Hank's balanced salt solution (HBSS; Invitrogen). Retinas were isolated and cut into two pieces. Each piece of retina was placed with the ganglion cell layer up on Millicell culture membrane (Millipore) and gently flattened with two end-blunted glass pipettes. The membrane was first transferred to 1.0 ml neurobasal medium (Invitrogen) supplemented with 2 mM L-glutamine (Sigma), 2% B27 (Invitrogen), 25 units/ml penicillin and 25 μg/ml streptomycin (Invitrogen),

incubated in 5% CO_2 incubator at 37°C for 24 hr, and then transferred to 1.0 ml medium 199 (Sigma) supplemented with 0.7 mM L-glutamine, 4 mM sodium bicarbonate (Sigma), 10 mM Hepes (Sigma), 20 mM D-glucose (Sigma), 2% B27, 0.1 mM beetle luciferin (Promega), 25 units/ml penicillin and 25 μg/ml streptomycin. Bioluminescence was measured with a LumiCycle (Actimetrics, Wilmette, IL). Cultures were maintained at 37°C. Peak-to-trough amplitudes were determined using LumiCycle data analysis software (Actimetrics).

SCN Explant Culture

At approximately ZT 4, *mPer2*^{Luc} knockin mice were killed by cervical dislocation, the brains rapidly extracted following decapitation, and immediately placed in cold HBSS. Coronal brain sections (250 μm thickness) were cut with a Vibratome (World Precision Instruments, Sarasota, FL), transferred to cold HBSS, and sorted under a dissecting microscope. Slices containing the SCN were trimmed to rectangles 3 X 3 mm with a pair of scalpels, and then cultured on Millicell culture membranes with 1.0 ml DMEM (Sigma), supplemented with 4 mM sodium bicarbonate, 10 mM Hepes, 20 mM D-glucose, 2% B27, 0.1 mM beetle luciferin, 25 units/ml penicillin and 25 μg/ml streptomycin. Bioluminescence was continuously measured with a LumiCycle. Cultures were maintained at 37°C.

Data Analysis

Student's t-test was routinely performed to compare the difference between drug- and vehicle-treated samples. For $GABA_A$ and $GABA_C$ antagonists treatment, two-way repeated-measures ANOVAs were run for both drug and vehicle control in which the variances were homogeneous as indicated by a significant Levene's test. For post hoc analysis of significant interactions, independent t-tests with a Bonferroni-corrected alpha (the alpha was Bonferroni-corrected by the number of comparisons) were used to compare the effects of drug for each cycle.

Results

To monitor the activity of the retinal circadian clock, we cultured retinal explants from PER2::LUC knockin mice and measured their bioluminescence rhythms as in Chapter III. This protocol allows for routine measurement of endogenous retinal molecular rhythms for 10 days without the need to change media, and for at least 30 days with media changes. In the current study, pharmacological reagents were generally applied in a bolus at the initiation of the rising phase of the 3rd circadian cycle recorded in the LumiCycle (i.e. 4th cycle *in vitro* with our preparation).

GABA Negatively Regulates the Amplitude of Retinal PER2::LUC Rhythms

The mammalian retina has pathways using all the major fast neurotransmitters: glutamate, GABA, glycine and acetylcholine (ACh). To examine the influence of these fast neurotransmitters on retinal PER2::LUC rhythms, we activated their receptors with corresponding agonists, or blocked these receptors with their antagonists. None of the agonists or antagonists for glutamate, glycine, or ACh receptors exhibited marked effects on the amplitude or period of retinal PER2::LUC rhythms (n = 3 each; Figure 4-1). In contrast to other faster neurotransmitters, manipulation of GABA neurotransmission had substantial effects on the amplitude of retinal PER2::LUC rhythms. Application of 0.1 mM GABA did not obviously alter PER2::LUC rhythms compared with vehicle (Figure 4-2A), however, at a higher concentration (0.5 mM), GABA acutely suppressed the level of PER2::LUC bioluminescence signals and greatly reduced the peak-to-trough

amplitude of PER2::LUC oscillations on subsequent circadian cycles (Figure 4-2B). At 1 mM or 3 mM, GABA further inhibited PER2::LUC luminescence levels and resulted in rapid damping of PER2::LUC rhythms (Figures 4-2C and 2D). Media changes that removed GABA restored the damped oscillations (see below). To further characterize the inhibitory action of GABA on retinal rhythms, we calculated the ratio of the peak-to-trough amplitude of the 4th circadian cycle (2nd cycle after treatment) to the amplitude of the 2nd cycle (A_4/A_2). This ratio was plotted as a function of GABA concentration (Figure 4-2E). The A_4/A_2 for 0.1 mM GABA was 0.82 ± 0.06 (mean ± SEM; n = 5), which was similar to that for vehicle treatment (0.75 ± 0.08; n = 5). As GABA concentration was increased, A_4/A_2 gradually decreased and at 3 mM GABA, most explants lacked clear rhythms and the few cycles that could be measured were of very low amplitude (0.07 ± 0.01; n = 7), indicating that GABA inhibits PER2::LUC rhythmic amplitude in a dose-dependent manner. However, GABA did not significantly change the period of retinal PER2::LUC rhythms compared with vehicle (τ = 23.80 ± 0.16 hr for 0.5 mM GABA, n = 6; τ = 24.33±0.22 hr for 1 mM GABA, n = 7; τ = 24.05 ± 0.09 hr for vehicle, n = 5; $P > 0.05$ for both; Student's t-test).

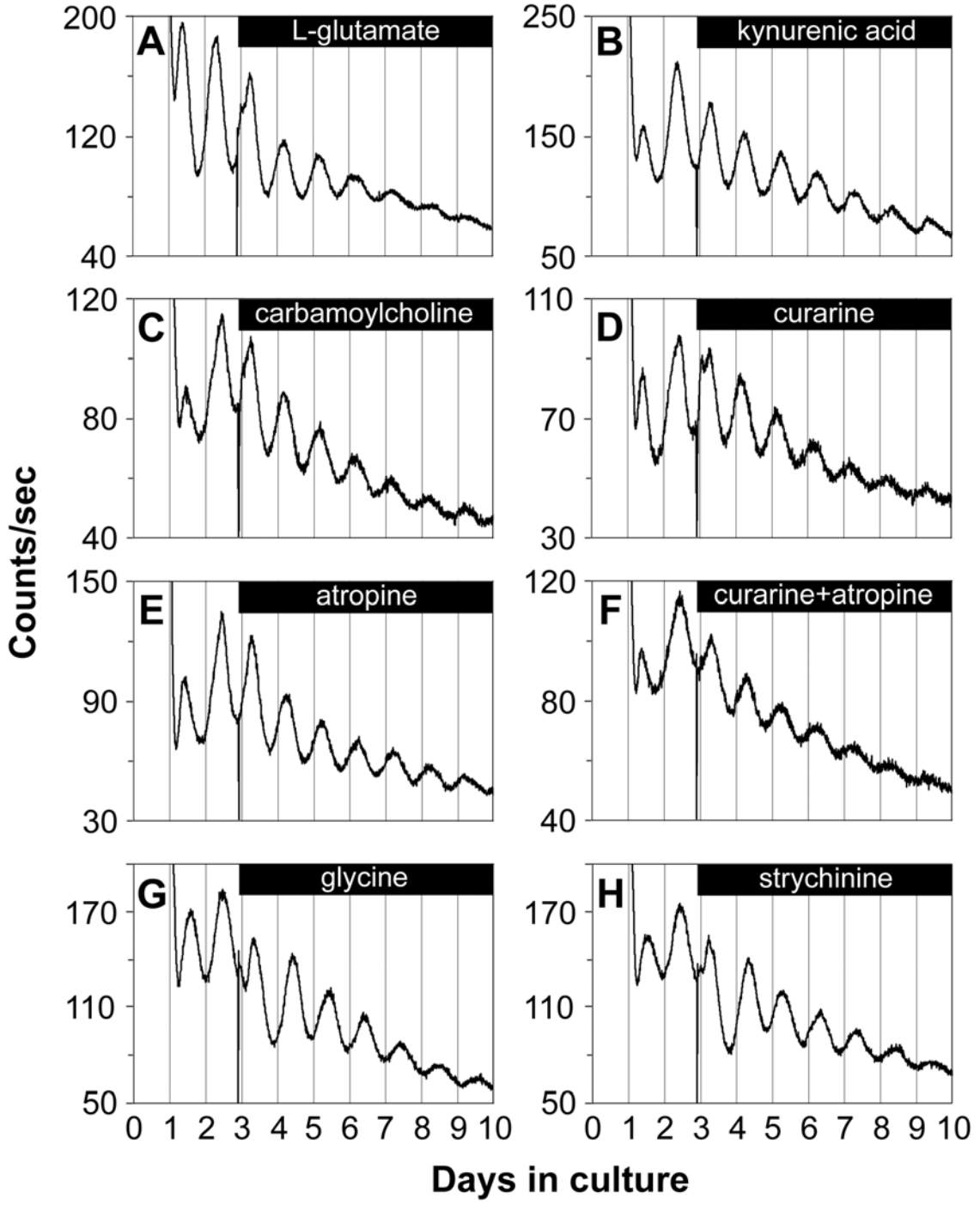

Figure 4-1: Fast neurotransmission by glutamate, acetylcholine, or glycine does not exhibit marked effect on retinal PER2::LUC rhythms.

Figure 4-1: Fast neurotransmission by glutamate, acetylcholine, or glycine does not exhibit marked effect on retinal PER2::LUC rhythms.
Shown are representative PER2::LUC bioluminescence traces of mouse retinal explants treated with: (A) L-glutamate (1 mM); (B) kynurenic acid (0.5 mM), a broad-spectrum glutamate receptor antagonist; (C) carbamoylcholine chloride (100 μM), a non-selective cholinergic agonist; (D) (+)-tubocurarine (100 μM), a nicotinic acetylcholine receptor antagonist; (E) atropine (100 μM), a muscarinic acetylcholine receptor antagonist; (F) (+)-tubocurarine (100 μM) along with atropine (100 μM); (G) glycine (3 mM); (H) strychnine hydrochloride (50 μM), a glycine receptor antagonist. None of these agonists or antagonists exhibited apparent effects on the amplitude or period of retinal PER2::LUC rhythms. Bars indicate the duration of treatment.

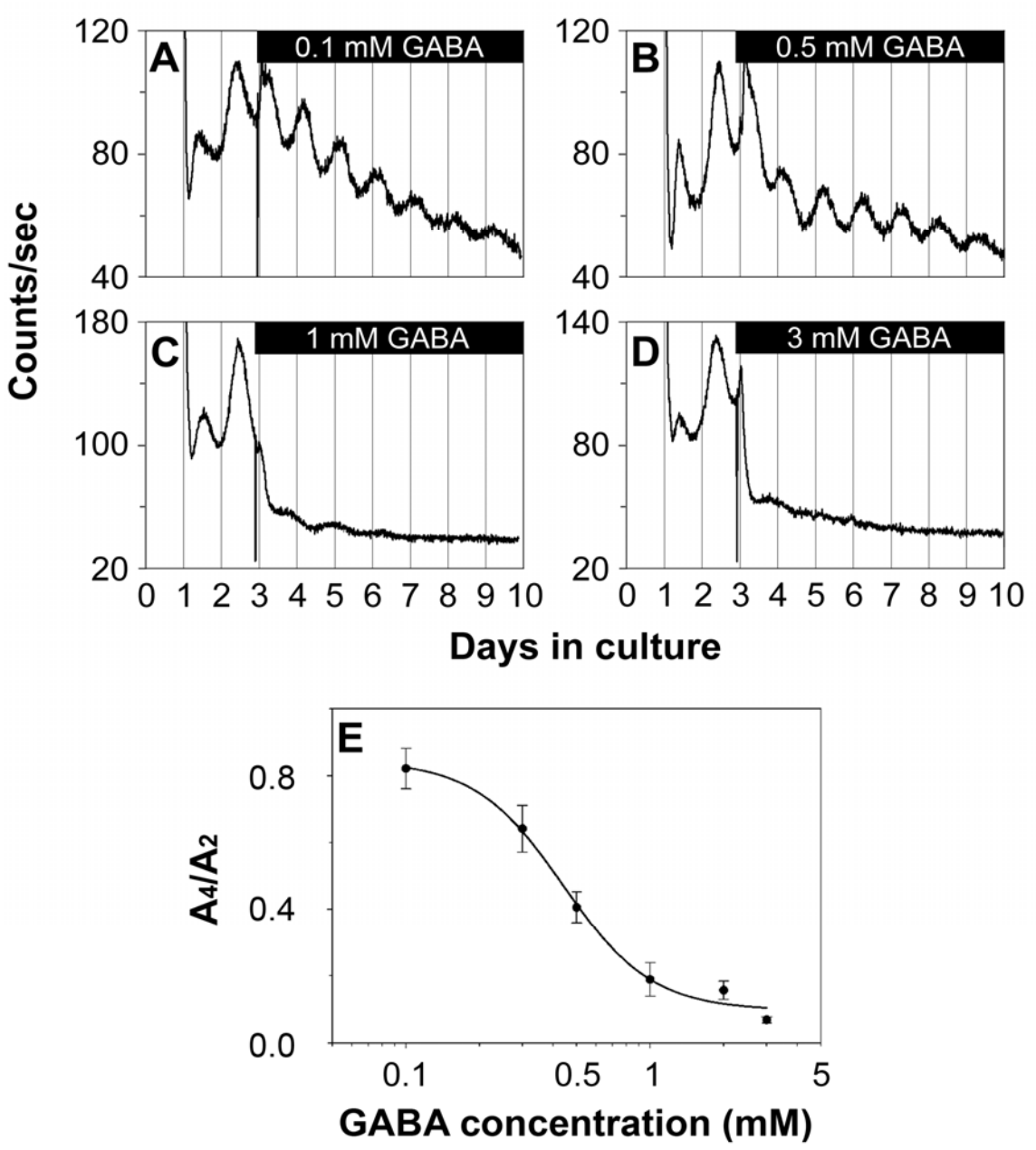

Figure 4-2: GABA suppresses the amplitude of retinal PER2::LUC rhythms in a dose-dependent manner.

(A-D) Representative PER2::LUC bioluminescence traces recorded from cultured *mPer2*^{Luc} retinal explants that received different doses of GABA treatment. Bars indicate the duration of GABA treatment.

(G) Dose-dependent inhibitory action of GABA on the amplitude of retinal PER2::LUC rhythms. The ratio of the peak-to-trough amplitude of the 4th cycle to that of the 2nd cycle (A_4/A_2) was plotted as a function of the external GABA concentration. Data are represented as means ± SEM (n = at least 3 explants per point).

To test whether the inhibitory action of GABA is specific to retina, or common to other neural clock tissues, we applied 1 mM or 3 mM GABA to mouse *mPer2*^{Luc} SCN explants. GABA did not significantly change the A_4/A_2 of SCN PER2::LUC rhythms compared to vehicle (0.91 ± 0.08 for 1 mM GABA; 0.86 ± 0.07 for 3 mM GABA; 0.84 ± 0.11 for vehicle treatment; $P > 0.05$ for both; n = 3 each; Figure 4-3); neither did GABA have significant effect on the period of SCN PER2::LUC rhythms (τ = 23.56 ± 0.26 hr, 23.96 ± 0.32 hr, and 23.63 ± 0.23 hr for 1 mM GABA, 3 mM GABA, and vehicle, respectively; $P > 0.05$ for both; n = 3 each). Therefore, GABA-induced inhibition of ensemble PER2::LUC rhythms is retina-specific.

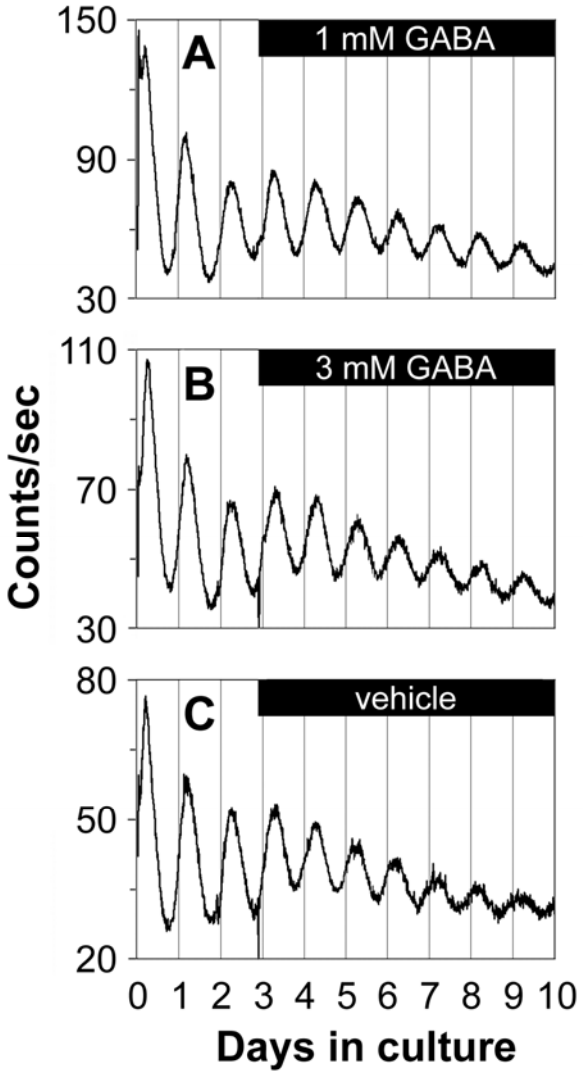

Figure 4-3: GABA does not suppress SCN ensemble PER2::LUC rhythms.

$GABA_A$ and $GABA_C$ Receptors Mediate the Effects of GABA on Retinal PER2::LUC Rhythms

Next, we characterized the receptors responsible for the inhibitory action of GABA. GABA receptors are classified as ionotropic, chloride-conducting $GABA_A$ and $GABA_C$ receptors, or as metabotropic $GABA_B$ receptors. All three types of GABA receptors are present in the mammalian retina (Yang, 2004). When the $GABA_A$ receptor agonist muscimol (200 μM), or the $GABA_C$ agonist *cis*-4-aminocrotonic acid (CACA, 50 μM) was applied individually to the retinal explants, the amplitude of retinal PER2::LUC rhythms was significantly reduced compared to that of vehicle-treated samples (A4/A2 = 0.46 ± 0.11 for muscimol, 0.40 ± 0.05 for CACA, and 0.68 ± 0.08 for vehicle treatment; $P < 0.05$; n = 4 each; Figures 4-4A and 4C). However, the amplitude of PER2::LUC rhythms was largely unaffected by the $GABA_B$ agonist baclofen (200 μM; A4/A2 = 0.63 ± 0.08; n = 4; Figure 4-4B). When muscimol and CACA were applied together they mimicked the inhibition of 1 mM GABA on retinal PER2::LUC rhythms ($A_4/A_2 = 0.30 \pm$ 0.05 for muscimol + CACA, 0.23 ± 0.04 for 1 mM GABA; $P > 0.05$; n = 5 each; Figure 4-5A). Baclofen co-applied with either muscimol or CACA, or baclofen co-applied with both muscimol and CACA, did not enhance the inhibition of the $GABA_A$ and $GABA_C$ agonists ($A_4/A_2 = 0.54 \pm 0.07$ for muscimol + baclofen, 0.36 ± 0.03 for baclofen + CACA, and 0.31 ± 0.03 for muscimol + baclofen + CACA; n = 5 each; Figures 4-4D-4F). *Trans*-4-aminocrotonic acid (TACA, 80 μM), an agonist for both $GABA_A$ and $GABA_C$ receptors, inhibited luminescence rhythms to a degree similar to 1 mM GABA ($A_4/A_2 =$

0.29 ± 0.05; n = 4; $P > 0.05$; Figure 4-5B). These results indicate that activation of both $GABA_A$ and $GABA_C$ receptors is necessary to fully mimic the action of GABA on retinal rhythms, with $GABA_B$ receptors playing no apparent role, either alone or in combination with the other receptors.

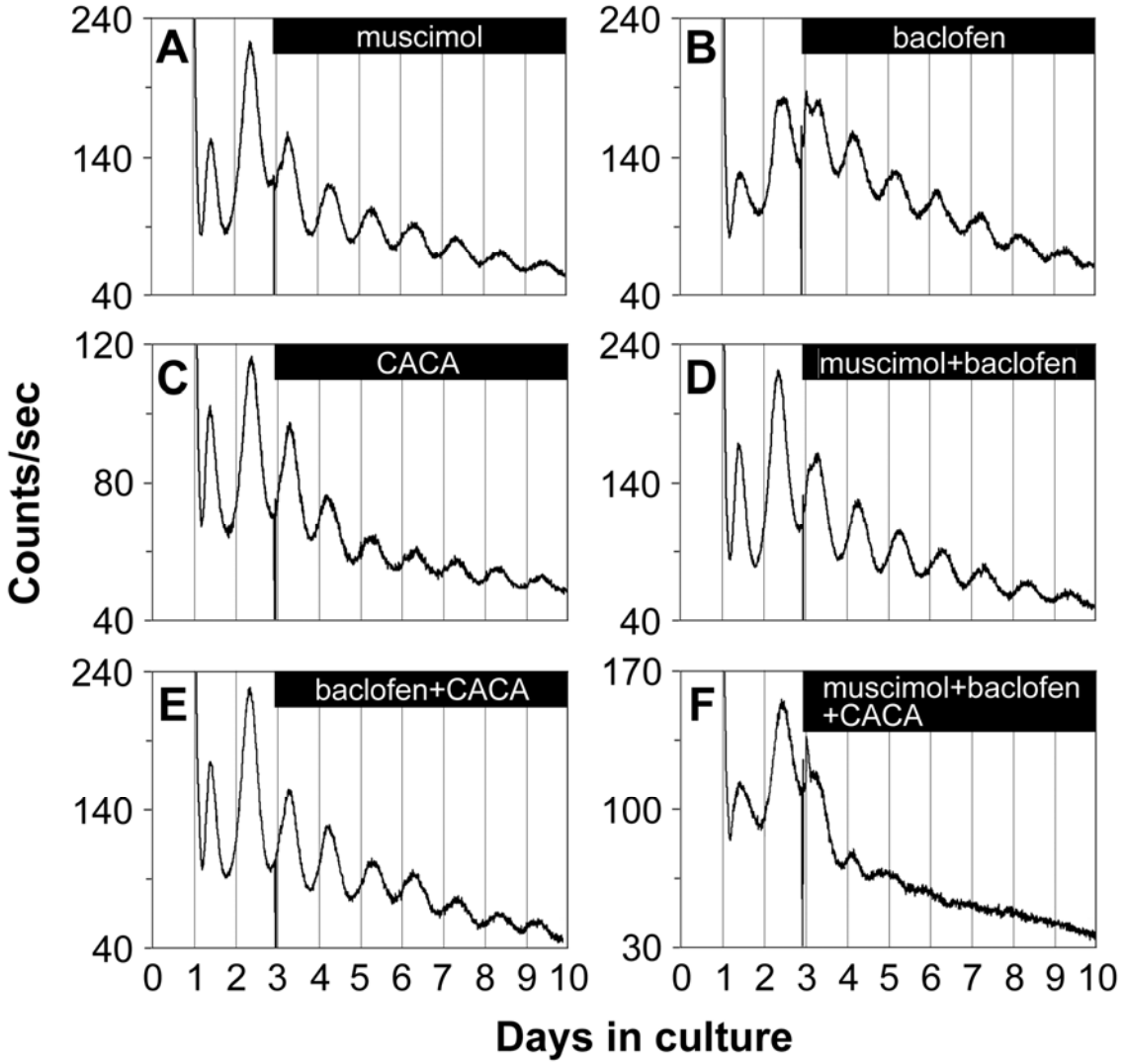

Figure 4-4: Actions of GABA receptor agonists on retinal PER2::LUC rhythms.
Shown are representative PER2::LUC bioluminescence traces of mouse retinal explants treated with: (A) the $GABA_A$ receptor agonist muscimol (200 μM); (B) the $GABA_B$ receptor agonist baclofen (200 μM); (C) the $GABA_C$ agonist CACA (50 μM); (D) muscimol (200 μM) and baclofen (200 μM); (E) baclofen (200 μM) and CACA (50 μM); (F) muscimol (200 μM), baclofen (200 μM), and CACA (50 μM). Bars indicate the duration of treatment.

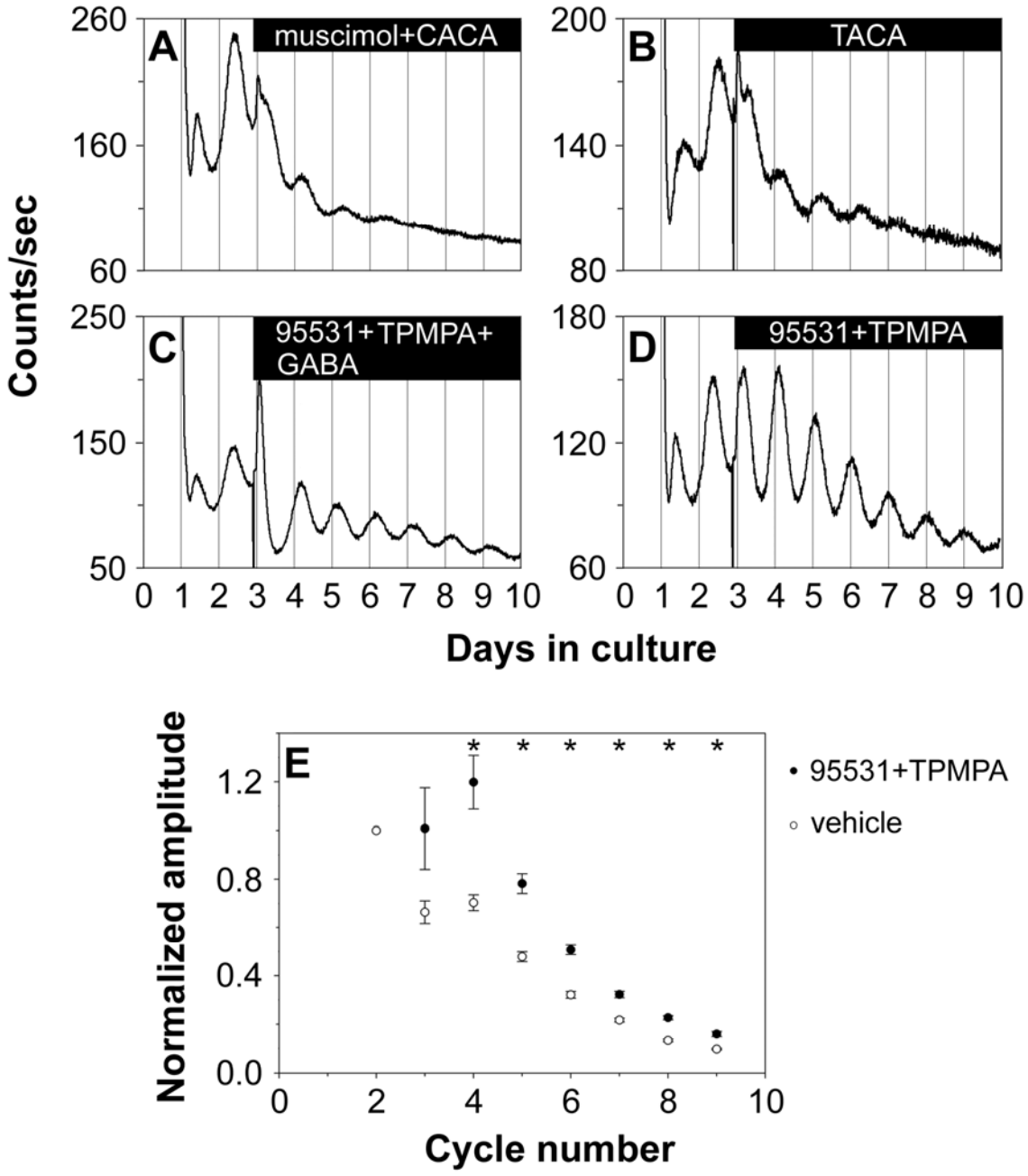

Figure 4-5: GABA acts through $GABA_A$ and $GABA_C$ receptors.

Figure 4-5: GABA acts through $GABA_A$ and $GABA_C$ receptors.
(A) Co-application of the $GABA_A$ receptor agonist muscimol (200 μM) and the $GABA_C$ receptor agonist CACA (50 μM) to retinal explants mimicked the inhibitory action of 1 mM GABA on retinal PER2::LUC rhythms.
(B) The $GABA_A$ and $GABA_C$ receptor agonist TACA (80 μM) inhibited PER2::LUC expression rhythms in a way similar to 1 mM GABA.
(C) The $GABA_A$ receptor antagonist SR-95531 (40 μM) and the $GABA_C$ receptor antagonist TPMPA (100 μM) greatly attenuated the action of GABA when they were co-applied with 1 mM GABA.
(D) Co-application of SR-95531 (40 μM) and TPMPA (100 μM) increased the amplitude of retinal PER2::LUC rhythms. Bars in (A-D) indicate the duration of treatment.
(E) Blockade of $GABA_A$ and $GABA_C$ receptors with SR-95531 (40 μM) and TPMPA (100 μM) significantly increased the amplitude of retinal PER2::LUC rhythms compared with vehicle-treated explants. Mean peak-to-trough amplitudes (± SEM, n = 5; normalized to the amplitude of the 2^{nd} circadian cycle) of retinal PER2::LUC rhythms were plotted as a function of PER2::LUC cycle number in the LumiCycle. * $P < 0.007$, Post hoc t-test with Bonferroni-corrected $\alpha = 0.007$.

We further examined whether specific antagonists could block the action of GABA. The inhibitory effect of 1 mM GABA persisted when it was applied in the presence of the $GABA_A$ receptor antagonist SR-95531 (40 μM) alone, the $GABA_B$ receptor antagonist CGP-35348 (100 μM) alone, or the $GABA_C$ receptor antagonist 1, 2, 5, 6-tetrahydropyridine-4-yl methyl phosphinic acid (TPMPA, 100 μM) alone (A4/A2 = 0.33 ± 0.08 for SR-95531 + GABA, 0.26 ± 0.05 for CGP-35348 + GABA, and 0.32 ± 0.10 for TPMPA + GABA; n = 4 each; Figures 4-6A-6C). However, the effect of 1 mM GABA was greatly attenuated by co-application of SR-95531 and TPMPA ($A_4/A_2 = 0.77$ ± 0.11; n = 5; Figure 4-5C). Co-application of either SR-95531 with CGP-35348, or TPMPA with CGP-35348 did not block the action of 1 mM GABA (A4/A2 = 0.35 ± 0.09 for SR-95531 + CGP-35348 + GABA, 0.28 ± 0.05 for CGP-35348 + TPMPA + GABA; n = 5 each; Figures 4-6D and 6E). CGP-35348 also did not significantly enhance the blocking ability of SR-95531 and TPMPA when it was co-applied with both antagonists (A4/A2 = 0.68 ± 0.06; n = 5; Figure 4-6F). These results indicate that blockade of both $GABA_A$ and $GABA_C$ receptors is necessary to attenuate GABA inhibition of retinal rhythms with, again, no dependence on $GABA_B$ receptors.

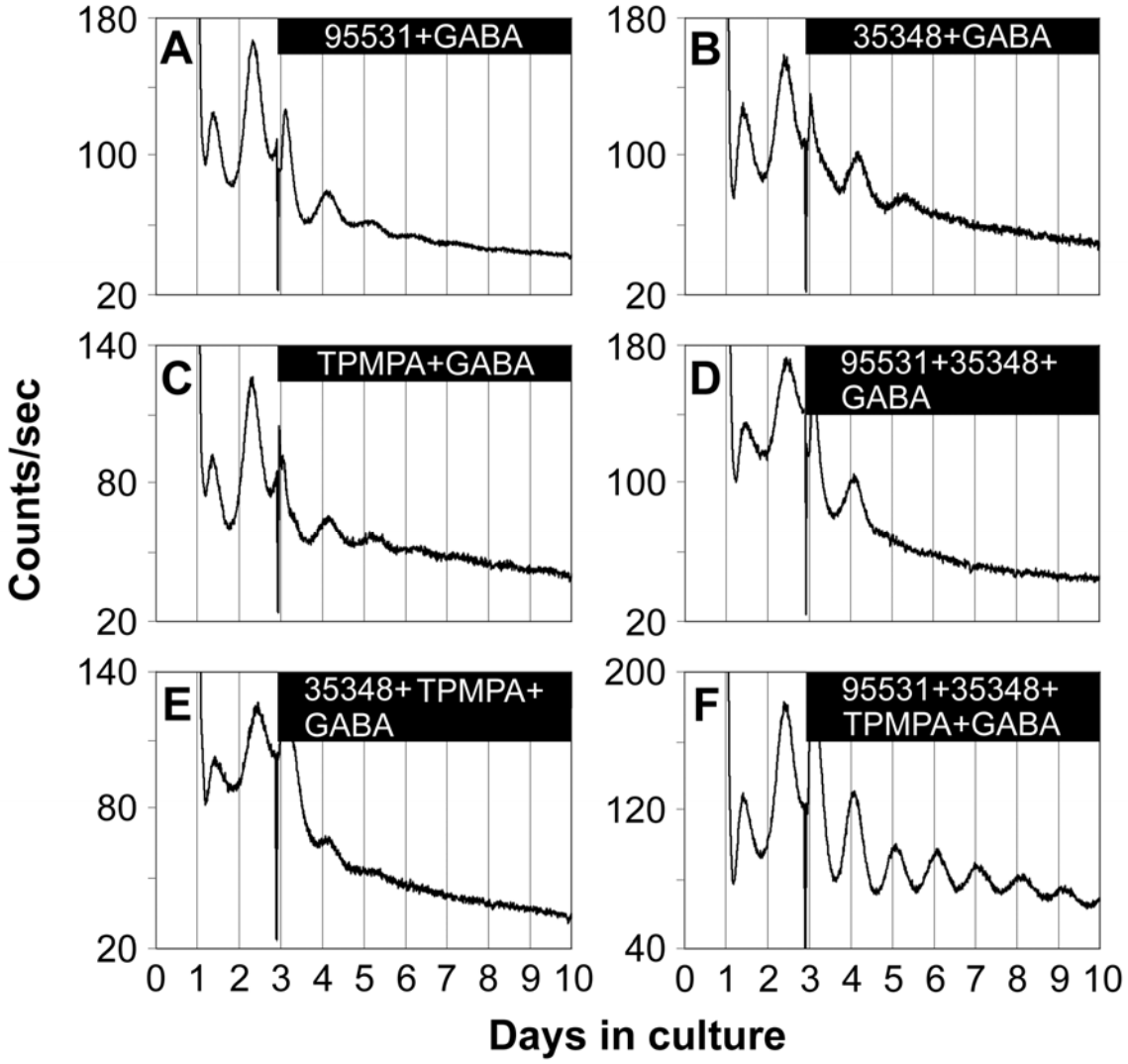

Figure 4-6: Actions of GABA receptor antagonists on the inhibitory effect of GABA on retinal PER2::LUC rhythms.

Shown are representative PER2::LUC bioluminescence traces of mouse retinal explants treated with 1 mM GABA along with: (A) the $GABA_A$ receptor antagonist SR-95531 (40 μM); (B) the $GABA_B$ receptor antagonist CGP-35348 (100 μM); (C) the $GABA_C$ antagonist TPMPA (100 μM); (D) SR-95531 (40 μM) and CGP-35348 (100 μM); (E) CGP-35348 (100 μM) and TPMPA (100 μM); (F) SR-95531 (40 μM), CGP-35348 (100 μM), and TPMPA (100 μM). Bars indicate the duration of treatment.

To assess the role of endogenous GABA in retinal rhythms generation, we next applied various GABA receptor antagonists to cultured *mPer2*^{Luc} retinal explants. When $GABA_A$ or $GABA_C$ antagonists SR-95531 (40 μM) and TPMPA (100 μM) were applied individually, each modestly increased the peak-to-trough amplitude of luminescence rhythms (A4/A2 = 0.78 ± 0.08 for SR-95531, 0.81 ± 0.07 for TPMPA, 0.68 ± 0.08 for vehicle; $P > 0.05$; n = 4 each; Figures 4-7A and 7C), whereas application of the $GABA_B$ antagonist CGP-35348 (100 μM) alone did not change amplitude (A4/A2 = 0.61 ± 0.08; n = 4; Figure 4-7B). However, antagonism of both $GABA_A$ and $GABA_C$ receptors with SR-95531 and TPMPA significantly increased the peak-to-trough amplitude of retinal ensemble PER2::LUC luminescence rhythms compared with vehicle (A4/A2 = 1.19 ± 0.11; n = 6; $P < 0.001$; Figures 4-5D and 5E), indicating that endogenous GABA indeed suppresses the amplitude of retinal PER2::LUC rhythms. Again, CGP-35348 did not enhance the effect of SR-95531 and TPMPA (A4/A2 = 0.72 ± 0.14 for SR-95531 + CGP-35348, 0.81 ± 0.11 for CGP-35348 + TPMPA, 1.01 + 0.10 for SR-95531 + CGP-35348 + TPMPA; n = 4 each; Figures 4-7D-7F). Taken together, these pharmacological studies indicate that endogenous retinal GABA reduces PER2::LUC signals and the amplitude of molecular retinal circadian rhythms through activation of both ionotropic $GABA_A$ and $GABA_C$ receptors.

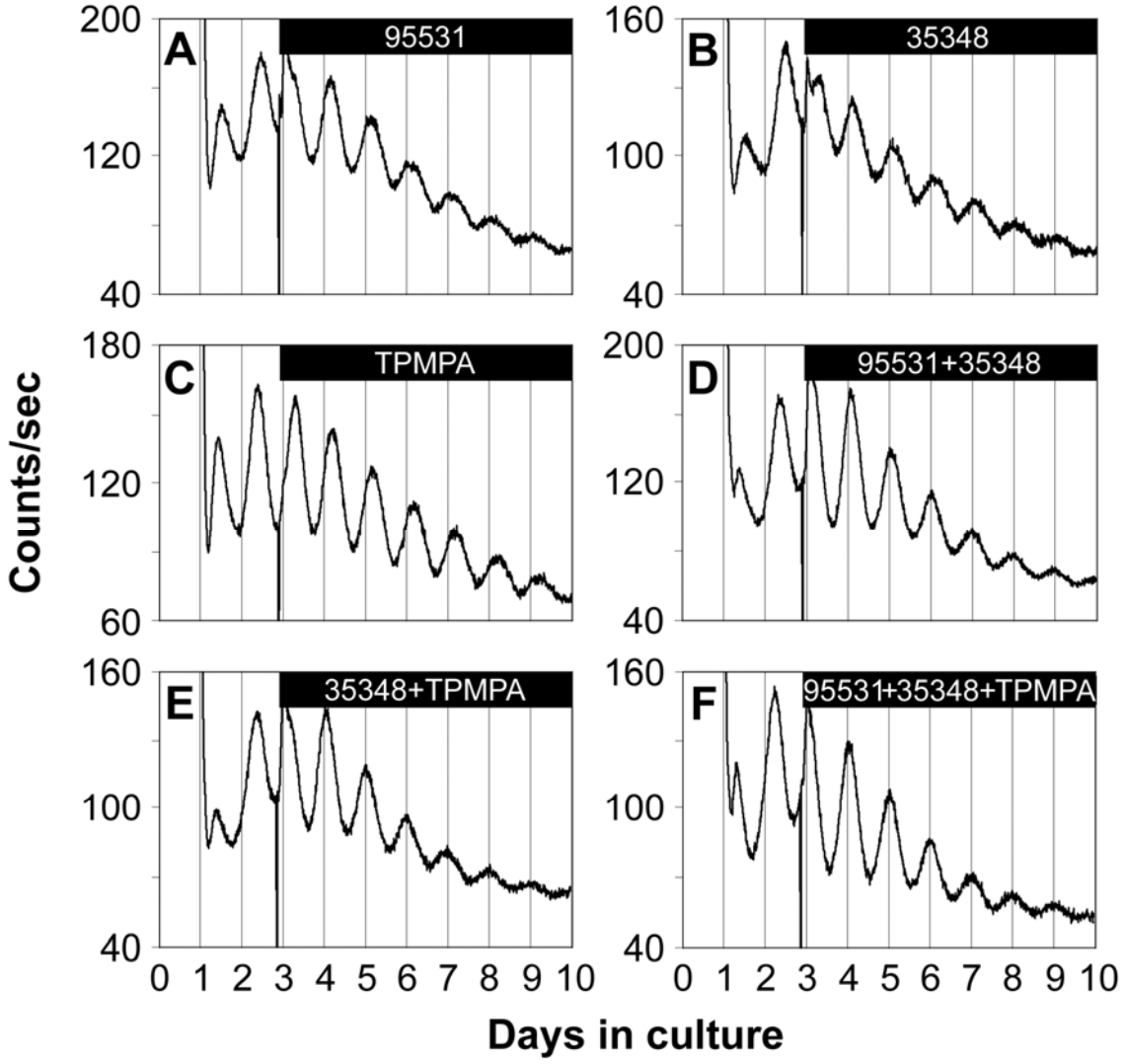

Figure 4-7: Actions of GABA receptor antagonists on retinal PER2::LUC rhythms.
Shown are representative PER2::LUC bioluminescence traces of mouse retinal explants treated with: (A) the $GABA_A$ receptor antagonist SR-95531 (40 μM); (B) the $GABA_B$ receptor antagonist CGP-35348 (100 μM); (C) the $GABA_C$ antagonist TPMPA (100 μM); (D) SR-95531 (40 μM) and CGP-35348 (100 μM); (E) CGP-35348 (100 μM) and TPMPA (100 μM); (F) SR-95531 (40 μM), CGP-35348 (100 μM), and TPMPA (100 μM). Bars indicate the duration of treatment.

Effect of Prolonged Application of GABA on Retinal PER2::LUC Rhythms

To further test whether GABAergic suppression of PER2::LUC luminescence rhythms is an effect on the core function of the retinal clock, we tested whether prolonged application of GABA can halt the molecular oscillation of the retinal clock. GABA (added to a final concentration of 3 mM in 1 μl water vehicle) was applied to retinal cultures for different durations (1, 7, 13, 19, 25, 31, 37, 43 hr), starting at the beginning of the third cycle *in vitro* (Figures 4-8A and 8B). We hypothesized that if retinal rhythms generation was stopped by prolonged GABA application, and restored upon washout, then the time of washout should predict the subsequent phase of restored rhythms. In contrast, if the observed suppression of PER2::LUC luminescence rhythms during GABA application was not an effect on the core clock, but merely on its output, then the phase of restored rhythms should be predicted by the projected phase of the baseline rhythm obtained prior to the treatment. Figure 4-8C shows the peak times of retinal PER2::LUC rhythms following GABA treatment and washout at the specified time intervals. For retinas exposed to GABA for 19 hr or greater, the first peak of the restored luminescence rhythm always appeared ca. 22 hr following GABA washout, with subsequent peaks occurring approximately at 24 hour intervals. The trend lines of peak times show that the phase of restored rhythms is indeed predicted by the time of washout, but not by projected continuation of previous rhythms. In a control experiment 1 μl water vehicle was applied to retinal cultures for 37 hr and then the media changed as in the GABA application experiments. In this case, the first peak of the ongoing PER2::LUC rhythms

occurred ca. 14 hr after the media change, not ca. 22 hr later as when GABA was washed out (n = 3).

One explanation to our results is that high concentration GABA has the ability to "stop" the retinal molecular clock mechanism. However, it is possible that GABA application perturbs retinal PER2::LUC rhythms from the limit cycle to a cycle on the isochron of ca. CT 17 where the amplitude is too small to be observed, and upon GABA washout, the perturbed retinal clock quickly (ca. 3.5 hr) moves back to the original limit cycle, resulting a steady-state (ca. 6 hr) phase shift. Moreover, the inhibitory action of GABA on the amplitude of retinal PER2::LUC rhythms could act through damping of circadian rhythms in individual retinal neurons, or through desynchronizing individual oscillators, and differentiating these mechanisms will require single-cell resolution studies of retinal circadian rhythms.

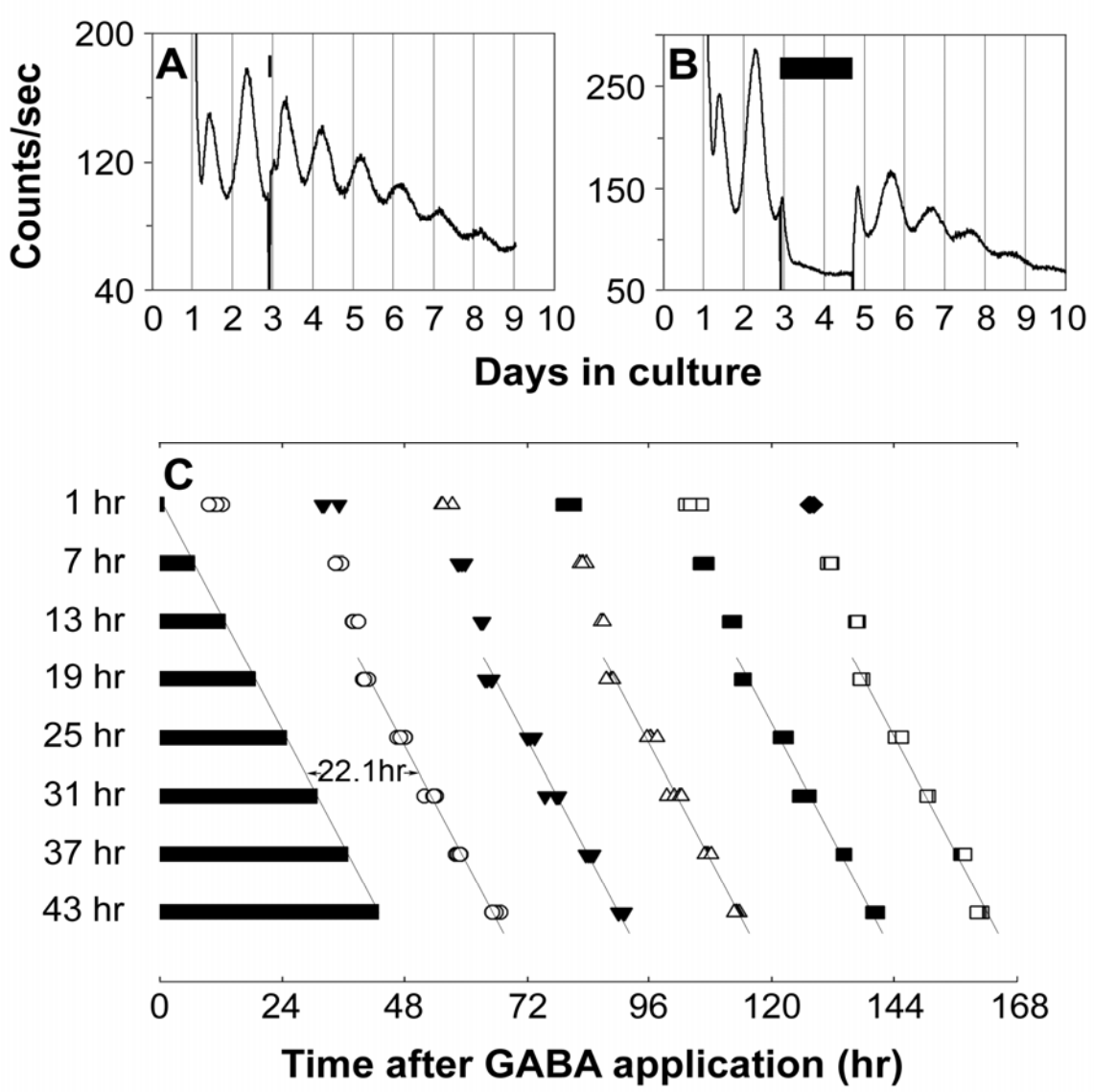

Figure 4-8: Effect of Prolonged Application of GABA on Retinal PER2::LUC Rhythms. (A and B) Representative retinal explant cultures that received 1 hr (A) or 43 hr (B) of 3 mM GABA treatment. GABA treatment was started at the beginning of the 3rd circadian cycle. Bars indicate the duration of GABA treatment. Treatment was terminated by fresh media change.

(C) Shown are PER2::LUC expression peaks following GABA washout. Four retinal explants were sampled for each duration of GABA treatment. Bars indicate the duration of GABA treatment. Time 0 corresponds to the start of GABA treatment. In the samples with 19-43 hr of GABA application, the first peaks appeared ca. 22 hr after media change, and the re-initiated rhythms were phase locked to the termination of the GABA pulse.

GABA and Membrane Polarization

The finding that GABA acts on the retinal PER2::LUC rhythms through ionotropic, Cl^--conducting receptors suggested that GABAergic modulation of the clock could be mediated by membrane hyperpolarization or, in the case of prolonged GABA application, by alteration of membrane ionic gradients leading to tonic depolarization (Ge et al., 2006). Depolarization with elevated K^+ media (4 mM) during 1 mM GABA application partially restored the rhythmic amplitude of PER2::LUC expression (A4/A2 = 0.70 ± 0.04 for vehicle, 0.20 ± 0.05 for GABA, 0.45 ± 0.07 for high K^+ + GABA; n = 5 each; Figures 4-9A and 9B), indicating that GABA acts in part through membrane hyperpolarization. Prolonged application of high K+ alone modestly increased the amplitude of PER2::LUC rhythms (A4/A2 = 0.90 ± 0.10; n = 5; Figure 4-9C), indicating that the inhibitory effect of GABA on retinal PER2::LUC rhythms is not due to tonic depolarization. GABA is not mediated through the simple cessation of neuronal spiking due to membrane hyperpolarization as blocking neuronal spiking with tetrodotoxin (TTX; 1 μM) did not affect retinal PER2::LUC rhythms (A4/A2 = 0.67 ± 0.09; n = 4; Figure 4-9D).

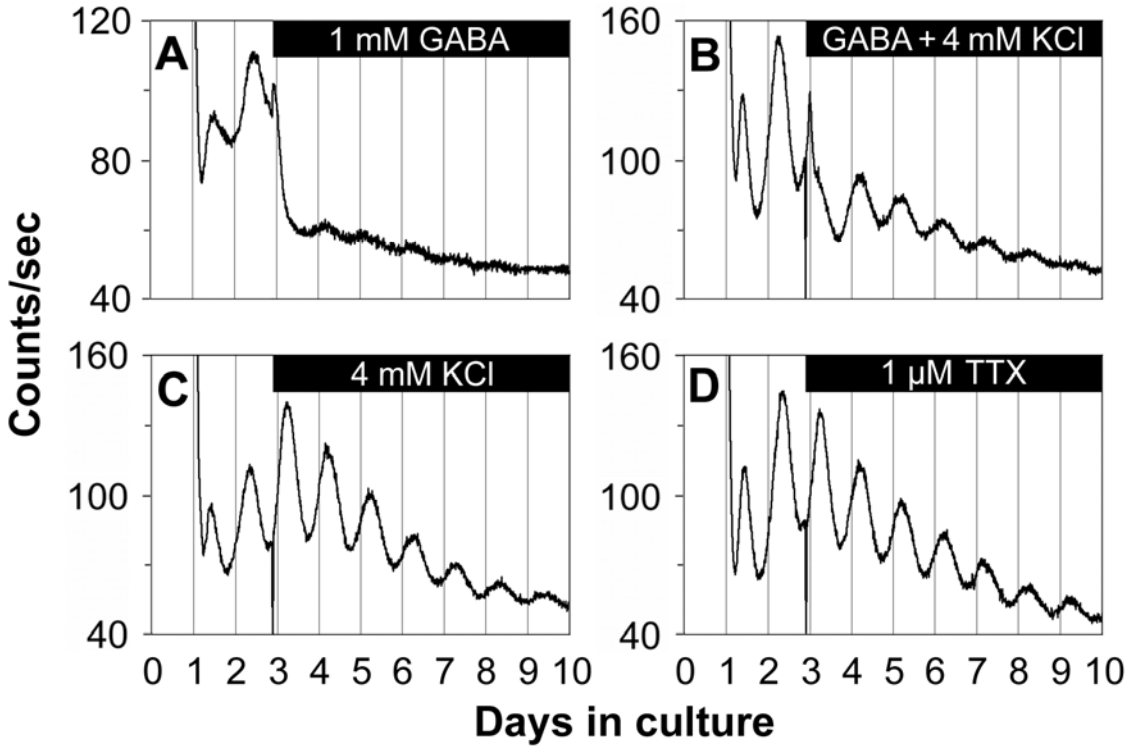

Figure 4-9: GABA and membrane polarization.
(A) GABA (1 mM) suppressed retinal PER2::LUC rhythms.
(B) Depolarization with elevated K^+ (4 mM) partially blocked the inhibitory effect of GABA (1 mM) on retinal PER2::LUC rhythms.
(C) Prolonged application of KCl (4 mM) modestly increased rhythmic PER2:LUC amplitudes.
(D) TTX (1 µM) did not affect the amplitude of retinal PER2::LUC rhythms.
Bars indicate the duration of GABA treatment.
Bars indicate the duration of treatment.

GABA and Casein Kinase Activation

Depolarization with elevated K^+ media in the presence of GABA only partially rescued the amplitude of retinal PER2::LUC rhythms, indicating that other mechanism(s) contribute to the inhibitory action of GABA. The epsilon and delta isoforms of casein kinase I are important regulators of PER protein stability that phosphorylate PER2 and target it for ubiquitin-mediated proteasomal degradation (Eide et al., 2005). In addition, casein kinases associate with $GABA_A$ and $GABA_C$ receptor subunits (Sedelnikova and Weiss, 2002; Ning et al., 2004). To test whether casein kinases are involved in the inhibitory effect of GABA on PER2::LUC levels and retinal rhythms, we applied the casein kinase inhibitor CKI-7 (50 μM) along with 1 mM GABA to *mPer2*^{Luc} retinal explant cultures. CKI-7 partially rescued rhythmic amplitude in the presence of GABA (A4/A2 = 0.35 ± 0.03 for CKI-7 + GABA vs 0.17 ± 0.04 for GABA; $P < 0.05$; n = 4 each; Figures 4-10A and 10B). When KCl (4 mM) and CKI-7 (50 μM) were co-applied with 1 mM GABA, the rescue of rhythmic PER2::LUC amplitude was complete (A4/A2 = 0.93 ± 0.15; n = 4; Figure 4-10C). When applied alone, CKI-7 did not significantly change the amplitude of PER2::LUC rhythms, however, it substantially lengthened retinal free-running period from 24.05 ± 0.09 hr (n = 5) to 25.77 ± 0.38 hr (n = 6; $P < 0.005$; Figure 4-10D). Taken together, these results suggest that GABA acts, in part, by stimulating casein kinase to suppress PER2 levels.

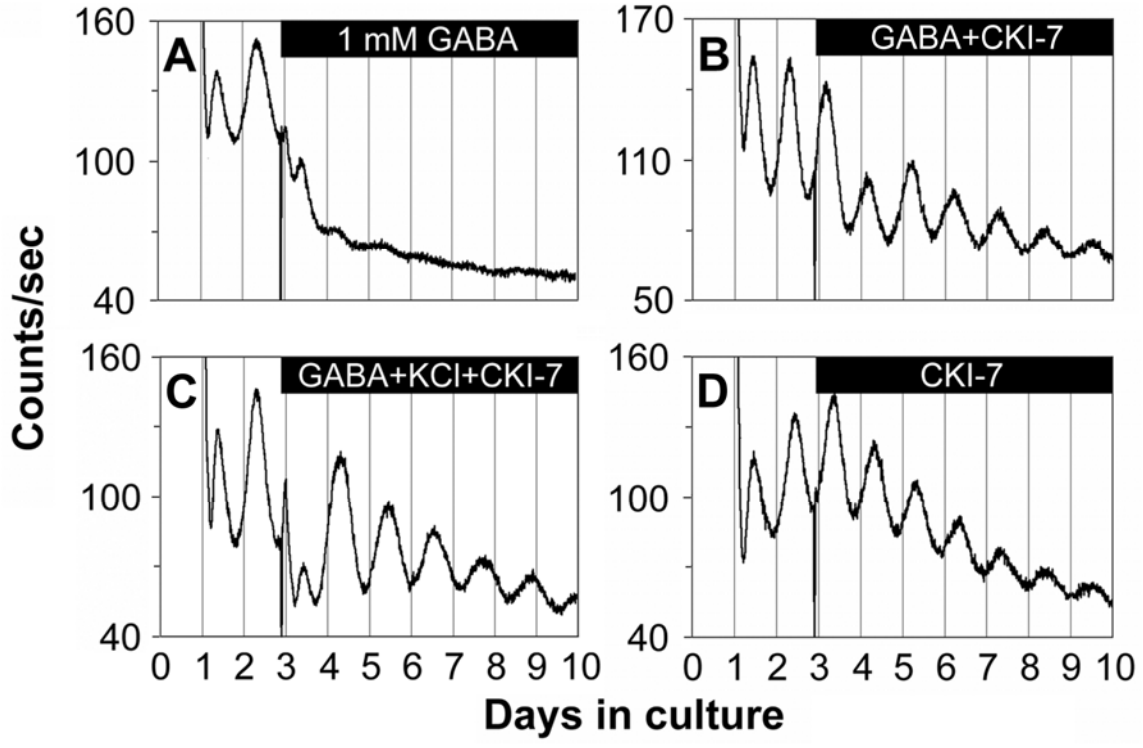

Figure 4-10: GABA and casein kinase.
(A) GABA (1 mM) suppressed retinal PER2::LUC rhythms.
(B) Inhibition of casein kinase activity with CKI-7 (50 μM) partially rescued the inhibitory action of 1 mM GABA on retinal PER2::LUC rhythms.
(C) KCl (4 mM) and CKI-7 (50 μM) completely rescued the inhibitory action of GABA (1 mM) on retinal PER2::LUC rhythms when they were co-applied with GABA.
(D) When CKI-7 (50 μM) was applied alone, it lengthened the period of retinal PER2::LUC rhythms.
Bars indicate the duration of treatment.

Discussion

In the present study, we examined the influence of GABA in the mouse retinal circadian clock using the retinal explant culture protocol described in Chapter III. There are three main findings of the current study: first, GABA inhibited the amplitude of retinal PER2::LUC rhythms in a dose-dependent manner through $GABA_A$ and $GABA_C$ receptors; second, prolonged GABA application stopped ensemble retinal PER2::LUC rhythms; and finally, the inhibitory function of GABA was blocked by elevated K^+ media along with the casein kinase inhibitor CKI-7. Our results indicate that GABA plays an important role in regulating the circadian clock in the mammalian retina, and imply that GABA acts at the molecular level of PER proteins to reinforce the autonomous generation of the retinal "night" state.

GABA and Retinal PER2::LUC Rhythms

In the current study, we found that $GABA_A$ and $GABA_C$ receptor blockade significantly increased the amplitude of retinal PER2::LUC rhythms, whereas application of exogenous GABA damped rhythmic amplitude, and with prolonged application, stopped the retinal circadian clock. The high concentrations of exogenous GABA (> 100 μM) necessary to modulate retinal PER2::LUC rhythms in these experiments are likely due to uptake by high-affinity GABA transporters present on a range of retinal neurons, as well as on retinal Müller cells (Johnson et al., 1996). Previous electrophysiological experiments have demonstrated, for example, that application of 100 μM GABA

produces near maximal effects on isolated retinal ganglion cells, but higher concentrations are necessary to produce detectable effect in retinal slice preparations (Pan and Slaughter, 1995). Our finding that the effects of GABA can be mimicked or blocked by $GABA_A$ and $GABA_C$ receptor agonists and antagonists indicates that the effects of GABA on retinal PER2::LUC rhythms are receptor-mediated and specific.

Our data establish that GABAergic regulation is a common mechanism across neural circadian clocks as blockade of GABA receptors in SCN explants also increases *Per1::luc* rhythmic amplitude (Aton et al., 2006) and application of GABA resets the phase of spike frequency rhythms in isolated SCN neurons (Liu and Reppert, 2000). However, the ability of exogenous GABA to substantially suppress PER2::LUC rhythms is unique to the retina, as similar experiments with SCN slices do not yield similar results. The increased effectiveness of GABA as a modulator of retinal PER2 expression is likely due to the specific retinal expression of the $GABA_C$ receptor, which engenders non-desensitizing responses and prolongs greatly the effectiveness of any GABA stimulation (Polenzani et al., 1991; Qian and Dowling, 1993; Fletcher et al., 1998).

The inhibitory action of GABA on the amplitude of retinal PER2::LUC rhythms could act through damping of circadian rhythms in individual retinal neurons, or through desynchronizing individual oscillators, and differentiating these mechanisms will require single-cell resolution studies of retinal circadian rhythms. A functional consequence reducing rhythmic amplitude, predicted by limit cycle models of circadian oscillators, is to increase sensitivity to phase resetting stimuli. Thus, a potential role of GABA in neural

circadian oscillators is to enhance the effectiveness of endogenous phase-communicating substances, such as vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP) in the SCN (Aida et al., 2002; Meyer-Spasche and Piggins, 2004), or dopamine in the retina (Cahill and Besharse, 1991), in resetting the rhythms of neuronal oscillators by limiting the amplitude of the intracellular molecular clock oscillations.

On a mechanistic level, our data suggest that GABA acts to suppress the amplitude of retinal molecular rhythms through membrane hyperpolarization and casein kinase activation. The mechanisms by which GABA may stimulate retinal casein kinases is unknown at this point. CKI has been shown to physically associate with $GABA_A$ receptors in a rhythmic manner in the SCN (Ning et al., 2004) and CKII has been shown to phosphorylate $GABA_C$ receptor subunits (Sedelnikova and Weiss, 2002). These types of physical associations between GABA receptors and casein kinases have been assumed to be for receptor modulation, but could provide a link between persistent activation of GABA receptors and activation of casein kinases as well. Alternatively, casein kinases are regulated by protein phosphatases and by metabotropic glutamate receptors (Rivers et al., 1998; Liu et al., 2002) and perhaps persistent GABA stimulation alters these pathways. It will be of interest in the future to elucidate more fully the mechanistic relationship of GABA to casein kinase and its effect on the retinal clock.

GABA and Retinal Circadian Clock Organization

Our finding that GABA plays key roles in the retinal circadian clock draws attention

to GABAergic neurons as sources of these retinal circadian signaling molecules and potential sites of rhythms generation. The full complement of core clock genes is expressed in GABAergic horizontal cells and dopaminergic amacrine cells which also express GABA (Ruan et al., 2006). In addition, transcription of the *Per1* clock gene has been shown to occur in most GABAergic amacrine cells, and rhythmically in the dopaminergic and NOS-positive subtypes of GABAergic amacrine cells (Witkovsky et al., 2003; Zhang et al., 2005). Thus, there is a strong correlation of core clock gene expression within GABAergic retinal neurons, suggesting a molecular basis for circadian regulation of this neurotransmitter. In contrast, *Per1* expression has been shown to be absent from cholinergic and glycinergic amacrine cells (Witkovsky et al., 2003; Zhang et al., 2005), two transmitter systems found not to be involved in the regulation of retinal PER2::LUC rhythms (Figure 4-1).

Retinal GABA turnover rate and release show rhythmic variations under constant darkness condition, with their levels higher in the subjective night (Jaliffa et al., 2001). Furthermore, GABA increases melatonin content in a dose-dependent manner in the hamster retina (Jaliffa et al., 1999). Our results suggest that GABA could, through membrane hyperpolarization and activation of casein kinase, stimulate the degradation of accumulated PER proteins on the falling phase of their rhythm (roughly corresponds to the night state), acting to remove feedback inhibition on *Per* transcription and thereby preparing retinal oscillators for transcriptional activation that characterizes the “day” state. Taken together, these data are consistent with the notion that one role of GABA in the

retinal circadian clock is to function as an analog for darkness and enforce the night state in the mammalian retina, along with melatonin.

Our finding that the $GABA_C$ receptor is a critical co-mediator of GABA influence on retinal PER2::LUC rhythms suggest roles for neurons in the outer half of the retina in circadian clock function. The $GABA_C$ receptor has a restricted distribution in the mammalian retina with the primary site of expression on bipolar cell synaptic terminals in the inner plexiform layer where it mediates recurrent feedback from GABAergic amacrine cells (Wassle et al., 1998), and a secondary site of expression in mouse cone photoreceptors (Pattnaik et al., 2000). Thus, the balance of results suggests that retinal bipolar cells, some of which coordinately express all the core clock genes (Ruan et al., 2006), and perhaps photoreceptors, are important targets of GABAergic regulation in the retinal circadian clock network. So far convincing evidence for the presence of $GABA_C$ receptors on rod photoreceptors, horizontal cells, amacrine cells and ganglion cells in the mammalian retina is still lacking, however, $GABA_A$ receptors may mediate the action of GABA on these cell types as the $GABA_A$ receptor agonist muscimol alone partially reduced rhythmic PER2::LUC amplitudes (Figure 4-4A) and $GABA_A$ receptors have been detected in these retinal cell types (Yang, 2004).

Summary

In summary, we have used our real-time clock gene expression assay for the isolated mouse retina to examine the influence of GABA on retinal PER2::LUC rhythms. Our

main finding is that GABA, acting through $GABA_A$ and $GABA_C$ receptors, functioned as an important negative regulator of the amplitude of retinal PER2::LUC expression rhythms. We propose that GABA plays a key role in mammalian retinal circadian organization by mediating the amplitude of the retinal clock, as well as serving as a rhythmic output signal by which the retinal clock establishes a functional "night" state in retinal circuits.

CHAPTER V

SUMMARY

In this dissertation I have described my work towards elucidating the cellular location and neural regulation of the circadian clock in the mouse retina. Several techniques and protocols have been modified or developed to address these questions, including long-term culture of adult mouse retina and single-cell real-time RT-PCR for clock genes. There are six main findings of this study: first, single-cell real-time PCR demonstrated that a significant portion of each neuron type in the inner nuclear layer and ganglion cell layer, but not photoreceptors, express the full complement of core clock genes and are thus potential circadian clock neurons; second, quantitative PCR and real-time gene expression assay of photoreceptor degenerate mouse retinas revealed that retinal molecular rhythms persist upon photoreceptor degeneration, indicating the inner nuclear and/or ganglion cell layers of the mouse retina contain endogenous, self-sustained circadian clock which is independent of photoreceptors and the master SCN clock; third, in the intact retina, molecular circadian rhythms are located to the inner nuclear layer; fourth, dopamine, acting through dopamine D1 receptors, resets the phase of retinal PER2::LUC rhythms; fifth, melatonin does not affect the phase or amplitude of retinal PER2::LUC rhythms; finally, but not least important, GABA, acting through $GABA_A$ and $GABA_C$ receptors, linked to membrane hyperpolarization and casein kinases, negatively

regulates the amplitude of retinal PER2::LUC rhythms. Therefore, my results have established a novel basis for the organization of the mammalian neural retina as a circadian pacemaker tissue, and support a model for neural regulation of the mouse retinal clock in which dopamine and GABA reinforce the "day" and "night" states of the endogenous retinal clock by regulating the phase and amplitude of retinal PER circadian rhythms, respectively.

Circadian Clocks in the Inner Nuclear and Ganglion Cell Layers

To localize potential circadian clock neurons in the mouse retina, I used single-cell real-time RT-PCR to profile circadian clock gene expression in individual neurons of each retinal neuron type. To avoid RNA contamination from culture medium, I introduced a perfusion system and modified it to make the outlet speed flow faster than the inlet speed so that there was very little fresh medium left near the target cell during cell harvesting. This modification effectively avoided RNA contamination from the medium. With this method and subsequent TaqMan real-time RT-PCR, individual horizontal, rod bipolar, dopaminergic amacrine, type 2 catecholamine amacrine, and ganglion cells were found to coordinately express all 6 core clock genes at varying rates according to cell type. However, individual photoreceptors expressed only 3 or less clock genes. In addition, imaging of PER2::LUC luminescence from intact retinal vertical slices of both B6C3 and C57BL/6J mice showed that PER2::LUC expression was predominantly localized to the inner nuclear layer of the retina, which contains the nuclei

of retinal horizontal, bipolar, and amacrine cells. Moreover, profiling of temporal core clock gene expression in photoreceptor degenerate mouse retinas with quantitative real-time PCR revealed that all core clock genes except *Clock* exhibited statistically significant variations in expression level over the 24 hr sampling period consistent with ongoing rhythms in both light-dark and constant darkness conditions. Consistently, PER2::LUC expression rhythms persisted *in vitro* following photoreceptor degeneration, indicating the clock gene-expressing neurons of the inner retinal layers comprise a self-sustained circadian clock that can generate rhythmicity independent of photoreceptors and the master SCN clock. These results, along with recent immunohistochemistry studies showing predominant localization of PER1, PER2 and CLOCK protein in the inner nuclear layer, form convergent lines of evidence supporting a novel paradigm for circadian organization of the mammalian retina in which retinal neurons other than rod or cone photoreceptors, neurons in the inner nuclear layer in particular, are a primary locus of endogenous circadian rhythms generation.

In the present study, I found that none of the major endogenous retinal neurotransmitter systems, including dopamine, melatonin, glutamate, acetylcholine, glycine and GABA, was required to support generation of retinal circadian PER2::LUC rhythms, nor was communication through action potentials or gap junctions (Figure 5-1). These data support cell-autonomous rhythms generation in the retinal circadian clock and indicate that rhythmic PER2::LUC expression in the inner nuclear layer is unlikely driven by possible circadian clocks located in photoreceptors (Tosini et al., 2007b), or by a

minority of pacemaker cells in other parts of the retina. The weak intercellular coupling in the retinal circadian organization is different from the master SCN clock, in which blocking action potentials with TTX or interrupting cell communication via vasoactive intestinal peptide (VIP) with genetic mutations causes both loss of intercellular coupling across SCN neurons, as well as reduction of amplitude within individual SCN neurons (Honma et al., 2000; Yamaguchi et al., 2003; Aton et al., 2005). The weak coupling exhibited by the retinal circadian clock under constant conditions in the current *in vitro* study may be a functional consequence of the retina having direct access to the principal *zeitgeber*, the external light/dark cycle, so that synchronization across retinal oscillators *in vivo* is accomplished via recurrent light cues, rather than by intercellular coupling mechanisms.

The wide distribution of potential circadian oscillators in the inner retina suggests that multiple physiological rhythms expressed in these cell types are controlled by circadian oscillators located in these cell types themselves. However, circadian rhythms in photoreceptors, such as disk shedding (LaVail, 1976), melatonin secretion (Tosini and Menaker, 1996), and Na^+/K^+ ATPase function (Shulman and Fox, 1996) are likely regulated by the inner retinal circadian signal dopamine (Nguyen-Legros et al., 1996; Iuvone et al., 2005).

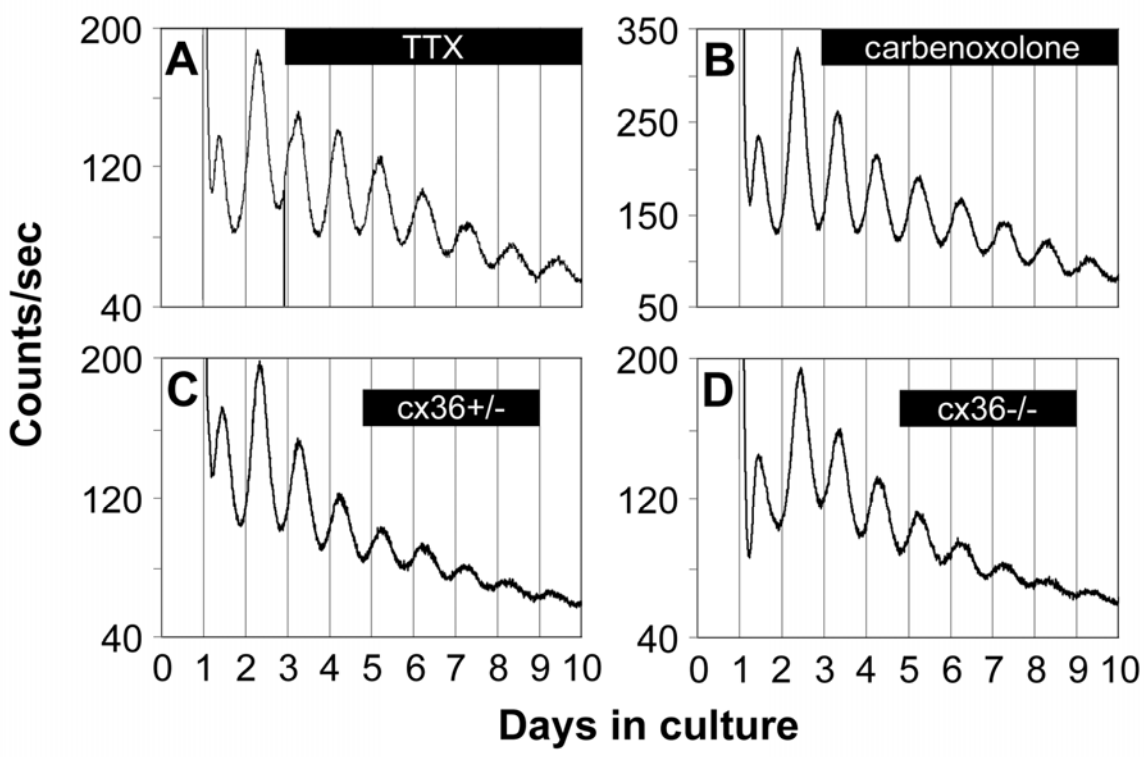

Figure 5-1: Generation of retinal PER2::LUC rhythms is independent of action potentials or gap junctions.

(A) Continuous application of the voltage-gated Na+ channel blocker tetrodotoxin (TTX; 1 μM) did not change the amplitude or damping rate of retinal ensemble PER2::LUC rhythms (n = 5).

(B) Continuous application of the gap junction blocker carbenoxolone (100 μM) did not change the amplitude or damping rate of retinal ensemble PER2::LUC rhythms (n = 4). Bars in (A-B) indicate the duration of treatment.

(C-D) Retinal explants cultured from connexin (cx) 36+/- mice (C) and cx36-/- mice (D) did not show difference in the amplitude or damping rate of PER2::LUC rhythms (n = 8 each).

Retinal Circadian Clock Regulated by Dopamine and GABA

Explant Culture of Intact Retina from Adult Mouse

In mammals, the neural retina is the most easily accessible part of the central nervous system. The structure, cellular interaction and physiological rhythms have been well characterized. However, information regarding the neural regulation of the mammalian retinal clock has been limited by the challenge of long-term culture of the mammalian retina and the interpretational caveats imposed by using melatonin or dopamine output rhythms as strategies for observing the clock mechanism itself. In the current study, I developed a new protocol for adult mouse retinal explant culture. Briefly, mouse retinal explants were first cultured in neurobasal medium in 5% CO_2 at 37°C for 24 hr, and subsequently transferred to medium 199 and sealed in petri dishes. Cultured retinal explants showed intact histological structure and the normal complement of dopamine neurons, as well as robust circadian rhythms of PER2::LUC expression for numerous circadian cycles without perturbation. This system has the advantage of measuring expression rhythmicity of a clock gene, rather than a neurochemical output of the retinal clock, thus allowing examination of the roles of neuronal communication in modulating core clock mechanisms in the mouse retina. In the present study, I have used this system to examine the influence of dopamine, melatonin, GABA, and other retinal neurotransmitters on the mouse retinal circadian clock.

Dopamine Regulates the Phase of Retinal Circadian Clock

In the current study I found that activation of dopamine D1 receptors reset the phase of retinal PER2::LUC rhythms, producing phase advances during treatments beginning in the early subjective day and phase delays during treatments beginning in the early subjective night. These effects on circadian phase demonstrate that retinal dopamine influences the core clockwork of mammalian retinas. Therefore, in the circadian organization of the retina, retinal dopamine not only serves as an output messenger that mediates a number of physiological rhythms, but also acts as an input signal to regulate the phase of the clock mechanism.

In the mammalian retina, dopamine D1 receptors are primarily expressed on horizontal, AII amacrine and bipolar cells (Nguyen-Legros et al., 1997; Nguyen-Legros et al., 1999; Witkovsky, 2004). Therefore, dopamine targets putative oscillator cell types in the inner nuclear layer to reset the retinal circadian clock. In contrast, activation of retinal dopamine D2/D4 receptors, which are expressed predominantly in photoreceptors (Witkovsky, 2004), did not change the phase of retinal PER2::LUC rhythms. The D1-dependent mouse retinal PER2::LUC phase resetting mechanism is distinct from the D2/D4-mediated phase resetting of amphibian retinal melatonin rhythms (Cahill and Besharse, 1991). These results further strengthen our conclusion that the inner retina is the primary location of the mouse retinal clock.

Melatonin Does Not Affect the Phase or Amplitude of Retinal Circadian Clock

In the current study, I found that PER2::LUC rhythms persisted in C57BL/6J mouse retinas with genetically blunted melatonin production and following activation of melatonin receptors or blockade of melatonin MT1 receptors. In addition, neither manipulation of melatonin, nor blockade of MT1 receptors, significantly altered the phase of retinal PER2::LUC rhythms. Taken together, these results suggest that melatonin's role in the mammalian retinal circadian clock is primarily as a rhythmic output. My findings are consistent with previous studies showing that melatonin is not required for circadian photoreceptor disk shedding rhythms in constant darkness (Grace et al., 1999). Melatonin secretion itself is likely regulated by the circadian neurotransmitters dopamine and GABA (Jaliffa et al., 1999; Tosini and Dirden, 2000). Similarly, the influence of melatonin on multiple retinal physiology might be indirect, through its inhibition of dopamine release (Dubocovich, 1983).

GABA Negatively Regulates the Amplitude of Retinal Circadian Clock

In the current study, one substantial finding is that GABA negatively regulates the amplitude of retinal PER2::LUC rhythms. Application of GABA reduced PER2::LUC expression and peak-to-trough amplitudes in a dose-dependent manner. This action was mimicked by simultaneous activation of $GABA_A$ and $GABA_C$ receptors. Blockade of $GABA_A$ and $GABA_C$ receptors increased the amplitude of retinal PER2::LUC rhythms, indicating that endogenous GABA suppresses rhythmic amplitude of retinal PER2

oscillation. Prolonged application of GABA reversibly suppressed PER2::LUC expression rhythms and re-initiated rhythms were phase locked to the termination of the GABA pulse, suggesting that GABA has direct access to the circadian clock mechanism. The inhibitory action of GABA on retinal PER2::LUC rhythms is likely mediated through membrane hyperpolarization and casein kinase. Thus, GABA plays a key role in regulating the circadian clock in the mammalian retina. Interestingly, core clock gene expression is strongly correlated with GABAergic retinal neurons (Witkovsky et al., 2003; Zhang et al., 2005; Ruan et al., 2006), suggesting a molecular basis for circadian regulation of retinal GABA transmission. In contrast, *Per1* expression is absent from cholinergic and glycinergic amacrine cells (Witkovsky et al., 2003; Zhang et al., 2005), which are not involved in the regulation of retinal PER2::LUC rhythms (Figure 4-1). Therefore, GABAergic amacrine cells not only are one source of retinal rhythms generation, but also maintain other retinal oscillators well-being through its circadian signaling molecule, GABA.

Overall Model for the Mouse Retinal Circadian Clock Organization

In summary, my data support a model for the mouse retinal circadian clock organization as shown in Figure 5-2. There are multiple putative circadian oscillators located in the inner nuclear layer of the mouse retina, and their rhythms generation is largely cell-autonomous and independent of major forms of retinal neural communication. However, GABAergic retinal neurons, GABAergic amacrine cells (including

dopaminergic amacrine cells) in particular, regulate the amplitude and phase of these autonomous oscillator cells through rhythmic secretion of dopamine and GABA. Dopamine transmission acts to reinforce the "day" state, while GABA transmission acts to reinforce the "night" state of the retinal oscillator cells (see Figure 5-2 legend). Future studies can employ multiple strategies to test this working hypothesis regarding the organization of the mammalian retinal circadian clock.

Future studies using our *in vitro* retinal explant culture system can further elucidate the cellular and molecular mechanisms of retinal circadian clocks, the mechanisms by which retinal circadian clock regulates multiple physiologically retinal rhythms, and their influence on normal retinal function. The highly ordered and well characterized property of retinal circuitry will for certain facilitate elucidation of the general principles of circadian pacemaking system.

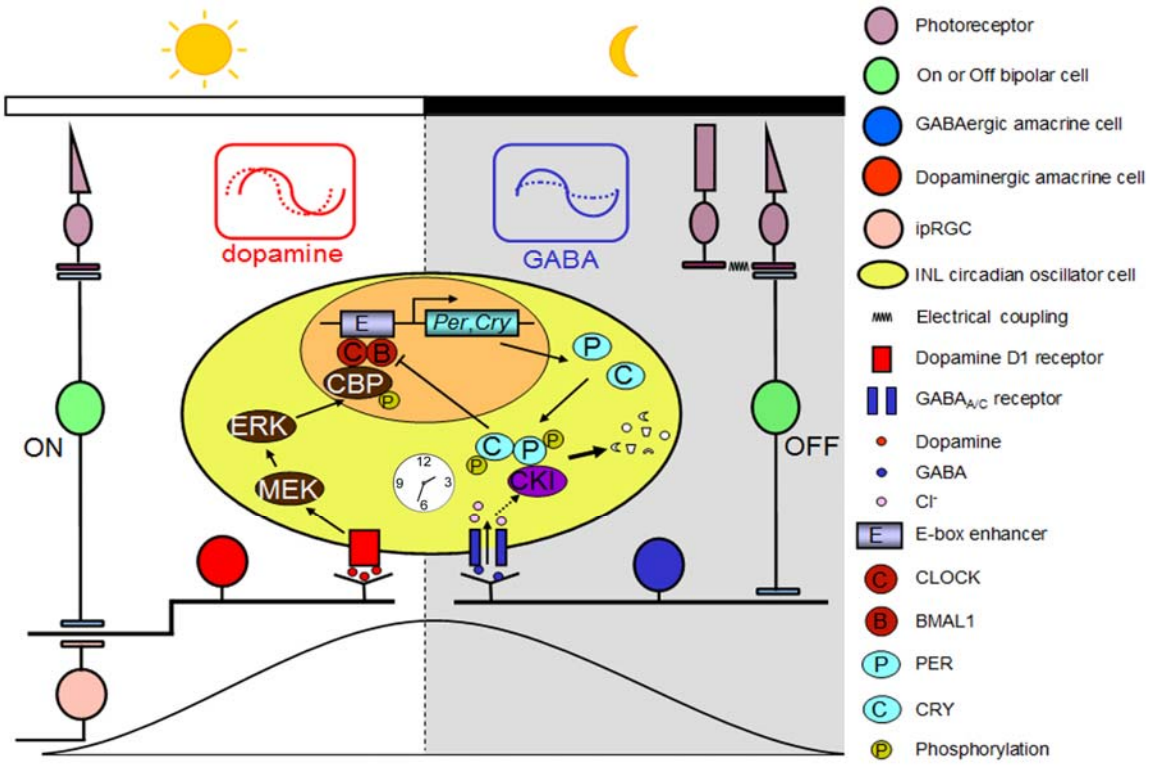

Figure 5-2: Model for the circadian clock organization in the mouse retina.
Dopaminergic amacrine cells and GABAergic amacrine cells reinforce the "day" and "night" states of multiple oscillator cells in the inner nuclear layer (INL) through rhythmic secretion of dopamine and GABA, respectively. During the day (i.e. molecularly, roughly the rising phase of PER2 rhythms), dopaminergic amacrine cells receive excitatory light input from M-cones via ON bipolar cells and/or from intrinsically photosensitive retinal ganglion cells (ipRGCs), and increase the secretion of dopamine to regulate the phase of the inner retinal clock via dopamine D1 receptors. In the early subjective day, dopamine phase advances the retinal clock; while in late subjective day and early subjective night, dopamine phase delays the retinal clock. Thus, dopamine transmission acts to reinforce the "day" state of the retinal clock. It has been reported that *Per1* expression upregulation by activation of D2 receptors is mediated through the mitogen-activated extracellular signal-regulated kinase (ERK) kinase (MEK) and the cAMP-responsive element-binding protein (CREB) binding protein (CBP) (Yujnovsky et al., 2006), which may be also involved in phase resetting of retinal PER2::LUC rhythms upon D1 receptor activation. In the night (i.e. molecularly, roughly the falling phase of PER2 rhythms), GABAergic amacrine cells receive excitatory input from OFF bipolar cell and increase the turnover and secretion of GABA, which in turn, through activation of $GABA_{A/C}$ receptors and casein kinase, stimulates the degradation of accumulated PER proteins on the falling phase of their rhythm, acting to remove feedback inhibition on *Per* transcription and thereby preparing retinal oscillators for next transcriptional activation. Thus, GABA transmission acts to reinforce the "night" state of the retinal clock.

APPENDIX

FUTURE DIRECTIONS

Although the current study has greatly improved our understanding of the mammalian retinal circadian clock system, many additional experiments are necessary to further elucidate the cellular and molecular mechanisms of retinal circadian clocks and their influence on visual function as well as reveal additional principles regarding the functional organization of neural circadian oscillators.

Explore the Location(s) of Cell-Autonomous Circadian Oscillators in the Mouse Retina

My examination of PER2::LUC bioluminescence in retinal vertical slices has revealed that PER2::LUC is expressed across the inner nuclear, indicating that horizontal cells, bipolar cells, and amacrine cells are all potential circadian oscillators. In addition, my single-cell real-time PCR study has demonstrated that significant ratios of horizontal, rod bipolar and dopaminergic amacrine cells of the inner nuclear layer express all six core clock genes and thus are putative self-sustained circadian oscillators (Ruan et al., 2006). Furthermore, *Per1* transcription has been detected in most GABAergic amacrine cells, and rhythmically in the dopaminergic and NOS-positive subtypes of GABAergic amacrine cells (Witkovsky et al., 2003; Zhang et al., 2005). However, all these findings

have not directly verified the existence of self-sustained oscillators in these cell types.

There are several strategies to determine whether certain cell types contain cell-autonomous oscillators or not. One is to prepare low-density primary retinal cell culture from reporter mice, use morphological characterizes or other transgenic reporters to identify dissociated cells, and then use high sensitive CCD camera to image these cells to determine whether they are cell-autonomous oscillators or not. The biggest concern about this strategy is that the PER2::LUC reporter, as a knockin reporter, does not yield high enough bioluminescence signals. *Per1*::luc transgenic reporter, on the other hand, does show much higher bioluminescence level compared to the PER2::LUC reporter, but its peak-to-trough amplitude was found to be quite low in the whole-mount mouse retina in my previous study. Generation of new *Per2*::luc or *Bmal1*::luc transgenic mice may enable detection of circadian oscillation in clock gene expression at single-cell level in primary retinal cell culture. In addition, a protocol for long-term culture of mouse retinal dissociated cells needs to be developed. An alternate strategy to locate cell-autonomous oscillators in the mouse retina is to establish conditionally immortalized cell lines using transgenic mice that harbor the PER2::LUC reporter and ubiquitously express the temperature-sensitive (ts) T-antigen gene of simian virus 40 (SV40), as the Tei lab did in the SCN (Kawaguchi et al., 2007), and then to assay PER2::LUC expression and circadian rhythmicity in the homogeneous cell populations, which are derived from individual retinal embryo cells. Another alternate way to address this question is to make new transgenic mice in which a luciferase reporter gene is driven by a retinal cell

type-specific promoter. Likewise, we can intravitreously inject mice or transfect cultured moue retinas with retrovirus that carries retinal cell type-specific promoter-driven luciferase gene.

My single-cell real-time RT-PCR study revealed that some but not all of horizontal, rod bipolar, dopaminergic amacrine cells, and ganglion cells expressed all six core clock genes (Ruan et al., 2006), suggesting that currently characterized retinal cell types/subtypes are heterogeneous in the regard of clock function. If this is verified by future studies, it will be of importance to further compare the difference in overall gene expression profile as well as cellular connection with other cells between oscillator cells and non-oscillator cells in the same retinal cell type/subtype to deepen our understanding of the functional organization of the mammalian retinal circadian clock.

Determine the Contribution of Circadian Clocks in Each Cell Population to the Overall Retinal Clock Network

My study indicates that horizontal cells, bipolar cells, and amacrine cells are all potential circadian oscillators. However, additional experiments are necessary to fully elucidate the specific role of the clock located in each cell type/subtype in the overall retinal clock network. The best way to achieve this goal might be to use a Cre/loxP system to specifically disrupt the clock function in each cell population and then examine its consequence to overall retinal molecular rhythms. For example, we can cross *TH*::Cre with *Bmal1*^{loxP} mice to specifically disrupt the clock function in dopaminergic neurons. In

this case, whereas dopamine will still be synthesized and released, its rhythmicity might be abolished if the rhythmicity is controlled by endogenous clock in dopaminergic amacrine cells. Ultimately, it will be possible to simultaneously disrupt clocks in several retinal cell types to see whether the remaining clocks are capable of maintaining molecular and physiological rhythms.

Determine Whether Dopamine Is A Mediator of Light Entrainment in the Mammalian Retinal Circadian Clock

My findings along with many other lines of evidence imply that dopamine serves as a chemical signal for light in the retina and is likely a mediator of light entrainment within the mammalian retinal clock system. Nevertheless, this assumption has not been directly demonstrated yet. One way to address this assumption is to test whether light can entrain PER2::LUC rhythms in retinal explants prepared from retina-specific dopamine-deficient mice. Retinal melatonin secretion rhythms has been reported to be entrained by light/dark cycles *in vitro* at 27°C (Tosini and Menaker, 1998). However, so far I have not succeeded in entraining retinal PER2::LUC rhythms with light/dark cycles at 37°C. This could be due to 11-cis retinol depletion during retinal culture preparation. It may be required to prepare retinal explant culture in complete darkness or under dim red light in order to preserve enough 11-cis retinol. Alternatively, preparation of pigment epithelium-attached retinal culture instead of pure retinal culture may be necessary to retain the retinol. Although temperature is another possible factor affecting entrainment, it is unadvisable to

lower the culturing temperature to 27°C, as it is not in the physiological range of mouse body temperature. Furthermore, in my previous study retinal PER2::LUC rhythms were not detected when retinal explants were cultured at 27°C. It is unclear to us why retinal cultures show circadian rhythms of melatonin release but not of PER2 expression at 27°C.

Melanopsinergic ganglion cells constitute the retinal projection to the SCN and are extremely important in entraining the SCN clock (Hannibal and Fahrenkrug, 2002; Hattar et al., 2002). However, it is unknown whether these cells participate in retinal clock entrainment as well. Therefore, once we are able to entrain our retinal PER2::LUC rhythms *in vitro* with light/dark cycles, it will be extremely interesting to test whether light is capable of entraining retinal PER2::LUC rhythms in melanopsinergic ganglion cell-deficient mice as well.

Examine the Role of Retinal Dopamine in Regulating Retinal Physiological Rhythms

There are multiple circadian rhythms at the physiological, cellular, and molecular levels in the mammalian retina (Table 1-1). However, it is unclear whether retinal clock controls these rhythms directly, or indirectly through its major rhythmic output, dopamine. Many *in vitro* studies suggest that retinal dopamine is a potent modulator of rhythmic retinal physiology and acts at multiple sites within the retinal circuitry to shape retinal function into “day” states. Nevertheless, this hypothesis has not been verified by *in vivo* study. One way to address this hypothesis is to compare between normal mice and

retina-specific dopamine-deficient mice in retinal physiological rhythms, e.g. circadian rhythms of electroretinogram (ERG) b-wave, which represents light-induced electrical activity in retinal cells post-synaptic to the photoreceptors and the major contribution of b-wave in the mammalian retina is ON bipolar cells. In the mouse retina, the amplitude of ERG b-wave shows circadian rhythmicity with higher level in the subjective day and lower level in the subjective night (Barnard et al., 2006; Storch et al., 2007). If circadian rhythmicity of ERG b-wave amplitude is altered in retina-specific dopamine-deficient mice, then dopamine should be involved in the regulation of ERG b-wave. Positive result obtained from this study will also answer the question of whether there is any retinal circadian rhythm that is not only controlled by local oscillators, but also by rhythmic neurotransmitter input(s).

Study the Mechanism by Which GABA Regulates Casein Kinase Activity

My current data suggest that GABA suppresses the amplitude of retinal molecular rhythms through casein kinase activation and degradation of PER2. However, it is not clear from my *in vitro* pharmacological studies how endogenous GABA modulates retinal CKI activity *in vivo*. $GABA_A$ and $GABA_C$ receptors have been shown to physically associate with casein kinase (Sedelnikova and Weiss, 2002; Ning et al., 2004). Associations between GABA receptors and casein kinases have been assumed to be for GABA receptor phosphorylation by casein kinases, but could provide a bridge for activation of casein kinases by activated GABA receptors. Alternatively, casein kinases

are regulated by protein phosphatases and by metabotropic glutamate receptors (Rivers et al., 1998; Liu et al., 2002) and perhaps persistent GABA stimulation alters these pathways. It will be of great importance in the future to elucidate more fully the mechanistic relationship of GABA to casein kinase.

Examine the Role of Casein Kinase in the Retinal Circadian Clock

My finding that casein kinases strongly influences the period of retinal PER2::LUC rhythms has potential implications for familial sleep disorders, such as familial advanced sleep phase syndrome (FASPS) and delayed sleep phase syndrome (DSPS), in which the phosphorylation of PER2 by casein kinases, or the activity of casein kinase Iδ (CKIδ) itself is altered and the entrained phase of the sleep/wake cycle is either substantially advanced or delayed (Toh et al., 2001; Xu et al., 2005). Retinal input potentially gated by the retinal circadian clock sets the entrained phase of the SCN to time sleep/wake cycles (Reppert and Weaver, 2002; Green and Besharse, 2004; Mistlberger, 2005), and influences the rhythmicity, free running period and developmental organization of the SCN clock (Yamazaki et al., 2002; Lee et al., 2003; Ohta et al., 2006). Thus, alterations to the retinal circadian clock by casein kinases associated mutations may contribute to the altered circadian organization and entrainment which typify these sleep disorders. One way to test this hypothesis is to use the tetracycline transactivator (tTA)/ tetracycline operator (tetO) system to specifically rescue the function of CKIδ in the retina of the FASPS mouse model, which carries the human CKIδ-T44A gene and mimics human

FASPS (Xu et al., 2005). In addition, analyzing retina-specific mutations in clock genes may identify new causes leading to human sleep disorder.

Compare the Retinal Clock Machinery with the SCN Clock Machinery

My study and some other studies all suggest that the retinal clock has many characteristics in common with the SCN clock at the molecular level. However, there might be some distinctions between the clock machinery of different tissues. Recent studies suggest that in *Clock*-deficient mice, NPAS2 is capable of maintaining circadian rhythmicity in the SCN, but not in the liver and lung (Debruyne et al., 2006; DeBruyne et al., 2007a, b). It will be useful to examine whether NPAS2 sustains retinal circadian molecular rhythms in *Clock*-deficient mice. In addition, imaging PER2::LUC expression in different tissues isolated from clock mutant mice reveal that *Per1* and *Cry1* are necessary for rhythmicity sustaining in some peripheral oscillators such as the lung, liver, and cornea. However, in the master SCN clock, oscillator network interactions is capable of compensating for *Per1* or *Cry1* deficiency (Liu et al., 2007). The mouse retina is different from most other peripheral oscillators in that it is a neural tissue, and neuronal connection in the retina is rather compact. It is important to examine whether cultured mouse retinas persist PER2::LUC rhythms in the absence of *Per1*, *Cry1*, or other important clock genes.

Determine the Role of *Per2* and *Cry1* in Retinopathy of Prematurity (ROP)

ROP is a retinal disease that occurs in premature infants who are born at a time when the development of retinal vascular and lung are not complete yet. Incompletely developed lungs require extra oxygen supply, however, incompletely formed retinal blood vessels are susceptible to high oxygen and this susceptibility will induce extensive angiogenesis, a process that leads to excessive growth of retinal blood vessels and can ultimately causes retinal detachment and blindness (Phelps, 1995). Accumulating evidence indicates that hypoxia-inducible vascular endothelial growth factor (VEGF) plays a central role in the pathogenesis of ROP (Campochiaro, 2000; Werdich and Penn, 2006). Interestingly, *Per2* and *Cry1* were recently found to be able to down-regulate the hypoxic induction of VEGF in a tumor cell line (Koyanagi et al., 2003). Thus, *Per2* and *Cry1* may play important roles in regulating the pathogenesis of ROP. To address this hypothesis, one experiment is to compare the VEGF level as well as retinal avascularity and neovascularization upon oxygen treatment (Werdich et al., 2004) between wild type mouse pups and *Per2* or *Cry1* knockout mouse pups. In addition, since Müller cells are the major retinal cell type that secrets VEGF during hypoxia-induced neovascularization (Pierce et al., 1995), an additional experiment is to examine the effect of over-expressing *Per2* or *Cry1* on VEGF expression in primary Müller cell culture or in Müller cell line (e.g. rMC-1 cells).

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