DEVELOPMENTAL REGULATION OF MELANOPSIN-CONTAINING RETINAL GANGLION CELLS AND ITS EFFECTS ON CIRCADIAN FUNCTION

by

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv
LIST OF FIGURES v
ABSTRACT vii

CHAPTER 1 Introduction 1

Circadian systems and photoentrainment 2
The Clock 2
Entrainment 5
Measuring circadian rhythms 9

Photoentrainment and the retina 13
Classical photoreceptive pathways 14
Photoentrainment and retinal degeneration 17

Discovery of melanopsin 21

Melanopsin-containing RGCs and the development of retinal architecture 24
Development of RGCs 28
Retinal innervation 30

Interaction among Photoreceptors 32
Potential regulation of ipRGCs 32

SCN organization and retinal input 34
"Core" and "shell" 35
Vasoactive Intestinal Peptide 37
Vasopressin 38
Additional molecules 39
SCN outputs 41

Course of Thesis 43

CHAPTER 2 The development of melanopsin-containing retinal ganglion cells in mice with early retinal degeneration 44

Abstract 46

Introduction 47

Materials and Methods 49
Animals 49
Wheel-running experiments 49
Tissue preparation 50
Characterizing retinal degeneration 50
Dendritic stratification 51
Cell numbers and distribution 52
Pupillometry 53
Statistics 53

Results 54
Light entrainment is altered in CBA/J mice 54
Time course of retinal degeneration in CBA/J mice . . 55
Dendritic stratification of ipRGCs is unaffected by loss of photoreceptors 55
Potential control of ipRGC number and distribution by photoreceptors 56
PLR is intact at high light intensities in CBA/J mice . . 57

Discussion 58

CHAPTER 3 Circadian behavior and central processing in mice with early postnatal retinal degeneration 70

Abstract 72

Introduction 73

Materials and Methods 76
Animals 76
Wheel-running experiments 77
Tissue preparation 77
Antibodies 78
Immunohistochemistry 78
Retinal innervation 79
Cell numbers 79
Light-induced c-Fos expression 80
Statistics 80

Results
CBA/J mice entrain to a narrower range of T cycles 81

CBA/J mice have greater numbers of VIP- and VP-positive cells 82
Retinal innervation does not differ between CBA/J and CBA/N mice 83
Light induction of c-Fos in CBA/J mice 83

Discussion 85
VIP- expressing cells 85
VP- expressing cells 87
Retinal innervation 87
Conclusion 89

CHAPTER 4 Summary and Discussion 95

Dendritic stratification of ipRGCs 96

IpRGC number 98

Physiology 99

Retinal innervation and the SCN 101

VIP-expressing cells 105

VP- expressing cells 107

Role of additional molecules 108

Implications for altered circadian behaviors 111

REFERENCES 114

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LIST OF FIGURES

CHAPTER 1:

Figure 1 Simplified model of *mPer1* induction 4

Figure 2 The SCN and peripheral clocks 5

Figure 3 Stable phase relationship between melatonin levels and the light/dark cycle 7

Figure 4 Phase response curve for light in rat 9

Figure 5 Idealized actogram showing light entrainment 10

Figure 6 Idealized actogram showing free-running rhythms 11

Figure 7 Representation of different T cycle exposures 13

Figure 8 Photic input to the retina 15

Figure 9 Layers of the Mammalian Retina 15

Figure 10 Stratification of RGC dendrites into the ON- and OFF layers 16

Figure 11 Phase-shifting in coneless (cl) mice 19

Figure 12 Melanopsin structure 22

Figure 13 Axonal projections of melanopsin-expressing RGCs 23

Figure 14 Melanopsin-expressing RGC dendrites 25

Figure 15 Dendritic stratification of a melanopsin-expressing RGC 25

Figure 16 Intrinsic light response of a RGC innervating the SCN 27

Figure 17 Dendritic stratification of RGCs after blocking glutamate 28

Figure 18 Melanopsin-positive cell dendrites are contacted by amacrine cell terminals 33

Figure 19 Mouse SCN 35

Figure 20 VP and VIP-expressing cells in the mouse SCN 37

CHAPTER 2:

Figure 1 Light entrainment, free-running rhythms and phase-shifting in CBA/J and CBA/N mice 63

Figure 2 Time course of outer retinal degeneration during development . .65

Figure 3 Dendritic stratification of melanopsin-positive RGCs during development 66

Figure 4 Cell number and distribution of melanopsin-positive RGCs in CBA/N and CBA/J mice 67

Figure 5 Pupillary light reflex in CBA/J and CBA/N mice 69

CHAPTER 3:

Figure 1 Entrainment of CBA/J, CBA/N and rd mice to T cycles 90

Figure 2 VIP-positive cells in the SCN of CBA/J and CBA/N mice 91

Figure 3 VP-positive cells in the SCN of CBA/J and CBA/N mice 92

Figure 4 Retinal innervation of the SCN in CBA/J and CBA/N mice . . . 93

Figure 5 Light-induced c-Fos expression in the SCN of CBA/J and CBA/N mice 94

CHAPTER 4:

Figure 1 A model for the proposed intercellular signaling pathways in the SCN 110

ABSTRACT

In mammals, light input through the retina is responsible for entraining the circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. This occurs via retinal projections from a subset of intrinsically photosensitive retinal ganglion cells (ipRGCs) which express the photopigment, melanopsin. Although studies have shown that the rod and cone photoreceptors that mediate image-forming vision are not required for photoentrainment, data suggest that these cells play a role in this process.

The goal of this project was to test the hypothesis that during development, maturation of the outer retina regulates the proper development of the ipRGCs. In this way, the rod and cone photoreceptors may influence the function of the circadian system in the adult. I used a strain of retinally degenerate mice (CBA/J) to look at the effects of early retinal degeneration on the circadian system. Wheel running experiments were done to examine photoentrainment in CBA/J mice. These mice show attenuated phase shifting behaviors and entrain to a narrower range of T cycles (non-24 hour cycles) compared to control (CBA/N) mice. Using immunohistochemistry I examined the effects of retinal degeneration on the development of ipRGCs by looking at their numbers, distribution and dendritic stratification. These studies show that dendritic stratification and cell distribution of ipRGCs are unaffected by photoreceptor loss. However, the CBA/J mice have greater numbers of ipRGCs suggesting that rods and cones may control the normal developmental death of ipRGCs. Using the pupillary light reflex (PLR) as

a functional assay, I show that the melanopsin pathway is functioning properly at the level of the retina, and therefore, functional changes in the retina are not sufficient to explain the differences in behavior.

To examine whether changes in central processing could explain the differences seen in the behaviors of the CBA/J mice, I used immunohistochemistry to examine the structural anatomy of the SCN and found that there are greater numbers of vasoactive intestinal peptide (VIP) -expressing cells, which receive direct retinal input. In addition, there are greater numbers of vasopressin (VP) -positive cells, which are responsible for SCN output. Using tracer studies I labeled the retinal projections to the SCN and found that there is no difference in the degree of anatomical innervation in the SCN of CBA/J mice. To test whether there were differences in functional innervation between the two strains, I conducted light-induced *c-fos* expression experiments. These studies show that CBA/J mice display a greater degree of *c-fos* induction compared to the controls. Further work is needed to understand how the differences seen in the CBA/J mice influence behavior. Together the findings in this work implicate a role of visual system maturation in influencing the function of the circadian system.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Circadian Systems and Photoentrainment

Almost all living organisms, from plants to primates, display rhythmic patterns of biological activity with a period close to twenty-four hours. This system is of great importance to an organism as it controls a number of biological functions including, but not limited to, bioluminescence in cyanobacteria, photosynthesis in plants, pupal eclosion in some insects and hormone production and secretion and the sleep/wake cycle in mammals (Dunlap 1999). Disruption of the circadian system leads to a number of problems such as the disconcerting experiences associated with jet lag and shift work in addition to insomnia and mood disorders in more serious cases (Monk 2000).

The circadian system is defined by three components. First, it contains an endogenous free-running clock that generates rhythmic outputs even in the absence of environmental cues. Second, it is capable of entrainment, a feature that enables an organism to synchronize its internal clock with the external world. Third, the circadian system exhibits temperature compensation, which allows the clock to properly function under variable temperature conditions (Johnson *et al*, 2003).

The Clock

In mammals the circadian clock is located in the suprachiasmatic nucleus (SCN) of the anteroventral hypothalamus (Silver and Moore 1998). The intrinsic physiological properties of SCN neurons are responsible for generating circadian oscillations. This rhythmicity has been demonstrated by electrophysiological recordings from both cultured SCN neurons (Welsh *et al*, 1995) and slice preparations (Chen *et al*, 1999), which show that oscillations in firing rates persist for several days *in vitro.* Synchronization of the neuronal activity of these cells produces circadian rhythms (Liu and Reppert 2000), though little is known about how this coordination is established. Ablation of the SCN results in animals with arrhythmic cycles (Moore and Eichler 1972; Stephan and Zucker 1972), and the blockade of SCN output by applying the sodium channel blocker tetrodotoxin (TTX) abolishes circadian rhythms (Schwartz 1991). In addition, when SCN neurons are transplanted into the brains of animals whose SCN have been ablated, behavioral rhythms are observed with a period corresponding to that of the donor animal (Ralph *et al*, 1990). Together these data demonstrate that neurons of the SCN are intrinsically rhythmic.

There is currently a large focus on understanding the molecular mechanisms underlying the intrinsic rhythmicity of SCN neurons. In 1971, Konopka and Benzer identified a mutation in *Drosophila* that led to a change in the length of the fly's period, or internal rhythms. As a result the gene was termed, *period*, and was the first gene isolated that displayed a circadian phenotype (Konopka and Benzer 1971). Additional so called, "clock genes" were soon discovered and

shown to be important in the mammalian circadian system. Core clock genes are genes whose protein products are necessary components for producing circadian rhythms as they comprise the molecular oscillatory mechanism in individual cells.

Clock genes and their protein products comprise a transcription-translation loop with positive and negative feedback regulation. In mammals, the transcription factors CLOCK and BMAL heterodimerize and activate the rhythmic expression of period genes (*mPer1)* (Figure 1) and cryptochrome (*mCry1* and *mCry2*) genes. mPER and mCRY outputs are important for maintaining a functioning circadian clock, because disruption of the *mPer1* and *mPer2* genes or the *mCry* genes causes arrhythmicity in constant conditions (Reppert and Weaver 2002).

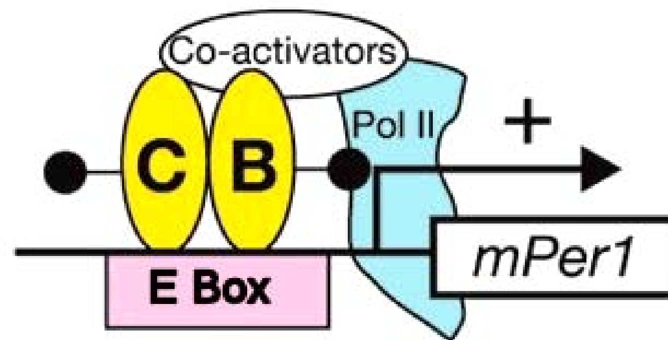

Figure 1. Simplified model of *mPer1* induction. Transcriptional activation of *mPer1* occurs when Clock (C) and BMAL (B) are bound to the E box. Coactivators bind to the phosphorylated transcription factors (black circles). This process is controlled by a complex negative and positive feedback loops (not shown) and is regulated in a circadian manner. Adapted from (Reppert and Weaver 2002).

Clock cells are now known to exist in both the SCN and peripheral tissues. The SCN is thought to be the master clock that serves to coordinate tissue-specific rhythms with external stimuli. The SCN is necessary for the synchronization of peripheral clocks; when isolated from the SCN, the cells drift out of phase with each other and the rhythms dampen (Figure 2). The way in which the SCN controls output and how peripheral tissues become coordinated is unclear and is an area under investigation.

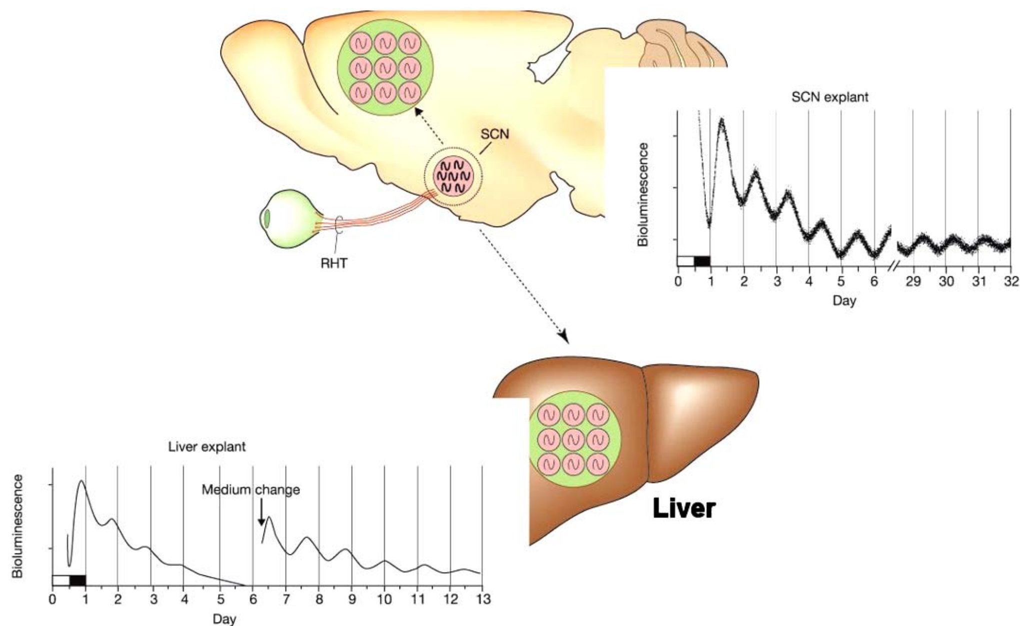

Figure 2. The SCN and peripheral clocks. Clock cells in SCN receive light information via the retinohypothalamic tract (RHT) to entrain the clock to the 24-h day. The entrained SCN, in turn, synchronizes oscillators in other brain areas and peripheral organs (for example, the liver). SCN (top) and liver (bottom) explants from rats expressing an *mPer1*-driven luciferase reporter gene

exhibit bioluminescence rhythms. In culture, the rhythms in the SCN persist for weeks, while that in liver decreases after days. Adapted from (Reppert and Weaver 2002).

Entrainment

Most organisms have adapted their physiology and behavior to take advantage of environmental cues that display rhythmic oscillations within a 24-hour period. These include lighting conditions, food availability, temperature and social cues. Such entraining stimuli are known as "zeitgebers", and, for mammals, the most effective is light. Even though an organism's internal clock can function in the absence of these variables, it uses these cues to synchronize the clock with the external world in a process known as entrainment. Entrainment is demonstrated when a stable phase relationship exists between the output rhythm, i.e. behavior, and the rhythm of the entraining stimulus, i.e. the zeitgeber. For example, in Figure 3, the melatonin levels of pregnant ewes and their fetuses show circadian expression (output rhythm) that are in phase with the light and dark cycle (the zeitgeber) (Richter *et al*, 2004). Another indication of entrainment is seen when the period length of the output rhythm is equal to that of the entraining stimulus. In addition, a biological rhythm is entrained if upon removal of the stimulus, the rhythm continues to oscillate with the same phase as the stimulus (Johnson *et al*, 2003). Proper entrainment of the biological clock to environmental cues is important as it confers selective evolutionary advantages, such as the avoidance of predators. (DeCoursey *et al*, 2000).

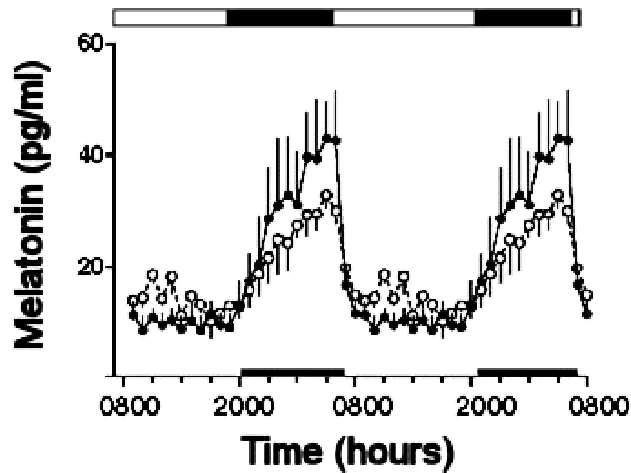

Figure 3. Stable phase relationship between melatonin levels and the light/dark cycle. The levels of melatonin in pregnant ewes (closed circles) and their fetuses (open circles) are in phase with the light/dark cycle, represent by the white (light) and black (dark) bars. Adapted from (Richter *et al*, 2004).

The endogenous clocks of many organisms do not keep perfect 24-hour time and need to be reset each day since the length of daylight varies with the season (Jud *et al* 2005). As a result, the clock must have a mechanism by which it responds to environmental cues. “Phase-shifting” allows an organism to synchronize its clock with the environment. The importance of this process can be seen in animals that need to shift their clocks because their internal periods are slightly shorter or slightly longer than 24 hours. For example, if an animal’s period is 23 hours, it must reset its clock each day to begin slightly later by delaying it by one hour. Similarly, if an animal’s endogenous period is 25 hours, it needs to advance it each day by one hour. Phase-shifting occurs when a cue alters the temporal relationship between the phase of the stimulus and the phase of

the endogenous rhythm. The degree and direction of the shift depends on both the time at which the stimulus is presented and its strength. For example, if an animal is entrained to a cycle of 12 hours of light and 12 hours of darkness (LD 12:12), a light pulse presented during the late subjective night (during darkness), will phase advance the clock. A light pulse given early during the subjective night will produce a phase delay (see phase-response curves below).

In order for entrainment to occur the clock must be able to phase shift at specific times during its cycle. While the animal is sensitive to light during the subjective night, the clock is insensitive to light pulses during the subjective day, when the lights are on. As a result, an animal's activity is not shifted when exposed to light during the subjective day. The period where light has no effect is known as the "dead zone". Phase response curves (PRCs) show how the timing of a stimulus affects the direction and strength of the phase shift. To construct a PRC for light, animals are entrained to a light/dark cycle then shifted to constant darkness (DD) and given a light pulse at different times during their free-running rhythms. The degree of phase shift (in hours) is plotted against the time during the circadian cycle in which the light pulse was given. If activity begins earlier, the value is shown as a positive number, and if it begins later, the value is expressed as a negative number. The PRC for light in rats is shown in Figure 4. This PRC shows that when light is presented early in the subjective night, shown by the black bar at the top, the animal shows a phase delay. When given a light pulse late in the night, the animal phase advances.

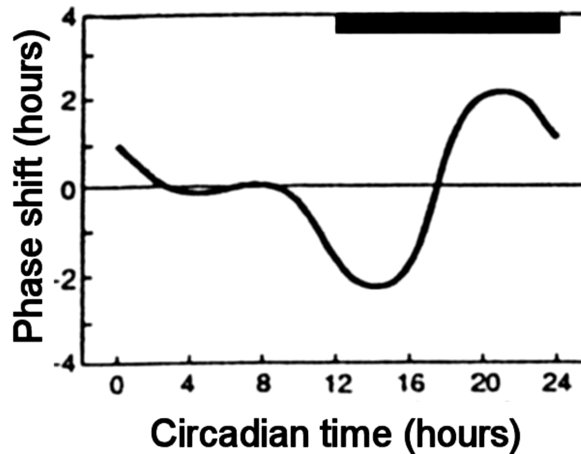

Figure 4. Phase response curve for light in rat. PRC for 1 hour light pulses to rats in DD. The behavioral output rhythm is wheel running. Black bar indicates subjective night. Positive values along the y-axis indicate phase advances and negative values are phase delays. Adapted from (Ding *et al*, 1994).

Measuring Circadian Rhythms

Photoentrainment occurs when an organism's internal clock is synchronized with the environmental lighting, i.e. there is a stable phase relationship between the output rhythm and the light cycle. Experimentally, photoentrainment is measured by determining the phase angle difference (ψ), which is the difference in time between the rhythm of the light/dark cycle and the observed internal rhythm. This can be analyzed by measuring an animal's locomotor activity within a running wheel (the observed internal rhythm) in a controlled light/dark cycle. When the activity onset is synchronized with the onset of darkness, and ψ is stable, the organism is said to be photoentrained. The actogram in Figure 5 represents an example of wheel running in a nocturnal animal entrained to a LD 12:12, indicated by the white and black bars at the top. Each horizontal line

represents one day, and the black vertical lines indicate wheel revolutions. The height of these black bars signifies the number of wheel revolutions for a particular amount of time (Jud *et al*, 2005). As expected for entrained nocturnal animals, the animal is active when the lights are off and inactive when the lights are on.

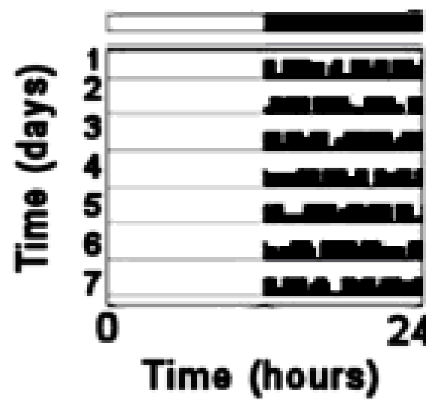

Figure 5. Idealized actogram showing light entrainment. Black vertical lines indicate wheel revolutions and signify periods of activity. The bar at the top represents light (white) and darkness (black) over a 24 hour period (12:12 LD). Adapted from (Jud *et al*, 2005).

In the absence of entraining stimuli, the organism relies solely on its endogenous clock, which has a period close to 24 hours. Because it varies slightly from individual to individual and does not exactly equal 24 hours, the organism will display "free-running" rhythms based on its endogenous period. Placing animals in constant conditions, such as complete darkness, allows the free-running periods of each individual to be measured. Eliminating external stimuli ideally leads to a behavioral rhythm that reflects the endogenous period. Animals

that have periods shorter than 24 hours will display free running behaviors in which activity onset occurs slightly earlier each day (Figure 6). Animals with longer periods will begin running slightly later each day. Observing activity onset for several days in constant conditions allows the endogenous period to be calculated (Jud *et al*, 2005).

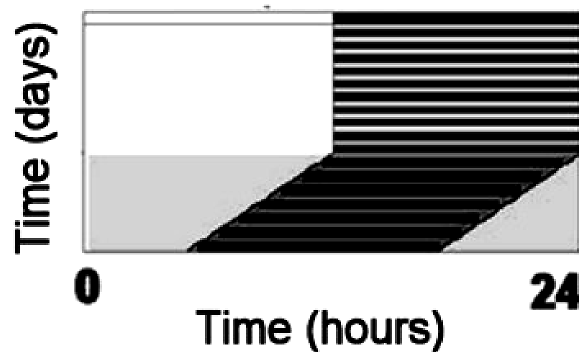

Figure 6. Idealized actogram showing free-running rhythms. Black lines indicate periods of activity. The bar at the top represents light (white) and darkness (black) over a 24 hour period (12:12 LD). When put into constant darkness, shown by the shaded area, the animal displays "free-running rhythms." In this example, the internal period is less than 24 hours and so activity begins slightly earlier each day. Adapted from (Jud *et al,* 2005).

In phase shifting studies a pulse of light is given during the subjective night of free-running animals kept in darkness, and the amount of time that the animal takes to begin its next period of activity is determined. This phase shift (φ) can manifest as either a phase advance or a phase delay, meaning that the organism's measured rhythm (wheel running activity) begins either earlier or later than it did before the introduction of the light pulse. Generally this depends on the time of

day the zeitgeber is applied (Jud *et al*, 2005; Pittendrigh 1981). Because entrainment relies on the daily resetting of the endogenous clock, phase shifting studies provide useful information about how well an animal can entrain to an external cue, such as lighting.

T cycle experiments are an additional way to test entrainment, and they consider the range of light cycles to which an individual can stably entrain. T cycles, in which T = L + D (where L = hours of light and D = hours of darkness) are periods of light and dark that deviate from the normal 24 hour period. For example, a T cycle in which T = 22 could consist of 11 hours of light and 11 hours of darkness (LD 11:11) (Figure 7). If an organism can stably entrain to a T cycle, then that cycle is within its limits. In order for it to do so, however, the organism must either accelerate (if the cycle is shorter than its endogenous period) or slow down (if internal period is longer) its rhythms. This adaptation can only occur within a specific range, and in mammals, the plasticity of the clock is approximately 2 hours. If the T cycle exceeds an organism's limits of entrainment, the animal would begin to free-run. Such studies provide information on how well an organism can adapt to new ranges of lighting conditions (Jud *et al*, 2005).

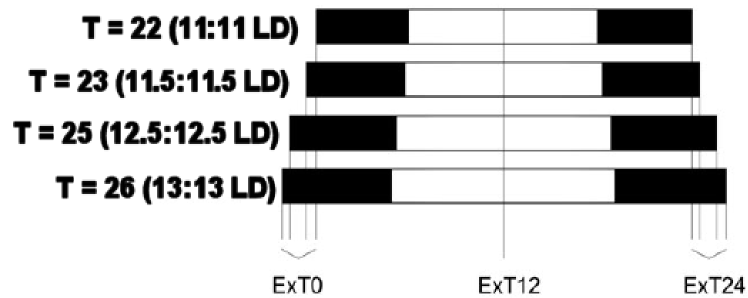

Figure 7. Representation of different T cycle exposures. White bars indicate lights on and black bars indicate lights off for different T cycles. Vertical black lines indicate the corresponding external time (ExT). Adapted from (Jud *et al*, 2005).

<u>Photoentrainment and the Retina</u>

There is strong evidence to suggest that retinal input to the SCN is required for proper entrainment in mammals. Transection of the optic nerve, removal of the eyes or blockade of optic nerve transmission with TTX eliminates shifts in the circadian phase in response to photic input (Schwartz *et al*, 1987; Johnson *et al*, 1988; Foster *et al*, 1991). These data indicate that, like the visual system, the circadian system is mediated by light-responsive synaptic pathways from the retina to the brain. While the visual system has a high sensitivity and is suited for fine spatial and temporal resolution, the circadian system displays optimal responses to brighter light of longer duration. The threshold intensity of light required to entrain the circadian pacemaker is much higher than that required for visual responses, and the circadian system is quite insensitive to brief pulses of light (Nelson and Takahashi 1991). These properties allow the circadian system to filter out spurious light signals, like those of moonlight and lightening, which are

not related to the environmental cycle of day and night. As a result of these differences, the photoreceptive mechanisms that are involved in circadian entrainment are likely to be quite different from those involved in visual function.

Classical Photoreceptive Pathways

Image-forming vision is mediated by the transmission of photic information from the external world through the cellular layers of the retina to the visual centers of the brain. In the classical view of photoreception, light enters the eye and passes through the retinal layers until it reaches the rods and cones located within the outer retina (Figure 8). Once they receive the photic signal, the rods and cones transmit the information through bipolar cells of the inner nuclear layer (INL) to retinal ganglion cells (RGCs). These synaptic pathways are modulated by interneurons, which include horizontal cells and amacrine cells. Horizontal cells synapse onto bipolar cells in the outer plexiform layer (OPL) and amacrine cells contact RGC dendrites in the inner plexiform layer (IPL) (Figure 9). Axons of RGCs comprise the optic nerve, which provides the sole output of the retina (Galli and Maffei 1988; Hubel 1995).

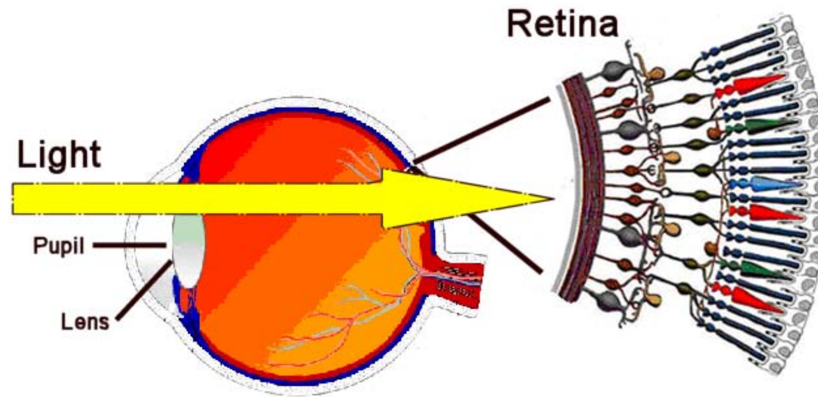

Figure 8. Photic input to the retina. Light entering the eye passes through all the layers of the retina until it reaches the rod and cone photoreceptors located at the back. Adapted from http://reu.uwosh.edu/images).

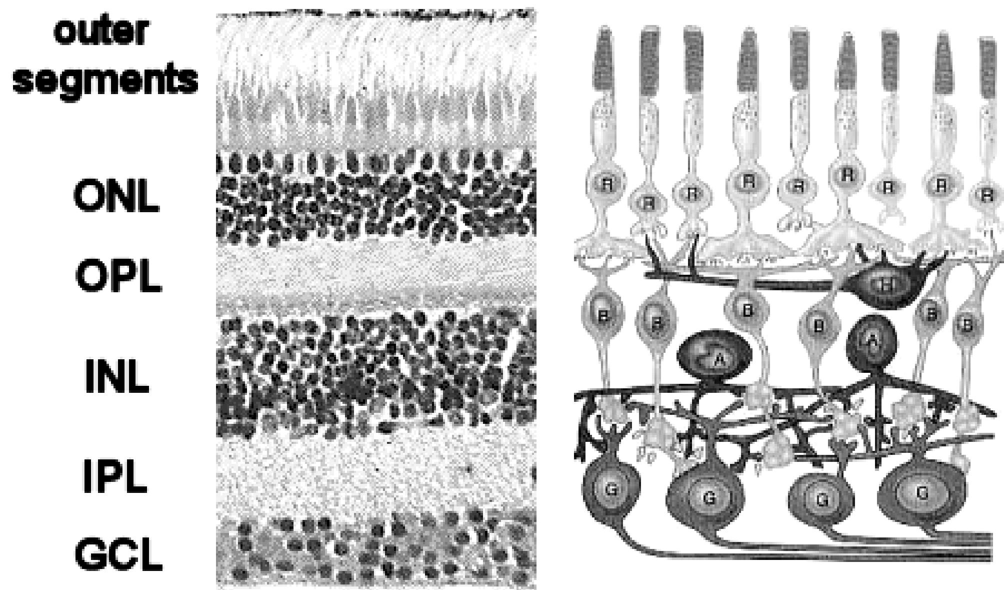

Figure 9. Layers of the Mammalian Retina. Image on the left depicts an H&E stained cross-section from a mouse retina. The cells corresponding to each layer are depicted on the right. The cell bodies of the rods (R) and cones (not shown) are found in the outer nuclear layer (ONL).

Horizontal (H), amacrine (A) and bipolar (B) cell bodies are found in the inner nuclear layer (INL), and ganglion (G) cell bodies are in the ganglion cell layer (GCL). Synaptic contacts among photoreceptors, bipolar and horizontal cells are found within the outer plexiform layer (OPL) and contacts among bipolar, amacrine and ganglion cells occur in the inner plexiform layer (IPL). Adapted from www.owlnet.rice.edu and http://thalamus.wustl.edu/course/eyeret.html.

The physiological responses of RGCs arise from synaptic connections within the IPL, where retinal circuitry segregates into ON and OFF pathways. RGCs with dendrites in the inner layer of the IPL are contacted by ON- bipolar cells and respond to the onset of light, while cells with dendrites in the outer layer of the IPL receive input from OFF-bipolar cells and respond to light offset (Figure 10). Some RGCs project into both sublayers and respond to both the onset and offset of light (Famiglietti, Jr. and Kolb 1976; Nelson *et al*, 1978; Chalupa and Gunhan 2004).

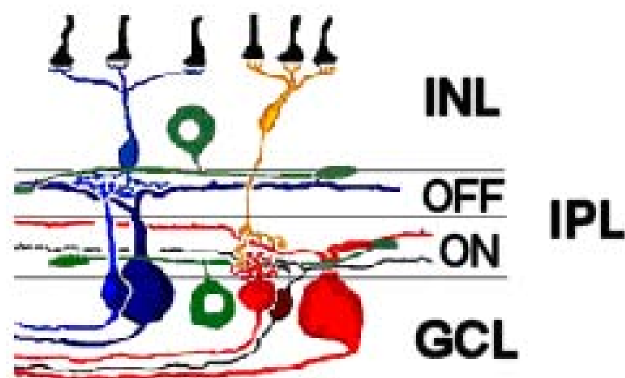

Figure 10. Stratification of RGC dendrites into the ON- and OFF layers. Ganglion cell dendrites stratify into the ON layer of the IPL (red) or the OFF layer (blue) or both (not shown) where they are contacted by ON bipolar cells (yellow) and OFF bipolar cells (blue). Adapted from (Nelson *et al*, 1978).

Photoentrainment and Retinal Degeneration

It was traditionally thought that the rod and cone photoreceptors were the only photoreceptive cells within the eye. The pioneering work of Foster *et al.* was integral in understanding that an additional photoreceptor was present. It demonstrated that mice with retinal degeneration (*rd*) effectively entrain their clocks, despite being blind in terms of visual perception (Foster *et al*, 1991). These rodless-coneless mice also retain several other physiological and behavioral measures of non-visual illuminance detection, including the pupillary light reflex (PLR) and suppression of pineal melatonin production (Lucas *et al*, 1999; Lucas *et al*, 2001b). At the time these experiments were conducted, the existence of an additional ocular photoreceptor seemed unlikely, and experiments began to more carefully examine the potential roles of rods and cones in regulating circadian responses to light. Phase-shifting studies in 80-day old C57BL/6J mice homozygous for the *rd* allele showed that these animals have light-induced circadian responses that are identical to control animals (*rd/*+ and +/+) (Foster *et al*, 1991). These mice exhibit an extreme loss of rod photoreceptors early in adult life, however, the cone photoreceptors degenerate much more slowly, leaving a small number of cones present in adults. A similar study showed that *rdta* mice, which express the gene for an attenuated diphtheria toxin under the control of a

portion of the rhodopsin promoter, also have intact light-induced phase-shifting responses (Lupi *et al*, 1999). In these animals, the rods are genetically ablated, but cone photoreceptors are still present (McCall *et al*, 1996). Because enucleation studies confirmed that the phototransduction required for photoentrainment takes place within the eye (Foster *et al*, 1991), it was hypothesized that the light-induced circadian responses were a result of the presence of cone photoreceptors. To address this issue, Freedman *et al.* (1999) performed phase-shifting studies on *cl* mice, which contain a construct made up of a portion of the human red cone opsin tagged to an attenuated diphtheria toxin gene. As a result *cl* mice have normal numbers of rods but a significantly reduced amount of cones (Wang *et al*, 1992; Soucy *et al*, 1998). The results from these experiments show that despite the loss of cones, *cl* mice show no differences in their abilities to phase shift in response to light (Figure 11) (Freedman *et al*, 1999). Taken together these data suggested that the elimination of either rods or cones does not alter the animal's ability to shift its circadian rhythms in response to light.

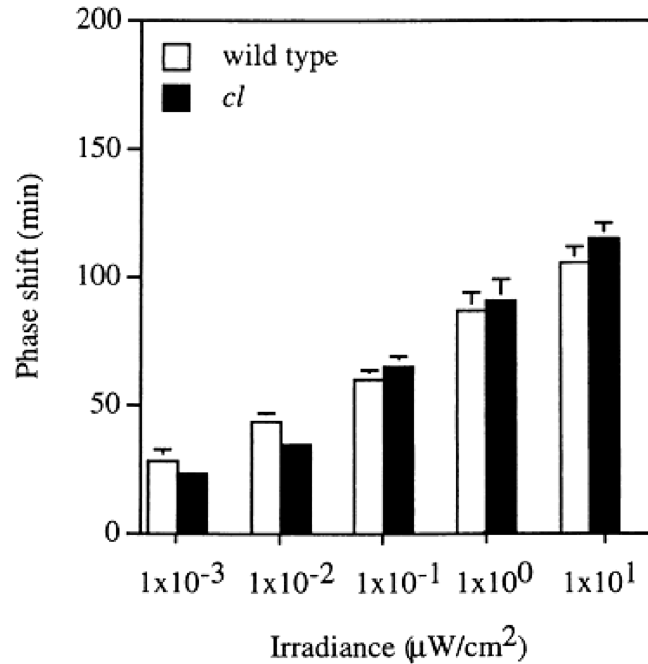

Figure 11. Phase-shifting in coneless (cl) mice. *Cl* (black bars) mice have no difference in their ability to phase shift (y-axis) at each light intensity (x-axis) compared to wild type mice (white bars). Similar results are seen in other animals lacking rod and/or cone photoreceptors (not shown) (see above text). Adapted from (Freedman *et al*, 1999).

From the results of these experiments, a new hypothesis emerged. It was postulated that perhaps the rod and cone pathways are redundant and that each pathway could compensate for the loss of the other. To test this, Freedman *et al.* constructed a mouse that lacked both photoreceptor cell types by introducing the *cl* transgene into the *rdta* mouse. Surprisingly, these mice, like the other models tested, showed intact phase shifting responses when given a pulse of light (Freedman *et al*, 1999). Studies on aged *rd/rd cl* mice (Semo *et al*, 2003b) and RCS/N-*rdy* rats, which have an adult degeneration (Tosini *et al*, 2007a), have yielded similar results.

Conflicting evidence comes from phase-shifting studies done on CBA/J mice, which have a mutation in the β subunit of the phosphodiesterase gene ($Pde6b^{rd1}$) that mediates rod transduction. This mutation causes an increase in cGMP levels that results in apoptosis of rod photoreceptors and secondary death of cone photoreceptors. Unlike the animals in the experiments discussed above, these retinally degenerate mice show attenuated phase shifting responses compared to controls (Yoshimura *et al* 1994). The difference between these mice and the other models is the time course of retinal degeneration. In the CBA/J mouse, the retinal degeneration is complete before the first month of life. This differs from the other models which display degeneration later in adulthood, after the retina has matured (see Chapter 2).

In addition to experiments on animal models of retinal degeneration, studies on humans showed that a subset of blind people can suppress melatonin synthesis and reliably photoentrain despite a total lack of image-forming visual perception (Czeisler *et al* 1995; Klerman *et al*, 2002). These data, along with studies on animal models with adult degeneration, suggested that rods and cones are not necessary for photoentrainment. It was then hypothesized that the mammalian retina contains additional ocular photoreceptors that are responsible for processing the light information needed for circadian entrainment. This was later confirmed by the discovery of the photopigment, melanopsin (Provencio *et al*, 1998b), which led to the understanding that there are two light responsive neural pathways within the retina: the image-forming visual pathway mediated by the

rod and cone photoreceptors, and the non-image forming pathway driven by melanopsin-containing RGCs.

Discovery of Melanopsin

In the conventional view of the retina, the rod and cone photoreceptors were the only cells with intrinsic sensitivity to light. As a result, it was thought that these photoreceptors must be responsible for transducing light information to both the visual and circadian systems. Studies, including the behavioral experiments described above, began to reveal an additional ocular photoreceptor that was responsible for directly mediating light input to the SCN (Berson *et al*, 2002). In 1998, Provencio *et al.* identified a photopigment expressed in the melanophores of *Xenopus laevis*, which they termed melanopsin. Melanopsin is structurally similar to all other known opsins in that it contains seven transmembrane domains and an extracelluar amino terminus (Figure 12). In addition to being found in melanophores, melanopsin mRNA transcripts were detected in the frog SCN and cells of the inner retina (Provencio *et al*, 1998b). In 2000, the same group found melanopsin expression in the inner retina of mice and primates. They noted that the distribution of melanopsin-positive cells was similar to that of a subset of RGCs that had been shown to project to the SCN via the retinohypothalamic tract (RHT), a dedicated monosynaptic pathway between the retina and the brain (Moore *et al*, 1995; Provencio *et al*, 2000). In addition, the action spectra for melanopsin is approximately 484 nm (Berson 2003), which is similar to that seen for circadian phase-shifting behavior in a strain of rodless coneless mice

(Yoshimura and Ebihara 1996). As a result, melanopsin became the candidate photopigment for light induced circadian rhythms.

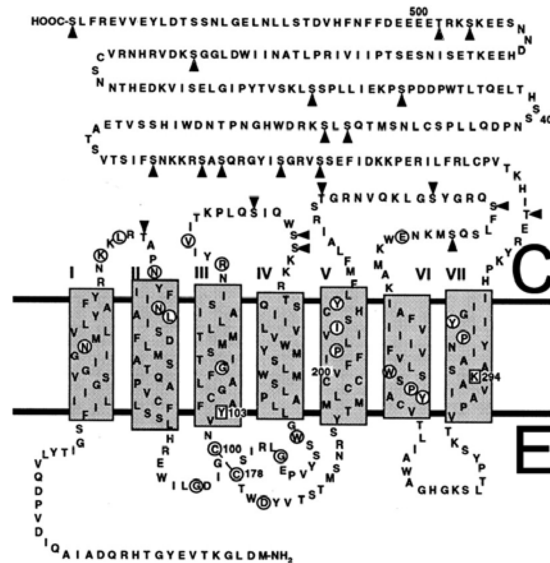

Figure 12. Melanopsin structure. Deduced amino acid sequence of melanopsin. Transmembrane domains are designated by shaded rectangles and circled residues are identical to at least 65 of 67 aligned vertebrate and invertebrate opsins. C = Cytoplasmic side; E = Extracellular. Adapted from (Provencio *et al*, 1998b).

Experiments began to focus on melanopsin as the photopigment responsible for transducing light information to the SCN to regulate circadian rhythms. In 2001, Gooley *et al.* injected FluoroGold into the SCN of rats and retrogradely labeled the small subset of RGCs that form the RHT. They found that 70% of the RGCs labeled for melanopsin were also labeled with the retrograde tracer. This suggested that the majority of RGCs that express melanopsin project to the SCN

(Gooley *et al*, 2001). To confirm this finding, a transgenic mouse strain with tau-lacZ targeted to the melanopsin gene was used to reveal projections to the SCN, the intergeniculate leaflet (IGL) and the olivary pretecal nucleus (OPN) (Figure 13) (Hattar *et al*, 2002; Hattar *et al*, 2006). It became clearer that these cells were likely involved in transmitting light information from the retina to the brain.

The final experiments that convincingly showed that melanopsin-expressing RGCs were the ocular photoreceptors involved in light induced circadian behaviors came from studies showing that targeted deletion of the melanopsin gene abolished the intrinsic photosensitivity (Hattar *et al*, 2003; Lucas *et al*, 2003; Panda *et al*, 2003), and heterologous expression of melanopsin in cells lacking light sensitivity rendered these cells intrinsically photosensitive (ip) (Panda *et al*, 2005; Melyan *et al*, 2005).

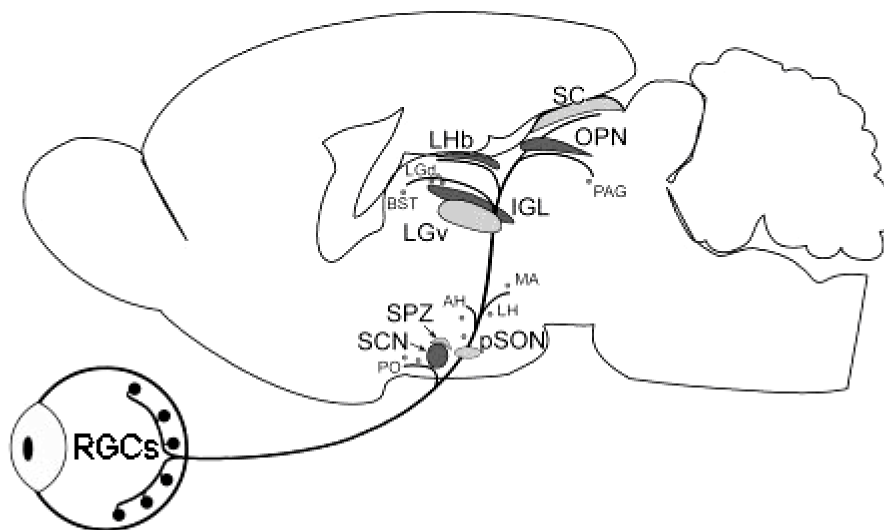

Figure 13. Axonal projections of melanopsin-expressing RGCs. Principle targets are shown in dark gray and secondary targets in light gray. Minor targets are indicated by small lettering and dots. RGCs = Retinal Ganglion Cells; SCN = Suprachiasmatic Nucleus; PO = Preoptic Area; pSON = Peri-supraoptic Nucleus; SPZ = Subparaventricular Zone; AH = Anterior Hypothalamic Nucleus ; LH = Lateral Hypothalamus; MA = Medial Amygdaloid Nucleus; LGd = Lateral Geniculate Nucleus, dorsal; LGv = Lateral Geniculate Nucleus, ventral; IGL = Intergeniculate Leaflet; BST = Bed Nucleus of the Stria Terminalis; LHb = Lateral Habenula; PAG = Periaqueductal Gray; OPN = Olivary Pretectal Nucleus; SC = Superior Colliculus Adapted from (Hattar *et al*, 2006).

Melanopsin-containing RGCs and the Development of Retinal Architecture

Melanopsin-containing RGCs make up approximately 1-2% of all RGCs in the mouse and rat retinas (Hattar *et al*, 2002). They project to areas of the brain involved in non-image forming responses to light, including the SCN, IGL and the OPN, which are responsible for circadian light entrainment and the pupillary light reflex (PLR). Other centers in the brain are targeted by melanopsin-containing RGCs (Figure 13), including some areas involved in image-forming vision, where these cells seem to play additional roles, including color detection (Dacey *et al*, 2005). These ipRGCs are relatively evenly distributed across the retina within the ganglion cell layer (GCL), though few are also found in the INL (Provencio *et al*, 1998b; Hattar *et al*, 2002; Hannibal *et al*, 2002). The dendrites project across the retina, forming a “photoreceptive net” (Figure 14) (Hattar *et al*, 2002; Provencio *et al*, 2002). Morphologically, melanopsin-containing RGCs have a small number of dendrites which, in cross-sections, appear uni- or

bistratified within the ON and OFF layers of the IPL (Figure 14) (Berson *et al*, 2002; Hattar *et al*, 2002; Provencio *et al*, 2002; Hannibal *et al,* 2002).

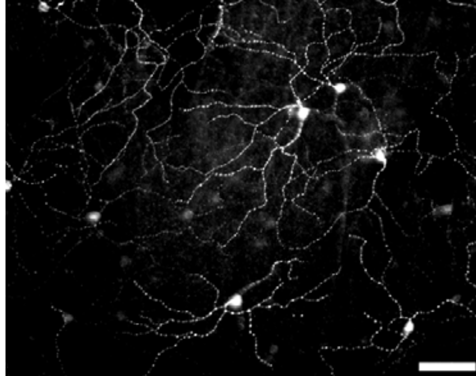

Figure 14. Melanopsin-expressing RGC dendrites. Whole mount retina stained with an antibody against melanopsin shows extensive network of dendrites across the retina. Scale bar, 100 µm. Adapted from (Provencio *et al*, 2002).

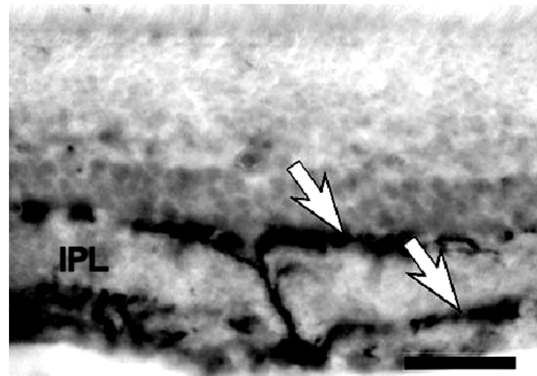

Figure 15. Dendritic stratification of a melanopsin-expressing RGC. Cross-section of a mouse retina, with a single melanopsin-positive RGC stratifying in 2 layers (arrows) within the inner plexiform layer (IPL). Scale bar, 50 µm. Adapted from (Provencio *et al*, 2002).

The intrinsic light response of melanopsin-containing RGCs is characterized by a slow depolarizing current. This has been shown by electrophysiological recordings from SCN-projecting cells labeled with a retrograde dye injected into the SCN of rats. A cocktail of antagonists to a number of receptors, including GABA, NMDA, AMPA/kainate and glycine, was used to block input from the rods and cones. In response to a pulse of bright white light, these RGCs exhibited a depolarized response with a long latency between time of stimulus and beginning of response (Figure 16) (Berson *et al*, 2002; Warren *et al*, 2003). The light-gated channel, which resembles a transient receptor potential (TRP) channel (Warren *et al*, 2006), is unknown. However, it appears that melanopsin activates a G protein, likely $G_{q/11}$, and stimulates phospholipase C (Graham *et al*, 2008). Although these cells respond to light independently of rods and cones, some cells exhibit photoreceptor-driven synaptic input. Also, these cells do receive glutamatergic and GABAergic input (Perez-Leon *et al*, 2006), though the degree of spontaneous synaptic inputs is relatively low.

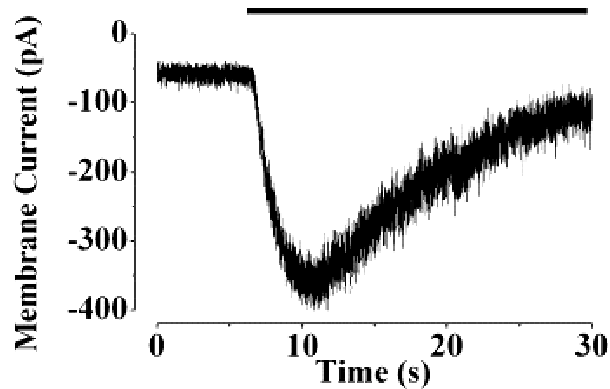

Figure 16. Intrinsic light response of RGC innervating the SCN. Whole-cell recording from an SCN-projecting RGC in rat in voltage-clamp mode. Black bar indicates lights on (Warren *et al*, 2003).

The role of melanopsin-containing RGCs in behavior has been demonstrated in melanopsin knockout mice (*Opn4-/-*). These animals are able to photoentrain, however, they have attenuated phase shifting responses to light compared to wild type mice (Panda *et al*, 2002; Ruby *et al*, 2002). In addition, *Opn4-/-* animals have incomplete PLR's at high light intensities (Lucas *et al*, 2003). These data suggest that melanopsin plays a role in non-image forming behavioral responses to light. Because the responses are not completely eliminated in mice lacking melanopsin, it was suggested that the rods and cones also contribute. Triple knockout mice, in which rods, cones and melanopsin are genetically removed, show no responses to light, suggesting that all three photoreceptors play a role. It is now known that the rods and cones use the melanopsin-containing cells as a way to convey light information to non-visual centers, because ablation of ipRGCs abolishes

responses to light in a manner similar to that seen in the triple knockout mouse (Hattar *et al*, 2003; Guler *et al*, 2008).

Development of RGCs

During development of the vertebrate retina, RGCs undergo massive changes in cell number and morphology. At birth there is an overproduction of RGCs (Young 1985) that is followed by a period of cell death as the animal matures (Young 1984). In addition, the dendritic stratification of RGCs undergoes substantial remodeling. In early development these neurons are unstratified and respond to both the onset and offset of light signals. As the animal matures, however, the dendrites of RGCs stratify into specific ON and OFF layers within the IPL, and the physiological responses they exhibit are determined by this stratification (Figure 10). This process is dependent on endogenous retinal activity, which is likely mediated by the release of glutamate from bipolar cells because blocking glutamate causes the cells to fail to properly stratify (Figure 17) (Chalupa and Gunhan 2004).

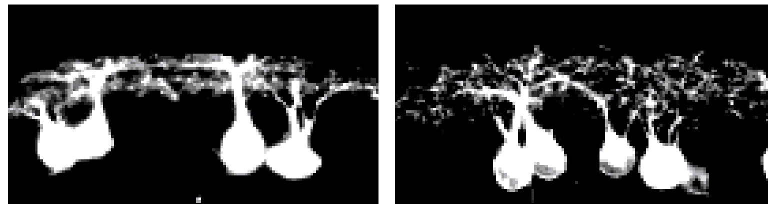

Figure 17. Dendritic stratification of RGCs after blocking glutamate. Left panel shows normal dendritic stratification of RGCs at P10. The right panel shows that blocking glutamate with 2-amino-4-phosphonobutyric acid (APB) causes the cells to remain unstratified. Adapted from (Bodnarenko and Chalupa 1993).

Studies have shown that like the RGCs of the visual system, ipRGCs also undergo changes in cell number and morphology during development. They display a similar reduction in number during early postnatal stages, and by P14 the number of these cells is similar to that seen in the adult (Sekaran *et al*, 2005). Although the significance of this change is unknown, it appears to be correlated with the maturation of photosensitivity in rods and cones in the outer retina (Tian and Copenhagen 2003), suggesting cross-talk between the two pathways. In addition, ipRGCs undergo restructuring with respect to dendrites, although the causes and consequences are unclear (see Chapter 2). Shortly after birth, ipRGCs have poorly organized dendritic arbors which extend throughout the IPL. The stratification of dendrites is apparent by P4 and becomes more defined by P6. By P19, the pattern is the same as in the adult, with dendrites from single cells extending into both the ON and OFF layers of the IPL (Tu *et al*, 2005). While the role of the anatomical and functional modification of RGCs involved in image-forming vision is well defined, how the dendritic stratification patterns of ipRGCs aid in light detection remains unclear. In Chapter 2, I examine the effects of early postnatal retinal degeneration on ipRGC number, distribution and dendritic stratification to test whether rods and cones play a role in the development of ipRGCs.

In addition to the morphological changes, developmental changes in the intrinsic membrane properties of RGCs, such as discharge patterns, have been observed and may reflect modifications in cellular function (Galli and Maffei

1988; Skaliora *et al*, 1993; Wang *et al*, 1997). Similarly, melanopsin-expressing RGCs display physiological changes during development, particularly with respect to light responsiveness. These cells display a greater sensitivity to light during P4-P6 than in the adult (Tu *et al*, 2005), however, it is not known what regulates the subsequent decrease in photosensitivity of individual cells nor how this change affects the overall sensitivity of the circadian system.

A greater understanding of how ipRGCs are regulated during development will provide insight into the functional significance. In Chapter 2 I examine the effects of retinal degeneration on cell number, morphology and dendritic stratification. Additional studies could be done to look at melanopsin expression in the cells. Whether developing animals require more sensitive circadian systems is unclear, and studies on the development of ipRGCs could present answers to such questions.

Retinal innervation

In addition to changes within the retina, the RGCs must undergo changes with regard to target innervation. This process must be regulated so the cells reach the proper targets. In the visual system, RGC axons terminate in the lateral geniculate nucleus (LGN), and early in development the projections of RGCs corresponding to both eyes overlap. As the retina matures, the projections segregate into specific layers of the LGN, corresponding to each eye. Spontaneous bursts of activity in the retina occur among RGCs, and these retinal waves contribute to proper axonal targeting because pharmacological blockade prevents appropriate projections

(Penn *et al*, 1998; Huberman *et al*, 2002). This suggests that endogenous activity among RGCs plays a role in driving axons to their appropriate targets. In addition, a number of guidance cues play a role in axonal targeting. For example, slits, which are secreted inhibitory factors, appear to play a role in axonal targeting. Slits bind to the receptor, robos, and Robo1 and Robo2 deficient mice have axon guidance defects in the visual pathway (Plachez *et al*, 2008). Together these studies show that retinal innervation is dependent on endogenous activity as well as external guidance cues. Additional research in this field is required to understand the complex interactions among activity within the retina and additional factors that are responsible for correct targeting of axons.

It is not clear how the ipRGCs know how to reach their targets and form functional connections during development and whether subsequent development alters retinal innervation. Studies of *c-fos* expression in the mouse show that ipRGCs have already functionally innervated the SCN at birth (Sekaran *et al*, 2005). While activity is important for proper target innervation by visual RGCs, the same does not appear to be the case for ipRGCs. In rat, dark rearing leads to a decrease in innervation by visual RGCs of the superior colliculus, as demonstrated by tracer studies. However, there is no change in the degree of retinal innervation of the SCN in dark-reared animals compared to controls kept on a normal light/dark cycle (Prichard *et al*, 2007). These data suggest that while light activity is required for projections to visual centers, it is not necessary for the anatomical innervation of the SCN. In Chapter 3, I examine retinal innervation of

the SCN in mice with developmental retinal degeneration and test if loss of rods and cones impacts innervation of the SCN.

Interaction Among Photoreceptors

Although the visual and circadian systems can be considered two functionally and anatomically distinct photoreceptive pathways in the retina, a number of studies suggest that they work together. Melanopsin knock-out (*Opn4 -/-*) mice show an attenuation of their ability to phase shift in response to light. In mice, loss of rods, cones and melanopsin abolishes all responses to light (Hattar *et al*, 2003; Panda *et al*, 2003; Peirson and Foster 2006). Interestingly, genetic ablation of ipRGCs leads to a decrease in the PLR and circadian behaviors that is more pronounced than that seen in the *Opn4 -/-* mouse because rods and cones use the ipRGCs to transmit information to the SCN (Guler *et al*, 2008). In addition, the PLR is controlled by all three photoreceptive cells. Mice lacking rods and cones have attenuated PLRs at low light intensities, and *Opn4-/-* mice have decreased PLRs at high light intensities, suggesting that the photoreceptors regulate the PLR at different light intensities but together contribute (Lucas *et al*, 2001a; Lucas *et al*, 2003). Together these studies suggest that all three photoreceptors interact to elicit behavioral light responses.

Potential Regulation of ipRGCs

There is anatomical and physiological evidence to suggest a role of photoreceptors in the regulation of ipRGCs. Electron micrographs show amacrine

and bipolar cell terminals contacting melanopsin-positive dendrites (Belenky *et al*, 2003). Similarly, immmunohistochemical studies that utilized antibodies against markers of bipolar and amacrine cell terminals show that the dendrites of melanopsin-containing RGCs are in close contact with bipolar and dopaminergic amacrine cell processes (Figure 18) (Ostergaard *et al*, 2007).

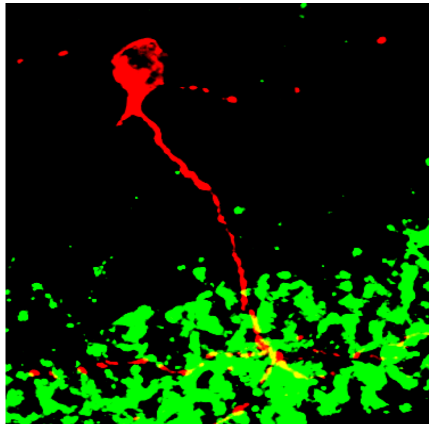

Figure 18. Melanopsin-positive cell dendrites are contacted by amacrine cell terminals. RGC stained for melanopsin (red) has dendrites in the IPL where dopaminergic amacrine cell terminals stained for tyrosine hydroxylase (TH) (green) are. Overlap shows potential contacts (yellow). Adapted from (Ostergaard *et al*, 2007).

The physiology of ipRGCs appears to be regulated by rod and cone photoreceptors. Electrophysiological data suggest that the melanopsin-containing cells in primate retinas are activated by rod and cone photoreceptors (Dacey *et al*, 2005). In addition, studies in rat suggest that rods and cones regulate melanopsin expression because adult photoreceptor degeneration leads to a decrease in overall melanopsin levels and an elimination of its circadian expression (Sakamoto *et al*, 2004). Together, these data suggest that melanopsin-expressing RGCs receive

input from photoreceptor-driven pathways, and this may serve to regulate their physiology.

Little is known about whether the rod and cone photoreceptors regulate the development of ipRGCs. The mechanisms by which changes in number, morphology and physiology occur are unclear, and whether changes in ipRGCs during development influence the photosensitivity of the circadian system is also not known. In Chapters 2 and 3, I examine the effects of early retinal degeneration on the development of ipRGCs and circadian system function in the adult.

SCN Organization and Retinal Input

The SCN is a paired structure with each nucleus containing about 10,000 cells. It sits dorsal to the optic chiasm (Figure 19). The retina innervates the SCN via a direct projection that occurs along the RHT (Moore and Lenn 1972) and an indirect pathway from the IGL of the thalamus via the geniculohypothalamic tract (GHT) (Hickey and Spear 1976). In hamster and mouse, the SCN is almost entirely innervated by the RHT, and the axonal projections from one retina extend to the SCN bilaterally (Johnson *et al*, 1988; Silver *et al*, 1999; Muscat *et al*, 2003; Morin and Allen 2006). Molecules released from RHT axons include glutamate (Shibata *et al*, 1992) and pituitary adenylate cyclase-activating peptide (PACAP) (Hannibal *et al*, 1997), and those released from the GHT include GABA and neuropeptide Y (NPY) (Card and Moore 1982; Card and Moore 1989). A serotonergic projection from the dorsal and medial raphe nuclei of the midbrain is also present (Azmitia and Segal 1978).

Photic stimulation induces the expression of the immediate early genes c-*fos* and *jun*-B in the SCN (Kornhauser *et al*, 1992; Masana *et al*, 1996). The proteins Fos and Jun associate to form the transcription factor AP-1, which regulates transcription. The downstream effects of these proteins on clock function are unclear, however, administration of *c-fos* and *jun*-B antisense oligonucleotides into the ventricle blocks light-induces phase shifts in rats (Wollnik *et al*, 1995). In addition, experimental light- induced *c-fos* expression is often used to look at retinal innervation of the SCN (Colwell and Foster 1992).

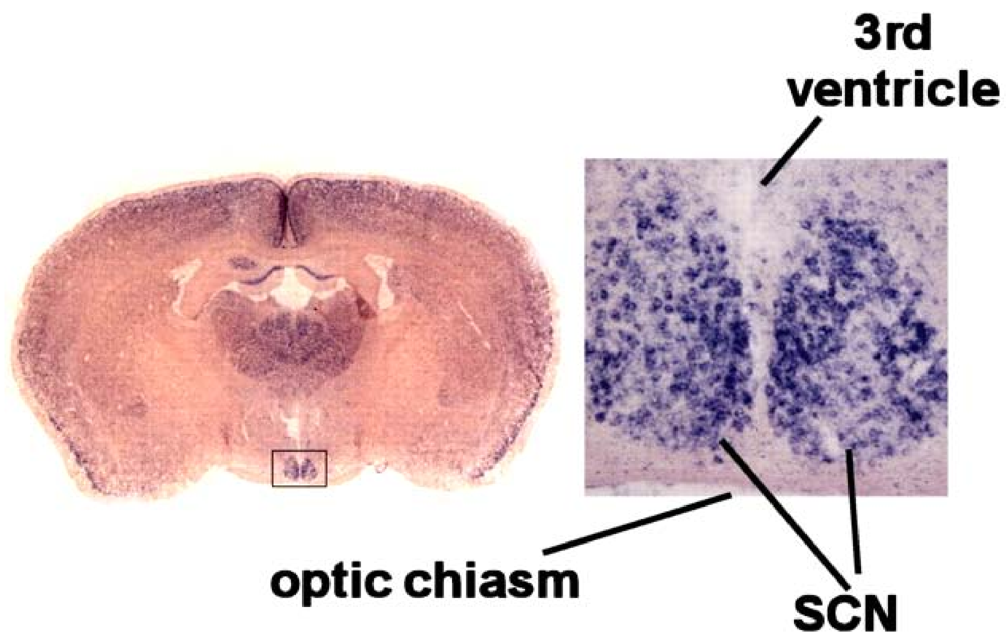

Figure 19. Mouse SCN. The left panel depicts a coronal section from mouse brain stained for *mPer-1* (marker for SCN). The inset is magnified on the right and shows staining of SCN neurons. Adapted from (Mieda *et al*, 2006).

"Core" and "Shell"

Cells of the SCN exhibit heterogeneity in the types of molecules and transmitters they express. It is thought that most SCN neurons are inhibitory, as many contain GABA (Moore and Speh 1993; Abrahamson and Moore 2001; Morin and Blanchard 2001). There appear to be two major divisions within the SCN: a dorsomedial area containing neurons expressing vasopressin (VP) and a ventral area with cells containing vasoactive intestinal peptide (VIP) (Figure 20). These divisions may be further divided. However, a major problem in defining the organization of the SCN is that the distribution of cell types within the nuclei differs among species. Although differences in organization exist among species, the presence of VP and VIP neuronal populations appears to be conserved (Moore *et al*, 2002; Morin and Allen 2006).

There have been a number of approaches to characterizing the SCN (Morin 2007). The "core/shell" terminology is commonly used to describe the organization of the SCN. Typically, the "core" refers to the area containing cells expressing VIP and gastrin-releasing peptide (GRP). In one model, the core also contains terminals of melanopsin-expressing RGCs, as it is the retinorecipient part of the SCN (Antle and Silver 2005). The "shell" is located dorsally and contains cells expressing VP (Moore *et al*, 2002). Cells of the core have been shown to project to the shell neurons, and both shell and core cells project to extra-SCN targets (Leak and Moore 2001).

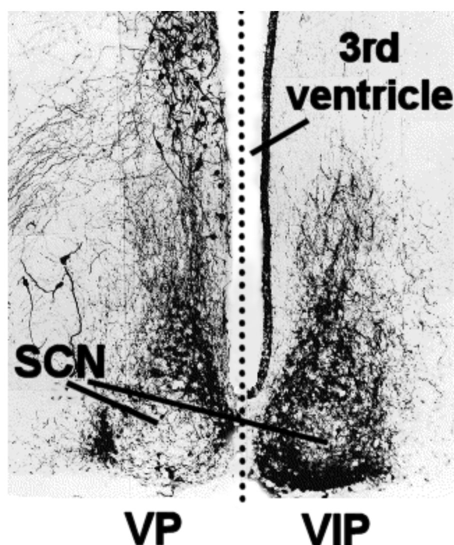

Figure 20. VP and VIP-expressing cells in the mouse SCN. Coronal sections of mouse SCN stained for VP (left) and VIP (right). VP cell bodies are localized to the dorsal part ("shell") of the SCN, while VIP cell bodies are found ventrally ("core"). Adapted from (Abrahamson and Moore 2001; Morin 2007).

The core/shell system also has functional significance. Cells in the core of the SCN are typically nonrhythmic, receive direct retinal input and express *c-fos* in response to light. Cells of the shell express self-sustained rhythmicity as a result of transcriptional feedback loops involving "clock" genes. These are the output cells of the SCN. As a result, their rhythmicity needs to be synchronized so that their outputs are coordinated. Shell cells receive little, if any, retinal innervation. For proper light entrainment, the photic signal must be transmitted from the light responsive cells of the core to the rhythmic cells of the shell (Antle and Silver 2005).

Vasoactive Intestinal Peptide

VIP is a neuropeptide expressed within the core of the mouse SCN (Figure 20). Cells expressing VIP, like most core neurons, receive retinal input and play a role in synchronizing SCN neurons with each other and with external lighting cues (Vosko *et al*, 2007). Though the mechanism by which these cells mediate cell-to-cell communication is unclear, there are data to support their importance in circadian function. SCN cells oscillate with different phases, which can be synchronized by application of GABA (Liu and Reppert 2000). In the SCN, GABA is modulated by VIP (Itri and Colwell 2003). Loss of VIP causes SCN cells to become arrhythmic and desynchronized (Aton *et al*, 2005). VIP knockout mice display behavioral arrythmicity as well as desynchronization of the clock with the environment (Hannibal and Fahrenkrug 2003; Colwell *et al*, 2003). In addition, loss of VPAC2, the receptor for VIP and PACAP, disrupts activity rhythms in mice (Harmar *et al*, 2002). It has also been shown that VPAC2 agonists can reset the SCN *in vitro* (Gourlet *et al*, 1997). These data suggest that VIP plays a role in coordinating inputs to and within the SCN and impacts behavior.

Vasopressin

Cells expressing VP are typically found dorsomedially, close to the third ventricle, in the shell of the SCN (Figure 20). VP is secreted in a circadian rhythm *in vivo* (Kalsbeek *et al*, 1995; Van der Zee *et al*, 2002). VP-expressing cells project to the paraventricular nucleus of the hypothalamus (PVN) and release VP to regulate the rhythmic secretion of corticotrophin releasing hormone (CRH)

(Buijs *et al*, 2003). In this way these cells exert a temporal influence over the body. The exact function of VP in the clock is unclear, however, it has been shown to modulate some circadian behaviors, such as the sleep/wake cycle, since animals lacking VP have a decrease in amplitude of sleep rhythms (Brown and Nunez 1989). In addition to its role in SCN output, VP appears to play a role in the actual clock mechanism. In VP-deficient rats, circadian rhythms of body temperature are abolished in the absence, but not presence, of entraining stimuli, suggesting that loss of VP causes a functional deficit in the endogenous pacemaker (Wideman *et al*, 1997). Similarly, mice lacking the vasopressin 1a receptor (Avpr1a) have altered free-running periods (Wersinger *et al*, 2007), supporting its role in the internal time-keeping system.

Additional molecules

Calbindin (CB) is a cytosolic calcium-binding protein involved in intracellular calcium signaling and buffering. There is a population of CB-positive cells which are concentrated in the core of the hamster SCN (Silver *et al*, 1996). As is characteristic of the core of the SCN, the CB-positive subnucleus is innervated by retinal terminals (Bryant *et al*, 2000), and the majority of the CB-positive cells express *c-fos* in response to light (Silver *et al*, 1996). In addition, these cells show no rhythmicity in spontaneous firing of action potentials (Jobst and Allen 2002). CB-positive cells play a role in circadian rhythms because if the CB-positive subnucleus in the hamster SCN is ablated, there is a disruption in the activity rhythms of the animal. Also, transplantation studies suggest that hamsters with lesions within the SCN can recover rhythmicity if the CB-positive cells are

present within the grafting tissue (LeSauter and Silver 1999). Although the role of CB is well established in the hamster, its function in the mouse circadian system is less clear. Adult mice lack the cluster of CB-positive neurons seen in hamster, (Ikeda and Allen 2003), however, CB knockout mice (CalB -/-) do not photoentrain properly, suggesting that this molecule plays a role in light-induced circadian behaviors (Kriegsfeld *et al*, 2008).

There is a group of cells in the mouse SCN containing gastrin-releasing peptide (GRP), which are thought to play an analogous role to CB cells. Like CB-positive cells, GRP-containing cells are located in the core, express *c-fos* in response to light and lack detectable rhythms in clock genes (Karatsoreos *et al*, 2004). *In vivo* application of GRP leads to behavioral phase shifts that are similar to light (Albers *et al*, 1995), suggesting that GRP plays a role in transmitting light information. In addition, mice lacking the GRP receptor show attenuated phase-shifting (Karatsoreos *et al*, 2006), which suggests that GRP-containing cells play a role in light-induced circadian behaviors. How GRP acts along with VIP and VP is unclear, but could provide insight into the mechanisms underlying circadian behaviors. Research on additional peptides, such as CB, GRP and GABA, within the SCN and the cells expressing them need to be examined in order to obtain a better understanding of how rhythmicity is established and how these cells work to coordinate outputs.

A key problem in understanding the function of the SCN is determining how the rhythmicity is maintained among the neurons and how this affects peripheral tissues. Individual cells oscillate with a wide range of periods and display

differences in timing of gene expression and firing patterns. As a result, SCN cells need to be synchronized to produce a coordinated output. A current model takes advantage of the idea that there are two general populations of cells: “gates”, which are directly sensitive to light and regulate the ability of the clock to respond to external cues and “oscillators”, which are intrinsically rhythmic and do not receive direct input from the retina. The gates and oscillators broadly reflect core and shell populations, respectively. The gates are retinorecipient and express *c-fos* and clock genes in response to light and are not rhythmic. Cells exhibiting these properties are the CB cells in the hamster and GRP cells in mice. Loss of these gate cells abolishes rhythmicity in the SCN, and when the ventral area of the SCN is removed, the putative location of gate cells, synchrony is lost (Antle *et al*, 2007). Cells corresponding to oscillators include VP-expressing cells. VIP-expressing cells also appear to be gate cells because application of VIP, at specific times, causes phase shifts of VP’s circadian expression (Watanabe *et al*, 2000).

SCN outputs

Outputs of the SCN project to other parts of the hypothalamus, where they induce rhythms by releasing peptides and transmitters and by producing rhythmic firing patterns (Inouye and Kawamura 1979; Green and Gillette 1982; Kalsbeek *et al*, 1995). The main output is to the subparaventricular zone (SPZ) and the dorsomedial nucleus. Cells in the ventral SPZ relay information to the dorsomedial nucleus, which is important in producing rhythms in sleep, locomotor activity, feeding and corticosterone production. Neurons in the dorsal SPZ control thermoregulation. A small number of axons reach additional areas

involved in hormone secretion, enabling the SCN to communicate temporal information throughout the body (Saper *et al*, 2005; Vosko *et al*, 2007).

Recent work has demonstrated that although the circadian and visual systems are two anatomically and functionally distinct systems, the two pathways interact within the retina. The purpose of this thesis was to examine the role of rod and cone photoreceptors in regulating the development of the ipRGCs involved in circadian function and whether this regulation influences the overall circadian system function in the adult.

COURSE OF THESIS

In the first part of this thesis, I use a strain of retinally degenerate mice, which have attenuated behavioral responses to light, to look at the effects of early retinal degeneration on the anatomical development of melanopsin-containing RGCs. In the second part, I explore the limits of entrainment of these mice by exposing them to different lighting conditions and examine whether differences seen in circadian behaviors are due to changes at the level of the SCN.

CHAPTER 2

THE DEVELOPMENT OF MELANOPSIN-CONTAINING RETINAL GANGLION CELLS IN MICE WITH EARLY RETINAL DEGENERATION

The development of melanopsin-containing retinal ganglion cells in mice with early retinal degeneration

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Abstract

In mammals, the neuronal pathways by which rod and cone photoreceptors mediate vision have been well documented. The roles that classical photoreceptors play in photoentrainment, however, have been less clear. In mammals, intrinsically photosensitive retinal ganglion cells (ipRGCs) that express the photopigment melanopsin project directly to the suprachiasmatic nucleus (SCN) of the hypothalamus, the site of the circadian clock, and thereby mediate non-image forming responses to light. Classical photoreceptors are not necessary for photoentrainment since loss of rods and cones does not eliminate light entrainment. Conflicting evidence arose, however, when attenuated phase-shifting responses were observed in the retinal degenerate CBA/J mouse. In this study, we examined the time course of retinal degeneration in CBA/J mice and used these animals to determine if maturation of the outer retina regulates the morphology, number and distribution of ipRGCs. We also examined whether degeneration during the early development of the outer retina can alter the function of the adult circadian system. We report that dendritic stratification and distribution of ipRGCs was unaltered in mice with early retinal degeneration, suggesting that normal development of the outer retina was not necessary for these processes. We found, however, that adult CBA/J mice have greater numbers of ipRGCs than controls, implicating a role for outer retinal photoreceptors in regulating developmental cell death of ipRGCs.

Introduction

In mammals, the photoreceptive mechanisms underlying vision have been known for sometime. Until recently, the pathways mediating non-image forming responses to light, such as circadian photoentrainment, were not well understood. It was traditionally thought that all photoreception in the eye was mediated by rod and cone photoreceptors. However, the discovery of melanopsin-containing retinal ganglion cells (RGCs) that exhibit light responses independent of rod and cone signaling (Berson *et al,* 2002; Hattar *et al*, 2002; Warren *et al*, 2003) led to an understanding that light reception within the retina is not exclusively mediated by rods and cones.

Studies have attempted to elucidate the respective roles of the ocular photoreceptors in photoentrainment. Mice and rats in which rods and cones degenerate during adulthood retain the ability to phase-shift in response to light (Foster *et al*, 1991; Semo *et al*, 2003; Tosini *et al*, 2007a), though some differences are seen at high light intensities (Freedman *et al*, 1999). Conversely, melanopsin knockout mice (*Opn4 -/-*) and mice in which the ipRGCs are genetically ablated show attenuated phase-shifting (Panda *et al*, 2002; Guler *et al*, 2008; Hatori *et al*, 2008). Loss of both melanopsin and rods and cones eliminates all non-visual light responses (Hattar *et al*, 2003). These data suggest that rods and cones are not necessary for photoentrainment, but do play a role.

Although photoentrainment is possible in the absence of rods and cones, CBA/J mice which are homozygous for the retinal degeneration allele $Pde6b^{rd1}$ display attenuated phase-shifting responses to light (Yoshimura *et al*, 1994). Most of the models used to isolate the role of photoreceptors in photoentrainment

display outer retinal degeneration during adulthood, after the retina has fully matured. However, CBA/J mice display degeneration that is complete before the first month of life. During this time, ipRGCs in wild-type mice undergo changes in cell number, morphology and physiology (Sekaran *et al*, 2005) (Tu *et al*, 2005). Whether photoreceptor degeneration influences these events is unclear.

During normal development, endogenous activity within the retina, which is likely mediated by the release of glutamate from bipolar cells, plays a role in directing the stratification of RGCs involved in vision (Chalupa and Gunhan 2004). In addition, RGCs undergo developmental cell death that is completed around the time rods and cones have matured (Tian and Copenhagen 2003). These findings led to the question: "Do classical photoreceptors play a role in the normal development of ipRGCs and, as a result, the circadian system as a whole?"

We set out to determine whether the differences in photoentrainment seen in CBA/J mice are a result of photoreceptor loss during development. If the photoreceptors play a role in regulating the number, distribution and/or dendritic stratification patterns of ipRGCs during early postnatal development it would be expected that the photosensitivity of the adult circadian system would be altered. We used immunohistochemistry and wheel running experiments to address the hypothesis that perturbation of the outer retina disrupts the proper development of ipRGCs and alters optimal circadian function.

Materials and Methods

Animals

All procedures were carried out in compliance with the guidelines of the National Institutes of Health. All protocols were approved in advance by the Institutional Animal Care and Use Committee of Oregon Health & Science University. Male CBA/J mice, which carry the *Pde6b*^{rd1} mutation and exhibit blindness by weaning age (Jackson Laboratory, Bar Harbor, ME, USA) were used to examine the effects of early retinal degeneration on the development of ipRGCs. For controls, we used male CBA/N (National Cancer Institute, Frederick, MD, USA) mice, which have the same genetic background as the CBA/J mice, but lack the *Pde6b*^{rd1} mutation and are, therefore, visually intact.

The mice were housed in facilities that permit the maintenance of a 12-hour light-dark cycle. Animals used in the behavioral studies were kept in separate chambers under the environmental conditions described below. Studies were restricted to male mice to avoid potential effects of fluctuations in female reproductive hormones on circadian behaviors. All mice used for entrainment studies were between 3 months and 7 months of age, to avoid variability in their abilities to entrain (Pittendrigh and Daan 1976).

Wheel-running experiments

Mice used in the behavioral experiments were housed in an Intellus Control System chamber (Percival Scientific, Perry, IA) that allows for constant lighting and temperature (24˚C) conditions. Within the chamber animals were kept in Nalgene cages equipped with running wheels and magnetic switches (Mini Mitter,

Bend, Oregon, USA) that allow for recording of wheel revolutions. Wheel-running data was collected continuously by VitalView (Mini Mitter) acquisition software. Actograms depicting activity and rest cycles during entrainment and free-running assays were generated using ClockLab (Actimetrics Software, Wilmette, IL, USA).

The ability of CBA/J and CBA/N mice to entrain to a photoperiod of 12 hours of light (1000 lux) followed by 12 hours of darkness (0 lux) (LD 12:12) was observed by measuring their wheel-running activity. Once entrained, the mice were kept in constant darkness (DD) to measure the period length of their free-running rhythms. To examine the ability of the mice to phase shift in response to light, a 15 min. white light pulse administered by a 32-W fluorescent bulb (300 lux) was given at circadian time 15 during the free-running activity. Phase-shifts and period lengths were calculated using ClockLab.

Tissue preparation

Animals were deeply anesthetized with isoflurane and sacrificed by cervical dislocation. Eyes were removed, placed in a Petri dish containing 0.1 M phosphate buffer (PB) (pH 7.4), and quickly hemisected. Using a Leica GZ4 dissection microscope, the retinas were carefully removed from the eyecup and fixed in 4% paraformaldehyde (PFA) (pH 7.4) overnight at 4˚C.

Characterizing retinal degeneration

To characterize the outer retina degeneration, retinas were obtained from mice at 4, 8, 10, 15, and 21 days of postnatal development (P4, P8, P10, P15, P21) and

from adults. Following fixation, the retinas were cryoprotected by successive immersion in phosphate-buffered solutions containing 10% and 30% sucrose at 4˚C overnight. The tissue was embedded in Thermo Shandon Cryochome (Thermo Scientific, Pittsburgh, PA, USA), fast-frozen over dry-ice mixed with 100% ethanol for 3-5 minutes and stored at -80˚C.

Vertical cross-sections (15 µm) of embedded retinas were cut on a Leica 1720 digital cryostat and thaw-mounted onto glass slides. Sections were rinsed with 0.1 M PB (pH 7.4) and incubated in a Modified Giemsa Stain (Sigma-Aldrich, St. Louis, MO, USA) (diluted 1:5 in dH_20) for 10 min at room temperature (RT; 20-22˚C). Sections were rinsed with 0.1 M PB for 5 min and then dehydrated in 20%, 80%, and 100% ethanol. Tissue was mounted in Entellan New (Electron Microscopy Sciences, Hatfield, PA, USA) and imaged by brightfield microscopy at 20X using a Zeiss Axioscope 2TM. Degeneration was quantified using AxioVision software (Carl Zeiss Microimaging, Germany) to measure the thickness of the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL) at each stage of development.

Dendritic stratification

To observe the dendritic stratification patterns of ipRGCs during development, retinas were processed as described above. To visualize ipRGC bodies and dendrites, sections were rinsed in 0.1 M PB with 0.3% Triton-X for 15 min, placed in blocking solution (1% BSA + 0.3% Triton-X) for 1 hour at RT and stained with an affinity-purified polyclonal antibody generated in rabbit and

directed against the amino-terminal peptide of mouse melanopsin O/N at 4˚C (Warren *et al*, 2003; Walker *et al*, 2008). Next, the tissue was incubated with a fluorophore-conjugated secondary antibody (Alexa-488 labeled goat anti-rabbit IgG; Molecular Probes, Eugene, OR, USA) diluted in blocking solution (1:2000) for 2 hours at RT in the dark. The retinas were then rinsed with 0.1 M PB, counterstained with the nuclear stain, DAPI (80 ng/ml) for 1-3 min at RT and mounted on a glass slide in Aqua Mount (Fisher Scientific, Pittsburgh, PA, USA). Immunostained tissue was imaged by fluorescence microscopy at 20X using a Zeiss Axioscope 2TM.

Cell numbers and distribution

To assess ipRGC number and distribution, whole mount retinas from CBA/J and CBA/N mice of the same developmental ages were fixed immediately after dissection for 30 min at RT. They were then rinsed in 0.1 M PB with 0.3% Triton-X and PFA (1:1 v/v) for 30 min and placed in blocking solution for 1 hour at RT. The retinas were then incubated with the α-melanopsin antibody in blocking solution (0.5 μg/ml) for 2 days at 4°C and rinsed with 0.1 M PB. Next, the tissue was incubated with a fluorophore-conjugated secondary antibody and DAPI and mounted as described above. Fluorescence microscopy was used to image melanopsin-positive cells with a 20X objective. Paintshop Pro (Corel Corporation, Fremont, CA, USA) was used to create a composite image of the entire whole mount. The number of melanopsin-positive cell bodies and their coordinates were calculated using ImageJ software (http://rsb.info.nih.gov/ij/).

To determine ipRGC distribution, the distance between melanopsin-positive cell bodies used in the cell count experiments was calculated and normalized to the maximum distance between cells. A smoothed non-parametric kernel density estimate was computed for each set of normalized distances. Density estimates were averaged for each strain at different stages of development.

Pupillometry

The pupillary light response was measured in both CBA/J and CBA/N adults. Mice were dark adapted for 1 hour prior to testing the PLR and experiments were conducted between 2 and 6 hours prior to lights off (12:12 LD) (Lucas *et al* 2001a). One eye of each animal was exposed to bright light for 30 seconds and was video taped under infrared light using a CCD camera. The light intensity was controlled by different combinations of neutral density filters placed in the light path. Pupil area was measured and normalized to area prior to light exposure.

Statistics

Averages are reported as the mean ± SD. Significance was determined using the Student's t-Test.

Results

Light entrainment is altered in CBA/J mice

To examine the potential role of outer retina maturation on the photosensitivity of the adult circadian system during development, wheel-running experiments were conducted with adult CBA/J (n= 6) and CBA/N mice (n = 6). The actograms in Figure 1A show that both strains can entrain to a photoperiod of 12 hours of light (1000 lux) followed by 12 hours of darkness (LD 12:12). When put into constant darkness (DD) (0 lux) for 17 days, the CBA/J mice show normal free-running rhythms. The period lengths of the CBA/J and CBA/N mice are 23.7 ± 0.4 hours and 23.1 ± 0.4 hours, respectively, and are significantly different ($p < 0.05$) (Figure 1B).

To examine the ability of light to shift the circadian phase of CBA/J and CBA/N mice, a pulse of light (300 lux) was administered at circadian time 15 during the period of free running activity. The animals were then maintained in DD for an additional 8 days. The degree to which the animals shifted their activity was measured. While both groups were able to phase shift in response to light, the shift seen in CBA/J mice was attenuated compared to the controls (CBA/N = 3 ± 0.6 hours; CBA/J = 1.67 ± 0.23 hours; $p < 0.05$). These data are in agreement with those previously reported (Yoshimura *et al*, 1994).

To determine the degree of degeneration in CBA/J animals that were used in the wheel running experiments, retinal cross-sections were stained with Giemsa (Figure 1C). The retinas from the CBA/J mice lack all outer cellular layers, indicating complete outer retinal degeneration.

Time course of retinal degeneration in CBA/J mice

To characterize the time course of degeneration during development, retinas were taken from mice at P4, P8, P10, P15, P21 and adults (3+ months). For CBA/J's, n = 3 - 5, and for CBA/N's, n = 3 - 6. Figure 2A shows cross-sections of representative retinas stained with Giemsa. From these images it is clear that in CBA/J mice, the ONL and OPL, the layers where the rod and cone photoreceptors reside, are present and intact until P10. By P15 these layers show signs of degeneration, which continues rapidly and is nearly complete by P21, when both layers are almost undetectable. This degeneration was quantified by using AxioVision software to measure the thickness of each retinal layer. By P15, The OPL (1.75 ± 2.0 μm, 6.39 ± 2.2 μm) and ONL (23.82 μm ± 8.1, 63.72 ± 13.5 μm) are significantly reduced in CBA/J compared to CBA/N ($p < 0.05$), and by P21 they are almost nonexistent (Figure 2B).

Dendritic stratification of ipRGCs is unaffected by loss of photoreceptors

During early stages of postnatal development (P4), the dendrites of ipRGCs are unstratified. Over time, however, they begin to stratify into distinct regions in the IPL. To determine whether the outer retinal degeneration affects the ability of ipRGC dendrites to properly stratify, retinal cross-sections were taken from mice at P4, P8, P10, P15, P21 and adults and immunostained for melanopsin (for CBA/J's, n = 3 - 5, and for CBA/N's, n = 3 - 5). Data from these studies show that in both strains the ipRGC dendrites are unstratified at P4. As the retina develops, the dendrites stratify into distinct regions in the IPL within the same time frame

for both CBA/J and CBA/N mice. The dendrites of the ipRGCs show no discernible differences in their ability to stratify compared to CBA/N controls at all time points analyzed (Figure 3). As a result, we conclude that despite the massive outer retinal degeneration in the CBA/J mice, the melanopsin-expressing RGCs can still extend their dendritic processes correctly, suggesting that photoreceptors are not necessary for this process.

Potential control of ipRGC number and distribution by photoreceptors

The results from the stratification studies described above demonstrate that photoreceptor loss during development does not affect stratification of ipRGCs. We conclude that the differences in the animals' behavioral responses to light are not the result of aberrant morphological development of melanopsin-containing RGCs. An additional possibility to consider is that outer retina maturation might control ipRGC development by regulating cell number and/or distribution. To examine this, we immunostained retinal whole mounts from CBA/J and CBA/N mice at P4, P8, P10, P15 and P21 for melanopsin (for CBA/J's, n = 4 - 8 and for CBA/N's, n = 4 - 5). The tissue was imaged by fluorescence microscopy, and the resulting images were stitched together to reconstruct the entire retina (Figure 4A). Data from these studies show that at P4, CBA/J and CBA/N mice have the same number of melanopsin-positive cells (CBA/J = 54.4 $\pm$ 29.7 cells/mm^2; CBA/N = 51.8 $\pm$ 17.2 cells/mm^2). However, the number of melanopsin-positive cells seen at P21, is greater in the CBA/J animals compared to the controls (CBA/J = 44.4 $\pm$ 9.0 cells/mm^2; CBA/N = 27.4 $\pm$ 6.6 cells/mm^2; $p < 0.05$) (Figure 4B), suggesting that the ipRGCs do not experience the same degree of cell death

in the CBA/J mice compared to CBA/N's. This suggests that the cells in the outer retina may play a role in regulating ipRGC number.

To examine the distribution of RGCs across the retina, ImageJ was used to calculate x, y-coordinates for each melanopsin-positive cell body. Normalized distance between cell bodies was calculated and used to determine differences in the distribution of cells. No significant differences in the distribution of cells in CBA/J mice were observed at any time points compared to CBA/N mice (Figure 4C). Moreover, within strains, the melanopsin-expressing cells remain evenly distributed across the retina throughout development. In addition, there are no differences found at any developmental time point. We conclude that although ipRGC number is changing, the relative distribution remains the same and is unaffected by photoreceptor loss.

PLR is intact at high light intensities in CBA/J mice

We examined the PLR in adult CBA/J mice and found that the response to bright light was attenuated at low light intensities. This is to be expected of animals lacking rods and cones (Lucas et al 03). However, at high light intensities, the CBA/J mice did not differ in their PLR from the controls, suggesting that the melanopsin pathway is functioning properly at the level of the retina (Figure 5). We conclude that developmental retinal degeneration does not affect the melanopsin-based contribution to the PLR and that the differences seen in behavior are not due to functional changes within the melanopsin-containing cells, at least at the level of the retina.

Discussion

A number of animal models have been used to determine the roles of rods and cones in the process of photoentrainment. Phase-shifting studies in 80-day old C57BL/6J mice homozygous for the *rd* allele showed that these animals have light-induced circadian responses that are identical to control animals (*rd/*+ and +/+) (Foster *et al*, 1991; Tosini *et al* 2007a). These mice exhibit an extreme loss of rod photoreceptors early in adult life, leaving a small number of cones present in adults. A similar study showed that *rdta* mice, which express the gene for an attenuated diphtheria toxin under the control of a portion of the rhodopsin promoter and exhibit photoreceptor loss, also show light-induced phase-shifting responses (Lupi *et al*, 1999). In addition, mice in which both the rods and cones are rendered nonfunctional but remain intact are also able to phase-shift in response to light (Hattar *et al*, 2003). Conversely, melanopsin knockout mice have attenuated phase-shifting behaviors, implicating their role in entrainment (Panda *et al*, 2002). In addition, genetic ablation of melanopsin-containing RGCs lead to a significant phase-shift reduction, suggesting that these cells serve as conduits for the rods and cones to the circadian clock (Guler *et al*, 2008; Hatori *et al*, 2008). Together these data suggest that the melanopsin-containing cells are responsible for light entrainment.

Despite work suggesting that normal phase shifting can occur in the absence of rods and cones, a study done by Yoshimura *et al*. on CBA/J mice led us to a different hypothesis. These mice display outer retinal degeneration as a result of a mutation in the B subunit of the phosophodiesterase gene that mediates rod transduction. This leads to the complete loss of both the outer plexiform layer

(OPL) and outer nuclear layer (ONL). However, unlike the animals in the experiments discussed above, these retinal degenerate mice show attenuated phase-shifting responses to light (Yoshimura *et al*, 1994). The difference between these mice and the other models is the time course of retinal degeneration. Our studies agree with the phase-shifting data reported by this group, and we characterize the time course of retinal degeneration and show that the ONL and OPL deteriorate during early postnatal development (P10-P21).

We have hypothesized that maturation of the outer retina is involved in regulating the proper development of ipRGCs and thereby dictates the photosensitivity of the adult circadian system. If true, this would explain the differences seen in the light-induced circadian behaviors of the CBA/J mice compared to the other models. There is significant anatomical and physiological evidence to suggest that photoreceptors regulate ipRGCs (Belenky *et al*, 2003; Sakamoto *et al*, 2004; Perez-Leon *et al*, 2006; Wong *et al*, 2007). Electron micrographs and immunohistological staining show that ipRGCs are contacted by amacrine and bipolar cell terminals (Belenky *et al,* 2003; Ostergaard *et al*, 2007) suggesting that these cells receive input from photoreceptor-driven pathways. In addition, in adult rats, photoreceptor degeneration leads to a decrease in overall melanopsin levels and an elimination of its circadian expression (Sakamoto *et al*, 2004; Ostergaard *et al*, 2007).

Because of the radical changes RGCs undergo during the maturation of the vertebrate retina, there are many potential points of regulation. During development, the dendritic stratification of RGCs experience substantial

remodeling. Early on these neurons are unstratified, but as the animal matures, RGC dendrites stratify into specific retinal layers. Glutamate released from bipolar cells plays a role in determining the stratification of RGCs involved in vision (Bodnarenko and Chalupa 1993). We reasoned that if glutamate played a similar role in ipRGC stratification, the loss of photoreceptors in CBA/J mice might impede this process. We find, however, that despite the massive degeneration of the outer retina, the dendrites of ipRGCs can still stratify as well as the controls, suggesting that photoreceptors are not required for this process. These data are consistent with an intrinsic mechanism being responsible for defining stratification. Additional possibilities would include signals from other cell types, such as amacrine and/or bipolar cells, as well as those from ipRGC targets.

RGCs also undergo changes in number. At birth there is an overproduction of RGCs that is followed by cell death as the animal matures (Young 1984). Studies have shown that ipRGCs also undergo a similar reduction in number during early postnatal stages (Sekaran *et al*, 2005). By P14, a time that coincides with the maturation of photosensitivity in rods and cones (Tian and Copenhagen 2003), the number of these cells is similar to that seen in the adult (Sekaran *et al*, 2005). We find that the number of ipRGCs is the same in both CBA/J and CBA/N mice at P4. However, the cell number does not change during the development of CBA/J mice. While the controls exhibit the expected decrease in number with increasing age, the number of ipRGCs seen in CBA/J mice at P21 is the same as it is as P4, suggesting that these cells do not experience the pruning seen in normal mice.

While the mechanisms of control of ipRGC developmental cell death are unclear, *in vitro* studies suggest RGCs require a number of survival factors during development (Mey and Thanos 1993; Meyer-Franke *et al*, 1995). Photoreceptor regulation might make sense if the rods and cones were responsible for regulating the availability of essential factors. This is an interesting finding given that a greater number of ipRGCs would suggest that the circadian system is more sensitive to light. However, CBA/J mice have attenuated phase-shifting. Future work might look at changes in central processing to more closely look at the differences in behavior.

We have shown that the distribution of ipRGCs was not altered by loss of photoreceptors. We see that throughout development, the relative distribution of ipRGCs remains constant. This suggests that the loss of photoreceptors does not result in clustering or in any significant change in cell distribution. We also observe this in the CBA/N mice, suggesting that although cell number changes in development, the relative distribution of cells is the same.

Studies have shown that all three photoreceptor cells contribute to the PLR in mice. The rods and cones are responsible for the PLR at low light intensities while the melanopsin-containing RGCs control the reflex at high light intensities (Lucas *et al*, 2003). Because the PLR is mediated by retinal input to the olivary pretectal nucleus, it can be used to test the function of the melanopsin pathway independent of the SCN. As expected, CBA/J mice show attenuated PLR's at low intensities, because they lack rods and cones. At high light intensities, the PLR is the same as that in CBA/N, suggesting that the melanopsin pathway is intact, at

least at the level of the retina. As a result, the attenuation in behavior seen in CBA/J mice does not appear to be due to functional changes in the retina.

Further research on central processing may provide more information on how development of photoreceptive pathways within the retina influences circadian function. Changes in the degree of retinal innervation of the SCN might account for differences seen in the behaviors of the CBA/J mice. In addition, altered neural networks of the SCN might also play a role. Together these provide areas for future research in understanding what dictates the proper functioning of the circadian system.

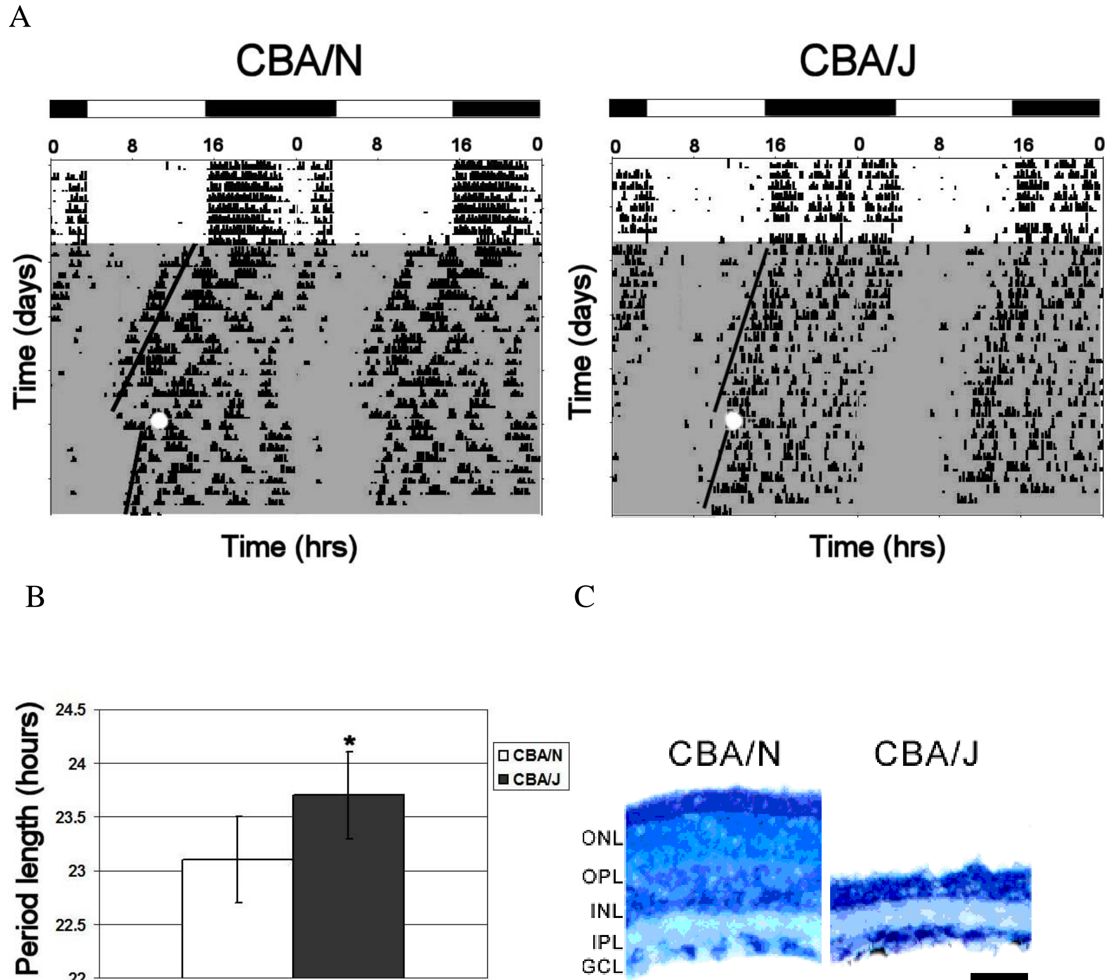
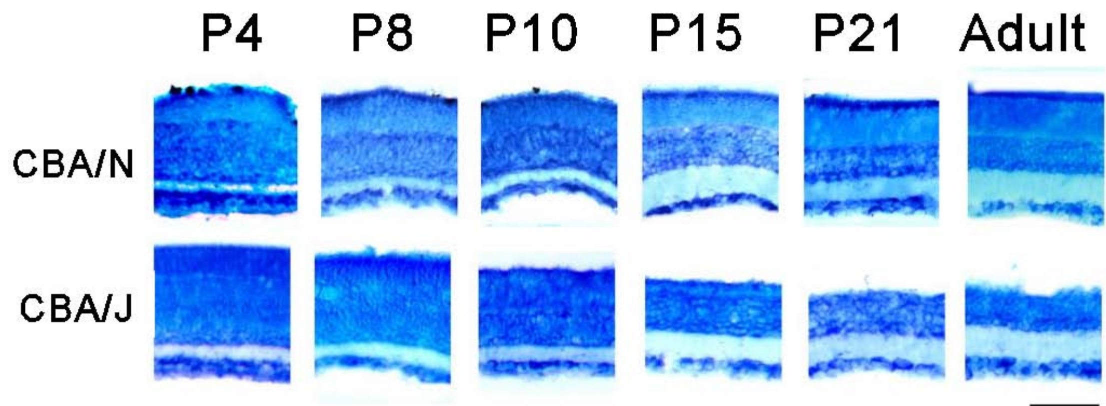


Figure 1. *Light entrainment, free-running rhythms and phase-shifting in CBA/J and CBA/N mice*. A. Actograms depicting wheel running behavior in CBA/J and CBA/N mice under 12:12 (LD). Activity and rest were double-plotted. White bars indicate lights on and black bars indicate lights off. The shaded region depicts constant darkness (12:12 (DD)) in which the animals exhibit free-running rhythms. The white circle indicates a light pulse given at CT 15 to cause a phase-shift. Both strains of mice exhibit a phase shift, though the response in CBA/J animals was slightly attenuated. The actograms and values reported are representative of data from 6 mice of each strain. B. Period lengths in CBA/J and

CBA/N mice are significantly different (* = $p < 0.05$). white bars = period length in CBA/N mice; dark grey bars = period length in CBA/J mice. C. Giemsa-stained retinal cross-sections taken from mice used in the wheel-running experiments confirm that CBA/J mice lack ONL and OPL. Scale bar = 50 µm.

A



B

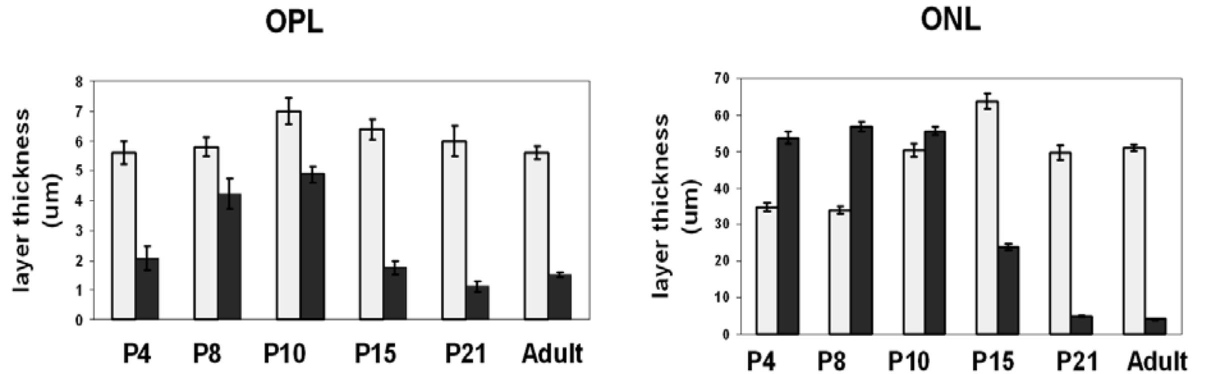


Figure 2. *Time course of outer retinal degeneration during development*. A. Retinal cross-sections taken from CBA/J and CBA/N mice at various stages of postnatal development show that the CBA/J mice display retinal degeneration that begins around P10 and is completed by P21. Scale bar = 100 µm. B. Quantification of the degeneration shows that by P21 the ONL and OPL are significantly reduced in CBA/J mice. Black bars = layer thickness in CBA/J mice; white bars = layer thickness in CBA/N mice.

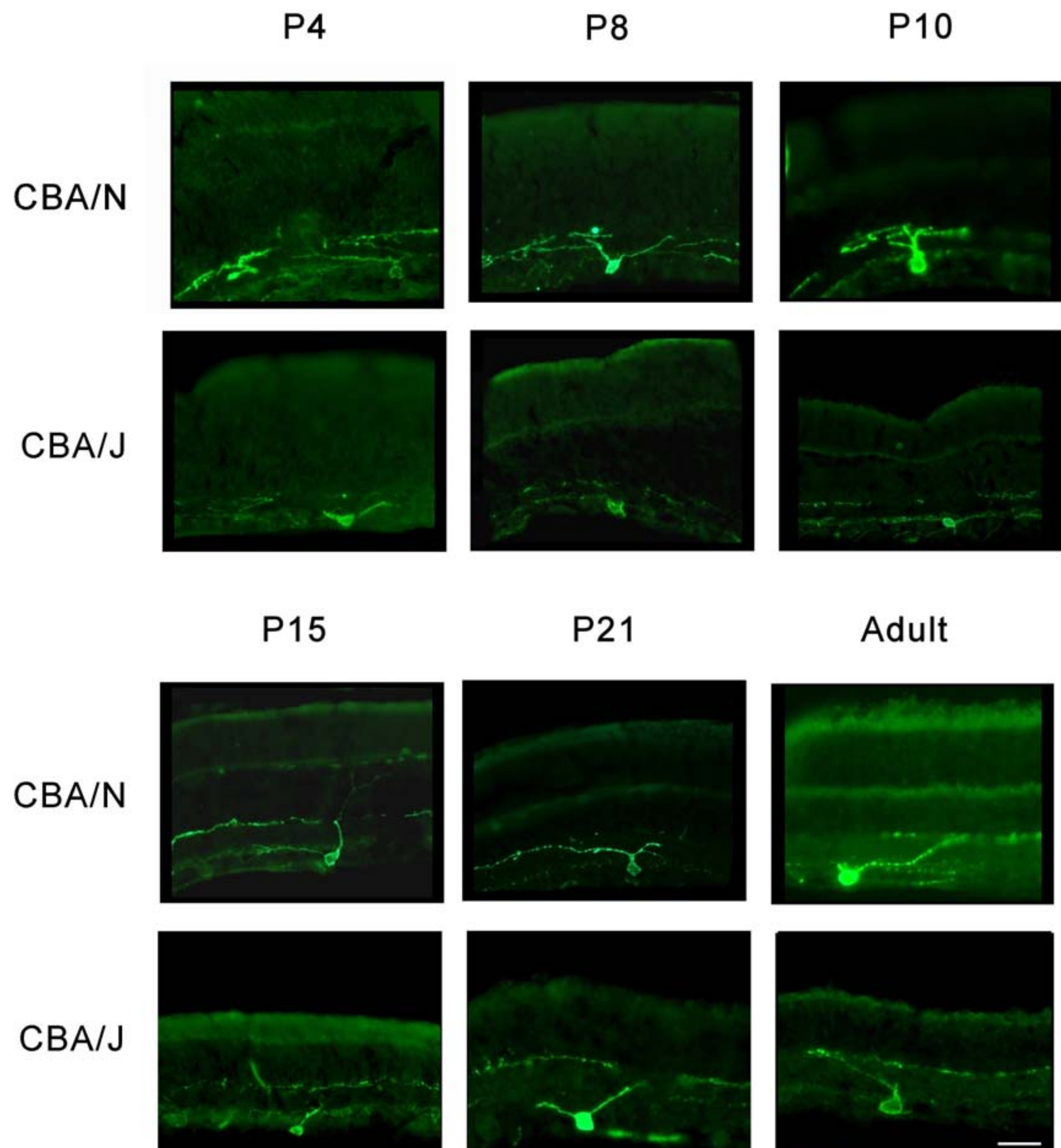
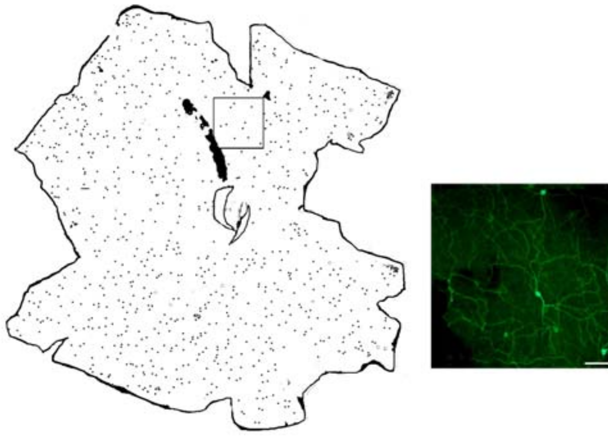


Figure 3. *Dendritic stratification of melanopsin-positive RGCs during development.* Retinal cross sections from CBA/N and CBA/J mice stained with an anti-melanopsin antibody. Scale bar = 50 µm.

A



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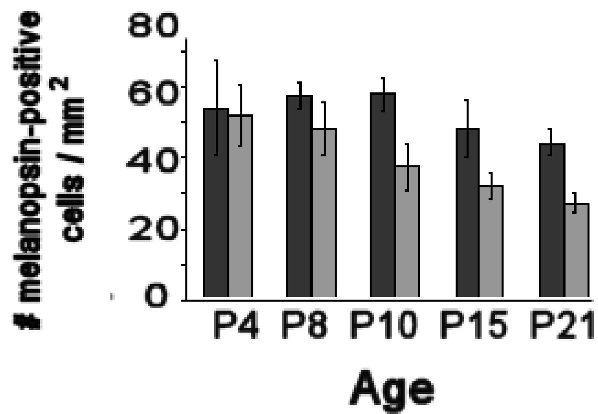

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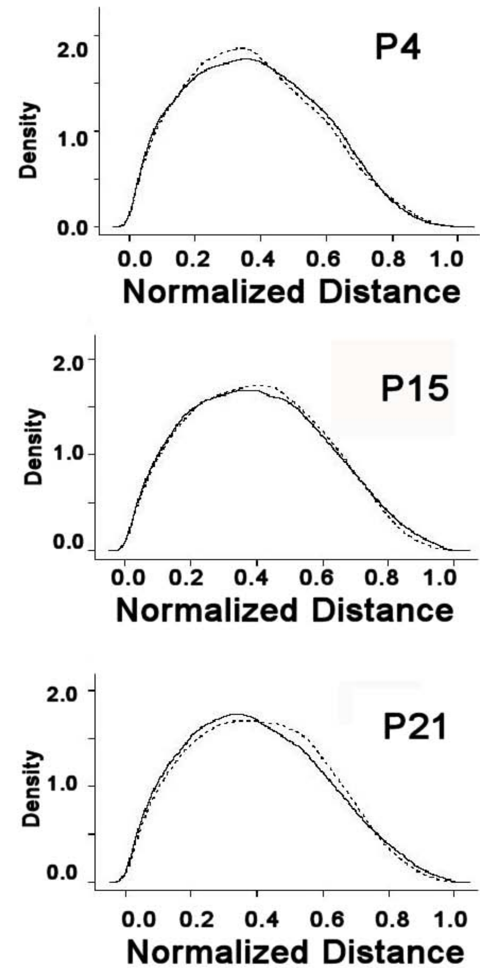

Figure 4. *Cell number and distribution of melanopsin-positive RGCs in CBA/N and CBA/J mice.* A. Whole mount retinas were stained with an anti-melanopsin antibody and imaged by fluorescence microscopy, and the location and number of cell bodies were mapped using ImageJ. Inset shows the actual staining of cells (20X). Scale bar = 100 µm. B. Quantification of melanopsin-positive cell number in developing mice. The y-axis represents the number of cells per unit area (µm).

Black bars = CBA/J mice; Gray bars = CBA/N mice. C. Distribution of melanopsin-positive cells in P4, P15 and P21 mice. Dotted lines depict distributon of cells in CBA/J mice and solid lines represent distribution in CBA/N mice.

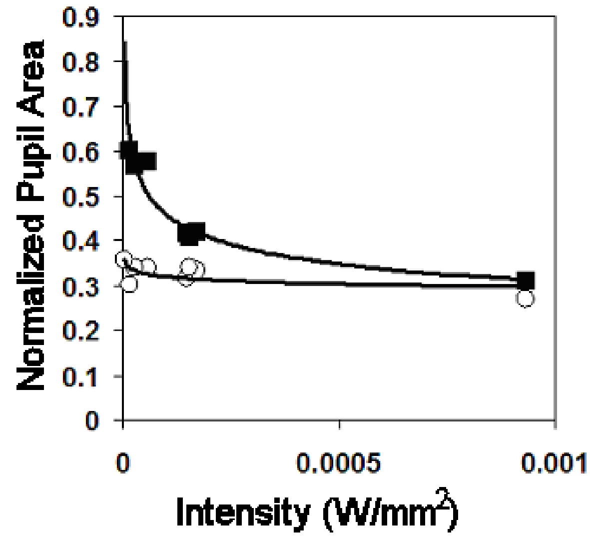

Figure 5. *Pupillary light reflex in CBA/J and CBA/N mice.* The y-axis depicts area of pupil normalized to pupil size at the start of the experiment. The x-axis shows increasing light intensities. Solid squares = CBA/J; Open circles = CBA/N.

CHAPTER 3

CIRCADIAN BEHAVIOR AND CENTRAL PROCESSING IN MICE WITH EARLY POSTNATAL RETINAL DEGENERATION

Circadian behavior and central processing in mice with early postnatal retinal degeneration

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Abstract

In mammals, the central clock responsible for generating circadian rhythms is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Silver and Moore 1998). Light entrainment of the clock occurs through a subset of intrinsically photosensitive retinal ganglion cells (ipRGCs) that project from the retina to the SCN via the retinohypothalamic tract (RHT) (Gooley *et al*, 2001) in a manner independent of rod and cone signaling (Berson *et al*, 2002; Hattar *et al*, 2002; Warren *et al*, 2003). Although ipRGCs are sufficient to drive photoentrainment, rod and cone photoreceptors also contribute (Hattar *et al*, 2003; Guler *et al*, 2008; Hatori *et al*, 2008). We have previously shown that the retinally degenerate CBA/J mouse has greater numbers of ipRGCs (Ruggiero *et al*, 2008, in press). An increase in photosensitive cells might argue for enhanced behavioral responses to light, however, these mice exhibit attenuated phase-shifting (Yoshimura *et al*, 1994). In this paper we further explore the limits of entrainment of CBA/J mice by exposing them to shorter T cycles. We also observe the retinal innervation of vasoactive intestinal peptide-containing SCN neurons and examine vasopressin-expressing cells as a marker for SCN output. In addition, we test the function of the circadian system by looking at light-induced *c-fos* expression in the SCN. We hypothesize that the differences in light-induced circadian behaviors of the CBA/J mice are due to changes in central processing.

Introduction

In mammals, retinal input to the circadian clock, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, is required for entrainment of circadian rhythms. Transection of the optic nerve, removal of the eyes or blockade of optic nerve transmission with TTX eliminates shifts in the circadian phase in response to photic input (Schwartz *et al*, 1987; Johnson *et al*, 1988; Foster *et al*, 1991). While it was traditionally thought that all photoreception within the retina was mediated by rod and cone photoreceptors, the discovery of intrinsically photosensitive retinal ganglion cells (ipRGCs), which express the photopigment, melanopsin, led to the understanding that these cells contribute to non-image forming responses to light (Gooley *et al*, 2001; Berson *et al*, 2002; Hattar *et al*, 2002; Panda *et al*, 2002). Although rods and cones are not required for entrainment, they do play a role because melanopsin-knock out mice retain non-image forming (NIF) responses to light that are eliminated in mice lacking rods, cones and melanopsin (Hattar *et al*, 2003; Lucas *et al*, 2003). Ablation of the ipRGCs produce mice with a similar phenotype (Guler *et al*, 2008; Hatori *et al*, 2008), suggesting that rods and cones use these cells as a means of transmitting light to NIF centers of the brain.

We have previously shown that CBA/J mice, which have an early retinal degeneration, have greater numbers of ipRGCs and attenuated phase-shifting responses compared to CBA/N control mice. A greater number of light sensitive cells might argue for enhanced light responses, however, these mice exhibit

attenuated phase shifting. Studies that assay the pupillary light reflex (PLR) in CBA/J mice suggest that the melanopsin pathway is functional. Therefore, we hypothesize that differences in circadian behaviors are due to changes in central processing.

The SCN is anatomically divided into a ventral "core" and a dorsal "shell" (Moore 1996; Hamada *et al,* 2001; Morin 2007). In one model, the cells of the core receive direct retinal input and express *c-fos* in response to light (Antle and Silver 2005). In mouse, the core contains a set of neurons which express vasoactive intestinal peptide (VIP) (Abrahamson and Moore 2001). VIP-expressing cells are thought to relay light information to the vasopressin (VP) -containing cells of the shell, because VIP induces phase-shifts in the circadian expression of VP in a similar manner to light (Watanabe *et al*, 2000). This transmission is important for photoentrainment because the cells of the core act to synchronize the intrinsically rhythmic shell neurons so that they produce a coordinated output.

The roles of VIP and VP in circadian behaviors have been demonstrated in mice lacking these peptides or their receptors. For example, VIP knockout mice display behavioral arrhythmicity and desynchronization of the clock with the environment (Colwell *et al*, 2003), and loss of its receptor, VPAC2, disrupts activity rhythms (Harmar *et al*, 2002). In addition, VP deficient rats exhibit disrupted circadian rhythms in body temperature in the absence of entraining stimuli (Wideman *et al*, 1997), and mice lacking the VP receptor, VP1a, have altered free-running periods (Wersinger *et al*, 2007). Together these data suggest

that changes in peptide content in the SCN can have significant effects on clock function and circadian behaviors.

In this paper, we explore the limits of entrainment in CBA/J mice by exposing them to shorter T cycles. To explain the differences in the circadian behaviors of these mice, we looked for potential changes in central processing by examining VIP- and VP- expressing cells in the SCN. We also examined the degree of retinal innervation of the SCN by ipRGCs. To test if the system is functioning, we looked for differences in light-induced *c-fos* expression in the SCN. Our data suggest that differences in VIP- and VP-positive cells exist in the SCN of the CBA/J mice, which may provide insight into the cellular pathways underlying circadian behaviors. Because CBA/J mice exhibit an early postnatal retinal degeneration, this work implicates a role for maturation of rods and cones in the development of circadian function.

Materials and Methods

Animals

All procedures were carried out in compliance with the guidelines of the National Institutes of Health. All protocols were approved in advance by the Institutional Animal Care and Use Committee of Oregon Health & Science University. Male CBA/J mice, which carry the $Pde6b^{rd1}$ mutation and exhibit blindness by weaning age (Jackson Laboratory, Bar Harbor, ME, USA) were used to examine the effects of early retinal degeneration on circadian function and retinal innervation of the SCN. For controls, we used male CBA/N (National Cancer Institute, Frederick, MD, USA) mice, which have the same genetic background as the CBA/J mice, but lack the phosphodiesterase mutation and are, therefore, visually intact. For wheel running experiments (see below) we also used the B6.C3Ga-$Mfrp^{rd6}$/J mouse (Jackson Laboratory, Bar Harbor, ME, USA), which we refer to as "rd". These mice display retinal degeneration later in adulthood, after the retina has matured. We use these mice to determine whether differences in the CBA/J mice are due to degeneration that is occurring specifically during development.

The mice were housed in facilities that permit the maintenance of a 12-hour light-dark (LD 12:12) cycle. Animals used in the behavioral studies were kept in separate chambers under the lighting conditions described below. Studies were restricted to male mice to avoid potential effects of fluctuations in female reproductive hormones on circadian behaviors. CBA/J and CBA/N mice used for entrainment studies were at least 3 months of age, to avoid variability in their

abilities to entrain (Pittendrigh and Daan 1976). Rd mice were at least 6 months of age to ensure retinal degeneration.

Wheel-running experiments

Mice used in the behavioral experiments were housed in an Intellus Control System chamber (Percival Scientific, Perry, IA, USA) with constant lighting and temperature (24˚C) conditions. Within the chamber animals were kept in Nalgene cages equipped with running wheels and magnetic switches (Mini Mitter, Bend, OR, USA) that allow for recording of wheel revolutions. Wheel-running data were collected continuously by VitalView (Mini Mitter, Bend, OR, USA) acquisition software. Actograms depicting activity and rest cycles during entrainment and free-running assays were generated using ClockLab (Actimetrics Software, Wilmette, IL, USA).

The ability of CBA/J, CBA/N and rd mice to entrain to a photoperiod of 12 hours of light (1000 lux) followed by 12 hours of darkness (0 lux) (LD 12:12) was observed by measuring their wheel-running activity. Once entrained, the mice were kept in constant darkness (DD) for at least 10 days to measure the period length of their free-running rhythms. To examine the ability of the mice to entrain to shorter T cycles, animals were placed on 22 hour (LD 11:11) and 23 hour (LD 11.5:11.5) T cycles for at least 2 weeks.

Tissue preparation

Animals were deeply anesthetized with isoflurane and perfused intracardially with 0.1 M 4% paraformaldehyde (PFA) (pH 7.4) for 10 min. The brains were

removed and postfixed in PFA overnight at 4˚C. Following fixation, the retinas were cryoprotected by successive immersion in phosphate-buffered solutions containing 10% and 30% sucrose at 4˚C overnight. The tissue was embedded in Thermo Shandon Cryochrome (Thermo Scientific, Pittsburgh, PA, USA), fast-frozen over dry-ice mixed with 100% ethanol for 3-5 minutes and stored at -80˚C.

Antibodies

Polyclonal antibodies generated in rabbit and directed against vasoactive intestinal peptide (VIP) (Peninsula Laboratories, San Carlos, CA, USA) or vasopressin (VP) (Abcam, Cambridge, MA, USA) were diluted in blocking solution (1% BSA + 0.3% Triton-X) (1:500). The polyclonal c-Fos antibody (Oncogene Science, Manhasset, NY, USA) was generated in rabbit and diluted in blocking solution (1:250). Alexa-488 labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) diluted in blocking solution (1:1000) was used for a secondary antibody.

Immunohistochemistry

Embedded brains were cross-sectioned (20 μm) on a Leica 1720 digital cryostat. Sections containing SCN were rinsed in 0.1 M PB with 0.3% Triton-X for 15 min. and placed in blocking solution for 1 hour at RT. For VIP and VP studies, the tissue was incubated in primary antibody for 48 hours at 4˚C. For the light-induced *c-fos* expression experiments, the tissue was incubated in primary antibody for 72 hours at 4˚C. Sections were rinsed with 0.1 M PB with 0.3% Triton and incubated in secondary antibody for 2 hours at RT. The tissue was

rinsed with 0.1 M PB, counterstained with the nuclear stain, DAPI (80 ng/ml) for 1-3 min. at RT and mounted on a glass slide in Aqua Mount (Fisher Scientific, Pittsburgh, PA, USA). Immunostained tissue was imaged by fluorescence microscopy at 20X using a Zeiss Axioscope 2TM.

Retinal innervation

To examine retinal innervation of the SCN, adult CBA/J and CBA/N mice were anesthetized with isoflurane and, using a Hamilton syringe, 2 μl of cholera toxin subunit B conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) was injected into the vitreous of one eye. Lidocaine (20 mg/ml) (Hospira, Inc., Lake Forest, IL, USA) was applied to the cornea prior to and after injection. Mice were left to recover for 48 hours and then sacrificed and perfused as described above. The tracer moves from the retina to the brain and labels the retinal terminals there. To examine retinal innervation, the brains were processed as described above. Brain sections containing SCN were stained with an antibody directed against VIP (see above). The tissue was imaged by fluorescent microscopy in both channels to look at retinal terminals and VIP-positive cells. Using Image J software (http://rsb.info.nih.gov/ij/), corresponding tissue sections were overlaid. Areas of overlap appear yellow, and the degree of retinal innervation of VIP-positive cells was calculated as the number of yellow cell bodies.

Cell numbers

VIP and VP staining were examined in CBA/J and CBA/N mice. Brain sections were stained as described above, and the average number of VIP- and VP- positive cell bodies was calculated in 2 - 4 20 μm coronal sections using ImageJ software (http://rsb.info.nih.gov/ij/).

Light-induced c-Fos expression

CBA/J and CBA/N mice were maintained in DD for at least 10 days, and the free-running periods were calculated. At circadian time (CT) 16, the mice were exposed to light (1000 lux) for 1 hour then returned to darkness for 90 min. Mice were anesthetized with isoflurane and perfused under dim red light. Brains were removed and processed as described above. Control mice were kept in the same lighting conditions without the presentation of a light pulse and sacrificed at the same CT. The number of c-Fos positive cells was calculated and the percent increase in c-Fos in response to light was calculated by dividing the number of cells in mice exposed to light by the number of cells in mice in DD.

Statistics

Averages are reported as the mean ± SD. Significance was determined using the Student's t-Test.

Results

CBA/J mice entrain to a narrower range of T cycles

CBA/J mice exhibit retinal degeneration during early postnatal development. By P21, the outer nuclear layer (ONL) and outer plexiform layer (OPL) are undetectable by light microscopy (Ruggiero *et al*, 2008, in press). These mice exhibit attenuated phase shifting responses (Yoshimura *et al*, 1994; Ruggiero *et al*, 2008, in press), while animals which lose photoreceptors during adulthood phase shift in a similar manner to controls (Foster *et al*, 1991; Freedman *et al*, 1999; Lupi *et al*, 1999; Semo *et al*, 2003b; Tosini *et al*, 2007a). As a result, we hypothesize that developmental loss of photoreceptors influences the development of ipRGCs thereby impacting circadian function in the adult.

To further explore photoentrainment in CBA/J mice, wheel-running experiments were conducted on adult CBA/J (n= 5), CBA/N (n = 4) and rd (n = 3) mice exposed to varying T cycles (non-24 hour cycles). The actograms in Figure 1A show that all three mouse strains can entrain to a photoperiod of LD 12:12. When put into constant darkness (DD) for at least 10 days, all 3 strains showed normal free-running rhythms, suggesting that the ability to entrain is not affected by loss of photoreceptors during development or adulthood. The period lengths of CBA/J and rd mice did not differ ($p = 0.87$), though the periods of both were longer than the CBA/N ($p < 0.05$) (CBA/J = 23.71 $\pm$ 0.23 hours; CBA/N = 22.9 $\pm$ 0.26 hours; rd = 23.7 $\pm$ 0.15 hours).

When the mice were housed in a T = 23 hour photoperiod (LD 11.5:11.5), all 3 strains of mice showed entrainment (data not shown). When T = 22 hours,

however, both the CBA/N and rd mice entrained, but the CBA/J mice did not, suggesting that developmental photoreceptor loss affects the lower limits of entrainment in these mice (Figure 1B). Though unable to entrain, CBA/J mice show signs of masking, which suggests that this behavior is unaffected by photoreceptor loss in development. The lack of an effect on masking is expected in animals with intact melanopsin systems, even in the absence of classical photoreceptors (Mrosovsky *et al*, 2000).

CBA/J mice have greater numbers of VIP- and VP-positive cells

VIP-containing cells are found in the core of the mouse SCN and receive direct retinal input. To examine whether mice with early retinal degeneration exhibit structural differences in the SCN, we examined the number of VIP-positive cells in CBA/J (n = 7) and CBA/N (n = 5) mice. Figure 2A shows that in both CBA/J and CBA/N mice, VIP-positive cell bodies are concentrated in the ventral core of the SCN with few to no cells in the shell. VIP-positive fibers project dorsally. We calculated the number of VIP-positive cells bodies and found that there are significantly greater numbers of cells in the CBA/J mice compared to CBA/N mice (CBA/J = 36.5 $\pm$ 7.7 cells; CBA/N = 27.2 $\pm$ 4.6 cells, $p < 0.05$) (Figure 2B).

To examine differences in output cells of the SCN of CBA/J mice, coronal brain sections from CBA/J (n = 8) and CBA/N (n = 4) mice were stained with an antibody against VP, a marker for the shell. In both strains, VP-positive cells are found dorsally, in the shell of the SCN. For the most part, cell bodies were not found in the core (Figure 3A). VP-positive cell bodies were counted, and we found that there are significantly greater numbers of cells in CBA/J mice

compared to controls (CBA/J = 73.0 $\pm$ 13.7 cells; CBA/N = 49.3 $\pm$ 8.0 cells, $p < 0.05$) (Figure 3B).

Retinal innervation does not differ between CBA/J and CBA/N mice

IpRGCs project via the retinohypothamalmic tract (RHT) to the SCN (Gooley *et al*, 2001). To examine the innervation of SCN core cells by ipRGCs, fluorophore-labeled cholera toxin was injected into the eyes of CBA/J (n = 6) and CBA/N (n = 5) mice. Brain sections containing labeled RGC terminals were counter-stained with an α-VIP antibody as a marker for cells receiving retinal input. The images obtained by fluorescent microscopy were overlaid, showing points of innervation (Figure 4A). Cell bodies that were contacted by retinal terminals were observed as yellow. The number of yellow cell bodies was carefully calculated to avoid overlap between VIP-positive and cholera toxin-positive fibers, which also appear yellow. When we calculated the number of VIP-positive cell bodies contacted by retinal terminals, we found that there is no significant difference in the anatomical innervation of VIP-positive cell bodies in CBA/J mice compared to controls (CBA/J ($p = 0.4$) (Figure 4B).

Light induction of c-Fos expression in CBA/J mice

The immunohistochemical data suggest that there is no difference in the degree of retinal innervation of VIP-positive cells. However, this method does not look at functional innervation and is limited to examining one set of cells receiving innervation. To examine functional innervation, we looked at light-induced *c-fos*

expression in the SCN. CBA/J (n = 5) and CBA/N (n= 5) mice kept in DD were exposed to a pulse of bright light for 1 hour at CT 16. In response to light, c-Fos was detected in the SCN of both strains. The gain of the fluorescent signal was increased equally in both mice in order to better visualize the staining. This causes some cells to appear brighter than others, which we do not claim to be a change in expression level of protein. Though concentrated in the ventral region, staining is present throughout the SCN, consistent with previously reported work (Colwell and Foster 1992; Rea *et al*, 1993; Ruby *et al*, 2002). This staining was low in mice kept in DD (CBA/J, n = 3; CBA/N, n = 3) with no exposure to light, suggesting that c-Fos induction is a result of exposure to light. These values did not differ significantly between the two mouse strains (CBA/J, 20.4 $\pm$ 3.6 cells; CBA/N, 26.1 $\pm$ 1.8 cells, $p = 0.07$) (Figure 5A). When we counted the number of c-Fos positive cells, we found that the percent increase in c-Fos positive cells in mice exposed to light compared DD was significantly greater in CBA/J mice compared to CBA/N (CBA/J, 4.8-fold increase; CBA/N, 2.1-fold increase) (CBA/J, 98.6 $\pm$ 12.5 cells; CBA/N, 55.5 $\pm$ 4.8, $p < 0.05$).

Discussion

CBA/J mice have attenuated phase-shifting responses to light compared to CBA/N controls (Yoshimura *et al*, 1994; Ruggiero *et al*, 2008, in press). To further explore the limits of entrainment of CBA/J mice we exposed them to shorter T cycles. We find that CBA/J mice can entrain to a narrower range of T cycles than CBA/N and rd mice. This supports the idea that these animals have altered circadian responses, as was suggested by the phase-shifting data. This is an interesting finding because our previous work showed that CBA/J mice have greater numbers of ipRGCs. This would suggest that these mice would be more sensitive to light. The work in this paper attempts to reconcile these finding by examining changes in central processing that could explain the differences in behavior.

VIP-expressing cells

To identify the connection between increased numbers of ipRGCs and the attenuated behaviors, we examined potential changes in central processing that may explain the differences seen in the CBA/J mice. The SCN is anatomically divided into a ventral core and a dorsal shell. In one model, cells of the core receive direct retinal input and transmit photic information to the cells of the shell (Antle and Silver 2005). In mouse, a number of core cells express VIP, which plays an important role in circadian rhythms. Injection of VIP into hamster SCN causes phase-shifts in locomotor activity that are similar to those produced by light (Albers *et al*, 1991; Piggins *et al*, 1995). VIP can induce clock gene expression and can phase shift the rhythmic activity of the VP-expressing cells of

the shell (Nielsen *et al*, 2002; Reed *et al*, 1002). The VIP receptor, $VPAC_{2,}$ is necessary for the synchronization of circadian periods among SCN neurons because mice with a null mutation in this gene (*Vipr2-/-*) have disrupted circadian rhythms in wheel running (Harmar *et al*, 2002) and do not express rhythms in clock gene or VP expression (Harmar 2003). Together these data suggest an important role for VIP in light transmission and coordination in the SCN and in circadian behaviors.

In these experiments, we sought to determine whether there are differences in the structural integrity of the SCN by using VIP as a “core” marker. We found that there are greater numbers of VIP-positive cells in the CBA/J mice. This is an interesting result because it has been shown that VIP deficient mice also have attenuated phase-shifting (Colwell *et al*, 2003). This would suggest that the increase in VIP-containing cells, seen in the CBA/J’s, has a similar effect on behavior as does a loss of VIP, seen in the VIP deficient mice. Together these data suggest that imbalance compromised function. In addition, these findings can be reconciled by examining further the contributions of other molecules to intercellular communication among cells of the SCN.

In the SCN, VIP enhances inhibitory transmission because adding VIP to the SCN increases the frequency of IPSCs mediated by GABA (Itri and Colwell 2003). The increase in VIP-positive cell numbers in CBA/J mice might lead to an increased modulation of GABAergic activity. The mechanism by which GABA may affect behavior is unclear, however, in hamster, injection of the GABA receptor agonist, baclofen, into the SCN decreases the animals’ phase-shifting

responses to light in wheel running paradigms (Gillespie *et al*, 1997). This suggests that activation of GABA receptors plays a role in attenuating behavioral responses to light. Further work would be needed to address the roles of VIP and GABA on light entrainment.

VP-expressing cells

VP is found in cells of the shell and is released in a circadian manner, implicating its role in circadian output. To examine potential changes in shell neurons and SCN output, I looked at the number of VP-positive cells and found that CBA/J mice have greater numbers of VP-expressing cells. Whether VP is involved in locomotor behavior is unclear, however, VP-deficient rats show coherent circadian rhythms in wheel-running behaviors (Groblewski *et al*, 1981). In the SCN, VP is often colocalized with GABA (Moore and Speh 1993). It is possible that the increase in VP cells could play a role in the behaviors of CBA/J mice if the effects are mediated by an increase in GABA levels. Though GABA clearly plays a role in SCN function and circadian rhythms, as it is expressed by almost all SCN cells, its sites of action are unclear. Further work would need to be done to better understand how VIP, VP and GABA influence circadian function.

Retinal innervation

Retinal input to the SCN is necessary for light entrainment, so we examined synaptic contacts between ipRGC axon terminals and VIP-positive cells in the core of the SCN. Given that our previous work suggests that there are greater

numbers of ipRGCs, and that there are greater numbers of VIP-positive cells in the SCN, we hypothesized that there would be a greater degree of retinal innervation in CBA/J mice compared to controls.

The data from the anatomical innervation studies suggest that there is no difference in the overlap of retinal terminals and VIP-positive cells between CBA/J and CBA/N mice, suggesting that retinal innervation is unaltered. This was surprising given that there are greater numbers of ipRGCs and VIP-positive cells in the CBA/J mice. This discrepancy can be due to the difficulties with interpreting the immunohistochemical data. The innervating fibers are dense and because there is no ideal way to specifically label ipRGC terminals, it is difficult to resolve the location of synapses.

To address this we conducted an additional assay to examine functional innervation. In these experiments we looked at light-induced *c-fos* expression in the SCN in CBA/J and CBA/N mice. We found that there are greater numbers of c-Fos positive cells in CBA/ mice compared to controls. In both strain, the distribution of cells is not concentrated in the core, which is inconsistent with the idea that cells of the core exclusively receive retinal input, though it is in agreement with previous studies (Colwell and Foster 1992; Rea *et al*, 1993; Ruby *et al*, 2002).). It does, however, suggest that there are greater numbers of light sensitive cells in the SCN of CBA/J mice, which is consistent with the greater number of ipRGCs.

Whether *c-fos* is involved in the entrainment pathway or if it is simply a marker for an early response to light is unclear. While injection of NMDA into

the SCN induces *c-fos* expression, it does not induce phase shifting (Rea *et al*, 1993). Though this suggests that c-Fos is not sufficient to produce phase shifts, blocking *c-fos* expression by injecting antisense oligonucleotides into the SCN of rat prevents phase-shifting in response to light (Wollnik *et al*, 1995). In addition, injection of excitatory amino acid antagonists into the hamster SCN at specific times blocks both light-induced *c-fos* expression and phase shifts, suggesting an association between the two processes (Rea *et al*, 1993). As a result a change in the number of cells expressing *c-fos* in response to light would suggest differences in functional innervation of the SCN, which could lead to changes in the light responsiveness of the circadian system.

Conclusion

Our data suggest that retinal degeneration during development seen in the CBA/J mice impacts the central processing responsible for circadian behaviors. It suggests a role for maturation of rods and cones in influencing circadian function. However, in order to better understand how changes in the retina and the SCN impact circadian behaviors, further work would need to look at the contributions of additional molecules.

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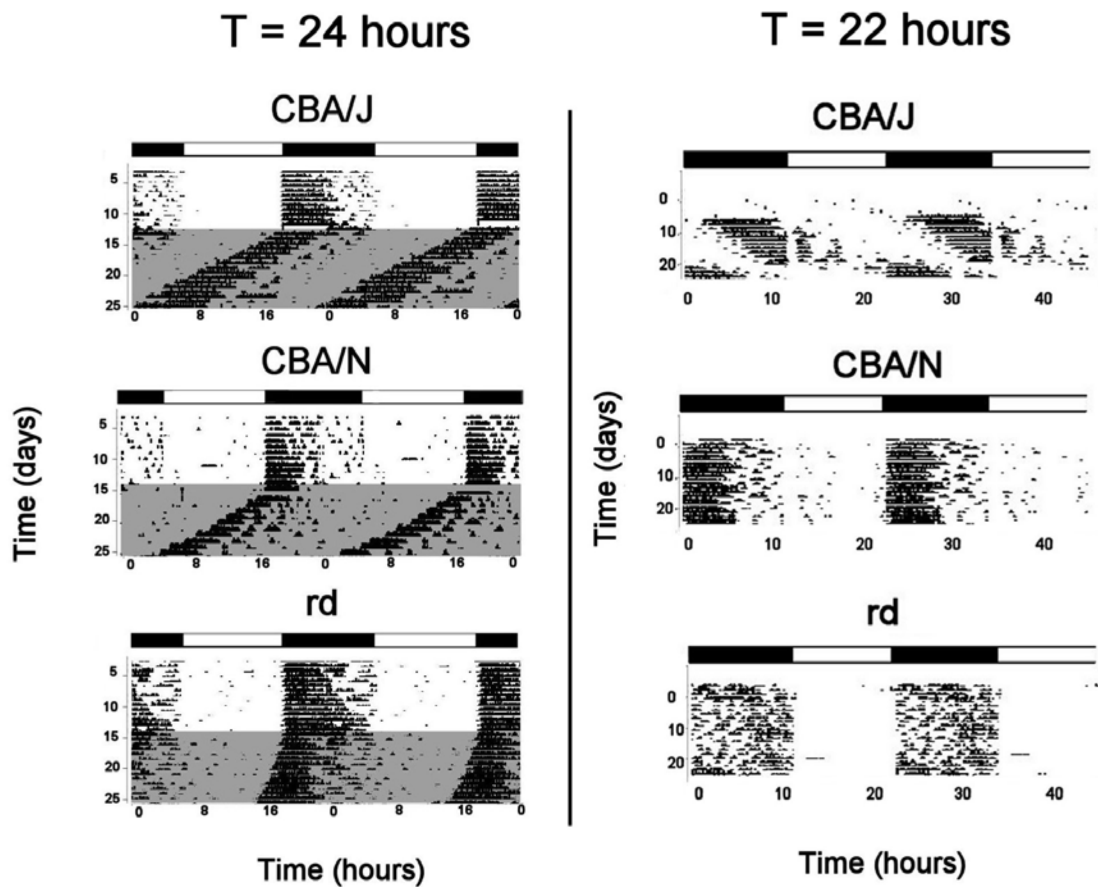

Figure 1. Entrainment of CBA/J, CBA/N and rd mice to T cycles. Actograms depicting wheel running behavior in CBA/J, CBA/N and rd mice. Activity and rest were double-plotted. White bars indicate lights on and black bars indicate lights off. Vertical lines indicate wheel revolutions and signify periods of activity. A. All 3 strains entrain to T = 24 hrs (LD 12:12). The shaded region depicts constant darkness (DD 12:12) in which the animals exhibit free-running rhythms. B. When placed on a 22 hour T cycle (LD 11:11) CBA/N and rd mice entrain, while CBA/J mice do not.

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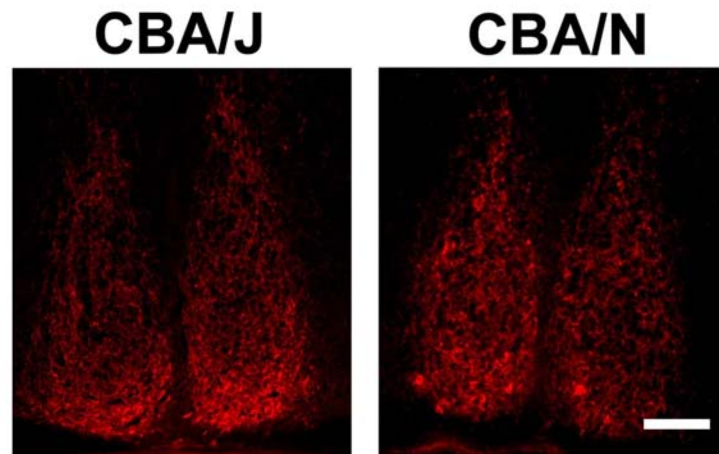

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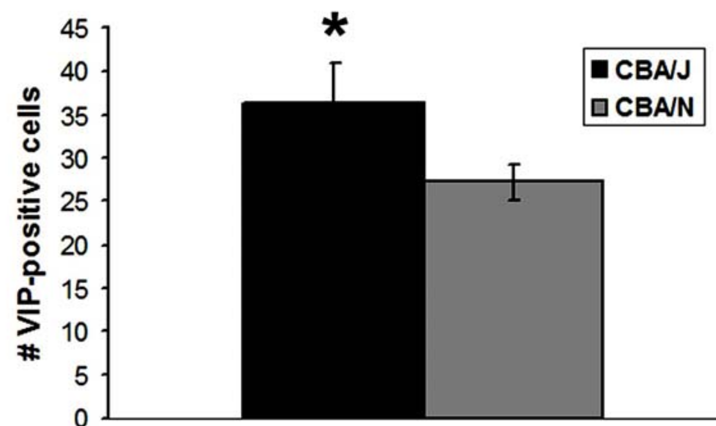

Figure 2. VIP-positive cells in the SCN of CBA/J and CBA/N mice. A. Coronal SCN sections stained with an antibody against VIP show that in both CBA/J and CBA/N mice, VIP-positive cells bodies are found primarily in the ventral core, with fibers projecting dorsally. B. The number of VIP-positive cells is greater in the SCN of CBA/J mice compared to CBA/N. Scale bar = 100 µm. * = $p < 0.05$.

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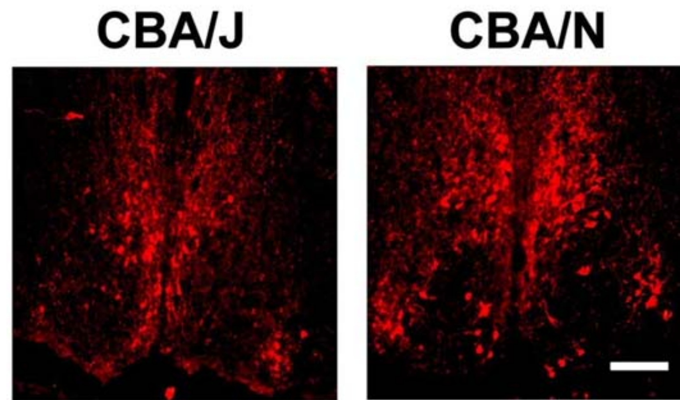

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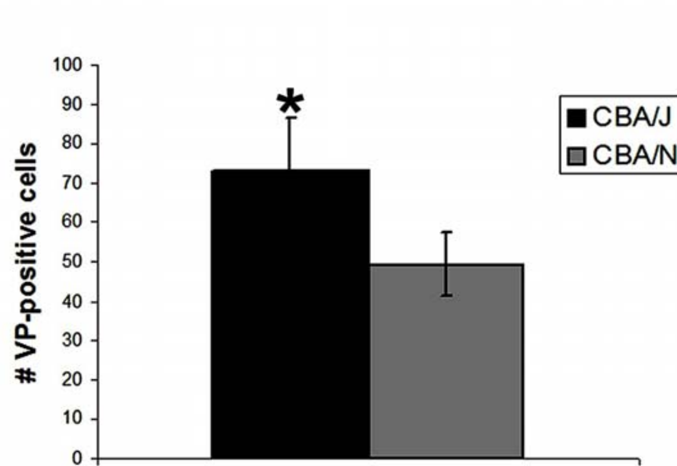

Figure 3. VP-positive cells in the SCN of CBA/J and CBA/N mice. A. Coronal SCN sections stained with an antibody against VP show that in both CBA/J and CBA/N mice, VP-positive cell bodies are found primarily in the dorsal shell. B. The number of VP-positive cells is greater in CBA/J mice compared to CBA/N. Scale bar = 100 µm. * = $p < 0.05$.

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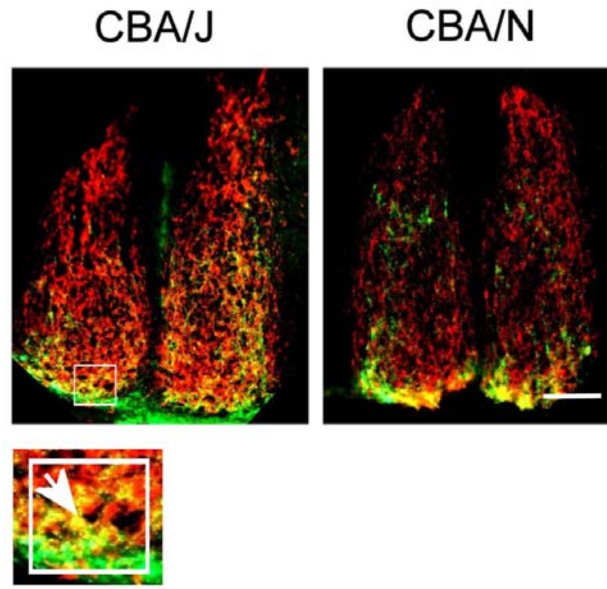

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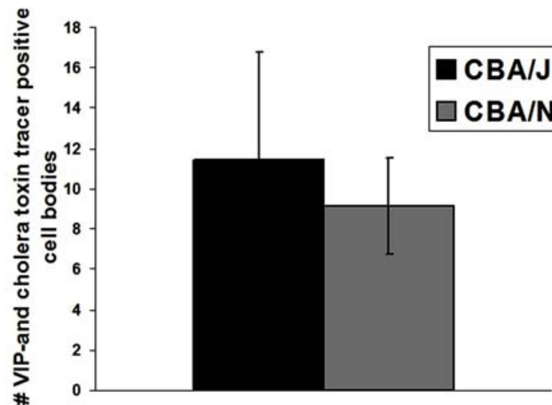

Figure 4. Retinal innervation of the SCN in CBA/J and CBA/N mice. A. Coronal sections of SCN from mice injected intraocularly with a fluorophore-labeled cholera toxin, which labels RGC terminals (shown in green), were stained with an antibody against VIP (shown in red). Images were overlaid and points of overlap are shown in yellow. Inset blown up to display cell body with terminal contact shown by arrowhead. B. The number of VIP-positive cells receiving retinal input does not differ between strains. Scale bar = 100 µm.

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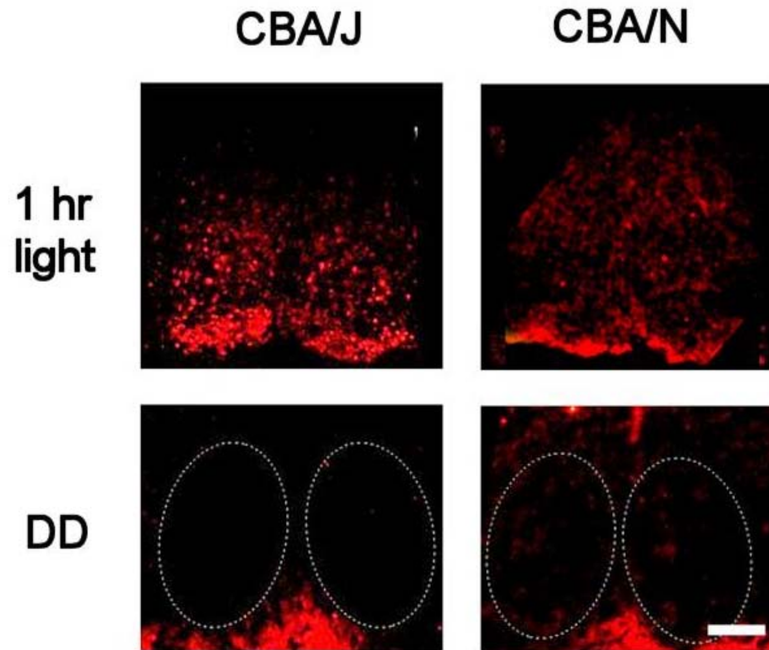

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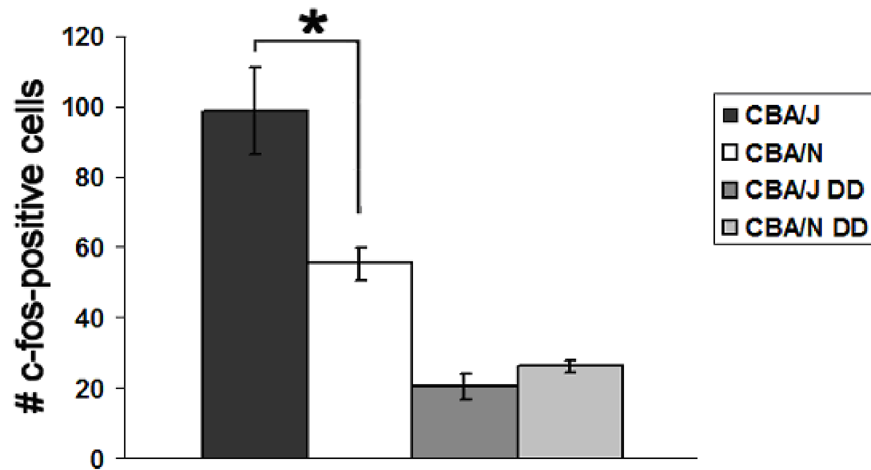

Figure 5. Light-induced c-Fos expression in the SCN of CBA/J and CBA/N mice. A. Top panels show c-Fos expression in CBA/J and CBA/N mice in response to a 1hr light pulse. Bottom panels show controls kept in DD without light exposure. B. There are greater numbers of c-Fos positive cells in CBA/J mice exposed to a light pulse than in CBA/N mice. Scale bar = 100 μm.

CHAPTER 4

SUMMARY AND DISCUSSION

SUMMARY AND DISCUSSION

The goal of my research was to examine the effects of developmental retinal degeneration on the proper development of melanopsin-expressing ipRGCs and to determine whether regulation of ipRGCs influences adult circadian system function. The first part of this work focused on changes at the level of the retina to explain differences seen in the circadian behaviors of CBA/J and CBA/N mice. I examined the effects of early retinal degeneration on the dendritic stratification, distribution and number of ipRGCs in both strains of mouse.

Dendritic stratification of ipRGCs

The dendrites of the RGCs of the image-forming visual system undergo substantial changes during development. Proper stratification is crucial as it determines the physiological functions of these cells. Visual stimulation is required for the proper stratification because dark rearing (DR) inhibits this process (Tian and Copenhagen 2003). This likely results from synaptic input from bipolar cells since blocking glutamate transmission from ON bipolar cells causes the RGC dendrites to remain unstratified. This data suggests that photoreceptor-driven input plays a role in dictating the segregation of RGC dendrites into distinct layers of the IPL (Bodnarenko and Chalupa 1993).

Because rod and cone inputs influence the stratification of the RGCs involved in vision, I hypothesized that photoreceptors play a similar role in ipRGCs and the degeneration seen in CBA/J mice might impede this process. However, despite

the massive degeneration in the outer retina, there were no differences in the stratification of ipRGC dendrites in the CBA/J mice, suggesting that rods and cones are not necessary for this process. An additional explanation is that the rod and cone photoreceptors dictate the stratification early in development, and that the photoreceptor loss in CBA/J mice occurs after stratification is determined.

Potential mechanisms exist whereby ipRGCs could stratify properly in the absence of photoreceptor input. Brain-derived neurotrophic factor (BDNF) has been shown to direct proper stratification in *Xenopus* RGCs (Lom and Cohen-Cory 1999; Cohen-Cory and Lom 2004). In mammals, BDNF mRNA is expressed within RGCs as is its receptor, TrkB (Perez and Caminos 1995; Vecino *et al*, 2002). It is possible that ipRGCs can autoregulate their own stratification by releasing BDNF onto themselves. Also, ipRGCs could respond to factors released by other cells in the retina, such as choline acetyltransferase (ChAT) positive amacrine cells, which also express BDNF (Fujieda and Sasaki 2008). It has been demonstrated that RGCs that lack the β2 nicotinic receptor subunit have altered dendritic stratification, suggesting input other than via BDNF from these amacrine cells is important in this process (Bansal *et al*, 2000). In addition, in mice with reduced expression of glycine receptors, the RGC dendrites fail to properly stratify, and the effects are similar to dark rearing (Xu and Tian 2008). Amacrine cells contain glycine (Hendrickson *et al*, 1988), providing a further reason to implicate their role in RGC dendritic stratification. These data suggest that input from amacrine cells may play a role in shaping the dendritic stratification of RGCs. Further investigation could look at the presence of BDNF

and glycine receptors on ipRGCs and whether their blockade affects the stratification of ipRGCs.

IpRGC numbers

Another potential level of regulation by photoreceptors would be the control of ipRGC number. At birth there is an overproduction of RGCs in the retina (Young 1985) that is followed by a period of apoptotic cell death (Young 1984). This has also been observed specifically in ipRGCs (Sekaran *et al*, 2005). The number of ipRGCs present at P14 is the same as that seen in the adult, suggesting that the period of cell death responsible for this decrease is completed at this developmental age. This is the same time as when the cells of the outer retina have become fully photosensitive (Tian and Copenhagen 2003). Because the time courses of these events coincide, I hypothesized that maturation of the outer retina plays a role in determining ipRGC number. I found that both strains have the same number of ipRGCs early in development (P4). However, there are greater numbers of melanopsin-expressing RGCs in the CBA/J adults than in CBA/N mice. This suggests that the retinal degeneration seen in the CBA/J mouse affects the developmental cell death responsible for pruning during maturation.

Developmental cell death is regulated by tropic factors, intercellular interactions, synaptic contacts and intracellular signaling. It is unclear how these factors interact in the retina to control RGC number. However, the role of tropic factors has been demonstrated in chick retina where BDNF released from both the target and the retina controls RGC number (Herzog and von Bartheld 1998). In addition, in rats, NMDA can activate both pro- and anti-apoptotic intracellular

pathways in RGCs, suggesting that glutamate can both induce and prevent cell death (Manabe and Lipton 2003). Apoptosis is regulated by members of the *Bcl2* family, including *Bax*, which seems to play a role in the death of RGCs. In *Bax*-deficient mice there is a decrease in apoptosis of RGCs during development (J.Mosinger Ogilvie 1998). The greater number of ipRGCs in the CBA/J mice might make sense if loss of rods and cones altered the availability of factors necessary for developmental cell death. Whether glutamate or BDNF play a role in developing ipRGCs is unknown, and future studies could look at the effects of blocking their receptors during development on dendritic stratification. In addition, looking at a model in which apoptosis is blocked, causing greater numbers of ipRGCs, we could test the circadian behaviors to see if the changes are due to an increase in ipRGCs.

Physiology

The increase in ipRGC number in CBA/J mice might argue for enhanced behavioral responses to light. However, the CBA/J mice show attenuated phase-shifting responses to light and narrower limits of entrainment to T cycles. One explanation is that there are changes in the physiology of ipRGCs as a result of the outer retina degeneration. It has been shown that the light sensitivity of melanopsin-expressing RGCs changes throughout development. Multielectrode array analysis of postnatal retinas show changes in the time course of action potential firing and sensitivity to light with an increase in age (Tu *et al*, 2005). Developmental changes in melanopsin levels, membrane properties and synaptic input of ipRGCs might influence light sensitivity.

In rats, adult photoreceptor degeneration leads to a decrease in *melanopsin* mRNA and an elimination of its circadian rhythmicity (Sakamoto *et al*, 2004), suggesting that the photoreceptors regulate melanopsin levels. It is possible that the attenuation seen in the behaviors of CBA/J mice is due to a decrease in melanopsin levels in the retina. This seems unlikely because during development, peak sensitivity to light is not correlated with peak melanopsin expression, suggesting that other factors play a role in dictating the light response (Tu *et al*, 2005). However, further work could quantify the levels of melanopsin in CBA/J and CBA/N mice to better understand how rods and cones influence the development of ipRGCs.

To examine differences in the physiology of ipRGCs in CBA/J mice, one could conduct electrophysiological recordings from these cells and look for differences in the properties of the light response. The problem with this approach is determining which properties to examine because there is a great deal of variability in the intrinsic light responses of individual ipRGCs. For example, the whole cell recordings from melanopsin-expressing RGCs in rats show variability in the amplitude of the intrinsic light response, as the reported value for the average peak is -135.3 ± 105.3 pA (Warren *et al*, 2003). Additionally, using spontaneous synaptic activity as a measure for the degree of input these cells are receiving could be another way to look at their physiology. This is also difficult, as these cells have little ongoing synaptic activity, and again, it is quite variable (Perez-Leon *et al*, 2006).

As a result of the difficulty in studying the physiology of individual ipRGCs, I measured the PLR in CBA/J and CBA/N mice as a way to look at gross functional changes in the melanopsin pathway in the retina. Because the PLR is mediated by projections to the OPN, it allows one to examine the melanopsin pathway independently of the SCN. It has been previously reported that the rod and cone photoreceptors and the ipRGCs contribute to the PLR. The rods and cones are responsible for the reflex at low light intensities, while the ipRGCS control the PLR at high intensities (Lucas et al 03). I hypothesized that, if the melanopsin pathway was functioning properly at the level of the retina, at high light intensities the PLR would be the same in both strains. The CBA/J and CBA/N mice showed no differences in their reflexes at high light intensities, suggesting that the melanopsin pathway is intact at the level of the retina. The problem associated with using this technique is that it is meant to examine the function of the melanopsin pathway independently of the SCN. This is possible because the PLR is controlled by projections to the OPN. However, although the densest projections are in the SCN and OPN (Hattar *et al*, 2006), it is unclear how many melanopsin-containing RGCs project to each area and whether or not individual cells bifurcate. As a result, although the PLR can be used to test if the system is working, it may not be an optimal measure of the entire network of ipRGCs, and additional functional measures, such as multielectrode arrays, would have to be conducted to examine this more closely.

<u>Retinal Innervation and the SCN</u>

The first part of this thesis focused on examining changes at the level of the retina to explain the differences seen in the circadian behaviors of CBA/J mice. Although there are greater numbers of ipRGCs in these animals, based on PLR studies, this does not appear to translate into a functional change in the retina. the mice did show attenuated behavioral responses to light. The second part of this project looked at potential dysfunction in central processing, at the level of the SCN. I examined the degree of retinal innervation in CBA/J mice and the expression of markers for SCN function.

Retinal innervation is an important part of establishing proper pathways during development. Retinal input to the SCN is necessary for mediating light induced behaviors. In these studies I examined the anatomical innervation of VIP-positive cells in the CBA/J mice. Labeled cholera toxin was injected into the eye of each mouse. The tracer moves from the retina to the SCN and labels the ipRGC terminals there. Colocalization with VIP can be detected by using an antibody against VIP. We found that in CBA/J mice there are no significant differences in the number of VIP-positive cells receiving synaptic contacts from ipRGC terminals. This is consistent with tracer studies showing that there are no differences in the degree of anatomical innervation of the SCN in adult rd mice compared to wild type controls (Provencio *et al*, 1998a). This suggests photoreceptor loss during adulthood or development does not impact the anatomical innervation of the SCN by ipRGCs. However, our finding was surprising given that CBA/J mice have greater numbers of ipRGCs. This immunohistochemical approach used to look at innervation of a specific set of

cells may be problematic. The innervating fibers are very dense and overlap cell bodies. There is no ideal way to label the terminals, and defining areas of overlap can lead to inaccurate cell counts. As a result I decided to use an additional method to examine retinal innervation.

To further examine the degree of innervation of the SCN by ipRGCs, I looked at light-induced c-Fos expression. *C-fos* is an immediate early gene turned on in response to a stimulus, such as light. Its expression is detected in the SCN in response to a pulse of light given at specific times in an animal's circadian rhythms, the same times at which light exposure induces phase shifts (Kornhauser *et al*, 1990; Rusak *et al*, 1990; Rea 1992). As a result it is used as a marker for functional innervation of the SCN.

I examined light-induced *c-fos* expression in CBA/J and CBA/N mice and found that when given a pulse of light at CT 16, c-Fos protein is detected within the SCN of both CBA/J and CBA/N mice. Staining is low in animals not exposed to light, consistent with previous studies (Colwell and Foster 1992). Interestingly we found that the CBA/J mice have a greater increase in c-Fos expression compared to CBA/N mice. This suggests that there are greater numbers of light sensitive cells in the SCN, which is consistent with the findings that there are greater numbers of ipRGCs.

It is unclear whether *c-fos* is involved in the process underlying phase shifting responses. While injection of NMDA into the SCN induces *c-fos* expression, it does not induce phase shifting (Rea *et al*, 1993). Though this suggests that c-Fos is not sufficient to produce phase shifts, blocking *c-fos* expression by injecting

antisense oligonucleotides into the SCN prevents the ability of the rat to phase-shift in response to light (Wollnik *et al*, 1995). In addition, injection of excitatory amino acid antagonists into the hamster SCN at specific times blocks both light-induced *c-fos* expression and phase shifts, suggesting an association between the two processes (Rea *et al*, 1993). As a result a change in the number of cells expressing c-Fos in response to light would suggest differences in functional innervation of the SCN, which could lead to changes in the light responsiveness of the circadian system.

In one model of SCN function, core cells receive retinal input and transmit the information to the shell (Antle and Silver 2005). Shell neurons generate near 24-hour rhythms in gene expression, firing rate and peptide release through a transcription-translation negative-feedback loop. These "clock" cells are responsible for regulating circadian output, but in order to do so, their activity must be coordinated. Cells of the SCN core receive light input from the retina and transmit the information to the rhythmic output cells of the shell. The degree of retinal input the SCN receives is an important component in circadian entrainment because blocking optic nerve transmission with TTX eliminates phase shifting responses to photic input (Schwartz *et al*, 1987). In addition, glutamate released from the RHT onto SCN core neurons produces phase-shifts similar to those evoked by light (Ding *et al*, 1994). The mechanisms by which glutamate functions in entrainment and behavioral output are unclear. However, data suggests that glutamate released from RGC terminals increases transmitter release from the core cells because application of NMDA increases VIP release (Shibata *et al*,

1994). VIP is expressed by core cells and synchronizes the cells of the shell, leading to entrainment. As a result, changes in retinal innervation could alter SCN output and might explain the behavioral differences seen in CBA/J mice.

VIP-expressing cells

A subset of core neurons release VIP (see Introduction), which is important in synchronizing the clock cells of the shell. Injection of VIP into hamster SCN causes phase-shifts in locomotor activity that are similar to those produced by light, suggesting that VIP mediates light transmission in the SCN (Albers *et al*, 1991; Piggins *et al*, 1995). In addition, VIP can induce clock gene expression and can phase shift the activity of the VP-expressing cells of the shell (Nielsen *et al*, 2002; Reed *et al*, 2002). The VIP receptor, $VPAC_{2,}$ is necessary for the synchronization of circadian periods among SCN neurons because mice with a null mutation in this gene (*Vipr2-/-*) have disrupted circadian rhythms in wheel running (Harmar *et al*, 2002) and do not express rhythms in clock gene or VP expression (Harmar 2003). Together these data suggest an important role for VIP in light transmission and coordination in the SCN.

In these experiments, I sought to determine whether there are differences in the structural integrity of the SCN by using VIP as a marker for core cells. I found that there are greater numbers of VIP-positive cells in the SCN of the CBA/J mice. This is an interesting result because it has been shown that VIP deficient mice also have attenuated phase-shifting (Colwell *et al*, 2003). This would suggest that loss of VIP, seen in the VIP deficient mice, has a similar effect on behavior as does an increase in VIP-containing cells, seen in the CBA/J's. An

understanding of how additional molecules influence circadian behaviors might reconcile these findings.

In the SCN, VIP enhances inhibitory transmission by GABA. Electrophysiological recordings show that adding VIP to the SCN increases the frequency of IPSCs mediated by GABA (Itri and Colwell 2003). The increase in ipRGC and VIP-positive cell numbers in CBA/J mice could translate into decreased behavioral responses if greater numbers of ipRGCs innervate the core cells and more VIP is released. This would presumably lead to an increased modulation of GABAergic activity and might lead to attenuated behavioral responses. The exact mechanism by which GABAergic synaptic transmission translates into a behavior is unclear, however, GABA can synchronize and phase-shift clock cells (Liu and Reppert 2000). In addition, blockade of $GABA_A$ receptors interferes with rhythm coordination between the core and the shell (Aton and Herzog 2005), suggesting an important role for GABA in transmitting information and coordinating activity among SCN cells. It would be interesting to examine levels of GABA and GABAergic transmission in CBA/J mice.

While GABA and VIP appear to mediate communication among SCN neurons, additional molecules released from the core may also be important. For example, in hamster, it is suggested that TGFα is a potential candidate that mediates intracellular communication in the SCN. It is found in the core area containing CB-positive neurons, is expressed rhythmically and blocks running wheel activity in hamster when infused into the third ventricle (Kramer *et al*, 2001; Jobst *et al*, 2004). Although I have not examined TGFα in the mouse SCN, these data suggest

that additional molecules may play a role in the altered circadian behaviors of the CBA/J mice and may provide potential for future research.

VP-expressing cells

In addition to looking at core cells of the SCN, I also examined the shell neurons, as a marker for SCN output. VP is expressed by shell neurons whose activity can be phase-shifted by VIP (Nielsen *et al*, 2002). In addition, VP is released in a circadian manner, suggesting its role in circadian output. To examine potential changes in shell neurons and SCN output, I looked at the number of VP-positive cells and found that CBA/J mice have greater numbers of VP-expressing cells. The role of VP in circadian behaviors is unclear, however, there is some evidence to suggest its importance. For example, in VP deficient rats, there is no change in the circadian rhythms of sleep, however the amplitudes of the rhythms are decreased, suggesting the gain of the rhythms might be affected by loss of VP (Brown and Nunez 1989). However, VP-deficient rats show coherent circadian rhythms in wheel-running behaviors (Groblewski *et al*, 1981).

Whether VP is involved in locomotor behavior is unclear. In the SCN, VP is often colocalized with GABA (Moore and Speh 1993). It is possible that the increase in VP cells could play a role in the behaviors of CBA/J mice if they are producing more GABA and if GABA has an effect on locomotor rhythms. In hamster, injection of the GABA receptor agonist, baclofen, into the SCN decreases the animals' phase-shifting responses to light in wheel running paradigms (Gillespie *et al*, 1997). This suggests that activation of GABA receptors plays a role in attenuating behavioral responses to light. The sites of

action of GABA are unclear, and further work would need to be done to better understand how VIP, VP and GABA influence circadian function.

Role of additional molecules

In these studies, I examined two molecules, VIP and VP, to examine potential changes in the core and shell of the SCN of the CBA/J mice. However, the behavioral phenotype seen in the CBA/J mice could be due to alterations in additional cells. Among these are CB-containing neurons. These cells receive direct retinal innervation and express *c-fos* in response to light (Bryant *et al*, 2000). Like many SCN neurons, these cells also express GABA. As a result changes in CB-expressing cells could modulate inhibitory responses, and the effects may be seen at the level of behavior as discussed above.

In hamster, knocking down CB levels results in attenuated phase shifting. CBA/J mice display attenuated phase-shifting, and a possible hypothesis would be that the CBA/J mice have decreased levels of CB. There are a number of reasons for and against this. For one, CB does appear to play a role in the circadian behaviors of mice, because CB knockout mice have disrupted entrainment (Kriegsfeld *et al*, 2008). However, mice lack the CB positive cluster of cells seen in the core of the hamster SCN (Ikeda and Allen 2003), and when I stained the SCN of CBA/J and CBA/N mice with an anti-CB antibody, there was little to no expression of CB in either strain (data not shown). This is not due to a failure of the antibody to stain, as there were CB-positive cells in other areas of the brain. In addition, in hamster, SCN ablation of CB cells leads to arrhythmic locomotor activity (LeSauter and Silver 1999). If CBA/J mice have fewer CB-containing

cells, we might expect these animals to be arrhythmic. However, although the CBA/J mice have altered light induced phase-shifting and entrainment, they are not arrhythmic.

An explanation for the behaviors of CBA/J mice could also be differences in GRP-containing cells, which are found in the core of the SCN in mouse (LeSauter *et al*, 2002). GRP-receptor deficient mice have attenuated phase-shifting responses to light (Aida *et al,* 2002). As a result, it is possible that CBA/J mice have altered numbers of GRP-expressing cells. Changes in GRP or its receptor could provide an explanation for why CBA/J mice have attenuated responses to light. Future experiments could further address the role of CB- and GRP-containing cells in the circadian system. Additional methods for looking at changes in expression could be employed, including different antibodies or in situ hybridization.

Although different cells clearly play important roles in circadian function, it is important to understand how these cells communicate to produce a coordinated output. This is an area of current research, and some models have been proposed based on current knowledge. Figure 1 shows a model of intercellular communication within the SCN.

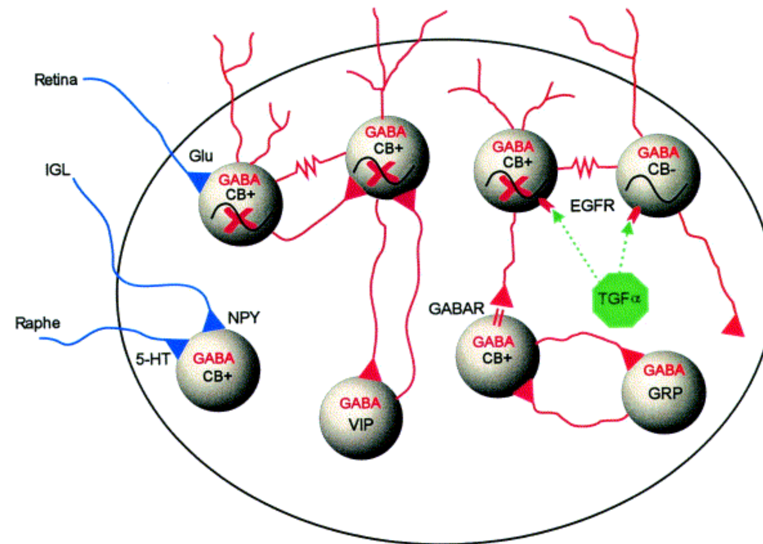

Figure 1. A model of proposed intercellular signaling pathways in the SCN. This model suggests that the non-rhythmic CB-positive cells (core) of the SCN receive glutamatergic (Glu) input from the retina. They communicate with each other and with rhythmic cells via gap junctions. Most cells express GABA, and synaptic transmission among cells, including VIP- and GRP- containing cells, plays an important role in transmitting information in the SCN. The receptor for TGFα, a potential signaling candidate mediating communication among cells, is present in some neurons. Communication with VP-expressing cells also occurs, though not shown. Additional inputs from the IGL and raphe nuclei are also shown (Jobst et al 04b).

The data presented here suggest a link between maturation of the visual system and development of the circadian system. More specifically, the work shows that animals with early outer retina degeneration during development have greater numbers of photosensitive cells in the retina (ipRGCs) and the SCN (VIP-expressing cells). In addition, these mice have greater numbers of circadian output cells (VP-containing cells). Though the link between this increase in cell number and the attenuated behaviors of CBA/J mice is unclear, we can begin to develop a

model for how communication between the retina and the SCN establishes the pathways responsible for generating circadian output.

If the hypothesis proposed here is true, that visual system maturation influences the function of the circadian system, then the retinal degeneration would result in circadian dysfunction. Our results support this theory if we conclude that during development, rod and cone photoreceptors control ipRGC death. Loss of rods and cones leads to an increase in ipRGC number. The link between proper targeting of axons and cell survival could explain why greater numbers of cells in the SCN survive. In the present study, these include VIP-containing cells, which receive retinal input and modulate GABAergic responses in the SCN. The VIP-expressing cells are known to communicate with the VP-containing output cells of the SCN, which also show an increase in number in CBA/J mice. Together the increase in GABAergic activity within the SCN could affect circadian function in a way that manifests as attenuated behaviors in response to light. However, as discussed earlier, additional work needs to address the roles of other molecules in the SCN. In addition, further studies would need to determine how changes at the molecular level influence behavior.

Implications for altered circadian behaviors

The work presented here discusses how the development of photoreceptive pathways in the retina and the brain influences circadian function. The behavioral experiments presented in this work suggest that the CBA/J mice have attenuated responses to light. Both the phase-shifting and T cycle data suggest that the

CBA/J mice are not able to reset their clocks as well as controls. Though research is still far from linking changes at the molecular level with behavior, understanding how the circadian system functions has significant implications.

Alterations in circadian rhythms may have profound effects on the health of an individual, as a number of disorders are associated with circadian dysfunction. In these experiments, CBA/J mice have attenuated phase-shifting responses. The significance of one's ability to phase shift is commonly seen in individuals experiencing jet lag. In this case, one's rhythms must undergo phase adjustment to account for the change in schedule. During this period of transition, the central and peripheral oscillators become desynchronized until they adapt to the new phase. As a result, an individual experiences fatigue, irritability and insomnia.

People who work night shifts also need to phase adjust, and the consequences of this appear to be more severe. Such individuals have schedules that are not in phase with the light/dark cycle of the common day and are therefore exposed to environmental light at the wrong time. When shift workers conform to the schedule of a typical day/night cycle on their days off, the rotation can lead to major disruptions of the circadian time organization. This commonly leads to sleep deprivation and, in more serious cases, cardiovascular dysfunction, altered metabolism, and mood disorders. Studies have shown that the incidence of on-the-job mistakes and industrial accidents is dramatically increased during night shifts, compared to the day shift (Coburn and Sirois 1999). Because of the growing need in our society for around-the-clock employment, understanding how to address the issues of circadian dysfunction is becoming increasingly

important. Light therapy has been used to treat circadian-based sleep disorders, seen in advanced and delayed sleep phase syndrome, jet lag, shift work and aging. Treatment protocols require careful attention to both the intensity of light and the circadian phase of light application. A greater understanding of how circadian rhythms are regulated is needed to develop effective treatments (Haus and Smolensky 2006).

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