Analysis of Behavioral, Cellular, and Anatomical Events Associated with Photic Entrainment of Circadian Rhythms in Rats

Christian Beaulé

A Thesis

In

The Department

Of

Psychology

Presented in Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy at
Concordia University
Montréal, Québec, Canada

February 2003

ABSTRACT

Analysis of Behavioral, Cellular, and Anatomical Events Associated with Photic Entrainment of Circadian Rhythms in Rats

Christian Beaulé, Ph.D.
Concordia University, 2003

Circadian rhythms in physiology and behavior are generated by a self sustaining endogenous pacemaker located within the cells of the hypothalamic suprachiasmatic nucleus (SCN). Photic entrainment is the process by which light resets the phase and the period of the oscillation of the SCN, thus generating circadian rhythms perfectly synchronized to the environmental light:dark cycle (24 hours). The SCN are divided into two compartments, a ventral retinorecipient core region surrounded by a dorsal shell region, each contributing to the entrainment process. The core integrates photic signals whereas the shell integrates non visual information. The shell is considered the major source of clock outputs to effector systems. Two general research questions were investigated in the present thesis. First, are there cellular changes in protein expression that are uniquely associated with photic entrainment? Second, which subset of retinal afferents is necessary for the transmission of photic information to the SCN? The experiments aimed at answering the first question revealed a unique pattern of Fos protein immunoreactivity in the SCN shell in response to entraining light. Specifically, light suppressed Fos protein expression in the shell region whilst inducing it in the core of rats receiving light at dawn or dusk. This complex pattern of Fos expression was also

present in animals treated neonatally with monosodium glutamate (MSG), an animal model of retinal degeneration that spares photic entrainment. Further, the expression of the protein product of the clock gene Per2 was found to be rhythmic within the SCN core and shell and unaffected by entraining light. Finally, behavioral arrhythmicity produced by constant bright light exposure was found to be associated with a complete disruption of the cellular rhythm of Per2 and Fos expression in the SCN. The experiments attempting to provide answers to the second question revealed that the retinal projection to the SCN is heterogeneous and that none of the retinal fiber phenotypes examined (fibers bearing the p75 neurotrophin receptor or melanopsin) is necessary to mediate photic entrainment. In conclusion, photic entrainment is a unique event characterized by cellular changes in protein expression that differ from those induced by discrete light-induced phase shifts. Further, retinal ganglion cells of all the different phenotypes appear to contribute to the transmission of the photic input to the circadian clock in a generally redundant manner.

ACKNOWLEDGMENTS

"How old are you? Wow, you are just a puppy". These are the words that Shimon Amir, the supervisor of my graduate studies used when I started my masters degree. I agree completely. Through his exceptional guidance, his undying curiosity, his trust and his confidence, the young and inexperienced puppy was able to grow and develop his skills and knowledge to the point where, well, he finally has to leave the nest and go roam the world as an adult scientist. And it is with confidence and eagerness that I start this new journey. I am extremely grateful that Shimmy was willing to give me my chance as a graduate student. I would have not wished that my graduate degree under his supervision have been any different. Thank you very much.

I wish to extend my gratitude to Jane Stewart and Barbara Woodside. Your genuine interest in the work I was doing has pushed me to come up with answers (and questions) that made me think in broader terms about my research. I am especially in debt to you for the last minute rush I imposed on you with the short deadline for the submission of the thesis. If, somehow, the quality of this thesis in not up to the level of what was expected of me, it is entirely of my doing.

Thanks to Barry Robinson for all his technical assistance and skills at making a lab run smoothly. The success of the experiments reported in this thesis is the reflection of his expertise.

To all the exceptional human beings who became my very close friends during my studies at Concordia: Lana, Elaine, Stephanie, Douglas, Larry, Alfonso. You were

responsible for making my stay at Concordia an exceptional one, you will be missed greatly.

I want to thank the staff of the CSBN for everything they did for me: Elizabeth, Phyllis, Aileen, Heshmat, Steve, Franc, Dave.

I want to acknowledge Dr. King-Wai Yau, (Johns Hopkins Univ Med Sch/HHMI) for the generous donation of the c-terminal melanopsin antibody.

Finally, I acknowledge the Canadian Institutes for Health Research and Concordia University for financial support during my studies.

TABLE OF CONTENTS

LIST OF FIGURES

Page

CONTRIBUTIONS OF AUTHORS

Dr. Shimon Amir acted as Ph.D. thesis supervisor and principal investigator and as such, appears as a co-author on every publication contained within this thesis.

Dr. Andreas Arvanitogiannis contributed to the experiments reported in Experiment 1. Specifically, he participated in the collection of the experimental data and to the editing of the scientific communication.

Ms. Jane Barry-Shaw was an undergraduate honours student and a summer research assistant. She participated in the collection of the experimental data of the experiment reported in Experiment 2.

Ms. Lisa Marie Houle was an undergraduate research assistant. She contributed to the collection of experimental data for the experiments reported in Experiment 3.

Mr. Barry Robinson is the laboratory technician in the lab of Dr. Amir. He contributed to the experiments reported in Experiment 6. Specifically, he determined the working dilutions of the melanopsin antibodies used, and participated in the collection of the experimental data.

Ms. Elaine Waddington Lamont is the other Ph.D. student in the lab of Dr. Amir. She contributed to the experiments reported in Experiment 6. Specifically, she was a great help in collecting the experimental data and in editing the manuscript.

NOTES ON NOMENCLATURE

Throughout this thesis, numerous references were made to genes, their transcripts and their protein products. To facilitate the distinction between the message (mRNA) and the signal (protein product), the following nomenclature was adopted as consistently as possible.

Genes, their mRNA and any other signal other than the final protein is in *Italics* with the first letter capitalized. For example: *c-Fos*, *Per1*, *Bmal1*.

Protein products of these genes and transcripts were written in CAPITAL letters. For example: FOS, PER1, BMAL1.

Note on clock genes:

Circadian clock genes have been cloned and described for a variety of species. Typically, the nomenclature suggests that the first letter of the species be used in order to differentiate between clock genes from different species. For example *Drosophila Period* gene would be *dPer*, rat *Period* would be *rPer*, and mouse *Period* would be *mPer*. The present work was performed exclusively in rats and as such, clock genes were presented without the species prefix. Whenever other's species clock genes are discussed, the type of species and the differences/similarities with rats will be made explicit.

LIST OF ABBREVIATIONS

AVP: Vasopressin pre-propressophysin

BDNF: Brain derived neurotrophic factor

CaBP: the calcium binding protein Calbindin-D28k

CKIε, CKIδ: Casein Kinase I (epsilon, delta)

CRY, *Cry*: Cryptochrome

CT: Circadian Time

CREB: Calcium/cAMP response element binding protein

DBP: Albumin D-element-binding protein

DD: Constant darkness.

GABA: γ-amino butyric acid

GHT: Geniculohypothalamic tract

IGL: Intergeniculate leaflet

LD: Light:Dark cycle

LL: Constant bright-ight

OPT: Olivary pretectal nucleus

PACAP: Pituitary adenylate cyclase activating peptide

P-CREB: Phosphorylated CREB

PER, *Per*: Period

PRC: Phase response curve

PVT: Paraventricular thalamic nucleus

RGC: Retinal ganglion cells

RHT: Retinohypothalamic tract

SCN: Suprachiasmatic nucleus

SPP: Skeleton photoperiod

ZT: Zeitgeber time

GENERAL INTRODUCTION

The revolution of the Earth in front of the sun creates days and nights and shapes the living conditions of all organisms. Numerous species ranging from mammals and birds to plants, insects and bacteria respond to this environmental fluctuation by adapting their biological rhythms to the day/night cycle. Rhythms that are in tune with the length of the solar day are called circadian rhythms (from latin *circa*: about, and *dies*: a day) and they entail both the harmonizing of internal biological rhythms with one another and with the external light/dark (LD) cycle. This process is performed by a circadian system that integrates photic information conveyed by the LD cycle and communicates it to the centers responsible for the generation of the specific components of biological rhythms.

In mammals, the circadian system that generates rhythms in physiology and behavior is made of a central clock that receives inputs from the retina (photic) and from the brain (non-photic) and that sends output timing information to the rest of the brain. Examples of such rhythms are core body temperature, blood pressure, hormonal secretion, sleep-wake cycle, and physical activity. In the absence of environmental input, the circadian clock generates "free-running" rhythms, with periods that approach but typically do not equal 24 hours. These endogenous rhythms are the result of interacting positive and negative molecular feedback loops involving a set of clock genes and their protein products. To exhibit rhythms with a 24 hour period, the circadian clock has to be synchronized (entrained) by cues from the external world. Although it has been shown that nonphotic cues such as feeding, temperature and social interactions can entrain

circadian rhythms (Takahashi & Zatz, 1982; Turek, 1985; Zucker, Rusak, & King, 1976), the most effective cue for entrainment is light (Rusak & Zucker, 1979).

Anatomy of the Circadian System

In mammals, the clock responsible for the generation and synchronization of circadian rhythms is located in the hypothalamic suprachiasmatic nucleus (SCN) (Moore, 1983; Rusak & Zucker, 1979). The SCN is a pair of nuclei located on either side of the third cerebral ventricle, just dorsal of the optic chiasm (hence *supra-chiasmatic*). In rats, each SCN contains about 10,000 tightly packed neurons (Güldner, 1983; Van den Pol, 1980) that are compartmentalized into a predominantly ventral core region and a more dorsal shell region (Moore, 1973; Moore & Lenn, 1972). The cells of the SCN core receive photic information and express vasoactive intestinal polypeptide, gastrin-releasing peptide, substance P, calbindin D28K (CaBP), and neurotensin II (Abrahamson & Moore, 2001; Moore, Speh, & Leak, 2002). The cells within the shell receive predominantly non-photic inputs from numerous thalamic and hypothalamic nuclei and express arginine vasopressin (AVP), calretinin, somatostatin, and enkephalin (Moore et al., 2002). Finally, a non-reciprocal core-to-shell projection connects the SCN's two compartments (Leak, Card, & Moore, 1999; Leak & Moore, 2001; Moore et al., 2002).

The circuitry responsible for the transmission of the photic input to the SCN originates in the retina. A small subset of retinal ganglion cells (RGC) form a dedicated monosynaptic retinal fiber pathway, the retinohypothalamic tract (RHT), that innervates the core of the SCN and that is both necessary and sufficient for the transmission of

photoperiodic information to the SCN (Johnson, Moore & Morin, 1988; Johnson, Morin & Moore, 1988; Moore, 1996; Moore & Lenn, 1972; Pickard, 1985). Recent advances in the study of circadian photoreception have shown that the RGC that form the RHT are heterogeneous with cells expressing neurotrophin receptors (the p75 low affinity neurotrophin receptor, p75NTR, and the high affinity tyrosine kinase-B receptor, Trk-B), and the cryptochromes and melanopsin photopigments (Bina, Rusak, & Semba, 1997; Hattar, Liao, Takao, Berson, & Yau, 2002; Liang, Sohrabji, Miranda, Earnest, & Earnest, 1998; Miyamoto & Sancar, 1998; Thresher et al., 1998). The role played by the neurotrophin receptor-bearing retinal afferents to the SCN is unclear. However, it has been shown that photic resetting of the rat circadian clock is modulated by brain-derived neurotrophic factor (BDNF) and occurs through its action on both p75NTR and Trk-B neurotrophin receptors (Liang, Allen, & Earnest, 2000; Liang, Sohrabji et al., 1998). Fibers of the RHT also convey photic information to the intergeniculate leaflet (IGL) and to the ventral lateral geniculate nucleus (vLGN) of the thalamus, two structures that are also part of the circadian system (Pickard, 1985). The primary neurotransmitter released in the SCN by the RHT following photic stimulation is glutamate (de Vries, Nunes Cardozo, van der Want, de Wolf, & Meijer, 1993; Ding et al., 1994; Ebling, 1996; Mikkelsen et al., 1995; Vindlacheruvu, Ebling, Maywood, & Hastings, 1992). In addition to glutamate, pituitary adenylate cyclase activating peptide (PACAP) is co-released from RHT terminals and modulates the effects of glutamate on retinorecipient SCN neurons (Akiyama, Minami, Nakajima, Moriya, & Shibata, 2001; Chen, Buchanan, Ding, Hannibal, & Gillette, 1999; Hannibal, Moller, Ottersen, & Fahrenkrug, 2000; Kopp, Meissl, Dehghani, & Korf, 2001).

A photic feedback fiber pathway, the geniculo-hypothalamic tract (GHT) projects from the IGL/vLGN back to the SCN (Mikkelsen, 1990; Mikkelsen & Moller, 1990; Moga & Moore, 1997). Both neuropeptide Y (NPY) and γ-amino-butyric-acid (GABA) are involved in neurotransmission in the GHT (Card & Moore, 1989; Harrington, Nance, & Rusak, 1987). The IGL is emerging as a modulator of photic signals that contribute to clock resetting but that is not required for the generation of circadian rhythms (Edelstein & Amir, 1996, 1999a, 1999b; Harrington & Rusak, 1989; Moore & Card, 1994; Pickard, Ralph, & Menaker, 1987). In addition, the SCN core receives a dense serotonergic (5-HT) input from the raphé nuclei (Amir et al., 1998; Meyer-Bernstein & Morin, 1996; Moore, Halaris, & Jones, 1978; Pickard & Rea, 1997). Finally, the entire SCN receives diffuse inputs from the paraventricular thalamic nucleus (PVT) (Ebling, Maywood, Humby, & Hastings, 1992; Moga, Weis, & Moore, 1995).

In contrast to the core, the SCN shell receives nonphotic inputs from the limbic system (infralimbic cortex, lateral septal nucleus, and ventral subiculum), the hypothalamus (median and medial preoptic nuclei; anteroventral periventricular, ventromedial, dorsomedial, tuberomammillary, and arcuate nuclei; subparaventricular zone; and the medial preoptic, lateral hypothalamic, and posterior hypothalamic areas), the basal forebrain, and the brainstem (Moga & Moore, 1997; Moore et al., 2002). The contribution of these nonphotic inputs to circadian clock function is poorly understood.

The SCN projects to a number of thalamic and hypothalamic nuclei including the subparaventricular zone, the paraventricular nucleus of the hypothalamus, the dorsomedial nucleus of hypothalamus, the medial preoptic nucleus; and the anterior PVT

(Leak & Moore, 2001; Thompson & Swanson, 1998; Watts & Swanson, 1987; Watts, Swanson, & Sanchez-Watts, 1987). Most of these nuclei are implicated in autonomic and visceral functions and are assumed to relay the circadian information generated by the SCN to effector centers such as the adrenal or pineal glands.

Molecular SCN Clockwork

The circadian oscillation originates within single cells of the SCN. It is believed that most, if not all SCN cells are clock cells that exhibit their own period length and sensitivity to resetting stimuli (Herzog, Takahashi, & Block, 1998; Liu & Reppert, 2000; Liu, Weaver, Strogatz, & Reppert, 1997; Welsh, Logothetis, Meister, & Reppert, 1995). The molecular mechanism responsible for the generation of the circadian oscillation within single SCN clock cells consists of interacting positive and negative transcriptional/translational-feedback loops involving the dynamic regulation of three *Period* genes (*Per1, Per2, Per3*), two *Cryptochrome* genes (*Cry1, Cry2*) and the orphan nuclear receptor *Rev-Erbα* (summarized in Figure 1). The rhythmic transcription of these genes is under the control of the transcription factors CLOCK and BMAL1 (Bunger et al., 2000; Gekakis et al., 1998; Hogenesch, Gu, Jain, & Bradfield, 1998; King et al., 1997). CLOCK and BMAL1 form heterodimers (CLOCK::BMAL1) that enhance transcription of other clock genes and of clock-controlled genes through binding to the E-

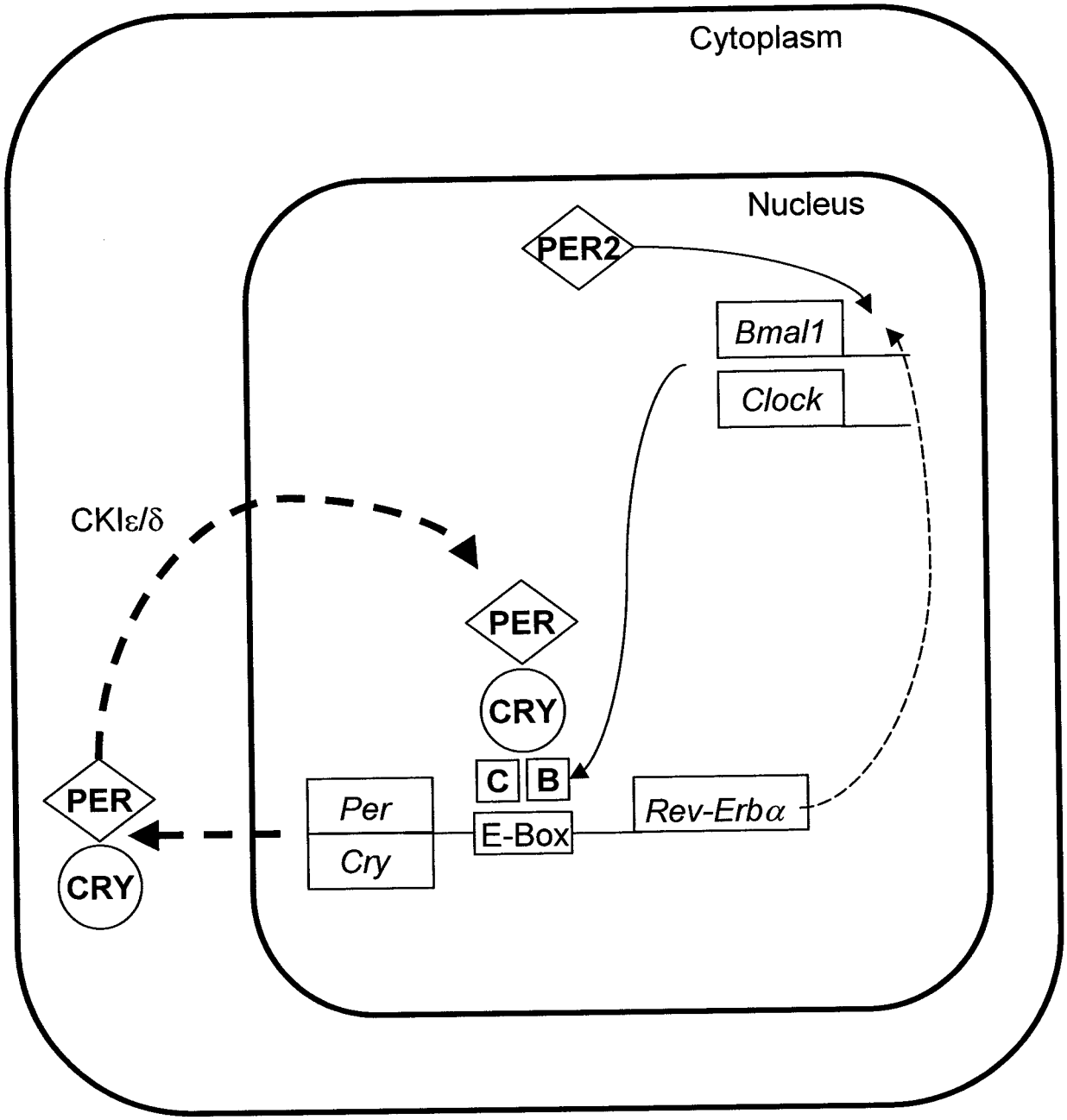


Figure 1. Simplified diagram illustrating the positive (full arrows) and negative (dashed arrows) transcriptional/translational feedback loops. mRNA for the clock genes is in *Italics* whereas proteins are in **Bold**. **C** = CLOCK protein. **B** = BMAL1 protein. See text for details. Diagram adapted from Reppert & Weaver, 2001.

Box elements on the gene promoter region (Gekakis et al., 1998; Hogenesch et al., 1998; Takahata et al., 1998).

Once activated, *Per* and *Cry* are translated into proteins which form multimeric complexes that are translocated back to the nucleus. In the nucleus, PER::CRY heterodimers act as negative regulators by preventing CLOCK::BMAL1 from activating *Per* and *Cry* transcription (Kume et al., 1999; Shearman et al., 2000; Okamura et al., 1999; Vitaterna et al., 1999). In this way, PER and CRY inhibit their own expression and close the negative limb of the feedback loop.

The positive feedback loop involves the periodic regulation of *Bmal1* transcription. *Bmal1* mRNA levels oscillate and peak 12 hours out of phase relative to the *Per* and *Cry* mRNAs (Oishi, Fukui, & Ishida, 2000; Shearman et al., 2000) in response to another source of negative feedback coming from REV-ERBα, which represses *Bmal1* transcription directly by acting through the Rev-Erb/ROR response elements on the *Bmal1* promoter (Preitner et al., 2002; Ueda et al., 2002). As a result of this suppression, *Bmal1* mRNA levels decrease whereas *Per* and *Cry* mRNA levels increase as a result of CLOCK::BMAL1 activation. Because PER::CRY proteins inhibit not only the transcription of *Per* and *Cry* but also *Rev-Erbα*, the resulting decrease in REV-ERBα levels disinhibits of *Bmal1* transcription (Preitner et al., 2002; Yu, Nomura, & Ikeda, 2002). The positive drive on *Bmal1* transcription is believed to come from the action of PER2 at the time when *Rev-Erbα* expression is inhibited (Shearman et al., 2000).

The results of the interaction between the positive and the negative feedback loops are rhythms in clock gene expression throughout the circadian cycle (Figure 2). At

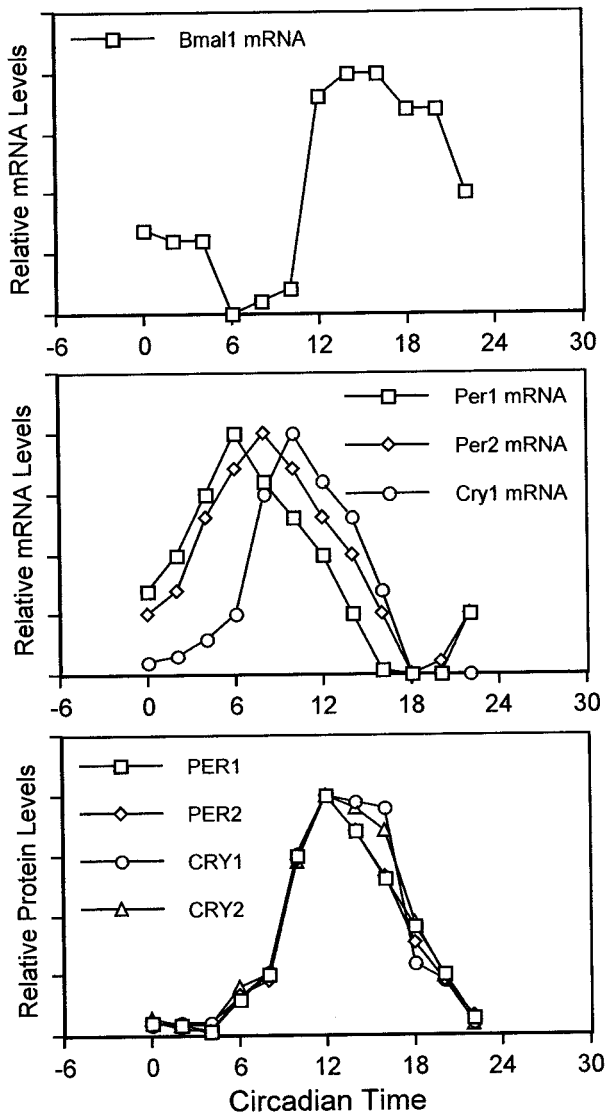


Figure 2. Graphs showing the relative mRNA and protein level throughout the length of the circadian cycle. See text for details. Graphs adapted from Reppert and Weaver, 2001.

the start of the circadian day (CT0, CT stands for circadian time with CT0 being the end of the active phase of a nocturnal rodent and CT12 the beginning of the active phase) *Per*, *Cry*, and *Rev-Erbα* transcriptions are activated by rising levels of CLOCK::BMAL1 protein heterodimers. *Per* and *Cry* mRNA oscillate in a circadian fashion with similar rhythms that are slightly out of phase with one another. *Per1* mRNA peaks from CT4 to CT6, *Per2* at CT8, *Per3* from CT4 to CT8, and *Cry1* peaks at CT10. By the middle of the circadian day (CT12), PER and CRY proteins are heavily expressed within the cell nucleus where PER::CRY proteins inhibit CLOCK::BMAL1 mediated transcription of *Per* and *Cry* mRNA. PER::CRY also suppresses REV-ERBα, allowing PER2 to activate *Bmal1* transcription, leading to peak *Bmal1* mRNA levels from CT15 through CT18. Following the rise in *Bmal1* mRNA, the levels of BMAL1 proteins rise, increasing CLOCK::BMAL1 heterodimer levels, restarting the circadian cycle. It is assumed that the availability of BMAL1 protein is the rate limiting factor in the formation of CLOCK::BMAL1 heterodimers, the critical step required to start the next loop at the beginning of the next circadian day (Reppert & Weaver, 2001).

The interaction between the positive and the negative feedback loops provide information about the phase of the circadian oscillation, not about its period. The nearly 24-hour time constant for the circadian oscillation is the result of phosphorylation and degradation of clock proteins. Although CRY proteins have been shown to be potent translocators of PER proteins, nuclear translocation can occur without the presence of CRY (Kume et al., 1999; Shearman et al., 2000; Yagita et al., 2000). The phosphorylation state of PER1 plays an important role in determining its nuclear entry.

The phosphorylating enzyme casein kinase I epsilon (CKIε) has been shown to facilitate PER1 translocation into the nucleus in vitro (Takano et al., 2000; Vielhaber, Eide, Rivers, Gao, & Virshup, 2000). Confirmation of the role played by phosphorylation of PER proteins in circadian physiology occurred following the cloning of the *tau* mutation in the Syrian hamster. Hamsters displaying this spontaneous, semidominant mutation exhibit shorter free-running periods when compared to wild type hamsters (Ralph & Menaker, 1988; Reppert & Weaver, 1997; Young, 2000). It was shown that the *tau* mutation codes for CKIε and that it reduced the enzyme capacity for autophosphorylation as well as its maximal phosphorylation velocity (Lowrey et al., 2000). In addition, it was recently shown that CKIε was capable of phosphorylating PER2, CRY1, CRY2, and BMAL1 in vitro, suggesting that any modifications in the phosphorylation states of these proteins might be responsible for the short period of the *tau* mutant hamsters. There is another member of the casein kinase family (casein kinase I delta, CKIδ) that is important for circadian clockwork. CKIδ is highly homologous to CKIε and it can phosphorylate PER1, PER2, CRY1, CRY2, and BMAL1 in vitro (Akashi, Tsuchiya, Yoshino, & Nishida, 2002; Eide, Vielhaber, Hinz, & Virshup, 2002; Lee, Etchegaray, Cagampang, Loudon, & Reppert, 2001). Other phosphorylating enzymes such as mitogen-activated protein kinase (MAP kinase) and glycogen synthase kinase-3 (in *Drosophila*) can directly change the phosphorylation state of clock protein. Complex and coordinated interaction between clock genes and phosphorylating enzymes results in the near 24-hour time constant for the cellular oscillations responsible for the generation of circadian rhythms.

The Circadian Response to Light

Because the RHT is anatomically and functionally distinct from the visual pathways involved in normal vision, it is described as part of the non image-forming visual pathway. The circadian system displays a sensitivity to light that is different from that of the higher visual centers. Cells of this system have a higher threshold for response to light and are thought to integrate photic stimuli over long time intervals (Nelson & Takahashi, 1991; Takahashi, DeCoursey, Bauman, & Menaker, 1984), however see (Arvanitogiannis & Amir, 1999). Furthermore, from the study of rodless-coneless mice (*rd/rd/cl*), it was made clear that circadian photoreception was distinct from classical visual photoreception. These mice show severe retinal degeneration due to the total loss of rods and cones (Freedman et al., 1999). Despite the retinal damage, these visually blind mice still entrain to light, show suppression of pineal melatonin secretion following light presentation at night and show an intact pupillary light reflex (Freedman et al., 1999; Lucas, Freedman et al., 2001), all responses believed to involve the non image-forming visual pathway. Additional evidence challenging the role played by classical photoreceptors in circadian photoreception comes from the human literature showing that some blind individuals who have eyes but no visual perception due to a retinal defect retain the circadian part of the photic response: light-induced melatonin suppression and entrainment of circadian rhythms (Czeisler et al., 1995). These results suggest that circadian photoreception occurs through novel, non-classical photoreceptors that are likely located within the inner retinal layers.

Recently, the opsin-based photopigment melanopsin was identified in both primates and rodents (Provencio et al., 2000). Melanopsin is a known photopigment in

lower vertebrates that is expressed in dermal melanophores, deep brain nuclei, iris, and in the nonphotoreceptor cells of the inner retina (Provencio, Jiang, De Grip, Hayes, & Rollag, 1998; Soni, Philp, Foster, & Knox, 1998). In mammals, melanopsin was shown to be localized in the inner layer of the retina, within ganglion and amacrine cells (Hannibal, Hindersson, Knudsen, Georg, & Fahrenkrug, 2002; Hattar et al., 2002; Provencio et al., 2000; Provencio, Rollag, & Castrucci, 2002). Unlike melanopsin expression in nonmammalian species, there are no extraretinal sources of melanopsin expression in mammals, suggesting that it functions as a novel non-rod non-cone based photopigment. Melanopsin has been found to be present in only 2.5% of RGC of rats and in 1% of RGC of mice (Hattar et al., 2002). Interestingly, those RGC that do express melanopsin form part of the RHT that projects to the SCN and IGL. In addition melanopsin is present in the retinal afferents to another structure that is part of the non image-forming visual pathway, the olivary pretectal nucleus (OPN) (Hattar et al., 2002). Furthermore, melanopsin was shown to colocalize with PACAP within RGC comprising the RHT (Hannibal et al., 2002), suggesting that these cells are responsible for the transmission of photic information to the SCN.

The consequences of photic entrainment of circadian rhythms are readily observed as small daily shifts in the phase of biological rhythms such as those of activity and temperature. It has been demonstrated that light produces behavioral phase shifts only when presented during the subjective night (the active phase of the cycle of a nocturnal animal housed in complete darkness, DD). Light presented during the subjective day (the inactive phase of the cycle of a nocturnal animal house in DD) does

not produce any effect on circadian rhythms (Rusak & Zucker, 1979). The magnitude and direction of light-induced phase shifts is not constant throughout the subjective night. In rodents, light can induce phase shifts up to approximately 120 minutes depending both on its intensity and when in the cycle it is presented. Maximal phase shifts are achieved during the subjective night with bright light around circadian times (CT) 14-16 and 20-22; smaller magnitude phase shifts are obtained close to the transition times corresponding to dawn (CT 0) and dusk (CT 12) (Takahashi & Zatz, 1982). Furthermore, light pulses of low intensity produce smaller phase shifts compared to brighter light presented at the same circadian times (Kornhauser, Nelson, Mayo, & Takahashi, 1990).

The direction of the phase shift is also a function of the CT at which light is presented during subjective night. Light falling early during the subjective night causes phase delays whereas light falling towards the end of subjective night causes phase advances (Pittendrigh & Daan, 1976; Rusak & Zucker, 1979). The plot of the circadian time versus the magnitude and direction of phase shifts yields the phase response curve (PRC). A given PRC will describe the relationship between light and phase shifts for a single individual. Once a PRC is determined for an animal, one can predict the direction and magnitude of a phase shift simply by knowing the CT at which the light pulse was administered.

The characteristics of the PRC obviously have implications for entrainment. The limits of entrainment depend on the period of the clock and on the maximal phase shift that can be obtained for an individual organism. For example, if the maximal phase shift (either advance or delay) that can be obtained for a given organism is 2 hours, and the clock's period is 24 hours, then the limits of entrainment will be between 22 and 26

hours. Failure to entrain to a stimulus outside the limits of entrainment will lead to free-running or disruption of rhythmicity (Pittendrigh & Daan, 1976).

Rodents entrain to a variety of lighting schedules and, experimentally, the most frequently used schedule is the 12h:12h LD cycle. However, this lighting schedule is artificial for nocturnal rodents (e.g., rats, hamsters and mice) since it is very unlikely that they will be subjected to such long periods of high illumination in their natural environment. These species can also entrain to lighting schedules that are more similar to those seen outside the laboratory, such as a skeleton photoperiod (SPP) and a T-Cycle. In a SPP, two short light pulses are given, separated by long periods of darkness usually ranging from 8-14 hours. These two light pulses represent the transition times corresponding to dawn and dusk. In a T-Cycle, a single light pulse is presented once a day (Pittendrigh & Daan, 1976; Schwartz & Zimmerman, 1990; Stephan, 1983).

The ability of a single light pulse presented at the same time each day (in the form of a T-Cycle) to produce stable entrainment of circadian rhythms is explained by the PRC. Entrainment to a 24 hour cycle in animals such as rats with long free-running periods (>24 h) is accomplished by a daily phase advance which would occur when light is presented at dawn (Stephan, 1983); whereas in animals such as mice with short periods (<24 h) entrainment is accomplished by a daily phase delay when light is presented at dusk (Schwartz & Zimmerman, 1990). For these different species, there is one point of the PRC, either around dawn or around dusk, which will produce the appropriate phase shift required for entrainment. In a SPP the second light pulse appears simply to stabilize

the behavioral rhythm by locking it between two light pulses and does not affect entrainment itself (Schwartz, Peters, Aronin, & Bennett, 1996).

One artificial manipulation of the LD cycle has dramatic impact on behavioral rhythms: the prolonged exposure to constant bright light (LL). Long periods of LL exposure will eventually disrupt circadian rhythms. This disruption takes the form of "split" activity rhythms in hamsters where usually two distinct activity components are visible (Abe et al., 2001; de la Iglesia, Meyer, Carpino, & Schwartz, 2000; Mason, 1991; Pickard, Kahn, & Silver, 1984; Shibuya, Melnyk, & Mrosovsky, 1980), and complete behavioral arrhythmicity in rats (Arvanitogiannis, Robinson, Beaulé, & Amir, 2000; Eastman & Rechtschaffen, 1983; Edelstein & Amir, 1999a; Edelstein, Pfaus, Rusak, & Amir, 1995). The mechanism mediating behavioral arrhythmicity in rats is unknown. Three working hypotheses can be formulated in order to explain this phenomenon. First, LL completely disrupts the cellular clock mechanism present within each SCN cell, essentially "stopping" the clock and preventing it from sending meaningful timing signals to effector systems. Second, the circadian clock is still functional after prolonged LL exposure but the coupling between the SCN and the effector systems is lost, resulting in a loss of rhythmicity in overt behavioral and physiological variables. Finally, coupling of the electrical activity among individual SCN cells is disrupted by LL, resulting in desynchronized cellular activity within the pacemaker.

Significant progress has been made toward the identification of the molecular and cellular mechanisms by which light resets the circadian clock. Light that is capable of resetting overt rhythms in physiology and behavior induces the expression of clock genes

and immediate-early genes (IEG). The photic activation of clock genes interacts with the molecular dynamics of the circadian oscillation, leading to photic phase shifts and entrainment of circadian rhythms. The activation of IEG expression results in late gene transcription that is ultimately responsible for behavioral and physiological entrainment (Earnest, Iadarola, Yeh, & Olschowka, 1990; Kornhauser, Mayo, & Takahashi, 1996; Meijer & Rietveld, 1989; Rusak, Robertson, Wisden, & Hunt, 1990).

Photic clock resetting implies that light can directly affect the genetic oscillatory feedback loops in order to induce a delay or an advance in the normal cycling of clock genes. Because the circadian system is only sensitive to light presented at night, there must be some aspects of the transcriptional/translational feedback loops that are acutely sensitive to light exposure at night. In fact, the transcription of *Per1* is rapidly induced by light presented either at the beginning or at the end of the night (Shigeyoshi et al., 1997). Further, *Per2* induction is robust following light exposure only during the beginning of the night (Zylka, Shearman, Weaver, & Reppert, 1998). The intracellular signaling cascade mediating light-induced *Per* transcription involve direct activation of the *Per* genes via the binding of phosphorylated cyclic AMP response element-binding protein (P-CREB) to the cAMP response elements (CRE) in the 5' flanking regions of the *Per1* and *Per2* genes (Hsu et al., 1996; Kuhlman, Quintero, & McMahon, 2000). It is hypothesized that *Per1* might be a mediator of light-induced phases advances whereas *Per2* would be a mediator of light-induced phase delays. However, thus far one report testing this hypothesis using mice with targeted disruptions of either *Per1* or *Per2* obtained results consistent with the hypothesis (Albrecht, Zheng, Larkin, Sun, & Lee,

2001) whereas another one showed that *Per1* is not necessary for light-induced phase shifts (Cermakian, Monaco, Pando, Dierich, & Sassone-Corsi, 2001).

In addition to the effects of light on clock genes, photic resetting of circadian rhythms induces the expression of IEG in the SCN. Several IEG, such as *c-Fos, Fos-B, Jun-B, Zif268, Nur77,* and *Egr3* were shown to be specifically induced within the rodent SCN by light given during subjective night (Kornhauser et al., 1996; Kornhauser et al., 1990; Kornhauser, Nelson, Mayo, & Takahashi, 1992; Morris, Viswanathan, Kuhlman, Davis, & Weitz, 1998; Rea, 1989; Rusak et al., 1990). Other immediate-early genes such as *c-Jun* and *Jun-D* are also present within the rodent SCN but their induction is relatively insensitive to photic input (Kornhauser et al., 1996; Kornhauser et al., 1992; Rusak, McNaughton, Robertson, & Hunt, 1992; Takeuchi, Shannon, Aronin, & Schwartz, 1993).

Among the IEG present within the SCN, FOS was the first IEG known to be inducible by light in the SCN (Rea, 1989, 1998; Rusak et al., 1990) and considerable efforts were made to characterize the circadian and light-induced expression patterns of *c-Fos* and its protein product FOS. Different patterns of FOS expression have been observed in the SCN core and shell. In the SCN core, basal FOS expression for animals housed in DD is low and generally non rhythmic (Edelstein, Beaulé, D'Abramo, & Amir, 2000; Guido, de Guido, Goguen, Robertson, & Rusak, 1999; Kornhauser et al., 1996). Furthermore, FOS expression is rapidly induced in the core following presentation of light that produces behavioral phase shifts where it has been closely linked, both temporally and functionally, to clock resetting and entrainment of circadian rhythms

(Aronin, Sagar, Sharp, & Schwartz, 1990; Beaulé & Amir, 1999; Edelstein et al., 2000; Hastings et al., 1995; Kornhauser et al., 1996; Kornhauser et al., 1990; Rea, 1989, 1998; Rusak et al., 1990; Wollnik et al., 1995). In the SCN shell region, basal FOS expression is rhythmic (Guido, de Guido et al., 1999; Guido, Goguen, De Guido, Robertson, & Rusak, 1999; Rusak et al., 1992; Sumova & Illnerova, 1998; Sumova, Travnickova, & Illnerova, 2000; Sumova, Travnickova, Mikkelsen, & Illnerova, 1998). Expression is higher during the subjective day when photic sensitivity of the core is minimal, and lower in the subjective night, when photic sensitivity of the core is maximal. Although it has been shown that the pattern of FOS expression in the shell tracks the photoperiod (Sumova et al., 2000), nothing is known about whether light influences the expression of FOS in the shell region or about the role of FOS expression in the shell in clock resetting and entrainment.

Although there is ample evidence to suggest that FOS induction is a correlate of photic resetting, the question of whether FOS in the SCN is necessary and/or sufficient to produce behavioral phase shifts remains. Advances in genetic manipulations have yielded a line of gene-targeted mice carrying a null mutation for the *c-Fos* gene (Johnson, Spiegelman, and Papaioannou, 1992). The investigation of the circadian system of this mutant mouse revealed that the period of locomotor rhythms was similar to that of wild-type mice, suggesting that FOS is not required for normal circadian rhythmicity. Mutant mice, however, took significantly longer to entrain to a 12:12 LD cycle, suggesting that photic entrainment was not as efficient in the absence of FOS. In addition, the magnitude of the light-induced phase shifts were attenuated in the mutant mice, even though the

timing of the PRC for phase advances and phase delays was not affected (Honrado et al., 1996). This suggests that a homozygous null mutation of the *c-Fos* gene is not sufficient to completely prevent photic entrainment and phase shifts.

The most convincing evidence for a causal role for FOS in phase shifts comes from a study in which microinjections of antisense oligonucleotides were made into the SCN to block the expression of both *c-Fos* and *Jun-B*. Application of the antisense was found to block light-induction of *Fos* and *Jun-B* mRNA specifically and to completely prevent phase shifts when light was presented at CT 15. Control animals not treated with antisense showed normal photic responses to the light pulse (Wollnik et al., 1995). These results suggest that functional FOS protein is required for photic clock resetting and that a compensatory mechanism might explain the results found for the *c-Fos* deficient mice.

The Present Thesis

Photic entrainment is a process by which light adjusts, on a daily basis, both the phase and the period of the circadian pacemaker. It is qualitatively different from the presentation of a single light pulse during the subjective night that produces only phase resetting without changes in the period. In addition, the two stimuli differ in their ecological validity. A bright light pulse given during the subjective night is a highly artificial situation that bears very little ecological significance to a nocturnal (or a diurnal) animal. In contrast, entraining light, especially when presented in the form of a SPP or a T-Cycle is more natural because it corresponds to the daily transitions associated with dawn and dusk. It is of interest, therefore, to know whether the changes in

clock gene and IEG expression seen following light stimulation in the phase shifting procedure will also occur following the presentation of an entraining light stimulus.

The experiments contained within the present thesis can be divided in two sections, each containing three sets of experiments. In the first section, the experiments were designed to provide answers to questions regarding the effects of presentation of entraining light on the expression of the IEG FOS and on the clock gene PER2. In the second section, the experiments were designed to provide answers to questions regarding the role played by different types of RGC during photic entrainment.

First, because photic entrainment is qualitatively different from nocturnal phase shifts, is there a pattern of FOS expression that could be associated only with the presentation of entraining light? In the first set of experiments, FOS expression was examined in the SCN core and shell of animals that were free-running and that were presented with light at four specific time points: dawn, dusk, middle of the day and middle of the night. Further, the time course of the FOS response was examined in another group of animals following the presentation of an entraining light pulse at dawn.

The second question had to do with whether the changes in FOS expression seen in the SCN shell and core were dependent on the integrity of the RHT. Neonatal treatment with the retinal toxin monosodium glutamate (MSG) severely compromises the integrity of the RHT by selectively destroying the RGC layer (Chambille, 1998a; Chambille & Serviere, 1993; Edelstein et al., 1995; Olney, 1969; Pickard, Turek, Lamperti, & Silverman, 1982). Despite retinal impairments, these animals entrain

normally to light (Arvanitogiannis et al., 2000; Edelstein et al., 1995, Pickard et al., 1982). In the second experiment, FOS expression patterns in response to entraining light were examined in animals treated with MSG and in control animals.

Both FOS and PER2 protein expression are expressed rhythmically within the SCN shell and induced by light in the SCN core, suggesting a relationship between the expression of these two proteins that could be revealed by studying them during photic entrainment. In the third set of experiments, therefore, the rhythmic and light-induced patterns of FOS and PER2 protein expression were examined in animals entrained to a 24-hour T-Cycle. Protein expression was assessed throughout the whole circadian cycle. Furthermore, the effects of constant bright-light induced behavioral arrhythmicity on FOS and PER2 protein expression in the SCN core and shell were also investigated.

A well-known effect of neonatal MSG treatment on the circadian system is a resistance to the disruptive effects of LL exposure (Arvanitogiannis et al., 2000; Edelstein et al., 1995). Because neonatal MSG treatment severely compromises the integrity of the RHT, is there a change in some RHT marker that could explain these effects? In the fourth experiment, the effects of neonatal MSG treatment on the expression of p75NTR within the SCN were investigated.

Next, because neonatal MSG treatment causes widespread damage to the retina, would there be a way of selectively destroying one type of RHT projection to the SCN and identify a role for that projection in circadian physiology? The fifth set of experiments examined in further detail the role played by p75NTR bearing RGC by selectively destroying the p75NTR immunoreactive plexus within the SCN core by using

the p75NTR-specific immunotoxin 192-IgG Saporin. The response to photic entrainment and the presence of FOS, p75NTR, and CaBP in the SCN were investigated.

Finally, melanopsin is emerging as a circadian photopigment that could mediate photic entrainment of circadian rhythms. Because photic entrainment was spared following MSG or 192-IgG Saporin treatments, could melanopsin containing RGC be spared following such treatments? The sixth and final set of experiments is preceded by an up to date review of the available information concerning the role played by melanopsin containing RGC in the photic response of the circadian system. In addition, melanopsin immunoreactivity both in the retina and in the SCN following either MSG or 192-IgG Saporin treatment was examined in order it clarify the role played by this novel photopigment.

SECTION 1: PROTEIN EXPRESSION AND THE ENTRAINMENT OF CIRCADIAN RHYTHMS IN THE RAT

EXPERIMENT 1

Light Suppresses FOS Expression in the Shell Region of the Suprachiasmatic Nucleus at Dusk and Dawn: Implications for Photic Entrainment of Circadian Rhythms

Christian Beaulé, Andreas Arvanitogiannis, and Shimon Amir

Neuroscience, 106(2), 249-254 (2001),

The transcription factor FOS is implicated in neuronal signaling in the suprachiasmatic nucleus, the mammalian circadian clock (Ikonomov & Stoynev, 1994; Kornhauser et al., 1996). FOS is expressed in two different regions within the suprachiasmatic nucleus. In the retinorecipient core region (Leak et al., 1999; Moga & Moore, 1997), FOS is induced by light and expression is closely linked, both temporally and functionally, to clock resetting and entrainment of circadian rhythms (Aronin et al., 1990; Beaulé & Amir, 1999; Hastings et al., 1995; Kornhauser et al., 1996; Kornhauser et al., 1990; Rea, 1989, 1998; Rusak et al., 1990; Wollnik et al., 1995). In the shell region (Leak et al., 1999), FOS expression is rhythmic (Guido, de Guido et al., 1999; Guido, Goguen et al., 1999; Rusak et al., 1992; Sumova & Illnerova, 1998; Sumova et al., 1998). Expression is high during the subjective day when photic sensitivity of the core is minimal, and low in the subjective night, when photic sensitivity of the core is maximal. Although it has been shown that the pattern of FOS expression in the shell tracks the photoperiod (Sumova et al., 2000), nothing is known about whether light influences the expression of FOS in the shell region or about the role of FOS expression in the shell in clock resetting and entrainment. In the present study we found that, in rats maintained in constant darkness, brief exposure to light in the early subjective day or night induced FOS in the core, as expected, and acutely suppressed the levels of FOS immunoreactivity in the shell region. Similar changes in FOS expression in the core and shell regions were seen after exposure to a brief entraining light. Light exposure in the mid-subjective day or night differentially affected FOS expression in the core, as previously shown, but had no effect on FOS expression in the shell region. The finding that FOS expression in the shell

region of the suprachiasmatic nucleus is suppressed by light at dawn and dusk suggests a critical role for the shell in photic entrainment of circadian rhythms.

The effect of light on the expression of FOS immunoreactivity in the shell and core regions of the suprachiasmatic nucleus (SCN) was assessed at four distinct phases of the cycle: dawn, dusk, which are the only phases implicated in photic entrainment, and the middle of the day or night. Groups of rats were housed under a 12h:12h light:dark (LD) cycle for 15 days and were then placed in complete darkness for 36-48 hours. FOS expression was assessed in 8 groups of rats (n=6/group) killed at projected circadian times (CT) corresponding to dawn (CT2; CT0 denotes to the time of onset of the light phase of the preceding LD cycle), day (CT8), dusk (CT14) and night (CT20) with or without exposure to 30-min of light (250 lux at cage level) at CT1, 7, 13 and 19.

Representative images of FOS expression in the SCN as a function of CT in rats killed either in complete darkness or after exposure to a 30-min light pulse are shown in Fig. 3. In rats killed in complete darkness, FOS expression in the shell region varied as a function of time with significantly more labeled nuclei seen at CT2, 8 and 14 than at CT20 (Fig. 4a). Exposure to 30 min of light suppressed FOS expression in the shell at CT2 and CT14, but light had no effect on FOS expression in the shell at CT 8 and 20. ANOVA revealed a significant main effect of circadian time ($F_{[3,40]}$=1.1, $p<0.0001$), a significant main effect of light ($F_{[1,40]}$=34.8, $p<0.0001$), and a significant time X light interaction ($F_{[3,40]}$=22.8, $p<0.0001$). In the core, Fos expression was consistently low in rats killed in complete darkness, with less than 25 FOS immunoreactive cells seen at any

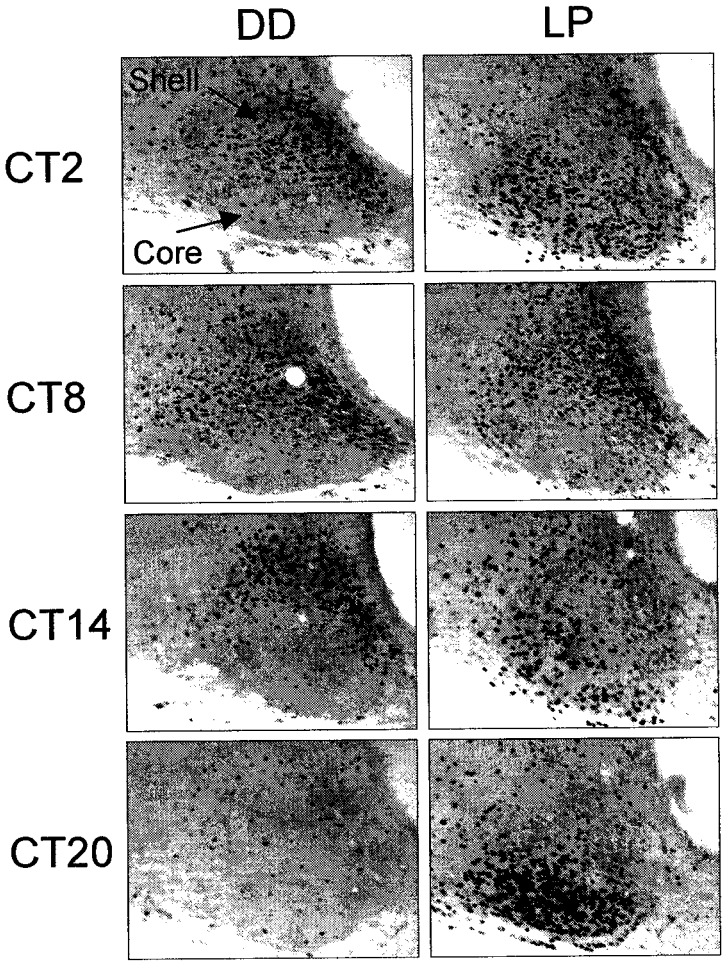


Figure 3. Photomicrographs showing examples of FOS immunoreactivity in the shell and core regions of the SCN as a function of circadian time (CT) in free running rats perfused in darkness (DD) or after exposure to a 30-min light pulse (LP).

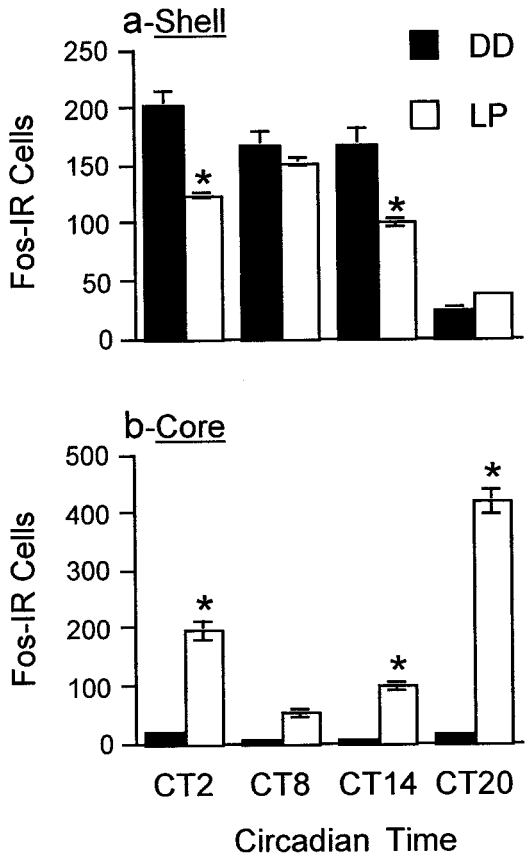


Figure 4. Mean number of FOS-immunoreactive cells (± s.e.m.) in the shell (a) and core (b) regions of the SCN as a function of circadian time in free running rats perfused in darkness (DD) or after exposure to a 30-min light pulse (LP) (n=6/group). Asterisks indicate significant difference from the corresponding group (Tukey test, $p<0.05$).

CT examined (Fig. 4b). As expected, exposure to light for 30 min significantly increased the number of FOS immunoreactive cells in the core at CT2, 14, and 20 but not at CT8 (Fig. 4b). Overall, photic induction of FOS in the core was maximal at CT20, minimal at CT8, and significantly greater at CT2 than at CT14. ANOVA revealed a significant main effect of time ($F_{[3,40]}$=1.3, $p<0.0001$), a significant main effect of light ($F_{[1,40]}$=5.8, $p<0.00010$, and a significant time x light interaction ($F_{[3,40]}$=1.2, $p<0.0001$).

These results show that FOS expression in the shell region of the SCN is suppressed by light in a phase-specific manner. Light suppressed FOS expression in the shell only in the early subjective day (CT2) and early subjective night (CT14). In contrast, as expected, light induced FOS expression in the core region at CT2 and CT14. Light had no effect on FOS in the shell in the middle of the subjective day (CT8), a period when basal FOS expression in the shell was maximal but light did not induce FOS in the core. Similarly, light had no effect in the shell at night (CT20) when basal levels were at a minimum, but when, in the core, light induced strong expression of FOS. Taken together, these results show that light exerts diametrically opposite effects on FOS expression within the core and shell regions of the SCN that are restricted temporally to times corresponding to dawn and dusk. This finding raises the interesting possibility that such reciprocal changes represent a unique correlate of the process underlying photic entrainment.

To directly study the effect of photic entraining stimulus on FOS expression in the core and shell, we assessed Fos in the SCN of rats entrained to a single daily presentation

of a 30-min light pulse (T-cycle). Stable entrainment to such a T-cycle involves daily clock resetting at the early subjective day (dawn) and is associated with an induction of FOS in the core (Beaulé & Amir, 1999). Rats were first entrained to a normal 12h:12h LD cycle (lights on from 8 am – 8 pm) for 10 days and then placed under a 0.5h:23.5h LD cycle (lights on from 8 am – 8:30 am) for 15 days. On day 15, groups of rats (n=4/group) were killed 1, 2, and 4 hours after the onset of the 30-min entraining light (zeitgeber times (ZT) 1, 2, and 4; ZT0 denotes light onset). Additional groups of rats (n=4/group) were killed at ZT0, and at each of the time points as above (ZT1, 2, 4), but were not exposed to the entraining light.

Representative images of FOS expression in the SCN as a function of ZT in entrained rats killed either in complete darkness or after exposure to the 30-min entraining light pulse are shown in Fig. 5. In the absence of light, FOS expression in the shell was high with significantly more labeled cells at projected ZT2 compared to ZT1 (Fig 6a). Presentation of the entraining light pulse significantly reduced the number of FOS immunoreactive cells within the shell. The light-induced suppression was transient, significant at ZT1 and ZT2, but not at ZT4. At ZT4 FOS returned to the high basal levels equal to those found in rats killed at the same time without exposure to the light pulse (Fig. 6a). ANOVA revealed a significant main effect of time ($F_{[3,21]}=15.8$, $p<0.0001$), a significant main effect of light ($F_{[1,21]}=41.3$, $p<0.0001$), and a significant time x light interaction ($F_{[2,21]}=12.3$, $p<0.0003$). FOS expression in the core was minimal in rats

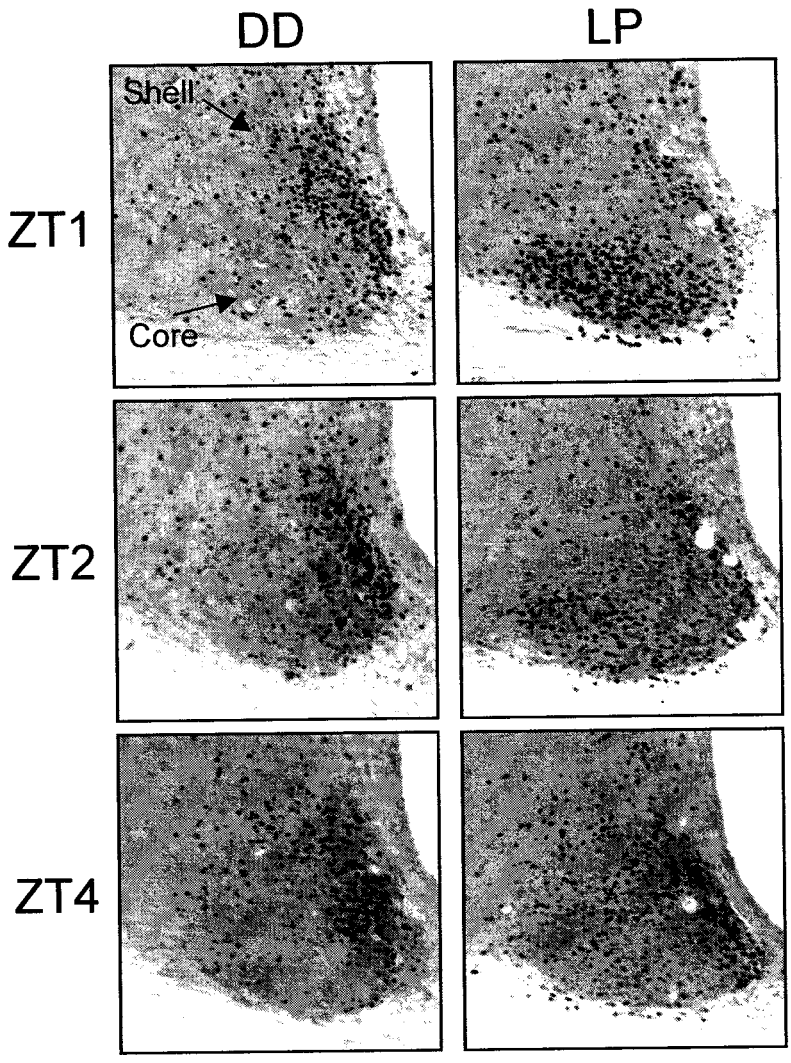


Figure 5. Photomicrographs showing examples of FOS immunoreactivity in the shell and core regions of the SCN as a function of zeitgeber time (ZT) in entrained rats perfused in darkness (DD) or after exposure to a 30-min entraining light pulse (LP).

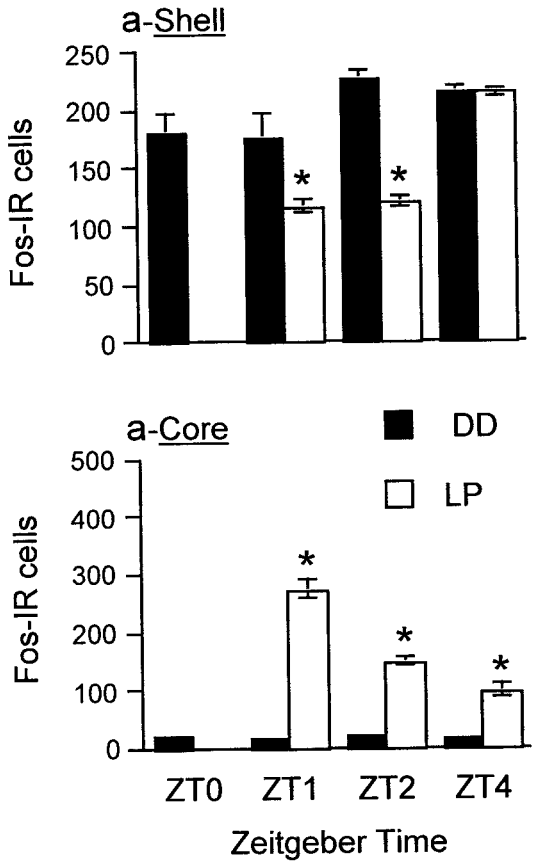


Figure 6. Mean number of FOS-immunoreactive cells (± s.e.m.) in the shell (a) and core (b) regions of the SCN as a function of zeitgeber time in entrained rats perfused in darkness (DD) or after exposure to a 30-min entraining light pulse (LP) (n=4/group). Asterisks indicate significant difference from the corresponding group (Tukey test, $p<0.05$).

perfused in the absence of light and, as expected, exposure to the entraining light pulse significantly increased FOS levels in the core region (Fig 6b). FOS expression in the core remained elevated at all time intervals, however, the number of FOS labeled nuclei in the core region significantly decreased with the passage of time. ANOVA revealed a significant main effect of time ($F_{[3,21]}$=37.1, $p<0.0001$), a significant main effect of light ($F_{[1,21]}$=4.9, $p<0.0001$), and a significant time x light interaction ($F_{[2,21]}$=59.1, $p<0.0001$). Thus, exposure to an entraining light stimulus transiently suppresses FOS expression within the SCN shell, and at the same time induces a strong and slow-decaying increase in FOS in the core region. These changes in FOS expression in the two regions follow a similar temporal time course. Taken together with those from the first study these results show that exposure to light at times uniquely associated with photic entrainment has opposite effects on FOS expression in the core and shell region.

How might light suppress FOS expression in the shell? Unlike the core, which receives direct excitatory input from the retina, the shell region is not innervated by the retina (Johnson, Morin, & Moore 1988; Moga & Moore, 1997; Moore, 1996). It does, however, receive direct innervation from the core (Leak et al., 1999). It is likely, therefore, that the transient suppression of FOS expression in the shell reflects diminished neuronal activity resulting from light-induced activation of the retinorecipient core. Indeed, it is known that the shell region of the SCN is innervated by a non-reciprocal projection from the core containing the inhibitory transmitter gamma-amino-butyric acid (GABA) (Leak et al., 1999; Moore & Speh, 1993; van den Pol, 1986, 1993; van den Pol & Dudek, 1993). Although the identity of the cells in the shell that express FOS and that

presumably receive the direct inhibitory innervation from the core is unknown (Sumova et al., 2000), it is noteworthy that the shell is a primary site of origin of projection neurons allowing communication from the clock to effector systems (Leak & Moore, 2001).

A great deal is known about the relationship between light-induced FOS expression in the core, phase shifts, and entrainment (Hastings et al., 1995; Ikonomov & Stoynev, 1994; Kornhauser et al., 1996; Rea, 1998). The present results add a new element to this relationship by emphasizing correlated events occurring in the shell region of the SCN and could provide further insight into the process whereby photic resetting of the clock is linked to entrainment. The complex relationships between light induced changes in FOS expression in the core and shell and light induced phase shifts are shown schematically in Fig 7. First, it can be seen in Fig. 7a, that the effect of light on clock resetting varies as a function of time of day. At dawn, light causes small advances and, at dusk, small delays in the phase of the clock. Light has no effect during the day, whereas at night it produces large delays or advances in clock phase. The effect of light on phase shifts is in tune, both temporally and functionally, with its effect on FOS expression in the core region of the SCN, as shown in Fig. 7b. In the absence of light FOS expression within the core is minimal throughout the day/night cycle. At dawn and dusk, when light induces small phase shifts, it induces only moderate levels of FOS in the core; at night, light induces strong phase shifts accompanied by strong expression of FOS in the core; lastly, light has no effect on FOS in the core during the day when the clock is not

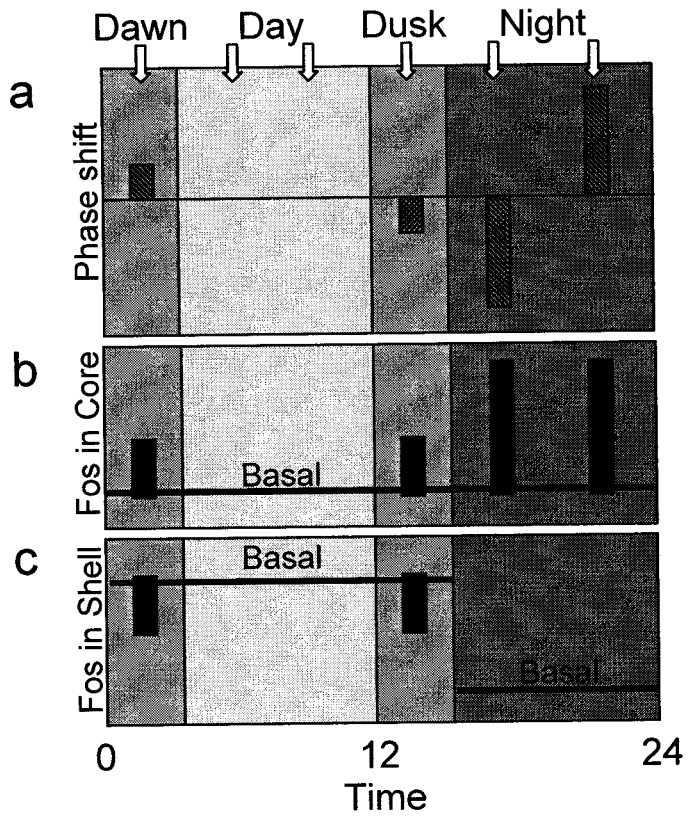


Figure 7. A schematic representation of the relationship between (a) light-induced phase shifts, (b) light-induced expression of FOS in the core region of the SCN, and (c) light-induced suppression of FOS expression in the shell region of the SCN as a function of time of day. White arrows represent light exposure. Basal in b and c refers to Fos expression in constant darkness in each of the two SCN compartments.

sensitive to light. This pattern of sensitivity to light is maintained in total darkness and is presumed to be under the control of the circadian clock (Kornhauser et al., 1996).

Fig. 7c shows the temporal pattern of basal and light induced suppression of FOS expression in the shell. First, note that basal expression of FOS is high at dawn and dusk as well as throughout the day, whereas at night it is minimal. This pattern, like that seen in the core in response to light, persists in constant darkness, consistent with the idea that it too is under the control of the clock. Second, light has a unique effect on FOS expression in the shell at dawn and at dusk: it acutely suppresses the high basal levels of FOS. Note that at the corresponding times light induces only moderate FOS expression in the core and small phase shifts. As Figure 7c also shows when basal FOS expression in the shell is minimal, light induces large phase shifts and strong FOS expression in the core.

As a rule, entrainment to the environmental light cycle requires only small daily shifts in clock phase in order to maintain stability. When the period of the clock is longer than the period of the external light cycle, entrainment requires small daily advances that can be achieved only at dawn, whereas when the period of the clock is shorter than that of the external cycle, entrainment requires small delays that can be achieved only at dusk. Thus, there are two circadian times when light induces FOS in the core, induces small phase shifts, and entrains circadian rhythms: dawn and dusk. At these times, and only at these times, light also suppresses basal FOS expression in the shell. To conclude, the transient decrease in FOS expression in the shell at dawn and dusk is a unique and

defining correlate of photic entrainment and as such, may well be central to the mechanism underlying photic entrainment.

Experimental Procedures

The experimental procedures followed the guidelines of the Canadian Council on Animal Care. The procedures were approved by the Animal Care Committee, Concordia University, and all efforts were made to minimize the number of animals used and their suffering. Experiments were carried out in male Wistar rats (275-325 g, Charles River Canada, St Constant, Québec). The rats were housed individually in plastic cages and placed in light-tight ventilated boxes equipped with a 6-W cool-white fluorescent tube. For FOS immunocytochemistry, the rats were anesthetized with sodium pentobarbital (100mg/kg i.p.) and perfused transcardially with 300 ml of cold physiological saline (0.9% NaCl) followed by 300 ml of cold 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). Brains were removed, postfixed in 4% paraformaldehyde (4°C) overnight, and cut on a vibratome in 50-μm-thick coronal sections. Immunostaining was carried-out on free-floating sections using a rabbit anti-FOS polyclonal antibody recognizing residue 4-17 of the FOS protein (Ab-5, Oncogene Science, Cambridge MA) diluted 1:150,000 with a solution of 0.3% Triton X-100 in tris-buffered saline (TBS) with 3% normal goat serum. Sections were incubated with the anti-FOS antibody for 48 h at 4°C, rinsed in TBS, and then transferred to a solution of 0.3% Triton X-100 in TBS containing biotinylated anti-rabbit secondary antibody (1:200, Vector Labs). FOS immunoreactivity was detected with a Vectastain Elite ABC Kit (Vector Labs) using nickel chloride-enhanced diaminobenzidine reaction. Counts of FOS-immunoreactive

neurons within the SCN were obtained using a computerized image acquisition and analysis system with NIH Image software. Labeled cells were individually marked and manually counted. The SCN was subdivided into the core and shell based on local distribution of FOS immunoreactivity, as previously described (Edelstein et al., 2000; Sumova et al., 2000; Sumova et al., 1998). For analysis, the mean number of FOS immunoreactive cells per SCN compartment was calculated from the counts of five alternate images showing the highest number of labeled nuclei. Group means were derived from these values and analyzed using a two factor ANOVA with light treatment and time as the independent variables. *Post-hoc* test were conducted using Tukey test with alpha set at 0.05.

Acknowledgements

Supported by grants from the Medical Research Council of Canada (now Canadian Institutes of Health Research), the Natural Sciences and Engineering Research Council of Canada, and from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR, Québec).

EXPERIMENT 2

Photic Entrainment of Circadian Rhythms and Suppression of FOS Protein Expression in the Suprachiasmatic Nucleus Shell of Monosodium Glutamate Treated Rats

Christian Beaulé, Jane Barry-Shaw and Shimon Amir[1]

Manuscript submitted to Brain Research, January 24, 2003

Abstract

The protein product of the immediate-early gene *c-Fos* is expressed rhythmically in the shell region of the suprachiasmatic nucleus (SCN), the mammalian circadian clock. Recently, we found that exposure to an entraining light pulse caused a suppression of FOS expression in the SCN shell in rats. To study the hypothesis that suppression of FOS in the shell is a correlate of photic entrainment, we used rats that were treated with the retinal neurotoxin monosodium glutamate (MSG) during the neonatal period. In spite of retinal degeneration, MSG treated rats entrained normally and displayed light-induced suppression of FOS within the SCN shell. These results support the view that light-induced suppression of FOS within the SCN shell is a cellular correlate of photic entrainment.

Introduction

There is considerable evidence that the immediate-early gene *c-Fos* is involved in photic signaling in the suprachiasmatic nucleus (SCN), the circadian clock in mammals (Hastings et al., 1995; Ikonomov & Stoynev, 1994; Klein, Moore, & Reppert, 1991; Kornhauser et al., 1996; Rea, 1998). In rodents, photic stimulation rapidly induces the expression of FOS protein in the retinorecipient (core) region of the SCN (Beaulé & Amir, 1999; Edelstein et al., 2000; Guido, Goguen et al., 1999; Kornhauser et al., 1990; Rea, 1989; Rusak et al., 1992; Rusak et al., 1990). In this region constitutive expression of FOS is normally low, and photic induction of FOS has been linked both temporally and functionally to photic resetting of the circadian clock and to photic entrainment of circadian rhythms (Beaulé & Amir, 1999; Edelstein et al., 2000; Guido, Goguen et al., 1999; Kornhauser et al., 1990; Rea, 1989; Rusak et al., 1992; Rusak et al., 1990; Schwartz et al., 1996; Schwartz, Takeuchi, Shannon, Davis, & Aronin, 1994). FOS is also expressed in the shell region of the SCN, the presumed location of the circadian oscillators driving behavioral and physiological rhythms (Kalsbeek & Buijs, 2002; Leak & Moore, 2001; Moore, 1996; Moore et al., 2002). FOS expression in the shell is rhythmic in animals housed in constant dark, with high levels during the subjective day (the inactive phase of a rat rest-activity cycle) and low levels during the subjective night (the active phase) (Beaulé, Arvanitogiannis, & Amir, 2001; Guido, de Guido et al., 1999; Guido, Goguen et al., 1999; Sumova & Illnerova, 1998; Sumova et al., 2000; Sumova et al., 1998). Surprisingly little is known about the role of FOS in the SCN shell in the regulation of circadian rhythms or about the effect of light on FOS expression in this region.

Recently, we found that brief exposure to light at the early subjective day or early subjective night, but not at other circadian times, acutely suppresses FOS expression within the shell region of the SCN in rats (Beaulé et al., 2001). Because the early subjective day and early subjective night are the only circadian times when daily light exposure leads to stable entrainment, we hypothesized that light-induced suppression of FOS in the SCN shell might be a significant cellular correlate of photic entrainment in rats. To further investigate the relationship between photic entrainment and FOS expression in the shell region of the SCN, in the present study we assessed the effect of an entraining light pulse in rats that were treated with the retinal neurotoxin monosodium glutamate (MSG) during the neonatal period. In these animals normal visual functions and circadian responses to constant light exposure are degraded as a result of widespread retinal damage, but photic entrainment and light-induced FOS expression in the SCN core are normal, in spite of severely compromised retinal input to the SCN (Beaulé & Amir, 2001; Chambille, 1998a, 1998b; Chambille & Serviere, 1993; Edelstein et al., 1995; Olney, 1969; Pickard et al., 1982; van Rijn, Marani, & Rietveld, 1986). We reasoned that if light-induced suppression of FOS expression in the shell region was a true correlate of photic entrainment, then MSG treated animals should show this unique expression pattern in response to entraining light despite severe damage to the retinal innervation of the SCN.

Methods

The experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia University. Wistar rat pups received five subcutaneous injections of either 2 mg/g MSG (Sigma) dissolved in distilled water, or 10% saline (to control for the osmolarity of the MSG) on postnatal days 1,3,5,7, and 9, as previously described (Beaulé & Amir, 2001; Edelstein et al., 1995). Animals were weaned at 21 days of age, and male rats were separated from the females and housed two per cage under a 12h:12h light-dark cycle with free access to food and water. Two months later, rats were housed individually in cages equipped with running wheels. The cages were housed in individual isolation chambers equipped with a ventilation system and a timer-controlled cool white fluorescent light source (300 lux at eye level). Wheel running activity rhythms were monitored continuously using Vitalview data acquisition hardware and software (Mini Mitter Co. Inc., Sunriver, OR). Activity data were displayed as actograms using Circadia software. Rats were first entrained to a 12h light 12h dark cycle (LD). Following the acquisition of stable entrainment to the LD cycle, animals were placed under a 0.5h light 23.5h dark cycle (24h T-Cycle) and maintained on this new cycle for 30 days. On the test day, animals were killed 60 minutes after the onset of the entraining light pulse by sodium pentobarbital anesthesia (100 mg/kg I.P.). Control animals were killed at the same time but without exposure to the entraining light pulse (DD). Rats were perfused, their brains collected, sliced and alternate sections from the SCN were stained for FOS immunoreactivity as previously described (Beaulé et al., 2001). The remaining SCN sections were stained for the low affinity p75 neurotrophin receptor (p75NTR), a marker for retinal ganglion cell axons

innervating the SCN shown previously to be sensitive to the toxic effect of neonatal MSG treatment (Beaulé & Amir, 2001). Briefly, coronal brain sections (50 μm in thickness) from each animal were incubated in either a rabbit polyclonal antibody against FOS (diluted 1:150,000, Oncogene Science) or a mouse monoclonal antibody against p75NTR (diluted 1:30,000, Chemicon). Immunoreactivities were detected with a Vectastain *Elite* ABC Kit (Vector Labs, ON, Canada) using diaminobenzidine and nickel chloride as the chromogens. Sections were inspected under a light microscope using a computerized image acquisition and analysis system with NIH Image software (v 1.62). The SCN was subdivided into the core and shell compartments as previously described (Beaulé et al., 2001). For the analysis, the mean number of FOS immunoreactive nuclei per SCN compartment was obtained for each animal. A two-factor analysis of variance (ANOVA) with treatment (MSG vs. Saline) and light (Light vs. DD) as the independent variables was performed on the mean number of FOS immunoreactive cells. Separate ANOVAs were performed for each SCN compartment. Post-hoc test were performed using the Scheffé method. In order to quantify the density of p75NTR immunostaining in the SCN, the total area encompassing the stained plexus was calculated (in pixels^2) and the mean grayscale density was obtained. Background reference values were obtained from an unstained area of similar size located dorsal to the SCN. Adjusted values for density of p75NTR immunostaining in the SCN were calculated as follows: $DENSITY_{SCN/adj} = AREA\ (DENSITY_{SCN} - DENSITY_{background})$.

As shown in Fig. 8, photic entrainment to the 12h LD cycle or to the 24h T-Cycle was normal in rats that were treated with MSG during the neonatal period despite a

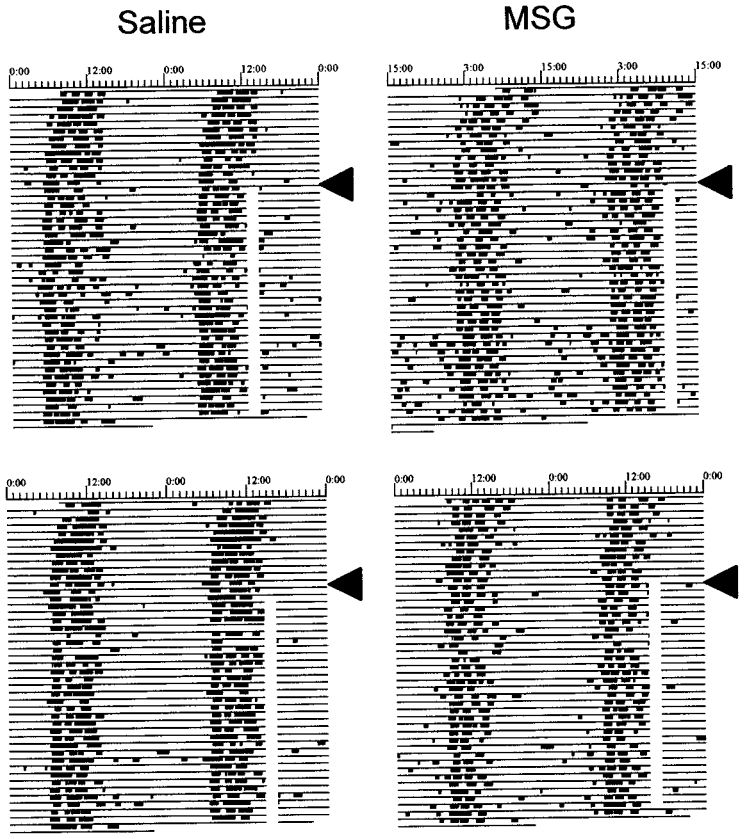


Figure 8. Representative double-plotted actograms showing entrained activity rhythms for Saline and MSG treated rats. Black triangle represent the start of the 24h T-Cycle, shown by the white vertical bar.

marked reduction in the retinal innervation of the SCN of these animals, as is evident from the dramatic decrease in p75NTR immunoreactivity within the SCN core ($F(1,13)=77.11$, $p<0.0001$; Fig. 9).

The effect of light exposure on FOS expression in the SCN is shown in Fig. 10. It can be seen that FOS protein immunoreactivity within the SCN was not affected by neonatal MSG treatment. Within the shell, basal FOS expression was high for animals killed in the dark (projected ZT1) and the overall levels were not different between MSG and Saline-treated animals. Entraining light produced a significant reduction of FOS protein immunoreactivity within the shell of both MSG-treated and control rats ($F(1,10)=17.797$, $p<.01$; Fig. 11). There was no difference between the magnitude of the suppression observed for MSG and for Saline treated animals (Fig. 11). Within the core, basal expression in complete darkness was low for both groups of animals (Fig. 10). Entraining light produced robust FOS induction that was limited to the retinorecipient region of the SCN ($F(1,10)=42.192$, $p<.001$, Figs. 10, 11). The magnitude of the photic induction in the core was not affected by MSG treatment: both the MSG and Saline treated animals showed the same levels of FOS protein induction in the core in response to entraining light (Fig. 11).

The present results confirm our previous finding that exposure to an entraining light pulse suppresses FOS expression in the SCN shell in rats (Beaulé et al., 2001). Furthermore, they show that photic suppression of FOS in the SCN shell persists in rats treated with MSG during the neonatal period. Because photic entrainment is normal in MSG-treated rats despite a substantial damage to the retinal innervation of the SCN in

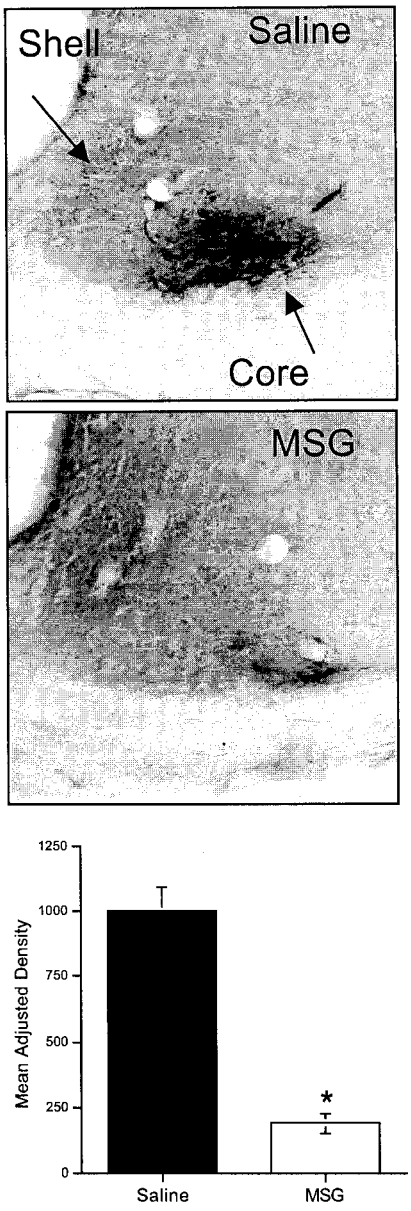


Figure 9. Representative photomicrographs showing p75NTR immunoreactivity within the SCN of Saline and MSG treated rats. Magnification 10X. The graph shows adjusted density values for the intensity of p75NTR immunostaining. The adjusted density values were divided by 1000 for clarity. Significant differences are denoted by an asterisk (*).

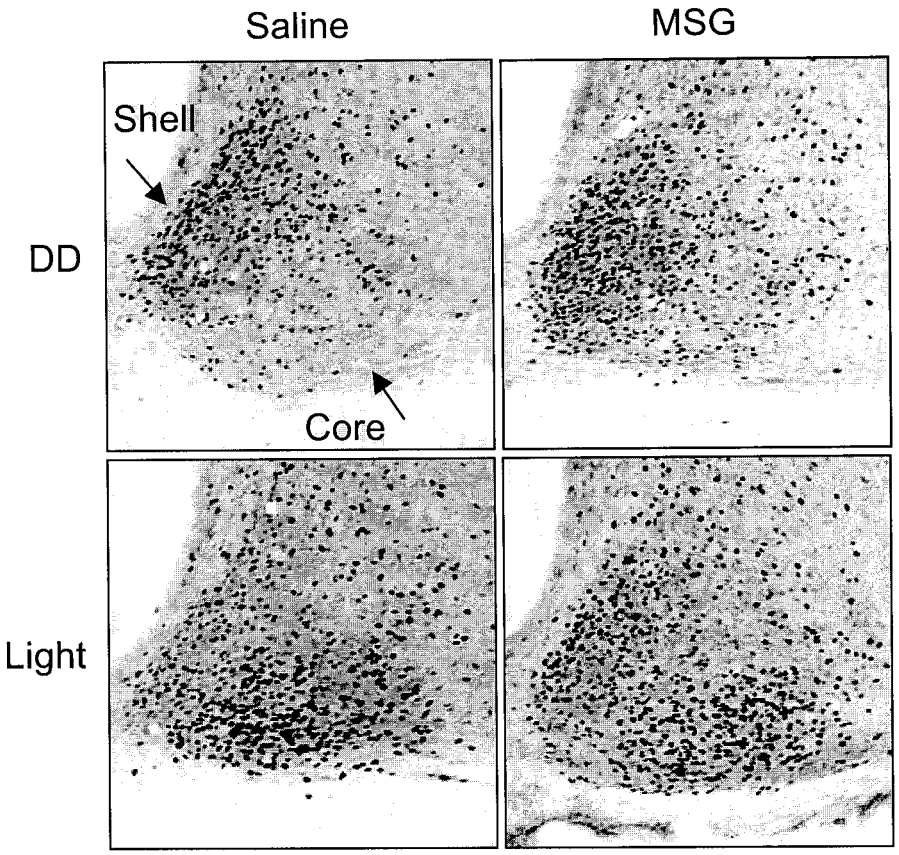


Figure 10. Representative photomicrographs showing FOS protein immunoreactivity in the SCN of Saline and MSG treated rats killed in darkness (DD) or 60 minutes after the entraining light pulse (Light). Magnification 10X.

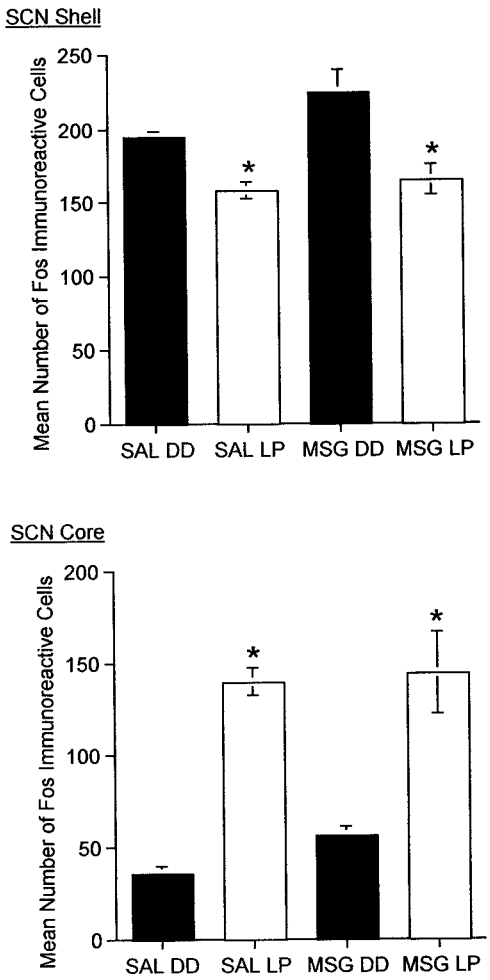


Figure 11. Graph showing the mean number of FOS immunoreactive nuclei in the SCN shell (top) and SCN core (bottom) of Saline and MSG treated rats killed in darkness (black bars) or 60 minutes after the entraining light pulse (white bars). Significant differences are denoted by an asterisk (*).

these animals, these results support the idea that suppression of FOS expression in the shell is a significant correlate of photic entrainment.

Photic entrainment to the environmental light cycle requires small daily shifts in the phase of the SCN clock to maintain a stable relationship between overt behavioral rhythms and the photocycle (Pittendrigh & Daan, 1976). These daily shifts take the form of small phase advances or delays depending on whether the period of the clock is longer or shorter than the period of the external light cycle. Phase advances are achieved by light presented at dawn whereas phase delays depend on light exposure around dusk. Light presented at any other time during the circadian cycle is incapable of sustaining entrainment of circadian rhythms. In rats the free-running period is normally greater that 24 hours and thus rats require light at dawn to produce the daily phase advances needed for stable entrainment. The present finding that FOS expression in the shell is sensitive to entraining light presented at dawn could have implications for the understanding of the relationship between clock resetting and the clock output mechanisms. The shell of the SCN is considered to be the location of the molecular oscillators generating circadian rhythmicity as well as a primary site of origin of projection neurons allowing the transmission of circadian information from the clock to effector systems (Leak et al., 1999; Leak & Moore, 2001; Moga & Moore, 1997; Moore et al., 2002). Previous electrophysiological studies have shown that SCN cell firing is high at dawn (Chen et al., 1999; Gribkoff et al., 1999; Herzog, Geusz, Khalsa, Straume, & Block, 1997; Lundkvist, Kristensson, & Hill, 2002; Shirakawa, Honma, & Honma, 2001). Further, the finding of high levels of FOS immunoreactivity within the shell at dawn is consistent with high

level of cellular activity within the shell during this time (Beaulé et al., 2001; Guido, de Guido et al., 1999; Guido, Goguen et al., 1999; Sumova et al., 2000; Sumova et al., 1998; and present experiment). The reduction of activity in the shell following exposure to the entraining light, as inferred from the decrease in FOS expression, suggests that the output signal emanating form the shell is reduced, possibly leading to a change in the activity in the SCN targets. The inhibitory neurotransmitter gamma-amino-butyric acid (GABA) is the dominant neurotransmitter present within the SCN (Gribkoff et al., 1999; Liu & Reppert, 2000; Moore & Speh, 1993; van den Pol, 1993). GABA is present both within interneurons of the SCN and in the SCN outputs (Leak & Moore, 2001; Moore & Speh, 1993; van den Pol, 1986, 1993). It is known that the SCN shell is innervated by a GABA containing projection from the core (Leak & Moore, 2001; Moore & Speh, 1993; van den Pol, 1986, 1993). This core-to-shell projection opens the possibility for GABA to mediate both the light-induced suppression of FOS protein expression in the SCN shell and the resetting of downstream targets. Such transient suppression of FOS expression might reflect diminished neuronal activity in the shell that results from light-induced activation of the retinorecipient core, and which in turn leads to GABA release within the shell. Resetting of the effector systems that receive input from the shell could occur through a reduction of the tonic GABAergic inhibition imposed by the sustained activity of the SCN shell. By reducing the activity of the shell, entraining light might lift the inhibitory drive on effector systems and lead to a daily resetting of the activity of the SCN target centers.

In conclusion, we found that FOS protein expression within the SCN shell is suppressed during exposure to an entraining light pulse in MSG-treated rats bearing substantial damage to retinal innervation of the SCN. This finding is consistent with the proposition that suppression of FOS expression in the shell is a unique and defining cellular correlate of photic entrainment in rats.

EXPERIMENT 3

Photic and Circadian Expression Profiles of Fos and Per2 Protein Expression within the Rat Suprachiasmatic Nucleus.

Christian Beaulé, Lisa Marie Houle, and Shimon Amir

Manuscript to be submitted to: The Journal of Neuroscience

Abstract

The mammalian circadian clock located in the suprachiasmatic nucleus (SCN) generates oscillations in physiology and behavior that are synchronized (entrained) by the external light:dark cycle. The SCN is compartmentalized into a retinorecipient core region surrounded by a more dorsal shell region. The rhythmic expression and photic induction of FOS and PER2 proteins have been implicated in photic clock resetting. Further, PER2 is an essential component of the cellular clock mechanism generating the circadian oscillation. In the present experiment, we examined the patterns of FOS and PER2 protein expression in the SCN core and shell under various entrainment conditions. We first show that in animals entrained to a 0.5h light 23.5h dark cycle, PER2 protein expression is rhythmic in the core and shell whereas FOS protein expression is rhythmic only within the shell. Further, presentation of entraining light only induced FOS protein expression in the core and did not affect PER2 expression in this compartment. Next, we show that presentation of a discrete light pulse around dusk did not induce PER2 protein expression in the SCN, even 6 hours after photic stimulation. Finally, in behaviorally arrhythmic animals resulting from prolonged bright light exposure, FOS and PER2 protein expression in the SCN is low and non rhythmic. These results show that rhythmic PER2 and FOS expression occurs predominantly within the SCN shell. Further, PER2 protein expression was not regulated by entraining light. Finally, constant-light induced arrhythmicity underlies a disruption of rhythmic PER2 and FOS protein expression in the whole SCN.

Introduction

Photic entrainment is a process by which light resets both the phase and the period of the circadian clock located within the cells of the hypothalamic suprachiasmatic nucleus (SCN) (Klein et al., 1991; Pittendrigh & Daan, 1976). In rats, dawn and dusk are the critical times for photic entrainment and light presented only at these times is both necessary and sufficient to stably entrain circadian rhythms to a 24 hour day (Klein et al., 1991; Pittendrigh & Daan, 1976). The SCN is divided into two anatomically and functionally distinct compartments, a ventral retinorecipient core region surrounded by a dorsal shell region, each contributing to the entrainment process (Leak et al., 1999; Leak & Moore, 2001; Moore et al., 2002). The core integrates photic signals incoming directly from the retina or indirectly through the thalamic intergeniculate leaflet and the raphé nuclei (Amir et al., 1998; Card & Moore, 1982, 1989; Johnson et al., 1988; Moore, 1973, 1996; Moore & Card, 1994; Moore et al., 1978; Moore & Lenn, 1972; Shen & Semba, 1994). The shell integrates non visual information from a range of thalamic and hypothalamic structures and is considered the major source of clock outputs to effector systems (Moga & Moore, 1997; Moore et al., 2002). Circadian (rhythmic) and light-induced cellular changes occur within the two SCN compartments. Of particular interest is the finding that the protein product of the immediate-early gene *c-fos* and the message for the clock gene *period 2* (*Per2* mRNA), show robust rhythmicity within the shell and photic inducibility in the core (Dunlap, 1999; Hastings et al., 1995; King & Takahashi, 2000; Kornhauser et al., 1996). Within the shell, FOS expression is high during the day and low during the night whereas *Per2* mRNA expression is high at midday/early night and lower at the end of the night/early day (Beaulé et al., 2001; Guido, de Guido et al.,

1999; Hamada, LeSauter, Venuti, & Silver, 2001; Miyake et al., 2000; Sumova et al., 2000; Sumova et al., 1998; L. Yan, Takekida, Shigeyoshi, & Okamura, 1999; Zylka et al., 1998). In the core, both FOS and *Per2* mRNA are induced by light presented during the subjective night (the active phase of a nocturnal animal's activity cycle) and photic induction of FOS and *Per2* in the core has been functionally linked to photic resetting of the circadian clock (Albrecht et al., 2001; Bae et al., 2001; Beaulé & Amir, 1999; Beaulé et al., 2001; Colwell & Foster, 1992; Earnest et al., 1990; Edelstein et al., 2000; Honrado et al., 1996; Kornhauser et al., 1990; Miyake et al., 2000; Namihira et al., 2001; Rea, 1989; Rea, Michel, & Lutton, 1993; Rusak et al., 1990; Wollnik et al., 1995). The role played by the rhythmic expression of FOS and *Per2* in the shell of the SCN is unclear. We have recently shown that light necessary for entrainment acutely and transiently suppressed basal FOS protein expression in the shell (Beaulé et al., 2001). The temporal restriction of light-induced FOS suppression to dawn and dusk suggests that it is a cellular event uniquely associated with photic entrainment of circadian rhythms in rats. Although *Per2* mRNA was shown to be light inducible in the core, little is known about light-induced PER2 protein expression in the core or basal PER2 protein expression in the shell since the majority of the work has been done using mRNA (Albrecht et al., 2001; Bae et al., 2001; Miyake et al., 2000; L. Yan et al., 1999). In the present experiments, we first compared FOS and PER2 protein expression patterns within the rat SCN during entrainment to a 0.5h light 23.5h dark cycle (24h T-Cycle) where light was presented at dawn. Because *Per2* mRNA has been shown to be most sensitive to light around dusk, in the second experiment we investigated the effects of a single light pulse presented at dusk on PER2 protein expression. Finally, we investigated PER2 and FOS

protein expression in the SCN of rats housed in constant bright light until complete behavioral arrhythmicity. The mechanism responsible for behavioral arrhythmicity following constant light exposure is unknown and might be mediated by changes in FOS and/or PER2 expression in the SCN.

Methods

Animals

Male Wistar rats (Charles River, St-Constant, Québec) were used in all experiments. All animals had ad lib access to food and water. Rats were housed in running-wheel equipped clear plastic cages and individually placed in ventilated sound and light tight boxes. Each box had a computer controlled lighting system (VitalView Mini Mitter Co. Inc., Sunriver, OR). Each running wheel was equipped with a magnetic microswitch connected to a computer and activity data was collected and analyzed with Circadia.

Entrainment to a 24h T-Cycle

Rats were first entrained to a 12h12h light:dark (LD) cycle for 15 days. Next animals were switched to a 24h T-Cycle (T24) consisting of a 0.5h light pulse followed by 23.5h of darkness. The onset of the 30 minute light pulse corresponded to light onset of the previous LD cycle. The T24 was maintained for 20-30 days. On test day, groups of rats (n = 4 per group) were killed at zeitgeber times 1, 4, 7, 10, 13, 16, 19, and 22 (zeitgeber time ZT, ZT0 corresponds to the onset of the entraining light pulse). Control animals (n = 4 per group) were killed at the same times but without exposure to the

entraining light pulse (in this case, circadian times, CT1, 4, 7, 10, 13, 16, 19, and 22 with CT12 corresponding to the onset of the activity rhythm of a nocturnal rodent).

Photic induction of Per2

Rats were initially entrained to a LD cycle followed by 48 hours of constant darkness (DD). After 48h in DD, groups of rats (n = 4 per group) received a 30 min light pulse at projected ZT13 and killed 2, 4, and 6 hours after light onset. Control animals were killed at the same times in DD.

Constant bright light

It is impossible to determine the phase of the activity cycle for animals that are arrhythmic following prolonged exposure to constant bright-light (LL). However, it is unknown whether the circadian clock is itself arrhythmic in LL or whether the clock is still rhythmic but the output signals are disrupted by LL. In order to differentiate between these two possibilities, we examined FOS and PER2 protein expression in two groups of rats that were first entrained to opposite light cycles. Each group (n = 6 per group) was either entrained to a normal LD cycle (lights ON at 08:00) or a reverse LD cycle (lights ON at 20:00) for 30 days. Next, all animals were subjected to constant bright light (~ 300 lux at eye level). The LL regimen was maintained until all animals became behaviorally arrhythmic (about 8 weeks). If the circadian clock is still cycling in LL, we expect a difference in FOS and PER2 protein immunoreactivity between animals entrained to a normal cycle compared to animals entrained to a reverse cycle because they initially started 180 degrees out of phase. If the clock is indeed arrhythmic following LL, no

effect of previous light history on FOS and PER2 protein expression is expected. All animals were killed on test day between 09:00 and 13:00, local time. To assess that animals were arrhythmic in LL, chi-square periodograms were generated using the Circadia software. Periodogram analysis assesses the period of rhythmicity of each animal by determining the probability that the period of the cycle is within a given range, in this case between 5h and 35h. The confidence interval is also generated and the line of best fit added to the periodogram. The presence of a significant rhythm is displayed on the periodogram as a large peak that is significantly superior to the line of best fit. Periodograms were generated at three time points during the experiments: the last 10 days of the LD cycle, the first 10 days of LL and the last 10 days prior to perfusions.

Tissue preparation

Rats were killed with an overdose of sodium pentobarbital (100 mg/kg approx). Deeply anesthetized rats were perfused intracardially with 300 ml of cold saline (0.9% NaCl) followed by 300 ml of cold, 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). The descending aorta was clamped to force the perfusate into the upper part of the body. Following perfusion, brains were removed and postfixed in 4% paraformaldehyde and stored at 4° C overnight. A total of 30 serial coronal brain sections (50 μm) containing the SCN were collected from each animal using a vibrotome. For each animal, one half of the brain sections were used for FOS immunocytochemistry and the other half for PER2 immunocytochemistry.

FOS *protein immunocytochemistry*

Free floating sections were washed in cold 50 mM Tris buffered saline (TBS; pH 7.6) and incubated at room temperature for 30 minutes in a quenching solution consisting of TBS and 30% w/w hydrogen peroxide (H_2O_2). Following the quenching phase, sections were rinsed in cold TBS and incubated for 1 hour at room temperature in a pre-blocking solution made of 0.3% Triton X 100 in TBS (Triton-TBS) and 3% normal goat serum. Following the pre-blocking phase, sections were transferred directly into a rabbit polyclonal antibody recognizing residue 4-17 of the FOS protein (Oncogene Science, Boston, MA) diluted 1:150 000 and with a solution of Triton-TBS with 3% normal goat serum and incubated for 48 hours at 4°C. Following incubation in the primary antibody, sections were rinsed in cold TBS and incubated for 1 hour at 4°C with a biotinylated anti-rabbit IgG made in goat (Vector Labs, Burlingame, CA), diluted 1:200 with Triton-TBS with 2% normal goat serum. Following incubation with secondary antibody, sections were rinsed in cold TBS and incubated for 2 hours at 4°C with an avidin-biotin-peroxidase complex (Vectastain *Elite* ABC Kit, Vector Labs). Following incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris-HCl (pH 7.6), and again for 10 minutes with 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 minutes in DAB/Tris-HCl with 0.01% H_2O_2 and 8% $NiCl_2$. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in Citrisolv, and cover-slipped with Permount (Fisher).

PER2 *protein immunocytochemistry*

Free-floating sections were washed in cold TBS and incubated at room temperature for 30 minutes in a quenching solution as above. Following the quenching phase, sections were rinsed in cold TBS and incubated for 1 hour at room temperature in a pre-blocking solution made of Triton-TBS, 3% normal goat serum and 5% milk buffer. Following the pre-blocking phase, sections were transferred directly into a rabbit polyclonal antibody raised against PER2 (ADI, San Antonio, TX) diluted 1:4000 with a solution of Triton-TBS with 3% normal goat serum in milk buffer. Following incubation in the primary antibody, sections were rinsed in cold TBS and incubated for 1 hour at 4°C with a biotinylated anti-rabbit IgG made in goat (Vector Labs), diluted 1:400 with Triton-TBS with 2% normal goat serum. Sections were then incubated in ABC reagents and stained using DAB as above.

Immunocytochemistry data analysis

Stained brain sections were examined under a light microscope and images captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and NIH Image software. SCN cells immunopositive for FOS and PER2 were counted manually using the captured images. For each animal, the SCN was subdivided into three areas within the rostro-caudal plane (rostral, medial, caudal). In addition, each SCN was subdivided into shell (dorsomedial) and core (ventrolateral) compartment as previously described (Beaulé et al., 2001; Edelstein et al., 2000). The score used in the analysis was the mean number of immunopositive cells obtained from each rostro-caudal division and each compartment: rostral shell and core; medial shell and core; caudal shell and core.

Differences between groups were revealed with two way analysis of variance (ANOVA) with time and light treatment as independent variables. Separate two-way ANOVA's were performed for each rostro-caudal division and compartments as previously descrived (Beaulé et al., 2001). Alpha level was set at 0.05 for all analyses.

Results

Entrainment to 24h T-Cycle

All animals entrained to the 24h T-Cycle. Examples of FOS and PER2 protein expression for the animals that received the entraining light pulse are shown in Fig. 12 and Fig. 13. PER2 protein expression is rhythmic within the rat SCN with maximal protein expression at ZT13 and minimal expression at ZT4. The amplitude of the rhythm in PER2 protein expression was approximately fourfold greater in the shell than in the core. Rhythmic PER2 expression was visible both in the SCN shell and core with similar timing of the peak and trough. Further, significant rhythmic PER2 protein expression was present at all rostro-caudal division for both SCN compartments (Fig.12 graphs). The rostral SCN is formed predominantly by the shell with only the beginning of a core region at the transition between rostral and medial SCN. Although the rostral SCN core is very limited in size, significant rhythmic PER2 expression was observed albeit with a very low amplitude (mean maximal peak expression of less that 10 immunoreactive cells). PER2 protein expression in animals that received the entraining light pulse did not differ from dark control animals at any time or rostro-caudal division (Fig. 12, graphs).

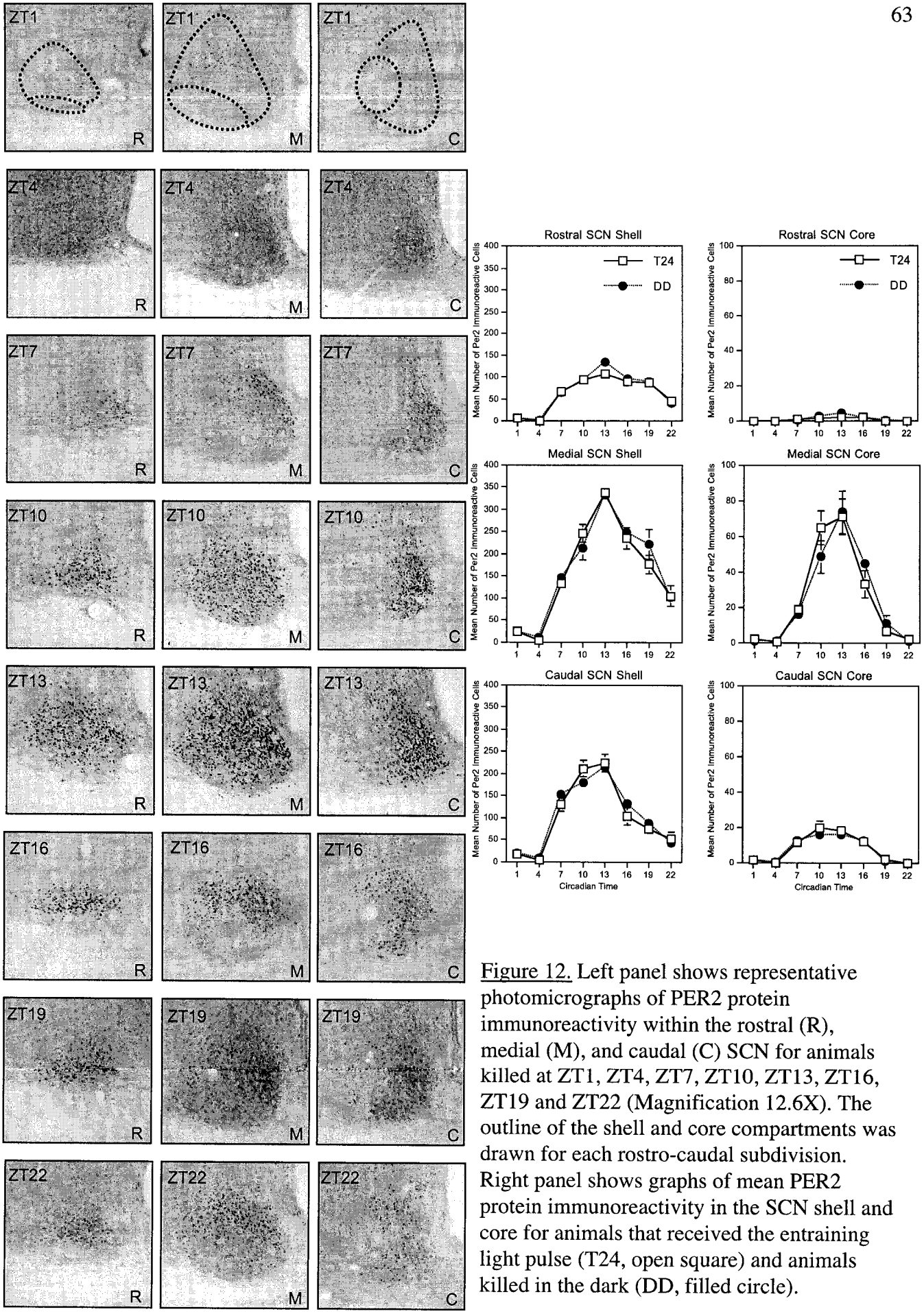
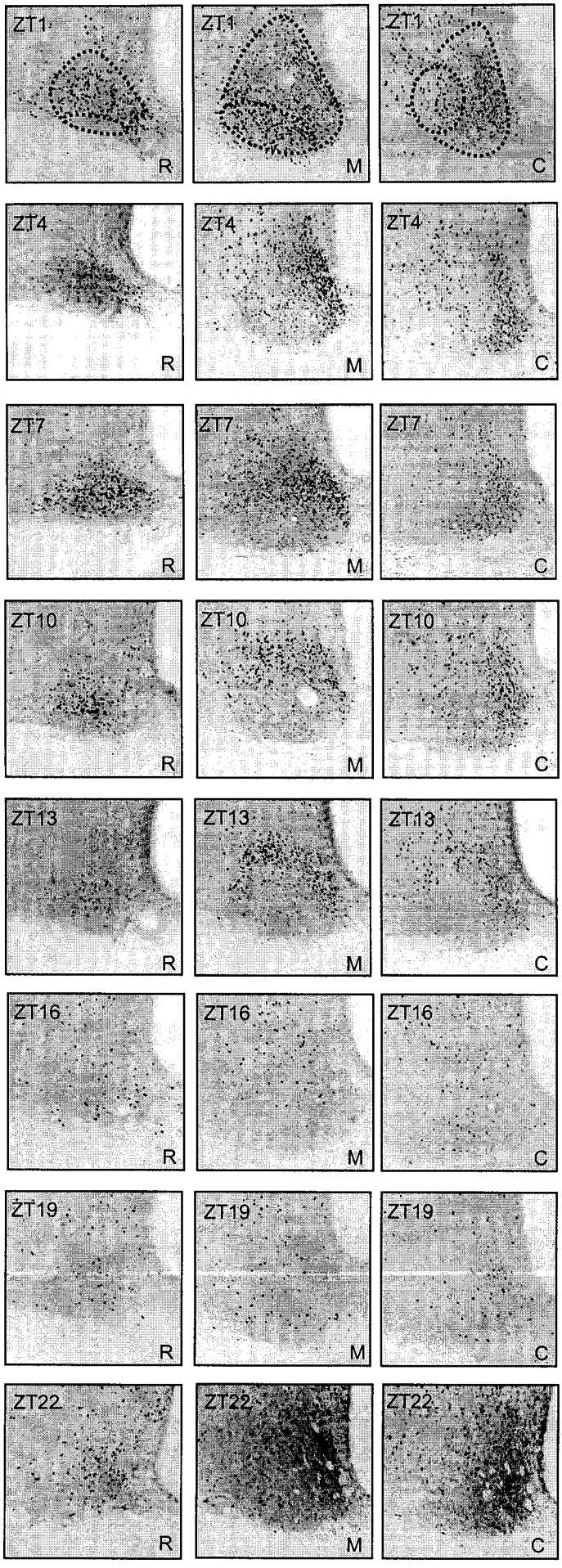


Figure 12. Left panel shows representative photomicrographs of PER2 protein immunoreactivity within the rostral (R), medial (M), and caudal (C) SCN for animals killed at ZT1, ZT4, ZT7, ZT10, ZT13, ZT16, ZT19 and ZT22 (Magnification 12.6X). The outline of the shell and core compartments was drawn for each rostro-caudal subdivision. Right panel shows graphs of mean PER2 protein immunoreactivity in the SCN shell and core for animals that received the entraining light pulse (T24, open square) and animals killed in the dark (DD, filled circle).



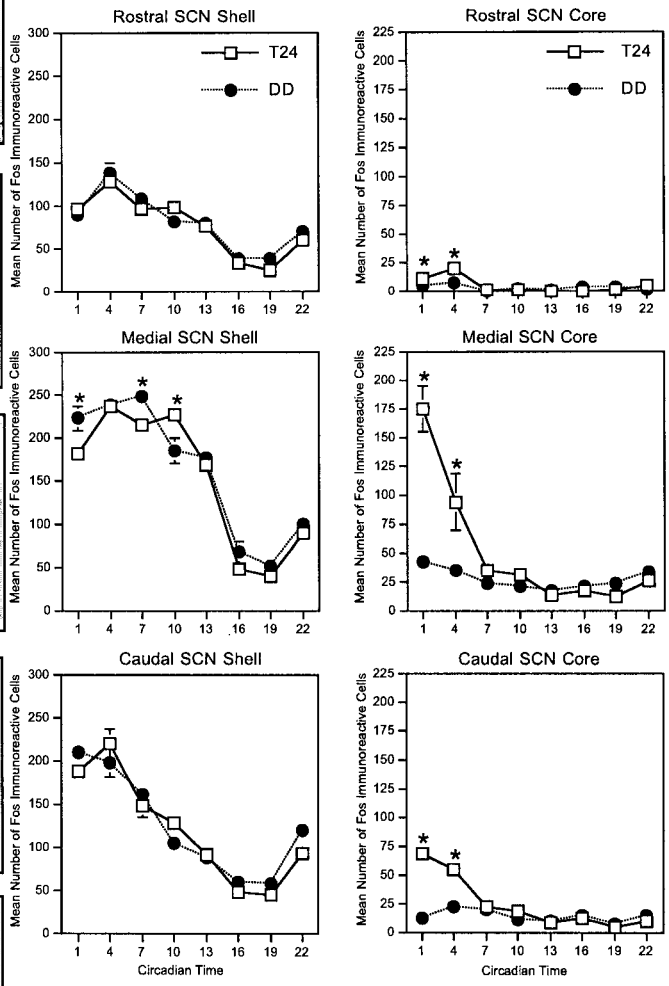


Figure 13. Left panel shows representative photomicrographs of FOS protein immunoreactivity within the rostral (R), medial (M), and caudal (C) SCN for animals killed at ZT1, ZT4, ZT7, ZT10, ZT13, ZT16, ZT19 and ZT22 (Magnification 12.6X). The outline of the shell and core compartments was drawn for each rostro-caudal subdivision. Right panel shows graphs of mean FOS protein immunoreactivity in the SCN shell and core for animals that received the entraining light pulse (T24, open square) and animals killed in the dark (DD, filled circle). The asterisk (*) denotes significant differenced between T24 and DD animals.

FOS protein expression is rhythmic only within the SCN shell with highest protein immunoreactivity at ZT4-7 and lowest at ZT19 (Fig. 13). As shown in the graphs in Fig. 13, the rhythm of FOS protein expression in the shell occurred at the same times, irrespective of location in the rostro-caudal plane. In contrast to PER2, light had a significant effect on FOS protein expression in the SCN shell but only in the medial SCN ($F(1, 43) = 4.868$, $p < .05$) and the Time by Light interaction was significant ($F(7, 43) = 3.671$, $p < .01$). Entraining light significantly suppressed FOS protein expression in the shell at ZT1. Further, significant differences were found between the animals receiving the entraining light pulse and the animals killed in DD at ZT7 and ZT10 (Fig. 13, Medial SCN shell). These later results, although significant, do appear to be the result of experimental variation since no logical pattern can be extracted from the data at these two time points: at ZT7, animals killed in the dark have higher levels of FOS than the animals receiving light and at ZT10, the opposite is true. Furthermore, it is unlikely that light-related changed in FOS protein immunoreactivity would still be visible within the shell 7 and 10 hours after the presentation of the entraining light pulse given the rapid and transient kinetics of this immediate-early gene. Within the SCN core, FOS protein immunoreactivity is low and non-rhythmic in animals killed in DD for any rostro-caudal SCN division (Fig. 13). Presentation of the entraining light pulse significantly increased FOS protein immunoreactivity within the SCN core for animals killed at ZT1 and ZT4, again, irrespective of rostro-caudal subdivision. In animals that received the entraining light pulse, FOS protein levels were undistinguishable from control animals killed in DD by ZT7.

Light-induced PER2

Presentation of a 30-minute light pulse at CT13 had no effect on PER2 protein expression within the rat SCN (Fig. 14). PER2 protein levels were identical for animals killed with or without the light pulse 2, 4 and 6 hours after light presentation.

Constant bright-light induced arrhythmicity

Fig. 15 shows double plotted behavioral actograms and periodogram for animals entrained to a Normal or a Reverse cycle prior to LL. All animals that were housed in LL became behaviorally arrhythmic and remained arrhythmic until they were killed. Periodogram analysis confirmed that all animals were rhythmic during the last 10 days under the LD cycle with a period of 24 hours (Fig. 15a). Following the first 10 days in LL, all animals were rhythmic but the period increased significantly, as expected from initial LL exposure (Fig. 15b). Finally, all animals displayed complete behavioral arrhythmicity within the last 10 days prior to perfusion (Fig 15c). FOS and PER protein immunoreactivity was low and diffuse throughout the SCN and there was no effect of previous light history on protein immunoreactivity within the SCN for any rostro-caudal subdivision (Fig. 16 and Fig. 17).

Discussion

The present results show that FOS and PER2 proteins are expressed rhythmically within the SCN. Rhythmic protein expression is robust within the SCN shell for both FOS and PER2. Within the core, only PER2 shows a significant rhythm that is in phase with the rhythmic expression of the shell. Further, light failed to induce the expression of

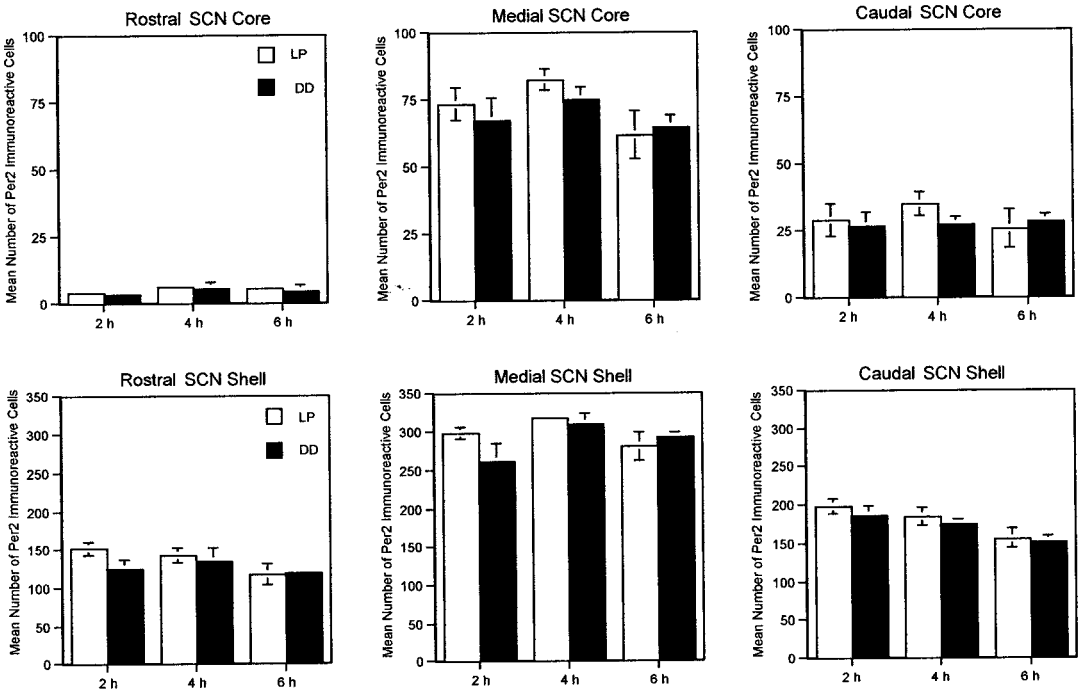


Figure 14. Graphs showing the mean number of PER2 immunoreactive cells within the rostral, medial, and caudal SCN core and shell 2, 4, and 6 hours following the presentation of a 30 minute light pulse at CT13 (LP, open bars) or without light presentation (DD, filled bars).

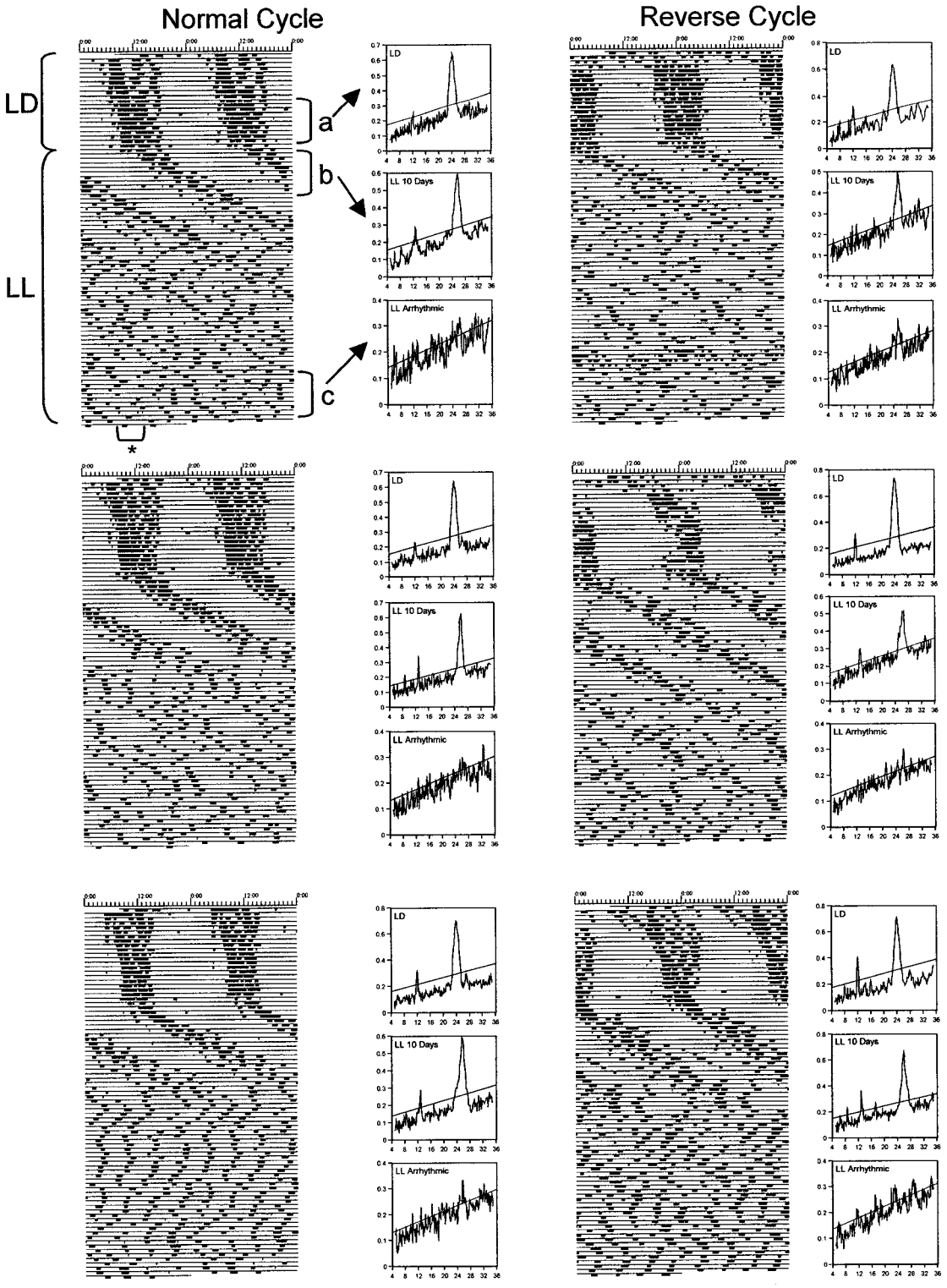


Figure 15. Double plotted actograms of the activity rhythms for 3 animals that were initially entrained to a normal cycle and 3 animals initially entrained to a reverse cycle. LD: entrainment to a 12h light:12h dark cycle. LL: constant bright light exposure. (a), (b), and (c) represent the ranges of days used for the generation of the periodograms. The periodograms on the right of the actograms show the presence of rhythmic behaviors for the last 10 days of the LD cycle (a), the first 10 days in LL (b) and the last 10 days in LL (c). The time at which all animals were perfused is shown by the asterisk (*)

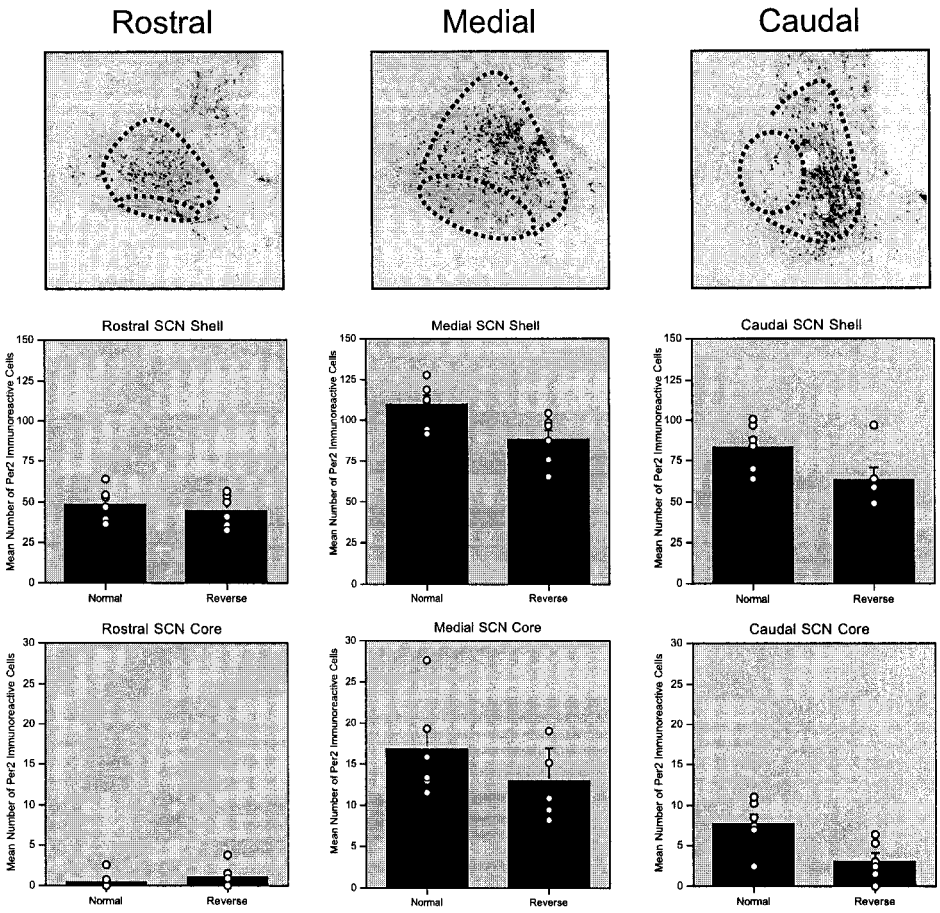


Figure 16. Representative photomicrograph showing PER2 protein immunoreactivity within the SCN of behaviorally arrhythmic animals (magnification 12.6X, top panel). The outline of the shell and core compartments was drawn for each rostro-caudal subdivision. Graphs show the mean number of PER2 immunoreactive cells within the SCN of animals that were initially entrained to a normal LD cycle (Normal) or a reverse LD cycle (Reverse). Open circles represent individual scores for each animal.

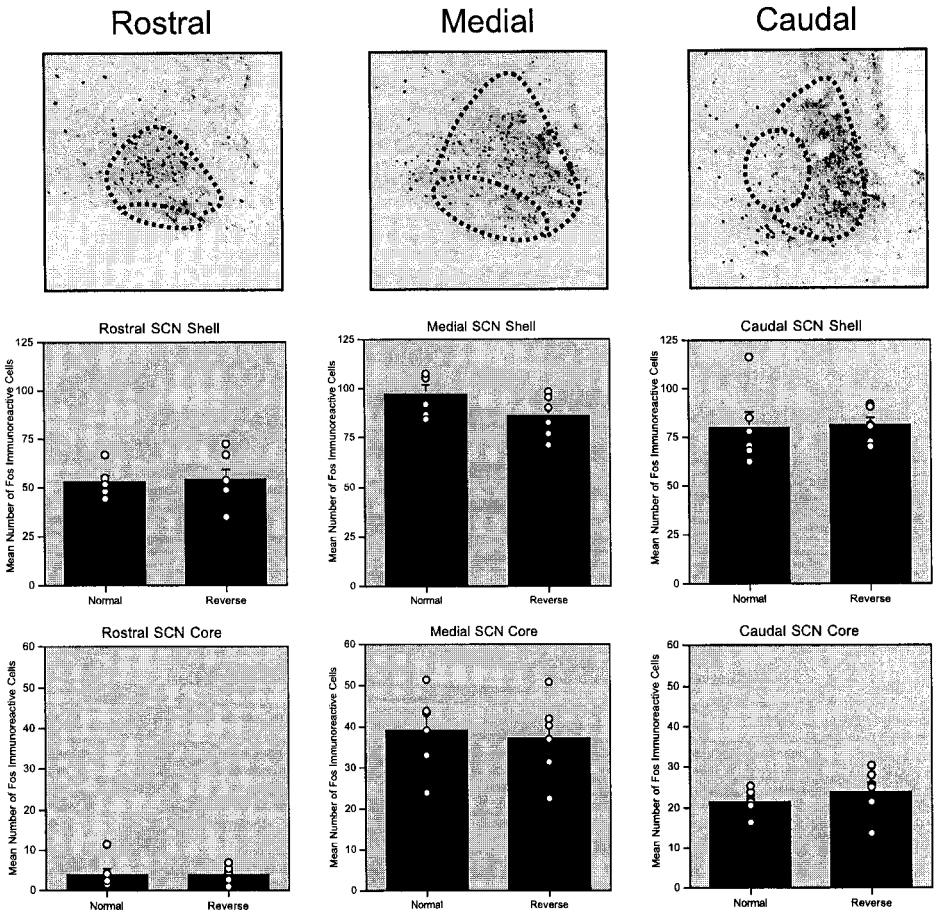


Figure 17. Representative photomicrograph showing FOS protein immunoreactivity within the SCN of behaviorally arrhythmic animals (magnification 12.6X, top panel). The outline of the shell and core compartments was drawn for each rostro-caudal subdivision. Graphs show the mean number of FOS immunoreactive cells within the SCN of animals that were initially entrained to a normal LD cycle (Normal) or a reverse LD cycle (Reverse). Open circles represent individual scores for each animal.

PER2 protein within the SCN core when it was presented at times associated with dawn and dusk. Furthermore, constant bright light exposure completely disrupts the rhythmic expression of PER2 and FOS proteins in the whole SCN. This loss of cellular rhythmicity in the SCN might be the underlying mechanism responsible for constant-bright light induced behavioral arrhythmicity.

In mammals, the clock gene *Per2* is implicated in the positive feedback loop generating the circadian oscillation. *Per2* knockout animals rapidly become arrhythmic in DD and the amplitude of rhythmic expression of the clock genes *Per1, Cry1,* and *Bmal1* is significantly blunted in these knockout animals (Bae et al., 2001). Several studies have suggested that PER2 can activate the transcription of *Bmal1*, one of the positive elements of the circadian molecular loop, driving the rhythm forward (Reppert, 2000; Reppert & Weaver, 2001, 2002; Yu et al., 2002; Zheng et al., 1999). CLOCK::BMAL1 heterodimers activates the transcription of the *period* (*Per1, Per2, Per3*), *cryptochrome* (*Cry1, Cry2*) and the orphan nuclear receptor gene *Rev-Erbα* (Reppert, 2000; Reppert & Weaver, 2001, 2002). REV-ERBα protein is known to repress *Bmal1* transcription through its action on the Rev-Erb/ROR response elements on the *Bmal1* promoter (Preitner et al., 2002; Yu et al., 2002). Rev-Erbα repression of *Bmal1* transcription is inhibited by Per/Cry heterodimers, in fact de-repressing *Bmal1* transcription. It is at this point of the molecular feedback loop that PER2 is thought to play its role. PER2 protein is assumed to provide the positive "drive" on *Bmal1* transcription when the *Bmal1* promoter is de-repressed in the absence of REV-ERBα (Reppert & Weaver, 2002; Yu et al., 2002). In the present experiment the observation that PER2 protein is expressed rhythmically

within the SCN core and shell, unaffected by entraining light and severely disrupted by LL is consistent with this proposed role of PER2.

The finding that PER2 protein is not induced by light presented at times associated with dawn and dusk suggest that in rats PER2 protein is not involved with photic entrainment, further supporting the idea that PER2 contributes to the generation of the circadian oscillation, not its resetting. The fact that a light pulse presented at CT13 did not induce PER2 expression in the SCN core is puzzling in light of the mRNA data. Numerous studies have shown rapid *Per2* mRNA induction following a light pulse presented early during the dark phase (Albrecht et al., 2001; Miyake et al., 2000; Namihira et al., 2001; Shearman, Zylka, Weaver, Kolakowski, & Reppert, 1997; Zylka et al., 1998). Peak mRNA expression occurred 90 minutes following light presentation suggesting that mRNA translation into protein is potentially initiated at least 90 minutes after photic stimulation. The fact that no photic induction of PER2 protein is visible 6 hours after photic stimulation at CT13 suggests that post-transcriptional regulatory events take place and prevent *Per2* mRNA from being translated into protein. The failure to observe light-induced PER2 protein up to 6 hours after the light pulse is also inconsistent with the rhythmic mRNA data. Rhythmic *Per2* mRNA expression peaks at CT8 (see (Reppert & Weaver, 2001) for review). In the present experiment, rhythmic PER2 protein expression was delayed by about 5 hours (peaked at CT13). Assuming a 6-hour delay between maximal rhythmic *Per2* mRNA expression and maximal rhythmic PER2 protein expression, we should have been able to observe photic induction of PER2 protein in the SCN core 6 hours after the light pulse. Although the kinetics of mRNA translation into

protein are unknown for PER2, it would be unlikely that the velocity of translation of light-induced *Per2* mRNA into protein in the core would be different than the velocity of translation for the rhythmic PER2 expression in the whole SCN. Failure to observe changes in protein levels following changes in mRNA levels is common. In fact, the relationship between mRNA and protein levels is doubtful in most cases. Several studies have shown no correlation between mRNA levels and protein levels (Anderson & Seilhamer, 1997; Gygi, Rochon, Franza, & Aebersold, 1999). The main difference between the present experiment and the reports of light-induced *Per2* mRNA is in the timing of the light pulse. We presented light at CT13, which is in the very early part of the subjective night whereas previous reports presented light at CT15-18 (Bae et al., 2001; Miyake et al., 2000; Shearman et al., 1997; L. Yan et al., 1999). We chose this time because it corresponds more closely to the transition time associated with dusk, a critical time for photic entrainment. The light-induced phase shift produced by presentation of light at CT13 is significantly smaller than the phase shift resulting from light at CT15 (Daan & Pittendrigh, 1976; Pittendrigh & Daan, 1976). It is possible that light-induced PER2 protein expression is associated with large phase delays and not smaller ones. If it is indeed the case, it still questions the role played by PER2 in photic entrainment of circadian rhythms since during normal entrainment conditions, the daily phase shift required for stable entrainment is generally small (Beaulé & Amir, 1999). Further, in rats that display an endogenous free-running period longer than 24h, light is specifically required at dawn in order to produce the required phase shift and in this case, PER2 protein expression is not enhanced following light presentation (Beaulé & Amir, 1999; and current data). Results from the present experiment using PER2 protein

immunocytochemistry suggests that PER2 is not involved in photic entrainment that require light presentation at dawn and dusk. Instead, photic regulation of other clock genes such as PER1 might be responsible for mediating clock resetting during photic entrainment.

Constant bright light-induced behavioral arrhythmicity is paralleled by a complete loss of rhythm of FOS and PER2 protein expression within the SCN. The loss of rhythmicity of PER2 is of significant importance in view of its proposed role in driving the positive limb of the cellular clock mechanism (Bae et al., 2001; Zheng et al., 1999). When animals are behaviorally arrhythmic, PER2 is no longer rhythmic in the SCN and levels are low. Low levels of PER2 protein would prevent the normal cycling of the circadian clock by lack of forward drive. These observations are in agreement with the *Per2* knockout data in which the animals become arrhythmic when housed in DD (Bae et al., 2001). Without rhythmic PER2 expression, there is no activation of *Bmal1* and loss of circadian oscillation. The lack of a rhythm in FOS protein immunoreactivity in LL is a reflection of the disrupted clock. FOS protein expression is clock dependent although it does not appear to depend on direct activation by the clock genes. As such, FOS protein rhythm might be considered an overt manifestation of clock functioning. In the case of LL-induced arrhythmicity, the clock is disrupted and clock dependent processes like FOS protein rhythmicity is disrupted as well. It is unlikely that the clock is still functioning at the time at which the animals were killed for two reasons. First, because the animals started the LL regimen 180 degrees out of phase due to prior entrainment, such relationship should have been maintained in LL if the clock had been working. At the

very least, a difference between animals initially entrained to a normal cycle and the animals entrained to the reverse cycle should have been observed. In the present case, all animals were basically identical, irrespective of prior light history. Second, the within group variability in the number of PER2 and FOS immunoreactive cells was low. Assuming that the circadian clock was still functional and that the animals were all at different points of their circadian cycle, we should have observed large differences in protein expression patterns. This was not the case, strongly suggesting that the circadian clock is completely disrupted during prolonged LL exposure.

In conclusion, there is a robust rhythm of FOS and PER2 protein expression within the shell of the SCN. Further, PER2 protein is not induced by light presented at times mediating entrainment, suggesting that PER2 is not involved in mediating photic entrainment in rats. Finally, constant bright-light induced arrhythmicity is the result of a complete disruption of the cellular clockwork that is highlighted by loss of PER2 and FOS rhythmic expression.

SECTION 2: THE RETINOHYPOTHALAMIC TRACT AND THE TRANSMISSION OF PHOTIC INPUT TO THE SUPRACHIASMATIC NUCLEUS

Photic entrainment requires the transmission of light signals from the retina to the retinorecipient region of the SCN. In rats, the retinorecipient compartment of the SCN is the ventrolateral core region. The experiments contained within the next section investigated the role played by the retinal fibers containing the p75NTR and melanopsin in the transmission of the entraining light signal.

The first two experiments investigated the role played by RHT fibers bearing the p75NTR. In the first experiment, neonatal administration of MSG was used to destroy the ganglion cell layer of the retina and the effects of MSG treatment on p75NTR expression in the SCN core were then assessed. In the second experiment, targeted, selective lesion of the p75NTR fiber plexus within the SCN core were performed using the immunotoxin 192-IgG Saporin (SAP). SAP is a ligand-bound immunotoxin specific for the p75NTR. The effects of SAP lesions on photic entrainment of circadian rhythms were also evaluated.

The third experiment looked at the effects of both MSG and SAP treatment on the expression of melanopsin within the SCN core. Melanopsin is believed to be a specific photopigment for the circadian system. As such, the role played by melanopsin

immunoreactivity in the SCN of animals in which the integrity of the RHT is compromised without effects on entrainment will provide important information for the photic regulation of circadian rhythms.

EXPERIMENT 4

Photic Regulation of Circadian Rhythms and the Expression of p75 Neurotrophin Receptor Immunoreactivity in the Suprachiasmatic Nucleus in Rats.

Christian Beaulé and Shimon Amir

Brain Research, 894, 301-306 (2001), © Elsevier Science, with permission

Abstract

Neurotrophic factors have been implicated in the mechanism underlying photic regulation of circadian rhythms in mammals. In rats, the most abundant neurotrophin receptor found in the suprachiasmatic nucleus (SCN), the circadian clock, is the low affinity p75 neurotrophin receptor (p75NTR). This receptor is expressed by retinal afferents of the SCN, but nothing is known about its role in photic regulation of circadian rhythms. We show here that neonatal treatment with the retinal neurotoxin, monosodium glutamate (MSG), which has no effect on photic entrainment of circadian rhythms, nearly completely abolished p75NTR immunoreactivity in the SCN in rats. These findings suggest that p75NTR from retinal sources do not play an essential role in the mechanism mediating photic entrainment of circadian rhythms in rats.

Introduction

The retinal afferents mediating photic entrainment of circadian rhythms in mammals terminate in the ventrolateral core region of the suprachiasmatic nucleus (SCN), the circadian clock. In rats, a proportion of these retinal afferents express the low affinity p75 neurotrophin receptor (p75NTR) and the retinorecipient core region contains a prominent plexus of afferent terminals and cells immunoreactive to p75NTR (Bina et al., 1997; Kiss, Patel, & Halasz, 1993; Koh, Oyler, & Higgins, 1989; Moga, 1998b; Pioro & Cuello, 1990; Sofroniew, Isacson, & O'Brien, 1989; Suzuki, Nomura, Morii, Fukuda, & Kosaka, 1998; Yan & Johnson, 1988). Although there is evidence to suggest that neurotrophic factors play a role in photic regulation of circadian rhythms (Bina & Rusak, 1996; Earnest, Liang, Ratcliff, & Cassone, 1999; Liang et al., 2000; Liang, Sohrabji et al., 1998; Liang, Walline, & Earnest, 1998; van den Pol, Decavel, Levi, & Paterson, 1989), virtually nothing is known about the role of p75NTR. In the present study we assessed the expression of p75NTR immunoreactivity in the SCN of rats treated with the retinal neurotoxin, monosodium glutamate (MSG), during the neonatal period. Such treatment produces widespread retinal damage, sufficient to block the disruptive effect of constant light on circadian rhythms (Edelstein & Amir, 1999a; Edelstein et al., 1995), but does not affect photic entrainment of circadian rhythms (Chambille, 1998a, 1998b; Edelstein & Amir, 1999a; Edelstein et al., 1995; Pickard et al., 1982). We found that neonatal treatment with MSG strongly suppressed the expression of p75NTR immunoreactivity in the SCN. Thus, it would appear that p75NTR from retinal sources do not play a critical role in the photic entrainment mechanism in rats. Rather, it appears

that these receptors are more likely to be involved in the pathways mediating the disruptive effect of constant light on circadian rhythms.

Methods

The experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia University. Wistar rat pups received five subcutaneous injections of either 2 mg/g MSG (Sigma) dissolved in distilled water, or 10% saline (to control for the osmolarity of the MSG) on postnatal days 1,3,5,7, and 9, as previously described (Edelstein et al., 1995). Animals were weaned at 21 days of age, and male rats were separated from the females and housed two per cage under a 12h:12h light-dark cycle with free access to food and water. Two months later, the rats were housed individually in isolated cages equipped with running wheels, a ventilation system, and a timer-controlled cool white fluorescent light source (300 lux at eye level). Wheel running activity rhythms were monitored continuously under different light schedule using DataCol data acquisition hardware and software (Mini Mitter Co. Inc., Sunriver, OR). Activity data were displayed as actograms using Circadia software. For p75NTR immunocytochemistry, the rats were anaesthetized with sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially with 300 ml of cold physiological saline (0.9% NaCl) followed by 300 ml of cold 4% paraformaldehyde in a 0.1M phosphate buffer (pH 7.3). Brains were removed, post fixed in 4% paraformaldehyde (4°C) overnight, and cut on a vibratome in 50-μm thick coronal sections. Immunostaining was carried-out on free-floating sections using a mouse anti-p75NTR monoclonal antibody (Sigma) diluted 1:30,000 with a solution of 0.3% Triton

X-100 in TBS with 1% normal horse serum. Sections were incubated with the anti-p75NTR antibody for 48 h at 4°C, rinsed in TBS, and then transferred to a solution of 0.3% Triton X-100 in TBS containing biotinylated anti-mouse secondary antibody (1:66; Vector Labs). p75NTR immunoreactivity was detected with a Vectastain *Elite* ABC Kit (Vector Labs, ON, Canada) using diaminobenzidine as the chromogen. Sections containing areas of interest were inspected under a microscope using a computerized image acquisition and analysis system with NIH Image software. In order to quantify the density of p75NTR immunostaining in the SCN the total area encompassing the stained plexus was calculated (in pixels^2) and the mean density was obtained. Background reference values were obtained from an unstained area of similar size (in pixels^2) located dorsal to the SCN. Adjusted values for density of p75NTR immunostaining in the SCN were calculated as follows: $DENSITY_{SCN/adj} = AREA\ (DENSITY_{SCN} - DENSITY_{background})$.

Results

Neonatal MSG had no effect on the period of free running activity rhythms in constant darkness and on entrainment of activity rhythms to a 12h:12h light-dark (LD) cycle. Furthermore, as shown in Fig. 18, it had no effect on entrainment of activity rhythms to a 0.5h:24.5h LD cycle, which requires particularly large daily delays in clock phase. As previously reported (Arvanitogiannis et al., 2000; Edelstein & Amir, 1999a; Edelstein et al., 1995), neonatal MSG blocked the effect of constant light on rhythms (Fig. 18). These results confirm that the retinal damage produced by neonatal MSG treatment is sufficient to block the effect of constant light on circadian activity rhythms

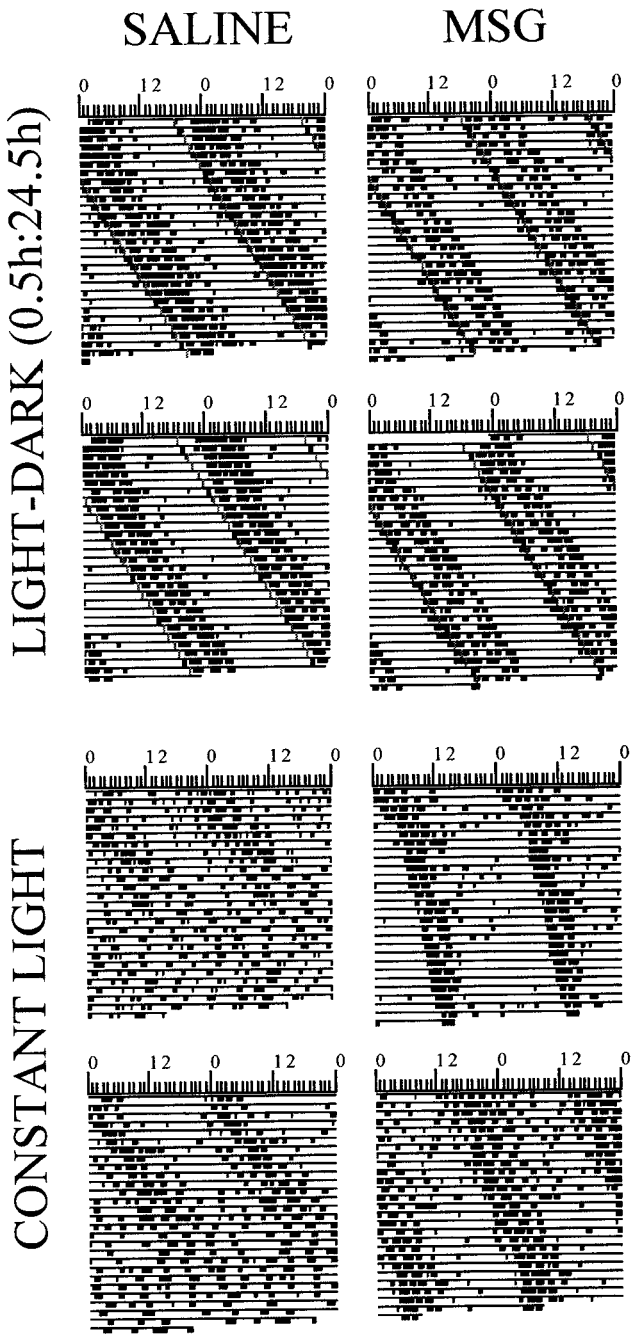


Figure 18. Double-plotted actograms showing examples of wheel-running activity rhythms of saline-treated and MSG-treated rats housed under a 0.5h:24.5h light-dark cycle or constant light. The vertical marks indicate periods of activity of at least 10 wheel revolutions/10 min. Successive days are double plotted from top to bottom.

but has no effect on photic pathways and neural mechanisms required for the generation and entrainment of circadian rhythms (Arvanitogiannis et al., 2000; Chambille, 1998a, 1998b; Chambille & Servière 1993; Edelstein & Amir 1999a; Edelstein et al., 1995; Pickard, 1982).

The effect of neonatal treatment with MSG on expression of p75NTR immunoreactivity in the SCN is shown in Fig. 19. In control rats immunostaining was robust and confined to the ventrolateral retinorecipient region of the SCN (Fig. 19a-c), as previously described (Bina et al., 1997; Moga, 1998b; Sofroniew et al., 1989). Afferent fibers emanating from the optic chiasm and coursing to the ventrolateral SCN region were also strongly labeled (Fig. 19d). Neonatal treatment with MSG diminished p75NTR immunostaining throughout the rostral-caudal extent of the SCN (Fig. 19e-g), and completely eliminated staining of fibers emanating from the optic chiasm (Fig 19h). A similar effect was noted in rats perfused 3 weeks after termination of MSG treatment. Semi-quantitative analysis of p75NTR immunostaining in the SCN revealed greater than 75% decrease in size and density of staining in the SCN of both young (30-days old) and adult rats (3-5 months old) treated neonatally with MSG.

In addition to its effect on p75NTR expression in the SCN, treatment with MSG strongly reduced p75NTR immunostaining in the ventral lateral geniculate nucleus (vLGN) and intergeniculate leaflet (IGL, Fig. 20a,e). These structures are innervated by the retina and have been implicated in photic regulation of circadian rhythms in rats

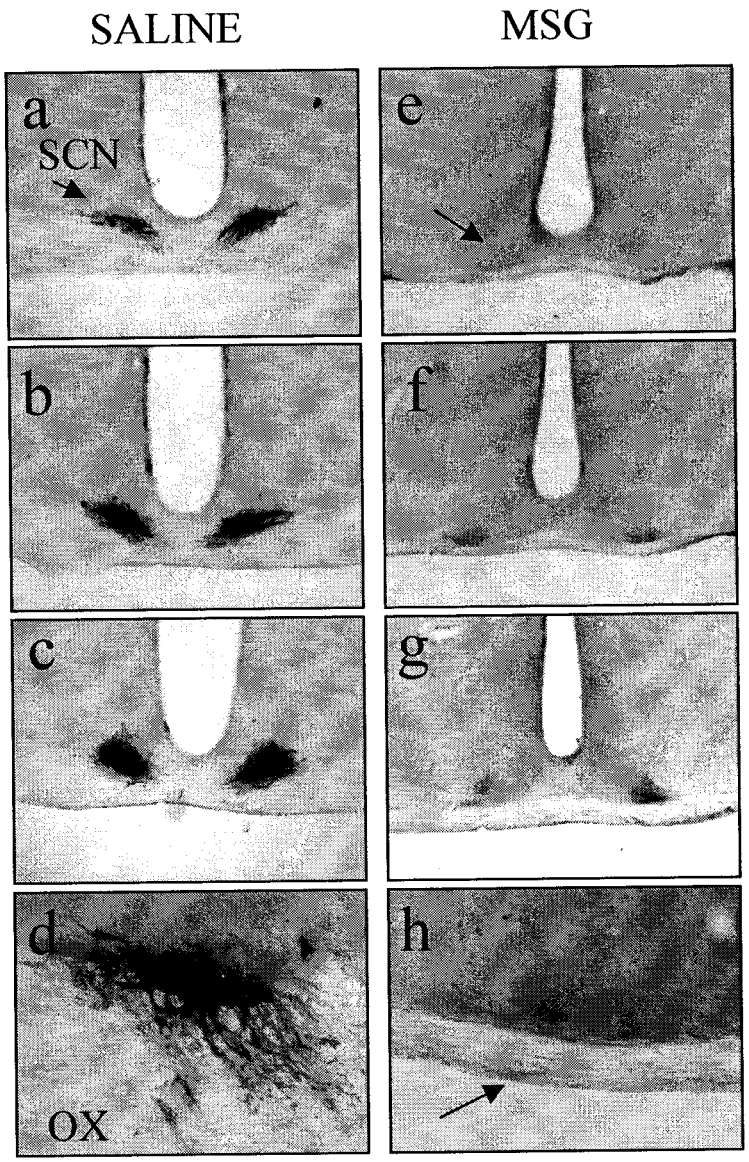


Figure 19. Photomicrograph showing examples of p75NTR immunostaining in the rostral (a, e), rostral-medial (b, f), and medial-caudal (c ,g) regions of the SCN and in the optic chiasm (OX) (d, h) of rats treated neonatally with saline or MSG. Magnification in a-c, e-g = x5; magnification in d, h = x20.

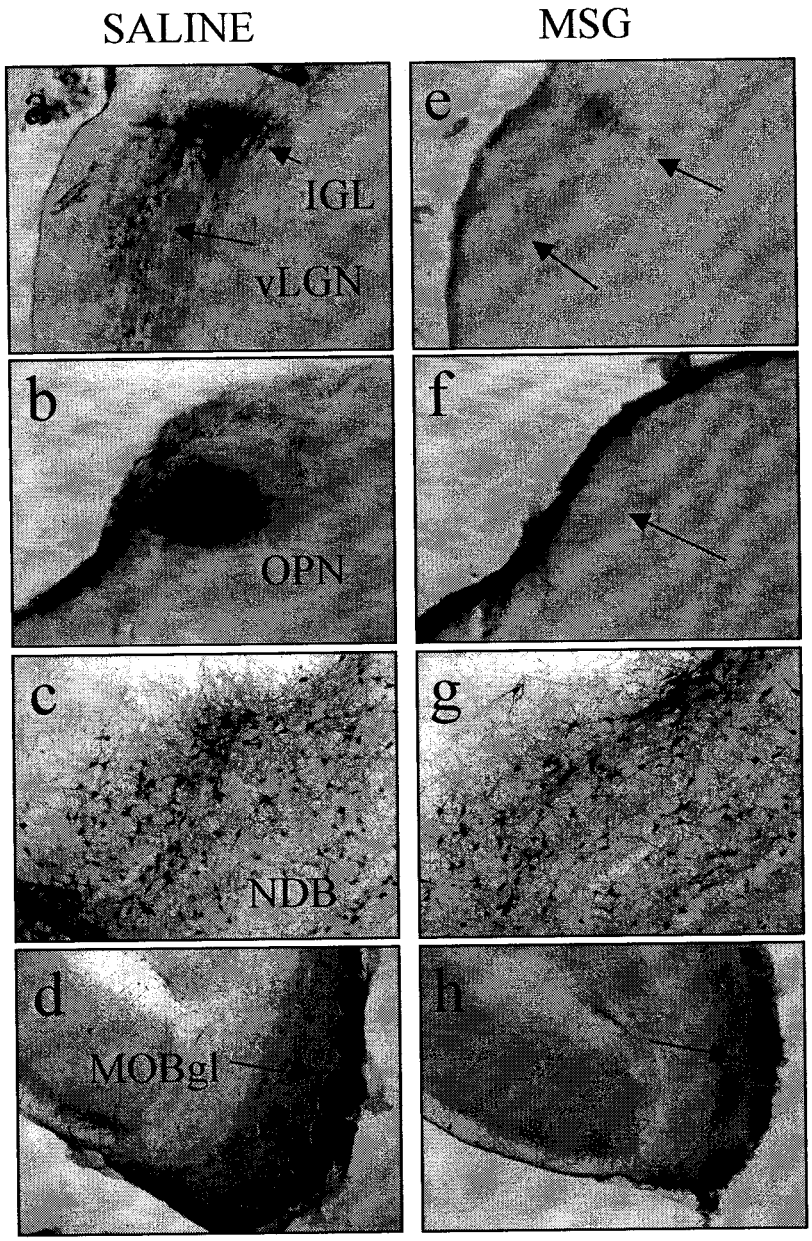


Figure 20. Photomicrograph showing examples of p75NTR immunostaining in the intergeniculate leaflet (IGL) and ventrolateral geniculate nucleus (vLGN) (a, e), olivary pretectal nucleus (OPN) (b, f), nucleus of the diagonal band (NDB) (c, g) and glomerular layer of the main olfactory bulb (MOBgl) (d, h) of rats treated neonatally with saline or MSG. Magnification = x10.

(Edelstein & Amir, 1999b; M. E. Harrington, 1997). Furthermore, this treatment completely eliminated staining in the olivary pretectal nucleus (OPN, Fig. 20b,f), a retinorecipient area known to project to the SCN (Mikkelsen & Vrang, 1994; Moga & Moore, 1997) and implicated in pupillo-constrictor light reflex. MSG had no apparent effect on p75NTR immunostaining in the basal forebrain, the main extraretinal source of p75NTR immunoreactivity in the SCN (Bina et al., 1997), and in the granular layer of the olfactory bulb (MOBgl). Examples of p75NTR in the nucleus of the diagonal band (NDB) and MOBgl are shown in Fig. 20.

Discussion

In the present study we found that neonatal MSG treatment, like bilateral orbital enucleation (Bina et al., 1997; Moga, 1998b), strongly suppressed the expression of p75NTR immunoreactivity in the SCN in rats. Because a primary target of MSG is the ganglion cell layer of the retina, and because retinal ganglion cells that project to the SCN express p75NTR (Bina et al., 1997), it is likely that the observed decrease in p75NTR expression in the SCN was linked specifically to retinal ganglion cell damage induced by MSG treatment. Consistent with this, MSG had no effect on p75NTR immunostaining either in the basal forebrain, an extraretinal source of p75NTR immunoreactivity in the SCN, or in the olfactory bulb. Taken together with the evidence that damage to ganglion cells induced by MSG does not compromise mechanisms required for photic entrainment, the present finding leads to the conclusion that retinal projections to the SCN that express p75NTR are not essential for photic entrainment of circadian rhythms in rats. This conclusion is of special interest in view of recent evidence that photic resetting of the

circadian clock in rats is modulated by brain-derived neurotrophic factor (BDNF) and involves signaling via high affinity tyrosine kinase B (TrkB) neurotrophin receptors. TrkB receptors, like p75NTR, are located on retinal afferents of the SCN (Liang, Sohrabji et al., 1998), and it has been speculated that signaling via TrkB in the SCN is coupled functionally to p75NTR signaling (Liang, Sohrabji et al., 1998). Such functional coupling between p75NTR and TrkB receptors has been described in other neuronal systems (Bibel, Hoppe, & Barde, 1999; Frade & Barde, 1998; Kaplan & Miller, 2000), including the visual cortex and retina (Allendoerfer et al., 1994; Frade, Bovolenta, & Rodriguez-Tebar, 1999; Sala et al., 1998). Contrary to this idea, the present results indicate that any role played by BDNF in photic resetting is probably independent of p75NTR signaling and of functional coupling between TrkB and p75NTR in the SCN.

The present results offer new insight into the mechanism underlying the effect of constant light on circadian rhythms. We have shown previously (Arvanitogiannis et al., 2000; Edelstein & Amir, 1999a; Edelstein et al., 1995) and in the present study, that neonatal treatment with MSG blocks the disruptive effect of constant light housing on circadian rhythms in rats. In view of these findings and the present results that neonatal MSG abolished p75NTR expression in retinorecipient areas of the circadian system, it would appear that the effect of constant light depends on the integrity of those retinal projections that express p75NTR. We can only speculate on how transmission via these afferents might contribute to loss of circadian rhythms in response to constant light exposure. It is thought, for example, that the primary cause of rhythm disruption in constant light is a sustained loss of coupling among individual pacemaker neurons in the

SCN. Putative mechanisms for coupling among SCN pacemaker neurons have been described (Colwell, 2000; Liu & Reppert, 2000; Shinohara, Hiruma, Funabashi, & Kimura, 2000; Welsh et al., 1995; Welsh & Reppert, 1996), and it is plausible that sustained activation of p75NTR during constant light exposure could interfere directly or indirectly with these mechanisms. Consistent with this, it is noteworthy that nerve growth factor has been shown to disrupt cell to cell communication in the ovary by affecting the functional integrity of gap junctions (Mayerhofer et al., 1996), and that signaling via p75NTR can mediate neuronal cell loss (Kaplan & Miller, 1997, 2000), including loss of retinal photoreceptors induced by constant light exposure in rats (Harada et al., 2000).

In summary, we found that neonatal treatment with MSG diminishes the expression of p75NTR immunoreactivity in the SCN. Because photic entrainment of circadian rhythms is not affected by MSG treatment it is unlikely that these receptors play any critical role in the photic entrainment pathway. Alternatively, we propose that p75NTR signaling in the SCN might play a role in mediating the disruptive effect of constant light on circadian rhythms in rats.

Acknowledgements

Supported by grants from the Medical Research Council of Canada, the Natural Sciences and Engineering Research Council of Canada, and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche.

EXPERIMENT 5

Effect of 192 IgG-Saporin on Circadian Activity Rhythms, Expression of p75 Neurotrophin Receptors, Calbindin-D28k, and Light-Induced FOS in the Suprachiasmatic Nucleus in Rats

Christian Beaulé and Shimon Amir

Experimental Neurology 176(2), 377-389 (2002), © Elsevier Science, with permission

Abstract

Photic entrainment of circadian rhythms in mammals is mediated through a direct retinal projection to the core region of the suprachiasmatic nucleus (SCN), the circadian clock. A proportion of this projection contains the low affinity p75 neurotrophic receptor (p75NTR). Neonatal monosodium glutamate (MSG) treatment, which dramatically reduces p75NTR immunoreactivity in the SCN, has no impact on photic entrainment. In order to clarify the contribution of p75NTR fibers in photic entrainment, targeted lesions of the p75NTR-immunoreactive SCN plexus were performed using intracerebroventricular (ICV) or intrahypothalamic injections of the immunotoxin, 192-IgG Saporin (SAP) in rats. SAP treatment effectively abolished p75NTR immunoreactivity within the SCN core. ICV-SAP treatment produced three different behavioral activity patterns: animals became either arrhythmic, displayed a shorter free-running period or remained rhythmic following the lesion. Arrhythmic animals had large hypothalamic lesion which encompassed the entire SCN. In rhythmic rats, ICV SAP significantly reduced immunostaining for calbindin-D28k (CaBP) in the SCN, and rats with shortened free-running periods had the lowest number of CaBP immunoreactive cells. ICV SAP also attenuated light-induced FOS expression in the SCN core. In spite of lack of p75NTR and reduced CaBP and FOS expression in the SCN, SAP-treated rhythmic rats displayed normal photic entrainment. Intrahypothalamic SAP treatment reduced CaBP expression in the SCN but had no effect on light induced For expression, free running rhythms or photic entrainment. The data show that p75NTR-immunoreactive elements in the SCN are not required for photic entrainment.

Introduction

Exposure to the environmental light cycle is essential for stable entrainment of circadian rhythms in mammals. Photic information necessary for entrainment is communicated to the circadian clock, the suprachiasmatic nucleus (SCN), via a direct projection from retinal ganglion cells that use glutamate as a primary transmitter (Ebling, 1996; Johnson et al., 1988; Mikkelsen et al., 1995; Moga & Moore, 1997; Moore & Lenn, 1972; Morin, 1994; Rea, 1998). In rats, a subpopulation of these axons express the low-affinity p75 neurotrophin receptor (p75NTR) (Bina et al., 1997; Moga, 1998b; Suzuki et al., 1998). The terminals of these axons form a dense plexus which is confined to, and completely overlaps, the core region of the SCN (Beaulé & Amir, 2001; Kiss et al., 1993; Koh et al., 1989; Moga, 1998b). This region contains light-responsive retinorecipient neurons that express NMDA and non-NMDA glutamate receptors (Ebling, 1996; Mikkelsen et al., 1995) and are immunoreactive to vasoactive intestinal polypeptide (VIP) and calbindin-D28k (CaBP), both of which have been implicated in the mechanism of photic entrainment of circadian rhythms (Arvanitogiannis et al., 2000; Bryant, LeSauter, Silver, & Romero, 2000; LeSauter & Silver, 1999; Reed, Meyer-Spasche, Cutler, Coen, & Piggins, 2001; Romijn, Sluiter, Pool, Wortel, & Buijs, 1996; Shinohara, Tominaga, & Inouye, 1999; Silver et al., 1996). The close association of the p75NTR-immunoreactive plexus with neurons involved in entrainment suggests a role for p75NTR in the transmission of entraining photic information to the SCN. Previous studies have indicated a role for p75NTR in several neuronal functions, including retrograde transport of neurotrophins and the modulation of high-affinity neurotrophin receptors (Bothwell, 1995; Chao, 1994; Chao & Hempstead, 1995). Surprisingly,

however, although there is some evidence that photic input to the SCN might be modulated by neurotrophic factors (Bina & Rusak, 1996; Kiss et al., 1993; Liang et al., 2000; Liang, Sohrabji et al., 1998; Liang, Walline et al., 1998; Paula-Barbosa, Silva, Andrade, Cadete-Leite, & Madeira, 2001; van den Pol et al., 1989), little is known about the specific role of p75NTR in these processes.

We have found that neonatal treatment with the retinal neurotoxin monosodium glutamate (MSG), which prevents the effect of constant light on circadian rhythms (Edelstein & Amir, 1999a; Edelstein et al., 1995), but, importantly, has no effect on entrainment to light cycles (Chambille, 1998a, 1998b; Chambille & Serviere, 1993; Edelstein & Amir, 1999a; Edelstein et al., 1995; Pickard et al., 1982), dramatically reduces the expression of p75NTR immunoreactivity within the SCN (Beaulé & Amir, 2001). This treatment also abolishes p75NTR immunostaining in the olivary pretectal nucleus (OPT) and intergeniculate leaflet (IGL) (Beaulé & Amir, 2001), retinorecipient structures implicated in the modulation of photic input to the SCN (Moga & Moore, 1997). Together, these data suggest either that the integrity of a very small proportion of the p75NTR-bearing retinal neurons are sufficient to maintain circadian function or that SCN afferents that express p75NTR do not play a role in normal circadian physiology. To investigate these possibilities further, in the present study we examined the effect of using the immunotoxin 192 IgG-saporin (SAP) to selectively target p75NTR-containing neurons and afferents of the SCN on circadian physiology. SAP is used widely to selectively lesion p75NTR-expressing neurons in the brain (Wiley, 1992, 1996), and microinjections of SAP into the third cerebral ventricle were found recently to completely

abolish p75NTR immunostaining in the SCN (Moga, 1998a). We used this approach in the first of the experiments described below. Because we found that 3rd ventricle SAP injections resulted in widespread hypothalamic damage in a proportion of the animals, in a second experiment, we also assessed the effect of SAP microinjections into the SCN region. The effect of both ICV and intrahypothalamic SAP microinjections on photic entrainment of circadian rhythms, the expression of p75NTR and CaBP immunoreactivity and light induced FOS expression in the core region of the SCN were assessed.

Methods

Animals and Behavioral Procedure

All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of Concordia University. Male Wistar rats (250-300g Charles River Canada, St. Constant, Québec) were housed individually in cages equipped with running wheels and had free access to food and water. Each cage was placed in a ventilated sound- and light-tight box equipped with a computer-controlled lighting system. All rats were housed in complete darkness (DD) for 10 to 20 days and their activity rhythms were monitored continuously using VitalView software (Mini Mitter Co, Sun River, OR), as previously described (Beaulé & Amir, 2001). They were then treated with SAP (see below) and returned to DD. Following recovery in DD, the rats were exposed to a 12 h:12 h light-dark (LD) cycle (light: 300 lux at cage level) or constant bright light (LL, 300 lux, for 15-30 days. They were then re-exposed to DD and entrained to a 0.5h:23.5h LD cycle. The rats were perfused 30 min after the offset of the entraining light pulse and their brains were processed for p75NTR, CaBP and Fos immunocytochemistry. Circadian wheel running activity rhythms were analyzed with ActiView software (Mini Mitter Co, Sun River, OR) and displayed in double-plotted actograms using Circadia software.

Intracerebroventricular 192IgG-Saporin injections

Animals were pre-treated with an intra peritoneal (IP) injection of atropine sulfate (0.12mg/animal) and anesthetized with sodium pentobarbital (65mg/kg, IP). Anesthetized rats were placed into a stereotaxic apparatus, their scalp incised and the skull exposed. A

small hole was drilled and a 31 gauge stainless steel cannula was inserted at the following coordinates: AP: -1.2 mm; L: ± 0 mm; V: -8.5 mm from dura (flat skull coordinates). 192IgG-Saporin (Chemicon, Temecula, CA) was diluted with 0.9 % saline and a dose of 2.5 μg of the drug was injected in a 4 μl volume (n=33) as previously described (Moga, 1998a). The injection was performed with a 10 μl Hamilton syringe connected to the cannula with polyethylene tubing at a rate of 1 μl/min. The cannula was left in place for an additional 20 min to ensure proper diffusion of the toxin. The cannula was withdrawn, the hole was sealed with bone wax, the skin sutured and the animals received an intramuscular injection of penicillin (7500 U of each of the following: benzathine penicillin G, procaine penicillin G). Sham animals (n = 6) underwent the same procedure except that a volume of 4 μl of 0.9% saline was injected.

Intrahypothalamic 192IgG-Saporin injections

Animals were pre-treated with an IP injection of atropine sulfate and anesthetized with sodium pentobarbital (65mg/kg). Anesthetized animals were placed into a stereotaxic apparatus, their scalp incised and the skull exposed. Two small holes were made 1.2 mm apart, at an equal distance from the midline. A 28 gauge double cannula system (distance between the two cannulae 1.2 mm, Plastic Ones, Roanoke, VA) with a 31 gauge internal injector extending 1.0 mm from the tip of each cannula was aimed to the dorsal surface of the SCN using the following coordinates: AP: -1.2 mm; L: ± 0.6 mm; VM: -8.5 mm from dura (flat skull coordinates). A dose of 200 ng of SAP in a total volume of 1 μl was injected on each side at a rate of 0.2 μl/min (n=15). The cannula system was left in place for an additional 10 minutes to prevent backflow. The cannula

system was withdrawn, the holes were sealed with bone wax, the skin sutured and the animals received an intramuscular injection of penicillin as above. Sham animals (n=7) underwent the same procedure except that a volume of 1 μl of 0.9% saline was injected on each side.

Tissue preparation

Deeply anesthetized rats were perfused intracardially with 300 ml of cold saline (0.9% NaCl) followed by 300 ml of cold, 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). The descending aorta was clamped to force the perfusate into the upper part of the body. Following perfusion, brains were removed and postfixed in 4% paraformaldehyde and stored at 4° C overnight. A total of 90 coronal brain sections (50 μm in thickness) containing the SCN, basal forebrain (BF), nucleus of the diagonal band (NDB), olivary pretectal nucleus (OPT) and intergeniculate leaflet (IGL) were obtained from each brain using a vibratome. For each animal, one-third of the sections from each region were used for p75NTR immunocytochemistry, one-third for CaBP immunocytochemistry and one-third for FOS immunocytochemistry.

p75NTR and CaBP immunocytochemistry

Free floating sections were washed in cold 50 mM Tris buffered saline (TBS; pH 7.6) and incubated for 48 hours at 4°C with a mouse monoclonal antibody against either CaBP (Sigma) or p75NTR (Chemicon, Temecula, CA), as previously described (Arvanitogiannis et al., 2000; Beaulé & Amir, 2001). Briefly, the antibodies were diluted 1:20 000 (CaBP) and 1:30 000 (p75NTR) with a solution of 0.3% Triton X 100 in TBS

with 1% normal horse serum. Following incubation in the primary antibody, sections were rinsed in cold TBS and incubated for 1 hour at 4°C with a rat-absorbed biotinylated anti-mouse IgG made in horse (Vector Labs, Burlington, Ontario, Canada), diluted 1:200 (CaBP) and 1:66 (p75NTR) with 0.3% triton X 100 in TBS with 1% normal horse serum. Following incubation with secondary antibody, sections were rinsed in cold TBS and incubated for two hours at 4°C with an avidin-biotin-peroxidase complex (Vectastain *Elite* ABC Kit, Vector Labs). Following incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris-HCl (pH 7.6), and again for 10 minutes with 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 minutes in DAB/Tris-HCl with 0.01% H_2O_2 and 8% $NiCl_2$. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in xylene, and cover-slipped with Permount (Fisher).

FOS immunocytochemistry

Free-floating sections were washed in cold TBS and incubated at room temperature for 30 minutes in a quenching solution consisting of TBS and 30% w/w H_2O_2. Following the quenching phase, sections were rinsed in cold TBS and incubated for 1 hour at room temperature in a pre-blocking solution made of 0.3% Triton X 100 and 3% normal goat serum. Following the pre-blocking phase, sections were transferred directly into a rabbit polyclonal antibody recognizing residue 4-17 of the FOS protein (Oncogene Science, Boston, MA). The antibody was diluted 1:150 000 with a solution of 0.3% Triton X 100 in TBS with 3% normal goat serum. Sections were incubated for 48

hours at 4°C. Following incubation in the primary antibody, sections were rinsed in cold TBS and incubated for 1 hour at 4°C with a biotinylated anti-rabbit IgG made in goat (Vector Labs), diluted 1:200 with 0.3% Triton X 100 in TBS with 2% normal goat serum. Following incubation with secondary antibody, sections were rinsed in cold TBS and incubated for two hours at 4°C with an avidin-biotin-peroxidase complex (Vectastain *Elite* ABC Kit, Vector Labs). Following incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris-HCl (pH 7.6), and again for 10 minutes with 0.05% DAB in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 minutes in DAB/Tris-HCl with 0.01% H_2O_2 and 8% $NiCl_2$. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in xylene, and cover-slipped with Permount (Fisher).

Immunocytochemistry data analysis

Stained brain sections were examined under a light microscope and images captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and NIH Image software. The intensity of p75NTR immunostaining was evaluated qualitatively using staining intensity in control rats as a reference. Cells within the SCN core immunopositive for CaBP and FOS were counted manually using the captured images. For each animal the score used in the analysis was the mean number of immunopositive cells obtained from the 3 sections through the SCN core exhibiting the highest number of counts. Differences between groups were revealed with one-way analysis of variance

(ANOVA). *Post hoc* comparisons were conducted using Scheffé tests and the alpha level was set at 0.05 for all analyses.

Results

Circadian Behavior after ICV SAP

ICV injections of SAP resulted in three different circadian activity patterns. Of the 33 SAP-treated rats, 13 became completely arrhythmic within 3-5 days following injection (Fig. 21a). These rats remained arrhythmic until the end of the study and failed to respond to entraining light cycles. The other 20 SAP-treated rats remained rhythmic, entrained normally to LD cycles and displayed typical disruption of circadian behavior in LL (Fig. 21b). Significantly, of the 20 rhythmic rats, 6 displayed a sharp and permanent shortening of about 60 min in their free-running period length within 5-10 days following treatment (Fig. 21c). ICV saline injections did not affect rhythmicity or entrainment (n = 6). ANOVA performed on period length before and after treatment revealed a significant effect of group (SAL, SAP-arrhythmic, SAP-normal period, SAP-short period; $F_{3,36}$ =14930, $p<.0001$) and a significant treatment effect (pre-treatment vs. post-treatment; $F_{1,3}$ =30934, $p<.0001$). Fig. 22 shows the periods of SAL-treated and SAP-treated rats before and after treatment.

p75NTR Immunostaining after ICV SAP

All arrhythmic rats exhibited large hypothalamic lesions which included the entire SCN and optic chiasm. In all cases, the boundary of the lesion exhibited dense staining for p75NTR, consistent with the presence of extensive vascularization in the lesioned

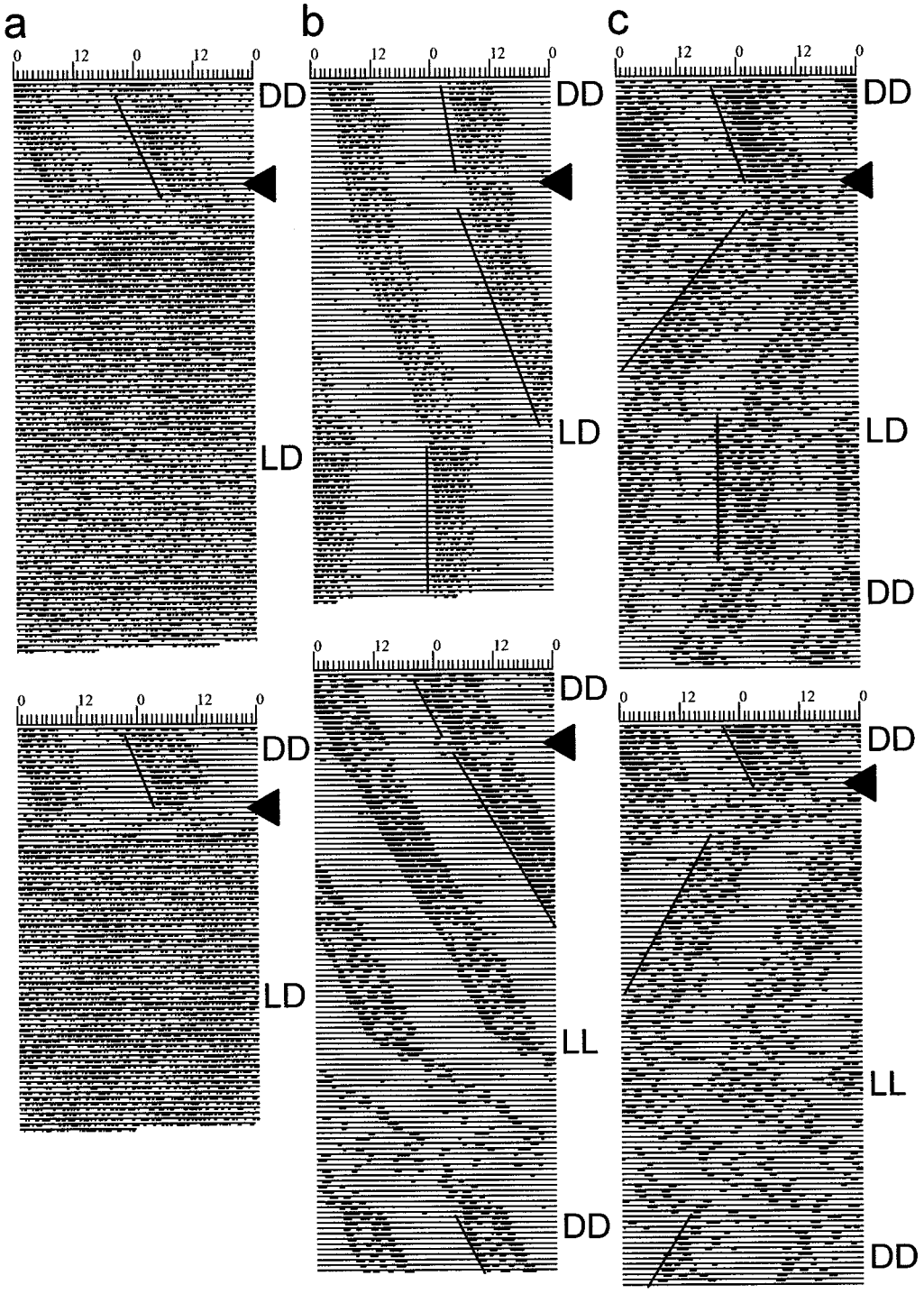


Figure 21. Double-plotted actograms showing examples of circadian wheel-running activity rhythms in rats following ICV SAP. The vertical marks indicate periods of activity of at least 10 wheel revolutions/10 min. Successive days are plotted from top to bottom. Black triangles indicate the time of ICV SAP treatment. (a) actograms from two rats that became arrhythmic following ICV SAP; (b) actograms from two rats that exhibited no significant change in free-running period and responded normally to photic stimuli; (c) actograms from two rats that exhibited a significant shortening of the free-running period and normal photic responses. DD, constant darkness; LL, constant light; LD, 12-h:12-h light-dark cycle.

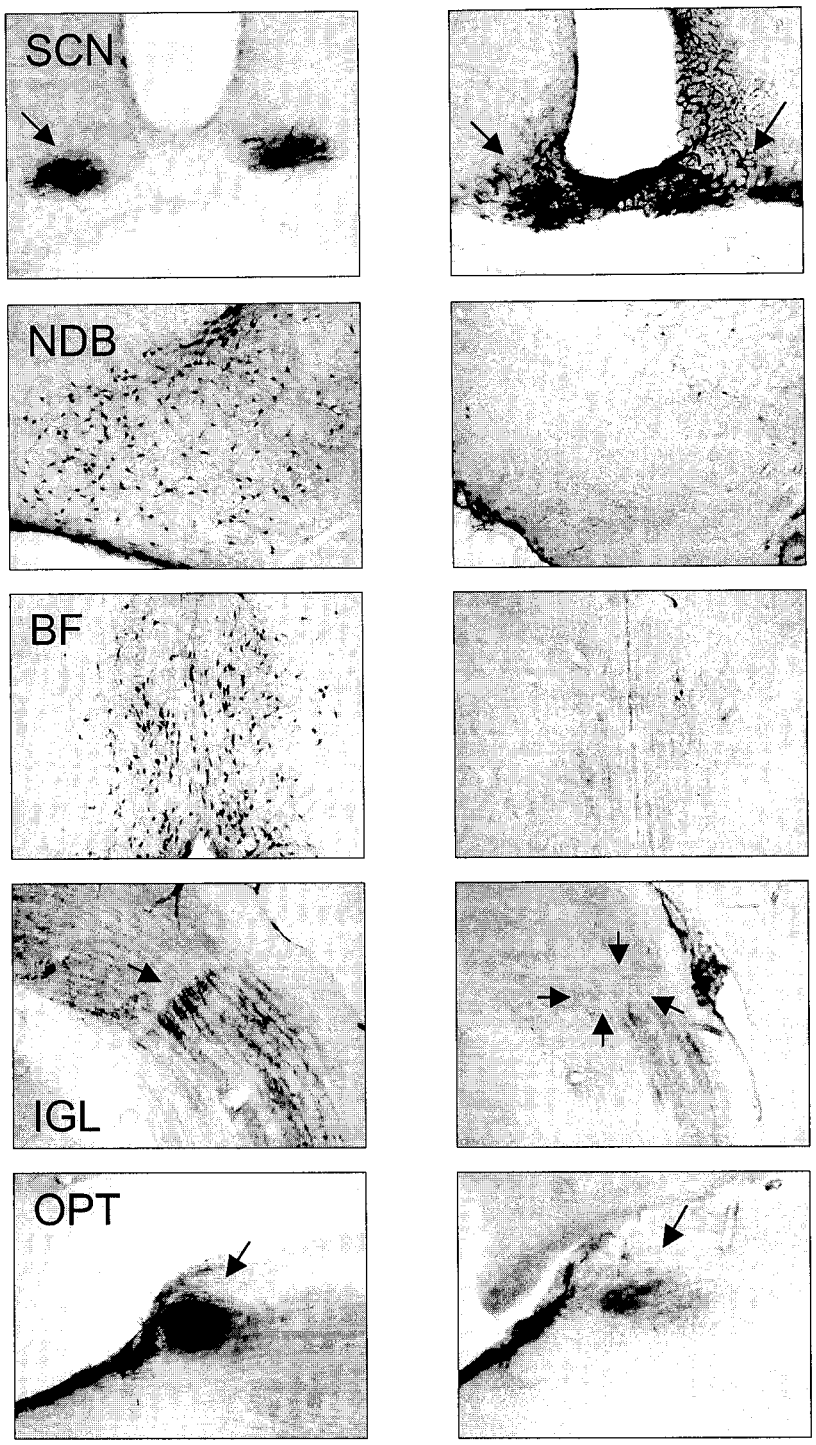


Figure 22. Photomicrograph showing examples of p75NTR immunostaining in the SCN, nucleus of the diagonal band (NDB), basal forebrain (BF), intergeniculate leaflet (IGL), and olivary pretectal nucleus (OPT) from an ICV saline-treated rat (ICV SAL) and from a rat treated ICV with SAP and that became arrhythmic following treatment. Note the massive lesion in and around the SCN following SAP. Magnification = X10.

area (Fig. 23). Immunostaining for p75NTR in the BF and NDB, believed to be extraretinal sources of p75NTR in the SCN (Bina et al., 1997), was minimal in all arrhythmic rats (Fig. 23). Likewise, the OPT and IGL, retinorecipient structures which project to the SCN (Moga & Moore, 1997) and which normally stain heavily for p75NTR, were only moderately stained (Fig. 23). SAP-treated rats which exhibited normal circadian periods in DD and which responded to entraining LD cycles had only small hypothalamic lesions, and, significantly, in all cases the bulk of the SCN remained intact. Immunostaining for p75NTR in the SCN was completely abolished and the pattern of p75NTR immunostaining outside the SCN in these animals was similar to that seen in rats with large lesions (Fig. 24). SAP-treated rats which showed a shortening of the circadian period had lesions similar in extent and localization to those showing normal circadian periodicity and also displayed reduced staining within the BF, NDB and IGL, and moderate to heavy stain in the OPT. Immunostaining for p75NTR in the SCN was abolished (Fig. 24).

CaBP and FOS Immunostaining after ICV SAP

Animals with large lesions that became arrhythmic were excluded from this part of the analysis. The number of CaBP-immunopositive neurons in the SCN of SAP-treated rats that exhibited shorten circadian period was significantly lower than that seen in treated rats that displayed normal periods, and, overall the SCN of SAP-treated rats contained fewer CaBP immunoreactive cells than saline-treated rats ($F_{2,14} = 45.699$, $p<.0001$) (Figs. 24, 25). Light-induced FOS was noted in the SCN of all rats (Fig. 24),

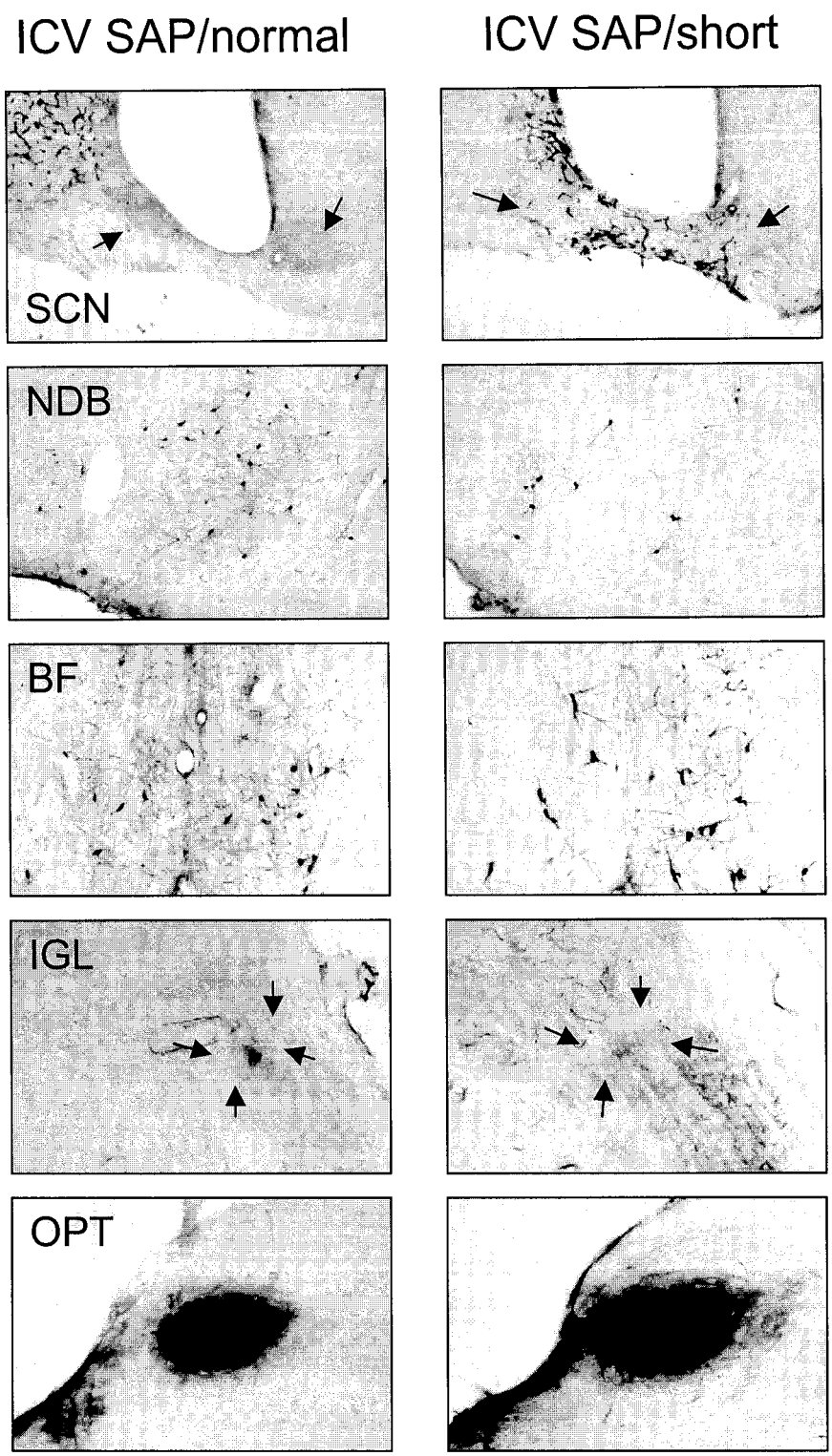


Figure 23. Photomicrograph showing examples of p75NTR immunostaining in the SCN, nucleus of the diagonal band (NDB), basal forebrain (BF), intergeniculate leaflet (IGL), and olivary pretectal nucleus (OPT) from a rat injected ICV with SAP that remained rhythmic following treatment (ICV SAP/normal) and from a SAP-treated rat that exhibited a sharp shortening of the free-running period following treatment (ICV SAP/short). Magnification = X10.

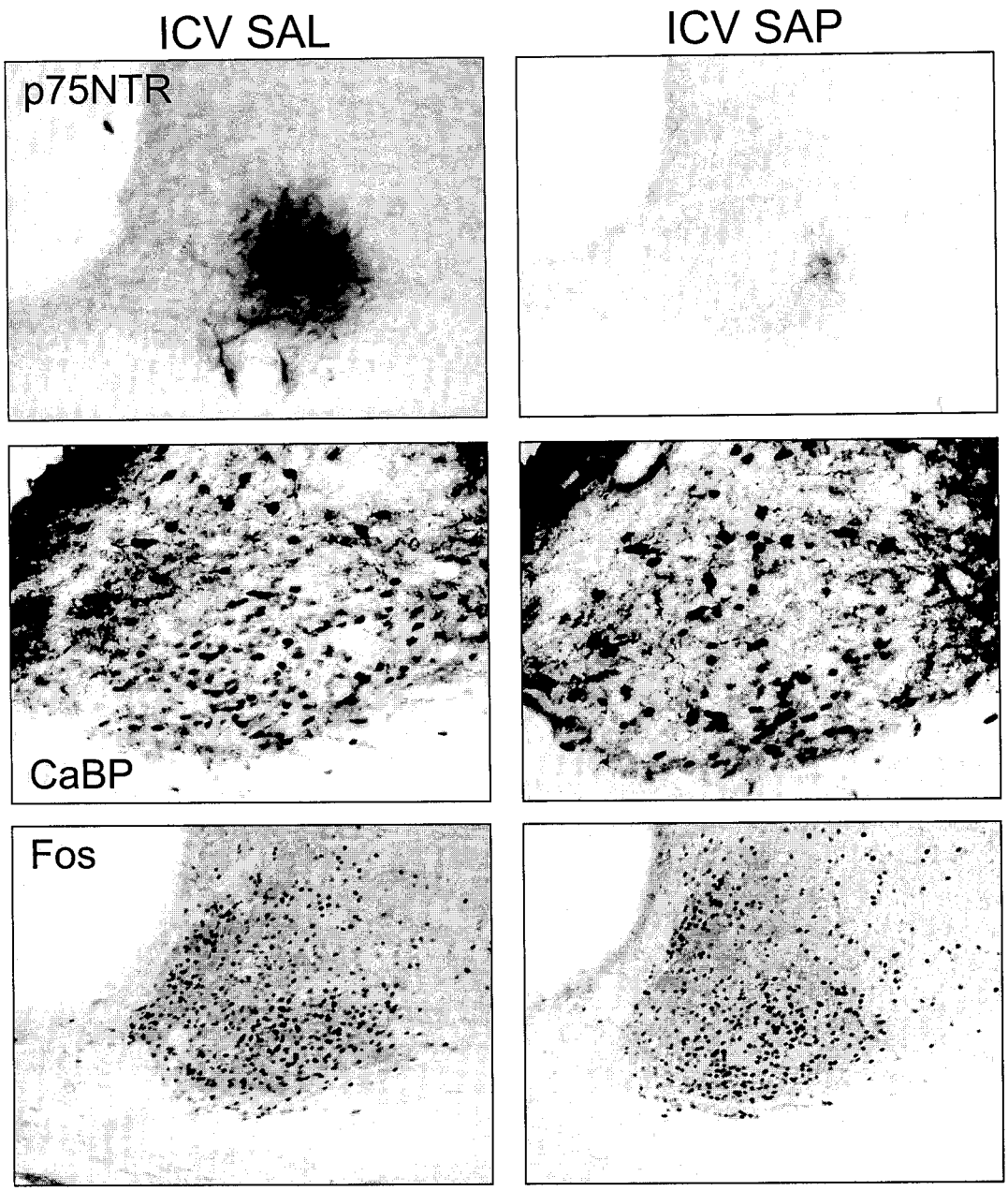


Figure 24. Photomicrograph showing examples of p75NTR, calbindin-D28k (CaBP) and FOS immunostaining in the SCN of rats treated ICV with saline (ICV SAL) or SAP (ICV SAP). Magnification: p75NTR and FOS images = X10; CaBP images = X20.

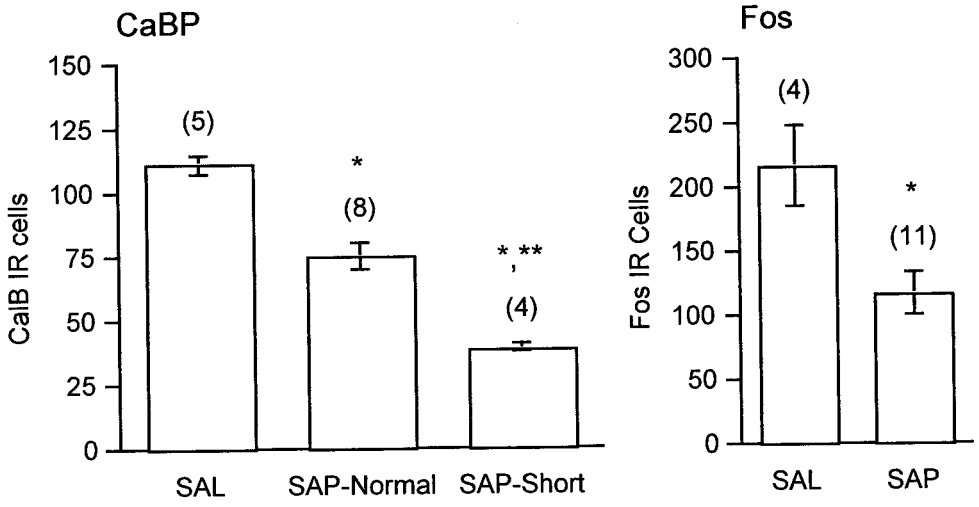


Figure 25. (Left) Mean ± SEM number of calbindin-D28k (CaBP)-immunoreactive cells on one side of the SCN in ICV saline-treated rats (SAL) and in ICV SAP-treated rats that exhibited normal (SAP-Normal) or short (SAP-Short) circadian period following treatment. (Right) Mean ± SEM number of light-induced FOS-immunoreactive cells on one side of the SCN in SAL and SAP-treated rats. The number in brackets indicate the number of animals assessed for each marker. Asterisks: *significant difference from SAL; **significant differences from SAP-Normal; $P < .05$, Scheffé's test.

however, SAP treated animals had lower levels of light-induced FOS when compared to controls ($F_{1,13} = 9.395$, $p<.01$) (Fig. 25).

Circadian Behavior and Hypothalamic Integrity After Intrahypothalamic SAP

All 15 rats given bilateral intrahypothalamic injections of SAP remained rhythmic and entrained to LD cycles. Of these, only one animal displayed shortened free-running period. Intrahypothalamic SAP produced small localized lesions marked by intense vascularization in 11 out of the 15 rats. Importantly, however, none of the rats displayed lesions which caused bilateral damage to the SCN. Intrahypothalamic injections of saline did not affect rhythmicity or entrainment (not shown).

p75NTR, CaBP and FOS Immunostaining after intrahypothalamic SAP

Bilateral intrahypothalamic injections of SAP abolished p75NTR immunostaining in the SCN (Fig. 26, 27). Immunostaining in extra-SCN regions was largely unaffected (Fig. 26). Overall, intrahypothalamic injections of SAP reduced CaBP immunostaining ($F_{2,17} =17.7$, $p<.0001$) but had no effect on light-induced FOS expression in the SCN ($F_{1,10} =1.973$, n.s.) (Figs. 27, 28).

Discussion

These studies demonstrate that rats with complete depletion of p75NTR immunostaining in the SCN, and intact or only partially-damaged SCN were rhythmic, responded normally to entraining light cycles, and exhibited light-induced Fos expression in the SCN. These results confirm and extend those of our previous studies showing that

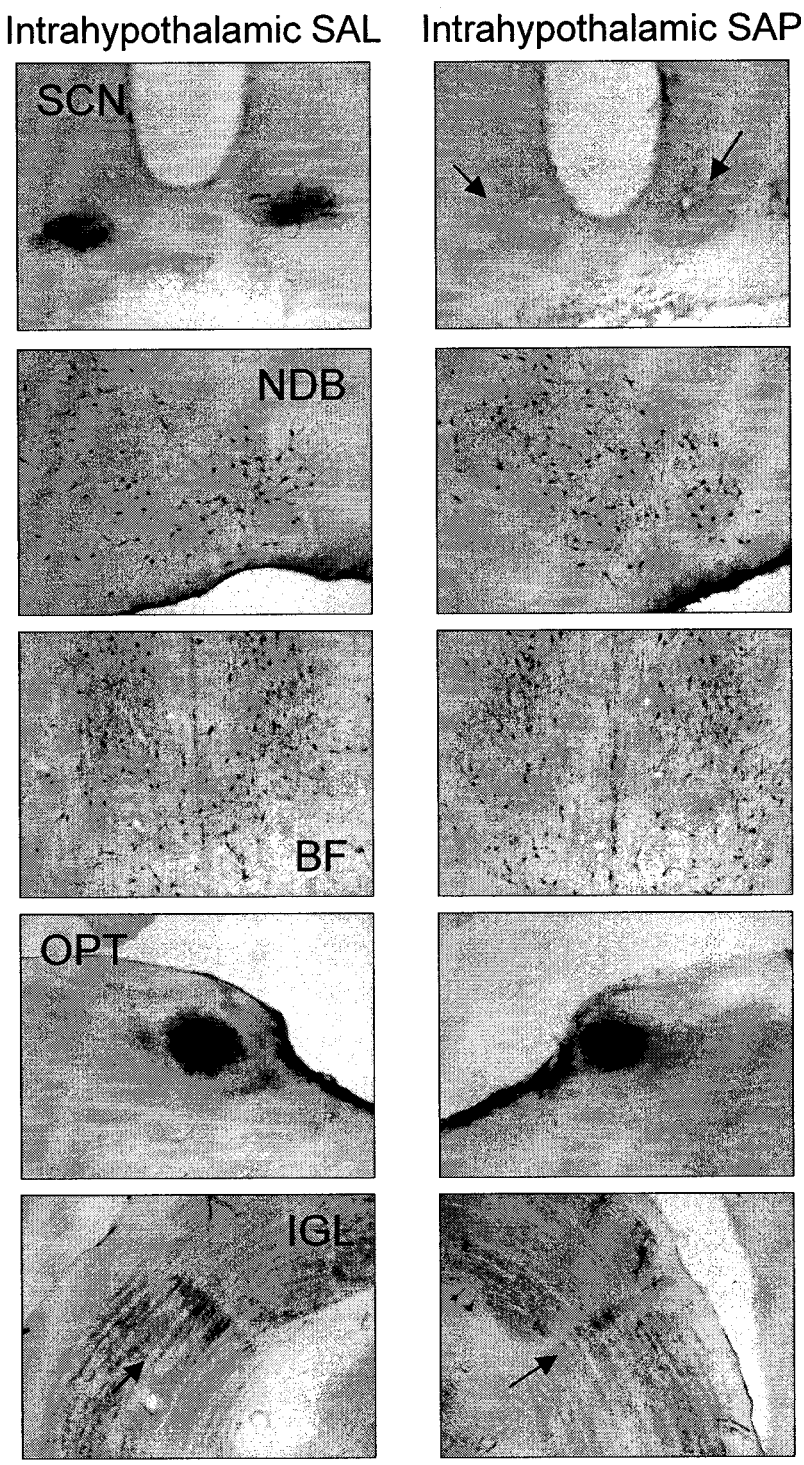


Figure 26. Photomicrograph showing examples of p75NTR immunostaining in the SCN, nucleus of the diagonal band (NDB), basal forebrain (BF), olivary pretectal nucleus (OPT), and intergeniculate leaflet (IGL) following bilateral intrahypothalamic injections of saline (intrahypothalamic SAL) or SAP (intrahypothalamic SAP). Magnification = X10.

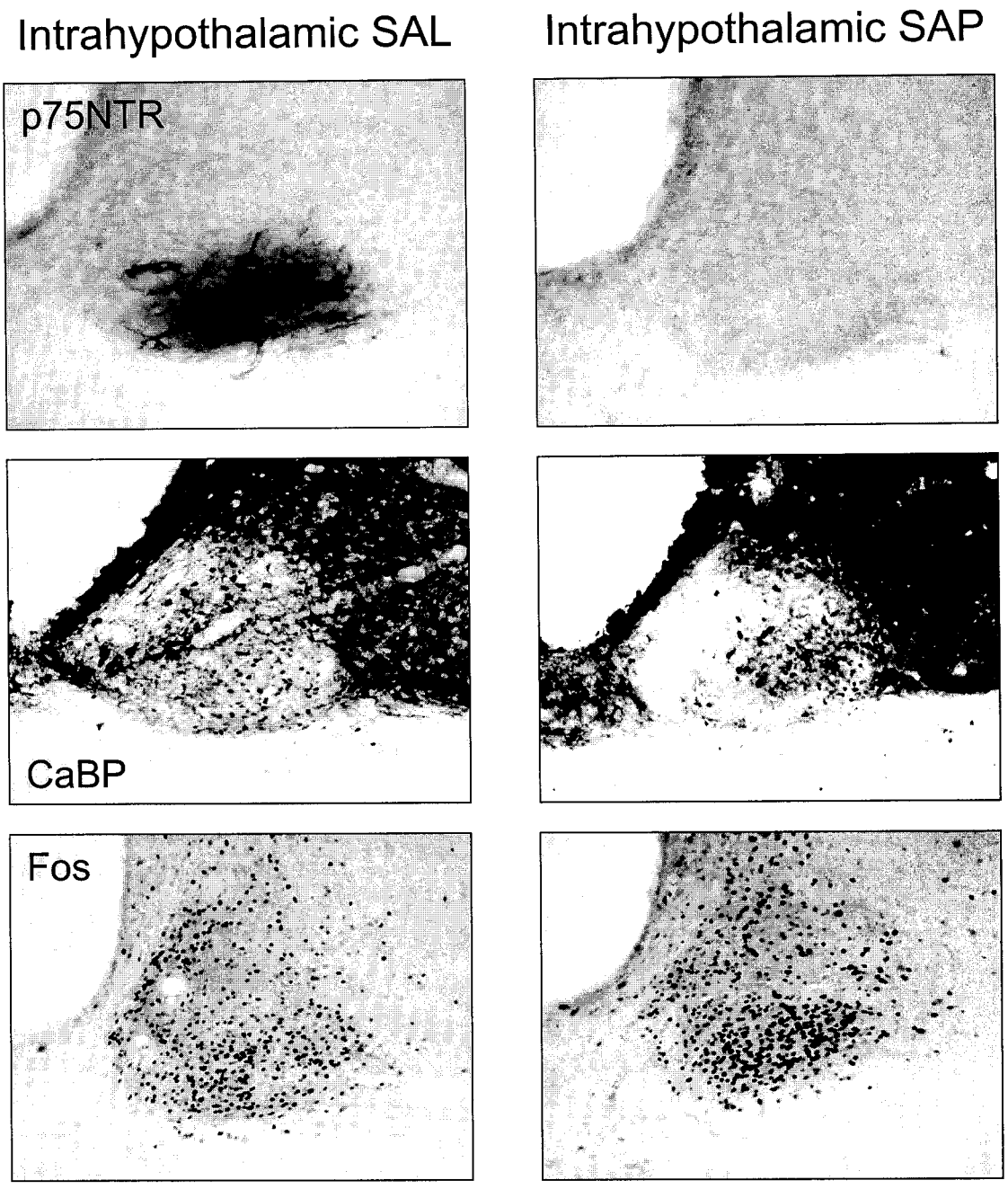


Figure 27. Photomicrograph showing examples of p75NTR, calbindin-D28k (CaBP), and FOS immunostaining in the SCN of rats following bilateral intrahypothalamic injections of saline (SAL) or SAP. Magnification = X10.

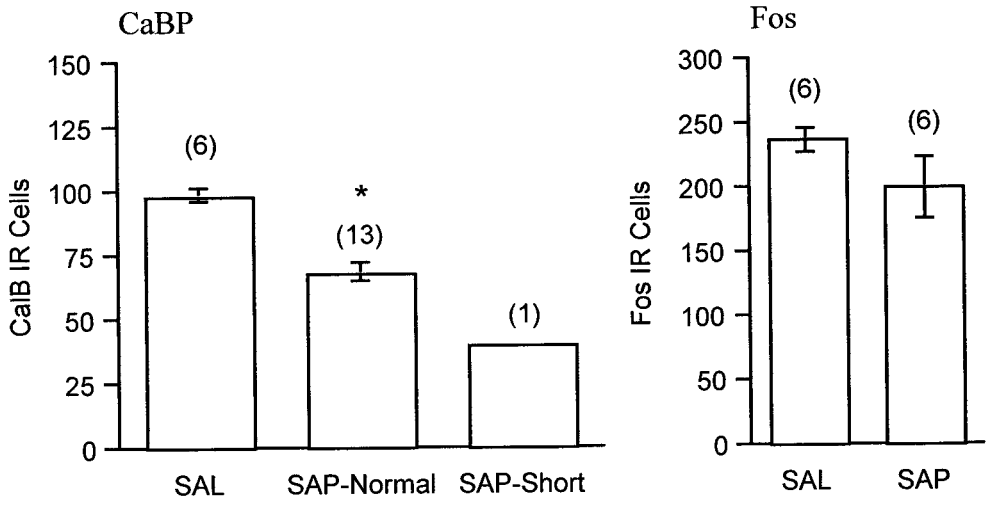


Figure 28. (Left) Mean ± SEM number of CaBP-immunoreactive cells on one side of the SCN in intrahypothalamic saline-treated rats (SAL) and in intrahypothalamic SAP-treated rats that exhibited normal (SAP-Normal) or short (SAP-Short) circadian periods following treatment. (Right) Mean ± SEM number of light-induced FOS-immunoreactive cells on one side of the SCN in intrahypothalamic SAL- and SAP-treated rats. The number in brackets indicate the number of animals assessed for each marker. Asterisk indicate significant difference from SAL, $P < .05$, Scheffé's test.

neonatal treatment with MSG almost completely diminished p75NTR immunostaining in the SCN, but had no effect on photic entrainment (Beaulé & Amir, 2001; Edelstein & Amir, 1999a; Edelstein et al., 1995). Together, these data indicate that the expression of p75NTR in the SCN is not required for the transmission of entraining photic information to the SCN. Our findings contrast with those showing that mice with a targeted deletion of the p75NTR gene show an attenuation in light-induced phase shifts of circadian rhythms (Golombek, Hurd, Lee, & Ralph, 1996). However, the full extent of this mutation, its developmental impact, and, most importantly, its effect on normal photic entrainment have not yet been clarified.

The results of the current study shed important new light on the pathways through which neurotrophic factors might modulate the circadian clock. Recently, it has been shown that the neurotrophic factor, brain-derived neurotrophic factor (BDNF) modulates photic input to the SCN and may be involved in the mechanism responsible for gating the temporal sensitivity of the circadian clock to light (Liang et al., 2000). Furthermore, it has been shown that retinal axons innervating the SCN express the high affinity tyrosine kinase B (Trk-B) neurotrophin receptor (Liang, Sohrabji et al., 1998), and that endogenous BDNF signaling via this receptor modulates photic input to the SCN (Liang et al., 2000; Liang, Walline et al., 1998). Interestingly, based on the apparent overlap in immunostaining for Trk-B and p75NTR in the SCN, it has been proposed that p75NTR modulates BDNF signaling via Trk-B in the SCN, as has been described in several neuronal systems, including the visual cortex and retina (Liang et al., 2000; Liang, Sohrabji et al., 1998). The results presented here indicate that the effect of BDNF on the

transmission of photic information to the SCN probably does not require p75NTR-mediated signaling. Nerve growth factor (NGF) also binds to Trk-B receptors as well as p75NTR, and, in hamsters, intra-SCN microinjections of NGF have been shown to induce phase shifts in free running rhythms (Bina & Rusak, 1996). The current results suggest that as with BDNF it is unlikely that these effects are mediated through p75NTR.

In the present study we used SAP to directly destroy p75NTR-expressing elements in the SCN. This approach was based on reports that a similar treatment abolished p75NTR immunostaining in the SCN without affecting neurons immunoreactive to VIP (Moga, 1998a). In view of this and of other reports that SAP has a selective effect on p75NTR-bearing neurons, it is puzzling that in many of our animals ICV injections of SAP produced hypothalamic damage that was often quite extensive. Other recent studies, however, have shown that SAP injections into regions of the brain rich with p75NTR-bearing neurons, such as the basal forebrain, produce local structural damage similar to that observed in the present study (Perry, Hodges, & Gray, 2001). As might be expected, SAP-treated rats with hypothalamic lesions extending to the entire SCN and optic chiasm rapidly became arrhythmic, whereas rats with only partial damage remained rhythmic and responded normally to entraining light cycles. Surprisingly, a proportion of the rhythmic rats with partial SCN lesions exhibited a sharp and sustained decrease in the length of the free-running period. Overall, we could not detect any consistent relationships between size and/or location of lesions and length of circadian period in DD. Thus these parameters do not provide a satisfying explanation for this behavioral outcome. One interesting possibility, however, is that the effect of SAP on

circadian period is linked to its effect on CaBP expression in the SCN. We found that, as a group, rhythmic rats that exhibited short circadian periods following ICV SAP had significantly fewer CaBP neurons in the SCN compared to rats with unaltered circadian period. The CaBP subnucleus within the core region of the SCN has been shown to play a key role in circadian rhythm generation and photic entrainment in hamsters (Bryant et al., 2000; LeSauter & Silver, 1999; Silver et al., 1996), and has been implicated in period determination in this species (LeSauter, Stevens, Jansen, Lehman, & Silver, 1999). Interestingly, however, whereas the present results point to a positive relationship between the number of CaBP-expressing cells and period length, data from hamsters suggest that period length is inversely related to the number of CaBP-bearing neurons in the SCN (LeSauter et al., 1999).

The current data strongly suggest that p75NTR-bearing retinal afferents are not necessary for transmission of entraining photic information to the SCN. The neurochemical identity of the afferents that do play a role remains unresolved. Recent studies have shown that a proportion of retinal afferents mediating photic input to the SCN in rats contain pituitary adenylate cyclase-activating polypeptide (PACAP) and, in addition, express the photopigment, melanopsin, a putative circadian photoreceptor (Berson, Dunn, & Takao, 2002; Hannibal et al., 1997; Hannibal et al., 2002; Hannibal et al., 2000; Hattar et al., 2002). PACAP-containing retinal afferents appear to play multiple roles in the transmission of photic information to the SCN. Photic stimulation induces Fos expression in PACAP-containing retinal ganglion cells both during the day and at night (Hannibal, Vrang, Card, & Fahrenkrug, 2001). PACAP, administered in nanomolar

concentrations mimics the phase-shifting effect of light on circadian activity rhythms as well as the effect of glutamate on neuronal activity rhythms and clock gene expression in the SCN (Akiyama et al., 2001; M. E. Harrington, Hoque, Hall, Golombek, & Biello, 1999; Piggins, Marchant, Goguen, & Rusak, 2001). At micromolar concentrations PACAP modulates the effect of glutamate on neuronal activity rhythms in the SCN (Chen et al., 1999). Furthermore, PACAP administration has been shown to induce phase shifts in vitro in SCN neuronal activity rhythms during the day, when neither light nor glutamate are effective (Hannibal et al., 1997). An interesting and possibly related finding is that chronic infusion of BDNF into the SCN of rats promotes light-induced phase shifts during the day when endogenous BDNF is normally low (Liang et al., 2000; Liang, Walline et al., 1998). Such a finding might suggest that BDNF modulates activity in PACAP containing retinal afferents. A stimulatory effect of PACAP on Trk-B receptor activity in hippocampal neurons has been described (Lee, Rajagopal, Kim, Chang, & Chao, 2002). Based on the above results and those obtained from the current study one would predict that PACAP and melanopsin containing retinal afferents express Trk-B but not p75NTR, and, furthermore, that they are resistant to the neurotoxic effect of SAP and neonatal MSG treatment. Indeed, a recent study indicates that neonatal MSG treatment reduces, but does not completely abolish PACAP immunostaining in the SCN in rats (Hannibal et al., 2001).

In conclusion, our findings show that the expression of p75NTR immunoreactivity in SCN is not required for photic entrainment of circadian rhythms. Because the retina is known to be the main source of p75NTR immunoreactivity in the

SCN, we propose that the *absence* of p75NTR expression might be a defining feature of the population of retinal afferents that mediate photic entrainment in rats. This information can be used to further identify photoreceptors and elements and processes within the SCN critical for photic entrainment of mammalian circadian rhythms.

Acknowledgments

Supported by grants to S.A. from the Natural Sciences and Engineering Council of Canada, the Canadian Institute of Health Research, and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR, Québec).

EXPERIMENT 6

Melanopsin in the Circadian Timing System

Christian Beaulé, Barry Robinson, Elaine Waddington Lamont, and Shimon Amir

The Journal of Molecular Neuroscience, *in Press*

Abstract

In mammals, circadian rhythms are generated by a light-entrainable oscillator located in the hypothalamic suprachiasmatic nucleus (SCN). Light signals reach the SCN via a dedicated retinal pathway, the retinohypothalamic tract (RHT). One question that continues to elude scientists is whether the circadian system has its own dedicated photoreceptor or photoreceptors. It is well established that conventional photoreceptors, rods and cones, are not required for circadian photoreception, suggesting that the inner retinal layer might contribute to circadian photoreception. Melanopsin, a novel photopigment expressed in retinal ganglion cells (RGC), has been proposed recently as a candidate circadian photoreceptor. Melanopsin containing RGC are intrinsically photosensitive, form part of the RHT, and contain neurotransmitters known to play a critical role in the circadian response to light. Furthermore, melanopsin containing RGC do not depend on inputs from rods and cones in order to transmit light signals to the SCN. However, based on a review of the available information about melanopsin and on new data from our laboratory, we propose that melanopsin, in itself, is not necessary for circadian photoreception. In fact, it appears that of the known photoreceptor systems, none, in and of itself, is necessary for circadian photoreception. Instead, it appears that within the photoreceptive systems there is some degree of redundancy, each contributing in some way to photic entrainment.

Introduction

The presence of circadian rhythms in physiology and behavior is one of the defining characteristics of living organisms. These rhythms are driven by cellular clocks that use autoregulatory molecular transcriptional/translational feedback loops and that are entrained to the environmental light cycle. Photic entrainment of circadian clocks is critical for optimal functioning allowing proper temporal alignment between physiological variables and the light cycle. In mammals, the master circadian clock is contained within the cells of the hypothalamic suprachiasmatic nucleus (SCN) (Klein et al., 1991). Photic signals reach the SCN directly from the retina through the retinohypothalamic tract (RHT) (Moore and Lenn, 1972; Johnson et al., 1988). Although the role of the SCN as the master light-entrainable circadian pacemaker has been recognized for more that three decades in mammals, the nature of the retinal cells essential for the transmission of photic information from the eye to the SCN has and continues to elude scientists. The recent discovery of novel photosensitive molecules within the mammalian retina was therefore regarded as an important breakthrough, potentially providing a mechanism for circadian photoreception.

The Retinohypothalamic Tract and Circadian Photoreception

The RHT is a dedicated, monosynaptic pathway between the retina and the SCN (Moore and Lenn, 1972; Johnson et al., 1988). The RHT is formed by a small subset of retinal ganglion cells (RGC) that do not contribute to the image formation pathway. RGC forming the RHT are anatomically homogeneous adopting the type III or W-cell morphology (Perry, 1979). The retinal ganglion cells forming the RHT terminate within

the ventrolateral region of the SCN, the retinorecipient core, where they interface with clock cells. Daily photic activation of the SCN core cells by environmental light occurs through the release of glutamate and pituitary adenylate cyclase activating peptide (PACAP) from retinal afferents, leading to clock resetting and stable entrainment of circadian rhythms (Ebling, 1996; Hannibal et al., 1997).

Unlike many non-mammalian species that have the capacity for extra-ocular photoreception, mammals require eyes in order to detect light. Although the visual and circadian pathways are anatomically and functionally distinct, both originate from the retina and use RGC to carry photic information. Consequently, the search for the mammalian circadian photoreceptor has focused on the retina. Initial studies were conducted to identify the optimal wavelength of light necessary for photic entrainment in rodents and humans. It was shown that photic entrainment in hamsters was most sensitive to a wavelength around 500 nm (light blue/turquoise color). In humans, light-induced melatonin suppression, an assay commonly used to monitor circadian photic sensitivity, is maximal for wavelengths around 480 nm (Takahashi et al., 1984; Provencio and Foster, 1995; Brainard et al., 2001). These two wavelengths are well within the range of an opsin-based photopigment (like rods and cones). However, from the study of rodless-coneless mice (*rd/rd/cl*), it was discovered that circadian photoreception was distinct from classical visual photoreception. These mice show severe retinal degeneration due to the total loss of rods and cones (Freedman et al., 1999). In spite of the retinal damage, these visually blind mice still entrain to light, show suppression of pineal melatonin secretion following light presentation at night, and show an intact pupillary light reflex (Freedman et al., 1999; Lucas et al., 2001), indicating sparing of the circadian visual

pathway. In the *rd/rd/cl* mice the pupillary light reflex is most sensitive to a wavelength of 479 nm (Lucas et al., 2001). This wavelength is again within the range of an opsin-based photoreceptor even though these mice lack such classical opsin-based photoreceptors. Additional evidence challenging the role played by classical photoreceptors in circadian photoreception comes from human literature showing that some blind individuals who have eyes but no visual perception due to a retinal defect, retain the circadian part of the photic response: light-induced melatonin suppression and entrainment of circadian rhythms (Czeisler et al., 1995).

In view of the fact that classical photoreceptors do not play a critical role in circadian photoreception, researchers began to focus on the inner layers of the retina, and in particular, on the ganglion cell layer. Indeed, it has now been shown that the RGC that project to the SCN are intrinsically photosensitive and continue to be so in spite of either surgical isolation or blocked neurotransmission from rods and cones (Berson et al., 2002; Hattar et al., 2002). RGC that are not part of the RHT lack such intrinsic photosensitivity. Furthermore, the peak absorption of the light-activated RGC was found to be 484 nm, again consistent with behavioral data on photic sensitivity of the circadian system and with opsin-based photoreceptors (Berson et al., 2002). The critical next step was to identify what makes some RGC photosensitive and to determine whether these photosensitive cells contribute to the circadian response to light.

Melanopsin as a Mammalian Circadian Photoreceptor

Many non-mammalian vertebrates have the capacity for extra-ocular photoreception. Within these species, the skin, several deep brain nuclei, and the pineal

gland contain functional photopigments that are photosensitive and that can mediate the effects of light in the absence of the eyes (Groos, 1982). Of these photopigments, melanopsin is of special interest. Melanopsin was first identified in photosensitive dermal melanophores, deep brain nuclei, and the iris of *Xenopus* (Provencio et al., 1998). Subsequently, melanopsin was found in the retinal pigment epithelium cells (RPE) of the inner retina of frog and fish (Provencio et al., 1998; Soni et al., 1998). In vertebrates, RPE is responsible for the re-isomerization of the chromophore. Once re-isomerized, the chromophore is carried back to the photoreceptor where it regenerates the bleached visual pigment. Melanopsin within the RPE of lower vertebrates does not appear to contribute to re-isomerization. Rather, it appears to be a phototransducing opsin, since it does not separate from its chromophore and does not appear to require third-party re-isomerization (Provencio et al., 1998).

Recently both the human and mouse melanopsins have been cloned and their expression shown to be localized to the inner layer of the retina, within ganglion and amacrine cells (Provencio et al., 2000). Melanopsin was found to be present in a small subset of RGC that project to the SCN, intergeniculate leaflet, and olivary pretectal nucleus, structures of the non-image forming visual pathway (Hattar et al., 2002). In rats approximately 2.5% of RGC were found to be melanopsin positive (Hattar et al., 2002), expressing immunoreactivity in the cell body, dendrite and axon. The dendritic fields of these cells were extensive, overlapping significantly with adjacent cells (Hannibal et al., 2002; Hattar et al., 2002; Provencio et al., 2002). Furthermore, melanopsin was shown to colocalize with PACAP (Hannibal et al., 2002), a circadian neuromodulator present exclusively in RGC forming the RHT (Hannibal et al., 2002). Finally, all RGC that were

shown to be directly light sensitive were melanopsin positive and their spectral sensitivity corresponded to the behavioral action spectrum of photic entrainment in rodents (Berson et al., 2002). RGC that did not respond to light and did not project to the SCN lacked melanopsin.

Melanopsin and the Circadian Response to Light

Recently, two lines of melanopsin deficient mice were developed; a melanopsin knockout (KO) and a melanopsin null (*Opn4*$^{-/-}$) (Panda et al., 2002; Ruby et al., 2002). Although melanopsin is non-functional in both these mouse lines, the circadian behavior of these animals did not differ from that of wild-type controls. Free-running circadian periods were identical for intact and melanopsin deficient mice (approximately 23.5 hours) suggesting that the presence of melanopsin is not required for the generation of the circadian oscillation (Panda et al., 2002; Ruby et al., 2002). Melanopsin deficient mice entrained to a 12h light/12h dark cycle with no difference in the phase angle of entrainment (the relationship between light offset and activity onset), total activity, or length of activity (Panda et al., 2002; Ruby et al., 2002). Furthermore, the ability of light to suppress activity during the dark phase of the cycle (masking) was intact in *Opn4*$^{-/-}$ mice (not tested in KO mice) (Panda et al., 2002). In addition, the ability of light to induce the expression of the immediate early gene *c-fos* was normal in the KO mice (not tested in *Opn4*$^{-/-}$) suggesting that light-activation of the retinorecipient SCN is not affected by lack of melanopsin (Ruby et al., 2002).

Slight, but significant, impairments were noted in the magnitude of the light induced phase shifts (Panda et al., 2002; Ruby et al., 2002). Melanopsin deficient mice

showed a reduced magnitude of light-induced phase shift for light pulses presented during the first half of the night (circadian times (CT) 15 and 16; CT12 is the onset of the subjective night, the active phase in nocturnal animals). In the KO mice, the magnitude of the phase shift in response to white light was decreased by 36% as compared to wild type (Ruby et al., 2002). Although this difference is statistically significant, KO mice still exhibited phase shifts exceeding 60 minutes, which is more than sufficient to explain the normal entrainment to a 24h light cycle. Phase shifts in *Opn4*$^{-/-}$ mice were produced by presentation of different irradiances of monochromatic light with a wavelength specific for melanopsin (480 nm). At this wavelength, melanopsin null mice still showed phase shifts but the magnitude was significantly lower than that in wild type mice (Panda et al., 2002). At maximal irradiance, *Opn4*$^{-/-}$ mice showed phase shifts of approximately 40 minutes, again more than sufficient to mediate photic entrainment to a 24-h day.

Finally, the role of melanopsin in the pupillary light reflex and the circadian response to constant light (LL) was investigated using melanopsin KO mice. Pupillary constriction in response to a brief light pulse was present, although the magnitude and velocity of the response were less than those in wild-type mice (Lucas et al., 2003). Similarly, both melanopsin deficient and intact mice showed significant lengthening of the free-running period when housed in LL (Panda et al., 2002; Ruby et al., 2002). However, the magnitude of the period increase was smaller in melanopsin deficient animals than in controls.

Melanopsin and the Circadian Response to Constant Light

The results from the experiments just described do not provide support for a critical role for melanopsin in circadian photoreception mediating normal photic entrainment. They do suggest, however, that melanopsin might be involved in mediating the acute effect of light on pupillary constriction and the disruptive effects of LL on circadian rhythms. To study the role of melanopsin in the circadian response to light we assess the expression of melanopsin in the circadian systems of rats that were treated with the neurotoxin monosodium glutamate (MSG) during the neonatal period.

Methods

The experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia University.

Animals and treatments

Wistar rat pups received five subcutaneous injections of either 2 mg/g MSG (Sigma) dissolved in distilled water, or 10% saline (to control for the osmolarity of the MSG) on postnatal days 1,3,5,7, and 9, as previously described (Edelstein et al., 1995). Neonatal enucleation was performed on 3 days old male Wistar rat pups, under Isoflurane anaesthesia, by severing the optic nerve, muscle and other connective tissues and removing the eyes. Animals were weaned at 21 days of age, and male rats were housed two per cage under a 12h:12h light-dark cycle with free access to food and water. Two months later, rats were housed individually in cages equipped with running wheels. The cages were housed in individual isolation chambers equipped with a ventilation system

and a timer-controlled cool white fluorescent light source (300 lux at eye level). Wheel running activity rhythms were monitored continuously using VitalView data acquisition hardware and software (Mini Mitter Co. Inc., Sunriver, OR).

Tissue preparation

The rats were anaesthetized with sodium pentobarbital (100 mg/kg i.p.), in the dark, and perfused transcardially with 300 ml of cold physiological saline (0.9% NaCl) followed by 300 ml of cold 4% paraformaldehyde in a 0.1M phosphate buffer (pH 7.3). Following perfusion, the brains were removed and postfixed in 4% paraformaldehyde at 4° C overnight. Serial coronal brain sections (50 μm) containing the SCN were collected from each animal using a vibrotome.

Immunocytochemistry

Free floating brain sections and whole retinas (for melanopsin immunocytochemistry) were washed in cold 50 Mm Tris buffered saline (TBS; pH 7.6) and incubated at room temperature for 30 minutes in a quenching solution consisting of TBS and 30% w/w H_2O_2. Next, the tissues were rinsed in cold TBS and incubated for 1 hour at room temperature in a pre-blocking solution made of 0.3% Triton X 100 in TBS with milk buffer (TBS+MB, 0.05g milk powder / ml TBS) and 5% normal goat serum. Next, tissues were transferred directly into a solution containing a rabbit polyclonal antibody raised against the c-terminus of rat melanopsin (1:800, Donated by Dr. King-Wai Yau, see Hattar et al., 2002). In some experiments, brain sections were incubated in a solution containing a rabbit polyclonal antibody raised against PACAP-38 (1:8000,

Peninsula, #IHC8920) or a mouse monoclonal antibody against p75NTR (1:30,000, Chemicon). The antibodies were diluted in a solution of 0.3% Triton X 100 in TBS+MB with 3% normal goat serum and sections were incubated for 64 hours at 4°C. Following incubation in the primary antibody, tissues were rinsed in cold TBS and incubated for 1 hour at 4°C with a biotinylated anti-rabbit IgG made in goat (Vector Labs), diluted 1:400 with 0.3% Triton X 100 in TBS with 2% normal goat serum. Following incubation with secondary antibody, tissues were rinsed in cold TBS and incubated for two hours at 4°C with an avidin-biotin-peroxidase complex (Vectastain *Elite* ABC Kit, Vector Labs). Following incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris-HCl (pH 7.6), and again for 10 minutes with 0.05% DAB in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 minutes in DAB/Tris-HCl with 0.01% H_2O_2 and 8% $NiCl_2$. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel-coated slides and allowed to dry overnight. Sections were then dehydrated through a series of alcohols, soaked in Citrisolv (Fisher), and cover-slipped with Permount (Fisher). Slides were inspected under a light microscope using a computerized image acquisition and analysis system with NIH Image software (v 1.62).

Results

Neonatal MSG treatment and the Circadian Response to Constant Light

In rats, the neonatal treatment with MSG produces a circadian phenotype strongly resembling that produced by genetic deletion of melanopsin in mice, that is, intact photic entrainment accompanied by diminished sensitivity to constant light (LL; Pickard et al.,

1982; Chambille and Serviere, 1993; Edelstein et al., 1995; Chambille, 1998; Beaulé and Amir, 2001). As shown in Fig. 29, neonatal MSG treatment dramatically reduces the number of retinal ganglion cells and causes atrophy of the optic nerves. In spite of severe degeneration of the retina and optic nerves and a substantial decrease in the number of RGC innervating the SCN (Chambille and Serviere, 1993; Chambille, 1998) MSG treated animals exhibit normal photic entrainment and show phase dependent light induction of Fos protein within the SCN (Fig. 29) (Pickard et al., 1982; Chambille and Serviere, 1993; Edelstein et al., 1995; Chambille, 1998; Beaulé and Amir, 2001). Furthermore, and consistent with the effect of genetic deletion of melanopsin in mice, neonatal MSG treatment significantly decreases sensitivity to the disruptive effects of LL (Edelstein et al., 1995; Edelstein and Amir, 1999; Beaulé and Amir, 2001). An example of the effect of neonatal MSG treatment on the circadian response to LL in rats is shown in Fig. 30. It can be seen that in normal rats exposure to LL initially causes a lengthening of the circadian period, similar to the period lengthening observed in mice. After several weeks in LL, rats become behaviorally arrhythmic. In contrast, MSG treated rats remain rhythmic in LL, with an increased period length, compared to their endogenous free-running rhythm (Fig. 30) (Edelstein et al., 1995; Edelstein and Amir, 1999; Beaulé and Amir, 2001).

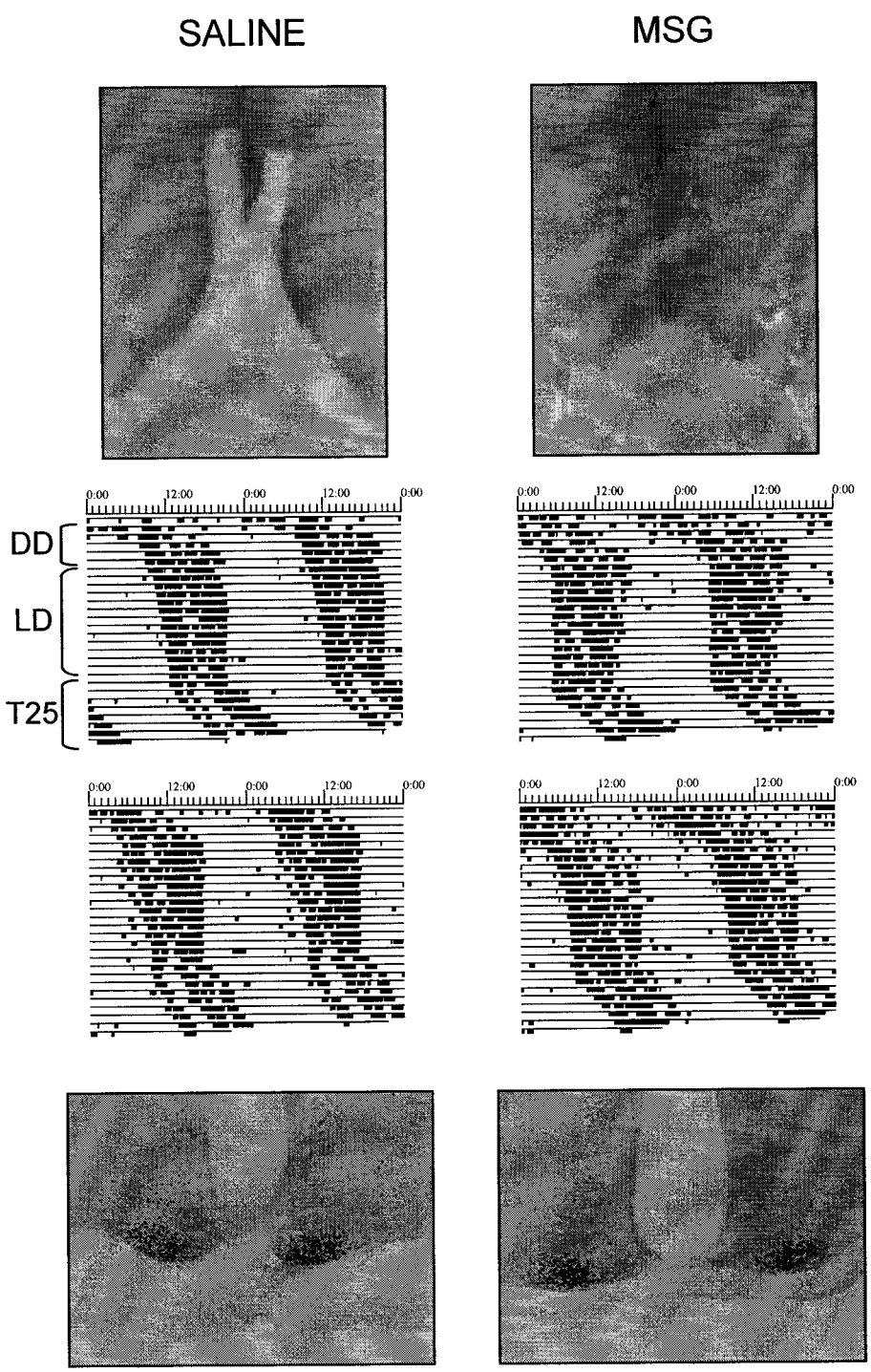


Figure 29. (Top) Photomicrographs showing the optic nerve and optic chiasm of a saline (SALINE) and a MSG treated rat. (Middle) Double plotted behavioral actograms showing free-running (DD), entrainment to a 12h light-12h dark (LD) cycle and entrainment to a 0.5h light-24.5h dark (25h T-Cycle, T25) cycle in two saline and two MSG treated rats. (Bottom) Representative photomicrographs showing light-induced FOS protein expression in the SCN of a saline and a MSG treated rat. Magnification X6.3.

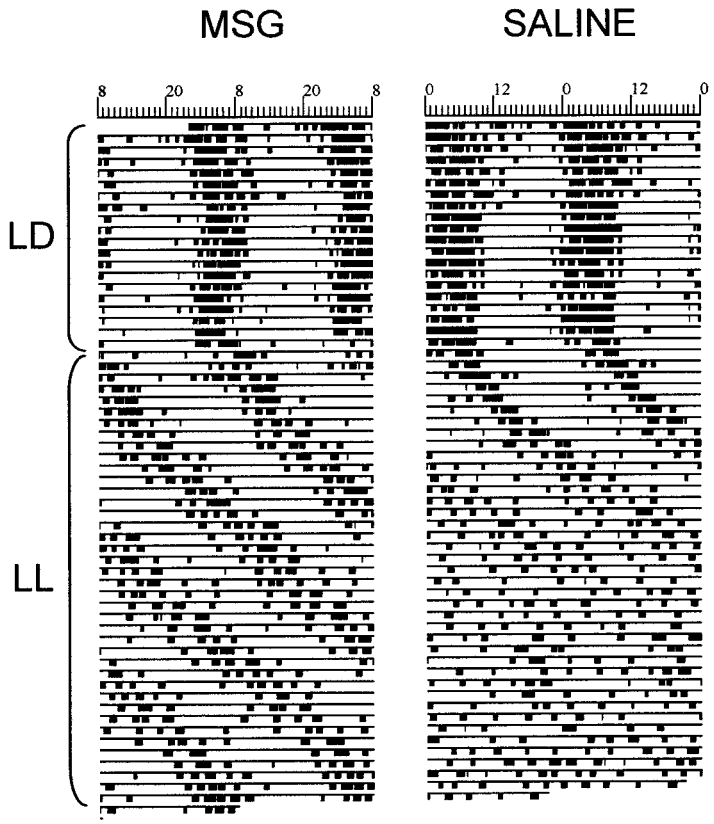


Figure 30. Representative double-plotted behavioral actograms showing the effects of constant-light (LL) exposure in an animal treated with MSG or saline.

Neonatal MSG Treatment and Melanopsin Expression in the Retina

Immunocytochemistry for melanopsin was performed using conventional methods (see Experimental Procedures), with a previously characterized antibody targeting the c-terminal region of rat melanopsin (Hattar et al., 2002). As shown in Fig. 31, and consistent with previous reports (Hannibal et al., 2002; Hattar et al., 2002; Provencio et al., 2002) melanopsin immunoreactivity was present in the rat retina. Cells immunoreactive for melanopsin were diffusely distributed, had large dendritic fields and showed visible axonal fibers coursing to the optic disk. Neonatal treatment with MSG dramatically reduced the number of melanopsin positive RGC (Fig. 32). Melanopsin immunoreactive RGC in the retina of MSG treated animals were morphologically identical to those in the saline treated controls. The dendritic arborization was visually more distinct in the retina of MSG treated animals likely due to the decrease in number of RGC and reduced thickness of the retina. Thus, although melanopsin immunoreactivity was not completely abolished in the retina, the substantial reduction in the melanopsin-mediated input to the central circadian structures could explain the resistance of MSG animals to the effects of LL.

Melanopsin Expression in the SCN

Melanopsin immunoreactivity was also present within the core region of the SCN. In three separate replications, immunostaining of brain sections from saline-treated rats revealed the presence of a dense plexus of melanopsin immunoreactive fibers in the SCN. Immunostaining was confined to the ventral lateral region of the SCN and was observed throughout the rostral-caudal extent of the nucleus (Fig. 33). The localization of the

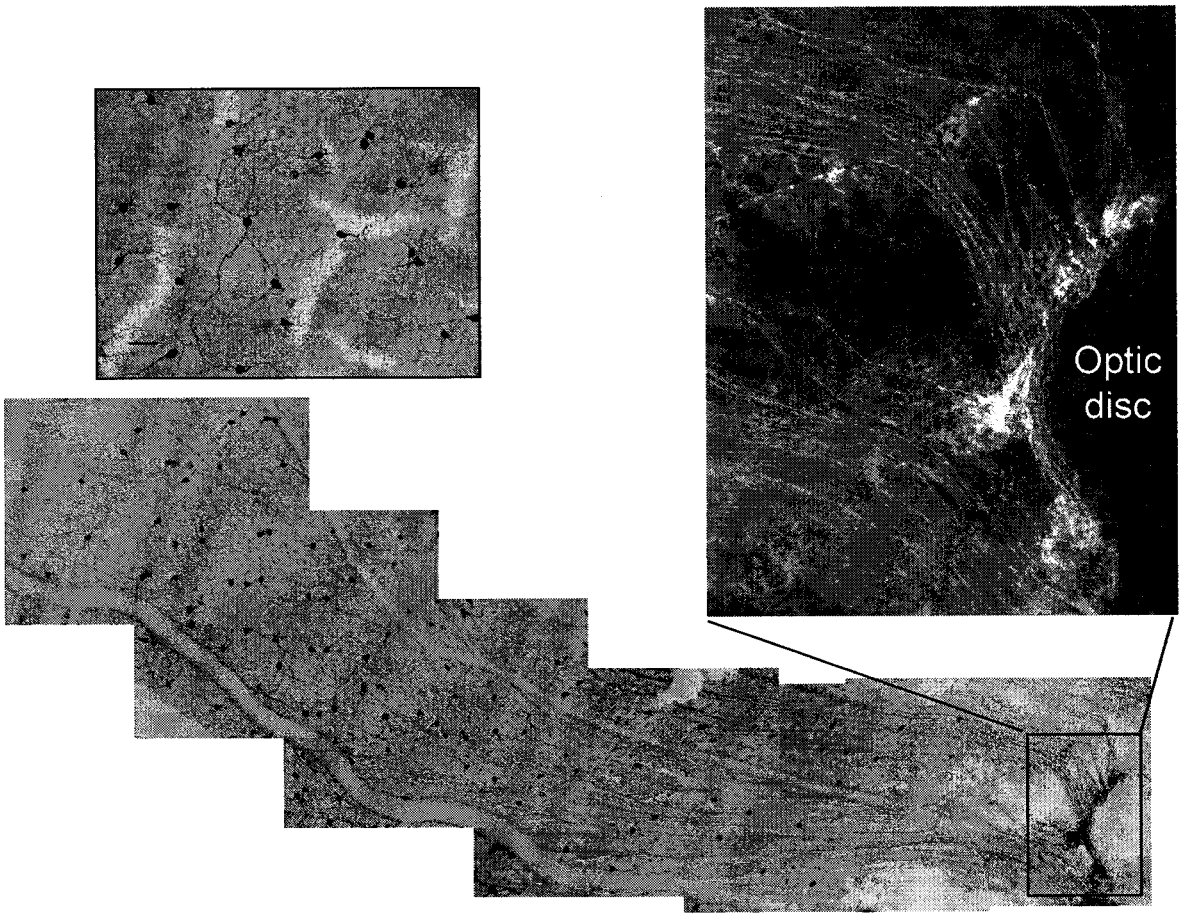


Figure 31. Composite photomicrographs showing the distribution of melanopsin-containing retinal ganglion cells and the converging of the axons towards the optic disk. Boxed area shows the exit point of the optic disk with a high power inverted photomicrograph showing the axons coursing to the optic disk. Scale bar 50 μm.

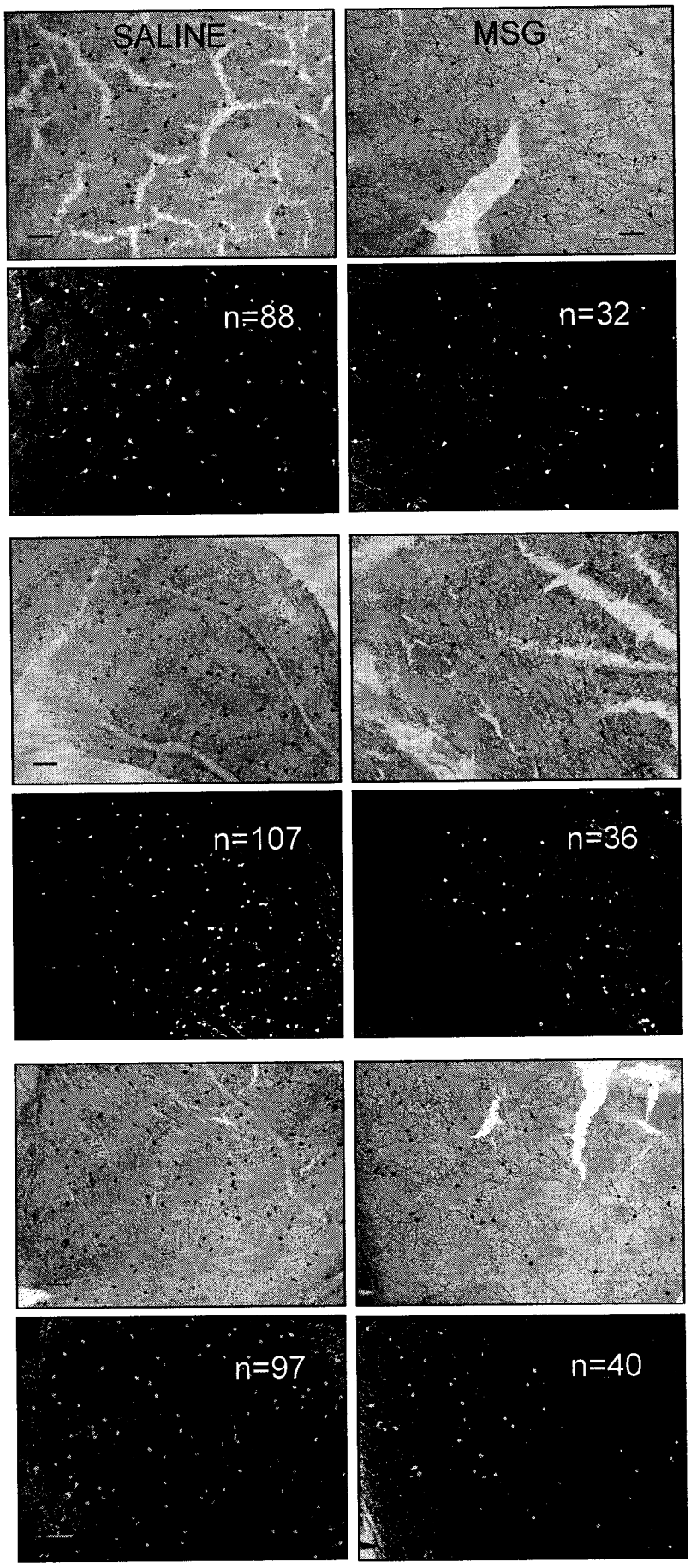


Figure 32. Bright field and inverted photomicrographs comparing the number of melanopsin containing retinal ganglion cells in saline and MSG treated rats. Numbers in the inverted images represent the total number of melanopsin immunoreactive cells present within the visual frame. Magnification **X10**.

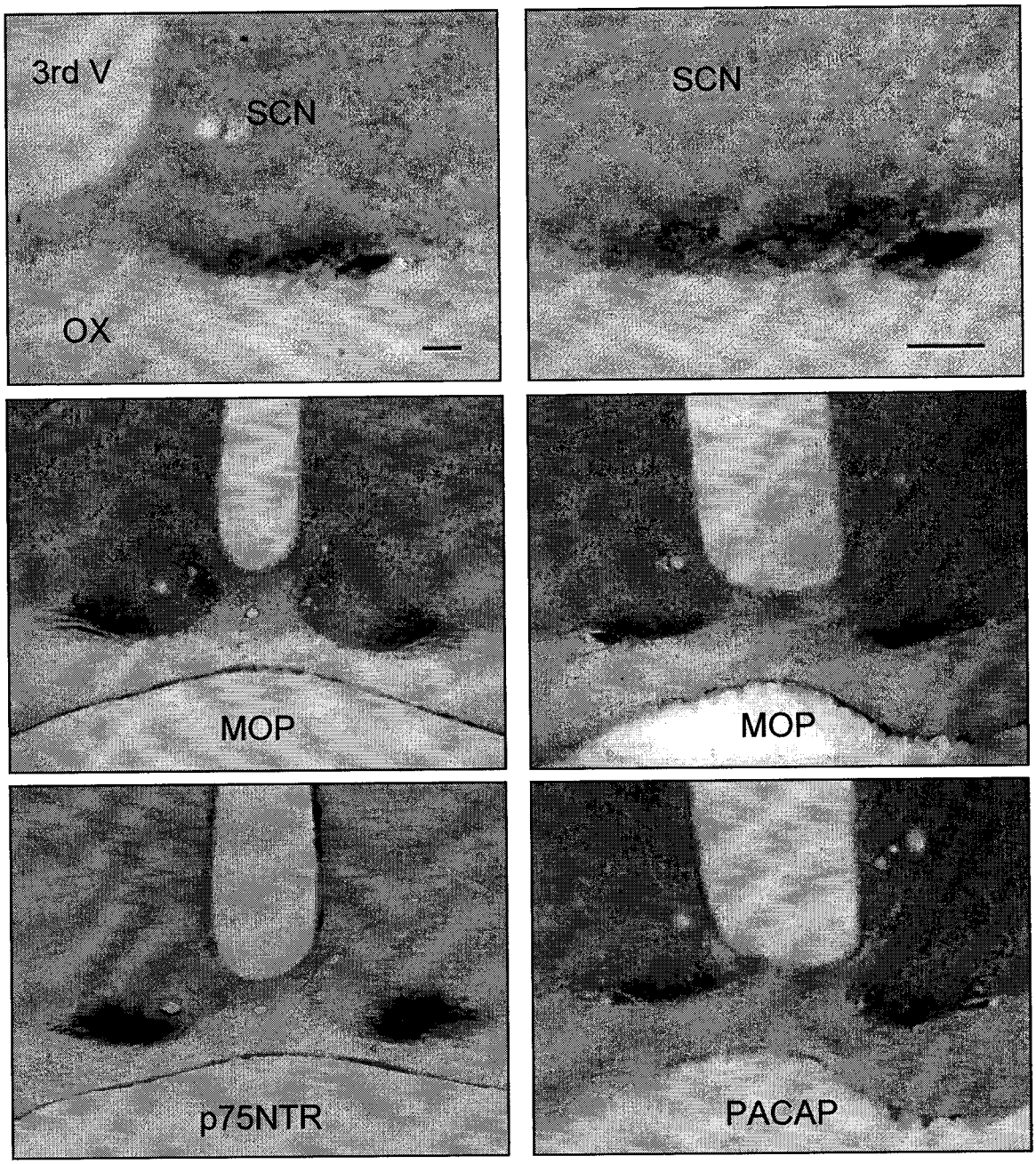


Figure 33. (Top) Representative photomicrographs showing melanopsin (MOP) immunoreativity within the rat SCN (Scale bars, 50 μm.). (Middle and Bottom) Representative photomicrographs showing that the melanopsin immunoreactive plexus is in the same location as the p75NTR and PACAP, within the core SCN region. 3rd V, third ventricle; OX, optic chiasm.

melanopsin immunoreactive plexus was consistent with other RHT markers such as p75NTR and PACAP (Fig. 33). In contrast, in each of the three replications, melanopsin immunostaining in the SCN of rats treated with MSG during the neonatal period was virtually eliminated (Fig. 34). The finding of a dense melanopsin immunoreactive fiber plexus in the SCN using the antibody raised against the c-terminal sequence of melanopsin is in conflict with the results obtained with the n-terminal antibody, where no melanopsin staining was visible in the brain (Hattar et al., 2002). It is possible that the melanopsin protein gets truncated and that only the c-terminal portion remains present within the axons projecting to the SCN core. Variations in the sensitivity of the immunocytochemical assays due to technical differences between laboratories may further explain this discrepancy.

The ganglion cell layer of the retina is a primary target of MSG during the neonatal period, and RGC that express melanopsin are the only likely source of melanopsin immunoreactivity in the SCN. Hence, the observed decrease of melanopsin immunostaining in the SCN following neonatal MSG treatment can be linked directly to the loss of melanopsin containing RGC. To gain further support for this contention, in the third replication we also assessed the effect of neonatal MSG treatment on PACAP expression in the SCN. PACAP is present in the cell bodies, dendrites and axon terminals of retinal afferents to the SCN, and retinal ganglion cells that express melanopsin also express PACAP (Hannibal et al., 1997; Hannibal et al., 2002). Consistent with our interpretation of the effect of MSG, and in agreement with a previous report (Hannibal et al., 2001), we found that neonatal treatment with MSG greatly diminished the expression of PACAP immunoreactivity in the ventral lateral SCN region (Fig. 35).

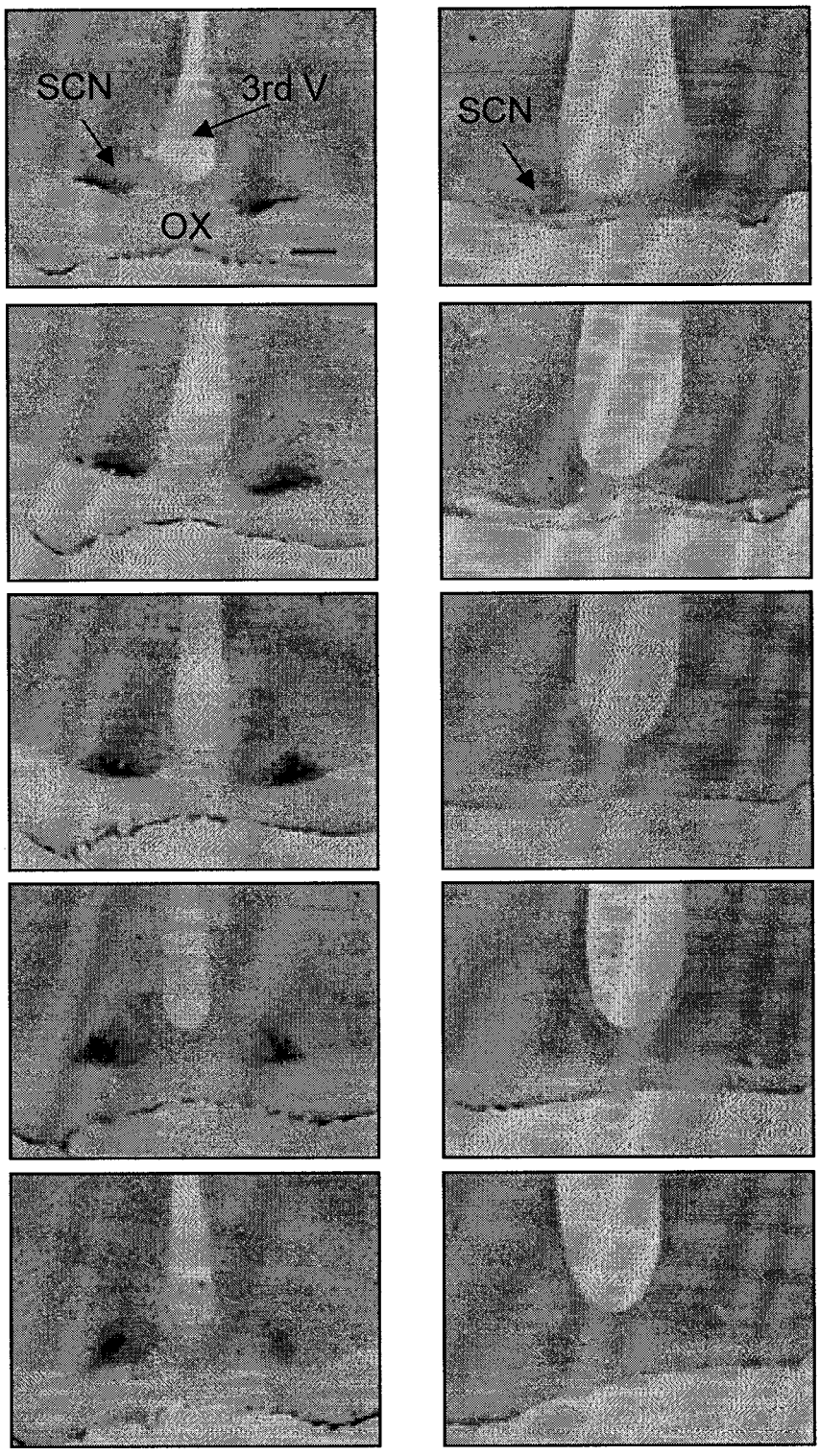


Figure 34. Photomicrographs showing immunostaining for melanopsin throughout the rostral-caudal extent of the SCN of adult rats treated with saline or MSG during the neonatal period. 3rd V, third ventricle; OX, optic chiasm. Scale bar, 100 μm.

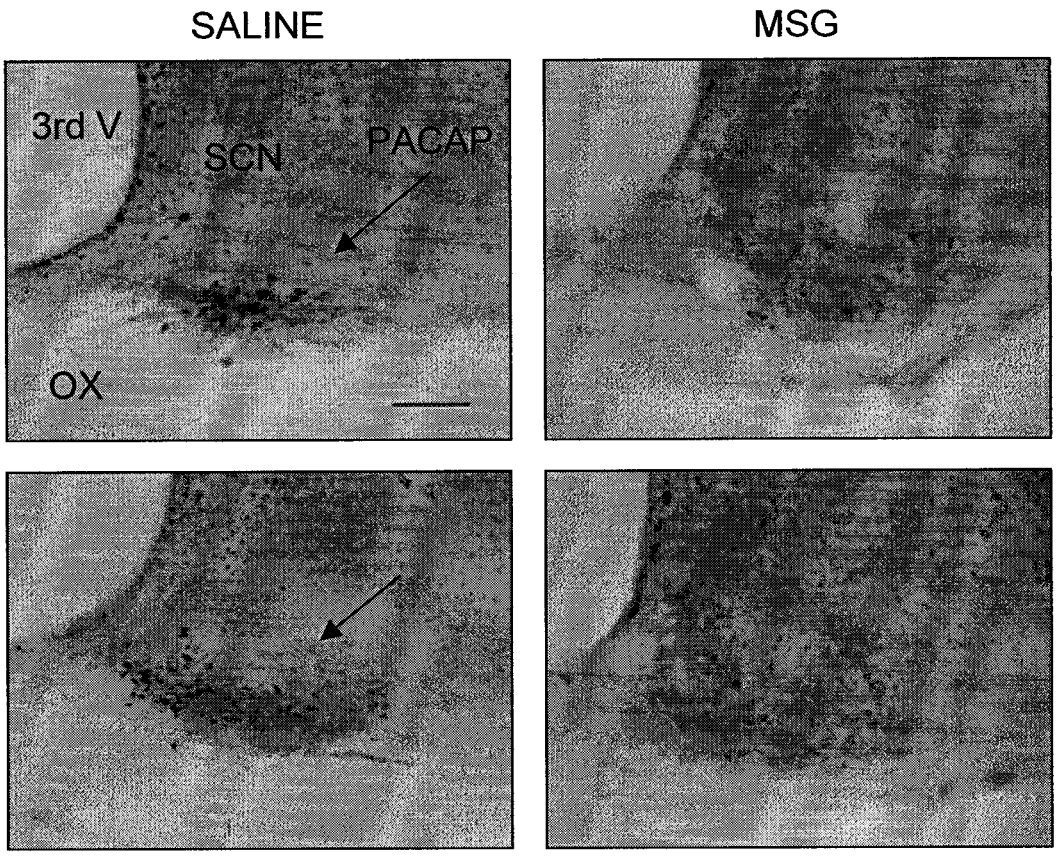


Figure 35. Photomicrographs showing immunostaining for PACAP in the SCN region of adult rats treated with saline or MSG during the neonatal period. 3rd V, third ventricle; OX, optic chiasm. Scale bar, 100 μm.

Effect of Unilateral Enucleation on Melanopsin Expression in the SCN

Next, we examined the expression of melanopsin and p75NTR immunoreactivity in the SCN of rats that were subjected to unilateral orbital enucleation (UOE) on postnatal day 3. As shown in Fig. 36, staining for both melanopsin and p75NTR is virtually eliminated in the SCN contralateral to the missing eye. Specifically, the SCN contralateral to the missing eye shows staining for melanopsin and p75NTR that is similar to that seen in MSG treated animals, whereas the SCN ipsilateral to the missing eye shows staining that is similar to saline-treated control rats. Furthermore, the optic chiasm of the UOE rat is thick on the side ipsilateral to the missing eye (similar to the optic chiasm of saline-treated rats), whereas its thickness is severely reduced on the side contralateral to the missing eye (similar to MSG animals).

Effect of 192IgG-Saporin on Melanopsin Expression in the SCN

Because the RHT is heterogeneous, with cells containing at least p75NTR, PACAP, and melanopsin, we decided to investigate the extent of the colocalization between the expression of these RHT markers within the SCN core. We have shown previously that complete removal of p75NTR immunoreactivity within the SCN core by use of the neurotoxin 192IgG-saporin (SAP) has little effects on photic entrainment (Beaulé and Amir, 2002). In order to determine whether p75NTR, and melanopsin are colocalized within axons of the RHT innervating the SCN, we lesioned the p75NTR plexus by intrahypothalamic infusion of SAP and stained the SCN for both p75NTR and melanopsin. We found that complete removal of p75NTR immunoreactivity from the

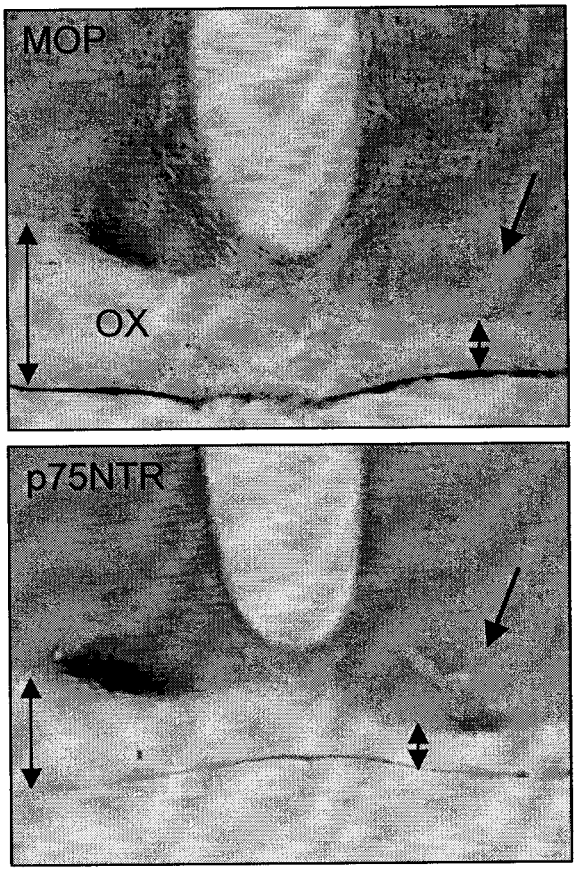


Figure 36. Photomicrographs showing melanopsin (MOP) and p75NTR immunoreactivity in animals that underwent unilateral orbital enucleation on postnatal day 3. Single arrows point to the SCN contralateral to the missing eye. Double arrows show the thickness of the optic chiasm (OX). Magnification X6.3.

SCN resulted in a decrease in melanopsin staining in the SCN (Fig. 37) that was smaller than that seen in MSG treated rats (Fig. 37).

Discussion

The fact that saporin treatment did not abolish melanopsin suggests that there are p75NTR containing cells that do not express this photopigment. These cells probably carry visual signals from classical photoreceptors to the SCN, highlighting the likelihood of parallel and redundant photic inputs from various types of photopigments to the SCN. Consistent with this idea, it has been shown recently that a proportion (10-20%) of RHT fibers projecting to the SCN do not contain melanopsin (Sollars et al. 2002). Finally, the finding that neonatal MSG treatment abolishes melanopsin staining in the SCN but has only a partial effect on melanopsin expression in the retina suggests the possibility that the retina contains two distinct populations of melanopsin-containing RGC: cells expressing the photopigment in their dendrite, cell body and entire axon length, and cells that express melanopsin only in their cell body and in their dendritic field. The cells containing melanopsin in their axons might be more sensitive to the neurotoxic effect of neonatal MSG treatment. RGC, which do not express melanopsin in their axons, may be more resistant to the toxic effect of MSG, but would not be visible in the SCN.

In conclusion, our findings, taken together with the evidence from melanopsin deficient mice lead to the conclusion that retinal afferents to the SCN that contain melanopsin are not necessary for photic entrainment. A more likely role for the melanopsin containing retinal ganglion cells is in the effect of constant light exposure on circadian rhythms. The evidence that retinal ganglion cells expressing melanopsin are intrinsically sensitive to

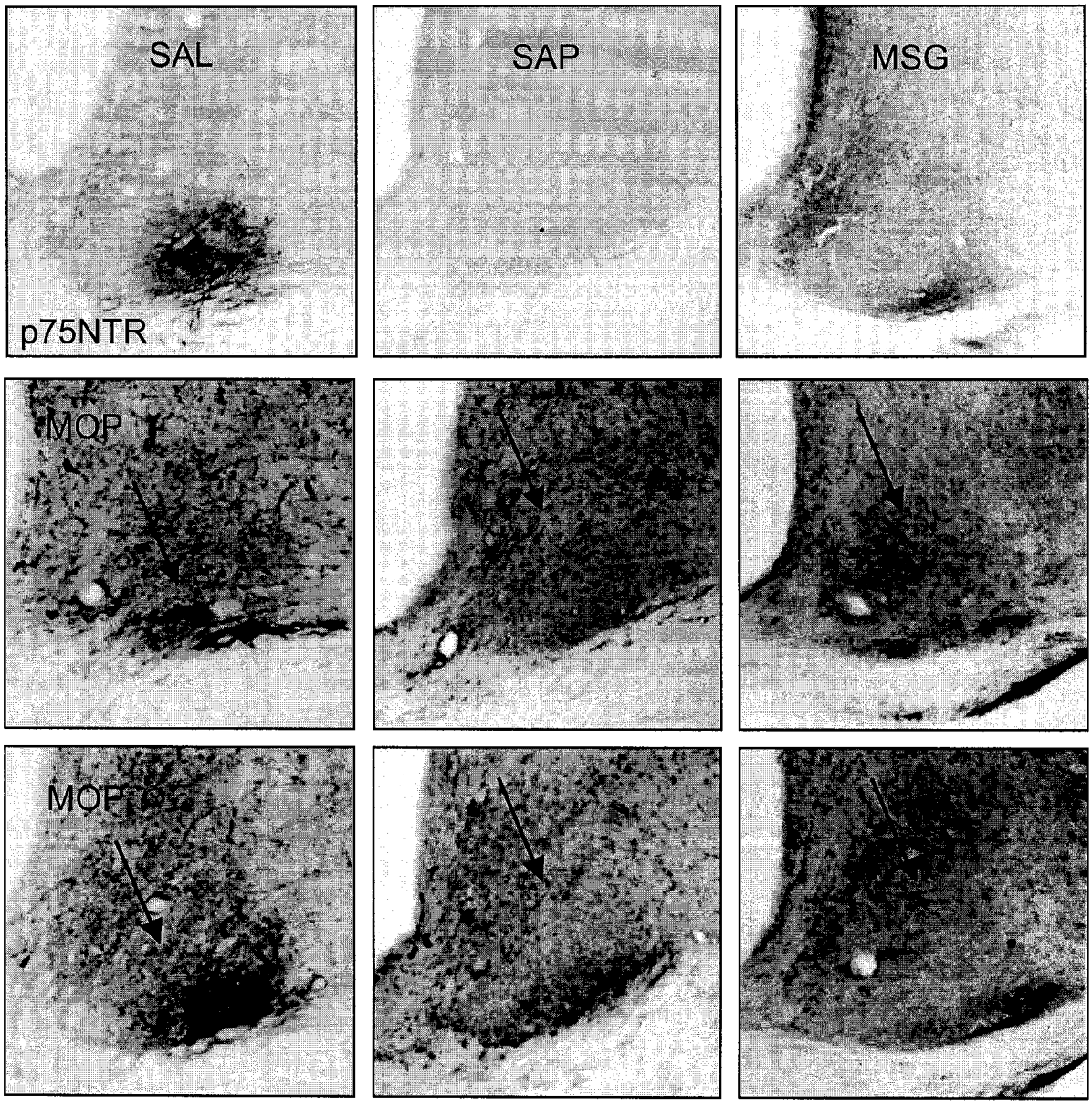


Figure 37. Representative photomicrographs showing p75NTR (Top) and melanopsin (MOP) immunoreactivity in the rostral (Middle) or medial (Bottom) SCN of saline (SAL), 192-IgG-Saporin (SAP), or MSG treated rats. Magnification X12.6.

light, respond tonically to light, and innervate structures such as the intergeniculate leaflet that also respond tonically to light, is consistent with this hypothesis (Edelstein and Amir, 1996; Berson et al., 2002; Hattar et al., 2002). The fact that neonatal treatment with MSG attenuates the disruptive effect of constant light exposure on circadian rhythms in rats, decreases substantially the number of melanopsin containing RGC, and virtually eliminates melanopsin-containing afferents to the SCN adds further support for the contention that melanopsin-containing retinal ganglion cells mediate the effects of tonic light exposure on the circadian system.

Other Circadian Photoreceptors?

If melanopsin is not the critical circadian photoreceptor, then other photosensitive molecules must serve that role. The mammalian retina contains another type of photopigment, different from the opsin-based pigments, the cryptochromes (CRY1 and CRY2). Cryptochromes are flavoprotein-based photopigments that are evolutionarily related to the light-activated repair enzyme photolyase and to one class of plant blue-light photoreceptors (Ahmad and Cashmore, 1993; Adams et al., 1995; Cashmore et al., 1999; Todo, 1999; Sancar, 2000). A role for cryptochromes in circadian photoreception has been proposed in plants (*Arabidopsis*) and insects (*Drosophila*) (Hall, 2000; Young, 2000; Williams and Sehgal, 2001). In mammals, cryptochromes are present both in the ganglion cell layer of the retina and in the SCN where they function as an integral part of the interacting transcriptional/translational feedback loops generating the circadian oscillation (Miyamoto and Sancar, 1998; Thresher et al., 1998; Miyamoto and Sancar, 1999; van der Horst et al., 1999). Although CRY double mutant mice are arrhythmic in

constant darkness due to disruption of the SCN molecular clockwork, they still show behavioral masking in response to light (van der Horst et al., 1999). In these animals, classical photoreceptors and melanopsin are intact and could mediate this response. Recently, triple mutants mice bearing the *rd/rd, cry1/cry1, cry2/cry2* mutations were generated (Selby et al., 2000). These triple mutant mice lack rods, cones, CRY1 and CRY2 in the retina and their circadian clocks are disabled due to a lack of cryptochromes. Unlike the CRY double mutants, the triple mutants fail to show masking (Selby et al., 2000). They do, however, show photic induction of Fos protein in the SCN, albeit severely reduced as compared to wild type mice. The residual Fos induction in triple mutant mice suggests that some other pigment, probably melanopsin, is mediating this effect (Selby et al., 2000).

A role for cryptochromes in the transmission of photic input to the SCN has been proposed as a result of experiments in which the regular opsin photopigments were disrupted. Vitamin A deprivation in retinol-binding protein deficient mice ($RBP^{-/-}$) leads to a complete loss of retinal activity due to inactivation of all opsin-based photopigments such as rhodopsin, color opsins and melanopsin (Thompson et al., 2001). In these mice, the light-induction of two of the period genes (*Per1* and *Per2*) is unaffected, suggesting that photic signals are able to reach, and reset, the SCN clock. Cryptochromes are sufficient to mediate photic transmission to the SCN in absence of functional opsin-based photopigments. Again, from this evidence, it can be proposed that regular opsins are not required for circadian photoreception. However, unlike regular opsin-based photopigments, melanopsin might not require re-isomerization of its chromophore (Provencio et al., 1998) and might be less sensitive to vitamin A deprivation.

Conclusion

In conclusion, the hope that melanopsin would prove to be the primary "circadian photoreceptor" in mammals has to date not been fulfilled. Rather, current evidence suggest that there are multiple, redundant photosensitive systems capable of transmitting photoperiodic information to the circadian system (Fig. 38). The ubiquity of circadian systems throughout the phylogenetic scale would explain the presence of the plant CRY proteins and invertebrate and amphibian melanopsin in the mammalian circadian visual pathways. The appearance of so-called classical photoreceptors (rods and cone) can be considered to be a later addition of light sensitive photoreceptive mechanisms capable of contributing to photic entrainment pathways of mammalian circadian rhythms. Because of this redundancy, it is likely that no single photopigment will be found to be necessary for photic entrainment.

Acknowledgements

We thank Jane Stewart for comments on the manuscript and Dr. King-Wai Yau, (Johns Hopkins University Medical School) for the generous gift of the c-terminal melanopsin antibody. Supported by grants from the Canadian Institute of Health Research and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche.

Types of Retinal Ganglion Cells, Photic Entrainment, and the Circadian Response to Constant Light

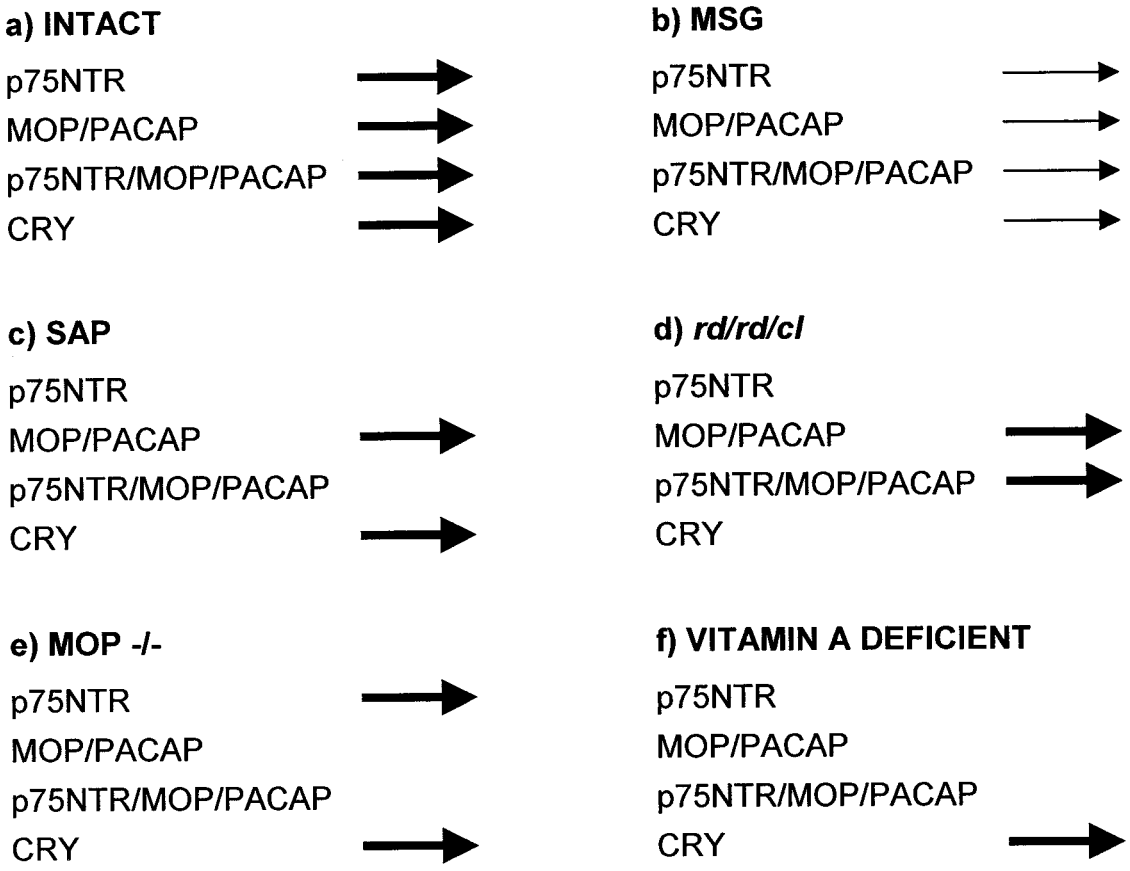


Figure 38. (a) In the intact animal, photic information necessary for entrainment is transmitted to the SCN via several distinct types of RGCs, including those that contain p75NTR; melanopsin and PACAP (MOP/PACAP); p75NTR, melanopsin, and PACAP (p75NTR/MOP/PACAP); and cryptochrome (CRY). These animals show normal photic entrainment and normal responses to constant light (i.e. arrhythmicity). (b) Following MSG treatment, the RGC layer is damaged and the number of SCN-projecting RGCs is dramatically reduced, irrespective of phenotype. MSG-treated animals show normal photic entrainment but an abnormal response to constant light. (c) Following 192IgG-Saporin (SAP) treatment, the RHT fibers bearing the p75NTR are eliminated but other RHT fibers are spared, allowing for normal entrainment and normal responses to constant light. (d) In rodless-coneless mice (*rd/rd/cl*), the only functional SCN-projecting RGCs are those that contain melanopsin. *rd/rd/cl* mice show normal photic entrainment and normal masking. (e) Like MSG-treated rats, MOP-/- mice show normal photic entrainment and abnormal responses to constant light. (f) In animals with vitamin A deficiency, which disables opsin-based photoreceptors, the transmission of photic information to the SCN is mediated by RHT fibers that contain cryptochromes. Vitamin A-deficient mice show normal photic entrainment.

GENERAL DISCUSSION

Three major findings emerge from the experiments contained in the present thesis. First, photic entrainment in rats is correlated with a suppression of FOS protein immunoreactivity in the SCN shell at a time when light normally induces FOS in the core. Secondly, constant light-induced behavioral arrhythmicity is accompanied by a disruption of the rhythmic expression of PER2 and FOS proteins in the SCN. Finally, drastic reduction in p75NTR, PACAP and melanopsin immunoreactivity within the SCN core does not prevent photic entrainment and suggests that the RHT conveys photic information to the SCN via redundant pathways. Taken together, these results show that coordinated changes in the activity of the SCN core and shell are important for both the generation of rhythmic behavior and for photic entrainment.

Measurement of FOS immunoreactivity within the SCN has proved a useful tool in the study of circadian function. The presence of FOS immunoreactivity in the SCN core is a direct correlate of photic resetting (Beaulé & Amir, 1999; Earnest et al., 1990; Edelstein et al., 2000; Ikonomov & Stoynev, 1994; Kornhauser et al., 1996; Rea, 1989; Rusak et al., 1990). The finding that, within the shell, high levels of FOS occur during the subjective day and lower levels occur during the subjective night (Guido, de Guido et al., 1999; Guido, Goguen et al., 1999; Jac, Sumova, & Illnerova, 2000; Sumova & Illnerova, 1998; Sumova et al., 2000; Sumova et al., 1998) means that expression of FOS within the shell can give an indication of SCN clock phase. The current results showing that light presented at dawn and dusk significantly suppresses FOS protein expression in the SCN shell are the first to demonstrate that light can affect this non-retinorecipient SCN

compartment and thus serves as a further example of the utility of studying FOS expression in investigating SCN function.

These findings raise a number of issues including the mechanism through which FOS expression in the shell is suppressed; why for example, is the ability of light to suppress FOS in the shell limited to dawn and dusk? And, what are the functional implications of this phenomenon? It is unlikely that light acts directly to modulate the activity of the shell in rats because it is well established that only the core receives retinal inputs (Johnson et al., 1988; Moore, 1973; Moore & Lenn, 1972; Pickard, 1982). Rather, it is more probable that photic activation of the retinorecipient core leads to an inhibition of the activity of a proportion of the cells of the shell through the activation of inhibitory interneurons that connect the core and shell (Leak et al., 1999; Leak & Moore, 2001; Moore et al., 2002) and that GABA mediates this inhibition. Both the core and shell of the SCN contain GABA and GABA-A receptors (Gao, Fritschy, & Moore, 1995; Moore & Speh, 1993; Okamura et al., 1989; van den Pol, 1986). In the SCN, activation of GABA-A receptors leads to a hyperpolarization of the membrane potential and inhibition of cell firing in vitro and in vivo (Gribkoff et al., 1999; Kim & Dudek, 1991; Liou, Shibata, Albers, & Ueki, 1990; Mason, Biello, & Harrington, 1991; Strecker, Wuarin, & Dudek, 1997). Furthermore, in hamsters, microinjection of the GABA-A agonist muscimol prevents light-induced FOS expression in the SCN core (Gillespie et al., 1999). With respect to the present results, inhibition of cell firing in the shell in response to GABA released from an inhibitory projection from the core could then lead to a suppression of the basal expression of FOS within the shell.

It is obviously of interest to identify the phenotype of those cells in which FOS expression is suppressed by light. The shell is comprised of cells that contain arginine-vasopressin (AVP), calretinin, enkephalin, somatostatin, and angiotensin II (Moore et al., 2002). A higher proportion of the cells in the shell express AVP than any other peptide, but there is no colocalization of AVP and FOS (Sumova et al., 2000). The core contains cells expressing vasoactive intestinal polypeptide, gastrin-releasing peptide, calbindin, and neurotensin (LeSauter & Silver, 1999; Moore et al., 2002; Silver et al., 1996). Given that GABA is present in all the cells in the SCN (Moore & Speh, 1993; van den Pol, 1986; but see also Castel & Morris, 2000), one would predict that these cells coexpress GABA and a peptide other than AVP and, in the absence of light, FOS.

A second pathway through which light could produce a suppression of activity within the SCN shell is via the geniculo-hypothalamic projection originating from the IGL. This projection releases the inhibitory neurotransmitters GABA and neuropeptide Y (NPY) (Card & Moore, 1989; Harrington, Nance, & Rusak, 1985; Moore, Weis, & Moga, 2000; Shibata & Moore, 1993). NPY immunoreactive fiber terminals are mostly restricted to the SCN core, but it is possible that the extent of this innervation overlaps with the beginning of the shell region. Because the cells showing a suppression of FOS expression are located primarily near the interface between the shell and the core, GABA and NPY released following photic stimulation of the IGL could in fact influence these cells directly and be responsible for the suppression of FOS expression. In addition, the fact that IGL lesioned animals fail to entrain to photocycles involving short light pulses (such as T-cycles and skeleton photoperiods, (Edelstein & Amir, 1999b)) could be an

indication that the IGL plays an important role during this process. An experiment using electrical stimulation of the GHT could be used to induce the release of NPY and GABA in the SCN and observe whether basal FOS expression in the shell is affected by such treatment. Another technique that could be used to investigate the role played by NPY and GABA in the suppression of FOS would be to infuse these substances into the SCN and see whether either or both would be sufficient to produce a suppression of FOS expression in the absence of light.

One aspect of light-induced FOS suppression in the shell demands further attention, its peculiar temporal restriction to dawn and dusk. It has been shown that the SCN gates its own sensitivity to incoming stimuli and that there are different pathways that are associated with this gating. Interestingly, these pathways are organized into three discrete "time domains" (Gillette & Mitchell, 2002) that are restricted to the day, the night, and dawn/dusk. Each domain is characterized by different intracellular signaling pathways that are mutually exclusive. Signaling during the dawn/dusk domain occurs through the action of melatonin on melatonin type 2 receptor and involves phospholipase C, diacylglyceride, and protein kinase G (Gillette & Mitchell, 2002). The SCN has been shown to be sensitive to melatonin at dawn and dusk (McArthur, Gillette, & Prosser, 1991; Starkey, Walker, Beresford, & Hagan, 1995; Stehle, Vanecek, & Vollrath, 1989), and administration of melatonin has been shown to cause phase advances at dawn and dusk in vitro (McArthur et al., 1991; McArthur, Hunt, & Gillette, 1997). Because pinealectomy was shown to have little impact on photic entrainment (Armstrong, Cassone, Chesworth, Redman, & Short, 1986; Cassone, Chesworth, & Armstrong, 1986),

studying the effect of pinealectomy on the expression of light-induced suppression of FOS protein in the SCN shell would provide additional information on the mechanism regulating this dawn/dusk time domain.

Functionally, light-induced suppression of cell firing in the shell offers one mechanism through which photic resetting of effector systems might be achieved. Once again, GABA is the probable mediator of these effects. The majority of SCN neurons are projection neurons that target predominantly the adjacent hypothalamus, preoptic area, subparaventricular zone, retrochiasmatic area and posterior hypothalamic areas (Leak & Moore, 2001; Thompson & Swanson, 1998; Watts & Swanson, 1987; Watts et al., 1987) and that would release GABA into these areas. Inhibition of cell firing in a proportion of the cells in the shell following photic entrainment would result in a disinhibition of these SCN targets, potentially leading to their resetting. Determining the hypothalamic targets of the cells that show suppressed activity in response to entraining light would provide information about the transmission of circadian outputs. Furthermore, it would render possible the investigation of the relationship between the resetting of the clock and resetting of effector centers.

Prolonged exposure to constant bright light produces complete behavioral arrhythmicity in rats. Three possible mechanism are thought to mediate this effect: (1) disruption of the molecular feedback loops within each SCN cell; (2) uncoupling between rhythmic SCN activity and effector systems; and (3) uncoupling between the rhythmic activity of individual clock cells. The results contained in the present thesis lend strong support to the first proposition and, to a lesser extent, the third. Following constant LL

exposure, the rhythmic expression of PER2 and FOS proteins is disrupted. The fact that the levels of these two proteins were low and essentially similar for all the animals assessed suggests that there is no longer any forward drive on the molecular oscillation and that the circadian clock is no longer rhythmic. The mechanism through which LL disrupts the rhythmic PER2 and FOS is unknown. It is now important to investigate the effects of LL on the expression of other clock genes, especially PER1 and the CRY since PER1 is induced by light and both PER1 and CRY are part of the negative component of the molecular feedback loop. LL-induced up regulation of PER1 protein expression could account for the suppression of PER2 levels and for the loss of circadian rhythmicity. As discussed in chapter 3, this hypothesis is consistent with the effects seen in PER2 knockout mice (Bae et al., 2001).

LL-induced behavioral arrhythmicity does not appear to be the result of a lack of coupling between the SCN and effector systems. This proposition assumes that the SCN is still rhythmic and that it is unable to send its timing signals to effector centers. If the SCN was indeed still rhythmic in LL, it should have been possible to observe PER2 and FOS protein expression levels that would have been comparable to the rhythmic expression seen under entrainment to a 24 T-Cycle, and this was not the case. It is also possible that during LL, the clock is sending "arrhythmic" timing signals to its effector centers and this results in behavioral arrhythmicity. Although this is more consistent with the first proposed mechanism, it would be interesting to investigate the nature of the timing output signals that the SCN sends under conditions of LL-induced arrhythmicity and see whether the SCN is sending an arrhythmic signal or no signal at all.

Finally, the coupled electrical activity among SCN cells might have been disrupted following LL exposure. The data presented in this thesis do not provide sufficient information to rule out this option. It is possible that at any given time in LL, a proportion of SCN cells are active and show PER2 and FOS protein expression. This proportion of cells could remain relatively constant at all time points, explaining the similar levels of PER2 and FOS protein expression in all the arrhythmic animals killed in LL. When SCN cells are dispersed and maintained in culture, they exhibit a wide range in the period of their firing rhythms (Welsh et al., 1995; Welsh & Reppert, 1996). In contrast, this firing pattern is highly synchronous in the whole slice and in vivo (Gillette, 1986; Gillette et al., 1995; Lundkvist et al., 2002). Coupling among SCN cells occurs both at the electrical and at the neurochemical level. SCN neurons contain connexins that are essential to the formation of gap junctions (Colwell, 2000; Welsh & Reppert, 1996). Further, SCN neurons were shown to be dye-coupled, an indication that there is a functional electrical coupling between adjacent SCN neurons (Colwell, 2000). Verifying whether SCN neurons are still dye coupled in arrhythmic animals would provide a way to determine if electrical coupling among SCN neurons persists in LL. Furthermore, it has been shown that glial cells within the SCN are rich in gap junctions and they could contribute to the synchronization seen among SCN neurons (Colwell, 2000; Welsh & Reppert, 1996). In fact, the role played by glial cells in modulating neuronal SCN function is unknown. There is emerging evidence that suggest that glial cells modulate neuronal neurotransmission and synaptic efficacy (Castonguay, Levesque, & Robitaille, 2001; Castonguay & Robitaille, 2001; Latour, Gee, Robitaille, & Lacaille, 2001; Rochon, Rousse, & Robitaille, 2001). Identifying the role played by glial-cell modulated

neurotransmission and determining the effects of light on glial cell function could open a whole new dimension to the investigation of photic resetting of the circadian clock. Finally, It is possible that prolonged LL exposure interferes with GABA or with its synthesis, causing a disruption of rhythmic SCN activity. In vitro cell firing is inhibited by bath application of GABA (Gribkoff et al., 1999; Mason et al., 1991) and timed, daily treatment with GABA has been shown to entrain desynchronized dispersed SCN clock cells in culture (Liu & Reppert, 2000).

The results contained in the second section of the present thesis show that anatomically, the SCN receive retinal innervation through redundant pathways. RGC that project to the SCN can be divided into at least 4 categories; cells that contain the p75NTR, cells that contain melanopsin and PACAP, cells that contain p75NTR/PACAP/melanopsin and, finally, cells that contain CRY. Both the p75NTR pathway and the melanopsin/PACAP pathway are severely compromised by neonatal MSG treatment and there is no evidence suggesting that there is a differential sensitivity to MSG among the different types of RGC. Further, SAP treatment that completely eliminates p75NTR spares a proportion of melanopsin containing retinal afferents and this proportion is greater than that spared by MSG treatment, consistent with non-overlapping retinal pathways. However, the effect of SAP injections in the SCN on the populations of RGC in the retina has not been determined and it would provide important information about the effects of such treatment on the retina. A complete phenotypical analysis of the RGC that are spared by MSG and SAP treatments would provide additional information about the respective susceptibilities to the toxic effects of

MSG/SAP in these cells and potentially determine if one type of RGC plays a more critical role in photic entrainment than another.

Functionally, the retinal pathways transmitting photic information to the SCN can be divided into two, based on the behavioral data from MSG and SAP animals. MSG animals are resistant to the disruptive effects of LL but show normal photic entrainment and levels of FOS in the SCN, suggesting that the cells that are spared by MSG treatment are necessary for photic entrainment and that the cells destroyed by MSG mediate the effects of LL. In animals injected with SAP, photic entrainment is normal in spite of a complete loss of the retinal afferents containing p75NTR. These results suggest that the cells that contain p75NTR are not necessary for photic entrainment. The effects of LL in SAP treated animals were not investigated up to complete behavioral arrhythmicity however and it would have provided important information about the role played by p75NTR bearing retinal afferents in mediating the disruptive effects of LL.

In conclusion, the first two sets of experiments described in this thesis have identified a new phenomenon, light-induced suppression of FOS immunoreactivity in the SCN shell, which is specifically linked to photic entrainment. This finding opens the way for a better understanding of the role played by the shell during photic entrainment and provide a starting point for the identification of SCN targets that might be involved in photic resetting at dawn and dusk. Further, the data obtained in arrhythmic animals provide a working hypothesis that could lead to an understanding of the mechanism through which constant bright light produces complete behavioral arrhythmicity in rats. Finally, the experiments contained in section 2 suggest that there is not a single circadian

photoreceptor system but rather multiple redundant pathways that transmit photic information to the circadian clock and that each pathway might contribute to specific aspects of the circadian response to light. Taken together, these results provide a base for future efforts aimed at further characterizing the role played by the SCN core, the SCN shell, and by the redundant RHT inputs during photic entrainment of circadian rhythm.

REFERENCES

Abe, H., Honma, S., Namihira, M., Masubuchi, S., Ikeda, M., Ebihara, S., et al. (2001). Clock gene expressions in the suprachiasmatic nucleus and other areas of the brain during rhythm splitting in CS mice. *Brain Res Mol Brain Res, 87*(1), 92-99.

Abrahamson, E. E., & Moore, R. Y. (2001). Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. *Brain Res, 916*(1-2), 172-191.

Adams, M. D., Kerlavage, A. R., Fleishmann, R. D., Fuldner, R. A., Bult, C. J., Lee, N. H., Kirkness, E. F., Weinstock, K. G., Gocayne, J. D., White, O., et al. (1995). Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence. *Nature, 377*(6547 Suppl), 3-174.

Ahmad, M., & Cashmore, A. R. (1993). HY4 gene of A. thaliana encodes a protein with characteristics of a blue-light photoreceptor. *Nature, 366*(6451), 162-166.

Akashi, M., Tsuchiya, Y., Yoshino, T., & Nishida, E. (2002). Control of intracellular dynamics of mammalian period proteins by casein kinase I epsilon (CKIepsilon) and CKIdelta in cultured cells. *Mol Cell Biol, 22*(6), 1693-1703.

Akiyama, M., Minami, Y., Nakajima, T., Moriya, T., & Shibata, S. (2001). Calcium and pituitary adenylate cyclase-activating polypeptide induced expression of circadian clock gene mPer1 in the mouse cerebellar granule cell culture. *J Neurochem, 78*(3), 499-508.

Albrecht, U., Zheng, B., Larkin, D., Sun, Z. S., & Lee, C. C. (2001). MPer1 and mper2 are essential for normal resetting of the circadian clock. *J Biol Rhythms, 16*(2), 100-104.

Allendoerfer, K. L., Cabelli, R. J., Escandon, E., Kaplan, D. R., Nikolics, K., & Shatz, C. J. (1994). Regulation of neurotrophin receptors during the maturation of the mammalian visual system. *J Neurosci, 14*(3 Pt 2), 1795-1811.

Amir, S., Robinson, B., Ratovitski, T., Rea, M. A., Stewart, J., & Simantov, R. (1998). A role for serotonin in the circadian system revealed by the distribution of serotonin transporter and light-induced Fos immunoreactivity in the suprachiasmatic nucleus and intergeniculate leaflet. *Neuroscience, 84*(4), 1059-1073.

Anderson, L., & Seilhamer, J. (1997). A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis, 18*(3-4), 533-537.

Armstrong, S. M., Cassone, V. M., Chesworth, M. J., Redman, J. R., & Short, R. V. (1986). Synchronization of mammalian circadian rhythms by melatonin. *J Neural Transm Suppl, 21*, 375-394.

Aronin, N., Sagar, S. M., Sharp, F. R., & Schwartz, W. J. (1990). Light regulates expression of a Fos-related protein in rat suprachiasmatic nuclei. *Proc Natl Acad Sci U S A, 87*(15), 5959-5962.

Arvanitogiannis, A., & Amir, S. (1999). Resetting the rat circadian clock by ultra-short light flashes. *Neurosci Lett, 261*(3), 159-162.

Arvanitogiannis, A., Robinson, B., Beaulé, C., & Amir, S. (2000). Calbindin-D28k immunoreactivity in the suprachiasmatic nucleus and the circadian response to constant light in the rat. *Neuroscience, 99*(3), 397-401.

Bae, K., Jin, X., Maywood, E. S., Hastings, M. H., Reppert, S. M., & Weaver, D. R. (2001). Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. *Neuron, 30*(2), 525-536.

Beaulé, C., & Amir, S. (1999). Photic entrainment and induction of immediate-early genes within the rat circadian system. *Brain Res, 821*(1), 95-100.

Beaulé, C., & Amir, S. (2001). Photic regulation of circadian rhythms and the expression of p75 neurotrophin receptor immunoreactivity in the suprachiasmatic nucleus in rats. *Brain Res, 894*(2), 301-306.

Beaulé, C., & Amir, S. (2002). Effects of 192 IgG-saporin on circadian activity rhythms, expression of p75 neurotrophin receptors, calbindin-D28K, and light-induced Fos in the suprachiasmatic nucleus in rats. *Exp. Neurol, 176*, 377-389.

Beaulé, C., Arvanitogiannis, A., & Amir, S. (2001). Light suppresses Fos expression in the shell region of the suprachiasmatic nucleus at dusk and dawn: implications for photic entrainment of circadian rhythms. *Neuroscience, 106*(2), 249-254.

Berson, D. M., Dunn, F. A., & Takao, M. (2002). Phototransduction by retinal ganglion cells that set the circadian clock. *Science, 295*(5557), 1070-1073.

Bibel, M., Hoppe, E., & Barde, Y. A. (1999). Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. *Embo J, 18*(3), 616-622.

Bina, K. G., & Rusak, B. (1996). Nerve growth factor phase shifts circadian activity rhythms in Syrian hamsters. *Neurosci Lett, 206*(2-3), 97-100.

Bina, K. G., Rusak, B., & Semba, K. (1997). Sources of p75-nerve growth factor receptor-like immunoreactivity in the rat suprachiasmatic nucleus. *Neuroscience, 77*(2), 461-472.

Bothwell, M. (1995). Functional interactions of neurotrophins and neurotrophin receptors. *Annu Rev Neurosci, 18*, 223-253.

Brainard, G. C., Hanifin, J. P., Greeson, J. M., Byrne, B., Glickman, G., Gerner, E., et al. (2001). Action spectrum for melatonin regulation in humans: evidence for a novel circadian photoreceptor. *J Neurosci, 21*(16), 6405-6412.

Bryant, D. N., LeSauter, J., Silver, R., & Romero, M. T. (2000). Retinal innervation of calbindin-D28K cells in the hamster suprachiasmatic nucleus: ultrastructural characterization. *J Biol Rhythms, 15*(2), 103-111.

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., et al. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell, 103*(7), 1009-1017.

Card, J. P., & Moore, R. Y. (1982). Ventral lateral geniculate nucleus efferents to the rat suprachiasmatic nucleus exhibit avian pancreatic polypeptide-like immunoreactivity. *J Comp Neurol, 206*(4), 390-396.

Card, J. P., & Moore, R. Y. (1989). Organization of lateral geniculate-hypothalamic connections in the rat. *J Comp Neurol, 284*(1), 135-147.

Cashmore, A. R., Jarillo, J. A., Wu, Y. J., & Liu, D. (1999). Chryptochromes: blue light receptors for plants and animals. *Science, 284*(5415), 760-765.

Cassone, V. M., Chesworth, M. J., & Armstrong, S. M. (1986). Entrainment of rat circadian rhythms by daily injection of melatonin depends upon the hypothalamic suprachiasmatic nuclei. *Physiol Behav, 36*(6), 1111-1121.

Castel, M., & Morris, J. F. (2000). Morphological heterogeneity of the GABAergic network in the suprachiasmatic nucleus, the brain's circadian pacemaker. *J Anat, 196 (Pt 1)*, 1-13.

Castonguay, A., Levesque, S., & Robitaille, R. (2001). Glial cells as active partners in synaptic functions. *Prog Brain Res, 132*, 227-240.

Castonguay, A., & Robitaille, R. (2001). Differential regulation of transmitter release by presynaptic and glial Ca2+ internal stores at the neuromuscular synapse. *J Neurosci, 21*(6), 1911-1922.

Cermakian, N., Monaco, L., Pando, M. P., Dierich, A., & Sassone-Corsi, P. (2001). Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene. *Embo J, 20*(15), 3967-3974.

Chambille, I. (1998a). Retinal ganglion cells expressing the FOS protein after light stimulation in the Syrian hamster are relatively insensitive to neonatal treatment with monosodium glutamate. *J Comp Neurol, 392*(4), 458-467.

Chambille, I. (1998b). Temporospatial characteristics of light-induced fos immunoreactivity in suprachiasmatic nuclei are not modified in Syrian hamsters treated neonatally with monosodium glutamate. *Brain Res, 808*(2), 250-261.

Chambille, I., & Serviere, J. (1993). Neurotoxic effects of neonatal injections of monosodium L-glutamate (L- MSG) on the retinal ganglion cell layer of the golden hamster: anatomical and functional consequences on the circadian system. *J Comp Neurol, 338*(1), 67-82.

Chao, M. V. (1994). The p75 neurotrophin receptor. *J Neurobiol, 25*(11), 1373-1385.

Chao, M. V., & Hempstead, B. L. (1995). p75 and Trk: a two-receptor system. *Trends Neurosci, 18*(7), 321-326.

Chen, D., Buchanan, G. F., Ding, J. M., Hannibal, J., & Gillette, M. U. (1999). Pituitary adenylyl cyclase-activating peptide: a pivotal modulator of glutamatergic regulation of the suprachiasmatic circadian clock. *Proc Natl Acad Sci U S A, 96*(23), 13468-13473.

Colwell, C. S. (2000). Rhythmic coupling among cells in the suprachiasmatic nucleus. *J Neurobiol, 43*(4), 379-388.

Colwell, C. S., & Foster, R. G. (1992). Photic regulation of Fos-like immunoreactivity in the suprachiasmatic nucleus of the mouse. *J Comp Neurol, 324*(2), 135-142.

Czeisler, C. A., Shanahan, T. L., Klerman, E. B., Martens, H., Brotman, D. J., Emens, J. S., et al. (1995). Suppression of melatonin secretion in some blind patients by exposure to bright light. *N Engl J Med, 332*(1), 6-11.

Daan, S., & Pittendrigh, C. S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents. II. The variability of phase response curves. *Journal of Comparative Physiology, 106*, 223-237.

de la Iglesia, H. O., Meyer, J., Carpino, A., Jr., & Schwartz, W. J. (2000). Antiphase oscillation of the left and right suprachiasmatic nuclei. *Science, 290*(5492), 799-801.

de Vries, M. J., Nunes Cardozo, B., van der Want, J., de Wolf, A., & Meijer, J. H. (1993). Glutamate immunoreactivity in terminals of the retinohypothalamic tract of the brown Norwegian rat. *Brain Research, 612*, 231-237.

Ding, J. M., Chen, D., Weber, E. T., Faiman, L. E., Rea, M. A., & Gillette, M. U. (1994). Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO. *Science, 266*(5191), 1713-1717.

Dunlap, J. C. (1999). Molecular bases for circadian clocks. *Cell, 96*(2), 271-290.

Earnest, D. J., Iadarola, M., Yeh, H. H., & Olschowka, J. A. (1990). Photic regulation of c-fos expression in neural components governing the entrainment of circadian rhythms. *Exp Neurol, 109*(3), 353-361.

Earnest, D. J., Liang, F. Q., Ratcliff, M., & Cassone, V. M. (1999). Immortal time: circadian clock properties of rat suprachiasmatic cell lines. *Science, 283*(5402), 693-695.

Eastman, C., & Rechtschaffen, A. (1983). Circadian temperature and wake rhythms of rats exposed to prolonged continuous illumination. *Physiol Behav, 31*(4), 417-427.

Ebling, F. J. (1996). The role of glutamate in the photic regulation of the suprachiasmatic nucleus. *Prog Neurobiol, 50*(2-3), 109-132.

Ebling, F. J., Maywood, E. S., Humby, T., & Hastings, M. H. (1992). Circadian and photoperiodic time measurement in male Syrian hamsters following lesions of the melatonin-binding sites of the paraventricular thalamus. *J Biol Rhythms, 7*(3), 241-254.

Edelstein, K., & Amir, S. (1996). Constant light induces persistent Fos expression in rat intergeniculate leaflet. *Brain Res, 731*(1-2), 221-225.

Edelstein, K., & Amir, S. (1999a). The intergeniculate leaflet does not mediate the disruptive effects of constant light on circadian rhythms in the rat. *Neuroscience, 90*(3), 1093-1101.

Edelstein, K., & Amir, S. (1999b). The role of the intergeniculate leaflet in entrainment of circadian rhythms to a skeleton photoperiod. *J Neurosci, 19*(1), 372-380.

Edelstein, K., Beaulé, C., D'Abramo, R., & Amir, S. (2000). Expression profiles of JunB and c-Fos proteins in the rat circadian system. *Brain Res, 870*(1-2), 54-65.

Edelstein, K., Pfaus, J. G., Rusak, B., & Amir, S. (1995). Neonatal monosodium glutamate treatment prevents effects of constant light on circadian temperature rhythms of adult rats. *Brain Res, 675*(1-2), 135-142.

Eide, E. J., Vielhaber, E. L., Hinz, W. A., & Virshup, D. M. (2002). The circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase Iepsilon. *J Biol Chem, 277*(19), 17248-17254.

Frade, J. M., & Barde, Y. A. (1998). Nerve growth factor: two receptors, multiple functions. *Bioessays, 20*(2), 137-145.

Frade, J. M., Bovolenta, P., & Rodriguez-Tebar, A. (1999). Neurotrophins and other growth factors in the generation of retinal neurons. *Microsc Res Tech, 45*(4-5), 243-251.

Freedman, M. S., Lucas, R. J., Soni, B., von Schantz, M., Munoz, M., David-Gray, Z., et al. (1999). Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. *Science, 284*(5413), 502-504.

Gao, B., Fritschy, J. M., & Moore, R. Y. (1995). GABAA-receptor subunit composition in the circadian timing system. *Brain Res, 700*(1-2), 142-156.

Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., et al. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. *Science, 280*(5369), 1564-1569.

Gillespie, C. F., Van Der Beek, E. M., Mintz, E. M., Mickley, N. C., Jasnow, A. M., Huhman, K. L., et al. (1999). GABAergic regulation of light-induced c-Fos immunoreactivity within the suprachiasmatic nucleus. *J Comp Neurol, 411*(4), 683-692.

Gillette, M. U. (1986). The suprachiasmatic nuclei: circadian phase-shifts induced at the time of hypothalamic slice preparation are preserved in vitro. *Brain Res, 379*(1), 176-181.

Gillette, M. U., Medanic, M., McArthur, A. J., Liu, C., Ding, J. M., Faiman, L. E., et al. (1995). Intrinsic neuronal rhythms in the suprachiasmatic nuclei and their adjustment. *Ciba Found Symp, 183*, 134-144; discussion 144-153.

Gillette, M. U., & Mitchell, J. W. (2002). Signaling in the suprachiasmatic nucleus: selectively responsive and integrative. *Cell Tissue Res, 309*(1), 99-107.

Golombek, D. A., Hurd, M. W., Lee, K., & Ralph, M. R. (1996). Mice lacking p75NGFR receptor exhibit abnormal responses to light. *Biol. Rhythm Res., 27*, 409-418.

Gribkoff, V. K., Pieschl, R. L., Wisialowski, T. A., Park, W. K., Strecker, G. J., de Jeu, M. T., et al. (1999). A reexamination of the role of GABA in the mammalian suprachiasmatic nucleus. *J Biol Rhythms, 14*(2), 126-130.

Groos, G. (1982). The comparative physiology of extraocular photoreception. *Experientia, 38*(9), 989-991.

Guido, M. E., de Guido, L. B., Goguen, D., Robertson, H. A., & Rusak, B. (1999). Daily rhythm of spontaneous immediate-early gene expression in the rat suprachiasmatic nucleus. *J Biol Rhythms, 14*(4), 275-280.

Guido, M. E., Goguen, D., De Guido, L., Robertson, H. A., & Rusak, B. (1999). Circadian and photic regulation of immediate-early gene expression in the hamster suprachiasmatic nucleus. *Neuroscience, 90*(2), 555-571.

Güldner, F. H. (1983). Numbers of neurons and astroglial cells in the suprachiasmatic nucleus of male and female rats. *Exp Brain Res, 50*(2-3), 373-376.

Gygi, S. P., Rochon, Y., Franza, B. R., & Aebersold, R. (1999). Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol, 19*(3), 1720-1730.

Hall, L. C. (2000). Chryptochromes: sensory reception, transduction, and clock functions subserving circadian systems. *Curr Opin Neurobiol, 10*(4), 456-466.

Hamada, T., LeSauter, J., Venuti, J. M., & Silver, R. (2001). Expression of Period genes: rhythmic and nonrhythmic compartments of the suprachiasmatic nucleus pacemaker. *J Neurosci, 21*(19), 7742-7750.

Hannibal, J., Ding, J. M., Chen, D., Fahrenkrug, J., Larsen, P. J., Gillette, M. U., et al. (1997). Pituitary adenylate cyclase-activating peptide (PACAP) in the retinohypothalamic tract: a potential daytime regulator of the biological clock. *J Neurosci, 17*(7), 2637-2644.

Hannibal, J., Hindersson, P., Knudsen, S. M., Georg, B., & Fahrenkrug, J. (2002). The photopigment melanopsin is exclusively present in pituitary adenylate cyclase-activating polypeptide-containing retinal ganglion cells of the retinohypothalamic tract. *J Neurosci, 22*(1), RC191.

Hannibal, J., Moller, M., Ottersen, O. P., & Fahrenkrug, J. (2000). PACAP and glutamate are co-stored in the retinohypothalamic tract. *J Comp Neurol, 418*(2), 147-155.

Hannibal, J., Vrang, N., Card, J. P., & Fahrenkrug, J. (2001). Light-dependent induction of cFos during subjective day and night in PACAP-containing ganglion cells of the retinohypothalamic tract. *J Biol Rhythms, 16*(5), 457-470.

Harada, T., Harada, C., Nakayama, N., Okuyama, S., Yoshida, K., Kohsaka, S., et al. (2000). Modification of glial-neuronal cell interactions prevents photoreceptor apoptosis during light-induced retinal degeneration [see comments]. *Neuron, 26*(2), 533-541.

Harrington, M. E. (1997). The ventral lateral geniculate nucleus and the intergeniculate leaflet: interrelated structures in the visual and circadian systems. *Neurosci Biobehav Rev, 21*(5), 705-727.

Harrington, M. E., Hoque, S., Hall, A., Golombek, D., & Biello, S. (1999). Pituitary adenylate cyclase activating peptide phase shifts circadian rhythms in a manner similar to light. *J Neurosci, 19*(15), 6637-6642.

Harrington, M. E., Nance, D. M., & Rusak, B. (1985). Neuropeptide Y immunoreactivity in the hamster geniculo-suprachiasmatic tract. *Brain Res Bull, 15*(5), 465-472.

Harrington, M. E., Nance, D. M., & Rusak, B. (1987). Double-labeling of neuropeptide Y-immunoreactive neurons which project from the geniculate to the suprachiasmatic nuclei. *Brain Res, 410*(2), 275-282.

Harrington, M. E., & Rusak, B. (1989). Lesions of the thalamic intergeniculate leaflet alter hamster circadian rhythms. *Journal of Biological Rhythms, 1*, 309-325.

Hastings, M. H., Ebling, F. J., Grosse, J., Herbert, J., Maywood, E. S., Mikkelsen, J. D., et al. (1995). Immediate-early genes and the neural bases of photic and non-photic entrainment. *Ciba Found Symp, 183*, 175-189.

Hattar, S., Liao, H. W., Takao, M., Berson, D. M., & Yau, K. W. (2002). Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science, 295*(5557), 1065-1070.

Herzog, E. D., Geusz, M. E., Khalsa, S. B., Straume, M., & Block, G. D. (1997). Circadian rhythms in mouse suprachiasmatic nucleus explants on multimicroelectrode plates. *Brain Res, 757*(2), 285-290.

Herzog, E. D., Takahashi, J. S., & Block, G. D. (1998). Clock controls circadian period in isolated suprachiasmatic nucleus neurons. *Nat Neurosci, 1*(8), 708-713.

Hogenesch, J. B., Gu, Y. Z., Jain, S., & Bradfield, C. A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc Natl Acad Sci U S A, 95*(10), 5474-5479.

Honrado, G. I., Johnson, R. S., Golombek, D. A., Spiegelman, B. M., Papaioannou, V. E., & Ralph, M. R. (1996). The circadian system of c-fos deficient mice. *J Comp Physiol [A], 178*(4), 563-570.

Hsu, D. S., Zhao, X., Zhao, S., Kazantsev, A., Wang, R. P., Todo, T., et al. (1996). Putative human blue-light photoreceptors hCRY1 and hCRY2 are flavoproteins. *Biochemistry, 35*(44), 13871-13877.

Ikonomov, O. C., & Stoynev, A. G. (1994). Gene expression in suprachiasmatic nucleus and circadian rhythms. *Neurosci Biobehav Rev, 18*(3), 305-312.

Jac, M., Sumova, A., & Illnerova, H. (2000). c-Fos rhythm in subdivisions of the rat suprachiasmatic nucleus under artificial and natural photoperiods. *Am J Physiol Regul Integr Comp Physiol, 279*(6), R2270-2276.

Johnson, R. F., Moore, R. Y., & Morin, L. P. (1988). Loss of entrainment and anatomical plasticity after lesions of the hamster retinohypothalamic tract. *Brain Res, 460*(2), 297-313.

Johnson, R. F., Morin, L. P., & Moore, R. Y. (1988). Retinohypothalamic projections in the hamster and rat demonstrated using cholera toxin. *Brain Research, 462*, 301-312.

Kalsbeek, A., & Buijs, R. M. (2002). Output pathways of the mammalian suprachiasmatic nucleus: coding circadian time by transmitter selection and specific targeting. *Cell Tissue Res, 309*(1), 109-118.

Kaplan, D. R., & Miller, F. D. (1997). Signal transduction by the neurotrophin receptors. *Curr Opin Cell Biol, 9*(2), 213-221.

Kaplan, D. R., & Miller, F. D. (2000). Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol, 10*(3), 381-391.

Kim, Y. I., & Dudek, F. E. (1991). Intracellular electrophysiological study of suprachiasmatic nucleus neurons in rodents: excitatory synaptic mechanisms. *J Physiol, 444*, 269-287.

King, D. P., & Takahashi, J. S. (2000). Molecular genetics of circadian rhythms in mammals. *Annu Rev Neurosci, 23*, 713-742.

King, D. P., Zhao, Y., Sangoram, A. M., Wilsbacher, L. D., Tanaka, M., Antoch, M. P., et al. (1997). Positional cloning of the mouse circadian clock gene. *Cell, 89*(4), 641-653.

Kiss, J., Patel, A. J., & Halasz, B. (1993). Colocalization of NGF receptor with VIP in rat suprachiasmatic neurones. *Neuroreport, 4*(12), 1315-1318.

Klein, D., Moore, R. Y., & Reppert, S. M. (Eds.). (1991). *Suprachiasmatic Nucleus: The Mind's Clock*. Oxford: Oxford University Press.

Koh, S., Oyler, G. A., & Higgins, G. A. (1989). Localization of nerve growth factor receptor messenger RNA and protein in the adult rat brain. *Exp Neurol, 106*(3), 209-221.

Kopp, M. D., Meissl, H., Dehghani, F., & Korf, H. W. (2001). The pituitary adenylate cyclase-activating polypeptide modulates glutamatergic calcium signalling: investigations on rat suprachiasmatic nucleus neurons. *J Neurochem, 79*(1), 161-171.

Kornhauser, J. M., Mayo, K. E., & Takahashi, J. S. (1996). Light, immediate-early genes, and circadian rhythms. *Behav Genet, 26*(3), 221-240.

Kornhauser, J. M., Nelson, D. E., Mayo, K. E., & Takahashi, J. S. (1990). Photic and circadian regulation of c-fos gene expression in the hamster suprachiasmatic nucleus. *Neuron, 5*, 127-134.

Kornhauser, J. M., Nelson, D. E., Mayo, K. E., & Takahashi, J. S. (1992). Regulation of jun-B messenger RNA and AP-1 activity by light and a circadian clock. *Science, 255*(5051), 1581-1584.

Kuhlman, S. J., Quintero, J. E., & McMahon, D. G. (2000). GFP fluorescence reports Period 1 circadian gene regulation in the mammalian biological clock. *Neuroreport, 11*(7), 1479-1482.

Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., et al. (1999). mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell, 98*(2), 193-205.

Latour, I., Gee, C. E., Robitaille, R., & Lacaille, J. C. (2001). Differential mechanisms of Ca2+ responses in glial cells evoked by exogenous and endogenous glutamate in rat hippocampus. *Hippocampus, 11*(2), 132-145.

Leak, R. K., Card, J. P., & Moore, R. Y. (1999). Suprachiasmatic pacemaker organization analyzed by viral transynaptic transport. *Brain Res, 819*(1-2), 23-32.

Leak, R. K., & Moore, R. Y. (2001). Topographic organization of suprachiasmatic nucleus projection neurons. *J Comp Neurol, 433*(3), 312-334.

Lee, C., Etchegaray, J. P., Cagampang, F. R., Loudon, A. S., & Reppert, S. M. (2001). Posttranslational mechanisms regulate the mammalian circadian clock. *Cell, 107*(7), 855-867.

Lee, F. S., Rajagopal, R., Kim, A. H., Chang, P. C., & Chao, M. V. (2002). Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides. *J Biol Chem, 277*(11), 9096-9102.

LeSauter, J., & Silver, R. (1999). Localization of a suprachiasmatic nucleus subregion regulating locomotor rhythmicity. *J Neurosci, 19*(13), 5574-5585.

LeSauter, J., Stevens, P., Jansen, H., Lehman, M. N., & Silver, R. (1999). Calbindin expression in the hamster SCN is influenced by circadian genotype and by photic conditions. *Neuroreport, 10*(15), 3159-3163.

Liang, F. Q., Allen, G., & Earnest, D. (2000). Role of brain-derived neurotrophic factor in the circadian regulation of the suprachiasmatic pacemaker by light. *J Neurosci, 20*(8), 2978-2987.

Liang, F. Q., Sohrabji, F., Miranda, R., Earnest, B., & Earnest, D. (1998). Expression of brain-derived neurotrophic factor and its cognate receptor, TrkB, in the rat suprachiasmatic nucleus. *Exp Neurol, 151*(2), 184-193.

Liang, F. Q., Walline, R., & Earnest, D. J. (1998). Circadian rhythm of brain-derived neurotrophic factor in the rat suprachiasmatic nucleus [published erratum appears in Neurosci Lett 1998 Jul 31;251(3):209]. *Neurosci Lett, 242*(2), 89-92.

Liou, S. Y., Shibata, S., Albers, H. E., & Ueki, S. (1990). Effects of GABA and anxiolytics on the single unit discharge of suprachiasmatic neurons in rat hypothalamic slices. *Brain Res Bull, 25*(1), 103-107.

Liu, C., & Reppert, S. M. (2000). GABA synchronizes clock cells within the suprachiasmatic circadian clock. *Neuron, 25*(1), 123-128.

Liu, C., Weaver, D. R., Strogatz, S. H., & Reppert, S. M. (1997). Cellular construction of a circadian clock: period determination in the suprachiasmatic nuclei. *Cell, 91*(6), 855-860.

Lowrey, P. L., Shimomura, K., Antoch, M. P., Yamazaki, S., Zemenides, P. D., Ralph, M. R., et al. (2000). Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science, 288*(5465), 483-492.

Lucas, R. J., Hattar, S., Takao, M., Berson, D. M., Foster, R. G., and Yau, K.-W. (2003). Diminished puppilary light reflex at high irradiances in melanopsin-knockout mice. *Science, 299*(5604), 245-247.

Lucas, R. J., Douglas, R. H., & Foster, R. G. (2001). Characterization of an ocular photopigment capable of driving pupillary constriction in mice. *Nat Neurosci, 4*(6), 621-626.

Lucas, R. J., Freedman, M. S., Lupi, D., Munoz, M., David-Gray, Z. K., & Foster, R. G. (2001). Identifying the photoreceptive inputs to the mammalian circadian system using transgenic and retinally degenerate mice. *Behav Brain Res, 125*(1-2), 97-102.

Lundkvist, G. B., Kristensson, K., & Hill, R. H. (2002). The suprachiasmatic nucleus exhibits diurnal variations in spontaneous excitatory postsynaptic activity. *J Biol Rhythms, 17*(1), 40-51.

Mason, R. (1991). The effects of continuous light exposure on Syrian hamster suprachiasmatic (SCN) neuronal discharge activity in vitro. *Neurosci Lett, 123*(2), 160-163.

Mason, R., Biello, S. M., & Harrington, M. E. (1991). The effects of GABA and benzodiazepines on neurones in the suprachiasmatic nucleus (SCN) of Syrian hamsters. *Brain Res, 552*(1), 53-57.

Mayerhofer, A., Dissen, G. A., Parrott, J. A., Hill, D. F., Mayerhofer, D., Garfield, R. E., et al. (1996). Involvement of nerve growth factor in the ovulatory cascade: trkA receptor activation inhibits gap junctional communication between thecal cells. *Endocrinology, 137*(12), 5662-5670.

McArthur, A. J., Gillette, M. U., & Prosser, R. A. (1991). Melatonin directly resets the rat suprachiasmatic circadian clock in vitro. *Brain Res, 565*(1), 158-161.

McArthur, A. J., Hunt, A. E., & Gillette, M. U. (1997). Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn. *Endocrinology, 138*(2), 627-634.

Meijer, J. H., & Rietveld, W. J. (1989). Neurophysiology of the suprachiasmatic circadian pacemaker in rodents. *Physiological Reviews, 69*, 671-707.

Meyer-Bernstein, E. L., & Morin, L. P. (1996). Differential serotonergic innervation of the suprachiasmatic nucleus and the intergeniculate leaflet and its role in circadian rhythm modulation. *J Neurosci, 16*(6), 2097-2111.

Mikkelsen, J. D. (1990). A neuronal projection from the lateral geniculate nucleus to the lateral hypothalamus of the rat demonstrated with Phaseolus vulgaris leucoagglutinin tracing. *Neurosci Lett, 116*(1-2), 58-63.

Mikkelsen, J. D., Larsen, P. J., Mick, G., Vrang, N., Ebling, F. J., Maywood, E. S., et al. (1995). Gating of retinal inputs through the suprachiasmatic nucleus: role of excitatory neurotransmission. *Neurochem Int, 27*(3), 263-272.

Mikkelsen, J. D., & Moller, M. (1990). A direct neural projection from the intergeniculate leaflet of the lateral geniculate nucleus to the deep pineal gland of the rat, demonstrated with Phaseolus vulgaris leucoagglutinin. *Brain Res, 520*(1-2), 342-346.

Mikkelsen, J. D., & Vrang, N. (1994). A direct pretectosuprachiasmatic projection in the rat. *Neuroscience, 62*(2), 497-505.

Miyake, S., Sumi, Y., Yan, L., Takekida, S., Fukuyama, T., Ishida, Y., et al. (2000). Phase-dependent responses of Per1 and Per2 genes to a light-stimulus in the suprachiasmatic nucleus of the rat. *Neurosci Lett, 294*(1), 41-44.

Miyamoto, Y., & Sancar, A. (1998). Vitamin B2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. *Proc Natl Acad Sci U S A, 95*(11), 6097-6102.

Moga, M. M. (1998a). 192 IgG-saporin abolishes p75 neurotrophin receptor immunoreactivity in rat SCN. *Neuroreport, 9*(14), 3197-3200.

Moga, M. M. (1998b). Delayed loss of p75 neurotrophin receptor-immunoreactivity in the rat suprachiasmatic nucleus and intergeniculate leaflet after binocular enucleation. *Neurosci Lett, 253*(3), 187-190.

Moga, M. M., & Moore, R. Y. (1997). Organization of neural inputs to the suprachiasmatic nucleus in the rat. *J Comp Neurol, 389*(3), 508-534.

Moga, M. M., Weis, R. P., & Moore, R. Y. (1995). Efferent projections of the paraventricular thalamic nucleus in the rat. *J Comp Neurol, 359*(2), 221-238.

Moore, R. Y. (1973). Retinohypothalamic projection in mammals: a comparative study. *Brain Res, 49*(2), 403-409.

Moore, R. Y. (1983). Organization and function of a central nervous system circadian oscillator: the suprachiasmatic hypothalamic nucleus. *Fed Proc, 42*(11), 2783-2789.

Moore, R. Y. (1996). Entrainment pathways and the functional organization of the circadian system. *Prog Brain Res, 111*, 103-119.

Moore, R. Y., & Card, J. P. (1994). Intergeniculate leaflet: an anatomically and functionally distinct subdivision of the lateral geniculate complex. *J Comp Neurol, 344*(3), 403-430.

Moore, R. Y., Halaris, A. E., & Jones, B. E. (1978). Serotonin neurons of the midbrain raphe: Ascending projections. *Journal of Comparative Neurology, 180*, 417-438.

Moore, R. Y., & Lenn, N. J. (1972). A retinohypothalamic projection in the rat. *Journal of Comparative Neurology, 146*, 1-14.

Moore, R. Y., & Speh, J. C. (1993). GABA is the principal neurotransmitter of the circadian system. *Neurosci Lett, 150*(1), 112-116.

Moore, R. Y., Speh, J. C., & Leak, R. K. (2002). Suprachiasmatic nucleus organization. *Cell Tissue Res, 309*(1), 89-98.

Moore, R. Y., Weis, R., & Moga, M. M. (2000). Efferent projections of the intergeniculate leaflet and the ventral lateral geniculate nucleus in the rat. *J Comp Neurol, 420*(3), 398-418.

Morin, L. P. (1994). The circadian visual system. *Brain Res Brain Res Rev, 19*(1), 102-127.

Morris, M. E., Viswanathan, N., Kuhlman, S., Davis, F. C., & Weitz, C. J. (1998). A screen for genes induced in the suprachiasmatic nucleus by light. *Science, 279*(5356), 1544-1547.

Namihira, M., Honma, S., Abe, H., Masubuchi, S., Ikeda, M., & Honmaca, K. (2001). Circadian pattern, light responsiveness and localization of rPer1 and rPer2 gene expression in the rat retina. *Neuroreport, 12*(3), 471-475.

Nelson, D. E., & Takahashi, J. S. (1991). Sensitivity and integration in a visual pathway for circadian entrainment in the hamster (Mesocricetus auratus). *J Physiol, 439*, 115-145.

Oishi, K., Fukui, H., & Ishida, N. (2000). Rhythmic expression of BMAL1 mRNA is altered in Clock mutant mice: differential regulation in the suprachiasmatic nucleus and peripheral tissues. *Biochem Biophys Res Commun, 268*(1), 164-171.

Okamura, H., Berod, A., Julien, J. F., Geffard, M., Kitahama, K., Mallet, J., et al. (1989). Demonstration of GABAergic cell bodies in the suprachiasmatic nucleus: in situ hybridization of glutamic acid decarboxylase (GAD) mRNA and immunocytochemistry of GAD and GABA. *Neurosci Lett, 102*(2-3), 131-136.

Olney, J. W. (1969). Glutamate-induced retinal degeneration in neonatal mice. Electron microscopy of the acutely evolving lesion. *J Neuropathol Exp Neurol, 28*(3), 455-474.

Panda, S., Sato, T. K., Castrucci, A. M., Rollag, M. D., DeGrip, W. J., Hogenesch, J. B., Provencio, I., and Kay, S. A. (2002). Melanopsin (*Opn4*) requirement for normal light-induced circadian phase shifting. *Science, 298*(5601), 2213-2216.

Paula-Barbosa, M. M., Silva, S. M., Andrade, J. P., Cadete-Leite, A., & Madeira, M. D. (2001). Nerve growth factor restores mRNA levels and the expression of neuropeptides in the suprachiasmatic nucleus of rats submitted to chronic ethanol treatment and withdrawal. *J Neurocytol, 30*(3), 195-207.

Perry, T., Hodges, H., & Gray, J. A. (2001). Behavioural, histological and immunocytochemical consequences following 192 IgG-saporin immunolesions of the basal forebrain cholinergic system. *Brain Res Bull, 54*(1), 29-48.

Perry, V. H. (1979). The ganglion cell layer of the retina of the rat: a Golgi study. *Proc R Soc Lond B Biol Sci, 204*(1156), 363-375.

Pickard, G. E. (1982). The afferent connections of the suprachiasmatic nucleus of the golden hamster with emphasis on the retinohypothalamic projection. *J Comp Neurol, 211*(1), 65-83.

Pickard, G. E. (1985). Bifurcating axons of retinal ganglion cells terminate in the hypothalamic suprachiasmatic nucleus and the intergeniculate leaflet of the thalamus. *Neuroscience Letters, 55*, 211-217.

Pickard, G. E., Kahn, R., & Silver, R. (1984). Splitting of the circadian rhythm of body temperature in the golden hamster. *Physiol Behav, 32*(5), 763-766.

Pickard, G. E., Ralph, M. R., & Menaker, M. (1987). The intergeniculate leaflet partially mediates effects of light on circadian rhythms. *Journal of Biological Rhythms, 2*, 35-56.

Pickard, G. E., & Rea, M. A. (1997). Serotonergic innervation of the hypothalamic suprachiasmatic nucleus and photic regulation of circadian rhythms. *Biol Cell, 89*(8), 513-523.

Pickard, G. E., Turek, F. W., Lamperti, A. A., & Silverman, A. J. (1982). The effect of neonatally administered monosodium glutamate (MSG) on the development of retinofugal projections and entrainment of circadian locomotor activity. *Behav Neural Biol, 34*(4), 433-444.

Piggins, H. D., Marchant, E. G., Goguen, D., & Rusak, B. (2001). Phase-shifting effects of pituitary adenylate cyclase activating polypeptide on hamster wheel-running rhythms. *Neurosci Lett, 305*(1), 25-28.

Pioro, E. P., & Cuello, A. C. (1990). Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system. Effect of colchicine and correlation with the cholinergic system--I. Forebrain. *Neuroscience, 34*(1), 57-87.

Pittendrigh, C. S., & Daan, S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: Pacemaker as clock. *J. Comp. Physiol., 106*, 291-331.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., et al. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell, 110*(2), 251-260.

Provencio, I., Cooper, H. M., & Foster, R. G. (1998). Retinal projections in mice with inherited retinal degeneration: implications for circadian photoentrainment. *J Comp Neurol, 395*(4), 417-439.

Provencio, I., & Foster, R. G. (1995). Circadian rhythms in mice can be regulated by photoreceptors with cone-like characteristics. *Brain Res, 694*(1-2), 183-190.

Provencio, I., Jiang, G., De Grip, W. J., Hayes, W. P., & Rollag, M. D. (1998). Melanopsin: An opsin in melanophores, brain, and eye. *Proc Natl Acad Sci U S A, 95*(1), 340-345.

Provencio, I., Rodriguez, I. R., Jiang, G., Hayes, W. P., Moreira, E. F., & Rollag, M. D. (2000). A novel human opsin in the inner retina. *J Neurosci, 20*(2), 600-605.

Provencio, I., Rollag, M. D., & Castrucci, A. M. (2002). Photoreceptive net in the mammalian retina. This mesh of cells may explain how some blind mice can still tell day from night. *Nature, 415*(6871), 493.

Ralph, M. R., & Menaker, M. (1988). A mutation of the circadian system in golden hamsters. *Science, 241*(4870), 1225-1227.

Rea, M. A. (1989). Light increases Fos-related protein immunoreactivity in the rat suprachiasmatic nuclei. *Brain Res Bull, 23*(6), 577-581.

Rea, M. A. (1998). Photic entrainment of circadian rhythms in rodents. *Chronobiol Int, 15*(5), 395-423.

Rea, M. A., Michel, A. M., & Lutton, L. M. (1993). Is Fos expression necessary and sufficient to mediate light-induced phase advances of the suprachiasmatic circadian oscillator? *J Biol Rhythms, 8*(Suppl), S59-64.

Reed, H. E., Meyer-Spasche, A., Cutler, D. J., Coen, C. W., & Piggins, H. D. (2001). Vasoactive intestinal polypeptide (VIP) phase-shifts the rat suprachiasmatic nucleus clock in vitro. *Eur J Neurosci, 13*(4), 839-843.

Reppert, S. M. (2000). Cellular and molecular basis of circadian timing in mammals. *Semin Perinatol, 24*(4), 243-246.

Reppert, S. M., & Weaver, D. R. (1997). Forward genetic approach strikes gold: cloning of a mammalian clock gene. *Cell, 89*(4), 487-490.

Reppert, S. M., & Weaver, D. R. (2001). Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol, 63*, 647-676.

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature, 418*(6901), 935-941.

Rochon, D., Rousse, I., & Robitaille, R. (2001). Synapse-glia interactions at the mammalian neuromuscular junction. *J Neurosci, 21*(11), 3819-3829.

Romijn, H. J., Sluiter, A. A., Pool, C. W., Wortel, J., & Buijs, R. M. (1996). Differences in colocalization between Fos and PHI, GRP, VIP and VP in neurons of the rat suprachiasmatic nucleus after a light stimulus during the phase delay versus the phase advance period of the night. *J Comp Neurol, 372*(1), 1-8.

Ruby, N. F., Brennan, T. J., Xie, X., Cao, V., Franken, P., Heller, H. C., & O'Hara, B. F. (2002). Role of melanopsin in circadian response to light. *Science, 298*(5601), 2211-2213.

Rusak, B., McNaughton, L., Robertson, H. A., & Hunt, S. P. (1992). Circadian variation in photic regulation of immediate-early gene mRNAs in rat suprachiasmatic nucleus cells. *Brain Res Mol Brain Res, 14*(1-2), 124-130.

Rusak, B., Robertson, H. A., Wisden, W., & Hunt, S. P. (1990). Light pulses that shift rhythms induce gene expression in the suprachiasmatic nucleus. *Science, 248*(4960), 1237-1240.

Rusak, B., & Zucker, I. (1979). Neural regulation of circadian rhythms. *Physiological Reviews, 59*, 449-526.

Sala, R., Viegi, A., Rossi, F. M., Pizzorusso, T., Bonanno, G., Raiteri, M., et al. (1998). Nerve growth factor and brain-derived neurotrophic factor increase neurotransmitter release in the rat visual cortex. *Eur J Neurosci, 10*(6), 2185-2191.

Sancar, A. (2000). Cryptochromes: the second photoactive pigment in the eye and its role in circadian photoreception. *Annu Rev Biochem, 69*, 31-67.

Schwartz, W. J., Peters, R. V., Aronin, N., & Bennett, M. R. (1996). Unexpected c-fos gene expression in the suprachiasmatic nucleus of mice entrained to a skeleton photoperiod. *J Biol Rhythms, 11*(1), 35-44.

Schwartz, W. J., Takeuchi, J., Shannon, W., Davis, E. M., & Aronin, N. (1994). Temporal regulation of light-induced Fos and Fos-like protein expression in the ventrolateral subdivision of the rat suprachiasmatic nucleus. *Neuroscience, 58*(3), 573-583.

Schwartz, W. J., & Zimmerman, P. (1990). Circadian timekeeping in BALB/c and C57BL/6 inbred mouse strains. *J Neurosci, 10*(11), 3685-3694.

Selby, C. P., Thompson, C., Schmitz, T. M., Van Gelder, R. N., & Sancar, A. (2000). Functional redundancy of cryptochromes and classical photoreceptors for nonvisual ocular photoreception in mice. *Proc Natl Acad Sci U S A, 97*(26), 14697-14702.

Shearman, L. P., Sriram, S., Weaver, D. R., Maywood, E. S., Chaves, I., Zheng, B., et al. (2000). Interacting molecular loops in the mammalian circadian clock. *Science, 288*(5468), 1013-1019.

Shearman, L. P., Zylka, M. J., Weaver, D. R., Kolakowski, L. F., Jr., & Reppert, S. M. (1997). Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron, 19*(6), 1261-1269.

Shen, H., & Semba, K. (1994). A direct retinal projection to the dorsal raphe nucleus in the rat. *Brain Res, 635*(1-2), 159-168.

Shibata, S., & Moore, R. Y. (1993). Neuropeptide Y and optic chiasm stimulation affect suprachiasmatic nucleus circadian function in vitro. *Brain Res, 615*(1), 95-100.

Shibuya, C. A., Melnyk, R. B., & Mrosovsky, N. (1980). Simultaneous splitting of drinking and locomotor activity rhythms in a golden hamster. *Naturwissenschaften, 67*(1), 45-47.

Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., et al. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell, 91*(7), 1043-1053.

Shinohara, K., Hiruma, H., Funabashi, T., & Kimura, F. (2000). GABAergic modulation of gap junction communication in slice cultures of the rat suprachiasmatic nucleus. *Neuroscience, 96*(3), 591-596.

Shinohara, K., Tominaga, K., & Inouye, S. T. (1999). Phase dependent response of vasoactive intestinal polypeptide to light and darkness in the suprachiasmatic nucleus. *Neurosci Res, 33*(2), 105-110.

Shirakawa, T., Honma, S., & Honma, K. (2001). Multiple oscillators in the suprachiasmatic nucleus. *Chronobiol Int, 18*(3), 371-387.

Silver, R., Romero, M. T., Besmer, H. R., Leak, R., Nunez, J. M., & LeSauter, J. (1996). Calbindin-D28K cells in the hamster SCN express light-induced Fos. *Neuroreport, 7*(6), 1224-1228.

Sofroniew, M. V., Isacson, O., & O'Brien, T. S. (1989). Nerve growth factor receptor immunoreactivity in the rat suprachiasmatic nucleus. *Brain Res, 476*(2), 358-362.

Sollars, P. J., Smeraski, C. A., Kaufman, J. D., Ogilvie, M. D., Provencio, I., Morin, L. P., & Pickard, G. E. Melanopsin and non-melanopsin expressing retinal ganglion cells innervate the suprachiasmatic nucleus. Program Number 371.21. *2002 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience, 2002. Online.

Soni, B. G., Philp, A. R., Foster, R. G., & Knox, B. E. (1998). Novel retinal photoreceptors. *Nature, 394*(6688), 27-28.

Starkey, S. J., Walker, M. P., Beresford, I. J., & Hagan, R. M. (1995). Modulation of the rat suprachiasmatic circadian clock by melatonin in vitro. *Neuroreport, 6*(14), 1947-1951.

Stehle, J., Vanecek, J., & Vollrath, L. (1989). Effects of melatonin on spontaneous electrical activity of neurons in rat suprachiasmatic nuclei: an in vitro iontophoretic study. *J Neural Transm, 78*(2), 173-177.

Stephan, F. K. (1983). Circadian rhythms in the rat: constant darkness, entrainment to T cycles and to skeleton photoperiods. *Physiol Behav, 30*(3), 451-462.

Strecker, G. J., Wuarin, J. P., & Dudek, F. E. (1997). GABAA-mediated local synaptic pathways connect neurons in the rat suprachiasmatic nucleus. *J Neurophysiol, 78*(4), 2217-2220.

Sumova, A., & Illnerova, H. (1998). Photic resetting of intrinsic rhythmicity of the rat suprachiasmatic nucleus under various photoperiods. *Am J Physiol, 274*(3 Pt 2), R857-863.

Sumova, A., Travnickova, Z., & Illnerova, H. (2000). Spontaneous c-Fos rhythm in the rat suprachiasmatic nucleus: location and effect of photoperiod. *Am J Physiol Regul Integr Comp Physiol, 279*(6), R2262-2269.

Sumova, A., Travnickova, Z., Mikkelsen, J. D., & Illnerova, H. (1998). Spontaneous rhythm in c-Fos immunoreactivity in the dorsomedial part of the rat suprachiasmatic nucleus. *Brain Res, 801*(1-2), 254-258.

Suzuki, A., Nomura, S., Morii, E., Fukuda, Y., & Kosaka, J. (1998). Localization of mRNAs for trkB isoforms and p75 in rat retinal ganglion cells [published erratum appears in J Neurosci Res 1999 Jan 1;55(1):135]. *J Neurosci Res, 54*(1), 27-37.

Takahashi, J. S., DeCoursey, P. J., Bauman, L., & Menaker, M. (1984). Spectral sensitivity of a novel photoreceptive system mediating entrainment of mammalian circadian rhythms. *Nature, 308*(5955), 186-188.

Takahashi, J. S., & Zatz, M. (1982). Regulation of circadian rhythmicity. *Science, 217*(4565), 1104-1111.

Takahata, S., Sogawa, K., Kobayashi, A., Ema, M., Mimura, J., Ozaki, N., et al. (1998). Transcriptionally active heterodimer formation of an Arnt-like PAS protein, Arnt3, with HIF-1a, HLF, and clock. *Biochem Biophys Res Commun, 248*(3), 789-794.

Takano, A., Shimizu, K., Kani, S., Buijs, R. M., Okada, M., & Nagai, K. (2000). Cloning and characterization of rat casein kinase 1epsilon. *FEBS Lett, 477*(1-2), 106-112.

Takeuchi, J., Shannon, W., Aronin, N., & Schwartz, W. J. (1993). Compositional changes of AP-1 DNA-binding proteins are regulated by light in a mammalian circadian clock. *Neuron, 11*(5), 825-836.

Thompson, C. L., Blaner, W. S., Van Gelder, R. N., Lai, K., Quadro, L., Colantuoni, V., et al. (2001). Preservation of light signaling to the suprachiasmatic nucleus in vitamin A-deficient mice. *Proc Natl Acad Sci U S A, 98*(20), 11708-11713.

Thompson, R. H., & Swanson, L. W. (1998). Organization of inputs to the dorsomedial nucleus of the hypothalamus: a reexamination with Fluorogold and PHAL in the rat. *Brain Res Brain Res Rev, 27*(2), 89-118.

Thresher, R. J., Vitaterna, M. H., Miyamoto, Y., Kazantsev, A., Hsu, D. S., Petit, C., et al. (1998). Role of mouse cryptochrome blue-light photoreceptor in circadian photoresponses. *Science, 282*(5393), 1490-1494.

Todo, T. (1999). Functional diversity of the DNA photolyase/blue light receptor family. *Mutat Res, 434*(2), 89-97.

Turek, F. W. (1985). Circadian neural rhythms in mammals. *Annu Rev Physiol, 47*, 49-64.

Ueda, H. R., Chen, W., Adachi, A., Wakamatsu, H., Hayashi, S., Takasugi, T., et al. (2002). A transcription factor response element for gene expression during circadian night. *Nature, 418*(6897), 534-539.

Van den Pol, A. N. (1980). The hypothalamic suprachiasmatic nucleus of rat: intrinsic anatomy. *J Comp Neurol, 191*(4), 661-702.

van den Pol, A. N. (1986). Gamma-aminobutyrate, gastrin releasing peptide, serotonin, somatostatin, and vasopressin: ultrastructural immunocytochemical localization in presynaptic axons in the suprachiasmatic nucleus. *Neuroscience, 17*(3), 643-659.

van den Pol, A. N. (1993). Glutamate and GABA presence and action in the suprachiasmatic nucleus. *J Biol Rhythms, 8*(Suppl), S11-15.

van den Pol, A. N., Decavel, C., Levi, A., & Paterson, B. (1989). Hypothalamic expression of a novel gene product, VGF: immunocytochemical analysis. *J Neurosci, 9*(12), 4122-4137.

van den Pol, A. N., & Dudek, F. E. (1993). Cellular communication in the circadian clock, the suprachiasmatic nucleus. *Neuroscience, 56*(4), 793-811.

Van der Horst, G. T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., Eker, A. P., van Leenen, D., Buijs, R., Bootsma, D., Hoeijmakers, J. H., and Yasui, A. (1999). Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature, 398*(6728), 627-630.

van Rijn, C. M., Marani, E., & Rietveld, W. J. (1986). The neurotoxic effect of monosodium glutamate (MSG) on the retinal ganglion cells of the albino rat. *Histol Histopathol, 1*(3), 291-295.

Vielhaber, E., Eide, E., Rivers, A., Gao, Z. H., & Virshup, D. M. (2000). Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I epsilon. *Mol Cell Biol, 20*(13), 4888-4899.

Vindlacheruvu, R. R., Ebling, F. J. P., Maywood, E. S., & Hastings, M. H. (1992). Blockade of glutamatergic neurotransmission in the suprachiasmatic nucleus prevents cellular and behavioural responses of the circadian system to light. *Europeean Journal of Neuroscience, 4*, 673-679.

Vitaterna, M. H., Selby, C. P., Todo, T., Niwa, H., Thompson, C., Fruechte, E. M., et al. (1999). Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc Natl Acad Sci U S A, 96*(21), 12114-12119.

Watts, A. G., & Swanson, L. W. (1987). Efferent projections of the suprachiasmatic nucleus: II. Studies using retrograde transport of fluorescent dyes and simultaneous peptide immunohistochemistry in the rat. *J Comp Neurol, 258*(2), 230-252.

Watts, A. G., Swanson, L. W., & Sanchez-Watts, G. (1987). Efferent projections of the suprachiasmatic nucleus: I. Studies using anterograde transport of Phaseolus vulgaris leucoagglutinin in the rat. *J Comp Neurol, 258*(2), 204-229.

Welsh, D. K., Logothetis, D. E., Meister, M., & Reppert, S. M. (1995). Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron, 14*(4), 697-706.

Welsh, D. K., & Reppert, S. M. (1996). Gap junctions couple astrocytes but not neurons in dissociated cultures of rat suprachiasmatic nucleus. *Brain Res, 706*(1), 30-36.

Wiley, R. G. (1992). Neural lesioning with ribosome-inactivating proteins: suicide transport and immunolesioning. *Trends Neurosci, 15*(8), 285-290.

Wiley, R. G. (1996). Targeting toxins to neural antigens and receptors. *Semin Cancer Biol, 7*(2), 71-77.

Williams, J. A., & Sehgal, A. (2001). Molecular components of the circadian system in Drosophila. *Annu Rev Physiol, 63*, 729-755.

Wollnik, F., Brysch, W., Uhlmann, E., Gillardon, F., Bravo, R., Zimmermann, M., et al. (1995). Block of c-Fos and JunB expression by antisense oligonucleotides inhibits light-induced phase shifts of the mammalian circadian clock. *Eur J Neurosci, 7*(3), 388-393.

Yagita, K., Yamaguchi, S., Tamanini, F., van Der Horst, G. T., Hoeijmakers, J. H., Yasui, A., et al. (2000). Dimerization and nuclear entry of mPER proteins in mammalian cells. *Genes Dev, 14*(11), 1353-1363.

Yan, L., Takekida, S., Shigeyoshi, Y., & Okamura, H. (1999). Per1 and Per2 gene expression in the rat suprachiasmatic nucleus: circadian profile and the compartment-specific response to light. *Neuroscience, 94*(1), 141-150.

Yan, Q., & Johnson, E. M., Jr. (1988). An immunohistochemical study of the nerve growth factor receptor in developing rats. *J Neurosci, 8*(9), 3481-3498.

Young, M. W. (2000). Circadian rhythms. Marking time for a kingdom. *Science, 288*(5465), 451-453.

Yu, W., Nomura, M., & Ikeda, M. (2002). Interactivating feedback loops within the mammalian clock: BMAL1 is negatively autoregulated and upregulated by CRY1, CRY2, and PER2. *Biochem Biophys Res Commun, 290*(3), 933-941.

Zheng, B., Larkin, D. W., Albrecht, U., Sun, Z. S., Sage, M., Eichele, G., et al. (1999). The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature, 400*(6740), 169-173.

Zucker, I., Rusak, B., & King, R. G., Jr. (1976). Neural bases for circadian rhythms in rodent behavior. *Adv Psychobiol, 3*, 35-74.

Zylka, M. J., Shearman, L. P., Weaver, D. R., & Reppert, S. M. (1998). Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron, 20*(6), 1103-1110.