Masters Theses 1911 - February 2014

January 2008

Combined Treatment With Npy Y5 Antagonists and Nan-190 Attenuates Transients in Light-induced Phase Shifts and Potentiates Phase Shifts Only During the Late Subjective Night

Mary K. Costello
University of Massachusetts Amherst

Follow this and additional works at: https://scholarworks.umass.edu/theses

COMBINED TREATMENT WITH NPY Y5 ANTAGONISTS AND NAN-190
ATTENUATES TRANSIENTS IN LIGHT-INDUCED PHASE SHIFTS AND
POTENTIATES PHASE SHIFTS ONLY DURING THE LATE SUBJECTIVE NIGHT

A Thesis Presented

By

MARY K. COSTELLO

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2008

Neuroscience and Behavior Program

COMBINED TREATMENT WITH NPY Y5 ANTAGONISTS AND NAN-190
ATTENUATES TRANSIENTS IN LIGHT-INDUCED PHASE SHIFTS AND
POTENTIATES PHASE SHIFTS ONLY DURING THE LATE SUBJECTIVE NIGHT

A Thesis Presented

By

MARY K. COSTELLO

Approved as to style and content by:

_____
Mary E. Harrington, Chair

_____
Nancy G. Forger

_____
Gordon A. Wyse

_____
Nancy G. Forger, Acting Director
Neuroscience and Behavior Program

DEDICATION

To my husband Alex and my children Katie, Alex and Halie for their love
and inspiration.

ACKNOWLEDGEMENT

ABSTRACT

COMBINED TREATMENT WITH NPY Y5 ANTAGONISTS AND NAN-190
ATTENUATES TRANSIENTS IN LIGHT-INDUCED PHASE SHIFTS AND
POTENTIATES PHASE SHIFTS ONLY DURING THE LATE SUBJECTIVE NIGHT

SEPTEMBER 2008

MARY K. COSTELLO, B.A., WELLS COLLEGE

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Mary E. Harrington

Circadian rhythms in physiology and behavior are synchronized by a central pacemaker, the suprachiasmatic nuclei (SCN) of the hypothalamus. Shift work, jet lag and sleep disorders can disrupt circadian rhythms, negatively impacting health and well-being. The SCN pacemaker resets rapidly in response to changes in the daily light cycle, however, adjustment of peripheral oscillators to changing time zones or work shifts is more gradual, leading to internal desynchrony. In addition, many diseases can impair the SCN's ability to adjust to changes in the light cycle. My research investigated whether combined pharmacological inhibition of neuropeptide Y and serotonin could enhance resetting and attenuate transient cycles in locomotor activity following a sudden change in light exposure. I found that simultaneously blocking neuropeptide Y and serotonin receptors potentiated phase shifts during the late subjective night and significantly reduced transient cycles of locomotor activity in hamsters. Development of treatments that enhance the circadian system's response to light may alleviate some of the negative health consequences experienced by travelers, shift workers and individuals with disease-related circadian desynchrony

TABLE OF CONTENTS   pages

LIST OF TABLES

CHAPTER I

INTRODUCTION

Circadian rhythms of behavior and physiology are coordinated by the master circadian pacemaker, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Klein, Moore and Reppert, 1991). The circadian timing system influences sleep-wake cycles, hepatic metabolism, endocrine secretion, body temperature, cognitive function, cardiovascular activity, cell growth and division as well as other processes (Schibler, Ripperger and Brown, 2003). Circadian rhythms are regulated by the daily light-dark cycle as well as by internal stimuli associated with behavioral and physiological status (reviewed in Reppert and Weaver, 2002; Yannielli and Harrington, 2004). Timing of daily rhythms allows optimal coordination of physiology and behavior with time of day. For example, a rise in glucose levels and corticosterone secretion in the early morning prepares the body for activity (La Fleur, Kalsbeek, Wortel, Fekkes and Buijs, 2001). Circadian rhythms also allow internal coordination, so that incompatible processes occur at different times of day.

There is increasing evidence that circadian disruption, particularly in a light-altered environment, may be responsible for increased incidence of human diseases including hypertension, diabetes, depression and breast cancer (Hastings, Reddy and Maywood, 2003; LaFleur, Kalsbee, Wortel, Fekkes and Buijs, 2001; Stevens, 2005). Travel to new time zones, lack of photic input, and an irregular circadian clock can disturb nightly sleep and reduce vigilance and cognitive function during the day (Cho 2001; Katz, Knobler, Laibel, Strauss and Durst, 2002; Waterhouse, Really and Atkinson. 1997). Seasonal affective disorder (SAD), which may be caused by a delayed circadian

system, affects 2-5% of the population in temperate climates (Lam et al., 2006). Symptoms of seasonal affective disorder include recurrent major depressive episodes, low energy and fatigue (Lewy, Lefler, Emens and Bauer, 2006).

Light is the most potent cue for entrainment of the circadian clock, however, non-photic stimuli, such as activity, sleep and feeding schedule can also influence circadian rhythms. The effects of light on circadian rhythms can be modified by non-photic stimuli. Non-photic information is conveyed to the SCN primarily via serotonergic input from the raphe nuclei and by neuropeptide Y input from the intergeniculate leaflet in the thalamus. Our lab recently found that simultaneously blocking NPY and serotonin input significantly potentiates photic phase shifts in hamsters (Lall and Harrington, 2006). This finding may have clinical value in treating disordered circadian rhythms. In human studies, light alone has not been able to produce the phase adjustment required to adjust to circadian dyschrony experienced during jet lag or shift work (Zeiter, Dijk, Kronauer, Brown and Czeisler, 2000). My work seeks to further characterize the potentiation of phase resetting by blocking neuropeptide Y (NPY) and serotonin (5-HT) input. I seek to extend the finding to another species, mice, and to evaluate at what circadian phases the light induced shifts are potentiated by this treatment. In addition, I will determine whether transient cycles, which underlie the malaise of jetlag, can be attenuated with this combined inhibition of NPY and 5-HT. Transient cycles are the unstable cycles in the days following an abrupt light change before the overt rhythm measured reaches a steady state. Phase adjustments may have a therapeutic role in treating depression and SAD as well as alleviating the negative effects of jet lag and shift work (Sollars, Ogilvie, Simpson and Pickard, 2006).

CHAPTER II

BACKGROUND AND SIGNIFICANCE

A.   The suprachiasmatic nucleus controls circadian rhythms of many behavioral and physiological responses

Circadian rhythms are defined by three fundamental properties. First, the rhythm must persist in constant conditions with a period of approximately 24 hours. Additionally, a circadian rhythm is temperature compensated, having a period that is approximately the same across different constant ambient temperatures. The third and most important criterion is that a circadian rhythm can be entrained to daily cues, known as zeitgebers, in the environment (Johnson, Elliott and Foster, 2003).

In mammals, a master pacemaker coordinates circadian rhythms of behavior and activity. Brain lesion, metabolic and electrophysiological studies determined that a central clock resides in the suprachiasmatic nucleus of the anterior hypothalamus of mammals. The SCN are small paired nuclei of ~10,000 neurons lying just above the optic chiasm. Lesions of the SCN extinguish circadian rhythms of locomotor activity, feeding, drinking, body temperature, and plasma adrenal corticosterone in rodents (Moore and Eichler, 1972; Stephan and Zucker, 1972; Abe, Kroning, Greer and Critchlow, 1979). The rhythmicity of the SCN persists *in vitro* in both hypothalamic slice and cell culture preparations (Shibata, Oomura, Hattori and Kita, 1982; Welsh, Logothetis, Meister and Reppert, 1995). Conclusive evidence for the autonomy of the SCN comes from transplant studies; transplants of fetal SCN to SCN-lesioned rodents reinstate wheel running activity rhythms with the period of the donor tissue (Ralph, Foster, Davis and Menaker, 1990)**.**

3
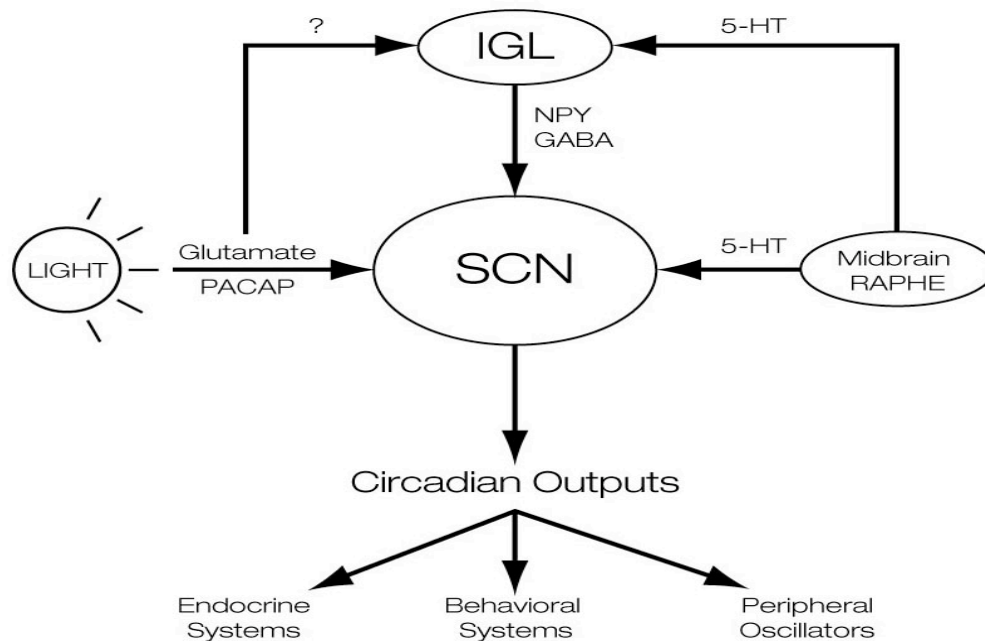
Figure 1. The principal input pathways to the suprachiasmatic nucleus (SCN). Photic input comes from the retina via the retinohypothalamic tract utilizing primarily glutamate and PACAP. Non-photic input arrives to the SCN from the intergeniculate leaflet (IGL) utilizing neuropeptide Y (NPY) and GABA and from serotonergic (5-HT) afferents arising from the midbrain raphe nuclei. (adapted from Yannielli and Harrington, 2004)

The SCN receives direct neural input from the retina as well as innervations from the midbrain raphe and the intergeniculate leaflet of the thalamus (IGL) (see Figure 1). Photic entraining stimuli reach the SCN via the retinohypothalamic tract while non-photic information is conveyed via the median raphe nuclei and via the geniculohypothalamic tract (GHT) from the IGL (Morin and Allen, 2006; Morin and Meyer-Bernstein, 1999; Harrington, 1997). It has also been suggested that the dorsal raphe nucleus might contribute to circadian phase setting in the Syrian hamster by enhancing serotonergic activity in the SCN, the IGL and/or the midbrain raphe nucleus (Glass, DiNardo and Ehlen, 2000). A number of IGL neurons projecting to the SCN are

photically responsive and some encode light intensity, suggesting the IGL also plays a role in photic entrainment and photoperiodic responses (Harrington, 1997).

Individual neurons in the SCN are autonomous oscillators and can generate circadian firing rhythms when dissociated from SCN tissue (Honma, Shirakawa, Katsuno, Namihira and Honma, 1998; Welsh, Logthetis, Meister and Reppert, 1995). The core oscillation of the SCN is driven by auto-regulating transcription-translation feedback loops (Dunlap, 1999; Reppert and Weaver, 2001). In brief, two positive activators, CLOCK and BMAL1, which are members of the bHLH-PAS domain family of transcription factors, dimerize and drive the transcription of the *Period (per)* and *Cryptochrome (cry)* genes. As PER and CRY protein levels increase, PER proteins dimerize with CRY proteins and are phosphorylated. The phosphorylated PER-CRY complex moves into the nucleus and binds to the CLOCK-BMAL1 heterodimer removing the positive drive to the *per* and *cry* genes. When the level of the PER-CRY complex is low, the transcription of *per* and *cry* is once again activated by the BMAL-CLOCK heterodimer. In addition, posttranslational modifications play a key role in the molecular generation of circadian rhythmicity in all eukaryotes (Gallego and Virshup, 2007; Lee, Etchegaray, Cagampang, Loudon and Reppert, 2001; Merrow, Spoelstra and Roenneberg 2005; Schibler, 2005). Phosphorylation can affect half-life and control the sub-cellular localization of a protein to regulate transcriptional activity. The major clock kinase, casein kinase I (CKI)ε and the related kinase, CKIδ, regulate the negative arm of the feedback loop through phosphorylation of PER proteins (Partch, Shields, Thompson, Selby and Sancar, 2006). The BMAL1 and cryptochrome proteins are also substrates for CKIε in vitro (Eide EJ, Vielhaber EL, Hinz WA and Virshup DM, 2002).

B.   Circadian rhythms are entrained by the environmental light-dark cycle

How does the circadian timing system coordinate internal rhythms with the external environment? The most consistent environmental cue is the 24-hour light cycle and the phase of the master pacemaker, the SCN, is set every day by light cues (Schibler, 2005). In mammals, rods, cones and intrinsically photosensitive retinal ganglion cells mediate circadian photoentrainment (Guler et al., 2008). The retinal ganglion cells, which are specialized for irradiance detection, use the photopigment melanopsin (Pierson and Foster, 2006). The axons of the melanopsin containing retinal ganglion cells project to the suprachiasmatic nucleus, the intergeniculate nucleus and the olivary pretectal nucleus. The SCN and IGL regulate circadian photoentrainment and the olivary pretectal nucleus regulates pupillary light reflex (Hattar et al., 2003). Mice lacking the melanopsin gene have diminished ability to phase-delay and lengthen circadian period, while mice lacking rods and cones have altered amplitude in their photic phase response curves. Together the melanopsin retinal ganglion cells and rod-cone cells have complementary roles in tuning of phase and period length for photoentrainment and normal pupillary light reflex (Hattar et al., 2003).

Light is transduced into a neural signal by retinal ganglion cells and the signal is conveyed to the SCN via the retinohypothalamic tract, triggering the release of the neurotransmitter glutamate and the neuromodulators, substance P and pituitary adenylyl cyclase-activating peptide (PACAP) onto retinorecipient cells in the SCN (see Fig. 2). Glutamate activates the excitatory NMDAR and mGluR receptors causing an influx of Ca^{2+} and initiating a cascade of intracellular signaling events (reviewed in Reppert and Weaver, 2002).
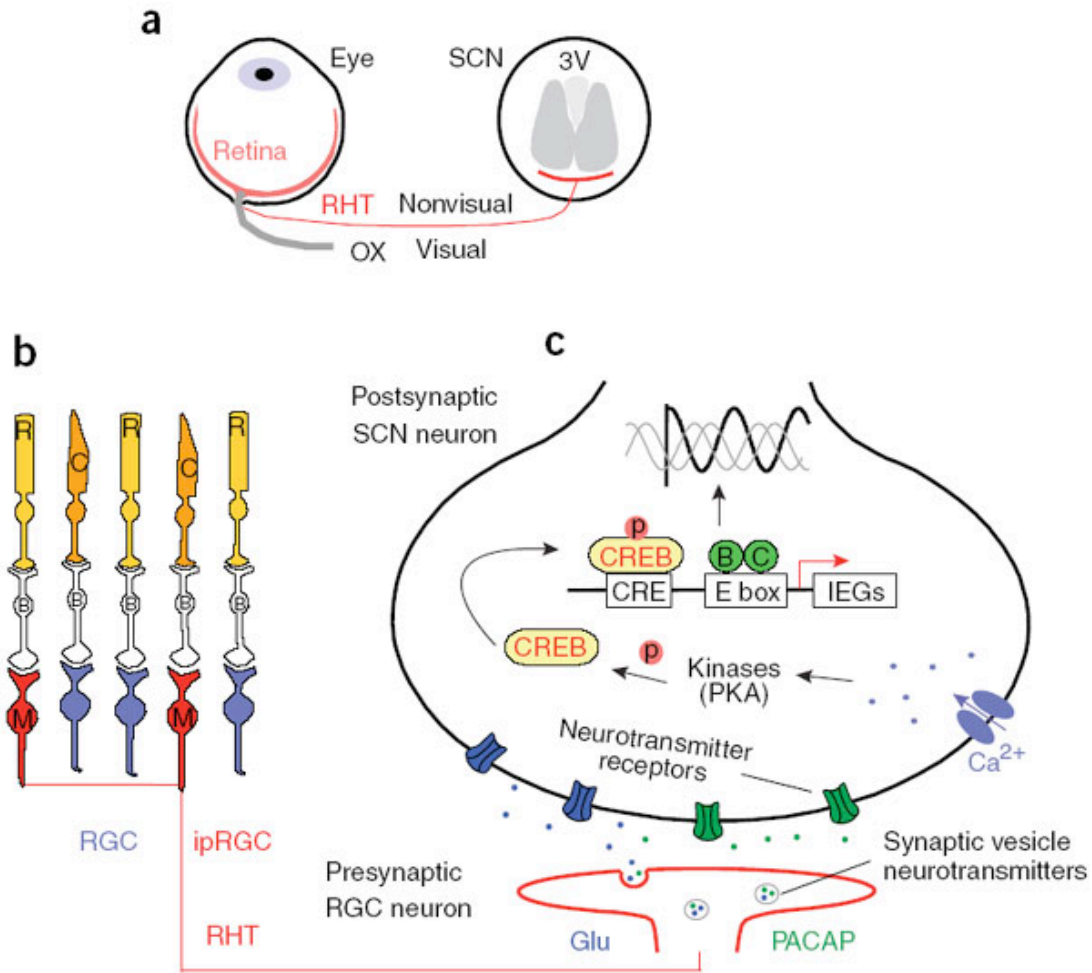
Figure 2. Light resets the clock. a. A subset of melanopsin-containing retinal ganglion cells (RCGs) form the retinohypothalamic tract projecting to neurons in the SCN. b. When stimulated by light, the melanopsin-containing RGCs release primarily PACAP and glutamate. c. Glutamate and PACAP bind to their respective receptors, triggering an influx of Ca^{2+}. This influx leads to increased cAMP levels, leading to phosphorytlation of CREB, and activation of the immediate early genes Per 1 and Per 2 resulting in a phase shift (shown in black). (Liu, Lewis and Kay, 2007)

Circadian entrainment depends on the activation of specific signal transduction pathways in the SCN including: protein kinase A, protein kinase G, protein kinase C, calcium-calmodulin protein kinase, and mitogen-activivated kinase (Butcher, Lee, Cheng and Obrietan, 2005; Jakubcakova et al., 2007). These signaling cascades activate the

Ca $^{2+}$/cyclic response elements (CRE) in the promoter region of the clock genes *Per1* and *Per* 2 (Butcher*,* 2005)*.* The clock genes *Per1* and *Per2* are induced in response to light stimuli presented during the subjective night, when their levels are minimal. Light during the day has little effect on *Per* levels; however light in the early night delays the feedback loop by inducing *Per1* expression as it falls, and light in the late night advances the clock cycle by accelerating the transcription of *Per* as levels are rising. Transcriptional activation of *Per* is followed by increased levels of the PER1 and PER2 proteins*.* (Yan and Silver, 2004). Significantly, light-induced *Per* induction during the night correlates with resetting behavior of the locomotor activity rhythms (Shigeyoshi et al., 1997).

The circadian clock responds differently to light cues at different phases of its endogenous period. Circadian phase is frequently determined by measuring activity onset in locomotor rhythms of small mammals (see Fig. 3). Animals are placed in constant dark conditions (DD) following entrainment to a light: dark cycle and daily locomotor activity rhythms are analyzed. The first half of the cycle (CT0-CT12) is identified as subjective day and the second half (CT12-CT24) identified as subjective night. Light stimulation during the early subjective night phase delays the circadian activity rhythm whereas light during the late subjective night phase advances the rhythm (Fig. 3). Light pulses received during the subjective day have little or no effect on the activity phase.
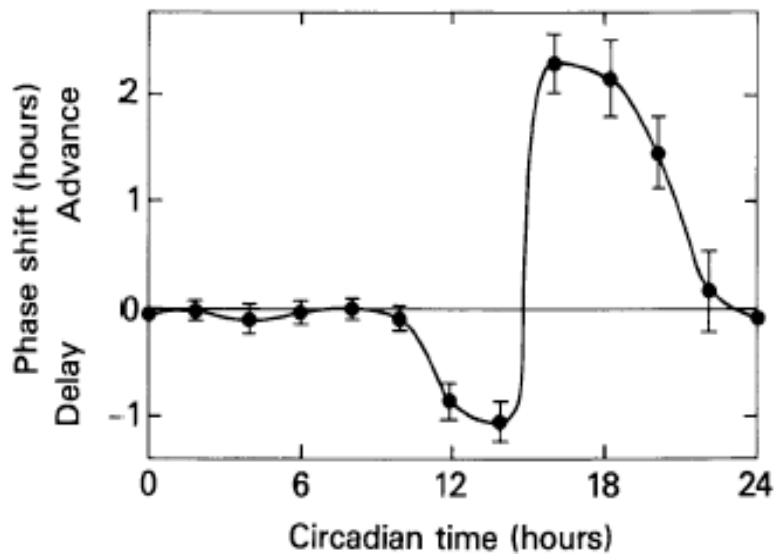
Figure 3. A photic phase response curve (PRC) determined for the Syrian hamster. Phase shifts are plotted as a function of the circadian phase when the light pulse is given as indicated on the x-axis. Light exposure during the early subjective night phase delays the clock and during the late night, light phase advances the clock. Phase delays are plotted as negative values and phase advances as positive values. Light during the day has little or no effect on phase. (Subjective day: CT 0 – CT 12, subjective night: CT 12 - CT 24)

The endogenous circadian clock can function in the absence of external time cues, however, it is important that the clock can also be entrained by environmental cues in order to provide an internal estimate of external time (Johnson, Elliott and Foster, 2003). Entrainment allows coordination of physiological processes and oscillations in peripheral tissues to the daily light schedule. The interval (measured in hours) between the entraining signal, referred to as a *zeitgeber,* and the onset of the rhythm (e.g. activity rhythm or body temperature) is referred to as the phase angle difference. Entrainment requires that a stable phase angle difference between the environmental cycle and the

overt rhythm be established (Jud, Schmutz, Hampp, Oster and Albrecht, 2005). The period of the internal rhythm becomes approximately equal to that of the oscillating environmental signal (Dunlap, Loros and DeCoursey, 2004). Light is the most significant environmental signal for synchronizing circadian rhythms with external time. The circadian pacemaker is entrained by light falling at a specific phase of an organism's phase response curve. For example, light during the late night results in an advance shift, allowing for an adjustment equal to the difference between the intrinsic free running period and the period of the entraining cycle (see Fig. 4).

During entrainment to new light-cycle, unstable cycles, known as transients can be observed in locomotor activity. Although the SCN resets rapidly in response to a changing light cycle, many overt rhythms require several cycles to reach the steady-state phase shift. Transient cycles, thought to reflect disequilibrium between the overt rhythm and the pacemaker, are observed for a few days following phase shifts to light stimuli (Johnson, Elliott and Foster, 2003). Pittendrigh (1958) suggested that transients occur as a light-driven oscillator gradually regains phase with the oscillating rhythm it drives. Advance and delay resetting by the pacemaker in response to light is rapidly accomplished within 2 hours of light onset (Best, Maywood, Smith and Hastings, 1999). However, it takes several days for the oscillations driven by the light-responsive pacemaker (the SCN in mammals) to reach this steady state. When the steady state in the overt rhythm is reached, it is in response to a signal that has occurred several days earlier. Transients are more pronounced for advance shifts than for delays and may contribute to the malaise experienced during jet lag and shift work.

A FRP = 26h; T = 24h

PRC model   Actogram

Timing of light for
+ 2h shift

26h
24h

24h
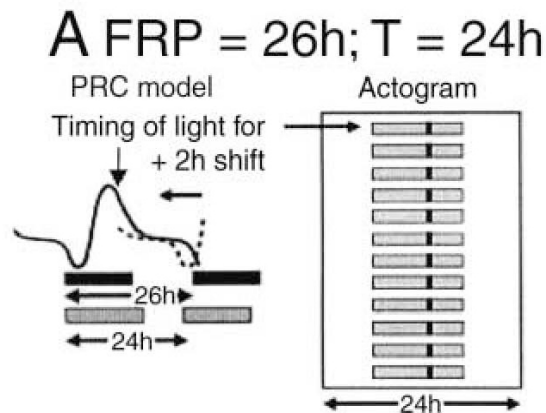
A. On the left**,** the solid PRC represents a 26- hour FRP of locomotor rhythm of a night-active animal, and the dotted PRC represents the necessary shift for entrainment to a 24-hour T cycle. The black bars on the actogram indicate the timing of the light necessary to bring about this shift.

B FRP = 22h; T = 24h

PRC model   Actogram

Timing of light for
– 2h shift

22h
24h

24h
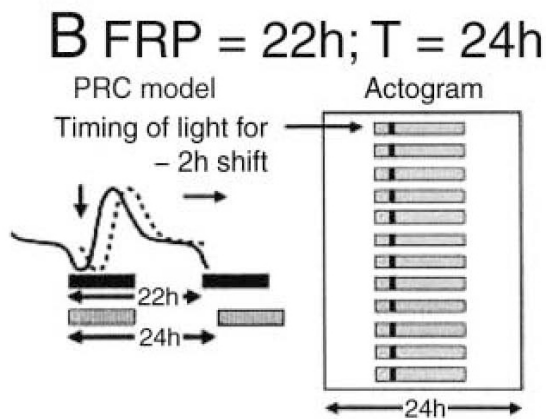
B. On the left**,** the solid PRC represents a 22- hour FRP of locomotor rhythm of a night-active animal, and the dotted PRC represents the necessary shift for entrainment to a 24-hour T cycle. The black bars on the actogram indicate the timing of the light necessary to bring about this shift.

Figure 4. The phase shift necessary for stable entrainment, phase shift = FRP – T, depends upon the length of the FRP. When the FRP is 2 hours longer than the T cycle as in A, light must strike the PRC during late subjective night to elicit a phase advance of two hours to establish stable entrainment. However, when the FRP is 2 hours less than the T cycle, light must coincide with early night to elicit a two- hour delay for stable entrainment. FRP: free running period, PRC: phase response curve, T: period of the environmental light/dark cycle (Johnson, Elliott and Foster, 2003)

C.   Nonphotic stimuli phase shift circadian rhythms during subjective day

Circadian rhythms can be phase shifted or entrained by non-photic stimuli during subjective day, a time when light has little effect (Reebs and Mrosovsky, 1989). Early studies first identified social stimuli as zeitgeibers that could influence phase and period of circadian rhythms in birds, mice and humans (Menaker and Eskin, 1966; Yamada, Shimomada, Takahasi, and Takahasi, 1986; Ehlers, Frank and Kupfer, 1988). Subsequent studies determined that social stimuli do not effectively entrain circadian rhythms in hamsters, mice or monkeys and it is likely that social stimuli instead affect circadian rhythms by regulating light exposure, inducing activity or stimulating arousal (Refinetti, Nelson and Menaker, 1992; Mistleberger and Skene, 2003). In addition, many studies established that nonphotic stimuli which increase arousal, including novelty-induced wheel running, sleep deprivation and saline injection will also produce large phase advances when presented during subjective day (Antle and Mistlberger, 2000; Edgar and Dement 1991; Hastings, Duffield, Smith, Maywood and Ebling, 1998; Mrosovsky, Reebs, Honrado and Salmon, 1989). Dark pulses, food deprivation, short acting benzodiazepines and morphine injections also produce phase advances, which depend upon induced behavioral activity (Van Reeth and Turek, 1989; Mistlberger, Sinclair, Marchant, and Neil, 1997; Mrosovsky and Salmon, 1990; Byku and Gannon, 2000). Phase delays elicited by non-photic manipulations may also be possible at the end of the animal's activity period or at the beginning of the rest period (see Fig. 5) but are smaller and not consistently observed (Marchant and Mistlberger, 1996; Reebs and Mrosovsky, 1989).
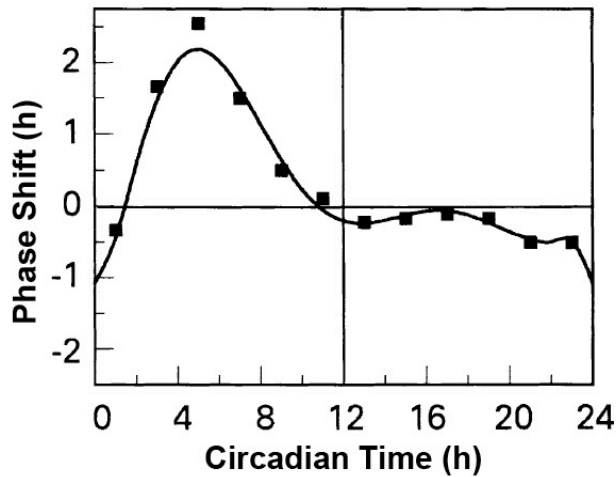
Non-photic signals are conveyed to the SCN via the GHT from the IGL utilizing projections containing neuropeptide Y, GABA and enkephalin (Harrington, 1997). There is considerable evidence that neuropeptide Y has a primary role in mediating non-photic input to the circadian system. Lesions of the GHT attenuate non-photic phase shifts in response to novel-running wheel induced activity (Wickland and Turek, 1994), dark pulses at circadian time 5-7 and 7-14 (Harrington and Rusak, 1986), confinement to a running wheel during the day (Janik and Mrosovsky, 1994) and benzodiazepines (Biello, Harrington and Mason, 1991; Johnson et al., 1998). NPY microinjected into the SCN induces a phase advance of circadian activity rhythms in hamsters (Albers and Ferris, 1984; Biello, Janik and Mrosovsky, 1994). Microinjections of antiserum raised against neuropeptide Y attenuate novelty-induced running wheel phase shifts (Biello, Janik and Mrosovsky, 1994). Treatment with NPY can advance the circadian clock in vitro as well as in vivo during subjective day (Shibata and Moore, 1993) but has no effect on circadian phase during the night (Harrington and Schak, 2000). Fos, a marker for neuronal

13

activation, is colocalized with neuropeptide Y in the intergeniculate leaflet following a non-photic phase shift (Janik, Mikkelsen and Mrosovsky, 1995). The NPY Y2 receptor mediates the phase advancing effects of NPY during the day (Golombek, Biello, Rendon and Harrington, 1996; Huhman, Gillespie, Marvel and Albers, 1996; Soscia and Harrington, 2005).

Serotonergic projections from the midbrain raphe also convey non-photic input to the SCN (Meyer-Bernstein and Morin, 1996). Serotonin agonists phase advance the clock during subjective day in vitro (Prossner, Miller and Heller, 1990) and in vivo (Edgar, Miller, Prossner, Dean and Dement, 1993; Horikawa et al., 2000). Both novelty-induced activity and sleep deprivation stimulate serotonin release during the day and novelty-induced locomotor activity suppresses serotonin release during the late subjective night in hamsters (Dudley, Di Nardo and Glass, 1998; Grossman et al., 2000). In addition, electrical stimulation of the dorsal and median raphe induces phase advances at circadian time (CT) 6 and phase delays at CT 14 in the Syrian hamster (Meyer-Bernstein and Morin, 1999). In the Syrian hamster, serotonergic afferents project to the SCN from the median raphe nucleus and to the IGL from the dorsal raphe nucleus and the midbrain raphe (see Fig. 6) (Meyer-Bernstein and Morin, 1996). Peripheral injections of serotonin agonist phase-shift the clock during daytime, but intracranial injections to the SCN have minimal effect (Challet, Scarbrough, Penev and Turek, 1998; Cutrera, Ouauour and Pevet, 1994). Serotonin exerts its phase-shifting effects by acting in the raphe or intergeniculate leaflet, rather than in the SCN (Duncan, Grear and Hoskins, 2004). Although serotonergic agonists are unable to elicit phase shifts when applied directly to the SCN, they readily phase shift the SCN when applied in vitro. The phase-shifting

effect of 5-HT agonists in vitro appears to depend on low endogenous levels of serotonin in the in vitro preparations, which allow the system to become more sensitive to treatments with the 5-HT agonists (Prossner, Lee and Wehner, 2006).
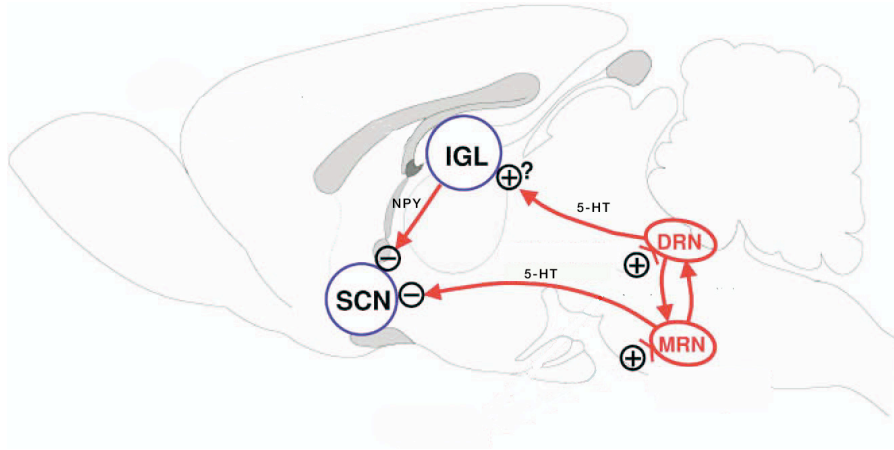
Figure 6. Afferent projections mediating non-photic input in the hamster circadian system.

How do non-photic phase shifts affect the master clock? The SCN is reset by non-photic phase shifts rapidly, within one to two hours of stimulus presentation (Hastings, Duffield, Smith, Maywood and Ebling, 1998; Mead et al., 1992). Non-photic stimulation suppresses basal c-fos expression in the SCN while increasing c-fos expression in the intergeniculate leaflet (Mikkelsen Vrang, and Mrosovsky, 1998; Antle and Mistlberger, 2000). Non-photic resetting acutely suppresses the clock genes *per1* and *per2*. Non-photic phase advances elicited by benzodiazepines treatment, novel-wheel induced activity, and saline injection, are associated with decreased *per* gene activity in the SCN (Maywood, Mrosovsky, Field and Hastings, 1999; Yokota et al., 2000;

Maywood and Mrosovsky, 2001). Neuropeptide Y1/Y5 and Y2 agonists decreased *per 1* and *per2* expression in SCN slices (Fukuhara, Brewer, Dirden, Bittman, Tosini and Harrington, 2001). Injections of antisense oligonucleotides to *per1* induce phase advances during subjective day, suggesting that suppression of period genes is the cause and not the consequence of non-photic phase shifts (Hamada, Antle and Silver, 2004). Light-induced phase shifts during the night have the opposite effect, enhancing *per* expression in the SCN.

Light during the day does not elicit a photic phase shift; however, light can inhibit the response of the clock to non-photic stimuli during the subjective day. A light pulse during the day blocks phase advances in response to novelty-induced activity, NPY, serotonin and sleep deprivation (Mrosovsky, 1991; Biello and Mrosovsky, 1995 and Penez, Zee and Turek, 1997; Grossman, Mistlberger, Antle, Ehlen and Glass. 2000). *Per1* expression is higher in animals given a light pulse directly after non-photic stimuli than in animals receiving non-photic stimuli alone (Maywood and Mrosovsky, 2001). Glutamate, replicating the actions of light, inhibits phase resetting in response to NPY in the SCN slice preparation (Biello, Golombek and Harrington, 1997).

D. **Light-induced phase shifts are inhibited by non-photic stimuli, serotonin and NPY**

The possibility that behavioral stimuli might interact with light-induced phase shifts was prompted by reports that (1) testes regression, which is prevented by a night time light pulse, will occur if the light is delivered with concurrent locomotor activity (Ferraro and McCormack, 1985) and (2) light pulses and locomotor activity compete in setting circadian phase in the scorpion (el Bakary and Fuzeau-Braesch, 1988).

Ralph and Mrosovsky (1992) determined that light-induced phase advances are attenuated by simultaneous locomotor activity in the Syrian hamster. Sleep deprivation, morphine-induced activity, and the neurochemicals that phase advance circadian rhythms during the day (NPY, 5-HT, and opioids) decrease the phase response to light stimuli during the late subjective night (Mistlberger and Antle, 1998; Mistlberger and Holmes, 2000; Biello et al., 1997a; Edelstein et al., 2003; Tierno, Fiore and Gannon, 2002). However, light-induced phase delays are not significantly attenuated by behavioral activation, NPY and serotonin (Challet, Turek, Laute and Van Reeth, 2001; Mistlberger and Antle, 1998; Weber and Rea, 1997).

Co-application of NPY with glutamate or NMDA (which mimic the effects of light) blocks both phase delays and advances to glutamate or NMDA in vitro (Biello et al., 1997;Yannielli and Harrington, 2000). In addition, NPY can be applied in vitro to block phase delays and advances to a light pulse administered before the slice preparation showing that its actions act on processes downstream of light input (Yannielli and Harrington, 2000). Effects of NPY on light-induced resetting are mediated by the NPY Y5 receptor (Lall and Biello, 2003; Yannielli and Harrington, 2001). Antiserum to NPY and the NPY Y5 receptor antagonist, RJW-57926, (RW Johnson Pharmaceutical Research Institute, Spring House), potentiate light-induced phase advances in the hamster (Biello, 1995; Lall and Biello, 2003;Yannielli and Harrington, 2004). In addition, the Y5 antagonist, RJW-57926, counteracts the inhibitory effects of NPY on light induced phase shifts in vivo and in vitro (Yannielli and Harrington, 2001a; Yannielli and Harrington, 2001b).

Serotonin can interact with light-induced phase shifts through the presynaptic 5-HT1B receptor on retinohypothalamic tract axon terminals and via the postsynaptic 5-HT1A/7 receptor (Pickard and Rea, 1997; Rea, Barrera, Glass and Gannon, 1995). An agonist at the 5-HT 1A/7 receptor, given systemically or microinjected into the SCN, inhibits light-induced phase advances in hamsters (Rea, Glass and Colwell, 1994; Weber, Gannon and Rea, 1998). Light exposure blocked phase advances induced by the 5-HT (1A/7) receptor agonist, 8-OH-DPAT (Ehlen, Grossman and Glass, 2001). A caveat to this finding is that 8-OH-DPAT did not induce daytime phase shifts and had no effect on light-induced phase shifts in mice (Antle, Ogilvie, Pickard and Mistlberger, 2003). In a subsequent study, 8-OH-DPAT induced phase advances when given to mice systemically during midday (Horikawa and Shibata, 2004). Additional studies are necessary to determine under what conditions serotonin regulates phase shifting in mice.

Several serotonergic compounds acting at the 5-HT1A receptors greatly enhance photic phase shifts in hamsters (Gannon, 2003; Gannon and Millan, 2006b). NAN-190, which acts as an agonist at the 5-HT1A somatodendritic autoreceptor and a partial agonist at the 5-HT1A/7 postsynaptic receptor, potentiates light-induced phase advances in hamsters by as much as 250% (Rea, Barrera, Glass and Gannon, 1995). Activation of a different class of receptors, the presynaptic 5-HT1B receptors located on the retinal axons in the SCN, attenuates light-induced phase shifts in hamsters (Rea, Buckley and Lutton, 1993; Pickard et al., 1999).

At the behavioral level, non-photic stimuli oppose the effects of photic stimuli. Novelty-induced activity attenuated the phase advance to a light pulse, but did not alter Per1 mRNA and Fos protein expression in the SCN (Edelstein, de la Iglesia, Schwartz

and Mrosovsky, 2003). However, NPY decreased light-induced *per1* mRNA expression and blocked light-induced *per2* mRNA expression in the SCN, although it did not alter c-Fos expression (McKinley Brewer, Yannielli and Harrington, 2001). Inhibition of photic responses by serotonergic agonists is accompanied by decreased c-Fos expression as well as decreased light-induced expression of both *per1* and *per2* mRNA (Yokota et al., 2000; Horikawa et al., 2000). The interactions of light and non-photic stimuli have opposing effects, which appear to converge on the core mechanism of the clock (Maywood and Mrosovsky, 2001).

Inhibition of photic-induced phase shifts by non-photic stimuli is more effective during the late subjective night (the phase advance portion of the PRC). Behavioral manipulations can attenuate light induced phase advances, but not phase delays (Ralph and Mrosovsky, 1992; Mistlberger and Antle, 1998). Triazolam, a benzodiazepine used for the treatment of anxiety and insomnia, induced phase advances during the day (CT6). However, during the nighttime, triazolam inhibits light-induced phase advances and increases light-induced phase delays (Joy and Turek, 1992). *In vitro* studies determined that NPY can inhibit phase delays as well as phase advances, but NPY is more effective in attenuating phase advances *in vivo* (Yannielli and Harrington, 2000; Lall and Biello, 2003; Weber and Rea, 1997). These differences may be due to endogenous tone of NPY in the early night *in vivo,* which is not a factor in the brain slice preparation (Yannielli and Harrington, 2004). Serotonin is able to oppose the effects of light throughout the subjective night. The 5-HT1A/7 agonist (8-OH-DPAT) and the 5-HT1B agonist (TFMPP) injected systemically inhibit light-induced phase delays as well as phase advances (Rea et al., 1994; Pickard et al., 1999; Weber et al., 1998).

E.   Combined inhibition of NPY and 5-HT input potentiates photic phase advances

Individually, both NPY and serotonin can interact with light to influence entrainment of the circadian pacemaker. NPY and 5-HT agonists block the phase shifting effects of nighttime light. Antagonists at the NPY Y5 receptor can potentiate the phase shifting effects of light during the subjective night (Yannielli and Harrington, 2004). In addition, drugs that act as antagonists at the postsynaptic 5-HT 1A/7 receptor and agonists at the 5-HT autoreceptors potentiate phase shifts to nighttime light pulses (Rea, Barrera, Glass and Gannon, 1993; Smart and Biello, 2001 and Gannon, 2003). Lall and Harrington (2006) measured the effects of combined inhibition of NPY and 5-HT input on photic shifts to determine whether these neural pathways provide independent or redundant input to the SCN. They found that pharmacological treatments that target both serotonergic and neuropeptide Y afferents to the SCN (Fig. 6) could significantly potentiate light-induced phase advances during the late subjective night in the Syrian hamster. Their work was the first report of the effect of combined inhibition of NPY and 5-HT input on light induced phase shifts *in vivo*.

F.   Introduction to the thesis experiments

It is important to extend the findings of Lall and Harrington (2006), that blocking both serotonergic and neuropeptide Y input (Fig. 7) significantly potentiated light-induced phase shifts in the Syrian hamster, to other species. In my first experiment I investigated whether combined treatment with an NPY Y5 antagonist, CP-781, 214, and

the 5-HT 1A partial agonist, NAN-190, would regulate light-induced phase shifting in mice. An advantage to using the mouse as a model is the ability to study the effects of the pharmacological treatment on the knockouts for specific clock genes, NPY, and 5-HT receptors. In addition, the Harrington Lab has recently acquired the knockin mouse, *Per2^{Luc}* (Yoo et al., 2004) and has the capability to perform bioluminescent imaging (Actimetrics Lumicycle System, Chicago, IL) in SCN slices and tissue dissected from peripheral organs. The Lumicycle can be used to study the effects that pharmacologically blocking non-photic input has on light induced PER 2 expression in the SCN and in peripheral organs.
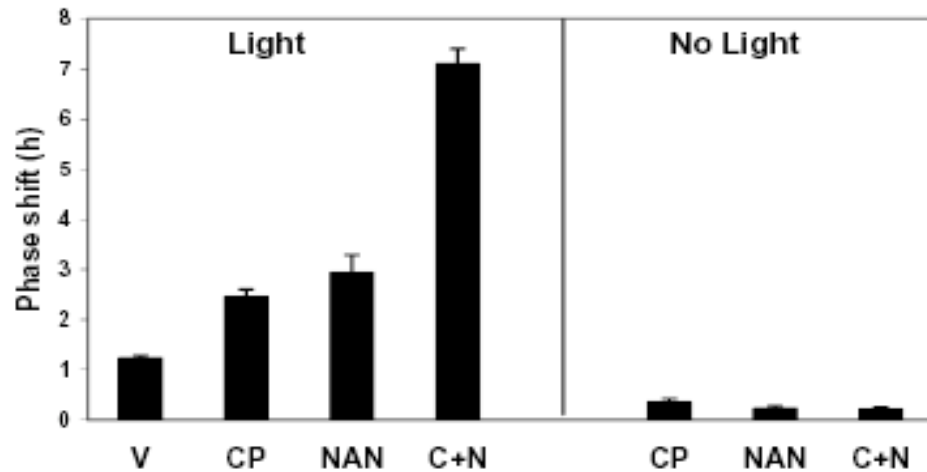
Figure 7. The average phase shifts of wheel-running activity in Syrian hamsters treated with NPY Y5 antagonist, CP-760, 542 and NAN-190 45 minutes before a light pulse at CT 19. The following abbreviations were used: V: vehicle-treated animals; CP: CP-760, 542, a NPY Y5 antagonist; NAN: NAN-190, a 5-HT1A partial agonist; C + N: combined treatment with CP-760, 542 and NAN-190. (Lall and Harrington, 2006)

In the first experiment, I measured the effects that the combined treatment (NPY Y5 antagonist and a 5-HT1A/7 partial agonist) had on photic phase shifts in mice. I administered light pulses to the mice during the early subjective night and measured the effects of combined treatment on phase delays. I chose to work with phase delays in mice because mice have a small advance region in their phase response curve making it difficult to attain consistent and sizeable phase advances with a light pulse administered in late subjective night (see Fig.8). Whether pharmacologically blocking both NPY and Y5 and 5-HT 1A/7 receptors will potentiate phase delays during the early subjective night has not yet been determined. Studies blocking either NPY or 5-HT in mice prior to a light pulse have yielded inconsistent results (Lall and Biello, 2003b; Weber and Rea, 1997). In addition, light-induced phase delays are not significantly attenuated by NPY or serotonin (Mistleberger and Antle, 1998;Weber and Rea, 1997).
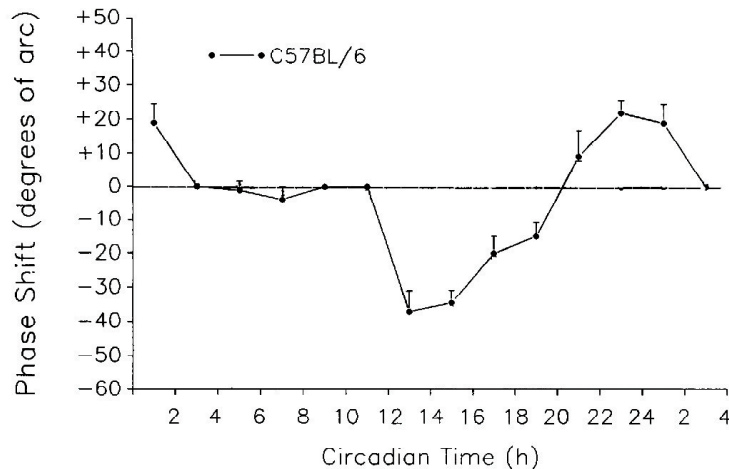
Figure 8. Phase response curve for C57BL/6 mice for 15- minute light pulse (100-200 lux). (Schwartz and Zimmerman, 1990)

22

I found that blocking NPY and 5-HT input with systemic injections of CP-781, 214 and NAN-190 did not modulate photic phase delays in mice (see Fig. 11). It was unclear whether the discrepancy of this finding from the previous results (Lall and Harrington, 2006) was due to species differences between mice and hamsters, or because the treatment was given to mice during the early subjective night. Although NPY can reduce the phase-resetting action of a light pulse during late subjective night, it does not block phase delays during the early subjective night in vivo in hamsters (Yannielli and Harrington, 2001). Serotonergic compounds are able to modulate phase advances (Gannon and Millan, 2006; Moriya et al., 1998; Rea et al., 1995) and phase delays (Lall and Biello, 2003b) in hamsters. Lall (2006) found that blocking both NPY and 5-HT input potentiated phase advances when light pulses were delivered during the late subjective night using hamsters, but did not study the effects on phase delays in the early night. In my second experiment, I sought to test the hypothesis that blocking both NPY and 5-HT input selectively affects photic phase shifting during the late subjective night (advances) and not phase shifts during the early subjective night (delays) in hamsters. I predicted that blocking NPY input with the NPY Y5 receptor antagonist (CP-760, 542 or CP- 781,214) in combination with blocking serotonin input with the 5-HT 1A partial agonist, NAN-190, would potentiate phase advances during the late subjective night, but would not significantly modulate light-induced phase delays during the early subjective night in hamsters. If, on the other hand, I found that blocking NPY and 5-HT input potentiates phase delays in hamsters, this would suggest that the discrepancy in my findings was due to species differences.

Treatments that modulate circadian responses to light-induced phase setting could facilitate entrainment and may have therapeutic value in treating circadian desynchronization disorders including jet lag, seasonal affective disorder and dysregulated circadian function in Alzheimer's patients. In real-life, photic and non-photic inputs interact and circadian entrainment will be influenced by behavioral stimuli as well as by the light/dark cycle (Dallman and Mrosovsky, 2006). This research will increase our basic understanding of circadian biology by characterizing the interaction of photic and non-photic inputs at different phases of the PRC.

CHAPTER III

MATERIALS AND METHODS

A.   General Methods

Animals. Young adult male C57BL/6 mice (Charles River Labs, Kingston, NY) and Syrian Hamsters (Charles River Labs, Kingston, NY) were individually housed in clear plastic cages with free access to food and water. Animal procedures were performed in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and the National Institute of Health; the Smith College Institutional Animal Care and Use Committee approved all experiments. Manipulations were carried out under dim red light and all efforts were made to minimize confounds with environmental cues, including noise. Cage changes were not scheduled for the three days immediately before and after treatments. Mice were entrained under a 12 hour light/12 hour dark cycle, while hamsters were entrained under a 14 hour light/10 hour dark cycle. An additional difference is that mice were placed in five days of complete darkness (DD) prior to treatment while hamsters were maintained in 10 days of DD prior to treatment.

Wheel running studies. Animals were housed in cages equipped with running wheels connected to a computer running Clock Lab software (Actimetrics, Evanston, IL, USA). Wheel running activity was detected by magnetic microswitches and was collected in one-minute bins. Wheel-running activity records (actograms) were analyzed using ClockLab software. Free running period of locomotor activity rhythm and light-induced phase shifts

were measured in constant dark conditions. Each day of the free-running period for an individual subject is divided into 24 hours of circadian time. Activity onset (CT 12) for the day of treatment was calculated by forward extrapolation of a regression line fitted to activity onsets for the three days prior to treatment for mice and for the seven days prior to treatment for hamsters. The circadian phase was determined by the addition of the subject's circadian hours (CH), calculated as free running period divided by 24, to their CT 12 on treatment day. For example, CT16 = CT12 + 4 X CH. Phase shifts in locomotor activity rhythms were calculated using the linear regression method (Pittendrigh and Daan, 1976).

Drugs and routes of administration. The selective neuropeptide Y Y5 antagonists, CP-781, 214 or CP-760, 542 (Pfizer, CT) and the 5-HT 1A partial agonist, NAN-190 (Sigma, MO), were dissolved in 32% cyclodextrin (Sigma, MO). Although CP-781, 214 was used in the pilot study and Experiment 1, it was necessary to use another NPY Y5 antagonist, CP-760, 542, in Experiment 2 because CP-781, 214 was not available in sufficient quantity from Pfizer at the time Experiment 2 was conducted. CP-760, 542 is given at a higher dose than CP-781, 214. The drugs were administered at a dose CP-781, 214 (10 mg/kg) or CP-760, 542 (40 mg/kg) in combination with 5 mg/kg (NAN-190*).* Vehicle injections consisted of an equivalent volume of 32% cyclodextrin. Injection volumes were approximately 0.1 ml for mice and 0.5 ml for hamsters. Calculations were based on the animal's weight as measured three to five days before each treatment. Drugs were prepared on the day of treatment. Each animal received a single intraperitoneally (i.p.) injection 45 minutes prior to a light pulse. Preliminary studies determined that the drugs could be administered in a single i.p. injection and that drug treatment alone did not result in a phase shift (see Fig. 9).

Statistical analysis. The design of the experiment included 4 treatment conditions at 6 time point for 30 animals and would have provided 5 data points for each condition (TX @ CT); however I only collected ½ the data and had only 2 data points at some conditions, so it was not possible to us ANOVA as planned. Instead, means for phase shifts in the different treatment groups were compared within a circadian phase (CT 14, CT 16, CT 18, CT 20 and CT 22). Differences in means were analyzed with the two-sample independent t-test (one-tailed) (Mendenhall, Beaver and Beaver, 2006). Means are represented as ± standard error of the mean in the text and figures.

B.   Pilot Study. Can CP-781, 214 and NAN-190 be delivered in a single intraperitoneal injection?

Light-induced phase shifts were measured in hamsters following a single combined i.p. injection with the NPY Y5 antagonist, CP-781, 214, and the 5-HT 1A partial agonist, NAN 190, prior to a 15 minute light pulse (500 lux) at CT 19. These shifts were compared to phase shifts in hamsters that received only an equivalent light pulse at CT 19. Regression lines were drawn through the activity onsets for seven days prior to treatment and through activity onsets for the seven days following treatment. Activity onsets for the three days following treatment were omitted to allow for transient cycles. Phase shifts were calculated as the horizontal distance between the two regression lines on the first day after treatment.

C.   Experiment 1. Does combined treatment with 781, 214 and NAN-190 modulate photic phase delays in mice?

Mice were maintained under a 24-hour light/dark cycle consisting of 12 hours of light and 12 hours of darkness for a minimum of three weeks. Animals were transferred into constant darkness (DD) for five days before each treatment. Immediately following treatment, mice were maintained under DD for 10 days. Mice were allowed to reentrain under a 12 hours light/ 12 hours dark schedule after receiving two successive treatments.. Mice were randomly assigned to control group (light + vehicle injection) or experimental group (light and NAN-190 + CP-781, 214). Two additional groups were injected with vehicle or drug combination 45 minutes prior to CT 16, without light pulse. Light pulses were given at circadian time

(CT) 16, the circadian time of maximal phase delay for the C57BL/6 strain (Schwartz and Zimmerman, 1990; preliminary studies in the Harrington lab, 2004). Mice were received a 15-minute light pulse (500 lux) in a clean cage without bedding cage or cover and were returned to their own cage. Mice remained in constant dark for ten days following treatment. Regression lines were drawn through the three activity onsets prior to treatment and through activity onsets for the seven days following treatment. Activity onsets for the three days following treatment were omitted to allow for transient cycles. Phase shifts were calculated as the horizontal distance between the two regression lines on the first day after treatment.

D.   Experiment 2. Do NPY Y5 and 5-HT 1A receptors regulate photic phase shifting only during a temporal window in late subjective night?

Experimental treatments. Hamsters were maintained under a 24-hour light/dark cycle consisting of 14 hours of light followed by 10 hours of darkness for at least three weeks. They were transferred to constant darkness (DD) for 10 days prior to each treatment and were maintained in constant darkness for ten days after each treatment. Hamsters were randomly assigned to receive two treatments in early subjective night and two treatments during late subjective night. Following two treatments, hamsters were allowed to reentrain under a 14 hours light/ 10 hours dark schedule. The order of treatments was varied so that no more than two animals received the same treatment sequence at a specific circadian phase. Light pulses were given at CT 14, CT 16, CT 18, CT 20 and CT 22. Each animal received a maximum of 2 light pulses and 4 treatments. Control conditions included 1) a light pulse with vehicle injection and 2) drug treatment without a light pulse. Animals received a 5-minute

light pulse (500 lux) in a clean cage without bedding and were returned to their home cage.

Phase shifts for hamsters were calculated as described in the Pilot Study.

E.   Experiment 3. Can blocking NPY and 5-HT input eliminate transient cycles in photic phase shifts?

Transient analysis. During my experiments, I observed that hamsters treated with CP-760, 542 and NAN-190 prior to the light pulse showed a rapid shift, without transient cycles (see Fig. 14 and Fig. 16). I quantified this observation by comparing the phase shift on the day immediately following treatment as a percent of the steady state phase shift measured on day 4. The percent of steady phase shift reached on day 1 for the drug + light treated animals was compared to this percent in the vehicle + light treated animals in Experiment 2.

CHAPTER IV

RESULTS

A. **Pilot Study. Can CP-781, 214 and NAN-190 be delivered in a single intraperitoneal injection?**

The average phase shifts of wheel running rhythms in hamsters given a single i.p injection of CP-781, 214 combined with NAN-190 in cyclodextrin (n =5) prior to light was 3.8 ± .7 h. The mean phase shift in the group receiving light alone was 1.4 ± .12 h. (n =5); hamsters receiving no light (n = 4) showed no phase shift. Injections were given 45 minutes prior to a 15-minute light pulse at CT 19. Animals receiving a single injection of the combined drugs show a significantly greater phase shift than the group given light alone ($P< 0.05$) (see Fig. 9).
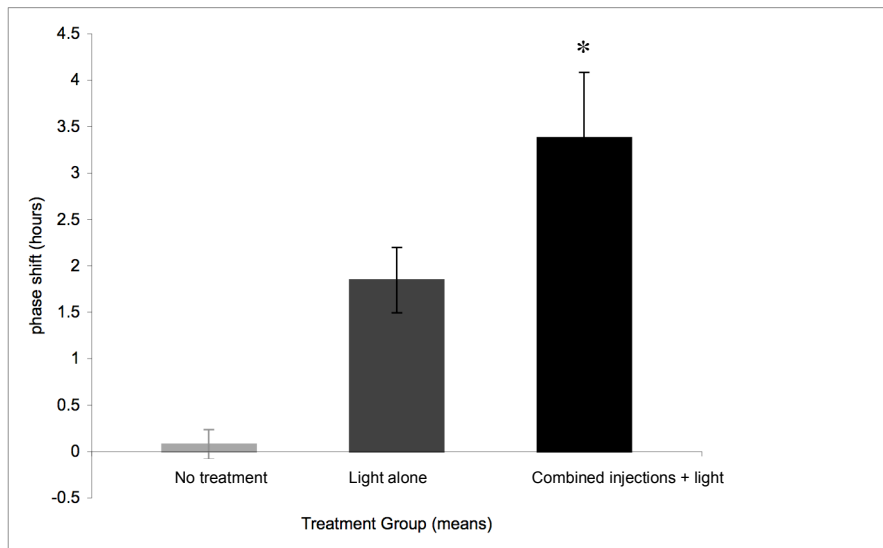
Figure 9. CP-781, 214 and NAN-190 given in cyclodextrin in single i.p. injection was effective in eliciting photic phase advances in hamsters. Treatments were given 45 minutes prior to a light pulse at CT 19. The mean phase shift in hamsters receiving single injection of NAN-190 + CP-781, 214 + light was greater than group receiving light alone ($P< 0.05$).

B. **Experiment 1: Does combined treatment with NPY and serotonin antagonists modulate photic phase delays in mice?**

Representative actograms for Experiment 1 are shown in Fig.10. Control animals, treated with vehicle injection 45 minutes prior to a light pulse at CT 16, had a mean phase delay of -1.035 ± 0.23 h (mean ± SEM, n = 5). Mice treated with CP-781, 214 and NAN-190 prior to a light pulse at CT 16 showed a mean phase shift of -1.1 ± 0.18 h (n =8). The difference in the mean phase shift between the mice treated with CP-781, 214 and NAN-190 prior to light pulse and the mice receiving vehicle injection prior to light pulse was not significant (p = 0.41; see Fig. 11). Treatment with either vehicle (n = 4) or combined CP-781, 214 and NAN-190 (n = 5) without light pulse did not result in a phase delay. Two mice were excluded from the study because of insufficient running wheel data.

A   No treatment

Time (h)

B   LP at CT 16

Time (h)

C   Drug + LP at CT 16

Time (h)
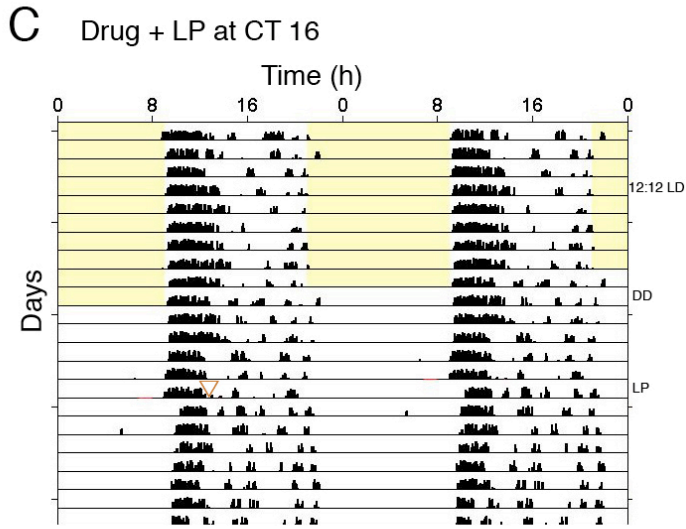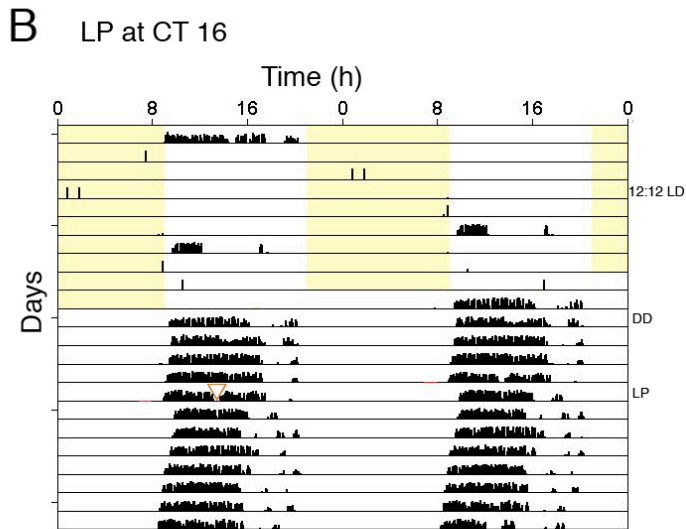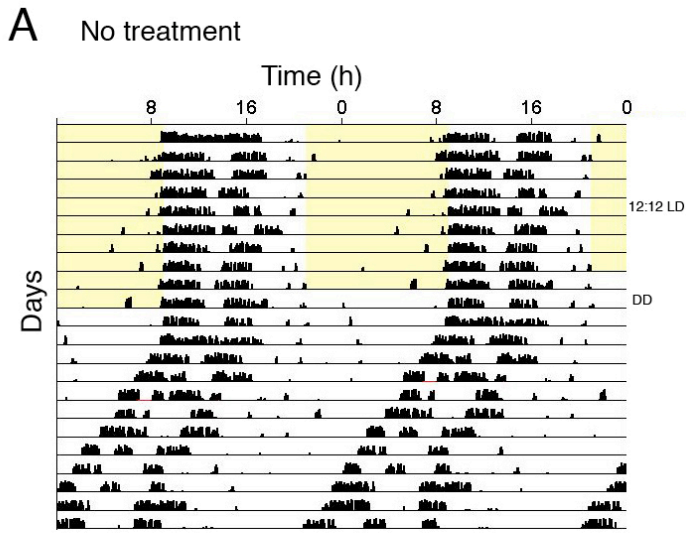
Figure 10. Representative double-plotted actograms from C57/BL6 mice released into DD after entrainment to a 12:12 light: dark (LD). Mice were released into constant darkness (DD) and received (A) no treatment (B) a 15-minute light pulse (LP) delivered at CT 16 without injection C) treatment with CP-781, 214 and NAN-190 in a single i.p. injection 45 minutes prior to a light pulse at CT 16. The black marks indicate revolutions of the running wheel of an individual mouse collected in one-minute bins. Two consecutive days of activity are plotted horizontally, with day 1 on the left and day 2 on the right. Day and time of the Light pulses (15 min, 500 lux) are indicated by the ($\triangledown$).
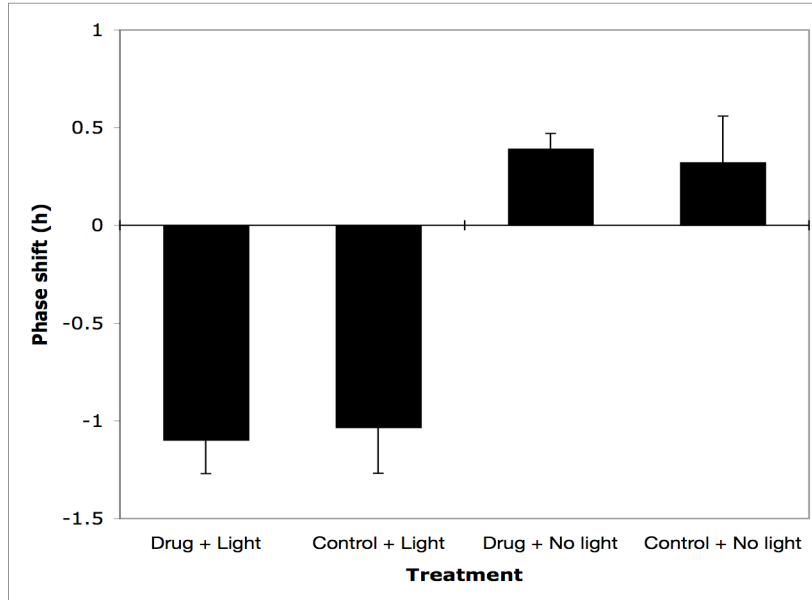
Figure 11. Average phase shifts of wheel running rhythms in mice following treatment with the 5-HT 1A/7 receptor mixed antagonist/agonist NAN-190 and the NPY Y5 antagonist, CP-781, 214) (n=8) prior to a light pulse at CT 16 (15 min pulse, 500 lux). Control group (n=5) received a vehicle injection prior to the light pulse at CT 16. Additional treatment groups received systemic injection with vehicle or CP-781, 214 + NAN-190 45 minutes prior to CT 16 without subsequent light pulse. Error bars identify standard error of the mean (SEM). Drug indicates combined CP-781, 214 + NAN-190 in a single i.p. injection.

C. Experiment 2: Do NPY Y5 and 5-HT 1A/7 receptors regulate photic phase shifting only during a temporal window in late subjective night?

Both vehicle and drug treated hamsters phase delayed following a light pulse at CT 14 (Fig.

12); hamsters receiving drug treatment without light did not phase shift. A light pulse

administered at CT 14 induced a mean phase delay of -1.39 ± .29 h (n = 3) in vehicle treated

hamsters and a mean delay of -0.9 ± 0.13 h (n = 4) in animals receiving CP-760, 542 and

NAN-190 (Fig. 13). Although the drug treatment attenuated the photic phase delay, the

difference in mean phase shifts was not statistically significant in vehicle treated and drug

treated animals (P > 0.05). These results supported my findings in Experiment 1, that

combined treatment with a NPY Y5 antagonist and NAN-190 did not potentiate phase delays

in mice. Animals receiving either vehicle or combined drug injection without light showed

minimal phase shifts (0.04 ± .06 h and 0.04 ± .04 h respectively).

Both vehicle and drug treated hamsters phase advanced following a light pulse at CT 16, CT

18 CT 20 and CT 22; hamsters receiving drug treatment without light at these circadian

phases did not phase shift (Fig.13). A trend was observed for drug treatment to potentiate

light induced phase shifts only during late subjective night. At CT 16, the light pulse elicited

a mean phase advance of 0.71 ± 0.35 h (n = 3) in vehicle treated animals and a mean advance

of 2 ± 1.08 h in the group administered CP-760, 542 and NAN-190 (Fig 13). The difference

in mean phase advances was not statistically significant (P > 0.05). Systemic injection of

CP-760, 542 + NAN-190 or vehicle without light resulted in phase shifts of - 0.135 ± 0.124

h (n = 4) or 0.092 ± .0188 h (n = 4) respectively. Mean photic phase advances following

treatment with vehicle and light at CT 18 were 1.78 ± 0.09 h (n = 2); treatment with CP-760,

542 and NAN-190 and light at CT 18 resulted in a phase advance of 3.05 ± .919 h (n=2); the

phase shift difference was not statistically significant (P > 0.05) (Fig 13). Treatment with

either combined vehicle or CP-760, 542 and NAN-190 without light resulted in phase delays

of - 0.2 ± 0.09 h (n = 2) or - 0.195 ± 0.141 h (n = 2) respectively. A light pulse administered

at CT 20 induced a mean phase advance of 1.61 ± 0.3 h (n = 2) in vehicle treated hamsters

and a mean advance of 2.815 ± 0.175 h (n = 2) in animals receiving CP-760, 542 and NAN-

190 (Fig. 13 and Fig. 14). The difference at CT 20 was statistically significant (P < 0.05).

Phase shifts measured in animals receiving drug or vehicle without light at CT 20 were 0.07

± 0.04 h (n = 2) and – 0.04 ± 0.02 h (n = 2) respectively. At CT 22, animals treated with

vehicle and light had a mean phase advance of 1 ± 0.23 h (n = 2) and animals receiving CP-

760, 542 and NAN-190 with light had a mean phase advance of 1.39 ± 0.45 h (n = 2) (Fig.

13). The difference in mean phase advances was not statistically significant $(P > 0.05)$. Treatment with either vehicle or combined CP-760, 542 and NAN-190 without light resulted in phase shifts of 0.16 h (n = 1) or 0.13 ± 0.227 h (n= 3) respectively. The results for Experiment 2 are summarized in Table 1.

The phase shift in the CP-760, 542 + NAN-190-treated group was significantly larger than the phase shift in the vehicle-treated group at CT 20 $(P< 0.05)$. The phase shift in the drug-treated + light group was smaller than in the vehicle + light group at CT 14. The average phase shifts elicited by treatment with the combined CP-760, 542 and NAN- 190 prior to the light pulse and vehicle injection prior to a light pulse at the different circadian phases: CT 14, CT 16, CT 18, CT 20 and CT 22 are shown in Figure 15. In summary, the combined inhibition of NPY and 5-HT input potentiated light induced phase shifts during late subjective night (at CT 20) and not during early subjective night (at CT 14).

A   Veh + no light at CT 14

Time (h)

B   Veh + LP at CT 14

Time (h)

C   Drug + LP at CT 14

Time (h)
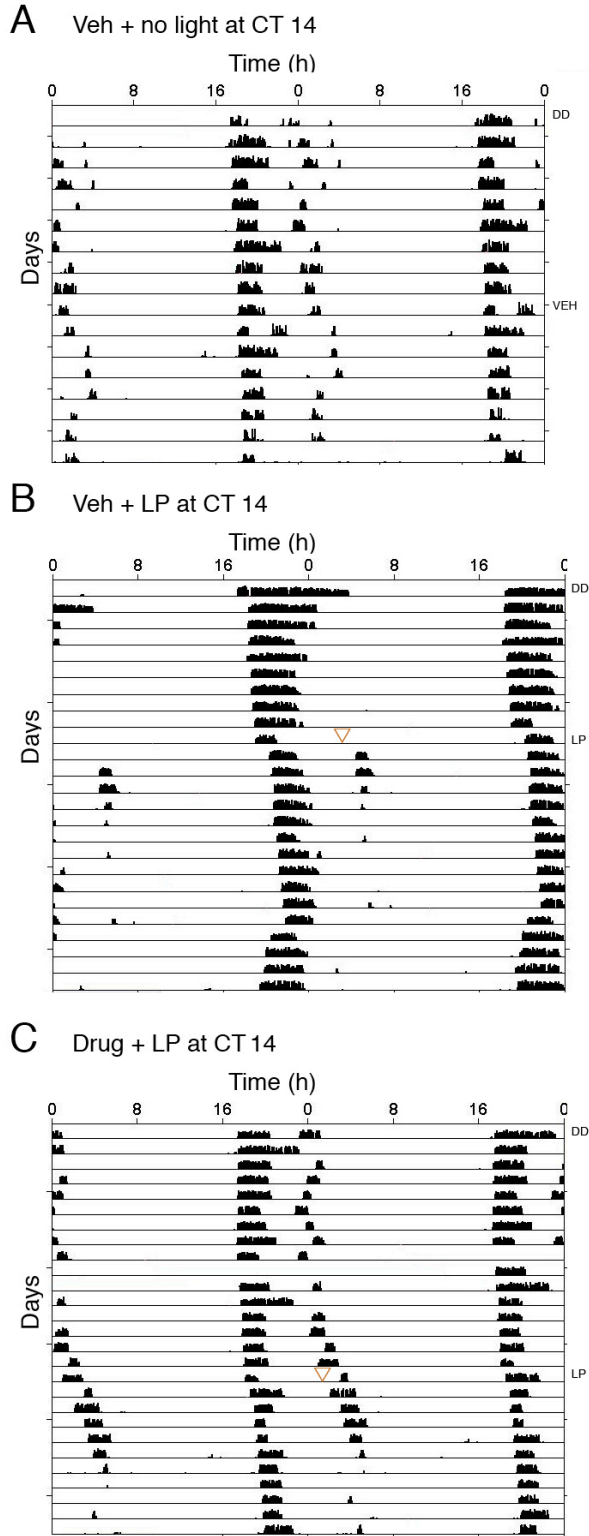
Figure 12. Representative double-plotted actograms showing wheel running activity in hamsters following: treatment with (A) vehicle, 32% cyclodextrin, without light pulse, (B) vehicle 45 minutes prior to a light pulse at CT 14 and (C) treatment with CP-760, 542 and NAN-190 45 minutes prior to a light pulse at CT 14. The black marks indicate revolutions of the running wheel of an individual hamster collected in one-minute bins. Animals were previously entrained to 14 h light: 10 h dark before, and then maintained in DD for 10 days prior to treatment. Times of light pulses (5 minute, 500 lux) are indicated by the triangle (▽).
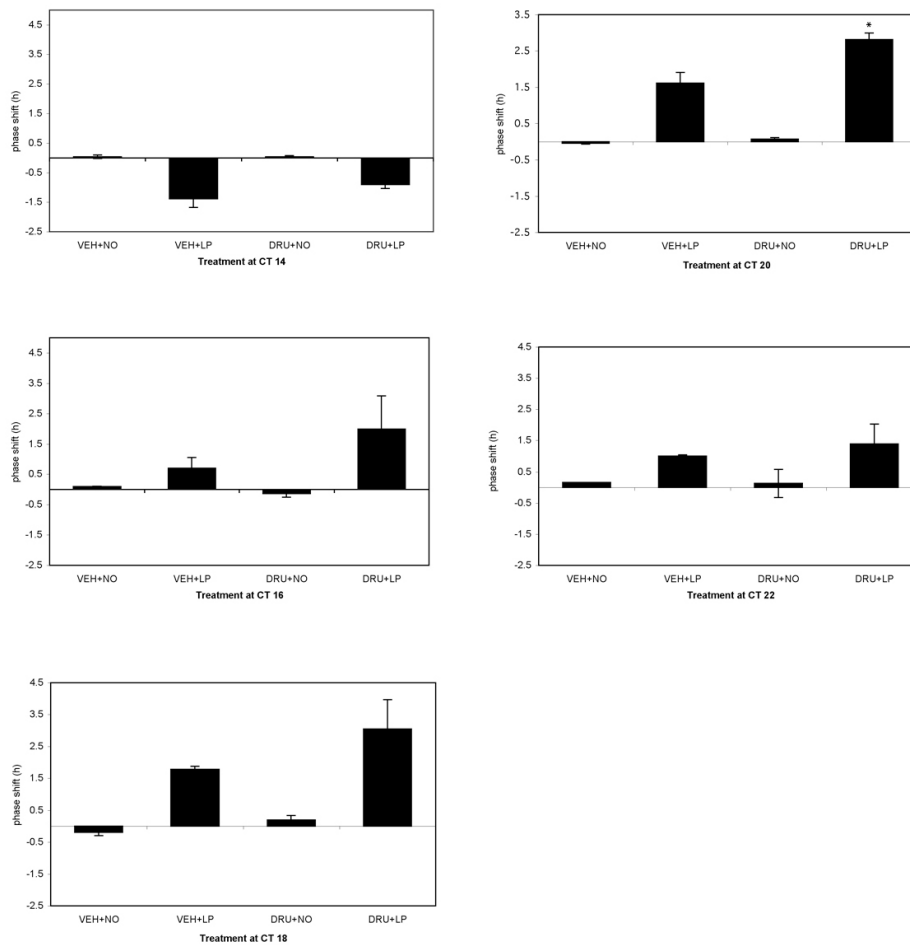
Figure 13. Average phase shift of wheel-running activity rhythms in hamsters treated with vehicle or combined CP-760, 542 + NAN-190 at circadian phases throughout the subjective night. In addition, some hamsters received a light pulse (5 minute 500 lux) 45 minutes after i.p. injection while others remained in constant darkness after receiving an injection. Error bars indicate standard error of the mean (SEM). Significance indicated by ∗, (P< 0.05).

Table 1: Mean phase shifts for all groups in Experiment 2.

	CT 14	CT 16	CT 18	CT 20	CT 22
Vehicle alone	0.04 ± .06	0.09 ± .02	-0.19 ± .09	-0.04 ± .02	0.16
Vehicle + light	-1.39 ± .29	-.71± .35	1.8 ± .09	1.61 ± 0.3	1.01 ± .02
Drug alone	0.04 ± .04	-.13 ± .12	-0.19 ± .14	0.07 ± .04	0.13 ± .23
Drug + light	-0.9 ± .13	2.0 ± 1.09	3.05 ± .92	2.82 ± .17	1.39 ± .45
P- values	0.110784	0.177614	0.216774	0.042097	0.280813
t-test between veh + light and drug + light	not significant	not significant	not significant	significant	not significant

A Drug + no light at CT 20

Time (h)

B Veh + LP at CT 20

Time (h)

C Drug + LP at CT 20

Time (h)
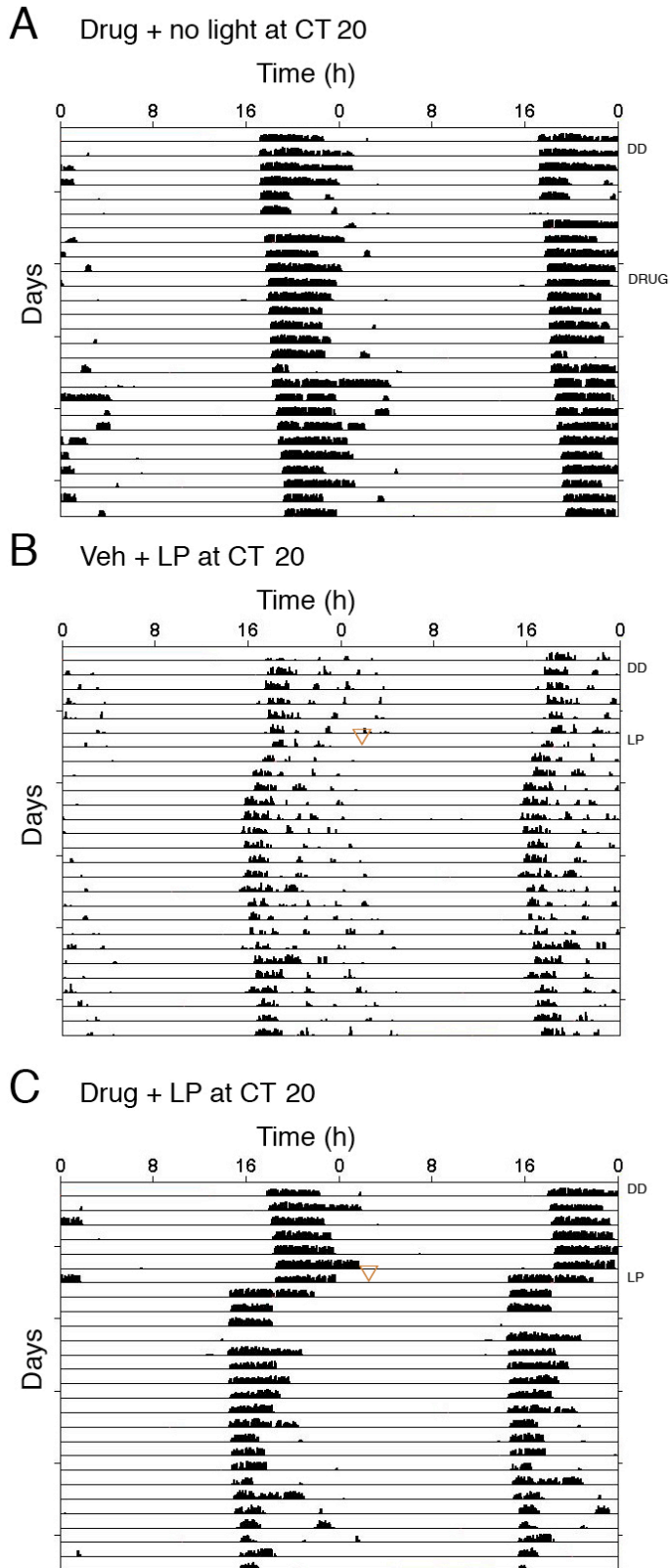
Figure 14. Representative double plotted-actograms showing the potentiation of light induced phase advances in hamsters by combined treatment with NPY Y5 antagonist, CP-760, 542, and 5-HT1A partial agonist, NAN-190. Treatment: (A) CP-760, 542 and NAN-190 45 minutes to CT 20 without light pulse, (B) treatment with vehicle, 32% cyclodextrin 45 minutes prior to a light pulse at CT 20 and (C) treatment with CP-760, 542 and NAN-190 45 minutes prior to a light pulse at CT 20. The black marks indicate revolutions of the running wheel of an individual hamster collected in one-minute bins. Animals were previously entrained to 14 h light: 10 h dark before, and then maintained in DD for 10 days prior to treatment. Light pulses (5 min, 500 lux) are indicated by the triangle (∇). It is significant that the phase advance following treatment with combined CP-760, 542 and NAN-190 and light was without transient cycles.

40
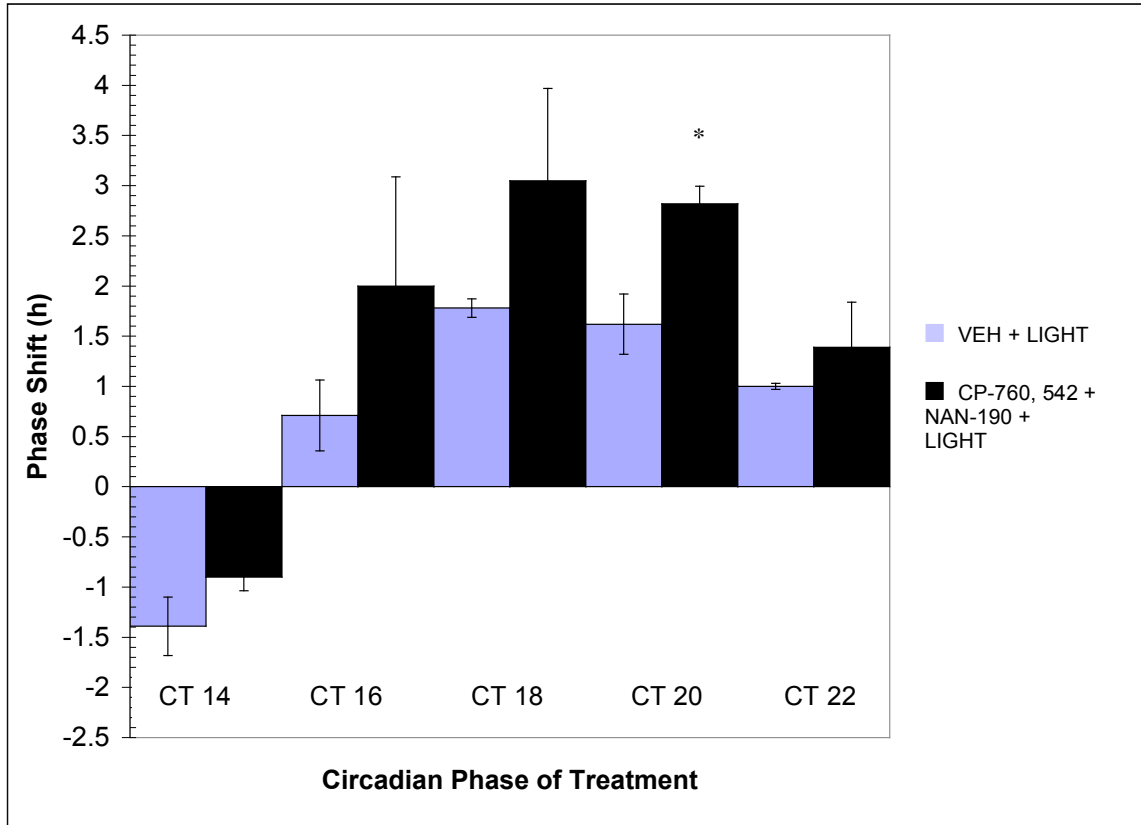
Figure 15. Average phase shift of wheel-running circadian rhythms of hamsters in response to treatment with CP-760, 542 and NAN-190 45 minutes prior to a 5 minute light pulse at the circadian phases CT 14, CT 16, CT 18, CT 20 and CT 22. Error bars indicate standard error of the mean (SEM). The asterisk indicates statistical significance in mean photic phase shifts between vehicle-treated and CP-760, 542 + NAN-190-tretaed groups at CT 20 (P< 0.05).

D. Experiment 3. Blocking NPY and 5-HT input attenuated transient cycles in photic phase shifts during subjective night

I noticed during my preliminary study, that there was a striking difference in the transient cycles in hamsters in the CP-781 214 + NAN-190 + light pulse group and the vehicle + light pulse group at CT 19. In the drug-treated group, the shift on the first day after a light pulse appeared to be almost equivalent to the stable phase shift achieved on the fourth day. The resetting seemed to be without transient cycles. (See a representative actogram from CT 19 in Fig. 16.) To quantify this phenomenon observed in Experiment 2, I calculated the phase shift on the first day after treatment as a percent of the steady state phase achieved on the fourth day after treatment (see Fig. 17 and Fig. 18). The vehicle-treated group (n = 10) achieved 42.9 % of the stable phase shift, as measured on Day 4, on the day immediately following treatment. The CP-760, 542 + NAN-190–treated group (n = 9) attained 82.6 % of the stable phase shift, measured on Day 4, on the day immediately following treatment. This difference was statistically significant ($P < 0.05$).

A Veh + no light at CT 19

B Veh + LP at CT 19

C Drug + LP at CT 19
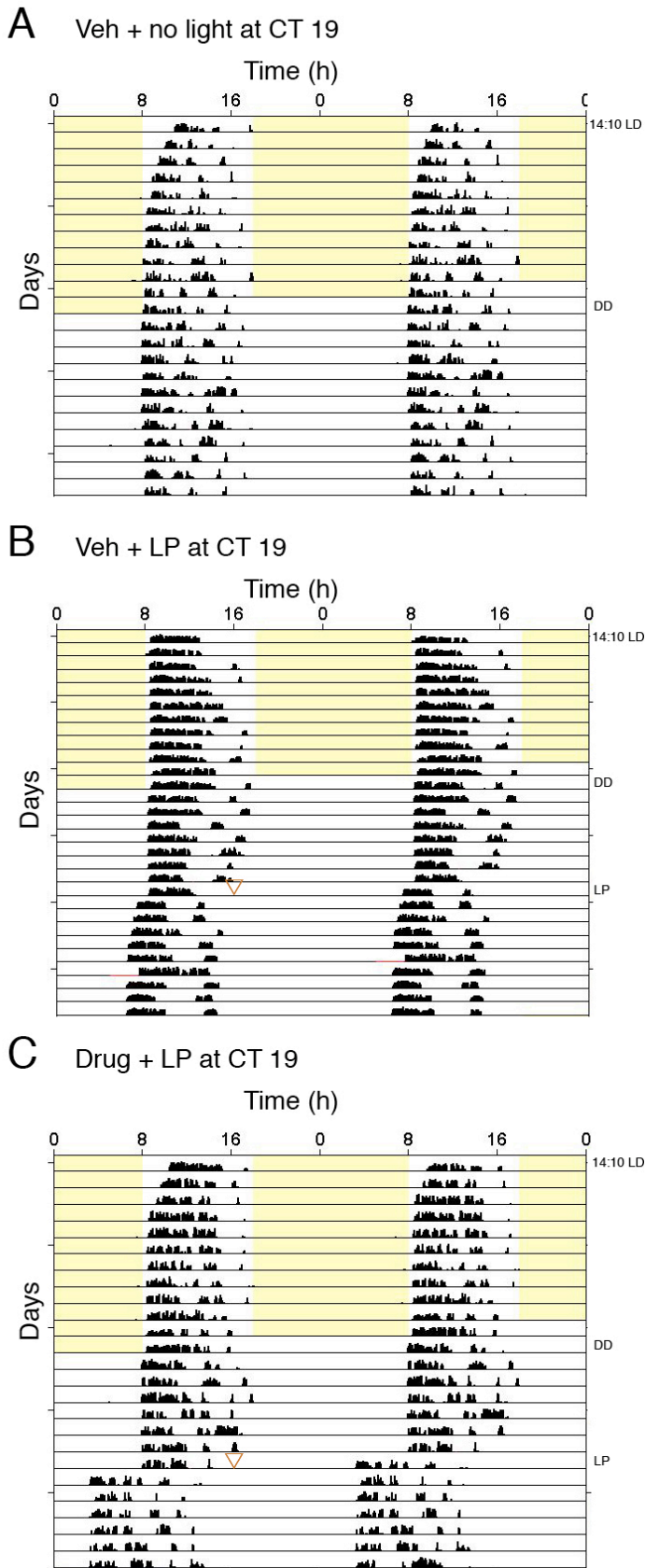
Figure 16. Representative double-plotted actograms showing the potentiation of light induced phase advances in hamsters by combined treatment with the NPY Y5 antagonist, CP-781-214, and the 5-HT1A partial agonist, NAN-190. Treatment: (A) Vehicle injection at CT 18.25 + no light at CT 19 (B) treatment with vehicle, 32% cyclodextrin 45 minutes prior to a light pulse at CT 19 and (C) treatment with CP-781-214 and NAN-190 45 minutes prior to a light pulse at CT 19. The black marks indicate revolutions of the running wheel of an individual hamster collected in one-minute bins. Animals were entrained to 14 h light: 10 h dark and then maintained in DD for 10 days prior to treatment. Light pulses are indicated by the triangle ($\triangledown$). Note that these hamsters were older (14 months at the time of this treatment as compared to young hamsters, less than 6 months, used in Experiment 2). It is significant that the phase advance following treatment with combined CP-781-214 and NAN-190 and light was without transient cycles.

A

Phase shift achieved on day 1 and day 4 following vehicle + light

B

Phase shift achieved on day 1 and day 4 follwoing CP-760,542 + NAN-190 + light
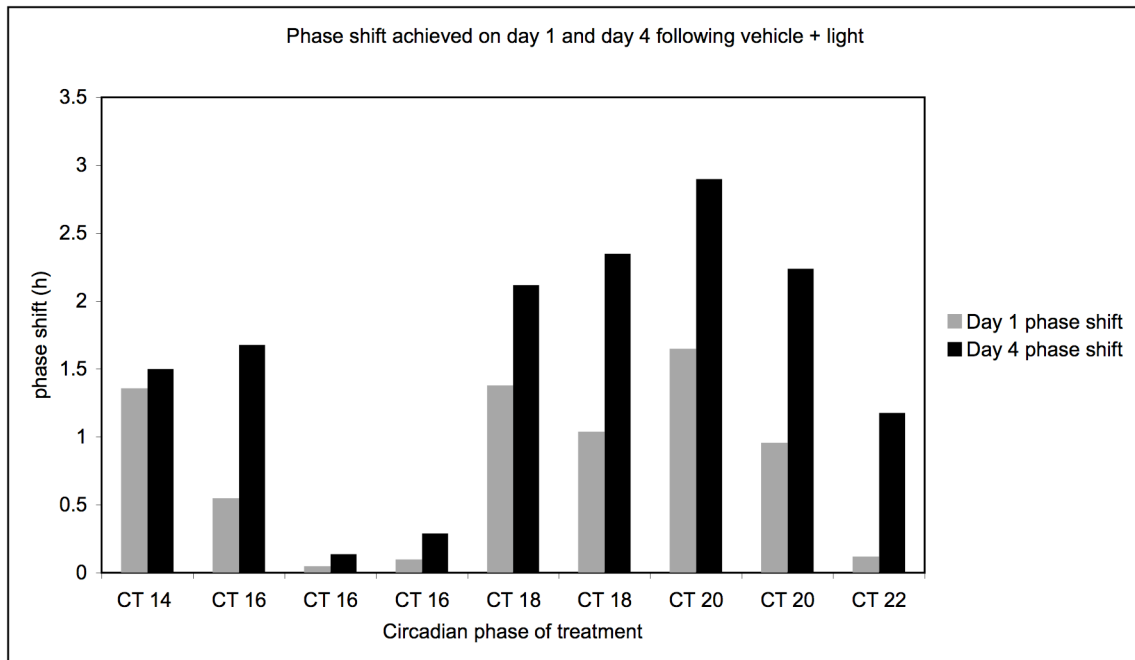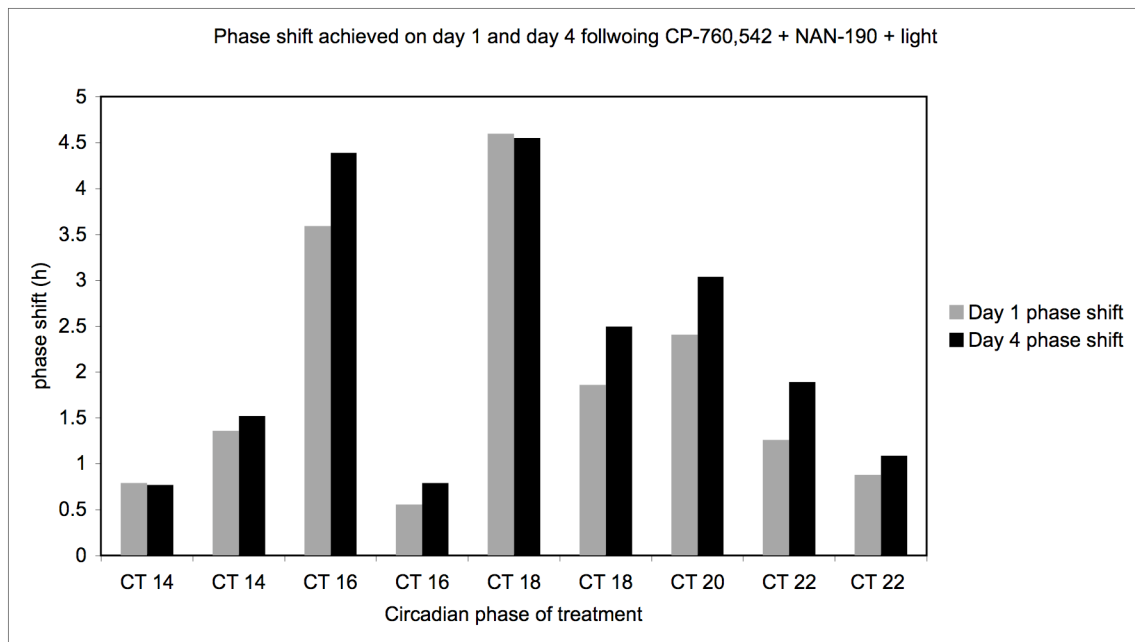
Figure 17. Transient cycles in hamsters treated with either (A) vehicle injection 45 minutes to light pulse and (B) CP-760, 542 + NAN-190 45 minutes to a light pulse. The bar on the left ■ represents the phase shift measured on the first day after the light pulse while the bar on the right ■ represents the steady state phase shift achieved on the fourth day after treatment.
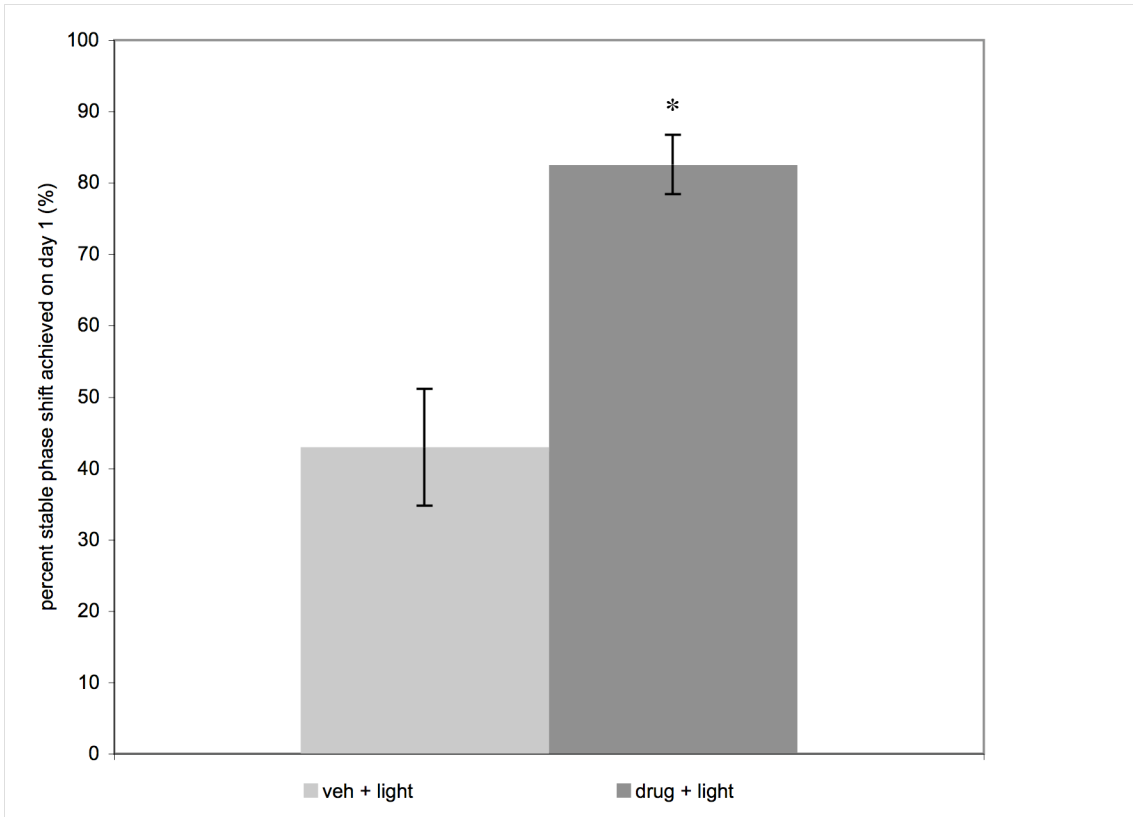
Figure 18. Percent of the stable phase shift, as measured on day 4 post-treatment, achieved on day 1.
The average percent of the final shift achieved on the first day after treatment was 42.9 % by the animals receiving vehicle + LP (n = 10); the average percent of the final shift achieved on the first day post treatment by the hamsters treated with CP-760, 542 + NAN-190 + LP (n = 9) was 82.6 %. The difference between the groups was statistically significant (P < 0.05). Error bars indicate S.E.M.

CHAPTER V

DISCUSSION

A.   Blocking NPY and 5-HT input does not potentiate photic phase delays in mice

This is the first study to investigate the effects of combined inhibition of NPY and 5-HT input on photic phase shifting in mice. Mice were given CP-781, 214 and NAN-190 in a single i.p. injection because my preliminary study determined combined injections of the drugs prior to a light pulse elicited statistically larger phase shifts in hamsters than light alone. The most significant finding from Experiment 1 was that blocking NPY and 5-HT input with combined injection of CP-781, 214 and NAN-190 did not potentiate photic phase delays in C57BL/6 mice (shown in Fig. 10). My finding suggests two possible interpretations. Lall (2006) reported that CP-760, 542 and NAN-190 potentiated phase shifts in hamsters when given during late subjective night, when light advances the clock. However, in my experiment mice were treated with the drug prior to light pulses during the early subjective night, a time when light delays the circadian clock. Does the combined inhibition of NPY and 5-HT input modulate photic phase shifts only during the late subjective night? (Experiment 2 was designed to address this question and it is discussed in detail in the next section.)

Another possibility is that there are significant species differences in the responsiveness of the circadian system to modulation by systemically delivered NPY and serotonin agents. There is substantial evidence that neuropeptide Y has a primary role in mediating the effects of non-photic input on photic phase shifting in the mouse and hamster circadian systems (Biello, 1995; Biello et al., 1997; Marchant et al., 1997; Maywood,

46

Okamura and Hastings, 2002). In addition, serotonin antagonists influence the responsiveness of the hamster circadian system to light stimuli (Smart and Biello, 2001). Gannon (2003) determined that blocking 5-HT1A input elicited large magnitude phase advances (5-6 hours) in Syrian hamsters. However, the serotonergic modulation of light-induced phase shifts has not been well established in mice. Systemic injection of the 5-HT1A/7 agonist, 8-OH-DPAT, failed to significantly attenuate light induced phase shifting in mice, although it inhibited photic phase advances in hamsters (Antle, Oglive, Pickard and Mistlberger, 2003). There are other controversies in the literature regarding the role of serotonin in entrainment in the mouse. In one study, lesions of serotonergic fibers in mice increased phase delays to nighttime light pulse at CT 14 (Bradbury, Dement and Edgar, 1997). However, Marchant (1997) determined that complete loss of 5-HT in mice (as a result of 5-HT lesions with neurotoxin 5,7-DHT) does not prevent modulations of the pacemaker by behavioral stimuli. A caveat to interpreting lesion studies is that destruction of the raphe-SCN afferent may also affect NPY input to the SCN, as the IGL is innervated by the dorsal raphe (Meyer-Bernstein and Morin, 1996).

An important future experiment should determine what effects serotonin input has on the regulation of photic phase shifting in mice. I think this could be addressed with pharmacological treatment using NAN-190 alone to determine the role the 5-HT1A receptor has in regulating circadian phase setting in the C57/BL6 mouse.

However, if we find that phase delays are not potentiated in hamsters then we will need to investigate the first interpretation that NPY and 5-HT modulate photic shifting only during late subjective night, in mice. We could readdress the question of phase advances in mice, although it is difficult to elicit consistent phase advances in the C57/BL6 species.

Specifically, treatment with the antagonists, CP-781, 214 and NAN-190, should be given prior to light pulses at CT 22.5 and CT 23; times when light elicits the largest advances phase in the C57/BL6. Although phase advances in mice are small and inconsistent, blocking NPY and 5-HT input may potentiate the advances. It would be even better to give the drug treatment to mice under an advancing light/dark cycle. For example, after mice are entrained for two weeks under a12:12 L:D cycle, the lights are advanced each day by coming on 6 hours earlier than on the previous day. We could determine whether blocking both 5-HT and NPY input eliminates transient cycles, accelerating the rate of re-entrainment in the mouse under advancing light cycles (see Future Directions section).

A third interpretation of the failure to potentiate phase shifts in the mouse may be due to the dose of the NPY Y5 antagonist, CP-781, 214, used in my work with mice. I used a dose for CP-781, 214 determined in pilot studies in our lab for Syrian hamsters (Lall, unpublished, 2004). Before it is concluded that combined treatment with NAN-190 and CP-781, 214 cannot potentiate phase shifts in mice, a dose response study for CP-781, 214 should be conducted in C57/BL6 mice to establish the effective dose. Finally, the sample size was small and the investigator was new to giving mice injections under dim red light. Potentiation of phase shifts with combined NPY Y5 antagonist and NAN-190 could be a valuable tool to investigate reentrainment in mice, especially given the availability of mouse knockout models and luciferase reporter mice. This important work should be pursued with larger sample sizes after dose response for the NPY Y5 antagonist for C57/BL6 mice is determined (specific suggestions may be found in the Future Studies section).

B.   NPY and 5-HT regulate photic phase shifting during a temporal window in late subjective night

One of the most significant findings of Experiment 2 was that the effects of blocking NPY

and 5-HT input might be phase-dependent. A statistically significance difference was found

between mean phase shifts in the drug-treated group and vehicle-treated group to a light

pulse at CT 20 but not at CT 14. Blocking non-photic input might potentiate phase resetting

only during the late subjective night; or more specifically, the combined inhibition of NPY

and 5-HT input with CP-760, 542 + NAN-190 might only be effective in potentiating phase

shifts to light during late subjective night. In fact, treatment with CP-760, 542 + NAN-190

attenuated the phase delay following light at CT 14, while CP-760, 542 + NAN-190

treatment augmented the light-induced phase shift at all phases tested during late subjective

night (see Fig. 15). While Lall and Harrington (2006) demonstrated significant potentiation

of light-induced phase advances with combined inhibition of NPY and 5-HT input, there are

no previous reports in the literature investigating the effects of combined inhibition of NPY

and 5-HT on photic phase delays. Novel wheel exposure as well as either NPY activation or

5-HT activation modulate photic phase advances during late subjective night (Lall and

Biello, 2002). However, the results of my experiments are consistent with many studies

reporting that light-induced phase delays are not significantly attenuated by behavioral

activation, NPY or serotonin (Challet, Turek, Laute and Van Reeth, 2001; Mistlberger and

Antle, 1998; Weber and Rea, 1997). Lall and Biello (2003), on the other hand, reported that

NPY agonists block phase advances (by about 90%) and inhibit phase delays (40%) in

hamsters via the NPY Y1 and/or the Y5 receptors. They are the first to report that NPY and

the NPY Y1/Y5 agonist, (Leu [31]Pro [34] NPY attenuated phase delays as well phase advances

in vivo. Neuropeptide Y applied in vitro attenuates light and NMDA induced phase delays (Yannielli and Harrington, 2001a; Yannielli and Harrington, 2001b). To address the role of NPY in modulating photic phase delays in the hamster, hamsters should be treated with the NPY Y5 CP-760, 542 alone prior to light pulses at multiple phases throughout the subjective night.

The finding that the circadian clock's responsiveness to NPY and 5-HT differ during early and late subjective night, adds to the growing body of literature illustrating cases where photic entrainment is influenced by biochemical pathways that regulate sensitivity in a phase-dependent manner. For example, light-induced phase delays but not advances are prevented with bicuculline, a GABA $_A$ receptor antagonist (Ralph and Menaker, 1985). Light-induced phase delays were increased by the benzodiazepine triazolam, a $GABA_A$ agonist, while light-induced phase advances were decreased by triazolam and by another $GABA_A$ agonist, diazepam (Joy and Turek, 1992; Subramanian and Subbaraj, 1996). During late subjective night, light pulses increase cyclic guanosine monophosphate (cGMP) levels in the SCN whereas light pulses given in early subjective night do not affect cGMP levels (Golombek, Agostino, Plano and Ferreyra, 2004). In addition, inhibitors of PKG (guanosine 3'5' –cyclic nucleotide-dependent kinase) attenuated light induced phase advances during late subjective night, but had no effect on light induced phase delays during early subjective night (Ferreyra and Golombek, 2001). Light input may be mediated at different circadian time points by functionally separate pathways. Moreover, nonphotic input may differentially regulate photic responsiveness of the circadian system throughout the night.

Another consideration is that, the lack of potentiation of light-induced phase delays with pharmacologically blocking NPY Y5 and 5-HT 1A receptors may be due to the higher

extracellular levels of endogenous serotonin and neuropeptide Y early in the night (Harrington, 2005). Additionally, light in the early night can increase levels of NPY in the SCN of the rat (Shinohara, Tominga, Isobe and Inouye, 1993). It is possible that treatment with CP-760, 542 and NAN-190 was not sufficient to block the higher levels of endogenous NPY and 5-HT during the early night, while capable of blocking the lower endogenous tone of the neuropeptides during the late night.

The results suggest that neuropeptide Y and serotonin regulate photic phase shifting only during a temporal window in late subjective night. However, a caveat to interpreting the results from Experiment 2 is that small subject numbers were used for each treatment. The difference in photic phase shifts between vehicle-treated animals and drug-treated animals reached statistical significance at CT 20. There was a trend for increased phase shift in drug treated subjects at CT 16, CT 18 and CT 22, however, the differences did not reach statistical significance at other circadian phases during the late subjective night. It is important to replicate the study across subjective night with larger numbers of subjects, and especially to replicate findings that phase shifts were not augmented, and may have been attenuated, at CT 14.

C. Blocking NPY and 5-HT input eliminated transient cycles in photic phase shifts

Phase advances after treatment with CP-760, 542 + NAN-190 antagonists were without transient cycles (see actograms Fig. 14 and Fig. 16). Transient cycles are thought to reflect disequilibrium between the SCN and the overt rhythm (locomotor activity in this case) (Johnson et al., 2003). Transients are more prevalent in phase advances and may underlie the fatigue, insomnia and gastrointestinal symptoms of jet lag. Jet lag is usually worse after traveling eastward, which requires the circadian pacemaker advance. Why were transients eliminated when NPY and 5-HT input was blocked prior light stimuli during the late night? Although the master circadian clock resets rapidly in response to light (Best, 1999), the oscillations synchronized by the clock are not immediately reset (Balsalobre, 2000; Yamazaki et al., 2000; Buijs and Kalsbeek, 2001). The SCN uses a variety of autonomic, paracrine and endocrine cues to regulate behavioral and physiological processes (Meyer-Bernstein et al., 1999; Cheng et al., 2002; Maywood, O'Neill, Wong, Reddy and Hastings, 2006). In this study, blocking NPY and 5-HT reduces the time required to reach a steady state resetting of locomotor activity rhythms after a light pulse during the late subjective night. I suggest that tonic levels of NPY and 5-HT may contribute to the prolonged resetting of locomotor activity and possibly other oscillations driven by the master clock. Photic information converges with non-photic information to determine circadian phase; this coordination between non-photic and photic information and the signals required to integrate the information may slow down the message relayed to peripheral oscillators. Eliminating the non-photic input may mean that light information is conveyed rapidly to other brain areas and to peripheral tissues, and transients (at least those observed in locomotor activity) are

thus eliminated. This rapid signaling would depend upon neural communication or possibly a gaseous transmitter, possibly nitric oxide, and not slower acting endocrine signals.

The SCN coordinates rhythmic timing cues for light entrainment to oscillators in peripheral tissues lacking photoreceptors. It is possible that blocking NPY and 5-HT input affects expression of clock genes and that this modulates the SCN's coordination of peripheral oscillators. In addition, a number of diffusible factors have been identified as timing signals including vasopressin, transforming growth factor-α, prokineticin 2 and cardiotropin-like cytokine (Liu, Lewis, and Kay, 2007). Possibly, blocking the NPY and serotonin pathways leads to an interaction with one or more of these factors to attenuate transient cycles during phase-resetting following light during the late night. We need to better understand why transients occur before we can begin to answer the question, "how does pharmacologically blocking NPY and 5-HT input eliminate transient cycles following phase advances?"

In my study, blocking NPY and 5-HT input eliminated transient cycles in locomotor activity following photic phase advances. Whether transients in other shifted rhythms (endocrine rhythms, metabolism, cell cycle for example) are eliminated or reduced warrants further investigation (see Future Studies). The elimination of transients is important for the clinical applications of this research as it might reduce the symptoms of jet lag, enhance rest-activity rhythms in the elderly and possibly attenuate the negative impact the '24-7 lifestyle' has on human health (Filipski et al., 2004).

D. **Experiment 2 failed to replicate large phase advances previously measured with CP-760, 542 + NAN-190 treatment**

My study did not replicate the large light-induced phase advances that were elicited in response to CP-760, 542 + NAN-190 treatment at CT 19 by Lall (2006). Lall reported a mean photic phase shift of 7.1 h after CP-760, 542 + NAN-190 treatment at CT 19; the largest mean shift in my study was 3.05 h at CT 18. There are several differences in protocol that may have affected the size of the phase shifts. I did not measure phase shifts following light at CT 19, the time for maximal photic phase advances, according to the hamster photic phase response curve. In addition, I combined CP-760, 542 and NAN-190 in a single intraperitoneal injection, while Lall gave separate injections (CP-760, 542 dissolved in DMSO delivered in a subcutaneous injection and NAN-190 dissolved in cyclodextrin in an intraperiotoneal injection). My preliminary study found that phase shifts with CP-781.214 + NAN-190 in a single i.p. injection significantly potentiated phase shifts compared to light alone at CT 19, (see Fig. 9) however I did not use CP-781, 214 for Experiment 2. The Harrington Lab has extensive preliminary data using the NPY Y5 antagonist, CP-760, 542 (Pfizer), and we chose to use CP-760, 542 for these studies in order to supplement the preliminary findings and because CP-760, 542 was more readily available from Pfizer at the time of this study. The Pfizer drug I used, CP-760, 542, is difficult to dissolve; maintaining a good solution with CP-760, 542 may have been confounded by the use of cyclodextrin as a vehicle and the addition of NAN-190 to the mixture. This may have resulted in decreased drug delivery.

I also want to consider the role that stress has in the outcome of these experiments. Is it possible that administration of two separate injections increased the stress response of the

hamsters in Lall's study? There is evidence that both neuropeptide Y and serotonin antagonize the behavioral consequences of stress through their actions within the brain. Activation of 5-HT1a receptors (with the agonists 8-OH-DPAT or WAY-100635) counteracted the behavioral effects of stress (Roja et al., 2004). Neuropeptide Y1 and Y5 agonists can mediate the anti-stress actions of NPY (Hellig, 2004). In addition, a recent study suggests that NPY functions as an integrator between different stress signals such as immobilization, cold and pain, and a neuroendocrine response (Dimitrov, DeJoseph, Brownfield and Urban, 2007). Is it possible that light at night is also a stressor? Might blocking NPY and 5-HT input allow an increased response to the stressor of the light pulse and thus potentiate the phase shift? In this case, anything that occurred during the experimental procedure to increase the animal's stress level might also increase the photic phase shift. Although, not fully studied, light stimuli during the night may be associated with emotionality (Valentinuzzi et al., 2000). Although only conjecture, I think it would be worthwhile to determine if additional stressors (i.e. pain, noise, cold, handling) increase the response to nighttime light pulses when NPY and 5-HT input are blocked. If this is the case, can we conclude that blocking NPY and 5-HT input may increase anxiety levels elicited by nighttime light? This question might be addressed by measuring anxious behavior levels in an elevated-plus maze in drug and control animals exposed to nighttime light.

Differences in the age of the hamsters at time of treatment, prior light treatment and individual variation in responses to light may also have influenced the magnitude of the phase shifts. One other issue is that Pfizer has discontinued production of the NPY Y5 antagonists, so that the drugs used in Experiment 2 were older than those used in the previous

55

study. Although Pfizer will no longer produce CP-760, 542 and CP-781, 214, new potent and selective NPY Y5 antagonists are available for future studies (including S 255853/4 and US Patent 6989379; for reference see Della-Zuana et al., 2004 and Patent Storm, 2006).

CHAPTER VI

FUTURE DIRECTIONS

A.   Will blocking NPY and 5-HT input eliminate transients of per 2 rhythms in the SCN and peripheral organs following phase advances?

It is noteworthy that treatment with CP-760, 542 + NAN-190 potentiated phase advances without the usual transient cycles. Transients may contribute to the malaise experienced during jet lag and shift work and are usually more pronounced for phase advances (Merrow et al., 2005). Although, it is difficult to study phase advances in mice due to their small and inconsistent shifts to light in late subjective night, a future study could determine whether treatment with CP-760, 542 + NAN-190 can be used to reaccelerate entrainment following advancing potentiate light-induced phase advances in mice. Under advancing light-cycles, the light comes on regular number of hours earlier each day while maintaining a 12:12 LD for mice. This could allow us to use the PER2:: LUC knockin mouse to measure bioluminescence rhythms of PER2 in the SCN and peripheral organs using the LumiCycle. Are these rhythms of PER::2 luminescence without transients? On the other hand, if we find that the pharmacological treatment does not affect mice, we could use in situ hybridization to measure levels of *per* expression in the SCN and quantitative RT-PCR to measure *per* levels in peripheral organs of hamsters to determine whether there are transient cycles in *per* expression following phase shifts elicited by light in combination with this pharmacological treatment. It is important to gain understanding of the factors regulating transient cycles in peripheral organs for in order to develop therapeutic agents to treat the misalignment of peripheral oscillations experienced in transient cycles.

B. **Will blocking NPY and 5-HT input eliminate transients in physiological rhythms following phase advances?**

In addition to measuring levels of per gene expression in peripheral oscillators it will be important to measure changes in physiological rhythms regulated by the circadian system following abrupt light changes in animals receiving both NPY Y5 antagonist and NAN-190. Body temperature and heart rate could be monitored in hamsters following light pulses during the late subjective night and under advancing light cycles which model jet lag conditions. Levels of plasma corticosterone secreted by the adrenal cortex are under circadian regulation, and could be measured in animals using this drug + light protocol.

Future studies should also include female hamsters (and mice) to investigate whether blocking NPY and 5-HT can affect circadian rhythms of behavior and physiology in female subjects as well as males. In women, increased breast cancer risk and menstrual irregularities have been associated with working the night shift and as flight attendants (Stevens, 2005; Nagata et al., 2008). The SCN has a central role in regulating timing of the luteinizing hormone (LH) surge, which may regulate circadian rhythmicity in the ovaries (Karman and Tischkau, 2006). Rhythms of hormone secretion and reproductive behavior could be studied in female subjects receiving the drug treatment under conditions simulating altered light cycles. The role that inhibition of NPY and serotonin input may have in regulating phase resetting to light and alleviating transient cycles, which may lead to circadian desynchrony in females, is an important area for investigation.

C.   Will combined inhibition of NPY and 5-HT input modulate light-induced phase shifts in the *tau* mutant hamster? Is CK Iε involved in the regulation of photic shifting by nonphotic input?

I think the *tau* mutant hamsters discovered by Ralph and Menaker (1988) would be a useful tool for future research investigating the effects of inhibition of NPY and 5-HT input on photic phase shifts. The tau mutation is a semi-dominant autosomal allele that shortens the circadian period length to 20 hours in the homozygous mutant animals and 22 hours in the heterozygous mutant (wild type hamsters have a period of approximately 24 hours). A missense mutation within the substrate region of casein kinase Iε is responsible for the *tau* mutation (Gietzen and Virshup, 1999). Recently the hamster *tau* mutation has been identified as a gain of function *in vivo*, increasing phosphorylation-dependent degradation of PER proteins and thus speeding up the circadian clock (Gallego, Eide, Woolf, Virshup and Forger, 2006). Hamsters with the *tau* mutation have an altered response to light exposure during the subjective night. The *tau* hamster responds to a 1 h light pulse at CT 15 with a large phase advance of approximately 11 hours, compared to the one hour advances measured for wild type hamsters (Scarbrough and Turek, 1996; Grosse, Loudon and Hastings, 2000). While the magnitude of the advance shift differed significantly between the two phenotypes, light induced phase delays were equivalent (approximately one hour). The *tau* mutation also alters response to nonphotic stimuli and to NPY (Mrosovsky, Salmon, Menaker and Ralph, 1992; Biello and Mrosovsky, 1996). The amplitude of the response of the PRC to nonphotic stimuli and NPY is greater in the homozygous tau mutants. I suggest treating the tau mutant hamsters with CP-760, 542 + NAN-190 to determine if phase shifts are regulated by these antagonists. If *tau* hamsters have an altered response to the antagonists than wildtype hamsters, than it is possible that CK Iε is involved in the regulation of photic shifting by nonphotic input. The effects of a novel CKIε inhibitor, PF-670462, (Pfizer, CT) on the inhibition of nonphotic input

on photic phase shifting might also be evaluated in wildtype hamsters and mice (for reference see

Badura et al., 2007).   The *tau* mutant hamsters and the ability to inhibit CKIε with PF-670462 are

useful tools for further study of the interaction between nonphotic and photic input in the circadian

system.

Why are tau hamsters more responsive to light during late subjective night than wild type

hamsters? Scarborough and Turek (1996) found that levels of vasopressin (AVP) and vasoactive

intestinal peptide (VIP) in the SCN were significantly lower in the *tau* mutant hamsters than wild

type following prolonged time in DD. They suggest that the differences in peptide levels may be

responsible for the qualitative differences in phase shifts. Measuring VIP and AVP levels with in

situ hybridization in brains collected from hamsters treated with CP-781, 214 (or CP-740, 542) +

NAN-190 could determine if the treatment increased levels of these peptides in the SCN.

Animals with the altered *tau* gene have reduced longevity, accompanied by severe

cardiovascular and renal disease. A recent study (Martino et al., 2008) demonstrated that circadian

disorganization caused by the *tau* mutation, and not the mutation itself, is responsible for

cardiomyopathy and renal disease. Significantly, when tau hamsters were maintained in a 12:10

LD, allowing them to entrain to an appropriate light cycle, abnormal cardiac and renal measures

were not observed.

D. **Does blocking NPY and 5-HT during the late subjective night potentiate phase shifting via enhanced activation of the PKG pathway?**

This pharmaceutical protocol could also be used to investigate the signal transduction pathways for photic phase shifting. Signal transduction pathways differ for photic phase advances and phase delays (Golombek et al., 2000). Light stimulation during the night induces clock resetting through an excitatory signal transduction pathway mediated by glutamate (Glu), NMDA receptor activation, stimulation of nitric oxide synthase (NOS), and intercellular movement of nitric oxide (NO) (for review see Ding et al., 1997). However, light stimulation activates the guanylyl cyclase-cGMP-cGMP-dependent kinase (PKG) pathway only during late subjective night. Light induced phase advances, but not delays, are blocked by pharmacological inhibition of cGMP-dependent protein kinase (Weber, Gannon and Rea, 1995; Ferreyra and Golombek, 2001). *In vitro,* cGMP analogs and activation of PKG induce phase shifts only during the late subjective night. Is it possible that a blockade of NPY and 5-HT input converges on the PKG pathway to potentiate the phase advancing effects of light on the SCN? To determine whether NPY and 5-HT inhibition activates the PKG pathway, PKG activity could be measured in hamsters treated with CP-760542 + NAN-190 with light and compared to PKG activity in hamsters treated with light alone with an *in vitro* phosphorylation assay using a PKG specific substrate (Peninsula Labs, Belmont, CA). In addition cGMP levels could be compared using an enzyme immunoassay kit (Correlate-EIA, Assay Designs, Ann Arbor, Michigan). Significantly, a recent study determined that administration of sildenafil, which inhibits c-GMP-specific phosphodiesterase V (an enzyme which degrades cGMP), allowing increased accumulation of cGMP, increases phase advances at CT 18 by 50% and accelerates reentrainment to advancing light cycles

(Agostino, Plano and Golombek, 2007). I think it is important to evaluate the possible role that activation of cGMP and PKG has in mediating the effects NPY and 5-HT on photic phase shifts during late subjective night. Identifying biochemical events associated with the potentiation of phase advances by these drugs may increase our basic understanding of the circadian clock.

CHAPTER VII

CONCLUSION

The potentiation of phase shifts may have important clinical value. Individuals with seasonal affective disorder, familial sleep disorders or Alzheimer's disease might benefit from treatment to phase shift a delayed circadian system. In addition, blocking non-photic input might allow the circadian system to adjust more quickly to shift work and travel across time zones. Any therapeutic value will depend upon minimizing the side effects of this treatment. This study also underscores the necessity to consider time of day for treatment with prescription drugs. In particular, the role of 5-HT in blocking phase advances and possibly impairing entrainment should be considered when prescribing serotonergic agents. Significantly, blocking NPY and 5-HT input eliminated the normal time lag required for a steady state phase shift to be reached after a light pulse during the late subjective night. The transient cycles before a stable phase relationship is reached between the circadian clock and peripheral oscillator may also contribute to human circadian dysynchrony. Some of the health risks associated with circadian dysynchrony may be alleviated by pharmacological treatments that target both NPY and serotonin levels.

In addition, the potentiation of phase shifts, which are elicited with the combined NPY Y5 antagonist and 5-HT1A partial agonist, provides an excellent opportunity to study differences between phase advances and delays, elucidate the interaction between photic and non-photic input to the circadian system, and to increase basic understanding of the circadian system.

VIII.   LITERATURE CITED

Abe K, Kroning J, Greer MA and Critchlow V (1979) Effects of destruction of the suprachiasmatic nuclei in plasma corticosterone, body temperature, feeding and plasma thyrotropin. Neuroendocrinology 29:119-131.

Agostino PA, Plano SA and Golombek DA (2007) Sildenafil accelerates reentrainmnet of circadian rhythms after advancing light schedules. Proc Nat Acad Sci 104:9834-9839.

Albers HE and Ferris CF (1984) Neuropeptide Y: role in light-dark cycle entrainment of hamster circadian rhythms. Neurosci Lett 50:163-168.

Albrecht U, Zheng B, Larkin D, Sun ZS and Lee CC (2001) MPer1 and mper2 are essential for normal resetting of the circadian clock. J Biol Rhythms16: 100-104.

Amir S, Robinson B, Ratovitski T, Rea MA, Stewart J and 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 84:1059-1073.

Antle MC, Marchant EG, Niel L and Mistleberger RE (1998) Serotonin antagonists do not attenuate activity-induced phase shifts of circadian rhythms in the Syrian hamster. Brain Res 813:139-149.

Antle, MC and Mistlberger, RE (2000) Circadian clock resetting by sleep deprivation without exercise in the Syrian hamster. J Neurosci 20: 9326—9332

Antle MC, Ogilvie MD, Pickard GE and Mistlberger RE (2003) Response of the mouse circadian system to serotonin 1A/2/7 agonists in vivo: surprisingly little. J Biol Rhythms 18:145-158.

Antle MC and Silver R (2005) Orchestrating time: arrangements of the brain circadian clock. Trends Neurosci. 28:145-51.

Badura L, Swanson T, Adamowicz W, Adams J, Cianfrogna J, Fisher K, Holland J, Kleiman R, Nelson F, Reynolds L, S t Germain K, Schaeffer E, Tate B and Sprouse J (2007) An inhibitor of casein kinase I {epsilon} induces phase delays in circadian rhythms under free-running and entrained conditions. J Pharmacol Exp Ther 107:

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM and Weaver DR (2001) Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 30:525-536.

Berger AM and Hobbs BB (2006) Impact of shift work on the health and safety of nurses and patients. Clin J Oncol Nurs 10:465-471.

Best JD, Maywood ES, Smith KL and Hastings MH (1999) Rapid resetting of the mammalian circadian clock. J Neurosci 19:828--835.

Biello SM, Harrington ME and Mason R (1991) Geniculo-hypothalamic tract lesions block chlordiazepoxide-induced phase advances in Syrian hamsters. Brain Research 552:47-52.

Biello SM, Janik D, and Mrosovsky N (1994) Neuropeptide Y and behaviorally induced phase shifts. Neuroscience 62:273--279.

Biello SM and Mrosovsky, N (1995a) Blocking the phase-shifting effect of neuropeptide Y with light. Proc R Soc Lond Biol Sci 259:178-187.

Biello SM (1995b) Enhanced photic phase shifting after treatment with antiserum to neuropeptide Y. Brain Res 673:25-29.

Biello SM and Mrosovsky NM (1996) Phase response curves to neuropeptide Y in wild type and tau mutant hamsters. J Biol Rhythms 11:27-34.

Biello S.M., Golombek D.A. and Harrington M.E. (1997a) Neuropeptide Y and glutamate block each other's phase shifts in the suprachiasmatic nucleus in vitro. Neuroscience 77:1049-1057.

Biello SM, Golombek D, Schak DM and Harrington ME (1997b) Circadian phase shifts to Neuropeptide Y in vitro: cellular communication and signal transduction. J Neurosci 17:8468-8475.

Bradbury MJ, Dement WC, and Edgar DM (1997) Serotonin-containing fibers in the suprachiasmatic hypothalamus attenuate light-induced phase delays in mice. Brain Research 768:125-134.

Butcher GQ, Lee B, Cheng HY and Obrietan K (2005) Light stimulates MSK1 activation in the suprachiasmatic nucleus via a PACAP-ERK/MAP kinase-dependent mechanism. J Neurosci 25:5305-5313.

Byku M and Gannon RL (2000) Opioid induced non-photic phase shifts of hamster circadian activity rhythms. Brain Res 873:189-196.

Challet E, Scarbrough K, Penev PD and Turek FW (1998) Roles of suprachiasmatic nuclei and intergeniculate leaflets in mediating the phase-shifting effects of a serotonergic agonist and their photic modulation during the day. J Biol Rhythms 13:410-421.

Challet E, Turek FW, Laute M and Van Reeth O (2001) Sleep deprivation decreases phase- shift responses of circadian rhythms to light in the mouse: role of serotonergic and metabolic signals. Brain Research 909:81-91.

Cho K (2001) Chronic 'jet lag' produces temporal lobe atrophy and spatial cognitive deficits. Nature Neurosci 4: 567-568.

Christian CA and Harrington ME (2002) Three days of novel wheel access diminishes light-induced phase delays in vitro with no effect on per1 induction by light. Chronobiol Int 19:671-682.

Coogan AN and Piggins HD (2003) Circadian and photic regulation of phosphorylation of ERK1/2 and Elk-1 in the suprachiasmatic nuclei of the Syrian hamster. 23:3085-3093.

Crowley SJ, Lee C, Tseng CY, Fogg LF and Eastman CI (2004) Complete or partial circadian re-entrainment improves performance, alertness, and mood during night-shift work. Sleep 27:1077-1087.

Cutrera RA, Ouarour A and Pevet P (1994) Effects of the 5-HT/1A receptor agonist 8-OH-DPAT and other non-photic stimuli on the circadian running wheel activity in hamsters under different constant conditions. Neurisci Lett 172:27-30.

Dallman R and Mrosovsky N (2006) Scheduled wheel access during daytime: A method for studying conflicting zeitgebers. Physiology and Behavior 88:459-465.

Davidson AJ, Sellix MT, Daniel J, Yamazaki S, Menaker M and Block GD (2006) Chronic jet-lag increases mortality in aged mice. Curr Biol 21:914-916.

Della-Zuana O, Revereault L, Beck-Sickinger A, Monge A, Caignard DH, Fauchers JL, Henlin JM, Audinot V, Boutin JA, Chamorro S, Feletou M and Levens N (2004) A potent and selective NPY Y5 antagonist reduces food intake but not through blockade of the NPY Y5 receptor. International Journal Obesity 28: 628-639.

Dimitrov EL, DeJoseph MR, Brownfield MS and Urban JH (2007) Involvement of neuropetide Y Y1 receptors in the regulation of neuroendocrine corticotropin-releasing hormone neuronal activity. Endocrinology 148: 3666-3673.

Dudley TE, Dinardo, LA and Glass D (1998) Endogenous regulation of serotonin release in the hamster suprachiasmatic nucleus. J Neurosci 18:5045-5052.

Duncan MJ, Grear KE and Hoskins MA (2004) Aging and SB-269970-A, a selective 5-HT 7 receptor antagonist, attenuate circadian phase advances induced by microinjections of serotonergic drugs in the hamster dorsal raphe nucleus. Brain Research 1008:40-48.

Duncan MJ, Franklin KM, Davis VA, Grossman GH, Knoch ME, Glass JD (2005) Short-term constant light potentiation of large-magnitude circadian phase shifts induced by 8-OH-DPAT: effects on serotonin receptors and gene expression in the hamster suprachiasmatic nucleus. Eur J Neurosci 22:2306-2314.

Dunlap, JC (1999) Molecular bases for circadian clocks. Cell 96:271-290.

Dunlap JC, Loros JL and DeCoursey PJ (2004) Chronobiology: Biological timekeeping. Sinauer Associates, Sunderland, MA, USA

Earnest DJ and Olschowka JA (1993) Circadian regulation of c-fos expression in the suprachiasmatic pacemaker by light. J Biol Rhythms 8:65-71.

Eastman CI, Gazda CJ, Burgess HJ, Crowley SJ, Fogg LF (2005) Advancing circadian rhythms before eastward flight: a strategy to prevent or reduce jet lag. Sleep 28: 33-44.

Edelstein K, de la Iglesia HO, Schwartz WJ and Mrosovsky N. (2003) Behavioral arousal blocks light-induced phase advances in locomotor rhythmicity but not light-induced Per1 and Fos expression in the hamster suprachiasmatic nucleus. Neuroscience 118:253-261.

Ehlen JC, Grossman GH and Glass JD (2001) In vivo resetting of the hamster circadian clock by 5-HT7 receptors in the suprachiasmatic nucleus. J Neurosci 21:5351-5357.

Ehlers CL, Frank E and Kupfer D (1989) Social zeitgebers and biological rhythms. Arch Gen Psychiat 45:948-952.

Eide EJ, Vielhaber EL, Hinz WA and Virshup DM (2002) The circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase I ε. J Biol Chemistry 277:17248-17254.

el Bakary Z and Fuzeau-Braesch S (1988) Circadian rhythms and time measurement in locomotor activity of the scorpion *Leiurus quinquestriatus* (Buthidae). Chronobiology International 5:167-174.

Ferraro JS and McCormack CE (1985) Gonadal regression despite light pulses coincident with locomotor activity in the Syrian hamster. Biol Reproduction 33:93-102.

Filipski E, Delaunay F, King VM, Wu MW, Claustrat B, Grechez-Cassiau A, Hastings MH and Francis L (2004) Effects of chronic jet lag on tumor progression in mice. Cancer Res 64:7879-7885.

Filipski E, Li M and Levi F (2006) Disruption of circadian coordination and malignant growth. Cancer Causes Control 17:509-514.

Freeman, DA, Teubner BJ and Goldman BD (2006) The thalamic intergeniculate leaflet mediates locomotor activity-induced reversal of phenotype in photoperiod nonresponsive Siberian hamsters. J Biol Rhythms 21:206-213.

Fukuhara C, Brewer JM, Dirden JC, Bittman EL, Tosini G and Harrington ME (2001) Neuropeptide Y rapidly reduces period 1 and Period 2 mRNA levels in the hamster suprachiasmatic nucleus. Neurosci Letters 314:119-122.

Gallego M, Eide EJ. Woolf MF, Virshp DM and Forger DB (2006) An opposite role for tau in circadian rhythms revealed by mathematical modeling. Proc Nat Acad Sci 103:10618-10623.

Gallego M and Virshup DM (2007) Post-translational modifications regulate the ticking of the circadian clock. Nat Rev Mol Cell Biol 8:139-148.

Gamble KL, Novak CM and Albers HE (2004) Neuropeptide Y and N-methyl-D-aspartic acid interact within the suprachiasmatic nuclei to alter circadian phase. Neuroscience 126(3): 559-65.

Gamble KL, Ehlen JC and Albers HE (2005) Circadian control during the day and night: Role of neuropeptide Y Y5 receptors in the suprachiasmatic nucleus. Brain Res Bull. 65:513-9.

Gamble KL, Paul KN, Karom MC, Tosini G and Albers HE (2006) Paradoxical effects of NPY in the suprachiasmatic nucleus. Eur J Neurosci 23:2488-94.

Gannon RL (2003) Serotonergic serotonin (1A) mixed agonists/antagonists elicit large-magnitude phase shifts in hamster circadian wheel-running rhythms. Neuroscience 119:567-76.

Gannon RL and Millan MJ (2006a) The corticotrophin-releasing factor (CRF)(1) receptor antagonists CP154,526 and DMP695 inhibit light-induced phase advances of hamster circadian activity rhythms. Brain Res 1083:96-102.

Gannon RL and Millan MJ (2006b) Serotonin1A autoreceptor activation by S 15535 enhances circadian activity rhythms in hamsters: evaluation of potential interactions with serotonin 2A and serotonin 2C receptors. Neuroscience 137:287-299.

Gallego M and Virshup DM (2007) Post-translational modifications regulate the ticking of the circadian clock. Nat Rev Mol Cell Biol 8:139-148.

Gannon RL (2003) Serotonergic serotonin (1A) mixed agonists/antagonists elicit large-magnitude phase shifts in hamster circadian wheel-running rhythms. Neuroscience 119:567-576.

Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, Takahasi JS and Greenberg ME (1993) Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science 260: 238-241.

Glass JD, Di Nardo LA and Ehlen JC (2000) Dorsal raphe nuclear stimulation of SCN serotonin release and circadian phase-resetting. Brain Research 859:224-232.

Glass JD, Grossman GH, Farnbauch L and DiNardo L (2003) Midbrain raphe modulation of nonphotic circadian clock resetting and 5-HT release in the mammalian suprachiasmatic nucleus. J Neurosci 23:7451-7460.

Golombek DA, Ferreyra GA, Marpegan L, Katz ME, de Tezanos Pinto F, and Yannielli P.C. (2000) The neurochemical basis of photic entrainment in mammals. Biol Rhythms Res 31:56-70.

Golombek DA, Ferreyra GA, Agostino PV, Murad AD, Rubio MF, Pizzio GA, Katz ME, Marpegan L, and Bekinschtein TA (2003) From light to genes: moving the hands of the circadian clock. Frontiers in Biosci 8:285-289.

Grossman GH, Mistlberger RE, Antle MC, Ehlen JC and Glass JD (2000) Sleep deprivation stimulates serotonin release in the suprachiasmatic nucleus. Neuroreport 11: 1921-1932.

Hamada T, Antle MC and Silver R (2004) The role of Period 1 in non-photic resetting of the hamster circadian pacemaker in the suprachiasmatic nucleus. Neurosci Lett 362:87-90.

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

Harrington ME and Schak KM (2000) Neuropeptide Y phase advances the in vitro hamster circadian clock during the subjective day with no effect on phase during the subjective night. Can J Physiol Pharmacol 78:89-92.

Hastings MH, Duffield GE, Smith EJ, Maywood ES and Ebling FJ (1998) Entrainment of the circadian system of mammals by nonphotic cues. Chronobiol Int 15:425-445.

Hastings MH, Reddy AB and Maywood ES (2003) A clockwork web: circadian timing in brain and periphery, in health and disease. Nat Rev Neurosci 4:649-661.

Hattar S, Lucas RJ, Mrosovsky N, Thompson S, Douglas RH, Hankins MW, Lem J, Hofmann F, Foster RG and Yau K-W. (2003) Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. Nature 424: 76–81.

Hellig M (2004) The NPY system in stress, anxiety and depression. Neuropeptides 38:213-224.

Honma K, Hashimito S, Nakao M and Honma S (2003) Period and phase adjustments of human circadian rhythms in the real world. J Biol Rhyhms 18:261-270.

Honma S, Shirakawa T, Katsuno Y, Namihira M and Honma K (1998) Circadian periods of single suprachiasmatic neurons in rats. Neurosci Letters 250:157-160.

Horikawa K, Yokota S, Fuji K, Akiyama M, Moriya T, Okamura H and Shibata S (2000) Nonphotic entrainment by 5-HT1A/7 receptor agonists accompanied by reduced Per1 and Per2 mRNA levels in the suprachiasmatic nuclei. J Neurosci 20, 5867-5873.

Horikawa K and Shibata S. (2004) Phase-resetting response to (+)8-OH-DPAT, a serotonin 1A/7 receptor agonist, in the mouse in vivo. Neurosci Lett 368:130-134.

Janik D and Mrosovsky, N (1994) Intergeniculate leaflet lesions and behaviorally-induced shifts of circadian rhythms. Brain Res 651:174--182.

Huhman KL, Gillespie CF, Marvel CL and Albers HE (1996) Neuropeptide Y phase shifts circadian rhythms in vivo via a Y2 receptor. 17:1249-1252.

Jakubcakova V, Oster H, Tamanini F, Cadenas C, Leitges M, van der Horst G, and Eichele G (2007) Light entrainment of the mammalian circadian clock by a PRKCA-dependent posttranslational mechanism. Neuron 54:831-843.

Janik D and Mrosovsky N (1994) Intergeniculate leaflet lesions and behaviorally-induced shifts of circadian rhythms. Brain Research 651:174-182.

Janik D, Mikkelsen JD, and Mrosovsky N (1995) Cellular colocalization of Fos and neuropeptide Y in the intergeniculate leaflet after non-photic phase shifting events. Brain Res 698:137-145.

Johnson CH, Elliott JA and Foster R (2003) Entrainment of circadian programs. Chonobiol Int 20:741-774.

Jud C, Schmutz I, Hampp G, Oster O and Albrecht U (2005) A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions. Biological Proced Online 7:101-116.
Karman BN and Tischkau SA (2006) Circadian gene expression in the ovary: effects of luteinizing hormone. Biol Reprod 75:624-632.

Klein DC, Moore MD and Reppert SM (1991) Suprachiasmatic nucleus: the mind's clock. Oxford University Press, New York.

Klerman EB, Rimmer DW, Dijk DJ, Kronauer RE, Rizzo JF and Czeisler CA (1998) Nonphotic entrainment of the human circadian pacemaker. Am J Physiol 274:R991-996.

Knoch ME, Siegel D, Duncan MJ and Glass JD (2006) Serotonergic mediation of constant light-potentiated nonphotic phase shifting of the circadian locomotor activity rhythm in Syrian hamsters. Am J Physiol Regul Integr Comp Physiol 291:R180-R188.
LaFleur SE, Kalsbee A, Wortel J, Fekkes ML and Buijs RM (2001) A daily rhythm in glucose tolerance: a role for the suprachiasmatic nucleus. Diabetes 50:1237-1243.

Lall GS and Biello SM (2002) Attenuation of phase shifts to light by activity or neuropeptide Y: a time course study. Brain Res 957: 109-116.

Lall GS and Biello SM (2003a) Attenuation of circadian light induced phase advances and delays by neuropeptide Y and a neuropeptide Y Y1/Y5 receptor agonist. Neuroscience 119:611-618.

Lall GS and Biello SM (2003b) Neuropeptide Y, GABA and circadian phase shifts to photic stimuli. Neuroscience 120:915-921.

Lall GS and Biello SM (2003c) Neuropeptide Y (NPY) attenuates photic phase shifts via the NPY Y5 receptor subtype. Program No. 510.14. Washington, D.C. Society for Neuroscience.

Lall GS and Harrington M (2006) Potentiation of the resetting effects of light on circadian rhythms of hamsters using serotonin and neuropeptide Y receptor antagonists. Neuroscience 141:1545-1552.

Lam RW, Levitt AJ, Levitan RD, Enns MW, Morehouse R, Michalak EE, and Tam EM (2006) The Can-SAD study: a randomized controlled trial of the effectiveness of light therapy and fluoxetine in patients with seasonal affective disorder. Am J Psychiatry 163:805-812.

Landry GJ and Mistleberger RE (2005) Differential effects of constant light on circadian clock resetting by photic and nonphotic stimuli in Syrian hamsters. Brain Research 1059:52-58.

Lee C, Etchegaray JP, Cagampang FR, Loudon AS and Reppert SM (2001) Posttranslational mechanisms regulate the mammalian circadian clock. Cell 107:855-867.

Levi F and Schibler U (2007) Circadian rhythms: mechanisms and therapeutic implications. Annu Rev Pharmacol Toxicol 47:593-628.

Lewy AJ, Lefler BJ, Emens JS and Bauer VK (2006) The circadian basis of winter depression. Proc Natl Acad Sci 103:7414-7419.

Liu AC, Lewis WG and Kay SA (2007) Mammalian circadian signaling networks and therapeutic targets. Nat Chem Bio 10:630-639.

Lowrey PL and Takahasi JS (2004) Mammalian circadian biology; elucidating genome-wide levels of temporal organization. Annu Rev Genomics Hum Genet 5:407-441.

Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR, Menaker M and Takahasi JS (2000) Positional syntenic cloning and functional characterization of the mammalian circadian mutation *tau*. Science 288:483-492.

Lovenberg TW, Baron B, de Lecea L, Miller JD, Prosser RA, Rea MA, Foye PE, Racke M, Slone AL, Siegel BW, Danielson PE, Sutcliffe JG and Erlander MG (1993) A novel adenylate cyclase-activating serotonin receptor ($5-HT_7$) implicated in the regulation of mammalian circadian rhythms. Neuron 11:449-458.

Marchant EG and Mistleberger RE (1995) Morphine phase-shifts circadian rhythms in mice: role of behavioral activation. Neuroreport 7:209-212.

Marchant EG, Watson NV and Mistleberger RE (1997) Both neuropeptide Y and serotonin are necessary for entrainment of circadian rhythms in mice by daily forced treadmill running schedules. J Neurosci 17:7974-7987.

Martino TA, Oudit Gy, Herzenberg AM, Tata N, Koletar MM, Kabir GM, Belsham DB, Backx PH, Ralph MR and Sole MJ (2008) Circadian rhythm disorganization produces profound cardiovascular and renal disease in hamsters. Am J Physiol Integr Comp Physiol 294:1675-1683.

Maywood ES, Mrosovsky N, Field MD and Hastings MH (1999) Rapid down-regulation of mammalian period genes during behavioral resetting of the circadian clock. Proc Natl Acad Sci 96: 15211-15216.

Maywood ES and Mrosovsky N (2001) A molecular explanation of interactions between photic and non-photic circadian clock resetting stimuli. Brain Res Gene Expr Patterns 1:27-31.

Maywood ES, Okamura H and Hastings MH (2002) Opposing actions of neuropeptide Y and light on the expression of circadian clock genes in the mouse suprachiasmatic nuclei. Eur J Neurosci 15216-15220.

McKinley Brewer J, Yannielli PC and Harrington ME (2002) Neuropeptide Y differentially suppresses *Per1* and *Per 2* mRNA induced by light in the suprachiasmatic nuclei of the golden hamster. J Biol Rhythms 17:28-29.

Mead S, Ebling F, Maywood ES, Humbly T, Herbert J and Hastings MH (1992) A nonphotic stimulus causes instantaneous phase advances of the light-entrainable circadian oscillator of the Syrian hamster but does not induce the expression of c-fos in the suprachiasmatic nucleus. J Neurosci 12:2516-2522.

Menaker M and Eskin A (1966) Entrainment of circadian rhythms by sound in Passer domesticus. Science 154:1579-1581.

Mendenhall W, Beaver RJ and Beaver B (2006) Introduction to probability and statistics. Thompson Higher Education, Belmont CA, USA (Chapter 10: Inferences from small samples).

Merrow M, Spoelstra K and Roenneberg T (2005) The circadian cycle: daily rhythms from behaviour to genes. EMBO Rep 6:930-5.

Meyer-Bernstein EL and Morin LP (1996) Differential serotonergic innervation of the suprachiasmatic nucleus and the intergeniculate leaflet and its role in circadian rhythm modulation. J Neurosci 16: 2097--2111.

Meyer-Bernstein EL, Blanchard JH and Morin LP (1997) The serotonergic projection from the median raphe nucleus to the suprachiasmatic nucleus modulates activity phase onset, but not other circadian rhythm parameters. Brain Research 755:112-120.

Meyer-Bernstein EL and Morin LP (1998) Destruction of serotonergic neurons in the median raphe nucleus blocks circadian rhythm phase shifts to triazolam but not to novel wheel access. J Biol Rhythms 13:494-505.

Mikkelsen JD, Vrang N and Mrosovsky (1998) Expression of Fos in the circadian system following nonphotic stimulation. Brain Res Bull 47:367-376.

Miller JD, Morin LP, Schwartz WJ and Moore RY (1996) New insights into the mammalian circadian clock. Sleep 8:641-667.

Mintz EM, Gillepsie CF, Marvel CL, Huhman KL and Albers HE (1997) Serotonergic regulation of circadian rhythms in Syrian hamsters. Neuroscience 79:563-569.

Mintz EM, Jasnow AM, Gillepsie CF, Huhman KL and Albers HE (2002) GABA interacts with photic signaling in the suprachiasmatic nucleus to regulate circadian phase shifts. Neuroscience 109:773-778.

Mistlberger RE, Landry GE and Marchant EG (1997) Sleep deprivation can attenuate light-induced phase shifts of circadian rhythms in hamsters. Neurosci Lett 238:5-8.

Mistlberger RE, Sinclair SV, Marchant EG and Neil L (1997) Circadian phase shifts to food deprivation and refeeding in the Syrian hamster are mediated by running activity. Physiol Behav 61:273-278.

Mistlberger RE and Antle MC (1998) Behavioral inhibition of light-induced circadian phase resetting is phase and serotonin dependent. Brain Res 786:31-38.

Mistlberger RE, Antle MC, Glass JD.and Miller JD (2000) Behavioral and serotonergic regulation of circacadian rhythms. Biol Rhythms Research 31, 240-283.

Mistlberger RE and Holmes MM (2000) Behavioral feedback regulation of circadian rhythm phase angle in light-dark entrained mice. Am J Physiol Regul Integr Physiol 279:813-821.

Mistlberger RE and Skene DJ (2003) Social influences on mammalian circadian rhythms: animal and human studies. Biol Rev Camb Philos Soc 79:533-556.

Mistlberger RE and Skene DJ (2005) Nonphotic entrainment in humans? J Biol Rhythms 20:339-352.

Mistlberger RE (2006) Illuminating serotonergic gateways for strong resetting of the mammalian circadian clock. Am J Physiol Regul Integr Physiol 1:177-179.

Morin LP and Meyer-Bernstein EL (1999) The ascending serotonergic system in the hamster: comparison with projections of the dorsal and median raphe nuclei. Neuroscience 91:81-105.

Moga MM and Moore RY (1997) Organization of neural inputs to the suprachiasmatic nucleus in the rat. J Comp Neurol 389:508-534.

Moore RY and Eichler VB (1972) Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res 13:201-206.

Morin LP and Allen CN (2006) The circadian visual system. Brain Research Rev 51:1-60.

Moriya T, Yoshinobu Y, Ikeda M, Yokota S, Akiyama M and Shibata S (1998) Potentiating action of MKC-242, a selective 5-HT1A receptor agonist, on the photic entrainment of the circadian activity rhythm in hamsters. Br J Pharmacol 125:1281-1287.

Mrosovsky N, Salmon PA, Menaker M and Ralph MR (1992) Nonphotic phase shifting in hamster clock mutants. J Biol Rhythms 7:41-49.

Mrosovsky N (1996) Locomotor activity and non-photic influences on circadian clocks. Biol Rev Camb Philos Soc 71:343-372.

Mrosovsky N and Salmon PA (1990) Triazolam and phase shifting acceleration re-evaluated. Chronobiol Int 7:35-41.

Muscat L, Tischler RC and Morin LP (2005) Functional analysis of the role of the median raphe as a regulator of hamster circadian system sensitivity to light. Brain Res 1044:59-66.

Muscat L and Morin LP (2006) Intergeniculate leaflet: contributions to photic and non-photic responsiveness of the hamster circadian system. Neurosci 140:305-320.

Nagata C, Nagano Y, Yamamoto S, Shibuya C, Kashiki Y and Shimuzu H (2008) Light exposure at night, urinary 6-sulfatoxymelatonin, and serum estrogens and androgens in postmenopausal Japanese women. Cancer Epidemiol Biomarkers Prev. 6:1418-23.
Panda S and Hogenesch JB (2004) It's all in the timing: many clocks, many outputs. J Biol Rhythms 19:374-387.

Partch CL, Shields KF, Thompson CL, Selby CP and Sancar A (2006) Postranslational regulation of the mammalian circadian clock by cryptochrome and protein phosphatase 5. Proc Nat Acad Sci 103:10467-10472.

Peirson S and Foster RG (2006) Melanopsin: another way of signaling light. Neuron 49:331-339.

Pickard GE, Ralph MR and Menaker M (1987) The intergeniculate leaflet partially mediates effects of light on circadian rhythms. J Biol Rhythms 2: 35-56.

Pickard GE and Rea MA (1997) Serotonergic innervation of the hypothalamic suprachiasmatic nucleus and photic regulation of circadian rhythms. Biol Cell 89:513-523.

Pittendrigh CS, Bruce V and Kaus P (1958) On the significance of transients in daily rhythms. Proc Natl Acad Sci USA 44:965-973.

Pittendrigh CS and Daan S (1976) A functional analysis of circadian pacemakers in nocturnal rodents. J Comp Physiol 106:291-331.

Prossner RA, Miller JD and Heller HC (1990) A serotonin agonist phase-shifts the circadian clock in the suprachiasmatic nuclei in vitro. Brain Research 534:336-339.

Prosser RA (1998) Neuropeptide Y blocks serotonergic phase advances of the suprachiasmatic circadian clock in vitro. Brain Research 808:31-41.

Prossner RA, Lee H-M and Wehner A (2006) Serotonergic pre-treatments block in vitro serotonergic phase shifts of the mouse suprachiasmatic nucleus circadian clock. Neuroscience 142:547-555.

Ralph MR and Menaker M (1985) Bisculline blocks circadian phase delays but not advances. Brain Res 325:405-408.

Ralph MR and Menaker M (1986) Effects of diazepam on circadian phase advances and delays. Brain Res 372:405-408.

Ralph MR and Menaker M (1988) A mutation of the circadian system in golden hamsters. Science 241:1225-1227.
Ralph MR, Foster RG, Davis FC and Menaker M (1990) Transplanted suprachiasmatic nucleus determines circadian period. Science 247:975-978.

Ralph MR and Mrosovsky N (1992) Behavioral inhibition of circadian responses to light. J Biol Rhythms 7:353-359.

Rea Ma, Buckley B and Lutton LM (1993) Local administration of EAA antagonists block light-induced phase shifts and c-fos expression in hamster SCN. Am J Physiol 265:1191-1198.

Rea MA, Glass JD and Colwell CS (1994) Serotonin modulates photic responses in the hamster suprachiasmatic nucleus. J Neuroscience 14:3635-3642.

Rea MA, Barrera J, Glass JD and Gannon RL (1995) Serotonergic potentiation of photic phase shifts of the circadian activity rhythm. NeuroReport 6: 1417-1420.

Reebs SG and Mrosovsky N (1989) Effects of induced wheel running on the circadian activity rhythms of Syrian hamsters: entrainment and phase response curve. J Biol Rhythms 4:39-48.

Refinetti R, Nelson DE and Menaker M (1992) Social stimuli fail to act as entraining agents of circadian rhythms in the golden hamster. J Comp Physiol 170:181-187.

Reppert S and Weaver D (2002) Coordination of circadian timing in mammals. Nature 418: 935 - 941.

Roenneberg T, Daan S and Merrow M (2003) The art of entrainment. J Biol Rhythms 18:183-194.

Roja J, Santin LJ, Garcia M, Dona A, de Pablos L, Cuadrado MI, Alcade FJ, Gonzalez-Baron S and Aguirre JA (2004) 5-HT1A receptor activation before acute stress counteracted the induced long-term behavioral effects. Ann NY Acad Sci 1018333-338.

Rusak B and Zucker I (1979) Neural regulation of circadian rhythms. Physiol Rev 59:449-526.

Scarbrough K and Turek (1996) Quantitative differences in the circadian rhythm of locomotor activity and vasopressin and vasoactive intestinal peptide gene expression in the suprachiasmatic nucleus of tau mutant hamsters compared to wildtype hamsters. Brain Res 736:251-259.

Schibler U (2005) The daily rhythms of genes, cells and organs. Biological clocks and circadian timing in cells. EMBO Rep 6:9-13.

Schwartz WJ and Zimmerman P (1990) Circadian timekeeping in BALBc and C57BL/6 inbred mouse strains. J Neuroscience 10:3685-3694.

Shibata S, Oomura Y, Hattori K and Kita H (1982) Circadian rhythm changes of neuronal activity in the suprachiasmatic nucleus of the rat hypothalamic slice. Brain Res 247:154-158.

Shigeyoshi Y, Taguchi K, Yamamoto S, Takekida S, Yan L, Tei H, Moriya T, Shibata S, Loros J, Dunlap J and Okamura H (1997) Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the *mPer1* transcript. Cell 91:1043-1053.

Shimomura K, Kornhauser JM, Wisor JP, Umezu T, Yamazaki S, Ihara NL, Takahashi JS and Menaker M (1998) Circadian behavior and plasticity of light-induced c-fos expression in SCN of tau mutant hamsters. J Biol Rhythms 13:305-314.

Shinohara K, Tominga K, Fukuhara C, Otori Y and Inouye SI (1993) Processing of photic information within the intergeniculate leaflet of the lateral geniculate body: assessed by neuropeptide Y immunoreactivity in the suprachiasmatic nucleus of rats. Neuroscience 56:813-822.

Smart CM and Biello SM (2001) WAY-100635, a specific 5-HT1A antagonist, can increase the responsiveness of the mammalian circadian pacemaker to photic stimuli. Neurosci Lett 305: 33-36.

Sollars PJ, Oglivie MD, Simpson AM and Pickard GE (2006) Photic entrainment is altered in the 5-HT1B receptor knockout mouse. J Biol Rhythms 21:21-32.

Soscia SJ and Harrington ME (2004) Neuropeptide Y attenuates NMDA-induced phase shifts in the SCN of NPY Y1 receptor knockout mice in vitro. Brain Res 1023:148-153.

Soscia SJ and Harrington ME (2005) Neuropeptide Y does not reset the circadian clock in NPY Y2 -/- mice. Neurosci Lett 373:175-178.

Stephan FK and Zucker I (1972) Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions Proc Nat Acad Sci USA 69:1583-1586.

Stevens RG (2005) Circadian disruption and breast cancer: from melatonin to clock genes. Epidemiology 16:254-258.

Stevens RG (2006) Artificial lighting in the industrialized world: circadian disruption and breast cancer. Cancer Causes Control 17:501-507.

Stopa EG, Johnson JK, Friedman DI, Ryer HI, Reidy J, Kuo-LeBlanc V and Albers HE (1995) Neuropeptide Y receptor distribution and regulation in the suprachiasmatic nucleus of the Syrian hamster. Pept Res 8:95-100.

Subramanian P and Subbaraj R (1996) Diazepam modulates the period of locomotor rhythm in mice and attenuates light-induced phase advances. Pharmacol Biochem Behav 54:393-398.

Takahashi S, Yoshinobu Y, Aida R, Shimomura H, Akiyama M, Moriya T, and Shibata S (2002) Extended action of MKC-242, a selective 5-HT (1A) receptor agonist, on light-induced Per gene expression in the suprachiasmatic nucleus in mice. J Neurosci Res 68:470-478.

Thankachan S and Rusak B (2005) Juxtacellular recording/labeling analysis of physiological and anatomical characteristics of rat intergeniculate leaflet neurons. J Neurosci 25:9195-9204.

Tierno A, Fiore P and Gannon RL (2002) Delta opioid inhibition of light-induced phase advances in hamster circadian activity rhythms. Brain Res 937:66-73.

Van Gelder RN and Hogenesch JB (2004) Clean thoughts about dirty genes. J Biol Rhythms 19: 3-6.

Valentinuzzi VS, Buxton OM, Chang OM, Scarbrough K, Ferrari EA, Takahashi JS and Turek FW (2000) Locomotor response to an open field during C57BL/6J active and inactive phases differences dependent on conditions of illumination. Physiol & Behav 69:269-275.

Weber ET, Gannon RL and Rea MA (1995) cGMP-dependent protein kinase inhibitor blocks light-induced phase advances of circadian rhythms in vivo. Neurosci Lett 197:227-230.

Weber ET and Rea MA (1997) Neuropeptide Y blocks light-induced phase advances but not delays of the circadian activity rhythm in hamsters. Neuroscience Letters 231: 159-162.

Weber ET, Gannon RL and Rea MA (1998) Local administration of serotonin agonists blocks light-induced phase advances of the circadian activity rhythm in hamsters. J Biol Rhythms 13:209-218.

Welsh DK, Logthetis DE, Meister M and Reppert SM (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 14:697-706.

Wickland C and Turek FW (1994) Lesions of the thalamic intergeniculate leaflet block activity-induced phase shifts in the circadian activity of the golden hamster. Brain Res 660:293-300.

Winkler D, Pjrek E, Iwaki R and Kasper S (2006) Treatment of seasonal affective disorder. Expert Rev Neurother 6:1039-1048.

Yamazki S Numano R Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, and Tei H (2000) Resetting central and peripheral circadian oscillators in transgeneeic rats. Science 288:682-685.

Yan L and Silver R (2004) Resetting the brain clock: time course and localization of mPER1 and mPER2 protein expression in suprachiasmatic nuclei during phase shifts. Eur J Neurosci 19:1105-1109.

Yannielli PC and Harrington ME (2000) Neuropeptide Y applied in vitro can block the phase shifts induced by light in vivo. NeuroReport 11:1587-1591.

Yannielli P and Harrington ME (2001a) Neuropeptide Y in the mammalian circadian system: effects on light-induced circadian responses. Peptides 22:547-556.

Yannielli PC and Harrington ME (2001b) The neuropeptide Y Y5 receptor mediates the blockade of "photic-like" NMDA-induced phase shifts in the golden hamster. J Neurosci 14:5367-5373.

Yannielli P, McKinley Brewer J and Harrington ME (2004a) Blockade of the NPY Y5 receptor potentiates circadian response to light: complementary in vivo and in vitro studies. Eur J Neurosci 19:891-897.

Yannielli P and Harrington ME (2004b) Let there be "more" light: enhancement of light actions on the circadian system through non-photic pathways. Prog Neurobiol 74:59-76.

Yokota SI, Horikawa K, Akiyama M, Moriya T, Ebihara S, Komuro G, Ohta T and Shibata S (2000) Inhibitory action of brotizolam on circadian and light-induced per1 and per2 expression in the hamster suprachiasmatic nucleus. Br J Pharmacol 131:1739-1747.

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci 101:5339-46.

Zhang Y, Van Reeth, Zee PC, Takahasi JS and Turek FW (1993) Fos protein expression in the circadian clock is not associated with phase shifts induced by a nonphotic stimulus, triazolam. Neurosci Lett 164:203-208.

Zeitzer JM, Dijk DJ, Kronauer R, Brown E, Czeisler C (2000) Sensitivity of the human circadian pacemaker to nocturnal light: melatonin phase resetting and suppression. J Physiol 526 695–702.