The suprachiasmatic nucleus (SCN) of the hypothalamus is the principle mammalian endogenous biological oscillator, generating and orchestrating a myriad of circadian rhythmic events and processes. This autonomous pacemaker can be entrained by both environmental cues (primarily via fluctuations in the light/dark cycle), as well as organismal influences (internal state feedback information), in order to coordinate and synchronize internal biochemical functions to solar time, which allows the organism to anticipate and therefore, prepare for upcoming events or tasks rather, than reflexively reacting to stimuli in the environment. In the present study we primarily utilized in vivo techniques to examine the regulation of non-photic (all stimuli other than light or the chemical events associated with photic signaling) phase-resetting of the circadian clock. The monoamine, serotonin (5-hydroxytryptamine; 5-HT) is a well described neurotransmitter in the central nervous system of vertebrates. Though it is discretely synthesized in the raphe complexes of the mid-brain, it is released from terminal fields of collaterals that innervate various portions of the brain and spinal cord. Its role in mediating non-photic-induced phase-alterations in the clock, as well as the respective output rhythms generated by the clock, has been investigated. However, it remains speculative if endogenous serotonergic signaling directly in the area of the clock, or in other regions of the circadian system is necessary and or sufficient to cause
phase-shifts. HPLC analysis of samples collected by in vivo microdialysis, provides the first direct evidence that general arousal, in the absence of vigorous locomotor activity, is sufficient to induce 5-HT release in the SCN, which is mediated by the activation of the dorsal raphe nucleus (DRN). The results also demonstrate that inhibition of the DRN, attenuates phase-shifts associated with activity. In addition, the results constitute the first direct evidence that activity/arousal induces 5-HT release in a brain region that has direct connections to the SCN, the intergeniculate leaflet (IGL), and that the release is also coupled to the activation of the DRN. The finding that an exogenous 5-HT agonist locally targeted in the IGL induces shifts in rhythms similar to activity/arousal behaviorally-induced shifting further implicates 5-HT in phase-alterations. In addition, post synaptically blocking 5-HT signaling in this region attenuates shifts associated with behavioral manipulations. In general, these results provide evidence that the serotonergic system plays a critical role in the regulation of non-photic phase-resetting of mammalian circadian rhythms.
REGULATION OF NON-PHOTIC PHASE-RESETTING
OF THE MAMMALIAN CIRCADIAN CLOCK

A dissertation submitted
to Kent State University in partial
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degree of Doctor of Philosophy

by

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ABBREVIATIONS

Alpha — the period of continuous activity during a 24 h period

AP — anterior-posterior coordinate

c-fos — an immediate early gene, various stimuli induces rapid transcription and translation, resulting in the Fos protein which primarily acts as a transcription factor

CT — circadian time; the time scale of a 24 h period used under constant conditions (i.e. DD or LL), in which CT 12 denotes the time of activity onset by convention

DAB — 3,3’-diaminobenzidine-HCL

DD — dark/dark cycle; the chamber is in constant darkness for this period

DMSO — dimethyl sulphoxide; a common solvent

DPAT — 8-OH-DPAT, 8-hydroxy-2[di-n-propylamino] - tetralin

DRN — dorsal median raphe nucleus

DV — dorsal-ventral coordinate

Fos — the protein produced from the c-fos gene

GABA — γ-amino butyric acid

IGL — intergeniculate leaflet of the lateral geniculate complex in the thalamus

i.p. — intraperitoneal

LD — light/dark; the chamber’s photic cycle mimics a solar day, with periods of light corresponding to day and a periods of darkness corresponding to night

LL — light/light; the chamber is in constant light during this period

ML — medial-lateral coordinate

MRN — median raphe nucleus
NPY — neuropeptide Y

$p$-CPA — $p$-chlorophenylalanine (tryptophan hydroxylase inhibitor)

per — the period gene

PER — period gene protein product

PRC — phase response curve; a plot showing the relationship between the time point at which a given stimulus is delivered, and the magnitude of the phase-shift induced by the stimulus

PSA — polysialic acid; its expression was used as a marker for the margins of the rostral intergeniculate leaflet

RHT — retinohypothalamic tract

SCN — suprachiasmatic nucleus; the site of the mammalian circadian clock

tau — the period of a circadian rhythm (roughly 24 h in length under DD conditions)

TRZ — triazolam (a benzodiazepine; class of drugs that inhibit neuronal activity via the GABA$_A$ receptor

ZT — zeitgeber time; the time scale of a 24 h period used under light/dark (LD) conditions, in which CT 12 denotes the time of activity onset by convention

5-HT — 5-hydroxytryptamine, serotonin

5-7-DHT — 5,7-dihydroxytryptamine (serotonin neuron-specific neurotoxin)

5-HIAA — 5-hydroxyindoleacetic acid (serotonin metabolite)
I would like to express my overwhelming gratitude to my dissertation advisor, mentor and friend Dr. J. David Glass. Without his support, mentorship and generosity, none of this could have been possible. The opportunities that Dr. Glass has afforded me through this endeavour have placed me among a group of graduate students that has been given the tools and resources necessary to answer any scientific question encountered. I would also like to thank my dissertation committee for their input, inspiration and guidance through this process. Dr. James Blank, chair of the department of biological sciences and previous head of the School of Biomedical Sciences, and Dr. Robert Dorman, current director of the School of Biomedical Sciences have helped me immensely throughout my academic career. I must thank them for all the money they lost at the pool table at Ray’s. As the outside member of my committee, Dr. Gail Frasier has been a wonderful inside source of assistance. I would also like to express my appreciation for Dr. Marilyn Duncan’s efforts in running in situ hybridization. I am indebted to the former students of this laboratory, particularly, Dr. Tom Dudley and Dr. Christopher Ehlen. The example you set forth, as well as the time and energy you gave in mentoring me, will never be forgotten (or forgiven). I am also indebted to Herculean efforts of the two best laboratory technicians a nascent scientist could hope to work with, Laure Farnbauch and Amelie Cornil. Finally, I wish to thank my friends Dr. Robert Clements, Dr. Michelle Martin, the late Dr. Crackers, Sandy, Dr. Locke and Dr. Robert Grossman for the good times had over the years, as well as their help and advice.
DEDICATION

I dedicate this dissertation to the two forces that not only have propelled me through this process, but have been a tireless source of financial and emotional support throughout the years, my parents Dr. Morton and Beatrice Grossman and the lovely Marybeth Carragher.
CHAPTER I

INTRODUCTION

As the earth undertakes its yearly revolution around the sun, the planet rotates about its axis, completing one turn every 24 h. Half the globe is in darkness at any one point, while the other half is illuminated by the sun. This 24 h light/dark (LD) cycle is the most overt daily fluctuation under which life on Earth has evolved. Daily rhythmic alterations in physiology, biochemistry and behavior allow organisms to synchronize internal biological events to daily changes in the environment. A circadian rhythm is defined as an activity, or biological process, that under constant conditions (i.e. constant light or darkness, where the organism looses reference to external photic timing cues) oscillates with a period close to, but often not precisely 24 h (Dunlap et al., 2003). The most overt rhythmic activity is the sleep/wake cycle. However, a multitude of circadian controlled rhythms have been reported, including body temperature (Albers, 1982), release of hormones (Moore and Eichler, 1972; Klein and Moore, 1979,) and locomotor activity (Binkley, 1971). These rhythms in biological activity are endogenously generated and sustained by oscillators or intrinsic clocks located within the organism (Dunlap et al., 2003).

As stated above, the period generated by the endogenous clock is approximate to, but often equal, to the 24 h cycle of the external environment. Therefore, organisms have evolved physiological means, in order to be synchronized with the light/dark external cycle. In other words, they synchronize biological time to solar time. This
allows the organism to anticipate, and therefore, prepare for upcoming events or tasks rather than reflexively reacting to stimuli in the environment.

The Suprachiasmatic Nucleus is the Mammalian Circadian Pacemaker

The site of the primary circadian clock is the suprachiasmatic nucleus of the anterior hypothalamus (Moore and Eichler, 1972; Stephan and Zucker, 1972) in mammals. Bilateral lesioning of the SCN abolishes a multitude of circadian rhythms, including behavioral, physiological, endocrine and biochemical fluctuations (Moore and Eichler, 1972; Klein and Moore, 1979; Binkley, 1983), while transplantation of donor SCN tissue into an area proximal to the lesioned host SCN restores rhythmicity. Perhaps the most convincing evidence that the SCN is the master circadian clock is the finding that the restored rhythms express the period of the donor, not the recipient (Lehman et al., 1987; Ralph et al., 1990).

It was shown that electrical activity of the SCN displays a 24 h rhythm, where activity is highest during the day and low at night. This rhythm is preserved in hypothalamic slices containing SCN (Inouye and Kawamura, 1979). That the rhythms persist in SCN slices maintained in vitro with ensemble firing rhythms approximately 24 h (Groos and Hendriks, 1982; Shibata et al., 1982; Prosser and Gillette, 1989; Green and Gillette, 1992) further demonstrates that the SCN is an autonomous pacemaker. In addition, individual rat SCN neurons in vitro demonstrate circadian rhythms in neuronal firing and vasopressin release for several days, indicating that the rhythms generated are cell-autonomous (Murakami et al., 1995; Welsh et al., 1995).

Although these cells show rhythms, it is not known if single SCN cells have the ability to phase-shift and entrain (Weaver, 1998). It was demonstrated, by using 2-
deoxyglucose uptake, that the SCN exhibits an oscillation in metabolic activity *in vivo*, with a pattern similar to that of electrical activity, high during the day in the absence of lighting cues (Schwartz and Gainer, 1977). Interestingly, the fetal SCN of most mammalian species displays a clear rhythm in metabolic activity like the mother’s SCN (Reppert and Schwartz, 1983). Taken together, these findings establish that the SCN meets several key criteria of a master circadian pacemaker: 1) the SCN generates an endogenous rhythm that is approximately 24 h, as evidenced by both *in vivo* and *in vitro* studies; 2) ablation of the SCN induces arrhythmia in a variety of circadian controlled biological rhythms; 3) transplantation of donor SCN restores rhythms, with a period of that of the donor. (Binkley, 1983).

**Photic Signaling: Entrainment and Phase Alteration of Rhythms by Light**

What is termed the “circadian timing system” comprises not only the clock, but also afferent pathways that transmit external and internal information to the SCN, and the neural and or humoral networks able to generate rhythmic outputs (Weaver, 1998). Inputs to the SCN that signal external environmental time cues or zeitgebers (time givers) are necessary to reset the clock on a daily basis. This is termed entrainment (Hastings, 1997). Locomotor activity can be used as an indicator of the rhythm generated by the SCN. Recordings of locomotor activity in hamsters placed under a 24 h LD cycle show a clear and concise phase relationship with the LD cycle (entrainment), with activity onset occurring at the time of lights-off, designated under LD conditions as zeitgeber time 12 (ZT 12). The free-running period (tau) of the animal’s activity, can be ascertained under constant dark (DD) environmental conditions, revealing the intrinsic rhythm of the clock. Animals with a tau slightly longer than 24 h will commence activity
later in respect to the previous day, while those with a tau less than 24 h exhibit advanced onset of activity day to day. Under constant conditions the ZT reference index is inappropriate. Thus, reference to time is denoted as circadian time (CT), with CT 12 designating the time of onset of activity by convention.

Light is the primary zeitgeber that can reset the SCN and its respective output rhythms. Photic pulses delivered to animals housed under DD can almost instantly reset the SCN (Best et al., 1999). However, the effect of these pulses is dependent upon the time at which they are administered. Light pulses have been shown to induce a bimodal (discontinuous) responses. They elicit phase delays in locomotor activity, when applied during the early subjective night, and they induce phase advances in late night (Takahashi and Zatz, 1982). Rhythms are unaffected by photic stimulation, during all other periods of the subjective night and day. Thus, light’s ability to phase-shift rhythms is gated by the circadian system, meaning that there are temporal windows through which photic signals must pass to alter the clock.

Photic information is relayed from the retina directly to the SCN via a monosynaptic pathway, the retinohypothalamic tract (RHT) (Moore RY et al., 1971). The RHT axons terminate primarily in the ventrolateral (core) portion of the SCN of the hamster (Hendrickson et al., 1972). Studies in which the RHT has been severed show that this treatment does not alter circadian rhythms, but prevents entrainment and photic-induced phase-shifts by light (Dunlap et al., 2003). Glutamate and the neuropeptide pituitary adenylate cyclase activating peptide (PACAP) are co-localized in the terminals of RHT axons (Hannibal et al., 2000). Glutamate released from the RHT has been implicated as the primary mediator for the direct effect of photic-induced phase-shifts. Glutamate or glutamate agonists injected into the SCN of hamsters can
mimic the effects of light pulses delivered during the subjective night (Mintz and Albers, 1997; Mintz et al., 1999;). Glutamate or electrical stimulation of the optic tract has also been shown to reset the SCN rhythms in a light-like manner in hypothalamic slices (Ding et al., 1994; Ding et al., 1997; Prosser, 2001).

Glutamate and light are believed to act through both NMDA and non-NMDA receptors whose activation ultimately modulate the clock (Prosser, 2001). The activation of these receptors by glutamate effects cellular responses that are critical for photic-induced shifts including; increasing intracellular Ca++ (Hamada et al., 1999), activating cAMP response element-binding protein (Ding et al., 1997; Obrietan et al., 1999), rapidly inducing immediate early gene expression (Best et al., 1999) and raising levels of Per1 and Per2, two clock-coupled gene proteins (Akiyama et al., 1999).

Photic information is also conveyed to the SCN by an indirect pathway, the geniculohypothalamic tract (GHT). The GHT arises solely from the intergeniculate leaflet (IGL), a narrow band of neurons of the lateral geniculate complex situated in the dorsal thalamus (Hickey and Spear 1976; Morin and Blanchard, 2001). The IGL receives direct projections from the retina (Hickey and Spear, 1976). The GHT is formed from IGL neurons co-localising neuropeptide Y (NPY), encephalin, γ-amino butyric acid (GABA) and neurotensin (Morin and Blanchard, 2001) in the hamster. While the GHT and IGL may contribute photic information to the clock, they are not essential for normal photic entrainment (Pickard et al., 1987). However, they appear to modulate entrainment to a short day photoperiod (Johnson, 1989), skeleton photoperiod (Edelstein and Amir, 1999) and modify the degree of the phase-response to light (Pickard et al., 1987). The IGL appears to be critical in modulating and conveying non-photic information to the SCN (Janik and Mrosovsky, 1992; Challet et al., 1996; Harrington, 1997; Marchant et al.,
Non-photic Signaling: Entrainment and Phase-resetting of Rhythms by Signals Other than Light

Manipulations of endogenous compounds not related to photic signaling can phase-shift the SCN circadian clock. The commonality between most of these non-photic influences is that they induce phase advances only during the subjective mid-day, unlike the pattern seen in photic-induced shifting. In the last decade, research has focused on the ability of behavioral activity to reset rhythms (Mrosovsky, 1995). Experimental manipulations such as 3 h of sleep deprivation (Antle et al., 2000; Grosssmann et al., 2000) or novel-wheel-induced running (Mrosovsky et al., 1989; Reeb and Mrosovsky, 1989) induce large phase-advances (~1.5 h) when delivered during the subjective day. Other non-photic stimuli that have demonstrated the ability to phase-advance rhythms in the day-time include; dark pulses (Boulos and Rusak, 1982), saline injections (Hastings et al., 1992), social interaction (Mrosovsky, 1988), bedding changes (Mrosovsky et al., 1989), application of NPY (Huhman and Albers, 1994), benzodiazepines (Turek and Van Reeth, 1988; Mistlberger et al., 1991) and serotonin (5-HT) agonists (Tominaga et al., 1992; Edgar et al., 1993; Cutrera et al., 1994; Ehlen et al., 2000). In addition to inducing advances, entrainment to non-photic stimuli, such as voluntary wheel running in mice (Edgar and Dement, 1991) and forced running in rat (Mistlberger, 1991) has been demonstrated. Finally, it has been shown that photic and non-photic stimuli are mutually inhibitory, attenuating each other’s advances or delays.
Non-photic Signaling: Phase-resetting of Rhythms by Serotonin

The ventral and ventromedial areas of the SCN display a dense plexus of serotonergic immunoreactive fibers (Ueda et al., 1983; Cassone et al, 1998). In the hamster, anterograde and retrograde tracing studies have revealed that serotonergic innervation of the SCN arises solely from the medial raphe nucleus (MRN) of the raphe nuclear complex of the midbrain (Meyer-Bernstein and Morin, 1996; Leander et al., 1998). Also, chemical or electrical stimulation of the dorsal raphe nucleus (DRN) or the MRN induce increases in 5-HT output in the SCN region as measured by in vivo microdialysis (Dudley et al., 1999; Glass et al., 2003). It has been proposed that while the DRN has no direct connection, it contributes to serotonergic influence of the SCN indirectly via a multi synaptic pathway involving the MRN (Glass et al., 2000; Glass et al., 2003).

In the SCN of hamsters, it has been demonstrated there exists a diurnal rhythm of 5-HT release in freely behaving animals (Dudley et al., 1998). The pattern of release is characterized by basal levels throughout the day with a sharp increase at the light/dark transition under LD conditions, or the onset of activity in animals housed under DD. This is consistent with studies that suggest that general arousal or activity is directly associated with increased neuronal activity of the serotonergic system (Wilkinson et al., 1991; Mendlin et al., 1996; Rueter and Jacobs, 1996; Gardner et al., 1997). Indeed, novel wheel running (activity) administered midday causes a dramatic increase in 5-HT release in the SCN (Dudley et al., 1998). Levels of 5-HT returned to baseline levels within 1 h after cessation of treatment.

There is a growing body of research supporting a critical role of 5-HT in circadian phase regulation. For example, there is evidence that 5-HT agonists delivered in vivo
phase advance rhythms during the day. Also, systemic (Cutrera et al., 1994; Tominaga et al., 1992) or ventricular injections (Edgar et al., 1993) of 2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydro-naphthalene (8-OH-DPAT), a selective 5-HT$_{1A/7}$ receptor agonist, induces phase advances of behavior. These advances are blocked by the pretreatment with pindolol, a selective 5-HT$_{1A}$ receptor antagonist (Tominaga et al., 1992).

Additionally, two studies point to the direct effect of in vivo administered 5-HT agonists in the SCN. Bilateral intercranial micro-injections of 8-OH-DPAT targeted at the SCN, cause significant phase advances in hamsters (Challet et al., 1998). Moreover, 8-OH-DPAT delivered unilaterally via reverse microdialysis perfusion aimed at the lateral margin of the SCN, induces advances in wheel running rhythms (Ehlen et al., 2001). These shifts are significantly potentiated by the pretreatment with the tryptophan hydroxylase (the rate limiting enzyme in the synthetic pathway of 5-HT) inhibitor $p$-chlorophenylalanine ($p$-CPA). In addition, shifts are reduced by the co-perfusion of 8-OH-DPAT with ritanserin, a selective 5-HT$_{2,7}$ receptor antagonist, or DR4004, a selective 5-HT$_7$ receptor antagonist. Consistent with these results are reports that destruction of 5-HT fibers in the SCN (Schuhler et al., 1998), or depletion of brain 5-HT (Sumova et al., 1996; Marchant et al., 1997) attenuates locomotor/arousal induced phase shifts.

Commensurate with studies providing evidence for a role of 5-HT in non-photic phase alterations of behavioral rhythms is a large body of evidence attesting to 5-HT’s ability to alter circadian rhythms within the clock itself. The majority of these studies utilized in vitro assessments of rat SCN containing hypothalamic slices. Application of 5-HT agonists has been reported to advance the circadian rhythm of firing rates (Prosser 1990; Medanic and Gillette, 1992; Shibata et al., 1992; Prosser et al., 1993; Prosser, 2000: Sprouse et al., 2004; Sprouse et al., 2005). In addition, 5-HT fibers in mouse
SCN (Marchant et al., 1997) or neuronal 5-HT in hamsters (Sumova et al., 1996) are necessary for entrainment by daily scheduled activity.

Conversely, there is also evidence refuting 5-HT’s ability to phase-shift rhythms. In the mouse, in vivo stimulation of 5-HT$_{1A/2/7}$ receptors of the circadian system is neither sufficient to attenuate light-induced phase shifts, nor reset the SCN (Antle et al., 2003). Significant phase-shifts were not observed after unilateral microinjections of 8-OH-DPAT in the SCN or IGL of Syrian Hamsters at CT 7 (Mintz et al., 1997). Bilateral injections in the SCN of serotonin specific neuron toxin 5,7-dihydroxytryptamine (5,7-DHT), reduced 5-HT and the 5-HT metabolite 5-hydroxyindole acetic acid (5-HIAA) by ~ 90%, but had no effect on the magnitude of phase-shifts produced by 3 h of novel wheel exposure at ZT 4 (Bobrzynska et al., 1996). In a study designed to examine 5-HT neuron-specific neurotoxic lesions in DRN or MRN prior to treatment with the benzodiazepine triazolam (TRZ) or novel wheel, it was found that MRN lesioned animals did not phase-advance when injected with TRZ at CT 6, but the DRN lesioned animals did. All MRN lesioned hamsters had comparable phase shifts to controls or DRN lesioned animals, with novel wheel access. It was proposed that it is unlikely that MRN neurons are necessary for phase-shifts associated with novel wheel access (Meyer-Bernstein and Morin, 1996).

The relationship between 5-HT release, behavioral state and circadian phase-resetting is evidence that 5-HT signaling may be critical in providing behavioral input to the circadian clock; specifically by participating in non-photic phase-resetting. The relative lack in the phase-resetting efficacy of unilateral microinjections of 8-OH-DPAT compared to long term dialysis or administration in a sensitized animal model (Ehlen et al., 2001) may be attributed to the length of the application of the agonist. Behavioral manipulations must be delivered for approximately 3 h for maximal phase-resetting to
occur (Mrosovsky et al., 1989). Activation of the raphe nuclei during this extended time
provides increases in 5-HT output in the SCN, which does not return to baseline levels
until after the cessation of the treatment (Dudley et al., 1998). Thus, serotonergic
receptor activation might be temporally insufficient in microinjection experiments to
induce shifting. The lack of parity in shifts elicited by unilateral microinjections in either
the SCN or IGL, compared with i.p. injections of 8-OH-DPAT, may be also explained by
temporal effects, or possibly, by the lack of serotonergic receptor activation in multiple
circadian rhythm centers. Serotonin is released in multiple brain regions during
activation of the raphe system. Systemically-applied 8-OH-DPAT injected i.p. should
have the ability to induce global effects throughout the brain. Thus, several nuclei could
be affected by 5-HT, in order for phase-resetting to occur. It is important, therefore, to
re-examine microinjection experiments, with the specific aim of co-delivering 8-OH-
DPAT and other serotonergics to the SCN, as well as other inputs to the clock, at the
same time point. The IGL is a good candidate due to its serotonergic innervation from
the DRN, as well as its suspected role in non-photic phase-resetting (Huhman and
Albers, 1994).

Stimulus Induced Cellular Signaling

Immediate early genes (IEGs), such as c-fos, belong to a category of proto-
oncogenes that are rapidly and transiently induced when triggered by a stimulus. Their
respective translated proteins form dimers, which enter the nucleus and induce the
transcription of other genes (Caputto and Guido, 2000). C-fos protein (Fos) is also able
to suppress its own transcription, thereby forming a negative feedback loop (Sassone-
Corsi et al., 1988).
The demonstration that light exposure can induce Fos in the SCN is a powerful marker for the cellular effects of photic input to the circadian clock (Aronin et al., 1990). Multiple efforts have confirmed that light exposure administered during the subjective night can induce c-fos mRNA and Fos in the SCN, as well as Fos in the IGL of nocturnal rodents (Rea, 1989; Kornhauser et al., 1990; Janik et al., 1995; Edelstein and Amir, 1996; Amir et al., 1998). Indeed, minimal luminescent levels light pulses as brief as 5 min elicit photic-induced phase-shifts, and they increase c-fos mRNA in SCN of hamsters (Kornhauser et al., 1990). This expression of Fos is mainly restricted to the ventrolateral region of the SCN, the primary site for retinal fiber innervation (Aronin et al., 1990). It is important to note that in one study, light exposure during the subjective night or subjective day induces Fos-immunoreactivity in the IGL (Edelstein and Amir, 1996), unlike observations in the SCN. This indicates that photic information transmitted by the GHT, though important in relaying photic information to the SCN, is not sufficient for light-induced Fos expression during the subjective day in the SCN, or that photic-induced Fos expression in the IGL is not gated by the circadian system as it appears to be in the SCN. This latter point is consistent with evidence that clock-related-genes exhibit little or no cycling in areas of the brain outside of the SCN (Dunlap et al., 2003).

The significance of Fos expression in the SCN after light exposure is revealed by studies demonstrating that in c-fos knock-out mice, phase-shifts are attenuated (Honrado et al., 1996), and in the rat, perfusion of antisense oligonucleotides to Fos blocks photic-induced resetting (Kornhauser et al., 1990; Rusak et al., 1990). Finally, it is important to note that expression of Fos might be necessary for phase-shifts induced by light, it has not been reported to be sufficient (Caputto and Guido, 2000). Thus, Fos expression in the SCN is a reliable marker for photic input to the circadian system, and is
essential for light-induced phase shifts. However, expression of additional immediate early genes or supplementary cellular events, presumably play key roles in light-induced phase shifting.

There is evidence that Fos expression in the SCN and IGL may not be limited to the photic signaling pathway. For example, spontaneous rhythmic expression of Fos without light exposure has been reported primarily in the dorsomedial portion (the shell) of the SCN in both rat and hamster (Sumova et al., 1998; Guido et al., 1999). Also, behavioral manipulations, such as sleep deprivation, novel wheel-induced running, cage changes or i.p. saline injections administered during the subjective mid-day induce increased Fos expression in the IGL of hamsters and rats (Janik and Mrosovsky, 1992; Edelstein and Amir, 1995; Janik et al., 1995; Mikkelsen et al., 1998; Antle and Mistlberger, 2000). However, it has been reported that wheel running induces Fos in the IGL at all circadian times, in contrast to a previous published findings (Mikkelsen et al., 1998). This observation suggests that expression in the IGL is not under circadian gating control, as light-induced expression appears to be. Interestingly, it was found that Fos was co-localized with NPY in cells of the IGL of hamsters only during the subjective day after non-photic phase-shifting manipulations (Janik et al., 1995). Fos-like immunoreactivity was not common in cells that are NPY positive in animals that received photic pulses during the subjective night. The significance of this finding is two fold. First, NPY expressing neurons are activated during behavioral-induced phase-shifting, thereby possibly enabling NPY via the GHT to be released in the SCN and ultimately induce a phase alteration of the clock. Second, the results suggest that photic and non-photic signals are conveyed to separate cell populations in the IGL.
The significance of behaviorally-induced Fos expression in the SCN remains a more complex and elusive issue. In the hamster, wheel running or sleep deprivation during the subjective day suppresses Fos expression in the SCN (Antle and Mistlberger, 2000; Mikkelsen et al., 1998). In the rat, cage changing, i.p. saline injections or stress restraint have been shown to increase Fos levels in the SCN (Edelstein and Amir, 1995). This differential expression may be due to species differences, or possibly due to variability in experimental manipulations. Notably, brief light pulses administered during early subjective day or night in rats maintained under constant conditions induce Fos in the core of the SCN, but suppress Fos in the shell, suggesting a role in photic entrainment by the shell (Beaule et al., 2001). Thus, the question remains whether Fos expression is an accurate marker for non-photic input to the circadian system, and more critically, if the observed Fos alterations are necessary or sufficient in the IGL and SCN for non-photic phase-shifting.

The role of the serotonergic system in mediating behaviorally-induced Fos expression in the SCN has not been fully established. To this date, its role in the IGL has not been assessed. One study showed that i.p. 8-OH-DPAT injections induce suppression of Fos in the hamster SCN during the subjective day, but no alterations in SCN Fos were detected in the mouse (Antle et al., 2003). In the rat SCN slice preparation, fundamental subjective day levels of fos mRNA are suppressed by application of 5-HT agonists (Prosser et al., 1994). Additionally, there is evidence that the serotonergic system might also play an important role in the mediation of light-induced Fos. Rats exposed to a 30 min light flash show increases of Fos in the SCN and the IGL Fos (Amir et al., 1998). These Fos-labeled cells are bounded by 5-HT transporter-immunoreactive fibers, indicating an anatomical means of by which activity-
induced 5-HT release could modify photic-induced phase-shifting. Complimenting this finding is the observation that electrical stimulation of the DRN or MRN reduces Fos-immunoreactivity in the SCN after a 10 min light pulse delivered at ZT 14. (Meyer-Bernstein and Morin, 1999). Finally, pretreatment with 8-OH-DPAT blocks increases in Fos expression in the SCN associated with light pulses (Glass et al., 1994).

As stated previously, 5-HT’s role in behaviorally-induced Fos expression has not been completely ascertained, although the evidence suggests that the serotonergic system would be an excellent candidate for study. Behavioral manipulations, such as sleep deprivation and wheel running, alter Fos levels and increase 5-HT release in the SCN and IGL (Dudley et al., 1999; Grossman et al., 2000; Grossman et al., 2003), as well as induce phase-shifts during the midday (Dudley et al., 1998; Reebs and Mrosovsky, 1989; Grossman et al., 2000). In addition, 5-HT antagonists injected locally in the DRN attenuate phase-advances induced by these experimental manipulations, as well as reduce 5-HT output in the SCN (Glass et al., 2003). It would be of interest to determine if 5-HT can reduce or block light-induced Fos expression in the IGL. In addition it will be critical to examine if 5-HT or 5-HT agonists are able to induce Fos expression in the IGL, particularly in neurons that are NPY positive.

**Aim 1. The effect of behavioral arousal on 5-HT release in the SCN.**

Characterize the effect of 3 h of sleep deprivation on the potentiation of 5-HT output in the SCN, in the presence or absence of photic influence.

Increased activity (locomotor activity) is associated with transient increases in the activity of the serotonergic system (Wilkinson et al., 1991; Rueter and Jacobs, 1996; Mendlin et al., 1996; Dudley et al., 1998). It has also been shown that the treatment of
sleep deprivation (arousal) reversibly stimulates serotonergic neuronal activity in multiple brain regions (Borbely et al., 1980; Asikainen et al., 1995; Gardner et al., 1997). It is therefore hypothesized that 3 h of sleep deprivation will significantly increase output of 5-HT in the region of the SCN. Such a finding would constitute the first direct in vivo evidence that sleep deprivation promotes the release of 5-HT in the SCN. In addition, it will be critical to attempt to block the sleep deprivation-induced release of 5-HT in the SCN by application of a 5-HT antagonist which has been shown to attenuate the increased output of 5-HT in the SCN associated with electrical stimulation of the DRN, as well as reducing basal levels of 5-HT release in the SCN (Glass et al., 2000). It is hypothesized that a localized microinjection of metergoline, a 5-HT$_{1,2,7}$ antagonist in the DRN will significantly reduce the increased serotonin output associated with the treatment of sleep deprivation.

**Aim 2. Role of the DRN in mediating locomotor-induced phase-shifts during midday.** Define the roles of 5-HT$_7$ and GABA$_A$ receptors in regulating DRN-mediated behavioral effects in the circadian system.

It has been shown that novel wheel running significantly increases serotonin output in the SCN (Dudley et al., 1999), and that 5-HT agonists administered in the SCN during the midday induce phase-advances in activity (Ehlen et al., 2001). Since intra-DRN microinjections of 5-HT antagonists have been shown to attenuate the increased release of 5-HT in the SCN associated with electrical stimulation of the DRN, as well as reducing basal levels of 5-HT release in the SCN (Glass et al., 2000), it is therefore hypothesized that intra-raphe microinjections of a 5-HT antagonist will reduce the output of 5-HT in the SCN associated with novel wheel running and, due to this decreased
serotonergic signaling, attenuate phase-advances associated with this treatment. It has been reported that GABA inhibits DRN activity (Gallager and Aghajanian, 1976). In addition, intra-DRN injections of the GABA<sub>A</sub> agonist muscimol reduce basal levels of 5-HT in the SCN of hamsters (Glass et al., 2003). It is, therefore, hypothesized that muscimol microinjections targeting the DRN will suppress DRN neuronal activity associated with locomotor activity (Gardner et al., 1997), thereby reducing 5-HT output in the SCN and ultimately attenuating phase-advances.

**Aim 3. Regulation of 5-HT release in the IGL region.** Characterize the circadian profile of, and effects of behavioral and DRN stimulation on, *in vivo* release of 5-HT in the IGL region.

3A. Characterize the daily profile of *in vivo* release of 5-HT in the IGL region under a 14:10 LD cycle in freely behaving animals.

While it has been shown that the IGL receives afferent projections from the DRN of the midbrain (Vrang et al., 2003), the daily rhythm of 5-HT release in the IGL has not been characterized. This study seeks to directly measure 5-HT output in this region over a 24 h period, as measured using *in vivo* microdialysis. It is hypothesized that during the dark phase of animals maintained under a 14:10 LD photoperiod, 5-HT output will be significantly higher vs. day-time levels. This is a pattern seen in other brain regions, including the SCN (Dudley et al., 1998).

3B. Effect of activity on 5-HT output in the IGL region.

This portion of the study seeks to determine if locomotor activity induced by exposure to a running wheel directly alters serotonergic activity in the IGL. Behavioral activity has been shown to be coupled with increases in the activity of the serotonergic
system (Wilkinson et al., 1991; Mendlin et al., 1996; Rueter and Jacobs, 1996; Dudley et al., 1998). It is hypothesized that 3 h exposure to novel wheels will induce significant increases in 5-HT output in the IGL.

3C. DRN regulation of 5-HT output in the IGL region.

This study will test the functionality of the serotonergic connection between the DRN and the IGL by electrical stimulation of the DRN. This study is based on the observation that electrical stimulation of the DRN or MRN increases 5-HT levels in multiple brain regions, including the SCN (Dudley et al., 1999) and that the IGL is innervated by serotonergic fibers of the DRN of the mid-brain (Vrang et al., 2003). It is hypothesized that 5-HT output in the IGL will be transiently increased after electrical stimulation of the DRN (20 min).

Aim 4. Serotonergic stimulation of neuronal activity in the intergeniculate region.

Analyze the effect of serotonergic stimulation on neuronal Fos expression in the IGL.

It has been shown that a variety of behavioral manipulations administered midday induce increased Fos expression in the IGL of hamsters and rats (Janik and Mrosovsky, 1992; Edelstein and Amir, 1995; Janik et al., 1995; Mikkelsen et al., 1998; Antle and Mistlberger, 2000). It is proposed that serotonin is a prime candidate for the non-photic-induced Fos expression, since increased arousal/activity is associated with increased 5-HT output in multiple brain regions including the SCN, and there exists a dense plexus of serotonergic fibers in the region of the IGL. It is hypothesized that application of the 5-HT$_{1A/7}$ agonist 8-OH-DPAT during the midday will significantly increase Fos expression in the IGL as compared to vehicle treated animals. In addition, this study will characterize the relationship between Fos expressing cells and NPY.
neurons in the IGL. It was found that in animals that received non-photic phase-shifting manipulations delivered during the midday, Fos co-localized with NPY (Janik et al., 1995), possibly indicating that 5-HT is responsible for the activation of NPY signaling to the SCN. It is therefore hypothesized that in the IGL of animals receiving i.p injections of 8-OH-DPAT, the expression of Fos and NPY will exhibit a high degree of co-localization.

**Aim 5. The role of the intergeniculate leaflet in non-photic circadian clock resetting.** Analyze the effect of serotonergic stimulation in the IGL, and the SCN on activity rhythms.

5A. The coordinated influence of the IGL and SCN in phase-shifts.

This experiment will involve unilateral dual microinjections of pharmacological agents in the SCN and IGL to test the effects of stimulation or inhibition of these two regions on phase-advances during the midday. Serotonergic and or GABAergic compounds will be administered simultaneously to the contralateral nucleus to better understand how these two areas work in conjunction to produce a phase response. It is hypothesized that the agonists of these compounds will work synergistically in the IGL and SCN to promote shifts, while the antagonists will produce the opposite effect, attenuating phase-shifts.

5B. Effects of blocking serotonergic signaling in the intergeniculate leaflet on behaviorally-induced phase-shifts.

This experiment is designed to test the ability of microinjections of a 5-HT antagonist, bilaterally targeted in the IGL to attenuate behavioral-induced phase-resetting. The animals will be exposed to the behavioral manipulation of sleep deprivation, which is effective for inducing phase advances, as well as promoting 5-HT
release (Grossman et al., 2000). Though 5-HT release in the IGL has not been examined with this treatment, another behavioral manipulation, wheel running, has shown to induce significant 5-HT signaling (Grossman et al., 2004). It is hypothesized that the pretreatment of the 5-HT antagonist metergoline will reduce 5-HT signaling in the IGL and block or attenuate phase advances associated with this treatment.

5C. Phase-shifting activity of 5-HT in the intergeniculate leaflet.

Here, a 5-HT agonist will be targeted bilaterally in the IGL region, in order to determine if 5-HT signaling in this region is sufficient to induce phase-advances. Animals will receive the 5-HT$_{1A/7}$ receptor agonist 8-OH-DPAT, or vehicle, over the course of 3 h by reverse microdialysis. It is hypothesized that the group exposed to the agonist will have significantly larger phase-advances as compared to the control group.

Aim 6. Serotonergic regulation of the circadian clock at the molecular level.

Examine the effects of localized serotonergic stimulation on Per1 and Per2 mRNA levels in the SCN.

This experiment was undertaken to further test the ability of 5-HT to directly alter the phase of the clock. Decreases of midday levels of mRNA transcripts of the clock-coupled genes Per1 and Per2, which exhibit a peak at this time have been associated with a variety of non-photic stimuli that advance the phase of the clock, as well as the respective detectable out rhythms (Maywood and Mrosovsky, 1999; Horikawa et al., 2000; Fukahara et al., 2001). Thus, a reduction in levels of mRNA in the SCN can be used as a marker for non-photic-induced alterations of the clock at this time point. It has been previously been shown that the 5-HT$_{1A/7}$ agonist 8-OH-DPAT significantly reduces Per1 and Per2 mRNA levels in the SCN when administered via i.p. injection (Horikawa
et al., 2000; Duncan et al., 2005). Since direct in vivo application of 5-HT or 5-HT agonists in the SCN, delivered over several hours during the midday, in the absence of photic signaling, induces phase-advances of locomotor activity onset (Ehlen et al., 2001), it is hypothesized that a targeted in vivo application of the 5HT$^{1A/7}$ receptor agonist 8-OH-DPAT applied under similar conditions, will significantly reduce Per1 and Per2 mRNA levels in the SCN of freely behaving hamsters.
CHAPTER II

MATERIALS AND METHODS

Animals

Adult male Syrian hamsters (*Mesocricetus auratus*) aged 8-10 weeks, raised from breeder pairs obtained from Harlan Sprague-Dawley (Madison, IL), were used in these studies. Animals were maintained under a 14L:10D photoperiod (LD; ~250 lux) in a climate-controlled vivarium (20-22\(^{\circ}\)C), individually housed and provided with food (Prolab 3000, PMI Feeds; St. Louis, Mo) and water *ad libitum*. All experiments were performed under the NIH guidelines for Care and Use of laboratory animals, as well as complying with Kent State University’s care and use protocols.

Activity Measurements and Analysis of Phase-shifts

Circadian wheel running activity. Animals were confined to polycarbonate cages with access to a running wheel (Nalge, 14” diameter, 1.5 kg). Wheel revolutions were recorded via magnetic switches interfaced with a data acquisition system (Dataquest III; Minimitter, Sunriver, OR). A modified Aschoff II procedure (Aschoff, 1965) was used to ascertain circadian phase-resetting. Animals were placed into LD until the time point of the experiment (ZT 6), whereby they were released into constant darkness (DD) for the remainder of the experiment. Activity was collected in 10 min bins, and activity onset was characterized by the initial 10 min period where the number of revolutions exceeded 50% of the maximum revolutions of that day, that continued for at least a 60 min period. Phase-shifts in wheel running were determined as the difference between the average
onset activity time for 5 days prior to the experiment and the predicted onsets from least squares regression lines of activity onset from day 3 to day 10 post experimentation.

**General circadian locomotor activity.** In the last two experiments animals were placed in square polycarbonate cages with individual infrared sensors located directly above the cage tops. These sensors were directly linked to the same acquisition system as the previous groups, and general activity was collected, measured and analyzed by the same methods.

**Surgical Technique**

All animals that received implants were placed under sodium pentobarbital anesthesia. The head was shaved and betadine was applied to the surface of the skin. After the initial incision was made to access the skull surface, Marvicane was applied to the site to act as a post-surgical analgesic. The animals were placed on a standard rodent stereotaxic frame, with the head leveled. All implants where anchored in place by 3 stainless screws inserted into pilot holes drilled into the skull. The screws and implants were then locked into place with dental acrylic. Coordinates for the implants varied due to location and nature of the experiment. Specific coordinates for each experiment are listed in the experimental protocols of each respective aim. All coordinates reported were in respect to the confluence of bregma and mid-sagittal sutures.

**Microdialysis**

Construction of microdialysis probes was performed at Kent State University using the same procedures outlined in previous studies (Dudley et al. 1998).
glass tubes (Polymicro Technologies; Phoenix, AZ) were inserted into 26-gauge outer cannulas (Small parts; Miami, FL). Hemicellulose dialysis tubing (12 kDa MW cutoff; 230 um OD; Spectra-por [Fisher Scientific, Pittsburgh, Pa]) was inserted into the cannula and secured with epoxy, such that it extended from the cannula with an active dialyzing length of 1 mm. The end of the tubing was capped with a drop of epoxy. Filtered artificial spinal fluid (ACSF; NaCl, 147 mM; KCl, 4.0 mM, CaCl2, 1.8 mM; pH=7.2) was continually perfused at a rate of 1.2 µL/min via a syringe pump (CMA/100; Bioanalytical Systems Inc.; West Lafayette, IN), which was attached by line to a liquid swivel (Instech; Plymouth Meeting Pa), which provided the animal with a complete range of motion within the cage. Citalipram (4 µM) was added to the perfusate for those experiments measuring extracellular 5-HT. Dialysis samples were collected at either 20 or 60 min intervals and stored at 5°C for HPLC analysis. In the experiment utilizing reverse in vivo microdialysis, perfusate was simply collected and discarded after the termination of perfusion.

Re-entry Cannula

In experiments 5B and 6, animals received re-entry cannulas during surgery (~6mm in length) that were constructed from 30-gauge blood sample collection needles (Vaccutaner). The vial piercing side was removed and ground flush with the plastic body. To this end a polyethylene sleeve was attached, in which a microdialysis probe could be inserted post surgery and prior to experimentation. The probe was secured with a plastic wire tie, acting as a collar. When inserted, the probe tip extended 3mm from the bottom of the cannula. A dummy cannula (26 gauge wire stylus) was inserted
and locked in place by wire tie Immediately following surgery. This stylus extended 1
mm past the re-entry cannula in order to prevent blockage before probe insertion.

Microinjections

In experiments utilizing microinjections, 24 gauge stainless steel guide cannulas
(Plastics One, Roanoke, VA) were implanted during surgery. A 31 gauge stylet was
inserted into the micro-injection cannula to prevent blockage. The tip extended 1 mm
beyond the bottom of the cannula. Injections were performed by the use of a 31 gauge
injection needle attached by polyethylene tubing to a Hamilton syringe. The stylet was
placed back into the cannula after each injection and the animals were returned to their
cages.

HPLC Analysis

Samples collected from the microdialysis studies were analyzed by HPLC (high
performance liquid chromatography; Bionanalytical Systems, Inc.) with amperometric
detection. Microdialysate was injected onto a 100 mm x 1 mm 3 µ C-18 reverse-phase
microbore column. The mobile phase consisted of 9.45g monochloroacetic acid (Fisher
Scientific; Pittsburgh, PA), 0.2 g octanesulfonic acid, and 0.25 g EDTA (Eastman Kodak
Co.; Rochester, NY) dissolved in 1 L HPLC-grade deionized water (pH=3.1).
Tetrahydrofuran (6.0 mL) was added after filtration. Flow rate of the mobile phase
through the column was 90 um/min. A 3.0 mm glassy carbon radial-flow electrochemical
detector (Bioanalytical Systems, Inc.) set at a potential of 590 mV relative to an AgCl
reference electrode was used to measure 5-HT. The lower sensitivity (signal >5x
background) was approximately 500 fg. Electrode output was interfaced with an IBM-
compatible computer which recorded and analyzed the data. Authenticity of the 5-HT
peak was verified by predictable changes in the size following electrical and pharmacological stimulations of the raphe and localized administration of pharmacological agents to the SCN via dialysis probe (Dudley et al 1998).

In Situ-Hybridization

In situ hybridization was performed in the laboratory of Dr. Marylin Duncan (Department of Anatomy and Neurobiology at the University of Kentucky Medical Center, Lexington, Ky). Experimental protocols were as follows: Coronal sections (12 mM) through the SCN were prepared with a cryostat, mounted onto positively-charged slides, and stored at –80°C. Immediately before in situ hybridization, the slides were equilibrated to room temperature, fixed for 5 min in 4% PFA in 0.1M PBS (pH 7.4), and rinsed in PBS (2 min). Then the sections were acetylated in triethanolamine (0.1 M, pH 8.0) and 0.25% acetic anhydride (10 min at room temperature), dehydrated, delipidated and air-dried.

Partial cDNA sequences of hamster Per1 (nucleotides 726-1367) and hamster Per2 (nucleotides 822-1601) in pGEM-T Easy Vector (Promega, Madison, WI) were generously provided by Dr. H. Okimura (Kobe University, Kobe, Japan) and Dr. S. Shibata (Waseda University, Saitama, Japan), respectively. Bacteria were transformed with these plasmids and DNA was purified for c-RNA synthesis. (The characteristics of these clones and their utility for in situ hybridization have been published previously [Horikawa et al, 2000]).

The sections were hybridized overnight at 55°C with one of the 35S-labeled riboprobes (0.5 x 10^6 cpm/section) diluted in hybridization cocktail containing yeast tRNA (250mg/ml), Tris-HCl (20 mM, pH 7.4) EDTA (1 mM), NaCl (300 mM), deionized
formamide (50% v/v), dextran sulphate (10% v/v), Denhardt’s solution (1x), and dithiothreitol (DTT, 100 mM). After 16 hours, sections were rinsed twice for 10 min each in 2xSSC (1xSSC=0.25M NaCl, 0.015M sodium citrate, pH 7.2) containing 10 mM DTT at 22°C, treated with RNAse (20 mg/ml) for 30 min at 37°C, washed for 15 min in 1xSSC at room temperature, and washed twice in 0.1xSSC (30 min ea at 63°C). Finally, the sections were rinsed in 0.1xSSC at 22°C, quickly dehydrated and air-dried. All solutions, except Tris buffer, were prepared with diethyl pyrocarbonate-treated water (0.1%) and autoclaved. Slides were exposed to X-ray film (Kodak Bio-max MR) for 1-2 weeks.

The hybridization signal of the film autoradiograms was assessed by semi-quantitative analysis using computer-assisted microdensitometry. The hybridization signal, defined as the difference between the relative optical density over the SCN and an adjacent brain region, was averaged for 4 replicates for each animal.

Experimental Protocols

**Experiment 1. The effect of behavioral arousal on 5-HT release in the SCN.**

Animals were anesthetized and received a unilateral microdialysis probe implant stereotaxically aimed at the lateral margin of the SCN (AP=0.4mm; ML=0.4mm; and -8.0mm from dura) one day prior to experimentation. On the day of each experiment, perfusion began in the animal’s home cage at ZT 2 for probe equilibration (n=12). Baseline samples were collected from ZT3 to ZT6. Visual inspection confirmed that animals slept through the baseline period. Animals were kept awake for three hours from ZT6 to ZT9 by gentle handling and light puffs of air. During the sleep deprivation treatment period animals were released into dim red light (<1.0 lux) for the remainder of the experiment. Animals were left undisturbed from ZT9 to ZT14 while collection of
microdialysis samples continued. After 2 days animals were placed in one of two groups (n=6/group). Animals within the first of these 2 groups received the same prior treatment, but underwent sleep deprivation under normal chamber lighting conditions. In the second group, animals received the same microdialysis procedure, but did not undergo sleep deprivation (control group). Implant locations were determined histologically after completion of experimentation. Microdialysates were analyzed for serotonin using HPLC. Data was normalized as a percentage of baseline mean. Treatment effects were determined by a one-way ANOVA followed by a Dunnett’s test. The level of significance was set at $p < 0.05$.

In a separate experiment, animals received a unilateral microdialysis probe implant stereotaxically aimed at the lateral margin of the SCN (AP=0.4mm; ML=0.4mm; and -8.0mm from dura) and a micro-injection cannula aimed at the DRN (AP=-4.7mm, ML=1.7mm; and -4.8mm from dura at a 20° angle). On the day of the experiment, perfusion began in the animal’s home cage at ZT 4 for probe equilibration. Baseline samples were collected from ZT5 to ZT6. Visual inspection confirmed that animals slept through the baseline period. Animals were kept awake for three hours from ZT6 to ZT9. Animals received either a micro-injection of the 5-HT$_{1,2,7}$ antagonist metergoline (2µg, 1µL volume, Sigma, St. Louis MO; n=5) or vehicle (1:1 ACSF/DMSO, 1 µL volume; n=5) just prior to ZT6, and again halfway through the treatment period at ZT 7.5. During the sleep deprivation treatment period, animals were released into dim red light (<1.0 lux) for the remainder of the experiment. Animals were left undisturbed from ZT9 to ZT 10 while collection of microdialysis sampling continued. Implant locations were determined histologically after completion of experimentation. Treatment effects were determined by Dunnett’s test. The level of significance was set at $p < 0.05$. 

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**Experiment 2.** Role of the DRN in mediating locomotor-induced phase-shifts during midday. Animals were anesthetized and received a microinjection cannula implant stereotaxically aimed at the DRN (AP=-4.7mm, ML=1.7mm: and -4.8mm from dura at a $20^\circ$ angle) and placed into cages with wheels about two weeks prior to experimentation. On the day of the experiment, chamber lights were turned off at ZT 6 and were kept off for the remainder of the experiment. Animals received either the GABA$_A$ agonist muscimol (25 ng, 500 nL volume, Sigma: n=10), the selective 5-HT$_7$ antagonist DR4004 (2 µg, 1 µL volume, Meiji Seika Kaisha, Yokohama, Japan: n=13) or vehicle (1:1 ACSF/DMSO, 1 µL volume; n=14) injections under dim red light (< 1 lux). Animals were then transferred from home cages to novel wheels. Novel wheel running activity was recorded by the same system. Chamber lights were turned off beginning at ZT6 placing animals in DD for the remainder of experiment. All groups received a second injection at ZT 7.5. At ZT 9 animals were returned to their cages for 10 days post treatment. Phase-advances were analyzed between groups of animals that ran at or exceeded the revolution range known to induce maximal shifting using a one-way ANOVA followed by the Student-Newman-Keuls test. The level of significance was set at $p<0.05$. Implant locations were determined histologically after completion of experimentation.

**Experiment 3.** Regulation of 5-HT release in the IGL region. Animals were anesthetized and received a unilateral microdialysis probe implant (1 mm tip; 12 kDa mol. wt cutoff, 230 µm o.d.) stereotaxically aimed at the medial margin of the IGL (AP=-0.5mm, ML=0.3mm: and -5.5mm from skull surface) (Fig. 8). In animals that received electrical dorsal raphe nucleus stimulation, a bipolar electrode (Plastics One, Roanoke,
VA) was implanted in the DRN (AP=-4.7mm, ML=1.7mm: and -4.8mm from dura at a 20° angle). Microdialysis was performed by continuously perfusing artificial cerebrospinal fluid (ACSF; flow rate 1.2 µL/min) through the probe. Implant locations were determined histologically after completion of experimentation. Microdialysate was analyzed for 5-HT using HPLC.

On the day of each experiment, perfusion began in home cages at ZT 3 for probe equilibration. For the 24 h profile experiment (n=4), hourly sample collections began at ZT 5 and were collected over a continuous 24 h period under LD. Baseline sample collection began at ZT 5 (n=5) for the electrical dorsal raphe nucleus stimulation and novel-wheel trials. Animals in the novel wheel group were transferred from their home cage and confined to a large running wheel at ZT 6 (n=6). At this time the animals were released into darkness for the remainder of the experiment. At ZT 9, animals were placed back into their home cages until the completion of sample collection at ZT 10. Animals used in the electrical dorsal raphe nucleus stimulation trials were kept in their home cages throughout experimentation. At ZT 6 a 20 min pulse of constant current (150 µA, 10 Hz stimulus frequency and 2.0 msec pulse duration) was delivered via a bipolar electrode from a stimulus isolator (World Precision Instruments, Sarasota, FLA) coupled to a Grass S11 stimulator.

**Experiment 4. Serotonergic stimulation of neuronal activity in the intergeniculate leaflet.** Fos analysis: Adult (8-10 weeks of age) female Syrian hamsters were used in these studies. On the day of the experiment, animals received either vehicle (DMSO; n=4) or 8-OH-DPAT (5mg/Kg, in 5mg/ml DMSO; n=4) i.p. injection at ZT 6. At this time animals were returned to their home cages and the lights were turned off thereafter.
One hour after injections, animals received a lethal dose of sodium pentobarbital, and 40 µm coronal brain slices containing the IGL were stained for Fos or polysialic acid (PSA) using standard immunocytochemical DAB staining procedures. Alternating sections were transferred to well plates to be immunologically stained for PSA or Fos. Brightfield photomicrographs were obtained by digital camera. The IGL of alternating Fos or PSA slices were aligned in Photoshop using the PSA-stained outline to map the IGL’s borders on the Fos sections. The IGL region was isolated and placed within Image Pro Plus for cell count analysis. Intensity range selection was used to determine Fos immunoreactive-cell counts. Treatment effects were determined by student t-test, with the level of significance set at $p < 0.05$.

**Experiment 5.** The role of the intergeniculate leaflet in non-photic circadian clock resetting. **Experiment 5A.** Animals were individually housed in cages with free access to running wheels and provided with food and water *ad libitum*. About two weeks prior to experimentation, animals were anesthetized and received a micro-injection cannula stereotaxically aimed at the IGL (AP=-0.5.mm, ML=0.3mm: and -5.5.mm from skull surface) and another cannula targeting the contralateral SCN (AP=0.4mm; ML=-0.4mm; and -8.0mm from dura). On the day of the experiment animals received microinjections of vehicle (1:1 ACSF/DMSO, 1 µL volume), the 5-HT$_{1,2,7}$ antagonist metergoline (2 µg, 1 µl volume; Sigma, St. Louis, MO), the GABA$_{A}$ agonist muscimol (25 ng, 500 nl volume, Sigma, St. Louis, MO), the GABA$_{A}$ antagonist bicuculline (125 ng, 500 nL volume) or the 5-HT$_{1A/7}$ agonist 8-OH-DPAT (5 mg/Kg, in 5 mg/mL DMSO), as per group. Animals were placed back into their home cages for the remainder of the experiment. Animals’ shifts were assessed as in previous studies and histology.
confirmed the location of the cannulas. Comparison of groups was analyzed using a one-way ANOVA followed by the Tukey/Kramer test. The level of significance was set at $p<0.05$.

**Experiment 5B.** Animals were housed in cages with infrared sensors and provided with food and water *ad libitum*. About two weeks prior to experimentation, animals were anesthetized and received bilateral micro-injection cannulas stereotaxically aimed at the IGLs (AP=-1.4mm; ML=+/−0.4mm; and -5.0mm from skull surface at a 10° angle). On the day of the experiment, subjects received either metergoline (2 µg, 1µL volume; Sigma, St. Louis, MO; n=4) or vehicle (1:1 ACSF/DMSO, 1 µL volume; n=4) microinjections at ZT6 in their home cages. Animals were then kept awake for 1 h from ZT6 to ZT7 by gentle handling and light puffs of air. Chamber lights were turned off beginning at ZT6 placing animals in DD for the entirety of experiment. Both groups received a second injection at ZT 7.5. At ZT 9 animals were returned to their cages for 7-10 days post treatment. Treatments were analyzed using a paired student t-test. The level of significance was set at $p<0.05$. Implant locations were determined histologically after completion of experimentation.

**Experiment 5C.** Animals were housed in cages with infrared sensors and provided with food and water *ad libitum*. About two weeks prior to experimentation, animals were anesthetized and received bilateral re-entry cannulas stereotaxically aimed at the IGLs (AP=0.4mm; ML=+/−.4mm; and -5.0mm from skull surface at a 10° angle). On the day prior to testing animals received microdialysis probes, for probe hydration. On the day of the experiment animals received either 3 h perfusion of vehicle (n=4) or 8-OH-DPAT (dissolved in 1 mL DMSO then in ACSF perfusate to obtain a 1.2 mM
concentration, yielding a ~35 µM concentration outside the dialysis membrane: n=5) from ZT6-ZT9. Chamber lights were turned off beginning at ZT6 placing animals in DD for the entirety of experiment. At ZT 9 the probes were be removed. Comparison of phase-advances was analyzed using a paired student t-test. The level of significance was set at \( p < 0.05 \). Implant locations were determined histologically after completion of experimentation.

**Experiment 6. Explore serotonin’s ability to shift the clock at the molecular level.**

Animals were housed at 22°C in a climate-controlled vivarium under a 14:10 LD cycle, in rectangular polycarbonate cages. Under anesthesia, animals received a guide cannula stereotaxically aimed such that when the dialysing membrane of the probe was inserted, it was positioned at the lateral border of the midpoint of the rostrocaudal axis of the SCN (AP=0.4mm; ML=0.4mm; and -5.0mm from dura; 3mm probe extension from cannula, with level head). Guide cannulae were anchored by three stainless screws inserted into pilot holes drilled in the skull. A 26 ga. wire stylus (1mm extension from cannula) was inserted in the reentry cannula to prevent blockage before and after probe insertion. Following surgery, animals were maintained on 14:10 LD cycle in clear plastic cages. Animals received two treatments of \( p \)-CPA by i.p. injection (150mg/kg; 75 mg/mL; in Tris buffered ACSF). One treatment was given four days prior to perfusion and the second one day prior to perfusion at the time of microdialysis probe insertion. On the day of experimentation 10-15 min prior to ZT 5, dialysis probes were connected to a syringe pump (CMA/100; Bioanalytical Systems, West Lafayette, IN) by means of a single channel liquid swivel (Instech; Plymouth Meeting, PA) mounted over the cage. Perfusions were performed for 3 h beginning at ZT 5, with artificial cerebral spinal fluid
(ACSF; NaCl, 147 mM; KCl, 4.0 mM, CaCl$_2$, 1.8 mM) containing 8-OH-DPAT (dissolved in 1 ml DMSO then in ACSF perfusate to obtain a 1.2 mM concentration, yielding a ~35 µM concentration outside the dialysis membrane) or vehicle, at a rate of 1.2 µl/min. Animals were released into DD within 10 min of the start of perfusion, for the remainder of the study. At ZT 8, animals were sacrificed (Nembutal, i.p. injection) in the dark. Brains were immediately extracted and put onto dry ice, dorsal side down. Powdered dry ice was sprinkled over the ventral surface to initiate rapid freezing of the hypothalamus. After several minutes the brains were covered in parafilm, to prevent desiccation, and transferred to –70ºC.

The samples were then packed with dry ice and shipped to the laboratory of Dr. Marylin Duncan (Department of Anatomy and Neurobiology at the University of Kentucky Medical Center, Lexington, Ky), where in situ hybridization was performed. Treatment effects were analyzed by paired Student t-test. The level of significance was set at $p<0.05$. 

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CHAPTER III

RESULTS

Experiment 1. The Effect of Behavioral Arousal on 5-HT Release in the SCN

Imposed 3 h sleep deprivation in dim red light (< 1 lux) during the midday used in this study produced a maximal 170±10% increase in 5-HT release as compared to baseline levels (Fig. 1). An average increase of 158±5% in 5-HT levels over baseline was observed during the 3 h period of sleep deprivation (Fig. 2). 5-HT output returned to baseline levels within 40 min after the cessation of treatment. In animals that received the same treatment, but under normal lighting (~500 lux), there was a maximal increase of 149±12% as compared to baseline levels (Fig. 1B). The average increase during the 3 h treatment period was 149±12% (Fig. 2). In the control group, 5-HT levels did not show an increase during the treatment time under dim red light (Fig. 1C & 2). In all groups 5-HT levels increased approaching the time of activity onset.

A separate experiment was conducted, which attempted to block the sleep deprivation-induced release of 5-HT in the SCN. In those animals that received vehicle microinjections during 3 h of sleep deprivation, 5-HT levels rose to a maximal 151±10% increase over the pretreatment period (Fig. 3). The average increase in 5-HT levels during this period was 134±3% vs. baseline levels (Fig. 4). In the group that received the 5HT1,2,7 antagonist metergoline, the increase in 5-HT output associated with sleep deprivation was abolished (Fig. 3). The average change in 5-HT measured during the
Figure 1. The effect of sleep deprivation from ZT6-9 (broken lines) on the release of 5-HT in the SCN under dim red light (A; n=12) or bright light (B; n=6). A lack of increase in 5-HT release was observed in the non-sleep-deprived control group (C; n=6). Shaded horizontal bars signify the period of exposure to dim red light. *p < 0.05 vs. averaged pretreatment baseline release.
Figure 2. Graphical representation of the mean of 5-HT release in the SCN during the 3 h sleep deprivation treatment (ZT6-9) designated by open bars, and the 3 h pretreatment period (ZT3-6) designated by filled bars. *p < 0.05 vs. pretreatment period; *p < 0.05 vs. non-sleep-deprived controls.
Figure 3. The suppressive effect of intra-DRN microinjection of metergoline on 5-HT release in the SCN evoked by 3 h of sleep deprivation from ZT6-9. 5-HT levels increased during treatment, with a maximal output ~150% of baseline, that was equivalent to previous reported experiments. The metergoline treatment abolished the induced increase in release (n=5); a$p < 0.05$ vs. pretreatment period; *$p < 0.05$ vs. vehicle controls.
treatment period in this group was -17±9% (Fig. 4). A two-way ANOVA revealed a significant difference the metergoline and control group (p<0.01).

Experiment 2. Role of the DRN in Mediating Locomotor-induced Phase-shifts During Midday

Animals were placed into three groups to investigate the role of the DRN in locomotor-induced circadian phase resetting. Each group was subjected to 3 h in a novel running wheel during the midday. Animals exhibited a wide range of revolutions during the experimental period (Fig. 5). Shifts in onset of activity were then recorded after the treatments. Animals received an intra-DRN microinjection of either vehicle, the GABA_A agonist muscimol, or the selective 5-HT_7 antagonist DR4004 (Fig. 5). Under the control conditions, animals that ran at or over 400 revolutions exhibited significant and maximal phase-advances (136±23 min; n=4). Thus, running above 400 revolutions over the 3 h treatment period was considered an index for inducing maximal phase alterations. In this range of revolutions during the treatment period, the group that received the muscimol treatment exhibited significantly attenuated phase-advances as compared to the control group (46±22 min; n=4; p <0.05 vs controls) (Fig. 6). The group that received the DR4004 treatment also exhibited significantly attenuated phase-advances as compared to the control group (31±15 min; n=3; p <0.05 vs controls) (Fig. 6). Actograms representing the three treatment groups are shown in Fig. 7. The mean revolutions of running for controls was not significantly different from the drug groups in this range (control, 630±93; muscimol, 628±51; DR4004, 612±106; p <0.98) (Fig. 6).
Figure 4. Graphical representation of integrated 5-HT release in the SCN during the 3 h sleep deprivation treatment. Note the decrease in 5-HT release in the metergoline treated group. *p < 0.05 vs. pretreatment period; *p < 0.05 vs. vehicle controls (n=5).
**Figure 5.** Relationship between the number of wheel revolutions during the 3 h novel wheel exposure and induced phase-advance shifts in animals that received intra-DRN injection of vehicle (VEH), muscimol (MUSC), or DR4004.
Figure 6. Effects of intra-DRN muscimol, vehicle or DR4004 injection on novel wheel-induced phase-resetting. Top: phase-advance shifts for animals that ran at or exceeded the 400 revolution index range for maximal shifting response. Bottom: number of wheel revolutions for groups of animals represented in the top. Vehicle, n=4; muscimol, n=4; Dr4004, n=3. For each graph, bars with different letters are significant different. Data are mean±SEM.
Figure 7. Double-plotted wheel-running activity records showing representative profiles from the three groups. Asterisks represent onset of novel wheel exposure. A,B, vehicle. C,D, muscimol. E,F, DR4004.
Experiment 3. Regulation of 5-HT Release in the IGL Region

**Experiment 3A.** Daily profile of *in vivo* release of 5-HT in the IGL region.

Microdialysis sampling of 5-HT from the region of the thalamus near the medial margin of the IGL (Fig. 8) revealed a diurnal pattern of release, with a nadir (70.8±1.5% of daily mean) that occurred during the midday (ZT 7), and a peak (140.5±23.7% of daily mean) close to the time of lights off (ZT 13). This peak period constituted a 70% increase in 5-HT output as compared the nadir (*p* <0.05) (Fig. 9). During the dark phase, 5-HT levels showed a significant increase as compared to the light phase (116.7±4.1% vs. 86.7±3.3% of daily mean, respectively; *p* <0.01) (Fig. 10).

**Experiment 3B.** Effect of activity on 5-HT output in the IGL region. Significant increases in 5-HT release were recorded in animals exposed to 3 h of novelty-induced wheel running (Fig. 11). This exposure produced an averaged 44.3±6.0% increase in 5-HT release over baseline levels (*p* <0.05). The peak in this output occurred midway through the exposure period (168.0±15.5% over baseline). Within 1 h of removal from the running wheel, 5-HT output levels returned to baseline. A strong correlation was not noted between the increase in 5-HT output over the 3 h treatment period, and total number of wheel revolutions (*r*=0.43; *p* < 0.2).

**Experiment 3C.** DRN regulation of 5-HT output in the IGL region. A 20 min duration of DRN electrical stimulation elicited an immediate peak in 5-HT output (206± of baseline; *p* <0.05) (Fig. 11). Output of 5-HT levels fell to baseline within 80 min after the cessation of electrical stimulation.
Figure 8. Diagrammatic coronal brain section showing the target location for microdialysis sampling of 5-HT from the IGL region. Probes were aimed at the medial margin of the rostral IGL. Data collected from probes located > 500 µm from this location were excluded from the analysis.
Figure 9. The 24 h profile of in vivo 5-HT release in the IGL region assessed under LD. Solid horizontal bar denotes the dark phase. Each point represents the mean±SEM (n=4). *p<0.05 versus averaged daytime release.
Figure 10. Bar chart depicting the integrated levels of 5-HT release during the light phase and dark phase of the 24 h sampling. *p < 0.05 vs. light phase.
Figure 11. Stimulation of 5-HT release in the IGL region by novel wheel exposure from ZT6-9 and by electrical stimulation of the dorsal raphe nucleus for 20 min at ZT 6. Horizontal bars denote the periods of wheel exposure and dorsal raphe nucleus stimulation. For both manipulations, *p < 0.05 vs. pre-treatment baseline (n=5-6 for both treatments).

Animals that received i.p. 8-OH-DPAT exhibited a significant increase in the number of cells exhibiting Fos-immunoreactivity in the IGL region as compared to those receiving vehicle (9.5±2 vs. 3.7±3.0; \( p < 0.05 \)) (Fig. 12). A low magnification photomicrograph of a coronal section of the thalamus shows the location of the IGL as revealed by immunohistological staining for PSA (Fig. 13). Photomicrographs of isolated IGLs immunohistologically stained for Fos representing the drug and control groups are shown in Fig. 14.

Experiment 5. The Role of the Intergeniculate Leaflet in Non-photic Circadian Clock Resetting.

Experiment 5A. The coordinated influence of the IGL and SCN in phase-shifts. Animals that received microinjections of bicuculline in IGL and metergoline in the SCN (67.38±14.20 min; \( n=5 \)), bicuculline in the IGL and DPAT in the SCN (69.81±13.68 min; \( n=5 \)) and muscimol in the IGL and DPAT in the SCN (66.05±9.49 min; \( n=7 \)) had significantly different phase-shift responses than those treated with muscimol in the IGL and vehicle in the SCN (14.76±4.68 min; \( n=7 \)) (\( p < 0.05 \)). Table 1 shows the mean±SEM of phase-shift and sample size for each treatment group (groups with different letters are significantly different; \( p < 0.05 \)).

Experiment 5B. Effect of blocking serotonergic signaling in the intergeniculate leaflet on behaviorally-induced phase-shifts. The group that received metergoline microinjections exhibited smaller behaviorally-induced phase-advances, as compared to
Figure 12. Graphical representation showing the effect of vehicle (n=4) or 8-OH-DPAT (n=4) i.p. injections on the number of cells expressing Fos-immunoreactivity in the intergeniculate region (*p <0.05).
Figure 13. Photomicrograph of a 40 µm coronal section of the dorsal thalamus immunohistologically stained for PSA. Note the darkly stained rostral IGL.
Figure 14. Photomicrographs of isolated IGLs showing Fos immunoreactive-cells after treatment with vehicle (A) or 8-OH-DPAT (B). Circles denote areas that were significantly denser than background as interpreted by image analysis software.
Table 1. Circadian phase-resetting effects of muscimol (MUS), metergoline (MET), bicuculline (BIC), 8-OH-DPAT (DPAT) or vehicle (VEH) unilateral microinjections in the IGL region and the contra-lateral SCN during midday. Values are means±SEM. Treatment groups with different letters are significantly different; \( p < 0.05 \). Numbers in parentheses indicate sample size for each group.

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<td>Phase-shift (min)</td>
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<td>67.38 ± 14.20</td>
<td>69.82 ± 13.68</td>
<td>66.05 ± 9.49</td>
<td>14.76 ± 4.68</td>
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vehicle controls (18.6±4.86 min, n=4 vs. 56.4±10.71 min, n=4; p < 0.05, Fig. 15). This represents a three fold decrease in the phase-shifting response.

**Experiment 5C.** Phase-shifting activity of 5-HT in the IGL. Animals that received the 3 h of bilateral reverse microdialysis of 8-OH-DPAT had greater phase-advances than vehicle controls (117.92±31.07 min, n=5 vs. 12.00±8.45 min n=4; p < 0.05, Fig. 16). Thus, the 8-OH-DPAT group displayed a 10 fold greater shift response than the control group.

Experiment 6. Serotonergic Regulation of the Circadian Clock at the Molecular Level. 

Semi-quantitive analysis using computer-assisted microdensitometry of the hybridization signal revealed that 3 h perfusion of 8-OH-DPAT significantly reduced hPer1 mRNA (not detectable, <1% of controls) and hPer2 mRNA (40.8±3.6% vs. controls, p < 0.05, Fig. 17). Fig. 18 shows representative autoradiograms of hPer the SCN at ZT8.
Figure 15. Graphical representation of the effects of bilateral microinjections of vehicle (n=4) or the 5-HT antagonist metergoline (n=5) on behaviorally-induced phase-shifts delivered during midday (*p <0.05).
Figure 16. Graphical representation of the phase-shifting effect of bilateral reverse microdialysis perfusion of vehicle (n=4) or 8-OH-DPAT (DPAT) (n=5) in the intergeniculate leaflet region during midday (*p < 0.05).
Figure 17. Bar charts plotted from semi-quantitative analysis using computer-assisted microdensitometry of the hybridization signal. Intra-SCN treatment with 8-OH-DPAT reduced hPer1 mRNA to below detectable levels (ND, non-detectable; <1% of controls) and reduced hPer2 mRNA 40.8±3.6% vs. control; *p <0.05.
Figure 18. Representative autoradiograms of the SCN at ZT 8. 8-OH-DPAT treated rostral SCN exhibited reduction of both hPer1 and hPer2 mRNA hybridization signal (B,D) vs. control (A,C).
CHAPTER IV

DISCUSSION

The Effect of Behavioral Arousal on 5-HT Release in the SCN

The present results constitute the first direct evidence that sleep deprivation can reversibly stimulate the release of 5-HT in the SCN circadian clock. This is significant because it confirms that 5-HT output in this region can be potentiated by a behavioral manipulation that induces general arousal without locomotor activity. Notably, it has been shown that dramatic increases in 5-HT release in the region of the SCN are induced by vigorous bouts of activity during this time of the day (Dudley et al., 1999). However, data from this present study confirm that robust activity is indeed not necessary for the induction of 5-HT release in the clock.

Targeted application of 5-HT or 5-HT agonists to the SCN over the course of 3 h have been shown to significantly phase-shift rhythms in activity when delivered during this portion of the day (Ehlen et al., 2001). This suggests that behavioral stimulation of 5-HT in the SCN could also reset clock phase. In several studies, the action of sleep deprivation induced significant phase-advances in subjects’ activity when delivered in the dark, during midday (Antle and Mistlberger, 2000; Grossman et al., 2000). Thus, it may be the case that sleep deprivation induces shifts in rhythms by increasing 5-HT signaling to the clock, although it is plausible that other agents could contribute to phase-shifting induced by sleep deprivation. For example, it has been shown that NPY levels in the region of the
SCN significantly rise during novel wheel running as detected by mass spectrometric analysis (Glass et al., 2005). Thus, it will be critical in future experiments to ascertain whether general arousal (i.e., sleep deprivation) can cause alterations in NPY signaling. It will also be important to explore the effects of blocking NPY communication to the clock in sleep deprivation-induced phase-shifts.

Sleep deprivation-induced 5-HT increases were not diminished in the presence of light (Fig. 1B), suggesting that the mechanism underlying behaviorally-induced release of 5-HT is independent of photic signaling. It has been shown that sleep deprivation conducted in the presence of light induces shifts that are significantly smaller compared to those induced in the absence of light (Antle and Mistlberger, 2000). This is consistent with findings that behavioral manipulations, or administration of 5-HT agonists, must be delivered in the absence of light to cause phase-shifts (Ehlen et al., 2000). Taken together, these results suggest that the attenuation of phase-shifts by light is not due to pre-synaptic blockage of 5-HT release, but an effect somewhere downstream of release. It is interesting to note however, that the group exposed to light displayed a higher degree in variance of response, as well as greater latency in attaining maximal output levels (Fig. 1A). This phenomenon may be in part due to the hamsters’ behavior when experiencing light during the midday.

A subsequent experiment was performed to further probe the effect of sleep deprivation on 5-HT output in the SCN. Previous experiments conducted in this laboratory have shown that electrical stimulation of the DRN induces significant and transient increases in 5-HT in the SCN region. Such stimulation-induced release of 5-HT is abolished by the simultaneous application of intra-DRN microinjections of 5-HT
antagonists (Glass et al., 2003). The present study revealed that intra-DRN microinjections of the 5-HT_{1,2,7} antagonist metergoline significantly reduced sleep deprivation-induced 5-HT output in the SCN (Figs. 3 & 4). This finding strengthens the case for the proposed multisynaptic raphe pathway to the SCN (Glass et al., 2003).

**DRN Control of Locomotor-induced Phase-shifts During Midday**

This experiment was designed to characterize the role of 5-HT release in the SCN in non-photic-induced phase-shifting. There is evidence that exposure to a novel wheel during the midday can cause significant phase-advances in activity onset (Bobzynska and Mrosovsky, 1998; Meyer-Bernstein and Morin, 1998; Dudley et al., 1998). Sleep deprivation has also been shown to induce similar shifts when delivered during that time of day (Antle and Mistlberger, 2000). The fact that both behavioral treatments induces release of 5-HT in the SCN region (Dudley et al., 1998, experiment 1, Fig. 1), strongly implicates 5-HT in mediating non-photic shifts. Here, novel wheel running was used instead of sleep deprivation to test the effects of serotonergics and other agents over a broader range of phase-resetting response. Using this method, subjects exhibited wide ranges of revolution counts and phase-shifting response (Fig. 5), which is consistent with other studies in which this technique was utilized (Bobzynska and Mrosovsky, 1998; Meyer-Bernstein and Morin, 1998). The selective 5-HT_7 antagonist, DR4004, and the GABA_A agonist, muscimol, were delivered via intra-DRN microinjection at concentrations shown to significantly reduce *in vivo* basal 5-HT levels in the SCN when injected into the DRN (Glass et al., 2003). It was noted that control subjects that ran at or over 400 revolutions exhibited maximal phase-advances (136±23 min). This level of exertion was thus considered as the threshold for inducing maximal
phase-shifts. The group that received intra-DRN muscimol exhibited significantly smaller phase-advances compared to the controls (46±22 min). Animals receiving intra-DRN DR4004 also exhibited small shifts (31±15 min). The mean revolution count for the controls was not significantly different from those of the drug groups (control, 630±93; muscimol, 628±51; DR4004, 612±106). Though these drugs act differently to inhibit neuronal firing of raphe cells, their ultimate effect of reducing or inhibiting serotonergic signaling in the SCN is similar (Glass et al., 2003).

The results of this study are significant in two regards. One is that the drugs did not suppress wheel running, but did significantly reduce the shifts associated with novel wheel exposure. Thus, the pharmacologic suppression of 5-HT release in the SCN per se attenuated the behaviorally-induced phase advances. This finding strongly supports the role of 5-HT signaling in the SCN in non-photic phase-resetting of the clock. Second is that this work is consistent with previous results, suggesting a multisynaptic pathway from the mid-brain raphe complex to the SCN (Glass et al., 2003). It is important to note here that phase-shifting in both drug groups was not completely abolished. Possibly the drug dosage or duration of exposure to the drugs (two pulses delivered by microinjection), were not adequate for the complete block of serotonergic signaling. A more likely explanation, however, is that other factors, such as NPY from the IGL may contribute to the phase-shifting response. This is supported by studies showing that NPY delivered in vivo can shift the clock (Huhman and Albers, 1998), and that novel wheel access increases NPY release in the region of the clock (Glass et al., 2005). An additional possibility is that the suppression of neuronal firing of DRN cells by these
drugs could affect other circadian-related brain regions, including the IGL, which is directly innervated by the DRN (Vrang et al., 2003).

Regulation of 5-HT Release in the IGL Region

The present data are the direct evidence 5-HT signaling in the IGL region in freely-behaving animals, pointing to a functional pathway from the DRN to the IGL. In addition, this study presents the first in vivo characterization of the daily rhythm of 5-HT release in the IGL. Sampling of 5-HT from the region of the thalamus near the margin of the IGL revealed a diurnal pattern of release, with a nadir at midday, and an acrophase close to the time of lights off. During the dark phase, 5-HT levels showed a significant increase as compared to the light phase. It is interesting to note that we observed a significant peak at the dark/light transition. The significance of this peak is not known, but interestingly, bimodal rhythms of in vivo NPY release by the GHT occurring at the same time points (light/dark & dark/light) have been observed under LD conditions (Shinohara and Inoue, 1997). The connection between the pattern of 5-HT release in the IGL and NPY release in the SCN will be of interest, and will provide insight into the control of non-photic input to the clock. The pattern seen in this study represents the daily rhythm of 5-HT release under a LD cycle, but does not expose the free-running pattern of release. It remains critical to examine the 24 h profile of release of 5-HT in the IGL under constant conditions, particularly under DD, to ascertain if this pattern is truly circadian in nature. It is likely, however, that the general pattern of release in DD would be similar to the one reported herein, with an increase an increase during the period of activity (normally associated with night) and a depression during the sleep/non-active
portion of the subjective day. Such a pattern is seen in other brain regions (Mendin et al., 1996; Rueter and Jacobs, 1996; Dudley et al., 1998).

Increases in locomotor activity are associated with transitory increases in the serotonergic tonus (Rueter and Jacobs, 1996; Mendlin et al., 1996; Dudley et al., 1998; Wilkinson et al., 1991). As novel wheel running has been reported to increase 5-HT within the circadian system, specifically in the region of the clock (Dudley et al., 1998), we sought to determine if novel wheel exposure increases 5-HT release in the IGL. As hypothesized, significant increases in 5-HT release were observed during novel wheel exposure, although there was not a strong correlation between the output of 5-HT measured and the number of wheel revolutions. This lack of correlation, together with the demonstration that general arousal (sleep deprivation) is sufficient to induce 5-HT release, is evidence that arousal during the midday (the time of sleep/inactivity) may be just as effective as locomotor activity in communicating non-photic information to the clock. This concept is supported by studies showing that several hours of behavioral manipulation are needed to elicit maximal phase-shifts during the midday (Reeb and Mrosovsky, 1989; Mrosovsky et al., 1989). Also experiments performed in this lab showed that 3 h of sleep deprivation induces larger phase-advances than those induced by one hour of the treatment.

From the present results it is evident that behavioral manipulation can increase IGL 5-HT release. As the IGL conveys non-photic, as well as photic input to the circadian clock, such changes in the 5-HT suggest that this transmitter could regulate these inputs (Marchant et al., 1997; Challet et al., 1996; Schuhler et al., 1999; Challet et al., 1998; Mintz et al., 1997; Antle and Mistlberger 2000; Janik and Mrosovsky 1992;
Specifically, as wheel running induces Fos expression in cells of the IGL that exhibit NPY immunoreactivity (Janik et al., 1995), serotonergic signaling in the IGL induced by activity/arousal could serve to modulate NPY signaling to the clock, thus ultimately inducing or potentiating phase-shifts.

The present finding that electrical stimulation of the DRN evokes an abrupt peak in 5-HT output confirms a physiologically functional connection from the DRN to the IGL, and suggests that the 5-HT release induced by activity/arousal, is due to the activation of the DRN.

Serotonergic Stimulation of Neuronal Activity in the Intergeniculate Leaflet

Animals treated with i.p. injection of the 5-HT$_{1A/7}$ agonist 8-OH-DPAT exhibited greater Fos-immunoreactivity in the IGL, compared to vehicle controls. Thus, serotonergic signaling in the IGL induced by behavioral DRN activation could serve to stimulate neuronal activity in the IGL. It is possible that the alterations in cellular activity observed were not solely mediated by post-synaptic 5-HT receptors, since the application of the agonist was not localized to the IGL region. Therefore, it remains speculative as to the direct effects of 5-HT in this region. Additionally, the cells that responding to the 8-OH-DPAT were not characterized. Our hypothesis that there would be a high degree of co-localization of Fos and NPY-immunoreactivity in animals exposed to a 5-HT agonist during the midday could therefore not be tested.

The Intergeniculate Leaflet’s Role in Non-photic Circadian Clock Resetting

Presently, there are few studies that have directly addressed the combined roles of the IGL and SCN in circadian phase-shifting. The purpose of this experiment was to
gain a better understanding of the competition, or cooperation between the IGL and the SCN in behavior-induced phase-shifting. Increased neuronal stimulation of the DRN associated with arousal has been shown to increase 5-HT signaling in the SCN as well as in the IGL. It has been suggested that behavioral information is consolidated into 5-HT signaling to the SCN via a multi-synaptic pathway that is under GABAergic and serotonergic control (Glass et al., 2003). Currently, the modulatory internal circuitry of the IGL is unknown. There is evidence, however, that 5-HT signaling in the IGL is critical for non-photic shifting. For example, the intact IGLs are necessary for the chronopharmacological effects of systemic 8-OH-DPAT (Schuler et al., 1999), and bilateral injection of 8-OH-DPAT in the IGL is sufficient to produce phase-shifts (Challet et al., 1998). There is also evidence that GABA may play a key role in modulating activity within the IGL. In fact, GABA has been considered as the primary neuromodulator of IGL neurons, since a vast proportion of IGL neurons appear to be GABAergic (Moore and Speh, 1993; Moore and Card, 1994). It is significant that electrical stimulation of the DRN induces a decrease in isoperiodic firing rates of cells of the IGL, in contrast to increases in IGL cell activity after lesioning of the DRN (Blasiak and Lewandowski, 2003). Thus, it is plausible that 5-HT signaling to the IGL blocks the GABAergic inhibition, thus inducing NPY signaling to the SCN ultimately producing a phase-advance.

Experiments in this section were designed to explore serotonergic and GABAergic influences in the IGL concurrently with serotonergic effects in the SCN on circadian phase regulation. Here, unilateral intercrainial microinjections, were undertaken allowing two pharmacological agents to be delivered to both (contralateral)
nuclei simultaneously. The dual vehicle injections yielded small shifts (~27 min), comparable with other studies, in where the physical manipulation of the animals associated with this procedure produced insignificant shifting. The group that received 8-OH-DPAT in both the SCN and IGL had slightly larger, although not significant phase-shifts (~36 min). Although in a previous study single injections of 8-OH-DPAT in either nucleus did not elicit significant shifts (Mintz et al., 1998), it was our contention that 8-OH-DPAT could work synergistically via a direct action in the SCN. This is supported by previous studies (Ehlen et al., 2001). It is also possible that 8-OH-DPAT–induced NPY signaling in the IGL could have also inhibited the phase-shifting effect of 8-OH-DPAT in the SCN. It has been shown that NPY inhibits 5-HT-induced shifts \textit{in vitro}, presumably via the Y2 receptor (Prosser, 1998). Also, injections of NPY anti-serum in the SCN attenuates shifts induced by running wheel and systemic 8-OH-DPAT (Biello et al., 1994; Albers and Mintz, 1997).

In animals that received vehicle in the IGL and 8-OH-DPAT in the SCN, a more pronounced shift occurred (~57 min). From a previous study, where a unilateral injections of 8-OH-DPAT were administered to the SCN (Mintz et al., 1998), we hypothesized that there would be negligible phase-shifting effect. It is interesting to note that this group displayed larger shifts than the preceding groups, possibly suggesting that NPY signaling attenuates the affect of 5-HT signaling in the SCN.

The remaining part of the study focused on the possible GABAergic inhibition of NPY neurons in the IGL, alone or in combination with serotonergic input the SCN. The group that received the GABA$_\alpha$ antagonist, bicuculline, in the IGL and vehicle in the SCN had small shifts, similar to those of the vehicle control group (~31 min). This
treatment was used to attenuate GABA inhibition, perhaps promoting NPY release downstream. However, the group that received the GABA_A agonist muscimol in the IGL and vehicle in the SCN, shifted the least of any group, refuting our proposed model. The groups that had the largest shifts, that were statistically different than the muscimol/vehicle group, all had shifts of approximately 70 min. The fact that treatment with either GABA antagonist or agonist in the IGL together with 8-OH-DPAT in the SCN produced similar shifts was not expected. Also unexpected was the finding that GABA antagonist injected into the IGL did not affect SCN response to 8-OH-DPAT, or the 5-HT_1,2,7 antagonist metergoline. One explanation for these results is that the inhibition of NPY-neurons is primarily mediated through the GABA_B or other 5-HT receptor subtypes not tested here. Another reason may be that the duration of exposure to the agents was insufficient. This might explain why 8-OH-DPAT does not elicit a phase-shifting response, when unilaterally delivered in the SCN via a single microinjection (Mintz et al., 1998), as compared to several hours by microdialysis (Ehlen et al., 2001). It is also important to note that the IGL is one of the longest nuclei in the hamster thalamus (~2.3cm in length; in respect to the anterior-posterior axis). It is plausible that the portion of the IGL we targeted does not contain a sufficient number of cells able to respond to the agents. A study examining the neuropeptide content of the IGL revealed that this region contains significantly fewer cell bodies of all cell population types analyzed; as compared to sections more caudally located (Morin et al., 2000).

A further confound in our experimental design that may be problematic, is that individuals received the drugs in only two locations, disregarding the complexity of the circadian system. Anatomical studies using retrograde and anterograde techniques
have revealed a highly complicated picture of inter- and intra-nuclei connections (Morin and Blanchard, 1998). There are reciprocal connections between the bilateral IGLs, and each IGL sends out collaterals that directly innervate the ipso-lateral and contra-lateral SCN. There are also fibers from the SCN that terminate in the ipso-lateral IGL (Morin and Blanchard, 1998). Thus, it may be that pharmacological agents need to be delivered bilaterally to the leaflets, as well as the SCNs, in order to produce the hypothesized effect. This proposal is supported by studies in which bilateral (Challet et al., 1999), but not unilateral (Mintz et al., 1998) microinjections of 8-OH-DPAT in the IGL and SCN are sufficient to induce phase-advances similar to other non-photic influences.

With these salient points in mind, an experiment was designed, where bilateral microinjections were utilized for drug delivery. Additionally, cannulae were aimed at a more central region of the IGL. In this experiment, animals received the behavioral manipulation of sleep deprivation shown to produce phase-shifts similar to other non-photic influences when delivered during the midday. Immediately before sleep deprivation, animals received either vehicle or metergoline bilateral microinjections. The group that received the metergoline microinjections exhibited smaller phase-advances as compared to the vehicle control group. This finding demonstrates that blocking 5-HT receptors in the IGL, attenuates shifting associated with the manipulation of sleep deprivation (Mistlberger et al., 2001), which been shown to induce 5-HT release in the IGL region. This current data may provide evidence, that 5-HT signaling in either region is sufficient to produce shifts, since a previous study reported that this same behavioral treatment also increases 5-HT release in the SCN (Grossman et al., 2000).
It is important to note that the duration of drug exposure used in previous experimental designs utilizing microinjections, was taken into account in the current experiment. Rather than using the standard 3 h of sleep deprivation shown to elicit large shifts (perhaps maximal), animals were exposed to 1 h of sleep deprivation. This shorter application has been shown to elicit significant phase-shifts, but should significantly limit the IGLs’ exposure to serotonergic signaling, providing a temporal window in which the exogenous antagonist and endogenous ligand could compete.

The last experiment of this section was designed to test 5-HT signaling in the IGL region on non-photic phase-shifts. After reviewing data from our previous studies, reverse-microdialysis perfusion using two separate probes was employed for the targeted delivery of the 5-HT agonist. The benefits of this approach are twofold: 1) dialysis enables the subjects to receive a continuous serotonergic stimulation over the course of several hours, mimicking endogenous signaling observed in wheel running for this length of time; 2) serotonergic stimulation could be delivered bilaterally, further simulating endogenous signaling. The animals that received the 8-OH-DPAT exhibited 10-fold larger phase-advances as compared to vehicle control animals. This result shows that the bilateral application of the agonist was effective in producing shifts, in part, due to the bilateral delivery, a result not seen with unilateral reverse-microdialysis perfusion over a 3 h period (data not shown), but seen in bilateral microinjections (Challet et al., 1998). In addition, the shifting response most likely was potentiated by the prolonged delivery, which yielded larger shifts then those seen with bilateral microinjections (Challet et al., 1998).
Serotonin’s Ability to Shift the Clock at the Molecular Level

This study was designed to confirm the ability of 5-HT to directly alter the phase of the clock as shown by Ehlen et al., 2001, by assessing the effect of prolonged 8-OH-DPAT exposure on clock gene transcripts. Here, we show that 3 h of \textit{in vivo} reverse-microdialysis perfusion of the SCN with 8-OH-DPAT reduced the \textit{hPer1} mRNA hybridization signal to undetectable levels. This treatment also significantly reduced \textit{hPer2} mRNA levels (40.8±3.6% vs. controls). This observed decrease in peak midday levels of mRNA transcripts of the clock-coupled genes Per 1 and Per 2 is similar to previous results, where non-photic manipulations that have been shown to advance the phase of the circadian clock and reduce expression of these genes (Fukahara et al., 2001; Horikawa et al., 2000; Maywood and Mrosovsky, 1999). The proteins of these and other clock genes are expressed in a rhythmic daily pattern in the SCN by individual cells. They are able to form dimers, whereby they can inhibit their own transcription, forming a negative feedback loop. This autoregulatory oscillations in transcription/translation/inhibition, generates circadian rhythms (Dunlap et al., 2004). These results reported in this present study provide additional evidence that 5-HT has a direct phase-resetting effect in the SCN (Ehlen et al., 2001), since reduction in levels of mRNA of these clock genes in the SCN has been shown to be an accurate marker for non-photic-induced alterations of the clock at this time point.
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