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Neural Basis of Arousal Signaling for Non-Photic Resetting of the Circadian Clock

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Neural Basis of Arousal Signaling for Non-Photic Resetting of the Circadian Clock

by

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Abstract

Input to the suprachiasmatic nucleus (SCN) from the intergeniculate leaflet (IGL) is necessary for non-photic entrainment. However, the underlying mechanisms of IGL activation remain unknown. There are several arousal centers in the brain that could be involved in bringing about non-photic entrainment. These include the lateral hypothalamus (LH) which contains clock-projecting orexin cells, and the cholinergic basal forebrain that directly communicates with the SCN. Even though arousal is the key component of non-photic entrainment, the relationship between the IGL and these arousal areas is unclear. We investigated the neural basis of arousal signaling by first studying the potential inputs to the IGL from the LH and the basal forebrain of Syrian hamsters. Projections to the IGL, from both the LH and the basal forebrain cholinergic cells were found. Next, we examined whether orexin is necessary and sufficient for non-photic phase shifting by both blocking orexin prior to an arousal-inducing protocol such as sleep deprivation and administering orexin in the IGL. It was found that orexin alone is not necessary or sufficient to cause shifts. Instead, it was found that dual administration of a glutamate receptor agonist with orexin is sufficient to cause significant shifts, suggesting an additive effect at the IGL. We next examined whether acetylcholine is necessary for non-photic entrainment. No attenuation of the arousal-induced response was observed by blocking acetylcholine at the IGL, suggesting that it is not necessary for non-photic entrainment, though it has been reported to be critical at the SCN level. Finally, accumulation of the sleep factor adenosine in the basal forebrain was mimicked as a potential signal for activating the basal forebrain. No significant phase shifts or cellular activation of the basal forebrain was observed after blocking adenosine at the basal forebrain. Taken together, the results present the first report of a dual role for orexin and glutamate in potentially gating the IGL's non-photic inputs to the SCN.

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To my mom for her unconditional support

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List of Symbols, Abbreviations and Nomenclature

5-HT: 5-hydroxytryptamine, or serotonin

8-CPT: 8-cyclopentyl-1,3-dimethylxanthine

ACh: Acetylcholine

aCSF: Artificial cerebrospinal fluid

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor

ANOVA: Analysis of variance

AVP: Arginine vasopressin

CalB: Calbindin

CalR: Calretinin

ChAT: Choline acetyl transferase

CT: Circadian time

CTβ: Cholera toxin β

DAG: Diacyl glycerol

DD: Constant darkness

dLGN: Dorsolateral geniculate nucleus

DMSO: Dimethylsulfoxide

DRN: Dorsal raphe nucleus

GABA: γ-aminobutyric acid

GHT: Geniculohypothalamic tract

GRP: Gastrin-releasing peptide

IGL: Intergeniculate leaflet

i.p. : Intraperitoneal

IP_3: inositol trisphosphate

LD: Light dark cycle

LH: Lateral hypothalamus

LL: Constant light

MK-6096: Orexin antagonist, Filorexant

MRN: Median raphe nucleus

NMDA: N-methyl-D-aspartate receptor

NPY: Neuropeptide Y

OX: Orexin

OX_A: Orexin A

$OX_{B:}$ Orexin B

OX_{A+B}: Orexin A and Orexin B

PACAP: Pituitary adenylate cyclase-activating polypeptide

PBS: Phosphate buffered saline

PBSx: PBS with 0.1% Triton-X-100

PEG: Polyethylene glycol

P-ERK: Extracellular signal-regulated kinase I/II

PKC: Protein Kinase C

PLCβ: Phospholipase C β

PRC: Phase response curve

RHT: Retinohypothalamic tract

SCN: Suprachiasmatic nucleus

SD: Sleep deprivation

SEM: Standard error of the mean

SI: Substantia innominata

VIP: Vasoactive intestinal polypeptide

vLGN: Ventrolateral geniculate nucleus

ZT: Zeitgeber time

Chapter One: General Introduction

1.1 Circadian Rhythms

1.1.1 Historical and evolutionary background

Life on earth has evolved to anticipate the rhythmic changes in the environment. These changes come about due to the earth's position as a planet in the solar system and its orbital characteristics (Dunlap et al., 2004). The earth's rotation around its own axis leads to the daily light-dark cycle while the earth's orbit around the sun leads to the cycle of seasonal changes. The moon's rotation around the earth also leads to shorter-term changes in tidal cycles (Dunlap et al., 2004). Fluctuating environmental conditions such as temperature and light availability present challenges to living organisms who must adapt to daily changes. In response, all organisms have evolved internal biological clocks that acts as temporal regulators in almost every aspect of their daily lives (Dunlap et al., 2004). From an evolutionary standpoint, it is most advantageous for an organism to anticipate environmental change and schedule behaviours such as feeding and sleep around optimal time points. This increases the likelihood of an organism persisting long enough to reproduce and pass along its genes to the next generation (Dunlap et al., 2004).

Circadian rhythms (Latin for *Circa* "around" and *Diem* "day") are endogenous oscillations with a length of about a day and have been observed in virtually all organisms, including both eukaryotes and prokaryotes (Dunlap et al., 2004; Kondo et al., 1993). The length of each endogenous cycle is referred to as the intrinsic circadian period and follows close to 24 hours. It has been proposed that early on in evolutionary history, metazoans evolved means of anticipating changing levels of sunlight, allowing them to protect their genetic material against the damaging effects of ultraviolet radiation by traveling deeper in the ocean during the day and coming back up at nighttime (Gehring & Rosbash, 2003). Despite our current knowledge of the endogenous nature of circadian rhythms, the historical view of daily rhythmic behaviour was that

it is a passive process, driven by the external environment (Edery, 2000). Early pivotal research examining plants helped to establish the knowledge that circadian rhythms are an endogenous property of an organism. In 1729, astronomer Jean-Jacques d'Ortous de Mairan housed species of a flowering plant in constant darkness and noted the persistence of daily leaf movements in the absence of any external light cues (de Mairan, 1729). Nonetheless, the prevailing view that the external environment generates rhythms remained for a long time after. Further experiments with plants replicated de Mairan's work (Hill, 1757) while seminal work measuring the period length of leaf movements provided further evidence for the endogenous nature of rhythms (de Candolle, 1832). More specifically, it was found that the period length of leaf movements was shorter (22 to 23 hrs) than the 24-hour day length, suggesting the intrinsic nature of these rhythms, and not simply as a response to environmental cues (de Candolle, 1832).

The functional significance of circadian rhythms has become apparent through several field studies observing a variety of species, including insects, birds, and mammals. German scientist Gustav Kramer studied the migratory patten of birds and observed that they use their internal biological clock to track and offset the sun's continuously changing location in order to fly from south to the north when the seasons changed (Kramer, 1952). Furthermore, the need for an accurate timekeeping system was demonstrated in the foraging behavoiur of the free-living chipmunk *Tamias striatus* (DeCoursey et al., 2000). Specifically, when conditions were unfavorable to the survival of the foraging chipmunk such as an increase in predator population, the presence of a functioning internal rhythm was critical to the temporal organization of behaviours such as foraging for food and therefore the survival of the chipmunk population (DeCoursey et al., 2000). Animals lacking a proper functioning timekeeping mechanism did not

choose the optimal times to leave their burrow in search of food or became more active during the nighttime, which led to an increased risk for predation (DeCoursey et al., 2000).

1.1.2 Circadian rhythm disruption and health consequences

There are a growing number of findings suggesting that circadian disruption leads to negative health consequences. There is an increasing demand in modern society for individuals to work at all hours of the day, mostly under artificial lighting. This has created a situation where many individuals have disrupted rhythms and sleep. The misalignment between the endogenous rhythm and the timing of sleep-wake cycles gives rise to a variety of health issues. Shift workers are more susceptible to negative health effects such as decreased mood and impaired cognitive functioning, which can lead to poorer work performance (Folkard et al., 2005). Long-term consequences of rotating shift work include cardiovascular events, reproductive issues, diabetes, and cancer (Wang et al., 2011; Zhao et al., 2022).

Although the demand for increased productivity has amplified in the last decade, the COVID-19 pandemic, in particular, highlighted the extra burden placed on the health sector with a growing dependence on around-the-clock activity among many frontline workers (Lin et al., 2021; San Martin et al., 2020). Circadian misalignment also places individuals at an elevated risk for viral diseases, including COVID-19. This is presumably due to the detrimental effect of misalignment on a properly functioning immune system (Ray & Reddy, 2020). Sleep-wake cycles are regulated through the interaction of circadian and homeostatic processes, with the latter being defined as the build-up of a sleep drive throughout the wakefulness period. When work is scheduled during the daytime, these processes function in synchrony with the environmental light cycle and allow for alertness during the day and quality sleep at night. However, staying awake at odd hours in the night or early morning results in a reduced circadian

drive for wakefulness. This results in decreased alertness during wakefulness and can lead to catastrophic consequences such as workplace accidents and injuries (Van Dongen et al., 2016). Hamsters that have disruptions in their light-dark cycles also show an increase in negative health consequences and have a shorter life span (Martino et al., 2008).

1.2 The SCN is the Central Pacemaker

While circadian rhythms can mimic external environmental cycles, they are also capable of persisting in the absence of any environmental conditions such as in constant darkness (DD). Under these circumstances, an organism's internal cycle maintains its own rhythmicity and is therefore said to be free-running (Holzberg & Albrecht, 2003). The fact that this rhythmicity can operate independently from any outside influences suggests its dependence on a timekeeping system driven by an internal biological clock. In mammals, the suprachiasmatic nuclei (SCN) in the anterior hypothalamus act as the central pacemaker of the endogenous rhythm (Antle & Silver, 2005; Miller et al., 1996). Research on the mammalian circadian system identified that SCN lesions eliminate sleep-wake cycles, drinking and locomotor activity (Ibuka & Kawamura, 1975; Stephan & Zucker, 1972). More data in support of the SCN as the main clock came from studies demonstrating restoration of locomotor rhythms in SCN-lesioned rodents who received SCN transplants from embryos or neonatal tissue (Lehman et al., 1987; Sawaki et al., 1984). More evidence came from the discovery of a single-gene mutation in a Syrian hamster model called the *tau* mutant hamster (Ralph & Menaker, 1988). In these animals, the circadian period is shortened from the usual 24 hours to approximately 22 hours in heterozygous and as short as 20 hours in homozygous animals (Ralph & Menaker, 1988). Shortly after this discovery, it was demonstrated that transplanted SCN from animals with normal circadian periods was sufficient to restore the period in the tau mutants to that of the donor animals (Ralph & Menaker, 1990).

Therefore, the mutants were able to attain 24-hour periodicity by virtue of the transplant. This result provided strong support for the role of the SCN as the central pacemaker.

1.2.1 SCN heterogeneity and gene expression

The SCN is a pair of nuclei with a total of about 20,000 individually rhythmic cells (Reppert & Weaver, 2001). Individual SCN cells express a range of different phases and periods and there is heterogeneity of function among cell populations (Antle & Silver, 2005; Welsh et al., 1995). Each SCN nucleus can be differentiated by a distinct dorsomedial shell and ventrolateral core (Antle & Silver, 2005). Cells in the core are principally in charge of relaying photic information to the shell for synchronization of the different phases of the rhythmic cells (Holzberg & Albrecht, 2003). The shell consists of individually rhythmic cells with an endogenous autoregulatory transcription-translation feedback loop of the clock genes, period and cryptochrome. Transcription of period (*Per*1 and *Per*2) and cryptochrome (*Cry*1 and *Cry*2) begins with the binding of the positive protein regulators, BMAL1 and CLOCK to the genes' E-box elements. The resultant mRNA exits the nucleus and is translated into the protein products, PER, and CRY, in the cytoplasm. These proteins then dimerize forming PER/PER or PER/CRY dimers and accumulate throughout the day (Reppert & Weaver, 2001). As they do so, they move back into the nucleus where they interact with BMAL1 and CLOCK, rendering them inactive. As a result, the accumulation of the protein products leads to the inhibition of their own genes (Kwon et al., 2011). PER degradation is facilitated by interaction with the enzyme casein kinase 1 ε in the cytoplasm (Kwon et al., 2011). Cells in the core do not inherently express clock genes but rather do so in response to a cue such as light (Hamada et al., 2001), while non-photic cues lead to a reduction in the expression of period genes in the SCN (Maywood & Mrosovsky, 2001; Mendoza et al., 2004).

Cells in the shell and core are also phenotypically distinct, with different cells expressing a variety of peptides. Generally, intrinsically rhythmic cells of the shell express arginine vasopressin (AVP) while cells in the core express vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP; Moore, 1996). This pattern is seen in different species including rodents and humans. However, there are a great deal of peptidergic cell types in the SCN, and several differences exist between species (Morin & Allen, 2006). For instance, the hamster SCN core is further characterized by calbindin (CalB), and extracellular signal-regulated kinase I/II (P-ERK) while the mouse core expresses little CalB in adulthood, calretinin (CalR), and the amino acid neurotransmitter γ-aminobutyric acid (GABA), among others (Abrahamson & Moore, 2001; Antle & Silver, 2005).

1.3 Entrainment

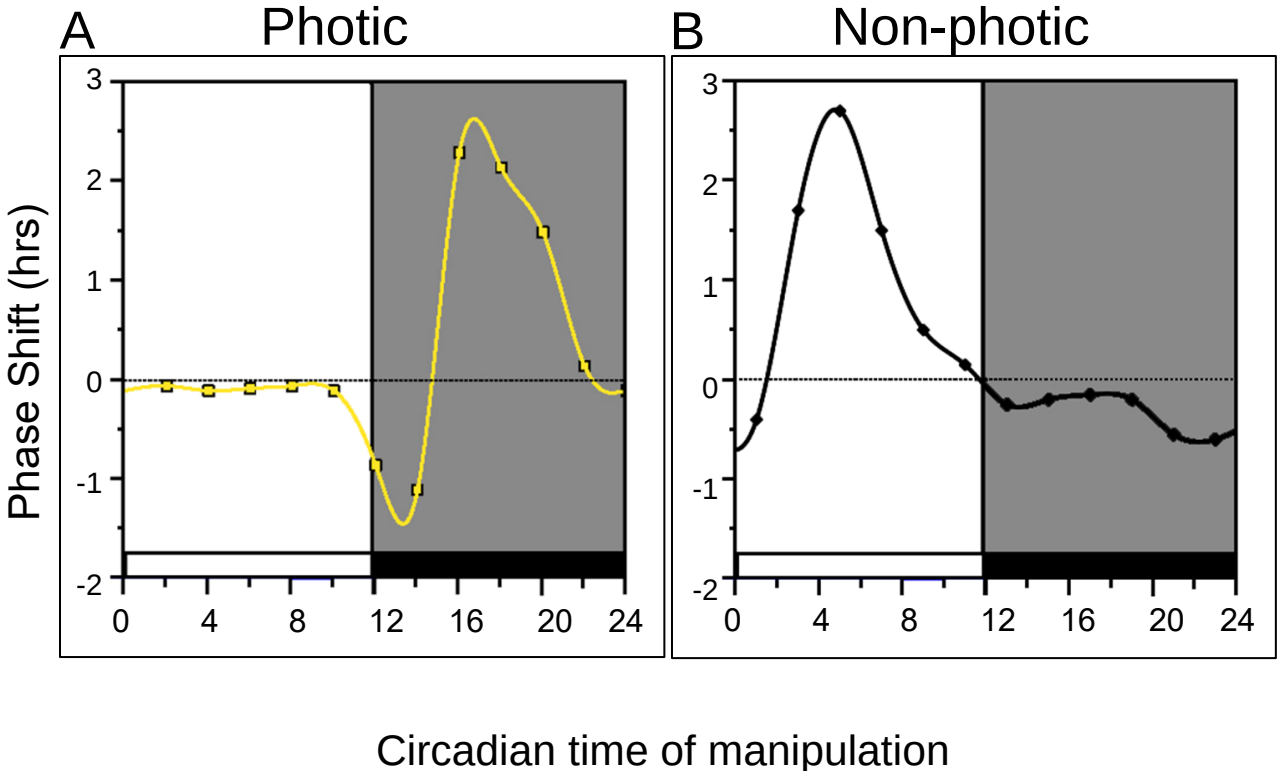
In unchanging environmental conditions such as in constant darkness (DD), endogenous rhythms can maintain their own rhythmicity, independent from outside influences (Holzberg & Albrecht, 2003). The length of each cycle is referred to as the intrinsic circadian period and follows close to 24 hours. However, there are species-specific differences in period length both in humans and other animals, with each having a species-specific mean ranging from slightly below to slightly above 24 hours (Pittendrigh & Daan, 1976). Synchronization with the environment, known as entrainment, takes place when the endogenous cycle follows the external cycle length which requires it to undergo a daily delay or advance. Entrainment occurs via the influence of an external environmental cue or zeitgeber (German for time giver; Dunlap et al., 2004)). The most prominent zeitgeber in mammals is the external light cycle (Berson et al., 2002; Hirota & Fukada, 2004; Rusak & Zucker, 1979). Nevertheless, a variety of other stimuli including behavioural arousal, food, and social interactions can reset the SCN rhythm (Mistlberger & Antle, 2011; Mrosovsky, 1996). Zeitgebers cause a change or shift in the phase

of the SCN which can be observed by measuring alterations in activity onset. In rodents, this is often accomplished through examining locomotor behaviours such as wheel running.

Phase shifts can be in the form of delays or advances depending on the zeitgeber and when it is delivered. A zeitgeber can either cause a phase delay, which refers to phase markers, such as activity onset, occurring later on subsequent days, or a phase advance, characterized by earlier onsets. The pattern of shifts in the clock phase can be plotted over time, producing a phase response curve (PRC; Daan & Pittendrigh, 1976; Figure 1.1A). The photic PRC illustrates how light shifts rhythms earlier or later when it is presented throughout time in a free-running organism. Shifts to various stimuli other than light (such as exercise, sleep deprivation, etc…) can also be plotted, creating what is referred to as a non-photic PRC (Figure 1.1B). In animals that display a stable free-running rhythm in constant conditions (such as DD conditions) including Syrian hamsters and some mice, an Aschoff Type I protocol can be employed to measure phase shifts (Jud et al., 2005). In this protocol, animals are maintained in DD conditions where they free run and the influence of zeitgebers can be assessed at different time points or circadian times, according to the phase of the endogenous cycle (Jud et al., 2005). Circadian time (CT) can be differentiated from zeitgeber time (ZT) as the latter defines the timing based on the phase relationship with a strong stimulus such as the light-dark cycle while the former defines timing based on the free-running period of the internal rhythm (Jud et al., 2005) . In an alternative design called the Aschoff Type II protocol, animals are entrained to a light-dark cycle before lights are turned off for the manipulation and remain off for a period of time to ensure that photic masking of locomotor activity does not occur (Aschoff, 1960; Jud et al., 2005). Masking refers to instances where the effect of a stimulus is obscured by an uncontrolled factor. For instance, locomotor activity can be suppressed by the presence of light in nocturnal animals and

so, it is important to keep the lights off in the Aschoff Type II design with nocturnal animals to ensure the observation of any effects from a manipulation (Aschoff, 1960; Jud et al., 2005).

Figure 1.1. Photic and non-photic phase response curves (PRC). An example of a photic **(A)** and non-photic **(B)** PRC. To generate a PRC, the effect of a stimulus is investigated to determine how an organism's response to the stimulus varies as a function of the phase of the subjective time (circadian time) that the stimulus is applied. More specifically, a stimulus can be presented at different time points in a free-running organism and the phase shift response can be plotted in hours creating a graph of the data. A general PRC can be plotted for a whole population or species of an organism. In the plots shown, phase advances are displayed as positive phase shifts and phase delays are shown as negative phase shifts. The shaded region represents the dark phase.


A
Photic
B
Non-photic
Phase Shift (hrs)
3
2
1
0
-1
-2
0
4
8
12
16
20
24
Circadian time of manipulation

1.4 Non-photic Entrainment

1.4.1 Dark pulses

The first line of evidence for non-photic entrainment came from experiments looking at the effect of bouts of darkness ("dark pulses") on rhythms of animals under constant lighting conditions (Subbaraj & Chandrashekaran, 1978). Locomotory activity patterns of an insectivorous bat species, *Taphozous melanopogon,* were analyzed following confinement in tilting cages (Subbaraj & Chandrashekaran, 1978). Bats were kept under dim constant light (LL) which was interrupted by pulses of darkness for 2-4 hours. Dark pulses administered during the animal's subjective day (time period that an animal considers as day, without external light-dark cues), resulted in phase advances while dark pulses given during the animal's subjective night (active period for the nocturnal bats) led to phase delays.

The non-photic PRC obtained from dark pulse experiments was shown to be different from the photic PRC which is characterized by phase advances in the late subjective night and phase delays in the early subjective night (Rosenwasser & Dwyer, 2001; Subbaraj & Chandrashekaran, 1978). Similar experiments in mice and hamsters provided more support for the presence of non-photic entrainment pathways to the circadian clock (Boulos & Rusak, 1982; Ellis et al., 1982; Marston et al., 2008). Dark pulses given during the late subjective day of Syrian hamsters housed in LL conditions led to phase advances while dark pulses in the late subjective night or early subjective day did not result in significant phase shifts (Ellis et al., 1982). However, the non-photic PRC is similar in both diurnal and nocturnal species with stimuli causing phase advances in the subjective day and phase delays in the subjective night, regardless of sleep and activity patterns (Glass et al., 2001; Hut et al., 1999). However, there are species-specific differences in the magnitude of phase shifts. For instance, non-photic stimuli lead to much larger phase advances in the mid-subjective day for Syrian hamsters than for mice and rats

and as a result, the PRC differs somewhat in these animals (Dallmann et al., 2007; Mistlberger, 1991).

1.4.2 Other stimuli causing non-photic shifts and relation to locomotor activity

Following dark pulses, a variety of other stimuli were shown to cause phase shifting in a manner resembling the dark pulse PRC pattern. Injections of benzodiazepines in Syrian hamsters were shown to cause similar phase shifts as dark pulses (Van Reeth & Turek, 1989). Triazolam, a drug commonly prescribed for insomnia was shown to cause phase advances when administered in the subjective day with smaller delays reported during the subjective night. Furthermore, triazolam administration facilitated re-entrainment to a light-dark cycle (Van Reeth & Turek, 1989). Similarly, hamsters given injections of the benzodiazepine chlordiazepoxide during the subjective day exhibited large phase shifts (Biello & Mrosovsky, 1993).

Many early experiments with dark pulses and triazolam injections reported an acute increase in locomotor activity (Boulos & Rusak, 1982; Ellis et al., 1982; Turek & Losee-Olson, 1986). This led to the re-examination of the critical stimuli in non-photic phase shifting mechanisms. Hamsters that were confined to nest boxes and therefore had restricted locomotor activity did not show phase shifts following triazolam injections (Mrosovsky & Salmon, 1990). Similarly, confinement to nest boxes led to attenuation of phase shifts in response to dark pulses (Reebs et al., 1989). Elsewhere, the importance of locomotor activity in non-photic phase shifting became clear with studies examining re-entrainment to a light-dark schedule similar to the study looking at triazolam. Specifically, hamsters that were confined to a novel running wheel in a new cage (away from their home environment), immediately following a change in their light cycle were able to entrain to the new schedule much faster than the control group (Mrosovsky & Salmon, 1987). This study provided insight into the role of locomotion as a non-

photic zeitgeber and demonstrated a potential alternative recovery for jetlag as opposed to drugs. Furthermore, confinement to a novel running wheel for a period of 1-3 hours resulted in phase advances in the subjective day (Mrosovsky et al., 1992).

Locomotor activity has also been shown to shorten period in rodents with a dependence on the amount of activity (Mistlberger et al., 1998; Mrosovsky, 1999). The amount of locomotor activity is also correlated with the magnitude of phase shifts; the greater the amount of activity, the larger the phase shift (Janik & Mrosovsky, 1993). This dose-dependent relationship is also seen for triazolam injections (Turek & Losee-Olson, 1986). Larger phase shifts are also observed with increasing dark pulse duration in hamsters (Canal & Piggins, 2006). Longer pulse duration in hamsters acutely increases wheel running activity, thereby presumably influencing phase shift magnitude (Canal & Piggins, 2006). However, the increase in locomotor activity in response to dark pulses is not correlated with phase shift magnitude in mice (Marston et al., 2008) Rather, behavioural arousal seems to be the critical stimuli for phase resetting (Marston et al., 2008).

1.4.3 Behavioural arousal is the main component of non-photic entrainment

Although greater phase advances in response to dark pulses have been reported when animals had higher levels of locomotor activity, phase shifts to dark pulses have also been observed in the absence of wheel running (Mendoza et al., 2004). Furthermore, hamsters injected with chlordiazepoxide showed phase shifts despite restricted locomotor activity (Biello & Mrosovsky, 1993). More importantly, studies looking at keeping an animals awake through methods other than wheel running, such as handling the animals, were still able to report large phase advances similar to past reported non-photic shifts. Phase advances were observed in sleep deprivation experiments that used a gentle handling protocol where running wheels were locked and hamsters were lightly poked to keep them awake (Antle & Mistlberger, 2000). While the

animals only travelled a small distance in the cage, these phase shifts were comparable to shifts seen in response to high bouts of locomotor activity such as in novel wheel confinement experiments (Antle & Mistlberger, 2000; Mistlberger et al., 2002). The amount of locomotor activity also does not accurately reflect non-photic phase shifting in diurnal species (Hut et al., 1999). Taken together, these findings provided support for behavioural arousal as the necessary stimulus for resetting the phase of the circadian clock. Therefore, when looking to past stimuli including dark pulses and novel wheel confinement, it was likely that any manipulation that influenced the arousal state of an animal led to non-photic entrainment of the circadian system (Antle & Mistlberger, 2000; Mistlberger et al., 2002, 2003; Yamakawa et al., 2016).

All manipulations discussed thus far involve some level of behavioural arousal. Sleep deprivation through gentle handling is sufficient to cause non-photic phase shifts without the need for the degree of locomotor activity reported in other studies (Antle & Mistlberger, 2000). However, other findings support the role of locomotion as a non-photic cue. Protocols that restrict locomotor activity but that are still arousing fail to give rise to significant phase shifts, although it has been suggested that anxiety-provoking procedures involved in confinement may have blocked non-photic entrainment (Mistlberger et al., 2003). The level of arousal has been shown to be critical in determining non-photic phase shifting (Yamakawa et al., 2016). Variations in levels of arousal for different studies may underlie the conflicting results obtained from locomotor activity and sleep deprivation studies. Animals that did not phase shift in response to a sleep deprivation protocol were shown to be drowsier than those that phase shifted (Yamakawa et al., 2016). Therefore, a threshold level of arousal seems necessary for resetting the circadian clock.

Non-photic phase shifting leads to a reduction in the expression of period genes in the SCN. All non-photic manipulations discussed so far including dark pulses, benzodiazepine administration, and novel wheel confinement appear to cause a downregulation of *Per*1 and *Per*2 genes (Maywood & Mrosovsky, 2001; Mendoza et al., 2004) . Decreasing *Per*1 expression during the day by injecting *Per*1 antisense oligodeoxynucleotides into the SCN also leads to non-photic phase shifts similar to other non-photic cues (Hamada et al., 2004). As well, arousal-induced stimuli such as spontaneous activity suppress SCN electrical activity during the day, a period during which neural activity is at its highest (Meijer et al., 1997; Oosterhout et al., 2012).

Behavioural arousal protocols also attenuate expression of the immediate early gene *Fos* in the SCN (Antle & Mistlberger, 2000). Furthermore, administration of neuropeptide Y (NPY) and many other neurotransmitters including γ-aminobutyric acid (GABA), serotonin, orexin, and acetylcholine also leads to similar decreases in gene expression and suppression of electrical activity (Belle et al., 2014; Besing et al., 2012; Fukuhara et al., 2001; Horikawa et al., 2000). The role of these neurotransmitters in non-photic entrainment is discussed further on in this chapter.

1.4.4 Interaction of non-photic and photic cues

Under regular light-dark (LD) cycles, arousal-inducing stimuli delivered in the midday do not lead to significant phase shifts in Syrian hamsters (Janik et al., 1994), although introducing a hamster to wheel running for the first time can lead to large phase advances (Gannon & Rea, 1995). Arousal stimuli generally act by competing with photic stimuli and lead to blocking of photic phase shifts. Behavioural arousal through locomotor activity can block phase advances in response to light pulses at late subjective night (Ralph & Mrosovsky, 1992). However, a study looking at whether novel wheel confinement prior to light exposure can also alter clock gene expression found that both *Per1* mRNA and c-Fos protein expression in the SCN were not

influenced by the non-photic manipulation (Edelstein et al., 2003). This suggests that there are separate mechanisms in the expression of clock genes for photic and non-photic entrainment. Also, the role of non-photic cues was shown to be more complex than previously thought and may not have the sole purpose of modulating photic entrainment. The inhibitory relationship between photic and non-photic cues also suggests separate pathways of entrainment for each type of stimulus. Non-photic entrainment pathways lead to opposite effects on the expression of critical clock genes. More specifically, non-photic cues lead to downregulation of period genes in mammals (Maywood et al., 1999). Furthermore, non-photic stimuli are associated with suppression of SCN cell activity which is highest during the subjective day (Oosterhout et al., 2012).

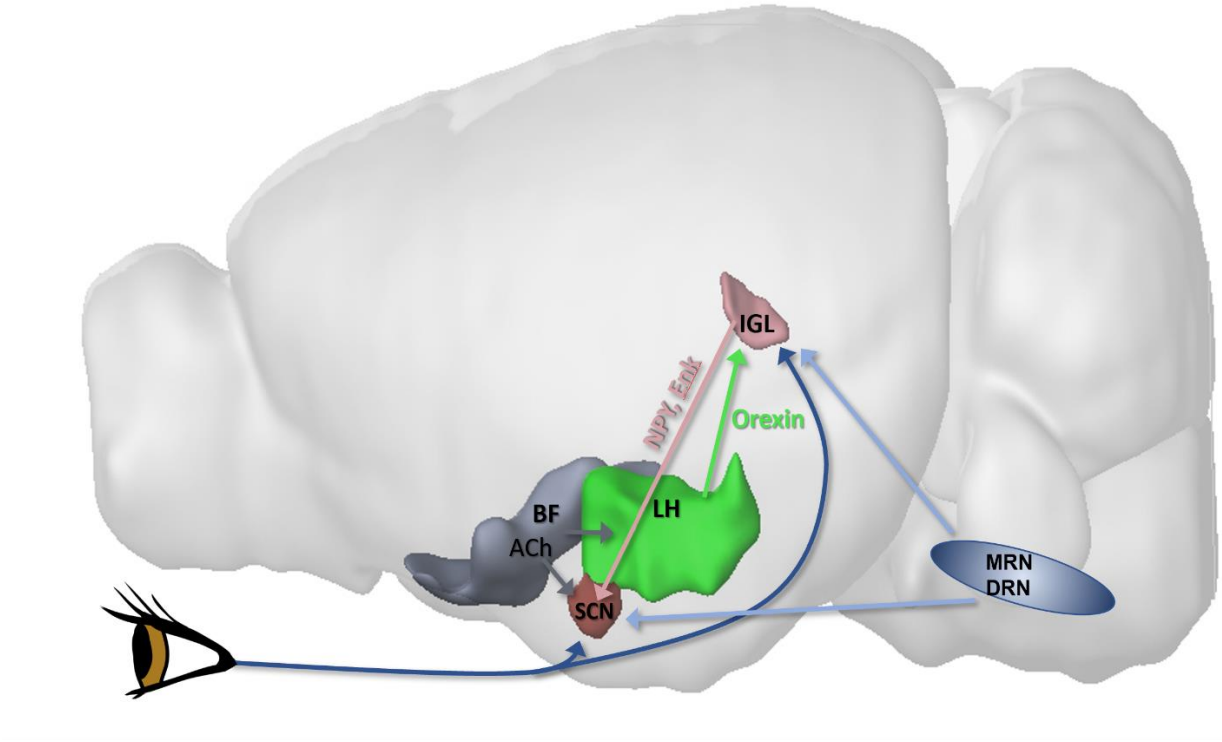
1.5 Projections to the SCN and Contributions of Neurotransmitters to Non-Photic Entrainment

The SCN receives both photic and non-photic information from various brain areas (Figure 1.2). Light reaches the retina and photic information is relayed to the SCN via a direct axonal pathway known as the retinohypothalamic tract (RHT; Morse & Sassone-Corsi, 2002). Principally, retinal cells release glutamate which acts on N-methyl-D-aspartate (NMDA) receptors present in SCN cells and eventually leads to the coordination of the clock's rhythm (Ebling, 1996; Morse & Sassone-Corsi, 2002). In addition, these cells contain pituitary adenylate cyclase-activating polypeptide (PACAP) which can also be involved in the photic pathway (Nielsen et al., 2001). Another major input pathway to the clock are the serotonergic projections from the raphe nuclei (Meyer-Bernstein & Morin, 1996). There is also a direct cholinergic projection from the basal forebrain to the SCN (Bina et al., 1993; Yamakawa et al., 2016). A major source of input to the SCN is from the intergeniculate leaflet (IGL) of the thalamus. The IGL plays a significant role in non-photic entrainment and is also involved in modulating photic

information and indirectly sends photic information to the SCN via the geniculohypothalamic tract (GHT; Hattar et al., 2002; Moore, 1996a; Moore & Card, 1994). The IGL is involved in mediating the effects of light on circadian activity (Pickard et al., 1987). However, the exact function of the GHT pathway remains unclear. The RHT alone seems to be sufficient for photic entrainment and studies looking at the effect of IGL destruction have reported little disruption to entrainment (Dark & Asdourian, 1975). IGL lesions in Syrian hamsters give rise to disrupted information and decreased discriminatory abilities about light wavelength and intensity (Pickard et al., 1987). Also, although the IGL does not appear to be necessary for photic entrainment, lesioned animals take longer to re-entrain to a new light-dark cycle (Johnson et al., 1989). Lesioning has also been shown to disrupt photic entrainment to a skeleton photoperiod, a lighting schedule that approximates the natural lighting conditions of nocturnal animals with brief light pulses at early and late subjective night (Edelstein & Amir, 1999).

Due to the relevance to this thesis, the focus from this point forward will be placed on the non-photic pathways to the SCN. More important will be the GHT and the role of the IGL in non-photic entrainment. From there, the eventual emphasis will be placed on the other non-photic pathways to the clock that also communicate with the IGL.

Figure 1.2. Schematic of the network showing pathways to the suprachiasmatic nucleus (SCN). Image of a rodent brain displaying the photic and non-photic pathways important for entrainment of the SCN. Light input from the eyes is relayed both directly to the SCN and indirectly, via the geniculohypothalamic tract (pink arrow) from the intergeniculate leaflet (IGL). Neuropeptide Y (NPY) and enkephalin (ENK) are involved in this pathway. There is also cholinergic input (ACh) from the basal forebrain (BF) to the SCN (grey arrow) as well as orexinergic input from the lateral hypothalamus (LH, green arrow) to both the SCN, and IGL regions. The cholinergic basal forebrain also communicates with the LH (grey arrow) arousal center. Finally, there are serotonergic projections (dark blue arrows) from the dorsal and median raphe brainstem nuclei (DRN, MRN) to both the SCN and the IGL.


IGL
NPY, Enk
Orexin
BF
ACh
LH
SCN
MRN
DRN

1.5.1 Serotonergic input from the raphe nuclei

The serotonergic input pathway has been shown to be necessary for non-photic phase shifting (Moore & Card, 1994; Yamakawa & Antle, 2010). Arousal-inducing protocols such as wheel confinement and sleep deprivation increase serotonin levels in the SCN while direct stimulation of the median raphe nucleus (MRN) results in serotonin (5-HT) release in the SCN (Dudley et al., 1998, 1999). Electrical stimulation of the MRN at mid-subjective day results in characteristic phase advances (Yamakawa & Antle, 2010). Injections of a serotonin neurotoxin have also been shown to block non-photic phase shifting due to the elimination of serotonergic inputs to the SCN (Yamakawa & Antle, 2010).

In vitro studies have also provided evidence for the role of serotonin in non-photic phase shifting. Administration of the 5-$HT_{1A/7}$ agonist 8-OH-DPAT shifts the cellular firing pattern of SCN cells (Prosser, 2003; Prosser et al., 1993). Furthermore, systemic administration of 8-OH-DPAT gives rise to non-photic phase shifts while 5-$HT_{1A/7}$ receptor knockout mice do not show non-photic phase shifts in response to 8-OH-DPAT (Smith et al., 2008). However, lesioning of the MRN fails to block phase shifts to novel wheel confinement protocols, although it significantly attenuates shifts to triazolam (Meyer-Bernstein & Morin, 1998). Similarly, serotonin antagonists such as the 5-HT_{1A} antagonist NAN-190, failed to block phase shifts to wheel confinement (Antle et al., 1998). It is important to note that serotonergic input to the SCN is also implicated in photic entrainment (Sterniczuk et al., 2008) and so it is likely not the sole or main player in non-photic entrainment. Nonetheless, there is evidence for a degree of non-photic modulation by the serotonergic pathway. For instance, serotonergic modulation has been shown to lead to similar inhibition of photic cues, consistent with other non-photic influences (Antle et al., 2003).

1.5.2 Cholinergic input from the basal forebrain

The basal forebrain is a major arousal area in the brain and receives inputs from several brainstem sleep-wake centers that also project to the circadian system (Bina et al., 1993; Brown et al., 2012; Lee & Dan, 2012). The basal forebrain receives cholinergic inputs from brainstem nuclei including the laterodorsal and pedunculopontine tegmenta and the SCN also receives brainstem cholinergic projections (Hallanger et al., 1987; Kiss & Halász, 1996). The study by Yamakawa et al. (2016) was the first to report direct cholinergic input in the hamster SCN and to highlight a significant functional link between a major arousal area and the circadian system. More specifically, c-Fos expression was observed directly in the basal forebrain in response to non-photic manipulations through, both novel wheel confinement, and sleep deprivation. Fos expression was present specifically in cholinergic cells, as evidenced by double labelling of c-Fos and choline acetyl transferase (ChAT), the enzyme responsible for the synthesis of acetylcholine (ACh; Yamakawa et al., 2016)

Intra-SCN injections of the ACh agonist carbachol during the subjective day have also been reported to lead to non-photic phase shifts (Basu et al., 2016; Bina & Rusak, 1996). Carbachol is a non-selective agonist and could exert its effects on the various nicotinic and muscarinic cholinergic receptors (M1-M5) that are all found in the SCN (Bina, Rusak, & Wilkinson, 1998; O'Hara et al., 1998). However, carbachol-induced phase shifts are blocked with pre-treatment of atropine, a muscarinic antagonist (Bina & Rusak, 1996). Administration of the M1/4 agonist McN-A-343 in the mid-subjective day results in phase advances that are comparable in magnitude to those induced by carbachol (Basu et al., 2016). However, intra-SCN injections of an M2/3 agonist fails to result in significant phase shifts. These findings provide

support for a significant contribution of acetylcholine in modulating arousal-induced phase shifting.

Both cholinergic and noncholinergic cells in the basal forebrain have been shown to project to the SCN, with a subset of SCN-projecting cholinergic cells also expressing c-Fos (Yamakawa et al., 2016). The majority of SCN-projecting cholinergic cells reside in the substantia innominata (SI), in the caudal basal forebrain (Bina et al., 1993). The cholinergic input to the SCN is both sufficient and necessary for non-photic phase shifting. Pre-treatment with atropine prior to wheel confinement significantly decreases phase shifts while electrical stimulation of the basal forebrain at mid-subjective day elicits significant phase advances (Yamakawa et al., 2016).

The finding that non-photic phase shifting can be blocked by atropine infusion in the SCN following electrical stimulation of the basal forebrain, provides strong support for the significance of the basal forebrain cholinergic input to non-photic entrainment (Yamakawa et al., 2016). However, it remains unknown how the basal forebrain gets activated and whether it interacts with other brain areas in the circadian circuitry that are also implicated in non-photic entrainment.

1.5.3 Adenosine and non-photic entrainment

Adenosine is a purinergic sleep factor that accumulates during wakefulness. Administration of adenosine promotes sleep, and its accumulation takes place in specific areas of the brain including the cortex and cortex-projecting neurons of the basal forebrain (Kalinchuk et al., 2011; Porkka-Heiskanen et al., 2000). There are four main subtypes of adenosine receptors, each of which are G-protein-coupled receptors that lead to diverse second messenger pathways (Dunwiddie & Masino, 2001). Like other non-photic stimuli, adenosine A_1 agonists attenuate

phase shifts to light (Elliott et al., 2001). As well, infusions of the A_1 receptor agonist N_6-cyclohexyladenosine (N-CHA) in the SCN cause significant non-photic phase shifts by inhibiting the activity of SCN neurons, while c-Fos expression is also characteristically reduced in the SCN following midday exposure to N-CHA (Antle et al., 2001).

Despite the above findings, it is unclear whether adenosine acts at the level of the SCN or whether it is involved in modulating the basal forebrain. Application of the specific adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dimethylxanthine (8-CPT) in the basal forebrain and cortex promotes wakefulness and decreases slow wave and rapid eye movement sleep (Strecker et al., 2000). Furthermore, adenosine infusions in the basal forebrain suppress the activity of cholinergic neurons in the substantia innominata (SI; Arrigoni et al., 2006).

1.5.4 Glycine and non-photic entrainment

SCN neural activity is also modulated by the inhibitory neurotransmitter glycine. Glycine activation of the chloride current of dissociated rat SCN cells is inhibited by the glycine ion channel antagonist strychnine (Ito et al., 1991). Fos expression is observed in the SCN shell region following application of exogenous glycine (Kawai et al., 2015). This contrasts with arousal-inducing cues that generally act to suppress SCN firing rate and gene expression during the inactive phase. Therefore, it is suggested that glycine acts to promote sleep by increasing SCN activity during the resting phase by modulating SCN glutamate receptors. Indirectly, glycinergic modulation of NMDA receptors is also suggested to lead to sleep-promoting changes in the periphery such as vasodilation which likely comes about through SCN outputs to other hypothalamic targets involved in regulating sleep and temperature (Kawai et al., 2015).

In vitro experiments in mice have reported non-photic phase shifting of SCN neurons following glycine administration (Mordel et al., 2011). Application of glycine gives rise to

characteristic non-photic shifts, with phase advances in the subjective day and delays in the subjective night. Phase shifts are blocked by administration of strychnine and the selective strychnine-sensitive receptor antagonist phenylbenzene ω-phosphono-α-amino acid (PMBA) suggesting that glycine may modulate non-photic responses through strychnine-sensitive glycine receptors in the SCN (Mordel et al., 2011). Also, at least one study has reported glycinergic input from the brainstem to the basal forebrain cholinergic neurons that can also project to the SCN (Bardoczi et al., 2017). Glycinergic modulation of the basal forebrain seems to be through strychnine-sensitive inhibitory post-synaptic potential currents. The presence of glycine in cholinergic cells suggests possible modulation of the basal forebrain arousal pathway.

1.6 The Intergeniculate Leaflet and Non-Photic Entrainment

A major source of input to the SCN is the intergeniculate leaflet (IGL) of the thalamus (Moore, 1996b; Moore & Card, 1994). The IGL is a long bilateral structure that lies in between the dorsolateral and ventrolateral geniculate nuclei of the thalamus. The IGL is described as a distinct region made up of morphologically diverse cell populations (Moore & Card, 1994). There is a direct projection from the IGL to the circadian clock, via the GHT (Morin & Blanchard, 2001). IGL cells that project to the SCN express a variety of neurochemicals, including NPY, GABA, enkephalin (ENK), and neurotensin (Morin & Blanchard, 2001). Importantly, arousal-inducing manipulations can increase c-Fos expression in the IGL (Mikkelsen et al., 1998).

NPYergic projection to the SCN is critical for mediating non-photic phase shifting and is by and large the most significant mediator of non-photic entrainment. Wheel confinement during the midday period results in a significant increase in c-Fos expression in NPY cells in the IGL (Janik et al., 1995) while intra-SCN injections of NPY gives rise to phase shifting that is similar to those seen with other non-photic manipulations previously described (Biello et al., 1994;

Huhman & Albers, 1994). NPY antiserum infusions to the SCN also significantly attenuate non-photic phase shifting in response to novel wheel-running (Biello et al., 1994). Intra-SCN NPY infusions can also block photic phase advances to light pulses administered at late subjective night (Weber & Rea, 1997). IGL lesions block phase shifts in response to wheel confinement while loss of NPYergic cells can eliminate arousal-induced phase shifts in response to stimuli (Janik & Mrosovsky, 1994; Maywood et al., 1997).

The non-photic effect of NPY at the SCN appears to be mediated by NPY Y_2 receptors in Syrian hamsters (Golombek et al., 1996; Huhman et al., 1996). Nevertheless, other NPY receptors including Y_1 and Y_5 have also been localized in the SCN while other studies have failed to report Y_2 receptors in the SCN of rats and mice (Fetissov et al., 2004; Wolak et al., 2003). However, Y_1 and Y_5 agonists fail to produce phase shifts while Y_2 receptor agonists lead to phase advances in the mid-subjective day (Huhman et al., 1996). Furthermore, Y_2 knockout mice are unable to phase shift following intra-SCN NPY injections (Soscia & Harrington, 2005).

Activation of NPY receptors involves G_q proteins and the activation of the primary effector enzyme phospholipase C β (PLC β; Biello et al., 1997). PLCβ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to the second messengers, diacyl glycerol (DAG) and inositol trisphosphate (IP_3). This leads to calcium release in the cytoplasm and activation of protein kinase C (PKC) by DAG. Pre-treatment with PKC inhibitors blocks NPY-induced phase shifts (Biello et al., 1997). However, blocking of sodium or calcium channels does not alter phase shifts to NPY, suggesting the influence of NPY at postsynaptic sites in the SCN. Therefore, non-photic phase shifting by NPY involves downstream second messenger pathways that can lead to altered neuronal activity and gene expression. NPY suppresses SCN neural activity and is also involved in downregulation of clock gene expression (Gribkoff et al., 1998; Maywood et al.,

2002). Other NPY receptor subtypes do not appear to mediate non-photic shifting in the SCN (Golombek et al., 1996; Huhman et al., 1996).

A subset of IGL cells are enkephalin-producing and project to the ventrolateral SCN as part of the GHT, potentially influencing non-photic phase shifting (Harrington, 1997; Moore & Card, 1994; Morin & Blanchard, 1995). Enkephalins are endogenous ligands of the body's opioid receptors, μ, δ, and κ (Byku et al., 2000). The IGL communicates with its contralateral counterpart through enkephalinergic neurons (Moore et al., 2000). Labelling of δ receptors in the SCN has shown the greatest distribution of the receptor in the ventral and medial regions, with staining revealing the location of receptors in presynaptic axon terminals (Byku et al., 2000). The presence of δ receptors in the medial SCN region suggests a role for ENK in regulating the activity of SCN cells (Abrahamson & Moore, 2001; Byku et al., 2000). Also, administration of δ receptor agonists result in phase advances in the mid-subjective day in hamsters, while κ receptor agonists fail to give rise to phase shifts. The exact mechanisms underlying enkephalinergic modulation of non-photic phase shifting remain unknown. Some studies have reported an indirect effect through an increase in locomotor activity following administration of receptor agonists while others have shown non-photic suppression of electrical activity in the SCN (Byku & Gannon, 2000; Vansteensel et al., 2005).

Another neurotransmitter present in nearly all IGL cells is GABA. GABAergic projections to the ventrolateral SCN are similar to and can overlap those of NPY and enkephalin (Harrington, 1997). Muscimol ($GABA_A$ agonist) injections in the IGL also lead to non-photic phase shifts that are independent from locomotor activity (Smith et al., 1989; Turek & Losee-Olson, 1986). It is unclear whether locomotor activity contributes to the non-photic influence of GABA given that some benzodiazepines including triazolam lead to increased locomotor activity

while others such as chlordiazepoxide do not (Biello & Mrosovsky, 1993; Turek & Losee-Olson, 1986). Injection of the competitive antagonist $GABA_A$ receptor bicuculline in the SCN block phase shifts to NPY (Mintz et al., 2002) suggesting the importance of GABA to NPY-mediated non-photic phase shifting.

A subset of IGL cells that project to the SCN also express neurotensin and SCN cell bodies contain the neurotensin receptors, neurotensin 1 and neurotensin 2 (Meyer-Spasche et al., 2002; Morin & Blanchard, 2001). Neurotensin has also been shown to increase cell firing rate in half of the rat SCN cells tested in vitro, while approximately 10% of cells respond with a decrease in their firing rate (Coogan et al., 2001). This contrasts with other manipulations such as NPY administration which tend to suppress SCN firing rate (Gribkoff et al., 1998). Furthermore, administration of neurotensin significantly reduces the duration of firing rate suppression mediated by NPY (Coogan et al., 2001).

1.6.1 Projections to the IGL from brain areas involved in entrainment of the clock

The IGL receives serotonergic input from the dorsal raphe nuclei and bilateral IGL injections of 8-OH-DPAT at midday have been shown to increase NPY release in the SCN (Glass et al., 2010). As well, bilateral intra-IGL injections of the $5\text{-}HT_{1A,2,7}$ receptor antagonist metergoline, blocked the release of NPY in the SCN in response to wheel running (Glass et al., 2010). Blocking of NPY release in the SCN was also demonstrated in the same study following administration of the $GABA_A$ agonist muscimol in the IGL. Taken together, these findings suggest that serotonergic input to the IGL modulates NPY release in the SCN which is critical to non-photic phase shifting. This modulation is presumed to occur through inhibition of the GABAergic cells of the IGL (Glass et al., 2010).

The IGL also receives cholinergic input from brainstem arousal centers (Moore & Card, 1994; Morin & Blanchard, 2005). Administration of the acetylcholine agonist carbachol in the hamster IGL results in consistent and previously characterized non-photic shifts (Cain et al., 2007). These shifts are still observed when carbachol is injected in surrounding areas outside the IGL, although their magnitude is smaller. Furthermore, pre-treatment with atropine blocks carbachol-induced phase shifts (Cain et al., 2007). Furthermore, orexin/hypocretin input to the IGL has been previously documented (McGranaghan & Piggins, 2001; Mintz et al., 2001). Orexin A and orexin B are hypothalamic neuropeptides that are derived from the precursor prepro-orexin and play a significant role in regulating arousal (de Lecea et al., 1998; Sakurai et al., 1998). There are two G-protein coupled orexin receptors found in the mammalian brain, orexin receptor 1 (OX_1) and orexin receptor 2 (OX_2; Sakurai et al., 1998). Orexin A and B are both capable of binding to both receptors, however orexin B has greater affinity to the OX_2 subtype. Furthermore, second messenger signaling pathways are likely mediated by the G_q class of G proteins that lead to mobilization of internal calcium stores (Sakurai et al., 1998).

Orexin-producing cells are found in the lateral hypothalamic area (LH) in various species, including rodents and humans (Chen et al., 1999; Nambu et al., 1999). Orexinergic fibers are found throughout the brain, including the hypothalamus, septal nuclei, and the thalamus, with dense projections throughout the rostro-caudal extent of the IGL (McGranaghan & Piggins, 2001; Nambu et al., 1999). Orexin has been implicated in a variety of physiological processes including modulation of arousal and feeding activity. Intraventricular orexin A infusions can modify feeding in a diurnal pattern with decreased feeding activity occurring during the night (Haynes et al., 1999). Also, there is a day-night variation in the expression of prepro-orexin mRNA in the LH with peak expression in the early morning period (Estabrook et

al., 2001; Haynes et al., 1999; Taheri et al., 2000). Orexin release is most prominent during the subjective night, the period of activity in nocturnal rodents such as rats and mice (Deboer et al., 2004).

There is also greater release of orexins during the active period of rodents (nighttime; Deboer et al., 2004). Orexin neurons are active during wakefulness and c-Fos expression in orexinergic neurons is positively correlated with the amount of wakefulness (Estabrook et al., 2001).Orexin A fibers have been found in the SCN periphery but not in its core region (McGranaghan & Piggins, 2001). However, a study in mice has found further orexinergic innervation of the SCN as well as the presence of OX_1 and OX_2 receptor mRNA(Belle et al., 2014). Orexin has a mainly excitatory postsynaptic effect, although it hyperpolarizes SCN neurons during the night (Belle et al., 2014). More specifically, orexin A decreases intracellular calcium levels and suppresses SCN neuronal activity with a day-night variation in the recruitment of inhibitory signaling in the SCN (Belle et al., 2014). However, orexin A application in the SCN does not lead to non-photic phase shifts (Belle et al., 2014).

1.7 Current Study: Overview and Objectives

Given the presence of a dense plexus of orexinergic fibers in the IGL, it is likely that orexin may be important for modulating IGL activity such as by influencing NPYergic projections to the circadian clock. More specifically, orexin can influence the activity of IGL NPYergic inputs at the level of the SCN (Belle et al., 2014). Like other non-photic cues, NPY suppresses SCN cell activity and orexin has been shown to enhance this suppression (Belle et al., 2014). Orexinergic input to IGL neurons has also been shown to regulate the basal firing rate of IGL neurons and to depolarize neurons through activation of non-selective cation channels (Palus-Chramiec et al., 2019). This may be the mechanism by which activation of NPYergic cells in the IGL occurs. NPYergic projections to the circadian clock can then directly affect SCN

cellular activity. The findings from the literature therefore demonstrate a possible role for orexin in modulating non-photic entrainment of the circadian clock by the IGL.

Since it has also been suggested that cholinergic input from the basal forebrain is necessary for non-photic entrainment (Yamakawa et al., 2016), it is important to examine the role of acetylcholine at the level of the IGL. This has not yet been undertaken and is important for better understanding the overall non-photic circuitry. This can aid in firmly establishing a link between arousal systems and the circadian system. There are questions that remain regarding the activation of the basal forebrain and cholinergic modulation of the SCN. Furthermore, it remains unclear whether the basal forebrain communicates with other components of the circadian circuitry, such as the IGL.

Although the role of the IGL as non-photic modulator of the clock is well known, it is not entirely clear how the IGL interacts with sleep-wake centers such as the basal forebrain. There are cholinergic inputs to the IGL from brainstem nuclei (Moore et al., 2000; Morin & Blanchard, 2005). However, these areas have not been implicated in non-photic entrainment. Nonetheless, arousal seems to be the key non-photic stimulus and given the importance of the IGL in modulating non-photic responses, it is likely that this area communicates with one or more arousal centers to bring about this modulation. The main arousal centers and their neurotransmitters to consider are orexinergic input from the lateral hypothalamus, and cholinergic input from the basal forebrain.

The aims of the proposed project are to understand how the IGL gets activated, and to investigate a link between the IGL and arousal areas of the brain. Inputs to the IGL that are both necessary and sufficient for non-photic entrainment are not well defined and in order to understand arousal-induced phase shifting, it is important to know where in the pathway, from

behavioral arousal to resetting of the SCN rhythm, gating takes place. To these ends, several experiments have been devised. Chapter 2 consists of a tract tracing study for the purpose of cataloging arousal centers that communicate with the IGL. Given past findings, it is hypothesized that there is a direct cholinergic projection from the basal forebrain to the IGL. Furthermore, in support of what has already been found, we expect to see orexinergic input from the LH also. Chapter 3 examines whether orexin is necessary and sufficient for non-photic entrainment. If so, then it is predicted that intra-IGL infusions of orexin should be enough to cause phase shifts. As well, blocking the activity of orexin should block phase shifts in response to an arousing procedure such as sleep deprivation. Furthermore, it is hypothesized that IGL-projecting orexinergic cells of the LH will be significantly activated in response to an arousal procedure.

Chapters 4 and 5 focus on the role of the basal forebrain in non-photic entrainment, with the former studying the influence of acetylcholine on phase shifts and IGL activation while the latter narrows in on a possible mechanism of basal forebrain activation. Given the existence of multiple arousal centers and their connections to the circadian clock, it is hypothesized that acetylcholine is another potential necessary signal for non-photic entrainment. If so, then it is predicted that blocking acetylcholine at the level of the IGL should lead to an attenuation of the non-photic response. Finally, activation of the basal forebrain by blocking adenosine at the source is examined. Given that adenosine accumulation seems to suppress the cholinergic cells of the basal forebrain, it is predicted that blocking adenosine with an antagonist is enough to cause phase shifts. Also, it is predicted that this will lead to activation of cholinergic cells of the basal forebrain.

Chapter Two: Inputs to the Intergeniculate Leaflet from Arousal Centers of the Brain

2.1 Introduction

In constant environmental conditions, organisms' endogenous biological cycles are capable of remaining rhythmic owing to the presence of an internal clock. In mammals, the suprachiasmatic nucleus (SCN) in the hypothalamus is a paired structure that houses individually rhythmic cells and acts as the body's main timekeeping center (Antle & Silver, 2005; Holzberg & Albrecht, 2003). This independent rhythmicity persists in the absence of environmental cues, referred to as zeitgebers (Dunlap et al., 2004). Although the light-dark cycle is the dominant entraining cue among terrestrial organisms, there are a whole host of other stimuli, collectively referred to as non-photic zeitgebers that can shift the phase of the SCN, including temperature, social interactions, food, exercise and sleep deprivation (Mistlberger & Antle, 2011; Mrosovsky, 1988, 1996).

Depending on the type of zeitgeber and the time of delivery, there can be different changes in the SCN phase. Regarding non-photic entrainment, a cue delivered during the subjective day (period that the animal considers as day in the absence of external environmental cues) causes a phase advance (the animal has a delayed onset of activity on the subsequent day) whereas exposure to a non-photic cue during the subjective night (period perceived by the animal as night; in the case of a nocturnal animal, this is the active period) causes phase delays (Glass et al., 2001; Hut et al., 1999). The SCN receives inputs from many brain areas that have been implicated in non-photic entrainment including the intergeniculate leaflet (IGL), the basal forebrain and the raphe nuclei (Bina et al., 1993; Meyer-Bernstein & Morin, 1996; Moore & Card, 1994; Yamakawa et al., 2016).

The IGL is a bilateral thalamic structure housed in the lateral geniculate complex and lies in between the dorsolateral geniculate nucleus (dLGN) and the ventrolateral geniculate nucleus

(vLGN). Historically, the IGL was considered as a part of the vLGN but with further examination, it was recognized as a distinct region characterized by a morphologically diverse population of cells (Hickey & Spear, 1976; Moore & Card, 1994). There are bilateral projections to the IGL from the retina and information about light reaching the eyes is indirectly sent to the SCN via the geniculohypothalamic tract (Hattar et al., 2002; Moore & Card, 1994). IGL cells that project to the SCN nearly all produce γ-aminobutyric acid (GABA), and can also express neuropeptide Y (NPY), enkephalin, and neurotensin (Morin & Blanchard, 2001).

NPYergic IGL input to the SCN is critical for mediating non-photic phase shifting. Confinement to a running wheel during the midday period results in a significant increase in the immediate early gene *Fos* in NPY cells of the IGL (Janik et al., 1995) while intra-SCN injections of NPY cause phase shifts that are comparable to those seen with other non-photic manipulations previously described (Biello et al., 1994; Huhman & Albers, 1994). Injection of NPY antiserum in the SCN also significantly attenuates non-photic phase shifting in response to novel wheel-running (Biello et al., 1994). Photic phase shifting can be blocked by application of NPY as shown in vitro, because of its antagonizing interaction with glutamate (Biello et al., 1997). Furthermore, NPY infusions in the SCN block photic phase advances to light pulses administered at late subjective night (Weber & Rea, 1997). This highlights the role of the IGL NPYergic projections in non-photic entrainment given the manner of its interaction with photic cues. Furthermore, IGL lesions give rise to smaller phase shifts to wheel confinement protocols and loss of NPYergic cells eliminates arousal-induced phase shifts to certain stimuli (Janik & Mrosovsky, 1994; Maywood et al., 1997).

Although the role of the IGL as a non-photic modulator of the circadian clock is well known, it is unknown how the IGL interacts with arousal centers in the brain including the basal

forebrain which contains cholinergic cells, and the lateral hypothalamus (LH), an area that produces orexin. The neuropeptide orexin is involved in a variety of physiological processes such as arousal, feeding activity and motivation (Haynes et al., 1999; McGranaghan & Piggins, 2001). There are cholinergic inputs to the IGL from brainstem nuclei (Moore et al., 2000; Morin & Blanchard, 2005). However, these areas have not been implicated in non-photic entrainment. Nonetheless, knowing that arousal is the main non-photic stimulus and given the importance of the IGL in modulating non-photic responses, it is likely that this area receives input from one or more arousal centers that are instrumental in influencing its modulation of non-photic entrainment.

The aim of the present study was to obtain a catalogue of sleep and arousal brain areas that communicate with the IGL. Specifically, the goal was to confirm and highlight direct afferent projections to the IGL from the basal forebrain and LH. Several arousal areas including several brainstem nuclei, and the LH have been observed as either receiving and/or sending projections to the IGL (Moore et al., 2000; Vidal et al., 2005; Vrang et al., 2003). However, there are some discrepancies among the various sources and some brain areas that are documented in one study are not listed or well-described in another source. Therefore, it is important to start with a tract tracing study to first look for possible projections to the IGL from areas such as the basal forebrain and LH. Although the latter has been better described as a source of input to the IGL (Vidal et al., 2005), it is less clear whether cholinergic cells, particularly in the substantia innominata (SI; a collection of cholinergic cells in the basal forebrain), are communicating with the IGL.

Given that cholinergic input to the SCN from the basal forebrain represents another source of non-photic modulation of the circadian clock (Yamakawa et al., 2016), it is likely that

this area also sends projections to the IGL and may be involved in NPYergic modulation of non-photic phase shifting (Cain et al., 2007). Using a tract tracing technique, the beta subunit of the cholera toxin (CTβ) was injected into the IGL of Syrian hamsters with the goal of collecting and staining the brain for choline acetyltransferase (ChAT), the enzyme that synthesizes acetylcholine, as well as orexin. It was hypothesized that there would be an input to the IGL from cholinergic cells of the basal forebrain. More specifically, it was proposed that double fluorescence immunohistochemical labelling of CTβ with ChAT would be observed in the basal forebrain, particularly in the SI where there is a dense collection of cholinergic cells. Similarly, it was predicted that there would be orexin labelling throughout the IGL, directly from the LH.

2.2 Methods

2.2.1 Animals

A total of 14 male Syrian hamsters (*Mesocricetus auratus*, 80-90g) were obtained from Charles River Laboratories (Kingston, NY, USA). As it was difficult to accurately target the IGL brain region, animals were continuously being added to the experiment. At least 4 females were also used but issues with the surgery did not allow for staining of the brains so they were not included in the total animal count. Animals were housed in a temperature and humidity-controlled room with a 14:10 light dark cycle. All animals were initially paired in cages and provided with food and water *ad libitum*. All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to policies of the Canadian Council of Animal Care. Following surgeries, each animal was transferred to an individual cage and allowed to recover for a period of one week under the same conditions. Animals were then euthanized and perfused after the seven-day survival period following surgery.

2.2.2 Surgeries

Prior to surgery, hamsters were administered a subcutaneous injection of the analgesic butorphanol (2 mg/kg; Wyeth, Madison, NJ, USA) and an intraperitoneal injection of the anesthetic sodium pentobarbital (90 mg/kg; CEVA Santé Animale, France), their head was shaved, and they were placed in the stereotaxic frame. The surgical procedure mostly followed that of a previously described protocol (Yamakawa et al., 2016). The incisor bar was set at 2.0 mm below the interaural level. Following incision of the scalp, a burr hole was drilled through the skull and a borosilicate capillary glass tube (with a tip of approximately 28-30 µm tip; with filament, original outer diameter:1.50 mm, inner diameter: 0.86 mm; Warner instruments, Hamden, CT, USA) was backfilled with CTβ (1% dissolved in dH_2O; List Biological Laboratories Inc, Campbell, CA, USA). An iridium and platinum (10% wt/wt) wire was carefully inserted into the glass tube. The lead electrode of a BAB-150 iontophoresis machine (Kation Scientific, Minneapolis, MN, USA) was attached to the wire while the ground electrode was attached to a skin flap on the animal. The coordinates used for unilateral tube insertion in the IGL were as follows: 1.9 mm posterior to bregma, 3.3 mm lateral to bregma and 4.6 mm ventral to dura. These coordinates were adapted from IGL coordinates used for cannulation surgery in experiments elsewhere in this thesis (adapted from Mintz et al., 1997; Yamakawa et al., 2016). The glass tube was slowly lowered through the hole under a continuous negative retaining current of -1.0 µA in order to avoid CTβ leakage along the tube track. Iontophoresis of CTβ occurred over a period of 10-12 minutes (positive current, 1.5µA, 5 second on/5 second off pulses). Subsequently, the current was turned off while the capillary tube was left in place for a period of 15 minutes. The tube was then slowly raised under a continuous negative retaining current of -1.0µA. Prior to suturing, some bone wax was used to fill up the hole in the skull.

2.2.3 Perfusions and brain slice preparations

Hamsters were administered an overdose of sodium pentobarbital (CEVA Santé Animale, France) and were then transcardially perfused with ~50ml of cold phosphate buffered saline (PBS) and subsequently, ~50ml of cold 4% paraformaldehyde in PBS. Brains were extracted and post-fixed at 4°C in 4% paraformaldehyde overnight and then transferred to 20% sucrose in PBS solution for 24 hours. Brains were then transferred to 30% sucrose and stored for another 24 hours. Storage in sucrose served the purpose of cryoprotecting the tissues until they were ready to be sliced. Brains were cut with the use of a Leica cryostat set at a temperature of -21°C, at a thickness of 35 microns. Alternate sections were collected into separate wells filled with PBS.

2.2.4 DAB immunohistochemistry

Sections were collected throughout the extent of the rostrocaudal IGL for purpose of confirming the CTβ infusion in the IGL. Brain sections were initially rinsed for 25 minutes in 0.5% (vol/vol) H_2O_2 in 0.3% Triton X-100 in PBS (PBSx) for the purpose of inactivating endogenous peroxidases. Sections were exposed to three 10-minute washes in 0.3% PBSx, followed by a 90-minute incubation at room temperature in blocking buffer (5% normal goat serum (NGS) in PBSx). Sections were further incubated for 48h in the CTβ primary antibody (mouse anti-CTβ, 1:5 Bio-Rad Laboratories, Hercules, CA, USA) diluted in blocking buffer (2% NGS in PBSx) at 4°C. The tissue was again rinsed in PBSx prior to a 90-minute incubation at room temperature in the secondary antibody (biotinylated goat anti-mouse, 1:200 in PBSx; Vector Laboratories, Burlingame, CA, USA). This was followed by more rinses with PBSx (3 x10 min each) and a 60-minute incubation at 4°C, in an avidin-biotin complex (1:100 Vector Vectastain Elite ABC kit, Vector Laboratories). A final set of PBSx rinses was performed and section were then developed with a 0.4% diaminobenzidine solution (DAB) with DAB as the

chromagen (12.5mg/mL in 0.1 M Tris buffer, 80 mg/mL of nickel chloride, and hydrogen peroxide). Sections were finally exposed to a series of rapid PBSx rinses and were mounted onto gelatin—coated microscope slides. Slide-mounted sections were dehydrated in an alcohol series (70% to 100% EtOH), cleared with xylene and coverslipped with Permount.

2.2.5 ChAT/Orexin (A+B) immunohistochemistry

Alternate sections (one 35 micron-thick section was kept and the next discarded) were collected through the basal forebrain, the lateral hypothalamus (LH), and IGL. For each brain area, sections were collected in separate wells. The alternate section of one brain region was kept as the section for another brain section given that these brain regions are all in close proximity. The basal forebrain and IGL, in particular, are long structures and sections were collected for the entire length. However, the part of the substantia innominata (SI) where cholinergic cells are clustered (area of interest for this work) is smaller and was the focus for the staining. Sections containing the SI were double labelled for CTβ (mouse 1:3000, Bio-Rad Laboratories, Hercules, CA, USA) and ChAT (goat 1:500, Millipore, Burlington, MA, USA). The secondary antibodies used were CY-2 donkey anti-mouse and CY-3 donkey anti-goat (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), all in 0.3% PBSx. Sections were initially rinsed in a series of PBSx washes (3x 10 min) and then incubated for 60 min in blocking buffer (5% normal donkey serum (NDS) in PBSx). Subsequently, sections were incubated for 48h at 4º C in the primary antibodies diluted in blocking buffer (2% NDS). Another round of PBSx washes (6x10 min) was followed by a 1h incubation in the secondary antibodies. Sections were protected from light from this point forward. The slides were rinsed in PBSx a final time (3x10 min) and were mounted on gelatin-coated slides. Slides were cleared with xylene and coverslipped with Krystalon. Labelling for CTβ and orexin (OX; goat anti-OXA, goat anti-OXB, 1:5000, Santa

Cruz, Dallas, TX, USA) was conducted separately for sections containing the LH and followed the procedure above with the exception that all brain sections were used for staining.

2.2.6 Imaging and analysis of brain sections

Analysis of section-mounted slides was conducted using an Olymppus BX51 microscope. Images were captured with a QI CAM Fast 1394 cooled CCD camera (QImaging, Burnaby, BC, Canada) and observed/analyzed using Image-Pro Plus software (Media Cybernetics, Inc. Rockville, MD) for double-labeled fluorescent immunohistochemistry with either CTβ and ChAT, or CTβ and OX_{A+B}. Cells were considered to be double labeled if the CTβ immunoreactivity of one channel matched the cytoplasmic shape defined by either the ChAT or OX_{A+B}, immunoreactivity, on their respective channels.

2.3 Results

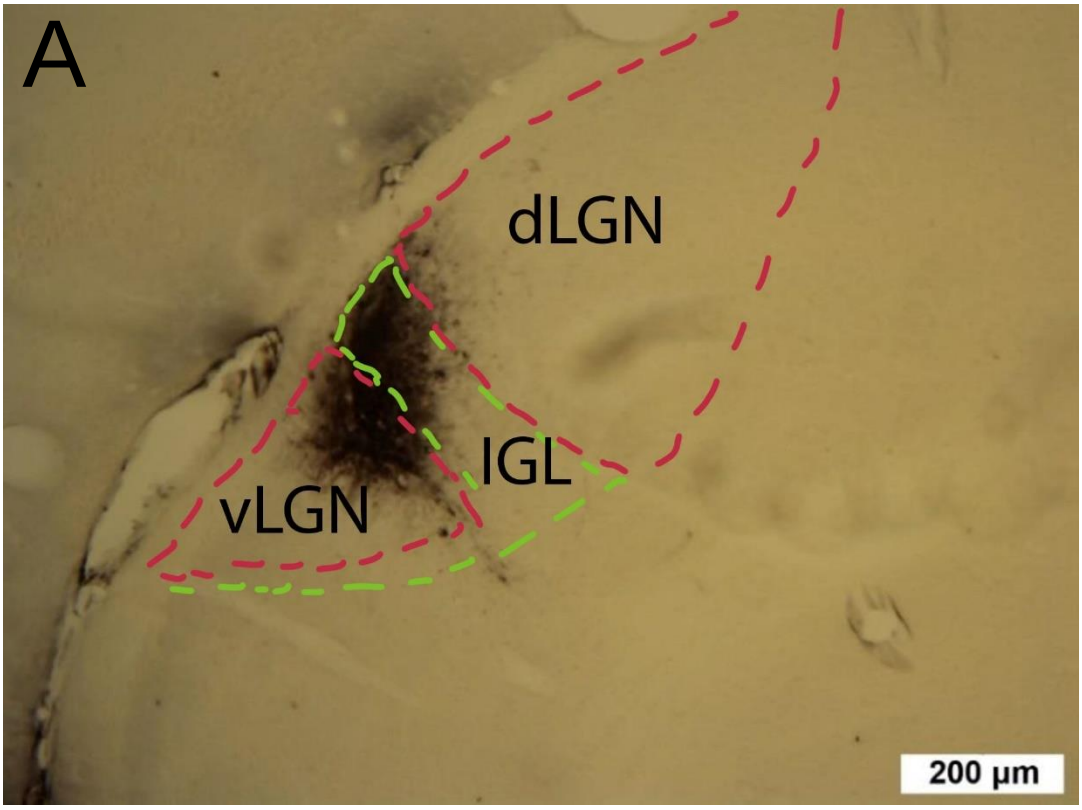
Overall, two out of the fourteen animals that underwent the tract tracing surgeries successfully received CTβ tracer in the mid IGL area. For the rest of the animals, either the tracer was deposited rostral or lateral to the IGL and/or no infusion was detected owing to issues with the iontophoresis pump. These surgeries took place continuously for the goal of getting more successful hits, but time constraints did not allow for further successful tracing. Through trial and error, coordinates of the surgery and other parameters were modified to hit the target location of the IGL with the glass tube. At any one time, surgeries were conducted on multiple animals and a successful hit was confirmed by at least two or more brains. Successful surgical injections were confirmed by observing CTβ DAB immunoreactivity (Figure 2.1A).

As shown in Figure 2.2, the IGL receives inputs from cholinergic cells in the basal forebrain as well as orexinergic inputs from the LH. CTβ ChAT double labelled cells are shown to be present in the basal forebrain, particularly localized to the SI. There were cells observed throughout the basal forebrain, but sections were particularly targeting the SI where there is a

collection of cholinergic cells, and this is where the most double labelled cells were found. As well, CTβ OX double labelled cells were also observed in the LH. A dense collection of orexinergic fibers was also found throughout the IGL's rostrocaudal extent (Figure 2.1B).

Figure 2.1. Successful CTβ infusion targeting the intergeniculate leaflet (IGL).

(A) Photomicrograph (10x magnification) of a representative hamster coronal slice (n=2) showing cholera toxin (CTβ) infusion in the mid IGL as evidenced by diaminobenzidine (DAB) immunocreactivity. The area of dark staining represents the site of infusion **(B)** Photomicrographs (10x magnification) of representative hamster coronal slices showing CTβ staining denoting the IGL and dense orexin (OX_{A+B}) fibers throughout the IGL as evidenced by fluorescent OX_{A+B} immunoreactivity; dLGN, dorsolateral geniculate nucleus; vLGN, ventrolateral geniculate nucleus.


A
dLGN
IGL
vLGN
200 µm

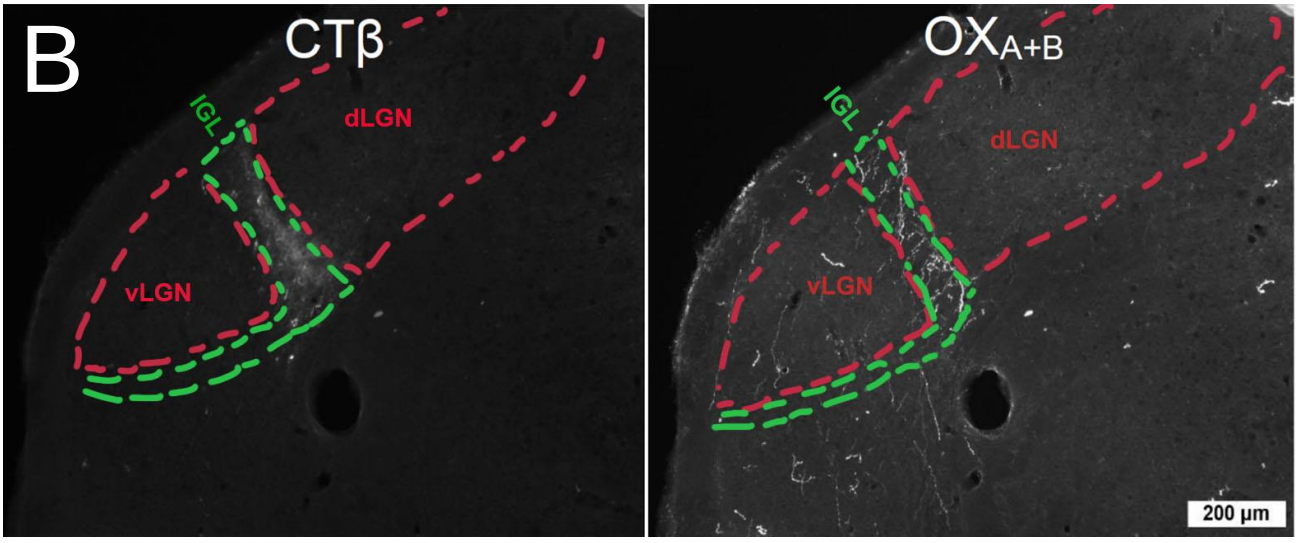
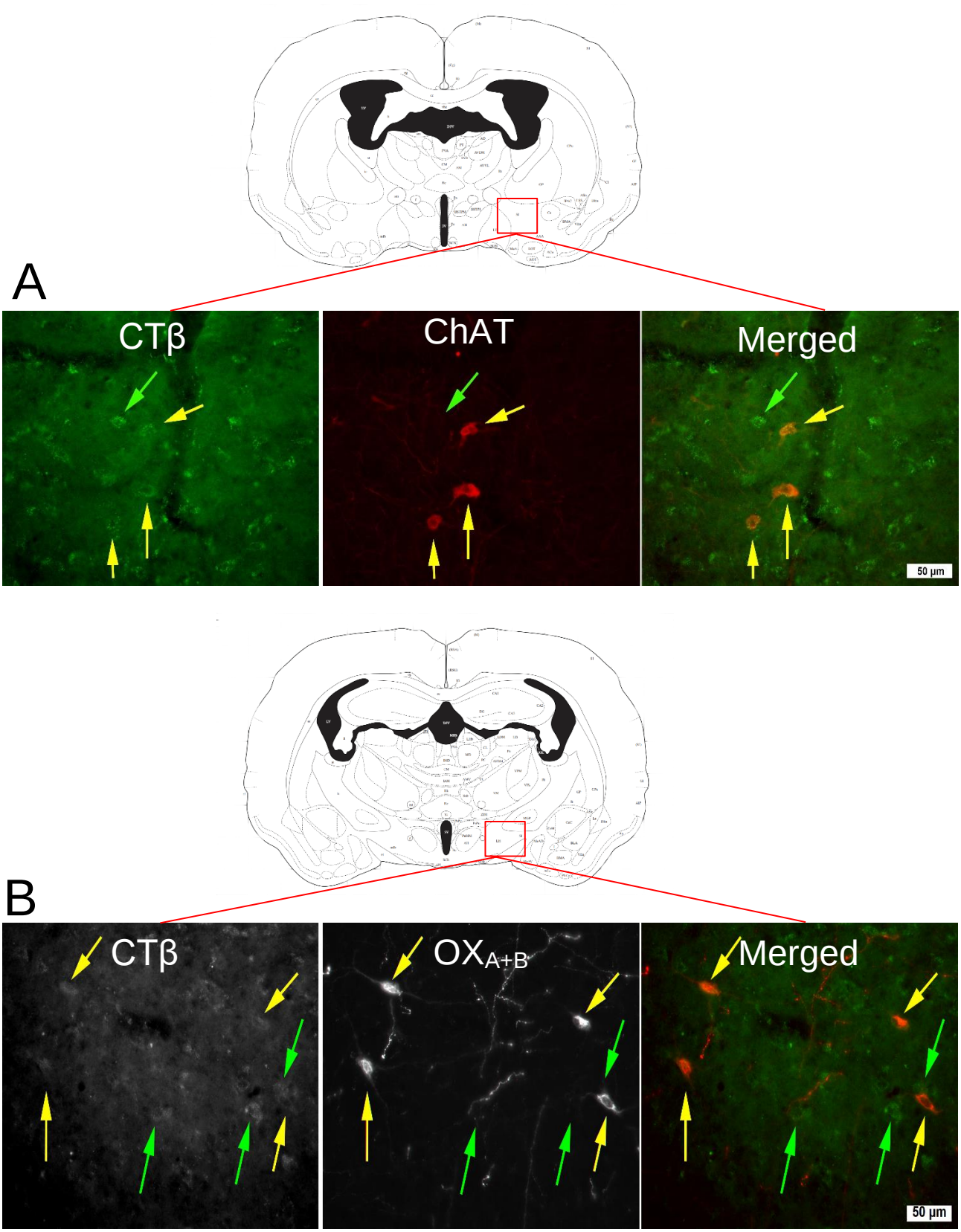

B
CTβ
IGL
dLGN
vLGN
OXA+B
IGL
dLGN
vLGN
200 µm

Figure 2.2. Fluorescent labelling of orexinergic and cholinergic projections to the intergeniculate leaflet (IGL). Photomicrograph (10x magnification) of representative hamster coronal slices showing double fluorescent labelling of **(A)** cholera toxin (CTβ) and choline acetyltransferase (ChAT) in the substantia innominata (SI) and **(B)** CTβ and orexin A+B (OX_{A+B}) in the lateral hypothalamus (LH). Green arrows point to single cells while yellow arrows point to a double-labelled cell. A cell that is double-labelled has the same shape and size on the CTβ panel as the same cell as viewed on the ChAT or OX_{A+B} panel. Coronal views of the brain areas are shown with a red box highlighting the zoomed in area for panels in (A) and (B).


A
CTβ
ChAT
Merged
50 µm
B
CTβ
OXA+B
Merged
50 µm

2.4 Discussion

The aim of this study was to visualize projections between the IGL and important arousal centers in the brain, including the basal forebrain and lateral hypothalamus. It was important to trace these areas to the IGL to establish the link between non-photic entrainment and arousal. Although the IGL's role in non-photic entrainment has been well accepted in the circadian research community, the IGL has not been defined as an arousal brain region. However, ample evidence suggests that the non-photic pathway is principally governed by arousal-inducing stimuli (Antle & Mistlberger, 2000; Marston et al., 2008; Yamakawa et al., 2016). To this end, CTβ was used as a retrograde tracer and the basal forebrain and LH were stained for double labelling of CTβ with ChAT and OX_{A+B}, respectively.

To our knowledge, this is the first report of a link between the cholinergic cells of the basal forebrain and the IGL. Specifically, CTβ and ChAT double labelled cells were identified throughout the basal forebrain, particularly localized in the substantia innominata region. The SI is the region of the basal forebrain that contains the greatest cluster of cholinergic cells (Bina et al., 1993) and this can be used for delineating this area in brain slices under the microscope. This finding provides support for the link between the IGL and the basal forebrain arousal center and provides evidence for potential cholinergic influence on the IGL's non-photic modulation of the circadian clock. It has previously been reported that the IGL receives cholinergic input from brainstem arousal centers (Moore et al., 2000; Morin & Blanchard, 2005). However, a functional role for this input pathway has not been described.

Given that cholinergic input from the basal forebrain to the SCN is both sufficient and necessary for non-photic phase shifting (Yamakawa et al., 2016), it is likely that the basal forebrain cells are also important for the role of the IGL in non-photic entrainment. It has been previously reported that intra-IGL injections of the acetylcholine receptor agonist carbachol

result in non-photic shifts while pre-treatment with the antagonist atropine blocks these shifts (Cain et al., 2007). Activation of IGL NPYergic non-photic modulation of the clock may be mediated by acetylcholine. To better understand this, it is important to follow up with an experiment looking to see whether atropine can block phase shifts to other arousal-inducing procedures such as sleep deprivation. This would provide evidence for the necessity of cholinergic input from the basal forebrain to the IGL in non-photic entrainment.

The results of this experiment are in support of past studies showing a dense plexus of orexinergic fibers throughout the rostrocaudal extent of the IGL (McGranaghan & Piggins, 2001; Mintz et al., 2001). This dense collection of fibers was most evident in the mid IGL region which is where cannulas and capillary tubes are aimed at based on the protocols used in this study and in the past. Furthermore, a collection of CTβ and OX_{A+B} double labelled cells were visualized in the LH as reported previously (Vidal et al., 2005). Although the study by Vidal et al (2005) reported sparse labelling of orexinergic cells that project to the IGL, dense double labelling was observed in this experiment. However, not all orexinergic or other cell types of the LH were visualized and so it is not certain what proportion of orexinergic cells project to the IGL. Nonetheless, cells were identified in most of the LH sections that were collected. This may have also been the case as labelling was undertaken for both orexin A and orexin B. This is particularly important considering the recent study showing that the IGL is unique in expressing both orexin A and B receptors, in contrast to other parts of the thalamus (Chrobok et al., 2020). To inquire about the influence of orexinergic input to the IGL in non-photic entrainment, it will be important to follow up this study by observing the effect of orexin administration in the IGL and measuring non-photic phase shifts. Furthermore, following a non-photic manipulation, it is important to observe c-Fos expression in orexinergic cells of the LH. Fos expression is

upregulated in the IGL following non-photic protocols such as wheel confinement (Janik et al., 1995). Furthermore, c-Fos expression increases in orexin-labelled cells in response to non-photic manipulations including wheel running and gentle handling (Webb et al., 2008). Therefore, upregulation of c-Fos in orexinergic neurons can implicate orexin in non-photic entrainment and provide a further link between the IGL and the LH.

It should be noted that the IGL also receives cholinergic input from the brainstem arousal areas and a catalogue of the various nuclei including the laterodorsal tegmental nucleus and the pedunculopontine tegmental nucleus would complement the findings of this study (Moore et al., 2000; Morin & Blanchard, 2005). Nonetheless, the basal forebrain remained the area of focus for cholinergic input given the functional significance of this area in modulating non-photic entrainment in the circadian clock (Yamakawa et al., 2016). The IGL also receives serotonergic (5-HT) input from the dorsal raphe and injections of the 5-$HT_{1A/7}$ agonist 8-OH-DPAT at midday results in an increase in NPY release in the SCN (Glass et al., 2010). These findings indicate that serotonin input to the IGL likely modulates NPY release in the SCN and is therefore implicated in non-photic entrainment. Given the established finding of serotonergic input to the IGL, this study focused on orexinergic and cholinergic inputs instead.

In summary, this study has shown the presence of a forebrain cholinergic input and a hypothalamic orexinergic input to the IGL. However, the functional role of these neurotransmitters in non-photic entrainment is not fully clear. Previous research and the findings of this study suggest that several neurotransmitters are likely involved in modulation of the IGL, in particular the NPYergic cells that project to the SCN. Arousal-induced non-photic entrainment causes a downregulation both in gene expression and electrical activity at the level of the SCN (Maywood & Mrosovsky, 2001; Meijer et al., 1997; Mendoza et al., 2004; Oosterhout et al.,

2012). NPY suppresses SCN cell activity and orexin has been shown to enhance this suppression. It is possible that input from arousal centers in the brain act directly to enhance the effect of NPY at the SCN. Since there are several arousal centers, each of these pathways may be a contributor (either through additive or redundant effects) to non-photic entrainment. Given the antagonizing relationship of photic and non-photic cues, it is possible that arousal centers send signals to activate the IGL and allow it to block responses to light by suppressing cellular activity. This is in line with recent findings showing that orexinergic input from the LH targets retinorecipient IGL neurons (Chrobok et al., 2020). If so, the functional link between the arousal centers and the IGL may be the modulation or inhibition of photic input to the circadian clock.

Chapter Three: Orexin Infusions in the Intergeniculate Leaflet to Examine Non-Photic Phase Shifting

3.1 Introduction

Orexin A (OX_A) and orexin B (OX_B) are hypothalamic neuropeptides that play an important role in regulating many physiological functions including sleep-wake states, feeding, sexual behaviour, and reward and motivation (Boutrel et al., 2005; Chemelli et al., 1999a; de Lecea et al., 1998; Muschamp et al., 2007; Sakurai et al., 1998; Yamanaka et al., 1999). Expression of the immediate early gene product c-Fos in orexin neurons is positively correlated with time spent awake while destruction of orexinergic cells leads to narcolepsy, a sleep disorder characterized by persistent drowsiness and the inability to stay awake or regulate sleep-wake cycles (Chemelli et al., 1999b; Estabrook et al., 2001; Thannickal et al., 2000).

Orexinergic cells are found in the lateral hypothalamus (LH) in various species, including rodents and humans and target G-protein coupled receptors (OX_1 and OX_2) found throughout the brain (Sakurai et al., 1998). OX fibers have been visualized in the hypothalamus, septal nuclei, and the thalamus, including in the areas of the brain that are critical for the production and maintenance of circadian rhythms such as the suprachiasmatic nucleus (SCN; McGranaghan & Piggins, 2001; Nambu et al., 1999). The SCN is a paired structure in the anterior hypothalamus and serves as the main endogenous pacemaker of the body (Antle & Silver, 2005; Dunlap et al., 2004; Miller et al., 1996). The SCN allows mammals to keep an internal rhythm that is free-running, independent of changing environmental conditions. However, many signals, including light (photic) and non-photic stimuli, can synchronize (entrain) the phase of the SCN (Daan & Pittendrigh, 1976; Pittendrigh & Daan, 1976).

The SCN increases the activity of orexinergic cells so that OX release is most prominent during an organism's period of activity (Deboer et al., 2004). Both OX_1 and OX_2 receptors have

been reported in the mouse SCN and OX_A specifically has been shown to suppress the activity of SCN cells during the night (Belle et al., 2014; McGranaghan & Piggins, 2001). This contrasts with the mainly excitatory influence of OX in other areas of the brain. Many OX neurons are glutamatergic which is consistent with its excitatory effects (Rosin et al., 2003). Expression of c-Fos in OX-labelled cells of the intergeniculate leaflet (IGL), a thalamic input pathway to the SCN, increases in response to non-photic manipulations including wheel running and gentle handling (Webb et al., 2008). The IGL is a bilateral region of the lateral geniculate complex that mediates both photic and non-photic entrainment (Hattar et al., 2002; Huhman & Albers, 1994; Janik et al., 1995; Moore & Card, 1994). IGL projections to the SCN mainly consists of γ-aminobutyric acid (GABA)ergic cells that co-express neuropeptide Y (NPY) and are critical for non-photic phase shifting (Janik et al., 1995; Morin & Blanchard, 2001). NPY infusions in the SCN result in non-photic phase shifts and non-photic manipulations such as wheel confinement during the midday result in a significant increase in c-Fos expression in NPY cells of the IGL (Biello et al., 1994; Huhman & Albers, 1994; Janik et al., 1995).

Orexin input to the IGL has been previously documented (McGranaghan & Piggins, 2001; Mintz et al., 2001) and it has been suggested that OX can directly influence the activity of IGL NPYergic inputs at the level of the SCN. NPY has an inhibitory effect on SCN cellular activity and orexin likely enhances this suppression (Belle et al., 2014). A more detailed functional link between OX and the circadian system has also been suggested. Specifically, OX may contribute to the maintenance of IGL neuronal activity through the activation of non-selective cation channels and by allowing NPY cells to receive and send signals to the SCN (Palus-Chramiec et al., 2019). The influence of OX in the IGL may help explain the mechanism by which the IGL gets activated and participates in non-photic entrainment.

Although the IGL has been established as an important non-photic modulator of the clock, it is not fully clear how the IGL gets activated by arousal procedures. Input from several brain areas involved in arousal such as the LH may be the source of IGL activation to bring about entrainment. If orexins play a role, then intra-IGL infusions of exogenous OX should be sufficient in causing phase shifts. Furthermore, if OX is necessary for non-photic entrainment, then blocking OX prior to an arousal-inducing manipulation should disrupt non-photic entrainment and decrease the phase shift response. Given what is known about c-Fos expression in response to non-photic stimuli, it is important to also observe levels of c-Fos in the IGL. Arousal-inducing manipulations lead to a significant elevation in c-Fos levels in OX neurons (Webb et al., 2008). However, in this study, a significant increase in c-Fos levels was also observed in response to a stressful manipulation such as physical restraint (Webb et al., 2008). This procedure has been previously documented and is unable to induce phase shifts (Mistlberger et al., 2003). Therefore, it has been suggested that OX is not sufficient for non-photic entrainment (Webb et al., 2008).

This study aimed to investigate a link between the IGL and the orexin arousal system in the LH. The main goal was to see if OX is both necessary and sufficient for non-photic phase shifting and to compare some of the findings with what has been previously shown regarding cellular activation of orexinergic neurons in response to non-photic manipulations (Hoyer & Jacobson, 2013). First, orexin was blocked prior to sleep deprivation through a gentle handling procedure to see whether this can lead to attenuation of the non-photic response. Orexin was blocked in the midday (six hours prior to the time of dark onset which by convention is zeitgeber time (ZT) 6, where dark onset is ZT12) with the use of the dual orexin receptor (OX_1 and OX_2) antagonist MK-6096 (Filorexant) which has been previously used for the treatment of insomnia,

among other disorders (Hoyer & Jacobson, 2013). Next, the effect of OX_{A+B} infusions in the IGL were examined. More specifically, orexin was administered every hour for three hours starting at midday (circadian time (CT) 6, which defines timing based on the free-running period of the internal rhythm and is during the inactive phase of the animal). The repeated infusions were given during the period of greatest sensitivity to non-photic cues (CT6-8) and were also meant to mimic the chronic in-vivo release of OX. Also, a previous pilot study from our lab showed that single infusions of OX are not enough to cause phase shifts. However, this was still a repeated injection and not a continuous release of orexin.

Furthermore, given the colocalization of OX and glutamate (Rosin et al., 2003), infusions of an OX and N-methyl-D-aspartic acid (NMDA) cocktail, were examined along with infusions of orexin and NMDA alone. Given previous suggestions that OX is not sufficient for non-photic phase shifting (Webb et al., 2008), it was important to investigate the possible role of glutamate being co-released. Finally, the proportion of orexinergic cells in the LH that project to the IGL and that stain positive for c-Fos following sleep deprivation was examined. It was hypothesized that blocking OX in the IGL would block arousal-induced phase shifts so that c-Fos levels in the IGL would also be attenuated. Furthermore, it was predicted that administration of OX and an OX+NMDA cocktail in the IGL would cause significant phase shifts and activate IGL cells. Lastly, it was hypothesized that the majority of OX cells in the LH would become active in response to an arousal-inducing procedure like sleep deprivation.

3.2 Methods

3.2.1 Experiment 1: Orexin antagonist MK-6096 infusions prior to sleep deprivation: Observing the phase shift response

3.2.1.1 Animals and drugs

A total of 20 male and female Syrian hamsters (*Mesocricetus auratus*, 80-90g; 10 males, 10 females) were obtained from Charles River Laboratories (Kingston, NY, USA). Animals were initially housed in pairs in a temperature and humidity-controlled room with a 14:10 light dark (LD) cycle and provided with food and water *ad libitum*. Animals were left in the room for at least two weeks, enough time for them to entrain to the LD cycle. Prior to the beginning of the experiment, all animals were provided with a running wheel (14 cm in diameter) and underwent a sleep deprivation protocol (SD; described below) to differentiate those that are "responders" (animals that had a phase advance to the SD) from the "non-responders", as done previously (Yamakawa et al., 2016). All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care.

The dual receptor antagonist MK-6906 (Cedarlane, ON, Canada) was administered intraperitoneally (i.p.) at a dose of 3 mg/kg ; dissolved in 10% DMSO0.9% and 90% polyethylene glycol (PEG). The dosages were determined from Raheem et al. (2015) where a higher dose interfered with sleep deprivation of animals. The vehicle control was DMSO and PEG (10% in 90% PEG).

3.2.1.2 Behavioural manipulations and phase shift calculations

Animals underwent four manipulation protocols in a counterbalanced design. Specifically, injections of the orexin antagonist MK-6096 were made, either alone, or 30 minutes prior to the start of a sleep deprivation protocol which took place from ZT 6 to ZT9 (6 hours prior to dark onset where ZT12 is the time of lights off, by convention) in dim red light (< 1 lux). Injections of the vehicle DMSO were also made both alone, and, prior to the same 3-hour SD. In

conditions without SD, animals were left undisturbed in constant darkness (DD). The manipulations took place with a modified Aschoff type II design previously described (Yamakawa et al., 2016). Animals were entrained to a 14:10 LD cycle and were placed into dim red light on the day of the manipulation and left in DD for three days after. The SD procedure was similar to that performed in past studies (Antle & Mistlberger, 2000; Yamakawa et al., 2016). Hamsters were awakened at ZT6 and provided with a new cage with fresh bedding and paper towels. Animals were observed with the cage top off for the first hour and there was minimal need for interventions by the researcher at this time. Responders are less drowsy during this time while non-responders require more frequent pokes to keep them awake (Antle & Mistlberger, 2000). Throughout the three hours, hamsters were monitored and given a combination of gentle pokes and/or paper towels to shred/for nest building, any time they were immobile and maintained a sleeping posture (i.e., curled up or hunched back). A qualitative approach was used to monitor hamsters (and their level of alertness) so that for every five-minute interval, the amount of time the animal spent moving around the cage (and the number of times it engaged in behaviours including chewing on paper towels, rearing, etc.…) was recorded.

Following the SD procedure, running wheels were returned for each animal. The wheel running activity of the animals was detected by means of a magnetic switch fastened to each wheel and monitored using the ClockLab analysis software (Actimetrics, Wilmette, IL). Phase shifts were calculated using ClockLab by determining the activity onsets for the five days of activity of animals prior to the SD and the activity onsets for the two days in DD after the manipulation. The difference between the activity onsets of the entrainment period (baseline in LD) and the onsets for the two days after the SD was calculated as the phase shift.

3.2.1.3 Perfusions and brain slice preparations

Animals were administered an overdose of sodium pentobarbital (~0.35 ml) (CEVA Santé Animale, France) in dim red light. Following the absence of reflexes, their heads were covered in aluminum foil to prevent any light input from the eyes, and they were then transcardially perfused (in the light), with ~50ml of cold phosphate buffered saline (PBS) and ~50ml of cold 4% paraformaldehyde in PBS. Brains were post-fixed at 4°C in 4% paraformaldehyde overnight and then transferred to 20% sucrose in PBS solution for 24 hours. Brains were cut with the use of a Leica cryostat set at a temperature of -19°C, at a thickness of 35 microns. Sections throughout the rostrocaudal extent of the IGL were collected into PBS baths with 2% sodium azide.

3.2.1.4 Statistical analyses

All comparisons were made using SigmaPlot (Systat Software, Inc; San Jose, CA). Statistical significance was set at $p<0.05$ for all tests. To determine if there was a statistically significant difference in mean phase shifts between the four manipulations (vehicle alone or prior to SD, and MK-6096 administration alone, and prior to SD), a one-way repeated measures analysis of variance (ANOVA) was used. All means are reported as ± standard error of the mean (SEM) in the figures and as ±standard deviation in the text.

3.2.2 Experiment 2: Orexin and NMDA infusions into the IGL: Observing phase shifts

3.2.2.1 Animals

A total of 26 male Syrian hamsters (*Mesocricetus auratus*, 80-90g) were obtained from Charles River Laboratories (Kingston, NY, USA). For the first part of the experiment, 12 males were used to study the effect of orexin infusions while 14 males were later used in a follow-up experiment with orexin and NMDA infusions as explained below. A timeline of Experiment 2 is

also shown in Figure 3.1 with the initial study design with orexin infusions shown in Figure 3.1A while the follow-up experiment looking at orexin and NMDA shown in Figure 3.1B. Animals were initially housed in pairs in a temperature and humidity-controlled room with a 14:10 light dark (LD) cycle and provided with food and water *ad libitum*. Following cannula implantation, each animal was transferred to an individual cage and allowed to recover for one week. Animals were then transferred to individual polycarbonate cages (20x45x22 cm) equipped with a running wheel (14 cm in diameter) and placed in DD for the duration of the experiment. Periodic cage changes took place seven days prior to the day of the manipulation.

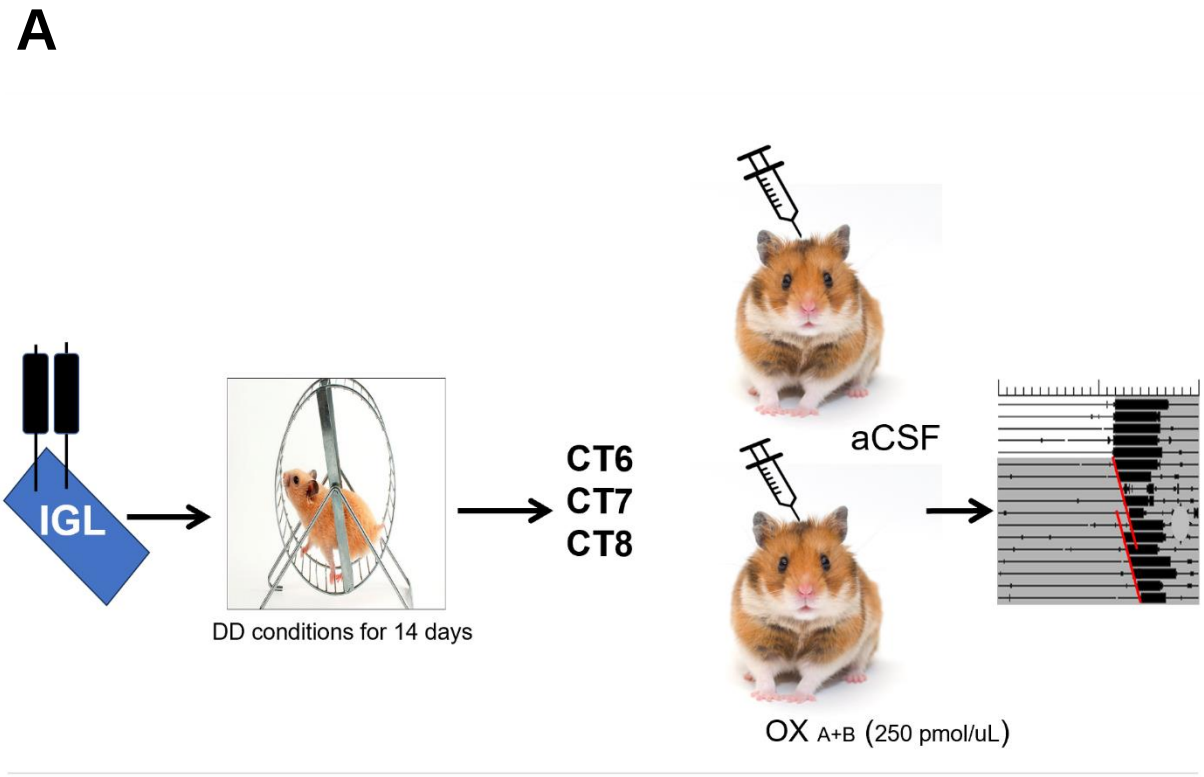
3.2.2.2 Surgeries and drugs

Stereotaxic surgery was performed for double cannula implantation in the left and right IGL. The surgical procedure for cannula implantation (22-gauge stainless steel guide cannula, PlasticsOne, VA, USA) and coordinates followed that of previous procedures done in this lab (Moshirpour et al., 2020). The coordinates for the IGL were 1.9mm posterior to bregma and 3.3 mm lateral to the midline. To determine depth, two targets were calculated below dura and skull (3.3 mm and 4.8 mm, respectively) and the average of the values was taken as the dorsoventral reading. The incisor bar was set to 2 mm below the interaural level.

OX_{A+B} (250 pmol/µL of OX_A and OX_B combined in artificial cerebrospinal fluid (aCSF); Tocris Bioscience, Bristol, UK), its vehicle control (aCSF), NMDA (1mM, in aCSF; Sigma, St. Louis, MO), and an OX+NMDA cocktail (1 mM in 90% OX_{A+B}) were administered to animals, with bilateral injections happening every hour starting at CT6 for three hours (CT6, CT7, CT8) for each animal. Drugs were administered to animals based on a counterbalanced design. Injections took place over a period of 30 seconds during each manipulation with a 1 µL Hamilton syringe attached to polyethylene 20 tubing with an injector tip (28-gauge internal

cannula injector, PlasticsOne, VA, USA) attached to the guide cannula. The injector was left in place for an additional 30 seconds after each injection. All injections were made in DD conditions with night-vision goggles.

Figure 3.1. A timeline of the study design and methods for Experiment 2. Stereotaxic surgery was performed for double cannulation of the Syrian hamster intergeniculate leaflet (IGL). Following recovery from surgery, animals were placed in constant darkness (DD) for at least 14 days prior to receiving injections (0.5 µL given over a period of 30 seconds) of **(A)** artificial cerebrospinal fluid (aCSF) or 250 pmol/µL of orexin (OX_{A+B} ; n=12) in the first experiment and **(B)** aCSF, 250 pmol/µL OX_{A+B}, N-methyl-D-aspartate (NMDA), or an OX_{A+B} and NMDA cocktail (n=14). All animals were administered all injections in a counterbalanced design with injections being administered at every hour for three hours starting at circadian time 6 (CT6, CT7, CT8). Running wheel activity was observed and phase shifts were calculated from corresponding actograms.


A
IGL
DD conditions for 14 days
CT6
CT7
CT8
aCSF
OX A+B (250 pmol/uL)
B
IGL
DD conditions for 14 days
0.5uL injection over 30s
CT6
CT7
CT8
aCSF
NMDA (1mM in aCSF)
Orexin A+B (250 pmol/uL)
Orexin/NMDA Cocktail (1 mM in 90% orexin)

3.2.2.3. Histology for cannula placements

To confirm the bilateral placement of the cannulas in the IGL, brains were collected for histology at the end of the experiment. All animals were administered an overdose of sodium pentobarbital and were then transcardially perfused with ~50ml of cold phosphate buffered saline (PBS) and subsequently, ~50ml of cold 4% paraformaldehyde in PBS. Brains were post-fixed at 4°C in 4% paraformaldehyde overnight and then transferred to 20% sucrose in PBS solution for 24 hours. Brains were cut with the use of a Leica cryostat set at a temperature of -19°C, at a thickness of 35 microns and thawed/collected directly on gelatin-coated microscope slides. Brain sections were subsequently stained using a cresyl violet Nissl stain and coverslipped in Permount. The slides were then viewed under an Olympus BX51 microscope to confirm cannula placements. Animals were excluded if the tip of either one of the two cannulas was more than 600µm away from the margin of the left and right IGL.

3.2.2.4 Phase shift calculations and statistical analyses

The wheel running activity of animals was detected by means of a magnetic switch fastened to each wheel and monitored using ClockLab analysis software (Actimetrics, Wilmette, IL). Phase shifts for each manipulation were calculated by fitting a line of best fit to activity onsets, following a previously described method of calculation (Sterniczuk et al., 2008). All comparisons were made using SigmaPlot and statistical significance was set at $p<0.05$ for all tests. A two-way repeated measures ANOVA was used to analyze the effect of presence of orexin and the presence of NMDA on mean phase shifts (in hours). All means are reported as ± standard error of the mean (SEM) in the figures and as ±standard deviation in the text.

3.2.3 Experiment 3: c-Fos expression in orexinergic neurons of the LH after sleep deprivation

3.2.3.1 Animals, sleep deprivation and phase shifts

A total of 10 male and female Syrian hamsters (*Mesocricetus auratus*, 80-90g; 5 males, 5 females) were obtained from Charles River Laboratories (Kingston, NY, USA). Hamsters were initially pair housed in a temperature and humidity-controlled room with a 14:10 LD cycle and provided with food and water *ad libitum*. Animals were left in the room for at least two weeks, enough time for them to entrain to the LD cycle. Prior to the beginning of the experiment, all animals were provided with a running wheel (14 cm in diameter) and underwent a sleep deprivation protocol (SD: described below) to identify animals that are "responders" (animals that had a phase advance to the SD) from the "non-responders" as done previously (Yamakawa et al., 2016). Out of the ten animals, four (2 males and 2 females) were identified as "responders" and showed significant phase advances to the SD procedure (Antle & Mistlberger, 2000; Yamakawa et al., 2016). The manipulation took place with a modified Aschoff type II design previously described (Yamakawa et al., 2016). The SD procedure was like that performed in past studies (Antle & Mistlberger, 2000; Yamakawa et al., 2016). Animals were awakened at ZT6 and provided with a new cage with fresh bedding and paper towels. Throughout the three hours, hamsters were monitored and given a combination of gentle pokes and/or paper towels to shred any time they were immobile and maintained a sleeping posture (i.e., curled up or hunched back). Following the SD procedure, running wheels were returned for each animal. The wheel running activity of the animals was detected and monitored using the ClockLab analysis software. Wheel revolutions were counted and summed into 10-minute bins (plotted as an actogram). The activity onsets for five days in the LD cycle prior to SD were determined along with the activity onset in DD two days following the SD. Phase shifts were calculated as the

difference between the baseline activity onset and activity onset after the SD protocol (Antle & Mistlberger, 2000). Following recovery from surgeries, hamsters underwent a second SD procedure and were euthanized immediately after the procedure for the collection of brain tissue.

3.2.3.2 Tract tracing surgeries

Animals were administered a subcutaneous injection of the analgesic butorphanol (2 mg/kg; Wyeth, Madison, NJ, USA) and an intraperitoneal injection of the anesthetic sodium pentobarbital (90 mg/kg; CEVA Santé Animale, France), their head was shaved, and they were placed in the stereotaxic frame. The surgical procedure mostly followed that of a previously described protocol (Yamakawa et al., 2016). The incisor bar was set at 2.0 mm below the interaural level. Following incision of the scalp, a burr hole was drilled into the skull and a borosilicate capillary glass tube (with a tip of approximately 28-30 µm tip; with filament, original outer diameter:1.50 mm, inner diameter: 0.86 mm, Warner instruments, Hamden, CT, USA) was backfilled with CTβ (1% dissolved in dH_2O; List Biological Laboratories Inc, Campbell, CA, USA). An iridium and platinum (10% wt/wt) wire was carefully inserted into the tube. The lead electrode of a BAB-150 iontophoresis machine (Kation Scientific, Minneapolis, MN, USA) was attached to the wire while the ground electrode was attached to a skin flap on the animal.

The coordinates used for unilateral capillary tube insertion in the IGL were as follows: 1.9 mm posterior to bregma, 3.3 mm lateral to bregma and 4.6 mm ventral to dura. These coordinates were adapted from IGL coordinates used for cannulation surgery in experiments elsewhere in this thesis (Moshirpour et al., 2020; Yamakawa et al., 2016). The capillary tube was slowly lowered through the hole under a continuous negative retaining current of -1.0 µA to avoid CTβ leakage along the glass tube track. Iontophoresis of CTβ occurred over a period of 10-

12 minutes (positive current, 1.5μA, 5 second on/5 second off pulses). Subsequently, the current was turned off while the tube was left in place for a period of 15 minutes. The tube was then slowly raised under a continuous negative retaining current of -1.0μA. Prior to suturing, some bone wax was used to fill up the hole in the skull.

3.2.3.3 Perfusions and brain slice preparations

Immediately following SD (ZT6-ZT9), hamsters were administered an overdose of sodium pentobarbital (~0.35 ml) in dim red light. Following the absence of reflexes, their heads were covered in aluminum foil to prevent any light input from the eyes, and they were then transcardially perfused (in the light), with ~50ml of cold phosphate buffered saline (PBS) and subsequently, ~50ml of cold 4% paraformaldehyde in PBS. Brains were post-fixed at 4°C in 4% paraformaldehyde overnight and then transferred to 20% sucrose in PBS solution for 24 hours. Brains were cut with the use of a Leica cryostat set at a temperature of -21°C, at a thickness of 35 microns sections throughout the rostrocaudal extent of the IGL and the lateral hypothalamus (LH) and were collected into PBS baths with 2% sodium azide.

3.2.3.4 Immunohistochemistry

To confirm the CTβ injection in the IGL, brain sections were collected throughout the rostrocaudal extent of the IGL. Sections were rinsed for 25 minutes in 0.5% (vol/vol) H_2O_2 in 0.3% Triton X-100 in PBS (PBSx) and subsequently exposed to three 10-minute washes in 0.3% PBSx, followed by a 90-minute incubation at room temperature in blocking buffer (5% normal goat serum (NGS) in PBSx). Sections were further incubated for 48h in the CTβ primary antibody (mouse anti-CTβ, 1:5 Bio-Rad Laboratories, Hercules, CA, USA) diluted in blocking buffer (2% NGS in PBSx) at 4°C. The tissue was again rinsed in PBSx prior to a 90-minute incubation at room temperature in the secondary antibody (biotinylated goat anti-mouse, 1:200 in

PBSx; Vector Laboratories, Burlingame, CA, USA). This was followed by more rinses with PBSx (3 x10 min each) and a 60-minute incubation at 4°C, in an avidin-biotin complex (1:100 Vector Vectastain Elite ABC kit, Vector Laboratories). A final set of PBSx rinses was performed and section were then developed with a 0.4% diaminobenzidine (DAB) solution (DAB as the chromagen, 12.5mg/mL in 0.1 M Tris buffer, 80 mg/mL of nickel chloride, and hydrogen peroxide). Sections were finally exposed to a series of rapid PBSx rinses and were mounted onto gelatin—coated microscope slides. Slides were dehydrated in an alcohol series (70% to 100% EtOH), cleared with xylene and coverslipped with Permount.

To visualize cells in the LH, sections collected throughout the LH underwent triple fluorescence staining for orexin, CTβ, and c-Fos. Free-floating sections were rinsed in 0.1% PBSx and incubated in 5% NDS in PBSx for 60 minutes. Sections were subsequently incubated for 48h in the following primary antibodies: mouse anti-cholera toxin (1:3000), goat anti-Fos (1:10000) and guinea pig anti-orexin (1:20000) diluted in blocking buffer (2% NDS in PBSx) at 4°C. The tissue was then rinsed in PBSx (6 x10 minutes) prior to a 60-minute incubation in the following fluorescent secondary antibodies: donkey anti-mouse Cy2, donkey anti-goat Cy3, and donkey anti-guinea pig Cy5 (each at 1:200). Lights were turned off from this point forward. Following the incubation, the tissue was mounted on gelatin-coated microscope slides, cleared with xylene and coverslipped with Krystalon.

3.2.3.5 Image analysis and cell counting

At least three representative LH sections per animal were examined using an Olympus BX51 microscope attached with a QI CAM Fast 1394 cooled CCD camera. Issues with mounting of the tissue did not allow for animals to have all representative LH sections but all available stained sections were included for each animal. The injection site of CTβ was confirmed with DAB staining before any cell counting such that animals were excluded if CTβ was not

deposited in the IGL. Cells in the LH were counted using Image Pro Plus software. Colocalization required the c-Fos positive nucleus to fill the space defined by the OX immunoreactivity, and the CTβ immunoreactivity had to match the cytoplasmic shape defined by the OX immunoreactivity.

3.3 Results

3.3.1 Experiment 1: Blocking orexin does not block phase shifts to sleep deprivation but increases cellular activation in the IGL

No significant differences in the mean phase shifts were observed between the vehicle (DMSO) with no SD and the drug (MK-6096) with no SD conditions (DMSO: 0.291±0.327, MK-6096: 0.236± 0.337), $t(17) = 0.582$, $p=0.568$; Figure 3.2). Negligible phase shifts were observed for both conditions. As a result, their data was combined into one group representing the no SD condition. A one-way repeated measure ANOVA revealed a significant difference in phase shifts between the conditions, $F(1.337, 22.726) =14.577$, $p <.001$, and Bonferroni post-hoc tests revealed that the only significant difference was between the no SD (0.267± 0.265) and the DMSO +SD (1.706± 1.478), and MK-6096 + SD (1.474±1.538) conditions, $p =0.001$ and $p = 0.007$, respectively. Most importantly, there was no significant difference between the DMSO +SD and MK-6096+SD conditions (mean difference =.232, $p =0.468$; Figure 3.2). Therefore, MK-6096 did not block phase shifts to sleep deprivation, as compared to the vehicle control. However, hamsters who were treated with MK-6096 displayed drowsiness, were more prone to adopting a sleeping posture and/or were more difficult to keep awake.

3.3.2 Experiment 2: Orexin and NMDA in the IGL have an additive effect on non-photic phase shifting

A pilot experiment looked at the difference in mean phase shifts between OX_{A+B} infusions (at CT6,7, 8) and a vehicle control (aCSF). The data from a total of eight male

hamsters (out of the 12) were used in the analysis. Histological examination led to two animals being excluded due to missed placements for one of the two cannulas aimed at the left and right IGL. Two further animals lost their headcaps prior to undergoing all manipulations. The results of a paired t-test revealed no significant phase shifts in response to orexin (0.189±0.105), as compared to the vehicle control (-0.0925±0.399), $t(7) = -1.849$, $p = 0.107$; Figure 3.3 showing actograms of representative animals).

A follow-up experiment looked at the effect of OX and NMDA on phase shifting. Data from a total of six male hamsters (out of the 14) were used in the analysis. Histological examination led to three animals being excluded due to missed placements for one of the two cannulas. Two further animals lost their headcaps prior to being administered both a vehicle control and drug condition and were excluded from the analysis and three further animals did not have enough consistent running data throughout and prior to the end of the experiment. A two-way repeated measures ANOVA did not reveal a significant interaction between the effect of presence of OX_{A+B} and the presence of NMDA, $F(1,5) = 0.225$, $p = 0.655$. However, there was a significant main effect of OX_{A+B} ($F(1,5) = 26.929$, $p = 0.003$) as well as NMDA ($F(1,5) = 9.085$, $p = 0.030$). OX_{A+B} infusions resulted in significantly larger phase shifts (0.178 ± 0.171) than to aCSF (-0.208 ± 0.405; Figure 3.4). Similarly, NMDA infusions resulted in significantly larger phase shifts (0.832± 0.576) than to aCSF. As shown in the actograms of representative animals (Figure 3.4A), despite the absence of a significant interaction, the magnitude of mean phase shifts to the OX+NMDA cocktail was the largest (1.432 ± 0.701; Figure 3.4 A,B). Cannula implantation in the IGL is shown in Figure 3.5.

3.3.3 Experiment 3: Orexinergic cells in the lateral hypothalamus are activated in response to sleep deprivation.

A total of four animals (out of the 10) were determined to be responders. Only these animals were selected for the sleep deprivation procedure and later had surgeries performed on them. Out of the four, two animals did not recover from the surgery during the seven-day recovery period. Therefore, two animals (one male and one female) were used for the analysis. While the original aim of this study was to determine the proportion of IGL-projecting orexin neurons in the LH that show c-Fos expression in response to a SD, examination of DAB-stained tissue for CTβ revealed that the infusions missed the IGL. In one animal, the infusion was too rostral while no infusion was successfully stained for in the other animal. Therefore, only the activation of OX cells in the LH was analyzed. Active OX cells were identified as double-labelled with immunoreactivity in the cytoplasm and a c-Fos positive nucleus (Figure 3.6A-C). A large proportion of OX cells in the LH had a c-Fos positive nucleus (77.9% ± 1.77) following the SD protocol (Figure 3.6D).

Figure 3.2. Blocking orexin does not block phase shifts to sleep deprivation.

Actograms from a representative animal depicting phase shifts following each treatment: The vehicle, DMSO alone, DMSO prior to a sleep deprivation (DMSO +SD), orexin antagonist (MK-6096) alone, and MK6-96 +SD (n=20, 10 males, 10 females). There was no significant difference between the DMSO +SD and MK-6096+SD conditions (p = 0.468). Injections took place just prior to a SD that lasted from ZT6-ZT9 (denoted by the green rectangles drawn on the actograms). Each horizontal line represents a day of wheel running as shown by the black vertical bars with subsequent days plotted below. The height of the bars is proportional to the number of wheel revolutions. A modified Aschoff Type II design was used whereby animals were in a 14:10 LD cycle but lights went off at ZT6 and remained off for the next three days (shaded area of the graph shows lights off).

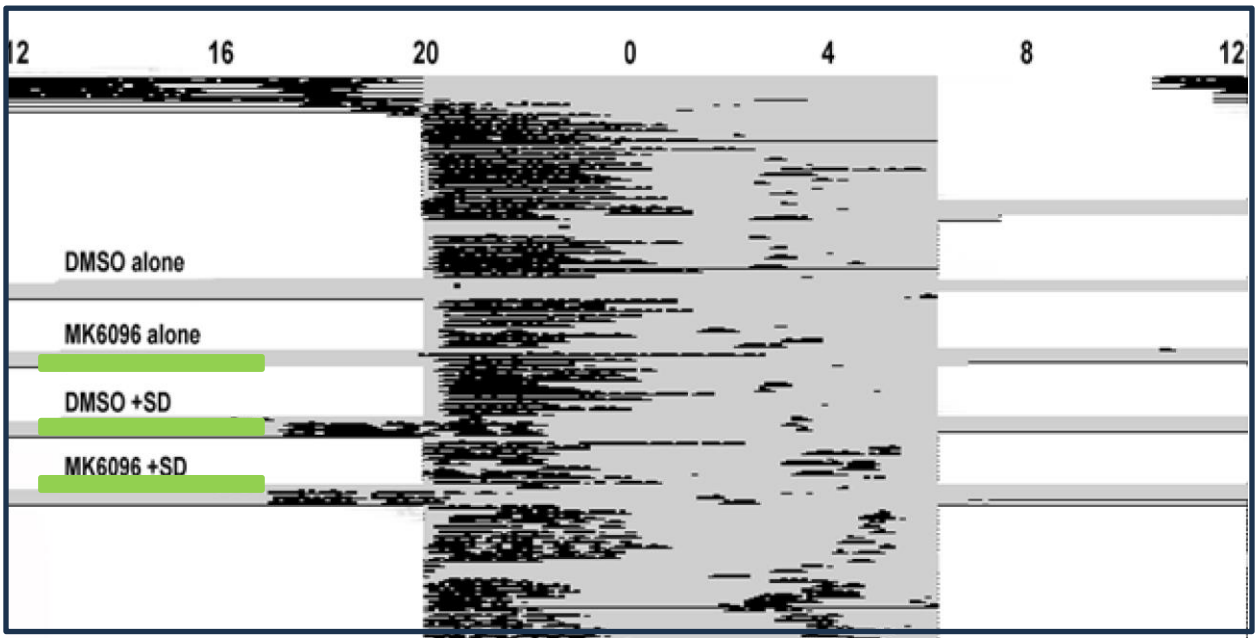

12
16
20
0
4
8
12
DMSO alone
MK6096 alone
DMSO +SD
MK6096 +SD

Figure 3.3. Orexin IGL infusions do not cause significant phase shifts as compared to vehicle control.

Actograms from representative animals depicting phase shifts following treatment with vehicle, aCSF, and orexin (OX_{A+B}; n=8).There was no significant difference between the two conditions (p>.05). Injections took place at CT6 as denoted by the green diamond (◊) and were repeated at CT7, and CT8. Each horizontal line represents a day of wheel running as shown by the black vertical bars with subsequent days plotted below. The height of the bars is proportional to the number of wheel revolutions. The red diagonal lines are lines of best fit drawn to represent activity onsets before and after the manipulations.

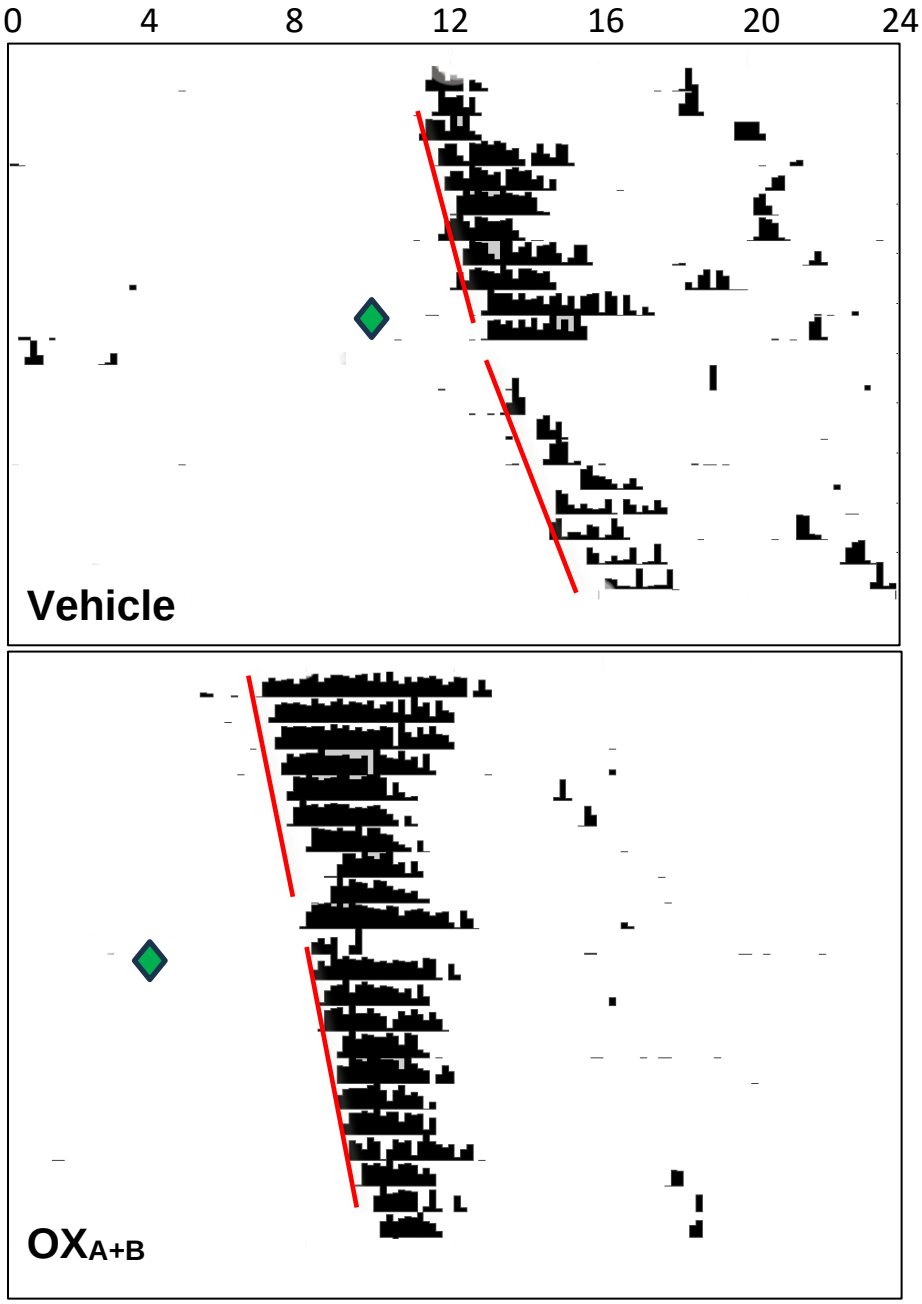
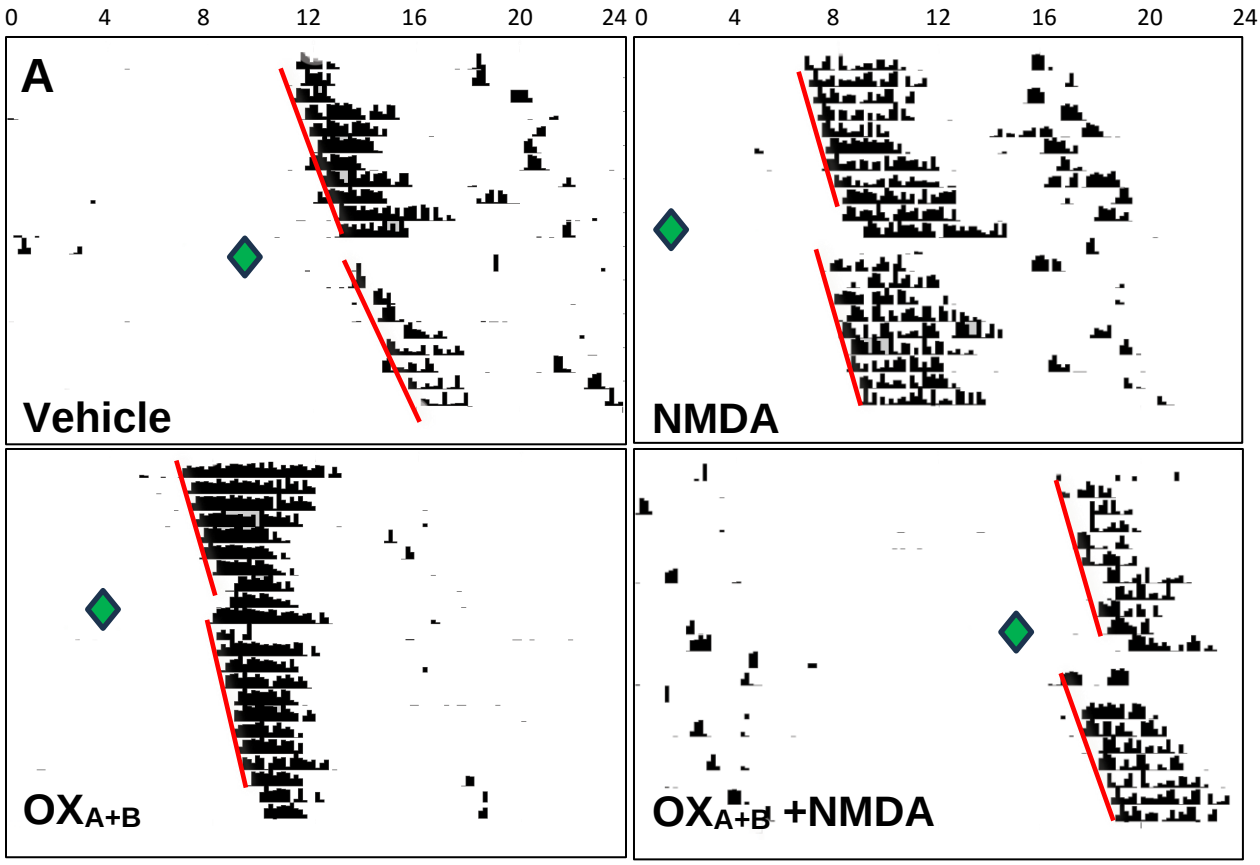

0
4
8
12
16
20
24
Vehicle
OXA+B

Figure 3.4. Orexin and NMDA in the IGL cause significant phase shifts as compared to vehicle control.

(A) Actograms from representative animals depicting phase shifts following each treatment: The vehicle, aCSF, orexin (OX_{A+B}), NMDA, and OX+NMDA cocktail (OX_{A+B}+ NMDA; n=6). The cocktail showed the greatest magnitude of phase advances as compared to the other conditions. There was no significant interaction ($p>.05$) while both orexin and NMDA resulted in significant phase shifts as compared to the control condition ($p<0.05$). Injections took place at CT6 as denoted by the green diamond (◊) and were repeated at CT7, and CT8. Each horizontal line represents a day of wheel running as shown by the black vertical bars with subsequent days plotted below. The height of the bars is proportional to the number of wheel revolutions. The red diagonal lines are lines of best fit drawn to represent activity onsets before and after the manipulations. **(B)** Mean phase shift (±SEM) of hamsters exposed to aCSF, orexin (OX_{A+B}), NMDA, and OX+NMDA, at CT6,7, and 8. The cocktail condition had the greatest magnitude of phase shifts but there was no significant interaction ($p>0.05$). Phase shifts were significantly greater for both the orexin, and NMDA treatments (as compared to the vehicle control, $p<0.05$).


A
0
4
8
12
16
20
24
Vehicle
NMDA
OXA+B
OXA+B +NMDA

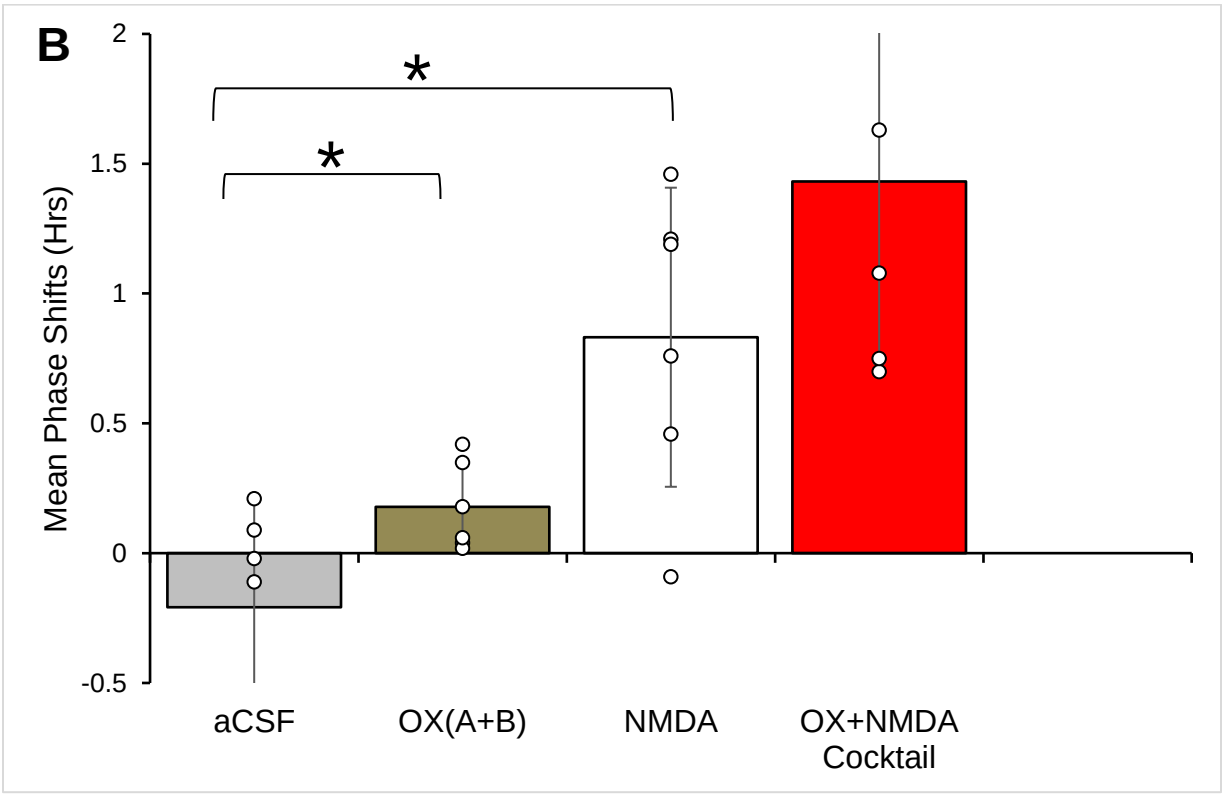

B
Mean Phase Shifts (Hrs)
2
1.5
1
0.5
0
-0.5
*
*
aCSF
OX(A+B)
NMDA
OX+NMDA
Cocktail

Figure 3.5. Cannulation in the intergeniculate leaflet (IGL).

Photomicrograph (1.25x magnification) of a cannula placement in the left IGL of a representative hamster coronal slice stained with cresyl violet. Bilateral cannulation was performed but only the left cannula is depicted in this section. The tip of the guide cannula which is targeting the IGL is denoted by the black arrow; 3V, 3rd ventricle, D3V, dorsal 3rd ventricle; dLGN, dorsolateral geniculate nucleus; fi, fimbria of hippocampus; hf, hippocampal fissure; IGL, intergeniculate leaflet; LV, lateral ventricle; vLGN, ventrolateral geniculate nucleus.

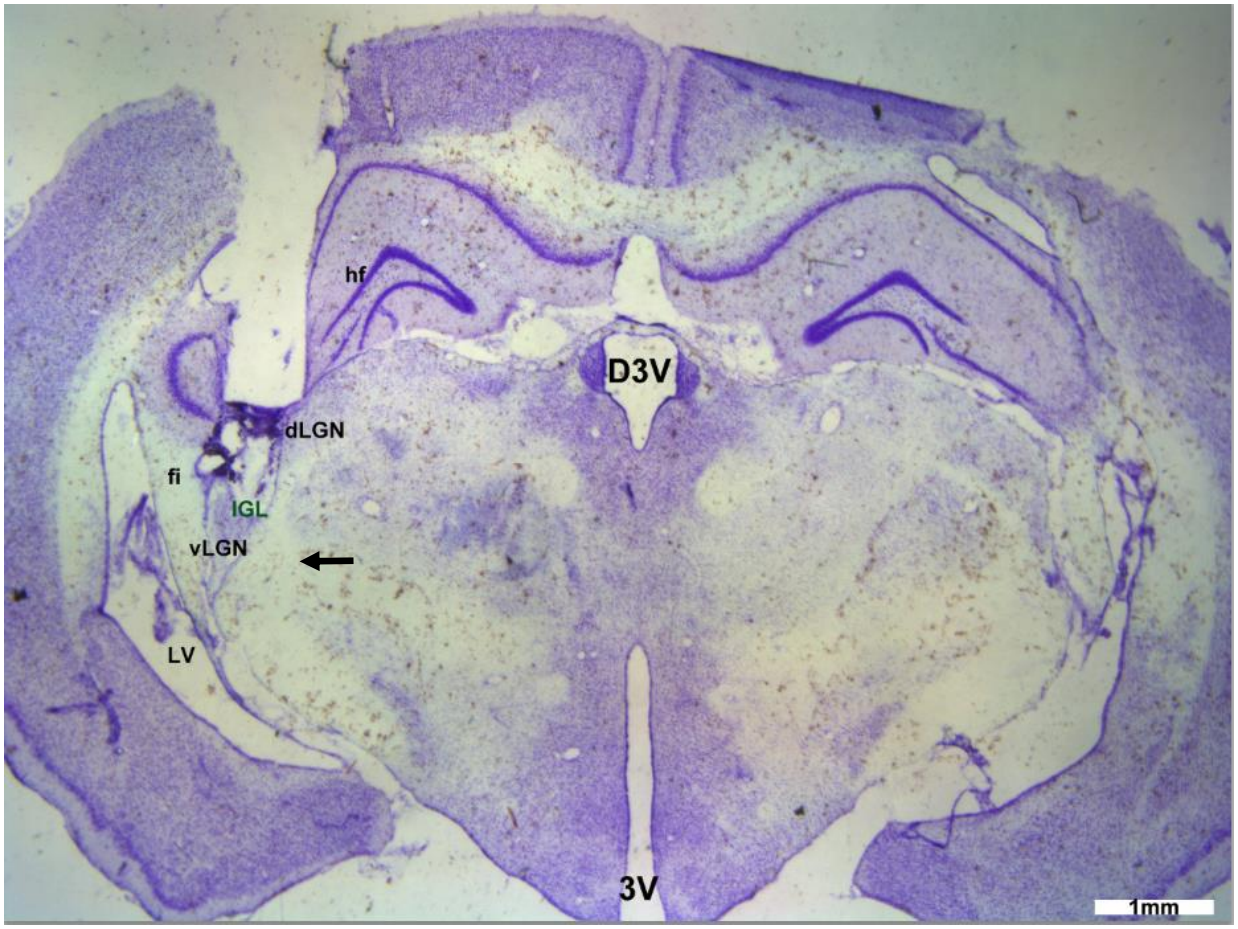
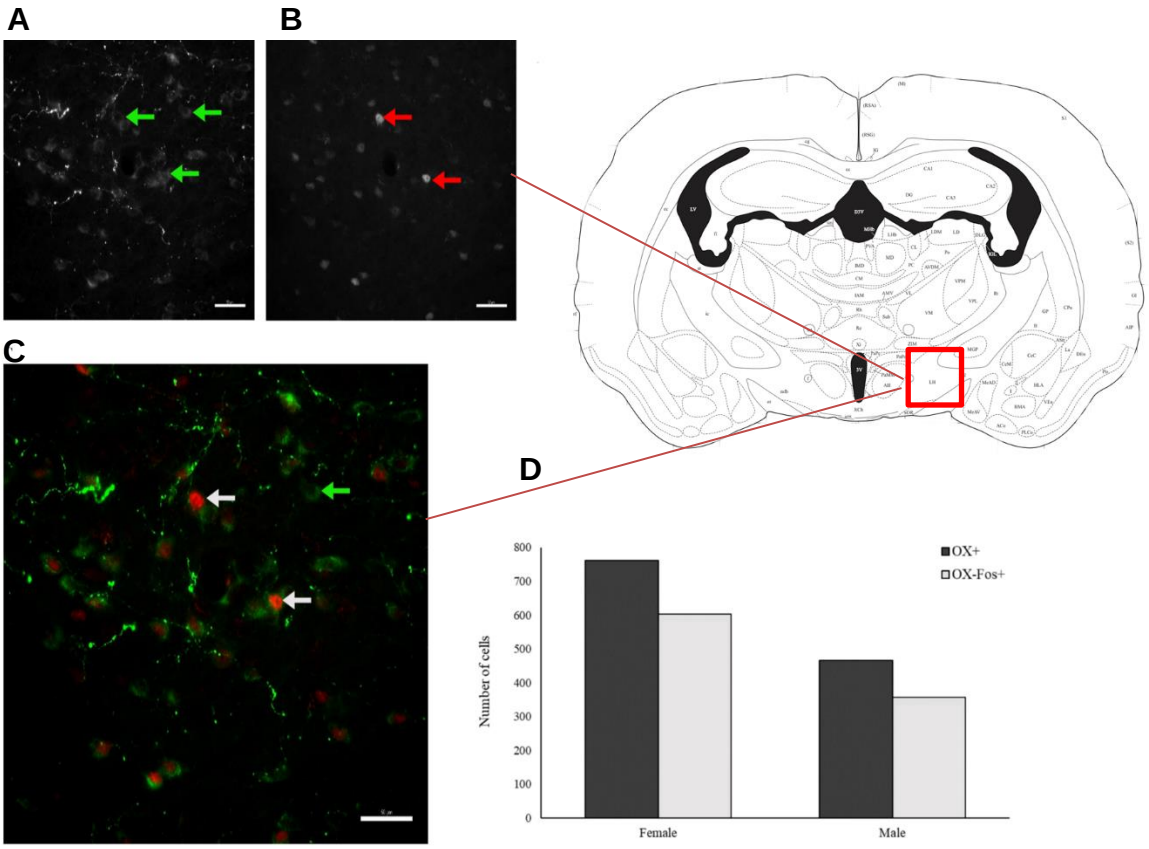

hf
D3V
dLGN
fi
IGL
vLGN
LV
3V
1mm

Figure 3.6. Orexinergic cells in the lateral hypothalamus (LH) express c-Fos in response to an arousal sleep deprivation (SD) procedure.

Representative photomicrographs (40x magnification) showing immunoreactivity of **(A)** orexin (OX) and **(B)** c-Fos, in the LH of a hamster following a 3-hour SD. **(C)** The merged image showing double-labelled cells (white arrows), as well as OX cells not activated by the SD procedure (green arrow). **(D)** Number of total OX immunoreactive cells and c-Fos positive OX cells counted in the LH of animals (n_{female} =1, n_{male} = 1). Most orexinergic cells identified (77.9% ± 1.77) stained positive for c-Fos.


A
B
C
D
Number of cells
800
700
600
500
400
300
200
100
0
Female
Male
OX+
OX-Fos+

3.4 Discussion

The present study examined the link between the IGL and one of the main arousal centers of the brain, the lateral hypothalamus. Given the critical role of the IGL in non-photic phase shifting, and the significance of the arousal component in entrainment, the aim was to see whether orexin is the necessary signal for gating phase shifts to arousal procedures. The main finding of this study is that orexin alone is likely not sufficient for inducing phase shifts. With the knowledge that most OX neurons are glutamatergic, we examined whether the presence of glutamate and OX was sufficient for inducing phase shifts. The results showed that glutamate and OX likely have an additive effect in causing non-photic shifts and activating IGL cells and that glutamate may mediate this response through NMDA receptors. Application of an OX_{A+B} + NMDA cocktail in the IGL gave rise to larger phase shifts when compared to all the other manipulations (aCSF control, NMDA, and OX_{A+B}). However, statistical analyses did not reveal a significant interaction between the presence of NMDA and OX_{A+B}. Nonetheless, when looking at the main effects of each injection, both the OX_{A+B} and the NMDA injections gave rise to significantly greater phase shifts when compared to the control condition.

These findings are in support of the study by Webb et al. (2008) suggesting that orexin is not sufficient for phase shifting. However, our findings report a possible role for glutamate as a necessary component for OX_{A+B} to modulate arousal responses. Past studies have reported the expression and release of orexin with glutamate (Rosin et al., 2003). It is not uncommon for neuropeptides to be co-released with other neurotransmitters and to have a direct influence on the neurotransmitters' postsynaptic effects. A functional role for orexin has been previously reported where orexin has been shown to increase suppression of SCN cellular activity by NPYergic IGL neurons (Belle et al., 2014). It is possible for glutamate to be co-released with orexin in the IGL and directly influence NPY neurons. This could provide a basis for gating the

IGL's modulation of non-photic entrainment. More specifically, the LH arousal center sends signals to, and activates the IGL NPYergic neurons and allows them to suppress cellular activity at the SCN, perhaps as part of a mechanism to block responses of the clock to light cues.

This study also examined whether orexin in the IGL is necessary for inducing phase shifts by blocking it with a dual OX receptor antagonist. Use of the antagonist MK-6096 which blocks both OX receptors (1 and 2) prior to an arousal procedure (SD) was not able to block phase shifts suggesting that OX alone is not necessary for entrainment. It should be noted however that systemic injections of MK-6096 were used to study the effects of blocking orexin and intra-cranial IGL injections have not been explored but should be undertaken in a follow-up experiment. However, as explored above, it is likely that other neurotransmitters such as glutamate are also components of this pathway and blocking NDMA receptors is an important follow-up step to explore this relationship further. It was important to not only look at behavioural phase shifts, but to also examine c-Fos expression in the IGL and LH in response to SD. To this end, c-Fos expression was examined in the IGL following SD with blocking of orexin. There was significantly lower c-Fos expression in the IGL of animals who received injections of MK-6096 as compared to the control conditions. As well, in response to a SD procedure, the majority of orexinergic neurons of the LH labelled were activated. Other findings from our lab using the same animals that received orexin infusions have also shown significantly more c-Fos positive cells in the IGL as compared to control animals who received aCSF. It is important to note that a no SD control group was not included in the results to show that there is no c-Fos expression in the IGL in the absence of an arousal procedure. However, this has been shown previously in the lab and is also consistently reported in the literature (Janik et al., 1995; Mikkelsen et al., 1998; Webb et al., 2008). Taken together, these findings further

support the role of orexin as an important, but non-sufficient contributor to entrainment (Lavoie & Liu, 2020).

It is important to follow up these studies with a tract tracing study that will identify activated orexin neurons of the LH that project to the IGL. This will complement the findings both in this study and those reported in Chapter 2. Although one of the aims of the present experiments was to trace these neurons, issues with the iontophoresis pump used with tracing surgeries as well as the small number of responders that had surgeries completed on them resulted in the inability to successfully target the IGL. A more targeted approach than retrograde tracing with CTβ can be undertaken for greater accuracy and confidence in the results. For instance, injections of a retrograde Cre vector (Canine Adenovirus 2 expressing *Cre ;*CAV2-Cre) can confirm Cre expression in cells throughout a neural pathway, ensuring that retrograde tracing takes place while only highlighting a specific cell subtype such as NPYergic IGL neurons (Lavoie & Liu, 2020). Furthermore, it will be important to further explore the role of glutamate by blocking its receptor and investigating the impact on phase shifting. If orexin and glutamate are both necessary for gating this response at the IGL, then blocking both will block phase shifts to an arousal procedure such as sleep deprivation through gentle handling or novel wheel confinement. This is an area of future work and time did not allow for the inclusion of this potential experiment in this thesis. However, it is important to note that glutamate input to the IGL may also be coming from other brain regions that have not been examined. As well, it should be noted that other glutamate receptors may be involved as well, and NMDA may not be the only subtype. NMDA has traditionally been used for the purposes of lesioning a brain area such as the IGL while the importance of glutamate (and specifically NMDARs) in photic entrainment has been well documented (Ebling, 1996; Mintz et al., 1999). However, the role of

glutamate in non-photic mechanisms has not been explored in the literature and given the novel findings of this thesis, it will be important to conduct future experiments and tweeze apart the influence on photic and non-photic entrainment.

To conclude, this study has shown that orexin alone is not sufficient for non-photic entrainment. Also, the corelease of orexin and glutamate is sufficient in inducing phase shifts and that both likely have an additive effect in activating the IGL. Findings of this study provide further support for the view that several neurotransmitters are likely involved in the modulation of the NPYergic IGL neurons. The lateral hypothalamus represents only one of several arousal centers of the brain and future experiments investigating the role of acetylcholine input to the IGL from the basal forebrain (as described in Chapter 2) will further aid in resolving the neural basis of arousal signaling.

Chapter Four: Atropine Infusions in the Intergeniculate Leaflet to Examine Non-Photic Phase Shifting

4.1 Introduction

There are several important sleep-wake centers in the mammalian brain such as the brainstem and forebrain. Some of these areas include the pedunculopontine tegmentum, the raphe nuclei, as well as the basal forebrain (Blanco-Centurion et al., 2006; Nishino, 2011; Schwarz & Luo, 2015). These arousal centers also receive input from orexinergic cells of the lateral hypothalamus (LH) and are critical for maintaining waking and regulating the transition from sleep to wakefulness (Marston et al., 2008; Sakurai et al., 1998). Although most of these arousal centers project to the brain's circadian circuitry and are intimately involved in modulating sleep-wake states, it is still unclear how arousal centers influence synchronization of the circadian system, a process referred to as entrainment (Mistlberger & Antle, 2011; Moore, 1996b). The suprachiasmatic nucleus (SCN) in the anterior hypothalamus is the mammalian central endogenous clock that maintains and coordinates various internal rhythms (Holzberg & Albrecht, 2003; Sawaki et al., 1984). While the light-dark cycle is the principal cue for entrainment, there are a variety of so-called non-photic cues that influence the clock including food, locomotion, and sleep deprivation (SD; Antle & Mistlberger, 2000; Mistlberger et al., 2002; Mistlberger & Antle, 2011). The element of arousal is key between these non-photic cues; therefore, it is likely that inputs from arousal centers in the brain such as the basal forebrain are involved.

It has been shown that input from the acetylcholine-producing (cholinergic) cells of the basal forebrain is necessary for arousal-induced phase shifting (Yamakawa et al., 2016). Expression of the immediate early gene product c-Fos, which can be used as an indicator for cellular activation, was observed in the basal forebrain of Syrian hamsters in response to arousal-

inducing manipulations including access to a novel running wheel and sleep deprivation by gentle handling (Yamakawa et al., 2016). More specifically, activation of cholinergic cells was observed by labelling of c-Fos and choline acetyl transferase (ChAT), the enzyme that synthesizes the neurotransmitter acetylcholine (ACh). More evidence for the role of acetylcholine (ACh) in non-photic entrainment comes from manipulations at the level of the SCN. Intra-SCN injections of the ACh agonist carbachol leads to phase shifting that is comparable to other non-photic responses (Basu et al., 2016). These phase shifts are successfully blocked with the ACh muscarinic receptor antagonist, atropine, suggesting that ACh is necessary for causing phase shifts (Basu et al., 2016). Carbachol injections in the intergeniculate leaflet (IGL), a backdoor to the SCN, also result in phase shifts (Cain et al., 2007). Neuropeptide Y (NPY) producing cells of the IGL are critical for non-photic entrainment, but it is not fully known how the IGL gets activated in the first place. The IGL also receives cholinergic input from the brainstem arousal centers and orexinergic input from the LH (Chapter 2; McGranaghan & Piggins, 2001; Moore et al., 2000; Morin & Blanchard, 2005). Therefore, it is likely that one or more of these areas provides the critical signal for gating arousal-induced phase shifts.

The goal of the current study was to investigate the role of ACh as the necessary signal for activation of the IGL. Given the presence of cholinergic input to the IGL from brainstem arousal centers, as well as the finding that there is cholinergic input from the ACh-dense area of the basal forebrain, the substantia innominata (SI; as reported in Chapter 2 of this thesis), it was important to investigate what happens when ACh is blocked in the IGL. Blocking ACh in the SCN blocks phase shifts to novel wheel confinement (Yamakawa et al., 2016) while application of atropine in the IGL blocks carbachol-specific phase shifts (Cain et al., 2007). However, it is unknown whether blocking ACh in the IGL can block phase shifts to an arousal-induced

manipulation. Therefore, we investigated changes in the phase shifts to a sleep deprivation (SD) procedure for Syrian hamsters who received atropine infusions in the IGL, prior to the arousal protocol. It was predicted that if cholinergic input to the IGL is necessary, then administration of atropine in the IGL would block phase shifts to sleep deprivation.

4.2 Methods

4.2.1 Animals and surgeries

A total of 25 male Syrian hamsters (*Mesocricetus auratus*, 80-90g) were obtained from Charles River Laboratories (Kingston, NY, USA). Animals were placed in pairs in a temperature and humidity-controlled room with a 14:10 light dark (LD) cycle and provided with food and water *ad libitum.* Hamsters were left in the room for at least two weeks to allow for entrainment to the LD cycle. Prior to the beginning of the experiment, all animals were provided with a running wheel (14 cm in diameter) and underwent a three-hour SD protocol at midday (six hours prior to the time of dark onset which by convention is zeitgeber time (ZT) 6, where dark onset is ZT12). This protocol served the purpose of differentiating "responders" (animals that had a phase advance to the SD) from the "non-responders" (see below and as described in Yamakawa et al. (2016)). Out of the 25 animals, eight were eventually used in the experiment (excluded animals were either non-responders or did not survive the surgeries/lost head caps). Responders underwent cannulation surgery (see below) and following surgeries, each animal was transferred to an individual cage and allowed to recover for a period of one week. Following the recovery period, hamsters were transferred to individual polycarbonate cages (20x45x22 cm) equipped with a running wheel and maintained in the same LD cycle as before. All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care.

Stereotaxic surgery was performed for bilateral cannula implantation (22-gauge stainless steel guide cannula, PlasticsOne, VA, USA) in the IGL. Surgical procedures followed that of previous surgeries done in our lab (Moshirpour et al., 2020). Coordinates for the IGL were 1.9 mm posterior to bregma, and 3.3 mm lateral to the midline. To determine depth, two targets were calculated below dura and skull (3.3 mm and 4.8 mm. respectively) and the average of the values was used as the dorsoventral reading. The incisor bar was set at 2.0 mm below the interaural level.

4.2.2 Injections, sleep deprivation manipulations, and phase shift calculations

The manipulations took place with a modified Aschoff type II design previously described (Yamakawa et al., 2016). Animals were entrained to a 14:10 LD cycle and placed into dim red light (<1 lux) on the day of the manipulation and left in constant darkness (DD) for three days after. Immediately prior to the SD protocol, animals were administered an injection of either physiological saline (0.9%) or atropine sulphate (10mM, dissolved in physiological saline; Sigma-Aldrich, St. Louis, MO). Drugs were administered to animals based on a counterbalanced design. Intracranial injections of 0.5µL of drugs were administered over a period of 30 seconds during each manipulation by means of a 1 µL Hamilton syringe attached to polyethylene 20 tubing with an injector tip (28-gauge internal cannula injector, PlasticsOne, VA, USA) attached to the guide cannulas. The injector was left in place for an extra 30 seconds following injections. The SD procedure took place following injections and was like that performed in past studies (Antle & Mistlberger, 2000; Yamakawa et al., 2016). Lights were turned off at ZT6 and animals were provided with a new cage with fresh bedding and paper towels and observed in dim red light. Throughout the three hours, hamsters were monitored and given a combination of gentle pokes and/or paper towels to shred/for nest building, any time they became immobile and/or

assumed a sleeping posture (i.e., curled up or with a hunched back). A qualitative approach was used to monitor hamsters (and their level of alertness) so that for every five-minute interval, the amount of time the animal spent moving around the cage (and the number of times it engaged in behaviours including chewing on paper towels, rearing, etc.…) was recorded.

Following the SD procedure, running wheels were returned for each animal. The wheel running activity of the animals was detected by means of a magnetic switch fastened to each wheel and monitored using the ClockLab analysis software (Actimetrics, Wilmette, IL). Phase shifts were calculated with ClockLab by determining the activity onsets for the five days of activity of animals prior to the SD and the activity onsets for the two days in DD after the manipulation. The difference between the activity onsets of the entrainment period (baseline in LD) and the onsets for the two days after the SD was calculated as the phase shift.

4.2.3 Histology for cannula placement and statistical analyses

To confirm placement of the two cannulas in the IGL, brains were collected for histology at the end of the experiment. All animals were administered an overdose of sodium pentobarbital (~0.4 ml) and were transcardially perfused with ~50ml of cold PBS and subsequently, ~50ml of cold 4% paraformaldehyde in PBS. Brains were post-fixed at 4°C in 4% paraformaldehyde overnight and then transferred to 20% sucrose in PBS solution for 24 hours. Brains were cut with the use of a Leica cryostat set at a temperature of -18°C, at a thickness of 35 microns and sections through the IGL were thawed/collected directly on gelatin-coated microscope slides. Sections were subsequently stained using a cresyl violet Nissl stain and coverslipped with Permount. The slides were then viewed under an Olympus BX51 microscope to confirm cannula placements. Images were captured with a QI CAM Fast 1394 cooled CCD camera (QImaging,

Burnaby, BC). Animals were excluded if the tip of either cannula was more than 600 µm away from the margin of the IGL.

All comparisons were made using SigmaPlot (Systat Software, Inc; San Jose, CA). Statistical significance was set at $p < 0.05$. A paired t-test was used to compare the mean phase shifts for animals between the saline and atropine conditions. All means are reported as ±standard deviation in the text.

4.3 Results

Data from a total of eight hamsters were used in the analysis. No significant differences in the mean phase shifts were observed between the saline (1.423±1.244 hrs.) and atropine conditions (1.688±1.443 hrs.), $t(7) = 0.885$, $p=0.658$; Figure 4.1). Therefore, atropine did not block phase shifts to sleep deprivation, as compared to the vehicle control. Also, hamsters who were treated with atropine did not display greater drowsiness, and were not more difficult to keep awake during the last hour of the SD (as determined by qualitative observations described in the methods section). Cannula implantation in the IGL is shown in Figure 4.2.

Figure 4.1. Atropine infusions in the intergeniculate leaflet (IGL) do not block arousal-induced phase shifting.

Actograms from representative animals depicting phase shifts following treatment with 0.9 % saline, and 10mM atropine sulfate. There was no significant difference between the two conditions (p>.05, n=8). Injections took place just prior to sleep deprivation (SD) that lasted from ZT6-ZT9 (denoted by the green rectangles drawn on the actogram). Each horizontal line represents a day of wheel running as shown by the black vertical bars with subsequent days plotted below. The height of the bars is proportional to the number of wheel revolutions. A modified Aschoff Type II design was used whereby animals were in a 14:10 LD cycle but lights went off at ZT6 and remained off for the next three days (shaded area of the graph shows lights off).

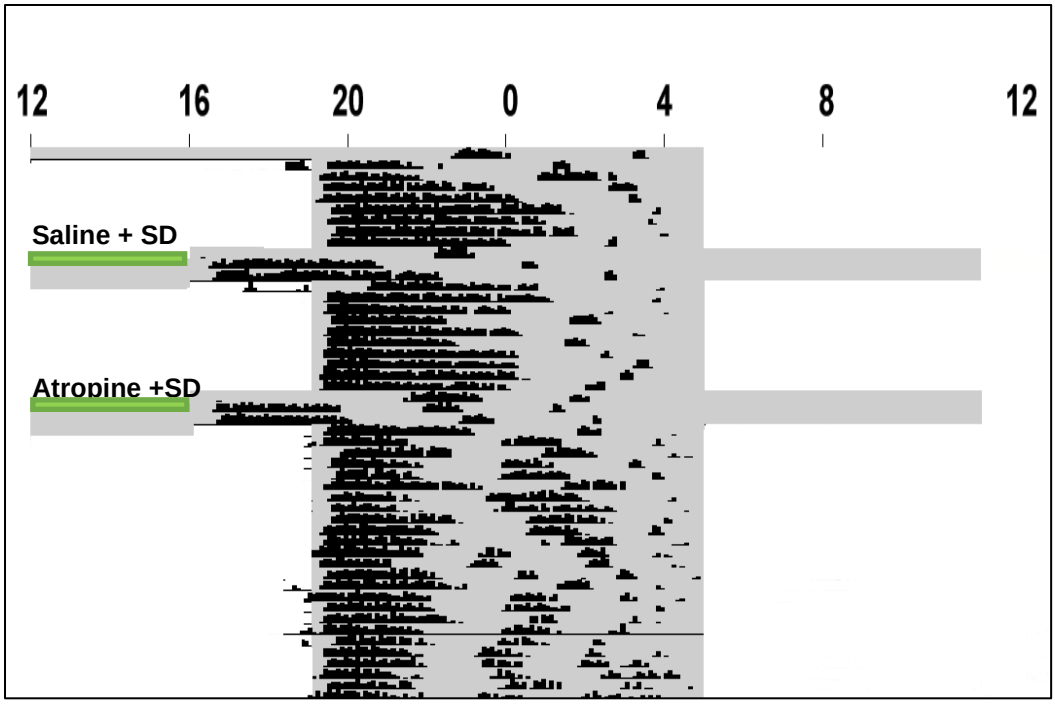
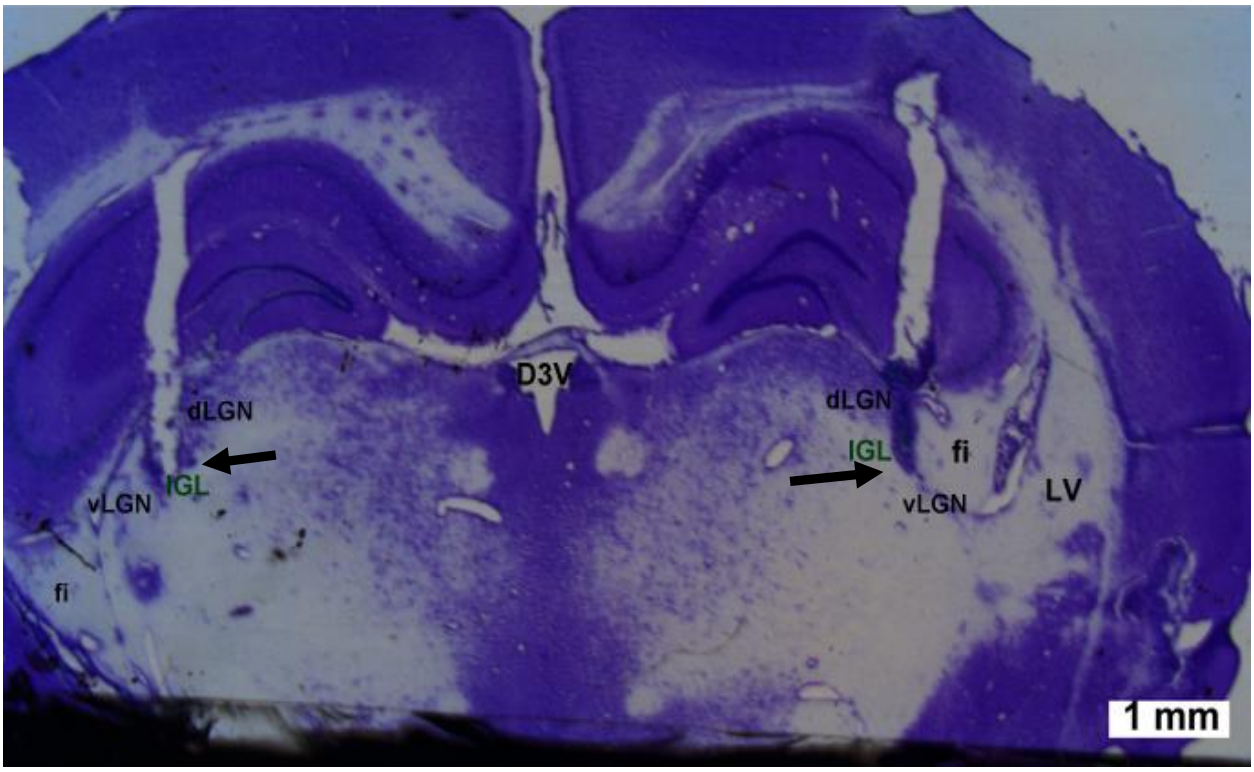

12
16
20
0
4
8
12
Saline + SD
Atropine +SD

Figure 4.2. Cannulation in the intergeniculate leaflet (IGL).

Photomicrograph (1.25x magnification) of bilateral cannula placements in the IGL of a representative hamster coronal slice stained with cresyl violet. The tip of the guide cannulas which are targeting the IGL are denoted by black arrows; D3V, dorsal 3rd ventricle; dLGN, dorsolateral geniculate nucleus; fi, fimbria of hippocampus; IGL, intergeniculate leaflet; LV, lateral ventricle; vLGN, ventrolateral geniculate nucleus.


D3V
dLGN
IGL
vLGN
fi
dLGN
IGL
fi
vLGN
LV
1 mm

4.4 Discussion

This experiment tested the hypothesis that the neurotransmitter acetylcholine is necessary for the IGL's role in non-photic entrainment. The main finding of this study is that blocking ACh in the IGL does not block phase shifts to an arousal-induced manipulation like sleep deprivation by gentle handling. This finding suggests that although ACh is both sufficient and necessary for non-photic phase shifting, it is not necessary for activation of IGL neurons and does not seem to play a primary role in NPYergic modulation of entrainment. To our knowledge, no other study has examined the effect of atropine infusions, specifically in the IGL, on sleep deprivation and arousal levels. Therefore, it is difficult to compare our findings with previous knowledge in the area. However, past findings have shown successful blocking of phase shifts to novel wheel confinement following atropine infusions in the SCN (Yamakawa et al., 2016). Therefore, it is evident that cholinergic activity at the SCN itself is necessary for arousal-induced phase shifting, rather than involvement with the IGL.

Nonetheless, there is evidence of phase shifting of the clock with direct application of ACh agonists in the IGL, suggesting a role for ACh in modulating IGL cellular activity (Cain et al., 2007). As well, this effect can be reversed with atropine. Yet the effect of blocking ACh at the IGL on behavioral manipulations like wheel confinement and sleep deprivation have not been explored and our results indicate that ACh is not required for this arousal response. However, an in vitro study examining the effect of atropine on IGL neuronal activity has suggested modulation of neuronal firing rate by ACh (Werhun & Lewandowski, 2009). Therefore, it is still possible that ACh is directly involved in modulating IGL cellular activity which can have consequences for phase resetting of the clock and/or blocking unwanted perturbations of the clock to photic stimuli. Given evidence of projections from the cholinergic cells of the basal forebrain to the IGL (see Chapter 2 of this thesis) as well as projections from

other arousal centers in the brain such as the lateral hypothalamus and the raphe nuclei (Glass et al., 2010; Mintz et al., 2001), it is likely that one or more of these areas are involved and that several neurotransmitters including ACh, glutamate, serotonin, and orexin play a role. For instance, glutamate is co-expressed with orexin of the LH and the cholinergic cells of the basal forebrain and as findings in Chapter 3 of this thesis illustrate, infusions of glutamate receptor agonists may be necessary to see an effect.

Despite the negative findings, there are several factors to keep in mind going forward. Firstly, it is important to examine the effect of blocking atropine on different types of arousal-inducing procedures such as novel-wheel confinement. This protocol involves quite a bit of locomotion while SD through gentle handling does not, and it will be intriguing to see if there is a different outcome on the phase shift response. However, it is presumed that the SD procedure is sufficient to achieve a high level of arousal given also that responders are chosen prior to the manipulation (Antle & Mistlberger, 2000; Yamakawa et al., 2016). It is understood that exercise is not necessarily required for achieving the required level of arousal so it would be predicted that atropine infusions in the IGL would also fail to block phase shifts to novel wheel confinement.

A limitation of this study was the small sample size which reduces the statistical power of the experiment. A future study with more animals would be needed to ensure that the lack of significance here is not due to the sample size. Another limitation of this study is that single bolus injections of atropine may not mimic the endogenous mechanisms and therefore may not allow for any response to be seen. Future work either looking at gradual application of atropine or a more receptor-selective antagonist would better answer the question of whether ACh is involved in modulating non-photic entrainment at the level of the IGL. Also, cellular activation

in the IGL could be examined separately from phase shifts by observing c-Fos expression in the IGL, although it is predicted that given the findings, there would be no significant attenuation in expression. Brains were collected for c-Fos staining but issues with staining prevented analysis of cell counts and so this is an area for future work. In conclusion, the findings from this study suggest that acetylcholine in the IGL is not necessary for arousal-induced phase shifting and that there are likely several neurotransmitters that are involved in IGL activation and the gating of this response.

Chapter Five: Investigating Basal Forebrain Activation with an Adenosine Antagonist

5.1 Introduction

The suprachiasmatic nucleus (SCN) is a paired hypothalamic structure that serves as the central timekeeping center in the mammalian brain (Holzberg & Albrecht, 2003). In the absence of signals from the environment, the SCN governs and maintains the rhythmicity of internal biological cycles. The existence of internal clocks as temporal regulators increases the evolutionary fitness of an organism by allowing the anticipation and organization of behaviour at the most advantageous times (Dunlap et al., 2004). Environmental cues can influence the phase of the clock and allow it to synchronize (entrain) to environmental cycles. These cues can be characterized as light (photic) and non-photic signals (Berson et al., 2002; Mrosovsky, 1996). Non-photic stimuli can include social interactions, food, and locomotion, all of which share the critical component of behavioural arousal (Antle & Mistlberger, 2000; Mistlberger & Antle, 2011; Yamakawa et al., 2016).

There are several inputs to the SCN that have been shown to be both necessary and sufficient for non-photic entrainment including neuropeptide Y (NPY) input from the intergeniculate leaflet of the thalamus (IGL) and acetylcholine from the basal forebrain (Janik & Mrosovsky, 1994; Moore & Card, 1994; Yamakawa et al., 2016). Given that there is cholinergic input from the basal forebrain to the SCN (Yamakawa et al., 2016), as well as evidence of input to the IGL (see Chapter 2 of this thesis), it is important to investigate influences on this cholinergic input to the circadian system. A possible factor is the neuromodulator adenosine. Adenosine is a homeostatic sleep factor and neuromodulator that accumulates throughout wakefulness. The administration of adenosine promotes the transition to sleep, and its accumulation occurs in specific areas of the brain including the cortex and basal forebrain (Kalinchuk et al., 2011; Porkka-Heiskanen et al., 2000). Injections of the adenosine A_1 receptor

agonist N_6-cyclohexyladenosine (N-CHA) in the SCN induces non-photic shifts (Antle et al., 2001). Like other non-photic cues, adenosine A_1 agonists suppress SCN cellular activity and attenuate phase shifts to photic stimuli (Elliott et al., 2001). Caffeine is a well-known A_1 receptor antagonist which has also been shown to block arousal-induced phase shifts, in a dose-dependent fashion (Antle et al., 2001).

Despite these findings, the site of action of adenosine is unknown. It is unclear if adenosine acts at the level of the SCN or whether it is involved in modulating the basal forebrain. The SCN itself is sensitive to caffeine, and this suggests an important role for adenosine in the clock's timekeeping mechanism (Antle et al., 2001; Burke et al., 2015; Oike et al., 2011). As well, in vitro application of adenosine has been shown to reduce the firing rate of cholinergic neurons of the substantia innominata (SI), a part of the basal forebrain that contains a dense cluster of cholinergic cells (Arrigoni et al., 2006). Subsequent application of the A_1 receptor antagonist 8-cyclopentyl-1,3-dimethylxanthine (8-CPT) blocked the effect of adenosine on firing rate (Arrigoni et al., 2006). However, it is unclear if adenosine is the specific activating signal for the cholinergic neurons of the SI, as in the mentioned study, a subset of noncholinergic neurons were also impacted by adenosine application.

This study examined the role of adenosine in one of the non-photic pathways of the circadian system. Specifically, the influence of adenosine on basal forebrain activation was investigated by targeting the A_1 receptor antagonist, 8-CPT, at the SI of the basal forebrain. It was predicted that blocking adenosine in the basal forebrain would be sufficient to cause a non-photic shift. Given that antagonists like 8-CPT promote wakefulness (Strecker et al., 2000) and that adenosine itself suppresses the activity of SI cholinergic cells, it was hypothesized that application of an antagonist would lead to disinhibition of cholinergic cells that project to the

SCN and lead to non-photic phase shifting. The expression of the immediate early gene product c-Fos was also examined in this study and taken as an indicator of cellular activation in the SI. It was predicted that administration of 8-CPT would lead to an increase in c-Fos expression in the SI. Given evidence of cholinergic input from the basal forebrain to the IGL (Chapter 2), it was also possible that blocking adenosine would lead to activation of IGL cells. Therefore, c-Fos expression was examined in this area with the prediction that 8-CPT administration would increase c-Fos levels in the IGL also.

5.2 Methods

5.2.1 Experiment 1: Intra-cranial injections of an adenosine antagonist in the basal forebrain

5.2.1.1 Animals

A total of 13 male Syrian hamsters (*Mesocricetus auratus*, 80-90g) were obtained from Charles River Laboratories (Kingston, NY, USA). Animals were placed in pairs in a temperature and humidity-controlled room with a 14:10 light dark cycle and provided with food and water *ad libitum.* Following cannula implantation, each animal was transferred to an individual cage and allowed to recover for a period of one week. Following the recovery time, hamsters were transferred to individual polycarbonate cages (20x45x22 cm) equipped with a running wheel (14 cm diameter) and kept in constant darkness (DD) for the remainder of the experiment. Periodic cage changes took place seven days prior to the day of the manipulation. All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care.

5.2.1.2 Cannulation surgeries and drugs

Stereotaxic surgery was performed for unilateral cannula implantation (22-gauge stainless steel guide cannula, PlasticsOne, VA, USA) of the basal forebrain. Specifically, the substantia innominata (SI) where there is a cluster of cholinergic cells was targeted. The surgical

procedure followed that of a previously outlined protocol (Moshirpour et al., 2020; Sterniczuk et al., 2008) and was adapted from past electrode implantations in the SI (Yamakawa et al., 2016). The coordinates used for the SI were 0.1 mm anterior to bregma, 1.9 mm lateral to bregma, and 6.2 mm ventral to the skull surface. The incisor bar was set at 2.0 mm below the interaural level.

8-CPT (8-cyclopentyl-1,3-dimethylxanthine; Tocris Bioscience, Minneapolis, MN, USA) was dissolved in 0.1% NaOH to final concentrations of 10μM and 15μM. Different concentrations were used based on the literature to observe if there is a dose-dependent effect (Blanco-Centurion et al., 2006; Strecker et al., 2000). The vehicle control was 0.1% NaOH dissolved in artificial cerebrospinal fluid (aCSF). Drugs were administered to animals based on a counterbalanced design. Intracranial injections of 0.5μL of drugs were administered over a period of 30 seconds during each manipulation by means of a 1 μL Hamilton syringe attached to polyethylene 20 tubing with an injector tip (28-gauge internal cannula injector, PlasticsOne, VA, USA) attached to the guide cannulas. The injector was left in place for an extra 30 seconds following injections. All manipulations took place in DD condition with night vision goggles.

5.2.1.3 Phase shift and statistical analyses

Injections were made at mid-subjective day (CT6). Wheel running activity of animals was monitored using ClockLab analysis software (Actimetrics, Wilmette, IL) and phase shifts were analyzed by fitting a line of best fit to activity onsets for a period of 10 days before the manipulation and another line to onsets occurring on subsequent days following the manipulation (3-10 days). The horizontal difference between the regression lines were taken as the phase shift. All statistical comparisons were made using SigmaPlot (Systat Software, Inc; San Jose, CA). A one-way repeated measures analysis of variance (ANOVA) was used to determine if there are

statistically significant differences in the phase shifts between the conditions (NaOH, 10µM and 15µM of 8-CPT). All means are reported as ± standard deviation in the text.

5.2.1.4 Histology for cannula placement

To confirm placement of the cannula in the SI, brains were collected for histology at the end of the experiment. All animals were administered an overdose of sodium pentobarbital (~0.35 ml) and were transcardially perfused with ~50ml of cold PBS and subsequently, ~50ml of cold 4% paraformaldehyde in PBS. Brains were post-fixed at 4°C in 4% paraformaldehyde overnight and then transferred to 20% sucrose in PBS solution for 24 hours. Brains were cut with the use of a Leica cryostat set at a temperature of -20°C, at a thickness of 35 microns and sections through the basal forebrain were thawed/collected directly on gelatin-coated microscope slides. Sections were subsequently stained using a cresyl violet Nissl stain and coverslipped with Permount. The slides were then viewed under an Olympus BX51 microscope to confirm cannula placements. Images were captured with a QI CAM Fast 1394 cooled CCD camera (QImaging, Burnaby, BC). Animals were excluded if the tip of the cannula was more than 600 µm away from the SI.

5.2.2 Experiment 2: Systemic injections of an adenosine antagonist for observation of c-Fos in the basal forebrain and intergeniculate leaflet

5.2.2.1 Animals and drugs

A total of ten male Syrian hamsters (*Mesocricetus auratus*, 80-90g) were obtained from Charles River Laboratories (Kingston, NY, USA). Animals were placed in pairs in a temperature and humidity-controlled room with a 14:10 light dark (LD) cycle and provided with food and water *ad libitum.* Hamsters were left in the room for at least two weeks, enough time for them to entrain to the LD cycle. On the day of the manipulation, animals were injected just prior to lights

being turned off at midday (six hours prior to the time of dark onset, which by convention is zeitgeber time (ZT) 6, where dark onset is ZT 12 by convention). Animals were left alone in DD and euthanized and perfused 90-minutes post injection for brain extraction and staining (peak expression of c-Fos protein is approximately 90-120 minutes post stimulus; Kovács, 1998).

8-CPT was dissolved in 100% DMSO (4 mg/ml) and administered to animals at a dose of 20mg/kg with DMSO serving as the vehicle control. Lower percentages of DMSO were tried but it was difficult to keep the drug from not coming out of solution and given the short duration between injections and perfusions, 100% DMSO was used. The dose was chosen based on published findings that there is greater selectivity of 8-CPT for the A_1 receptor over the A_2 receptor (Stenberg et al., 2003).

5.2.2.2 Perfusions and immunohistochemistry

Animals were administered an overdose of sodium pentobarbital (~0.35 ml) in the dark with the aid of night vision goggles. Following the absence of reflexes, their heads were wrapped in aluminum foil to prevent light input to the eyes, and they were taken out in the light. Hamsters were perfused with ~50ml of cold PBS and then ~50ml of cold 4% paraformaldehyde in PBS. Brains were extracted and post-fixed at 4°C in 4% paraformaldehyde overnight and then transferred to a series of sucrose solutions, each for 24 hours (20% to 25% to 30% sucrose in PBS). Brains were cut with the use of a Leica cryostat set at a temperature of -19°C, at a thickness of 35 microns. Sections were sliced throughout the intergeniculate leaflet (IGL) and the basal forebrain (SI) and were collected into PBS baths with 2% sodium azide.

To visualize cellular activity in the SI and IGL, sections underwent fluorescent staining c-Fos. Free -floating sections were rinsed in 0.1% PBSx (3x10 minutes) and incubated in the primary antibody (rabbit anti-cFos (1:500) diluted in blocking buffer: 3% normal donkey serum,

NDS) for 24 hours at room temperature (on a shaker tray). The tissue was then rinsed again in PBSx (3x10 min) prior to a 24-hour incubation in the secondary antibody, Alexa Fluor 594 Donkey anti-rabbit (1:500) at room temperature. The tissue was protected from light from addition of the secondary antibody onward. Sections were once again rinsed with PBSx (2x10 min) prior to being mounted and coverslipped with mounting media and stored in the dark.

5.2.2.3 c-Fos cell counts and statistical analyses

Mounted brain sections were photographed using an Olympus BX51 microscope attached with a QI CAM Fast 1394 cooled CCD camera (QImaging, Burnaby, BC, Canada) and observed/analyzed using Image-Pro Plus software (Media Cybernetics, Inc. Rockville, MD). All collected IGL sections from the rostral, mid, and caudal regions (at least four sections), were selected for analysis and all representative sections from the basal forebrain were selected which included the cluster of cholinergic cells in the SI (at least three sections). Alternate sections were collected for NPY and ChAT staining to confirm the boundaries of the IGL and SI, respectively and with the use of a brain atlas, *A Stereotaxic Atlas of the Golden Hamster Brain* (Morin & Wood, 2001). Fos cells were counted within the IGL and BF boundaries by using Image J (NIH, Bethesda, MD, USA).

All comparisons were made using SigmaPlot (Systat Software, Inc; San Jose, CA). Statistical significance was set at $p<0.05$ for all tests. The mean cell counts for the IGL (with cell counts averaged for the left and right IGL) were compared for the two manipulations (DMSO, 8-CPT) using an independent sample's t-test. The same analysis was also used to compare mean cell counts for the SI (averaged for the left and right SI) All means are reported as ± standard error of the mean (SEM) in the figures and as ±standard deviation in the text.

5.3 Results

5.3.1 Experiments 1 and 2: Blocking adenosine does not cause non-photic phase shifting or significant cellular activation in the SI or IGL

Data from a total of ten hamsters (out of 13) were used in the analysis due to various reasons including loss of head caps, animal death, and/or missed cannula placements. Furthermore, due to the above-mentioned cases, not all animals received all three treatments. However, all animals included in the analysis received at least one vehicle and one treatment condition (10µM or 15µM 8-CPT). There was no significant difference in phase shift responses between the treatments, $F(2,18) = 0.857$, $p = 0.441$ and negligible phase shifts were recorded between all conditions (Figure 5.1). A successful cannula placement targeting the SI is shown in Figure 5.2.

For experiment 2, data from seven animals (out of ten; n=3 DMSO, n= 4 8-CPT) was used in the analysis as the tissue from the other three animals was not representable. The number of c-Fos positive cells in the SI was not significantly different in the drug condition (8-CPT; 115.453±21.359) as compared to the vehicle group (DMSO; 126.243 ±14.965, $t(5) = .741$, $p = .429$), Similarly, there was no significant difference in c-Fos expression in the IGL between 8-CPT (12.883 ±10.314) and DMSO (12.419 ±9.997) conditions, $t(5) = -0.060$, $p = .955$ (Figure 5.3).

Figure 5.1. Infusions of the adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dimethylxhanthin (8-CPT) in the basal forebrain (substantia innominata, SI) do not cause significant phase shifts as compared to vehicle control.

Actograms from representative animals depicting phase shifts following treatment with vehicle, 0.1% NaOH, 10µM 8-CPT, and 15 µM 8-CPT. There was no significant difference between the three conditions (p>.05, n=10). Injections took place at CT6 as denoted by the grey diamond (◊). Each horizontal line represents a day of wheel running as shown by the black vertical bars with subsequent days plotted below. The height of the bars is proportional to the number of wheel revolutions. The red diagonal lines are lines of best fit drawn to represent activity onsets before and after the manipulations.

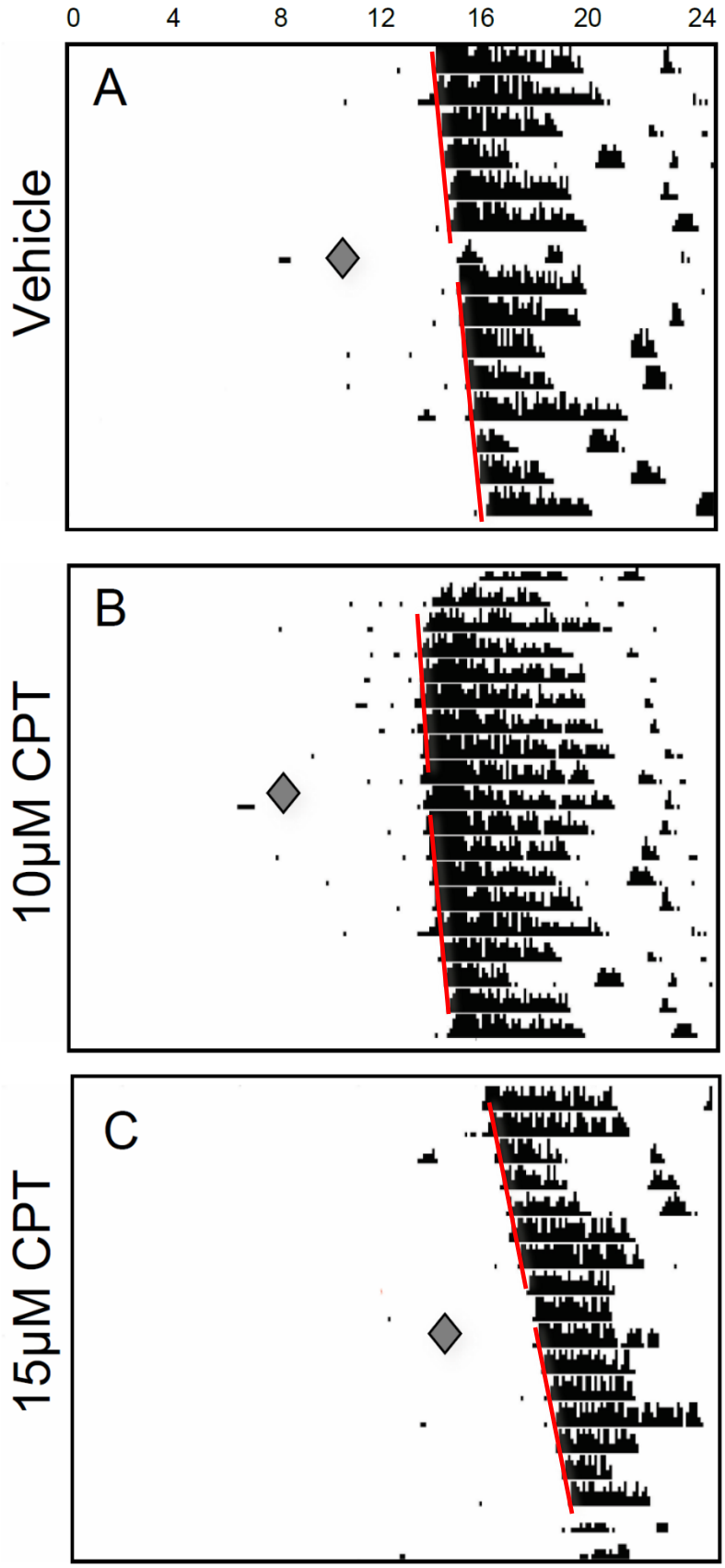

0
4
8
12
16
20
24
A
Vehicle
B
10μM CPT
C
15μM CPT

Figure 5.2. Unilateral cannulation in the substantia innominata (SI) of the basal forebrain. Photomicrograph (1.25x magnification) of a successful cannula placement in the SI of a representative hamster coronal slice stained with cresyl violet. The tip of the guide cannula is denoted by the black arrow which is targeting the SI; 3V, 3rd ventricle, D3V, dorsal 3rd ventricle; ic, internal capsule; fi, fimbria of hippocampus; hf, hippocampal fissure; LV, lateral ventricle; ot, optic tract; SI, substantia innominata.

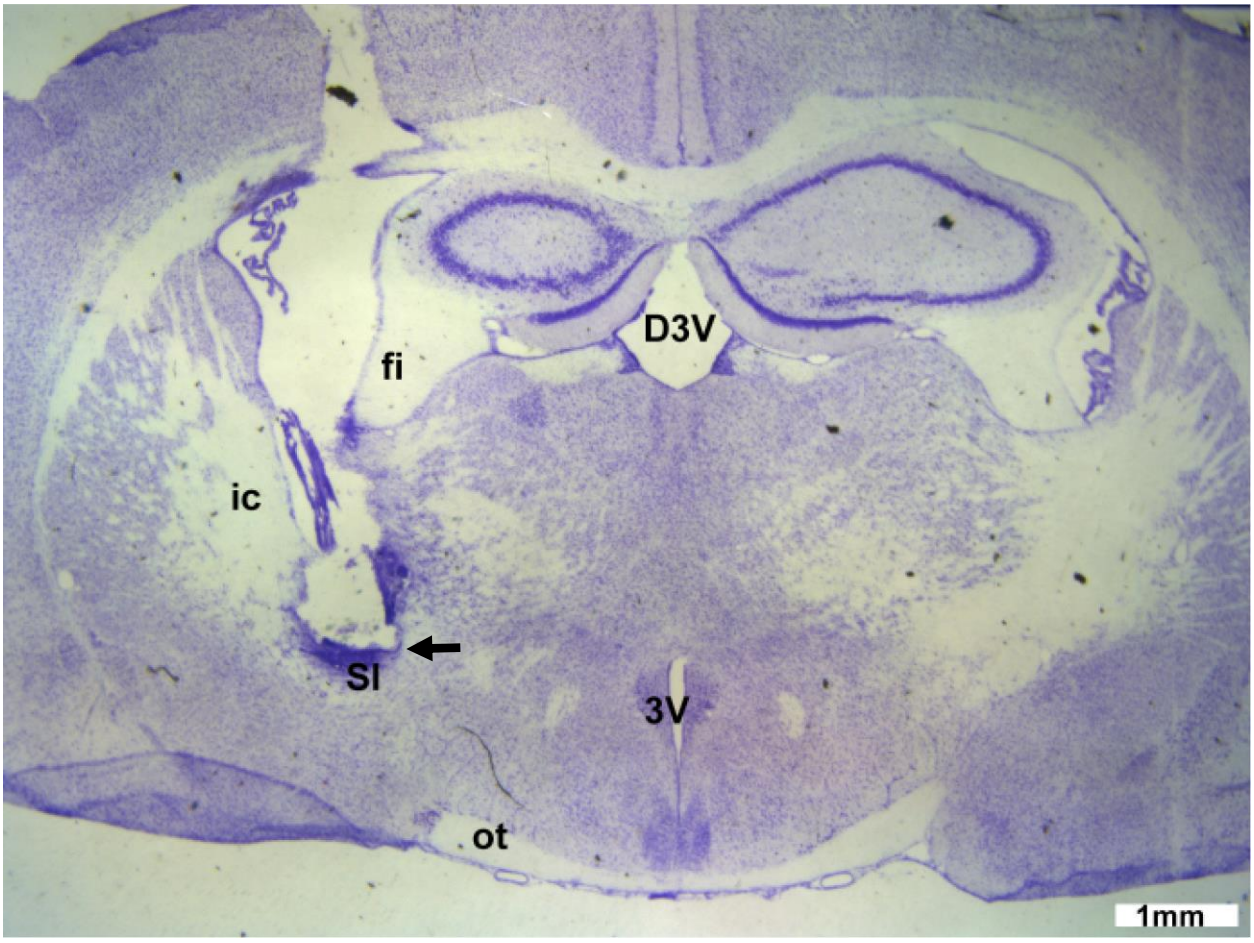
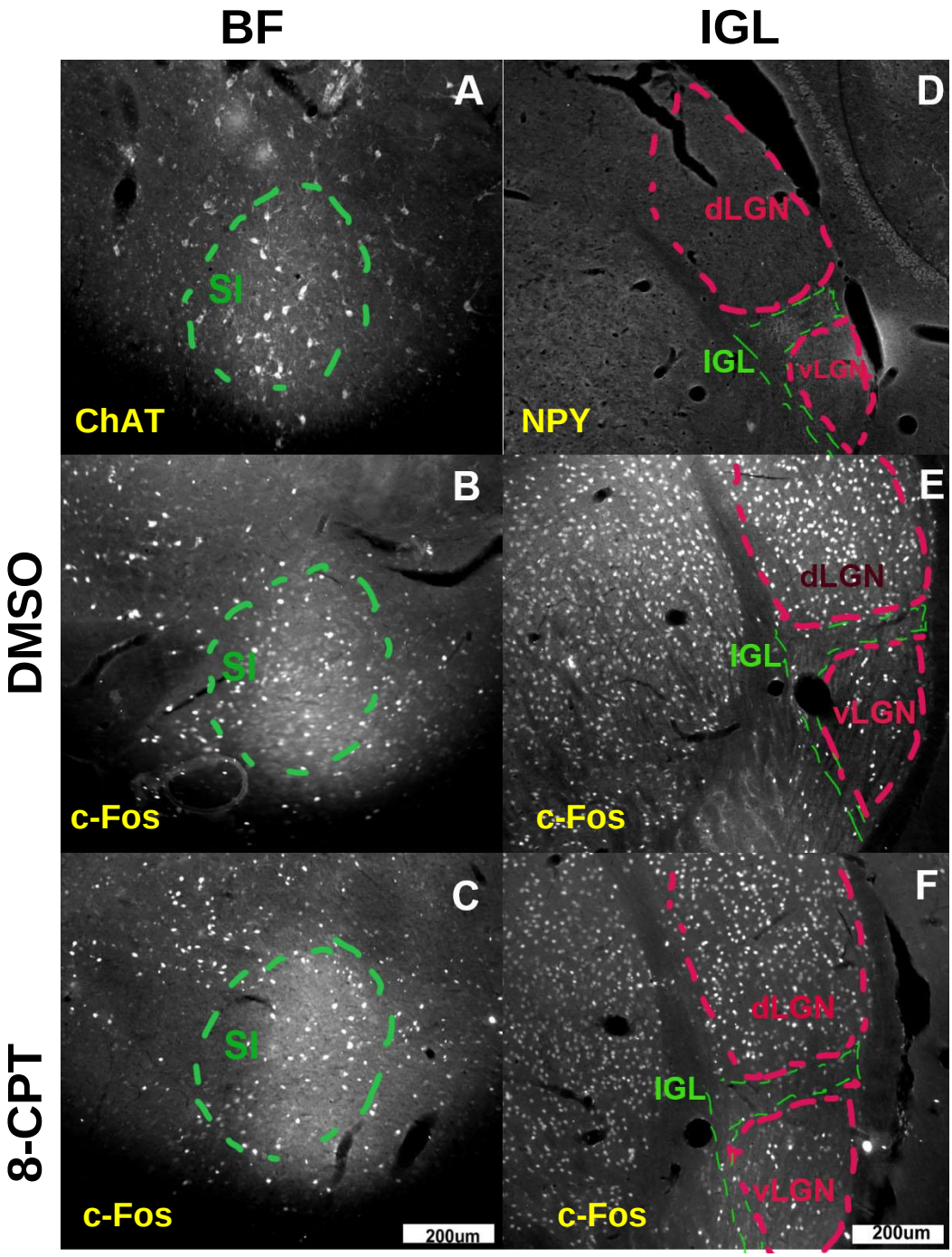

D3V
fi
ic
Sl
3V
ot
1mm

Figure 5.3. Systemic infusions of the adenosine A_1 receptor antagonist, cyclopentyl-1,3-dimethylxhanthin (8-CPT) does not enhance cellular activation in the substantia innominata (SI) of the basal forebrain, as compared to vehicle control.

Photomicrograph (10x magnification) of representative hamster coronal slices showing **(A)** ChAT-staining representing the boundaries of the SI **(B)** c-Fos expression in the SI for the vehicle control (DMSO) and **(C)** 8-CPT. **(D)** NPY-staining delineating the intergeniculate leaflet (IGL), and **(E)** c-Fos expression in the IGL for DMSO, and **(F)** 8-CPT. No significant difference in c-Fos expression was found between the two conditions for both the SI, and IGL ($p > 0.05$, n=8). dLGN, dorsolateral geniculate nucleus; vLGN, ventrolateral geniculate nucleus.


BF
IGL
A
D
SI
dLGN
IGL
vLGN
ChAT
NPY
DMSO
B
E
SI
dLGN
IGL
vLGN
c-Fos
c-Fos
8-CPT
C
F
SI
dLGN
IGL
vLGN
c-Fos
c-Fos
200um
200um

5.4 Discussion

This study examined the role of the neuromodulator adenosine in non-photic entrainment by examining a phase shift response and activation of the cholinergic basal forebrain in response to infusion of an adenosine receptor antagonist. The main finding of this experiment is that blocking adenosine is not sufficient in inducing phase shifts and therefore the method of basal forebrain activation has not been made clear by this study. Also, systemic infusions of the antagonist, 8-CPT did not cause noticeable activation of the basal forebrain or the IGL. Taken together, these findings suggest that adenosine is not the primary signal that is able to activate cholinergic cells that project to the circadian system. However, despite the negative findings, a role for adenosine can still be possible considering what has already been reported in the literature.

Findings from this study are not in line with past reports of adenosine activation of SI cholinergic cells (Arrigoni et al., 2006). However, not much has been investigated in this area, and in relation to the circadian system, so it is difficult to compare the findings here to past reports. Though the role of adenosine as a homeostatic sleep factor has been well documented (Blanco-Centurion et al., 2006; Strecker et al., 2000), its role in arousal-induced phase shifting is not understood. However more recently, a role for adenosine in entrainment has been described where adenosine has been shown to regulate the clock genes *Per1* and *Per2* and inhibit entrainment by light cues (Jagannath et al., 2021). Thus, sleep information has been shown as an important contributor to modulation of photic pathways to the circadian clock. This is in line with the explanations in Chapter 3 of this thesis which discussed the ability of arousal centers to suppress cellular activity at the SCN, as a mechanism for blocking responses to light signals. However, this was discussed in the context of acetylcholine from the basal forebrain, but it may well involve adenosine given that it is an important sleep factor. Accumulation of adenosine

which takes place in the basal forebrain may be important for blocking or modulating the clock's response to light cues. It would be intriguing to study the effects of the same A_1 antagonist used in this study on photic phase shifting by conducting a similar experiment but with injections prior to a brief light pulse. According to the study by Jagannath et al. (2021), an antagonist should enhance phase shifting to light and it will be important to examine this at the level of the basal forebrain.

The examination of c-Fos expression in the SI also did not yield significant findings in this study, further suggesting that adenosine is not sufficient for activating the basal forebrain. However, it should be noted that there are several study limitations that make it difficult to draw any definitive conclusions. Firstly, the behavioral experiment with intra-cranial 8-CPT infusions involved unilateral cannulation and given the bilateral structure of the basal forebrain, it is likely that not all adenosine receptors were blocked. Nonetheless, past studies in our lab with unilateral IGL injections were sufficient to see a phase shift effect so bilateral cannulas may not have been required. Given the angle at which the cannulas had to be implanted to avoid contact with one another, the procedure in this lab was simplified with the use of a single cannula. However, a future study with bilateral drug administration may be necessary. Also, no significant difference was observed between the different drug doses which were chosen based on what has been used in the past but different concentrations and/or various antagonists may need to be tested to further understand the role of adenosine in entrainment. This study, like most others, concentrated on the A_1 receptor antagonist. However, there are also A_2 adenosine receptors expressed in the SCN (Dunwiddie & Masino, 2001). However, at least one study has suggested that A_2 receptor agonists and antagonists have the opposite effect to the A_1 receptor and so they may not be involved in the modulatory arousal pathways that influence clock responses

(Jagannath et al., 2021). However, another study has also found that the adenosine A_1 receptor does not prevent the regulation of sleep (Stenberg et al., 2003).

A further limitation of this study is that intracranial 8-CPT injections may not have activated the necessary proportion of cholinergic cells in the SI. Although cannula placements were confirmed and successful targeting of the basal forebrain region where the SI is most likely found was supported by histology, there is no way to confirm that actual cholinergic cells were targeted. A future study using a more targeted approach would be an important follow-up step. Looking at triple labeling of IGL or SCN projecting cholinergic cells that are activated following a manipulation (labeling with a tracer like CTβ, choline acetyltransferase, ChAT, and c-Fos) will ensure that the population of cells that are interesting to look at are being targeted.

To conclude, the findings from this study suggest that there is likely no role for adenosine in non-photic entrainment, and, for activation of the basal forebrain. It is not fully known how this area gets activated and whether the site of action of adenosine is at the clock itself, or at some farther away input like the IGL or SI. Nonetheless, evidence from recent published findings support a role for adenosine as a part of a regulatory mechanism for photic entrainment and given the possible role of several neurotransmitters in arousal-induced phase shifting, including acetylcholine, glutamate, and orexin (as discussed in Chapters 2-4 of this thesis) it is likely that one or more of these arousal signals act (and perhaps synergistically) to modulate the clock phase and influence clock responses to stimuli.

Chapter Six: General Discussion

6.1 Summary and Conclusions

The aim of the studies outlined in this thesis was to establish a link between the intergeniculate leaflet, a necessary component of the circadian circuitry, and the major arousal centers of the brain. Despite the well-known role of the IGL in non-photic entrainment, how this area gets activated and what mechanisms underlie gating of arousal-induced phase shifting has not been established in the circadian research community. Nonetheless, increased evidence is emerging that is linking sleep-wake centers with phase resetting mechanisms of the circadian clock. To make sense of these findings, this thesis examined a potential link between the IGL and three main arousal signals: orexin from the lateral hypothalamus, acetylcholine from the basal forebrain, and the neuromodulator adenosine that accumulates among other areas, in the basal forebrain. The four main findings of this work are that: (1) orexin and glutamate likely have a dual effect on IGL activation and the presence of both neurotransmitters is sufficient for non-photic phase shifting, (2) the IGL receives cholinergic input from the substantia innominata, which also projects to the central clock and has been shown in the literature to be both sufficient and necessary for phase shifting, (3) the cholinergic input to the IGL does not seem to be necessary for non-photic phase shifting, and (4) blocking adenosine is also not sufficient in inducing phase shifts.

The main novel finding of this work is that glutamatergic input is likely a necessary component and that in addition to this, orexin can act as an arousal signal and lead to activation of IGL NPYergic neurons. Arousal-inducing manipulations have also been shown to lead to activation of a significant portion of orexinergic cells of the lateral hypothalamus. Experiments in Chapter 2 were able to provide further support for the existence of an orexinergic pathway to the IGL which provides evidence on top of what has already been reported in the literature

(McGranaghan & Piggins, 2001; Mintz et al., 2001). Chapter 3 further explored this orexinergic pathway and the outlined experiments suggest that the co-release of orexin and glutamate is sufficient to cause phase shifts like those seen with arousal procedures and stimuli. Although tracing of activated orexin neurons in the LH (in response to the sleep deprivation protocol) was unsuccessful, activation of the orexin cells nonetheless indicates that the neurotransmitter is involved in non-photic entrainment.

The interesting finding from Chapter 3 is that orexin alone is unable to cause phase shifts but that the presence of glutamate (acting via NMDA receptors) is important for resetting the phase of the clock. Given that these injections were made in the IGL, it is likely that orexin and glutamate act directly on NPY neurons that project to the SCN. Figures 6.1 and 6.2 outline the circadian circuitry as discussed in Chapter 1 with updated pathways to the IGL (from the BF) and present a summary of the proposed model of IGL activation. Based on the findings of this thesis, the proposed model of IGL activation involves direct glutamatergic input to the IGL NPY cells from orexinergic neurons of the LH. Glutamate release is likely sufficient for activating NPY cells as was evident by significant phase shifts in response to NMDA infusions. The more gradual release of orexin from these cells is then likely to augment the response. Past findings showing that orexinergic input regulates firing rate of IGL neurons provide further support for a role for orexin as an activating arousal signal to the IGL. Perhaps studies by Palus-Chramiec et al. (2019) and Belle et al. (2014) are the best sources for linking our findings with a functional role for orexin at the IGL.

Figure 6.1. Schematic of the circadian network with a proposed model of IGL activation. Image of a rodent brain displaying the photic and non-photic pathways important for entrainment of the suprachiasmatic nucleus (SCN) including light input from the eyes, inputs from the intergeniculate leaflet (IGL), basal forebrain (BF), lateral hypothalamus (LH), the median raphe nucleus (MRN) and dorsal raphe nucleus (DRN). According to the findings of this thesis, there is direct cholinergic input (ACh) from the basal forebrain (BF) to the intergeniculate leaflet (IGL; grey arrows) in addition to the SCN. Orexinergic input from the lateral hypothalamus (LH, green arrow) to both the SCN, and IGL regions is also shown. These orexin cells are glutamatergic and their input to the IGL is proposed to activate neuropeptidergic (NPY) cells of the IGL that then project to the SCN and are necessary for non-photic entrainment. Glutamate input is likely sufficient for activation with orexin likely adding to IGL activation.

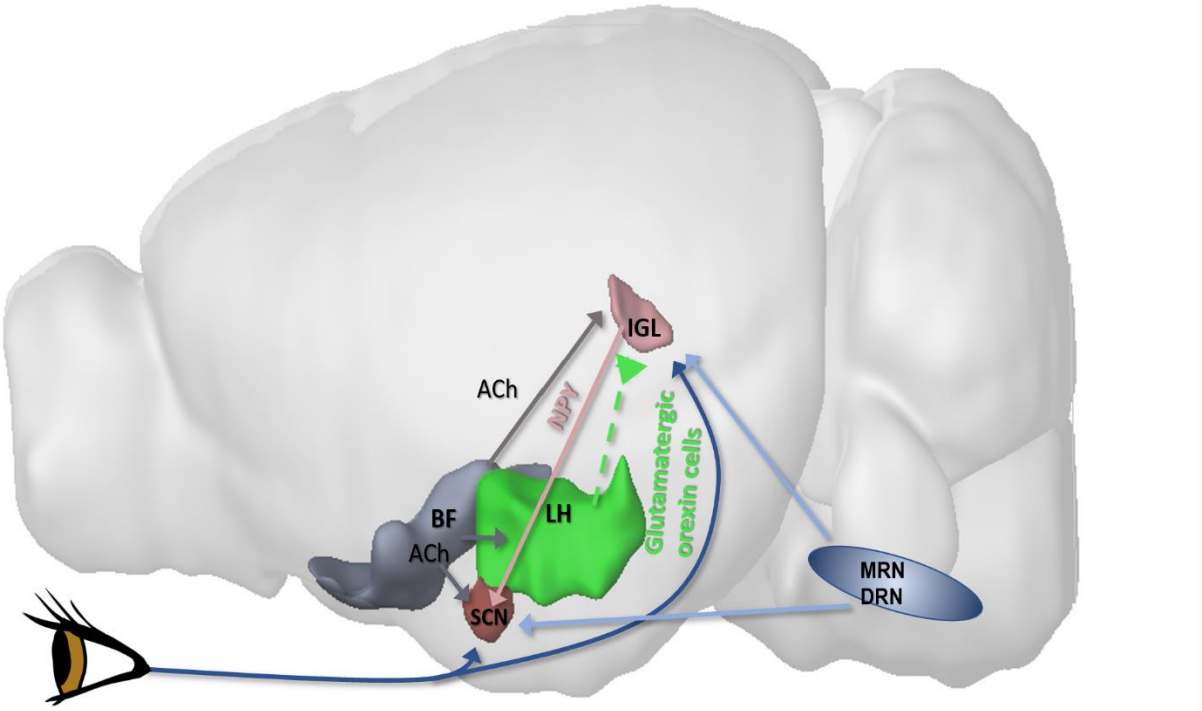
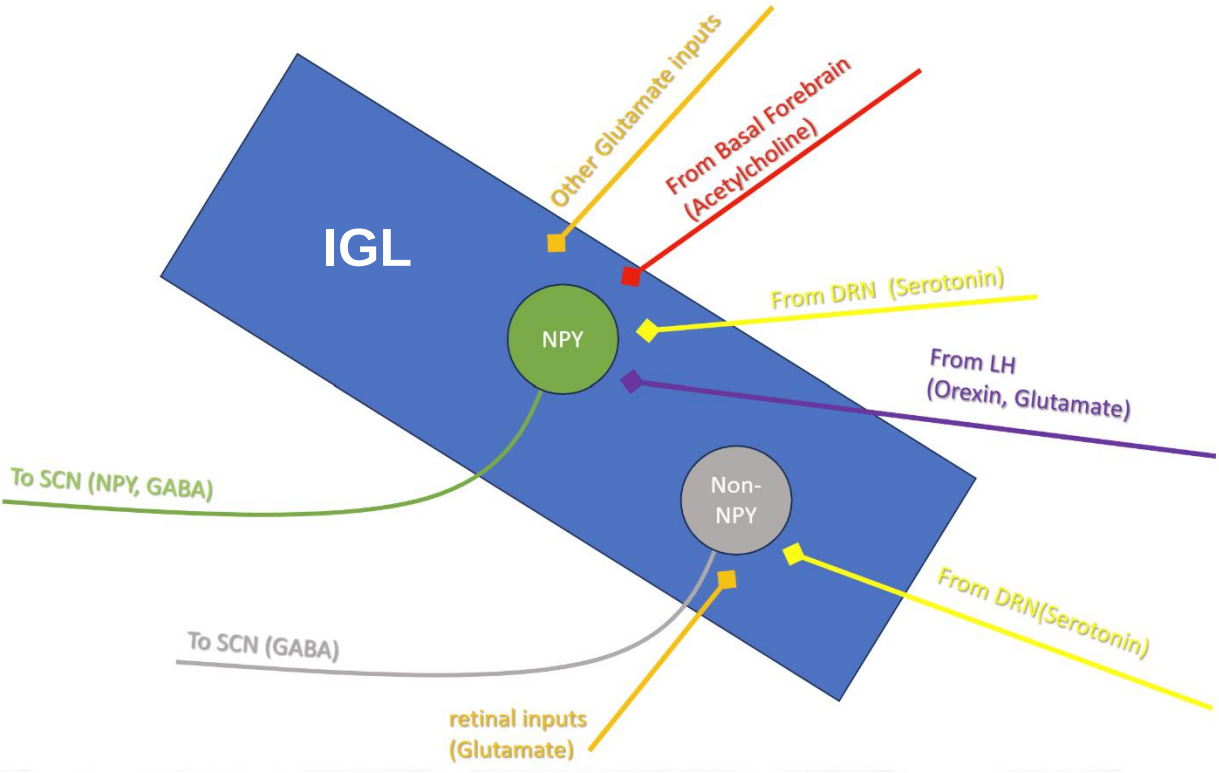

IGL
ACh
NPY
Glutamatergic orexin cells
BF
ACh
LH
SCN
MRN
DRN

Figure 6.2. Schematic of the IGL as part of the circadian circuitry. Schematic of the intergeniculate leaflet (IGL; blue rectangle) displaying inputs from important wakefulness centers in the brain including glutamatergic and orexinergic input from lateral hypothalamus (LH; purple arrow), cholinergic input from the basal forebrain (red arrow) and serotonergic input from the dorsal raphe nucleus (DRN; yellow arrow). There are likely other sources of glutamatergic input to the IGL (orange arrow) from brain areas other than the LH. It is proposed that glutamatergic and orexinergic input from the LH is important for the activation of neuropeptide Y (NPY) cells (shown in green) of the IGL that also release GABA. These cells project to the suprachiasmatic nucleus (SCN) and induce phase shifts by inhibiting cellular activity in the SCN. Other cells in the IGL (shown in grey) receive glutamatergic retinal inputs (orange arrow) and are important for the modulation of photic responses. There are also serotonergic inputs from the DRN to these cells (yellow arrow). These cells are principally GABAergic and modulate the SCN's response to light information.


IGL
Other Glutamate inputs
From Basal Forebrain
(Acetylcholine)
From DRN (Serotonin)
NPY
From LH
(Orexin, Glutamate)
To SCN (NPY, GABA)
Non-
NPY
From DRN(Serotonin)
To SCN (GABA)
retinal inputs
(Glutamate)

More specifically, orexin depolarizes IGL neurons and seems to enhance the actions of NPY neurons on the SCN's cellular activity (specifically, by increasing suppression of cellular activity). Given that arousal cues for the most part, suppress SCN cellular activity and inhibit clock gene expression, it makes sense for orexin to be the gating signal for NPY cells of the IGL. The role of NPYergic neurons is to suppress SCN cellular activity, perhaps as a mechanism for blocking phase resetting by light. And if orexin is the arousal signal, then its influence on NPY cells of the IGL would be to increase their activity. This is in line with the phase shifts that we saw after orexin infusions. Also, in a pilot study in the lab (unreported findings), the blocking of orexin by the antagonist, MK-6096, resulted in significantly lower c-Fos expression in the IGL as compared to the control condition. As well, other findings from the lab have shown an increase in c-Fos expression post-orexin IGL infusions. All of this provides support for the role of orexin in non-photic entrainment. However, since orexin on its own is not sufficient for phase shifting, most likely other neurotransmitters are intimately involved, glutamate being one of them.

The experiments in Chapter 4 of this thesis were designed and conducted partly due to past findings highlighting the importance of cholinergic input to the SCN as well as findings showing phase shifts in response to carbachol injections in the IGL. Chapter 4 experiments were also planned partly due to the findings in Chapter 2 reporting cholinergic input from the basal forebrain to the IGL. This is the first report of such a projection and complements findings of a projection to the SCN (Yamakawa et al., 2016). This is also not that surprising considering that the IGL receives input from other cholinergic arousal centers such as some brainstem nuclei in the reticular activating system (Moore et al., 2000; Morin & Blanchard, 2005). However, the role of these areas in non-photic entrainment has not been studied, while the basal forebrain seems to

be necessary for phase shifting. To investigate the role of acetylcholine at the IGL, the muscarinic receptor antagonist, atropine, was injected prior to SD. However, atropine was unable to block phase shifts suggesting that ACh is not the necessary signal for activating the IGL. Similarly, Chapter 5 looked at adenosine as another signal that is present in the basal forebrain and may participate in non-photic entrainment. However, despite findings that adenosine suppresses the activity of the basal forebrain cholinergic cells, blocking adenosine with an A_1 receptor antagonist (8-CPT) did not activate the basal forebrain and did not lead to non-photic shifts. Taken together, findings from Chapters 4 and 5 do not provide evidence for a necessary role of basal forebrain input to the IGL, in non-photic entrainment. However, these findings also do not rule out a possible role of this area in IGL activation, especially considering the projections of cholinergic cells to the IGL (Chapter 2).

6.2 Limitations and Future Directions

There are several limitations to the studies presented here that should be considered when reflecting upon the findings of this thesis. Firstly, pertaining to the tract tracing study in Chapter 2, although brain sections were collected throughout the brainstem area as well, these tissues were not representable and therefore staining of these areas did not take place. However, this should be taken into consideration for future research as it will be important to understand the extent of cholinergic input to the IGL from brainstem arousal centers. Furthermore, although cholinergic cells in the SI of the basal forebrain were clearly visualized, the proportion of these cells that project to the IGL was not determined. There are several known cell types in the basal forebrain including glutamatergic neurons (Gritti et al., 2003). To get a better understanding of the dual role of glutamate and other neurotransmitters, it will be important to compare the number of projections from cholinergic cells as compared to other cell types. Also, owing to issues with the tract tracing procedures, the sample size for this experiment was small. It will be

important to follow up this experiment with more animals to provide further support for a link between the basal forebrain and the IGL and to determine the proportion of cells that project to the IGL. Furthermore, pertaining to findings in Chapter 3, it will be important to follow up the experiments with a tract tracing study that links the activated orexinergic neurons of the lateral hypothalamus directly to the IGL. In this way, a stronger link can be established between orexin and the IGL's activation.

Another limitation which is relevant to Chapter 3 is that although blocking orexin did not attenuate arousal-induced phase shifting, given the evidence that multiple neurotransmitters are involved, this may have not accomplished much. To better understand what goes on internally, it would make sense to block glutamate release also. However, blocking glutamate on its own can prevent anything further from happening so it becomes a bit more complicated to interpret the findings. Also, experiments in Chapter 3 only focused on the NMDA receptor while it is well known that there are a variety of glutamate receptors and so focusing on another ionotropic receptor such as AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptors) is important given their relevance. More importantly, despite the co-release of glutamate and orexin, there are likely multiple sources of glutamate to the IGL, other than from orexinergic cells of the LH. This should be the focus of a more targeted future study looking at the sources of input to the NPY cells. However, our findings lend support for the glutamatergic modulation of the IGL from orexin cells in the LH. Another limitation of the experiments throughout this thesis which involved intra-cranial drug infusions is that the manipulation may not mimic what takes place internally. Specifically, with intra-IGL infusions, administration of a single bolus may not be sufficient to initiate a response while repeated injections (or continuous and gradual infusions) may be necessary. This was considered in Chapter 3 which is why orexin administration was

repeated every hour for three hours and why for all IGL surgeries, double cannulas were implanted. This would also explain why shifts to orexin were observed as compared to negligible differences in a pilot study with single IGL injections. The same argument can be made with regards to atropine and 8-CPT injections as described in Chapters 4, and 5, respectively. However, in the former case, the sleep deprivation procedure followed immediately after the injection and the goal was to block arousal phase shifts while in the latter case, logistical issues and time prevented adjustments to the study design. A study tracing neuronal pathways with the aid of CAV (Canine adenovirus) vectors would be a better approach for studying the afferent pathways of the IGL. Also, stimulation of specific neuronal cell types with the use of Designer Receptors Exclusively Activated by Designer Drugs (DREADDS), or electrical stimulation, would be a more targeted approach and a logical future direction. An approach that specifically targets NPY cells of the IGL would be most helpful in uncovering the role of signals like orexin and acetylcholine.

Finally, pertaining to Chapter 4, another limitation is the use of a non-selective receptor antagonist for studying the role of acetylcholine. Although histology confirmed that cannulas targeted the IGL, it is unclear why atropine injections do not block phase shifts to sleep deprivation but are able to block similar phase shifts to carbachol. Repeating this study with a more selective antagonist along with another experimental group with novel wheel confinement can help uncover the role of ACh in the IGL. Also, given the findings of the possible additive role of orexin and glutamate, it will be important to observe what happens with injections of NMDA/carbachol cocktail as well as orexin/carbachol cocktail. The latter has been done already in a pilot study in the lab but yielded negative findings. However, this study had logistical issues and should be replicated with more animals.

It is important to note that although pilot studies were conducted for the experiments in Chapter 3, this was not undertaken for the experiments in Chapters 4 and 5 and more importantly, a power analysis was not performed to get a better idea of the sample sizes. Given the negative findings in both Chapters 4 and 5, the low sample size may have been an issue. Determining an ideal sample size by conducting a power analysis would ensure adequate statistical power to detect significance and decrease the chances of failing to reject a false null hypothesis which assumes no difference between conditions. In terms of the findings in Chapter 3, the tract tracing experiments need to be conducted with more animals while the behavioural experiments looking at phase shifts in response to the orexin and NMDA cocktail yielded positive findings despite a relatively small sample size. Furthermore, the majority of the experiments outlined in this thesis utilized male animals with the exception of Chapter 3 and therefore sex differences were not examined. However, the results of orexin and NMDA infusions in female hamsters has also been done in a previous related experiment in our lab and showed significant phase shifts also. However, the female hamsters used were too old and their running data was not consistent, so this data was not included in this thesis. However, this should be followed up with an emphasis on any differences between the sexes in terms of the phase shift response.

In conclusion, the findings of this thesis point towards a role for orexin and glutamate in non-photic entrainment and activation of the intergeniculate leaflet. The IGL is a major source of input to the circadian clock and understanding what underlies its activation warrants further examination. This thesis points towards a role for multiple arousal centers in non-photic entrainment including the lateral hypothalamus and basal forebrain. Although the novel positive findings of this thesis pertain to the lateral hypothalamus, our findings also suggest a link

between the cholinergic forebrain and the IGL. Understanding how the clock receives information about arousal cues is crucial for determining the adaptive role of arousal cues in combination with photic cues. If the external light-dark cycle is the principal entraining cue, why then do a whole host of other signals influence the clock and do so in an antagonizing fashion with photic cues? Understanding how the IGL gets activated will help answer this question and can lead to the further development of therapeutic interventions for individuals with circadian dysregulation. Knowing what signals can allow the clock to be more or less sensitive to light input can aid in the regulation of the sleep-wake cycles of individuals suffering from sleep disturbances such as shift workers or patients suffering from neurological disorders like Alzheimer's disease.

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