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Non-Photic Phase Shifting: Acetylcholine and Arousal

Yamakawa, Glenn

UNIVERSITY OF CALGARY

Non-Photic Phase Shifting: Acetylcholine and Arousal

by

Glenn R.S. Yamakawa

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Abstract

Non-photic stimuli are capable of producing large phase advances of the suprachiasmatic nuclei (SCN) during the midday in a manner that is different than light. Two prominent non-photic stimuli include confinement to a novel running wheel, and sleep deprivation by gentle handling. It was hypothesized that these stimuli both provide a sustained level of behavioral arousal to the animals in order to bring about phase shifts. If this was the case, then neurotransmitters involved in behavioral arousal should also participate in non-photic phase shifting. Neurotransmission of acetylcholine (ACh) is involved in bringing about arousal and also has been shown to play a role in the circadian system. The purpose of these experiments was to determine if ACh plays a role in non-photic phase shifting and to what extent arousal is involved. First, it was shown that Fos expression is increased in general areas containing ACh, orexin and histamine in Syrian hamsters undergoing sleep deprivation. Next, it was found that Fos expression specifically in cholinergic cells of the forebrain is increased in animals undergoing novel wheel confinement, but not sleep deprivation. It was then found that *in vivo* electrical stimulation of the basal forebrain region containing cholinergic neurons phase advanced circadian rhythms of wheel running activity at midday. The phase shifts to these stimulations was found to be dependent on a direct cholinergic input into the SCN. Finally, it was shown that blocking cholinergic neurotransmission in the SCN attenuates phase advances to novel wheel confinement. Taken together, these results show that ACh participates in non-photic phase shifting of the SCN through direct projections from forebrain cells. This also provides evidence for a role of behavioral arousal in novel wheel confinement induced phase shifts and a system that can feedback information on behavioral state onto the SCN.

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Dedication

To Tara for her love and support.

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List of Abbreviations

Measurement terms

ºC	degrees Celsius
cm	centimeters
lux	a unit of luminance
µm	micrometer
mM	millimolar

Symbol	*Definition*
ACh	Acetylcholine
BMAL1	Bmal1 gene
ChAT	Choline acetyltransferase
CRY	Cryptochrome gene
CT	Circadian time
Cy	Cyanine
DAB	Diaminobenzidine
DD	Constant darkness
ERK	Extracellular signal responsive kinases
GABA	Gamma amino butyric acid
H202	Hydrogen peroxide
HA	Histamine
IGL	Intergeniculate leaflet of the thalamus
LD	Light/dark
NDS	Normal donkey serum
NGS	Normal goat serum
NHS	Normal horse serum
NPY	Neuropeptide-Y
OX	Orexin/hypocretin
PBS	Phosphate buffered saline
PER	Period gene
PRC	Phase response curve
TTFL	Transcription translation feedback loop
ZT	Zeitgeber time

1. General introduction

1.1 Circadian rhythms are adaptive

Life on this planet has adapted ways to keep track of time. Since the earth takes approximately 24 hours to complete a full rotation, organisms have developed cyclic patterns of behavior that vary in the day and night and cycle every 24 hours. Biological processes that take approximately one day to cycle are referred to as circadian coming from the Latin "circa" for around and "diem" for a day. Within an organism, a circadian system works to coordinate internal biological processes with the external world. There are also other biological rhythms that oscillate with periods less than 24 hours known as ultradian rhythms, greater than 24 hours known as infradian rhythms, or yearly known as circannual rhythms. The circadian system is highly studied due to the ubiquitous nature it in all biological processes. Some researchers suggest that the circadian system remains highly plastic in many species, particularly ones that experience extreme seasonal changes, such as arctic or other eusocial creatures (Bloch et al., 2013). So although many organisms possess biological processes that cycle every 24 hours, this may not always be advantageous and some flexibility is involved in synchronizing to the environment. The circadian system is responsible for coordination of internal biological processes with the external world. It provides an enhancement to reproductive fitness; it is highly flexible and adaptive and can influence many biological processes.

It has been hypothesized that early forms of biological timing systems evolved for primordial organisms to escape from damaging ultraviolet light (Gehring and Rosbash, 2003). These organisms lacked the ability to filter out damaging rays from the sun and therefore needed some means of anticipating when the sun would rise in order to escape to the depths of the ocean. Since simple synchrony to the rising and setting of the sun was not sufficient, these

organisms needed to develop some way of anticipating when these events would occur. The circadian time keeping system was believed to enhance reproductive fitness and therefore has been found across all domains of life. Since the components of this biological time keeping system tend to vary across domains in life, it has been difficult to study the evolution of such a system. Intriguingly, recent work has provided evidence that rhythms in oxidation of peroxiredoxin proteins may be a universal marker of circadian machinery across all domains of life (Edgar et al., 2012).

There have been few studies on the adaptive purpose of circadian rhythms. It is generally thought that internal biological clocks could provide increases in adaptive fitness through providing synchronicity of biological functions with the external world, coordination of internal biological functioning as well as coordination with the changes throughout the year (Vaze and Sharma, 2013). One study examining the effects of different light dark schedules on strains of cyanobacteria expressing different endogenous periods showed that those strains that were placed in light dark cycles that were most similar to their endogenous periods could outcompete strains that did not (Ouyang et al., 1998).

Several field studies have also been conducted. In one study, chipmunks were captured and radio collared in the mountains, then released and placed in either a brain lesion group that had a disrupted circadian system, a sham surgery control, or an intact control group. Another group was also used as an in laboratory lesion group to measure the degree of disruption lesions of the circadian system, or partial lesions had on rhythmicity in chipmunks. A significantly higher number of lesioned chipmunks suffered from predation in free living conditions followed by sham controls and then intact controls (DeCoursey et al., 2000). One explanation for the results of this study was that the lesioned animals engaged in more trips outside of their burrows

at night, suffered from more nighttime restlessness and engaged in more bouts of activity during the night and that may have attracted predators. In another study, ground squirrels were measured first in laboratory conditions in order to determine if they could entrain to light dark cycles. Next, 12 animals had lesions of the circadian system, and some intact controls were released into the site of origin and monitored using tracking chips and video surveillance. Among the ground squirrels in an enclosed desert area, there were more deaths in the group that did not have a functioning circadian system following an unplanned attack on the animals from a feral cat that wandered into the enclosure (DeCoursey et al., 1997). The interpretation of these studies was that the lesion animals had no consolidation of activity and were thus active during the night while in their burrows attracting the attention of nocturnal predators. It is in this manner that a circadian pacemaker may have enhanced reproductive fitness in some species. Another study utilizing eastern chipmunks also used a circadian lesioned group, surgical control and intact control group to measure survival and reproduction. Measurements of chipmunk mortality over short-term and long-term conditions found no differences in survival or reproductive success, however (DeCoursey and Krulas, 1998). There was a statistical difference between the lesioned group compared to the pooled control groups in terms of survival though. The lack of major differences in this case was attributed to conditions favorable to survival such as adequate food supply and lack of significant predation.

1.2 Circadian rhythms and health issues

Modern society has placed increasing demands on humans to be productive at all hours of the day. Shiftwork has become more common and the increasing use of artificial lighting has caused exposure to dim-lighting at night to become commonplace. These workplace schedules

and demands for productivity have created condition where we are no longer synchronized with the natural environment and many people are sleep deprived. This can result in numerous consequences and industrial accidents.

It is now becoming apparent that the disruptive effects of shift working can have health consequences. A large study of retired shift workers showed links to poor sleep quality, diabetes, and hypertension suggesting the effects of circadian disruption are long-term (Guo et al., 2013). Studies in shift workers have also shown that there is an increase in oxidative stress due to the production of free radicals in the body, and that this may be in part mediated by a circadian disruption of the pineal hormone melatonin which normally acts as an anti-oxidant (Faraut et al., 2013). This increase in oxidative stress may have a negative impact on the cardiovascular system. A review on studies involving workers placed in rotating shifts found these workers are more likely to suffer from sleepiness and insomnia, decreases in productivity, diabetes, cancer, cardiovascular issues and more on the job accidents (Figueiro and White, 2013). Shift work has also been shown to have negative effects on reproduction in women resulting in lower birth weights, and higher chances of miscarriage (Gamble et al., 2013). In a study examining indiscriminate exposure to light during the night, a positive association was also found between incidences of breast cancer (Bauer et al., 2013).

It is hypothesized that humans evolved closed to the equator where light/dark (LD) cycles are very close to 24 hours and there is relatively little change throughout the year resulting in a natural circadian period for humans being the same. Modern technological advancements have allowed us to create environments to live in that are quite dissimilar to our ancestral one. Resonance refers to the agreement between environmental light and internal circadian system functioning. The invention of artificial light has created a lack of resonance between self-selected

LD cycles and the natural period of the human circadian clock. One example a problem this lack of resonance may have created is a disruption of metabolic state due to the consumption of food at less than optimal times. This may have contributed to the current obesity epidemic facing many countries in the world (Wyse, 2012). Also of importance is the extreme societal pressure placed on us to sleep or not, regardless of our internal biology, which is a phenomenon that has been described as social jet-lag. A large self-report questionnaire has found that over 80% of people use alarm clocks, the number of people using sleep aids is increasing and most of us are not sleeping at times that are optimal based on internal physiological needs (Foster et al., 2013).

There is other evidence that disrupted circadian functioning can lead to negative consequences. Experimental evidence using animal models has found that hamsters kept in disruptive light dark cycles showed decreased life spans, extensive cardiovascular problems and severe renal problems (Martino et al., 2008). Recent evidence from invertebrate species has also suggested that lack of alignment to environmental cycles can lead to decreased lifespans (Evans and Davidson, 2013). In humans, there appears to be extensive circadian control over the immune system and evidence that immune activity in turn, can modulate the circadian system. Not only are macrophages and other immune natural killer cells under circadian control, but immune challenge can also affect the circadian system (Cermakian et al., 2013). These interactions between the circadian and immune system may have important implications regarding disease if circadian disruption can lead to altered immune function and may help to explain the reduction in life span.

There have also been several links made between circadian system functioning and mental disorders. Since the circadian system regulates normal timing of brain function, then disrupted circadian functioning may lead to disordered brain function. Patients suffering from

depressive disorders often display disrupted circadian rhythmicity and treatment with antidepressants, or keeping to strict daily schedules often help to normalize these problems (Albrecht, 2013). It may be the case that a disruption of the normal functioning of the circadian system leads to loss of precision in the timing of neurotransmission and this may contribute to depression or other mental disorders. A natural disruption of circadian rhythms occurs during the normal aging process as well. As the circadian system becomes less precise, there is a weakening of the control over other oscillating structures in the periphery, and this disruption is thought to result in a number of conditions such as age associated neurodegeneration (Farajnia et al., 2014). So either circadian disruption can contribute to mental disorders, or mental disorders can lead to disruption in circadian function. Either way, alleviating circadian disruption may present an important therapeutic opportunity.

Through developing a thorough understanding of the underlying mechanisms of how the circadian system functions, we can begin to develop therapeutic interventions to deal with some of the health and aging related issues with regards to disrupted circadian functioning. In order to understand how the circadian system functions, it is important to explore the way by which it normally keeps time. An understanding of the structures, neurotransmitters, receptors, and pathways involved in normal circadian function is a necessary precursor to manipulating it.

1.3 The suprachiasmatic nucleus is a master clock

The suprachiasmatic nucleus (SCN) of the anterior hypothalamus is known as the mammalian master circadian clock because it entrains internal biological function to the external world and sets the time throughout the rest of the organism. This process importantly constrains biological activity to the appropriate time of the day or night. Diurnal organisms, for example,

are awake, consume and digest food during the day but generally rest, or sleep during the night when digestive and elimination processes become inactive. The SCN maintains a vast array of connections with the rest of the brain and has been found to communicate through a wide variety of projections and humoral factors in order to transmit time information to the rest of the brain and body (LeSauter and Silver, 1998). The primary way by which the SCN receives time information from the external world is through exposure to light. The SCN then sets the time throughout the rest of the organism. Light has therefore been referred to as the primary 'zeitgeber' or time giver.

Intraocular anterograde tracing studies have confirmed that fibres from the retina terminate in the SCN using an animal model (Kita and Oomura, 1982). This has also been confirmed using post-mortem human brain tissue (Sadun et al., 1984). In the hamster and rat, this retinohypothalamic projection has been described as dense collection of terminals along the optic chiasm in the rostral SCN but spreading out more to the more ventral and lateral SCN throughout the rest of the nucleus (Johnson et al., 1988). The SCN already shows circadian variation in glucose metabolism prior to development and innervation by the retinohypothalamic tract indicating that it is endogenously rhythmic and does not require environmental input (Fuchs and Moore, 1980).

Lesions of the SCN in rats disrupted rhythms of sleep and wake, locomotor activity, drinking rhythms and corticosterone release (Moore and Eichler, 1972; Stephan and Zucker, 1972; Ibuka and Kawamura, 1975). A genetic mutant strain of mice showing varying degrees of hypogenesis in the mediobasal hypothalamus showed lack of entrainment to a light dark cycle, and in some cases were arrhythmic in wheel running rhythms (Scheuch et al., 1982). When bilaterally enucleated animals received knife cuts creating an "island" of hypothalamic tissue

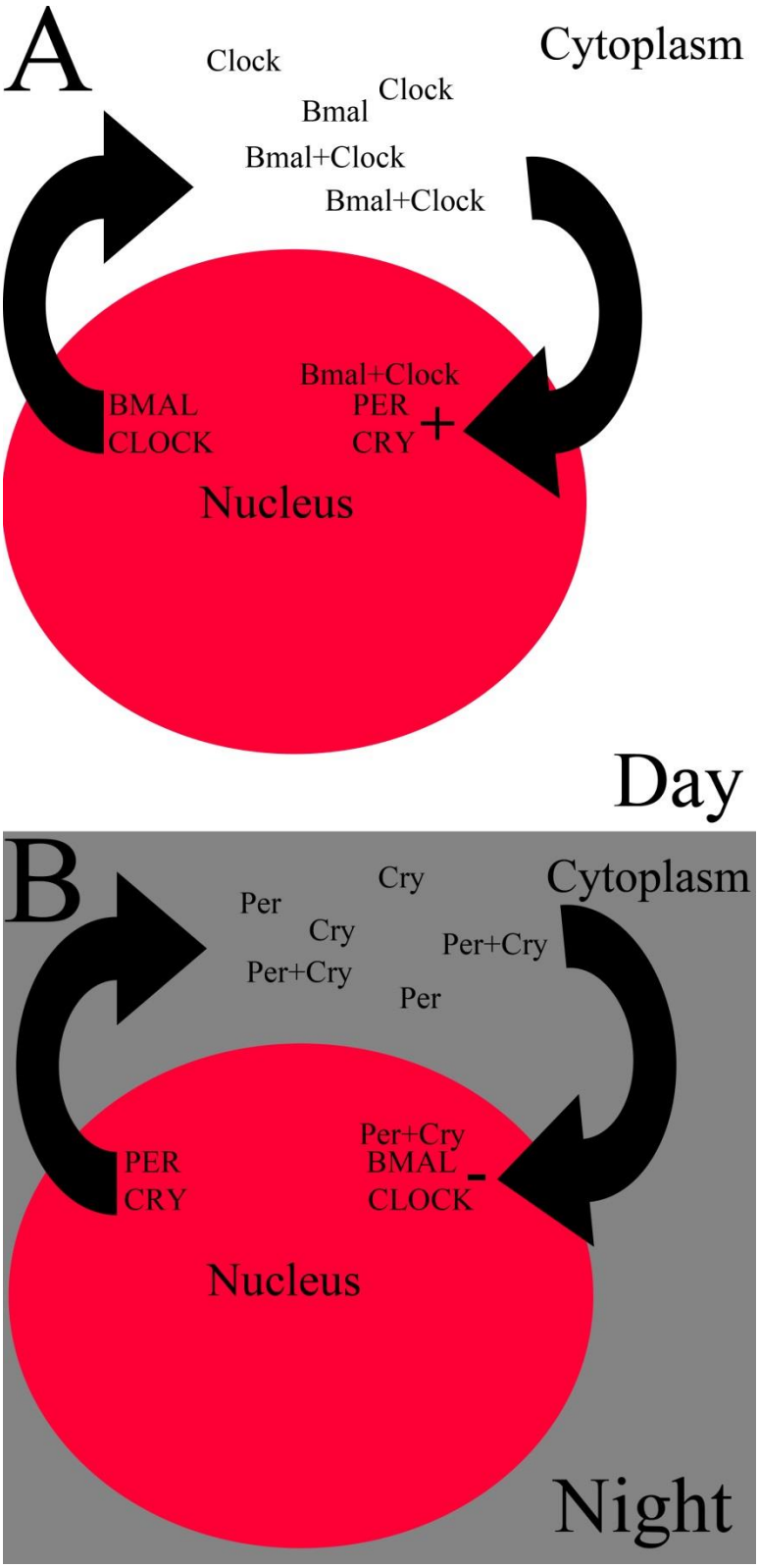
containing the SCN, it was found that cells within the island still retained spontaneous circadian rhythms of electrical activity (Inouye and Kawamura, 1979). This indicates that the SCN functions as an autonomous circadian pacemaker and it not simply being driven by some sort of external stimulus.

Numerous studies have also been published showing the restoration of circadian function in SCN lesioned animals. Rats rendered arrhythmic by SCN lesions received transplants of fetal SCN tissue into the 3rd ventricle and showed a restoration of circadian patterns of locomotor activity consistent with re-innervation of the periventricular zone (Sawaki et al., 1984). This restoration of rhythms was only found in animals that received SCN transplants into the 3rd ventricle as opposed to other tissue (DeCoursey and Buggy, 1989).

The SCN is able to produce 24 hour self-sustained oscillations through the transcription, translation feedback loop (TTFL). The basic TTFL consists of a genetic negative feedback loop where clock gene products feedback to repress their own transcription as is summarized in Figure 1. This loop is said to consist of a positive arm and a negative arm. On the positive arm the genes Bmal1 and Clock express their protein products that dimerize in the cytoplasm and translocate back to the nucleus. This BMAL1 CLOCK heterodimer binds to an E-Box promoter for the Per and Cry genes that comprise the negative arm of the feedback loop. PER and CRY also form a complex that repress their own activation by BMAL1 and CLOCK (Isojima et al., 2003; Kwon et al., 2011). Other important components of the TTFL involve casein kinase 1 ε that degrades the PER protein, and induction of Rev-erbα and Rorα by BMAL1/CLOCK that can repress, or activate the transcription of Bmal1 (Kwon et al., 2011).

Figure 1: The basic transcription translation feedback loop of clock genes

Displayed in A is the positive arm of the feedback loop of circadian clock gene expression. The red circle represents the nucleus. During the day, the genes Bmal and Clock express their protein products that form heterodimers in the cytoplasm. These heterodimers translocate back into the nucleus where they induce the expression of the Per and Cry genes. Displayed in B is the negative arm of the feedback loop that occurs during the night. PER and CRY form a heterodimer in the cytoplasm and translocate back into the nucleus where they shut off their own expression through inhibiting the expression of Bmal and Clock.


A
Cytoplasm
Clock
Clock
Bmal
Bmal+Clock
Bmal+Clock
Bmal+Clock
BMAL
CLOCK
PER
CRY
+
Nucleus
Day
B
Cytoplasm
Cry
Per
Cry
Per+Cry
Per+Cry
Per
Per+Cry
PER
CRY
BMAL
CLOCK
-
Nucleus
Night

Perhaps some of the most profound evidence with regards to the SCN functioning as a cell autonomous master circadian pacemaker came with the discovery of the *tau* mutation in Syrian hamsters. Heterozygous animals for this mutation were found to have circadian periods that ran close to 22 hours, whereas the homozygous mutants had periods in wheel running rhythms closer to 20 hours as opposed to the usual 24 hours (Ralph and Menaker, 1988). *In vitro* examinations of SCN firing rates using homozygous *tau* mutants has also confirmed that the circadian rhythm in firing rate is also approximately 20 hours (Davies and Mason, 1994). This mutation was traced to substitution in the gene for the enzyme casein kinase 1 ε which normally acts to speed the degradation of PER (Lowrey et al., 2000). This mutation has also been reproduced and acts in a similar manner in mice (Loudon et al., 2007). Importantly, when normal SCN ablated hamsters received SCN transplants from *tau* mutants, they adopted the unusually short periods of the mutants and did not revert to the usual 24 hour rhythms (Ralph et al., 1990).

1.4 The phase response curves

The normal function of the circadian system can be understood by examining how the SCN keeps time. The stimuli that are capable of shifting the clock are known as zeitgebers. Light can alter the phase of the SCN in what is known as a phase shift. Phase shifts are measured as changes in the onset of activity. Using rodents in laboratory conditions, this is often measured by examining changes in the onset of wheel running activity. A phase delay entails a zeitgeber that causes the activity onset of an animal to occur later than expected on subsequent days. A phase advance, then, involves a zeitgeber that causes the activity onset of an animal to occur earlier than expected on subsequent days.

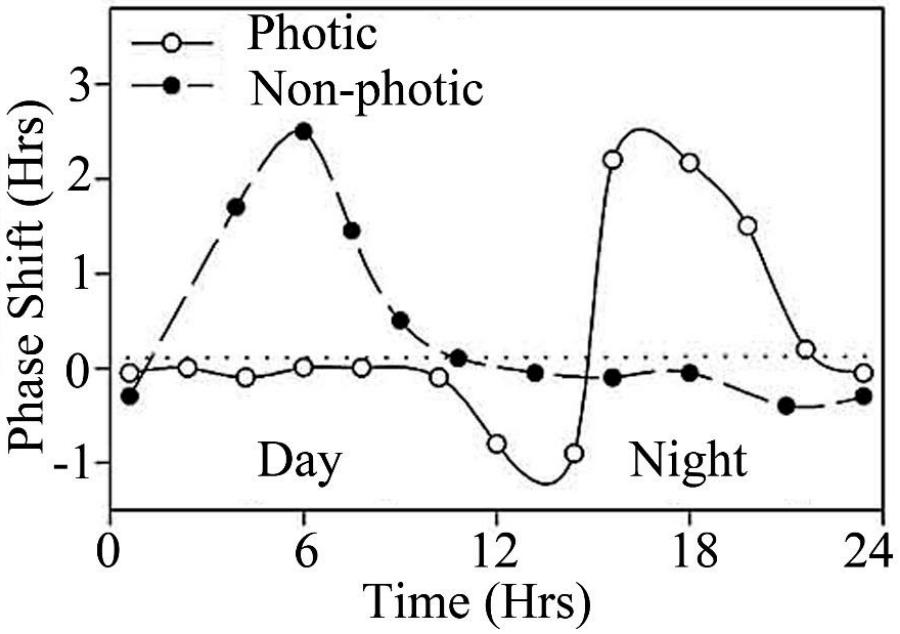
When phase shifts of the locomotor activity rhythm to stimuli are averaged at numerous time points across the 24 hour period, a phase response curve (PRC) is the result and is displayed in Figure 2. The data for these PRCs are usually collected from animals in constant darkness to avoid the masking effects of light. The photic PRC shows that light produces no phase shifts during the day, phase delays in the early night and phase advances during the late night (Smith et al., 1992). The photic PRC has been widely defined in a variety of species.

There are also a group of stimuli other than light that are capable of shifting the SCN that are referred to as non-photic stimuli. The non-photic PRC shows that large phase advances are produced during the day with smaller phase delays sometimes occurring during the night. Those stimuli producing responses similar to the non-photic PRC are presumed to act through the same mechanisms at the level of the SCN (Rosenwasser and Dwyer, 2001).

Two methods of examining the phase shifting effects to various stimuli have been employed. In the Aschoff Type 1 protocol, animals are kept in constant darkness have rhythms that are said to be free running. Then the phase shifting effects of various stimuli are tested throughout many circadian times (CT) throughout the free running rhythm (Jud et al., 2005). CT refers to the endogenous phase that the SCN is set to since it is no longer entrained by external stimuli. There is also the Aschoff Type 2 protocol that may be employed. In this method, animals are first entrained to a stable light dark cycle and then on the day of the manipulation, the lights are turned off and left off for several days to avoid the suppression of activity, or masking effects of light normally seen in nocturnal rodents (Jud et al., 2005).

Figure 2: The photic and non-photic phase response curves

Displayed are the photic and non-photic phase response curves. Phase advances are displayed as positive phase shifts whereas phase delays are displayed as negative phase shifts.


Photic
Non-photic
Phase Shift (Hrs)
3
2
1
0
-1
Day
Night
0
6
12
18
24
Time (Hrs)

1.5 Non-photic phase shifting

1.5.1 Dark pulses and arousal

Early work exploring how stimuli other than light could affect the circadian clock examined the effects of dark pulses. When nocturnal animals are kept in constant lighting, their rhythms become free running. Exposure to a period of darkness during the mid-subjective day, also called dark pulses, has been shown to phase advance circadian rhythms of wheel running activity (Ellis et al., 1982). This was a novel finding because it was one of the first stimuli discovered to phase shift the SCN during day. The effects of short, medium or long dark pulses in hamsters were examined. It was found that medium or long dark pulses induced running activity that was highly related to the magnitude of the phase shifts (Canal and Piggins, 2006). This suggested that the motor activity that the animals undertook during the dark pulses was the key factor in bringing about these phase shifts. Another study found that restrained hamsters still phase shifted to dark pulses and the shifts were highly related to the intensity of light prior to the midday dark pulse though (Dwyer and Rosenwasser, 2000). Yet another study reported that there were phase shifts to dark pulses without access to a home cage running wheel indicating that dark pulses are more complex of a stimulus than was initially thought and may include both photic and non-photic phase resetting components (Mendoza et al., 2004). Since this was a stimulus that was capable of shifting the SCN in a manner different than that of light, it became known as a non-photic stimulus. The adaptive significance of non-photic modulation of the circadian clock seem to support the idea that they do so through a modulation of the circadian response to light exposure (Edelstein et al., 2003).

1.5.2 Novel wheel confinement

Initial observations involving phase shifts and behavioral arousal to cage changes or disturbances lead to experiments involving the arousal of animals during the mid-subjective day. One study found that repeated social interactions at the same time each day was capable of entraining most hamsters in a manner consistent with the non-photic phase response curve (Mrosovsky, 1988). Hamsters undergoing a shift in the light dark cycle similar to the phenomenon of jet-lag were found to re-entrain faster to the new schedule when they became active during the middle of the day (Mrosovsky and Salmon, 1987). These hamsters are typically were made active when they are given access to a novel running wheel outside of their usual home cage. 2 hour pulses of novel wheel exposure during the day resulted in phase advances while smaller phase delays occurred at night in a manner that was consistent with the non-photic PRC (Reebs and Mrosovsky, 1989). Hamsters heterozygous or homozygous for the *tau* mutation also show altered responses to novel wheel confinement, showing larger phase advances and altered phase response curves (Mrosovsky et al., 1992). Surprisingly, single bouts of sustained activity in excess of 4 hours have been shown to shift circadian rhythms by greater than 12 hours (Gannon and Rea, 1995). Forced treadmill running, or restricted wheel access has also been shown to be able to entrain mice (Marchant and Mistlberger, 1996).

It has also been found that wheel confinement results in *tau* changes similar to what occurs during other non-photic stimuli (Mrosovsky, 1993). Animals exposed to novel running wheels during the day for two weeks in an LD cycle that were then transferred to constant darkness (DD) showed rhythms that had split into two components, one during the day when the novel wheel access was expected and the other during the night when they would be normally

active (Mrosovsky and Janik, 1993). This indicated that novel wheel confinement could even entrain the circadian pacemaker.

Importantly, it was shown that shifts to novel wheel confinement were not due to masking. Masking refers to the suppressive effect that light can have on locomotor activity in nocturnal animals. Masking could occur if a nocturnal animal is exposed to a phase shifting stimulus, but remains in a light dark cycle. Since the light will suppress activity, it is said to be masking the phase shift. These phase shifting effects have been thoroughly quantified. Maximal phase shifts usually occurred at circadian time (CT; by convention, CT12 is defined as activity onset) 2, 4, or 6 (early to mid-subjective day) with maximal phase shifts only occurring if the animal ran more than 5000 revolutions (Bobrzynska and Mrosovsky, 1998).

Sustained locomotor activity therefore appeared to be a potent zeitgeber of the circadian clock. The interpretation of the dark pulses was also re-examined. Hamsters receiving dark pulses with locked running wheels phase advanced relatively little, whereas those that ran vigorously, or were sleep deprived showed very large phase advances dependent on prior exposure to constant light (Mistlberger et al., 2002). This suggested that a large component of the phase shift involved in dark pulses was due to running in the wheel.

1.5.3 Sleep deprivation by gentle handling

Soon, other studies began to show large phase advances during the day independent of significant wheel running, or locomotor activity. Hamsters sleep deprived using a gentle handling procedure, only engaged in an average of 0.08 km of forward momentum and showed phase advances upwards of 4 hours, comparable to the shifts seen to novel wheel confinement (Antle and Mistlberger, 2000). The wheel confinement threshold of forward momentum required

to produce non-photic phase shifts was estimated to be around 2.5 km, or 5000 wheel revolutions (Bobrzynska and Mrosovsky, 1998). But the sleep deprivation by gentle handling procedure showed that excessive wheel running activity was not necessary to bring about phase shifts.

The possibility that dark pulses, wheel confinement and sleep deprivation by gentle handling had a common factor was explored due to the fact that were conducted in order to determine if the exact mechanism necessary to produce non-photic phase shifts to sleep deprivation by gentle handling. The possibility that the SCN was shifted due to a stress response was examined. Phase shifts to gentle handling were enhanced by cortisol inhibition, but restraint induced stress where arousal was maintained that was highly stressful did not produce phase shifts (Mistlberger et al., 2003). Interestingly, open field exposure, or resident intruder experiments also produced large phase shifts (Mistlberger et al., 2003). This seemed to indicate that stress did not induce or inhibit phase shifts to non-photic stimuli, but that sustained arousal allowing free movement of the animal was a necessary component of non-photic shifting. It could be the case that dark pulses, wheel confinement and sleep deprivation all phase shift the SCN because they are arousing and allow the animals to move about freely.

1.5.6 NPY and the IGL are involved in non-photic shifting

The intergeniculate leaflet (IGL) of the thalamus appears to have extensive connections throughout the forebrain including a significant projection to the SCN (Mikkelsen, 1990; Horowitz et al., 2004). The IGL also appears to receive some projections directly from the retina (Mikkelsen, 1990). The projection from the IGL to the SCN is known as the geniculohypothalamic tract and contains neuropeptide-Y (NPY), enkephalin, gamma amino-butyric acid (GABA) and neurotensin (Morin and Blanchard, 2001).

There is strong evidence that NPY from the IGL is critical in inducing non-photic phase shifts. Early work found that animals showing maximal responses to novel wheel confinement also showed increases in Fos expression in the geniculate of the thalamus (Janik and Mrosovsky, 1992). Further, it was determined that Fos expression following novel wheel confinement was common in neurons containing NPY (Janik et al., 1995). An examination of the IGL projection to the SCN showed that NPY fibres in the SCN appear to be ventrally located, with higher densities in the rostral portions (Mikkelsen and O'Hare, 1991). Intra-SCN injections of NPY results in phase shifts resembling the PRC for non-photic stimuli in constant light and constant dark (Albers and Ferris, 1984; Biello and Mrosovsky, 1996; Huhman et al., 1996). These NPY induced phase shifts did not appear to require behavioural arousal of the animal as confinement to a nest-box still resulted in phase shifts (Biello et al., 1994). Importantly, when an NPY anti-serum was injected into the SCN, phase shifts to novel wheel confinement were significantly attenuated (Biello et al., 1994). Non-photic attenuation of light induced phase advances during the night likely occurs through the NPY Y5 receptor, but non-photic phase advances during the day do not appear to rely on this receptor activation (Gamble et al., 2005).

In vitro slice preparations have shown that NPY has a tendency to excite hamster SCN neuron firing rates and the SCN is more responsive to NPY during the subjective day (Mason et al., 1987). However later studies using patch clamp recordings and calcium imaging showed NPY application induced a long term suppression of electrical activity and intracellular calcium levels for over an hour (van den Pol et al., 1996). *In vitro* studies showed that NPY phase advanced the firing rates of SCN neurons that seemed to be protein kinase C dependent (Biello et al., 1997). Further slice work showed that the an agonist for the NPY Y2 receptor could produce a similar phase advance, while a Y1 agonist had no effect (Golombek et al., 1996). The

importance of the Y2 receptor in producing non-photic shifts has also been replicated *in vivo* (Huhman et al., 1996)

Lesions of the IGL reduced phase shifts to novel wheel confinement, but also lowered overall activity making these findings difficult to interpret (Janik and Mrosovsky, 1994). Lesions of the IGL resulting in loss of NPY immunoreactivity in the SCN eliminated phase shifts to serial arousal by saline injection and benzodiazepines (Maywood et al., 1997). Benzodiazepines have been found to produce behavioral arousal in hamsters. One study utilizing lesions of the SCN, IGL, or serotonergic input into the SCN found that mice rendered arrhythmic with SCN lesions failed to entrain to scheduled treadmill running, but mice with IGL lesions or serotonergic lesions also failed to entrain (Marchant et al., 1997). This study indicated that entrainment to daily exercise involves the SCN and requires both NPY and serotonin. There may be an interaction between NPY and serotonin at the level of the SCN to mediate entrainment to exercise. Some animals with large SCN ablations of the entire region and surrounding areas still showed entrainment to daily cage changes indicating that not all non-photic stimuli may act directly on the SCN (Mistlberger, 1992).

Using clock gene reporters in cultured SCN cells, it was found that NPY produced the largest phase advances from CT0-6, and induced long term suppression of clock genes and firing rates (Besing et al., 2012). NPY release in the SCN of hamsters under a 14:10 LD cycle normally shows a nadir around midday, and novel wheel confinement stimulates NPY release (Glass et al., 2010). It has been suggested that serotonergic activity in the IGL inhibits GABA to promote NPY release in the SCN to bring about these shifts (Glass et al., 2010).

1.5.7 Other neurotransmitters involved in non-photic shifting

There is also some evidence for the role of GABA in non-photic resetting of the clock. Injection of the $GABA_A$ receptor agonist muscimol into the SCN produces phase advances during the midday and also suppresses Per1 and Per2 mRNA (Ehlen et al., 2006). A role for polysialic acid in the SCN has also been postulated. *In vivo* enzymatic removal of polysialic acid from neural cell adhesion molecules in the SCN potentiates phase shifting to sleep deprivation by gentle handling (Fedorkova et al., 2002). Inhibition of the Ca2+/calmodulin-dependent protein phosphatase PP2B produces a non-photic phase response curve and chronic administration of the drug disrupted circadian rhythmicity (Katz et al., 2008). A novel neuropeptide has also been localized in the SCN that may be involved in circadian entrainment. Neuromedin S has been described in rat brain tissue as the ligand for an orphan G-protein coupled receptor in the SCN and ventricular administration has been found to mimic non-photic phase shifts (Mori et al., 2005).

1.5.8 Gene expression and non-photic phase shifting

Non-photic stimuli are thought to suppress the usually high levels of gene expression in the SCN during the day in order to bring about phase advances. Per1 and Per2 show circadian oscillations in the hamster SCN and both showed significant suppression immediately following exposure to a novel running wheel during the early subjective day (Maywood et al., 1999). In response to midday novel running wheel exposure, Fos was up-regulated in the IGL and pretectum but down-regulated in the SCN (Mikkelsen et al., 1998). These Fos activation patterns are in contrast to what occurs during photic phase shifting. Dark pulses applied during the late subjective day transiently suppressed levels of Per1 and Per2 but not Cry1 in hamsters (Mendoza

et al., 2004). Fos expression has also been found to be down-regulated in the SCN during a sleep deprivation (Antle and Mistlberger, 2000). In order to determine if this suppression of gene expression was a cause of the non-photic phase shifts, antisense oligodeoxynucleotides to Per1 were injected into the SCN. Injections of Per1 antisense produced non-photic phase shifts similar to non-photic stimuli indicating that clock gene suppression is the cause and not merely a result of non-photic shifting (Hamada et al., 2004). Extracellular signal responsive kinases I/II (ERK) are also supressed and also may play a role. Animals that phase shifted in response to sleep deprivation also showed decreased levels of phosphorylated ERK in the SCN (Antle et al., 2008).

Selective deletion of the gene for the G-protein Dexras1 in mice diminishes the circadian response to light, but also greatly potentiates the phase shifting effects of NPY and non-photic stimuli even producing large phase advances during the night (Cheng et al., 2004; Koletar et al., 2011). These suggest that Dexras1 modulates non-photic shifts through control of arousal induced shifts of the SCN (Koletar et al., 2011). What seemed to be of interest in these Dexras1 animals are that mice typically do not respond to non-photic stimuli and these knockouts appeared to respond preferentially to these stimuli. Another study found that Dexras1 knockouts did not respond preferentially to non-photic stimuli when they were in conflict with photic stimuli and there was no difference in novel wheel confinement induced shifts when compared to wildtypes though (Dallmann and Mrosovsky, 2007). In sum, non-photic stimuli appear to phase advance the SCN during the midday through a suppression of the normally high levels of activity at that time.

1.6 Arousal as the non-photic zeitgeber

Arousal can be defined as a behavioral state taking place on a continuum. The lower end of this continuum would include sleep states where there may be a stereotypical sleep posture or very little, if any, reactivity to environmental stimuli, whereas the higher end of this continuum would include being awake and in a highly reactive state. Taken together with the novel wheel confinement and sleep deprivation data, it is hypothesized that any stimulus capable of producing sustained arousal during the midday at a particular level should phase advance the circadian clock through a suppression of activity in the SCN.

More evidence for the role of arousal has come from experiments using stimuli other than the wheel confinement and sleep deprivations. One study examined the influence of highly rewarding lateral hypothalamic brain stimulation and aversive foot shocks in hamsters. These experiments showed that both stimuli produced similar phase shifts following the non-photic PRC (Cain et al., 2004). It has been established that older animals do not phase shift as well to non-photic stimuli and hypothesized that this is due to lack of arousal. A study examining this showed phase advances in younger animals could be increased by placing a receptive female nearby, or increasing overall arousal using injections of benzodiazepines (Mrosovsky and Biello, 1994).

It has been found that arousal by handling or saline injection could phase shift the SCN at CT8 and 10 (Mead et al., 1992). Subcutaneous injections of melatonin or saline produced very similar phase advances during the late subjective day but importantly, these effects were eliminated when the animals received injections through remote cannulas that did not require the animals to be handled (Hastings et al., 1992). This lack of handling presumably did not produce arousal in the animals of the appropriate level to bring about phase shifts to these injections. In

hamsters, benzodiazepines have been found to be highly arousing (Biello and Mrosovsky, 1993). Intraperitoneal injections of benzodiazepines also resulted in phase advances when applied during the subjective day (Vansteensel et al., 2003).

Temperature and hoarding have also been found to produce arousal. It was hypothesized that cold temperatures would produce a greater motivation to run. Hamsters exposed to colder temperatures at midday were observed to begin eating, drinking and exhibiting some wheel running but also exhibited phase advances greater than 1.5 hours (Mistlberger et al., 1996). Some animals were even able to entrain to a 30 minute opportunity to hoard sunflower seeds in an open field outside of the home cage (Rusak et al., 1988). These results are consistent with the necessity of some kind of behavioral arousal being necessary in order to bring about non-photic phase shifts. They also point to the necessity of having animals being allowed to move about freely.

The interaction of brain systems responsible for the control of sleep and wake states is complex. Although it is of no doubt that NPY from the IGL is involved in non-photic phase shifting, it is not known how the IGL is activated to produce these shifts, or what other neurotransmitters can modulate, or act with NPY at the SCN. Furthermore, experiments using NPY have only found phase shifts during the day that are maximally around 50 minutes to 1.5 hours (Huhman and Albers, 1994; Huhman et al., 1996). Typical non-photic phase shifts seen are between 3-4 hours and sometimes larger. It has also been observed and quantified in the sleep deprivation studies that those animals more likely to phase shift during those procedures require fewer interventions to keep awake and appear more aroused (Antle and Mistlberger, 2000). This could indicate that those animals that phase shift are more aroused than those that are not. Similarly, those animals not running the requisite numbers of wheel revolutions in during wheel

confinement do not shift. This could be because they are not aroused enough as well. It is hypothesized that neurotransmitters typically involved in producing behavioral arousal also will play a role in non-photic phase shifting through feedback onto the SCN.

1.7 Acetylcholine plays a role in arousal

1.7.1 Neuronal phenotype in the basal forebrain

The acetylcholine (ACh) system has been thoroughly investigated due to the role it plays in many physiological processes including cortical arousal. Cholinergic neurons in the pedunculopontine tegmentum and laterodorsal tegmentum send major projections to the thalamus and basal forebrain (Hallanger et al., 1987). Cholinergic neurons are present throughout the basal forebrain where they project widely throughout the cortex and hypothalamus. Interestingly enough, forebrain cholinergic neurons also have been found to project to the SCN in rats (Bina et al., 1993).

Recordings of neurons in the basal forebrain also suggested that there were neurons containing phosphate activated glutaminase, an enzyme required for glutamate synthesis, and that these neurons fired at a lower frequency than cholinergic neurons suggesting that they were, at least partially, a separate population (Manns et al., 2003). Extensive stereological analysis of the rat basal forebrain suggested that approximately 5% of the cells synthesize ACh (approximately 22000), 35% contained GABA and 90% of the cells can produce glutamate (Gritti et al., 2006). This suggested that a large portion of both GABAergic and cholinergic cells in the basal forebrain could produce glutamate and was later confirmed through an examination of co-localization with vesicular glutamate transporter-3 (Gritti et al., 2006). Some of the basal

forebrain cholinergic neurons are co-localized with N-acetyl-aspartyl-glutamate (NAAG) (Forloni et al., 1987). NAAG can act as its own neurotransmitter, also act at certain glutamate receptors as well as engage in the production of glutamate (Neale et al., 2000).

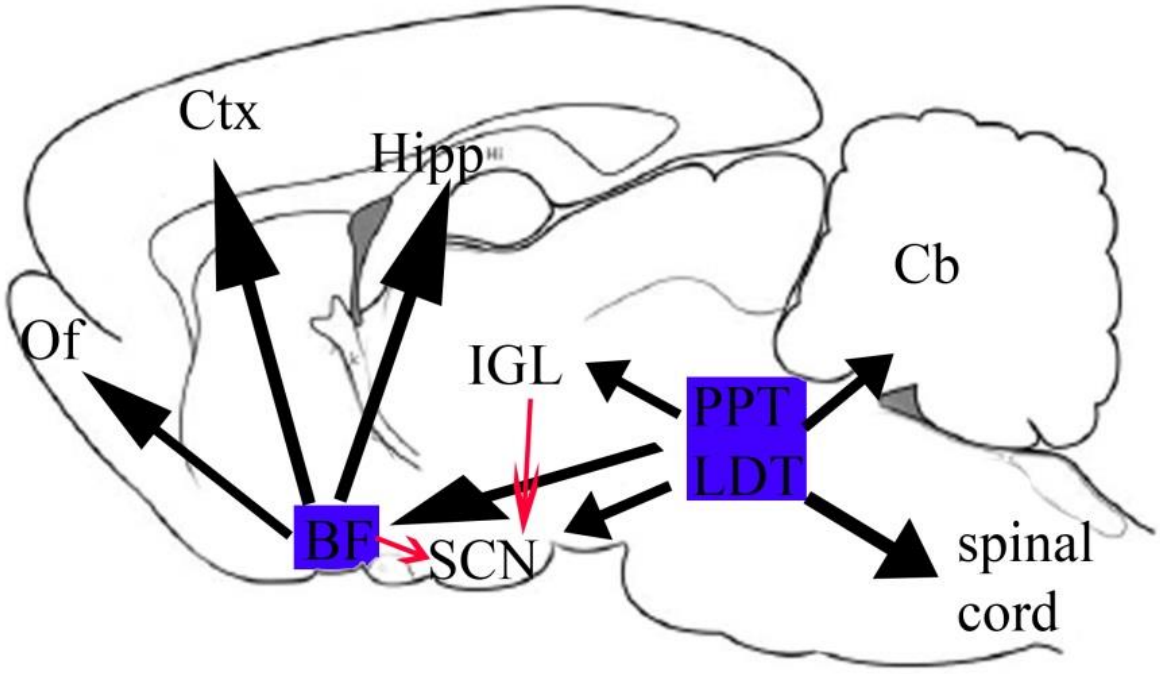
Basal forebrain neurons have also been shown to contain other substances. They have been found to contain a low affinity nerve growth factor receptor that is co-expressed with nerve growth factor and a high affinity tyrosine kinase A receptor (Lee et al., 1998). Many of the cholinergic neurons that project to the SCN are immunoreactive for P75-nerve growth factor (Bina et al., 1997). Throughout the entire forebrain, there appeared to be no evidence of co-localization of GABA containing neurons with those that were immunoreactive for choline acetyltransferase (ChAT). Due to the size, number and location of many of the GABA containing neurons that were in proximity to the cholinergic neurons, they appeared to be inhibitory interneurons (Gritti et al., 1993).

1.7.2 Activity of the basal forebrain

ACh neurons in the basal forebrain discharge more readily during waking, decrease firing rates during slow wave sleep and increase firing again during paradoxical sleep, a time associated with high cortical activity (Jones, 2005). A summary of cholinergic nuclei and their major projections is displayed in Figure 3. The brainstem laterodorsal and pedunculopontine cholinergic nuclei have major ascending projections through a dorsal projection to the thalamus and a ventral path to the hypothalamus and basal forebrain (Hallanger et al., 1987). The basal forebrain becomes activated during arousal and the forebrain cholinergic neurons project widely throughout the cortex, thalamus, hippocampus, and olfactory bulb where they contribute to arousal (Mesulam et al., 1983).

Figure 3: Major cholinergic projections of the rodent brain and the interactions with the SCN

Displayed is a sagittal view of the rodent brain. The purple highlighted nuclei contain acetylcholine. They are the pedunculopontine nuclei (PPT), the laterodorsal tegmentum (LDT) and basal forebrain (BF). The cholinergic nuclei project to many brain regions including the cerebellum (Cb), the intergeniculate leaflet in the thalamus (IGL), all of the major cortices (Ctx), the hippocampus (Hipp) and olfactory bulb (Of). The IGL sends a projection to the SCN that is important in non-photic phase shifting. The BF also projects to the SCN that may be involved in non-photic phase shifting as well.


Ctx
Hipp
Cb
Of
IGL
PPT
LDT
BF
SCN
spinal
cord

The discharge from these cholinergic neurons is highly correlated with theta oscillations in the cortex (Lee et al., 2005). *In vitro* intracellular recordings from cholinergic forebrain neurons showed rhythmic bursting and tonic firing with maximal frequencies of around 20 Hz (Khateb et al., 1992). However other studies using urethane anesthetized rats have suggested that cholinergic cells can fire maximally around 75 Hz, GABAergic neurons around 40 Hz and glutamatergic neurons in the basal forebrain can fire around 20 Hz and that together the basal forebrain works to modulate coherent cortical activity (Manns et al., 2003).

Electroencephalograms of rats showed basal forebrain injection of a glutamate agonist induced waking, increased gamma and theta waves (typically associated with waking states) whereas deactivation of the basal forebrain through injection of procaine decreased gamma and theta waves and paradoxical sleep (Cape and Jones, 2000). When the non-specific cholinergic agonist carbachol was applied to basal forebrain ACh neurons *in vitro*, there was a hyperpolarizing effect, likely through the muscarinic receptors and in the presence of NMDA was found to induce rhythmic bursting through a reduction in after hyperpolarizations (Khateb et al., 1997). Using the anterograde tracer biotinylated dextran amine, it was determined that the basal forebrain sends a prominent projection to the hypothalamic cells containing orexin, an important neuropeptide involved in arousal and a majority of these projections were glutamatergic, or GABAergic (Henny and Jones, 2006).

The release of ACh in the brain of mammals appears to follow a circadian pattern of activity. Rats kept entrained to a 12:12 LD cycle showed a diurnal peak in overall brain ACh levels at CT2 and a trough at CT18 (Hanin et al., 1970). Other studies measuring free and bound ACh employing slightly different approaches also showed diurnal variation in the levels with a trough occurring at CT 18 (Saito, 1971; Saito et al., 1975). Specific dialysis in the somatosensory

and motor cortices as well as the hippocampus showed an overall peak concentrations of ACh occurring at night suggesting that release was correlated to motor activity and arousal (Kametani and Kawamura, 1991; Mizuno et al., 1991; Jimenez-Capdeville and Dykes, 1993, 1996). These rhythms of ACh release persist in constant conditions (Kametani and Kawamura, 1991). In the SCN, radioimmunoassays have shown that light pulses in the early night increased ACh concentrations in the SCN after 30-60 min (Murakami et al., 1984).

Similarly, forebrain muscarinic receptors show a circadian rhythm of expression that also varies throughout the year with changing LD cycles in rats (Kafka et al., 1981). Binding to muscarinic receptors shows a peak in the late light phase extending into the dark during the early February to June, but during October, a second peak emerged during the light phase. The olfactory bulb, parietal cortex and caudate-putamen were all shown to have circadian rhythms of muscarinic receptor binding with peaks during the subjective night, a time also correlating with higher levels of motor activity (Kafka et al., 1986). These circadian rhythms of receptor expression are abolished with a lesion to the SCN (Kafka et al., 1985). Another study using radioligand labelled muscarinic antagonists only found circadian variation in the hypothalamus and hippocampus, but not the cortex, striatum or cerebellum (Por and Bondy, 1981). This suggests direct SCN control of the cholinergic system.

1.7.3 Cholinergic modulation of the circadian system

Electrical stimulation of the nucleus basilis magnocellularis results in an increase in ACh release and has been shown to increase Fos expression in thalamic and cortical areas implicating its role in producing arousal (Casamenti et al., 1986; Boix-Trelis et al., 2009). It has also been found that there is direct cholinergic innervation into the SCN. Using a dual retrograde, and

anterograde tracing approach, cholinergic cells in the substantia innominata (SI), nucleus basilis magnocellularis of the forebrain and the septum and tegmentum of the hindbrain were found to project to the SCN (Bina et al., 1993). This innervation may be important in neuropeptide synthesis. Bilateral lesions if the cholinergic nucleus basilis magnocellularis caused a significant reduction in SCN cells producing vasopressin and vasoactive intestinal polypeptide (Madeira et al., 2004).

There are many studies that report evidence for cholinergic activity directly in the SCN. Vesicular acetylcholine transporter immunoreactivity has also been reported in the lower subparaventricular zone and SCN of rats (Castillo-Ruiz and Nunez, 2007). Electron microscopy has revealed that choline acetyltransferase labelled fibres make axosomatic and axodendritic synapses directly onto SCN neurons (Kiss and Halasz, 1996). Nicotinic receptors are present in the SCN of the mouse and rat, although their expression appears to vary greatly across development (O'Hara et al., 1999). There is abundant mRNA, particularly for the alpha 7 subunit of nicotinic receptors in the SCN and some *in vivo* work has shown nicotine acts in a manner similar to light (O'Hara et al., 1999). Another study found that there is a nearly complete co-localization of muscarinic and nicotinic receptors in the SCN (van der Zee et al., 1991). Recently, it was shown that incubation in a primary antibody that is highly selective for ChAT at a high temperature revealed that there may be some local cholinergic neurons in the SCN (Hut and Van der Zee, 2011). It was suggested that previous studies have failed to find cholinergic neurons in the SCN because the antibodies have a higher affinity for ChAT that is located in terminals and fibers. This shows that there is a direct projection of ACh into the SCN where it can influence activity through the nicotinic or muscarinic receptors and there may even be some local cholinergic cells in the SCN.

1.7.4 Pharmacology of acetylcholine on the circadian system

Ventricular administration of the non-specific ACh agonist carbachol into golden hamsters resulted phase advances during the day and a photic like phase shifts at night (Earnest and Turek, 1985). These photic like phase shifts were found to be dose dependent and did not appear to be producing the shifts due to direct action on the SCN (Wee et al., 1992). Another study using ventricular carbachol showed large phase advances during the day with smaller phase advances at night similar to the non-photic PRC (Meijer et al., 1988). Furthermore, Djungarian hamsters that received ventricular infusions of carbachol showed phase advances during the day, and phase delays during the early night, but no consistent responses at other times (Wee and Turek, 1989). Bilateral intraventricular injections of carbachol into mice also resulted in photic-like phase shifts however time points during the day were not tested (Zatz and Brownstein, 1979; Zatz and Herkenham, 1981). Patch clamp recordings from rat SCN neurons showed that carbachol acted in a mainly inhibitory manner, likely through the M1 or M4 receptors (Yang et al., 2010)

Ventricular infusion of the nicotinic antagonist Mecamylamine also significantly reduced both the phase delays and phase advances to light pulses during the subjective night (Keefe et al., 1987). Mecamylamine has also been shown to block light induced Fos expression in the dorsomedial SCN at CT 19 (Zhang et al., 1993). *In vitro*, one study found that carbachol caused large phase advances to SCN firing rates only during the subjective night and the effect was blocked by M1 antagonists implicating the muscarinic receptors (Liu and Gillette, 1996). Further work using analogs and inhibitors showed that this muscarinic activation was likely tied to guanylyl cyclase, cyclic GMP, protein kinase G signal transduction mechanism (Liu et al., 1997).

Interestingly, the IGL also receives cholinergic input from the brainstem pedunculopontine, a major source of ACh in the brain (Moore et al., 2000). Extracellular recordings from IGL neurons showed oscillatory disruptions following intravenous administration of atropine (Werhun and Lewandowski, 2009). Unilateral injections of carbachol into the IGL produce small phase advances averaging around 43 minutes when applied at midday (Cain et al., 2007). *In vitro* extracellular brain slice recordings from the IGL show that application of carbachol reduced glutamate induced activity consistent with its role as a non-photic stimulus (Pekala et al., 2007).

Intra-SCN 192-IgG Saporin lesions of P75-nerve growth receptors in the SCN resulted in no changes to photic entrainment or Fos expression (Beaule and Amir, 2002). In addition, administration of the choline uptake blocker hemicholinium-3 into the SCN of rats failed to block light induced phase shifts (Pauly and Horseman, 1985). This seems to rule out involvement of P75-NGF in photic entrainment.

1.8 Other arousal related neurotransmitters in the SCN

Little research has been done as the role of histamine (HA) in the circadian system. Histamine has long been known to play a role in the production of wake and arousal (Chikahisa et al., 2013; Gondard et al., 2013) Histamine-immunoreactive cells show Fos expression during periods of activity in nocturnal rodents and appear to be involved in the sleep wake switch (Novak et al., 2000; Saper et al., 2001). HA immunoreactivity is present in the SCN, but a lack of histidine decarboxylase in the SCN indicates that it is synthesized elsewhere and trafficked into the SCN (Michelsen et al., 2005). A high density of H2 receptors has been reported in the SCN (Karlstedt et al., 2001). Administration of the histamine synthesis inhibitor α-

fluoromethylhistidine prior to light pulses resulted in significantly smaller phase shifts suggesting that it plays a role in photic phase shifting (Eaton et al., 1995). However, systemic injections of numerous histamine antagonists in hamsters had no effect on the phase shifts to light pulses making the conclusions unclear (Eaton et al., 1996). A compelling argument has even been made that histamine is the final entrainment pathway for both the photic and non-photic pathways before the phase of the SCN is reset (Jacobs et al., 2000). *In vitro* work has shown that application of histamine to SCN slices resulted in mainly an inhibitory effect, consistent with a potential role in non-photic phase shifting (Liou et al., 1983). But other studies have reported weak or inconsistent effects of histamine or the agonists when applied to the SCN (Meyer et al., 1998; Scott et al., 1998).

More recently, the role of orexin/hypocretin (OX) has been explored as a major neuropeptide involved in behavioral arousal. The SCN communicates phase information to the dorsomedial and lateral hypothalamus OX cells that can produce behavioral arousal partially through activation of the noradrenergic locus coeruleus (Abrahamson et al., 2001; Mahoney et al., 2013). Furthermore, Fos immunoreactivity is increased in OX containing neurons and the IGL during novel wheel confinement, sleep deprivation and restraint procedures (Webb et al., 2008). Further information regarding the role of OX can be found in Appendix 1.

1.9 Rationale and hypotheses

Non-photic phase shifting initially received a great amount of interest in the research community. Since the discovery of NPY, serotonin and the influences that they have had on non-photic phase shifting, research into the topic seems to have waned. Although environmental light is the principal entraining cue for the mammalian master circadian clock, it is behavior that

influences exposure to light. Behavior itself has also been shown to affect the circadian pacemaker (Mrosovsky and Salmon, 1987; Antle and Mistlberger, 2000). The IGL has been hypothesized as a structure that integrates behavioral state with light information and it has been shown that behavior interacts with the photic entrainment system (Mikkelsen, 1990; Mikkelsen et al., 1998). The hypothesized role that non-photic phase shifting plays in circadian rhythmicity is that it influences when the animal is exposed to light and can interact to modulate the effects of light on the pacemaker (Edelstein et al., 2003). Therefore, entrainment to environmental lighting conditions cannot be completely understood unless the behavioral state of the animal and interaction of contributing pathways at the level of the SCN is known.

The wide variety of findings with to the role of ACh in the circadian system have arisen likely due to species, sex, dose, circadian time of administration and differences in the drugs used (Reghunandanan et al., 1993). This has resulted in the role of ACh in circadian rhythmicity being unclear. There is also a lack of consensus as to the non-photic zeitgeber that phase shifts the SCN. The general hypothesis of this thesis is that behavioral arousal induces non-photic phase shifts and therefore neurotransmitters such as ACh that are involved in bringing about behavioral arousal will also be involved in non-photic phase shifting.

It was important to examine ACh because different studies have shown that it can produce photic phase shifts, non-photic shifts, or has inconsistent results. The IGL receives cholinergic and serotonergic input and in turn plays a crucial role in non-photic phase shifting through release of NPY (Cain et al., 2007; Glass et al., 2010). Since serotonin and acetylcholine are active when the animal is awake and aroused, this provides ways in which arousal can influence non-photic phase shifting. The basal forebrain also receives projections from brainstem cholinergic nuclei and has a direct projection to the SCN (Hallanger et al., 1987; Bina et al.,

1993). This could allow for the pathway from the basal forebrain to receive information about behavioral state and to the influence circadian timing through a projection to the SCN similar to how the IGL functions. If this hypothesis is correct, it would provide novel evidence for a role of behavioral arousal in non-photic phase shifting, it would provide evidence for a role of a third direct pathway to the SCN influencing non-photic shifting and finally, it would provide evidence for a direct role of ACh in the SCN to produce phase shifts.

The first hypothesis was that there would be a higher degree of activation in brain regions expressing ACh, during exposure to a non-photic stimulus. It was also hypothesized that there would be a higher degree of activation in brain areas containing orexin and histamine that is described in Appendix 1. In particular, it was expected that animals that were expected to phase shift to sleep deprivations, or novel wheel confinements would show higher specific activation in neurons expressing ACh, OX, or HA.

The second hypothesis was that direct electrical activation of the basal forebrain would produce phase advances when applied during the middle of the day. It was expected that the electrical stimulation would activate cholinergic cells that project to the SCN to produce phase advances during the midday.

Finally, it was hypothesized that if ACh played a role in non-photic phase shifting, then it would directly contribute to phase shifting during a known non-photic stimulus. It was expected that administration of an ACh antagonist to the SCN during novel wheel confinement would attenuate phase shifts to this non-photic stimulus. These experiments would provide evidence that sustained behavioral arousal was the common zeitgeber for non-photic stimuli and resolve the controversy regarding the role of ACh in the SCN.

Syrian hamsters were chosen to be used in these experiments due to the fact that they show large phase shifts to non-photic phase stimuli. Hamsters show extremely precise onsets of activity when provided access to a home cage running wheel. These onsets occur very closely to lights off in an LD cycle. This provides a very accurate and easy means by which circadian phase can be measured in baseline and experimental conditions. Although there are many genetic knockout mice lines that may provide important experimental means to elucidate the components of non-photic shifting, voluntary wheel running in mice is less accurate than hamsters and it would be more difficult to measure circadian phase. Additionally, mice show inconsistent responses to voluntary, or involuntary activity, known non-photic stimuli (Marchant and Mistlberger, 1996). Rats also show less than precise wheel running activity onsets and have been shown to be extremely difficult to entrain to bouts of exercise (Mistlberger, 1991).

2. Gene expression in arousal related brain areas

2.1 Introduction

Non-photic stimuli are capable of phase shifting the SCN, and do so in a stereotypical manner, producing large phase advances during the day with smaller phase delays sometimes occurring during the night (Yannielli and Harrington, 2004). These non-photic stimuli can involve midday exposure to a novel running wheel, or sleep deprivation by gentle handling (Mrosovsky and Salmon, 1987; Antle and Mistlberger, 2000). The common factor is that these two stimuli both produce behavioral arousal in animals at a time when they are normally at rest. Animals that fail to shift to the sleep deprivation procedure require more interventions and sooner interventions in order to keep them awake (Antle and Mistlberger, 2000). Similarly, those animals that do not run a threshold number of wheel revolutions during novel wheel confinement do no shift to the novel wheel confinement procedure (Bobrzynska and Mrosovsky, 1998). This indicates that those animals that do no shift to non-photic stimuli may not be sufficiently aroused. If this is true, then neurotransmitters typically involved in producing wake and arousal in the brain may also be involved in bringing about non-photic phase shifts. ACh is a neurotransmitter that is involved in producing behavioral arousal and was explored as potential contributor to non-photic phase shifting.

There is strong evidence that NPY from the IGL is involved in non-photic phase shifting. Intra-SCN injections of NPY result in phase shifts resembling the non-photic phase response curve with large phase advances during the day, and smaller phase delays occurring during the night (Albers and Ferris, 1984; Biello and Mrosovsky, 1996). It is not known how the IGL becomes activated during these stimuli. The IGL receives cholinergic input from the pedunculopontine nucleus (Moore et al., 2000). Unilateral injections of the cholinergic agonist

carbachol into the IGL produced small phase advances during the midday indicating that ACh may activate the IGL (Cain et al., 2007). This indicates that behavioral arousal could be a potential contributor to non-photic phase shifting through activation of the IGL. But arousal may also play a role in non-photic shifting through other pathways as well.

The role of ACh in the circadian system remains to be firmly established. Ventricular administration of carbachol results in phase shifts consistent with the photic PRC (Earnest and Turek, 1985). Another experiment using ventricular carbachol showed non-photic like phase shifts though (Meijer et al., 1988). Cholinergic cells from the forebrain, tegmentum and septum project to the SCN (Bina et al., 1993). Cholinergic projections throughout the forebrain and cortex contribute to the induction of wake, and brain waves associated with arousal (Cape and Jones, 2000).

The purpose of these experiments was to examine Fos expression as an index of cellular activation in regions containing ACh, in response to non-photic stimuli. ACh is a neurotransmitter involved in bringing about behavioral arousal that is also known to affect the circadian system but have no clearly defined role, or mixed findings. It was hypothesized that animals exposed to non-photic stimuli would show increased levels of Fos expression in areas of the brain containing ACh that is released during wake and responsible for bringing about arousal. Specifically, cholinergic cells of the basal forebrain were examined more thoroughly due to the debated role of ACh in the circadian system, and the contribution of cholinergic output to bringing about behavioral arousal.

In the first experiment, hamsters were classified as either non-responders or responders to sleep deprivation by gentle handling as is explained below. Next, they were placed into the non-responder, non-manipulated control group, the non-responder sleep deprived group, or the

responder sleep deprived group. Fos expression was examined in the cholinergic basal forebrain. In the second experiment, Fos expression was examined in response to sleep deprivation for cells specifically labelled for ChAT. In the third experiment, Fos expression was also examined in ChAT containing neurons of the basal forebrain in animals undergoing a novel wheel confinement procedure. It was hypothesized that non-photic stimuli would increase Fos expression in brain areas containing ACh, neurons. Furthermore, it was hypothesized that Fos expression would be specifically increased in neurons containing ChAT in the basal forebrain in response to sleep deprivation. Those animals that would be expected to phase shift to sleep deprivations and novel wheel confinements were predicted to show higher expression of Fos in ChAT containing neurons.

2.2 Materials and methods

2.2.1 Animals

Adult male Syrian hamsters were obtained from Charles River Laboratories (Saint-Constant, QC, Canada). Animals were initially group-housed 2-3 per cage. They were acclimatized to laboratory conditions in a 14:10 light/dark (LD) cycle with a temperature maintained around 21±1° C. At all times, food and water was available *ad libitum*. Care was taken to avoid pain and discomfort for the animals used in the study. All procedures were approved by the Life and Environmental Sciences University of Calgary Animal Care Committee. Ethical protocols were in accordance with the guidelines of the Canadian Council of Animal Care.

2.3 Experiment 1 – Fos expression in the cholinergic forebrain following a sleep deprivation

2.3.1 Sleep deprivations and phase shift analysis

Sleep deprivation by gentle handling was performed on young hamsters as a pre-screening. The sleep deprivation procedures took place in a modified Aschoff Type II procedure where first the animals were entrained to a 14:10 LD cycle with access to a novel running wheel. Then, prior to manipulation, the hamsters were placed into constant darkness (DD) and received a 3 hour sleep deprivation under dim red light (< 1 lux at cage bottom). Any time that the animal tried to adopt a stereotypical sleep posture, or would enter into a state of quiescence, the experimenters would lightly touch, or otherwise disturb the animal. This procedure continued from circadian time (CT) 6-9 where CT12 is defined as activity onset by convention. At the conclusion of the sleep deprivations, the cage lids with attached home cage running wheels were returned and the hamsters were left in DD for 5-7 days. Wheel running activity was continuously collected and summed into ten minute bins on a PC computer using ClockLab data collection software (Actimetrics, Evanston, IL, USA).

Phase shifts to the sleep deprivation procedure were calculated using ClockLab data analysis software (Actimetrics, Evanston, IL, USA) on a PC computer. The ClockLab algorithms were used to calculate the activity onsets for 5-7 days prior to the sleep deprivation as a measure of baseline activity. Leaving 2-3 days after the sleep deprivation to account for possible transients in wheel running activity, the activity onsets were also calculated for 5-7 days following the sleep deprivation. Regression lines were automatically fit to the activity onsets prior to and after the sleep deprivation, and the difference between these two regression lines on the day of the sleep deprivation was determined to be the phase shift. Based on the results of the

phase shifts, hamsters were separated into groups as responders (those that phase advanced), or non-responders (those that did not phase advance).

2.3.2 Perfusion and immunohistochemistry

Following pre-screening as responders, or non-responders, the hamsters were returned to an LD cycle and allowed to re-entrain for at least 7 days. When it had become apparent that the activity onsets were coinciding with lights off again, the animals were once again sleep deprived from CT6-9 in the activity cycle.

At the conclusion of the sleep deprivation, the hamsters were administered an overdose of sodium pentobarbital (~400mg/kg; Bimeda-MTC, Cambridge, ON, Canada). Their eyes were shielded from light using tin foil, they were brought to a fumehood and perfused transcardially using 50 ml of cold phosphate buffered saline (PBS), pH 7.4 followed by 50 ml of cold 4% paraformaldehyde in PBS. The brains were post-fixed overnight using the same fixative and then transferred to 20% sucrose in PBS for cryoprotection for at least one more day. The brains were frozen at -18°C and sliced using a cryostat into 35 µm coronal sections from the beginning of the SI in the forebrain until the end of the histamine containing cells near the mammillary recess. The sections were collected into wells containing 0.02% sodium azide in PBS.

In order to define the boundaries of the cell groups of interest, intact animals were processed for ChAT immunohistochemistry. The hamster brain atlas as well as previously conducted immunohistochemistry on intact animals for ChAT was used to create templates to judge the boundaries of particular cell groups of interest. Three sections were chosen based on ChAT immunoreactivity in the basal forebrain in slices containing the anterior, mid and posterior sections of the substantia innominata. These sections were at approximately +0.2 mm anterior to

bregma, -0.3 mm posterior to bregma and -0.8 mm posterior to bregma for the anterior, mid and posterior sections respectively. Adobe photoshop was used to create templates from each of the sections by tracing over the base of the brain, third ventricle and area that contained immunoreactivity for ChAT along one side of the brain (Adobe systems, San Jose, CA, USA). In order to create the template to count for the other side of the brain, the first template was flipped horizontally and then overlaid as described below.

2.3.3 Immunohistochemistry for ChAT

For the ChAT immunohistochemistry, alternate sections were processed through the entire length of the SI. The slices were incubated in 0.5% H_20_2 to eliminate the endogenous peroxidase in 0.5% TritonX-100 in PBS (PBSx). The sections were rinsed and then incubated in a 5% normal horse serum as a blocking agent (Vector Laboratories Inc., Burlingame, CA, USA). The primary antibody was a goat anti-ChAT (1:1000; Millipore) and incubation took place in a 2% solution of NHS in PBSx for ~48 hrs at 4°C on a shaker tray. The sections were rinsed and then incubated in a biotinylated horse anti-goat secondary antibody (Vector Laboratories Inc) for 1 hr. The sections were rinsed again, and then incubated in an avidin-biotin complex (ABC; 1:100; Vector Vectastain Elite ABC kit; Vector Laboratories Inc.) The tissue was rinsed thoroughly for a final time before being reacted using diaminobenzidine (DAB; 0.5mg/ml) as a chromagen with 0.02% nickel chloride to intensify the reaction product in tris buffer using H_20_2 to start the reaction. The tissue was mounted onto gelatin coated slides, dehydrated in a series of alcohol rinses and cleared in Xylenes before being coverslipped with Permount (Fisher Scientific, Pittsburgh, PA, USA).

2.3.4 Fos immunohistochemistry

Alternate sections of each of the ChAT containing regions were processed from the sleep deprived animals for Fos DAB immunohistochemistry as above, with the following exceptions. The tissue was blocked in a 1% normal goat serum (NGS; Vector, Laboratories). The tissue was incubated in the primary antibody a rabbit anti-fos (1:20000 in 1% NGS in PBSx; Santa Cruz biotechnology, Santa Cruz, CA, USA) for 48 hours at 4ºC on a shaker tray. The secondary antibody, was a biontinylated goat anti-rabbit (1:200 in PBSx; Vector Laboratories, USA) for one hour at room temperature on a shaker tray. The tissue was reacted in DAB as described above for 3-5 minutes, dehydrated, cleared in Xylenes and coverslipped using Permount (VWR).

2.3.5 Analysis

The brain sections were photographed using an Olympus BX51 microscope with an attached cooled CCD camera (QICAM 1394; QImaging, Burnaby, BC, Canada). Three sections were chosen from the sleep deprived animals that corresponded to the anterior, mid and posterior sections that were used to create the templates. In order to line up the boundary of the ChAT cells of the template to the sleep deprived sections, the templates were overlaid by lining up the middle of the third ventricle and base of the brain on the templates to the sleep deprived sections. Fos cells were counted within the ChAT cell group boundaries by using the Image J (NIH, Bethesda, MD, USA) The sleep deprivation treatment effect was examined using a one-way analysis of variance (ANOVA) and Tukeys test to examine follow-up pairwise comparisons.

2.4 Experiment 2- Fos expression in ChAT cells during sleep deprivation

As described above, animals were pre-screened as responders or non-responders using the sleep deprivation by gentle handling procedure. This time, the animals were sleep deprived using a dim light, and not a dim-red light as in experiment 1. The hamsters were then sleep deprived again following re-entrainment and sacrificed as above by administration of an overdose of sodium pentobarbital. The brains were removed, fixed and sectioned as above. The free floating brain sections were then processed for double label Fos, ChAT fluorescent immunohistochemistry. The free floating brain sections were processed as described above using 5% NDS as a blocking serum in PBSx for 1 hour at room temperature on a shaker tray. Following the blocking, the sections were incubated in the primary antibodies, a goat anti-ChAT (1:500; Millipore, USA), and a rabbit anti-c-fos (1:20000; Santa Cruz Laboratories) in a 2% solution of NDS in PBSx for 48 hours at 4°C on a shaker tray. The secondary antibodies were a Cy-3 conjugated donkey anti-rabbit and a Cy-5 conjugated donkey anti-goat (1:200; Jackson ImmunoResearch). The sections were rinsed a final time before being mounted onto gelatin coated slides and dehydrated in a successive series of alcohol rinses, cleared in Xylenes and coverslipped with Krystalon (VWR).

The fluorescently labelled brain sections were photographed using an Olympus BX51 microscope with an attached cooled CCD camera (QICAM 1394; QImaging, Burnaby, BC, Canada). Three sections were chosen from each animal at the same approximate anterior, mid and posterior locations. These sections were representative of ChAT expressing cells in the rostral, mid and caudal levels of the SI. Double labelled cells were counted bilaterally on each slice for all of the animals using Image J (NIH, Bethesda MD, USA). Cells were determined to be double labelled if they displayed a bright fusiform shape of a cell with a bright dense nucleus

indicating c-fos was also present in the cells. The differences between the anterior, mid and posterior sections for the non-responder and responder sleep deprived groups were examined using a one-way ANOVA with Tukeys follow-up pairwise comparisons to examine specific mean differences.

2.5 Experiment 3- Fos Expression in ChAT cells during a wheel confinement

2.5.1 Wheel Confinement

Single housed animals (n=6) without access to running wheels were entrained to a 14:10 LD cycle. The animals were then transferred to a recording room at zeitgeber time (ZT) 6 where ZT12 is defined as the time of lights off. The lights were turned out and the animals (n=3) were confined to novel running wheels while the control group (n=3) were left in their home cages in the dark without access to a wheel. At ZT9, all animals were sacrificed and perfused as described above. The tissue was then processed for double label ChAT/Fos fluorescent immunohistochemistry as described above, and the tissue was photographed and analyzed as in experiment 2.

2.6 Results

2.6.1 Experiment 1- Fos expression in the cholinergic region of the forebrain

Figure 4 displays representative actograms of non-responders and responders to the sleep deprivation procedure.

Figure 4: Sleep deprivation actograms

A: Displays a representative actogram of an animal that was determined to be a non-responder to the sleep deprivation procedure. The time of day when the lights were off is displayed as grey and when the lights were on is displayed as white. The phase shift was measured as the difference between the red and blue regression lines. B: displays an actogram of an animal that was determined to be a responder to the sleep deprivation procedure.

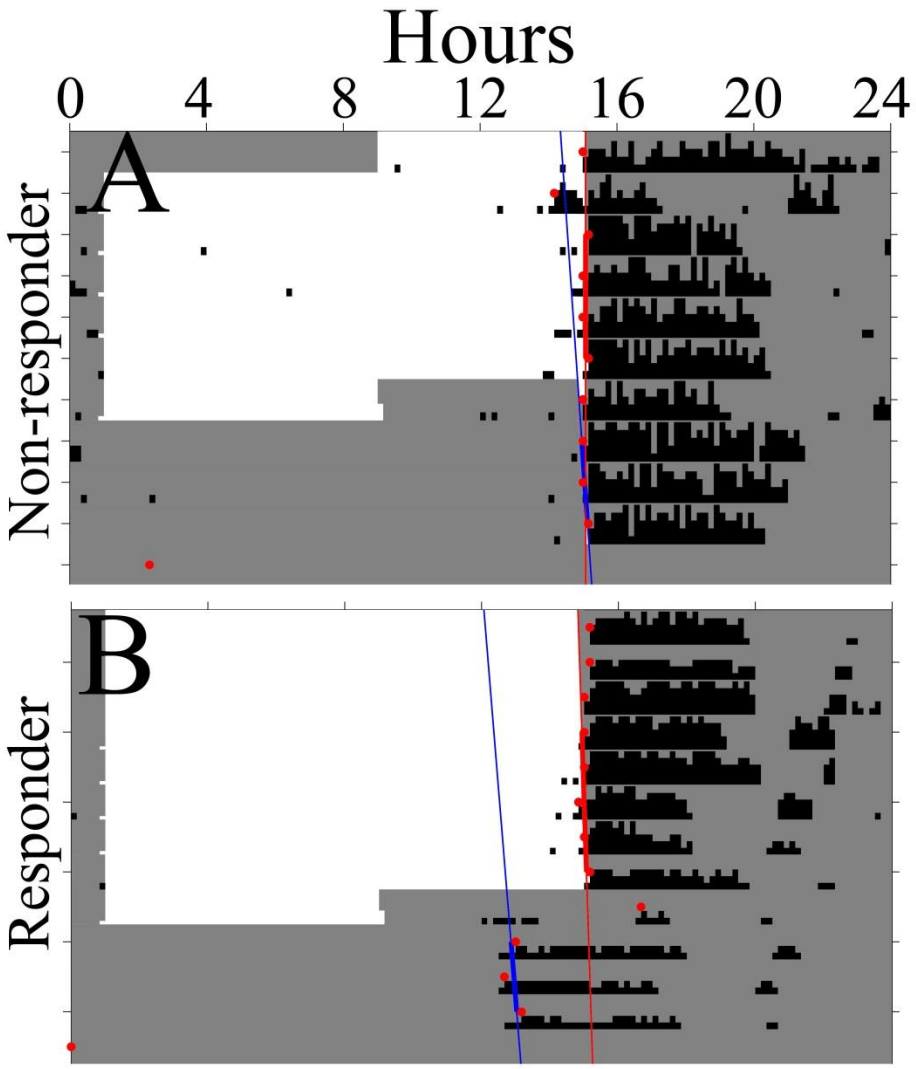

Hours
0
4
8
12
16
20
24
A
Non-responder
B
Responder

Figure 5, A-C displays the intact ChAT immunoreactive slices of the basal forebrain that were chosen to be counted as the anterior, mid and posterior sections with the templates for one side of the brain overlaid on them. Higher resolution representative sections of anterior, mid and posterior Fos labelled sections in the ACh, regions in the non-responder control, non-responder sleep deprived and responder sleep deprived groups are displayed in Figure 5 D-F, G-I and K-L respectively.

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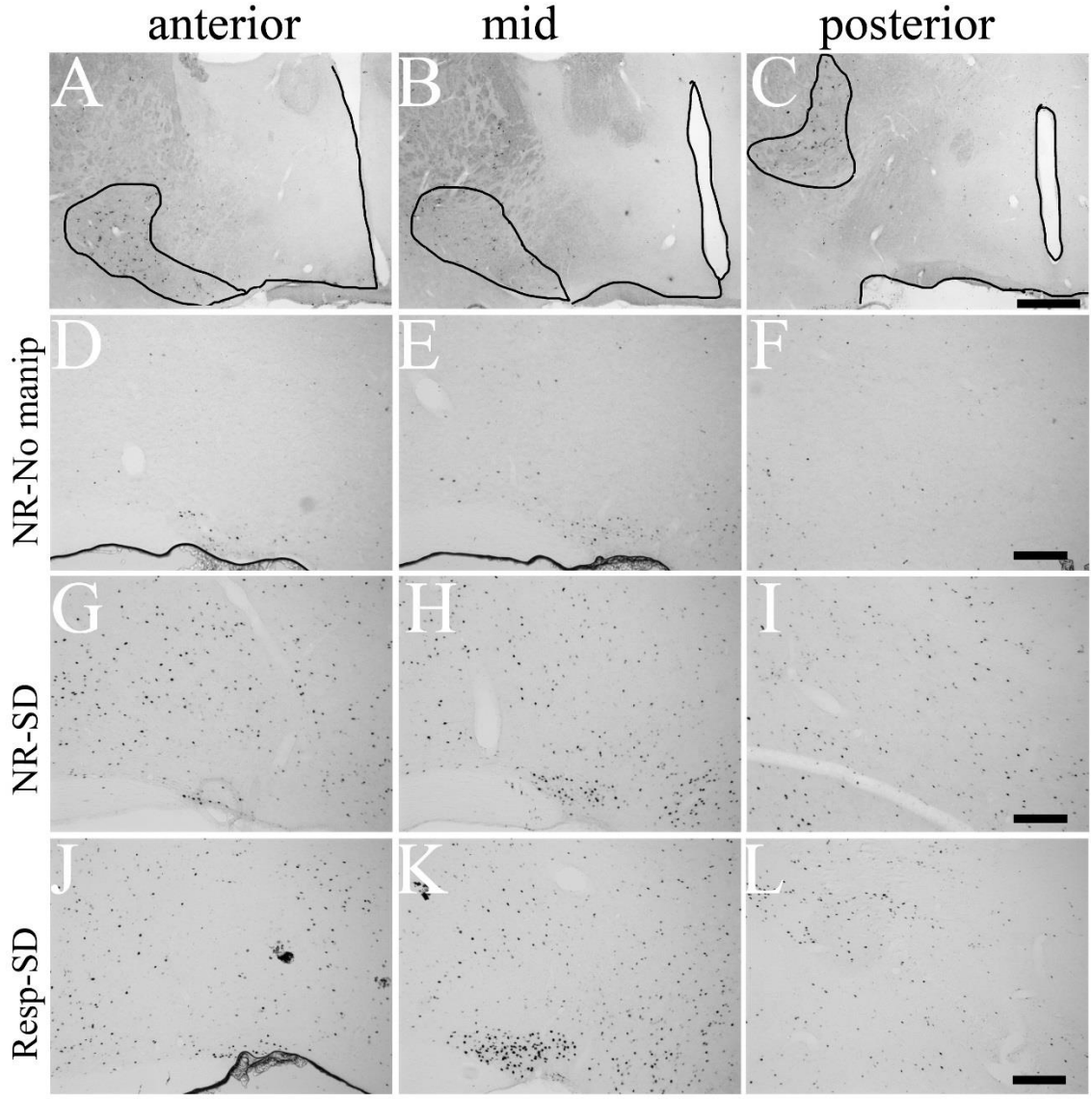
Figure 5: Fos expression in forebrain regions containing ACh

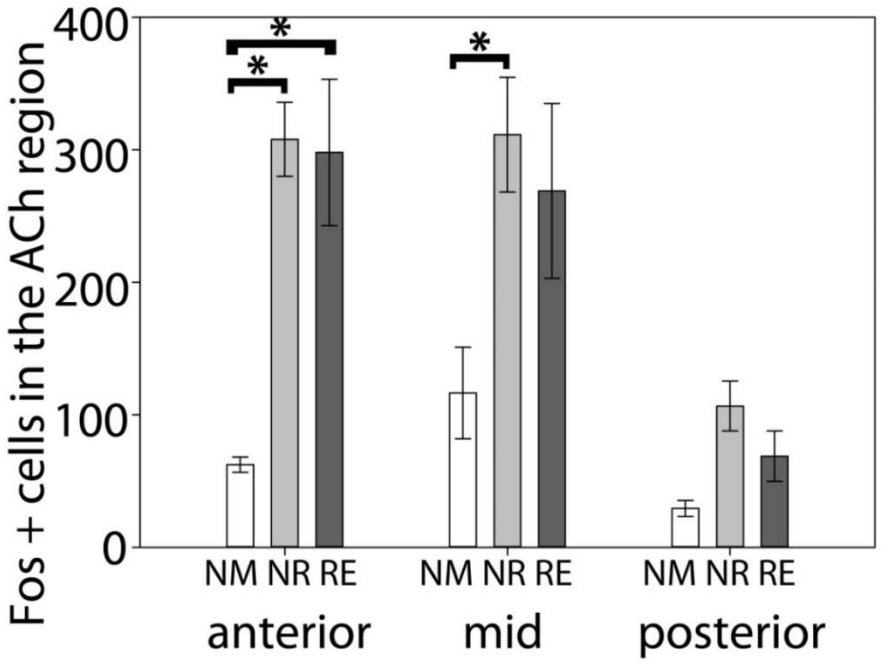
A-C displays the anterior, mid and posterior (respectively) sections of the cholinergic region of the basal forebrain of a control hamster that was a non-responder to a sleep deprivation from CT6-9 that did not undergo any manipulation at the time of sacrifice. The black dots are cells that are immunoreactive for ChAT. The black lines are the templates that were created for one side of the brain and used to determine the regions to count Fos positive cells in the sleep deprived animals. The scale bar is 600 μm. D-L displays higher magnification photomicrographs of the counted areas for each condition. Scale bars are 200 μm. D-F displays an animal that was pre-screened to be a non-responder and did not undergo any sleep deprivation. G-I represents an animal that was pre-screened to be a non-responder that received a sleep deprivation. J-L represents an animal that was pre-screened to be a responder to the sleep deprivation procedure that underwent a sleep deprivation prior to sacrifice.


anterior
mid
posterior
A
B
C
D
E
F
G
H
I
J
K
L
NR-No manip
NR-SD
Resp-SD

In the ACh containing region of the forebrain, there was an average of 62.4 ± 5.78 cells in the anterior sections, 116.4 ± 34.55 cells in the mid sections and 29.4 ± 6.04 cells in the posterior sections of the control animals. The average across all sections of the control animals was 69.4 ± 25.4 cells (n=5). In the non-responder, sleep deprived group, there was an average of 307.86 ± 27.97 cells in the anterior sections, 311.43 ± 43.24 cells in the mid sections and 106.57 ± 18.83 cells in the posterior sections. The average number of cells across all sections was 241.95 ± 67.7 for the non-responder sleep deprived group (n=7). In the responder sleep deprived group, there was an average of 298 ± 55.26 cells in the anterior sections, 269 ± 65.9 cells in the mid sections and 68.75 ± 19.03 cells in the posterior sections. The average number of cells across all sections was 211.92 ± 72.08 cells for the responder sleep deprived group (n=4). There was a significant effect for sleep deprivation on Fos expression in the ACh region of the forebrain ($F_{(8, 47)} = 7.164$; $p < 0.001$). Tukeys follow-up pairwise comparisons revealed a significant difference between the anterior sections for the control and non-responder sleep deprived group ($p<0.001$), the control and the responder sleep deprived group ($p=0.002$). There was also a significant difference between the control group and the non-responder sleep deprived group in the mid sections ($p=0.004$). The average cell counts are displayed for the anterior, mid and posterior regions for each group in Figure 6.

Figure 6: Mean Fos counts for cells in the ACh region

Displays the mean ± SEM single label Fos counts of control non-responder, no manipulation animals (NM) along with non-responder, sleep deprived animals (NR) as well as responder, sleep deprived animals (RE). These averages were calculated for the cholinergic substantia innominata region. The brackets and asterisks indicate that the cell counts were significant ($p<0.05$).

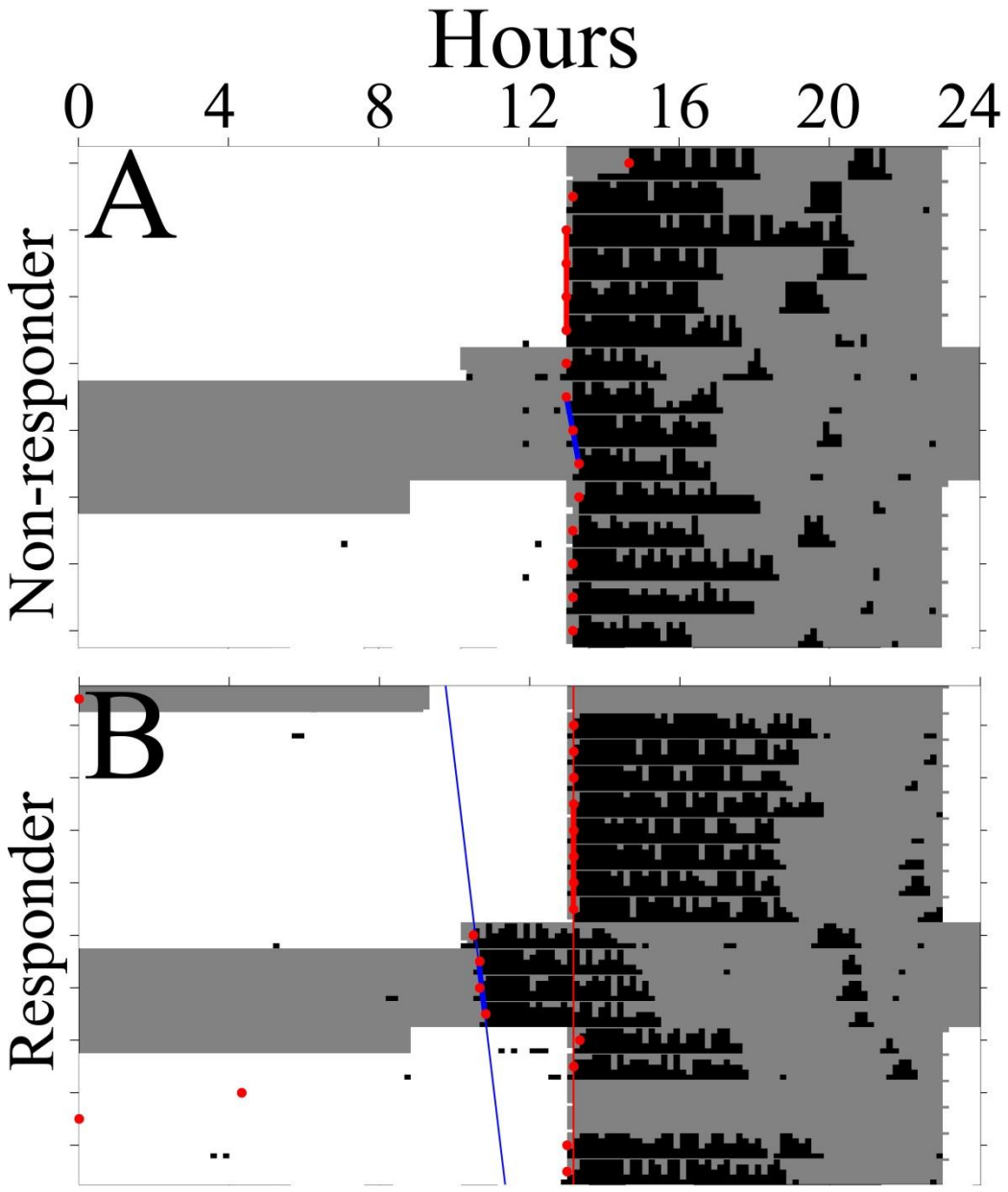

Fos + cells in the ACh region
400
300
200
100
0
*
*
*
NM NR RE
NM NR RE
NM NR RE
anterior
mid
posterior

2.6.2 Experiment 2 – Fos expression in ChAT cells of sleep deprived animals

Representative actograms of non-responders and responders to the sleep deprivation procedure are displayed in Figure 7.

Figure 7: Sleep deprivation actograms

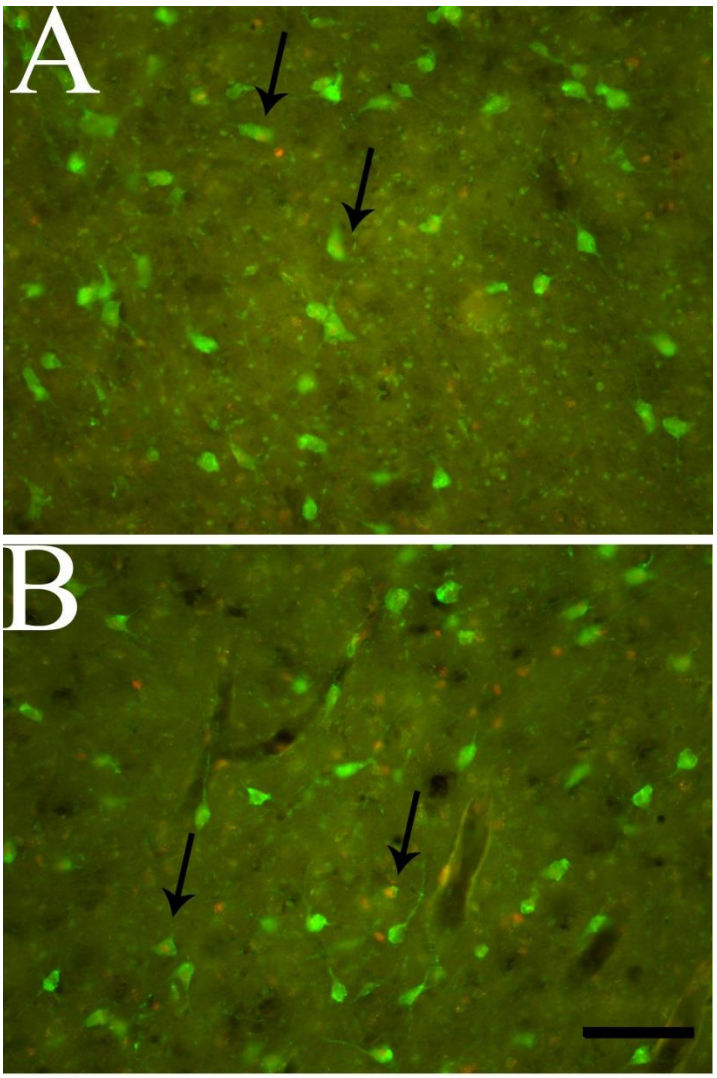
A: Displays a representative actogram of an animal that was determined to be a non-responder to the sleep deprivation procedure. The time of day when the lights were off is displayed as grey and when the lights were on is displayed as white. The phase shift was measured as the difference between the red and blue regression lines. B: displays an actogram of an animal that was determined to be a responder to the sleep deprivation procedure.


Hours
0
4
8
12
16
20
24
A
Non-responder
B
Responder

A representative section from a responder and non-responder from the anterior sections where there is the most ChAT immunoreactivity is displayed in Figure 8A and B. The mean number of Fos immunoreactive neurons in ChAT positive cells for the non-responders (n=2) in the anterior sections was 8 ± 1 cells, 4.5 ± 2.5 cells in the mid sections and 3.5 ± 1.5 cells in the posterior sections. The overall average of double label cells for the non-responders across all the sections was 5.33 ± 1.67 cells**.** The mean number of Fos immunoreactive neurons in ChAT positive cells for responders (n=8) was 8.25 ± 1.38 cells in the anterior sections, 6.88 ± 1.47 cells in the mid sections and 5 ± 1.45 cells in the posterior sections. The overall average of double label cells for the responder sleep deprived group across all the sections was 6.71 ± 0.58 cells**.** There was no significant main effect on Fos expression for responder versus non-responder in ChAT cells of the forebrain $F_{(5,25)} = 0.97$; $p=0.45$). The averages are displayed in Figure 8C.

Figure 8: Fos /ChAT cells and mean cell counts for sleep deprived hamsters

Displayed is the double labelled immunohistochemistry for ChAT labelled cells in the forebrain in animals that are either non-responders or responders to a sleep deprivation by gentle handling at midday CT6-9 (A or B). The scale bar is 200 μm. The means ± SEM are displayed in C. Double label cells are indicated by the black arrows.

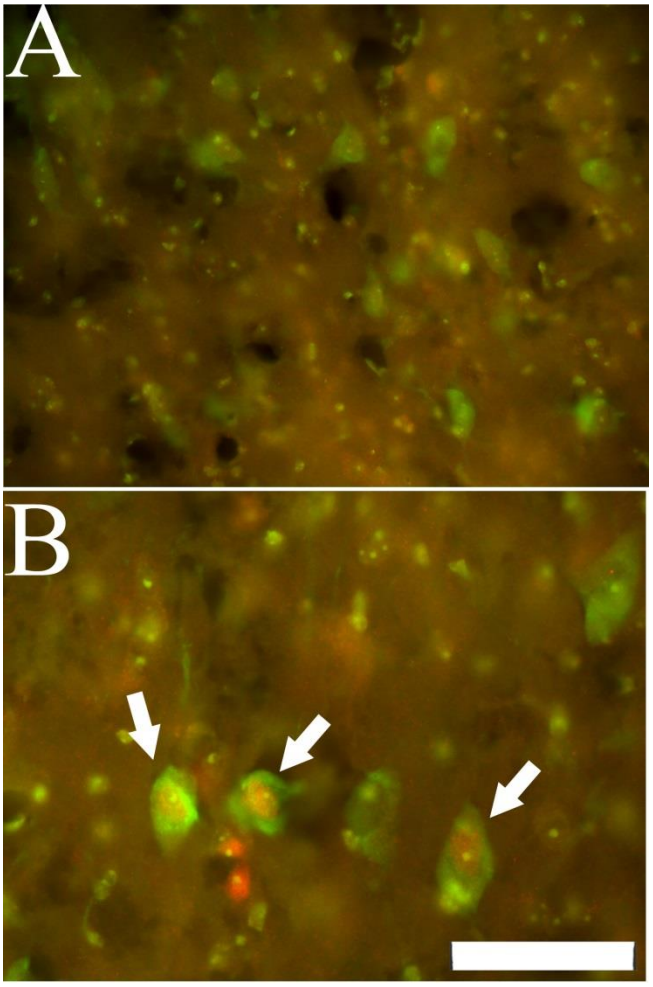

A
B
C
12
10
8
6
4
2
0
ChAT/Fos + Cells
NR
RE
NR
RE
NR
RE
anterior
mid
posterior

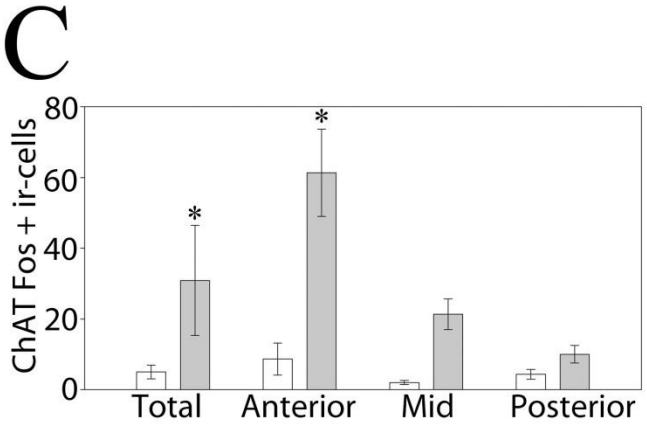
2.6.3 Experiment 3 – Fos expression in ChAT neurons during wheel confinement

All of the experimental animals ran continuously throughout the novel wheel confinement. The mean number of wheel revolutions was 3541 ± 262 in the novel running wheels. A representative section of fluorescent double label is displayed from the control animals that did not receive a manipulation and a wheel confined animal Figure 9A and B. In the control animals the averages for the anterior, mid and posterior sections were 8.67 ± 4.53, 2 ± 0.58 and 4.33 ± 1.34 double label cells respectively. The anterior sections of the cholinergic region of the forebrain in the novel wheel confinement animals showed an average of 61.33 ± 12.37 while the mid sections showed an average 21.33 ± 4.35 cells and the posterior sections showed 10 ± 2.48 cells. In total, across all brain sections, the control group showed an average Fos expression in 5 ± 3.9 cells in cells containing ChAT and the wheel confinement group showed an average of 30.89 ± 14.8 ChAT containing cells. There was a significant main effect for wheel confinement on Fos expression in ChAT containing neurons in the forebrain ($F_{(5,17)}$ =15.15; $p < 0.01$) Tukeys follow-up multiple pairwise comparisons revealed a significant difference between the cells in the anterior sections of control and wheel confined animals ($p<0.001$). The average cell counts are displayed in Figure 9C.

Figure 9: Fos /ChAT cells and mean cell counts for wheel confined animals

Displayed is the double labelled immunohistochemistry for ChAT labelled cells in the forebrain in animals that received no manipulation (A), or a one-time confinement to a novel running wheel at midday CT6-9 (B). Double label cells are indicated by the white arrows. The scale bar is 100 µm. The mean cell counts for ChAT and Fos double labelled cells are displayed ± SEM in Figure 9C.


A
B


C
ChAT Fos + ir-cells
80
60
40
20
0
*
*
Total
Anterior
Mid
Posterior

2.7 Discussion

In the first experiment, general Fos expression was examined in brain areas roughly corresponding to regions that express ChAT in the basal forebrain. It was found that sleep depriving hamsters during the middle of their usual rest period increases Fos expression in the cholinergic forebrain. For the experiments conducted involving Fos expression in the orexin and histamine containing brain regions of sleep deprived animals, please refer to Appendix 1.

In the control animals, Fos expression was present in all of the examined regions. In the sleep deprived groups, Fos expression was increased in most of the areas examined when compared to no-manipulation controls, however there did not appear to be any difference between non-responders and responders. This result did not appear to be consistent with the hypothesis. If arousal was the key-factor involved in non-photic phase shifting, then it would have been predicted that responders to the sleep deprivation procedure would have had higher Fos expression the cholinergic basal forebrain as it is strongly associated with bringing about a waking, aroused, behavioral state. Since all that was examined was single label, Fos, it still may have been the case that in the responders, more cholinergic cells were activated and this was not reflected in the overall cell counts of single label Fos expression though. In the non-responder sleep deprived group, it is possible that non-cholinergic cells express more Fos, but in the responder sleep deprived group, these cells do not become active and cholinergic cells do instead. Therefore, a possible explanation of the lack of difference in responders and non-responders for Fos expression in the forebrain could have been that the responders showed greater expression of Fos in those cells expressing ACh. If this hypothesis was valid, then there would also have to be a concurrent reduction in Fos expression in other cells around the active

ACh cells in order for there to be no detectable differences between the non-responders and responders.

Another potential explanation for the lack of differences between the non-responders and responders undergoing sleep deprivation in experiment 1 could be the fact that phase shifts to the sleep deprivation procedure were found with a lower frequency than would be expected based in previous literature, and that these phase shifts were often not as large as expected (Antle and Mistlberger, 2000). It could have been the case that the hamsters that were classified as responders actually belonged to the population of non-responders in terms of their abilities to phase shift. A similar issue was the pre-screening for responders and non-responders. This experiment was also conducted under the assumption that if an animal was classified as a non-responder, or responder, they would continue to be so for future exposures to the stimuli.

In order to determine if there was specific activation in neurons that expressed ChAT, Fos expression in neurons specifically labelled for ChAT in the forebrain was examined in sleep deprived non-responders and responders in Experiment 2. Particularly, cholinergic cells that were in and around the region of the SI were examined as there has been previous evidence that ACh containing neurons from these regions may project to the SCN (Bina et al., 1993). Surprisingly, relatively little double labelling was found for Fos expression in ChAT containing neurons of the basal forebrain. Unlike the sleep deprivations performed in experiment 1 the sleep deprivations performed in this experiment had an effect on most of the animals and the phase shifts were ~3 hours as would be expected. Also unlike experiment 1, it was rare in which animals did not phase shift to the sleep deprivation procedure. One of the differences in procedures was the use of a dim red light in the first experiment and the use of a dim light in the second experiment. The purpose of using a dim red light in the first experiment was to avoid the

masking effects of light as it is known to counteract non-photic stimuli (Mrosovsky, 1991). One possible explanation was that the hamsters in the first experiment that were sleep deprived under dim red light had too dim conditions to become thoroughly aroused enough. Behavioral observations during the first experiment did not seem to confirm this possibility though as animals in the first experiment seemed just as aroused as in the second experiment. Another possibility could be that animals in the first sleep deprivation experiment were in a different room than the animals in the second experiment and environmental conditions such as excessive daytime noise may have played a role.

In experiment 3, wheel confinement was also used as a non-photic manipulation to examine Fos expression in cholinergic neurons of the forebrain in order to investigate the role of ACh in non-photic phase shifting and to explore the role of arousal. The wheel confined group showed significantly greater Fos expression in cholinergic neurons of the basal forebrain than non-manipulated controls. This occurred in the anterior section, likely because the most ChAT immunoreactivity is found here. This provided evidence for the hypothesis that specific activation of the cholinergic cells in the forebrain participate in non-photic phase shifting of the circadian clock. This result could be interpreted in several ways. It could indicate that either the responders showed a higher degree of arousal due to greater ascending cholinergic activation of the cortex or this arousal produced the phase shifts through activation of some other structure such as the IGL. It is also possible that the cholinergic cells in the forebrain become active and directly release acetylcholine on the SCN in order to bring about these phase shifts. Or, a third interpretation is that the cholinergic neurons from the tegmentum are responsible for bringing about these shifts to novel wheel confinement as it was found they also project to the SCN (Bina et al., 1993). It was unexpected that there would be so much more Fos expression in ChAT

neurons in the wheel confinement as opposed to the sleep deprivation as it was hypothesized that they may have worked through similar mechanisms. This may have been due to the fact that the wheel confinement procedure is a lot more arousing than the sleep deprivations. So one interpretation could be that very arousing non-photic stimuli may rely more heavily on arousal related neurotransmission, and less arousing stimuli do not. One final issue with the interpretation to these results lies in the fact that the animals in this study did not have prior access to a novel running wheel before the experiment. Previous work has shown that entrained animals without access to running wheel given access to a wheel and then placed in DD can show very large phase shifts (Gannon and Rea, 1995).

In sum, the non-photic stimulus of sleep deprivation by gentle handling increases Fos expression in arousal related areas, but there does not appear to be a difference between non-responders and responders. This activation of arousal related cells appears to be specific to the highly arousing stimulus of novel wheel confinement particularly in the cholinergic cells of the basal forebrain, but is not involved in the less arousing non-photic stimulus of sleep deprivation by gentle handling. This suggests that arousal, in the case of sleep deprivations may act on the SCN in a different manner than during the wheel confinement procedure. Fos expression in the case of the sleep deprivation experiments may also be detectable given a more sensitive procedure as well.

3. Electrical stimulations of the forebrain

3.1 Introduction

Non-photic stimuli capable of phase shifting the circadian clock typically involve large phase advances during the day (Antle and Mistlberger, 2000). Typically, the stimuli capable of producing non-photic shifts produce high degrees of behavioural arousal in the animals at a time when they are normally at rest, and this arousal is not simply a stress response (Mistlberger et al., 2003).

Several input pathways into the SCN have been explored with regards to how these non-photic shifts are produced. Direct electrical stimulation of the SCN has been shown to produce phase shifts (Rusak and Groos, 1982). Electrical stimulation of the geniculohypothalamic tract has been shown to produce phase advances during the day, with smaller phase delays during the night (Rusak et al., 1989). Phase advances shift the phase of the circadian clock to an earlier time, whereas delays shift the phase to a later time. Similarly, electrical stimulation of the IGL can produce non-photic like phase shifts (Kaur and Rusak, 2007).

Other input pathways to the SCN have also been explored. Electrical stimulation of the median or dorsal raphe nuclei resulted in small phase advances during the day (Meyer-Bernstein and Morin, 1999). These median raphe nucleus stimulation induced phase shifts appear to be dependent on serotonin (Yamakawa and Antle, 2010). Interestingly, longer stimulations of the dorsal raphe nucleus resulted in larger phase shifts (Glass et al., 2000). This is consistent with non-photic stimuli requiring sustained arousal in order to produce phase shifts.

Non-photic stimuli involve behavioural arousal during the normal rest period of nocturnal animals. ACh plays a role in producing behavioral arousal, and also appears to make direct connections to the SCN from the forebrain, tegmentum and septum (Bina et al., 1993; Cape and

Jones, 2000). The brainstem laterodorsal and pedunculopontine project dorsally through the thalamus and send a ventral path to the hypothalamus and basal forebrain (Hallanger et al., 1987). The forebrain cholinergic neurons project widely throughout the cortex, thalamus, hippocampus, and olfactory bulb among others (Mesulam et al., 1983). Cholinergic neurons fire at high rates during wake and cortical activation, promoting wake and stimulating gamma activity (Jones, 2004). Another study found basal forebrain neurons discharge maximally during wake and paradoxical sleep (Lee et al., 2005). Recent work with optogenetics on cholinergic basal forebrain neurons showed that photo-stimulation induces immediate transitions from slow wave sleep to wake, or paradoxical sleep and sustain cortical activation (Han et al., 2014).

The role of ACh in the circadian system is still in debate (Earnest and Turek, 1985; Meijer et al., 1988). Since in experiment 1 and 3, it was found that forebrain cholinergic neurons were activated in response to non-photic stimuli, the role of the forebrain in phase shifting was examined. In order to determine the role of the basal forebrain cholinergic neurons in phase shifting, unilateral electrical stimulations of the substantia innominata were performed. To determine if activation of the forebrain resulted in cholinergic neurotransmission directly to the SCN to produce phase shifts, the stimulations were repeated again with administration of the muscarinic antagonist Atropine into the SCN. It was hypothesized that electrical stimulation of the cholinergic region of the forebrain would produce phase advances during the midday. It was further hypothesized that blocking ACh from binding in the SCN would eliminate these phase shifts.

3.2 Experiment 4 – Electrical stimulation of the basal forebrain

3.2.1 Electrode implantation

Syrian hamsters (n=13) were anesthetized, received analgesic, and placed in the stereotaxic frame as described above. Stainless steel insulated twisted bipolar electrodes were implanted through a burr hole drilled in the skull at the following coordinates from bregma AP+ 1.05, ML+ 2.4, DV– 7.7 mm (MS303/3, Plastics One, Inc, Roanoke, VA). The electrode tips were flattened and separated slightly with approximately 0.1 mm of the insulation removed at the tips using a scalpel blade. The electrodes were anchored in place using screws drilled into the skull and a dental acrylic headcap (Dentsply,York, PA).

3.2.2 Electrical stimulation

Following recovery from surgery (2-4) days, the hamsters were placed in cages equipped with running wheels as described above and released into DD. Animals free ran in DD for at least 8 days before the activity onsets were calculated and a regression line was fit to these onsets in order to predict circadian time 12 (CT12) on the day of the stimulation (by convention, CT12 is defined as activity onset).

The implanted electrodes were connected to either a Grass SD88 stimulator (Grass Instrument Company, Quincy, MA) coupled to a photic stimulus isolation unit (PSIU6) or an isolated pulse stimulator (Model 2100, A-M Systems Inc., USA) immediately prior to stimulation. Electrical stimulation of SI took place from CT5-7 in the activity rhythm. 200 µA, 0.5 ms biphasic constant current pulses were delivered at 50 Hz in the home cage. These stimulation parameters were chosen based on experiments conducted showing electrical stimulation of the nucleus basilis magnocellularis results in ACh release in the cortex (Casamenti et al., 1986). The animals freely moved about their cages throughout the stimulation. A control

procedure also took place where animals (n=5) were connected to the stimulator from CT5-7 but no current was passed. The differences were examined using a two-group t test.

3.2.3 Perfusion and ChAT immunohistochemistry

At the conclusion of the study the animals were perfused and sliced as described above. Cresyl violet staining as well as immunohistochemical staining for ChAT was conducted in order to determine the electrode placements. Alternate sections throughout the entire SI were collected into trays to be processed for immunohistochemistry. The slices were incubated in 0.5% H_20_2 to eliminate the endogenous peroxidase. The sections were rinsed and then incubated in a 5% normal horse serum as a blocking agent (Vector Laboratories Inc., Burlingame, CA, USA). The primary antibody was a goat anti-ChAT (1:1000; Millipore) and incubation took place in a 2% solution of NHS in PBSx for ~48 hrs at 4°C on a shaker tray. The sections were rinsed and then incubated in a biotinylated horse anti-goat secondary antibody (Vector Laboratories Inc) for 1 hr. The sections were rinsed again, and then incubated in an avidin-biotin complex (ABC; 1:200; Vector Vectastain Elite ABC kit; Vector Laboratories Inc.) The tissue was rinsed for a final time before being reacted using diaminobenzidine (0.5mg/ml) as a chromagen with 0.02% nickel chloride to intensify the reaction product in tris buffer using H_20_2 to start the reaction. Finally, the brain slices were mounted on gelatin- coated slides, dehydrated in a series of alcohol rinses, cleared in Xylenes and then cover slipped with Permount (Fisher Scientific).

3.2.4 Analysis

Activity onsets and regression lines before and after the manipulation were calculated using ClockLab data analysis software (Actimetrics). A regression line was fit to the activity

onsets at least 3 days following the manipulation to account for possible transients in the wheel running rhythms. The difference between the regression line prior to manipulation and following the stimulation was calculated as the phase shift in hours.

3.3 Experiment 5- Electrical stimulation of the SI with or without atropine

Syrian hamsters (n=23) were implanted cannula in the SCN as described above using a stereotaxic instrument equipped with two arms. They also received electrode implantations into the cholinergic cells of the forebrain at the following coordinates from bregma: AP+ 1.4, ML+ 2.4, DV-7.6 mm from the skull, or AP+ 0.6, ML+ 2.4, DV- 7.2. The implants were cemented in place using dental acrylic (Dentsply).

Following recovery from surgery, the animals were placed in constant darkness in running wheels similar to the other experiments already described. 10 minutes prior to CT5, the experimenters entered the room and injected either 1µl of physiological saline or atropine (10mM; Sigma Aldrich, St Louis, MO, USA) into the SCN of the hamsters slowly over the course of 2 minutes. The hamsters were then attached to the isolated pulse stimulator (Model 2100, A-M Systems Inc., USA) the lights were turned out and stimulation took place as described above for two hours. The animals were sacrificed and perfused as above and the cannula tips were localized using a cresyl violet stain and electrode tips were localized using ChAT immunohistochemistry. Only animals with cannula tips in the SCN and electrode tips adjacent to ChAT cell groups were used for analysis. Phase shifts were calculated as described above.

3.4 Results

3.4.1 Experiment 4 - Electrical stimulation of the basal forebrain

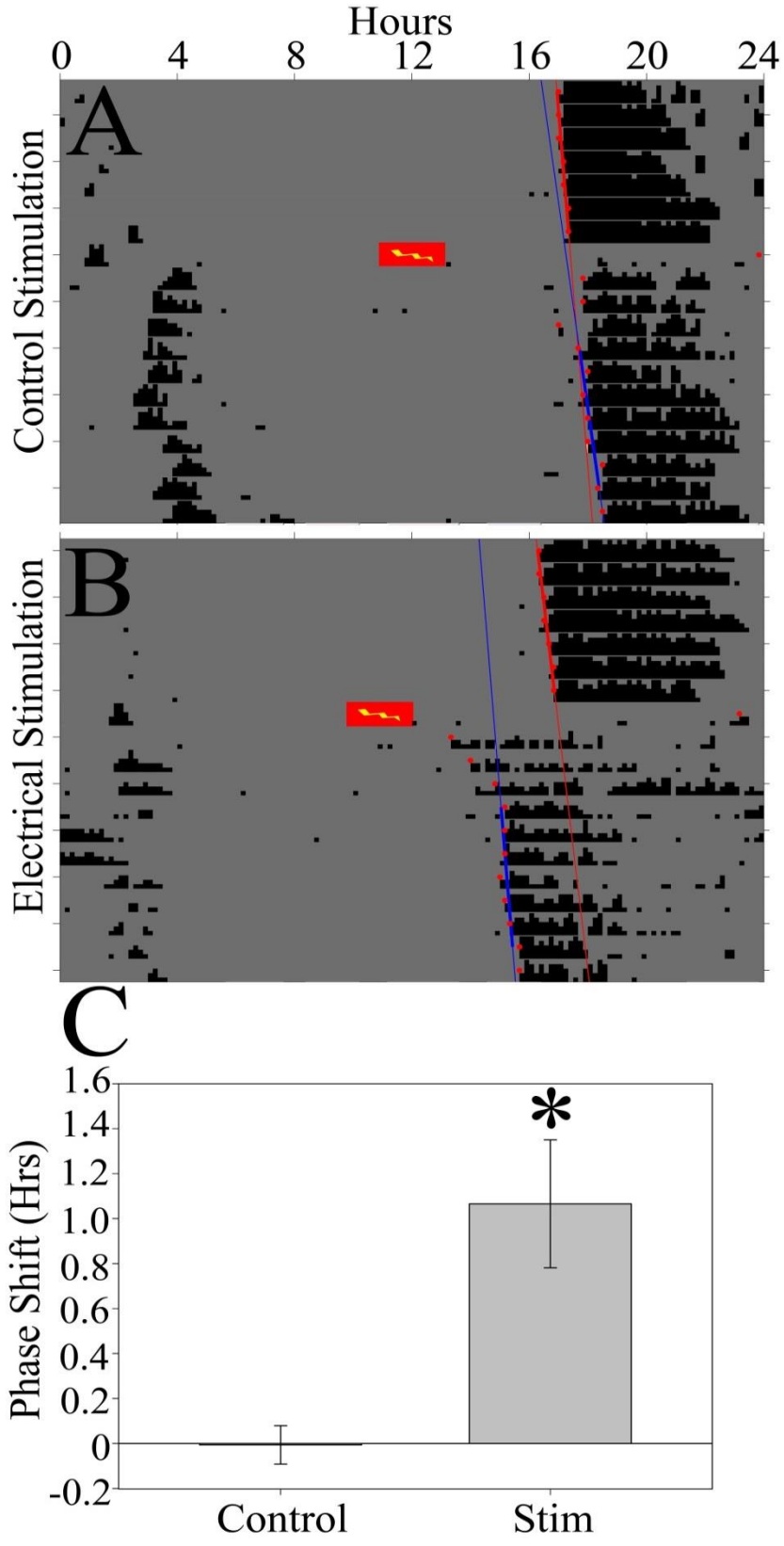
Before being connected to the stimulator, all of the animals were in sleeping postures. Among those hamsters that received the sham stimulation procedure (n=5), the animals became initially aroused upon being handled to connect the implanted electrodes to the stimulator. They would often engage in exploratory behaviors. All of the hamsters that received the sham stimulation procedure adopted stereotypical sleep postures, or became quiescent usually within about 15 minutes of being handled and remained that way until they were disconnected at the end of the procedure. The mean phase shift for these hamsters was -0.006 ± 0.08 hrs. Behaviors in those animals that were determined to have electrodes not located in the basal forebrain (n=8) showed very little behavioral response throughout the entire length of the stimulation or engaged in naturalistic exploratory behaviors such as digging, and sniffing or grooming throughout the stimulation. Some animals showed stereotyped repetitive behaviors such as circling, jaw movements, or forelimb clasping. These animals were more likely to have electrodes located too far dorsal of the basal forebrain. The mean phase shift for these hamsters was -0.07 ± 0.17 hrs.

Only those animals (n=5) with electrode tips localized within the basal forebrain were used for analysis. In these animals, there was only one animal that did not have a major behavioral response to the stimulation that appeared to be awake, but inactive throughout the stimulation. There was also one animal that appeared to engage in repetitive jaw movements. The remaining animals became active and engaged in normal exploratory behaviors and remained active throughout most of the stimulations usually adopting a sternal quiescent position near the end. A representative actogram of an animal that received the control stimulation is displayed in Figure 10A. A representative actogram displaying an animal that received

stimulation of the SI is displayed in Figure 10B. The control condition resulted in an average phase shift of -0.01 ± 0.20 hrs. Those animals that received electrical stimulation of the SI showed an average phase advance of 1.07 ± 0.28 hrs. This difference was significant ($t_{(8)}$=3.61 ; p=0.007). The mean phase shifts ± SEM for each group are displayed in Figure 10C.

Figure 10: Electrical stimulations of the basal forebrain and mean phase shifts

A: displays an actogram from a control stimulated animal. The hamster was implanted with an electrode into the cholinergic region of the forebrain and attached to the stimulator from CT5-7 however no current was passed. Days are displayed vertical whereas hours are displayed horizontal. Vertical deflections on the lines of the actogram are wheel running counts that are summed into ten minute bins. The red regression line shows activity onsets prior to the electrical stimulation and the blue regression line shows the activity onsets after the stimulation. The red box with the yellow lightning bolt shows the time in the activity rhythm that the animal was attached to the stimulator. B: displays the actogram for an animal that underwent electrical stimulation of the cholinergic region of the forebrain. C: displays the average phase shifts ± SEM for the animals that underwent the control, or electrical stimulation procedures. The asterisk indicates that the difference between these two groups was significant ($p<0.05$).


Hours
0
4
8
12
16
20
24
A
Control Stimulation
B
Electrical Stimulation
C
Phase Shift (Hrs)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
-0.2
*
Control
Stim

3.4.2 Experiment 5 - Electrical stimulation of the SI with atropine

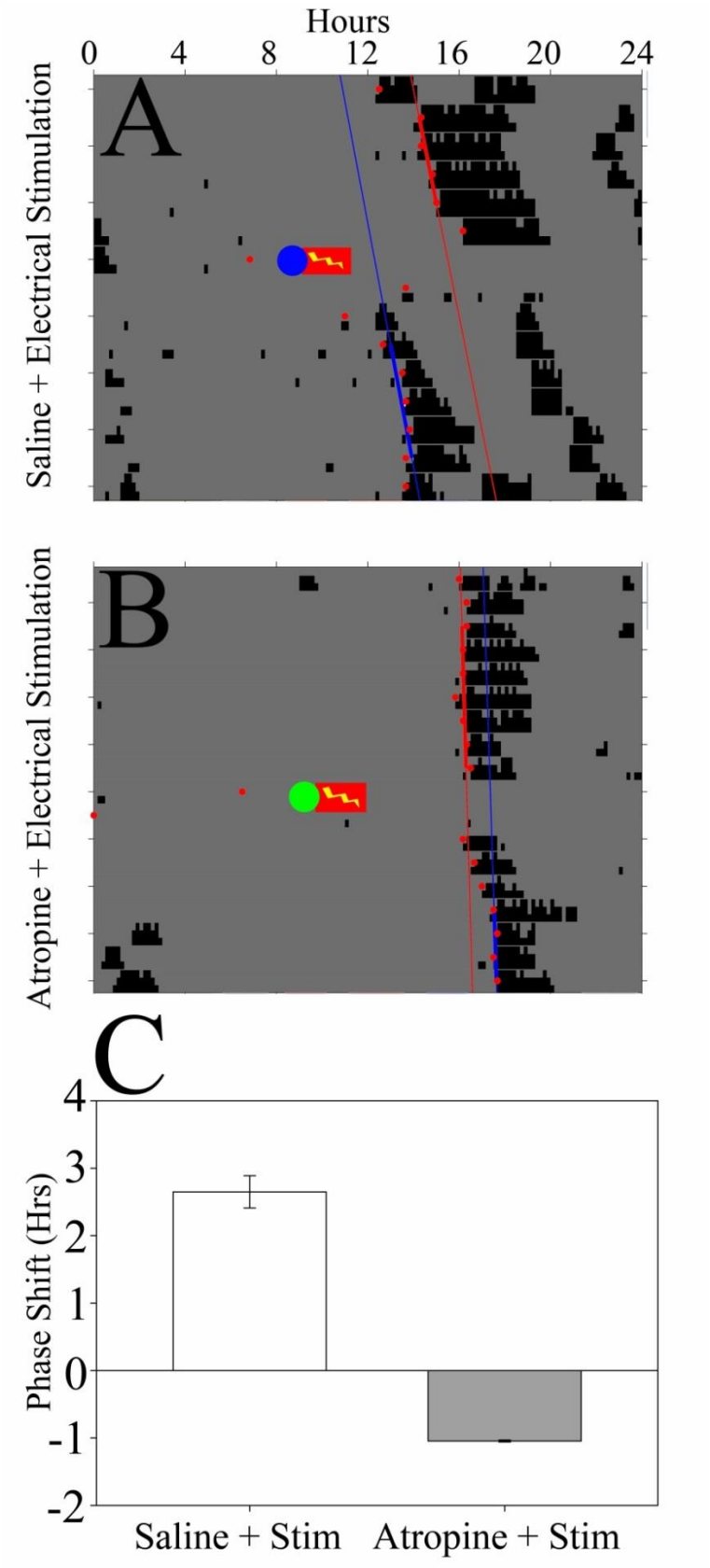
These animals received a cannula implant directly aimed at the SCN, as well as an electrode implanted in the cholinergic region of the forebrain. These surgeries had a higher degree of mortality due to the dual implants. These implants caused a higher degree of damage due to the fact that they were both in the ventral areas of the brain and in close relative proximity to each other. Of the total number of animals that received double implants of cannulas into the SCN (n=23), (n=2) lost their headcaps, (n=8) died, or the experiments could not be completed, (n=8) had incorrect placements of either the cannula, or electrode not being in proximity to the cholinergic cells. Only animals with electrode tips located in the area containing ChAT cells in the SI and peri-SI area showed phase advances in response to electrical stimulation (n=5). This effect did not appear to be mediated by a large increase in motor activity (behavioral observations). Only those animals with electrode tips located in direct proximity to ChAT containing neurons of the forebrain as assessed by immunohistochemical staining for ChAT that also had cannula tips located near the SCN as assessed by cresyl violet were used for analysis. There was one animal that had a correct placement of the electrode in the cholinergic region of the forebrain that phase shifted 2.41 hours that received atropine. The cannula, in this case, was determined to be in the third ventricle, well dorsal of the SCN.

Hamsters (n=2) that received saline prior to stimulation showed an average phase advance of 2.65 ± 0.24 hrs. These hamsters showed behaviors similar to described above. Those animals that received atropine prior to stimulation (n=2) showed an average phase delay of -1.05 ± 0.02 hrs. These hamsters showed little response to stimulation, or rhythmic motor movements as described above. Representative actograms of animals receiving saline and atropine, respectively into the SCN with electrical activation of cells containing ACh are displayed in

Figure 11, A and B. The average phase shifts for the saline group and the atropine group are displayed in Figure 11C.

Figure 11: Electrical stimulations of the basal forebrain with saline or atropine and mean phase shifts

A: Displays an actogram of an animal that was implanted with a cannula into the SCN and an electrode into the cholinergic region of the forebrain. This animal received a control injection of physiological saline (blue circle) into the SCN 10 minutes prior to receiving electrical stimulation (red bar with yellow lightning bolt). B: displays the actogram of an animal that was also implanted with a cannula in the SCN and an electrode in the cholinergic region of the forebrain. This animal received an injection of atropine (green circle) into the SCN prior to electrical stimulation (red bar with yellow lightning bolt). C: displays the average phase shift in hours for the saline stimulation animals, and the atropine stimulation animals.


Hours
0
4
8
12
16
20
24
A
Saline + Electrical Stimulation
B
Atropine + Electrical Stimulation
C
Phase Shift (Hrs)
4
3
2
1
0
-1
-2
Saline + Stim
Atropine + Stim

3.5 Discussion

In experiment 4, electrical stimulation of the cholinergic cells in the forebrain was found to phase advance the circadian rhythms of wheel running activity in Syrian hamsters during the mid-subjective day. The coordinates for these electrode implants were chosen to be in close proximity of the largest group of ChAT containing cells in sections anterior to the SCN. These results could be interpreted as an increase in ACh release acting on some other structure, or pathway in order to contribute to the phase advance, or a direct release of ACh in the SCN bringing about these shifts.

In order to determine if the direct release of ACh into the SCN was responsible for bringing about these phase shifts, the electrical stimulations were conducted again in experiment 5. The coordinates used for the electrodes in this case were more posterior than those used in experiment 4. The reason for this was to target cholinergic cell groups that were found to directly project to the SCN in the rat (Bina et al., 1993). This may also explain the differences in phase shifts between experiment 4 (1.07 hours) and experiment 5 (2.23 hours). In experiment 4, although more cholinergic cells may have been activated due to the location of the electrode in a denser region, fewer cells that projected to the SCN could also have been activated. In experiment 5, more cells that project directly the SCN may have been activated leading to the larger phase shifts as they released more ACh into the SCN. Evidence for this interpretation comes from the fact that non-photic stimuli have a suppressive effect in the SCN, and ACh also suppresses activity in the SCN (Maywood et al., 1999; Yang et al., 2010). Therefore, the more ACh that is released onto the SCN, the more gene expression would be suppressed, likely through muscarinic receptors, and the larger the resulting phase shift (Yang et al., 2010).

Another issue that may confuse interpretation of these results is that there may be non-specific activation of fibers of passage, or non-cholinergic cells near the electrode tip. There is also a lack of means to determine the relative size of the area activated. This was controlled for by making the electrode tips as similar as possible. While it may be the case that the stimulation of the cholinergic cells of the basal forebrain activate another pathway to bring about the phase shifts, the final output pathway appears to be cholinergic based on the results of Experiment 5 even though the ACh released may not be from the SI. Other possibilities include the septum of tegmentum as the source of acetylcholine in the SCN (Bina et al., 1993). Interestingly, the tegmental cholinergic neurons also input into the IGL, so it may be the case that NPY from the IGL and ACh are co-released from multiple pathways onto the SCN during highly arousing non-photic stimulation (Moore and Card, 1994; Cain et al., 2007). A recent experiment examined the brainstem cholinergic projections to the SCN. Electrical stimulation of either the laterodorsal, or pedunculopontine tegmental areas increased ACh release in the SCN, however only phase delays were found during the early subjective night in these mice with very small phase delays, or advances occurring at midday that were non-significant (Abbott et al., 2013). The reasons for these differences may represent a species difference, or the differences in stimulation parameters used in their study and the current one. The primary difference was that stimulations in their experiment only occurred for 20 minutes. Another alternative is that the brainstem cholinergic projection to the SCN differentially regulates circadian timing in a manner that is different than that of the forebrain. It could be the case that the brainstem projection acts on different cells in the SCN, or different receptors than the forebrain cholinergic neurons.

A final issue is that ACh cells in the forebrain are maximally active during wake, and paradoxical sleep (Jones, 2005). At the time of the stimulations, the hamsters would most likely

have highly active ACh neurons already as they would be engaging in paradoxical sleep throughout the rest period. Is this is true then it seems unlikely that these ACh cells could be acting directly on the SCN to produce these phase shifts as activity would be sporadically high throughout the rest period as the animal entered paradoxical sleep. If all cholinergic cells in the basal forebrain were activated equally, then every time the animal would enter paradoxical sleep ACh would be released onto the SCN and could contribute to a suppression of activity in the SCN through the M1 and M4 muscarinic receptors (Yang et al., 2010). As has been shown previously, suppression of activity in the SCN appears to be responsible for phase advances (Hamada et al., 2004). However, the presence of all 5 muscarinic as well as nicotinic receptors in the SCN has the possibility of ACh released at the time having an effect on any one though (van der Zee et al., 1991)

One explanation is that the neurons that project to the SCN are responsible for monitoring behavioral state and relaying that information to the SCN and the SCN in turn, relays that information to basal arousal networks. This would mean that the cholinergic neurons that project to the SCN do not become active during paradoxical sleep. Of the approximately 355000 neurons in the basal forebrain, only about 22000 are estimated to be cholinergic (Gritti et al., 2006). The results of the tracing studies from the SCN show that there appears to only be sparse and a relatively small subset of these cholinergic neurons that actually do project to the SCN which fits with this hypothesis (Bina et al., 1993).

Another explanation could be that the release of ACh onto SCN neurons during paradoxical sleep is too brief in order to bring about any kind of phase shift, or suppression of activity. Typical non-photic stimuli take place over several hours. This suggests that non-photic stimuli require sustained release of neurotransmitters at the SCN in order to bring about phase

shifts. This sustained activity of ACh on muscarinic receptors at the SCN would not occur during occasional bouts of paradoxical sleep throughout the rest period.

In sum, these experiments show that midday activation of forebrain regions containing cholinergic cells produce non-photic-like phase advances in the circadian rhythms of wheel running in Syrian hamsters. These phase shifts appeared to be larger when the substantia innominata was stimulated, likely because it is a region that contains the most cholinergic cells that project to the SCN. Phase shifts still occurred when the electrodes were placed in more anterior sections of the cholinergic regions of the forebrain but were smaller. This suggests that ACh cells that directly project to the SCN become active during exposure to a non-photic stimulation in order to relay information about behavioral state to the SCN. This hypothesis was further confirmed because blocking ACh binding in the SCN seemed to block phase shifts to electrical stimulation of the cholinergic neurons in the substantia innominata.

4. Novel wheel confinement in atropine treated animals

4.1 Introduction

Initial observations about phase shifting the circadian rhythms began with changes in the LD cycle (Summer et al., 1984). Soon, it was discovered that nocturnal animals kept in constant light and then exposed to several hours of darkness during the usual subjective rest period phase advanced the circadian rhythms of wheel running (Ellis et al., 1982). This was at a time when light normally did not have a phase shifting effect on the circadian rhythms of wheel running. Due to observations that minor cage disturbances during midday could produce phase shifts, novel wheel exposure was used in a re-entrainment paradigm. It was found that hamsters undergoing a shift in the light dark cycle could re-entrain faster if they were given the opportunity to become active during the midday (Mrosovsky and Salmon, 1987). It was found that novel wheel confinement during the midday could produce large phase advances if the animals ran more than a threshold level of revolutions (Bobrzynska and Mrosovsky, 1998).

Despite the evidence from the novel wheel confinement studies, motor activity was not necessary in order to bring about the midday phase advances. Sleep deprivation by gentle handling in the absence the threshold of motor activity resulted in phase advances very similar to those brought about novel wheel confinement (Antle and Mistlberger, 2000). This indicates that the necessary stimulus that brings about midday phase advances could be an increase in behavioral arousal. This hypothesis is supported by data that those animals that do not phase shift to the sleep deprivation procedure require more interventions and a lower latency to the first intervention in order to keep them awake. Similarly, those animals in the wheel confinement procedure that do not sustain enough activity to reach a threshold level of wheel revolutions also

do not phase shift. Behavioral arousal may be a necessary component of non-photic phase shifting.

Behavioral arousal is produced by ACh release (Jones, 2005). Interestingly enough, cholinergic neurons in the basal forebrain, and in particular, the SI appear to project to the SCN (Bina et al., 1993). ChAT fibres and vesicular acetylcholine transporter have also been reported in the SCN (Kiss and Halasz, 1996; Castillo-Ruiz and Nunez, 2007). Muscarinic and nicotinic receptors have also been found in the SCN (van der Zee et al., 1991). Previous reports have found mixed results regarding the role of ACh in the circadian system. Intraventricular injections of the cholinergic agonist carbachol resulted in phase shifts more resembling exposure to light (Zatz and Herkenham, 1981). Another study using ventricular carbachol showed non-photic like phase advances during the day, however (Meijer et al., 1988). Another study found large phase advances to intra-SCN carbachol injections at CT 6 and 22 but phase delays in the early night at CT14 (Bina and Rusak, 1996).

The purpose of this experiment was to determine if ACh from cells in the forebrain were involved in novel wheel confinement and non-photic phase shifting. In this experiment hamsters were implanted with cannulas into the SCN. The hamsters then received either an injection of saline or atropine prior to confinement during a novel running wheel in a counter-balanced fashion. The hypothesis was that since ACh is involved in arousal, and also appears to be involved in the circadian system, then blocking it during a non-photic stimulus would reduce phase shifts.

4.2 Experiment 6- Atropine injection to the SCN during novel wheel confinement

4.2.1 Cannula implantation

Prior to surgical procedures, the animals (n=18) were anesthetized with an intraperitoneal injection of sodium pentobarbital (~120 mg/kg; Ceva Sante Animale, France) and received Torbugesic subcutaneously as an analgesic (2 mg/kg; Butorphanol; Wyeth Canada, Que). The animals were placed in a stereotaxic apparatus, an incision was made in the skin and the skull leveled with respect to lambda and bregma. 9 mm long 22 gauge stainless steel cannulas (Plastics One, Roanoke VA) were implanted on a 10° angle at the following coordinates from bregma AP+ 0.4 ML+ 1.3 DV- 6.8 from the dura. The cannulae were anchored in place using screws drilled into the skull and a dental acrylic headcap (Dentsply,York, PA, USA). A dummy cannula that extended 1 mm beyond the implanted cannula was then put in place to maintain patency.

4.2.2 Novel wheel confinement

Following recovery from surgery, the animals were placed in a 14:10 LD cycle in clear polypropylene cages equipped with 14 cm diameter running wheels attached to the cage lid. Revolutions of the running wheels were continuously collected and summed into 10 minute bins by a PC desktop computer running ClockLab data collection software (Actimetrics, USA).

10 minutes prior to the wheel confinement, the experimenters entered the room in the light and unilaterally injected 1 µl of either physiological saline or atropine sulfate (10 mM, Sigma Aldrich, St Louis, MO, USA) into the SCN. The injections occurred over approximately 2 min and the injector tip was left in place for around 30 seconds afterwards to minimize flow-back. The hamsters were then confined to novel running wheels 24.2 cm in diameter. The wheel confinement took place in an Aschoff Type II design as above where the lights were turned off

and the animals were placed in constant darkness immediately prior to the manipulation. All wheel confinements took place from CT 6-9 in activity rhythms.

The hamsters were removed from their novel wheels and returned to the home cages at the conclusion of the experiment. The lights remained off for 4-10 days following the manipulation. The animals were then returned to the 14:10 LD cycle and re-entrained. Following re-entrainment, the conditions were counter balanced with half of the animals (n=6) that received saline and wheel confinement first then receiving atropine and wheel confinement while the other half of the animals (n=6) underwent the conditions in the reverse order. A control condition was also conducted where animals only received atropine injections into the SCN without a wheel confinement (n=7).

At the conclusion of the study, the animals were perfused and the brains sliced as above. Alternate sections of the SCN were mounted onto gelatin coated slides and stained for cresyl violet in order to confirm cannula placement in the SCN then cover-slipped using Permount (Fisher Scientific, Pittsburgh, PA, USA).

4.2.3 Phase shift analysis

Activity onsets for 5-7 days prior to the wheel confinement were calculated using ClockLab data analysis software (Actimetrics, USA). A regression line was fit to these onsets in order to calculate the baseline activity onset. After the wheel confinement, animals were kept in constant darkness for at least 3 days and the activity onsets were once again calculated. The average onset prior to the manipulation was compared to the activity onset on day 2 of constant darkness as the phase shift in hours (Mistlberger et al., 2003). The phase shifts in the saline condition were compared to the atropine condition using a paired group t-test. The wheel

revolutions during the novel wheel confinement group in the saline group condition were also compared to the atropine group using a paired group t-test in order to determine if atropine was affecting motor activity to eliminate phase shifts.

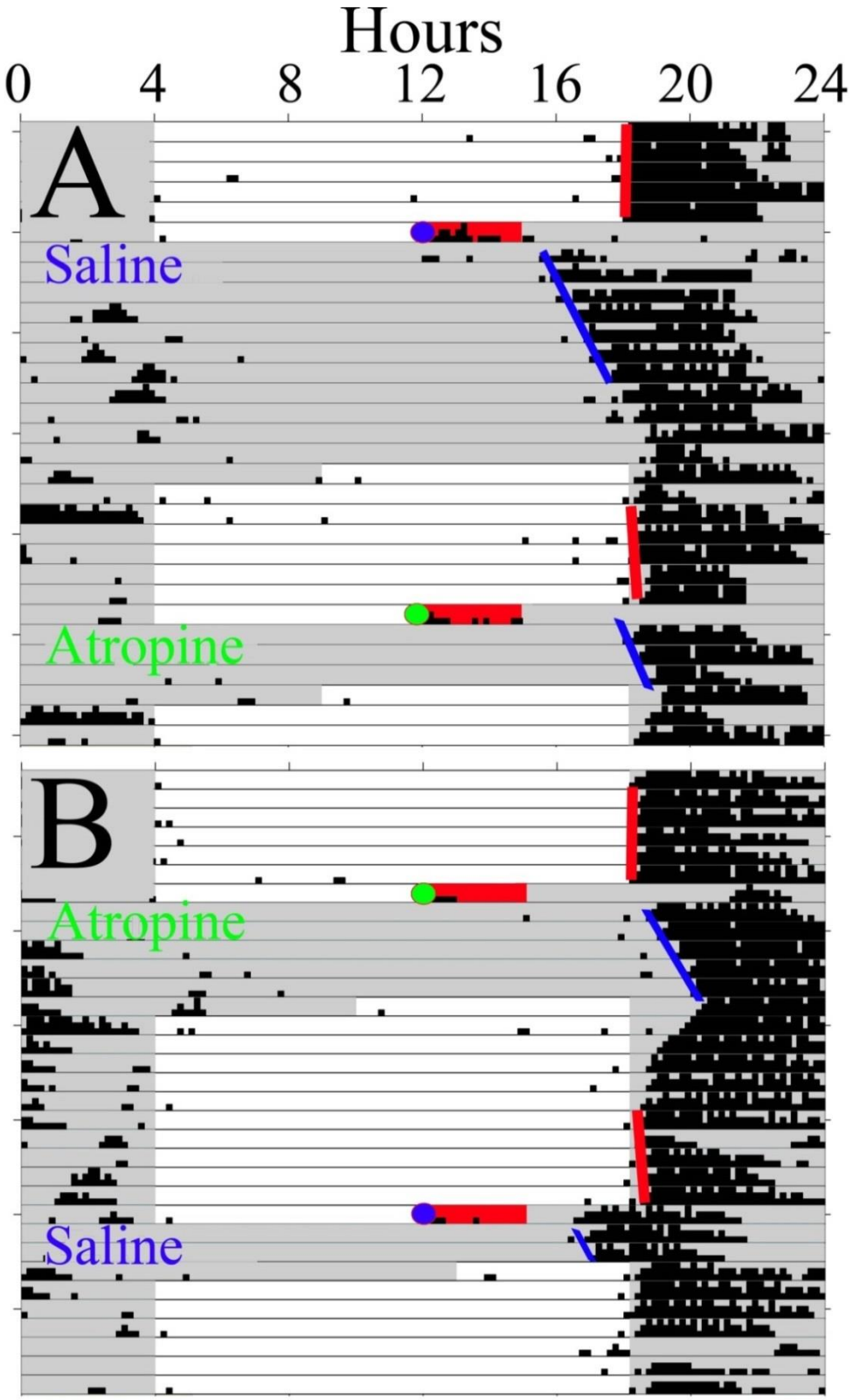
4.3 Results

4.3.1 Experiment 6 – Novel wheel confinement in atropine treated animals

There were (n=3) animals that were used in the analysis that had the saline treatment first, and (n=3) animals that had the atropine treatment first that then received the counterbalanced condition. A representative actogram of an animal that underwent saline injection followed by wheel confinement and then the atropine injection followed by wheel confinement is displayed in Figure 12A. A representative actogram of an animal that received the atropine manipulation first followed by the control saline injection is displayed in Figure 12B.

Figure 12: Actograms of animals administered saline or atropine into the SCN prior to novel wheel confinement

A: Displays the actogram of an animal that underwent the saline injection (blue circles) into the SCN followed by wheel confinement (red bar) first, followed by atropine injection (green circles) into the SCN followed by wheel confinement. The red regression lines display the baseline B: Displays the actogram of an animal that underwent atropine injection followed by wheel confinement, followed by saline injection and then wheel confinement.


Hours
0
4
8
12
16
20
24
A
Saline
Atropine
B
Atropine
Saline

Administration of atropine alone into the SCN resulted in an average phase delay of -0.09 ± 0.1 hrs. Only those animals in the saline condition that had cannula tips verified to be located within 500 µm of the SCN were used for analysis (n=6). When receiving saline before the wheel confinement, the hamsters showed an average phase advance of 1.64 ± 0.41 hrs. The mean number of wheel revolutions that were recorded during the wheel confinement for the saline group was 937 ± 197.31 revolutions. When atropine was administered prior to the wheel confinement, the average phase shift was 0.04 ± 0.17 hrs. The mean number of wheel revolutions that were recorded during the wheel confinement for the atropine condition was 752 ± 202.69 revolutions. This difference in phase shifts was found to be significant between the saline wheel confinement condition and the atropine wheel confinement condition ($t_{(5)}$= 3.06; p=0.03). There was no significant difference found between the wheel revolutions between the saline wheel confinement condition and the atropine wheel confinement condition ($t_{(5)}$= 0.65; p= 0.55). The mean number of wheel revolutions for the atropine alone condition, the saline and wheel confinement condition and the atropine and wheel confinement condition is displayed in Figure 13A. The wheel confinement revolutions for the saline and wheel confinement condition along with the atropine and wheel confinement condition area also displayed in Figure 13A. A graphical representation of the changes in wheel revolutions from the saline condition to the atropine condition is displayed in Figure 13B. There were three animals that decreased wheel revolutions from the saline condition to the atropine condition, there were two animals that showed increased numbers of wheel revolutions from the saline condition to the atropine condition and there was one animal that showed the same number of wheel revolutions from one condition to the other. Finally, a scatterplot of all of the recorded phase shifts along with the wheel confinement is displayed in Figure 14.

Figure 13: Mean phase shifts and wheel revolutions of animals confined to novel running wheels and graphical representation of changes in wheel revolutions

A: Displays the average phase shifts (blue bars) and wheel revolutions (red bars) ± SEM. The grey bar (AT) displays the phase shifts for the group (n=7) that received the atropine alone condition. The asterisks shows that the difference in phase shifts for the saline condition and atropine condition was significant ($p<0.05$). B: Displays the change in wheel revolutions from the saline condition to the atropine condition for each of the animals. Two animals increased in wheel running from the saline condition to the atropine condition, one stayed the same, while the other two animals decreased in revolutions from the saline condition to the atropine condition.

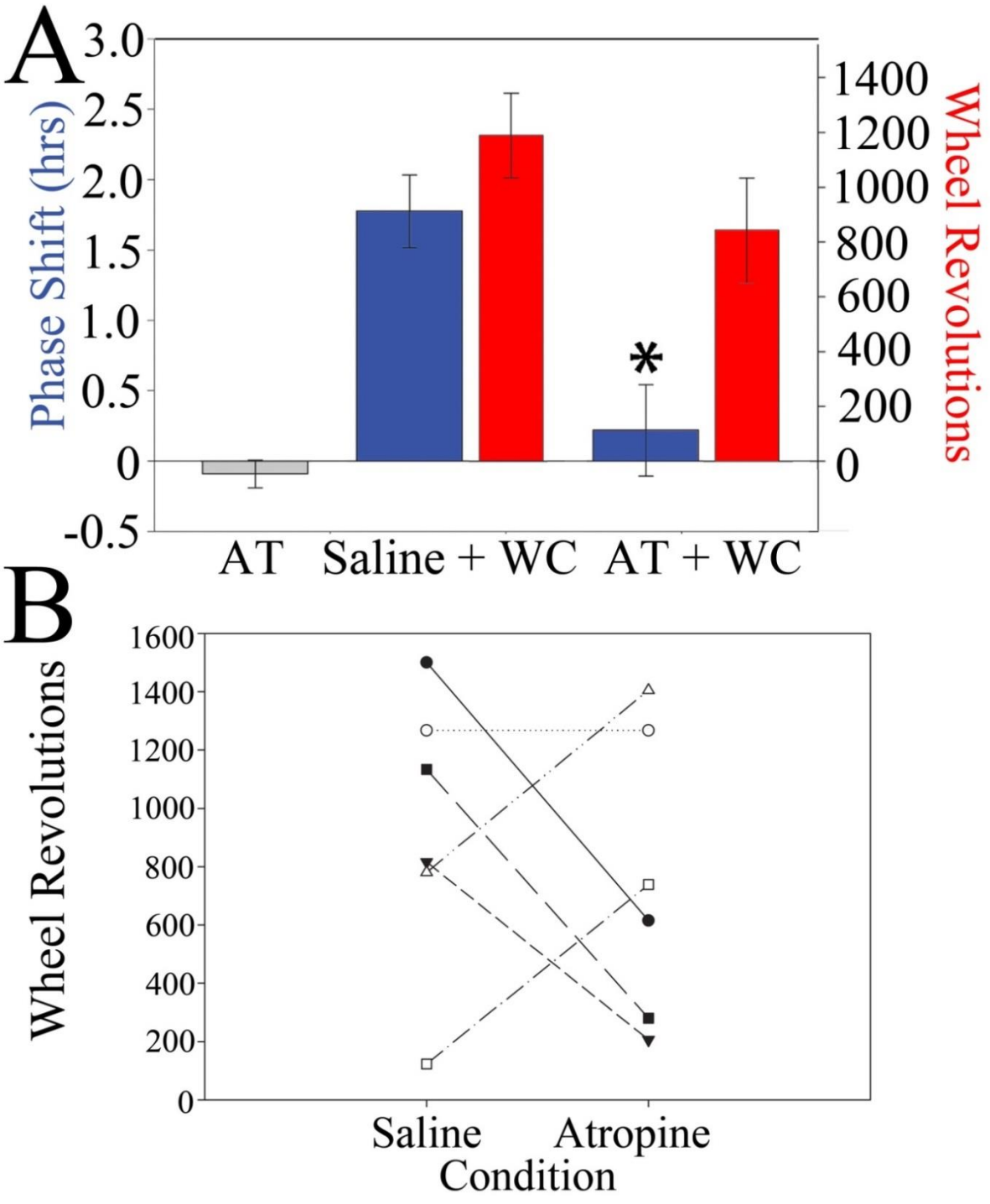
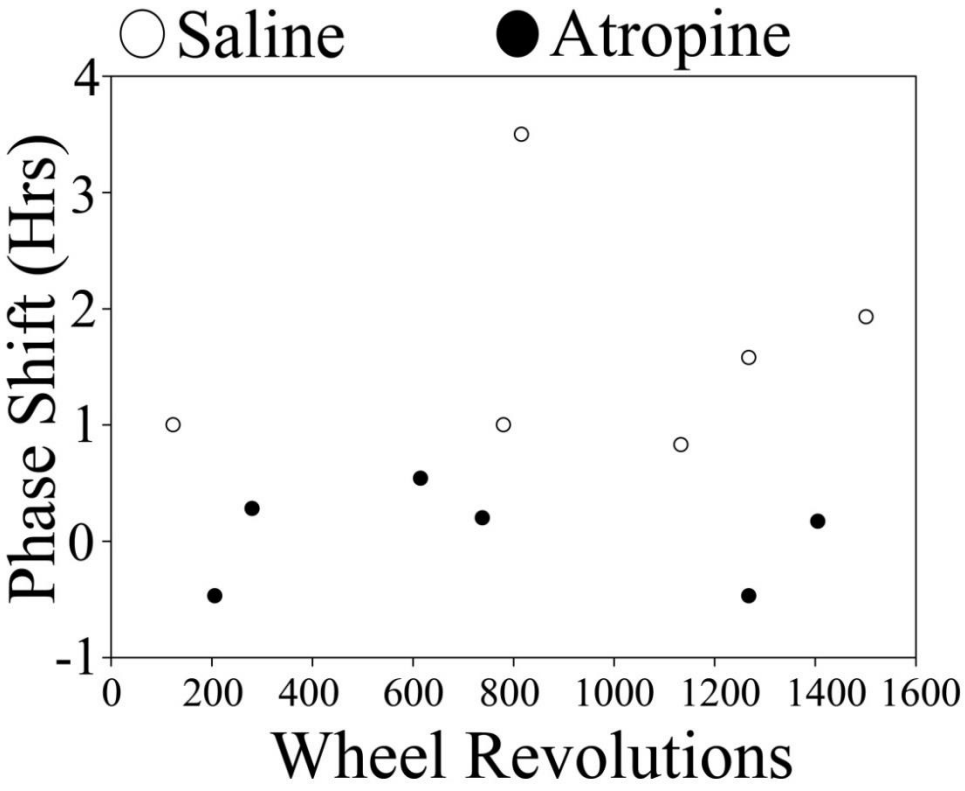

A
Phase Shift (hrs)
3.0
2.5
2.0
1.5
1.0
0.5
0
-0.5
1400
1200
1000
800
600
400
200
0
Wheel Revolutions
*
AT
Saline + WC
AT + WC
B
Wheel Revolutions
1600
1400
1200
1000
800
600
400
200
0
Saline
Atropine
Condition

Figure 14: Scatterplot showing the relationship between drug condition, wheel revolutions and phase shifts

Displayed in this figure is a scatterplot of all the phase shifts and wheel revolutions for the animals (n=6) that were recorded. The saline wheel confinement treatment is plotted with open circles and the atropine wheel confinement condition is plotted with the closed circles.


Saline
Atropine
Phase Shift (Hrs)
4
3
2
1
0
-1
0
200
400
600
800
1000
1200
1400
1600
Wheel Revolutions

4.4 Discussion

In Experiment 6, it was found that atropine injection into the SCN significantly attenuated phase shifts to novel wheel confinement. This effect did not appear to be due to atropine injection simply lowering the motor activity of the hamsters enough so that they did not run enough during the novel wheel confinement. This effect did not appear to be due to the actions of atropine alone in the SCN either. Unexpectedly, there did not appear to be a relationship between the number of wheel revolutions run and the phase shift as seen in Figure 14. This data confirmed the hypothesis that ACh participates in non-photic phase shifting during a novel wheel confinement.

One issue that arose during this study was the relative lack of running and the phase shift sizes when compared to previous literature. In other studies, maximal phase shifts occurred between 5000-9000 revolutions, and were generally around 3 hours (Bobrzynska and Mrosovsky, 1998). In this study, the largest observed phase shift was 3.5 hours and the maximal number of wheel revolutions was 1501. In general, phase shifts were less than 2 hours though. In the previous reported studies, the wheel diameters used were 17.5 cm. The novel wheels used in this study were 24.2 cm in diameter, which may partially explain the lower number of revolutions. It has also been found that older hamsters (21-23 months) often show reduced phase shifting to non-photic stimuli (Mrosovsky and Biello, 1994). It may have been the case that some of the animals used in this study were too old to phase shift, however a range of different aged hamsters were used, and little difference was noted between young hamsters (6 months) and older hamsters (12-14 months).

Finally, another issue with the experimental design that may have caused a lack of sustained running was that the novel wheels were covered with a soft plastic mesh. The purpose

of this mesh was to be softer on the hamsters' feet as assessed by pilot work and other experiments that have shown hamsters prefer to run in plastic mesh to metal rods (Mrosovsky et al., 1998). One of the drawbacks to this mesh is that it may have limited airflow while the animals were running. This may have caused the animals to get too warm thus limiting running wheel activity.

Previous work has shown that IGL lesions also eliminate phase shifts to novel wheel running however also reduced both the running activity during the wheel confinement and overall locomotor activity (Janik and Mrosovsky, 1994). Interestingly, when these animals were motivated to run by lowering the temperature in the room, they still did not phase shift to the wheel confinements. This finding received support from another study that applied NPY-antiserum to the SCN and found that it attenuated phase shifts to novel wheel confinement (Biello et al., 1994).

Other work was done with an examination of serotonergic agonists in the mice. A study using agonists for the serotonin receptors 1A, 2 and7 into the in SCN of mice found there was no phase shifts during midday (Antle et al., 2003). These results were in contrast to findings with hamsters (Antle et al., 2003). Another study that conducted lesions of the SCN, IGL, or serotonergic projection to the SCN found that serotonin and NPY are necessary for mice to show entrainment to scheduled exercise (Marchant et al., 1997). This indicates that an interaction of NPY and serotonin in the SCN may be necessary for non-photic phase shifts in mice. The evidence also points to important species differences.

This experiment showed a cholinergic input directly into the SCN participates in phase advances brought about by novel wheel exposure. This experiment provided evidence of another important input into the SCN and suggests some functional redundancy with regards to phase

shifting in the SCN. The NPY input into the SCN provides an important input into the SCN allowing for the integration of behavioral state with light information, the serotonergic input may interact with the NPY input and now the cholinergic input into the SCN has been shown to play a role in producing phase advances to the highly arousing stimulus of novel wheel confinement. This provides further evidence for the hypothesis that behavioral arousal is the common factor involved in non-photic phase shifting but important species differences cannot be overlooked. Future work on NPY, serotonin, and cholinergic co-localization of receptors and ultrastructural studies of the inputs into the SCN will be important to understand species differences and the interaction of various neurotransmitters in the SCN.

5. General discussion

5.1 Summary and conclusions

These studies show that ACh cells in the forebrain of the hamster that project directly to the SCN contribute to phase advances brought about by non-photic stimuli. These cells in the forebrain become active in response to highly arousing stimuli such as novel wheel confinement and release ACh onto the SCN to produce midday shifts. This suggests that there are multiple redundant pathways that project to the SCN in order to communicate information about behavioral state. These pathways may be redundant as serotonin inputs into the SCN produce similar phase advances to stimulations of the cholinergic basal forebrain and NPYergic IGL although the ability of one neurotransmitter to take over for another has not yet been shown (Rusak et al., 1989; Yamakawa and Antle, 2010). These pathways may be work in an additive fashion at the level of the SCN to bring about large (3 hour) phase advances during the midday.

Non-photic phase shifting of the SCN typically involves behavioral arousal of the animal during the middle of the subjective day (Mistlberger et al., 2003) . For nocturnal rodents, this time is the middle of the usual rest period. The most common means of arousing an animal and bringing about large phase advances is confinement to a novel running wheel and sleep deprivation by gentle handling (Bobrzynska and Mrosovsky, 1998; Antle and Mistlberger, 2000). During both wheel confinement experiments, and sleep deprivation experiments, there is usually a set of animals that do not phase shift in response to non-photic stimuli. During the novel wheel confinements, the animals that do not shift usually sustain a threshold level of activity in order to run the amount required for phase shifting (Bobrzynska and Mrosovsky, 1998). During the sleep deprivation procedures, those animals that did not phase shift required more interventions in order to keep awake, and a lower latency to the first intervention in order to keep them awake

(Antle and Mistlberger, 2000). This lead to the hypothesis that behavioral arousal was involved in non-photic phase shifting.

Specifically, it was hypothesized for experiment 1 that hamsters undergoing sleep deprivation during the midday would show greater Fos expression in brain areas that contained neurotransmitters that were associated with bringing about behavioral arousal during wake. In particular, it was expected that sleep deprivation would produce greater Fos expression in the brain regions that contained ACh, OX and HA when compared to non-manipulated controls. It was also hypothesized that those animals that were pre-screened to be responders to the sleep deprivation procedure would show higher expression of Fos in these regions as they would also show a higher degree of arousal in order to produce these non-photic phase shifts. It was found that animals undergoing sleep deprivation showed a higher degree of Fos expression in most of the areas examined, however there was no difference in Fos expression between non-responder and responders. This experiment showed that exposure to the non-photic stimulus increases Fos expression in ACh, OX and HA containing areas. This provided some evidence that arousal was involved in exposure to non-photic stimuli to match behavioral observations, but did not show that there was greater activation of these cell groups in responder versus non-responder groups.

Experiments 2 and 3 were conducted in order to determine if cells containing ChAT in the forebrain would show greater Fos expression in response to non-photic stimuli. These experiments would provide evidence on whether ChAT cells are specifically activated in the forebrain to add to evidence collected in experiment 1. In order to determine if there was specific activation of ChAT containing cells in the forebrain during sleep deprivation, double labelling for Fos and ChAT was performed on sleep deprived animals in experiment 2. Unexpectedly, there was very little Fos expression found in cells containing ChAT in any of the regions

examined and there was again, no difference between non-responders and responders. Although the number of non-responders used in this experiment was low, the general lack of double label cells in all sections examined seemed to indicate this was not an issue. It could be the case that the immunohistochemical techniques used to measure Fos expression were not sensitive enough to detect a difference.

In experiment 3, ChAT cells in the forebrain were examined for Fos expression following wheel confinement in home cage control DD animals, and novel wheel confined animals. Unlike the sleep deprived animals, these animals were not first pre-screened to be responders or non-responders as it was assumed that the number of wheel revolutions they engaged in the novel wheel confinement would be sufficient to determine if they would be phase shifters. It was found that there was significantly more Fos expression in animals confined to novel running wheels than the home cage controls. This provided evidence for the hypothesis that the non-photic stimulus of novel wheel confinement was highly arousing, and recruited a population of cholinergic cells in the forebrain in order to bring about a phase shift. An alternative explanation is that the activation of the cholinergic cells in this case was simply because the wheel confinement was arousing and was not related to bringing about a non-photic phase shift.

In experiment 4, direct electrical activation of the cholinergic cells in the forebrain was conducted in order to determine if this could bring about a non-photic phase shift. 2 hour electrical stimulations of the cholinergic cells in the basal forebrain were conducted. It was found that electrical stimulation produced phase shifts of around 1 hour. This effect did not appear to be dependent on an increase in motor activity (behavioral observations). This effect may have been due to activation of fibres projecting directly to the SCN, or elsewhere throughout the many projections acting in a secondary manner.

In experiment 5, direct electrical stimulation of cholinergic cells in the forebrain was conducted along with concurrent antagonism of muscarinic receptors in the SCN. This experiment was conducted in order to determine if the activation of cholinergic cells in the forebrain produced a general arousal effect that fed-back onto SCN neurons through some other pathway to produce the phase shift, or there was a direct action of ACh on the SCN. It was found that blocking ACh binding in the SCN during these electrical stimulations also blocked the phase shifts. This indicated that ACh release in the SCN is directly responsible for bringing about phase advances during the midday. This data fits with the results from experiment 3 where wheel confined animals showed increased Fos expression in the cholinergic neurons of the forebrain.

Finally, experiment 6 brought the findings of experiments 1-5 into a behavioral context. Experiment 3 showed that there was increased Fos expression in ChAT cells of the forebrain during midday exposure to a novel running wheel. Experiment 4 and 5 showed that electrical activation of these cholinergic regions produced phase advances that were dependent on cholinergic neurotransmission in the SCN. Experiment 6 showed that atropine injection into the SCN attenuated the phase shifts to novel wheel confinement.

The converging evidence from these experiments suggests that there is direct cholinergic neurotransmission from the basal forebrain to the SCN during novel wheel confinement to participate in producing phase advances. It is unlikely that these observed effects are due to ACh neurotransmission from another region such as the tegmentum. Unexpectedly, ACh did not appear to be involved in sleep deprivation by gentle handling. This may have been the case due to the fact that sleep deprivations are not as arousing as wheel confinements. These data along with the established roles for NPY and serotonergic inputs into the SCN show that there is complex regulation of behaviorally induced phase shifts (Janik et al., 1995; Yamakawa and

Antle, 2010). It also points to the intriguing possibility that different pathways are recruited depending on the type of non-photic stimulus.

There is an intricate network of brain systems involved in bringing about behavioral arousal during wake involving cholinergic projections from the tegmentum that interact with forebrain targets, histamine, noradrenaline from the locus coeruleus, and serotonin from the raphe (Saper et al., 2001). There are important links to the circadian system from the brain systems related to bringing about arousal. There is a cholinergic projection from the tegmentum to the IGL (Moore and Card, 1994). There are also cholinergic projections from the tegmentum to the SCN and basal forebrain (Hallanger et al., 1987; Bina et al., 1993). Within the basal forebrain specifically, there are cholinergic neurons mainly in the SI of the hamster that also project to the SCN. The neuropeptide OX_A also was found to have fibers near the periphery of the SCN, the IGL the dorsal and median raphe and the noradrenergic locus coeruleus (McGranaghan and Piggins, 2001). There also appears to be some noradrenergic fibers present in the IGL (Papadopoulos and Parnavelas, 1990). Therefore the systems that influence arousal are intricately tied back to the circadian system. The IGL is known to be an important relay back to the SCN in order to influence circadian phase through NPY release (Biello et al., 1994). The IGL monitors behavioral state through the serotonergic, noradrenergic, orexinergic, and cholinergic inputs and relays that information back to the SCN in order to keep time.

The evidence from these experiments points to cholinergic neurons in the basal forebrain accomplishing a similar task as the IGL in that they monitor behavioral state and feedback onto the SCN. Recent work involving sustained attention tasks in rats has provided consistent results with this hypothesis. Rats kept under LD that had undergone operant conditioning to receive a water reward would show entrainment and anticipation of this task that was dependent on

cholinergic input into the SCN from basal forebrain neurons (Gritton et al., 2013). The experimenters in this case attributed the increased cognitive demands on the rats as the zeitgeber that was affecting photic entrainment to allow for diurnal functioning. They concluded that this cognitive performance was being transmitted to the SCN via cholinergic signalling. An alternative explanation for this result could be that the rats were sufficiently aroused during the normal rest period and cholinergic signalling to the SCN phase advanced activity rhythms to phase advance to this diurnal stimulus, a finding that is consistent with the results from these experiments.

When an animal becomes awake, the brainstem cholinergic system becomes active (Jones, 2005). This brainstem cholinergic activity activates a basal forebrain cholinergic system that projects diffusely and is involved with sustaining behavioral arousal in the cortex, olfactory bulb and other major structures (Jones, 2008). Since the basal forebrain systems are also maximally active during paradoxical sleep, this would pose a problem for the interpretation of these results (Lee et al., 2005). The time these manipulations took place was during the middle of the rest period, a time when the animals would be engaging in bouts of paradoxical sleep. This would suggest that ACh is being released onto the SCN throughout the rest period anyways and could not be causing the shifts that we observed. One possibility is that there are particular subsets of cholinergic neurons that project to the SCN that do not become active during paradoxical sleep. These neurons would become active during highly arousing events in order to feedback onto the SCN about behavioral state to influence circadian timing of wake. Another possibility is that paradoxical sleep does not release ACh onto the SCN for a sustained enough time to bring about a phase shift.

The results of these experiments can also be interpreted along with the results of the other pharmacological findings. Early work showed that intraventricular carbachol caused phase dependent shifts in wheel running activity and pineal gland activity in a manner that is consistent with the photic PRC (Zatz and Brownstein, 1979; Zatz and Herkenham, 1981). One study found ventricular administration of carbachol during the day resulted in non-photic shifts during the day with photic-like phase shifts during the night (Earnest and Turek, 1985). It is possible that the phase shifts found during the day in this case were mediated by the SCN and at night the photic-like phase shifts were mediated outside of the SCN as ACh activity would already be high during wake in the SCN. Another study also found no relationship between photic-like phase shifts induced by carbachol injection into the SCN and the distance of the cannula tip from the SCN boundary (Wee et al., 1992). This could indicate that studies showing photic-like phase shifts using injections into the SCN actually are acting on an input pathway into the SCN, or some peri-SCN region as even a cannula tip measured to 600 µm from the SCN boundary still produced similar phase shifts to those cannulas that were closer to the SCN. The results of another study using ventricular carbachol found large phase shifts during the day, but relatively little phase advances during the night and attributed these to a strain difference (Meijer et al., 1988).

Additional evidence for carbachol acting in a manner different than light came from a study showing it does not induce Fos expression in the SCN like light pulses do (Colwell et al., 1993). A more recent study showed that whereas *in vivo* ventricular injection of carbachol was consistent with the photic PRC, *in vitro* direct application to the SCN of mice produced phase advances throughout the subjective night (Buchanan and Gillette, 2005). This means that ACh acts directly on the SCN during the day to participate in non-photic phase shifting in hamsters

but can also act outside of the SCN at night to influence photic phase shifting. There also may be important strain and species differences that remain to be elucidated and this may relate to the ability of that species to respond to non-photic stimuli.

Finally, although these experiments did not address the issue, there also must be some mechanism by which ACh can bring about these phase advances in the SCN during the day. *In vitro* rat SCN slice preparations that were saturated in carbachol showed mainly inhibitory effects on SCN neurons that appeared to rely in the muscarinic 1 and 4 receptors (Yang et al., 2010). Work has also shown that nicotinic receptor stimulation in the SCN does not alter Fos expression (O'Hara et al., 1999). This seems to indicate that the muscarinic receptors are playing a role in the phase shifting effects of ACh. All 5 of the muscarinic receptors (M1-M5) have been localized in the SCN (Kobayashi et al., 1978; Carsi-Gabrenas et al., 1997). At night, *in vitro* studies have shown that muscarinic receptor activity is linked to guanylyl cyclase, cyclic GMP and protein kinase G (Liu et al., 1997). Specifically, It appears that nearly all SCN neurons express the M1 receptor and that the M2 receptors are present on cholinergic axons innervating the SCN (Hut and Van der Zee, 2011). Since the M1 receptors activate G_q type g-proteins they would also work to activate phospholipase C and inositol triphosphate increasing calcium signalling. The M3 and M5 receptors work in a similar fashion. The M2 and M4 receptors work to decrease cyclic AMP. A potential way that ACh can act on SCN neurons is through the M2 and M4 receptors to inhibit activity in the SCN through a reduction in cyclic AMP.

Previous data suggests that the IGL plays an important role in non-photic phase shifting. The IGL receives input from OX, serotonin, and brainstem cholinergic nuclei (Hallanger et al., 1987; McGranaghan and Piggins, 2001; Yamakawa and Antle, 2010). The actions of NPY from the IGL in particular have been found to be critical for non-photic shifting (Albers and Ferris,

1984; Besing et al., 2012). The serotonergic projections to the SCN from the raphe nuclei contribute to non-photic phase shifting (Yamakawa and Antle, 2010). The cholinergic neurons of the basal forebrain also receive a projection from the cholinergic nuclei in the tegmentum (Hallanger et al., 1987). A specific subgroup of these neurons in the basal forebrain, particularly in the substantia innominata have been shown to project directly to the SCN (Bina et al., 1993). The results of these experiments have shown evidence that the direct projection from the basal forebrain cholinergic neurons to the SCN can also contribute to non-photic phase shifting of the SCN. ACh has a suppressive effect on electrical firing in the SCN through actions on the muscarinic receptors (Yang et al., 2010). Suppression of the normal high firing rate in the SCN is necessary to produce midday non-photic phase advances (Hamada et al., 2004). Therefore, it appears that non-photic phase shifting involves actions of serotonin and ACh on the IGL to release NPY on the SCN (Cain et al., 2007; Glass et al., 2010). It also appears that serotonin can act directly on the SCN to contribute to non-photic phase shifts (Yamakawa and Antle, 2010). Finally, the results of these studies show that basal forebrain cholinergic neurons that project directly to the SCN can be engaged during periods of high arousal during the normal rest period of hamsters to release ACh onto the SCN and also contribute to non-photic shifting.

5.2 Future directions

There are several important follow-up experiments that could be undertaken in order to confirm the role of ACh in non-photic phase shifting. Retrograde tracing could be performed from the hamster SCN to confirm which ACh cells project to the SCN and if it is similar to the rat. If this tracing was performed in conjunction to a novel wheel confinement, then the forebrain could be examined for cells that contain ChAT, express Fos and project to the SCN. It would be

hypothesized that cholinergic cells, mainly in the SI would project to the SCN and become highly active during novel wheel confinement. This experiment would show direct involvement of cholinergic cells in producing phase advances. A good follow-up experiment would be to perform *in vivo* microdialysis in the SCN during a non-photic stimulus in order to determine if ACh is released onto the SCN.

Another experiment that could be conducted would be to deplete ACh in the nerve terminals of the SCN. The choline uptake blocker hemicholinum-3 could be administered to the SCN to deplete ACh and novel wheel confinements administered. If ACh is sufficiently depleted then it should not be released onto the SCN and phase shifts would be expected to be lower. It also might be of interest to determine if ACh depletion in the SCN would have any effect of the amount of paradoxical sleep.

There is also the possibility that other neurotransmitters could be involved in non-photic phase shifting. Histamine presents a good candidate to be involved in non-photic phase shifting. Histamine antagonists could be applied to the SCN and non-photic stimuli could be tested. One possibility is that histamine regulates photic like phase shifts directly in the SCN and modulates cortical arousal via its projections to the thalamus (McCormick and Williamson, 1991; Michelsen et al., 2005).

In order to understand the consequences of circadian dysfunction, it is important to understand how the circadian system normally functions. One way of understanding the normal function is to explore ways in which the SCN is phase shifted. The phase shifting effects of light are still being thoroughly explored, however recent work in non-photic phase shifting has been lacking. The understanding of the mechanisms of how these stimuli can phase shift circadian rhythms has grown from understanding that NPY regulates these shifts to multiple pathways

converging on the SCN. These pathways may play modulatory roles, or be recruited during different conditions. Future work will understand how each of these pathways acts and interacts on SCN neurons and receptors. It will also be important to understand the receptors, biochemical mechanisms and the effects on clock genes in order to have an integrated understanding of how non-photic phase shifting functions.

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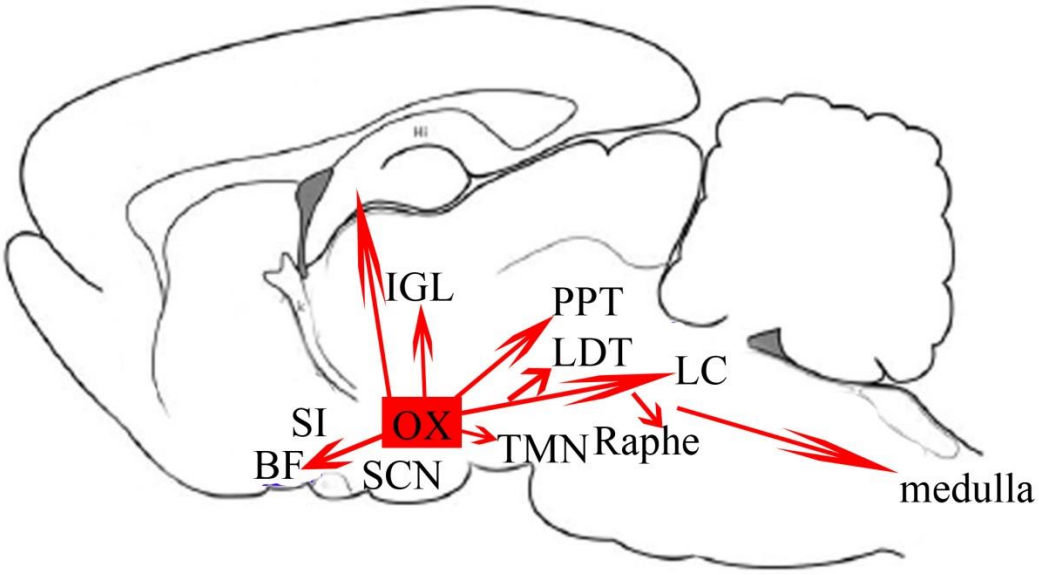
6. Appendix 1

6.1 Introduction

Orexin/hypocretin is a neuropeptide produced in the dorsal and lateral hypothalamic areas (de Lecea et al., 1998). The OX cells send major projections to the histaminergic and noradrenergic cell groups, but also project to the brain stem and forebrain cholinergic cells, serotonergic raphe, IGL and hippocampus as is displayed in Figure A1 (Tsujino and Sakurai, 2013).

Figure A1: Projections from orexin cells

Displayed is a sagittal section of a rodent brain. The Orexin cell group along with the projections are represented by red arrows. OX: Orexin, IGL: intergeniculate leaflet of the thalamus, SI/BF: Substantia innominata / basal forebrain, SCN: suprachiasmatic nucleus, PPT: pedunculopontine tegmentum, LDT: laterodorsal tegmentum, LC: locus coeruleus, TMN: tuberomammillary nucleus. This figure is not to scale.


IGL
PPT
LDT
LC
SI
OX
TMN
Raphe
BF
SCN
medulla

The SCN sends a projection to the dorsomedial hypothalamic OX cells and from there, OX can partially modulate arousal through a projection to the noradrenergic locus coeruleus (Abrahamson et al., 2001; Mahoney et al., 2013). Neurons in the IGL and orexinergic hypothalamus show increased Fos expression to novel wheel confinement, sleep deprivation and restraint procedures (Webb et al., 2008).

HA has long been known to play a role in wake and arousal (Chikahisa et al., 2013). HA does appear to be present to the SCN, but interestingly, it appears to be synthesized outside of the SCN and trafficked in (Michelsen et al., 2005). Application of HA to slices of the SCN has a mainly inhibitory effect, consistent with a role in non-photic phase shifting (Liou et al., 1983). Application of a histidine decarboxylase inhibitor prior to light pulses attenuated phase shifts, suggesting a role in photic phase shifting, however (Eaton et al., 1995).

The purpose of these experiments was to examine Fos expression in regions of the brain containing orexin and histamine in response to sleep deprivation by gentle handling. It was hypothesized that hamsters that were sleep deprived would show greater Fos expression in those regions containing orexin and histamine. Furthermore, it was hypothesized that hamsters that were pre-screened to be responders to the sleep deprivation procedure would show greater expression of Fos than non-responders.

6.2 Experiment 7 – Fos Expression in brain regions containing OX and HA

6.2.1 Perfusion and Immunohistochemistry

Following pre-screening as responders, or non-responders, the hamsters were returned to an LD cycle and allowed to re-entrain for at least 7 days. When it had become apparent that the

activity onsets were coinciding with lights off again, the animals were once again sleep deprived from CT6-9 in the activity cycle.

At the conclusion of the sleep deprivation, the hamsters were administered an overdose of Sodium pentobarbital (~400mg/kg; Bimeda-MTC, Cambridge, ON, Canada). Their eyes were shielded from light using tin foil, they were brought to a fume hood and perfused transcardially using 50 ml of cold phosphate buffered saline (PBS), pH 7.4 followed by 50 ml of cold 4% paraformaldehyde in PBS. The brains were post-fixed overnight using the same fixative and then transferred to 20% sucrose in PBS for cryoprotection for at least one more day. The brains were frozen at -18°C and sliced into 35 µm coronal sections from the beginning of the substantia innominata in the forebrain until the end of the histamine containing cells near the mammillary recess using a cryostat. The sections were collected into wells containing 0.02% sodium azide in PBS.

In order to define the boundaries of the cell groups of interest, intact animals were processed for OX and HA immunohistochemistry. The hamster brain atlas as well as previously conducted immunohistochemistry on intact animals for Orexin A and B and histamine was used to create templates to judge the boundaries of particular cell groups of interest.

For the OX immunohistochemistry, an intact animal was perfused, sacrificed and sliced as described above. Alternate sections throughout the lateral and dorsal hypothalamus were processed as described above except for the following differences. 5% normal horse serum was used as a blocking agent (Vector). The primary antibodies used were a goat anti- OX_A and OX_B (1:5000; Santa Cruz). The secondary antibody used was a biotinylated horse anti-goat (1:200 in PBSx; Vector).

In order to determine the boundaries for the HA cells, the tissue was processed for fluorescent immunohistochemistry. The intact animal was sacrificed as described above. For the perfusions, the hamster was first perfused using 50 ml of PBS followed by 100 ml of the fixative *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC; 4% solution in PBS; Sigma Aldrich) slowly over the course of 10 minutes. The brains were post-fixed for 24 hrs in the same fixative before being cryoprotected as described above. The brains were sectioned using the hamster brain atlas as a guide through the region of brains containing histamine neurons. The sections were dry mounted onto gelatin coated slides and thoroughly allowed to dry before being processed for HA immunohistochemistry. The slides containing the brain tissue were rinsed in PBSx and then placed in a normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, Inc.,West Grove, PA, USA 3% solution in PBSx). The sections were then incubated in the primary antibody a rabbit anti-HA for 48 hrs at 4ºC on a shaker tray (1:1000 in PBSx in 1% NDS; Immunostar Hudson, WI, USA). The slides were thoroughly rinsed again before being placed in PBSx overnight on a shaker tray at 4ºC again. The slides were rinsed thoroughly again before being placed in the secondary antibody, a donkey anti-rabbit conjugated to a cyanine-2 fluorophore (Cy2; 1:200; Jackson ImmunoResearch). The slides were rinsed for a final time before being coverslipped with Krystalon (VWR International, Edmonton, AB, Canada).

Three sections were chosen from the intact orexin reacted slices to correspond to the anterior, mid and posterior levels. These slices were at approximately -1.5 mm posterior from bregma, -1.8 mm posterior to bregma and -2.0 mm posterior to bregma. For the histamine reacted sections the anterior, mid and posterior sections were at the -2.7 mm, -3.0 mm, and -3.4 mm posterior to bregma respectively. Adobe photoshop (Adobe systems, San Jose, CA,USA)

was used to create templates from each of the sections by tracing over the base of the brain, third ventricle and area that contained immunoreactivity for orexin and histamine along one side of the brain. In order to create the template to count for the other side of the brain, the first template was flipped horizontally and then overlaid onto the sleep deprived brain sections as described below

6.2.2 Analysis

The brain sections were photographed using an Olympus BX51 microscope with an attached cooled CCD camera (QICAM 1394; QImaging, Burnaby, BC, Canada). Three sections were chosen from the orexin cell group and histaminergic cell groups corresponding to roughly the anterior, mid and posterior parts of those brain regions. Using previously conducted immunohistochemistry in intact animals, templates encompassing the areas of immunoreactivity for those three chosen sections were constructed using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA). These templates were overlaid onto the Fos Cells were counted using the Image J (NIH, Bethesda, MD, USA) The sleep deprivation treatment effect was examined using a one-way ANOVA and Tukeys test was used to examine follow-up pairwise comparisons.

6.3 Results

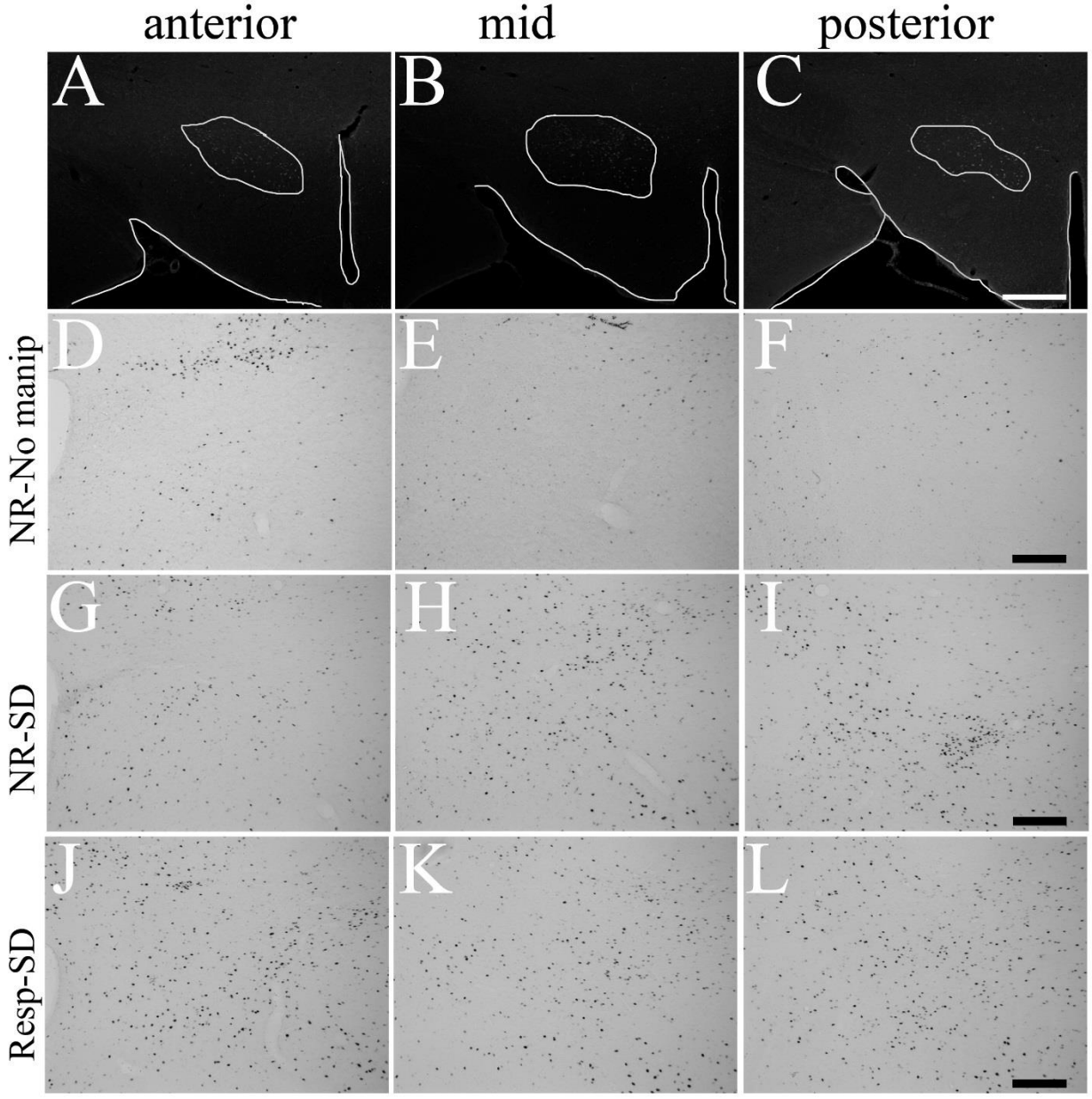
6.3.1 Fos expression in regions containing orexin and histamine

Representative sections of anterior, mid and posterior Fos labelled sections in the OX regions in the non-responder control, non-responder sleep deprived and responder sleep deprived groups are displayed in Figure A2 A-C along with the general areas that were counted. Higher

magnification photomicrographs are displayed in Figure A2, D-L that displays Fos immunoreactivity in each of the conditions.

Figure A2: Fos expression in forebrain regions containing OX

A-C displays the anterior, mid and posterior (respectively) sections of the orexinergic region of the lateral and dorsal hypothalamus of a control hamster that was a non-responder to a sleep deprivation from CT6-9 that did not undergo any manipulation at the time of sacrifice. Fos immunoreactivity is displayed as the dark black nuclei. The black circles represent the approximate regions that were counted for single labelled Fos cells. Scale bar is 600 µm. D-L displays higher magnification photomicrographs of the counted areas for each condition. Scale bars are 200 µm. D-F displays an animal that was pre-screened to be a non-responder and did not undergo any sleep deprivation. G-I represents an animal that was pre-screened to be a non-responder that received a sleep deprivation. J-L represents an animal that was pre-screened to be a responder to the sleep deprivation procedure that underwent a sleep deprivation prior to sacrifice.

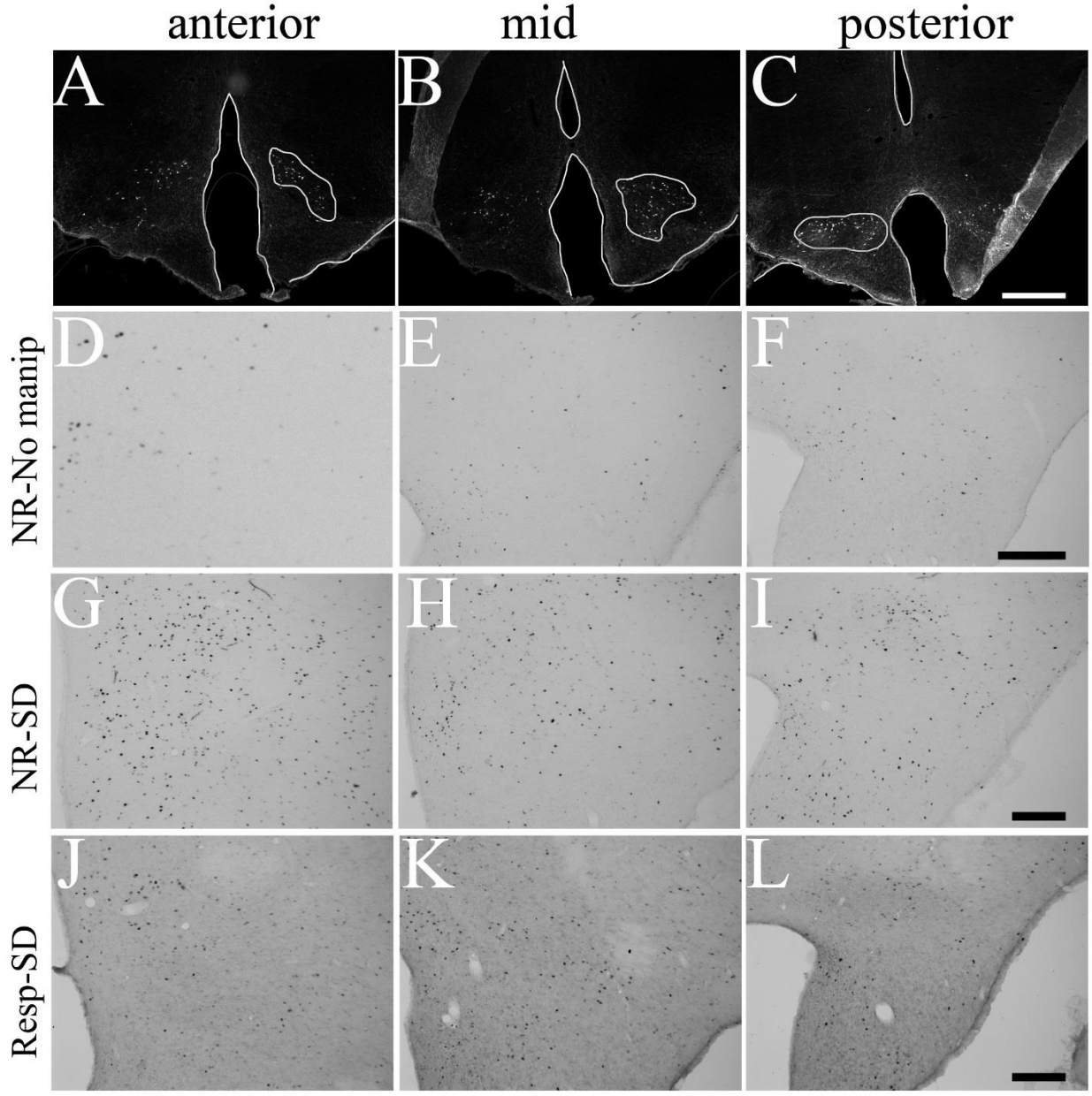

anterior
mid
posterior
A
B
C
NR-No manip
D
E
F
NR-SD
G
H
I
Resp-SD
J
K
L

In the orexin containing region of the control animals, there was an average of 102.25 ± 42.68 cells in the anterior region, 109 ± 42 cells in the mid region, and 65.25 ± 24.37 cells in the posterior region. The average number of cells across all sections in the control animals was 92.17 ± 13.6 cells (n=4). In the non-responder sleep deprived group, there was an average of 275 ± 26.02 cells in the anterior region, 424.14 ± 39.26 cells in the mid sections and 264.86 ± 26.66 cells in the posterior sections. The average number of cells across all sections in the non-responder sleep deprived group was 321.33 ± 51.49 cells (n=7). In the responder sleep deprived group, there was an average of 272.75 cells in the anterior sections, 447.25 ± 87.67 cells in the mid sections and 204.5 ± 68.61 cells in the posterior sections. The average number of cells across all sections in the responder sleep deprived group was 308.17 ± 72.23 cells (n=4). There was a significant effect for sleep deprivation on Fos expression in the Orexin region ($F_{(8,44)} = 9.1$; $p < 0.001$). Tukeys multiple comparisons revealed a significant difference between the control group, and the non-responder sleep deprived group ($p<0.001$) as well as the responder sleep deprived group in the mid sections ($p<0.001$). The average cell counts are displayed for the anterior, mid and posterior regions for each group in Figure A4A.

Representative sections of anterior, mid and posterior Fos labelled sections in the HA regions in the non-responder control, non-responder sleep deprived and responder sleep deprived groups are displayed in Figure A3 A-C along with the general areas that were counted. Higher magnification photomicrographs are displayed in Figure A3, D-L that clearly displays Fos immunoreactivity in each of the conditions.

Figure A3: Fos expression in forebrain regions containing HA

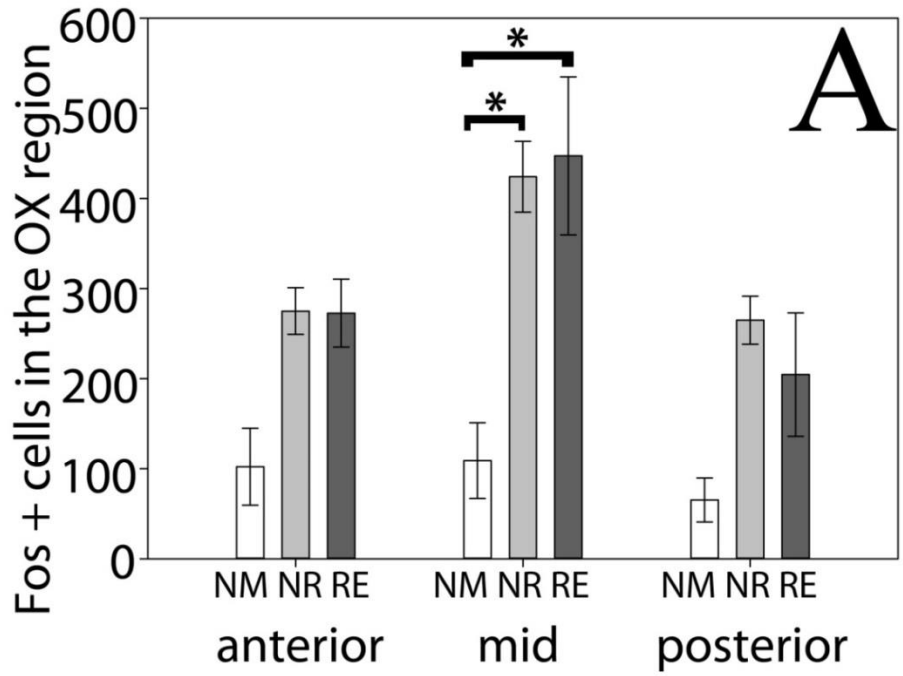
A-C displays the anterior, mid and posterior (respectively) sections of the histaminergic region of a control hamster that was a non-responder to a sleep deprivation from CT6-9 that did not undergo any manipulation at the time of sacrifice. Fos immunoreactivity is displayed as the dark black nuclei. The black circles represent the approximate regions that were counted for single labelled Fos cells. Scale bar is 600 µm. D-L displays higher magnification photomicrographs of the counted areas for each condition. Scale bars are 200 µm. D-F displays an animal that was pre-screened to be a non-responder and did not undergo any sleep deprivation. G-I represents an animal that was pre-screened to be a non-responder that received a sleep deprivation. J-L represents an animal that was pre-screened to be a responder to the sleep deprivation procedure that underwent a sleep deprivation prior to sacrifice.

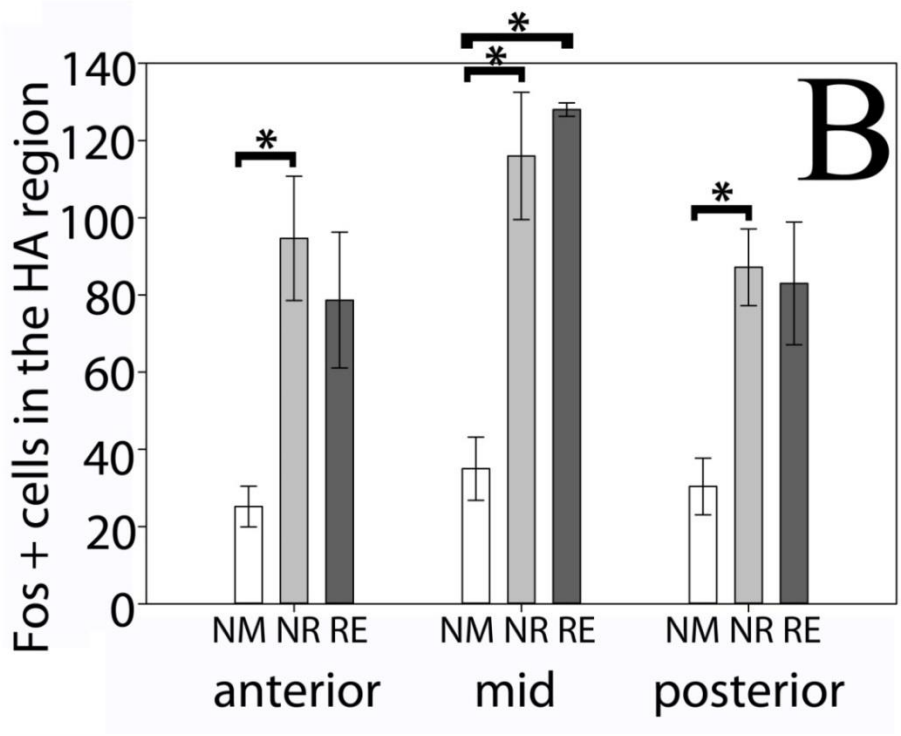

anterior
mid
posterior
A
B
C
NR-No manip
D
E
F
NR-SD
G
H
I
Resp-SD
J
K
L

In the HA containing regions for the control group there was an average of 25.2 ± 5.26 cells in the anterior sections, 35 ± 8.12 cells in the mid sections and 30.4 ± 15.89 cells in the posterior sections. The average number of cells across all sections in the control animals was 30.2 ± 2.83 cells (n=3). In the non-responder sleep deprived group, there was an average of 94.67 ± 16.08 cells in the anterior sections, 116 ± 16.48 cells in the mid sections and 87.17 ± 9.95 cells in the posterior sections. The average number of cells across all sections in the non-responder sleep deprived animals was 99.28 ± 8.64 cells (n=5). In the responder sleep deprived group, there was an average of 78.67 ± 17.61 cells in the anterior sections, 128 ± 1.73 cells in the mid sections and 83 ± 15.89 cells in the posterior sections. The average number of cells across all sections in the responder sleep deprived group was 96.56 ± 15.77 cells (n=3). There was a significant effect for sleep deprivation on Fos expression in the histaminergic region ($F_{(8,41)} = 8.54$; $p < 0.001$). Tukeys follow-up pairwise comparisons revealed a significant difference between the control animals and the non-responder, sleep deprived animals in the anterior section ($p=0.006$), mid-section ($p<0.001$), and posterior section ($p=0.042$). The control animals differed significantly from the responder sleep deprived group in the mid-section ($p=0.002$). The average cell counts are displayed for the anterior, mid and posterior regions for each group in Figure A4B.

Figure A4: Mean Fos counts in the OX and HA regions

Displays the mean ± SEM single label Fos counts of control non-responder, no manipulation animals (NM) along with non-responder, sleep deprived animals (NR) as well as responder, sleep deprived animals (RE). These averages were calculated for the orexinergic region of the hypothalamus and peri-hypothalamic region (A) and the histaminergic region of the hamster (B). The brackets with asterisks represent comparisons that were found to be statistically significant ($p<0.05$).


A
Fos + cells in the OX region
600
500
400
300
200
100
0
*
*
NM NR RE
NM NR RE
NM NR RE
anterior
mid
posterior


B
Fos + cells in the HA region
140
120
100
80
60
40
20
0
*
*
*
*
NM NR RE
NM NR RE
NM NR RE
anterior
mid
posterior

6.4 Discussion

In this experiment, general Fos expression was examined in brain areas roughly corresponding to regions that express, OX in the lateral hypothalamus and areas around it, and HA near the 3rd ventricle and mammillary recess. It was found that sleep depriving hamsters during the middle of their usual rest period increases Fos expression in the orexinergic hypothalamus and histaminergic regions, both areas associated with bringing about an aroused behavioral state.

In sum, the non-photic stimulus of sleep deprivation by gentle handling increases Fos expression in arousal related areas, but there does not appear to be a difference between non-responders and responders.