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An Exploration into the Non-Photic Influence of Acetylcholinesterase Inhibitors on Circadian Rhythms

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An Exploration into the Non-Photic Influence of Acetylcholinesterase Inhibitors on Circadian Rhythms

by

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Abstract

While light is the dominant zeitgeber ("time giver") for the circadian system, nonphotic cues, such as exercise and arousal, also affect circadian rhythmicity. Our lab has reported that cholinergic innervation of the suprachiasmatic nucleus arising from the basal forebrain is both necessary and sufficient for phase shifting circadian rhythms in a nonphotic manner. Therefore, the present study investigated a new avenue for modulating cholinergic activity in a less invasive manner by testing whether enhancing acetylcholine neurotransmission with acetylcholinesterase (AChE) inhibitors will cause nonphotic-like phase shifts of the circadian system. Three different AChE inhibitors were explored (Donepezil, Rivastigmine, and Tacrine) at varying doses. First, Syrian hamsters were housed in constant darkness (DD), and were administered an intraperitoneal (IP) injection of one of the AChE inhibitors or vehicle control in counterbalanced order six hours before their activity onset, a phase when nonphotic treatments elicit phase advances. The second hypothesis was that nonphotic phase shifts elicited by AChE inhibitor administration requires acetylcholine activity at the SCN. Hamsters were given a microinjection of either saline or the acetylcholine antagonist Atropine to the SCN 10 minutes prior to a CT6 injection of donepezil (10mg/kg). In experiment one, Donepezil at 10mg/kg had the most robust phase advances relative to the controls. In experiment two, the results were mixed with no significant differences were found between conditions, including the baseline measurement of Donepezil/control.

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List of Abbreviations

Symbol	*Definition*
ACh	Acetylcholine
AChE	Acetylcholinesterase
BF	Basal forebrain
BMAL1	Bmal1 gene
CalB	Calbindin
CRY	Cryptochrome gene
CG	Clock gene
CNS	Central nervous system
CT	Circadian time
CLOCK	Circadian Locomotor Output Cycles Kaput
DD	Constant darkness
GHT	Geniculohypothalamic tract
GPCR	G-protein coupled receptor
GRP	Gastrin-releasing peptide
IC	Intracranial
IGL	Intergeniculate leaflet
IP	Intraperitoneal
LD	Light/Dark
LH	Lateral hypothalamus
LL	Constant light
LDTg	Laterodorsal tegmental nucleus
NPY	Neuropeptide-Y
PER	Period gene
PRC	Phase response curve
PPTg	Pedunculopontine tegmental nucleus
RHT	Retinohypothalamic tract
SCN	Suprachiasmatic nucleus
SWA	Slow wave activity
TTFL	Transcription translation feedback loop
VIP	Vasoactive intestinal peptide
ZT	Zeitgeber time

1. Introduction

1.1 Circadian rhythms

In mammalian physiology, critical bodily processes such as sleep, hormone regulation, temperature regulation, and metabolism are rhythmic and occur within a 24-hour period. These processes are called circadian rhythms and are essential for an organism's survival. In order to maintain this elegant rhythmic organization, a cluster of cells in the anterior hypothalamus called the suprachiasmatic nucleus (SCN) controls and synchronizes these processes (Klein et al., 1991). The SCN is known as the central oscillator as it synchronizes oscillators in peripheral tissues throughout the body by receiving information from afferent sources and communicating relevant information to those respective systems (Antle & Silver, 2005). The SCN has evolved to track, as well as estimate day and night in the absence of salient cues. The structure is largely influenced by light, as light is the primary zeitgeber ("time giver"), to reset and synchronize SCN (Antle & Silver, 2005). While there is a consensus of light being the most salient external cue, there are also robust nonphotic cues that can influence rhythmicity in the absence of light.

1.2 Circadian terminology

Mammalian circadian researchers often assess the effect of manipulations on circadian rhythms by tracking changes in wheel-running activity before and after it occurs. Wheel-running activity is indicative of an animal's active (i.e. when the animals are awake) and inactive period (i.e. when the animals are asleep), as the animals' active period are gauged as the times that there is stable wheel-running activity. This can be done by quantifying wheel revolutions via magnetic switch closures between the wheel rungs and the cage top. Thereby, whenever the wheel is in motion, each revolution is counted when the magnetic switch is closed. The wheel revolutions are collected for the timeframe that the animal has access to the running wheel. This activity is

compiled onto a chart called an actogram (for example, see Figure 1), where each thin horizontal black line indicates a 24-hour day, and the thick black bars are the wheel-running activity of the animal.

In the lab, daytime and nighttime are mimicked by lights being on and off, respectively. How animals respond to the day and night depends on their chronotype, or whether they are nocturnal, diurnal, or crepuscular. For example, Syrian hamsters are nocturnal, which means that they are inactive during the day and active at night. Typically, animals entrain, or have a fixed phase relationship, to light. This relationship is between the external light cycle and the period of the animal. When an animal is being housed in a typical light:dark (LD) cycle, the animals' rhythms will follow zeitgeber time (ZT), with ZT12 being the time that the lights go off and the animals' active phase begins. In contrast, when an animal is experiencing constant conditions, the animals' rhythms are endogenously generated and free running, as they have no external cues indicating time of day. When an animal is in constant conditions, the subjective day is when the animal is behaving as if it were day (e.g. sleeping if the animal is nocturnal). Similarly, the subjective night is when the animal is behaving as if it were night (e.g. awake if the animal is nocturnal). These animals' rhythms will follow circadian time (CT), with CT12 being the beginning of the animals' active phase. In order to predict these times, a regression line will be fit for the beginning of these active periods leading up to the manipulation day to predict CT12.

On an actogram, the white portion indicates when the lights are on, and the gray portion indicate when the lights are off (see Figure 1). For example, if a section of the actogram is entirely white across the 24-hour timespan, it means that the animal is experiencing constant light (LL). Additionally, if a section is entirely gray across the 24-hour timespan, the animal is

experiencing constant darkness (DD). In both of these constant conditions, the animals' rhythms are free running and are relying entirely on their own internal rhythm, governed by the SCN.

The start of an animal's active periods is called activity onset, and these time points are necessary to predict the animal's future onset times, and to also measure changes in rhythmicity caused by an experimental manipulation. These onsets can be used for animals across different chronotypes and is indicated by the start of wheel-running activity. Photic and nonphotic manipulations can change the timing of activity onset relative to activity onset before the manipulation. These changes are called phase shifts, and there are two types of shifts that are of importance: a phase delay and a phase advance. A phase delay occurs when a manipulation causes the activity onsets of the animal to begin significantly and consistently later for the animal after the manipulation. An example of a phase delay is shown in Figure 2. in contrast, the other shift is called a phase advance, and this occurs when a manipulation causes the activity onsets of the animal to begin significantly and consistently earlier for the animal after the manipulation. An example of a phase advance is shown in Figure 3**.**

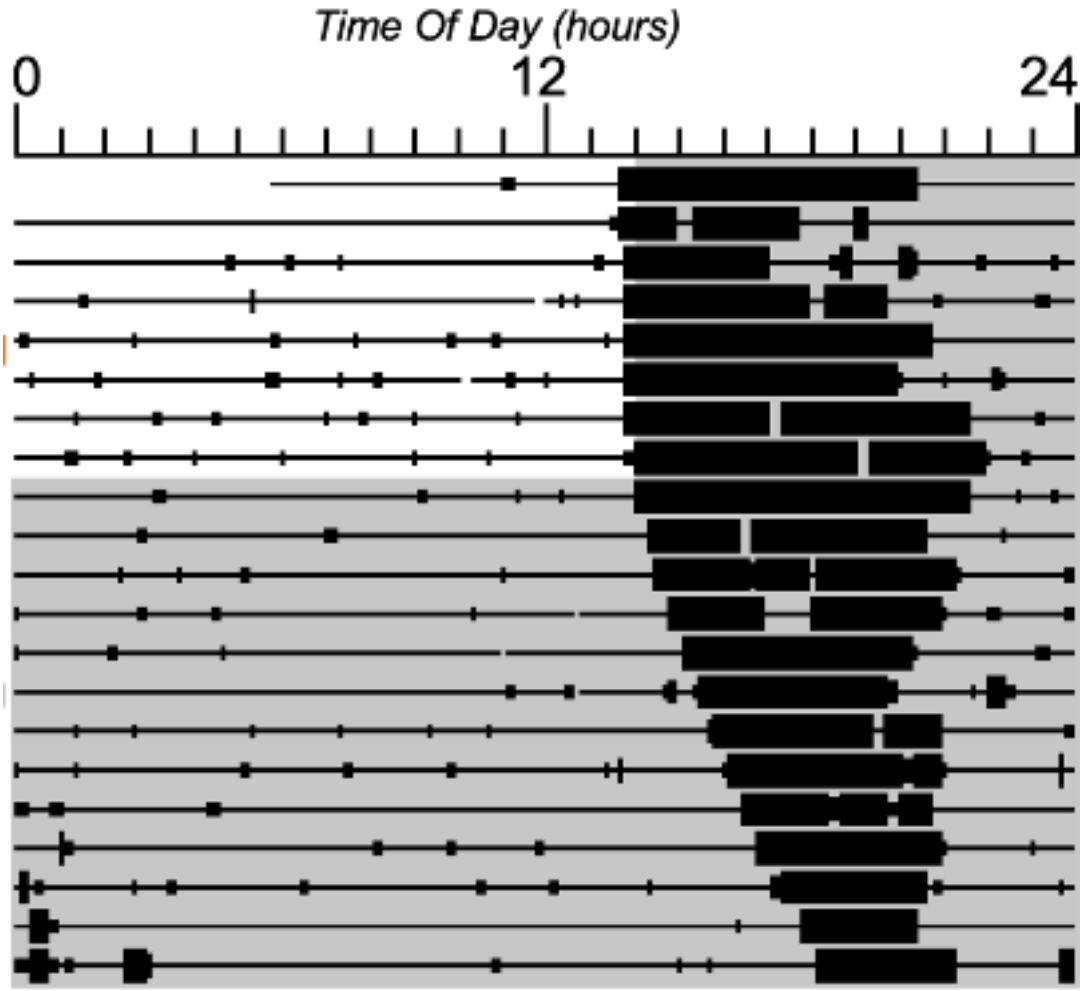

Figure 1: A sample actogram for a nocturnal animal. Each thin horizontal black line indicates a 24-hour day, and the thick black bars indicate wheel-running activity. The white portion of the actogram is when the lights are on, which mimics daytime. The gray portion is when the lights are off, which mimics nighttime.

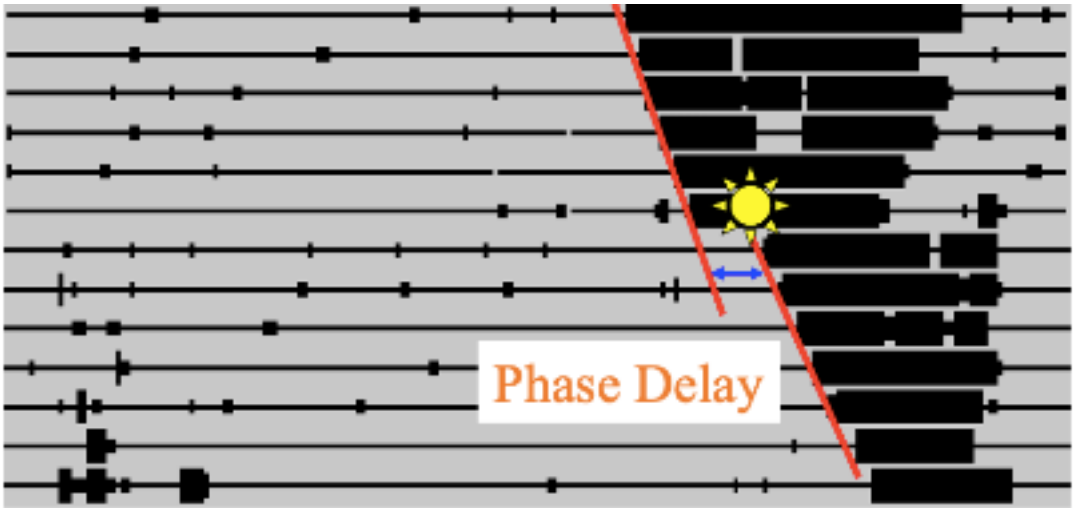

Figure 2: A sample actogram depicting a phase delay caused by a light pulse during the early subjective night in a nocturnal animal. Consistently later activity-onset times are observed post-manipulation.

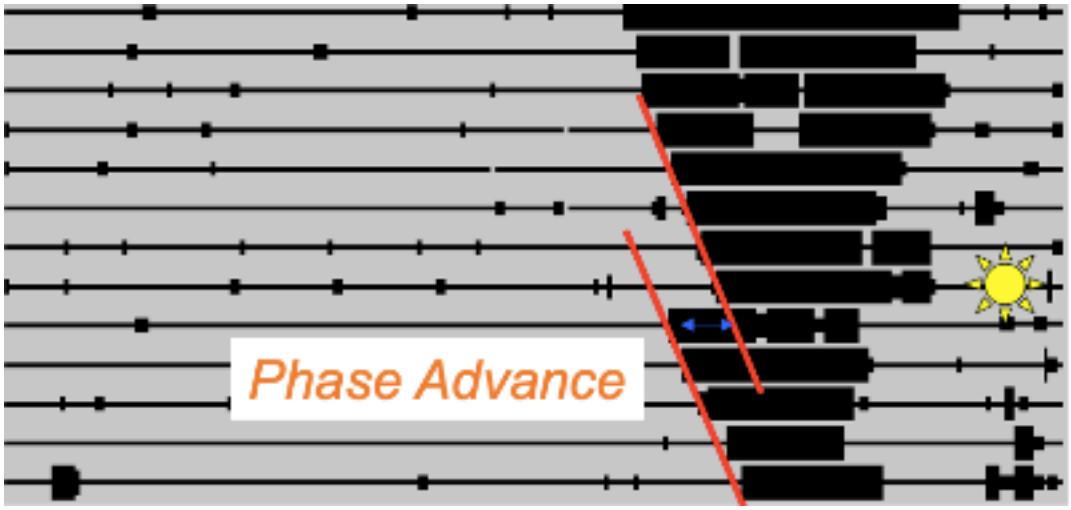

Figure 3: A sample actogram depicting a phase advance caused by a light pulse during the late subjective night in a nocturnal animal. Consistently earlier activity-onset times are observed post-manipulation.

1.3 Nonphotic cues

Investigation into the nonphotic zeitgebers stemmed from the discovery that there are cues beyond light that can significantly influence rhythmicity, and these cues involve both behavioural and pharmacological manipulations. Some examples of behavioural cues that influence nonphotic phase shifting include social interaction (e.g. communication between a neonatal offspring and its mother), novelty with environment (e.g. cage changes), dark pulses (i.e. putting an animal that was held in constant light in a dark box for a period of time), and also changes in motor activity (e.g. access to a running wheel) (Hastings et al., 1998; Rosenwasser & Dwyer, 2001). In addition, pharmacological agents including Triazolam, a benzodiazepine and CNS depressant (Mrosovsky & Salmon, 1990) and carbachol, a cholinergic agonist, have also been investigated (Bina & Rusak, 1996; Meijer, Zee, & Dietz, 1988; Zatz & Herkenham, 1981). Notably, nonphotic cues can influence circadian rhythms differently depending on when they are administered and for how long. A commonality among these nonphotic stimuli is that the most robust effects are typically observed when they are administered during a nocturnal animal's inactive phase, or mid-subjective day, as they have been shown to cause significantly large phase advances (Rosenwasser & Dwyer, 2001).

With respect to behavioural zeitgebers, some of the most robust nonphotic cues that have been investigated are dark pulses, novelty, and motor activity. Like a light pulse, which is the administration of a short pulse of light for animals being held in constant darkness, a dark pulse can influence rhythms differently depending on when during a 24-hour period they are administered (Canal & Piggins, 2006; Rosenwasser & Dwyer, 2001). For example, when hamsters experience dark pulses during their mid-subjective day, significant phase advances are observed (Boulos & Rusak, 1982; Ellis, McKlveen, & Turek, 1982). Changes in rhythmicity that

occur when these pulses are administered at different times over a 24-hour period can be quantified using a phase response curve (PRC). Notably, the PRC of dark pulses was initially considered to mirror the light pulse PRCs because peak advancing effects are observed when dark pulses are administered during the mid-subjective day, whereas advancing effects with light pulses are observed during the mid-subjective night (Canal & Piggins, 2006; Rosenwasser & Dwyer, 2001). However, they are different because dark and light pulses are not mirrored in phase delays. The phase delays caused by a light pulse during the early subjective night are large and can span 2-3 hours, but phase delays induced by dark pulses are minimal (Canal & Piggins, 2006; Rosenwasser & Dwyer, 2001). In addition to a dark pulse PRC, a dose response curve for dark pulses has also been identified, which indicates that the longer an animal receives a dark pulse, the larger the changes in rhythmicity are predicted to be (Canal & Piggins, 2006; Rosenwasser & Dwyer, 2001). One particularly important discovery about dark pulses was that there were marked differences in wheel running activity during and after the pulse itself. The differences in activity were related to the subsequent changes in circadian rhythms, where higher activity resulted in/was correlated to a larger phase shift (Canal & Piggins, 2006; Rosenwasser & Dwyer, 2001). Moreover, these findings confirmed dark pulses could not be considered a different kind of photic manipulation because they are affecting behavioural states. Additional evidence supporting this claim comes from when hamsters held in LL were administered dark pulses during their mid-subjective day with no wheel-running opportunities and kept locked in a nest box during the pulse, phase shifts were significantly attenuated (Reebs, Lavery, & Mrosovsky, 1989). The absence of light is not the only salient cue able to influence rhythmicity, but rather there are many underlying factors that are contributing to this phenomenon.

In addition to dark pulses, novelty has also been investigated within the nonphotic subfield. Some examples include cage changes, foreign odours, and exposure to a novel running wheel, which is the most studied cue (Hastings et al., 1998). Previous studies have found that when hamsters are given access to a novel wheel on a daily basis while being held in constant DD, they were able to entrain to wheel-running opportunities (Reebs et al., 1989). Entrainment has also been observed in mice that have experienced scheduled forced treadmill activity. In this example, after the forced activity, if the mice were blocked from using the wheels in their home cages, changes in circadian rhythms were significantly greater (Marchant & Mistlberger, 1996). Like dark pulses, a PRC has also been established for wheel-running activity, and peak phase advances were observed when wheel access was administered during the mid-subjective day (Bobrzynska & Mrosovsky, 1998; Reebs et al., 1989). Furthermore, duration of novel wheel access also influenced the magnitude of its subsequent phase changes; specifically, the longer an animal was in the wheel if the pulse was administered during the mid-subjective day, the larger their phase advances were. Like dark pulses, differences in activity levels during the manipulations played a significant role in how much rhythmicity changed post-manipulation. In these experiments, variable animal activity was noted between the tested animals, in that there were some animals that were more active than others, and the ones that were more active had larger phase shifts (Bobrzynska & Mrosovsky, 1998; Canal & Piggins, 2006; Reebs et al., 1989). In fact, the magnitude of these shifts could be predicted by measuring the number of wheel revolutions the animal performed within the first hour of being exposed to the running wheel (Bobrzynska & Mrosovsky, 1998). Specifically, when an animal is exposed to a novel wheel during their mid-subjective day and had more than 4000 revolutions within the first hour of

wheel exposure, they were predicted to experience maximum phase shifting post-manipulation (Bobrzynska & Mrosovsky, 1998; Canal & Piggins, 2006).

Since the majority of the dark pulse and novel wheel-running studies were occurring during nocturnal animals' inactive phases, whether the wakefulness levels of the animals contribute to the changes in rhythmicity was subsequently investigated. One study induced wakefulness in hamsters by sleep-depriving then with gentle handling them during their mid-subjective day for one to three hours, after which animals had no wheel access and were held in DD. Similar to the previous studies, significant phase advances were observed after handling ranging from 165 to 190 minutes (Antle & Mistlberger, 2000). However, similar to the previous studies, there was variability in wakefulness levels for this experiment, in that animals who were more awake displayed larger phase advances compared to animals that were less alert and required more effort to keep awake. These animals had smaller shifts relative to the other group (Antle & Mistlberger, 2000).

As such, investigations into the role that arousal levels play in modulating rhythmicity was pursued, and there are a few pharmacological agents that have been studied that induces such changes. The benzodiazepine Triazolam, which is prescribed for individuals who suffered from insomnia, was one of these agents. In hamsters that were being held in DD, significant phase advances were observed when this drug was administered during their mid-subjective day. Increased motor activity was also observed in parallel to these phase shifts (Mrosovsky & Salmon, 1990). Another example is with the drug Carbachol, which is a non-specific cholinergic muscarinic agonist, and this drug also induces significant phase advances when it is directly administered to the SCN (Bina & Rusak, 1996; Meijer et al., 1988; Wee & Turek, 1989). Carbachol was investigated further in arousal and nonphotic context, and like the dark pulses and

novel wheel exposure, the types of changes this drug caused were dependent on when it was administered during an animal's active and inactive periods. For example, when administered during a hamster's mid-subjective day, significant phase advances (~1.5 hours) were observed. In contrast, when it is administered during the mid-subjective night, significant phase delays were observed (Meijer et al., 1988).

Investigating the behavioural changes nonphotic cues are able to induce has led to subsequent studies investigating the neurobiology behind them. It is critical to investigate the structural, synaptic, and molecular dynamics underlying these changes in order to gain a complete understanding of circadian rhythms.

1.4 Molecular mechanisms underlying rhythmicity

The SCN itself is can be classified into heterogenous cell types, specifically breaking the nucleus down into the core and shell, as seen in Figure 4. The core of SCN receives afferent information from many brain regions, including direct retinal input via the retinohypothalamic tract (RHT) (Abbott et al., 2013; Antle & Silver, 2005; Yang et al., 2010). Furthermore, the primary cell types within this region include vasoactive intestinal peptide (VIP), gastrin-releasing peptide (GRP), and calbindin (CalB) cells (Moore, Speh, & Leak, 2002). The shell is involved with efferent communication to other neural structures. The afferent information that comes from the core is then relayed to the shell of the nucleus, and this information is then sent out to their respective systems, which enables the SCN to synchronize the oscillators in peripheral tissue throughout the body (Antle & Silver, 2005). Furthermore, the shell is also the region where the majority of the autoregulatory feedback loops for clock gene (CGs) expression occurs (Antle & Silver, 2005). The expression of CGs is where rhythmicity in all living organisms can be narrowed down to, as different homologues of these genes are expressed in different species. In

mammals, the clock genes include *Period* (*PER*) 1, 2, and 3, *Cryptochrome* (*CRY*) 1 and 2, along with transcription factors *CLOCK* (Circadian Locomotor Output Cycles Kaput), and *BMAL1* (Kwon et al., 2011). Expression of these CGs go through a transcriptional-translational feedback loop (TTFL), where there is a positive element and negative element in the CGs expression cycle. The positive element involves the *CLOCK/BMAL1* transcription factors to come together and form a heterodimer that binds to the E-box site on the genes for either *Per* or *Cry*. The negative element to this cycle is when the *Per* and *Cry* protein products form their own complexes (*Per/Per*, *Per/Cry*, or *Cry/Cry*), and they travel back into the nucleus of the cell and inhibit their own expression. Other critical clock components at the molecular level include the Casein Kinase 1 members (CK1δ and CK1ε), as these elements are involved with the phosphorylation of the *Per/Cry* complexes and translocating them back into the nucleus in a timely manner (Kwon et al., 2011). As time passes, the proteins degrade, and once the complexes are no longer present within the nucleus, transcription of these genes starts once again. This feedback loop takes approximately 24 hours to occur (Isojima, Okumura, & Nagai, 2003; Kwon et al., 2011), and when an animal is held in constant conditions (e.g. LL or DD), rhythmic CG gene expression persists (Tei et al., 1997).

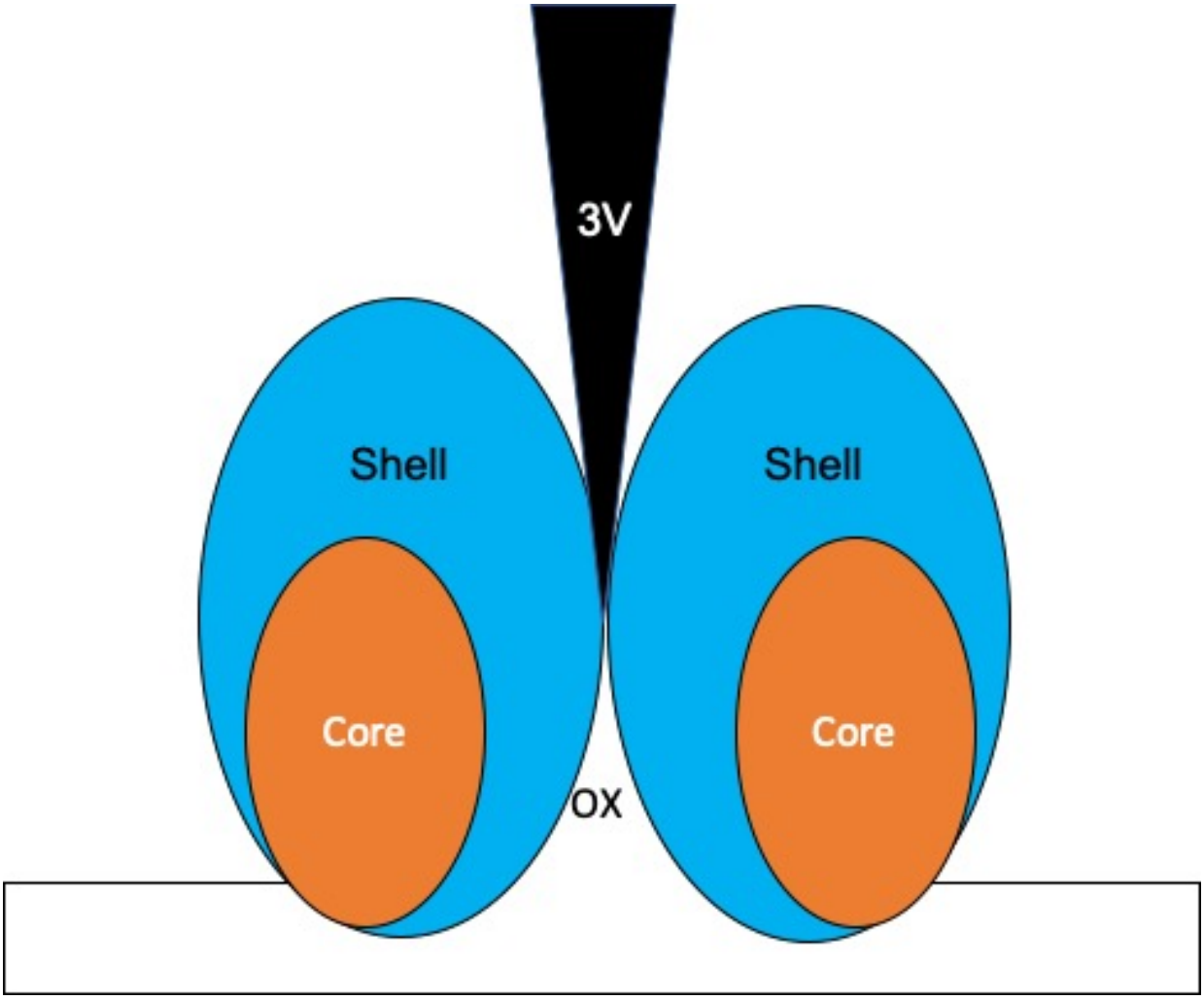

Figure 4: A simplified diagram of the SCN, depicting the core and shell's location within the nucleus. The OX denotes the optic chiasm and the 3V denotes the third ventricle.

Changes in spatial and temporal expression of clock genes underlies both photic and nonphotic phase shifts (Antle & Silver, 2005). For example, in mice, changes in *mPer1* and *mPer2* gene expression have been observed in different regions within the SCN when photic cues are administered at different time points. Specifically, in mice there is an upregulation of *mPer1* and *mPer2* mRNA presence in the core of the SCN when a light pulse is administered during the early subjective night, which causes a phase delay, followed by *mPer2* expression in the shell (Yan & Silver, 2002). Furthermore, light pulses administered during the late subjective night, which causes a phase advance, and induces the expression of *mPer1* only. Its expression also first occurs in the core, followed by the shell of the SCN (Yan & Silver, 2002). Similar findings can also be seen with PER protein expression within the SCN (Yan & Silver, 2004). Experiencing a typical light-dark cycle is what enables the SCN to synchronize mammals' endogenous rhythms to the external environment, no matter what their chronotype, and patterns of CGs expression remain the same across them.

1.5 Neural underpinnings of nonphotic cues

Within the central nervous system (CNS), the main path that communicates nonphotic information to the SCN is the geniculohypothalamic tract (GHT) (Horowitz, Blanchard, & Morin, 2004). Specifically, the intergeniculate leaflet (IGL) in this pathway, its primary neurotransmitter neuropeptide-Y (NPY), and this structure's direct projections to the SCN play a significant role in modulating changes in response to nonphotic cues (Card & Moore, 1989; Horowitz et al., 2004). The IGL relays both photic and nonphotic information, as there are retinal afferents that project to this structure; there is, however, a separate cell population within the IGL that relays nonphotic information to the SCN, and those projections are NPY-ergic (Biello & Mrosovsky, 1995). Furthermore, the photic and nonphotic networks interact to oppose each

other. Studies have investigated the interactions between wheel-running and a light pulse administration during the mid-subjective day found that phase advances from a 3-hour long wheel-running pulse were significantly attenuated when a light pulse was administered within the first hour after the activity pulse (Biello & Mrosovsky, 1995; Mrosovsky, 1991). Furthermore, overall wheel-running activity in the animals that received a light pulse immediately after the activity pulse was lower than the animals who received the activity pulse alone (Mrosovsky, 1991). Also, previous studies have found that inducible shifts caused by an intraSCN injection of NPY during the mid-subjective day can be attenuated if a light pulse is administered immediately after the injection (Biello & Mrosovsky, 1995).

Numerous lines of evidence support the hypothesis that NPY and the IGL are involved with nonphotic shift. On a molecular level, expression of the immediate early gene c-Fos, activity in the IGL has been found to increase with a nonphotic stimulus like wheel-running confinement during an animal's mid-subjective day. In addition, wheel running induced phase shifts can be attenuated with the administration of an NPY antiserum to the SCN prior to novel wheel exposure (Biello, Janik, & Mrosovsky, 1994). Furthermore, lesions of the IGL prevent phase shifts to nonphotic manipulations such as wheel confinement**,** indicating this structure's is importance in modulating nonphotic information to the SCN (Biello et al., 1994). Interestingly, these researchers also found that the number of wheel revolutions was not attenuated with the IGL lesion, further indicating that the IGL's importance in relaying activity information to the SCN (Biello et al., 1994).

In the hamster SCN, c-Fos expression has been seen to decrease when a nonphotic stimulus is administered during the mid-subjective day (Mikkelsen, Vrang, & Mrosovsky, 1998). Gene expression levels for *Per1* in the SCN can also be attenuated with a nonphotic stimulus.

When cues such as wheel running confinement and dark pulses are administered during an animal's mid-subjective day, levels of *Per1* expression in hamsters were markedly lower than the controls not administered these manipulations (Maywood et al.,, 1999; Mendoza et al., 2004). It has been postulated that the inhibition of *Per1* expression causes the CG cycle to reset and begin earlier for subsequent cycles. These changes are long-lasting and can be translated to overall bodily rhythms. This is the theorized molecular basis behind phase advances (Antle & Silver, 2005; Maywood et al., 1999). The mid-subjective day is when gene expression for *Per1* is at its peak across chronotypes, no matter if the animal is experiencing a typical LD cycle or is in constant conditions (Maywood et al., 1999; Tei et al., 1997; Yan & Silver, 2002). This evidence indicates that the expression of *Per1,* especially in the shell of the SCN, is sensitive to nonphotic cues, and administering such cues during an animal's mid-subjective day suppresses its expression, which can then lead to an advanced phase change in rhythmicity (Antle & Silver, 2005; Maywood et al., 1999).

Other neural systems that influence circadian rhythms in a nonphotic manner includes serotonin (5-HT) from the dorsal raphe nucleus (Morin, 1992), hypocretin from the lateral hypothalamus (LH) (Mintz et al., 2001), and acetylcholine (ACh) from the laterodorsal tegmental nucleus (LDTg), the pedunculopontine tegmental nucleus (PPTg), and the basal forebrain (BF) (Moore et al., 2002). These areas, except for the BF, project to the IGL, but the LDTg and the PPTg also project directly to the SCN (Bna et al., 1993). Modulating ACh activity in particular has been shown to significantly influence circadian rhythms in a nonphotic manner, but it is also one of the least well known and investigated neurotransmitters within the circadian field.

1.6 Acetylcholine

While there are connections between the cholinergic centers in the brainstem and the IGL, there is also direct cholinergic input from these aforementioned areas, as well as the substantia innominata and nucleus basalis magnocellularis in the BF to the SCN itself (Bina, Rusak, & Semba, 1993). ACh binds to and interacts with two receptor subtypes on the post-synaptic cell: nicotinic and muscarinic receptors, with nicotinic receptors being ionotropic, and muscarinic receptors being metabotropic G-protein coupled receptors (GPCRs). ACh is critical for many biological processes such as neuromuscular junctions, emotion, motivation, attention, and memory (Ma et al., 2018), and the circadian component of ACh is also crucial. For example, activity of cholinergic neurons in the BF are at their maximum firing rates during REM sleep and wakefulness (Davis & Sadik, 2006; Jones, 2008), which could contribute to enhanced cortical arousal during these states due to increased theta wave oscillations seen in these structures. Bursts of theta wave oscillations contribute to enhanced plasticity during attentive periods with wake and paradoxical sleep (Lee et al., 2005). Additionally, cholinergic cells within the BF are activated during nonphotic manipulations that induce arousal—increases in ChAT and c-Fos levels in the BF have been observed in hamsters that underwent wheel-confinement during the mid-subjective day (Yamakawa et al., 2016). Furthermore, cholinergic cells that innervate the SCN are also activated during an arousal-inducting nonphotic manipulation (Yamakwawa et al., 2016). Also, *in vivo* electrical stimulation to the BF during the mid-subjective day show similar results to the carbachol literature, in that significant phase advances are observed post-stimulation (Yamakawa et al., 2016). In addition, if an intraSCN administration of the competitive muscarinic ACh receptor antagonist Atropine occurs prior to BF stimulation, Atropine is able to attenuate inducible shifts caused by the stimulation (Yamakawa et al., 2016).

Furthermore, blocking muscarinic receptors with Atropine in the SCN in hamsters has been shown to attenuate arousal-induced phase shifts caused by novel wheel confinement (Yamakawa et al., 2016).

Early investigations and the use of carbachol, a nonspecific cholinergic muscarinic agonist, in a nonphotic context, found that the muscarinic receptor subtypes are the receptors underlying the induced changes caused by this agent. In addition to this, when the PRC for carbachol was formulated, its effects also varied depending on when during an animal's active and inactive phase it was administered. Specifically, when hamsters were held in constant darkness and administered intraSCN injections of carbachol, significant phase advances were observed when it was administered during the mid-subjective day (CT6 – 9) and mid-subjective night (CT18-22). In contrast, significant phase delays were observed when administered during an animal's early subjective night (CT12-15) (Bina & Rusak, 1996; Meijer et al., 1988; Zatz & Herkenham, 1981). More consistent findings came from the studies investigating these changes during the mid-subjective day. However, one caveat to the findings was that the phase shifts were smaller (~1.5 hours) than those observed with the previously discussed behavioural seminal studies with wheel-running, dark pulses, and sleep deprivation (2-3 hours).

Because carbachol is a nonspecific muscarinic agonist, it was unclear which of the muscarinic receptors were involved with rhythmicity and if it was more than one of the receptor subtypes working in conjunction. There are 5 different subtypes (M1-5), and each of them respond differently when activated. *In vitro* studies investigating spontaneous cell firing rates before and after carbachol is administered found that this agent, along with an agonist for M1/4 receptors, primarily inhibits spontaneous cell firing when applied (Yang et al., 2010), which is a similar to the phenomenon seen with *in vitro* applications of NPY and 5-HT (Liou & Albers,

1991; Shibata et al.,1983). Furthermore, firing rates were unaffected when simultaneous administration of carbachol and an M1/4 antagonist are applied to the cells (Yang et al., 2010). These results coincide with what has been seen in similar *in vitro* and *in vivo* studies with NPY (Liou & Albers, 1991; Mikkelsen et al., 1998) and 5-HT (Shibata, Liou, & Ueki, 1983; Ying & Rusak, 1994).

As previously discussed with the Yang et al., (2010) carbachol study, no increases in firing rate were observed when an M2/3/5 antagonist was administered with carbachol (Yang et al., 2010). This suggests that either the M1, the M4, or both receptors subtypes are involved in modulating cholinergic activity to the SCN. Investigations into whether activating these receptors *in vivo* have shown that when an M1/4 agonist is administered during a hamster's mid-subjective day while they are being held in DD, significant phase advances are observed, and these phase advances were similar in magnitude to phase shifts induced by carbachol (Basu et al., 2016). Moreover, no notable changes in rhythmicity were observed when an M2/3/5 agonist was administered during the mid-subjective day when compared to a vehicle control (Basu et al., 2016). These findings provide convergent evidence that, modulating cholinergic activity during an animal's mid-subjective day is able to sufficiently and significantly influence circadian rhythms in a nonphotic context. That being said, there are still unknowns regarding the cholinergic influence over circadian rhythms, which range from the molecular to the systems levels.

1.7 Acetylcholinesterase Inhibitors

There are multiple methods that have been utilized to manipulate levels of ACh in the CNS. One approach is the use of acetylcholinesterase (AChE) inhibitors. When AChE is active, it breaks down ACh into choline and acetate in the synaptic cleft (Colovic et al., 2013). AChE

inhibitors slow down the rate of metabolism by AChE, thereby prolonging the activity and effects of the neurotransmitter within the synapse (Colovic et al., 2013). One of the current uses for these inhibitors is for patients suffering from mild to moderate Alzheimer's disease (AD).

Some key AChE inhibitors that have been used in the clinical setting include Donepezil Hydrochloride, (Aricept), Tacrine Hydrochloride (Cognex), and Rivastigmine Tartrate (Exelon) (Jiang et al., 2019). Donepezil is the most prescribed drug for patients suffering from AD (Francis et al., 1999), as it is a selective and reversible AChE inhibitor that is active only in the CNS. Tacrine is a reversible cholinesterase inhibitor and was one of the first inhibitors to be used in the clinical setting, however due to some negative side effects, it is not commonly used to tread AD (Colovic et al., 2013). Rivastigmine is also selective to the CNS just like Donepezil, and it inhibits both AChE as well as pseudocholinesterase, the enzyme used to metabolize and breakdown butytyrlcholine, which is found mostly in non-neural tissue (Colovic et al., 2013). At the synaptic level, these drugs have some differences in their mechanisms of action, but their function is the same, in that they increase and prolong cholinergic activity at the synapse by slowing down the metabolism of ACh. Given the functions of these drugs, along with the convergent evidence surrounding ACh and circadian rhythms, it is possible that these inhibitors could significantly influence rhythmicity within a nonphotic context. Carbachol, electrical stimulation of cholinergic centers, and selective muscarinic receptor activation during the mid-subjective day all while animals are being held in constant conditions have been shown to significantly influence rhythmicity in an advancing manner. Theoretically, these AChE inhibitors could display similar effects if given the right dose at the right time. Furthermore, these drugs have not been directly investigated or utilized within the circadian field.

1.8 Hypothesis and rationale

The goal of the present experiments was to introduce and test a new**,** less invasive method of modulating ACh activity within the circadian context. For this study, it was hypothesized that AChE inhibitors could significantly phase advance circadian rhythms they were administered during an animal's mid-subjective day. I also hypothesized that the effects of these drugs can be localized to the SCN itself given the cholinergic projections to the SCN (Bina et al., 1993).

This project utilized male Syrian hamsters (*Mesocricetus auratus*) as the research model and was split into two experiments: Experiment one entailed administering intraperitoneal (IP) injections of three different AChE inhibitors: Donepezil Hydrochloride, Tacrine Hydrochloride, and Rivastigmine Tartrate at various doses, which were then compared to vehicle controls. Experiment two entailed the use of the AChE inhibitor at its respective dose that elicited the most robust phase shifts from experiment one. Hamsters were pretreated with intraSCN injections of the ACh agonist Atropine (or vehicle) prior to an IP injection of Donepezil. Administration of these drugs during both experiments occurred during the animals' mid-subjective day (CT6).

2. General methods

2.1 Animals

The model organisms of choice were Syrian hamsters (*Mesocricetus* auratus), and this study was split into two different experiments. Overall, the data from 43 animals were used split across two experiments, specifically 33 animals in experiment one and 10 animals in experiment two. The hamsters were bred within the Administration building Viviarium under LESARC at the University of Calgary. The ages of the animals ranged from 10 weeks to 5 months old for both experiments, and they weighed between 110 grams and 180 grams. Prior to the experiments, the animals were pair-housed and lived in a 14:10 light-dark cycle (lights on at 2:00 h and lights off at 16:00 h standard time). The animals had access to water and food *ad libitum*. For the experiments, they were single-housed and moved into constant darkness (DD) conditions. Experimenter interventions such as entry and exit, cage changes, and troubleshooting were tracked throughout the experiments. All protocols were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the Canadian Council on Animal Care guidelines.

2.2 Behavioural data

Wheel-running activity was the primary method of data collection across both experiments. The wheels were installed onto the lids for polycarbonate cages (20 x 45 x 22 cm). A computer program called Clocklab data collection software (Coulbourn Instruments, Allentown, PA) that was able to track the daily wheel-running activity was used. The manipulation time was kept constant at CT6 across both experiments, and this time was obtained by subsequently subtracting 6 hours from the predicted CT12.

Phase shifts are consistent changes in wheel-running activity after a manipulation (see Figure 2 and 3), and these shifts can vary depending on what kinds of manipulation is administered and when it is administered during an animal's 24-hour period. In order to quantify phase shifts, actograms are created, and two regression lines are plotted over the activity onsets—one line for before the manipulation, and a separate line for afterwards. The distance between the two plotted lines from a consistent time point (for this project, it was the day after the injection) was looked at, and that distance would indicate the phase shift the animal had whether it was a phase advance or a phase delay. The manipulations themselves were spaced out to occur at least 14 days apart. The animals were initially given 10-14 days to free run when put in DD to obtain enough data to make adequate predictions. From there, a minimum of 14 days in between each manipulation was needed in order to perform the subsequent phase shift calculations, along with giving the drugs enough time to clear out from the animals' systems.

2.3 Overview of Experiments

In the first experiment, global effect of three different AChE inhibitors at varying doses was explored. The goal of the first experiment was to see if modulating levels of ACh in a less invasive manner would have a significant effect on the circadian system. Subsequently, the second experiment of this project involved investigating whether the site of activity for these inhibitors could be localized to the SCN itself. This was done by blocking cholinergic muscarinic receptors within the SCN via a competitive antagonist while simultaneously administering the best performing inhibitor from the first experiment.

3. Experiment 1

3.1 Introduction

Acetylcholine has been shown to significantly influence rhythmicity in a nonphotic manner, as it is one of the neurotransmitters involved in arousal. Other systems involved with modulating arousal includes NPY, serotonin (5-HT), and hypocretin. Previous studies investigating these underlying networks found that there were afferent cholinergic projections from the substantia **i**nnominata and the nucleus basalis magnocellularis in the BF, along with projections from the LDTg and the PPTg within the brainstem (Bina et al., 1993). Moreover, firing of ACh cells have been noted to be highest during wake states and paradoxical sleep in the BF, which is involved with enhanced cortical arousal during these states due to increased theta wave oscillations seen in cortical structures (Lee et al., 2005). In parallel to this, SCN activity has peak firing during an animal's mid-subjective day (Deboer et al., 2003)**.**

On a systems level, cholinergic cells in the BF are activated during an arousing nonphotic manipulation like wheel confinement (Yamakawa et al., 2016). Additionally, nonphotic phase shifts have been observed if the BF is electrically stimulated during an animal's mid-subjective day while being held in DD (Yamakawa et al., 2016)**.** These phase shifts were comparable in direction and magnitude to phase shifts caused by intraSCN carbachol injections. Furthermore, those shifts were attenuated if the competitive cholinergic muscarinic antagonist Atropine was injected to the SCN prior to BF stimulation (Yamakawa et al., 2016). The effects of carbachol vary depending on the time of day the drug is administered, with the most prominent phase advances (~1.5 hours) being observed when directly injected to the SCN during the animals' mid-subjective day while being held DD (Bina & Rusak, 1996; Meijer et al., 1988; Wee & Turek, 1989). At the synaptic level, it is the M1/4 receptors underlying these nonphotic shifts

(Bina & Rusak, 1996**;** Basu et al., 2016)**.** When an agonist for these receptors was directly administered to the SCN of Syrian hamsters during their mid-subjective day, similar to the intraSCN carbachol injections, significant phase advances were observed (Basu et al., 2016). In short, ACh is both necessary and sufficient to significantly influence circadian rhythms in a nonphotic manner.

The use of AChE inhibitors within a circadian context has not been performed until this project. Additionally, investigating whether these inhibitors have a nonphotic influence over rhythmicity, similar to what has been seen in the previous literature, has not been addressed. There is a range of AChE inhibitors' used in the clinical context, including agents that are currently being used for patients who have AD. For this experiment, the effects of three specific AChE inhibitors used in the clinical setting were tested. The inhibitors include: Tacrine Hydrochloride, Rivastigmine Tartrate, and Donepezil Hydrochloride. All three of these inhibitors have different mechanisms of action, such as binding to different sites on the AChE molecule and differences in duration of activity. Donepezil binds to the peripheral anionic site of the AChE molecule, which slows down the inactivation of ACh by AChE before it gets metabolized within the esteratic site of the molecule. It is also non-competitive and has been shown to be the best tolerated AChE inhibitor in terms of side effects (Colovic et al., 2013). Tacrine binds to the active anionic site, and provides similar functions to Donepezil, but its duration of activity is shorter (Colovic et al., 2013). Rivastigmine binds directly to the esteratic site and inhibits the metabolism of ACh (Colovic et al., 2013). Furthermore, in humans, the half-lives of these drugs vary, specifically with Donepezil having a half-life of 70 hours, Tacrine having a variable half-life of 1.4-3.6 hours, and Rivastigmine having a half-life of 1 hour. Regardless of these mechanistic differences**,** they are functionally similar, in that they all slow down AChE from

metabolizing ACh in the synapse. Only half of the ACh molecules that are released from their vesicles make it to the post-synaptic terminal, whereas the other half are metabolized by AChE (Colovic et al., 2013); thereby, inhibiting AChE is another method to increase and prolong the activity of ACh within the synapse. However, due to the novelty of this experiment, which of these drugs and at what doses influence circadian rhythms was unknown. In terms of dosing, previous rodent studies that utilized these drugs in a cognitive and memory context were referenced. There was, however, variability across studies in this matter. However, the initial doses chosen were based on midpoints of what was published in the literature. For this experiment, these studies were used as a starting point for testing doses and expanded from there. For Donepezil, the dose was at 3mg/kg (Lian et al., 2017; Shin et al., 2018), Tacrine was 8mg/kg (Chopin & Briley, 1992; Jackson & Soliman, 1995), and Rivastigmine was 2mg/kg (Gawel et al., 2016; Papp, Gruca, Lason-Tyburkiewicz, & Willner, 2016).

Given the exploratory nature of this first experiment, the goal was to use these AChE inhibitors in a circadian context to see if this new method could emulate and add to the convergent evidence surrounding ACh and circadian rhythms. It was hypothesized that these inhibitors would have significant advancing effects on rhythmicity relative to a vehicle control when these inhibitors are administered during an animal's mid-subjective day, as seen in the previous literature.

3.2 Materials and Methods

3.2.1 Animals and housing

Male Syrian hamsters (n = 33; *Mesocrietus auratus, ~110 – 180g)* were used for this experiment. The animals were initially pair-housed and maintained in a 14:10 light:dark (LD) cycle. Animals had full access to food and water *ad libitum*. The age of the animals ranged from 10 weeks to 5 months old. When it came time for the experiment, the animals were single-housed and held in constant darkness (DD). Experimenter interventions such as entry and exit, cage changes, and troubleshooting were tracked throughout the experiments.

3.2.2 Behavioural data

Wheel-running activity was used to observe changes in circadian rhythms, and this activity was recorded in 10-minute bins by Clocklab data collection software (Coulbourn Instruments, Allentown, PA). Daily activity was recorded while the animals were in DD, which provided us comprehensive actograms to do our subsequent activity-onset predictions and phase shift calculations. At least 10 consecutive days were considered in order to predict activity onset for a manipulation, which were administered at CT6 with at least 14 days in between each manipulation.

3.3.3 Drugs and injections

Three different AChE inhibitors were tested in this experiment: Tacrine Hydrochloride (9-Amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate), Rivastigmine Tartrate, and Donepezil Hydrochloride. All the drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA). This study was exploratory in nature since these drugs have never been investigated within the circadian field. Due to this, a range of doses were tested for each of the inhibitors. There were four groups of animals used to study the effects of intraperitoneal

injections of these drugs. The doses used included**:** Tacrine Hydrochloride at 8mg/kg and 24 mg/kg, Rivastigmine Tartrate at 2mg/kg and 6mg/kg, and Donepezil Hydrochloride at 3mg/kg, 10mg/kg, 15mg/kg, and 30mg/kg.

The first set of animals (n = 12), received Donepezil at 3mg/kg, 10mg/kg, 15mg/kg, and a vehicle control. The second set of animals (n = 5) received Tacrine at 8mg/kg, Rivastigmine at 2mg/kg, Donepezil at 10mg/kg, and a vehicle control. The third set of animals (n = 12) received Tacrine at 24mg/kg, Rivastigmine at 6mg/kg, Donepezil at 10mg/kg, and a vehicle control. The fourth set of animals (n = 4) received higher doses of Donepezil at 10mg/kg, 15mg/kg, and 30mg/kg. Each set of animals received the manipulations in a counterbalanced fashion. The lower doses of the inhibitors were tested together first, followed by the higher doses. Donepezil at 10mg/kg was used when testing both the higher and lower doses of Tacrine and Rivastigmine because of the pilot data from the first round, which indicated this dose had the most robust effects over rhythmicity. Because all the manipulations were administered in a counterbalanced fashion, each animal experienced a different order of the administered drugs in order to mitigate order effects. An example of what manipulations an animal would have been administered within their respective rounds is shown in Figure 5. All the drugs were dissolved in a sterile saline solution and injected IP with the goal of testing the effects of 1mL/kg.

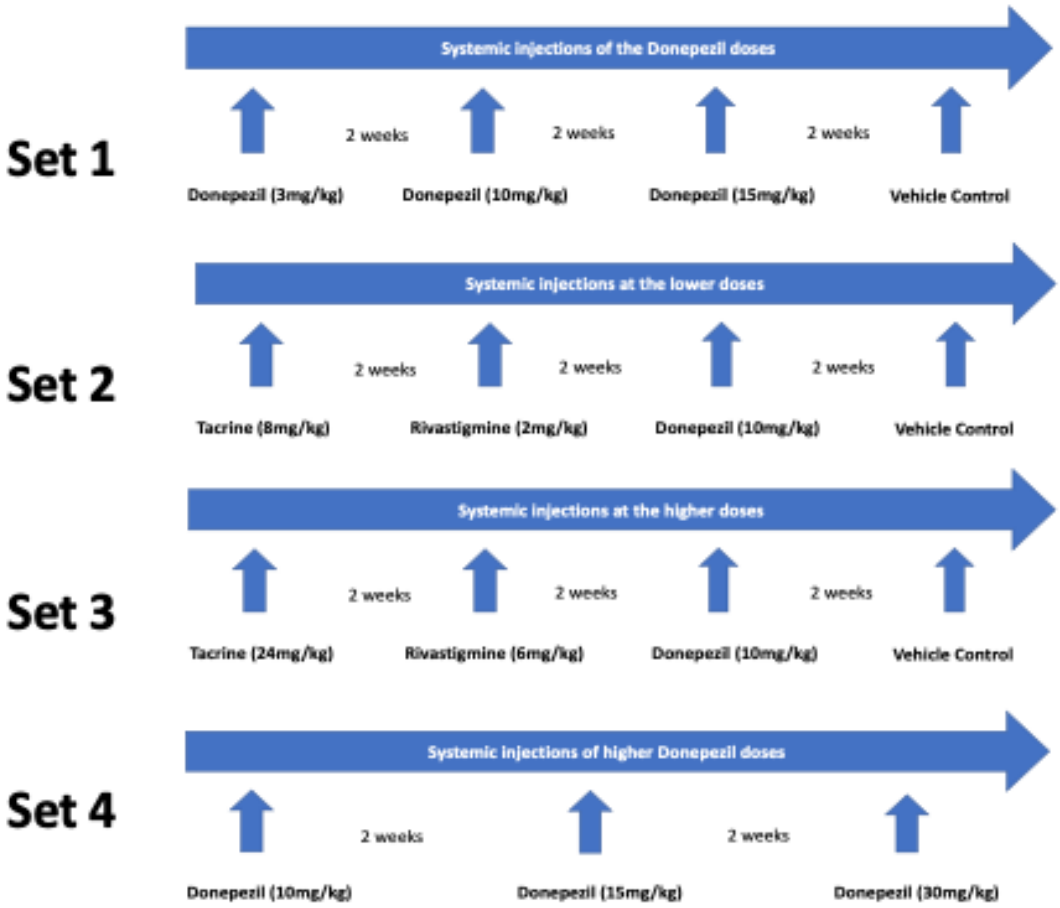

Figure 5: A visual representation of samples of the four rounds performed in experiment one containing the different drugs and doses tested in each. Separate sets of animals were used in each of these rounds, and the orders displayed in this figure are examples of what one animal would have been administered during the experiments.

3.3 Results

For this experiment, seven paired samples t-tests and one independent samples t-test were performed to analyze the datasets. The data from thirty-three animals were used in the analyses. Because of the exploratory nature of this experiment, how the drugs and their respective doses performed compared to the vehicle controls was investigated. Furthermore, since each set had different animals, the doses of those the drugs within those sets were compared to their respective control data.

The paired samples t-tests performed for Donepezil were at the doses of 3mg/kg versus the vehicle control (n = 12), at 10mg/kg versus the vehicle control (n = 28), and at 15mg/kg versus the vehicle control (n = 12). Donepezil at 10mg/kg has a larger sample size because this dose was tested across the first three rounds. For Tacrine, paired samples t-tests were performed for the doses of 8mg/kg versus the vehicle control (n = 5) and at 24mg/kg versus the vehicle control (n = 12). For Rivastigmine, paired samples t-tests were performed for the doses of 2mg/kg versus the vehicle control (n = 5) and at 6mg/kg versus the vehicle control (n = 12). The only independent samples t-test was performed for Donepezil at its 30mg/kg dose, which was compared to four of the vehicle controls from the first set of animals (n = 8).

Out of the inhibitors tested, Donepezil Hydrochloride at 10mg/kg had the largest phase advances observed relative to vehicle controls (paired t-test, $t_{(27)} = 2.17$, $p = 0.019$). However, the doses of Donepezil at 3mg/kg, 15mg/kg, and 30mg/kg did not elicit phase shifts that significantly differed from controls (paired t-test, $t_{(11)} = 0.918$, $p = 0.189$; $t_{(11)} = 0.292$, $p = 0.388$; independent samples t-test $t_{(6)} = 0.100$, $p = 0.462$).

Phase shifts elicited by Tacrine at both the lower and higher doses did not differ from the vehicle controls (paired t-test 8mg/kg, $t_{(4)} = -0.163$, $p = 0.439$; 24mg/kg, $t_{(11)} = 0.403$, $p = 0.348$).

Phase shifts elicited by Rivastigmine at both the lower and higher doses also did not differ from controls (paired t-test 2mg/kg, $t_{(4)} = 0.217$, $p = 0.420$**;** 6mg/kg, $t_{(11)} = 1.20$, $p = 0.127$).

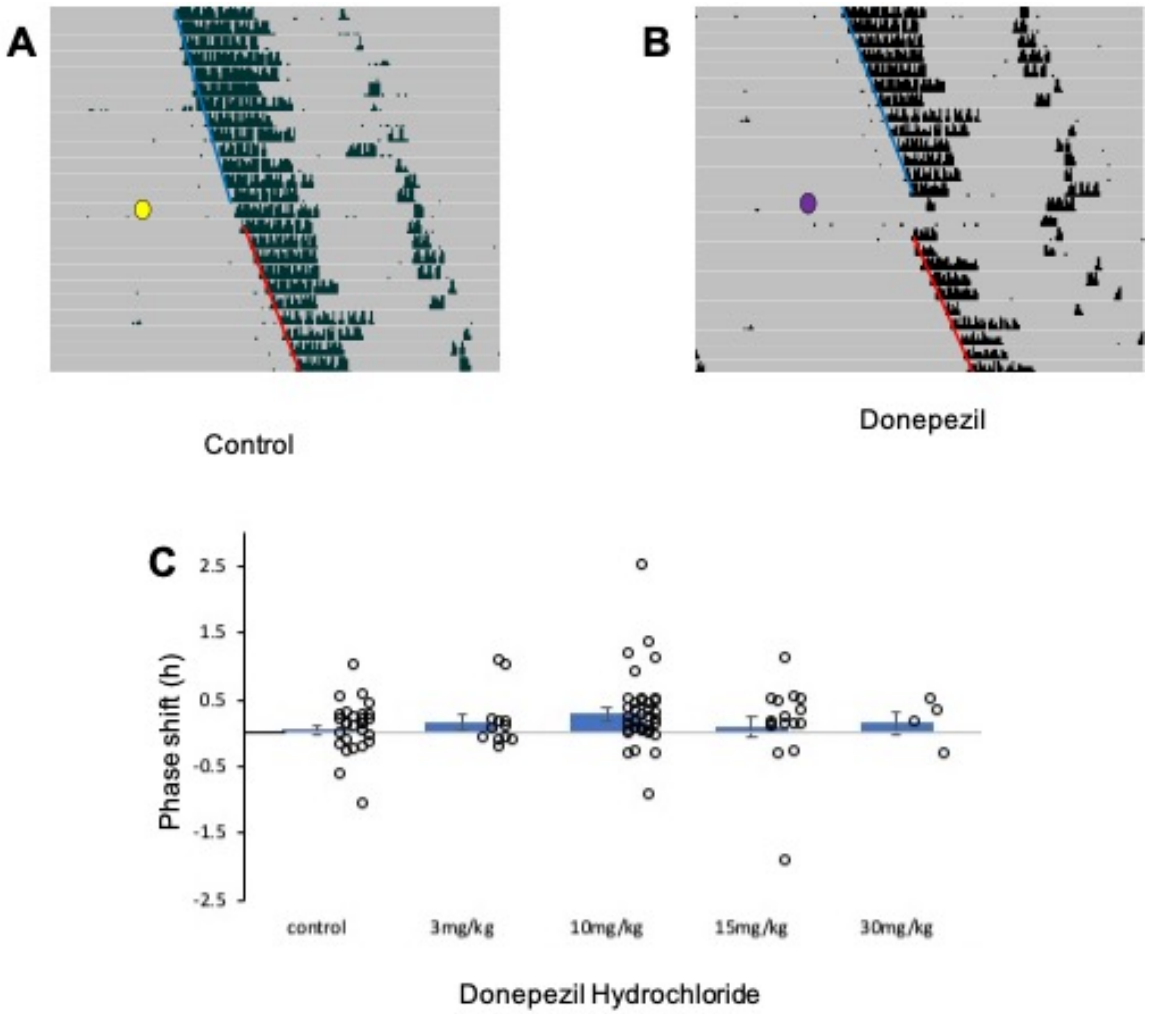

Figure 6: A) Representative actogram of wheel running activity from an animal that was administered the systemic injection of a vehicle control, with the yellow circle indicating the time (CT6) and day the injection was administered. The two diagonal regression lines have been fit to the activity onsets for the animals before and after the manipulation was administered, and the horizontal distance between the two lines indicates the phase shift. The horizontal distance between the lines the day after the injection was used in order to quantify the phase shifts. B) Representative actogram of an animal that was administered a systemic injection of Donepezil Hydrochloride at a dose of 10mg/kg at CT6 (indicated by the purple circle). C) Scatter plot of individual and mean (±SEM) phase shifts observed for Donepezil at varying doses relative to the vehicle control.

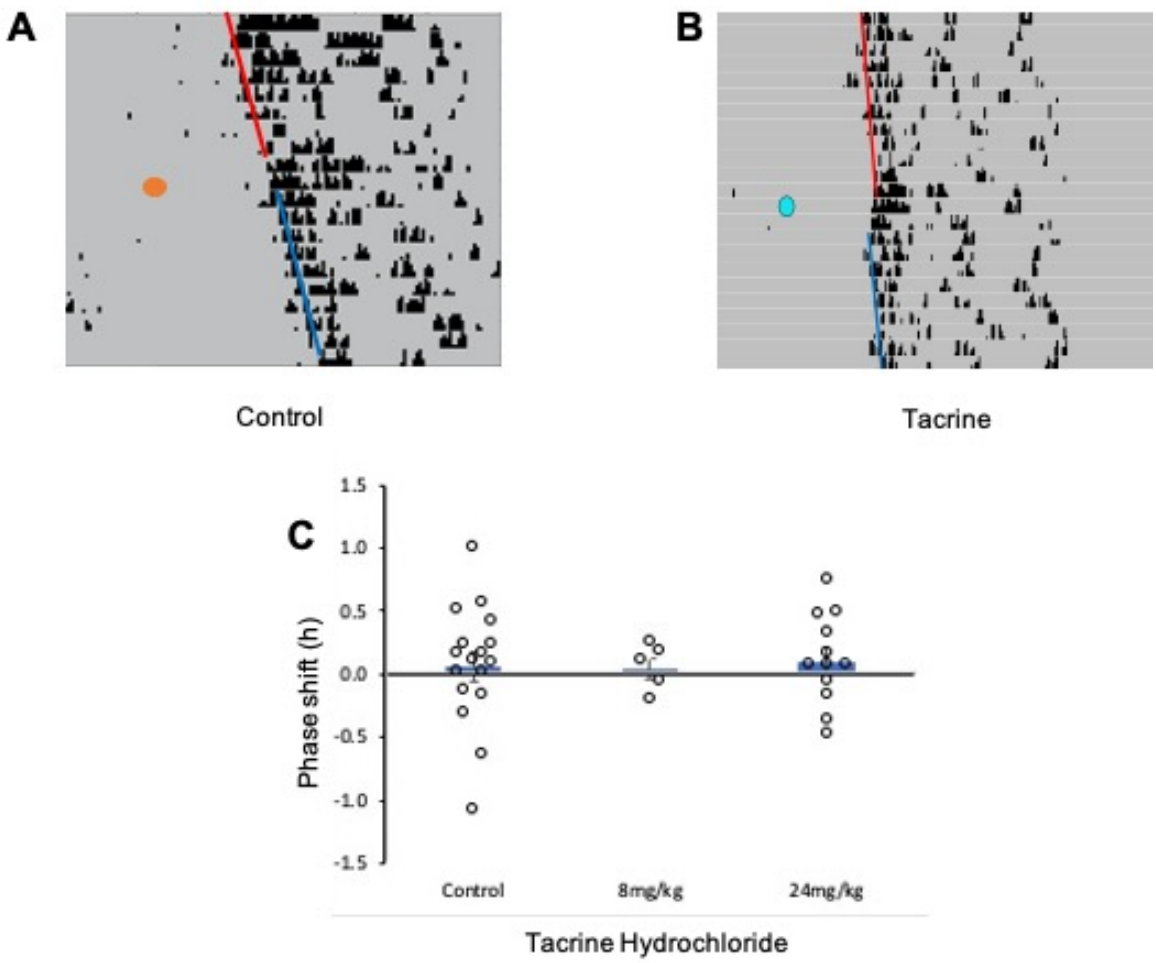

Figure 7: A) Representative actogram of wheel running activity from an animal that was administered the systemic injection of a vehicle control, with the orange circle indicating the time (CT6). B) Representative actogram of an animal that was administered a systemic injection of Tacrine Hydrochloride at a dose of 24mg/kg at CT6 (indicated by the blue circle). C) Scatter plot of individual and mean (±SEM) phase shifts observed for Tacrine at the lower and higher doses tested relative to the vehicle control.

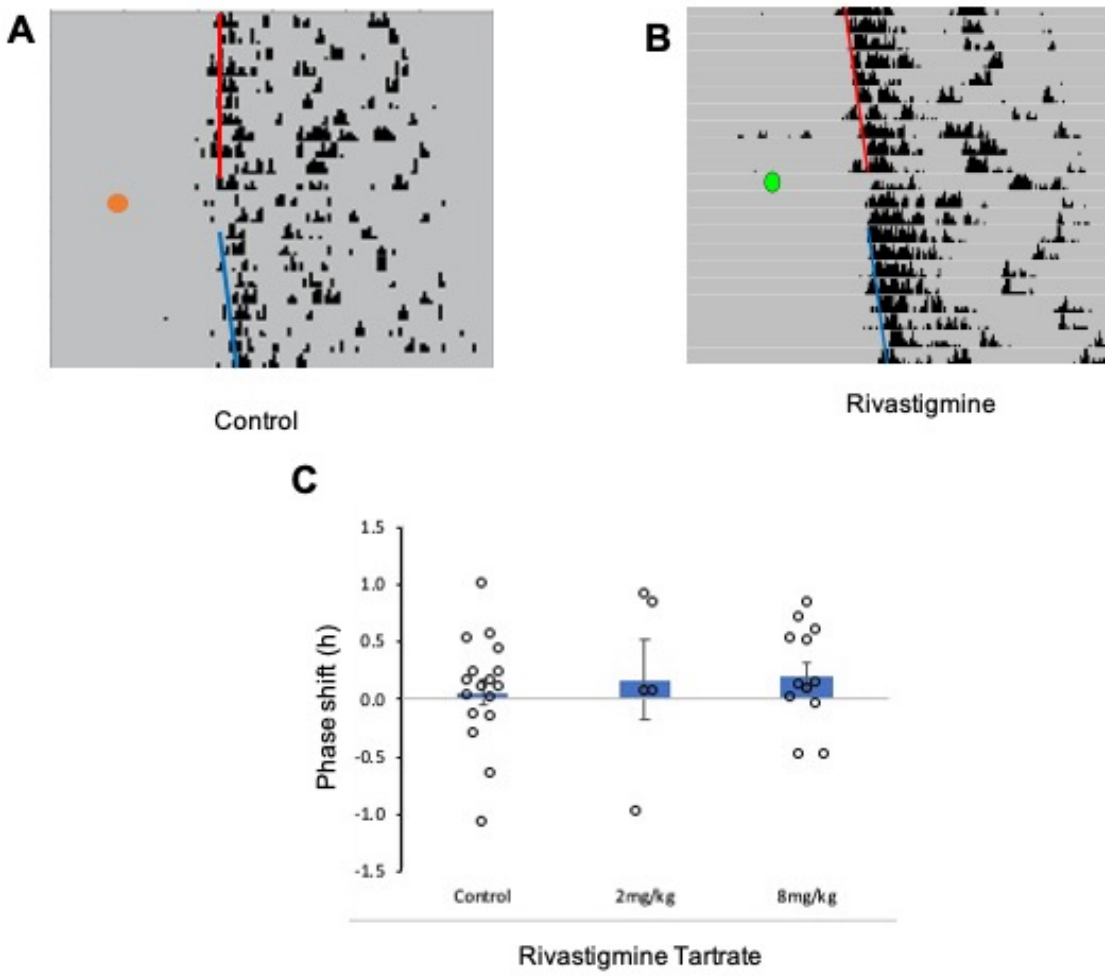

Figure 8: A) Representative actogram of wheel running activity from an animal that was administered the systemic injection of a vehicle control, with the orange circle indicating the time (CT6). B) Representative actogram of an animal that was administered a systemic injection of Rivastigmine Tartrate at a dose of 6mg/kg at CT6 (indicated by the green circle). C) Scatter plot of individual and mean (±SEM) phase shifts observed for Rivastigmine at the lower and higher doses tested relative to the vehicle control.

3.4 Discussion

In this experiment, an investigation into which of the AChE inhibitors tested had the most robust influence over circadian rhythms when compared to a vehicle control was performed. Out of the three that were used, Donepezil Hydrochloride at a dose of 10mg/kg performed the best when compared to a vehicle control. Specifically, significant phase advances were elicited by Donepezil when administered by IP injection during the animals' mid-subjective day (CT6); however, there were multiple paired samples t-tests performed on this dataset. Therefore, even though this specific comparison was found to be statistically significant, when multiple comparisons' corrections are taken into consideration, that is no longer the case.

That said, with increased power and further replication, if these drugs can significantly influence rhythmicity in a nonphotic manner, there are potential therapeutic avenues that can be investigated due to the less invasive nature of these drugs and their current used in the clinical setting. Furthermore, because the doses chosen and tested were exploratory, more troubleshooting is required in order to formulate an accurate dose response curve for this context. Future studies can expand the doses tested, along with perform direct statistical comparisons between the them to see if there were any statistically significant differences versus comparing them only to the vehicle controls.

These drugs in-and-of themselves have different mechanisms of action. For instance, bioavailability ratios could also play a role in the differences between them. Donepezil is selective to the CNS and has a 100% bioavailability ratio at starting doses of 5 - 10mg with oral administration in humans, along with being able to pass through the blood-brain-barrier easily. This means that after undergoing first pass metabolism, the active metabolites remaining are proportional to the amount of Donepezil that was initially ingested**.** Furthermore, it has slow

excretion and has a half-life of approximately 70 hours in humans (Colovic et al., 2013; Mcgleenon et al., 1999). Rivastigmine, which is also selective to the CNS, has a 40% bioavailability at the 3mg dose when administered orally, and a half-life of approximately 1 hour. Its duration of action, however, can last upwards of 10h (Colovic et al., 2013; Mcgleenon et al., 1999). Tacrine, however, has the lowest and most disproportionate bioavailability ratio, with 2-3% availability when administered orally. Furthermore, there is not a linear relationship between dose and half-life due to the disproportionate bioavailability ratio, and this drug has a variable half-life of approximately 1.4-3.6 hours (Colovic et al., 2013; Mcgleenon et al., 1999). When this drug undergoes first pass metabolism, the amount of active metabolites remaining do not match the initial dose administered, and because of this, it is difficult to predict how long Tacrine will remain in the body, along with how much of an effect certain doses will have (Colovic et al., 2013; Mcgleenon et al., 1999). These pharmacological differences could have contributed to the variability in this dataset—more specifically, it could be the duration of the drugs' actions that could be involved. Considering half-lives, it takes six half-lives for a drug to be completely excreted from the body. Since Donepezil does have a half-life of 70 hours in humans, it would take approximately 18 days for it to clear out of the body. This is long, especially if compared to Rivastigmine, which has a half-life of 1 hour. Further investigation would need to occur in order to see if the duration of action for these agents contributes to behavioural differences**.**

The results from this experiment show that Donepezil's effects at 10mg/kg were consistent with the convergent evidence for ACh's role in nonphotic phase shifting in causing phase advances when administered during the mid-subjective day in constant conditions. The use of these AChE inhibitors was another way to investigate the effects of increased cholinergic

activity on circadian rhythms, and like the intraSCN carbachol injections (Bina & Rusak, 1996; Meijer et al., 1988; Wee & Turek, 1989)**,** stimulation of the BF (Yamakawa et al., 2016), and activating specific muscarinic receptors within the SCN **(**Basu et al., 2016), overall advancing effects were observed. Future directions for this experiment could include characterizing a PRC for these inhibitors, specifically mapping what sorts of effects would be seen when these drugs are administered at different points in a 24-hour period. Given the evidence surrounding carbachol and its PRC, it could follow by inducing prominent phase advances if administered during an animal's mid-subjective day, along with phase delays if administered during the early subjective night (Wee & Turek, 1989).

The evidence surrounding ACh and circadian rhythms suggests that when ACh or ACh-like activity is increased during an animal's inactive phase, rhythms are reset in an advancing manner. However, nonphotic influences over circadian rhythms go beyond ACh. NPY, serotonin, and hypocretin also play a significant role in modulating arousal levels. Like ACh, modulation of both NPY and 5-HT levels have been shown to significantly decrease spontaneous firing in SCN cells *in vitro* when applied during the mid-subjective day (Liou & Albers, 1991; Shibata et al., 1983)**.** Similarly, increasing levels of these neurochemicals during the mid-subjective day *in vivo* significantly influence circadian rhythms in an advancing manner (Huhman & Albers, 1994; Ying & Rusak, 1994)**.** Hypocretin is also strongly linked to arousal and locomotor behavior (Mieda et al., 2004), as the presence of hypocretin-1 receptors, and projections from the LH are present in the IGL and areas closely surrounding the SCN (Chen et al., 1999; McGranaghan & Piggins, 2001)**.** All of these neurochemicals act through GPCRs, which could subsequently cause changes in gene expression through secondary messenger pathways and signaling cascades (Webb, Antle, & Mistlberger, 2014). Since ACh interacts with

muscarinic receptors, and these are the receptor subtypes that underly its circadian influence, the molecular mechanisms could be similar. Therefore, understanding ACh and its cellular dynamics within a circadian context will provide more insight to the large nonphotic network influencing rhythmicity. Given the findings of Yamakawa et al., (2016), a likely location of action for AChE mediated phase shifts would be the SCN, a hypothesis tested in the following chapter.

4. Experiment 2

4.1 Introduction

Following the first experiment investigating if the effects of different AChE inhibitors administered during animals' mid-subjective day would cause phase advances, Donepezil Hydrochloride at 10mg/kg had the most robust influence over circadian rhythms. A caveat to the first experiment is that since these inhibitors were administered IP, it was unclear where in the brain Donepezil was acting. The present experiment tested the hypothesis that Donepezil produced phase shifts by increasing cholinergic activity in the SCN. Given that the SCN controls all endogenous rhythms, and previous studies have found that targeting the SCN while manipulating afferent cholinergic activity to the nucleus displayed significant changes to circadian rhythms, it is the first area of interest for this project**.**

At the synaptic level, the cholinergic influence over rhythmicity has been narrowed to the muscarinic receptors (Basu et al., 2016; Bina & Rusak, 1996; Yang et al., 2010)**,** as the activation of these receptors can influence second messenger pathways and signaling cascades, which can ultimately lead to changes in gene expression (Webb et al., 2014)**.** Out of the five muscarinic receptor subtypes, the M1/4 receptors are especially important (Basu et al., 2016; Yang et al., 2010). For instance, activating these receptors with carbachol has been shown to inhibit SCN cell firing *in vitro.* Furthermore, the inducible phase shifts caused by carbachol can be attenuated with a M1/4 antagonist (Yang et al., 2010), adding to the current evidence that the cholinergic muscarinic receptors subtypes are the ones primarily involved with modulating rhythms. The mechanisms of these receptors are functionally similar to NPY and 5-HT, as these two neurochemicals also act on GPCRs, as silencing of spontaneous cell firing *in vitro* has been observed across all of these receptor subtypes (Liou & Albers, 1991; Shibata et al.,1983).

Furthermore, modulating activity of these two systems *in vivo* also leads to rhythms being significantly advanced (Huhman & Albers, 1994; Ying & Rusak, 1994)

While there are multiple systems and structures involved with arousal, the area of interest in investigating what is occurring at the SCN itself. This was done by observing whether any inducible changes to rhythmicity caused by an IP administration of Donepezil at 10mg/kg during the mid-subjective day could be attenuated by blocking the muscarinic receptors within the SCN. Specifically, the competitive cholinergic antagonist Atropine, which acts on muscarinic receptors**,** was utilized and administered directly to the SCN prior to the IP injection of Donepezil. It was hypothesized that no significant changes to rhythmicity would be observed with the combination of the Donepezil/Atropine injections versus significant phase advances with the injections of Donepezil/vehicle control. The Donepezil/Atropine condition is of interest, as it would be compared to the Donepezil/control condition, which is the baseline measurement for this experiment.

4.2 Materials and Methods

4.2.1 Animals and housing

Male Syrian hamsters (n = 20; *Mesocricetus auratus, ~110 – 180g)* were used for this experiment. The animals were initially pair-housed and maintained in a 14:10 light:dark (LD) cycle. Animals had access to food and water *ad libitum*. The animals were 10 weeks old to 5 months old before they underwent the surgeries for this experiment. Following the surgeries, the animals were single housed, and after they had adequately recovered from the procedure (at least a week of recovery), they were then put into 24-hour constant darkness condition (DD).

4.2.2 Behavioural data

Wheel-running activity was used to observe changes in rhythms, and like the previous experiment, activity was recorded in 10-minute bins by the Clocklab data collection software (Coulbourn Instruments, Allentown, PA). Daily activity was recorded while the animals were in DD, which provided us the same comprehensive actograms to do the subsequent activity-onset predictions and phase shift calculations. At least 10 consecutive days were considered in order to predict activity onset for manipulations, which were administered at CT6 with at least 14 days in between each manipulation.

4.2.3 Surgeries

A guide cannula implantation was required to gain access to the SCN for the intracranial injections. Animals were at least 10 weeks of age before undergoing the surgery and weighed at least 110g before the procedure. An IP injection of general anesthetic sodium pentobarbital (120mg/kg; CEVA) was administered, followed by a subcutaneous injection of the analgesic Meloxicam (1mg/kg). A gaseous anesthetic (Isoflurane) was supplemented continuously throughout the surgery. For the insertion of the 9 mm 22-gauge stainless steel guide cannula

(Plastics One Inc., Roanoke, VA, USA), the coordinates used were 0.3 mm anterior to bregma, 0.3 mm lateral to the midline, and 7.0 mm ventral from the surface of the skull. The injection cannula used extended 1mm beyond the guide cannula tip, and a dummy cannula was inserted into the guide cannula to prevent obstructions from occurring within the guide cannula. An example of this insertion is seen in Figure 9**.** After the surgeries were performed, the hamsters required at least one week of recovery time, where they would be experiencing a typical 14:10 light-dark cycle. When the hamsters recovered, they were provided new cages with wheels installed into the cage tops with the magnetic switches. When the hamsters were in DD, they were given 10-14 days to free-run or establish their own rhythms.

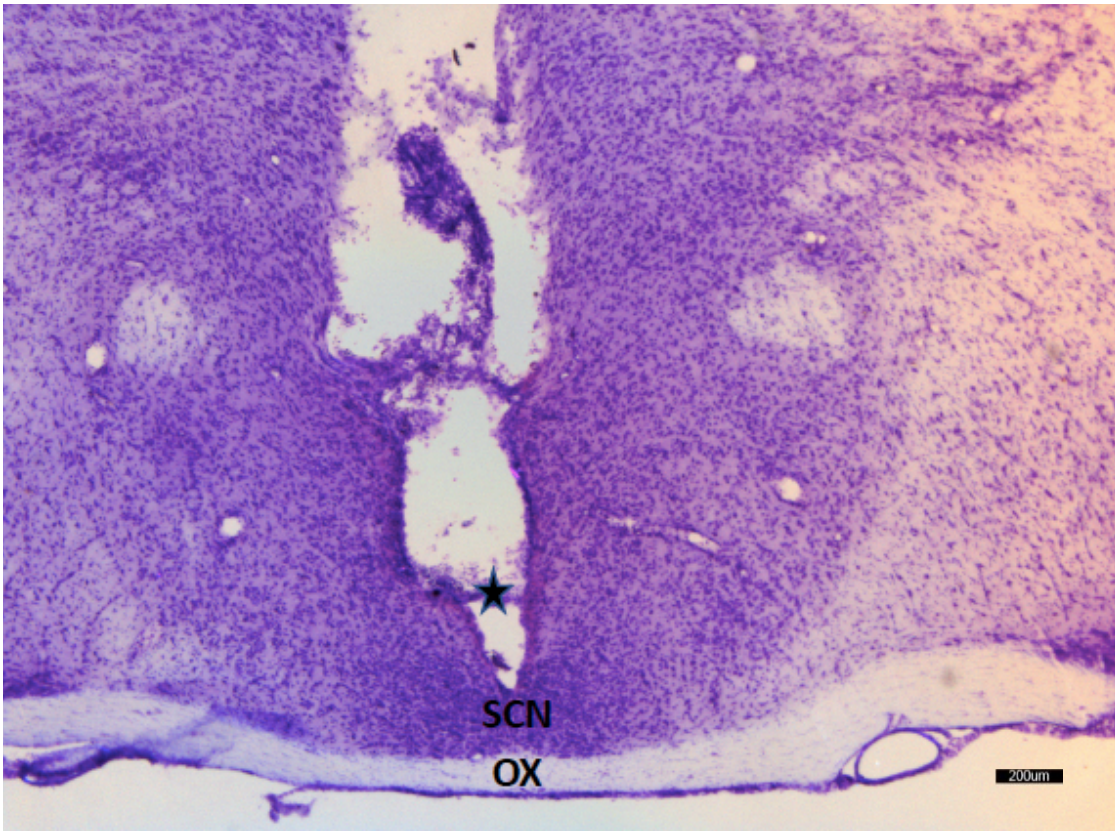

Figure 9: Representative image of a Cresyl violet-stained Syrian hamster brain with an intraSCN guide cannula insertion and injector tip that falls within 500-µm of the SCN. The star marks the injector tip, SCN marks the nucleus itself, and OX marks the optic chiasm.

4.2.4 Drugs and injections

For this experiment, there were two different drugs that were utilized—Donepezil Hydrochloride and Atropine sulfate**.** Both the drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA), and they were both dissolved in sterile saline (0.9%). For the IP injections, Donepezil was the inhibitor of choice, and the dose tested was 10mg/kg. For the IC injections, Atropine was used. The concentration was 3mg/mL and 0.5µL was administered to the animal intracranially. Atropine's activity in the brain lasts for ~3 hours. Both injections were administered at CT6, and the injections occurred with at least 14 days in between each manipulation. A regression line was fit for the activity onsets of the animals in order to obtain the predicted activity onset time (CT12).

On the day of the manipulations, the IC injection was performed first to block the muscarinic receptors within the SCN, and within 10 minutes, the IP injection followed. The IC injection required restraint of the animal, followed by the removal of the dummy cannula present in the guide cannula. Once completed, an injector tip was inserted into the guide, and this injector tip was attached to 1 µL Hamilton syringe via PE20 tubing. A 0.5µL injection of either Atropine or the vehicle control was administered directly to the SCN. The injection was administered over 1 minute, after which the injector tip was left in the guide cannula for at least another minute to permit the diffusion of the drug. Once complete, the injector tip was removed, and the animal was restrained for the IP injection of either Donepezil or a vehicle control. When the IP injection was done, the dummy cannula was placed back in the guide cannula.

There were 4 manipulations for this experiment, which were all administered in a counterbalanced fashion. The manipulations included a) an IP injection of Donepezil with an IC injection of Atropine, b) an IP injection of Donepezil with an IC injection of a vehicle control, c)

an IP injection of a vehicle control with an IC injection of Atropine, and d) a vehicle control for both the IP and IC injections. All the manipulations were administered in a counterbalanced fashion. An example of what manipulations an animal would have been administered is shown in Figure 10.

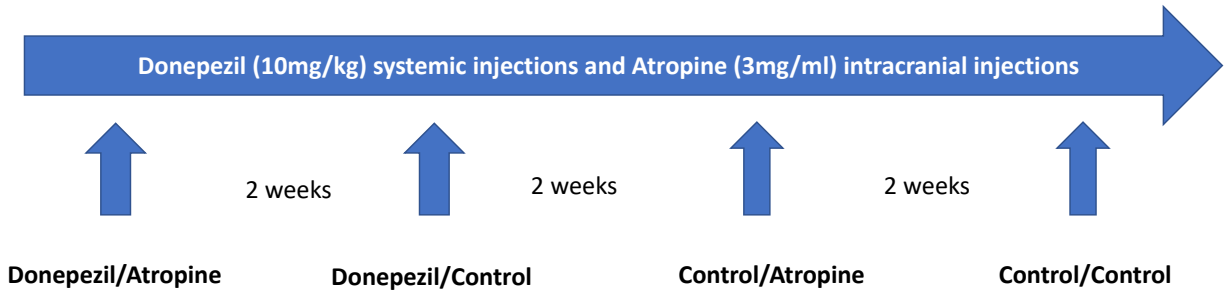

Figure 10: A visual representation of the IP/IC injection round performed in experiment two containing the different injection conditions. The order displayed in this figure is an example of what one animal would have been administered during the experiments.

4.2.5 Histology

To confirm the SCN was targeted accurately, histology was performed to measure the distance between the cannula tip and the perimeter of the SCN. This was performed to decide which animals would be included/excluded in the data analysis. For the brain collection, the hamsters were perfused using 4% paraformaldehyde, followed by being stored in paraformaldehyde for a minimum of 24 hours before being transferred to a 20% sucrose solution for cryoprotection. A cryostat was used to slice and collect the tissue**.** Coronal sections of the anterior hypothalamus were collected in 35-µm sections. The sections of interest were then mounted on gelatin-coated slides and were subsequently stained with cresyl-violet. If the tips of the intraSCN injection cannulas fell within 500-µm of the margin of the SCN, the animals' data were included in the analyses (see Figure 9).

4.3 Results

From the 20 animals that underwent and survived the cannula surgeries, there were 13 animals that received all 4 of the IC/IP injections. Eight of those 13 animals had cannulae appropriately placed and counted towards the final analysis. The remaining seven animals were administered only the Donepezil/Atropine and Donepezil/control injections. Two of those seven animals' data had their cannulae appropriately placed. This left an overall sample size of n = 10. Using a 2x2 repeated measures ANOVA to analyze the dataset, the combination of Donepezil/Atropine did not elicit any phase shifts different from the vehicle control ($F_{(1,7)}$ = 0.0127, p = 0.914). However, the Donepezil/control condition did not elicit any phase shifts that differed from the controls ($F_{(1,7)}$ = 0.258, p = 0.627).

The Donepezil/control condition was a control or baseline measurement to compare the other experimental findings to. With these results being mixed, it is difficult to make such comparisons. Because of this disconnect, a subgroup analysis was performed to explore the original hypothesis in those animals that had phase shifts to Donepezil and intraSCN saline matched the phase shifts to Donepezil in experiment one. Results of this subgroup analysis are presented in Figure 12. While the sample was too small to allow statistical analysis, phase shifts to Donepezil were smaller when animals were administered intraSCN injections of Atropine than when administered intraSCN injections of saline.

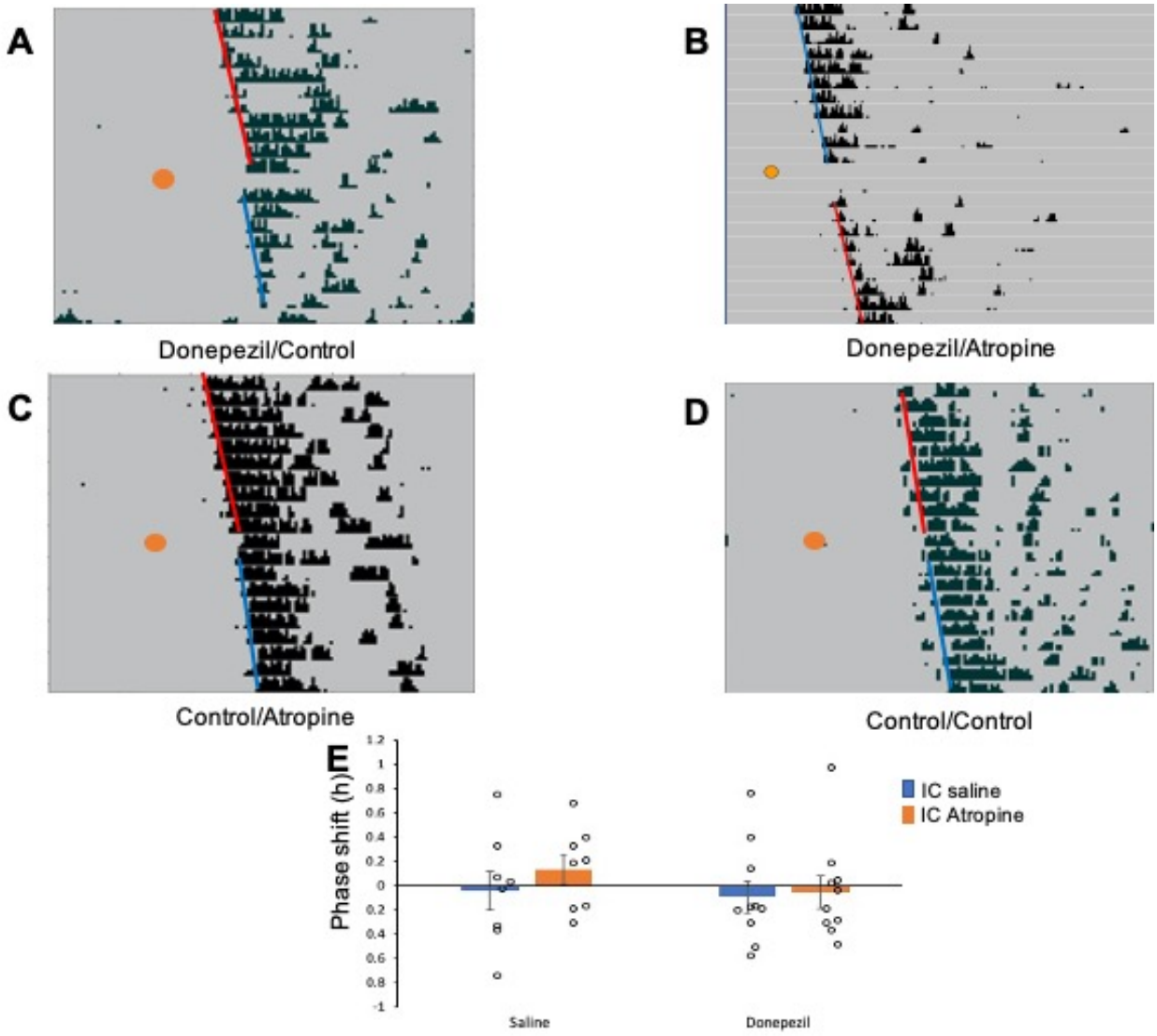

Figure 11: A) Representative actogram of wheel running activity from an animal that was administered an IP injection of Donepezil at 10mg/kg and an IC injection of a vehicle control, with the orange circle indicating the time of the injection (CT6). B) Representative actogram of an animal that was administered an intraSCN 0.5µL injection of Atropine (3mg/mL) and an IP injection of Donepezil 10mg/kg with the orange circle indicating the time of the injection (CT6). C) Representative actogram of wheel running activity from an animal that was administered an IP injection of the vehicle control and an intraSCN 0.5 µL injection Atropine (3mg/mL), with the orange circle indicating the time of the injection (CT6). D) Representative actogram of an animal that was administered an intraSCN and an IP injection of the vehicle control with the orange circle indicating the time of the injection (CT6). E) Scatter plot of individual and mean (±SEM) phase shifts observed for the four different IC/IP injections administered.

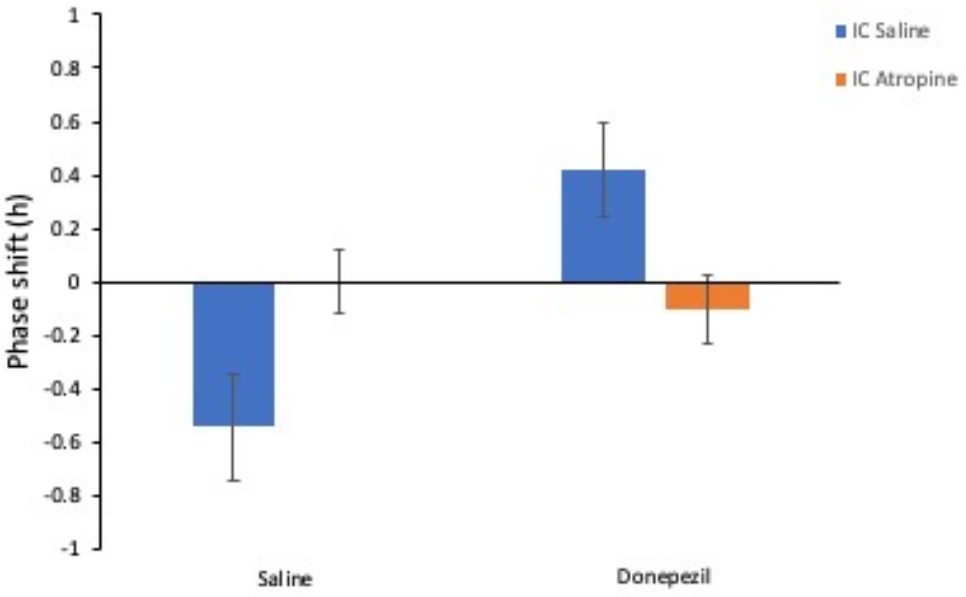

Figure 12: Means (±SEM) of the subgroup (n = 3) phase shifts observed for the 4 different IC/IP injections relative to the vehicle control. Selection criteria for this subgroup was based on how much the animals shifted for the baseline measurement of the Donepezil/control condition.

4.4 Discussion

In this experiment, an investigation into whether the effects of Donepezil Hydrochloride could be localized to the SCN by attempting to attenuate inducible phase shifts caused by an IP injection of this inhibitor by using an intraSCN injection of the muscarinic ACh antagonist Atropine. In the first experiment, since an IP administration of Donepezil is acting everywhere in the body, it was unclear if the phase shifts were elicited due to direct activity at the SCN or were secondary to increased cortical arousal due to enhanced ACh activity elsewhere in the brain. Out of the four experimental conditions that were tested, the two conditions of experimental focus were the Donepezil/Atropine and the Donepezil/vehicle control conditions. The purpose of the Donepezil/control condition was to be a baseline treatment for the Donepezil/Atropine findings to be compared to. However, in this study, the Donepezil/control condition yielded results that did not match the first experiment, in that no robust differences in rhythms were observed relative to the controls. Because the baseline measurement in this experiment had mixed results, it makes it difficult to compare and make conclusions about the other conditions of interest. There were, however, notable findings with further inspection of the data. As mentioned in the results, a subgroup analysis was performed looking at the animals that did respond to the Donepezil/control injections. In these same animals, the phase shifts in the other conditions were minimal, including the Donepezil/Atropine condition**.** No statistical claims can be made about this analysis, however this experiment should be replicated and investigated further.

The inconsistency between the experimental findings can be attributed to different factors. One of the issues with this experiment was the lack of power. Out of the 20 animals that were subjected to the cannula surgeries, a sample size of n = 10 remained. Furthermore, the second round of this experiment was interrupted due to university closures in response to the

COVID-19 pandemic. There were four rounds of manipulations planned, with one additional carbachol injection that was going to be performed for functional confirmation; unfortunately, only two of the manipulations could be performed, and that too performed in two weeks provided to students for emergency research designation. There were no other opportunities to modify this experiment or increase the sample size after those manipulations were performed. Another issue that contributed to the mixed findings is with the injections themselves. Receiving repeated IP and IC injections have inadvertent effects, specifically with receiving multiple IC injections over a prolonged period. These injections, if the guide cannulae are within the 500-μm range, can damage the SCN over time, which could play a part in the deterioration of their rhythms. Furthermore, the longer the animals remain in DD, the more their rhythms deteriorate as well. In addition, the guide cannula itself is invasive, so it is possible that the damage from its insertion could have caused behavioural deficits. Whether other afferent projections to the SCN, such as the IGL or LH, are damaged with this procedure would require further investigation**.**

Future investigations regarding AChE inhibitors in a circadian context will add to the convergent evidence surrounding the cholinergic influence on circadian rhythms in a nonphotic manner. Additionally, using this technique to modulate levels of ACh in a less invasive manner will be beneficial for future chronotherapeutics. Specifically, the use of these inhibitors can be utilized for future investigations on the deterioration of rhythms (e.g. with a neurodegenerative disorder like AD) within a circadian context, and whether these inhibitors can significantly influence rhythms in a nonphotic manner**.** ACh not only influences circadian rhythms, but also contributes to the maintenance of them under normal conditions. The evidence supporting claim includes peak firing rates being seen in both the BF and the SCN during REM sleep and regular

wake states (Deboer et al., 2003; Lee et al., 2005). Additionally, the LDTg and PPTg relay information about vigilance states to the SCN during slow wave activity (SWA) during sleep (Abbott et al., 2013; Deurveilher & Hennevin, 2001). In addition, there are projections from the BF and the brainstem to the SCN, along with the presence of cholinergic receptors in the nucleus itself (Bina et al., 1993). Also, both cholinergic projections to the SCN and cells within the BF are activated during an arousal-inducting nonphotic manipulation (Yamakawa et al., 2016). Furthermore, within the SCN, it is the M1/4 receptors that underly nonphotic phase shifts (Basu et al., 2016; Bina & Rusak, 1996; Liu & Gillette, 1996; Yang et al., 2010). Considering all this evidence**,** deeper investigations into ACh in the nonphotic context is crucial for understanding circadian rhythms as a whole.

5. General Discussion

5.1 Summary

For these experiments, we investigated if different AChE inhibitors could significantly phase advance circadian rhythms. Furthermore, whether the effects of these inhibitors could be localized to the SCN was also a question of interest. In the first experiment, three different inhibitors at varying doses were tested: Tacrine Hydrochloride, Rivastigmine Tartrate, and Donepezil Hydrochloride. Donepezil at 10mg/kg had the most robust influence over circadian rhythms when compared to a vehicle control. Even with the promising pilot data that emerged early on in this study**,** it is no longer significant when multiple comparisons are taken into consideration, leaving the results mixed. For the second experiment, which was conducted in parallel with the first, since the AChE inhibitors were administered systemically, it was unknown whether Donepezil was acting directly at the SCN, or if the phase shifts were secondary to arousal caused by the activity of Donepezil working elsewhere in the brain. Cholinergic muscarinic receptors were targeted and blocked with Atropine in the SCN to see if any inducible phase shifts caused by Donepezil would be attenuated by the blockage of these receptors. The condition where systemic Donepezil with an intraSCN injection of a vehicle control was administered did not match the findings from the previous experiment, in that significant phase shifts were not observed. Even though, statistically speaking, there was no significant difference, it is still a research question worth replicating.

5.2 Limitations

There are other overall issues that affected both experiments, with the first one being the age of the animals. While the minimum age of the animals was kept at least 10 weeks old before beginning the experiments, there were animals used throughout this study that were much older

than that. The age discrepancy could be contributing to the variability seen in the data, as rhythms do deteriorate with increasing age. Along those lines, the length the animals were kept in DD also play a role in rhythmicity, as the longer animals are held in constant conditions, the worse their rhythms get. While there were attempts to maintain the 14-day period in between manipulations, issues with obtaining consistent activity were inevitable, especially with the animals who received the cannula surgeries. Furthermore, the guide cannula surgeries could have caused tissue damage, which could lead to behavioural deficits**.**

5.3 Future directions

The use of these AChE inhibitors within the circadian field was a new endeavor and had not been investigated prior to the present experiments. Future amendments for this project include using animals of consistent younger ages and ensuring enough animals per experiment in order to account for power, while also maintaining exceptional animal use, ethics, and care. Also, for the guide cannula insertions, different lengths of guides can be used in the future to investigate if there are any observable differences in behaviour. Future studies can also investigate whether there are differences between the doses of the drugs, along with whether the inhibitors themselves differ substantially. Repeated measure one-way analyses of variances (ANOVA) could be used for the statistical analyses for such research endeavors. In addition, only male hamsters were used across both experiments. While the circadian field has strayed away from using female hamsters and rats for experiments relying on endogenous rhythms due females' robust estrus cycles, it does not dismiss the possibility of sex effects associated with these inhibitors. Future studies should include females into the experimental design and test for sex effects, however additional measures will be required in order to track the estrus cycles of the females. Other biological models, such as mice, can be utilized to investigate this research

question, as the estrus cycles in mice does not impact the circadian locomotor rhythms as severely as it does with rats and hamsters. In addition, the phase shifts observed could have been due to the manipulations causing wakefulness rather than fluctuating levels of ACh at the SCN. Further investigation would require looking deeper into the changes in wheel-running activity (e.g. measuring changes in wheel revolution) before the manipulation and for 2-3 days post-injection to see if there are any immediate differences in behaviour. In addition, different time points can also be investigated to administer the AChE inhibitors while an animal is in DD. Referring to the carbachol PRC, the mid-subjective night is another time when significant phase advances have been observed in hamsters (Wee & Turek, 1989). Given that CT6 is in the middle of their inactive phase, their wakefulness levels could vary when the injections were administered compared to activity during an animal's active phase. Wakefulness levels do play a role in how much an animal shifts, with increased levels of wakefulness leading to larger phase shifts, and vice versa with lower wakefulness levels (Antle & Mistleberger, 2000). This ties back into formulating a PRC for these inhibitors and finding that optimal time where its effects are at their strongest. Lastly**,** intraSCN injections of the AChE inhibitor itself during the mid-subjective day can be performed and then compared to a vehicle control. At the systems level, different structures outside of the SCN can be targeted for these intracranial injections. For example, to gain more insight into the connections between the BF and the SCN, these inhibitors could be directly administered to the BF, with an intraSCN injection of Atropine administered prior to the AChE injection.

Changes in molecular mechanisms can also be investigated in future studies. For instance, an IP injection of AChE inhibitor during an animal's mid-subjective day can be administered, after which immunofluorescence staining cane be performed to see if there is

expression of the immediate early gene c-Fos the SCN. Following that study if positive results are observed, specific genes and their respective expressions can be investigated. Since the M1/4 receptors are GPCRs, changes in gene expression of PER can be looked into. For instance, immunohistochemistry for *Per1* levels within the SCN can be performed. These experiments can be extended to similar methods that were utilized in this study with the simultaneous administration of the AChE inhibitor systemically and an intraSCN injection of Atropine during the animal's mid-subjective day, followed by immunohistochemistry for *Per1*. Increased cholinergic activity with these AChE inhibitors during the mid-subjective day could lead to increased activation of M1/4 receptors, which causes a suppression of *mPer1* gene expression, which could lead to an advanced phase in rhythmicity. From the c-Fos and *Per1* immunohistochemistry studies, it is predicted that cell activity and expression for animals receiving the IP injection of the AChE inhibitor would be significantly lower than controls.

There are also techniques that can be used in order to investigate the cellular dynamics involved with cholinergic activity modulation and rhythmicity. The use of transgenic mice, specifically with *Cre*-Lox system is an example of such a technique. Theoretically, a specific *Cre* line could be created that would conditionally knockout or silence cholinergic neurons (e.g. by knocking out the gene for vAChT) via an adeno-associated virus (AAV) in areas like the BF or brainstem that project to the SCN, investigating whether long-term silencing of these projections could significantly influence rhythms. Furthermore, enhanced characterization of the cholinergic network and the circadian system can also be done using a retrograde transducing Canine Adenovirus 2 that expresses *Cre* (CAV2-Cre) and providing dual injections of CAV2-Cre and a retrograde AAV (Gore, Soden, & Zweifel, 2014) into areas like the BF and the SCN to visualize the connections in more detail. This CAV2-*Cre* model can also be used for other sophisticated

genetic techniques such as selective gene expression and deletion (Gore et al., 2014). Additionaly, the effects of AChE inhibitors can be investigated using in-vivo live cell imaging technology in mice. Using fluorescent calcium signaling, a fiber-optic mini camera can be implanted into the region of interest so that it detects activation of cells via calcium signaling and gCAMP expression (e.g. in the SCN). Usjng selective live cellular microscopy techniques, an IP injection of Donepezil Hydrochloride at 10mg/kg can be administered, and then the changes in cellular activity in real time can be observed.

5.4 Conclusion

To summarize, the goal of the present experiments was to test a new method of modulating cholinergic activity in a less invasive manner by using AChE inhibitors to investigate whether these drugs could significantly influence circadian rhythms in a nonphotic manner when administered to hamsters during the mid-subjective day. Donepezil Hydrochloride at 10mg/kg produced significant phase advances relative to controls. However, these results should be interpreted cautiously, as, in the context of the wider experiment, this difference was no longer significant when corrections for multiple comparisons were applied. The effects of these inhibitors could be localized to the SCN by administering an intraSCN injection of Atropine with the simultaneous systemic injection of Donepezil was also investigated, and those findings were variable. While the results were mixed, further replication is encouraged given the novelty of this technique and the translational aspect of utilizing these drugs. Overall, modulating cholinergic activity is a part of the larger nonphotic network involving arousal, which significantly influences circadian rhythms as a whole. Understanding the complex interplay between the cholinergic system and rhythmicity is one step closer in understanding how organisms are able to function on a daily basis.

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