Bi-directional interactions between cognition and circadian rhythms

by

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In loving memory of Howard Earl Gritton

My father passed far too early but I feel his presence everyday of my life and I thank him for his guidance, wisdom, and love.

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Abstract

Circadian rhythms are ubiquitous and dynamic, allowing organisms to adapt and anticipate key environmental cues under a variety of conditions. Circadian systems are also complex regulatory mechanisms, involving the synchronization and coherent activity of many individual oscillators to maintain normal function. The loss of coherence amongst individual oscillators, characteristic of shift-work and some neuropsychiatric illnesses, results in a state of internal desynchrony that has been proposed to impact human health and productivity. Research described in this dissertation focuses on the interaction between forebrain cholinergic signaling and circadian rhythms. Furthermore, this research presents an animal model that forms the basis for studying the risk of prolonged shift-work on cognitive performance and physiological wellbeing.

While a variety of environmental cues are able to influence circadian rhythms, relatively little is known about how this modulation occurs or the mechanisms that underlie entrainment. Data demonstrated that cholinergic neurotransmission in the suprachiasmatic nucleus (SCN) acts as a prominent non-photic entrainment signal that alters circadian timing during tasks requiring sustained attentional effort. Changes in entrainment were strongly correlated with conditions where performance demands taxed the cognitive control of attention. Surprisingly, cognitive activity alone is capable of entraining circadian rhythms in animals lacking a functional SCN. However, SCN-lesioned animals also exhibit significant impairments in cognitive performance suggesting SCN function is important for cognitive learning.

The data also reveal that circadian processes interact with learning and memory consolidation in a bidirectional manner, particularly for tasks requiring attentional effort. Nocturnal rodents demonstrate better acquisition and performance when trained during the dark-phase as compared to daytime trained animals.

Collectively, these results illustrate that cholinergic signaling influences entrainment through interactions with the SCN and still unidentified oscillators outside of the SCN. Further, circadian rhythmcity can be modified through adaptive processes in the service of cognitive performance. These findings reveal that multiple clocks co-exist in the CNS and compete for control of circadian output. Animal models are important for understanding the mechanisms that lead to disruption of circadian oscillators and the testing of putative treatments to attenuate or prevent circadian desynchronies commonly present in shift-workers, the aging population, and some neuropsychiatric disorders.

Chapter 1

Introduction

The connection between environmental events and time-of-day may represent some of the earliest associations on earth. The capacity to time activity based on the availability of light is thought to have evolved over 3.5 billion years ago with the regulation of photosensitive proteins in single celled organisms (for review see: Gehring & Rosbash, 2003; Golden, Ishiura, Johnson, & Kondo, 1997). Prokaryotic life forms have been credited with the origin of circadian rhythms as a means to augment photosynthesis and to time DNA replication. Replication evolved to occur during the dark phase so as to protect replicating DNA from the harmful effects of *ultraviolent* radiation that ravaged the early earth landscape. Cyanobacteria are still thought to have the simplest molecular clock and are capable of sustaining 22-hour rhythms with a simple three protein compilation that comprise their oscillator (Nakajima, et al., 2005). Genetic mutations with periods other than 24 hours are readily produced in the laboratory, suggesting that while the presence of these mutant phenotypes can certainly exist, evolution has selected against clocks with periods other than approximately 24 hours. As life evolved, the ability of plants and animals to make complex associations that included the organization of circadian time on physiology and behavior also evolved; the role of circadian timing expanded from synchronization with the external world to synchronization of activities within the individual organism. Plants relied on circadian timing to regulate photosynthetic machinery to light availability and honeybees in turn modulated their foraging behavior for access to nectar and pollen during specific times of day (Beling, 1930). Plants and animals of coastal ecosystems adapted their feeding and reproduction cycles to tidal changes brought on by the periodic changes in the gravitational pull of the moon. This entrainment of biological organisms to a specific time-of-day imparted fitness by allowing them to anticipate environmental events and respond even in the absence of

that cue (*e.g*. initiation of foraging behavior from a burrowing animal). Through the synchronization of behavior to light-dark cues, animals developed activity cycles to meet daily and seasonal changes in their environment. Species typically have preferred temporal *niches* (*e.g.* diurnal, nocturnal or crepuscular activity). These temporal *niches* enhance fitness by coordinating timing of necessary behavioral activity including feeding, hunting, and breeding. In addition to the physiological and behavioral adaptations of organisms to meet a specific predictable *niche*, they are also able to adapt to unpredictable changes. The evolution of learning mechanisms fall into this category and are ubiquitously shared by simple and complex organisms alike. Habituation, sensitization, and simple conditioning are all psychological constructs that represent simple associations between environmental stimuli and their consequences. The sensitization of the withdrawal reflexes in Aplysia (Cleary, Byrne, & Frost, 1995) and the conditioned eye blink response classically demonstrated in rabbits (Gormezano, Kehoe, & Marshall, 1983) illustrate the ubiquitous nature of these associations across the phylogenetic spectrum. These specializations have become an essential prerequisite for survival as animals are under constant competition for scarce resources in pursuit of reproductive success. The selective pressures for these traits are as old as the evolutionary timeline and are evident today in the natural behaviors of all organisms.

The goal of my thesis is to understand the interactions of long-term and short-term adaptive processes, cognition, and circadian cycles. Following a brief introduction to the circadian system and the key players involved in attentionally mediated cognitive performance, I will discuss and support an argument for attention as a prominent non-photic regulator of circadian entrainment modulated by self-generated cognitive activity through the release of acetylcholine. I will argue that time-of-day activity interacts with processes of learning and memory and that acetylcholine release at the level of the superchiasmatic nucleus provides a key timing signal that allows time to serve as a contextual cue to improve future performance by altering circadian timing in the service of learning and cognitive performance. Finally, I will address where in the brain mechanisms driven by cognitive activity interact with SCN mediated circadian rhythms and present a model for how multiple clocks can co-exist and compete for control of

internal synchrony, albeit, sometimes to the detriment of the animal. In the conclusion section of my thesis I will argue that these interactions have important implications for human productivity and health as disruptions of circadian processes through shift-work, normal aging, or disease, have profound implications for measures of quality of life and societal wellbeing.

Introduction to circadian systems and entrainment

Circadian rhythms occur with a period of approximately 24 hours (ranging from 23–25 hrs, varying by species) and are endogenously generated - meaning that under conditions in which time setting cues from the outside world are no longer present, the circadian system will continue to generate daily rhythms on its own. Circadian rhythms in the absence of environmental cues will appear to "drift" a little each day, because the period is usually slightly longer or shorter than 24 hours (***figure 1.1A, 1.1C***). **Figure 1.1A** represents an activity record from a common house mouse over a period of approximately 60 days. This animal shows a robust entrainment to the light-dark (LD) cycle but under constant conditions - total darkness or dark/dark (DD) the endogenous period for this animal's central clock is revealed (slightly greater than 24 hours). Under normal circumstances, circadian systems require entrainment by external cues to maintain a 24-hour period (Roenneberg, Daan, & Merrow, 2003; Sulzman, Fuller, & Moore-Ede, 1982). Light is the preeminent environmental cue or zeitgeber (German for "time-giver") for the circadian system and most laboratory studies involving circadian rhythms reference zeitgeber time (ZT) or the hours of the day relative to zeitgeber exposure. Entrainment is characterized by daily activity that occurs coincident with or in advance of the daily cue and usually extends beyond the actual presentation of the cue. If the activity persists in the absence of the cue and absence of all external cues, including the light-dark cycle, the activity synchronized by the signal is considered to be entrained. Once a rhythm is entrained there is a stable relationship that exists between phase markers of the rhythm (*e.g.,* rhythm onset, peak, offset, and trough) and the zeitgeber event. (*e.g.* lights on = ZT0). Under conditions where environmental or laboratory cues are removed (*free-run*), entrainment is measured by the amplitude and persistence of phase markers relative

to the time the cue would have occurred. For the conditions of my studies, and for many in laboratory settings, the phase of biological and physiological rhythms is defined by the relationship of phase markers to the laboratory light-dark (LD) cycle. Non-photic cues are thought to entrain activity best in the absence of a LD cycle; however some daily events are significant enough that they cause entrainment of activity in the presence of a LD cycle. For example, novel wheel access in hamsters (Gorman & Lee, 2001) and palatable food access in rats (Mistlberger & Rusak, 1987) are both strong entraining signals in the presence of a LD cycle. Other non-photic cues are less effective at entrainment during the LD cycle, including forced treadmill running in mice (Marchant & Mistlberger, 1996) and daily timed water access in rats (Mistlberger & Rechtschaffen, 1985).

Suprachiasmatic nucleus (SCN)

In mammals, circadian rhythms are generated by a central pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore, 1983). Bilateral lesions of the SCN eliminate circadian rhythmicity, supporting its role as the essential nucleus in circadian regulation (Stephan & Zucker, 1972). Direct light information reaches the SCN via the retinohypothalamic tract (RHT) which projects directly from the retina to the SCN (Card & Moore, 1984; Moore & Lenn, 1972). Light input to the SCN is conveyed by the release of glutamate and results in the expression of immediate early genes, such as *cFos* (Meijer & Schwartz, 2003). Ultimately this transcription cycle leads to expression of the *PER* components of the clock gene regulatory feedback loop described below (Miyake, et al., 2000; Shigeyoshi, et al., 1997). The RHT provides the strongest signal influencing circadian activity patterns and is considered the preeminent driver of circadian rhythms; however, there are many non-retinal inputs to the SCN that can influence circadian entrainment (Card & Moore, 1984; Moore & Lenn, 1972). Afferent inputs to the SCN hypothesized to influence circadian rhythms include cortical projection neurons from neocortical regions involved in executive function (Hurley, Herbert, Moga, & Saper, 1991; Vertes, 2004), serotonergic neurons originating in the dorsal raphe nucleus (Edgar,

Miller, Prosser, Dean, & Dement, 1993; Medanic & Gillette, 1992), and cholinergic projections from midbrain and basal forebrain nuclei (Bina & Rusak, 1996a; Bina, Rusak, & Semba, 1993, 1997; Erhardt, et al., 2004a; Gillette, et al., 2001; figure 1.2). The mammalian circadian pacemaker was thought to have evolved to act as a central coordinator of activity for disparate tissues involved in essential functions such as metabolic homeostasis (Rutter, Reick, & McKnight, 2002), immune function (Oishi, et al., 2003), cell development and proliferation (Meerlo, Mistlberger, Jacobs, Heller, & McGinty, 2009), and cell signaling (Barnes, McNaughton, Goddard, Douglas, & Adamec, 1977).

The mechanisms of circadian regulation are generated within individual cells of the SCN through a transcriptional-translational feedback loop involving a group of regulatory proteins (Hastings and Herzog 2004). The feed forward portion of the loop is modulated by the proteins *BMAL* and *CLOCK* that up-regulate the feedback pathway (*PER, CRY*) whose expression act to inhibit the further transcription of *BMAL*; by doing so, *PER* and *CRY* protein production act to inhibit their own future transcription (Bae, et al., 2001; Zheng, et al., 2001). **Figure 1.1B** demonstrates the asynchronous activity records of a double knock-out mouse with targeted deletions of the *PER1* and *PER2* clock genes. In the presence of a light-dark cycle the animal shows activity primarily during the dark-phase but no entrainment to the light signal is present; this effect is referred to as masking and demonstrates that the underlying rhythm may not always be manifest in the presence of zeitgebers. Note that only under constant conditions (DD), in the absence of a timing-signal, is the asynchronous behavior fully revealed. The regulation of the clock mechanism happens in a cell-autonomous manner but coherent modulation of the SCN network is accomplished through the coupling of individual cells via electrical gap junctions (Yamaguchi et al. 2003). Cell output from the SCN involves the coordinated release of the amino-acid neurotransmitter GABA and the neuropeptides vasoactive intestinal polypeptide (*VIP*) and arginine vasopressin (*AVP*), although not necessarily from the same cell (Aton, Colwell, Harmar, Waschek, & Herzog, 2005).

Recent evidence has also provided support for the role of circadian timing through clock gene expression in regions other than the SCN. Collectively these regions are referred to as peripheral oscillators and include brain regions important for learning and

memory (*e.g.*, cortex, striatum, hippocampus, amygdala; Guilding & Piggins, 2007; Schibler, 2009; Wyse & Coogan, 2010). This expression is thought to occur semi-autonomously, although the entrainment of these oscillators is thought to be under control of the SCN, and some appear to be subject to entrainment by non-photic zeitgebers such as novelty or food. The influence of peripheral oscillators on circadian activity are sometimes only revealed in the absence of a light-dark (LD) cycle, or following the elimination of the SCN (Garcia-Allegue, Lax, Madariaga, & Madrid, 1999; Madrid, et al., 1998).

Interactions between the SCN and cholinergic systems

There are a significant number of cholinergic terminals throughout the SCN and researchers originally hypothesized that acetylcholine played a direct role in the mediation of light entrainment of the SCN via the activation of nicotinic acetylcholine receptors (nAChRs). Data that initially supported this hypothesis included the demonstration that SCN neurons responded to carbachol (a non-selective cholinergic agonist) in a manner similar to light and that cholinergic antagonist blocked the circadian effects of light (Earnest & Turek, 1983; Mistlberger & Rusak, 1986; Murakami, Furukawa, Yokawa, Etoh, & Takahashi, 1986; Wee, Anderson, Kouchis, & Turek, 1992; Zatz & Brownstein, 1979; Zatz & Herkenham, 1981; Zhang, Zee, Kirby, Takahashi, & Turek, 1993). A summary of these effects are presented in **figure 1.1C**. This hypothesis was eventually rejected and we now know that light affects the SCN through the release of glutamate as described earlier.

A comprehensive analysis of cholinergic signaling in the SCN was later provided by Bina et al. (1993) that provided conclusive evidence that forebrain cholinergic neurons of the medial septum, nucleus basalis, diagonal band, substantia inominata, and brainstem cholinergic neurons of the pedunculopontine, laterodorsal tegmental and parabigeminal nuclei all send projections to the SCN. A detailed set of immunolabeling for key markers of cholinergic synthesis, signaling and catabolism in the SCN are represented in **figures 1.3A-1.3E.** While immunohistochemistry and tract tracing studies have added to our

understanding of the distribution and origin of anatomical input to the SCN, the functional role of cholinergic inputs, particularly from the basal forebrain, has remained unclear. Experimental studies *in vivo* and *in vitro* suggest that acetylcholine (ACh) can have significantly influence on both cellular and behavioral measures of circadian activity. Intracranial injections of carbachol into either the lateral ventricle or SCN induce subjective night phase advances and delays similar to those produced by light (Buchanan & Gillette, 2005; Liu, Ding, Faiman, & Gillette, 1997; ***figure 1.1C***). Saporin lesions of the basal forebrain attenuate the behavioral response to the phase shifting effects of light (Erhardt, et al., 2004b) and block entrainment to timed daily handling in rodents (Hummer, Meixner, & Lee, 2010). Furthermore, experiments attenuating cholinergic signaling in the SCN, via lesions of the nucleus basalis, result in the reduction in the expression of SCN neuropeptides (*VIP* and *AVP*) important for circadian signaling (Madeira, Pereira, Silva, Cadete-Leite, & Paula-Barbosa, 2004). *In vitro* electrophysiological data from the SCN also demonstrates that activation of AChRs by carbachol produces shifts in the neural firing activity of *AVP* expressing neurons in the SCN and modifies the period at which endogenous peak firing rate occurs in SCN slices (Gillette, et al., 2001). The effects of ACh release on circadian physiology have been proposed to be mediated by the activation of mAChRs as nearly all neurons of the SCN have been shown to express at least M1 receptors (van der Zee, Streefland, Strosberg, Schroder, & Luiten, 1991). More recently the use of RT-PCR has demonstrated that the mRNA for all five mAChR subtypes is present in the SCN, although the distribution amongst particular cell types and pre and post-synaptic locations is unknown (Yang, Wang, Cheng, Kuo, & Huang, 2010). Alpha-7 containing-nAChR have also been shown to be present in the SCN and are co-localized with the distribution of cholinergic fibers (Fuchs & Hoppens, 1987); however, it is currently believed that nicotinic receptors play a less significant role in SCN signaling, particularly in the adult SCN. In support of this hypothesis, it has been demonstrated that the application of nicotine produces a robust *c-fos* expression in the prenatal rat SCN, but not in the adult rat SCN (O'Hara, et al., 1999). Additionally, the application of nicotine to the adult rat SCN produces only minor responses in cell excitability and only at relatively high concentrations (Artinian, Ding, & Gillette, 2001; Liu, et al., 1997).

An abundance of physiological and anatomical data suggest that pharmacological application of acetylcholine and cholinergic agonists can have major effects on circadian rhythms; however, the *in vivo* conditions and importance of endogenous ACh release at the SCN is not well understood.

Circadian factors influencing acetylcholine (ACh) release

Acetylcholine release in the nocturnal rodent cortex is significantly higher during the active (dark) phase as compared to the inactive (light) phase (Jimenez-Capdeville & Dykes, 1993; Kametani & Kawamura, 1991). Circadian variations can also be seen in acetylcholinesterase (AChE) expression in nocturnal rodents with the highest expression corresponding to the inactive phase, and lowest expression levels coincident with the active phase (Perry, Perry, Gibson, Blessed, & Tomlinson, 1977; Rao, et al., 1987). The production of acetylcholine also possesses circadian variations in expression with the highest expression of the enzyme choline-acetyl transferase (ChAT) in rats and humans coincident with their respective active phases (Jenni-Eiermann, von Hahn, & Honegger, 1985; Nordberg & Wahlstrom, 1980). In addition, the amount of mAChR transcript is highest in the cortex when dialysate levels of ACh is lowest and is reversed when the animal is most active (Mash, Flynn, Kalinoski, & Potter, 1985). A comparable pattern is found in human post-mortem tissue (Perry, et al., 1977). Collectively data from a variety of species demonstrates that daily variations exist in cholinergic markers that are characterized by high cortical ACh release during the active phase (the dark phase for nocturnal species and the light phase for diurnal species) is concurrent with enhanced ChAT activity, and reduced AChE activity and mAChR expression (see, ***figure 1.4*** *for a summary of these interactions and relationships*). Perhaps unexpectedly, ACh release in the SCN does not demonstrate circadian variations in release, but does show fivefold increases in baseline levels in response to 30 minutes of arousal (Murakami, Takahashi, & Kawashima, 1984). Further, the expression of mAChRs in the SCN does not reveal daily fluctuations in expression in rats and hamsters (Bina, Rusak, & Wilkinson, 1998; van der Zee, et al., 1991); however, brief exposure (5–20 min) to a novel environment is

sufficient to enhance muscarinic acetylcholine receptor immunoreactivity in the rat SCN at periodic intervals of 24 hours beginning 22-24 hours after the novel event and continuing for days to weeks as long as the subject is not exposed to interfering events (Van der Zee, Biemans, Gerkema, & Daan, 2004).

Circadian factors influencing cell excitability and plasticity

In nocturnal rodents, neurons of the SCN have circadian variations in cell excitability as measured by spontaneous firing rates and resting membrane potentials (de Jeu, Hermes, & Pennartz, 1998; Green & Gillette, 1982; Groos & Hendriks, 1982; Kononenko, Kuehl-Kovarik, Partin, & Dudek, 2008; Kuhlman & McMahon, 2004; Pennartz, de Jeu, Bos, Schaap, & Geurtsen, 2002). Time-of-day-dependent changes in long-term potentiation (LTP) has also been demonstrated in a variety of rodent species, including rats, hamsters and mice with variations in time of day depending on the region of induction (***figure 1.5A and figure 1.5B***). For example, LTP is augmented during the dark phase in the hippocampus perforant pathway while the Schaffer-collateral pathway has enhanced LTP during the light phase (Barnes, et al., 1977; Chaudhury, Wang, & Colwell, 2005; Dana & Martinez, 1984; Harris & Teyler, 1983; Nishikawa, Shibata, & Watanabe, 1995; West & Deadwyler, 1980). LTP has also been show to be time-of-day dependent in the SCN of rats, with a lower threshold for potentiation during the inactive period as compared to the active phase (Nishikawa, et al., 1995). Interestingly, these changes in LTP threshold have been shown to be dependent on the endogenous circadian timing mechanisms of the tissue independent of the time of tissue harvesting in both rats and hamsters (Harris & Teyler, 1983; Raghavan, Horowitz, & Fuller, 1999). This finding suggests that a variety of regions outside of the SCN act as autonomous oscillators themselves and show circadian variations in plasticity even in the absence of timing signals from the SCN (***figure 1.5B***).

Circadian factors influence on learning, acquisition, and performance

The role of circadian processes in learning and memory has been studied for decades. Early findings by Holloway and Wansley demonstrated that passive avoidance performance was optimized periodically at 24-hour intervals following learning (Holloway & Wansley, 1973a, 1973b). Later, it was determined that this periodic performance was dependent upon an intact SCN (Stephan & Kovacevic, 1978). Time-of-day studies have also looked at how SCN driven biological rhythms interact with learning and performance. Habituation to auditory cues in pigeons (Valentinuzzi & Ferrari, 1997) and habituation to spatial novelty in mice (Valentinuzzi, et al., 2000) were found to be more robust during the animal's endogenous active phase. Hoffman and Balschun also showed that mice when trained on an alternating T-maze showed fewer errors and faster rates of acquisition when trained during the dark phase (1992). In studies of contextual and cued fear conditioning, time of day effects have been reported sometimes with conflicting results (Chaudhury & Colwell, 2002; Valentinuzzi, Menna-Barreto, & Xavier, 2004). Lesions of the SCN also produce profound deficits in novel object recognition (Ruby, et al., 2008) and conditioned place preference in middle aged hamsters (Antoniadis, Ko, Ralph, & McDonald, 2000). These studies suggest that timing information is important for task acquisition and performance, and that timing signals may be stored in the SCN through a mechanism of unknown origin. It has recently been proposed that release of acetylcholine at the level of the SCN may provide the timing signal important for 'time-stamping' singular events that can be later used to interweave context, time, and place associations in the service of memory consolidation (for review see: Daan, 2000; Hut & Van der Zee, 2010). In support of this theory, it has been demonstrated that the expression of cholinergic receptors in the SCN following exposure to a novel stimulus shows no changes in expression until 24 hours later and follows a continuous periodic pattern of expression at 24 hour intervals for several days. Further, output as measured by changes in *AVP* release is time-locked to the change in cholinergic receptor expression profile and runs independent of the light entrained SCN *AVP* rhythm (van Esseveldt, Lehman, & Boer, 2000). It has been determined that only a subset of *AVP* expressing cells actually entrain output to a particular event and that other non-entrained

AVP cells will entrain to subsequent events if they occur. Mice, for example, can entrain to a minimum of three such events in addition to the LD entrained rhythm of *AVP* release (Van der Zee, et al., 2008). This information is likely to be conveyed to areas involved in memory storage and consolidation through release of vasopressin which modulates arousal and vigilance networks via projections to the dorsomedial hypothalamic nucleus (DMN), locus coeruleus (LC), orexin/hypocretin neurons of the hypothalamus, and the basal forebrain (Hajszan & Zaborszky, 2002; Lee, Kim, & Waterhouse, 2005; Novak, Harris, Smale, & Nunez, 2000; Novak & Nunez, 2000; Salazar-Juarez, Escobar, & Aguilar-Roblero, 2002; ***figure 1.2***). Overall, this anatomical organization suggests that the forebrain structures involved in time signaling to the SCN could potentially regulate their own activity through feedback mechanisms.

Acetylcholine influences on learning and attention

A considerable amount of information exists for the role of acetylcholine in the regulation of learning and memory and in particular for regions involved in hippocampal based learning. Acetylcholine has been shown to be elevated during tasks of learning and memory in a variety of brain regions including the hippocampus, striatum, and cortex (Chang & Gold, 2003; Fadda, Melis, & Stancampiano, 1996; McIntyre, Marriott, & Gold, 2003; McIntyre, Pal, Marriott, & Gold, 2002; Orsetti, Casamenti, & Pepeu, 1996; Ragozzino, Pal, Unick, Stefani, & Gold, 1998; Ragozzino, Unick, & Gold, 1996; Stancampiano, Cocco, Cugusi, Sarais, & Fadda, 1999; Stefani & Gold, 2001; Yamamuro, Hori, Tanaka, Iwano, & Nomura, 1995). This elevation is correlated with the type and intensity of learning and the time course over which training occurs and cannot be accounted for by activity, arousal, or handling. Agonists of cholinergic receptors facilitate hippocampal-dependent learning and memory (Farr, Flood, & Morley, 2000; Farr, Uezu, Flood, & Morley, 1999; Izquierdo, et al., 1992), presumably because ACh binding predominantly acts to lower the threshold for cell excitability, thereby increasing the effectiveness of other coincident synaptic inputs (for review, see: Ridley, Thornley, Baker, & Fine, 1991). Selective lesions of the basal forebrain cholinergic system using the neurotoxin 192 IgG saporin reduces neurogenesis in the dentate gyrus that coincides

with impairments in spatial memory (Mohapel, Leanza, Kokaia, & Lindvall, 2005). Additionally, deficits in memory associated with lesions of the medial septum can be rescued with the restoration of cholinergic tone in aged or lesioned animals (Cassel, Duconseille, Jeltsch, & Will, 1997; Cassel, et al., 2002; Hodges, Allen, Sinden, Lantos, & Gray, 1991; Howard, et al., 1989; Maho, Dutrieux, & Ammassari-Teule, 1988; Mohapel, et al., 2005; Tarricone, Simon, Li, & Low, 1996).

While some studies have shown substantial deficits in learning and memory following selective 192 IgG-saporin lesions of the basal forebrain cholinergic system (Cassel, et al., 2002; Lehmann, Bertrand, et al., 2002; Lehmann, Grottick, Cassel, & Higgins, 2003; Lehmann, Jeltsch, et al., 2002; Pang, Nocera, Secor, & Yoder, 2001; Shen, Barnes, Wenk, & McNaughton, 1996; Walsh, Herzog, Gandhi, Stackman, & Wiley, 1996), others have shown minimal impairments or no detectable impairments at all (Baxter, Bucci, Gorman, Wiley, & Gallagher, 1995; Baxter, et al., 1996; Baxter & Gallagher, 1996; Berger-Sweeney, et al., 1994; Chappell, McMahan, Chiba, & Gallagher, 1998; Dornan, et al., 1996; Frick, Kim, & Baxter, 2004; Kirby & Rawlins, 2003; McMahan, Sobel, & Baxter, 1997). These differences have led to substantial debate as to the role of cholinergic signaling in the process of learning and memory. The fact that 192 IgG-saporin fails to produce noticeable deficits in performance in some studies suggests deficits may only be exposed under conditions of near complete cholinergic depletion on many types of tasks. It has also been proposed that compensatory mechanisms may increase output from the remaining terminals to compensate for cholinergic lesions (Chang & Gold, 2003; Waite & Chen, 2001). In support of this theory, Chang and Gold (2002) reported that rats with 70% depletion of cholinergic terminals using192 IgG-saporin in the MS/VDB, had behaviorally induced increase in acetylcholine that was actually higher in lesioned rats than in controls relative to baseline. Another intriguing possibility is that the deficits in some tasks of learning and memory associated with cholinergic depletion studies could be directly related to deficits in aspects of attentional processing. As cholinergic lesions of the MS/VDB and in the NBM/substantia inominata can produce substantial deficits in attention (Baxter & Chiba, 1999; Baxter, Gallagher, & Holland, 1999; Baxter, Holland, & Gallagher, 1997; McGaughy, Dalley, Morrison,

Everitt, & Robbins, 2002; McGaughy, Kaiser, & Sarter, 1996; McGaughy & Sarter, 1998; Sarter, Bruno, & Turchi, 1999; Turchi & Sarter, 1997) it is possible that task specific deficits reported in some studies are a result of decreased capacity for attentional effort.

In summary, overwhelming support exists for cholinergic systems being actively engaged during normal memory processes, and it seems that acetylcholine has a powerful effect on the modulation of learning, particularly in aged or lesioned animals. However, cholinergic depletion studies do not always produce substantial deficits in studies of learning and memory and provide evidence that the cholinergic system may not be necessary for learning in some tasks. It has been proposed that differences in attentional requirements may account for these disparities and it has recently been hypothesized that the cholinergic system may facilitate flexibility in tasks requiring greater cognitive demands including the application of behavioral rules or the transfer of previous experiences to novel conditions (Janisiewicz & Baxter, 2003; Sarter, Draut, Herzog, & Bruno, 2002). In support of this theory data from place cell recordings in cholinergic lesioned rats show normal spatial selectivity and electrophysiological properties; however, individual place fields exhibit less flexibility when cues present in the environment are relocated (Ikonen, McMahan, Gallagher, Eichenbaum, & Tanila, 2002).

The basal forebrain and sustained attentional performance

The basal forebrain provides the principal source of cholinergic input to the neocortex, hippocampus, amygdala, and the SCN, while the brainstem provides the main cholinergic innervation for the thalamus. Cholinergic signaling is coincident with the presentation of behaviorally salient stimuli and mediates both cue detection and arousal by environmental cues important for associative learning (Acquas, Wilson, & Fibiger, 1996; Sarter, Hasselmo, Bruno, & Givens, 2005; Wenk, 1997). While cholinergic circuits have long been implicated in learning and memory systems associated with reinforcement and conditioning, recent evidence has emerged to support the cholinergic system as an important mediator of cognition and attention (Himmelheber, Sarter, & Bruno, 1997; Wilson & Rolls, 1990). One condition under which ACh release from the

basal forebrain is reliably elevated is during performance of tasks of sustained attention; sustained attention is characterized by a subject's readiness to detect an infrequent and unpredictable signal(s) over a prolonged period of time, to discriminate this signal from non-signal events or "noise" and to report the presence or absence of such a signal. **Figure 1.6** illustrates the conditions of task performance in the sustained attention task (SAT) used throughout the chapters of this thesis. Normal sustained attention performance, above chance levels, is dependent upon the integrity of the basal forebrain cholinergic projections to the cortex (McGaughy, et al., 1996). Sustained attention performance robustly increases cortical cholinergic transmission; ACh release is further augmented under challenging conditions (e.g. distracter presentation or fatigue) that require top-down optimization of input processing (Kozak, Bruno, & Sarter, 2006; Parikh, Kozak, Martinez, & Sarter, 2007; Sarter, Gehring, & Kozak, 2006; Sarter, et al., 2005). The ability to sustain attention is critical to a variety of cognitive functions, including: stimulus detection, discrimination, and signal processing (Kozak, et al., 2006).

Questions:

My thesis explores how self-sustained, internally motivated, cognitive activity imparts selective change on the behavioral output of circadian systems and the consequences of this interaction. I will explore this relationship in detail in three data chapters that are briefly outlined below.

Does cognitive performance modulate circadian rhythms?

The first goal of my thesis is to determine and quantify how cognitive performance influences circadian rhythms and to compare these changes to other non-photic cues that have previously been described in the literature as modulators of circadian activity. I hypothesize that the selective up-regulation of attention in the service of cognitive performance, mediated via the basal forebrain cholinergic system and mechanisms of attention, alter circadian rhythms to produce stable new patterns of entrainment. Chapter 2 answers the question of how daily task performance modulates

circadian rhythms, and provides evidence that changes in circadian markers of activity are directly tied to cognitive effort that cannot be accounted for by stress, activity, handling, novelty, general arousal, or even the process of learning complex associations in their environment.

Is there an interaction between time-of-day and performance and can strength of entrainment predict performance in a reliable way?

The second goal of my thesis was to determine how acquisition and performance of cognitive tasks is impacted by circadian entrainment to the light-dark cycle and to compare time-of-day effects on performance across a variety of tasks important in neuroscience research. I hypothesize that the level of entrainment will be correlated with the requirements for attentional demand, and tasks requiring lower cognitive effort will have less pronounced effects on circadian entrainment. Experiments in chapter 3 tests the hypothesis that time-of-day impacts learning in both the Morris water maze and on a task of sustained attention (SAT). The results indicate that cognitive performance and remote memory are dependent on time-of-day for cognitively demanding tasks. Additionally, behavioral flexibility, as measured by anticipation to timed daily training to learned cognitive tasks, acts as a predictor of performance on those tasks and suggests that entrainment counteracts the deleterious effects on performance that occurs when animals perform during normally inactive periods of their light-entrained activity cycle.

Is the cholinergic system responsible for cognitive influences on entrainment through signaling in the SCN?

The final goal of my thesis is to determine whether increased acetylcholine release, dependent upon and concurrent with cognitive task performance, acts as the critical timing event for non-photic cognition-induced entrainment of circadian rhythms. I hypothesize that attentional effort, manifest through the release of sustained levels of acetylcholine, presumably at the SCN, mediates entrainment to tasks of cognitive learning. Chapter 4 experiments, using selective cholinergic depletion lesions and

electrolytic ablation of the SCN, test how cholinergic influence at the SCN mediates entrainment. Data suggest that the SCN serves an important purpose in the acquisition of cognitive learning. Further, the results support a proposed model of competitive interactions between peripheral brain oscillators, entrained to daily cognitive activity, and the LD-entrained SCN oscillator, in rats trained during the light-phase. It is posited that a state of internal desynchrony characterized by this interaction is responsible for acquisition and performance deficits seen in animals performing cognitive tasks outside their endogenous active period.

Figure 1.1: **Double-plotted actograms (48 hours) demonstrating circadian markers of activity and cholinergic influences on the circadian system. A.** Locomotor activity of standard house mouse in a 12:12 light-dark cycle that shows robust nocturnal activity preference when in LD. When this animal was released into constant conditions (total darkness - DD) on 17th day of actogram, the mouse exhibits a free running period slightly more than 24 hours based on a daily delay in activity onset (approximately 20-30 min later onset of activity each day). **B.** Mouse homozygous for mutation in *PER1* and *PER2* circadian clock genes. Animal shows normal patterns of activity in the presence of a light-dark cycle (masking); however when released into DD this animal reveals an arrhythmic activity phenotype. This same phenotype is observed in animals with lesions of the SCN. **C.** Effects of carbachol on entrainment. Actogram showing a hamster maintained in constant darkness that has been implanted with bilateral cannula into the SCN. Yellow highlighting represents day of drug infusion. Carbachol (**C**) produces, in a concentration and time-of-day dependent manner, a change in animal activity onset. Infusions of carbachol at CT14 produce a 2 hour phase delay that were not blocked by the nicotinic receptor antagonist mecamylamine (**M**) but was blocked by the muscarinic receptor antagonist atropine (**A**). These findings suggest that cholinergic effects on circadian rhythms are mediated through muscarinic receptors. Overhead bars represent the phase of the light-dark cycle in the environment (white bars = lights on; black bars = lights off). Figures adapted from (Bae, et al., 2001; Bina & Rusak, 1996b; Vitaterna, et al., 1994).

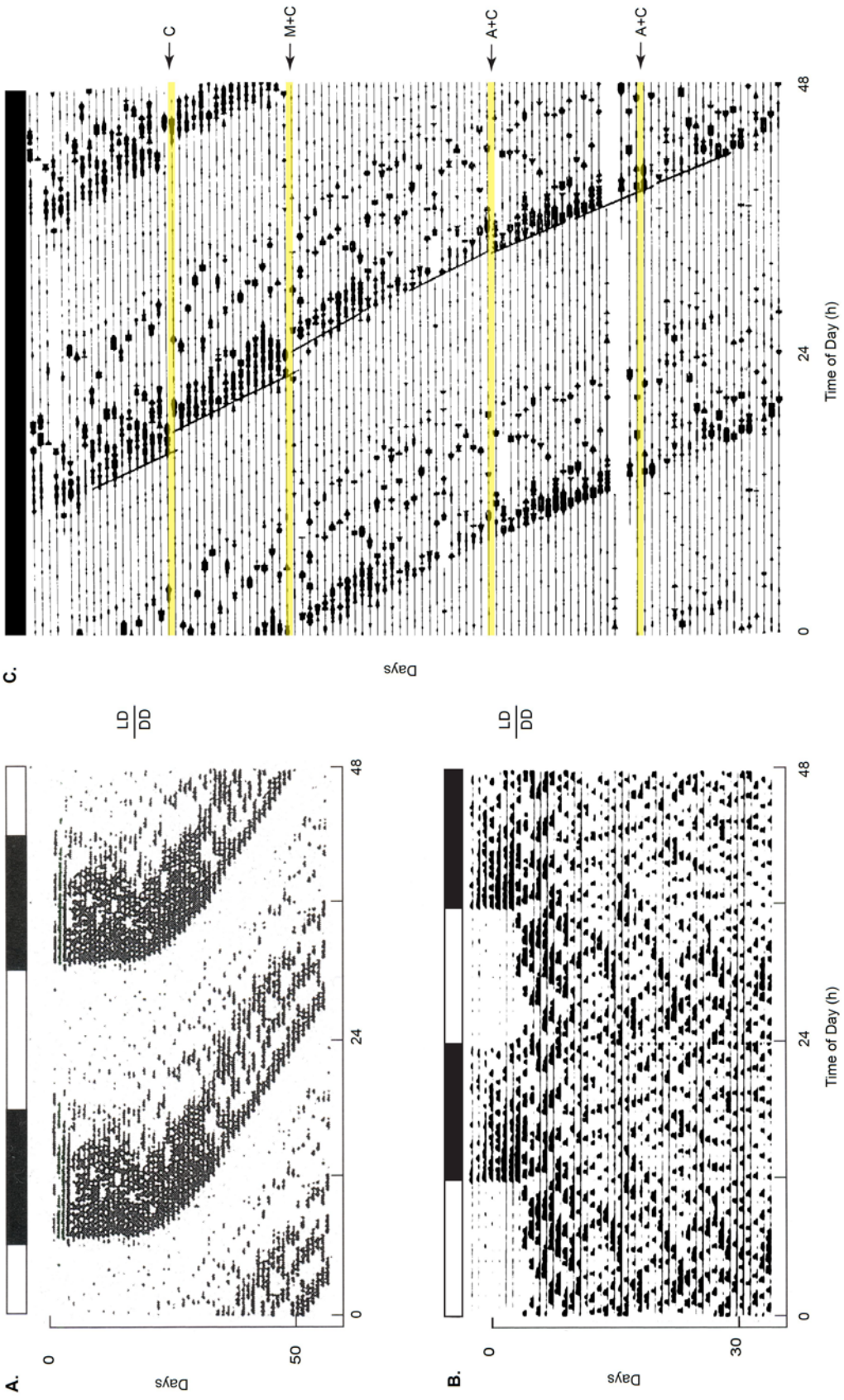
A.
LD
DD
Days
0
50
0
24
48
B.
LD
DD
Days
0
30
0
24
48
Time of Day (h)
C.
Days
C
M+C
A+C
A+C
0
24
48
Time of Day (h)

Figure 1.1

Figure 1.2: **Anatomical connections of the SCN.** Signals responsible for photic-entrainment reach the SCN via the retinohypothalamic tract (RHT). Non-photic functional inputs to the SCN theorized to have meaningful effects on entrainment include the basal forebrain cholinergic system (BFC), modulatory regions of the midbrain and brain stem including the dorsal raphe nucleus (RN), the locus coereleus (LC), cholinergic nuclei of the brainstem (LDT and PPT), and some cortical regions including the pre-frontal cortex (PFC). The SCN conveys output through various regions of the hypothalamus including the dorsal medial hypothalamus (DMH), ventrolateral preoptic area (VLPO), the paraventricular nucleus (PVN), and the tuberomammilary nucleus (TMN). How timing information from the SCN is conveyed to subcortical and cortical regions important for attentional control and learning are not well understood but are presumed to be at least in part gated through hypocretin or orexin-expressing cells of the LH and by melatonin secretion from the pineal gland. Light blue = cholinergic output, dark-blue dashed = SCN output, dark blue = other known neuroanatomical connections with arrows indicating direction of flow. Modified figure originally published by (Gerstner & Yin, 2010)

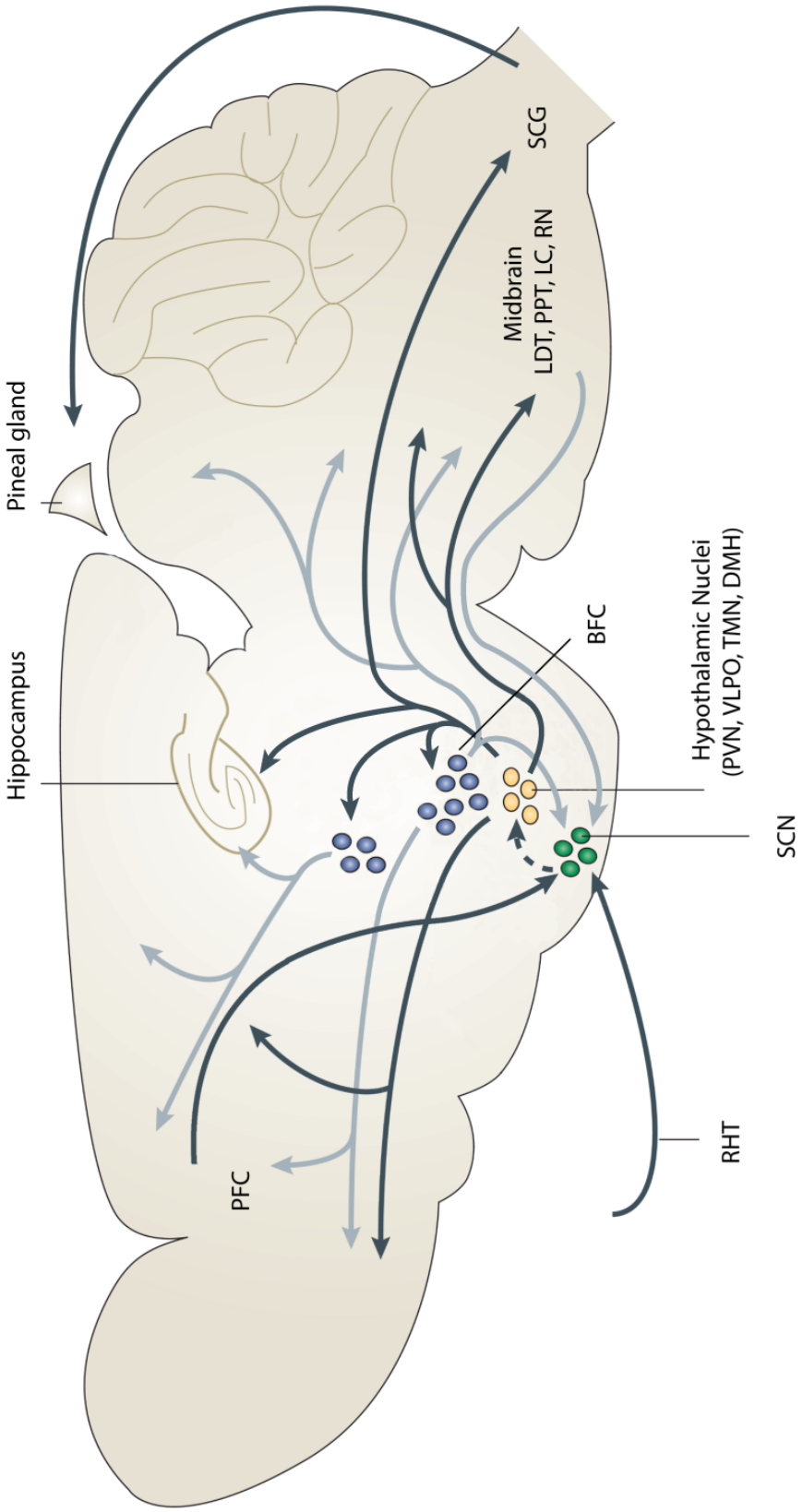
Pineal gland
Hippocampus
Midbrain
LDT, PPT, LC, RN
SCG
BFC
Hypothalamic Nuclei
(PVN, VLPO, TMN, DMH)
SCN
PFC
RHT

Figure 1.2

Figure 1.3: **Markers of cholinergic neurotransmission in the SCN. A.** Illustration of SCN location in coronal rat slice for immunohistology shown in 1.3B-1.3E; higher magnification from highlighted area shown in box is shown below. **B.** Labeling for choline-acetyltransferase (**ChAT**) positive fibers (arrowheads) and cell bodies (arrows) located in the SCN. **C.** Acetylcholinesterase (**AChE**) staining demonstrates dense staining around the SCN and throughout the dorsal SCN while ventral SCN shows a relatively lighter pattern of AChE expression . **D.** Muscarinic acetylcholine receptor (**mAChR**) positive neurons and associated dendrites show an even pattern of expression throughout the SCN. **E.** Labeling for vesicular acetylcholine transporter (**vAChT**) shows a robust pattern of expression through the dorsal and lateral portions of the SCN. Staining can easily be identified by varicosities (arrowhead – bottom). OC = optic chiasm; PVN = paraventricular nucleus; 3V = third ventricle. Scale bar = 90 µm. Some images have been adapted from original publications (Castillo-Ruiz & Nunez, 2007; Hut & Van der Zee, 2010).

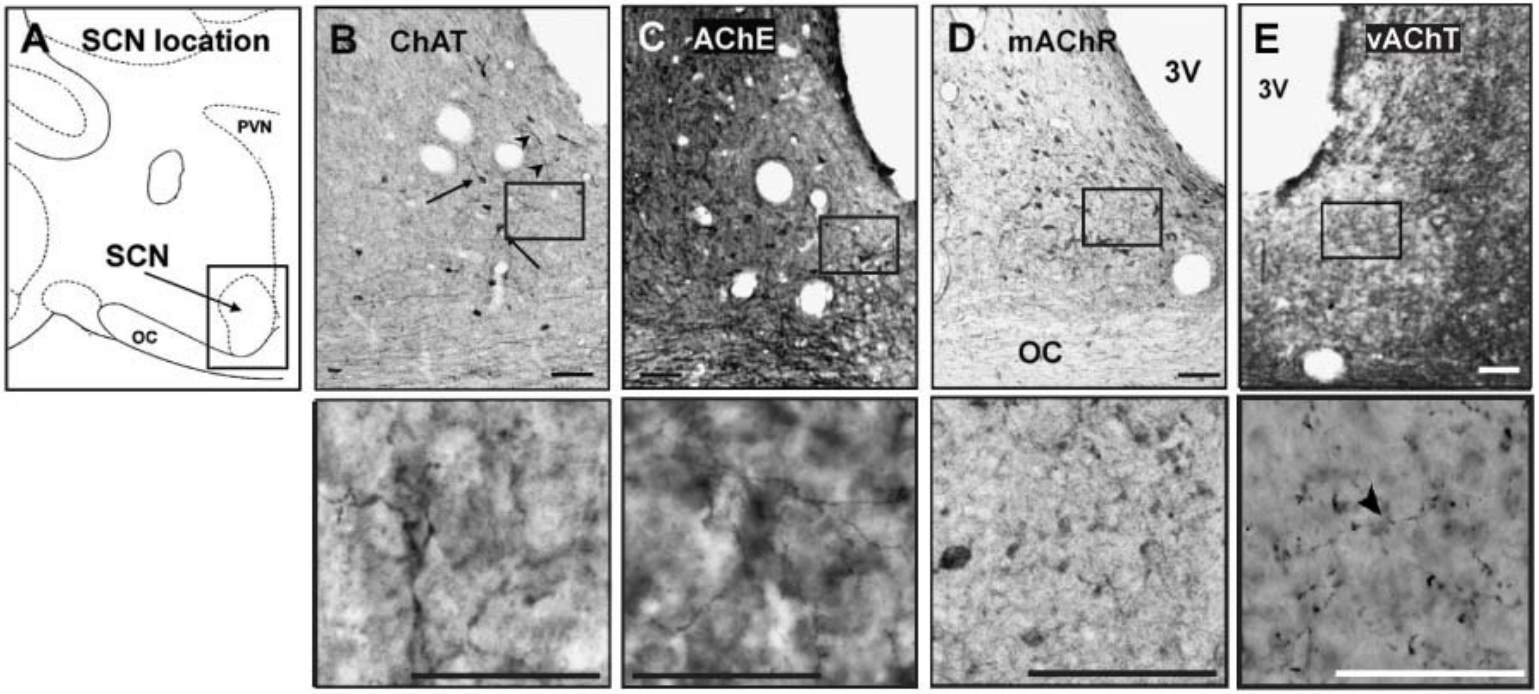

Figure 1.3

Figure 1.4

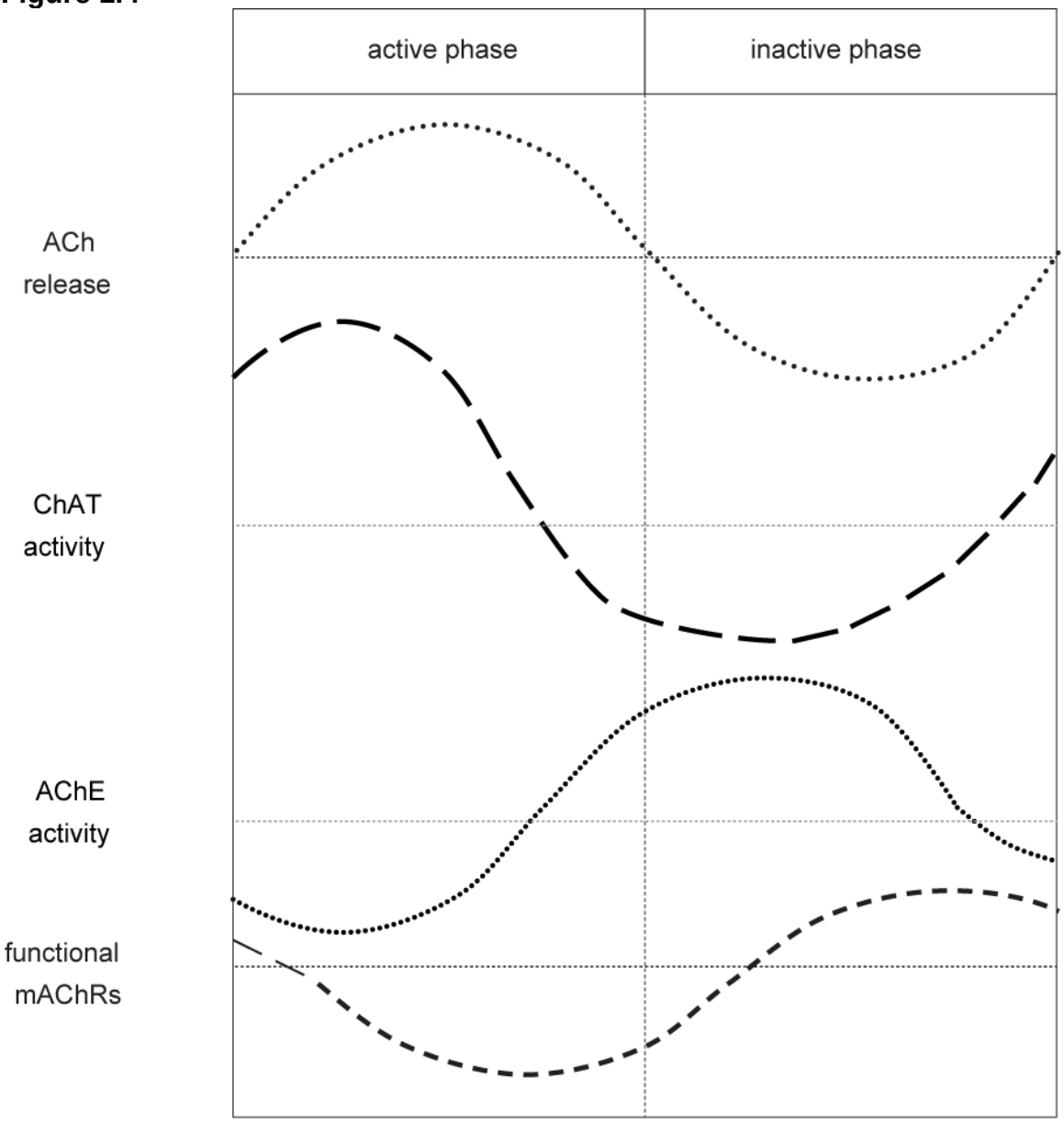

Figure 1.4: **Markers of cholinergic activity in the CNS show circadian patterns of expression.** Schematic illustration representing known circadian variations in acetylcholine release (**ACh**), ChAT expression, AChE immunoreactivity, and functional mAChRs in relation to the active and inactive phase of the individual. Data is based on studies of diurnal rodents, nocturnal rodents, humans and non-human primates. Diagram illustrates consensus findings; however, differences in amplitudes and phase relationships have been reported in some strains and between brain regions. Figure adapted from (Hut & Van der Zee, 2010).

Figure 1.5

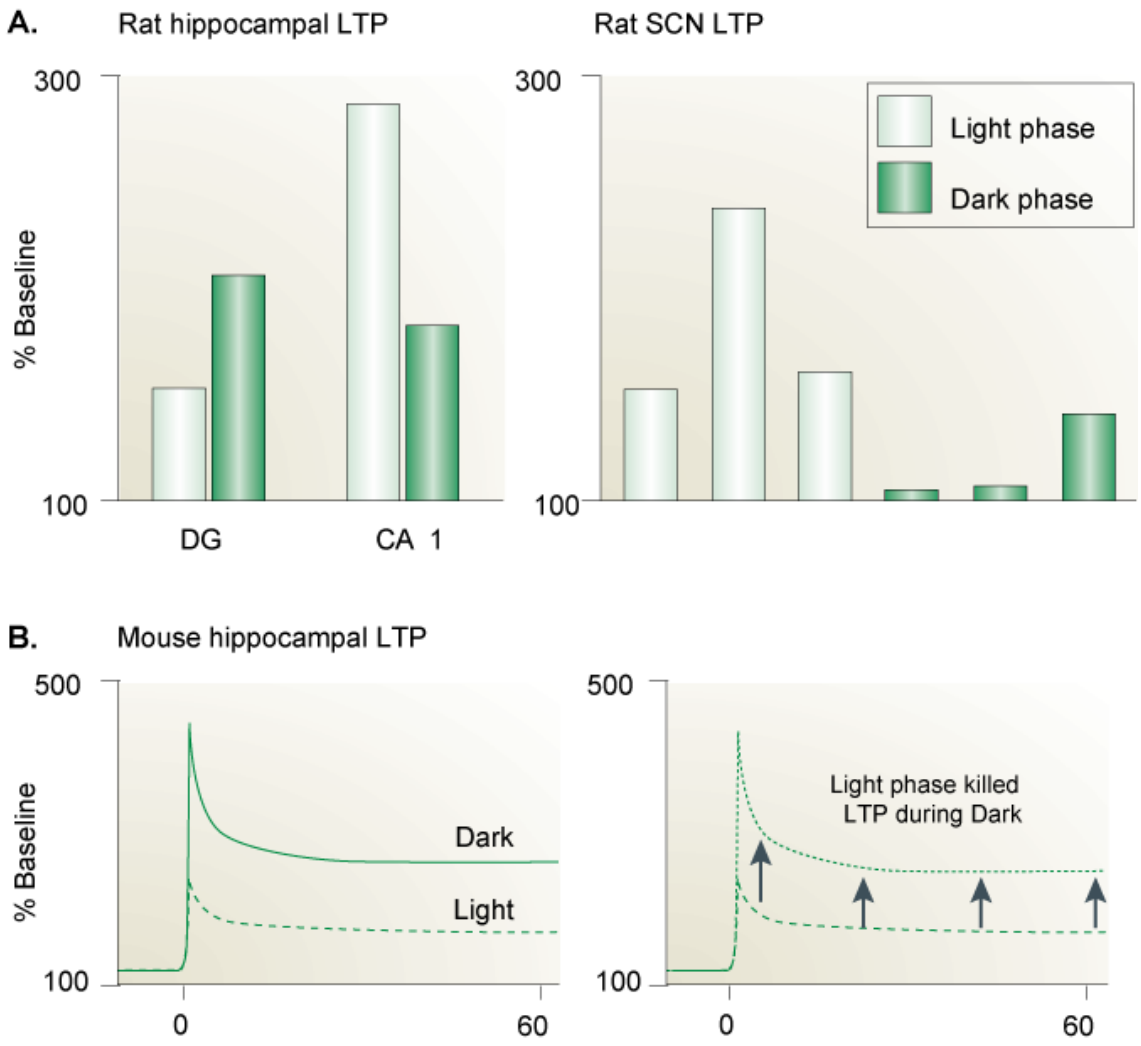

Figure 1.5: **Time of day effects on synaptic plasticity. A.** Hippocampal and SCN long-term potentiation (**LTP**) shows time-of-day effects in the rat. Schaffer collateral LTP is greater during the light-phase in rats (CA1) while perforant pathway LTP is greatest during the dark-phase (DG). The SCN also shows circadian variations in LTP strength with light-phase LTP induction showing a more robust change in amplitude than dark-phase induced LTP. **B.** LTP in the mouse hippocampus is time-of-day sensitive with dark-phase induction producing more robust changes in amplitude than light-phase induction. In addition LTP strength depends on time-of-day and not on the time that the animal was harvested. Hippocampal slices from animals killed during the light phase showed more robust LTP if LTP is not induced until after the dark-phase transition would have occurred suggesting endogenous circadian oscillators in hippocampal tissue drive time-of-day-dependent changes in synaptic plasticity. Figure adapted from (Chaudhury, et al., 2005; Gerstner & Yin, 2010).

Figure 1.6

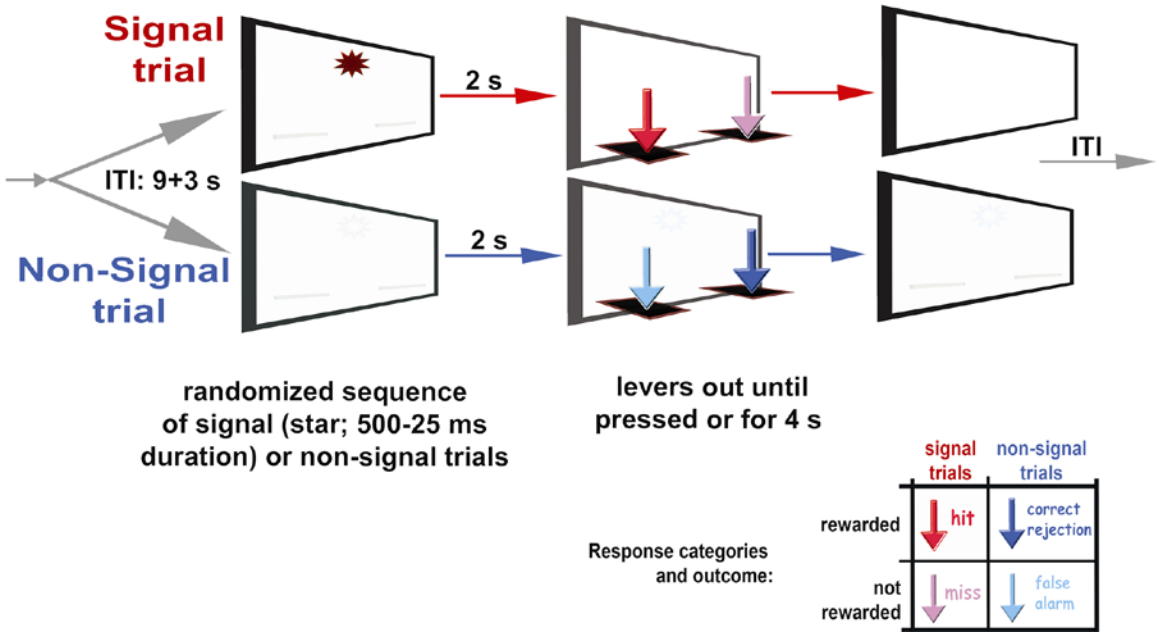

Figure 1.6: **Sustained Attention Task (SAT).** Illustration demonstrating the two trial types and the sequence of events of the sustained attention task (SAT) used in experiments in chapters 2-4. A training session consists of 162 trials (81 signal and 81 non-signal trials presented in a randomized order) and lasts approximately 40 minutes: signal trials consist of 3 signal durations (500ms, 50 ms, or 25 ms; 27 presentations of each in a daily training session) Correct responses in signal trials (hits – red arrow) and non-signal trials (correct rejections – dark blue arrow) are rewarded, while incorrect responses (misses and false alarms, respectively) are not. The inter-trial interval is randomized (9±3 sec) to prevent anticipation of next trial onset. Figure originally published by (Sarter, Parikh, & Howe, 2009).

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Chapter 2

Interactions between cognition and circadian rhythms: attentional demands modify circadian entrainment

Abstract

Animals and humans are able to predict and synchronize their daily activity to signals present in their environments. Environmental cues are most often associated with signaling the beginning or the end of a daily activity cycle but they can also be used to time the presentation or availability of scarce resources. If the signal occurs consistently, animals can begin to anticipate its arrival and ultimately become entrained to its presence. While many stimuli can produce anticipation for a daily event, these events rarely lead to changes in activity patterns during the rest of the circadian cycle. We demonstrate that performance of a task requiring sustained attention not only produces entrainment, but produces a robust modification in the animals' activity throughout the entire circadian cycle. In particular, normally nocturnal rats, when trained during the light phase (ZT4) adopted a significant and reversible diurnal activity pattern. Importantly, control experiments demonstrated that this entrainment could not be attributed to the non-cognitive components of task performance, such as handling, water deprivation, access to water used as a reward, or animal activity associated with operant training. These findings additionally indicate that levels of cognitive performance are modulated by the circadian cycle and that such activity can act as a highly effective entrainment signal. These results form the basis for future research on the role of neuronal systems mediating interactions between cognitive activity and circadian cycles.

Introduction

A variety of daily photic and non-photic environmental signals are able to synchronize (entrain) animals to a 24 hour period. Entrainment to these cues is characterized by daily activity that occurs coincident with or in advance of the daily cue and extends well beyond the actual presentation of the cue. If the activity persists in the absence of the cue and absence of all external cues, including the light-dark (LD) cycle, the activity synchronized by the signal is considered to be entrained. Non-photic cues are thought to entrain activity best in the absence of a LD cycle, however some daily events are significant enough that they cause entrainment of activity in the presence of a LD cycle. For example, novel wheel access in hamsters (Gorman & Lee, 2001) and palatable food access in rats (Mistlberger & Rusak, 1987) are both strong entraining signals in the presence of a LD cycle. Other non-photic cues are less effective at entrainment during the LD cycle, including forced treadmill running in mice (Marchant & Mistlberger, 1996) and daily timed water access in rats (Mistlberger & Rechtschaffen, 1985).

The primary circadian pacemaker in mammals resides in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore, 1983). The SCN produces endogenous biological rhythms, with a period of approximately 24 hours in duration, that are self-sustained in the absence of environmental input (Moore, 1989). Direct light information reaches the SCN via the retinohypothalamic tract (RHT) which projects directly from the retina to the SCN (Card & Moore, 1984; Moore & Lenn, 1972). This input provides the strongest signal influencing circadian activity patterns and is considered the preeminent zeitgeber or universal time-giver. However, the SCN also receives several other non-photic inputs that act to influence circadian patterns. For example, in the hamster non-photic information is thought to be conveyed via the intergeniculate leaflet (IGL) which receives neural input from various brain regions, including the visual system, the prefrontal cortex, and the ascending neuromodulatory systems (Vrang, Mrosovsky, & Mikkelsen, 2003). In addition, oscillators outside of the SCN sensitive to non-photic zeitgebers (*e.g.,* food entrainable oscillator) are thought to project to the SCN to influence entrainment (Guilding & Piggins, 2007).

Other non-retinal inputs to the SCN presumed to influence circadian rhythms include pyramidal projection neurons from neocortical regions involved in action selection and executive function (Hurley, Herbert, Moga, & Saper, 1991; Vertes, 2004), serotonergic neurons originating in the dorsal raphe nucleus (Edgar, Miller, Prosser, Dean, & Dement, 1993; Medanic & Gillette, 1992; Meyer-Bernstein & Morin, 1996; Ying & Rusak, 1994), and cholinergic projections from midbrain and basal forebrain nuclei (Bina & Rusak, 1996; Bina, Rusak, & Semba, 1993, 1997; Erhardt, et al., 2004; Gillette, et al., 2001). The role of cholinergic inputs, particularly from the basal forebrain, has remained unclear, although experimental *in vivo* and *in vitro* data suggest that acetylcholine (ACh) can significantly alter circadian rhythm expression. Injections of carbachol, a non-specific acetylcholine receptor (AChR) agonist, into either the lateral ventricle or SCN produces a phase-dependent shift of activity patterns (Bina & Rusak, 1996). *In vitro* electrophysiological data from the SCN also demonstrate that stimulation of AChRs by carbachol can modify the time at which endogenous peak firing rate occurs in SCN slices (Gillette, et al., 2001). These physiological and anatomical data suggest that ACh can have major effects on circadian rhythms; however, the *in vivo* conditions and importance of endogenous ACh release at the SCN have yet to be explored.

One condition under which ACh release from the basal forebrain is elevated is during performance of tasks of sustained attention; sustained attention is characterized by a subject's readiness to detect an infrequent and unpredictable signal(s) over a prolonged period of time, to discriminate this signal from non-signal events or "noise" and to report the presence or absence of such a signal. Normal sustained attention performance, above chance levels, is dependent upon the integrity of the basal forebrain cholinergic projections to the cortex. Sustained attention performance robustly increases cortical cholinergic transmission; ACh release is further augmented under challenging conditions (*e.g.*, distracter presentation or fatigue) that require top-down optimization of input processing (Kozak, Bruno, & Sarter, 2006; Parikh, Kozak, Martinez, & Sarter, 2007; Sarter, Gehring, & Kozak, 2006; Sarter, Hasselmo, Bruno, & Givens, 2005).

Conversely, the SCN is likely to influence the cognitive and motivational requirements of attentional processing via modulation of circadian sleep/wake/arousal states. The SCN acts to modulate attention networks via projections to the dorsomedial

hypothalamic nucleus (DMN), locus coeruleus (LC), and orexin/hypocretin neurons in the hypothalamus, which in turn project to the basal forebrain (Gabbott, Warner, Jays, & Bacon, 2003; Hajszan & Zaborszky, 2002; Lee, Kim, & Waterhouse, 2005; Novak, Harris, Smale, & Nunez, 2000; Novak & Nunez, 2000; Salazar-Juarez, Escobar, & Aguilar-Roblero, 2002). Overall, this anatomical organization suggests that the forebrain structures involved in attention and the SCN might interact in a bi-directional manner.

The present study was designed to examine the effects of cognitive training, using a task known to activate the basal forebrain cholinergic system, on circadian activity. Although nocturnal rats are naturally more alert during the dark phase, experimenters typically train and test animals during the light phase. Given this natural paradox we chose to look at how daily training on a cognitive task during the light phase influences circadian rhythmicity. We reasoned that such training would provide a non-photic signal, much like food (R. Mistlberger & B. Rusak, 1987) or exposure to novel wheel running (Gorman & Lee, 2001). It is important to note that water alone provides only a weak non-photic signal (Mistlberger & Rechtschaffen, 1985) and thus was chosen over food as the conditional reward for correct responses during training. Our findings demonstrate that sustained attention performance in rats produced a powerful and reversible entraining influence on circadian activity. Control experiments indicate that the elements of training and task performance outside of the requirement for attentional up-regulation were insufficient to drive the changes seen in circadian phase preference. Collectively, the effects of attention on circadian entrainment could provide a useful model for exploring cognitive therapies for circadian disorders as well as ameliorating the effects of nightshift work on circadian rhythms in humans.

Methods

Subjects

Twenty-four male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 350 g at the start of testing were housed individually in opaque single standard cages (27.7cm X 20.3cm). Cages were lined with corn cob bedding and kept in a humidity- and temperature-controlled environment. Animals were allowed to acclimate

for two weeks and were then mildly water deprived to ~95% of their free feeding weight and had *ad libitum* access to food (Purina 5001; supplier: Frontier, Oxford, MI). Animals were allowed access to water for the twelve-hour lighted period beginning on day one and gradually over the next 6 days were titrated down to a single one hour water access period from ZT4 to ZT 5. Rats acclimated for two weeks prior to experimentation and were subsequently divided into two groups designated to either undergo operant training (*i.e.*, task-performing group n=16) or to undergo handling procedures without operant training (*i.e.,* non-performing group n=8). All task-performing animals were given free access to water for twenty minutes following daily experimentation in addition to the quantity of water (~5 ml) obtained as reward during operant testing. Non-performing animals were given one hour of water access daily (ZT4). Standard housing conditions for all animals followed a 12:12 LD cycle with lights on at 1200 h. The task performing group consisted of two subsets of animals: a control group that underwent operant training on a simple reaction time task (SRTT) and an experimental group trained on a task designed to require and test sustained attentional (SAT) performance. Locomotor activity during training was recorded using either infrared (IR) motion detectors placed above the home cages (Slimline PIR; SmartHome, Irvine, CA) or via running wheel activity (Minimitter, Bend, OR). Activity data were collected in 10-min bins using Vitalview software (Minimitter, Bend, OR). Cages were cleaned and animals were weighed once-weekly during the light phase. All procedures were in accordance with protocols approved by the University Committee for the Care and Use of Animals at the University of Michigan.

Experimental timeline: SAT group

Following the two-week acclimation period and gradual water deprivation, task-performing rats began operant training procedures to facilitate shaping on a task that measures sustained attention (SAT). Upon achieving a performance criteria (described below), rats underwent additional operant testing in the presence of a visual distracter (*i.e.,* a flashing house light). Distracter presentation occurred for a total of 10 sessions. Five sessions occurred under standard lighting conditions and five were given following a 6 hour photic phase advance. The two sets of distracter sessions were separated by two

weeks of daily training on the standard task version. This order of events was reversed for half the rats, such that the first exposure to the visual distracter occurred during the phase shift and the second exposure occurred under conditions of stable circadian entrainment. Following the final distracter session animals continued to train daily until all animals had recovered stable entrainment on the new light-dark cycle. The operant component of the experiment was then terminated. On the last day of training the animals entered constant dark conditions (DD) for a period of 9 days to determine whether free-running rhythms would originate from the diurnal phase.

SAT operant methods

Operant training took place 7 days per week. Training and testing was conducted in individual operant chambers (MedAssociates, St. Albans, Vermont) each equipped with two retractable levers, a central panel white light (2.8 W), a ceiling mounted white house-light (2.8 W) and a water dispenser (located on the same wall as the panel lights and levers). Operant chambers were housed within individual sound-attenuating cabinets. Animals were transported from their home cages to the operant chambers and then placed in the unlit chambers for 3 minutes prior to task onset. Animals were first trained to press a lever for a water reward in accordance with a modified fixed-ratio 1 schedule of reinforcement. During this phase of training every lever press results in the delivery of a water reward. In most instances, animals show no side bias with regard to which lever is pressed; however, if more than 5 presses occured on any one lever in a row, the FR1 schedule is modified to require the animal to press the opposite lever before the next reward can be obtained. Rats were next trained to detect signals and discriminate between signal events and non-signal events (i.e. illumination of the central panel light for 1 s vs. non-illumination of the light). Two seconds following a signal or non-signal event, both levers extended into the operant chamber and remained active for four seconds or until a lever press occurred. If the animal failed to respond within 4 seconds, the levers were retracted and an omission was scored. Immediately following a response (either correct or incorrect), both levers were retracted and the variable inter-trial interval or ITI (12±3 s) was reset. During signal trials, a left-lever press indicated a correct response and was scored as a hit whereas a right-

lever press indicated an incorrect response was scored as a miss. Conversely, during non-signal trials a left-lever press indicated an incorrect response and was scored as a false-alarm and a right-lever press indicated a correct response and was scored as a correct rejection. Half the animals were trained in the opposite pattern to address the possibility of any selection bias. Animals received water rewards only for correct responses (30 μL for each hit and correct rejection) whereas incorrect responses (misses and false alarms) were not rewarded. During this phase of shaping incorrect responses resulted in the trial being repeated up to three times in the form of correction trials. If the animal responded incorrectly to three consecutive correction trials, a forced-choice trial was initiated. A forced-choice trial consisted of a signal or non-signal event followed by extension of only the correct lever into the operant chamber for 90 s or until a lever press occurred. In the event that the forced-choice trial was a signal trial, the signal light remained illuminated for as long as the lever was extended. The house light was off during this shaping phase. Behavioral sessions consisted of 162 trials per session. Animals progressed to the subsequent step of shaping if they responded correctly to≥ 59% of both signal - and non-signal trials for three consecutive days.

During the third phase of shaping, signal durations were shortened to 500, 50, or 25 ms (27 trials per signal duration) and the ITI was reduced to 9±3 s. Correction and forced-choice trials were also eliminated. Sessions were divided into three blocks of 54 trials each with all signal durations occurring randomly 9 times per block. Animals were advanced to the final stage of shaping when their performance met or exceeded a performance criterion of 70% hits to the 500 ms signal trials, 70% correct rejections and fewer than 20 omitted trials per session.

Throughout the final stage of testing (referred to as the 'SAT'), the house-light was illuminated throughout the entire session. The addition of the illuminated house-light represents a crucial element of testing sustained attention as it requires the animal to constrain its behavior and focus on the central panel light during task performance. Acquisition of the final stage of training is considered complete once animals reach the final criterion performance of ≥ 70% correct responses to the 500 ms signal trials, ≥ 70% correct responses to non-signal trials and fewer than that 25 omissions per sessions for a

minimum of 3 consecutive sessions. Animals then advanced to the visual distracter phase of training (described below). The latency to reach final performance criteria varied between animals, but was achieved in less than three months for all animals. One animal was ultimately excluded from all performance analysis due to a high level of inconsistency across repeated days independent of condition.

After reaching performance criteria, rats were exposed to a total of ten sessions that included the presentation of a visual distracter as a performance challenge (0.5 Hz flashing house light, referred to as 'dSAT' sessions; (Kozak, et al., 2006; Nuechterlein, Luck, Lustig, & Sarter, 2009). dSAT sessions were broken into two separate sets of 5 continuous daily sessions; each set was separated by two weeks of standard task performance. During distracter sessions, a visual distracter (*i.e.,* a house-light flashing at 0.5 Hz) was presented during the second block of trials (middle 54 trials). Distracter presentation during task performance typically results in reduced correct rejection rates for non-signal trials. Performing during distracter presentation is thought to necessitate the implementation of top-down mechanisms required for the successful detection of signals and the filtering of extraneous stimuli. All task-performing animals received 5 distracter sessions under conditions of stable circadian entrainment and 5 sessions beginning on the first day of a 6-hour phase-advance.

Phase shift

Circadian data collection (described above) began as animals approached performance criteria on the SAT. Once at criteria performance, the LD cycle was advanced 6 h (lights on at 0600 h from 1200 h). The actual timing of task performance in the 24 hour day was not shifted with the LD cycle and therefore now occurred at ZT10 (2 hours before lights off) following the phase advance. The number of days required to recover stable entrained activity following the phase shift of the light cycle was determined as previously described (Goel & Lee, 1996). Briefly, animals were considered stable when the phase (ψ) of activity onset and offset relative to the light cycle and daily SAT training remained unchanged for at

least 3 consecutive days after the LD shift. The period of reentrainment was defined as latency to achieve ψ stability following the phase shift. In most studies employing a phase shift, reentrainment is considered complete only when the animals also recover the same ψ that they had prior to the LD shift. However, this criteria is insufficient in this experiment due to the influence of SAT training now occurring at ZT10. Because of task influence on entrainment, many animals never returned to the same ψ, rather they developed a new stable relationship to the LD cycle associated with the new time of SAT training.

Entrained phase or masking

After animals regained stable entrainment, the possibility of masking (expression of an activity rhythm that is not controlled by entrainment of the SCN) rather than entrainment by the SAT was tested by releasing animals into conditions with no circadian time signals: constant darkness (DD) with no daily SAT training. DD conditions facilitated the assessment of the circadian entrainment underlying the observed patterns of activity. Animals were placed under DD conditions for 9 days. During the initial 48 h, animals underwent complete water deprivation to eliminate the time of water access as a potential circadian cue. During the subsequent 7 days rats were given *ad libitum* access to water. Dim red lights were used in order to assess animal health and to supply food and water at random times of day during the 7 day period.

Attention data analysis

SAT performance yielded measures of hits (H), misses (M), false alarms (FA), correct rejections (CR) and omissions. Statistical analyses were carried out on the relative number of hits (%H=H/H+M), the relative number of correct rejections (%CR=CR/CR+FA), and the number of omissions. Additionally, a Vigilance Index (VI) was also calculated as an overall measure of sustained performance. VI is calculated using the formula VI= (%H-%FA)/ [2(%H+%FA) – (%H+%FA)^2]. This calculation is similar to the Sensitivity Index (Frey & Colliver, 1973) except that omitted trials are excluded from the calculation. VI values can range from -1 to +1, with +1 indicating that all trials were either hits or correct rejections, 0 being a complete

lack of ability to discriminate between signal and non-signal events, and -1 being all trials scored as misses or false alarms. Prior to statistical analyses all percentage data underwent arcsine transformation (Zar, 1974). Multiple within-subjects analyses of variance (*ANOVAs*) were used to determine the effects of distracter on task performance, and to determine if diurnality attenuates the deleterious effects of the visual distracter. Mixed analyses tested the main effects and interactions of entrainment conditions (entrained vs. phase-shifted), signal duration (where applicable: 500, 50 and 25 ms) and distracter/trial block (3 blocks of 54 trials: blocks 1, 2 and 3) on the relative number of hits (%H), correct rejections (%CR), overall vigilance index (VI), and percent omissions (%O). During a distracter session, distracter presentation occurred during the second block of trials, and is thus represented in subsequent analyses as the factor 'trial-block'. *Post hoc* analyses for within subjects comparisons were carried out using the Least Significant Difference test (LSD). All analyses were performed using SPSS V16.

Control Treatment: entrainment to restricted water access

A second group of rats (n=8) was used to determine whether the altered circadian activity produced by SAT training could occur in animals undergoing all procedures except training in the operant chamber. Daily restricted access to water typically acts as a weak zeitgeber for rats (Mistlberger & Rechtschaffen, 1985), and daily handling can also act as a zeitgeber (Hummer, Meixner, & Lee, 2010). We hypothesized that together the two cues might be sufficient to cause the circadian reorganization detected in animals undergoing SAT training during the light phase. Experimental animals were housed and circadian rhythms monitored as described previously.

Control water restriction and handling procedures

Water deprived rats are known to anticipate water availability during restricted water access and daily handling by experimenters (Mistlberger & Rechtschaffen, 1985). In order to test the hypothesis that task-performing rats would entrain to a task of sustained attention, a separate group of non-performing rats was used to ensure that the circadian entrainment observed in task-performing rats could not be attributed to the

handling procedures or restricted water access which accompanied task training. Animals in the control treatment group underwent water deprivation and handling procedures identical to those of the task-performing group; however these rats were never trained to perform the sustained attention task. Control procedures were carried out in two phases: First, animals remained in the home cage environment and received one hour of daily water access from ZT4 to ZT 5. Animals were maintained on this daily schedule for 3 weeks. Entrainment was assessed by releasing the animals into free-running conditions (total darkness) and measuring circadian activity for a period of 10 days. Animals resumed a 12:12 LD cycle with daily 1 hour water access from ZT4 to ZT 5 for a period of 2 weeks followed by 2 weeks of handling procedures consistent with those used with task-performing animals: non-performing rats were removed from their home cages and placed into transport tubs (an opaque 50.8 X 50.8 cm cage) at ZT4. Animals were transported on a rolling cart from the cage-room to the testing chambers over a period of ~3 minutes after which they were returned to their home cages and given access to water for 1 h. Following 2 weeks of daily handling and water restriction, animals were released into free-running conditions with *ad libitum* water access for a period of 10 days to assess the level of circadian entrainment produced by the control treatment.

Control Treatment: entrainment to restricted water access with operant conditioning

A third group of rats (*n*=4) served as a control to test whether the altered circadian activity produced by SAT training was a result of activity associated with operant task performance. The physical activity associated with timed treadmill running in mice (Marchant & Mistlberger, 1996) and the physical activity/rewarding stimuli associated with novel running wheel access in hamsters (Gorman & Lee, 2001) is sufficient to produce entrainment in the presence of a LD cycle. Therefore, we chose a task that mimics the SAT in number of trials, physical activity, and amount of reward, but requires a minimal level of cognitive vigilance.

Control operant methods - SRTT group:

Operant training mirrored the training of animals in the SAT group. All animals were trained 7 days per week on a simple reaction time task (SRTT) in the same operant chambers used for the sustained attention task. After a two-week period of acclimation to the laboratory, animals experienced a week of gradual water deprivation consistent with animals trained on the SAT. Once animals reached one hour of daily access between ZT4 and ZT5, training on the SRTT was initiated. Controls were first trained to press levers for a water reward on the same fixed-ratio 1 schedule of reinforcement as animals trained on the SAT. After 3 days of shaping on the FR1 schedule, animals were advanced to training on the simple reaction time task. The SRTT is designed to mimic the final version of the SAT, albeit without the attentional demand required of the sustained attention task. This version of the task has been previously shown to produce only modest increases in cortical ACh release compared to training on the SAT (Arnold, Burk, Hodgson, Sarter, & Bruno, 2002). The SRTT differs from the SAT in only one way – that is, on every trial *only* the correct lever extends into the operant chamber. Animals are not forced to attend to the signal cue in order to maximize reward on the task. On each trial, as long as a lever press occurs, animals gain access to the same amount of reward as animals performing the SAT. All other conditions, including lighting, number of trials, signal cue duration, and the number of signal to non-signal trials remains consistent between the two versions of the task. Training continued on the operant task for a period of 20 days before the control experiment was terminated.

Circadian analysis

The phases (ψ) of onset and offset of activity relative to dark onset and SAT training onset were determined prior to the phase advance and after reentrainment. The activity onset was defined as 3 or more 10-minute bins of activity that was more than 10% of daily mean activity and was followed by a period of sustained activity. The phase of the onset of free-running activity was described relative to the previous phase of training (ZT4 or ZT10) and the previous phase of lights off (ZT 12). One animal was excluded from analysis because sensor sensitivity was too weak for circadian rhythms to be scored. Repeated measures, multi-variate analysis of variance (*MANOVA*; Systat V10, Systat Software, Inc., San Jose, CA) was used to assess the effect of SAT time (ZT4 vs. ZT10)

on onset and offset to phase of dark onset, onset and offset to phase of SAT onset, and ratio of locomotor activity between the light and dark phases. Activity ratios (LD Ratio) were determined using counts of the total number of 10 min bins in which activity was recorded during the light and dark period, rather than absolute number of movements during the light or dark periods to reduce the variability in amount of activity between animals and within animals across the circadian day. LD ratio values greater than one indicate diurnal activity pattern, whereas levels less than one indicate nocturnal activity pattern. Post-hoc *Tukey* tests were used to compare SAT time effects on specific variables when there was an overall effect on entrainment to LD or SAT. *Paired t-tests* were also used to assess significance between paired comparisons of repeated measures on the SAT and control groups.

Correlations

Correlations between circadian measures of entrainment at ZT4 and ZT10 and attention performance data were performed for hits (%H), correct rejections (%CR), vigilance index (VI) and percent omissions (% O). The possibility of correlations between performance variables altered during the phase shift were tested against four circadian measures: activity phase to SAT time (ZT4 and ZT10), activity phase to dark onset, activity phase to rate of re-entrainment, and activity phase to overall LD ratio. Significance of all correlations was tested using a Bartlett chi-square test with Bonferroni-adjusted probabilities.

Comparison between controls and SAT trained animals

LD ratio comparisons for within subjects tests between SAT(ZT4) and SAT(ZT10) and between H_20 and H_20+handling were made using a *paired t-test*. In addition, the entrained activity phase to the LD cycle and non-photic zeitgebers of daily SAT training at ZT4, daily 1 h water access at ZT4, daily handling with 1 h water access at ZT4, and daily SRTT training at ZT4 were compared across groups with *MANOVA*. *Post-hoc Tukey* tests used to determine significant group differences between treatments for variables with significant overall effects.

Results

Performance at baseline

Baseline levels of performance were calculated using data from the final five sessions prior to experimental manipulations (distracter presentation with or without phase-shift). Hit rates were signal duration-dependent ($F(2,20)= 73.55$, $p<0.001$; 500 ms: 76.9±4.5%, 50 ms: 49.9±0.3%, 25 ms: 38.8±4.3%) and animals responded correctly to 81.31±2.3% of all non-signal trials. Animals omitted an average of 7.34±2.7% of trials per session.

Correlations between circadian entrainment and baseline performance

The level of diurnal entrainment, as measured by LD activity ratio, was positively correlated with SAT performance as measured by vigilance index for the 5 day baseline period measured prior to the beginning of distracter training (Bartlett Chi-square = 3.983, $p=0.046$; *data not shown*). This finding indicates that animals with a more robust diurnal entrainment level during the final stages of operant training at ZT4 showed better overall performance on the SAT.

Effects of distracter presentation during conditions of stable entrainment

During conditions of stable circadian entrainment, animals received 5 consecutive distracter sessions. Performance during distracter presentation (block 2) was contrasted with performance of standard trials from the same session (blocks 1 and 3). Distracter presentation disrupted animals' performance on non-signal trials ($F(2,20)=26.28$, $p<0.001$). *Post-hoc* analyses determined that correct rejection rates were impaired during the distracter block relative to the two blocks of standard trials (block 1 vs. distracter: LSD=0.34, $p\leq0.001$; distracter vs. block 3: LSD=0.471, $p\leq0.001$). Performance of signal trials was dependent on signal duration ($F(2,20)=81.15$, $p\leq0.001$). Hit rates were not affected during the block of distracter trials, but were reduced during trial block 3 following exposure to the distracter ($F(2,20)=6.56$, $p=0.006$; block 1 vs. block 3: LSD=0.205, $p=0.025$; distracter vs.

block 3: LSD=0.252, p=0.003; block 1 vs. distracter: LSD=0.048, p=0.556). Block 3 consists of the final 54 trials of a daily session and measures the ability of the animal to recover from the deleterious effects of distracter trials presented during block 2. Distracter presentation did not affect the number of omitted trials.

Correlations between circadian entrainment and distracter performance

Performance during the 5 continuous days of distracter presentation was compared with the level of diurnal entrainment during the same period. Although distracter presentation impaired performance in all animals, diurnal behavior was a reliable predictor of performance during the recovery phase of distracter sessions. Performance during blocks 1 and 2 were not significantly correlated with entrainment; however, during block 3, the recovery block, performance on the composite measure of vigilance index was correlated with LD ratio. **Figure 2.1** represents the correlation between LD ratio and vigilance index for the longest signal duration (VI_{500}) and suggests that animals with a more diurnal activity pattern during task training show enhanced performance recovery in the trials following the distracter block (Bartlett Chi-square = 3.875, p= 0.048; ***figure 2.1***).

Effects of the phase-shift on SAT performance

The phase advance did not significantly affect animals' SAT performance for the variables measured. Animals' ability to respond correctly to signal or non-signal events was unchanged relative to baseline: hits ($F(1,10)=2.48$, p=0.14); correct rejections: ($F(1,10)=0.932$, p=0.357). Performance on signal trials remained signal duration dependent ($F(2,20)=38.93$, $p\leq0.001$; 500 ms: 82.5±5.4%, 50 ms: 54.8±7.9%, 25 ms: 41.1±6.6%). Similarly, errors of omission were not affected by the phase shift ($p>0.05$). Following the shift, animals responded correctly to 82.9±3.1% of all non-signal trials and omitted only 4.8±1.5% of all trials per session.

Effect of SAT training and control treatment on circadian rhythms

Circadian analysis revealed a robust effect of daily SAT training on locomotor timing. All animals (n=11) exhibited diurnal behavior when the training period was at ZT4 with the average amount of activity during the light phase, divided by that during the dark phase (LD ratio) being 1.69 ± 0.23 and no animals having an LD ratio below 1.0, which would indicate a nocturnal activity pattern (***figure 2.2 and figure 2.3***). When the daily test sessions occurred at ZT10 and circadian activity rhythms regained stable entrainment, robust diurnal activity was still present, and animals became more robustly diurnal as measured by LD ratio (***figures 2.2-2.4***). The increased preference for diurnal activity when SAT was shifted to ZT10 was reflected in a significant advance in daily activity onset relative to the SAT training time ($F(1,16)= 5.633$; $p = 0.03$) but not relative to the LD cycle (***figure 2.5***). Additionally, the LD ratio with SAT at ZT4 correlated strongly with the LD ratio with SAT at ZT10 ($r = 0.961$, Chi-square = 21.760, $p < 0.001$) demonstrating a consistent impact of SAT on circadian entrainment during the light phase both before and after the phase shift.

The free running data collected in constant conditions following SAT training at ZT10 indicated that, while all subjects displayed robust diurnal behavior, a varying amount of diurnal entrainment was evident after release into DD. The range of responses is demonstrated in **figure 2.6**. Activity from Rat 110 represents the entrainment pattern most commonly presented by animals released into constant conditions. Ten of the eleven animals scored, including Rat 110, expressed two clear activity components: one that free ran from the onset of light or the onset of a sustained diurnal activity and one from the high amplitude activity around the ZT10 SAT session. Rat 110 also demonstrates the reemergence of a nocturnal activity phase component soon after release into DD, suggesting that some negative masking has occurred as a result of SAT training. Rat 113 shows a robust level of diurnal entrainment that free ran from the period of lights on and continues until the end of the actogram. This animal also demonstrates very little, if any, nocturnal masking. In some animals (n=4) significant behavior was also immediately apparent during early subjective night, suggesting that the SAT session may also reduce activity during the dark phase (*i.e.,* negative masking). Rat 106 represents the opposite end of the spectrum, showing the least robust level of diurnal entrainment and a significant shift to a nocturnal activity presence shortly after being released into DD.

However, it is important to note that on the first day of DD and no water access, all animals demonstrated a robust period of activity during what would be the subjective day. Overall, the free running behavior following the phase shift to ZT10 training, suggests that circadian activity was entrained by light onset, onset of the SAT training period, and to a lesser extent, light offset.

Interestingly, the majority of animals did not quickly re-entrain to the LD cycle when it was reinstated after the DD period. In **figure 2.6**, only Rat 106 demonstrates a nocturnal phase preference, although it is delayed relative to rats that have never undergone SAT training. Rats 110 and 113 continue shifting the time of activity onset throughout the duration of the actogram. Even after another 10 days in the LD cycle to which they were previously exposed, without any further operant training, they have not established a normal nocturnal phase relationship of activity onset at or just after lights off. Rat 113, for example, maintains considerable diurnal activity 20 days after SAT training has ceased.

The control treatment of one hour daily water access at ZT4 produced no significant effect on circadian entrainment of activity. Only a few counts of binned activity occurred prior to the time of water access after 3 weeks, and no free-running activity occurred at the time of water access when animals were released into constant conditions. This control demonstrates that diurnal circadian entrainment associated with the SAT task is not caused by daily timed water access.

Weak daily entrainment occurred during the light phase when animals were handled prior to daily access to water for 1 h at ZT4 (***figure 2.7***). The small diurnal entrainment effects were apparent in the few minutes of activity prior to daily handling and water access, and the few days that the effect lasted during constant conditions (2.8 ± 0.986 days - *data not shown*). Overall, the second control treatment produced a greater entrained diurnal activity component, but was substantially less than that seen in animals performing the SAT. All animals remained robustly nocturnal throughout the duration of the experiment as measured by the LD activity ratio (***figures 2.3-2.5***).

The simplified reaction time task (SRTT) serves as an essential control for understanding how cognitive activity interacts with circadian rhythms. This task controls

for the critical characteristics of task performance, including physical activity and the rewarding mechanisms inherit in working for reward during operant conditioning. Even after 20 days of continuous training, all subjects (n=4) showed a robust nocturnal activity pattern as measured by LD ratio (0.67 ± 0.35), that did not differ significantly from either the water deprivation group (p=0.890) or the combined water deprivation and handling control group (p=0.550). Figure 8 shows a typical actogram for an animal training on the SRTT. The first 8 days of the actogram represent timed access to water alone occurring between ZT4 and ZT5. The arrow on day 9 indicates the first day of operant training. While no significant increase in diurnality was present, a small amount of diurnal entrainment was present based on the increase in activity in anticipation of the daily training sessions. This level of anticipation was not significantly different from animals that underwent handling and water deprivation alone.

When LD ratio of activity was analyzed for differences between the animals when trained at SAT-ZT4 or SAT-ZT10, the strength of diurnality was significantly after the shift to ZT10 (t(1,10) = -2.427, p = 0.036; ***figure 2.3***). Also, diurnal activity after exposure to the two non-operant control conditions, handling + water access ZT4 and water access ZT4, significantly differed from each other (t(1,7) = -4.052, p=0.007; ***figure 2.3***). When all five groups were compared, the SAT-ZT10 condition was significantly different from every control condition tested (p < 0.004; ***figure 2.3***).

The phase of activity onset and offset to the SAT differed significantly between SAT-ZT4 and SAT-ZT10 (F(1,20)= 31.789; F(1,20)= 69.067; for both p< 0.001; ***figure 2.4***). Significant differences were also found between the treatment and control groups (SAT-ZT4 and handling + water control-ZT4) with respect to onset (p< 0.050) and offset (p <0.001; ***figure 2.4***) of activity to treatment.

The two SAT treatment groups (SAT-ZT4 and SAT-ZT10) also differed in phase onset relative to darkness (F(1,20) = 4.743; p<0.05; ***figure 2.5***). Phase of activity for groups SAT-ZT4 and handling + water control ZT4 significantly differed with respect to onset and offset to darkness (p< 0.001 for both; ***figure 2.5***).

Correlations among circadian measures

Entrainment to the SAT training at both ZT4 and ZT10 were strongly correlated with entrainment to the LD cycle. The effect was strongly driven by a single daily activity onset during the light phase and little activity during the dark phase for most animals (***figure 2.2***). For example, when animals were entrained to SAT training at ZT4 there was a strong correlation between the phase of activity onset relative to time of SAT training and the activity at lights off (r = 0.845, p= 0.004; Chi-square = 43.404, p< 0.001). When animals completed re-entrainment and SAT occurred at ZT10, the correlation was strengthened (r = 0.969, p< 0.001; Chi-square = 154.225, p< 0.001). Taken as a whole, these correlations demonstrate that animals that anticipated the SAT task most strongly also had the most diurnal activity patterns. Somewhat surprisingly, there was no correlation between the phase angle of activity to treatment at ZT4 and ZT10, or between the phase angle of entrainment with dark onset before and after the phase shift.

The phase of activity entrainment relative to the LD cycle during the SAT-ZT4 treatment correlated with re-entrainment rate (r = -0.476, r = -0.577, activity onset and offset relative to dark, respectively; overall Chi-square = 12.916, p= 0.002; ***figure 2.9***). There was also a trend for phase of activity entrainment relative to the SAT-ZT4 training time and re-entrainment rate (r = -0.324, r = -0.262, activity onset and offset relative to SAT onset, respectively; overall Chi-square = 5.338, p= 0.069). There were no correlations between the post-shift entrainment of activity during the SAT-ZT10 and re-entrainment rate.

Discussion

These data support the hypothesis that a demanding cognitive task can produce a robust and significant effect on the organization of circadian locomotor behavior. Daily SAT practice at ZT4 produced a dramatic reversal in the activity pattern, such that all rats exhibited diurnal (predominantly light phase) activity. Additionally, this diurnal activity pattern was maintained after a 6 hour phase advance in the light cycle and additional training at ZT10. This effect was not due to the restriction of water access, daily social contact, activity associated with operant training, or working for access to a reward. SAT performance and dSAT performance also correlated with phase of entrainment at ZT4, suggesting that

entrainment may be an important condition of performance during tasks of cognition. Also, the correlations between ZT10 entrainment phase and performance indicate that performance during the phase advance may predict circadian entrainment patterns following re-entrainment, although this correlation may be driven entirely by the extent of diurnality produced by the SAT at ZT4.

Future experiments are necessary to fully analyze the relationship between SAT performance and phase of circadian recovery as well as the potential for the HPA axis to interact with the attentional system during periods of phase shifting. Mohawk *et al*. demonstrated that depressing the stress response improved recovery time after a phase advance (Mohawk, Cashen, & Lee, 2005). Under such conditions the animals had larger changes in phase each day. This raises the interesting question of whether performance on the SAT would be worse on days individuals are making the largest changes in phase, but might have fewer days of poor performance. This hypothesis supports a condition where animals slower to change phase would perform more poorly on the SAT if the task was advanced in coincidence with the light cycle.

The robustness and size of the effect of SAT training on circadian activity were surprising. Previous studies formed the basis for our theory that there would be entrained anticipatory locomotor activity surrounding the training period but that animals would still retain a predominantly nocturnal activity pattern (R. Mistlberger & B. Rusak, 1987; Mistlberger, 1992, 1993). All subjects, however, showed a robust diurnal activity pattern that extended well beyond the time immediately surrounding the training paradigm (***figure 2.2***). The significance of the SAT was further validated by the control treatments that demonstrated that these findings cannot be due to handling, the presence of daily timed water access, or activity associated with task performance. While all of the control groups show some level of anticipation during the light phase, each group remained primarily nocturnal. The results of these studies provide additional evidence that conclude that there is a significant impact of sustained attention practice on entrained circadian activity patterns. We suggest that the most plausible explanation for a change in circadian activity pattern is due to the activation of the cholinergic basal forebrain circuits recruited by the

SAT task. This theory is bolstered by the finding that performance of a simple reinforcement schedule, known to produce only small increases in ACh levels (Arnold, et al., 2002), is insufficient to produce the diurnal circadian preference shown by the SAT animals in this study. We suggest that this modulatory input could arrive via multiple mechanisms in order to influence circadian entrainment. One possibility is that top-down cortical regulation of SCN output could occur directly via projection systems known to be involved in decision making and cognitive control. Axon tract tracing studies show that projections from these cortical regions innervate not just the SCN, but multiple hypothalamic nuclei (Hurley, et al., 1991; Vertes, 2004) essential for autonomic function. Lesion studies designed to de-afferent neocortical regions essential for task performance are necessary to test the role of top-down regulation in modulation of the circadian clock. Additional studies are addressing the hypothesis that increased ACh release from the basal forebrain circuits, specifically activated with SAT training (Kozak, et al., 2006), directly influence the phase of activity entrainment via the anatomical connectivity that exists between the basal forebrain and the SCN (Erhardt, et al., 2004; Madeira, Pereira, Silva, Cadete-Leite, & Paula-Barbosa, 2004). These studies, in combination with microdialysis experiments in task performing animals, should offer insight into how ACh is being regulated across discrete brain regions in anticipation of daily training. Finally, the question of how cognition influences circadian activity at the level of the SCN remains to be explored. Future experiments will address if the modification of behavior is the result of changes in the expression of clock genes at the level of the SCN itself, or if the changes in phase activity seen in SAT animals occurs via regulation of SCN downstream targets.

An additional surprise in the data was the increased diurnality of animals training at ZT10 compared to ZT4. When the animals were shifted we expected to see the phase relationship shift such that activity would begin at ZT8 or 9, prior to onset of training each day, resulting in the animals being less diurnal after the shift. In other unpublished work from the laboratory (Hummer, et al., 2010), handling between ZT8 and 10 produces a phase advanced activity onset for the day, but the

animals maintain a predominantly nocturnal activity pattern. This leads to the hypothesis that the history of the animal's previous training schedule has an impact on subsequent entrainment as the LD cycle of timing of cognitive training is shifted. Experiments are needed to further explore these possibilities as they may have important consequences for humans that are shifting work schedules.

While light may be the strongest zeitgeber, the data presented here provide evidence that entrainment can be influenced substantially via mechanisms of sustained attention. Furthermore, the effects seen are not simple masking, but rather entrainment to the SAT that is clear in the absence of all external cues. The present experimental approach serves as an animal model for investigating the neuronal mechanisms that mediate the effects of cognitive performance on modulation of circadian activity. As circadian abnormalities contribute to the cognitive symptoms of major neuropsychiatric disorders (Bunney & Bunney, 2000; Monteleone & Maj, 2008; Van den Bergh, Van Calster, Pinna Puissant, & Van Huffel, 2008; Wirz-Justice & Van den Hoofdakker, 1999; Wu & Bunney, 1990), understanding the bidirectional interactions between cognitive performance and circadian control may be key to developing more conclusive neuroscientific theories of these disorders.

Acknowledgements

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Figure 2.1

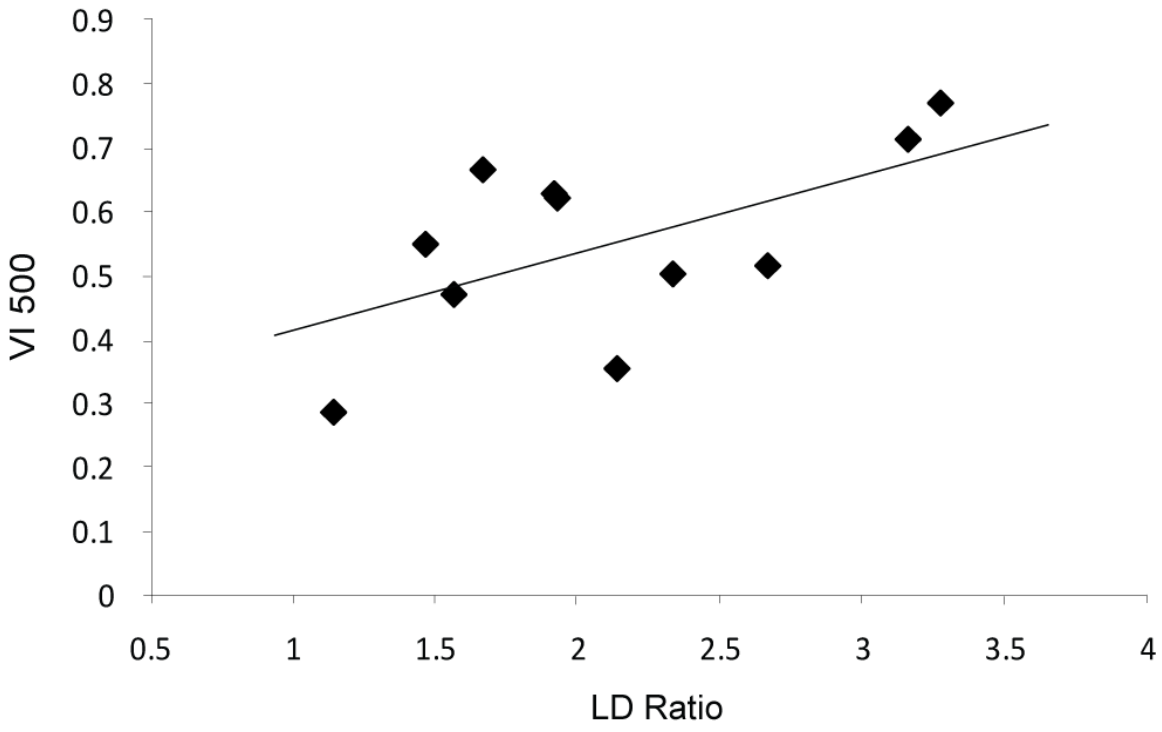

Figure 2.1: **Regression analysis between performance and diurnality.** Analysis demonstrates positive correlation between diurnality, as measured by LD ratio, and VI_{500} performance in the recovery block following distracter training (dSAT). Individual points represent each animals (n=11) average performance from block 3 (final 54 trials representing recovery from distracter) over the 5 day period of distracter presentation plotted against LD Ratio from the same 5 day period. Slope = 0.138; r^2= 0.366; p= 0.048. Performance during blocks 1 and 2 (before and during distracter, respectively) were not significantly correlated with entrainment (p = 0.13 and p = 0.17).

Figure 2.2: **Representative activity record for ZT4 animal shifted to training at ZT10.** Double-plotted actogram for locomotor activity collected with infrared motion detector with 48 hours per line. The actogram shows SAT training at ZT4 relative to the topmost LD bar (where dark bar = lights off) and the open column represents the approximate 40 min SAT training session in which animals are absent from their home cages. Dark phases each day are represented as shaded regions on the figure. The phase shift occurs on the 10^{th} day of the actogram as indicated by the arrow on the left hand side. Following the 6 h phase advance, SAT training remains unchanged but now occurs at ZT10. Re-entrainment to lights-off stabilizes by the 6^{th} day post shift; however, the animal continues to advance its daily onset of activity until day 12 following the shift. This rate of re-entrainment represents the average rate of recovery for all animals in this study.

Figure 2.2

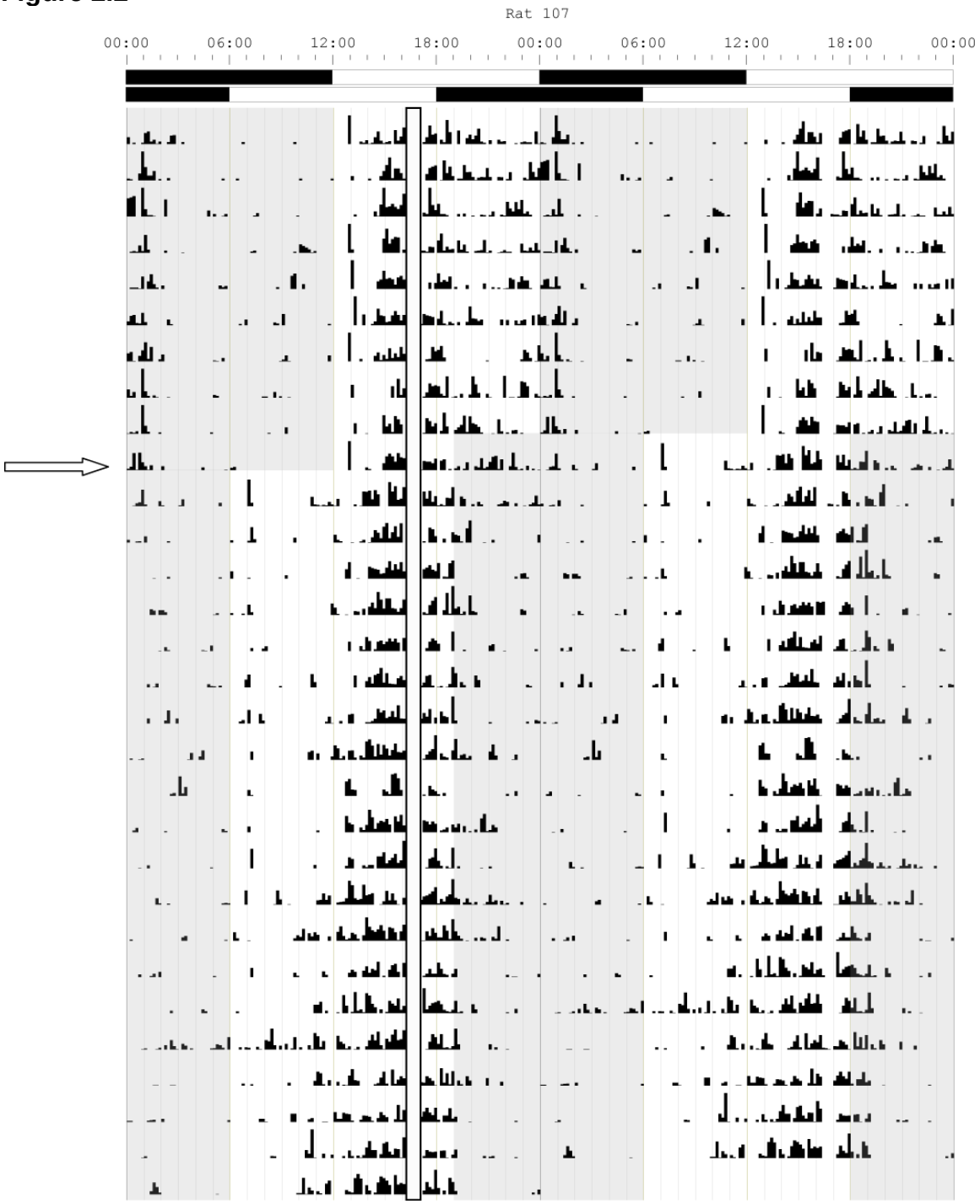

Figure 2.3

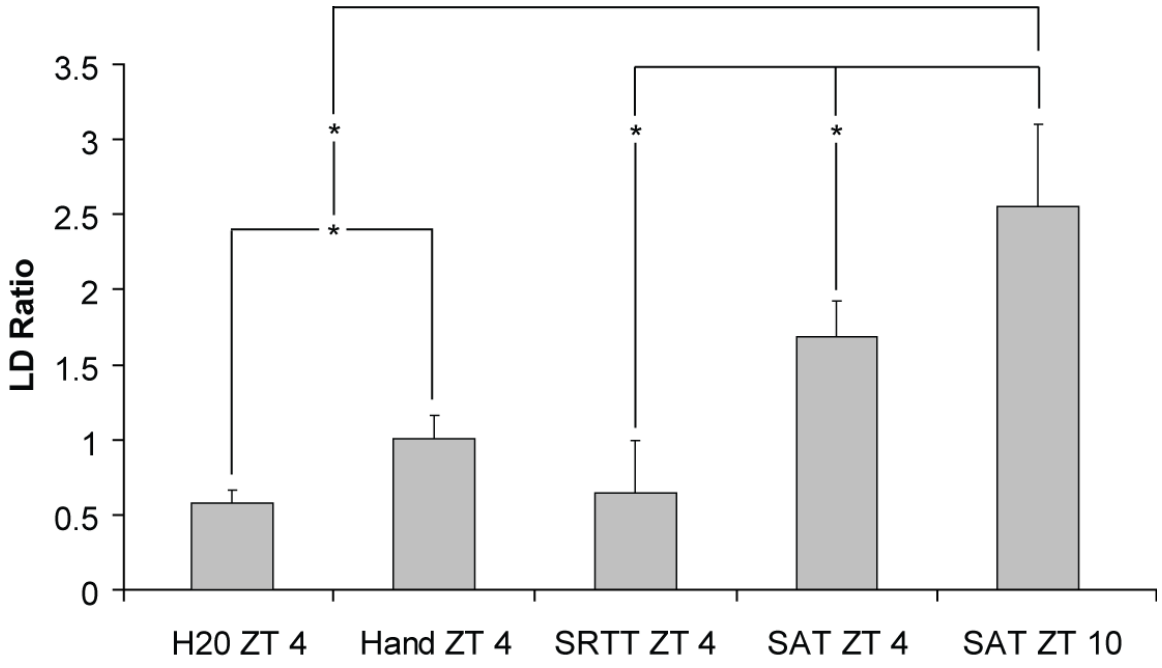

Figure 2.3: **LD Ratio by treatment group for all subjects in this study.** Light:Dark ratio of activity from animals with stable entrainment from before and after phase shifting (SAT at ZT4 and SAT at ZT10) as well as three control conditions (water access-ZT4, handling + water access-ZT4, and SRTT-ZT4). Both SAT groups and non-task performing control groups show significant differences from one another, but only SAT-ZT10 significantly differs from all the control groups (* indicates a significant difference between bracketed bars with a $p < 0.05$).

Figure 2.4

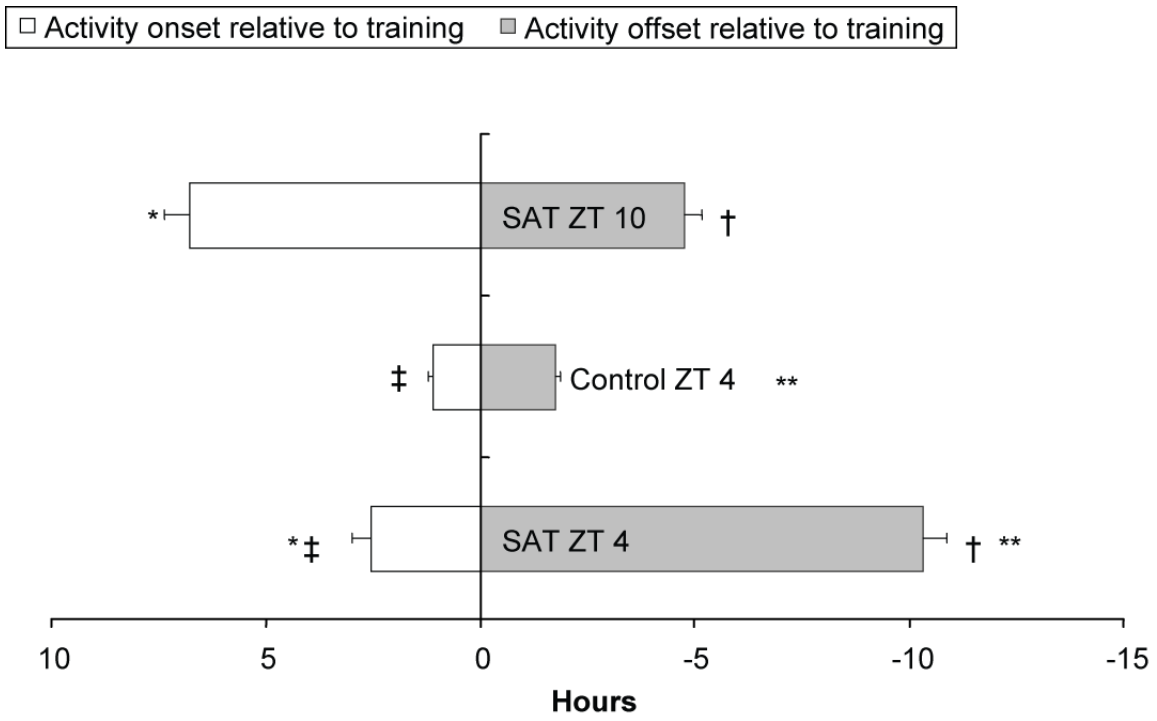

Figure 2.4: **Mean phase relationship between time of diurnal locomotor activity and time of SAT training.** Activity began and ended significantly earlier, relative to training, for animals with SAT at ZT10 than SAT at ZT4. The control animals that were handled and provided with water at ZT4 differ significantly from both SAT groups for activity onset, cessation and duration (* $p < 0.001$, SAT ZT10 and SAT ZT4 differ for activity onset; ** $p < 0.001$, Control and SAT ZT4 differ for activity onset; † $p< 0.001$, SAT ZT10 and SAT ZT4 differ for activity offset; ‡ $p < 0.05$, Control and SAT ZT4 differ for activity offset).

Figure 2.5

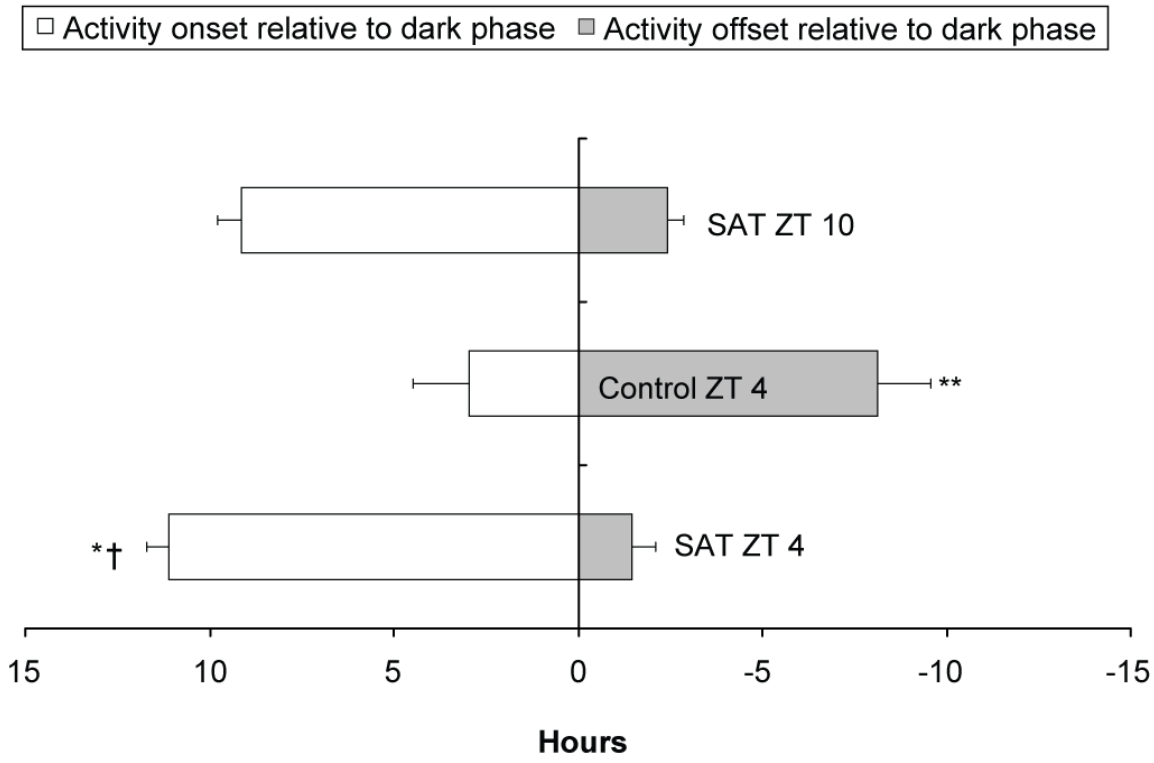

Figure 2.5: **Mean phase relationship between time of locomotor activity and lights-off.** The animals experiencing SAT at ZT4 and ZT10 began and ended activity significantly earlier than the control animals that were handled and provided with water at ZT4. SAT ZT4 animals also began activity earlier than animals with SAT at ZT10, but they did not end activity earlier after lights off (* $p < 0.05$, activity onset before dark differs between SAT ZT10 and SAT ZT4, † $p < 0.001$; activity onset before dark differs between Control and SAT at ZT4; ** $p < 0.001$, activity offset differs between Control and SAT at ZT4).

Figure 2.6: **Activity records of 3 subjects training at ZT10 released into DD (dark:dark).** Double-plotted actogram representing the locomotor activity collected with infrared motion detectors of three animals when released into constant conditions (DD). DD begins at the day indicated by the first open arrow. The second filled arrow indicates when the LD cycle was reinstated. Shaded regions above the open arrow and below the closed arrow represent the dark phase of the 24 hour period. The shaded region below the open arrow and before the closed arrow indicates the period of time when lights-off previously occurred. All animals following training at ZT10 show robust activity during subjective day on the first day of DD, and activity bouts on subsequent days that begin during the light phase periods prior to release into total darkness. Rat 110 demonstrates an example of diurnal activity that continues to occur at the prior phase, although some disappeared (e.g, the bout at lights-on, and activity between the onset of the main diurnal bout and the heavy bout at SAT training time) after being released into DD. While some level of nocturnal masking is apparent, at least two diurnal activity bouts also free-ran from the initial phase and are marked with superimposed solid lines demonstrating circadian changes resulting from SAT training are a product of entrainment and not masking alone. Rat 113 demonstrates a primarily diurnal level of entrainment to SAT training. This animal continues to show free-run activity from the light phase until the end of the actogram as demonstrated by the superimposed solid line with little indication of nocturnal masking. Rat 106 shows entrainment to the SAT task; however, nocturnal activity returns robustly within a few days of being released into DD, suggesting that masking may be more important for this animal than the others. Both Rats 110 and 113 continue to free-run after the LD cycle is reinstated, whereas Rat 106 is entrained with a nocturnal phase.

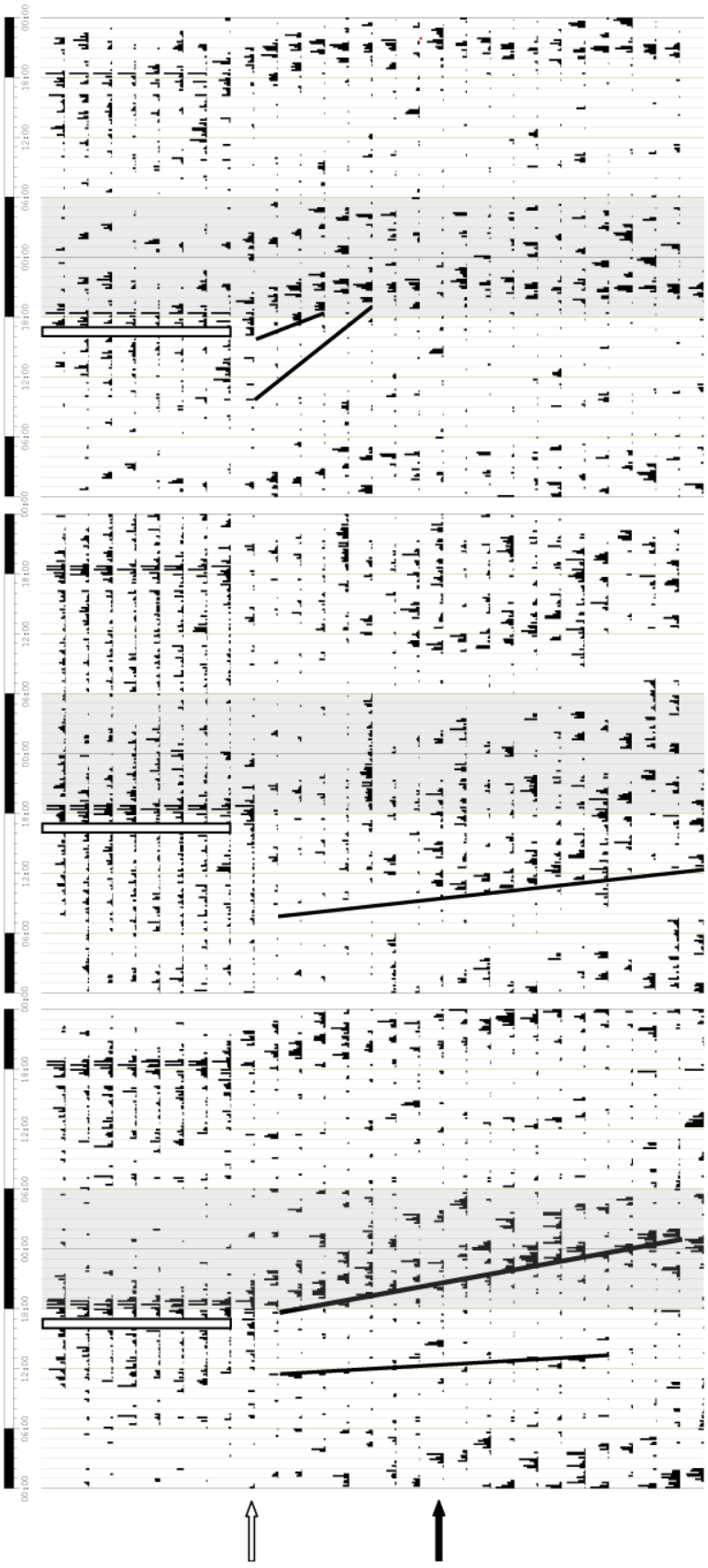

Figure 2.6

Figure 2.7: **Representative activity record for animal undergoing handling and water restriction.** Double plotted locomotor activity actogram collected by infrared motion detector of an animal experiencing both daily handling followed by 1 h of water access. The shaded regions represent the dark phase of the 24 hour period. Although there is an increase in diurnal locomotor behavior that accompanies handling, the animal remained strongly nocturnal. See Figure 3 for quantitative analysis of LD Ratio. Open rectangular column indicates the time of daily water access.

Figure 2.7

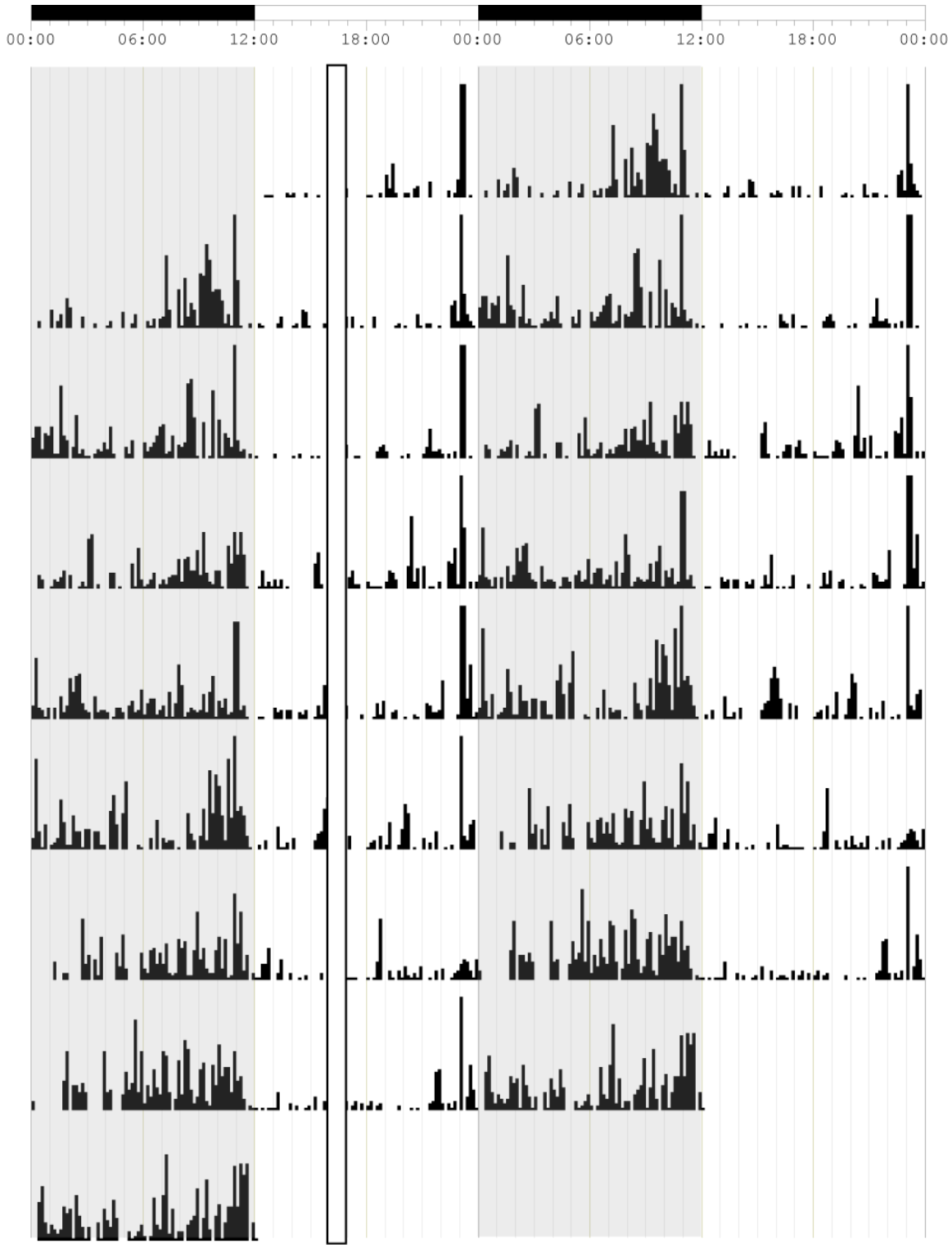

Figure 2.8: **Representative activity record for animal trained on SRTT (simple reaction time task).** Double plotted running wheel actogram of typical animal training on a simplified low-cognitive demand operant task (SRTT – see methods for details). During the first 8 days of the actogram, the animal is receiving 1 h of daily water access from ZT4-ZT5. Operant conditioning begins on the 9^{th} day of the actogram as indicated by the open arrow on the left hand side. The shaded region denotes the 12 hour dark phase of the experiment where the open box indicates the time of daily training. Training on the SRTT continued for 21 days with no overall shift in diurnality ratio.

Figure 2.8

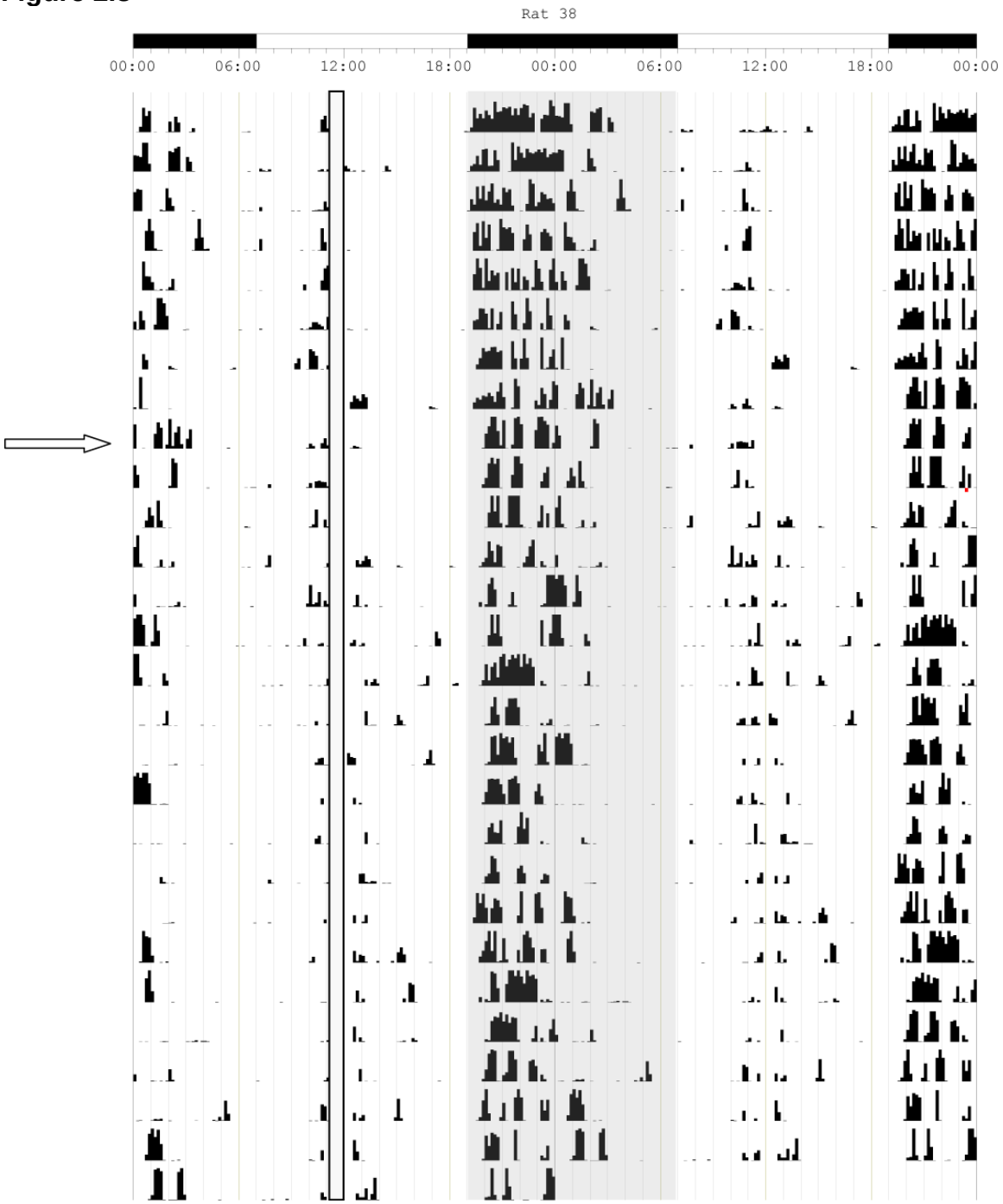

Figure 2.9

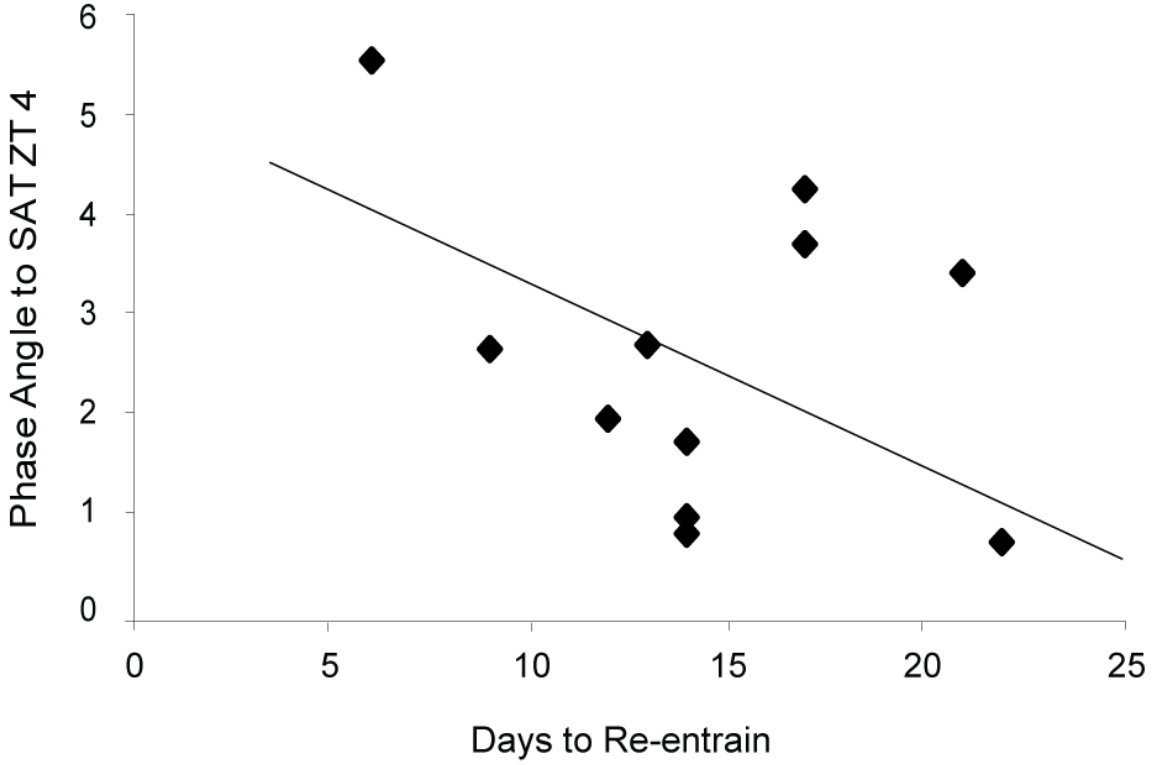

Figure 2.9: **Regression analysis between re-entrainment rate and strength of entrainment to previous training time**. Correlation between reentrainment rate after a 6 h phase advance in the LD cycle and entrained activity onset before the phase shift when entrained with SAT at ZT4. See text for statistical analysis.

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Chapter 3

Circadian phase and cognitive performance: bidirectional interactions

Abstract

Circadian rhythms influence a variety of physiological and behavioral processes. However, relatively little is known about how the states of these processes interact with the organisms' ability to acquire and retain information about their environment. This set of studies was designed to determine whether rats trained during the light-phase, outside their endogenous active period, demonstrate the same level of daily performance, rate of acquisition, and remote memory ability as their nocturnally-trained counterparts in tasks of sustained attention and spatial memory. Furthermore, we explored how daily task training influenced circadian patterns of activity and if those changes interact with performance. Our results indicate that rats demonstrate robustly better acquisition and performance on an operant task requiring elevated levels of attentional effort when trained during the dark-phase. This time-of-day effect on performance could further be dissociated with animals trained closer to the onset of the dark-phase performing better than animals trained earlier in the light-phase. Additionally, when animals showing asymptotic performance in the light-phase were switched to training during the dark-phase, they showed a significant increase in daily performance, but poorer daily performance if moved from the dark-phase to the light-phase. Time-of-day did not affect acquisition or performance on the Morris-water maze; however, when animals were tested two weeks after their last day of training, animals showed significantly better remote memory if training occurred during the dark-phase. Finally, attentional, but not spatial, performance during the light phase caused a shift toward diurnal activity patterns; this shift was most robust when performance taxed the cognitive control of attention. Our findings support a theory of bi-directional interactions between certain types of cognitive performance and circadian processes. These results are consistent with the view that the

circadian abnormalities associated with shift work, aging and neuropsychiatric illnesses may contribute to the deleterious effects on cognition often present in these populations. Furthermore, these findings suggest that the consideration of time of daily cognitive practice is an important variable for a variety of cognitive tasks principally used in psychological and neuroscience research.

Introduction

Endogenous circadian oscillators are responsible for daily changes in both physiological and behavior systems. The role of circadian rhythms in physiological processes has been well characterized and include daily regulation of genes important for metabolic homeostasis (Rutter, Reick, & McKnight, 2002), immune function (Oishi, et al., 2003), cell development and proliferation (Meerlo, Mistlberger, Jacobs, Heller, & McGinty, 2009), and cell signaling (Barnes, McNaughton, Goddard, Douglas, & Adamec, 1977). Furthermore, circadian dysregulation has been linked to a variety of systemic and biological pathologies (Folkard & Akerstedt, 2004; Lange, Dimitrov, & Born, 2010; Waage, et al., 2009). While much of the basic physiology under control of circadian pacemakers has been well studied, the interactions between these processes and their influence on cognitive behavior have been relatively unexplored. Although there is speculation that performance and learning may be influenced by circadian processes (*e.g.* Daan, 2000), almost nothing is known about whether regularly timed cognitive processes may exert influences on circadian oscillators, perhaps in service of optimizing task acquisition and augmenting performance.

The role of circadian effects on learning and memory has long been of interest to researchers. Early findings by Holloway and Wansley demonstrated that passive avoidance performance was optimized periodically at 24-hour intervals following learning (Holloway & Wansley, 1973a, 1973b; Wansley & Holloway, 1976), and it was later determined that this periodic performance was dependent upon an intact suprachiasmatic nucleus (SCN; Stephan & Kovacevic, 1978). Investigators have also queried how SCN-driven biological rhythms interact with performance through time-of-day studies on learning. For example, habituation to auditory cues in pigeons (Valentinuzzi & Ferrari, 1997) and habituation to spatial novelty in mice (Valentinuzzi, et al., 2000) were found to be more robust during the animal's endogenous active phase. Hoffman and Balschun (1992) illustrated that mice, when trained on an alternating T-maze, showed fewer errors and faster rates of acquisition when training occurred during the dark-phase, and in studies of contextual and cued fear conditioning, time-of-day

effects have been reported in acquisition, recall, and extinction learning (Chaudhury & Colwell, 2002; Eckel-Mahan, et al., 2008).

We have previously demonstrated that cognitive effort on a sustained attention task can have profound influence on circadian organization of activity (Gritton, Sutton, Martinez, Sarter, & Lee, 2009). This influence could not be attributed to daily handling, reward conditions, or the process of operant conditioning. We proposed that the changes in circadian re-organization produced by cognitive activity were mediated by augmented release of the neurotransmitter acetylcholine (ACh) based on anatomical and physiological evidence. Cholinergic projections from the midbrain and nuclei of the basal forebrain including the medial septum, nucleus basalis, and diagonal bands project to and innervate clock-gene expressing neurons of the SCN (Bina, Rusak, & Semba, 1993, 1997; Castillo-Ruiz & Nunez, 2007). Pharmacological evidence and lesion data also provide compelling evidence for cholinergic signaling at the SCN influencing circadian activity. For example, injections of carbachol, a non-specific acetylcholine receptor (AChR) agonist, into either the lateral ventricle or SCN produce phase-dependent shifts of activity patterns (Bina & Rusak, 1996; Buchanan & Gillette, 2005; Gillette, et al., 2001), and lesions of the basal forebrain cholinergic system block entrainment to timed daily handling in rodents (Hummer, Meixner, & Lee, 2010). Although ACh neurotransmission in the SCN does not show circadian variations in release, ACh levels as measured from microdialysis studies shows fivefold increases in response to 30 minutes of arousal (Murakami, Takahashi, & Kawashima, 1984). It is possible that timing information about salient events may be conveyed to the SCN through the release of acetylcholine. In support of this theory, Hut and Van der Zee (2010) have proposed that acetylcholine release at the level of the SCN may act as a ‘time-stamp’ that can be later used to interweave context, time, and place associations in the service of memory consolidation.

The present study was designed to examine the interaction of circadian factors on performance in two tasks of cognitive learning. The first task is a discrimination-based operant task requiring sustained periods of attentional effort and is dependent upon the basal forebrain cholinergic system for above chance levels of performance (McGaughy,

Kaiser, & Sarter, 1996). The second task was the Morris Water Maze (MWM), a commonly used task of hippocampal-dependent spatial learning in rodents. We trained groups of animals on a 12:12 light-dark schedule four hours after the onset of the dark-phase (ZT16) or four or ten hours after the onset of the light-phase (ZT4 and ZT10, respectively). We measured whether the rate of acquisition and level of performance was influenced by time-of-day as has been described in some performance tasks. Secondly, we tested how timed daily task performance influences circadian patterns of activity acutely and how these patterns of activity change over long-periods of continuous daily training. Finally, we determined whether the strength of entrainment, as measured by phase markers of activity, predict animal performance in a useful or meaningful way.

Methods

Subjects

Seventy-six male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 350 g at the start of the behavioral training were used for these studies. All animals, following arrival from the supplier, were housed individually in standard opaque single cages (27.7cm X 20.3cm) maintained on a LD 12:12 cycle. Cages were lined with corn cob bedding and kept in a humidity- and temperature-controlled environment at 21 ± 1° C. Animals had *ad libitum* access to food (Purina 5001; supplier: Frontier, Oxford, MI) and water throughout the habituation phase of the study. Activity was monitored with intra-abdominal transmitters (pdt 4000 e-mitter-telemetry implants, Mini-mitter Inc., Sunriver, OR; n=18), or via running wheel activity (n=58). Animals were allowed to acclimate for a minimum of two weeks before undergoing surgery or having running wheels introduced to their cages. Animals with surgical procedures (see below) were given two additional weeks of recovery before being introduced to running wheels. All procedures were in accordance with protocols approved by the University Committee for the Care and Use of Animals at the University of Michigan.

Surgeries and data collection

Transmitters were intraperitoneally implanted in half of all animals used in SAT operant training group I; the remaining animals in this study underwent sham surgical

procedures identical to animals given telemetry implants. No surgeries were performed for subjects used in the water maze studies. Animals were anesthetized using Isoflurane (5% iso, 95% oxygen). Incisions were made in the ventral abdominal area and transmitters were placed in the abdominal cavity. Muscle walls were sutured with 4.0 chromic gut and skin incisions closed with wound clips. Clips were removed 10 days post-surgery.

Locomotor activity during baseline and training phases was collected in 10-min bins using Vitalview software (Minimitter, Bend, OR). Cages were cleaned and animals were weighed twice-weekly during the animal's training phase when they were absent from their home cage. All circadian data was analyzed off-line using Actiview software (Minimitter, Bend, OR). The experimental outline for this study is presented in **figure 3.1**.

Experimental procedure: SAT groups I and II

Animals that were designated for operant training were allowed to acclimate for two weeks after arrival before undergoing surgery to implant telemetry devices. Following recovery (~2weeks), animals were introduced to running wheels and allowed to acclimate for two weeks before beginning the baseline portion of the experiment. During the two-week baseline period that followed animals continued to have *ad libitum* access to food and water during while running wheel data was collected. Afterwards, animals were mildly water deprived to ~95% of their free feeding weight while having *ad libitum* access to food. Adjustment to water deprivation occurred over seven days beginning with twelve-hour access on day one (starting 6 hours prior to their training time; ZT4, ZT10, or ZT16 and continuing for 12 hours). Gradually over the next 6 days, animals were titrated down to a single one-hour water access period from ZT4 to ZT 5, ZT10 to ZT11, or ZT16 to ZT17, respectively. Following the two-week acclimation period and gradual water deprivation, rats began operant training procedures to facilitate shaping on a task that measures sustained attention (SAT). All task-performing animals were given free access to water for twenty minutes following daily training sessions in addition to the quantity of water (~5 ml) obtained as reward during operant testing. Upon achieving a performance criteria (described below), SAT group II

rats were given additional operant testing in the presence of a visual distracter (*i.e.* a flashing house light). Distracter presentation occurred for a total of 4 sessions. Individual distracter sessions were separated by a minimum of three consecutive days on the standard task version with above criterion performance to ensure recovery before an additional distracter session was administered. Following recovery from the second distracter, SAT group II animals were removed from daily training until a stable nocturnal circadian pattern of activity re-emerged for all animals (34 days). Animals were then trained with their training times reversed (ZT4 animals trained at ZT16, and ZT16 animals trained at ZT4; see ***figure 3.1B***) for at least 30 days. SAT Group II rats had two more distracter sessions during the reversal training phase to compare the effects of distracter before and after the reversal.

Sustained attention task (SAT)

Operant training took place seven days per week. Behavioral training and testing was conducted in individual operant chambers (MedAssociates, St. Albans, Vermont) outfitted with two retractable levers, three red panel lights (2.8 W), and one red house light (2.8 W). The water dispenser was located on the same wall as the panel lights and levers. Animals were transported from their home cages to a room housing operant chambers in a light-tight shuttle box. The experiment room was maintained in dim red light. Animals were removed from the shuttle box and placed in the unlit operant chamber for 5 minutes prior to task onset. Operant chambers were housed within individual sound-attenuating cabinets. The shaping protocol for this task has been published in detail previously (Gritton, et al., 2009). Briefly, animals were first trained to press a lever for a water reward in accordance with a modified fixed-ratio-1 schedule of reinforcement. The FR1 schedule is modified in that it requires animals to respond to both levers and deters a selection bias if one exists.

Animals were next trained to discriminate between signal and non-signal trial types (*i.e.*, illumination of the central panel light for 1 s vs. non-illumination of the light). During the discrimination-learning (DL) phase, two seconds following a signal or non-

signal event, both levers extended into the chamber and remain active until a lever press occurred or 4 seconds had passed. If no response occurred within the allotted period, the trial would be scored as an omission and the ITI (12±3 s) would reset. During signal trials, a right-lever press indicated a correct response and was scored as a hit whereas a left-lever press indicated an incorrect response was scored as a miss. Conversely, during non-signal trials a right-lever press indicated an incorrect response and was scored as a false-alarm and a left-lever press indicated a correct response and was scored as a correct rejection. The lever rules were reversed for half of the animals to account for possibilities of handedness or selection bias. Animals received water rewards only for correct responses (30 μL for each hit and correct rejection) whereas incorrect responses (misses and false alarms) were not rewarded. The house light was off during this shaping phase. Behavioral sessions consisted of 162 trials per session. Animals progressed to the subsequent step of shaping if they responded correctly to≥ 70% of both signal - and non-signal trials for three consecutive days with fewer than 20 omitted trials per session.

The next phase of training introduced abbreviated signal durations of three different durations (shortened to 500, 50, or 25 ms; 27 trials of each duration during a daily training session) and the ITI was further reduced to 9±3 s. Individual sessions were divided into three blocks of 54 trials, each with all signal durations occurring randomly 9 times per block. Animals were advanced to the final stage of training when their performance met or exceeded a performance criterion of 70% hits to the 500 ms signal trials, better than 70% correct rejections, and less than 20 omitted trials.

During the final stage of testing (referred to as the ‘SAT’), the overhead red house-light was illuminated to increase the requirement for focused attention. The addition of the illuminated house-light requires the animal to constrain their visual focus to the central panel during testing to optimize performance. Animals were required to maintain criterion performance (≥ 70% correct responses to the 500 ms signal trials, ≥ 70% correct responses to non-signal trials and fewer than that 20 omissions per session) for 3 consecutive sessions before task acquisition was considered complete.

SAT group II animals were additionally presented with visual distracter sessions after reaching criterion (referred to as the 'dSAT'). Rats were exposed to a total of 4 sessions that included the presentation of a visual distracter as a performance challenge (*i.e.,* a house-light flashing at 0.5 Hz) during the second block of 54 trials. Distracter presentation typically results in reduced correct rejection rates for non-signal trials and a bias for the hit lever on all signal trials.

SAT data analysis and statistics

SAT performance yielded measures of hits (H), misses (M), false alarms (FA), correct rejections (CR) and omissions. Statistical analyses were carried out on the relative number of hits (%H=H/H+M), the relative number of correct rejections (%CR=CR/CR+FA), and the number of omissions. Additionally, a Vigilance Index (VI) was also calculated as an overall measure of attentional performance. VI is calculated using the formula VI= (%H-%FA)/ [2(%H+%FA) – (%H+%FA)^2]. VI values can range from -1 to +1, with +1 indicating that all trials were either hits or correct rejections, 0 being a complete lack of ability to discriminate between signal and non-signal events, and -1 being all trials scored as misses or false alarms. Statistical analysis of hits (%H), correct rejections (%CR), overall vigilance index (VI), and percent omissions (% O) was tested using a one-way *ANOVA* with time of daily training as the treatment factor. Significant main effects were further analyzed using *Tukey post-hoc* analysis. Multiple within-subjects analyses of variance (*MANOVA*s) were used to determine the effects of distracter on task performance and mixed analyses tested the main effects and interactions of signal duration (where applicable: 500, 50 and 25 ms) and distracter/trial block (3 blocks of 54 trials: blocks 1, 2 and 3) on the relative number of hits, correct rejections, vigilance index, and omissions. During a distracter session, distracter presentation occurred during the second block of trials, and is represented in subsequent analyses as the factor 'trial-block'. *Post-hoc* analyses for within subjects' comparisons were carried out using *Tukey post-hoc* analysis. All analyses were performed using SPSS V16.

Experimental procedure: water-maze group III

Following arrival from the supplier and acclimation to their housing environment, rats (*n*=24) were introduced to running wheels and allowed to acclimate for two weeks while undergoing 5 min of random daily handling before beginning the baseline portion of the experiment. Animals had *ad libitum* access to food and water throughout the course of the experiment. Water maze training took place 7 days per week at either ZT4 or ZT16 based on treatment group (*n*=12/group). Animals underwent daily training sessions (1 trial/day) for 28 consecutive days. Animals were given only one trial each day in order to increase task difficulty so that time-of-day effects, if they existed, could be better dissociated. Rats were also given an 8th probe trial 14 days after the last training session to test remote memory for the platform location. Following the last training trial (day 28), rats were released into constant darkness and were maintained in DD until after the remote memory test had been concluded. Animals were thus given the remote memory test while in free-running conditions and not tested at a particular time *per se*.

Water maze acquisition and performance

Animals were trained in a room 3.2 m^2 in dim red light (16.2 lux). All wall cues consisted of black shapes on white walls to allow for heightened contrast under red-light conditions. Morris water maze (MWM) training was conducted in a 1.6 meter diameter pool made of black acrylonitrile butadiene styrene (ABS) centered in the middle of the training room. Water was filled to a depth of 38 cm. The platform consisted of black neoprene glued to a 4 cm platform that was placed 1.75 cm below the water surface. Water temperature was maintained at 26±5 C° throughout the course of the experiment. Training occurred for 28 days with 1 trial per day except on probe trial days. Probe trial days were initiated with a probe trial followed by a standard trial to reduce the possibility of extinction learning. Standard trials began with the rat being placed on the platform for 15 sec. The rat was then moved to 1 of 5 possible start locations and placed into the water facing the wall of the pool. Once the experimenter released the animal, the trial timer was started and ended either when the rat reached the platform location or after 60 sec had elapsed. In the event of an unsuccessful trial, animals were led by the experimenter to the platform location. At the conclusion of each trial, the animal was allowed to remain on

the platform for 15 sec before being dried and returned to their opaque transport enclosure. Starting positions for each day were chosen randomly among five start positions; however, every animal was placed into the water from the same location each day. Probe trials were conducted every fourth day. During the probe trial, the escape platform was removed and rats initiated a trial by being placed in the pool at the start location directly opposite of the platform and allowed to swim for 60 sec. At the conclusion of the probe trial, animals were dried off and returned to their transport enclosure long enough for the platform to be re-inserted (~15sec). After which a standard trial was initiated. A final probe trial (probe trial 8) was conducted 14 days after the last standard training trial as a measure of remote memory.

Water maze data analysis and statistics

A video camera placed above the pool with a DVD recorder was used to record animal movement and location information. Watermaze data were analyzed off-line using a motion-tracking software package (*Actimetrics*, Wilmette, IL). Metrics analyzed included: path length, time to platform, and swim speed. For probe trials, the additional variables of Gallagher proximity and time spent in quadrant were quantified. Multiple within-subjects repeated analyses of variance (*MANOVAs*) were used to determine the effects of time of daily training on maze performance. Significant main effects were further analyzed using *Tukey post-hoc* analysis. Remote memory was assessed by analyzing block performance using *paired t-tests* and between-subjects effects were tested using *independent t-tests* for time-of-day. A probability value of $p<0.05$ was used as the criteria to determine statistical significance.

Circadian analysis and statistics

The binning procedure resulted in absolute counts of activity binned into 144 data points over a 24-hour period. Light-dark (LD) activity ratios were calculated by summing the total activity (movement or wheel revolutions) collected in 10 min bins during the light-phase divided by the summed activity of the dark-phase across the 24 hour cycle. LD ratios greater than one indicate animals with diurnal activity pattern,

whereas LD ratios less than one indicated a nocturnal activity pattern. Repeated measures, within-subjects analyses of variance were used to assess the effect of SAT time (ZT4 vs. ZT10 vs. ZT16) and the ratio of locomotor activity between the light and dark-phases across different phases of task acquisition. Statistical analysis of LD ratio between groups was tested using a one-way *ANOVA* with training time-of-day as the factor. Significant main effects were analyzed using *Tukey post-hoc* analysis. A probability value of $p<0.05$ was used as the criteria to determine statistical significance.

Correlations between circadian measures of entrainment at ZT4, ZT10, and ZT16 and attention performance were performed for hits (%H), correct rejections (%CR), vigilance index (VI) and percent omissions (% O). Significance of correlations was tested using a Pearson correlation with $p<0.01$ as the criteria for statistical significance.

Results

Acquisition rate and performance in a task of sustained attention (SAT) is dependent on time-of-day

All animals reached criterion performance on this task within 144 days, demonstrating the ability to acquire the task regardless of the time that training occurs; however, the rate of acquisition was greatly influenced by the time of daily training (***figure 3.2A, figure 3.2B***). **Figure 3.2A** denotes absolute time to criterion for each animal plotted along the x-axis from first to last for all 36 animals trained (n=12 per group) sorted by training time. Animal trained at ZT16 consistently showed fewer days to reach criterion with animals trained at ZT4 taking the longest time to achieve criterion and animals trained at ZT10 falling in between. ZT16 animals on average showed the fastest acquisition rate (40.2 ± 6.8 days) followed by ZT10 (50.5 ±7.8 days), and ZT4 (77.6 ± 11.0 days). There was a significant main effect of training time on acquisition ($F(2,33) = 4.847$, $p = 0.014$). *Post-hoc* analysis revealed significant differences in the number of days to reach criterion between ZT4 and ZT10 ($p = 0.036$) and between ZT4 and ZT16 ($p = 0.013$), but not between ZT10 and ZT16 (***figure 3.2B***). This basic pattern

was conserved for all animals in this study with the slowest ZT16 animals generally reaching criteria faster than the slowest ZT4 animals, and the ZT10 animals falling in between as demonstrated in **figure 3.2A**. These data demonstrate that in tasks requiring sustained levels of attentional demand, animals trained outside of their endogenously driven period of activity show robust deficiencies in acquisition.

We also assessed whether animals trained at different times-of-day would show relative differences in peak performance on the well trained task. Post-criterion performance of ZT16 animals were significantly better than the ZT4 training groups and marginally better than the ZT10 training group (***figure 3.2C and figure 3.2D***). There was an expected within-subjects effect of signal duration ($F(2,64)= 372.097$, $p<0.001$) and a between subjects main effect of time-of-daily training on performance as characterized by vigilance score ($F(2,32)= 3.371$, $p<0.047$; ***figure 3.2D***). *Post-hoc* analysis revealed ZT16 animals showed significantly better performance on the longest and shortest signal trials when compared to animals training at ZT4 (500 ms: $p = 0.009$; 50ms: $p = 0.092$; 25ms: $p = 0.047$). This was driven primarily by signal duration differences in hit rates ($F(2,32)= 3.551$, $p=0.040$), as no significant effects on non-signal trial responses were observed ($F(2,32) = 0.592$, $p = 0.559$; ***figure 3.2C***), suggesting that animals training at ZT4 have more difficulty detecting short unpredictable signals when they occur. ZT10 animals had intermediate performance at all signal durations that did not differ significantly from the ZT4 or ZT16 training groups.

Task performance in well trained animals is dynamic

In addition to testing how rate of acquisition and absolute performance differed by time-of-day, we evaluated how performance in animals would change in response to being reversed from one training time to another. After reaching stable performance and undergoing two dSAT training sessions, group II animals were removed from training and allowed to return to a stable nocturnal pattern of entrainment. Afterwards, animals were switched to training during the opposite phase of their previous training experience: ZT4 animals were trained at ZT16 and ZT16 animals were trained at ZT4. The goal was to determine if asymptotic performance was influenced by time of training once the

acquisition period was over. We determined that under either condition, there was a significant main effect of reversal on performance as measured by vigilance score ($F(1,19) = 8.844$, $p = 0.008$; ***figure 3.3A and figure 3.3B***). ZT16 animals showed a robust decrease in performance when trained at ZT4 ($F(1,10) = 7.927$, $p = 0.018$; Figure 3a inset). ZT4 animals showed robust increases in performance when trained at ZT16 ($F(1,9) = 11.277$, $p = 0.008$; ***figure 3.3B inset***). Interestingly, we noted that animals originally trained at ZT4, when trained at ZT16, were incapable of reaching the same performance level as animals that were originally trained at ZT16 ($t(1,19)= 2.738$ $p=0.013$; ZT4 trained at ZT16: vi=0.5867±0.127; ZT16: vi=0.7213±0.079; ***figure 3.3C***). This was also true for animals training at ZT16 originally; their performance when training at ZT4 never fell to the level of animals that were originally trained at ZT4 ($t(1,19)= 2.527$ p=0.021; ZT16 trained at ZT4: vi=0.6150±0.113; ZT4: vi=0.4766±0.131; ***figure 3.3C***). This finding reveals a training history effect that may play an important role in future performance and suggests that deficits associated with rest phase (daytime) acquisition may be long-lasting or even permanent.

Distracter performance is time-of-day sensitive

We next compared how animals training at different phases of the circadian cycle performed on trials with unexpected challenge sessions. Two "distracter" sessions (dSAT) were administered before the shift in training time, separated by several days; followed by two more sessions, separated by several days, at the conclusion of the 30 day training period. The challenge session was presented during the middle block (54 trials) of the 162 trial training session. Sustained attention performance under challenging conditions (*e.g.* distracter presentation or fatigue) is thought to emphasize top-down optimization of input processing in order to maintain performance (Kozak, Bruno, & Sarter, 2006; Parikh, Kozak, Martinez, & Sarter, 2007; Sarter, Gehring, & Kozak, 2006; Sarter, Hasselmo, Bruno, & Givens, 2005). There was no interaction of order by treatment group on dSAT performance and therefore dSAT sessions were binned by training time for all animals. Animals, when trained at ZT16, showed significantly better performance as compared to their performance when training at ZT4 based on SAT score ($t(1,40)= -4.130$, $p<0.001$; ***figure 3.3D***). There was a within-subjects effect of block and a main effect of treatment

group on performance ($F(1,40)= 4.138$, $p<0.049$). *Paired t-test* analysis revealed that this effect was significant at all signal durations; 500 ms: $p<0.006$, 50 ms: $p<0.001$, 25 ms: $p=0.020$).

Daily SAT performance entrains circadian rhythms and entrainment predicts performance

We quantified the change in diurnality at each stage of training to assess how task acquisition influences circadian rhythms. As expected and based on previous results, animals trained at ZT16 showed a decrease in light-dark ratio and a condensing of activity during the lights-off portion of the 24 hour day as animals reached the final phase of task acquisition (Gritton, et al., 2009). ZT4 and ZT10 animals both showed increases in diurnal activity over the course of training (***figure 3.4***). Unexpectedly, we discovered sharp changes in circadian activity patterns at transition points in task acquisition. Both daytime trained groups showed modest increases in diurnal activity during water shaping and lever training (***figure 3.5A***), however, a profound shift in light-dark ratio was correlated with the transition to the final stage of training that requires orienting toward and maintaining attentional focus on the intelligence panel (SAT). During the final stage of training, because of the illumination of the house light, animals are required to attend to the signal cue directly as orienting away from the intelligence panel increases the likelihood of missing signal cues. **Figure 3.4** depicts representative circadian actograms spanning 60 days from two ZT4 (Rat 11, Rat 14; early-day) and one ZT16 (Rat 30; early-night) trained animals. Baseline data collected during the first 10 day period demonstrates the nocturnal pattern of activity endogenous to all rats. Purple shading denotes gradual water deprivation and a 3 day water-shaping phase (learning to press levers). Green shading denotes a variable training phase where animals are introduced to discriminating between signal and non-signal trials with the house light off. The following non-shaded area represents training on the final version of the SAT task and is characterized by a robust increase in anticipation for the daily training session and changes in nocturnal activity distribution. Yellow shading represents the three day period in which the animal reaches criterion performance on the SAT (see methods). Animals training at ZT4 generally adopt one of two strategies for daytime training as depicted in **figure 3.4**: Rat

11 is an example of a strategy where animals extend their late night activity through the time of task performance and delay the onset of their nighttime activity until several hours after lights-off; the more common strategy represented by Rat 14 is to increase activity before task onset and remain somewhat day-active until several hours after lights-off. These animals generally show decreased late-night/early-morning activity. Neither rat 11 nor rat 14 reached criterion within the 60 day period shown – criterion performance was met on day 91 for rat 11, and day 80 for rat 14. Time histograms at the bottom of **figure 3.4** provide ten day averages of the daily activity shown for each time-of-day group for the baseline and SAT phases of training. **Figure 3.5C** baseline shows the average collective histogram distribution of activity for all 36 animals used in this study. ZT4, ZT10, and ZT16 activity distributions during the SAT training phase for all animals within each respective group are plotted below the baseline and represent the circadian activity profile from the final 10 days of cognitive training.

We quantified the changes in circadian activity by analyzing the LD ratio. *ANOVA* revealed a significant main effect of time-of-day training on LD Ratio ($F(2,33) = 10.448$, $p < 0.001$; ***figure 3.5A***) and revealed a group by stage of training interaction ($F(6,99) = 13.947$, $p < 0.001$; *Post-hoc* analysis determined that ZT4 training group differed from the ZT16 training group at all points other than baseline (H_20 Shaping, $p = 0.029$; DL, $p = 0.024$; SAT, $p = 0.018$). ZT4 animals did not differ from ZT10 animals until the final stage of training (***figure 3.5A***; $p = 0.048$).

We also assessed the relationship between diurnality and performance when animals reached criterion on the SAT. **Figure 3.5b** plots performance as measured by overall SAT score vs. LD ratio for all animals. Animals trained at ZT4 show a significant positive correlation between diurnality and performance (Pearson Correlation = 0.809, $p<0.001$). No significant correlations were observed for ZT10 or ZT16 training groups (ZT10: Pearson Correlation = 0.208, $p=0.540$; ZT16: Pearson Correlation = -0.225, $p=0.482$).

Water maze acquisition rate and performance are time-of-day insensitive while remote memory is time-of-day sensitive

In addition to testing the effects of cognitive learning on time-of-day performance in a task of sustained attention, we tested how performance in a hippocampal-dependent spatial memory task was impacted by time-of-day. We found no significant differences in task acquisition by treatment group in time to platform for the animals trained in the MWM ($F(1,14) = 0.606$, $p = 0.449$; ***figure 3.6A***). Repeated-measures *ANOVA* also revealed no significant main effect of treatment group for swim speed ($F(1,14) = 0.513$ $p = 0.486$), or path length ($F(1,14) = 0.751$, $p = 0.401$). Probe trial analysis did not reveal a main effect of training time during the acquisition phase on Gallagher proximity ($F(1,14) = 0.261$, $p = 0.618$; ***figure 3.6B***), or quadrant analysis ($F(1,14) = 0.064$, $p = 0.805$). Differences in remote memory were made by comparing performance between probe trials 7 and 8. Paired samples *t-tests* revealed a significant change in performance as assayed by time spent in target quadrant (qT) after the two-weeks without training for ZT4 ($t(1,7) = 2.901$, $p = 0.023$) but not for ZT16 animals ($t(1,7) = -0.414$, $p = 0.691$). Further analysis revealed significant differences in overall performance for platform location between groups ($t(1,14) = -2.274$, $p = 0.039$; ZT4 qT=24.84±4.3%; ZT16 qT=36.30±2.6%) on probe trial 8 despite non-significant differences in performance on probe trial 7 ($t(1,14) = 0.025$, $p = 0.980$; ZT4 qT=35.26±4.4%; ZT16 qT=35.11±4.0%; ***figure 3.6C***). Similar results were found for Gallagher proximity with ZT4 animals showing impairment during the retention period ($t(1,7) = -3.740$, $p = 0.007$) and significant differences between groups for probe trial 8 only ($t(1,14) = 2.249$, $p = 0.041$; ***figure 3.6B***).

Water maze training does not alter circadian activity patterns

Animals trained on the Morris water maze showed slight changes in LD ratio in response to daily training. Similar to ZT16 animals on the SAT, ZT16 MWM trained animals showed a subtle decrease in light-dark ratio and a tightening of activity during the lights-off portion of the LD cycle (***figure 3.7***). ZT16 animals also showed an increase in activity in anticipation for daily water maze training relative to handling alone. ZT4 animals showed subtle increases in diurnal activity as a result of daily handling and water

maze training. **Figure 3.7** shows representative actograms spanning 45 days from a daytime (ZT4) and nighttime (ZT16) trained animal. Baseline data for two weeks is un-shaded and indicates strong nocturnal patterns of activity in both animals. Yellow shading represents seven continuous days of timed-daily handling prior to MWM training. The gray shading denotes the daily MWM training portion of the experiment and represents the first 24 days of the 28 day training period. Time histograms at the bottom of **figure 3.7** represent the averaged activity for the baseline period and MWM periods shown above.

We quantified the effects of daily timed training in the MWM on the circadian activity for all animals in the study. Although ZT4 animals displayed increases in diurnal activity at the onset of training that extended throughout the training period, these changes were minimal compared to animals training on the SAT and were not significantly different from animals training at ZT16 on the MWM ($F(1,14) = 1.228$, $p=0.286$; ***figure 3.8A***). **Figure 3.8b** represents the collective circadian activity profile of all animals by training time over the final 10 days of water maze training (ZT4, $n=12$; ZT16, $n=12$). Note baseline data is from all 24 animals in study.

Discussion

The purpose of this study was to address three questions: (1) is there a time-of-day effect on the acquisition, daily performance, and remote memory ability of animals across multiple tasks of cognitive learning? (2) Does the daily performance of these tasks influence circadian patterns of activity and do they change as a function of task acquisition? (3) Does the underlying level of entrainment interact with performance in a meaningful and performance predictive way? Differences in acquisition associated with time-of-day have been reported for some tasks in rodents and humans (Allen, Grabbe, McCarthy, Bush, & Wallace, 2008; Chaudhury & Colwell, 2002; Hoffmann & Balschun, 1992; Hogan, et al., 2009) but not for others (Cain, Ko, Chalmers, & Ralph, 2004; Devan, et al., 2001; Ralph, et al., 2002; Valentinuzzi, Menna-Barreto, & Xavier, 2004). Our results indicate that animals show a robust dissociation in their ability to acquire and

perform a sustained attention task depending on time-of-day training (***figure 3.2A-figure 3.2D***). Animals trained daily 4 hours after lights-off (ZT16) reached criterion performance twice as fast as animals trained 4 hours after lights-on (ZT4) and demonstrated higher asymptotic performance than both daytime training groups (ZT4, ZT10). Differences in post-criterion performance was not limited to the standard versions of the task, as ZT16 animals also performed better on unexpected dSAT challenge sessions when compared to ZT4 animals (***figure 3.3C and figure 3.3D***). This effect was in contrast to our findings with animals undergoing daily training in the MWM; animals showed no differences in acquisition or performance between time-of-day training groups over the 28-day MWM acquisition period, but, when tested two weeks after their last day of training, animals showed a significant difference in remote memory, dependent on the time of day they were originally trained.

It is possible that the time-of-day differences on performance during the acquisition phase between the two tasks can be explained by the reliance on different neural networks utilized for task performance or the attentional demands placed on the animal during task acquisition. The SAT requires focused attentional effort that remains high throughout the testing period and produces increases in cortical ACh of 140 percent or more throughout the duration of the task (Kozak, et al., 2006). In contrast, the MWM is a hippocampal-dependent spatial learning task that requires a relatively brief application of cognitive effort, particularly for well-trained animals, and no longer than 60 seconds, assuming the animal is integrating proprioceptive information relative to external special cues throughout the duration of the training trial. Under these conditions, water maze trials with a fixed platform location across multiple training days may not evoke the same level of cognitive effort required by the SAT. It is possible if animals had been trained with varying across-day platform locations designed to increase the reliance on spatial working memory that more effectively taxed cognitive effort, time-of-day training effects would have been observed. In support of this theory, Winocur and Hasher (2004) showed a time-of-day training effect on performance between early in the dark-phase and late in the dark-phase on a working memory non-matching-to-sample (NMTS) variation in the water maze. Additionally, the largest time-of-day effects shown in human

subjects have been reported for cognitive tasks that challenge attentional processes or executive functions linked to the prefrontal areas (Yoon, May, & Hasher, 1999), which the SAT is shown to recruit in both humans and rodents (Demeter, Hernandez-Garcia, Sarter, & Lustig, 2011; Demeter, Sarter, & Lustig, 2008; Kozak, et al., 2006; Parikh, et al., 2007; Sarter, et al., 2006; Sarter, et al., 2005).

In response to the second question, we demonstrated that animals show robust changes in circadian activity in anticipation of the daily SAT training but not for water-maze training. ZT16 SAT animals exhibited a consolidated period of activity with activity onset occurring later in the dark-phase and activity offset occurring earlier during the dark-phase (***figure 3.4 and figure 3.5C***). Animals when trained during the light-phase (ZT4 and ZT10), adopt a significantly more diurnal activity pattern as predicted by earlier findings (Gritton, et al., 2009). Interestingly, we noted that for daytime-trained animals, the most significant changes in diurnal activity profile was correlated with transitions to increasingly difficult stages of training with peaks in LD ratios corresponding to the periods in which cognitive demands on attention are the highest, when animals advance to the final phase of SAT training (***figure 3.4A, figure 3.4B,and figure 3.5A***). Importantly, despite shifting their circadian activity profile to accommodate daytime training, animals trained outside of their endogenous circadian active period were incapable of matching the performance of their dark-phase trained counterparts. In order to dissociate performance deficits associated with daytime training from daytime task acquisition, a second group of animals underwent reversal training once they reached asymptotic performance. The deleterious effect of daytime training was not limited to the phase of the light-dark cycle during which the animals originally acquired the task, as animals trained during the dark-phase when shifted to daytime training showed consistently worse performance in every metric measured. Correspondingly, light-phase trained animals when switched to dark-phase training showed substantial improvements in performance (***figure 3.3A and figure 3.3B***). Reversal training occurred over 30 days and most animals established their new ceiling performance in 3-6 days, with animals moved from ZT16 to ZT4 training taking longer to reach asymptotic performance. It is unlikely that continued training at the new phase would have allowed the eventual return

to previous performance, as no animal showed even marginal improvement after the 7th day of training. Somewhat surprising, were the differences in asymptotic performance between the two training groups based on training history. ZT4 animals, when training at ZT16 never performed as well as animals originally trained at ZT16. In fact, their performance when training at ZT16 was on par with ZT16 animals training at ZT4 as described in **figure 3.3C**. This finding presents a compelling argument for an interaction between time-of-acquisition and future performance and suggests the conditions under which training is acquired has consequences for performance long after the initial learning has occurred. Future studies will test the length or permanency of this condition.

In contrast, we saw almost no effect of daily water maze training on animals' circadian rhythms, with only minor increases in daytime activity for ZT4 trained animals characteristic of handling alone for animals housed in a LD cycle (Gritton, et al., 2009; Hummer, et al., 2010). While other experiments have demonstrated that entrainment produced by handling using procedures that are stressful (Hastings, et al., 1997) or evoke high levels of activity/arousal (Mrosovsky, 1996), water maze trained animals showed lower overall levels of entrainment than were reported in these studies. We have also demonstrated that even tasks requiring sustained periods of activity (40 minutes or more), that lack a need for attentional effort, result in no significant changes in underlying circadian activity or entrainment as measured by circadian periodicity markers (Gritton, et al., 2009). The stark difference on circadian activity between animals training in the MWM and animals training on the SAT are striking, although perhaps not unexpected, given the before mentioned differences in cognitive effort between the two tasks.

Our findings allow us to propose a model where activation of cholinergic projections of the basal forebrain cholinergic system that mediate attentional effort and performance on tasks of cognitive learning conveys the signal that influences entrainment of the circadian clock. This is in part supported by our findings that demonstrate that animals with slower acquisition rates and lower performance levels initially fail or only weakly entrain to the time of daily task training, and suggests the rate of entrainment may predict how quickly animals can acquire the task. Given the purported role of ACh in

spatial learning and memory, it is interesting that water maze training does not more significantly influence circadian activity. While some level of cholinergic activity may facilitate spatial learning in the water maze, ample evidence exists that cholinergic signaling is not an essential component of task acquisition or performance (Baxter, Bucci, Gorman, Wiley, & Gallagher, 1995; Frielingsdorf, Thal, & Pizzo, 2006; Wisman, Sahin, Maingay, Leanza, & Kirik, 2008). We suspect that the recruitment of this system was not sufficiently large enough under our training conditions to evoke robust changes. The inclusion of a working memory variant of the water maze task, to increase cognitive effort, may be more successful in producing entrainment as noted earlier.

Finally, we have demonstrated that in tasks requiring cortical cholinergic signaling, performance is modulated by the circadian cycle and that such activity may be an important condition of performance during tasks of cognition. Our findings provide evidence that the neural networks mediating cognition have the ability to modulate the underlying circadian physiology, and by doing so, allow some animals to attenuate the deleterious effects of working outside of their endogenous active period. We suggest that these findings argue for a model of bi-directional interactions between cognitive learning and circadian rhythms. Lastly, we have demonstrated for the first time, to our knowledge, time-of-day training difference in remote memory for the MWM, and suggest that the factors that influence memory consolidation could be subject to circadian regulation.

In summary, our results demonstrate an interaction that exists between time-of-daily training and performance in which time-of-day could represent a factor as important as treatment group or dose and circadian factors should be considered in study design. Ongoing experiments are aimed at understanding the interactions between cognitive performance and circadian control and how non-photic entrainment cues could be used to consolidate or prevent desynchrony in shift-work models. Shift-work, when it includes night work, has detrimental effects on both measures of subjective and physiological sleepiness, cognitive performance, accident risk, immune function, and a variety of health outcomes such as cardiovascular disease and some forms of cancer in humans (Folkard &

Akerstedt, 2004; Lange, et al., 2010; Waage, et al., 2009). Although a variety of countermeasures may be used to attenuate the negative impact of shift-work on nighttime sleepiness and daytime insomnia, there are few options to eliminate the majority of the negative effects of shift-work on physiological processes and cognition. Furthermore, our findings suggest that at least for some tasks in rodents, night-phase performance can never be equaled by light-phase performance regardless of how strong entrainment is to the work schedule. It is interesting to speculate how this finding carries over to human shift-workers and what it means for performance in highly demanding contexts like EMT, police, and emergency care providers where the consequences for mistakes are high and attention to detail is a critical factor in positive outcomes.

Acknowledgements

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Figure 3.1

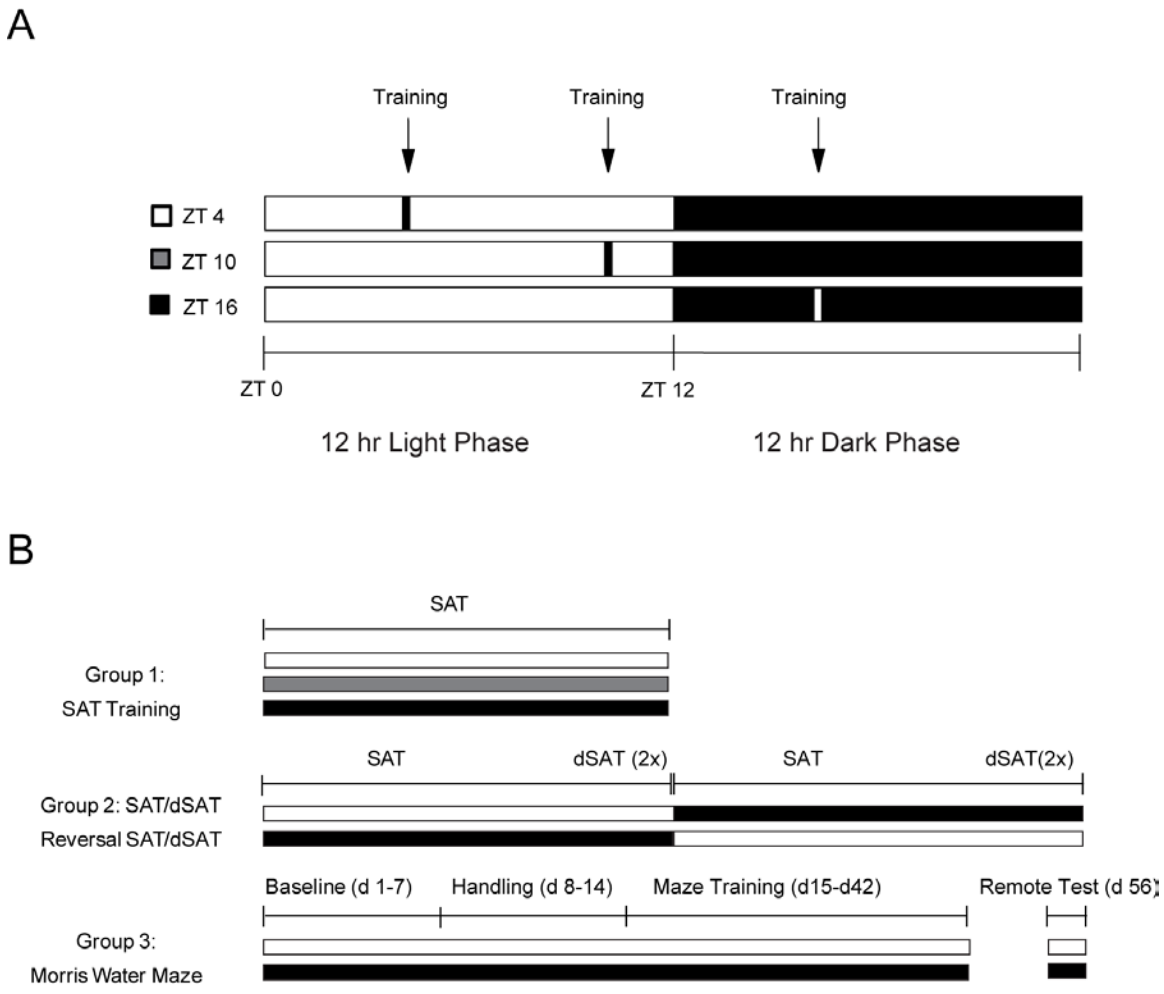

Figure 3.1: **Study design and timeline: A.** Training schedule for animals training on the sustained attention task (SAT). All animals were maintained on a 12:12 LD schedule with animals training at one of three training times (ZT4; four hours after lights-on, ZT10; ten hours after lights-on, and ZT16; four hours after lights-off). **B.** Design for three experimental groups used in this study: Group I animals consisted of three time-of-day training groups. Group II and Group III animals consisted of animals training at ZT4 and ZT16 only. SAT: Sustained Attention Task; dSAT: distracter Sustained Attention Task. White bars represent training at ZT4, grey bars represent training at ZT10, black bars represent training at ZT16.

Figure 3.2

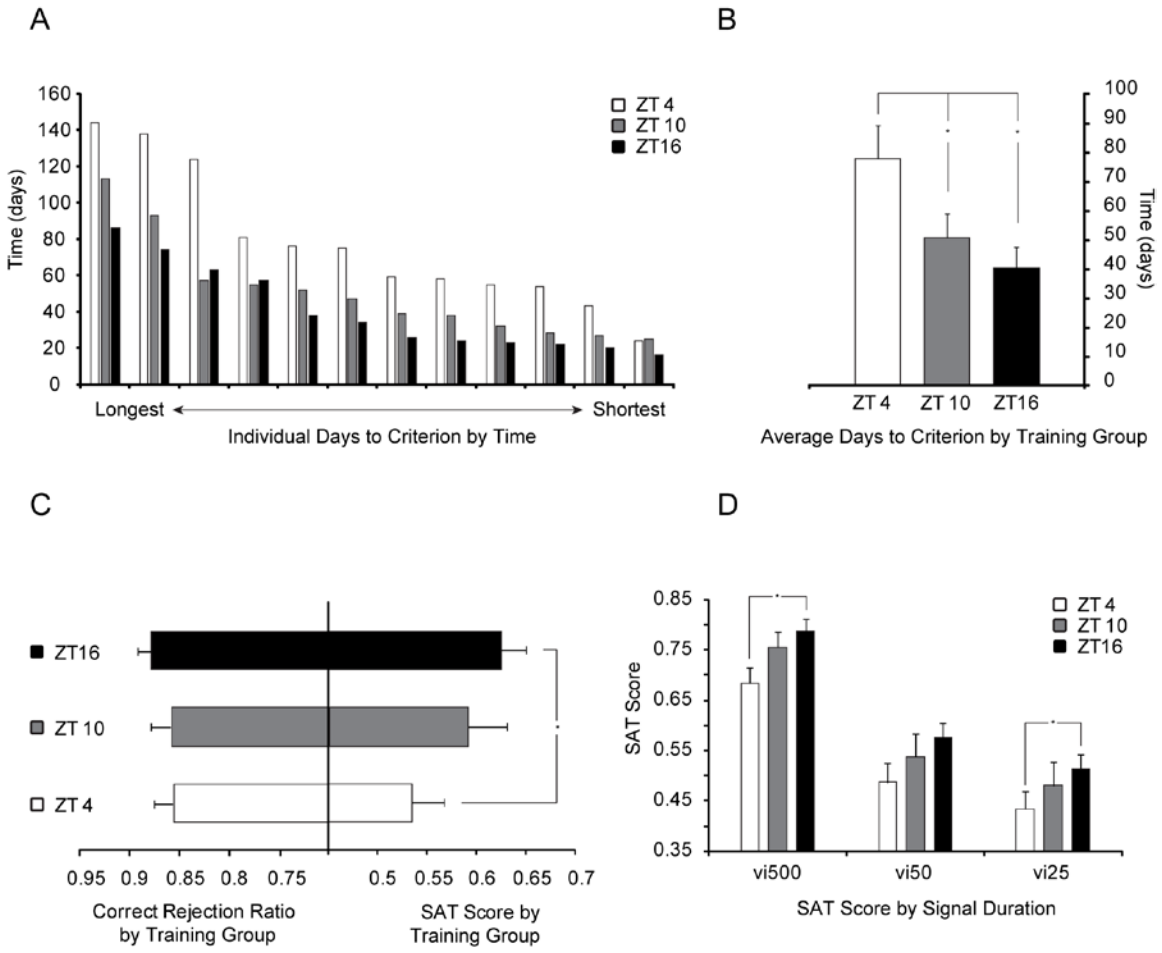

Figure 3.2: **Acquisition and criterion performance for Group I: A.** Days to criterion for individual animals plotted from longest to shortest for all animals in this study by time-of-day (*n*=36; 12 animals/group). **B.** Mean days to criterion by training time ± SEM, * $p < 0.05$. **C.** Correct rejection ratio and overall SAT score (see methods) by training time ± SEM, * $p < 0.05$. **D.** SAT score by signal duration across all three training groups ± SEM, * $p < 0.05$. (SAT score = vigilance index (vi) at 500ms, 50ms, 25ms).

Figure 3.3: **Time-of-day reversal training and dSAT performance for Group II: A.** SAT score of ZT16 trained animals by signal duration switched to training at ZT4. Baseline period consists of final 10 days of training at asymptotic performance. Interval between baseline and restarting training for both groups was 34 days. Time reversed training occurred over 30 days (reversal days 1-10 = (RV1), reversal days 11-20 = (RV2), reversal days 21-30 = (RV3). (Inset) Overall SAT score by block ± SEM, * $p < 0.05$; B=baseline, RB= reversal block. **B.** SAT score of ZT4 trained animals by signal duration switched to training at ZT16. **C.** History effect comparing performance of animals from before and after reversal of training time. SAT score by baseline or combined performance blocks 1-3 ± SEM, * $p < 0.05$. **D.** dSAT performance for animals in Group II by block ± SEM, * $p < 0.05$. Blocks 1 and 3 consist of standard trial types. Block 2 consists of trials where distracter is present.

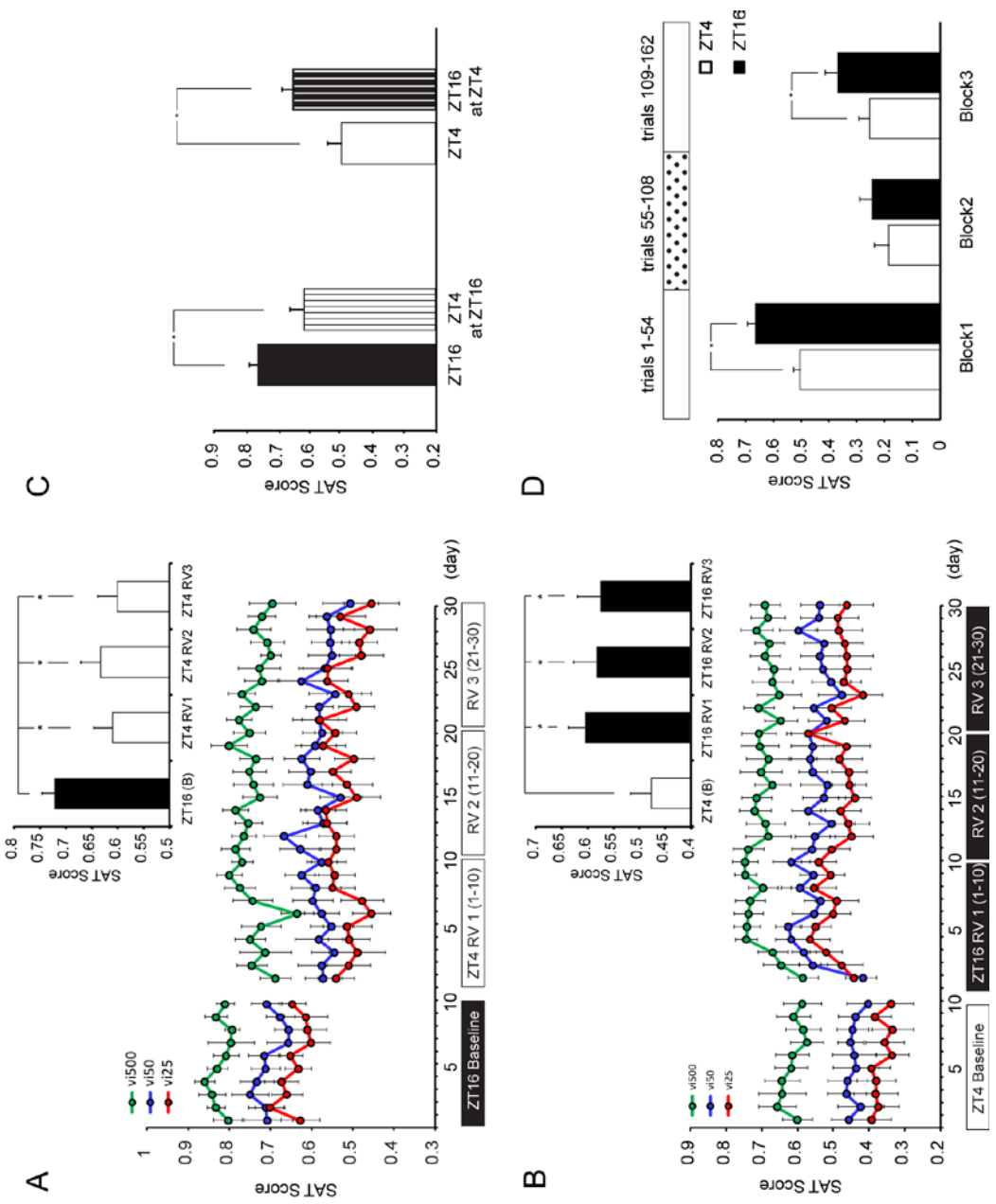
A
B
C
D
SAT Score
(day)
vi500
vi50
vi25
ZT16 Baseline
ZT4 RV 1 (1-10)
RV 2 (11-20)
RV 3 (21-30)
ZT16 (B)
ZT4 RV1
ZT4 RV2
ZT4 RV3
ZT4 (B)
ZT16 RV1
ZT16 RV2
ZT16 RV3
ZT4 Baseline
ZT16 RV 1 (1-10)
ZT16
ZT4
at ZT16
ZT16
at ZT4
trials 1-54
trials 55-108
trials 109-162
Block1
Block2
Block3
ZT4
ZT16

Figure 3.3

Figure 3.4: **Representative double-plotted actogram for three animals training on the SAT:** SAT training is relative to the topmost LD bar (where dark bar = lights off) and the open column represents the approximate 40 min SAT training period in which animals are absent from their home cages. Circadian actograms span 60 days with the baseline period reflecting the first 10 plotted days. Purple shading denotes water deprivation and shaping phase (learning to press levers). Green shading is a variable training phase where animals are only given rewarded conditioned trials to correct for lever biases (discrimination learning). Yellow shading represents when the animal reaches criterion performance on the SAT. Time histograms provide averages of the daily training shown above from both baseline and SAT phases of training with 48 hours per line.

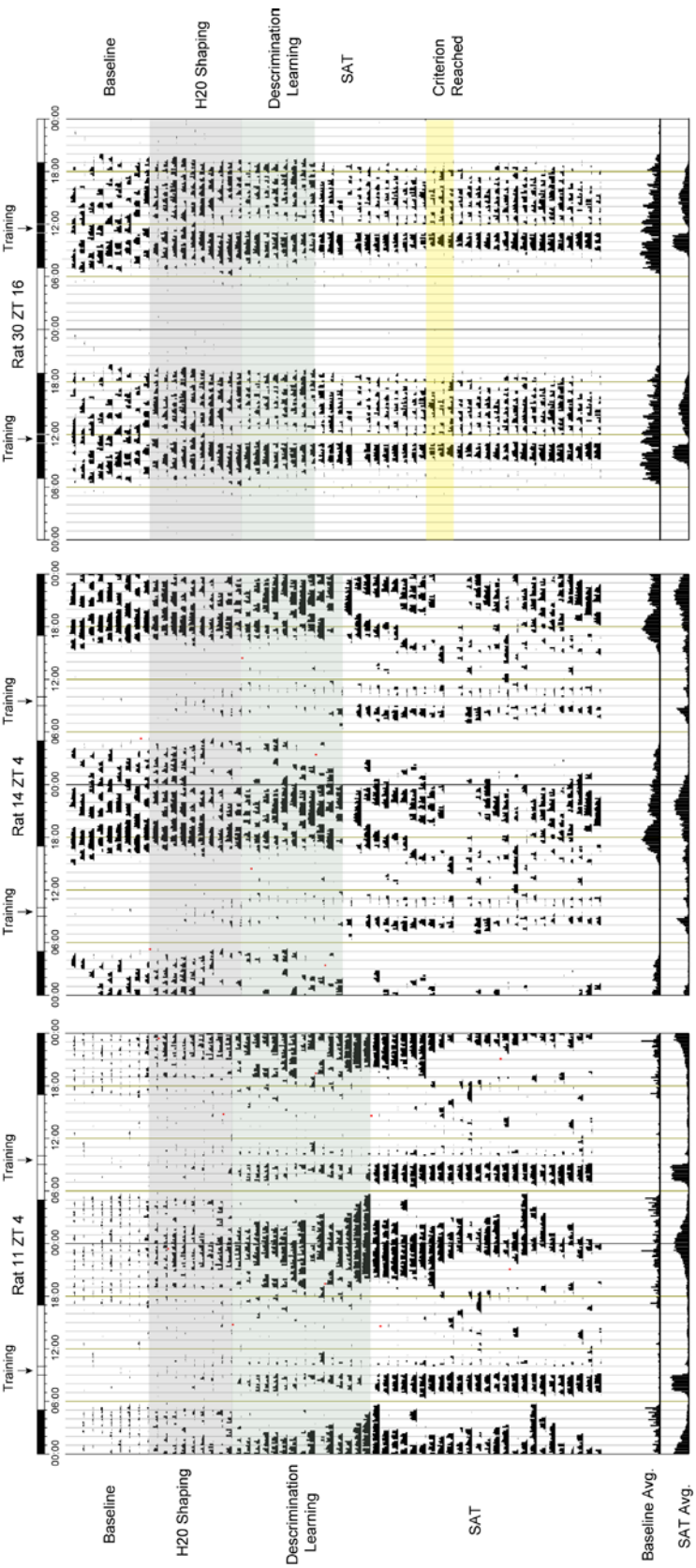
Rat 11 ZT 4
Rat 14 ZT 4
Rat 30 ZT 16
Training
Baseline
H20 Shaping
Descrimination Learning
SAT
Criterion Reached
Baseline Avg.
SAT Avg.

Figure 3.4

Figure 3.5: **Mean activity ratios and effects of entrainment on performance: A.** Light-dark ratio of activity from animals across stages of SAT training (left) and at criterion (right) for Group I animals LD Ratio ± SEM, * $p < 0.05$. **B.** Correlation between performance (SAT Score) at criterion and LD Ratio. **C.** Mean double-plotted histograms for all animals from Group I± SEM: X-axis is plotted in zeitgeber time (ZT) and y-axis represents wheel revolutions with activity grouped into 10 min bins. Baseline activity represents activity from all animals prior to training. SAT training is relative to the topmost LD bar (dark bar = lights off). ZT4 (white), ZT10 (grey), and ZT16 (black) mean histograms are taken from the last 10 days of the experiment with all animals at criterion performance. Inner line represents mean activity and upper shading represents SEM).

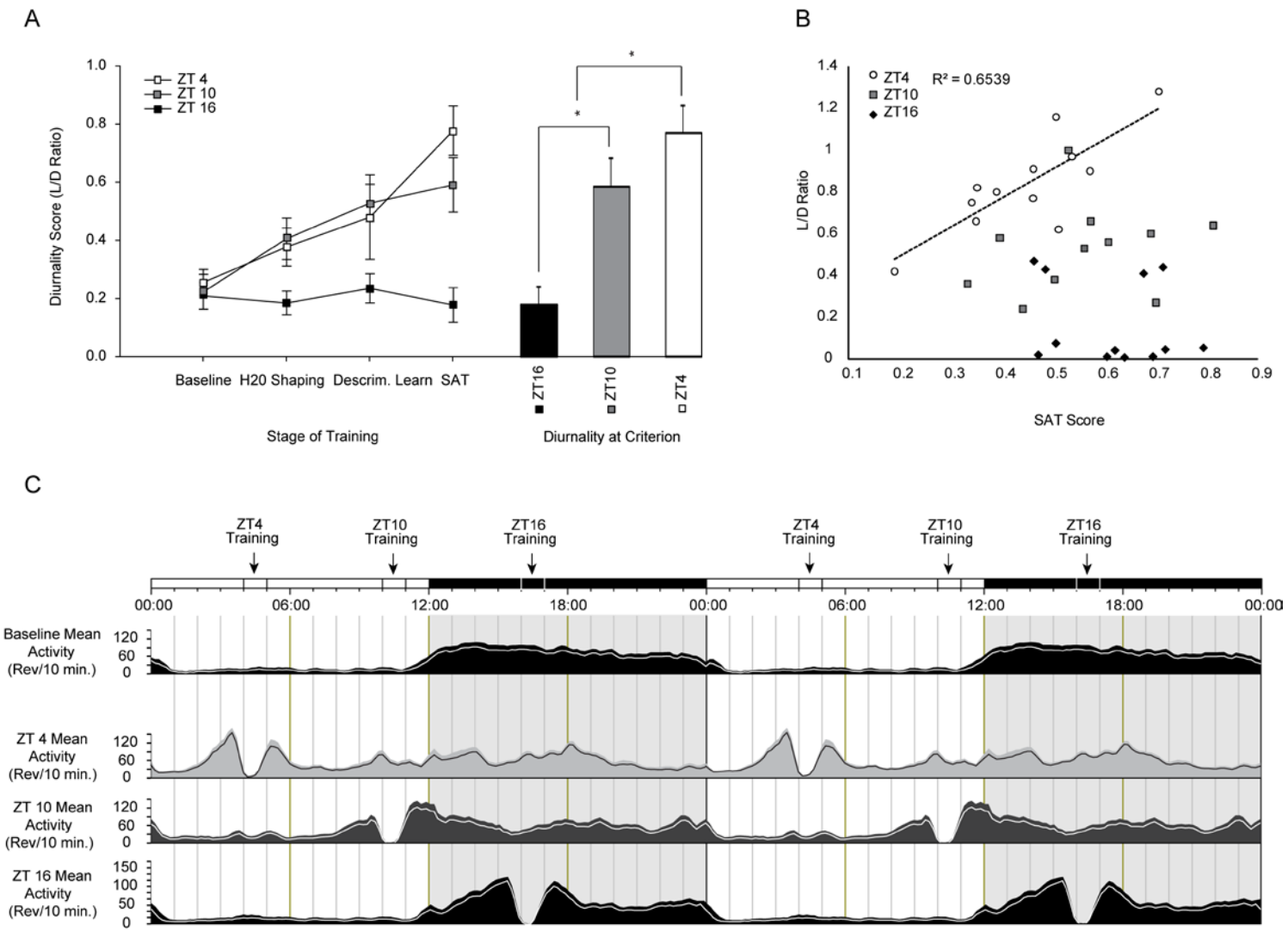
A
Diurnality Score (L/D Ratio)
ZT 4
ZT 10
ZT 16
Baseline H20 Shaping Descrim. Learn SAT
Stage of Training
ZT16
ZT10
ZT4
Diurnality at Criterion
B
ZT4
ZT10
ZT16
R² = 0.6539
L/D Ratio
SAT Score
C
ZT4 Training
ZT10 Training
ZT16 Training
00:00
06:00
12:00
18:00
Baseline Mean Activity (Rev/10 min.)
ZT 4 Mean Activity (Rev/10 min.)
ZT 10 Mean Activity (Rev/10 min.)
ZT 16 Mean Activity (Rev/10 min.)

Figure 3.5

Figure 3.6: **Morris water maze acquisition and remote memory: A.** Acquisition rate as measured by time to platform for all animals in the study grouped by time of training (ZT4, *n*=8; ZT16, *n*=8). Data was binned across 3 days and represents time in seconds ± SEM. No significant differences were found between groups. **B.** Probe trial performance as measured by average distance to platform over entire 60 second duration (Gallagher proximity) ± SEM; * $p < 0.05$. (**Insets**) 3-D heat maps represent relative location density during probe trial (warm colors=higher density of activity, cooler colors=little or no time spent in that location). Heat maps for probe trials 1 and 7 combine location density for all animals (*n*=16). Heat maps for probe trial 8 are separated by time-of-daily training (top) ZT4 (*n*=8), (bottom) ZT16 (*n*=8). **C.** Quadrant analysis during probe trials 7 and 8 ± SEM; * $p < 0.05$. Note non-significant differences for treatment groups for time spent in target quadrant on probe trial 7. During the remote memory test (probe trial 8), ZT4 animals returned to chance performance (~25%), while ZT16 animals did not differ from their probe trial 7 performance (qT=target quadrant; qO= quadrant opposite target; qRT= quadrant to right of target; qLT= quadrant to left of target).

Figure 3.6

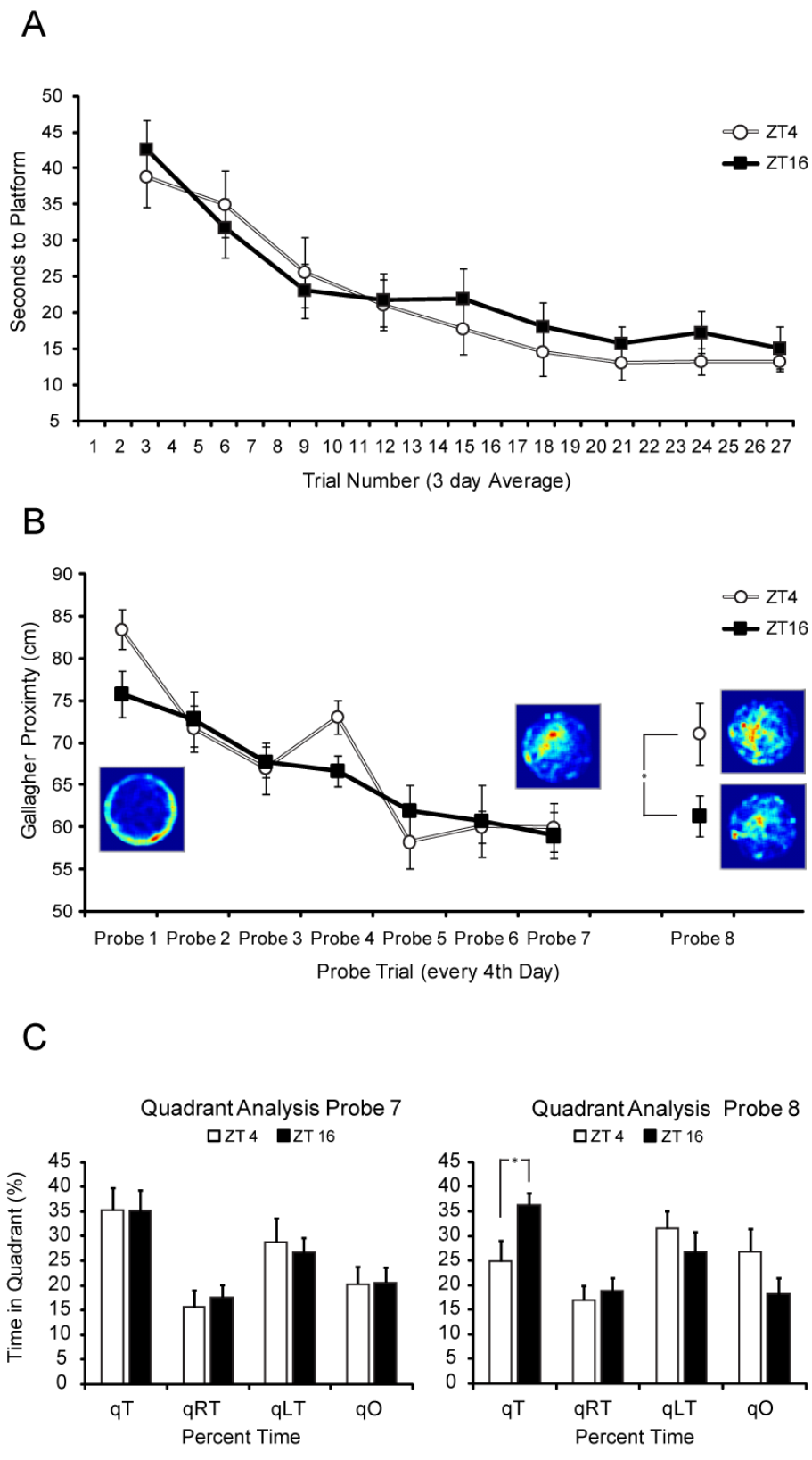
A
Seconds to Platform
ZT4
ZT16
Trial Number (3 day Average)
B
Gallagher Proximty (cm)
ZT4
ZT16
Probe 1 Probe 2 Probe 3 Probe 4 Probe 5 Probe 6 Probe 7
Probe 8
Probe Trial (every 4th Day)
C
Quadrant Analysis Probe 7
Quadrant Analysis Probe 8
ZT 4
ZT 16
Time in Quadrant (%)
qT qRT qLT qO
Percent Time

Figure 3.7: **Representative double-plotted actogram for animals training on the MWM:** Training is relative to the topmost LD bar (where dark bar = lights off) and the open column represents the approximate 15 min MWM training session in which animals are absent from their home cages (animals are carted to and from the testing area in groups of 8 and tested 1 at a time). Circadian actograms span 40 days with the baseline period reflecting the first 10 plotted days. Yellow shading represents 1-week of timed daily handling and green shading represents the first 24 days of the 28-day long MWM training phase. Time histograms at the bottom provide averages of the highlighted daily training phases shown above.

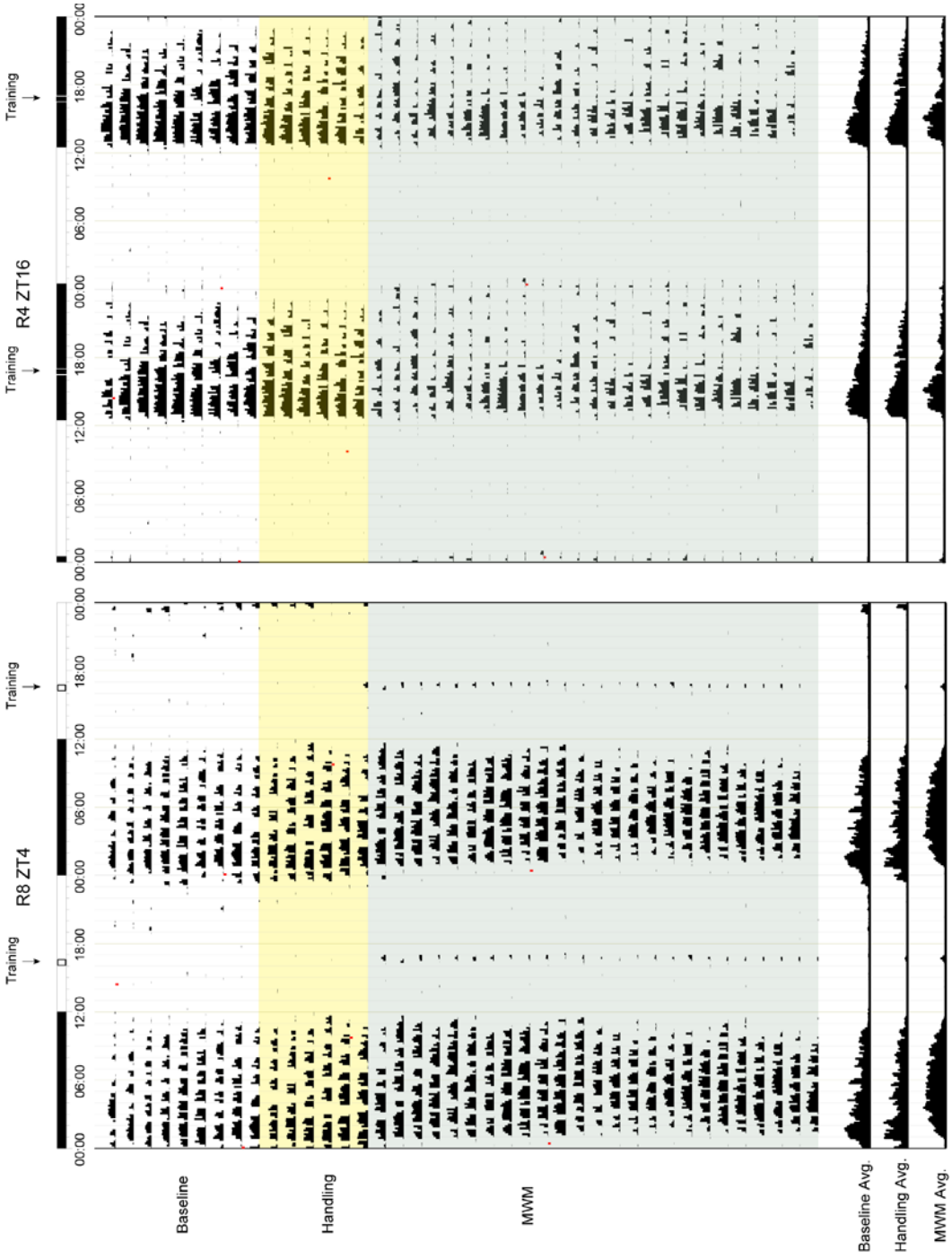
R8 ZT4
R4 ZT16
Training
00:00
06:00
12:00
18:00
Baseline
Handling
MWM
Baseline Avg.
Handling Avg.
MWM Avg.

Figure 3.7

Figure 3.8: **Mean activity ratios across task training for animals on the MWM: A.** Light-dark activity ratio from animals during a baseline condition, 1 week of timed daily handling, and 28 days of MWM training ± SEM (left). No significant differences were noted in LD ratio across the three phases tested. LD ratio for MWM phase of testing is plotted on the right ± SEM. **B.** Mean double-plotted histograms for all animals from Group III: X-axis is plotted in zeitgeber time (ZT) and y-axis represents wheel revolutions with activity grouped into 10 min bins. Baseline activity represents activity from all animals prior to training. MWM training is relative to the topmost LD bar (dark bar = lights off). ZT4 (white) and ZT16 (black) mean histograms are taken from the last 10 days of watermaze training. Inner line represents mean activity and upper shading represents SEM).

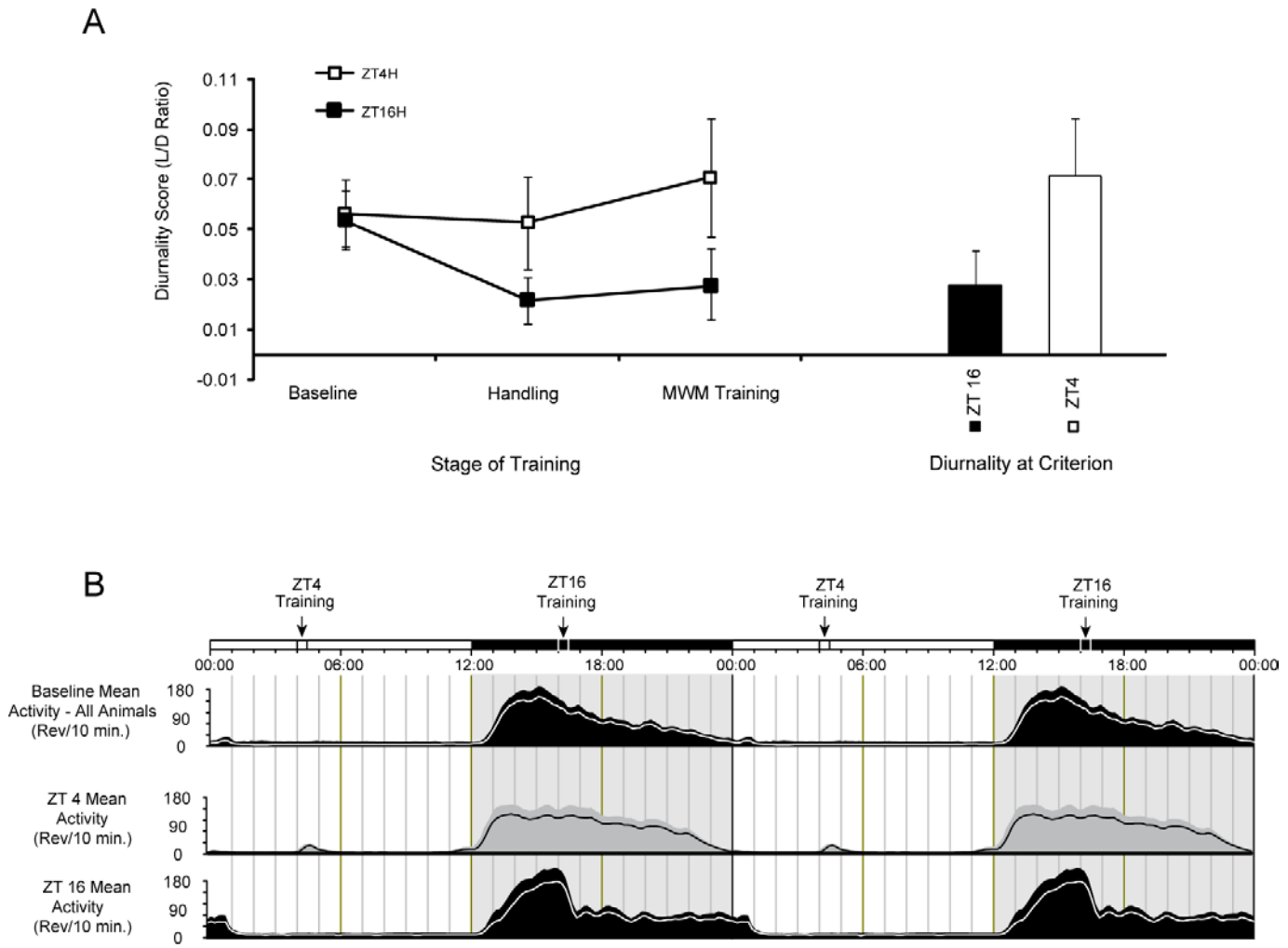
A
ZT4H
ZT16H
Diurnality Score (L/D Ratio)
0.11
0.09
0.07
0.05
0.03
0.01
-0.01
Baseline
Handling
MWM Training
ZT 16
ZT4
Stage of Training
Diurnality at Criterion
B
ZT4 Training
ZT16 Training
ZT4 Training
ZT16 Training
00:00
06:00
12:00
18:00
00:00
06:00
12:00
18:00
00:00
Baseline Mean Activity - All Animals (Rev/10 min.)
ZT 4 Mean Activity (Rev/10 min.)
ZT 16 Mean Activity (Rev/10 min.)
180
90
0

Figure 3.8

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Chapter 4

Cognition-induced circadian entrainment is mediated through cholinergic signaling at multiple oscillators

Abstract

The suprachiasmatic nucleus (SCN) is the primary circadian pacemaker in mammals and can become synchronized, or entrained, to environmental cues. Although anatomical and physiological data support a relationship between the cholinergic regions of the brain and the SCN, little is known about how endogenous basal forebrain acetylcholine release influences SCN function in behaving animals. We recently demonstrated that performance on an operant task requiring sustained periods of 'attentional effort' evokes a diurnal activity pattern through putative interactions of cholinergic systems on circadian oscillators. In this series of studies we tested how cholinergic systems influence entrainment in animals with two types of lesions of the SCN. Our results from animals with cholinergic deafferentation of the SCN, using 192IgG-saporin, demonstrate that cholinergic input to the SCN is the primary component for entrainment to daily cognitive task training. However, in arrhythmic rodents with electrolytic ablation of the SCN, cognitive task performance produces robust entrainment, suggesting that the SCN is unnecessary for cognitive entrainment. Interestingly, these animals exhibited significant impairments in task acquisition and performance accompanied by a delayed rate of entrainment to the time-of-daily training. Although animals can show unparalleled entrainment to cognitive tasks without a functional SCN, our results suggest that daily timing signals at the level of the SCN provide an important 'time-stamp' for learning and task performance. Collectively, these results suggest that daily timed cognitive practice evokes cholinergic signals that influence entrainment through interactions with the SCN and unidentified oscillators outside of the SCN. Finally, as internal desychrony between circadian oscillators is a hallmark of shift-workers, and some neuropsychiatric disorders, we compared the coherence of body

temperature rhythms and activity rhythms in both night performing and day performing animals. Our findings revealed that daily task training outside of the endogenous active period in rodents leads to a state of internal desychrony that mimics the condition of phase-shifting or chronic shift-work.

Introduction

Nearly all organisms express daily, twenty-four hour patterns of activity, or circadian rhythms, which are fundamental components of biological existence. A variety of daily behavioral and physiological activities including fluctuations in hormone levels, sleep cycles, food intake, and body temperature are modulated by circadian rhythms (*for review see:* Mendoza & Challet, 2009). The suprachiasmatic nucleus of the hypothalamus (SCN) acts as the central pacemaker in circadian regulation and is synchronized to the external twenty-four hour period by environmental cues (Moore, 1983). Bilateral lesions of the SCN eliminate this rhythmicity and the ability of animals to entrain to the light:dark (LD) cycle (Stephan & Zucker, 1972). Entrainment is characterized by a continued daily pattern of activity that persists for a period of several days even after the entraining cue is removed (Sulzman, Fuller, & Moore-Ede, 1982). Animals and humans can entrain to a variety of environmental cues: the strongest being light (photic); however, food, temperature, social interaction, sound, atmospheric pressure, novel exposure to a running wheel, methamphetamine access, and, more recently, cognitive activity have been shown to have entraining influences on circadian behavior (Gorman & Lee, 2001; Gritton, Sutton, Martinez, Sarter, & Lee, 2009; Honma, Honma, & Hiroshige, 1987; Mistlberger & Rusak, 1987; Moore-Ede, Sulzman, & Fuller, 1982). Although the mechanisms of photic entrainment have been fairly well described, the complex nature of non-photic entrainment, including the structures important for their expression, and how they interact with the SCN to influence biological rhythms remains poorly understood.

Recent evidence has also provided support for the role of circadian activity modifying entrainment in areas outside of the SCN. A variety of tissues in the periphery and non-SCN brain regions show persistent autonomous circadian expression of clock genes when isolated *in vitro* (Abe, et al., 2002; Balsalobre, Damiola, & Schibler, 1998; Davidson, Castanon-Cervantes, Leise, Molyneux, & Harrington, 2009). In some tissues, periodic expression of clock genes has been demonstrated to persist for up to 20 cycles (Yoo, et al., 2004). Although the expression of circadian markers in non-SCN oscillators is thought to be semi-autonomous, entrainment of these oscillators is thought to be under control of the SCN. The SCN conveys time of day information in order to synchronize

peripheral and CNS oscillators through other nuclei of the hypothalamus (Berk & Finkelstein, 1981; Watts & Swanson, 1987). This is evident in mice with lesions of the SCN, where peripheral tissues continue to show daily oscillations but the expression of these rhythms is no longer synchronized (Pezuk, Mohawk, Yoshikawa, Sellix, & Menaker, 2010). A temporary state of desynchrony amongst oscillators is evident in animals or humans undergoing phase-shifts or shift-work. During conditions of a phase shift, the circadian clock and peripheral oscillators do not entrain to the changes immediately and require several cycles for re-entrainment to occur. This period of time between the initial disturbance and the reemergence of a stable entrainment pattern represents a condition of internal desynchrony (ID), that is characterized by changes in amplitude or phase relationships between core body temperature, hormone release, sleep drive, or activity (Haus & Smolensky, 2006; Reid, Chang, & Zee, 2004; Salgado-Delgado, Nadia, Angeles-Castellanos, Buijs, & Escobar, 2010). Sustained periods of desynchrony, as noted in chronic shift-workers, are associated with increased risk for a variety of disorders including cardiovascular disease, obesity, diabetes, infertility, sleep disorders and some forms of cancer (Folkard & Akerstedt, 2004; Haus & Smolensky, 2006; Knutsson, 2003; Lange, Dimitrov, & Born, 2010; Waage, et al., 2009).

We recently demonstrated that animals trained at the same time each day on a task of sustained attention (SAT) develop profound changes in activity even in the presence of a light dark cycle. Sustained attention is characterized by a subject's readiness to detect an infrequent and unpredictable signal(s) over a prolonged period of time, to discriminate this signal from non-signal events or "noise" and to report the presence or absence of such a signal. Normal sustained attention performance, above chance levels, is dependent upon the integrity of the basal forebrain cholinergic (BFC) projections to the cortex. Sustained attention performance robustly increases cortical cholinergic transmission; ACh release is further augmented under challenging conditions (*e.g.*, distracter presentation or fatigue) that require top-down optimization of input processing (Kozak, Bruno, & Sarter, 2006; Parikh, Kozak, Martinez, & Sarter, 2007; Sarter, Gehring, & Kozak, 2006; Sarter, Hasselmo, Bruno, & Givens, 2005). The period of sustained attention is critical to cognitive functions, including: stimulus detection, discrimination,

and signal processing, all of which are necessary to maintain performance under exigent conditions (Kozak, et al., 2006). As animals undergo daily SAT training, acetylcholine release is elevated in service of task performance. SAT performing animals also, when trained during the light-phase, adopt a diurnal activity pattern that cannot be attributed to restricted water access, daily handling, or operant training for a reward (Gritton, et al., 2009). Additionally, the cholinergic system projects to and influences circadian entrainment. The SCN receives cholinergic input from forebrain cholinergic neurons of the medial septum, nucleus basalis, diagonal band, substantia inominata, and brainstem cholinergic neurons of the pedunculopontine, laterodorsal tegmental and parabigeminal nuclei (Bina, Rusak, & Semba, 1993). The exact role of these projections is still unknown, but data suggests that they can influence both cellular and behavioral activity through modulatory effects on the SCN. Injections of carbachol, a nonspecific acetylcholine agonist, into the SCN induce subjective night phase advances and delays similar to those produced by light (Bina & Rusak, 1996; Earnest & Turek, 1983). Furthermore, *in vitro* application of carbachol to SCN brain slices produces shifts in the neural firing activity of pacemaker neurons, and intraventricular injections of the cholinergic neurotoxin ethylcholine aziridinium ion produces aging-like changes to rat circadian rhythms (Endo, Shinohara, Fueta, & Irie, 2001; Liu, Ding, Faiman, & Gillette, 1997; Liu & Gillette, 1996). The correlations between attentional demand, known cholinergic signaling associated with SAT performance, and anatomical evidence, led us to hypothesize that cholinergic signaling could mediate the previously described changes in circadian entrainment associated with cognitive training.

The present study was designed to examine how attention-demanding performance and circadian activity interact at the level of the primary circadian pacemaker. We used site-specific injections of the immunotoxin 192 IgG-saporin to eliminate basal forebrain cholinergic projections to the SCN thereby testing if cholinergic afferents are necessary for SAT entrained activity patterns. Additionally we tested whether a cognition-induced circadian oscillator exists, independent of the SCN, by training animals with electrolytic lesions of the SCN on the SAT. Furthermore, we compared performance of animals with 192-IgG-saporin and electrolytic lesions to

determine how entrainment, or lack thereof, influences learning and/or performance. Finally, as internal desychrony is a hallmark of shift-workers, and some neuropsychiatric disorders, we looked at how body temperature rhythms are impacted by the performance of the daily sustained attention task in both night performing and day performing animals. We found that lesions of BFC afferents to the SCN blocked most of the effects of SAT training on circadian activity.

Our results also indicate that SCN electrolytic lesioned animals show robust entrainment and anticipation for daily task performance although they show profound deficits in task acquisition. These results provide evidence that a cognition-induced circadian oscillator exists, independent of the SCN, as has been reported for food and methamphetamine availability (Angeles-Castellanos, Salgado-Delgado, Rodriguez, Buijs, & Escobar, 2010; Pezuk, et al., 2010; Stephan, Swann, & Sisk, 1979a, 1979b). Lastly, we show that task performance outside of an animal's endogenous active period promotes a profound level of ID as characterized by out-of-phase markers of temperature and activity that may explain differences in task acquisition and performance in daytime trained nocturnal animals.

Methods

Subjects

Forty-eight male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 260 g at the time of arrival were used for these studies. All animals, following arrival from the supplier, were housed individually in opaque single standard cages (27.7cm X 20.3cm) maintained on a LD 12:12 cycle. Cages were lined with corn cob bedding and kept in a humidity- and temperature-controlled environment at 21 ± 1° C. Core body temperature was monitored in a subset of animals with intra-abdominal transmitters (pdt 4000 e-mitter-telemetry implants, Mini-mitter Inc., Bend, OR; n=20), while all animal had running wheels for monitoring of activity (n=48). Cages were cleaned and animals were weighed once-weekly during the animal's training phase when they were absent from their home cage. All circadian data was analyzed off-line using Actiview software (Minimitter, Bend, OR)

Experimental timeline

Animals were allowed to acclimate for a minimum of two weeks before undergoing surgery. All animals designated to undergo infusions of artificial cerebral spinal fluid (ACSF) or 192-IgG-saporin underwent surgery for telemetry implants or sham surgeries for this procedure, followed by two weeks for recovery, before being introduced to running wheels. Animals with SCN electrolytic ablation were allowed two weeks for acclimation to their environment before being introduced to running wheels. All animals were recorded for a minimum of two weeks under baseline conditions before undergoing surgery for lesions as described below. Electrolytic lesion (EL) rats trained four hours after lights-on (ZT4), and the saporin-lesion (SL) and surgical control (C) rats trained at one of two times: four hours after lights-on (ZT4) or four hours after lights-off (ZT16). Locomotor activity during baseline and training phases was collected in 10-min bins using Vitalview software (Minimitter, Bend, OR). Following recovery (~2weeks) animals were introduced to running wheels and allowed to acclimate for two weeks before beginning the baseline portion of the experiment. Animals had *ad libitum* access to food and water during the two-week baseline period. Afterwards, animals were mildly water deprived to ~95% of their free feeding weight while having ad libitum access to food (Purina 5001; supplier: Frontier, Oxford, MI). Water deprivation occurred over the seven days beginning with twelve-hour access on day one (starting 6 hours prior to their training time; ZT4 or ZT16, and continuing for 12 hours). Gradually over the next six days, animals were titrated down to a single one hour water access period from ZT4 to ZT 5 or ZT16 to ZT17, respectively. Following the two-week acclimation period and gradual water deprivation, task-performing rats began operant training procedures to facilitate shaping on a task that measures sustained attention (SAT). All task-performing animals were given free access to water for twenty minutes following daily training in addition to the quantity of water (~5 ml) obtained as reward during operant testing. Upon achieving a performance criteria (described below), rats were released into 14 days of constant darkness (DD) to assess strength of entrainment under constant conditions.

Surgeries and data collection

Transmitters were implanted intraperitoneally (i.p.) in three-fourths of animals scheduled to undergo chemical induced lesions or corresponding sham procedure (n=20). The remaining animals underwent sham surgical procedures identical to animals given telemetry implants (n=4). Animals designated to undergo SCN ablation via electrolytic lesioning did not undergo intraperitoneal surgery. Animals were anesthetized using isoflurane (5% iso, 95% oxygen) and incisions were made in the ventral abdominal area so that transmitters could be inserted into the abdominal cavity. Animals had muscle walls sutured with 4.0 chromic gut and skin incisions closed with wound clips. Clips were removed 10 days post-surgery.

Saporin lesioned animals, and corresponding controls, underwent a second surgery at approximately six-eight weeks after arrival (four-six weeks after the initial surgery for i.p. telemetry implants). Lesions using 192-IgG-saporin (Advanced Targeting Systems, San Diego, CA) were designed to selectively deafferent cholinergic projections to the SCN. Animals were anesthetized using isoflurane (5% iso, 95% oxygen) and placed in a stereotaxic instrument adjusted to level between bregma and lambda. Bilateralal injections were made using a 30 gauge needle attached to a 1 μL Hamilton syringe lowered once per hemisphere into the SCN (coordinates: anteroposterior: -1.0 mm from bregma, lateral: $\pm$ 1.1 mm from the midline, dorsoventral: -8.5 mm (at 6 °) ventral to dura). Rats received 0.4 μL of a 200 ng/μL bolus of 192 IgG-saporin suspended in artificial cerebro-spinal fluid (ACSF) per hemisphere. The syringe was lowered over a period of one minute to final coordinates and remained there for two minutes prior to the injection. Following the chemical infusion, the needle remained in place for four additional minutes to allow for chemical diffusion and then slowly removed. Sham-control animals received surgery under the same protocol except that vehicle alone was infused (ACSF).

Animals with electrolytic ablation of the SCN underwent a single surgery when they reached 350-400g to selectively ablate the SCN. A 50 μm stainless steel lesioning

electrode (FHC; Bowdoin, ME) with a 25 μm exposed tip was lowered to four locations at the following coordinates: anteroposterior: -0.8 mm and -1.2 mm from bregma, lateral: ± 1.1 mm from the midline, dorsoventral: -8.5 mm (at 6 °) ventral to dura. Electrolytic lesions were produced for SCN ablation by a Grass lesion maker by passing DC current at 0.5 mA for 20 seconds. A total of 24 rats underwent electrolytic lesioning of the SCN, and the 12 rats that showed the most arrhythmic behavior based on running wheel activity were selected for this study. Four animals from the SCN ablation group that began training were eventually excluded from analysis based on actogram data demonstrating entrainment to the LD cycle and histological verification that supported damage to the optic chiasm or extensive damage outside of the SCN. All procedures were in accordance with protocols approved by the University Committee for the Care and Use of Animals at the University of Michigan.

SAT operant methods

Operant training took place seven days per week. Behavioral training and testing was conducted in individual operant chambers (MedAssociates; St. Albans, VT) outfitted with two retractable levers, three red panel lights (2.8 W), and one red house light (2.8 W). Additionally, a water dispenser attached to a syringe pump, was located on the same wall as the panel lights and levers. Rats were removed from their home cages and placed in unlit chambers for five minutes prior to task onset. Operant chambers were housed within individual sound-attenuating cabinets. The shaping protocol for this task has been published in detail previously (Gritton, et al., 2009). Briefly, animals were first trained to press a lever for a water reward in accordance with a modified fixed-ratio-1 schedule of reinforcement. The FR1 schedule is modified in that it requires animals to respond to both levers and corrects selection bias if one exists; if rats press a single lever five times in a row they were required to press the opposite lever before receiving another reward in order to discourage lever bias. The rats were next trained to detect, discriminate, and respond to signal and non-signal events. During each session, levers extended for 4 seconds or until lever press. With one-second illumination of the central panel light (signal trials), a left lever press was counted as a hit, and a right lever press was counted as a miss. In the absence of the central panel light (non-signal trials), a left lever press

was counted as a false alarm, and a right lever press was counted as a correct rejection. Each correct response produced a water reward (30 μL for each hit or correct rejection) and incorrect responses (miss or false alarm) were not rewarded. Half of the rats were trained in a counterbalanced fashion with the lever rules reversed. The overhead house light was off during this second phase of training, and incorrect responses resulted in trial repetition up to three times in the form of correction trials. If the rat continued to respond incorrectly after three consecutive correction trials, a forced-choice trial was initiated: a signal or nonsignal event followed by extension of only the correct lever for 90 seconds or until the lever press occurred. In forced-choice signal trials, the central panel light remained illuminated for the duration of the lever extension. Each operant session consisted of 162 trials. Progression to the final training phase occurred after three consecutive days of correct responses of $\geq$ 69% to both signal- and non-signal trials. The last phase of operant shaping, the sustained attention task (SAT), consisted of shortened signal durations (500, 50, or 25 ms), shortened intertrial intervals (from the original 12 $\pm$ 3 seconds to 9 $\pm$ 3 seconds), and the elimination of correction and forced-choice trials. During the final stage of testing (referred to as the 'SAT'), the house-light was illuminated to increase the requirement for focused attention. The addition of the illuminated house-light requires the animal to constrain their behavior on the central panel during testing to optimize performance. Animals were required to maintain criterion performance ($\geq$ 70% correct responses to the 500 ms signal trials, $\geq$ 70% correct responses to non-signal trials and fewer than that 20 omissions per session) for 3 consecutive sessions before the task acquisition was considered complete.

SAT data analysis and statistics

Statistical analyses were carried out to determine differences in the number of days to criterion on the SAT (task acquisiton) and overall task performance at criterion. Days to criterion were tested using independent t-tests between lesion condition within time of day or between time of day within a lesion condition. Mixed analyses tested the main effects and interactions of treatment conditions and signal duration (where applicable: 500, 50 and 25 ms). SAT performance yielded measures of hits (H), misses (M), false alarms (FA), correct rejections (CR), omissions (O), and vigilance index (VI).

VI was calculated using the following formula: VI = (%H - %FA)/[2(%H + %FA) – (%H +%FA)^2]. This is similar to that of the Sensitivity Index (Frey & Colliver, 1973) except that omitted trials were excluded from the calculation. Statistical analysis of hit percentage (%H), correct rejection percentage (%CR), percent omissions (% O), and VI was tested using independent t-tests using the criteria stated above. Comparison of group means between experimental groups and controls was performed using SPSS (SPSS Statistics Version 17.0; Chicago, IL)

Circadian analysis and statistics

The phase onset (start) and offset (end) of activity relative to light-phase onset and SAT training onset were determined by the presence of 3 or more consecutive 10-min bins of activity (BOA) that were more than 10% of the daily mean activity and were analyzed for the last ten days of recorded activity following task acquisition and prior to release into constant conditions (dark:dark; DD). A repeated measure, multivariate analysis of variance (*MANOVA*) was used to assess treatment differences in the onset and offset of activities as well as the ratio of locomotor activity (LD ratio) between the light and dark-phases. Activity ratios were determined using BOA counts (number of 10-min bins in which activity was present) during the light and dark period, rather than the total activity or peak of amplitude (since rats run most intensively during any light transition) to best assess the distribution of activity in relation to the light and dark-phase. LD ratios greater than 1.0 indicate a diurnal activity pattern whereas those less than 1.0 indicate a nocturnal activity pattern. Days of entrainment, determined by the number of days in constant darkness that showed activity around the time of task onset, and overall LD ratio were compared between treatments using a one-way *ANOVA*. Repeated within-subjects analyses of variance (*ANOVAs*) were used to determine the main effects and interactions of time of day and treatment condition for differences between activity and temperature. Significant main effects were further analyzed using *Tukey post-hoc* analysis. A probability value of $p<0.05$ was used as the criteria to determine statistical significance. Statistical analysis was performed using SYSTAT (Systat V13, Systat Software, Inc., Chicago, IL).

Histology and immunohistochemistry

At the completion of testing, animals were euthanized and perfused with 300 mL buffered saline, followed by 300 mL 4% paraformaldehyde. Brains were carefully removed and post-fixed overnight in 4% paraformaldehyde before being transferred to a 30% sucrose solution. After three days in sucrose, brains were sectioned coronally in 40µm slices with a freezing microtome (CM 2000R; Leica) and tissue sections were collected throughout the basal forebrain and SCN. Sections with SCN ablation using DC current were stained with Nissl substance only. For all other animals, alternate sections were stained for Nissl substance (cresyl violet acetate - Fisher Scientific), acetylcholinesterase (AChE), or choline-acetyl transferase (ChAT). AChE staining was by a modified method of Tago et al. (1986). Briefly, after rinsing in 0.1 M phosphate buffer (pH 7.4), sections were incubated in 0.1% H_2O_2 for 30 min, and then rinsed in 0.1 M maleate buffer (pH 6.0). Sections were then immersed in a solution of: 14.70 mg sodium citrate, 1.65 mg potassium ferricyanide, 7.49 mg copper sulfate, and 5 mg acetylthiocholine iodide in 200 ml of 0.1 M maleate buffer. After rinsing with 50.0 mM Tris buffer (pH 7.6), sections were incubated in a solution of 50.0 mg of 3,3′-diaminobenzidine (DAB) and 375.0 mg of nickel ammonium sulfate in 125.0 ml of 50.0 mM Tris buffer (pH 6.2). After 10 min, 10µL of 30% H_2O_2 was added to the sections will they continued to incubate. Staining was considered complete once cortical layering could be detected. Sections were then rinsed with 5 mM Tris buffer and mounted on gelatin-coated slides and allowed to dry overnight. Following dehydration in an ascending series of alcohol rinses and clearing with xylene, slides was coverslipped with DPX.

Sections designated to undergo ChAT staining were rinsed three times in 0.1 m phosphate-buffered saline (PBS, pH=7.4) for 5 min each, incubated with 0.3% peroxide for 30 minutes, followed by blocking buffer (10% goat serum in 0.1 m PBS) for 60 min under conditions of constant shaking, followed by an overnight incubation with rabbit anti-CHT antibody (polyclonal goat anti-choline acetyltransferase; Millipore; Temecula,

CA) diluted 1:500 in dilution buffer (0.1 m PBS containing 1% goat serum and 0.1% triton X-100) at 4°C. The following day, sections were washed in wash buffer (0.1 m PBS containing 0.1% triton X-100) four times for 5 min each and incubated with a biotinylated goat anti-rabbit IgG (Vectastain Elite ABC; PK-6105; Vector Laboratories; Burlingame, CA) 1:200 for 2 h at 25°C. Sections were then rinsed three times for 5 minutes each in 0.2% Triton-X in 0.1M PBS and then incubated with the avidin-biotin complex (Vectastain) for 30 minutes. Following rinsing in 0.1M PBS, tissue was incubated in a peroxidase substrate solution containing 0.4% DAB and 0.19% nickel (II) chloride in 125 mL 0.1M PBS. 10µL of 30% hydrogen peroxide H_2O_2 was added after the tissue began the incubation. Once sections showed a significant level of background stain (5-10 min), sections were treated with four 5-min rinses with 0.1 M PBS and mounted on gelatin-coated slides to dry overnight. Following dehydrated in an ascending series of alcohol rinses and clearing with xylene, slides were coverslipped with DPX.

Quantification of lesion effects and statistics

In order to quantify the distribution of AChE+ stained axonal fibers, 40X images were taken from two mid-SCN sections (AP-1.0 to -1.2), two sections from the central region of the anterior hypothalamus (AP-1.6 to -1.8), and two sections from layers3/5 of primary motor cortex (AP-1.0 to -1.4) by an experimenter blinded to animal treatment groups using a Leica DM4000B light microscope equipped with a Spot Digital Camera and using Spot Software (Diagnostics Inc., Sterling Heights, MI, USA). We used a counting grid method to determine the number of fibers in each quantified region of interest. Briefly, using Adobe Photoshop CS3 software, a grid containing 50-µm squares was superimposed over each image, making a five-by-five grid (250x250 µm). Crossing of the horizontal and the vertical lines of the grid were counted by two researchers, blinded to the treatment condition. Overall counts by the researchers were averaged for statistical analysis.

Images of sections (5X) containing ChAT immunopositive neurons from the five regions of the basal forebrain were taken from the medial septum (AP+0.7 to +0.2), the

horizontal/vertical diagonal band (DB; AP+0.5 to -0.2), and the substantia inominata/nuc. Basalis of Meynert (SI/nBM; AP-1.1 to -1.6) by an experimenter blinded to the treatment group of each animal. The numbers of ChAT immunopositive neurons in each field of view were counted in both shams and 192-IgG-saporin injected animals. Average counts were based on two sections per region from each animal in this study. Cell counts were used to estimate lesion-induced decreases in cholinergic signaling, but were not intended as an absolute measure of cholinergic cell number.

The area of SCN ablation by electrolytic lesion was quantified by superimposing a stereotaxic atlas image over images of nissel stained sections containing the SCN ablation. Traces of the lesion outline were made using Adobe Photoshop CS3 and lesion size was quantified as the number of pixels remaining unlesioned against the stereotaxic outline of the SCN (number of overlapping pixels within the two defined lesion regions/the number of pixels within the SCN region). Animals with less than 39% ablation or damage to the optic chiasm were excluded from further analysis. The 39% cutoff was chosen based on the minimal lesion necessary for animals to show an arrhythmic phenotype in the presence of an LD cycle.

Statistical analysis of laminar and regional differences in AChE+ stained axonal fibers and ChAT cell counts were performed using *MANOVA* with region as a factor. Significant main effects were further analyzed using Tukey *post-hoc* analysis. Between-subjects effects were tested using independent t-tests for lesion condition. Alpha was set at 0.05 to determine statistical significance. Pearson correlation analyses was additionally done to determine relationships between days to criterion performance and cholinergic depletion based on number of AChE-stained fibers or ChAT positive cells of the basal forebrain.

Results

Quantification of lesions

Figure 4.1 provides histology results for both cholinergic deafferentation of the SCN (***figure 4.1A and figure 4.1B***) and size and location of electrolytic lesions of the SCN (***figure 4.1C***). We quantified the thoroughness of the 192-IgG-saporin lesion using fiber density counts of acetylcholinesterase (AChE) in the SCN. We also addressed the specificity of our SCN targeted infusion by quantifying changes in cholinergic expression on surrounding areas of the hypothalamus (anterior hypothalamus) and a control area (motor cortex) to rule out non-specific effects. Repeated measures *ANOVA* for AChE+ fiber counts revealed a within-subjects effect of region ($F(2,42) = 360.056$, $p < 0.001$, and a region by group interaction ($F(2,42) = 18.827$, $p < 0.001$). Between subjects comparisons revealed a main effect of treatment group ($F(1,21) = 19.568$, $p < 0.001$; ***figure 4.1B***), and *paired t-tests* analysis between lesion and sham groups revealed significant differences in fiber counts for the SCN ($p<0.001$), but not for the motor cortex ($p=0.276$), or the anterior hypothalamus ($p<0.310$).

Additionally, we quantified how the localized infusion of 192-IgG-saporin influenced the number of cholinergic cell bodies throughout the basal forebrain using immunohistochemistry directed against the cholinergic specific marker choline-acetyltransferase (ChAT). Repeated measures *ANOVA* for ChAT positive cell bodies revealed a within-subjects effect of region ($F(4,84) = 34.851$, $p < 0.001$, but not a region by group interaction ($F(4,84) = 0.984$, $p < 0.421$). Between subjects comparisons revealed a main effect of treatment group ($F(1,21) = 51.258$, $p < 0.001$; (***figure 4.1A***), and *paired t-tests* analysis between lesion and sham groups revealed significant difference in cell counts for all regions quantified: septum ($p<0.001$); Left DB ($p=0.008$); Right DB ($p=0.003$); Left SI/nBM ($p=0.001$); and the Right SI/nBM($p=0.015$). Our results reveal a loss of cholinergic cell bodies that did not appear to be localized to one particular region. These findings are consistent with published results demonstrating basal forebrain cholinergic innervation of the SCN arises from a distributed network of cells in disparate locations (Bina, et al., 1993; Erhardt, et al., 2004).

Lastly, we calculated the location and extent of electrolytic lesions in SCN ablated animals. **Figure 4.1C** demonstrates the most severe (92% - middle panel), and least severe (39% - right panel) for two of the eight animals that met inclusion criteria for this study (group average: 61.3% $\pm$ 6.6%). For inclusion, animals had to have damage primary isolated to the SCN with the additional mandate of showing an arrhythmic phenotype in the presence of a normally entraining LD cycle both before and after the study was concluded. Animals with visible damage to the optic chiasm were also excluded from further analysis.

Lesions of the SCN influence SAT mediated entrainment

We quantified the effects of localized 192-IgG-saporin (SL) infusions into the SCN on activity records of both ZT4 and ZT16 task performing animals as well as the effects of SCN electrolytic ablation (EL) on SAT entrainment. Representative running wheel records for animals with SL of the SCN training at ZT4 or ZT16 can be seen in ***figure 4.2 and figure 4.3***, respectively. **Figure 4.4** shows circadian activity from the two EL animals shown in **figure 4.1C**. *ANOVA* revealed significant treatment differences in total activity (10 min bins) during the light-phase ($F(4,26) = 17.794$, $p < 0.001$) and dark-phase ($F(4,26) = 11.587$, $p < 0.001$). Animals in the ZT4 EL treatment showed the greatest bins of activity during the light-phase (18.06 $\pm$ 1.62 bins), and they differed significantly from ZT4 SL (9.48 $\pm$ 1.21 bins; $p = 0.002$), ZT16 SL (4.19 $\pm$ 0.68 bins; $p < 0.001$), and ZT16 shams (4.72 $\pm$ 0.95 bins; $p < 0.001$) but not from ZT4 shams. ZT4 shams (14.58 $\pm$ 2.60 bins) differed significantly from control and saporin-lesioned animals that trained at ZT16 ($p = 0.003$; $p = 0.001$, respectively).

Conversely, dark-phase activity was greatest for ZT16 SL animals (27.60 $\pm$ 2.53 bins), differing significantly from ZT4 treatments (ZT4 EL = 9.84 $\pm$ 0.63 bins, $p < 0.001$; ZT4 SL = 17.57 $\pm$ 3.59 bins, $p = 0.039$; ZT4 shams= 11.90 $\pm$ 1.46 bins, $p = 0.001$). ZT16 shams (25.34 $\pm$ 3.14 bins) differed significantly from ZT4 EL ($p = 0.001$) and the ZT4 shams ($p = 0.011$), but not ZT4 SL animals. The ZT16 treatment groups did not differ from one another. Under all circumstances, SL animals showed robust entrainment to the

LD cycle following surgery and prior to the initiation of task training that did not differ from ACSF infused sham animals (***figure 4.2, figure 4.3***; *baseline*).

We further quantified entrainment by looking at the ratio of light-activity to dark-activity. The LD ratio, represented the average activity in the light-phase divided by the average activity in the dark-phase, with a value greater than 1.0 representing a diurnal phase preference. As expected, based on previous published results, ZT4 trained animals showed increases in diurnal activity over the course of training and strong activity during the light-phase based on LD ratio (***figure 4.5C***). *ANOVA* revealed a significant main effect of time of day on LD ratio ($F(4,26) = 37.849$, $p < 0.001$). The LD ratio was greater than 1.0 for all ZT4 EL and ZT4 sham animals. The LD ratio was severely attenuated in ZT4 SL animals. ZT16 sham animals, alternatively, showed a decrease in LD ratio (0.18 $\pm$ 0.02) relative to their baseline condition (baseline = 0.20 $\pm$ 0.024) and a condensing of activity during the lights-off portion of the 24 hour day as animals neared the final phase of task acquisition. As expected, all ZT16 and ZT16 SL animals had LD ratios of less than 1.0 indicative of nocturnal behavior. ZT4 SL animals showed behavior consistent with a more nocturnal phenotype (0.63 $\pm$ 0.13). *Post-hoc* analysis showed significant differences between the ZT4 EL animals and all other treatment groups (ZT16 sham, $p < 0.001$; ZT16 SL, $p < 0.001$; ZT4 sham, $p = 0.007$; ZT4 SL, $p < 0.001$). ZT4 shams differed significantly from both ZT16 groups (ZT16 sham, $p < 0.001$; ZT16 SL, $p < 0.001$) and from ZT4 SL animals ($p = 0.041$). ZT4 SL animals did not differ significantly from ZT16 SL ($p = 0.076$) or ZT16 shams ($p = 0.160$).

In order to quantify entrainment, as measured by anticipation, for daily task training and the light cycle, animals' activity was quantified for phase angle of entrainment for three measures: activity onset to task, activity offset to task, and activity offset to light-phase onset. *ANOVA* revealed a main effect of treatment condition for activity onset to task ($F(4,26) = 2.90$, $p = 0.041$; ***figure 4.5D: black bar***), activity offset to task ($F(4,26) = 5.50$, $p = 0.002$; ***figure 4.5D: white bar***), and activity offset to light-phase onset ($F(4,26) = 6.01$, $p = 0.001$; *not-shown*). *Post-hoc* analysis revealed the largest anticipation activity, as defined previously, to the onset of the task (in minutes) was in ZT4 EL animals which differed significantly from ZT4 SL animals ($p = 0.034$). Mean

activity anticipation to task for ZT16 SL and ZT4 SL treatments approached a significant difference (p = 0.060), but all other means for activity to task were not statistically different (*see* ***figure 4.5D***).

ZT16 sham and ZT16 SL animals showed the largest activity offset to the task, followed by ZT4 EL, ZT4 sham, and ZT4 SL animals showed the least activity following the task (***figure 4.5D***). *Post-hoc* analysis showed significant differences between ZT16 sham and ZT4 SL (p = 0.015), ZT16 sham and ZT4 sham (p = 0.047), and between the two SL treatments (ZT4 SL and ZT16 SL; p = 0.005; ***figure 4.5D***).

Activity following the onset of the light-phase (*not-shown*) was greatest in ZT4 EL (15.50 $\pm$ 2.75 min*)* and least in animals that trained at ZT16 (sham = 1.80 $\pm$ 0.80 min; SL = 1.29 $\pm$ 0.52 min). *Post-hoc* analysis showed significant differences between ZT4 EL animals and the three treatments with the lowest means: ZT4 SL (p = 0.048*)*, ZT16 sham (p = 0.014), and ZT16 SL (p = 0.004).

Lastly we quantified entrainment based on number of days of activity at the time training would have occurred under conditions of total darkness (DD). Days of entrainment under *free-run* conditions, were greatest for animals training at ZT16 (SL = 13.9 $\pm$ 0.1 days; C = 13.8 $\pm$ 0.2 days). All ZT16 averages differed from all ZT4 averages (ZT4 sham = 3.6 $\pm$ 1.0 days; ZT4 EL = 2.9 $\pm$ .8 days; ZT4 SL = 2.5 $\pm$ 0.3 days; p < 0.001). Activity for the *free-run* condition are represented in the individual actograms as the bottom 10-14 lines of activity for each treatment condition in **figures 4.2-4.4**. Each of these animals shows anticipation of the SAT, and LD activity patterns representative of their treatment.

Lesions of the SCN influence SAT task acquisition and performance

Although the time to final performance criterion varied for each animal, all animals reached final task performance standards within 132 days. *ANOVA* revealed a significant effect of time of day on task acquisition irrespective of lesion condition with ZT16 animals acquiring the task faster than ZT4 trained animals (F(1,29) = 4.456, p = 0.044). *Paired t-tests* were used to compare lesion condition to time of day matched

controls and revealed a significant difference between ZT4 EL and the ZT4 sham animals ($t(1,11) = -2.251$, $p = 0.049$; ***figure 4.6A***) Animals with electrolytic lesions (EL) trained at ZT4, took the longest time to achieve criterion on the final stage of the task. SL and sham animals trained at ZT4 did not differ significantly ($p = 0.384$). Animals training at ZT16 took the least amount of time to reach final stage criterion and did not differ from one another statistically ($p = 0.854$).

We also quantified asymptotic performance in SAT trained animals. Hit rates were signal duration dependent for all treatment groups. The highest hit rate was seen during 500 ms trials (85.7 $\pm$ 1.4%), with hit rate decreasing as signal durations shortened ($F(2,52) = 182.089$, $p < 0.001$; 50ms: 60.8±2.7 %, 25ms: 54.4±2.6 %). *Paired t-tests* between lesion and time of day matched controls showed significant difference between ZT4 EL and ZT4 shams for overall hit rate ($t(1,11) = -2.514$, $p = 0.032$; ***figure 4.6B: white bar***), however there was no significant differences between ZT4 SL and ZT4 controls ($t(1,9) = -0.391$, $p = 0.705$). ZT16 shams and ZT16 SL did not differ from one another ($t(1,10) = 1.607$. $p = 0.146$).

No significant differences across treatment groups were observed for correct rejections (ZT4 EL and ZT4 controls; $t(1,11) = -0.949$, $p = 0.367$; ZT4 SL and ZT4 controls; $t(1,9) = 1.071$, $p = 0.312$); and ZT16 C and ZT16 SL; $t(1,10) = 0.505$. $p = 0.625$; ***figure 4.6B: dark bar***) or omissions during baseline performance (*not-shown*).

In order to further quantify how lesion size may or may not interact with performance, we assessed the relationship between days to criterion and the extent or effectiveness of lesion in both 192-IgG-saporin infused and SCN ablated animals. **Figure 4.7A** plots acquisition, as measured by days to criterion versus the extent of cholinergic depletion, against AChE+ fiber counts and ChAT positive cell number. The result of the correlation analysis revealed no significant interaction between cholinergic depletion and acquisition for AChE+ fibers (*Pearson Correlation* = 0.286, p=0.583) or for ChAT positive cell bodies (Pearson Correlation = 0.546, p=0.262). Next we looked at the relationship between the extent of SCN damage and the rate of task acquisition. We

found that SCN damage was positively correlated with rate of task acquisition (*Pearson Correlation* = 0.718, *p*=0.045; ***figure 4.7B***).

Because SCN ablated animals acquire the task more slowly, but eventually show the highest level of entrainment, we wanted to capture how LD ratio changes as a function of task acquisition between these animals and controls. **Figure 4.7C** plots normalized diurnality, relative to each animal's own pre-training baseline, over the 19 weeks of training for ZT4 shams and ZT4EL animals. Repeated-measures *ANOVA* revealed a significant interaction between treatment group and time over the first seven weeks of training, ($F(6,66) = 4.058$, $p = 0.004$), that did not exist for the remainder of task training ($F(11,121) = 1.245$, $p = 0.277$). Control animals show a more rapid change from their baseline starting LD ratio that is maximally diurnal near the time that the group reaches criterion performance on average. *Post-hoc* analysis between treatment groups revealed significant differences in week 6 ($p = 0.028$), week 7 ($p = 0.009$), and near significant differences in week 18 ($p = 0.052$). SCN ablated animals are slower to acquire the task and their peak diurnality is not expressed until the final weeks of training. Ultimately these animals become even more diurnal as reflected in LD ratio by the end of the training (***figure 4.6A***).

Cholinergic lesions of the SCN influence temperature rhythms in SAT trained animals

As body temperature rhythms are directly influenced by the output of the SCN, we examined whether daily cognitive training influences the circadian expression of temperature rhythms in task performing animals in a way that reflects activity (***figure 4.8A*** *and* ***figure 4.8B***). Because body temperature is considered a more reliable marker of SCN output than activity, we also assessed how body temperature was impacted by cholinergic deafferentation of the SCN in SAT performing animals. Repeated measures *ANOVA* revealed a significant effect of time of day on temperature in both the lights-on phase ($F(71,1207) = 16.395$, $p < 0.001$), and lights-off phase ($F(71,1207) = 19.097$, $p < 0.001$) of training. Furthermore there was a significant time of day interaction between treatment group and temperature during lights-on ($F(213,1207) = 9.887$, $p < 0.001$) and lights-off phase of the LD cycle ($F(213,1207) = 3.570$, $p < 0.001$). A between-subjects

main effect of treatment group was found for the lights-on phase ($F(3,17) = 35.736$, $p < 0.001$), but not for the lights-off phase ($F(3,17) = 0.727$, $p < 0.550$). *Post-hoc* analysis during the lights-on phase revealed significant differences between ZT4 shams and all other treatment groups (ZT16 shams, $p < 0.001$; ZT16 SL, $p < 0.001$; and ZT4SL, $p = 0.044$). ZT16 SL and ZT16 shams did not differ significantly ($p = 0.367$).

We additionally quantified entrainment for daily task training by looking at how rises in body-temperature, relative to the daily-mean, differed for task onset and dark-phase onset across treatment groups. *ANOVA* revealed a main effect of treatment condition for activity onset to task ($F(3,20) = 2.468$, $p = 0.002$; ***figure 4.8C***). *Post-hoc* analysis revealed the largest anticipation activity, as defined previously, to the onset of the task (in minutes) was for ZT16 sham animals differing from both daytime training groups (ZT4 sham, $p = 0.039$; ZT4 SL, $p < 0.001$). ZT4 sham animals also differed from ZT4 SL animals ($p = 0.026$). We also looked at how body temperature rises in anticipation of the dark-phase onset, relative to the inactive period. *ANOVA* revealed a main effect of treatment condition for dark-phase onset ($F(3,20) = 6.841$, $p < 0.001$; ***figure 4.8D***). For dark-phase trained animals, this threshold crossing occurs at or about the dark-phase transition, with ZT16 sham animals rising slightly earlier than SL animals (***figure 4.8B***). *Post-hoc* analysis revealed the largest anticipation activity, as defined previously, to the onset of the dark-phase (in minutes) was found in ZT16 sham animals. These animals differed significantly from both daytime training groups (ZT4 shams, $p = 0.005$; ZT4 SL, $p < 0.001$) and ZT16 SL animals also differed from both daytime training groups (ZT4 shams, $p = 0.008$; ZT4 SL, $p < 0.001$). The two daytime training groups also differed from each another (ZT4 sham vs. ZT4 SL, $p < 0.015$).

Based on the analysis thus far, temperature rhythms and activity rhythms in task-performing animals do not share the same phase relationship. In particular, it appears that daytime trained animals show a higher level of internal desynchrony (ID) between body temperature and activity. In order to quantify this, we normalized body temperature and activity to daily minimums and maximums for each animal and quantified difference scores bin by bin across the light-cycle and the dark-cycle (***figure 4.9***). Repeated

measures *ANOVA* revealed significant differences in time of day on ID during both the light-on phase ($F(71,1207) = 7.234$, $p < 0.001$), and lights-off phase ($F(71,1207) = 2.681$, $p < 0.001$) of the 24 hour day. Furthermore there was a significant interaction between treatment group and time of day for lights-on ($F(213,1207) = 4.993$, $p < 0.001$ and lights-off $F(213,1207) = 1.890$, $p < 0.001$) phases of training. A between subject effect of group was found for the lights-on phase ($F(3,20) = 18.497$, $p < 0.001$) and lights-off phase ($F(3,20) = 3.242$, $p < 0.048$). *Post-hoc* analysis of the lights-on phase revealed significant differences between ZT4 shams and ZT16 shams ($p<0.001$) and ZT16 SL ($p<0.001$), but not for ZT4 SL ($p=0.446$). *Post-hoc* analysis of the dark-phase revealed significant differences between ZT4 shams and all other treatment groups (ZT16 shams, $p=0.015$; ZT16 SL, $p=0.029$, and ZT4 SL, $p=0.022$); however, ZT4 SL animals did not differ from either ZT16 training group during the dark-phase. These findings suggest that sham animals training at ZT4 have the most profound level of ID of any of the training groups. ZT4 SL animals maintain a high nighttime level of activity, comparable to animals training at ZT16, and a substantially lower level of activity during the lights-on phase compared to ZT4 sham animals.

Discussion

The collective results from our experiments indicate that animals, when trained on a task of sustained attention during the lights-on phase, adopt a significantly more diurnal activity pattern, coincident with profound internal desynchrony between activity and core body temperature, that is not seen in animals trained during the dark-phase. Furthermore, we provide evidence indicating that basal forebrain cholinergic input to the SCN is necessary for entrainment by cognitive activity. Intra-SCN injections of 192 IgG-saporin are reported to abolish all p75 expressing axons in the SCN (Erhardt, et al., 2004). Loss of markers for cholinergic innervation of the SCN was associated with only minor losses in adjacent regions of the hypothalamus and outlying cortical areas (***figure 4.1***). The remaining cholinergic activity in the SCN is presumed to be inputs from the brainstem cholinergic system including the pedunculopontine, laterodorsal tegmental and parabigeminal nuclei (Bina, et al., 1993) which are spared by infusions of 192 IgG-

saporin (Heckers, et al., 1994), and are thought to be important in the generation of sleep and generalized arousal (Steriade & McCarley, 2005).

Deafferentation of the SCN did not disrupt SAT acquisition or performance; however, deafferentation did result in significant decreases in the amounts of entrainment for daily training as measured by anticipation to activity onset, LD ratio, and *free-run* activity under constant conditions (***figures 4.2-4.3, figure 4.5***). Moreover, ZT4 trained SL animals were significantly less diurnal than ZT4 trained shams. As a group, ZT4 SL rats were the only ZT4 trained group to maintain an LD ratio below 1.0, differing significantly from other ZT4 groups, but not from the ZT16 training groups. These findings suggest that basal forebrain cholinergic projections to the SCN play an important role in the timing of entrainment for daily cognitive training. 192 IgG-saporin lesioned animals training at ZT4 show a minimal level of diurnal activity compared to non-lesioned controls and comparable to levels of diurnality seen in animals undergoing daily handling alone or performing a simplified operant task that requires limited cognitive effort (Gritton, et al., 2009; Hastings, et al., 1997; Hummer, Meixner, & Lee, 2010). In addition, the level of entrainment, as quantified by *free-run* activity to time of task onset, for ZT4 SL animals (2.5 $\pm$ 0.3 days) was similar to measures of entrainment for daily handling (2.8 + 0.986 days; Gritton, et al., 2009). These findings provide support for the hypothesis that direct innervation of the SCN by cholinergic inputs of the basal forebrain substantially regulates entrainment of circadian rhythms by cognition.

As 192 IgG-saporin-lesioned animals also maintain a small amount of entrainment to daily task performance without basal forebrain cholinergic input to the SCN, it suggests that cognition induced entrainment involves synchronization of a secondary oscillator outside of the SCN under normal conditions. Furthermore, as complete basal forebrain lesions also effectively block the anticipatory activity seen in animals undergoing timed daily handling (Hummer, et al., 2010), it is interesting to speculate if cholinergic release at structures outside of the SCN also acts as an entraining signal for handling or cognition-induced entrainment at non-SCN oscillators. A number of potential cholinergic targets associated with task performance have been shown to express clock genes, and could be entrained by daily task performance or handling (*e.g.*,

prefrontal cortex, hippocampus; Abe, et al., 2002; Angeles-Castellanos, Mendoza, & Escobar, 2007; Wang, et al., 2009). If this is true, it would suggest that the SCN, under normal conditions, is sensitive to direct cholinergic influences; however, in the absence of these direct inputs, non-SCN oscillators entrained by acetylcholine release are sufficient to mediate non-photic entrainment even in the presence of a strongly entraining LD cycle.

We also report, perhaps surprisingly, that animals with full electrolytic lesions of the SCN showed the highest levels of entrainment to the SAT at ZT4. This level of entrainment was greater than for animals with an intact SCN as measured by amount of activity to task onset, amount of activity to light-phase onset, and LD ratio. Moreover, the ZT4 EL animals' activity in anticipation of, and following the SAT training period, is most similar to animals that train at ZT16, although total activity duration may have been shorter and suggests an underlying desynchrony not seen in ZT16 trained animals (see ***Figure 4.4***). These findings are similar to recent findings for other non-photic entrainable oscillators including those entrained by food or methamphetamine, in that animals show enhanced anticipatory activity to non-photic cues in SCN-lesioned rats when compared to intact controls (Angeles-Castellanos, et al., 2010; Pezuk, et al., 2010; Stephan, et al., 1979a, 1979b). Entrainment that occurs in the absence of the SCN is thought to be under the control of non-SCN brain oscillators that have a more prominent role in modulating circadian output when uncoupled from the SCN. As SCN ablated animals show increased activity in the light-phase compared to all other treatments, our results support the hypothesis that a cognition-induced circadian oscillator(s) exist outside the SCN and is capable of organizing circadian activity. Our findings suggest that the cognition-induced circadian oscillator has a profound effect on the timing of organized behavioral activity that appears to be as robust as food or methamphetamine availability.

We propose that these data support a model of competitive interactions between non-SCN brain oscillators and the photic-driven SCN in rodents trained on a cognitively demanding task during the day. While non-SCN oscillators can apparently be entrained to a multitude of external cues including daily handling, food availability, or

methamphetamine access, cognition-induced entrainment represents a unique condition: it is internally motivated and the maximum effect is not driven by externally presented cues. It is interesting to note that non-SCN oscillators were not well synchronized in the absence of sustained cognitive performance; the ZT4 EL animals had arrhythmic wheel activity prior to SAT training, but eventually became entrained to the SAT training including a highly anticipatory diurnal rhythm which quickly disappeared following the cessation of training and a return to arrhythmia within several days (*see* ***figure 4.4***). The quick return of desynchronized activity in these rats, suggests that these oscillators are unable to maintain synchrony without external periodic cues (*i.e.,* sustained attention task) as has been noted for other non-SCN oscillator driven activity (Stephan, et al., 1979b).

The results of our studies with ZT4 EL animals also validate the hypothesis that the SCN is important for task acquisition and performance. Lesions of the SCN have profound effects on novel object recognition in hamsters (Ruby, et al., 2008), and age related dampening of circadian rhythms impairs development of conditioned place preference in middle aged hamsters (Antoniadis, Ko, Ralph, & McDonald, 2000). These findings suggest that timing information about salient events or the frequency of salient events may be conveyed to the SCN through a mechanism of unknown origin. It has recently been proposed that release of acetylcholine at the level of the SCN may provide a timing signal important for 'time-stamping' singular events in the service of memory consolidation (for review see Daan, 2000; Hut & Van der Zee, 2010). Both acquisition and performance were compromised in ZT4 EL animals with acquisition taking 35 percent longer on average. It is also interesting to note that ZT4 EL animals took substantially longer to entrain to daily task training. Given that the SCN has the important role of synchronizing various brain oscillators; it is possible that in the absence of an SCN, peripheral oscillators sensitive to daily cognitive training take much longer to become synchronized to the signal and each other. We hypothesize that as more independent oscillators become synchronized, by SAT performance, a pattern of entrainment emerges for the most prominent zeitgeber (*i.e.*, SAT performance; *see* ***figure 4.7C***).

As unintended damage to the optic chiasm caused by the SCN ablation could produce detection deficits that would explain differences in acquisition or performance, animals were only included in this study after careful histological examination. Quantification of lesion size and placement resulted in the exclusion of any animals with damage to the optic tract. Further, the robust ZT4 EL activity following light-phase onset (***figure 4.5A***) provides support for the integrity of the retinal hypothalamic tract (RHT), as well as the thoroughness of SCN lesions, since light-phase onset in intact animals profoundly depresses activity (see shams in ***figure 4.5A*** *and* ***figure 4.5B***). As ZT4 EL animals eventually reach criterion performance (*see methods*), it seems unlikely that high levels of performance could be concurrent with damage to the RHT. As a final note, ZT4 EL animals show a masking effect around each change in light-phase, which was not reported in other studies where large lesions of the SCN extended into the optic chiasm (Stephan, et al., 1979b). These data provide support for the size and placement of the lesions, as well as the integrity of the optic chiasm. Based on this study, the size of lesion correlated negatively with the rate of acquisition (*see* ***figure 4.7B***). It is interesting to speculate whether the remaining portion of the SCN, although not subject to light-induced entrainment, would be sufficient to support synchrony amongst non-SCN oscillators in the service of promoting task acquisition. Future studies will address how the SCN sub-regions overlap with cholinergic inputs to the SCN and whether or not those areas innervate SCN regions known to have interconnectivity with output regions of the hypothalamus.

Figure 4.10 summarizes a model by which daily performance of a cognitively demanding task could result in cognition-induced circadian entrainment. We suggest cholinergic signaling from the basal forebrain influences circadian oscillators, both at the SCN, and in unidentified regions of the central nervous system. Non-SCN oscillators can influence the SCN directly to produce circadian effects on entrainment. Over time, as in the case of SCN ablated animals, various independent oscillators come to express a coherent state of synchrony that can be measured by entrained phase markers. Animals trained during the dark-phase (ZT16) share a common phase of synchrony as non-SCN oscillators entrained to task performance and light-driven oscillators of the SCN promote activity at the same time of day. ZT4 training results in a state in which cognition-

induced oscillators actively work against light mediated activity rhythms to influence behavior. This state of internal desychrony (ID) is manifest by the dissimilarity of activity and temperature rhythms demonstrated by ZT4 shams (***figure 4.9***). ZT4 SCN ablated animals over time eventually become robustly entrained to daily task performance without the light-driven influence of the SCN, whereas 192 IgG-saporin lesioned animals, despite entraining non-SCN oscillators with daily task performance, never overcome the influence of SCN light-mediated entrainment.

Our findings offer insight into the consequences of chronic shift-work and provide an animal model for the risk of prolonged shift-work on physiological conditions described in this population. We have discovered that rats performing cognitive tasks during the light-phase, experience a state of desynchrony, exemplified by incoherence between activity and temperature that is consistent with a state of ID noted in chronic shift-workers (Haus & Smolensky, 2006; Reid, et al., 2004; Salgado-Delgado, et al., 2010). Future research with this model offers the potential to explore the long-term risks of circadian disorganization and test prospective therapies to attenuate this condition.

Finally, research on interactions between the basal forebrain cholinergic systems and circadian rhythms offer insight into neuropsychiatric disorders such as schizophrenia, bipolar disorder, and Alzheimer's disease that often present with symptomatic circadian disruptions and co-morbid cholinergic dysregulation (Costa e Silva, 2006; Morgan & Cheadle, 1976; Ross, et al., 2010; Sitaram, Nurnberger, Gershon, & Gillin, 1982; Vitiello & Prinz, 1989). Positive correlations have been found between cholinergic cell-death in the basal forebrain, decreases in choline acetyltransferase (ChAT), and impaired cognition in older individuals with dementia and Alzheimer's (Bartus, Dean, Beer, & Lippa, 1982). Decreased depolarization in cortical cholinergic neurons related to impaired learning and memory and a dissociation of core body temperature and daily activity are also found in old age, particularly in individuals with Alzheimer's disease (Ancoli-Israel, et al., 1997; Harper, et al., 2005; Satlin, Volicer, Stopa, & Harper, 1995). Of importance to this study is the change in SCN activity seen with old age: consequences of which include severe sleep disturbances, decreased sensitivity to environmental cues, a slowed SCN resynchronization speed, and weakened internal

coupling of peripheral oscillators (Van Someren, 2000). Recent studies suggest that age-related effects on the SCN may be stimuli specific (Biello, 2009), and the close relationship between circadian activity and cholinergic mediated entrainment suggested by these results calls for a continued focus and additional research on this topic.

Acknowledgements

Many thanks to Nick Lusk for his help with animal training, tissue processing, and lesion quantification efforts in the course of bringing this study to publication. We would also like to thank Ana Kantorowski and Sofya Rozenblat for their help with animal training and care. This research was supported by PHS Grant RO1 MH079084 (TL).

Figure 4.1: **Anatomical results of neurotoxic lesions and electrolytic ablation for animals used in this study** (anterior-posterior levels based on Bregma are shown in schematics; scale for all photomicrographs is indicated in the lower right of image): ***(A)*** Left side provides schematic illustration of coronal sections shown in microphotographs on right. Box outline in schematic represents the boundary of the microphotographs for areas in which ChAT-positive cell bodies were counted. Images are representative for animals that received ACSF infusions (shams) or 192 IgG-saporin infusions. The bar graphs on the right indicate ChAT-positive cell counts for animals by group for each region quantified (* = $p<0.05$). ***(B)*** Left side represents schematic of regional sections shown in microphotographs (center). Box outline in regional photomicrographs represent 40X images shown on right from ACSF and 192 IgG-saporin infused animals, respectively. Bar graph on the right indicate AChE+ fiber counts for animals by group for each region quantified (M1: layer 3/5, SCN, and AHC; * = $p<0.01$). ***(C)*** Left: schematic of SCN-lesioned section shown in microphotographs on right. Shaded area corresponds to the maximally outer boundary of lesions for all animals included in this study. The animal with the largest individual lesion (92% SCN ablation; *figure 4*) is shown in the center photomicrograph. Gray hatched area represents animal with minimal lesion included in this study (39% SCN ablation; *figure 4*) shown in photomicrograph on right. See methods for exclusion criteria. Abbreviations: 3V, third ventricle; AHC, anterior hypothalamus; B, basal nuc. of Meynert; BMA, basal medial amygdala; cc, corpus callosum; Cg, cingulate cortex; CPu, Caudate/Putamen; HDB horizontal diagonal band of Broca; LA, lateral anterior hypothalamus; LGP, lateral globus pallidus; LH, lateral hypothalamus; LV, lateral ventricle; M1, primary motor cortex; M2, secondary motor cortex; MS, medial septal nuc.; ot, optic tract; ox, optic chiasm; RCh, retrochiasmatic area; SCN, superchiasmatic nuc.; SI, substantia innominata; VDB, vertical diagonal band of Broca.

Figure 4.1

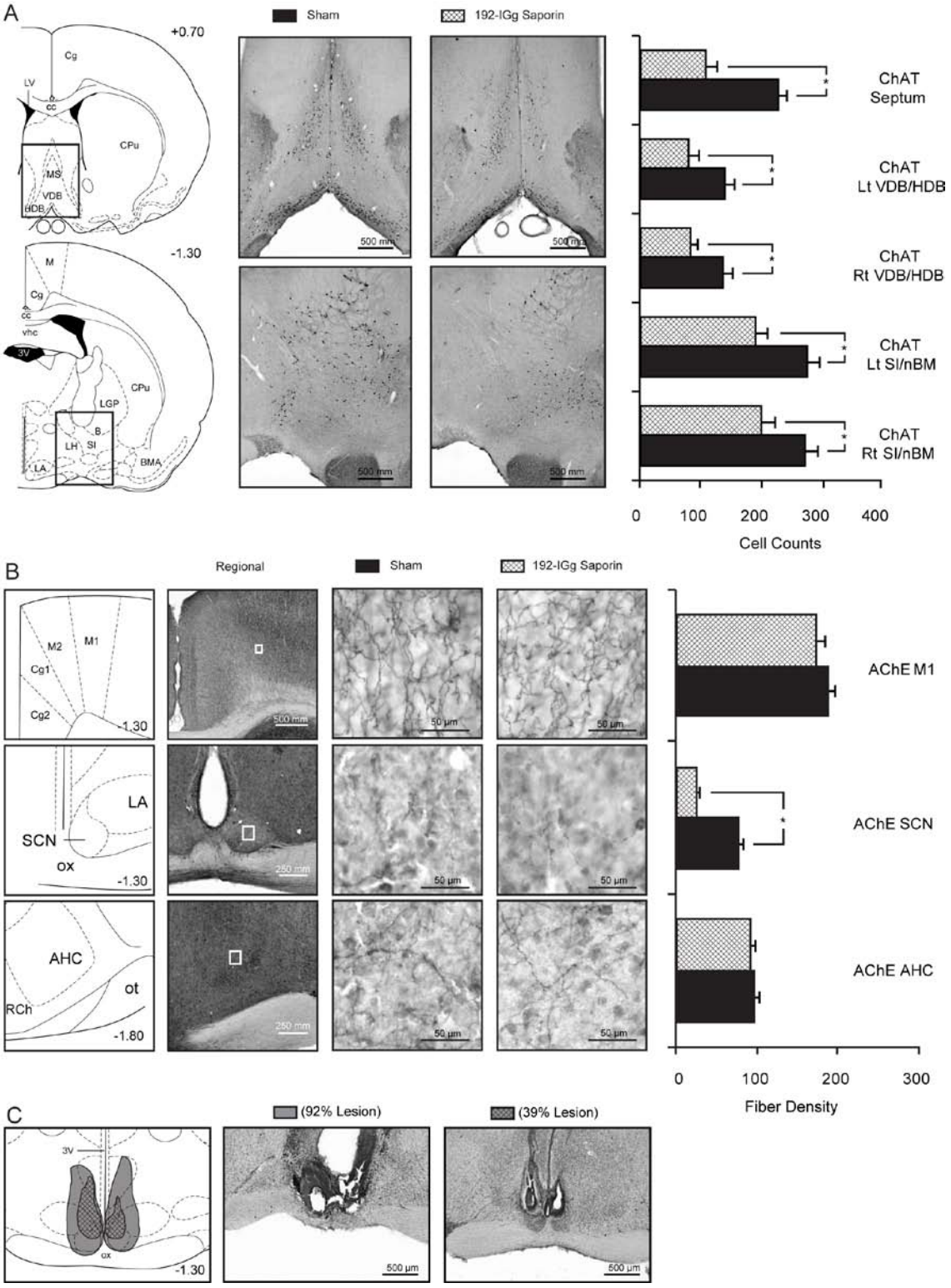
A
+0.70
Cg
LV
cc
CPu
MS
VDB
HDB
-1.30
M
Cg
cc
vhc
3V
CPu
LGP
B
LH
SI
LA
BMA
Sham
192-IGg Saporin
500 mm
ChAT Septum
ChAT Lt VDB/HDB
ChAT Rt VDB/HDB
ChAT Lt SI/nBM
ChAT Rt SI/nBM
0
100
200
300
400
Cell Counts
B
Regional
Sham
192-IGg Saporin
M2
M1
Cg1
Cg2
-1.30
LA
SCN
ox
-1.30
AHC
ot
RCh
-1.80
250 mm
50 µm
AChE M1
AChE SCN
AChE AHC
0
100
200
300
Fiber Density
C
(92% Lesion)
(39% Lesion)
3V
ox
-1.30
500 µm

Figure 4.2: **Representative double-plotted running wheel actograms for animals training at ZT4 on the SAT following ACSF or 192 IgG-saporin infusions:** Each line represents 48 hours of activity. SAT training (ZT4) is relative to the topmost LD bar (where dark bar = lights-off) and the shaded column represents the approximate 40 min SAT training period in which animals are absent from their home cages. Circadian actograms are separated into 2 phases: The first phase represents 14 days of baseline activity prior to SAT training but following surgery for ACSF or saporin infusions into the SCN. The second portion of the actogram illustrates the final 25 days of SAT training and the subsequent 14 days of constant conditions (DD). Time histograms below provide averages of running wheel activity for the final 25 days of training shown above on a 24-hour scale. Shaded column represents time of training. *(Left)* Actogram of ZT4 animal with ACSF (sham) infusions into the SCN. Note that animal shows a substantial daily anticipation for time of daily training and a primarily diurnal activity pattern. *(Right)* Actogram for ZT4 animal with 192 IgG-saporin infusion into the SCN. Daily anticipation is substantially reduced and this animal maintains a primarily nocturnal phenotype. Free-run activity from the time of training is about half of what is seen in sham animals.

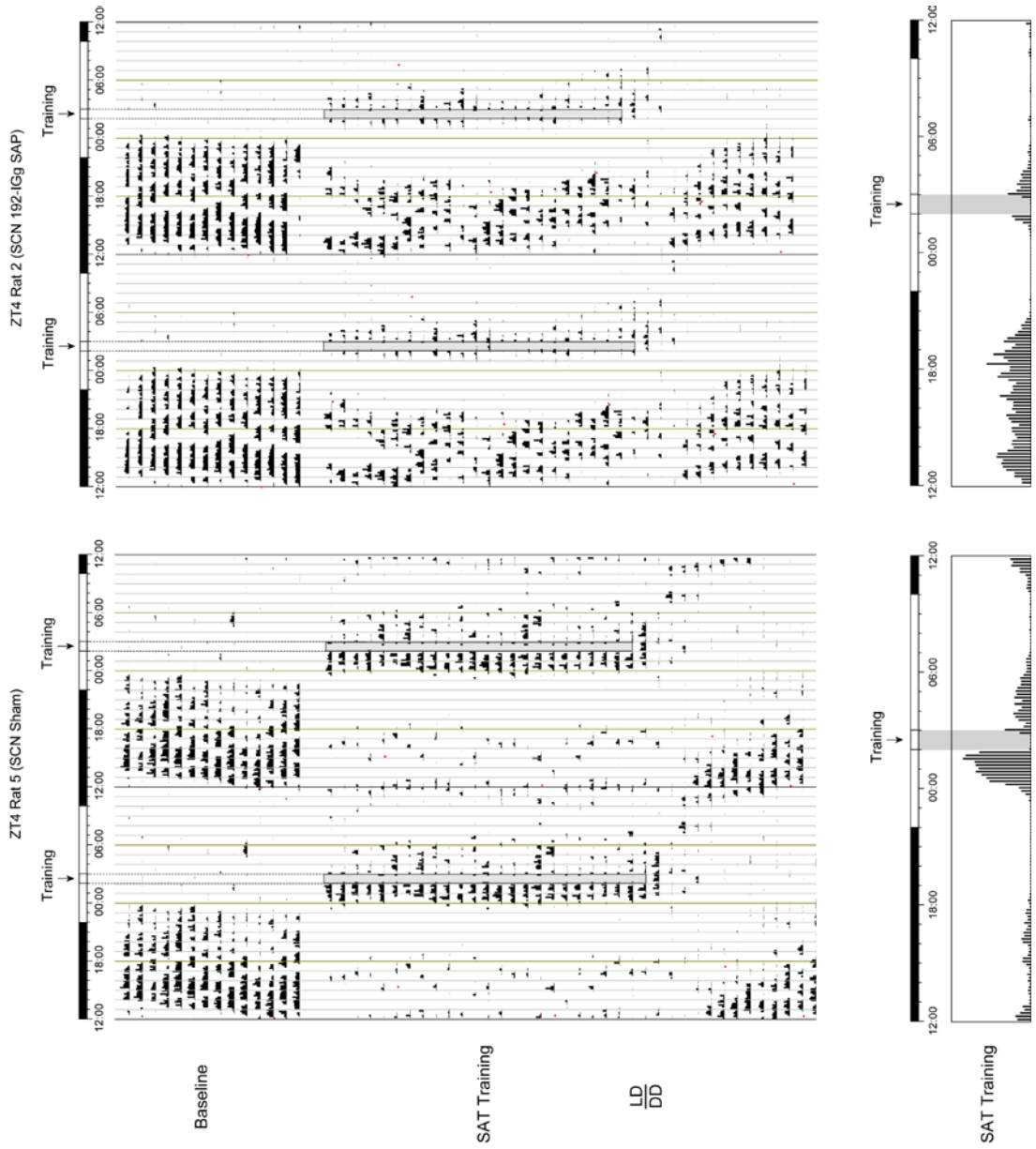
ZT4 Rat 2 (SCN 192-IGg SAP)
ZT4 Rat 5 (SCN Sham)
Training
Baseline
SAT Training
LD
DD
SAT Training

Figure 4.2

Figure 4.3: **Representative 48 hour double-plotted running wheel actograms for sham or 192 IgG-saporin infused animals training on the SAT at ZT16:** SAT training is relative to the topmost LD bar (where dark bar = lights-off) and the shaded column represents the approximate 40 min SAT training period in which animals are absent from their home cages. The first 14 days represent baseline activity of animals with ACSF or saporin infusions into the SCN prior to SAT training. The second portion of the actogram illustrates the final 25 days of training of SAT training and the subsequent period of constant conditions (DD). Time histograms below provide averages of running wheel activity for the final 25 days of training shown above on a 24-hour scale. Shaded column represents time of training. *(Left)* Double-plotted actogram of ZT16 animal with sham lesion. Note that animal shows a more robust amplitude of activity prior to daily training. *(Right)* Actogram of ZT16 animal with 192 IgG-saporin infusion into the SCN. Daily anticipation for task training is reduced with primary activity after the animal is returned from daily training.

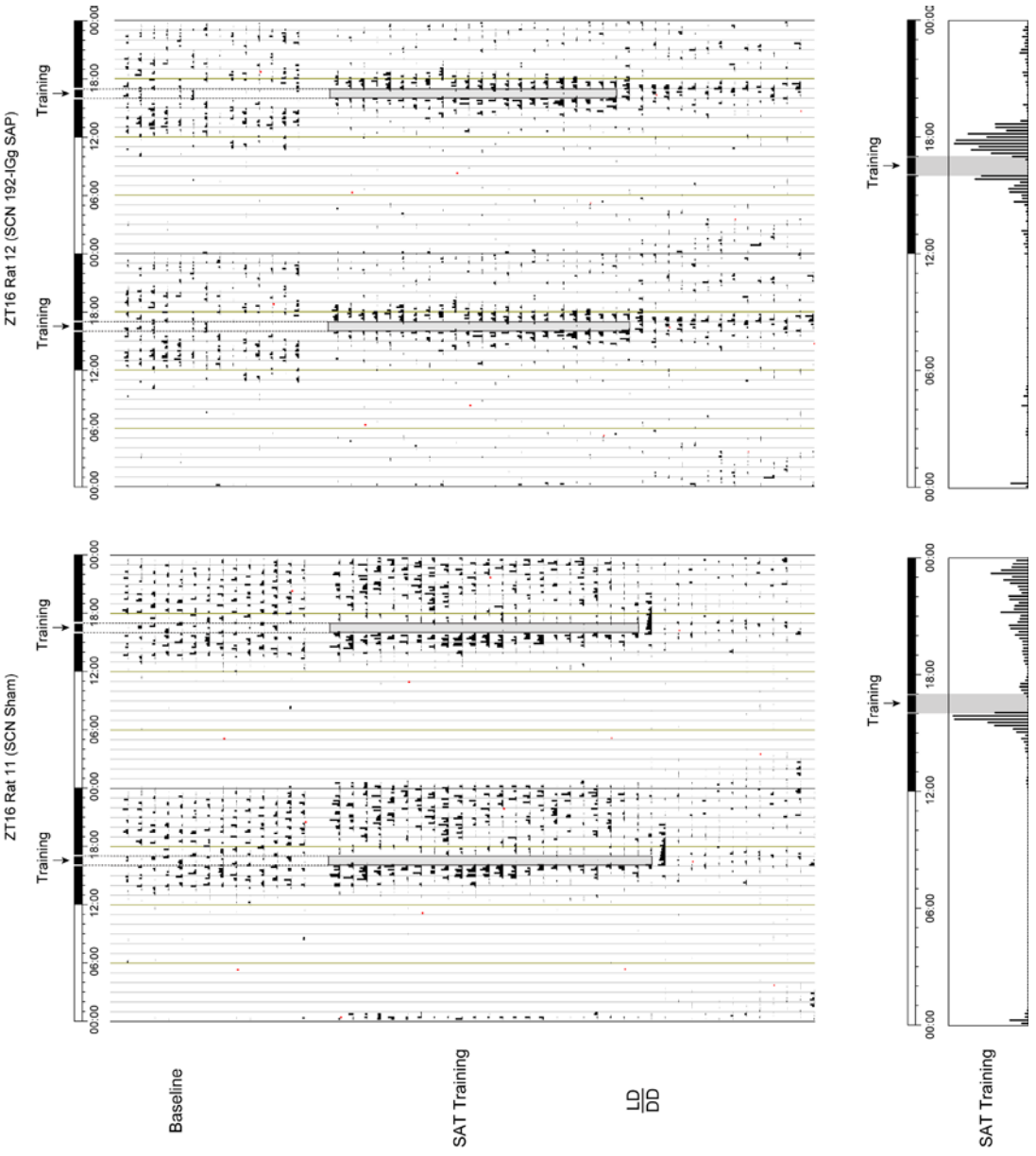
ZT16 Rat 11 (SCN Sham)
ZT16 Rat 12 (SCN 192-IGg SAP)
Training
Training
Training
Training
Training
Training
Baseline
SAT Training
LD
DD
SAT Training

Figure 4.3

Figure 4.4: **Double-plotted running wheel actograms for SCN ablated (electrolytic lesion - EL) animals with largest and smallest lesions used in this study training on the SAT at ZT4** (lesions from these animals are represented in *figure 1C*): Each line represents 48 hours of locomotor activity collected via running wheel activity. SAT training (ZT4) is relative to the topmost LD bar (where dark bar = lights-off) and the shaded column represents the approximate 40 min SAT training period in which animals are absent from their home cages. Circadian actograms are separated into 2 phases with 4 observable activity states: The first phase represents the final 8 days of baseline activity prior to surgery and lesioning of the SCN, followed by the first 10 days of post-lesion activity prior to onset of training. The second portion of the actogram illustrates the final 17 days of SAT training and the subsequent 10 days of constant conditions (DD). Time histograms below provide averages of running wheel activity for the final 17 days of training shown above on a 24-hour scale. Shaded column represents time of training relative to the LD cycle. In both cases animals show an arrhythmic phenotype following lesioning of the SCN in both LD and DD environments. *(Left)* Actogram of ZT4 animal with 92% lesion of the SCN representing the most severe lesion for an animal used in this study. Under conditions of daily task performance, this animal shows a profound daily anticipation for daily task training that persists even after the animal stops training. *(Right)* Actogram of ZT4 animal with 39% lesion of the SCN representing the least severe lesion for an animal used in this study. Animal shows a strong anticipation for daily task training that persists in constant conditions.

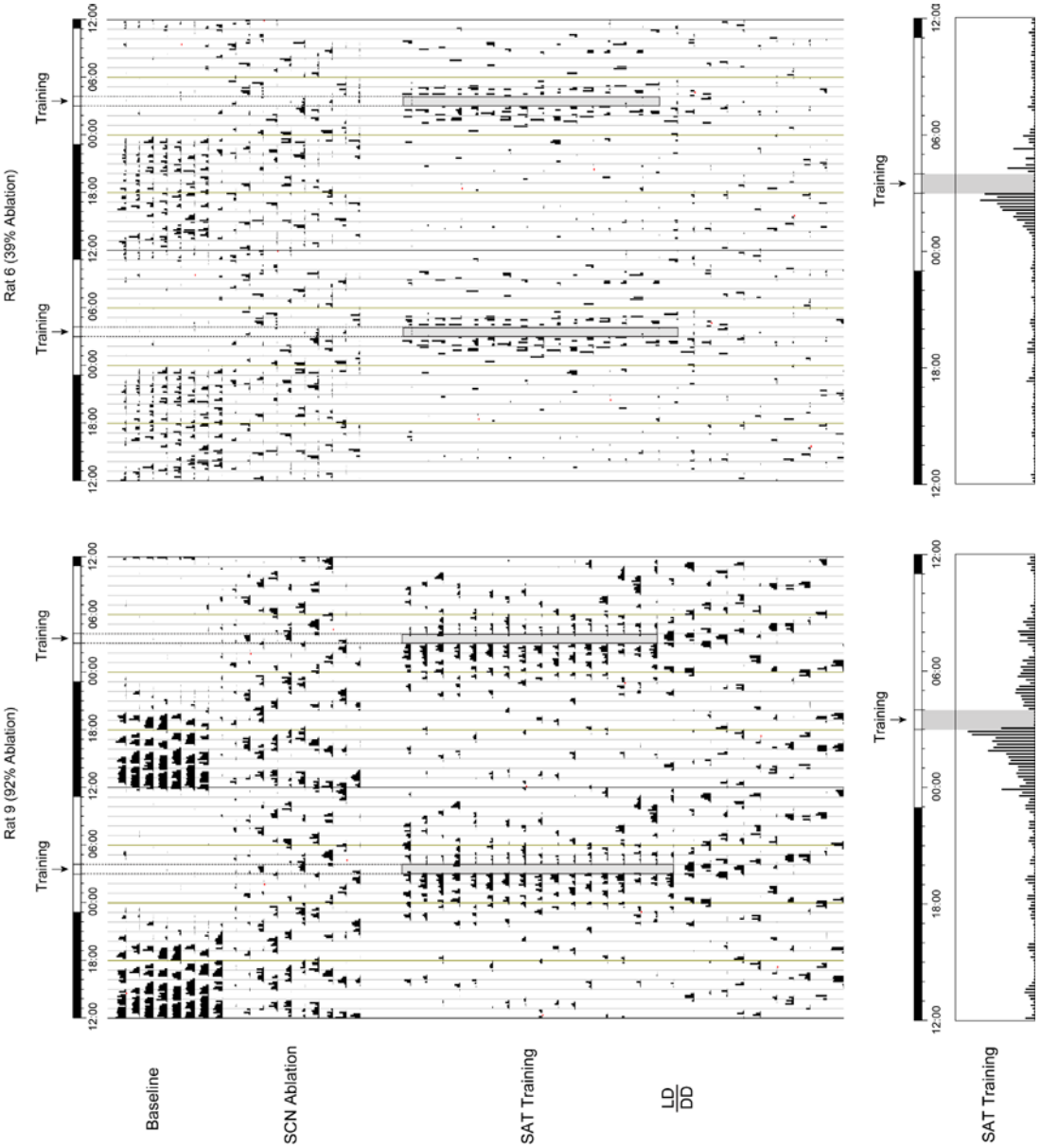
Rat 6 (39% Ablation)
Rat 9 (92% Ablation)
Training
12:00
18:00
00:00
06:00
12:00
Baseline
SCN Ablation
SAT Training
LD
DD
SAT Training

Figure 4.4

Figure 4.5: **Mean activity records and phase markers of entrainment for SAT trained animals by treatment group:** ***(A)*** 24 hour binned average of ZT4 trained animals. SAT training (ZT4) is relative to the topmost LD bar (where dark bar = lights-off) and the shaded column represents the approximate 40 min SAT training period in which animals are absent from their home cages. X-axis is plotted in zeitgeber time (ZT) and y-axis represents wheel revolutions with activity grouped into 10 min bins. Mean histograms are taken from the last 10 days of SAT training before entering constant conditions. ZT4 EL (red), ZT4 SAP (light gray), and ZT4 sham (blue) mean averages are plotted ± SEM (shading). ***(B)*** 24 hour binned average of ZT16 trained animals. Training is relative to the topmost LD bar and the shaded column represents the SAT training period in which animals are absent from their home cages. Mean histograms are taken from the last 10 days of SAT training before entering constant conditions. ZT16 SL (dark gray), and ZT16 sham (black) mean averages are plotted ± SEM (shading). (***C)*** Mean Light-Dark (LD) activity ratios from final 10 days of training on the SAT ± SEM (* = $p < 0.05$). LD ratio is calculated by taking the number of 10-min bins of activity during the light-phase and dividing by the number of 10-min bins of activity during the dark-phase with LD activity ratios less than 1.0 being indicative of nocturnal behavior. ZT4 EL animals show an LD ratio significantly higher than all other treatments. ZT4 shams also differed significantly from ZT4 SL animals and animals that trained at ZT16. ***(D)*** Mean phase relationship between time of locomotor activity and time of sustained attention task (SAT) training. Activity onset and offset in minutes relative to training is plotted for each condition (minutes ± SEM; * = $p < 0.05$).

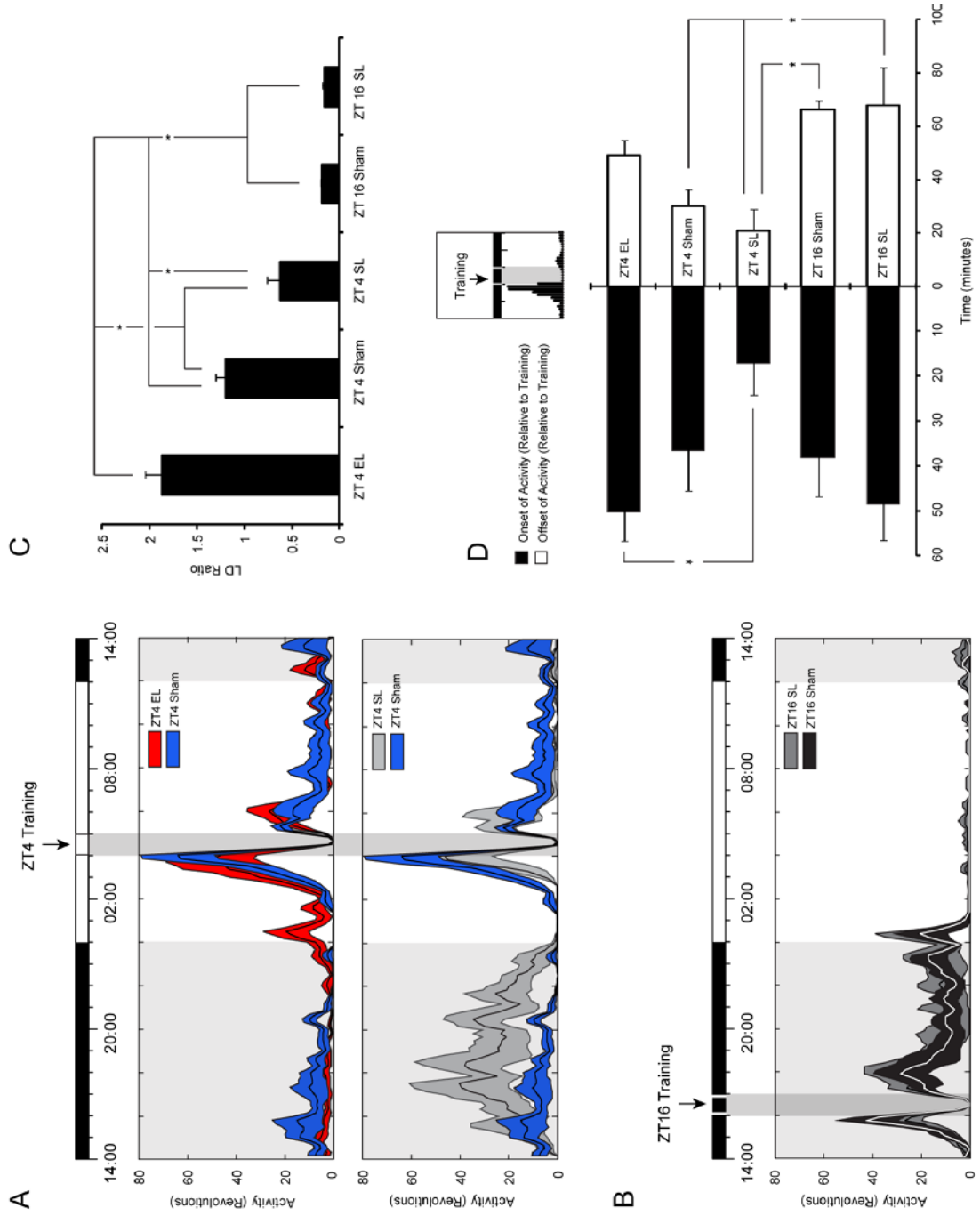
A
ZT4 Training
14:00
20:00
02:00
08:00
14:00
ZT4 EL
ZT4 Sham
ZT4 SL
ZT4 Sham
Activity (Revolutions)
0
20
40
60
80
B
ZT16 Training
ZT16 SL
ZT16 Sham
C
LD Ratio
0
0.5
1
1.5
2
2.5
ZT 4 EL
ZT 4 Sham
ZT 4 SL
ZT 16 Sham
ZT 16 SL
D
Training
Onset of Activity (Relative to Training)
Offset of Activity (Relative to Training)
Time (minutes)
60
50
40
30
20
10
0
20
40
60
80

Figure 4.5

Figure 4.6

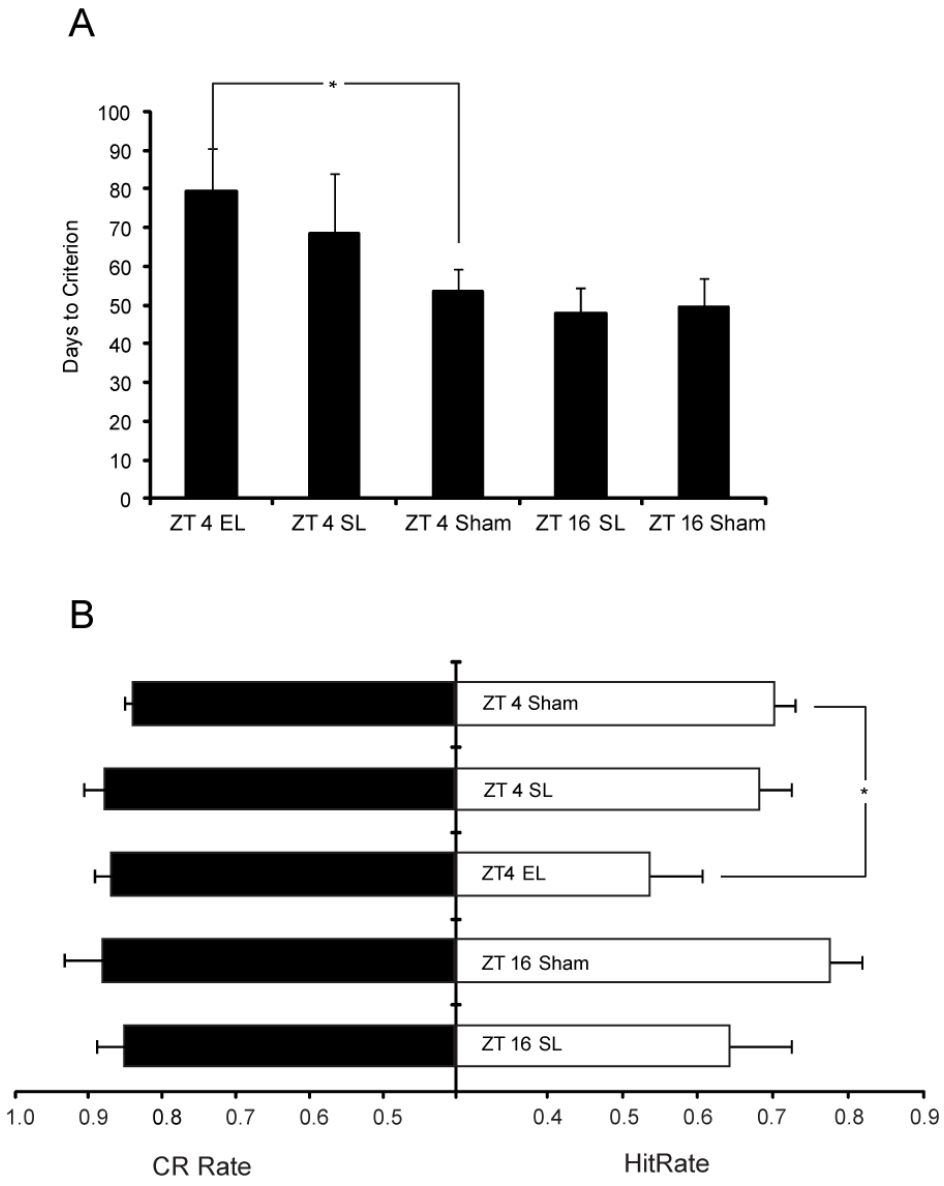

Figure 4.6: **Acquisition and criterion performance for SAT trained animals:** ***(A)*** Mean days to criterion by training time ± SEM (* = $p < 0.05$). ZT4 EL animals took the longest to reach criterion performance and differed significantly from sham controls. ***(B)*** Asymptotic performance at criterion for animals by treatment group training on the SAT. Correct rejection (CR) rate and hit rate (see methods) by training time ± SEM (* $p < 0.05$). ZT4 EL animals responded correctly to significantly fewer signals than their sham operated controls. Saporin animals did not differ significantly from their time-matched controls.

Figure 4.7: **Correlations between lesion types and acquisition rate for ZT4 SAT trained animals:** ***(A)*** Correlation between days to criterion (x-axis) and lesion effectiveness based on AChE fiber counts (y-axis – left) and cholinergic cell number (y-axis – right). Note that cholinergic depletion does not influence task acquisition in SL animals training at ZT4 (AChE, p=0.583; p=0.262). ***(B)*** Correlation between days to criterion (x-axis) and lesion effectiveness based on SCN ablation size (y-axis). There was a significant correlation between lesion size and task acquisition in ZT4 EL animals (p=0.045; see text for discussion). ***(C)*** Days to criterion plotted as a function of diurnality for ZT4 EL animals and controls. Weeks of training are represented on the x-axis and diurnality normalized to each animal's baseline is represented on the y-axis. Week 19 indicates the end of the training period for all animals in this study. Gray bars represent mean time to criterion for each treatment group noted. ZT4 sham animals reached a stabilized level of diurnality at or around the time animals reach criterion performance. ZT4 EL animals show increased diurnality over the entire training period but do not reach the same normalized level of diurnality until approximately the 14^{th} week of training.

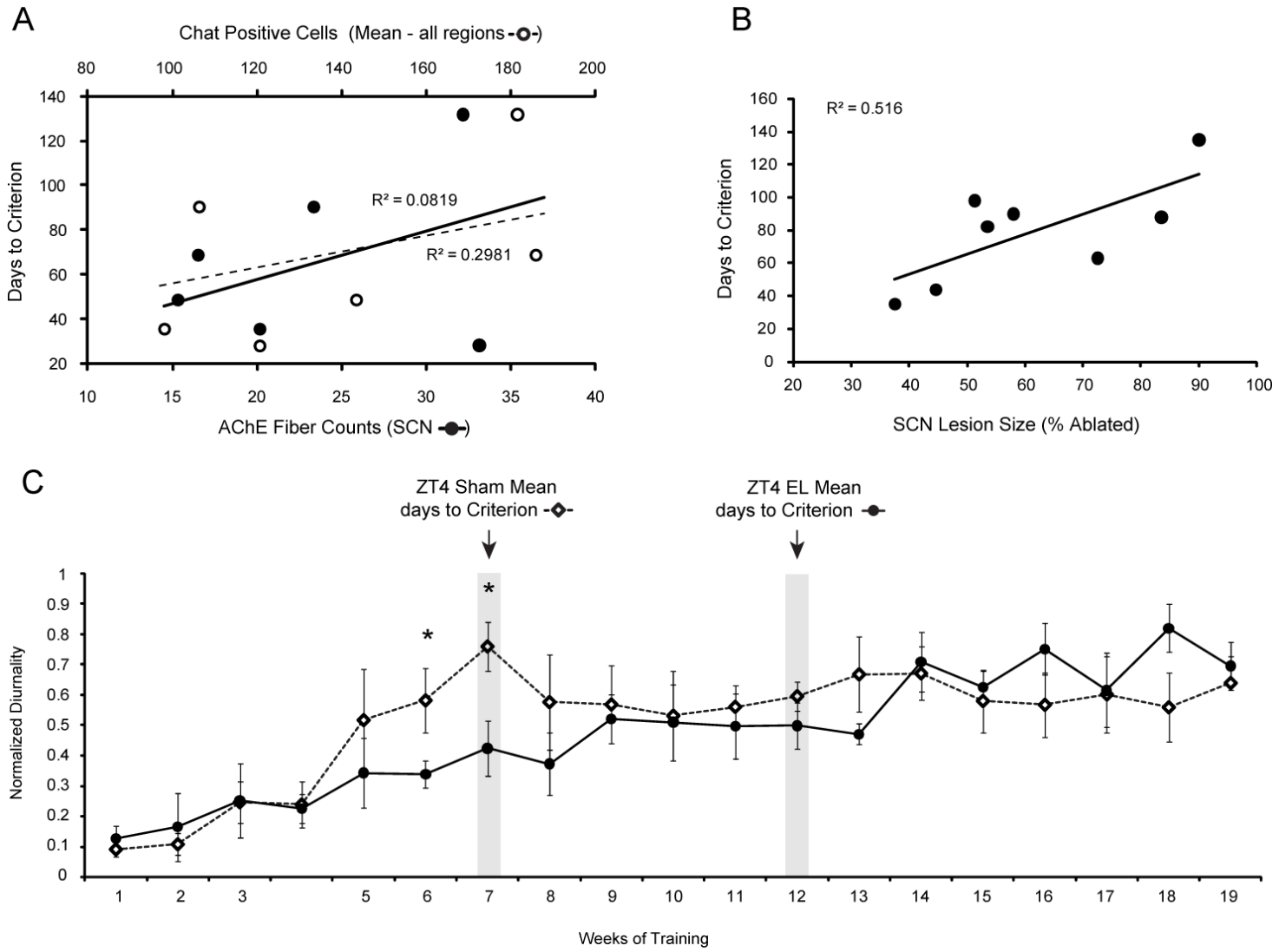
A
Chat Positive Cells (Mean - all regions)
Days to Criterion
AChE Fiber Counts (SCN)
R² = 0.0819
R² = 0.2981
B
R² = 0.516
Days to Criterion
SCN Lesion Size (% Ablated)
C
ZT4 Sham Mean days to Criterion
ZT4 EL Mean days to Criterion
Normalized Diurnality
Weeks of Training

Figure 4.7

Figure 4.8: **Mean temperature records and markers of entrainment for SAT trained animals by treatment group**: ***(A)*** 24 hour binned average of ZT4 trained animals. SAT training (ZT4) is relative to the topmost LD bar (where dark bar = lights-off) and the shaded column represents the approximate 40 min SAT training period in which animals are absent from their home cages. Zero values, when animals have been removed from their home cages for training (ZT4-ZT5, or ZT16-ZT17 for ZT4 and ZT16 trained animals, respectively), were adjusted to reflect daily mean values. X-axis is plotted in zeitgeber time (ZT) and y-axis represents mean body temperature grouped into 10 min bins. Histograms are taken from the last 10 days of SAT training, before entering constant conditions. ZT4 SAP (light gray), and ZT4 sham (blue) mean averages are plotted ± SEM (shading). ***(B)*** 24 hour binned average of ZT16 trained animals. ZT16 SAP (dark gray), and ZT16 sham (black) mean averages are plotted ± SEM (shading). (***C)*** Mean phase relationship between increase in temperature (above daily light-phase mean; ZT4, or above daily dark-phase mean; ZT16) to time of SAT training. Activity onset in minutes relative to training time is plotted for each condition (minutes ± SEM; * = $p < 0.05$). Note all animals show daily increase in anticipation of task training with the exception of ZT4 SAP lesioned animals. (***D)*** Mean phase relationship between increase in temperature (above daily dark-phase mean; ZT4, or above daily light-phase mean; ZT16) to time of lights-off. Activity onset in minutes relative to dark-phase is plotted for each condition (minutes ± SEM; * = $p < 0.05$).

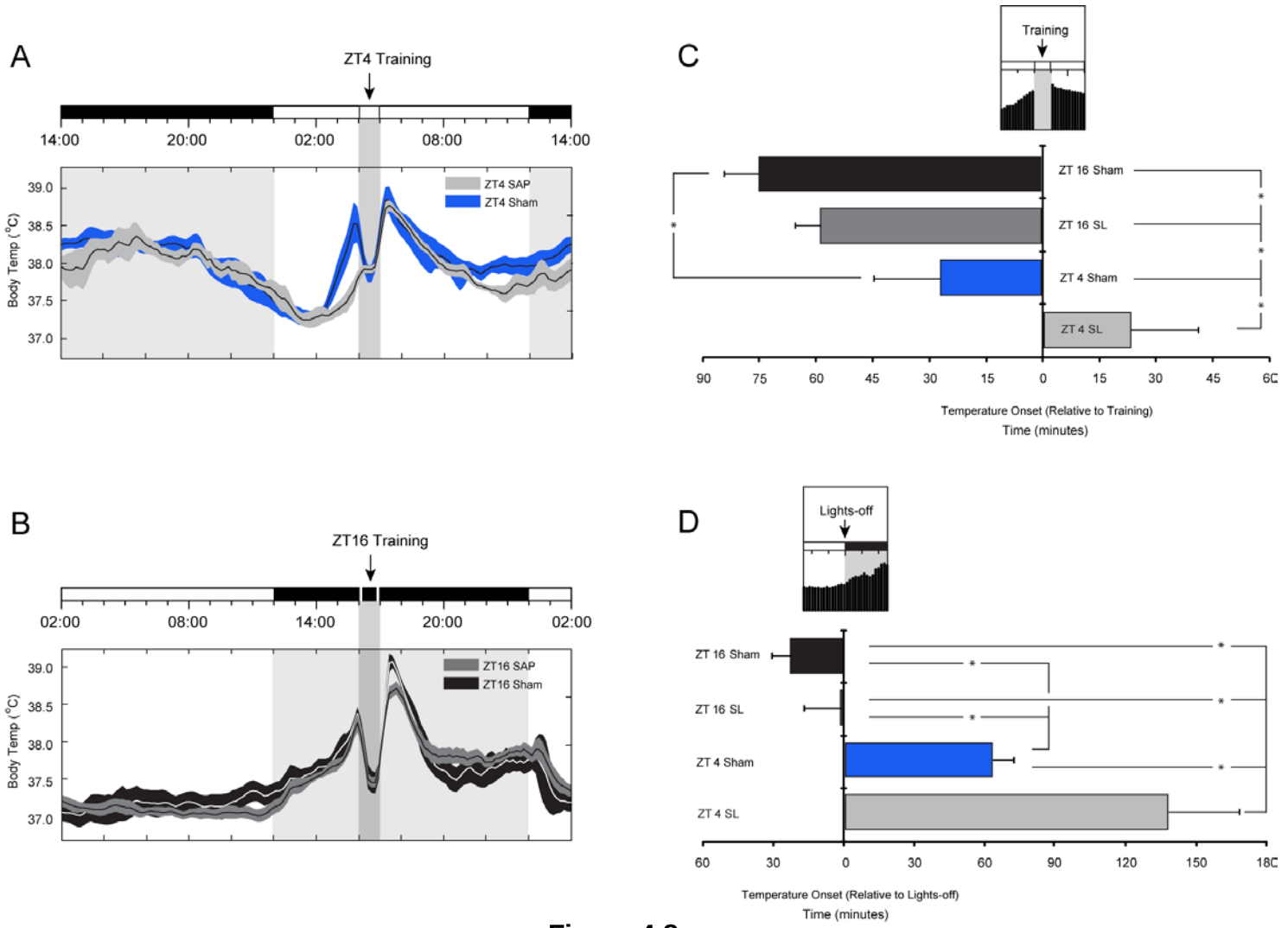
A
ZT4 Training
14:00
20:00
02:00
08:00
14:00
39.0
38.5
38.0
37.5
37.0
Body Temp (°C)
ZT4 SAP
ZT4 Sham
B
ZT16 Training
02:00
08:00
14:00
20:00
02:00
39.0
38.5
38.0
37.5
37.0
Body Temp (°C)
ZT16 SAP
ZT16 Sham
C
Training
ZT 16 Sham
ZT 16 SL
ZT 4 Sham
ZT 4 SL
90
75
60
45
30
15
0
15
30
45
60
Temperature Onset (Relative to Training)
Time (minutes)
D
Lights-off
ZT 16 Sham
ZT 16 SL
ZT 4 Sham
ZT 4 SL
60
30
0
30
60
90
120
150
180
Temperature Onset (Relative to Lights-off)
Time (minutes)

Figure 4.8

Figure 4.9: **Internal desynchrony in temperature and activity rhythms as a result of daily SAT training:** 24 hour binned average of activity and temperature relative to the time of daily training referenced in the topmost LD bar (where dark bar = lights-off). Shaded column represents the SAT training period in which animals are absent from their home cages. Difference scores represent an index of the absolute value of the mean normalized average of activity – the mean normalized average of temperature ± SEM (shading). Values range from +1 to -1, with +1 representing no difference between activity and temperature and -1 representing complete desynchrony between activity and temperature. Dashed line represents daily mean for all animals within that group. *(Left)* Comparisons of mean body temperature and activity for ZT4 trained animals. *(Right)* Comparisons of mean body temperature and activity for ZT16 trained animals. Note that ZT16 trained animals show less desynchrony whether it be in the light-phase or the dark-phase irrespective of lesion condition. ZT4 SAP infused animals show less desynchrony than non-lesioned controls in both the light-phase and the dark-phase.

Figure 4.9

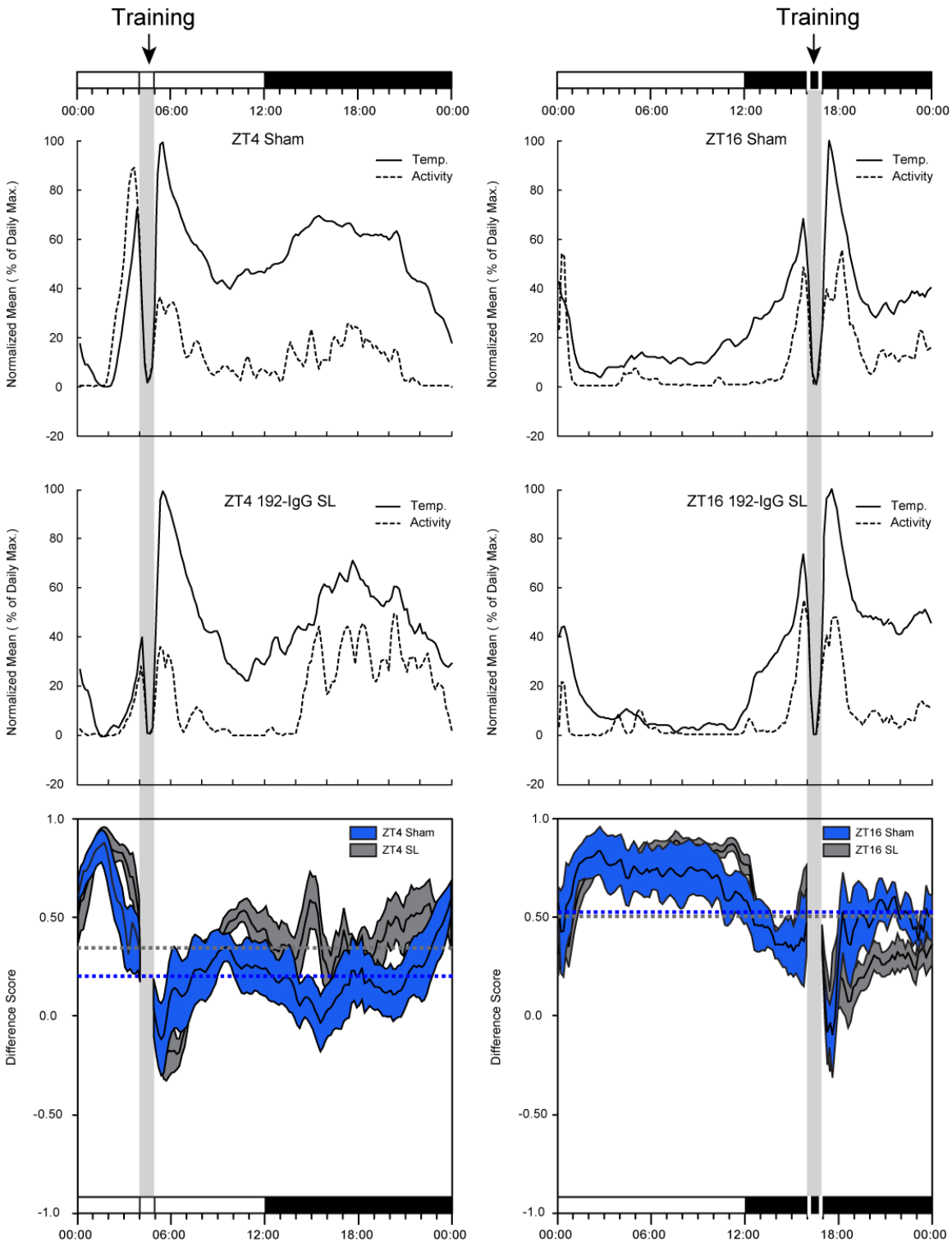
Training
Training
00:00
06:00
12:00
18:00
00:00
ZT4 Sham
ZT16 Sham
ZT4 192-IgG SL
ZT16 192-IgG SL
Temp.
Activity
Normalized Mean (% of Daily Max.)
100
80
60
40
20
0
-20
ZT4 Sham
ZT4 SL
ZT16 Sham
ZT16 SL
Difference Score
1.0
0.50
0.0
-0.50
-1.0

Figure 4.10: **Schematic illustration of potential mechanisms of cholinergic signaling influencing circadian rhythms:** *(Left)* Cholinergic signaling has the potential to influence oscillators of the SCN or peripheral oscillators of the CNS. Feedback of peripheral oscillators in turn, can influence circadian rhythms by modulating SCN output or via non-identified mechanisms. SCN output is relayed through nuclei of the hypothalamus in order to synchronize non-SCN oscillators and regulate physiology and activity. *(Right)* Illustration represents internal conflict of shift-work model represented by SAT training. Under normal conditions, ZT16 training results in entrainment mechanisms that increase activity concurrent with the natural activity cycle of a nocturnal rodent. ZT4 animals are influenced by task entrainment mechanisms that result in an internally desynchronized state that leaves the animal neither entirely diurnal nor nocturnal. ZT4 SCN ablated animals show a strong diurnal phenotype without retinal influences entraining the SCN. ZT4 SAP infused animals, although they show some level of entrainment to daily task training, lack cholinergic signaling at the SCN (that dampens light induced promotion of inactivity) results in animals driven primarily by retinal influences.

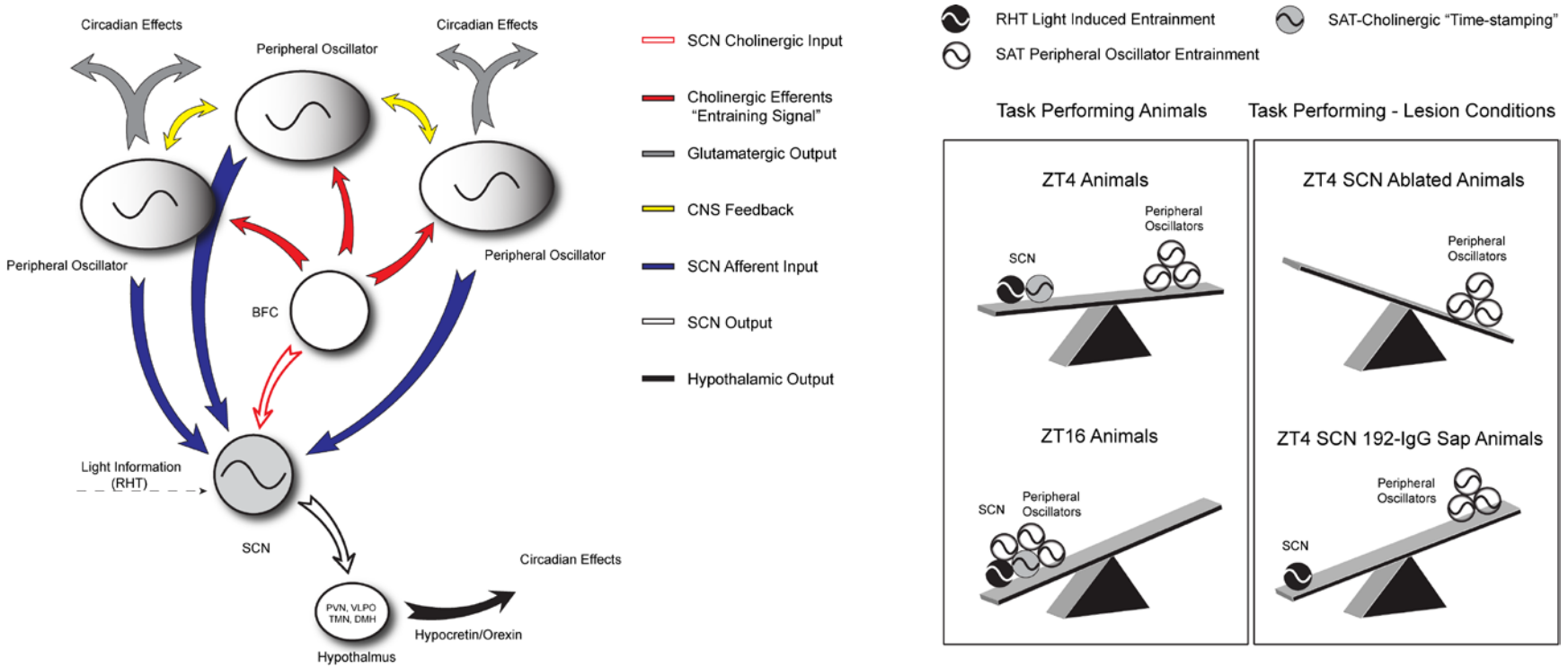
Circadian Effects
Peripheral Oscillator
Circadian Effects
Peripheral Oscillator
Peripheral Oscillator
BFC
Light Information
(RHT)
SCN
Circadian Effects
PVN, VLPO
TMN, DMH
Hypocretin/Orexin
Hypothalmus
SCN Cholinergic Input
Cholinergic Efferents
"Entraining Signal"
Glutamatergic Output
CNS Feedback
SCN Afferent Input
SCN Output
Hypothalamic Output
RHT Light Induced Entrainment
SAT-Cholinergic "Time-stamping"
SAT Peripheral Oscillator Entrainment
Task Performing Animals
Task Performing - Lesion Conditions
ZT4 Animals
Peripheral
Oscillators
SCN
ZT4 SCN Ablated Animals
Peripheral
Oscillators
ZT16 Animals
SCN
Peripheral
Oscillators
ZT4 SCN 192-IgG Sap Animals
Peripheral
Oscillators
SCN

Figure 4.10

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Chapter 5

Conclusion

The goal of this dissertation was to explore the interactions of cognitive activity, time of day performance, and circadian physiology. I have discovered alterations in circadian measures of entrainment evoked by timed daily performance of a cognitively demanding task that manifests in time of day differences in acquisition and performance. I also tested the hypothesis that cholinergic afferents to the SCN are necessary for the circadian changes mediated by cognitive performance with anatomical, behavioral, and lesion evidence. The discovery of a cognition-induced circadian oscillator further adds to our understanding of the complexity and importance of circadian oscillators and timekeeping in biological behavior. The flexibility of circadian systems to adapt and reflect the timing of non-photic signals speaks to the power with which these cues influence behavior. Finally, and importantly, because cognition is an internally driven state modulated by the individual, it has a special place in the entrainment of circadian rhythms. During cognitive activity, the zeitgeber is *not* the presentation of a cue, a change in a light-dark cycle, or the presentation of a novel stimulus; it is instead modulated by the end user in the service of cognitive performance.

In this chapter, I will summarize the findings presented in the three data chapters of my thesis and introduce a series of future experiments that can further validate my hypothesis. Finally, I'll discuss how these results, and how the model of multiple oscillator interactions, could offer insight into the consequences of shift-work, daytime sleepiness, and circadian dysregulation on human health and productivity.

Chapter 2 summary: cognition modulates entrainment

The results from my first data chapter demonstrate that the performance of a demanding cognitive task produces a profound change in the organization of circadian locomotor behavior. Daily sustained attention task (SAT) performance at ZT4 reverses the endogenous activity pattern of nocturnal rodents, such that animals trained at ZT4 exhibit diurnal (predominantly light-phase) activity. The overall strength of the effect of SAT training on circadian activity was unexpected based on previous findings. We speculated that entrained anticipatory locomotor activity might occur at time of daily handling, but animals would still retain a predominantly nocturnal activity pattern, as described for other types of non-photic entrainment including restricted daily access to food or timed methamphetamine delivery (Mistlberger, 1992, 1993; Mistlberger & Rusak, 1987). Control treatments revealed that this change in diurnal activity patterns could not be accounted for by daily handling, the presence of daily timed water access, or operant training for a reward.

We additionally demonstrated that entrainment patterns following a 6 hour phase advance in the light cycle and additional training at ZT10 are modulated by previous entrainment. When the light-dark cycle was shifted to accommodate a later training time, we expected to see the phase relationship shift such that activity would begin at ZT8 or 9, prior to onset of daily training. As the new training time now occurred closer to the dark phase, we predicted animals would be less diurnal after the shift. All ZT10 subjects, however, showed a robust diurnal activity pattern that began at or near their previous ZT4 training time (***figure 2.2***). These results differed dramatically from animals that have only trained at ZT10 (*see chapter 3 and discussion of history effects below*).

Chapter 3 summary: bidirectional interactions between circadian rhythms and cognitive performance

In chapter three, I demonstrate that *nocturnal* animals trained daily during the dark-phase of the circadian cycle acquire the SAT more quickly, and perform with fewer

errors than light-phase trained animals (***figure 3.2A-3.2D***): when training occurred daily 4 hours after lights-off (ZT16), subjects reached criterion performance twice as fast as those training 4 hours after lights-on (ZT4). Differences in post-criterion performance was not limited to the standard versions of the task, as ZT16 trained animals performed better on unexpected distracter challenge sessions (dSAT) than ZT4 trained animals (***figure 3.3C, 3.3D***). When switched to the opposite training time, there was a rapid reversal in performance for both training groups. Dark-phase animals, when shifted to daytime training, showed consistently worse performance in every metric measured. Correspondingly, ZT4 trained animals, when switched to dark-phase training, showed substantial improvements in performance (***figure 3.3A, 3.3B***). Performance could additionally be predicted by the level of entrainment, suggesting that flexibility of behavioral activity (sleep/wake) serves to augment learning and performance. For rats training on the Morris water maze (MWM), acquisition was not dependent on entrainment or time of day; however, remote memory was time of day sensitive. This is the first time to our knowledge that differences in remote memory have been demonstrated in the MWM for time of day and suggests that the factors that influence memory consolidation could be subject to circadian regulation.

The results of this study also revealed that robust changes in circadian activity, as measured by anticipation, was specific to daily SAT training and did not occur for water maze training. Activity records from ZT16 SAT trained animals showed consolidated periods of activity with onset of activity coming later in the dark-phase and cession of activity occurring earlier during the dark-phase (***figure 3.4, 3.5C***). Animals when trained during the light-phase (ZT4 and ZT10), adopt a significantly more diurnal activity pattern as shown previously (*chapter 2*). Unexpectedly, we noted that for daytime-trained animals, the most significant change in diurnal activity was correlated with transitions to increasingly difficult stages of training with peaks in diurnality (LD ratio) corresponding to the periods in which cognitive demands on attention are the highest – when subjects advance to the final phase of SAT training (***figure 3.4A, 3.4B, 3.5A***). In contrast, we saw almost no effect of daily water maze training on circadian rhythms with only minor

increases in daytime activity characteristic of handling alone for animals housed in a LD cycle (Hummer, Meixner, & Lee, 2010).

Chapter 4 summary: cholinergic systems mediate cognition induced entrainment

Results from the last data chapter demonstrate that tasks requiring sustained attention modulate circadian activity at multiple levels. We produced animals with two types of SCN lesions: SCN electrolytic ablation (EL) and animals with pharmacological deafferentation of cholinergic projections to the SCN using 192IgG-saporin (SL). SL animals, when trained at ZT4, show normal acquisition and performance; however, they demonstrate minimal amounts of entrainment to SAT training as measured by anticipation to task onset, LD ratio, and *free-run* activity in constant conditions (***figures 4.2-4.3, figure 4.5***). These results show that ACh at the level of the SCN promotes entrainment to daily task training under normal conditions of cognitive performance. Although SL animals were able to maintain a small amount of entrainment, as measured by LD ratio, it was comparable to levels of entrainment seen in animals undergoing daily handling (Gritton, Sutton, Martinez, Sarter, & Lee, 2009; Hummer, et al., 2010) or performing on an operant task that requires minimal cognitive effort (Gritton, et al., 2009). Moreover, ZT4 SL rats were the only ZT4 treatment group to maintain an LD ratio below 1.0: differing significantly from other ZT4 groups, but not from the ZT16 training groups. These findings provide support for a model that includes direct innervation of the SCN by cholinergic inputs of the basal forebrain in the entrainment of circadian rhythms by attentionally demanding tasks.

We also found that electrolytic lesions of the SCN produced animals with the most profound levels of entrainment to the SAT relative to any of the daytime trained groups in our study (***figure4.4***). The finding that SCN ablated animals show cognitively entrained activity provides evidence that a cognition-induced circadian oscillator exists independent of the SCN. Furthermore, the entraining properties of this oscillator have profound effects on the timing of organized behavioral activity that parallels the strength of any previously identified non-photic oscillator. Our results also revealed that damage

to the SCN slows task acquisition and results in lower overall performance, despite the profound entrainment that these animals show to daily task training.

Cholinergic signaling, time-stamping, and entrainment signals at the SCN

Because an endogenous circadian pattern of acetylcholine release exists throughout the majority of the CNS (Jimenez-Capdeville & Dykes, 1993; Kametani & Kawamura, 1991), it is possible that signaling via ACh may be less efficient during periods of time where it is already high in nocturnal rodents (*dark-phase: active-phase;* ***see figure 1.4***). These findings would suggest that for regions outside of the SCN to act as oscillators, they may be more receptive to cholinergic mediated entrainment at certain phases of the LD cycle relative to others. Furthermore, the observation that the SCN is one of the few regions of the brain where no demonstrable circadian pattern of acetylcholine release exists suggests that cholinergic signaling at the SCN may be equally effective at any time of day. It has also been proposed that acetylcholine release at the level of the SCN may act as a timing signal important for 'time-stamping' singular events that allows for time to represent a factor in the process of learning and memory consolidation (for review see Daan, 2000; Hut & Van der Zee, 2010).

A functioning circadian system appears to be an important prerequisite for object recognition and associative learning, as lesions of the SCN have been shown to have negative effects on the interaction of timekeeping during novel object recognition in hamsters (Ruby, et al., 2008) and repeated novelty exposure in rats (Buijs, Wortel, Van Heerikhuize, & Kalsbeek, 1997). Middle-aged hamsters with dampened circadian rhythms show impaired conditioned place preference compared to animals with high amplitude circadian rhythms (Antoniadis, Ko, Ralph, & McDonald, 2000). The loss of memory for the time of day that an event occurs (time-stamping) is coincident with SCN-lesioning in tasks of passive shock avoidance in rats: SCN-intact animals show strongest aversion tendencies when the training-test interval is 24 h whereas animals with SCN lesions show comparable aversion levels independent of time of day (Stephan & Kovacevic, 1978). Performance of time-place association is additionally diminished in

older mice (Van der Zee EA, 2009) suggesting that the mechanisms of time-stamping could be attenuated through the process of aging. As cholinergic cell loss and decreases in ChAT activity are hallmarks of dementia and Alzheimer's disease (Bartus, Dean, Beer, & Lippa, 1982), it is possible that cholinergic mediated time-stamping would be compromised in this population as well.

History effects influence subsequent entraining mechanisms and cognitive performance

Over the course of these studies I noted two interactions of history effects: one on activity and the other on performance. The first involves the role of previous patterns of circadian activity influance future patterns of entrainment. In the experiments conducted in chapter 2, animals training at ZT10, compared to ZT4, showed *increased* diurnality when the light cycle was advanced, effectively delaying the time of daily training (***figure 2.2***). This level of diurnality was not evident in animals that began training at ZT10 to begin with (*chapter 3*). What was surprising is that the history effect lasted throughout the remainder of the experiment (30+ days), and suggests a greater complexity, and perhaps inflexibility, of circadian oscillators than has previously been thought. Future studies will be needed to determine the mechanisms underlying this phenomenon.

The second history effect reflects differences in performance associated with acquisition history. ZT4 animals, when subsequently trained at ZT16, never performed as well as animals originally trained at ZT16. In fact, their performance when training at ZT16 was on par with ZT16 animals reversed to training at ZT4 as described in **figure 3.3C**. This finding presents an argument for the interaction between time-of-acquisition and future performance and suggests the conditions under which training is acquired has consequences for performance long after the initial learning has occurred. It will require future studies to test the duration or permanency of this condition; however, these results provide compelling evidence for the consideration of training ramifications on human productivity. Cognitive training based on our findings, should be limited to times of day when subjects are most alert and naturally active. Even under conditions where

administrators know human shift-workers will work night-shifts, training during the light-phase may be important for maximizing future potential. These findings would also predict that the process of learning (*e.g.*, studying for an exam), during periods when students are most alert, would result in better future performance regardless of the time of testing.

Model of cognitive entrainment:

Cholinergic signaling

Although circadian rhythms are endogenously driven, they are shaped and modulated by environmental cues. Under normal conditions, the circadian system receives periodic light information that acts to regularly entrain the clock facilitating a stable phase relationship with the outside world (Roenneberg, Daan, & Merrow, 2003). As has been mentioned, biological organisms can entrain to a variety of environmental cues: the strongest being light (photic-cues); however, a variety of non-photic cues including food, temperature, social interaction, sound, atmospheric pressure, novel exposure to wheel running in rodents, and now, elevated cognitive activity, have been shown to have influence or synchronize (entrain) circadian behavior (Gorman & Lee, 2001; Gritton, et al., 2009; Mistlberger & Rusak, 1987; Moore, 1983). Currently, however, little is known about how these events modify circadian phase markers of entrainment or if they share a common mechanism for doing so.

Mrosovsky has argued that the majority of these entraining signals mediate their effects via mechanisms of general arousal (1988, 1996); however, evidence from the data chapters of this thesis suggest that arousal alone cannot account for the entrainment effects in rats. Arousal as a general term is used to describe behavioral and physiological conditions of stimulus driven activation evoked by unexpected stimuli or other stressors. The term “arousal” has been associated with activation of the locus coeruleus (LC) as part of the need to generate a ‘rapid response’ to environmental stimuli (Aston-Jones, Chen, Zhu, & Oshinsky, 2001; Aston-Jones, Rajkowski, Kubiak, Valentino, & Shipley,

1996). While other experiments have demonstrated that entrainment can be produced by handling using procedures that are stressful (Hastings, et al., 1997) or evoke high levels of activity/arousal (Mrosovsky, 1996), the water maze trained animals in our studies showed lower overall levels of entrainment than was found in these studies. Our findings are consistent with the results of Hut et al. (1999), who also concluded high levels of activity do not necessarily produce entrainment through mechanisms of general arousal. However, the question remains: is it possible that non-photic stimuli still share a common regulatory mechanism for producing entrainment? As it appears that basal forebrain cholinergic systems may be involved in the anticipatory activity seen in timed daily handling (Hummer, et al., 2010), perhaps all types of non-photic entrainment relies in-part on cholinergic signaling. If so, then the robustness of entrainment might be predicted by the level of attentional focus which regulates ACh release, dictated by cue salience. However, if cholinergic signaling is a key to non-photic entrainment then one might expect that water maze training could influence circadian activity given that cholinergic release increases coincident with training in tasks of spatial learning (Chang & Gold, 2003; Fadda, Melis, & Stancampiano, 1996; Yamamuro, Hori, Tanaka, Iwano, & Nomura, 1995). While cholinergic activity may compliment spatial learning in the water maze, we suspect that the recruitment of this system; and particularly the necessity for demands on attention, were not sufficiently large in this training condition to evoke robust changes in circadian physiology. Contrastingly, tasks requiring sustained attentional effort, the process of "top-down" cortical recruitment of cholinergic systems is critical to cognition, including: stimulus detection, discrimination, and signal processing, particularly under exigent and distracting conditions (Kozak, Bruno, & Sarter, 2006). As SAT entrainment increases with required cognitive effort (***figure 3.5A***), the strength of cholinergic signaling seems to play an important role in the modulation of circadian rhythms.

Non-SCN oscillators of the brain

The observation that animals training on the SAT with lesions of the SCN show unparalleled activity and anticipation of training time during the light-phase compared to

all other daytime training groups supports the hypothesis that a cognition-induced circadian oscillator exists outside of the SCN and is capable of organizing circadian activity independently. Under normal circumstances, the SCN has the important role of maintaining a level of internal synchrony between various peripheral and non-SCN brain oscillators. The central pacemaker, through phase coherence, synchronizes a multitude of non-SCN oscillators or "slave oscillators" on a daily basis. This is evident in mice with lesions of the SCN, where peripheral tissues continue to show daily oscillations, but the expression of these rhythms does not occur in a synchronized way (Pezuk, Mohawk, Yoshikawa, Sellix, & Menaker, 2010). Our results with ZT4 EL animals suggest that cognitive activity can synchronize behavioral activity in the absence of the SCN. This finding is similar to those shown for other entrainable oscillators, including the food-entrainable oscillator (FEO) and the methamphetamine sensitive circadian oscillator (MASCO), in that SCN-lesioned animals show enhanced anticipatory activity to non-photic cues in the presence of a light-dark cycle when compared to intact controls (Angeles-Castellanos, Salgado-Delgado, Rodriguez, Buijs, & Escobar, 2010; Pezuk, et al., 2010; Stephan, Swann, & Sisk, 1979a, 1979b). The ZT4 EL animals had arrhythmic wheel activity prior to SAT training, but became entrained to the SAT as they acquired the task. These animals showed a highly anticipatory diurnal rhythm which quickly disappeared following the cessation of training and a return to arrhythmia within several days (***see figure 4.4***). The quick return of desynchronized activity in these rats suggests that these oscillators are unable to maintain synchrony without external periodic cues which is a shared condition of other noted peripheral oscillator driven activity (Stephan, et al., 1979b). The short continuation of entrainment that occurs in the ZT EL animals after entering constant conditions is also consistent with studies of animals entrained to food and daily handling (Hummer, et al., 2010; Stephan, et al., 1979a) and supports the theory that the non-SCN oscillators involved in daily cognitive activity are unable to remain entrained in the absence of external cues or daily input from the SCN. As the SCN, under normal conditions, plays a role in synchronizing various brain oscillators; it is likely that in the absence of an SCN, non-SCN oscillators sensitive to daily task performance would take much longer to synchronize. Presumably as more independent

oscillators become synchronized to the task, a pattern of entrainment would emerge for the most prominent zeitgeber (*i.e.*, SAT performance; ***see figure 4.7C***).

The results from ZT4 EL animals also provide evidence in support of an untested hypothesis of this thesis, that cholinergic release associated with daily handling or training acts to *entrain non-SCN oscillators* that influence daily activity independent of signaling at the SCN. Cholinergic signaling has been identified as an essential component in reinforcement and conditioning as well as the primary mediator of cognitive performance and attention (Himmelheber, Sarter, & Bruno, 1997; Wilson & Rolls, 1990). Additionally, the basal forebrain cholinergic system is the primary source of cholinergic input to many potential targets associated with task acquisition and performance that have also been shown to express clock genes (*e.g.*, prefrontal cortex, posterior parietal cortex, hippocampus; Abe, et al., 2002; Angeles-Castellanos, Mendoza, & Escobar, 2007; Wang, et al., 2009). Thus, timed-daily training, through acetylcholine release, could entrain peripheral clocks in a way that is sufficient to mediate non-photic entrainment even in the presence of a strongly entraining LD cycle.

The results of studies of animals with SCN lesions, as outlined in chapter 4, also support a theory of competitive interactions between peripheral brain oscillators and the light driven SCN oscillator (***figure 4.10***). In the case of light-phase trained animals, our data supports a state of competition between oscillators that results in internal desynchrony (ID) characterized by out-of-phase rhythms of temperature and activity, as well as attenuated rhythms of peripheral oscillators (***see figure 4.9, figure 4.10****, and future directions:* ***figure 5.2B***).

Future Directions

Future experiments can be broken into two sets of studies: studies designed to understand the consequences of circadian rhythms on performance and studies designed to reveal the mechanisms that allow cognitive activity to entrain circadian rhythms.

With regards to the first question, more experiments are required to fully analyze the relationship between time of day and SAT acquisition and performance. Experiments with animals training at ZT4 and ZT10 have uncovered a clear dissociation of performance in time of day training across the light-phase. It is also possible that a similar dissociation exists across the dark-phase. Winocur and Hasher (2004) found a time of day training effect on performance during the dark-phase between late and early testing on a working memory non-matching-to-sample (NMTS) variation of the water maze. I would predict, based on these results and my own, that temporal windows where performance is likely to be optimized or attenuated across the light-dark cycle exist, perhaps based on underlying circadian markers of activity. Such data could offer insight into optimization of performance or productivity based on individual chronotypes. The use of a the sustained attention task also offers the advantage of studying how performance in animals interacts with circadian rhythms during periods of phase shifting which can represent a state of short term desychrony (*e.g.*, jet-lag). The results from chapter 2 provide evidence that performance during a phase-shift is diminished; however, performance during the shift can be predicted based on the entrainment patterns following re-entrainment. This raises the interesting question of whether performance on the SAT would be worse on days individuals are making the largest changes in phase, but result in fewer days of poor performance.

To address the question of how cognition and circadian rhythms interact at a systems level, future studies are necessary to determine how cholinergic signaling at the SCN modulates circadian entrainment. Further studies are also necessary to identify where, and through what mechanism cognitive activity entrains putative non-SCN oscillators. Modification of circadian activity at the SCN could be the result of changes in the expression of clock genes in the SCN itself, or through a yet undiscovered mechanism that regulates SCN output to influence downstream targets. Interestingly, very few manipulations that produce changes in entrainment, aside from photic-cues, result in changes in clock gene expression in the SCN (Damiola, et al., 2000; Masubuchi, et al., 2000; Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001). This is despite strong

entrainment of locomotor activity and entrainment of clock-gene expression in peripheral organs and non-SCN brain areas such as the motor cortex. Future studies, some of which are ongoing, are designed to identify whether clock-gene patterns of expression in the SCN are modified by cognition-induced entrainment. Even if clock-gene expression is unaltered, cholinergic signaling at the SCN may alter neurotransmitter release from the SCN directly. As cell output from the SCN involves the release of several neuropeptides including vasoactive intestinal polypeptide (*VIP*) and arginine vasopressin (*AVP*) which are subject to entrainment by non-photic environmental cues (van Esseveldt, Lehman, & Boer, 2000), it is possible that changes in integrated output from the SCN are modified by cognition-induced cholinergic release even if the pattern of underlying clock gene expression is not.

Additional experiments are also required to identify which non-SCN oscillators are entrained by cognitive performance and by what mechanism they become entrained. It is likely that non-SCN oscillators are entrained by daily cognitive training in regions important for task acquisition and performance, presumably via acetylcholine release. On-going experiments by Jin Yan, a PhD candidate, are being conducted with the goal of identifying brain regions that show task entrainment, as measured by changes in clock-gene expression, in SAT performing animals. Preliminary evidence from a small number of regions indicates that SAT task performance alters clock-gene expression in light-phase trained animals (***figure 5.1A, figure 5.1B***). Her studies will be important for determining which areas are entrained by task performance, and they could lay the ground-work for future studies exploring how the timing information is conveyed to the rest of the nervous system in a way that impacts circadian architecture. These studies, in combination with experiments designed to measure neurotransmitter release, specifically acetylcholine, in task performing animals would offer the additional benefit of how, and where, neurotransmitter signaling is being regulated across discrete brain regions in anticipation of daily training. Dr. Giovanna Paolone, a post-doctoral researcher, found that acetylcholine release in the medial prefrontal cortex is phase-locked to the time of daily task performance (***figure 5.2A***). Increases in ACh begin approximately 30 minutes before daily task performance, and suggests

that augmentation of acetylcholine release is under control of circadian oscillators. A distinct possibility is that cortical areas involved in executive functioning, serving as peripheral oscillators, through "top-down" mechanisms exert influence on the basal forebrain to upregulate cholinergic release in anticipation of daily training.

Another study of great interest will be identifying whether cholinergic release is a shared mechanism of non-photic oscillators. Future studies involving lesions of the basal forebrain cholinergic system in animals given timed daily access to food or methamphetamine access could be conducted to explore this possibility. I would predict that as animals attend to the cues associated with these daily manipulations (FEO and MASCO), cholinergic signaling would play a role in both types of entrainment, and thus the strength of daily entrainment would be diminished in animals with cholinergic lesions.

The results from the preliminary evidence presented in this chapter along with experimental data from the chapters of this thesis provide support for a compelling hypothesis that cholinergic activity is responsible for entrainment via two mechanisms: modulating SCN by direct innervation, and providing an entrainment signal to synchronize non-SCN oscillators. While lesion studies of putative non-SCN oscillators could test the necessity or contribution to the circadian changes noted in SAT performing animals, many of the region's most likely to be entrained by the task play a critical role in task acquisition and performance. This interaction creates a dilemma, as animals that show weak levels of entrainment also perform poorly (***figure 2.1, figure 4.7C***). Ideally the use of genetic models might offer the best solution to addressing this question. Recently the SAT has been adapted to mice allowing the use of transgenic animals to test fundamentals of cognition and task-performance. As mice also become robustly entrained to daily SAT performance during the light-cycle (*personal communication*; Paolone, 2011), the use of tetracycline transactivator mice to bi-directionally regulate the expression of clock-genes allows for the dissociation of acquisition, performance and entrainment from one-another.

Social Relevance

The experimental training program described in this thesis can serve as a translational animal model for investigating the mechanisms that mediate the interaction of cognitive performance and circadian activity. Animals performing cognitive tasks during the light-phase experience a robust and maintained desynchrony of internal biological rhythms (***figure 5.2B, figure 5.2B)*** consistent with that reported during phase shifting (*i.e.*, jet-lag) or as a result of chronic shift work (Haus & Smolensky, 2006; Reid, Chang, & Zee, 2004; Salgado-Delgado, Nadia, Angeles-Castellanos, Buijs, & Escobar, 2010). During conditions of a phase shift, the circadian clock and peripheral oscillators, do not entrain to the changes immediately and require several cycles for re-entrainment before internal synchrony can re-emerge. This period of time between the initial disturbance and the reemergence of a stable entrainment pattern represents a state of circadian internal desynchrony (ID) that is characterized by out-of-phase circadian markers of rhythms including temperature, hormone release, sleep drive, or activity. Animals training on the SAT at ZT4 experience a permanent state of desynchrony exemplified by incoherence between activity and temperature (***figure 4.9***) as well as dampened rhythms in peripheral oscillators (***figure 5.2A***). A state of ID in human shift-workers includes the dampening of metabolic and endocrine rhythms (Knutson, Spiegel, Penev, & Van Cauter, 2007) which has been linked to increased risk for a variety of disorders including cardiovascular disease, obesity, diabetes, infertility, sleep fragmentation and cancer (Folkard & Akerstedt, 2004; Haus & Smolensky, 2006; Knutsson, 2003; Lange, Dimitrov, & Born, 2010; Waage, et al., 2009). As the loss of daily coherence between rhythms is a shared condition between shift-workers and light-phase trained nocturnal rodents performing the SAT, these animals provide an excellent model studying the risk of prolonged shift-work on physiological conditions, and putative treatments to minimize the negative effects.

Of additional importance, is how these findings may integrate SCN activity, circadian biology, and changes concurrent with old age: consequences of which include severe sleep disturbances, decreased sensitivity to environmental cues, an attenuated SCN

mediated re-synchronization, and weakened internal coupling (Van Someren, 2000). In studies of aged rats, changes in muscarinic receptor expression in the SCN associated with exposure to novel or arousing events is attenuated (Biemans, Van der Zee, & Daan, 2003). Aged animals also show significantly reduced expression of AVP levels suggesting that a consequence of aging may be the inability to store information about events concurrent with time of day (Roozendaal, van Gool, Swaab, Hoogendijk, & Mirmiran, 1987; Van der Zee, Jansen, & Gerkema, 1999). In addition, circadian rhythms often show deterioration with age and markers for cholinergic synthesis and release including choline acetyltransferase (ChAT), extracellular levels of ACh, and muscarinic AChRs immunoreactivity rhythms are dampened or lost with age (Jenni-Eiermann, von Hahn, & Honegger, 1985; Mitsushima, Mizuno, & Kimura, 1996; Mizuno, Arita, & Kimura, 1994; van der Zee, Streefland, Strosberg, Schroder, & Luiten, 1991). Recent studies suggest that age-related dampening of SCN rhythms does not occur equally for all entrainment cues (Biello, 2009), and the close relationship between the SCN and its cholinergic projections suggested by the results of this thesis call for continued research into cognitive mechanisms as a potential therapy for circadian disorders in compromised individuals.

As circadian abnormalities may also contribute to the common cognitive symptoms of major neuropsychiatric disorders (Bunney & Bunney, 2000; Monteleone & Maj, 2008; Van den Bergh, Van Calster, Pinna Puissant, & Van Huffel, 2008; Wirz-Justice & Van den Hoofdakker, 1999; Wu & Bunney, 1990) understanding the bidirectional interactions between cognitive performance and circadian control may be important for developing more conclusive neuroscientific theories of these disorders. The results obtained from these studies could elucidate the mechanisms by which the SCN interacts with both cortical and cholinergic brain regions. Studies looking at the influences of cholinergic systems on circadian rhythms are crucial in our search for a deeper understanding of neuropsychiatric disorders such as Schizophrenia and Alzheimer's disease (AD) - both of which have symptomatic circadian disruptions and are co-morbid with cholinergic dysregulation. Positive correlations have been found between cholinergic cell-death in the basal forebrain, decreases in ChAT, and

impaired cognition in older individuals with dementia and Alzheimer's (Bartus, et al., 1982). Circadian rhythmicity in ACh release is critical for optimal memory processing and a loss of this rhythmicity has been theorized to contribute to cognitive problems in AD (*for review see*: Nieoullon, Bentue-Ferrer, Bordet, Tsolaki, & Forstl, 2008). Additionally, memory deficits in aged rats have been shown to be concomitant with decreases of cholinergic release and neurotransmission (Aubert, Rowe, Meaney, Gauthier, & Quirion, 1995; Baxter & Gallagher, 1996; Ikegami, 1994; Lippa, et al., 1980; Scali, Casamenti, Pazzagli, Bartolini, & Pepeu, 1994; Sherman, Kuster, Dean, Bartus, & Friedman, 1981). Coincident with this loss, the ability of cholinergic signaling to influence circadian rhythms is likely to be lost as well. Decreased depolarization in cortical cholinergic neurons related to impaired learning and memory and a dissociation of core body temperature and daily activity are also found in old age, particularly in individuals with Alzheimer's disease (Ancoli-Israel, et al., 1997; Harper, et al., 2005; Satlin, Volicer, Stopa, & Harper, 1995).

By better understanding how endogenously driven changes in circadian markers are evoked, we may better be able apply these mechanisms to consolidate or prevent desynchronies commonly present in shift-workers, the aging population, and many neuropsychiatric disorders.

Figure 5.1

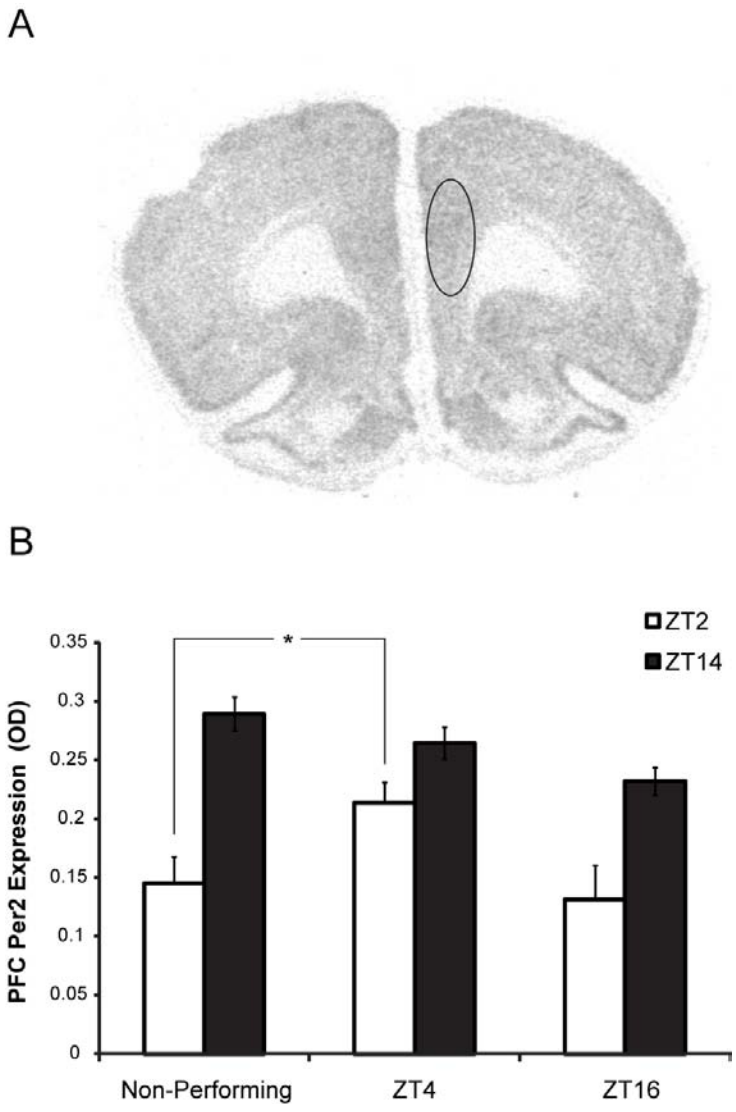

***Figure 5.1*: Prefrontal cortical measures of *per2* using in-situ hybridization: A.** Representative audioradiograph of coronal section of rat medial prefrontal cortex (mPFC). Black circle represents region of interest quantified for all animals in this study **B.** *Per2* expression quantified using optical density (OD) measurements in the pre-frontal cortex across two time-points for three treatment groups. ZT4 animals show eleveated *per2* levels relative to non-performing controls and ZT16 performing animals at ZT2. There was no significant difference in expression at ZT14 between treatment condition (* = $p<0.05$). Figure adapted from (Yan, Paolone, Hagenauer, Gritton, & Lee, 2010).

Figure 5.2

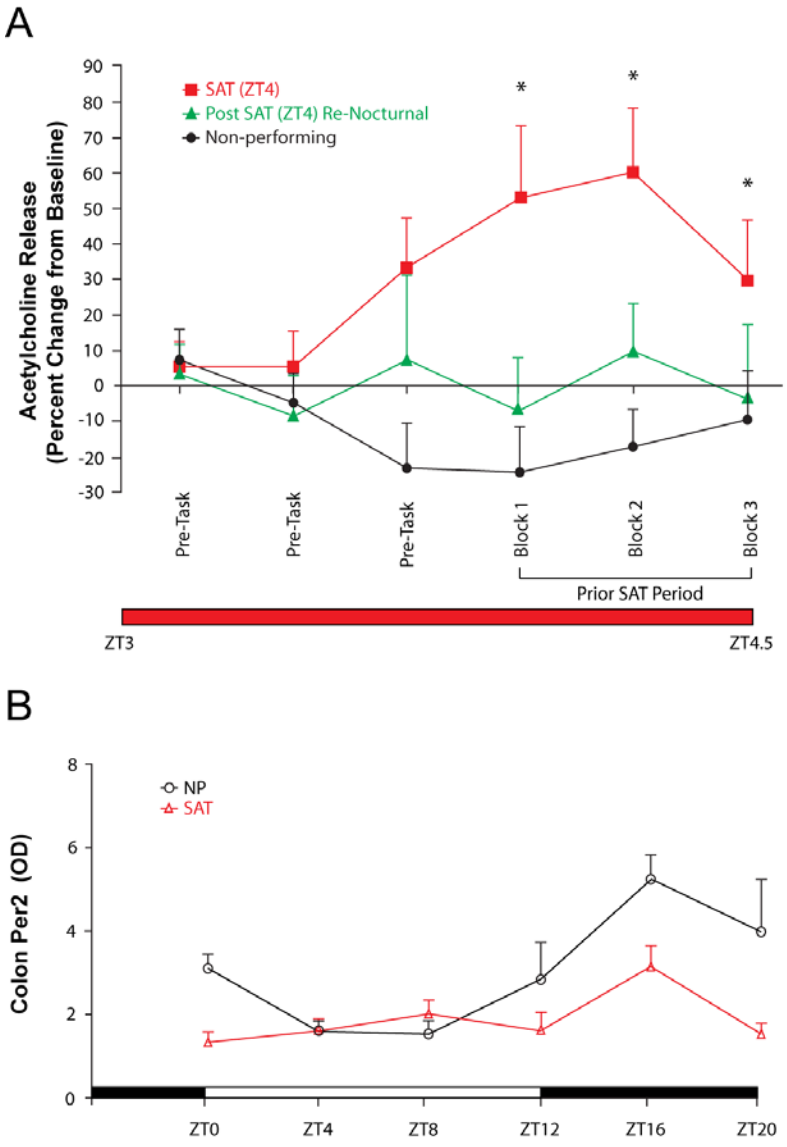

***Figure 5.2*: Effects of daily task performance on physiological measures of entrainment: A.** Microdialysis measurement of acetylcholine release from the medial prefrontal cortex of task performing animals three days after the last day of training on the SAT, or age-matched controls. Animals previously trained at ZT4 show increased cholinergic release ~30 minutes before prior task training time in anticipation of task activity. Animals that trained at ZT4, after returning to a nocturnal activity pattern (8-10 days of non-performance) no longer showed synchronized increases in ACh release (green line ; * = $p<0.05$). **B.** *Per2* rhythms quantified with RT-PCR from colon tissue of ZT4 SAT performing animals and non-performing controls (NP) across the light-dark cycle. ZT4 animals when compared to NP controls show dampened *per2* rhythms indicative of a state of internal desynchrony. Figures adapted from (Lee, et al., 2010)

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