A TIME TO FORGET: CIRCADIAN AND COGNITIVE COSTS OF NOCTURNAL ACTIVITY FOR A DIURNAL BRAIN.

By

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ABSTRACT

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Entrainment of circadian rhythms to the day-night cycle serves to maintain appropriate relationships between behavioral and physiological processes and the external environment. In today's 24hour society, the newly attained capability to be active around the clock has challenged the body's ability to maintain entrainment to the day-night cycle. Humans, who are chronically active at night, have a higher risk for physiological and psychological pathologies, including a reduction in cognitive abilities. Investigations to improve our understanding of the neural underpinnings of the pathologies associated with nocturnal activity in humans have been stunted by the lack of a diurnal animal model and by the use of forced-activity paradigms. The experiments in this dissertation address these issues with the use of a diurnal mammalian model, the grass rat (*Arvicanthis niloticus*), that has the propensity to shift voluntarily its activity to match that of a nocturnal mammal.

In the first set of experiments, the adoption of a nocturnal profile by grass rats resulted in a phase reversal of the rhythmic expression of clock genes in the hypothalamic paraventricular nucleus, but with no disruption of the nocturnal rise in plasma melatonin levels. These differences in the phase of clock gene expression, but not in the nocturnal rise in melatonin, indicate that nocturnal activity in a diurnal species is disruptive of some rhythmic processes, while others appear insensitive to such disruptions.

In the second set of experiments, the Morris water maze (MWM) was used to investigate the optimal phase of retention for a hippocampal dependent task in sedentary diurnal grass rats. The rhythmic expression of plasticity gene products in areas important for learning and memory was also monitored. The optimal phase for long-term retention of a hippocampal task was found to be out of phase with that of the nocturnal lab rat. No time-of-testing effects were found for the acquisition curve or for short-term retention of the task, as previously reported for nocturnal lab rats. Plasticity gene product rhythms showed peak expression during the light phase in grass rats, while peak expression for nocturnal lab rats has been reported to occur in the dark phase.

In the third set of experiments, the night-active grass rat was utilized as a model for understanding the cognitive deficits associated with nocturnal activity in humans. While the overall learning curve for the MWM did not show time-of-training or chronotype differences, the night active grass rat exhibited dramatic deficits in both short-and long-term retention. There was a chronotype difference in the phase of the rhythm of hippocampal expression of only one of two plasticity gene products. In the hippocampus cFOS expression was affected by chronotype only in the CA1. These observations indicate that nocturnal activity disrupts the circadian regulation of key hippocampal functions. Thus, circadian desynchrony in the hippocampus may be responsible for the cognitive deficits seen in humans who choose to be active at night. Taken together the present work links voluntary activity during the rest phase in a diurnal species to physiological and cognitive pathologies, and argues against the claim that mammalian chronotype is a completely malleable trait of the circadian system.

It has truly taken a village of faculty, students and friends to complete the doctorate. I dedicate this dissertation to my mom Alyce Martin. I hope that I have made you proud. While 'My journey hasn't been no glass stair case', every step of the way I persevered using the life skills and values you taught me. As I complete this final degree I reflect on how grateful and blessed I have been to have such wonderful colleagues, lab mates, friends, sorority sisters, and family in my life along the way. I have become colleagues with a group of really talented and genuine neuroscientist who have shaped my thoughts about neuroscience and life. I have had the honor of working with many highly motivated undergraduates and graduate students, who really made it possible for me to complete my dissertation work. The undergraduates I have worked with in the lab and in class I consider my seeds. Seeds that will hopefully be even more successful than I. My AGEP community members really supported me in ways I could never imagine, making sure I was successful and happy. My sorority sisters have blazed such paths here at MSU and throughout the world, while empowering me to be my best in all things and blaze my own paths. Finally, last but certainly not least I dedicate this dissertation to my family. My sister who showed me what a PhD looked like and encouraged me when I needed it. My husband, Donta Fairey, who pushed, pulled or held me when I needed understanding, motivation or resilience. Without my husband's support and plasticity I could not have achieved the doctorate. My fur and feather babies, Simone and Ali, who kept me company on many a long night of working and made me walk around outside in the real world. Overall, I would like to dedicate this dissertation to the ancestors that came before me and the seeds I have planted along my journey.

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KEY TO ABBREVIATIONS

PER	period
SCN	suprachiasmatic nucleus
PVN	paraventricular nucleus
aPVN	anterior regions of the PVN
pPVN	posterior regions of the PVN
DMN	dorsomedial hypothalamus
dTMN	dorsal tuberommamillary nuclei
3V	third ventricle
mt	mammillothalamic tract
Sub	submedius thalamic nucleus
AHP	anterior hypothalamic area posterior part
AHC	anterior hypothalamic area central part
SOX	supraoptic decussation
OPT	optic tract
ArcM	arcuate nucleus medial portion
ME	median eminence
ZI	zona incerta
ir	immunoreactive
ZT	zeitgeber time
DAS	day active sedentary
DA	day active grass rats
NA	night active grass rats

LSD	least significant difference
ANOVA	analysis of variance
vSPZ	ventral subparaventricular zone
SEM	standard error of the mean
BDNF	brain derived neurotrophic factor
TrkB	tyrosine kinase B
LD	light/dark
DG	dentate gyrus
CA1	cornus Ammon 1
ICC	Immunocytochemical
MWM	Morris water maze
cAMP	cyclic adenosine monophosphate
Arc	activity-regulated cytoskeleton-associated
MAPK	mitogen activated protein kinase
pCREB	phosphorylated cyclic adenosine monophosphate response element-binding protein
pERK	phosphorylated extracellular signal-regulated kinase
SCOP	suprachiasmatic nucleus circadian oscillatory Protein
CLOCK	Circadian Locomotor Output Cycles Kaput
BMAL1	brain and muscle aryl hydrocarbon receptor nuclear translocator like
CRY 1 and 2	Cryptochrome
hipp	hippocampus

DSt	dorsal striatum
BLA	basolateral amygdala
PI3-K	phosphatidylinositol 3-kinase
IMG	amygdaloid intramedullary gray
DAB	diaminobenzidine
MSU	Michigan State University
μm	micrometer
PLC-$_{\gamma}$	phospholipase C-$_{\gamma}$

CHAPTER 1: INTRODUCTION

Background and Significance: Many physiological and behavioral processes show daily rhythms that are generated by endogenous mechanisms and synchronized to environmental stimuli. These endogenous rhythms with periods close to 24 hours are referred to as circadian rhythms (Ko and Takahashi, 2006). Examples of functions that show circadian rhythms include blood pressure (Scheer et al., 2003; Schnell and Wood, 1993; Vukolic et al., 2010), body temperature (McElhinny et al., 1997; Petrovski et al., 2010), hormone levels (Bonnefont, 2010; Shechter and Boivin, 2010), the number of immune cells in blood (Scheff et al., 2010), and the sleep-wake cycle (Elmore et al., 1994), among many others. Rhythmicity in these functions is important for maintaining homeostasis (Appelbaum et al., 2010; Valdez et al., 2010). Synchronization of these rhythms to key environmental events, such as the day-night cycle, is critical to ensure that behaviors and physiological functions match the demands of an environment that changes across each 24-hour period (Bonnefont, 2010; Wright et al., 2006)

In mammals, circadian rhythms in physiology and behavior are generated by a central clock residing within the hypothalamic suprachiasmatic nucleus (SCN)(Stephan and Zucker, 1972).The SCN receives inputs from specialized photoreceptive retinal ganglion cells via the retinohypothalmic tract, and this retinal projection entrains the SCN clock to the light-dark cycle (Welsh et al., 2010). Individual neurons within the SCN can generate circadian oscillations, but the expression of robust circadian rhythms depends upon the coupling of multiple cell-autonomous oscillators within the SCN (Herzog et al., 1998; Herzog and Schwartz, 2002). In neurons of the SCN, several genes are expressed in a circadian rhythmic fashion. A portion of these genes are called clock genes. Clock genes are a group of genes that include Circadian Locomotor Output Cycles Kaput (CLOCK), brain and muscle aryl hydrocarbon receptor nuclear translocator like (BMAL1), Period (PER1-3) and Cryptochrome (CRY 1 and 2).Oscillation of the SCN molecular clock has been described as an autoregulatory transcriptional and translational feedback loop (Ko and Takahashi, 2006).

Rhythmic expression of clock genes is not limited to the SCN, but is found in many other brain regions and in the periphery (Dibner et al., 2010; Duncan et al., 2013). The SCN modulates the rhythms of these extra-SCN oscillators. In fact, with the exception of the olfactory bulb, extra-SCN oscillators in the brain and periphery require an intact SCN to maintain rhythms in clock-gene expression (Granados-Fuentes et al., 2004a; Granados-Fuentes et al., 2004b), and all extra-SCN oscillators depend upon SCN signals to remain synchronized to the light-dark cycle (Abe et al., 2002; Amir and Stewart, 2009a). Brain extra-SCN oscillators also respond with changes in phase to non-photic rhythmic inputs including hormonal rhythms and scheduled feeding (Escobar et al., 2007; Perrin et al., 2006; Segall and Amir, 2010). Thus, extra-SCN oscillators may control site specific rhythmic functions by integrating inputs from multiple sources including the SCN.

The Grass Rat as a Model: One notable limitation in our knowledge of mammalian chronobiology is that, almost exclusively, researchers have used nocturnal rodent models to study circadian biology, asserting that the evidence provided by these studies can be directly translated to human health issues. Laboratories at Michigan State University (MSU) have the ability to study circadian rhythms in a rodent model that is naturally diurnal in the wild and in captivity. The ability to utilize the diurnal grass rat (the Nile grass rat or *Arvicanthis niloticus*), as an animal model has enabled our MSU chronobiology group to identify many noteworthy similarities between nocturnal and diurnal species, such as the phase of rhythms in clock-gene and c-Fos expression within the SCN, and those of several molecules that serve as outputs of neurons of the SCN (Smale et al., 2003; Smale et al., 2008).

While the use of a diurnal model has allowed us to identify species similarities in the SCN, it has also given us insights into species differences that may determine a species chronotype. Among those is the observation that the rhythms in clock-gene expression in most brain extra-SCN oscillators

are 180^0 out of phase when grass rats are compared to nocturnal rodents. Specifically, Ramanathan et al (Ramanathan et al., 2008a; Ramanathan et al., 2008b; Ramanathan et al., 2010; Ramanathan et al., 2010b) reported that PER1 and 2 expression peaks during the late light period in diurnal grass rats and in the late night in nocturnal species (Abe et al., 2002; Amir and Stewart, 2009a; Amir and Stewart, 2009b) in several regions that contain extra-SCN oscillators, including the oval nucleus of the bed nucleus of the stria terminalis, central amygdala (CEA),basolateral amygdala (BLA), dentate gyrus (DG), CA1 region of the hippocampus, piriform cortex (PC), shell of the nucleus accumbens and dorsal striatum (DSt). These and other findings support the hypothesis that differences between diurnal and nocturnal mammals are due to changes in neural systems downstream from the SCN (Smale et al., 2003).

The Night-Active Grass Rat as a Model of Circadian Disruption: Interestingly, when grass rats are given access to a running wheel their activity patterns bifurcate into two subpopulations; day active (DA) animals that remain active during the day and night active (NA) animals that switch to being active primarily at night (Blanchong et al., 1999). The phase preference for the display of activity at night is the opposite of what is shown by sedentary (no wheels) grass rats, which are universally diurnal. This remarkably shift in activity by NA grass rats is accompanied by a phase reversal in the rhythmic expression of PER1 and 2 in most extra-SCN oscillators. In this subpopulation of animals, the rhythms of most extra-SCN oscillators are out of phase with those of the DA animals. Thus, this phase reversal results in PER rhythms that are similar to those normally displayed by nocturnal mammals. However, not all extra-SCN oscillators switch uniformly or at all in NA grass rats, some rhythms of clock-gene expression remain faithful to the diurnal pattern that exists in DA animals (Nunez et al., 2012; Ramanathan et al., 2006). This apparent desynchrony among extra-SCN oscillators makes the grass rat a powerful model for the investigation of pathologies

that accompany circadian disruption in humans who chose to be active during their natural rest phase.

Approach: The work presented here first explored the possible circadian disruptions that may be endured by NA grass rats when the phase of multiple extra-SCN oscillators is reversed, but some retain their diurnal phases. In particular, for the work presented as Chapter two, I monitored the phase of a local oscillator in the hypothalamic paraventricular nucleus (PVN) and also determined the phase of the melatonin rhythm in DA and NA grass rats. Melatonin production is under strong circadian control by the SCN, with high melatonin levels seen at night in both diurnal and nocturnal mammals (Challet, 2007; Kalsbeek et al., 2000); but signals from the SCN to the pineal gland are relayed via a multi-synaptic pathway that includes the PVN (Moore, 1996). Thus, changes in the phase of the PVN oscillator may disrupt the circadian control of the melatonin rhythm. Alterations in melatonin production patterns have been linked to various health problems including sleep and immunological disorders, jet lag, dementia, cancer, and anorexia nervosa (Brambilla et al., 1988; Carrillo-Vico et al., 2013; Erren, 2013; Haimov et al., 1997; Lammers and Ahmed, 2013; Ruhwald and Claesson, 1998). Many of these conditions have also been reported to have a high incidence among shift workers, who voluntarily remain active during their rest phase (Angersbach et al., 1980; Kaliterna et al., 1990; Pietroiusti et al., 2010; Suwazono et al., 2006). Investigation into possible phase reversals in melatonin secretion and in components of the multi-synaptic pathway that regulates melatonin secretion is important for understanding the extent of desynchrony among elements of the circadian system endured by NA grass rats. The experiments of Chapter two showed that the phase of the oscillator of the PVN displays remarkable plasticity across chronotypes of the same or different species, while the nocturnal pulse of melatonin production is relatively unaffected by chronotype or by the phase of the oscillator of the PVN. This

profile then results in what is a clear mismatch, for a naturally diurnal species, between wakefulness and elevated levels of melatonin.

Areas of the brain, such as the hippocampus, that house extra-SCN oscillators also play important roles in the regulation of cognitive functions. Since the phase of those oscillators is reversed between diurnal and nocturnal species, the research plan examined, whether that phase reversal extended to a reversal in the optimal phase for learning a hippocampal-dependent task. Thus, one aim of this work was to determine how the optimal phase for learning in grass rats compares to that of nocturnal species (Chapter 3), as well as how it may be disrupted when grass rats become NA (Chapter 4). Most studies support the existence of a night-time advantage for learning and memory in nocturnal rodents (Chaudhury and Colwell, 2002; Hoffmann, 1992; Valentinuzzi et al., 2000; Valentinuzzi et al., 2004) and in diurnal species there is consistent evidence of a diurnal advantage present in most learning and memory paradigms; but the work with diurnal animals has been restricted to experiments with bees, birds and non-human primates (Haley et al., 2009; Lehmann et al., 2011; Valentinuzzi and Ferrari, 1997), and the field is lacking a diurnal rodent model. When considering hippocampal dependent tasks in nocturnal rodents, there appears to be an advantage when the animals learn during their active nocturnal phase (Gritton et al., 2012; Hoffmann, 1992; Valentinuzzi et al., 2004).

The behavioral work of Chapters 3 and 4 was complemented by immunohistological studies that investigated, in grass rats, the rhythmic expression of gene products that mediate neural plasticity in the hippocampus and in other regions of the brain that mediate different types of learning and memory (Bekinschtein et al., 2014; Callaghan and Kelly, 2013; Golini et al., 2012). My aim was to determine if the observed phase reversal in the rhythmic expression of clock genes, evident when nocturnal and diurnal species are compared, extended to the rhythmic expression of gene products

that have been implicated in neural plasticity. Attaining that aim is an important step in understanding the neural mechanisms responsible for the presence of optimal times of day for learning as well as for retention of what is learned (Smarr et al., 2014).

Overall, the work of Chapter 3 served to determine the optimal phase of learning and memory in a diurnal species, and that of Chapter 4 demonstrated how that optimal phase is affected by being active during the rest phase. My results also showed how nocturnal activity in grass rats affects the phase of rhythms in gene products that contribute to the neural plasticity of the hippocampus. Additionally, Chapter 4 documented how nocturnal activity affects rhythms in hippocampal neural activity as assessed by the expression of the immediate early-gene product cFOS (Dragunow, 1996).

In summary, the work included in this dissertation showed that when naturally diurnal grass rats adopt a nocturnal profile of activity, the phase of the melatonin rhythm remains the same as it is in diurnal grass rats. This creates a mismatch between the display of wakefulness and the presence of high levels of melatonin that may contribute to circadian desynchrony in these animals. My research also identified rhythms in hippocampal dependent memory in a diurnal species and showed how the adoption of a nocturnal phase preference for activity affects cognitive functions and the phase relationships among hippocampal rhythms that are likely to affect the circadian regulation of learning and memory (Smarr et al., 2014). These results help us to understand the cognitive and physiological consequences of being nocturnally active for a diurnal species like ours. This is particularly important in reference to the cognitive and general health deficits associated with the growing portion of the population whose lifestyles include repeated travel across time zones, non-circadian activity schedules and night- or shift-work (Barnard and Nolan, 2008; Gold et al., 1992).

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CHAPTER 2

Plastic oscillators and fixed rhythms: Changes in the phase of clock-gene rhythms in the PVN are not reflected in the phase of the melatonin rhythm of grass rats.

The work presented in this chapter has been submitted in manuscript form and is currently under review.

Introduction

Multiple cell-autonomous oscillators that rhythmically express a multitude of genes comprise the master circadian oscillator of the mammalian suprachiasmatic nucleus (Welsh et al., 2010). The molecular core of these cellular oscillators is an autoregulatory transcriptional and translational feedback loop (Ko and Takahashi, 2006), which includes several clock genes such as Period (PER) 1 and 2. Rhythms in the expression of PER1 and 2 proteins have been reported for many brain regions outside the SCN (Granados-Fuentes et al., 2004, Duncan et al., 2013) and also in several peripheral organs (Scheer et al., 2001, Escobar et al., 2009, Dibner et al., 2010, Bonaconsa et al., 2014). These and other observations suggest that local oscillators outside the SCN may contribute to region and tissue specific rhythmic functions throughout the brain and body.

In the hypothalamus of laboratory rats, rhythmic expression of PER1, but not PER2, has been described in the paraventricular nucleus (PVN; Asai et al. 2001; Minana-Solis et al., 2009). The PVN receives direct inputs from the SCN (Munch et al., 2002) and is part of a multi-synaptic pathway that is responsible for the circadian and photic regulation of the pineal gland and the pineal's melatonin rhythm (Moore, 1996). Thus the phase of a local neural oscillator in the PVN could contribute to the phase of the melatonin rhythm.

Irrespective of phase preference for the display of activity, the phase of the melatonin rhythm is more or less the same across mammalian species (Reiter, 1991b), yet recent observations about the phases of extra-SCN, non-hypothalamic oscillators demonstrate that those of diurnal grass rats (Ramanathan et al. 2010b), Octodon degus (Otalora et al., 2013) and humans (Li et al., 2013) are 180° out of phase compared to those of nocturnal rodents (Amir et al., 2004, Amir and Robinson, 2006). Thus, given the common phase of the melatonin rhythm across diurnal and nocturnal species

and the involvement of the PVN in the mediation of that rhythm, one question addressed here relates to potential differences as well as similarities between the phase of the PVN oscillator in diurnal and nocturnal species. For nocturnal species, mRNA for PER1 peaks around zeitgeber time (ZT) 16 (Asai et al., 2001, Minana-Solis et al., 2009); suggesting that the peak of PER1 protein occurs during the late night, which is the time of peak PER protein expression in several areas of the brain of nocturnal species (Amir et al., 2004, Lamont et al., 2005, Angeles-Castellanos et al., 2007, Feillet et al., 2008); comparable data for the PVN are not available from diurnal species.

Here, PER1 and PER2 expression is characterized at two anatomical levels, the anterior and posterior regions of the PVN,(aPVN and pPVN respectively), where pre-autonomic neurons reside (Swanson and Kuypers, 1980, Swanson and Sawchenko, 1980, Swanson et al., 1980, (Smale et al., 1989; Teclemariam-Mesbah et al., 1999) in diurnal grass rats (*Arvicanthis niloticus*) kept in standard laboratory conditions (Experiment 1A). The aim was to determine if the phase profile of PER1 and 2 rhythms described for other brain regions of the grass rats (Ramanathan et al., 2010a, Ramanathan et al., 2010b) generalizes to a nucleus that regulates autonomic functions, including the rhythm of melatonin production by the pineal gland.

Grass rats are clearly diurnal in their natural habitat (Blanchong and Smale, 2000) and in captivity (Blanchong et al., 1999), but under some conditions they show remarkable plasticity and individual differences in their phase preference for the display of activity. Particularly, when given access to running wheels a proportion of diurnal grass rats become predominantly night-active (NA), whereas other individuals retain their day-active (DA) profile, even when wheels are available (Blanchong et al., 1999). The phase of brain extra-hypothalamic oscillators of NA grass rats appears to be 180^{0} out of phase with respect to that of DA animals, thus resembling the phase typical of nocturnal species (Ramanathan et al., 2010b). However, at least in the ventral subparaventricular

zone [vSPZ;(Ramanathan et al., 2006)], the hypothalamic dorsal tuberommamillary nuclei (dTMN) and the dorsomedial hypothalamus (DMN;Nunez et al. 2012), that reversal of phase is not complete, and thus the circadian profile of the hypothalamus of NA grass rats retains features typical of diurnal animals.

In Experiment 1B involved a comparison of the rhythmic expression of PER1 and 2 in the aPVN and pPVN of DA and NA grass rats, to determine if the oscillator of the PVN of NA animals adopts a nocturnal profile, as is the case for extra-hypothalamic oscillators (Ramanathan et al., 2010a, Ramanathan et al., 2010b) or, if alternatively, it behaves like other hypothalamic sites that retain diurnal features (Ramanathan et al., 2006, Nunez et al., 2012). Any putative change in the oscillator of the PVN would raise the question of how the nocturnal rise in melatonin would be affected. Therefore, melatonin was also measured during the day or night in diurnal grass rats with or without wheels (Experiment 2), to determine if melatonin profiles remained nocturnal regardless of changes in the preferred phase for the display of activity and the potential reversal of the phase of the PVN oscillator.

Methods

Experiment 1A and 1B

Animals and housing

Adult male grass rats (*Arvicanthis niloticus*) from our breeding colony at Michigan State University, East Lansing, MI, USA, were housed individually in Plexiglas cages (34×28×17 cm^3), under a 12:12-h LD cycle [lights on at ZT 0], with a red light (<5 lx) on throughout the dark phase, and with free access to food (PMI Nutrition Prolab RMH 2000, Brentwood, MO, USA) and water. Grass rats were initially divided into two groups: Day active sedentary (DAS; Experiment 1A) and

Wheel runners. Wheel runners were housed in the same Plexiglas cages, but equipped with running wheels (26 cm diameter; 8 cm width). Wheel runners underwent chronotype determination in order to be classified as a day or night active grass rats (DA and NA respectively; Experiment 1B) using methods previously published (Blanchong et al., 1999; Ramanathan, 2010b) and described below. All experiments were performed in compliance with guidelines established by the Michigan State University All University Committee on Animal Use and Care, and the National Institute of Health guide for the Care and Use of Laboratory Animals.

Chronotype determination: Experiment 1B

Wheel running data were continuously collected using a DSI Dataquest 3 system (MiniMitter, Sunriver, OR, USA) and actograms were generated using the Vital View program (MiniMitter, Sunriver, OR, USA). Activity profiles were used to classify grass rats as DA or NA after obtaining a stable wheel running rhythm for at least two weeks. An animal was classified as DA if daily activity ceased within 2 h after lights-out and classified as NA if activity continued for more than 4 h after lights-out (Blanchong et al., 1999). Animals exhibiting crepuscular activity patterns were omitted from the study.

PER1 and 2 protein expression in the aPVN and pPVN

Perfusion and tissue preparation

Animals (n=5-8 per ZT for each group of Experiments 1A and 1B) were perfused at ZTs 2, 6, 10, 14, 18, and 22. At the prescribed ZT, the animals were deeply anesthetized with sodium pentobarbital (Ovation Pharmaceutical, Deerfield, IL, USA) and perfused transcardially with 0.01 M phosphate buffered saline (PBS), pH 7.2, followed by 4% paraformaldehyde (Sigma, St. Louis,

MO, USA) in 0.1 M phosphate buffer. An aluminum hood covered the heads of the animals perfused during the dark period to prevent exposure to light. Brains were removed and post-fixed for 4 h in 4% paraformaldehyde and transferred to 20% sucrose solution overnight, then stored in cryoprotectant at −20 °C until sectioning. Brains were sectioned coronally at 30 μm using a freezing microtome and the sections stored in cryoprotectant at −20 °C for future processing.

Immunocytochemical procedures

Adjacent sections containing the PVN were subjected to Immunocytochemical procedures for detecting either PER1 or PER2 expression as previously described (Ramanathan et al., 2006). The procedure was the same for Experiment 1A and 1B, but the two tissue sets were processed separately. Briefly, free-floating sections were rinsed 3×10 min in 0.01 M PBS before the first as well as all subsequent incubations. All sections were incubated in 5% Normal Donkey serum (Jackson Labs, West Grove,PA) in 0.01 M PBS with 0.3% Triton X-100 for 1 h at room temperature. The sections were incubated in the primary antibody (mPER1# 1177, made in rabbit, 1:20,000, a gift from Dr. D. R. Weaver, University of Massachusetts Medical School, MA, USA) on a rotator for 48 h at 4 °C. On the third day, they were incubated in biotinylated secondary antibody (Donkey anti Rabbit; 1:200, Jackson Labs, West Grove,PA), followed by Avidin–Biotin peroxidase complex (ABC Vectastain Kit; Vector Laboratories, Burlingame, CA, USA). Protein was visualized by reacting with diaminobenzidine (0.5 mg/mL Sigma) in sodium acetate buffer (pH 7.2) and 3% hydrogen peroxide. Nickel sulfate was added to yield a purple reaction product. For detecting PER2 immunoreactivity, the same procedure was followed except that for PER2, the primary antibody (made in rabbit, and generously provided by Dr. D. R. Weaver, University of Massachusetts Medical School, MA, USA) was diluted to 1:10,000. All tissue was mounted onto gelatin-coated slides, dehydrated and cover slipped. To control for non-specific staining resulting

from the procedure, all the steps of the protocol were replicated excluding the incubation with the primary antibody. This control procedure produced no staining in any of the anatomical areas examined in the two experiments.

Data collection and analysis

Single sections were selected containing either the anterior or posterior paraventricular nucleus (aPVN and pPVN respectively, Figure 1.1) using the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 2007). For each region sampled, the approximate level of the section is given in reference to bregma from that atlas. To quantify PER1 and PER2 in both brain regions, immunoreactive cell nuclei were counted bilaterally in the aPVN (Bregma=-1.88mm) and pPVN (Bregma=-2.12mm), using counting boxes with the following dimensions: aPVN: 250×250 μm^2 and pPVN: 75×125μm^2 by an investigator who was not aware of the time of perfusion nor the chronotype of each animal. Total bilateral counts of each area were used for statistical analysis. For all experiments, statistical significance was set at p value less than or equal to 0.05, and for all data sets, SPSS version 22 was the software used for all statistical analyses. For the DAS group in Experiment 1A, one-way analysis of variance (ANOVA) was used to assess the effect of ZT on PER1 and 2 expression in each region. Significant *F* ratios were followed by comparisons between ZTs using least significant difference (LSD) *post hoc* tests. For Experiment 1B data from the DA and NA animals were analyzed using individual two-way ANOVAs (ZT×group) for each brain region and each PER protein, using total bilateral counts of labeled cells as the dependent variable. Significant interactions were followed by analyses of simple main effects and, when appropriate, post hoc comparisons of individual means used Fisher's LSD tests.

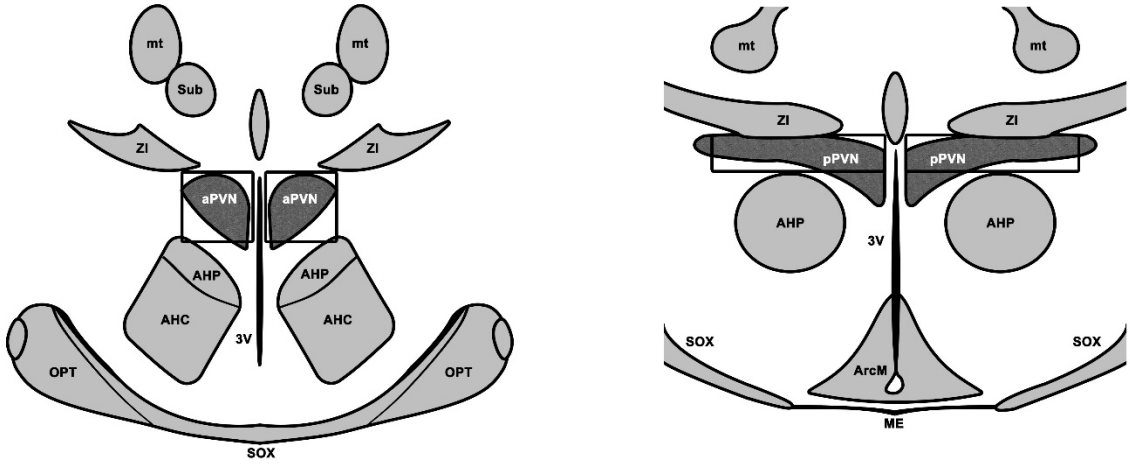

Figure 1.1: Line drawings depicting the location and levels of PER1 and 2 counts in grass rats
Illustrations adapted from Paxinos and Watson (2007) depicting representative sampling levels of paraventricular nucleus (PVN) areas used to quantify PER1 and 2 expression in the anterior PVN (aPVN, left panel) and the posterior PVN (pPVN, right panel) with sampling boxes affixed in the areas sampled (aPVN: 250×250 μm^2 and pPVN: 75×125μm^2). anterior PVN, aPVN; posterior PVN, pPVN; third ventricle, 3V; mammillothalamic tract, mt; submedius thalamic nucleus, Sub; anterior hypothalamic area posterior part, AHP; anterior hypothalamic area central part. AHC; supraoptic decussation, SOX; optic tract, OPT; Arcuate nucleus medial portion, ArcM; median eminence, ME; zona incerta, ZI

Experiment 2: Plasma melatonin levels in grass rats with and without access to wheels

Adult male grass rats (n=5-8 per group/ZT) from the MSU breeding colony housed and chronotyped as described above were sampled at either ZT6 or ZT18. At the prescribed ZTs, the animals were deeply anesthetized with sodium pentobarbital (Ovation Pharmaceutical, Deerfield, IL, USA). Whole blood samples were obtained via cardiac puncture using a heparinized 25 gauge needle. Samples were immediately centrifuged for 15min at 1500x g at 4^0C. Plasma was removed and stored at -80^0C for final processing.

Data collection and analysis

Plasma levels of melatonin were quantified using the competitive enzyme linked immunosorbant assay procedure from a commercial source (Geneway Biotech Inc., San Diego, CA). Initially, each sample was passed through a C18 reversed phase column, extracted with methanol, evaporated to dryness, and reconstituted with water. Next, 50 µl of each sample was pipetted into the corresponding well coated with the goat-anti-rabbit antibody on a microtiter plate. An unknown amount of antigen present in the sample and a fixed amount of enzyme-labeled antigen competed for the binding sites of the antibodies coated onto the wells of the microtiter plate. After incubation for at least 20 hrs at 4^0C, the wells were washed three times with a phosphate buffer washing solution. 150 µl of enzyme conjugate was added to each well and the plate was incubated for 120 minutes at room temperature on an orbital shaker set to 500 rpm. A *p*-nitrophenyl phosphate substrate solution was added to the wells, and the plate was incubated for an additional 20 minutes on an orbital shaker set to 500 rpm. Finally, a *p*-nitrophenyl phosphate stop solution was added into each well to stop the reaction. The concentration of antigen was inversely proportional to the optical density and was measured at 405nm in a photometer. The standard curve and sample values were calculated using Sigma Plot. Melatonin standards provided with the kit were used to construct a calibration curve against which the unknown samples were calculated. The sensitivity of the assay was 1.6 pg/mL.

Two-way ANOVAs (Group x ZT) were used to assess the effect of ZT and group on plasma melatonin levels. A significant interaction was followed by analysis of the simple main effects of Group and ZT, and by comparison of individual means Fisher's LSD tests for comparisons between groups within each ZT and independent-group t-tests (2-tailed) for evaluating the effects of ZT within groups.

Results

Experiment 1A

For PER1 expressing cells, ANOVAs revealed no significant main effect of ZT in either the aPVN ($F_{5,27}$=1.343; p=0.277) or the pPVN ($F_{5,29}$=2.225; p=0.079) of DAS grass rats (data not shown). Figure 1.2 (left panel) shows the mean (± standard error of the mean (SEM)) number of PER2 positive cells in the aPVN and the right panels show representative photomicrographs depicting the peak and trough of PER2 expression in the aPVN of DAS grass rats. For these data, ANOVAs revealed a significant effect of ZT with a peak at ZT10 within the aPVN ($F_{5,29}$=5.655; p=0.001). In the pPVN there was an almost significant main effect of ZT on PER2 expression with a peak at ZT10 ($F_{5,29}$=2.493; p=0.054; data not shown).

Experiment 1B

For the number of cells expressing PER1 in the aPVN the two-way ANOVA revealed a significant ZT x chronotype interaction ($F_{5,74}$=3.192; p=0.012;Figure 1.3). Follow up analyses of the simple main effect of ZT within group revealed no significant effect of ZT in DA grass rats ($F_{5,33}$=1.532; p=0.207) and a significant simple main effect of ZT in NA grass rats with a peak at ZT22 ($F_{5,41}$=2.744; p=0.031). For the number of cells expressing PER1 in the pPVN, the two way ANOVA detected a significant ZT x chronotype interaction ($F_{5,76}$=2.809; p=0.022). Follow up analyses of the simple main effect of ZT within chronotype found a significant effect of ZT in DA grass rats with a peak between ZT6 and 10($F_{5,34}$=2.582; p=0.044) and no significant main effect of ZT in NA grass rats ($F_{5,42}$=0.554; p=0.735).

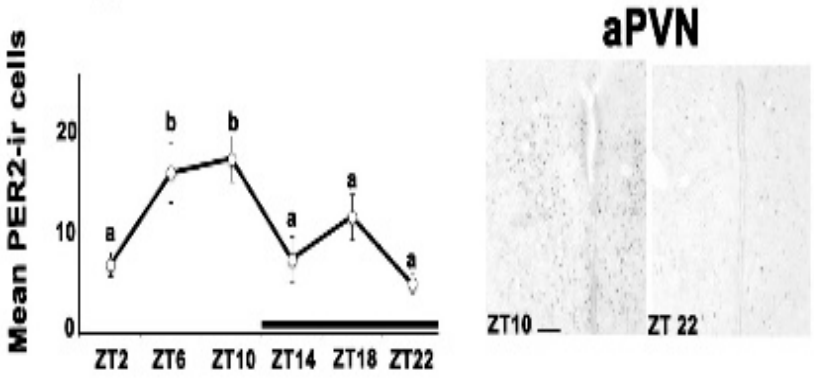

Figure 1.2: Diurnal variations in PER2 expression within the aPVN of DAS grass rats
Late light phase peak in PER2 expression in diurnal grass rat aPVN. Line charts showing mean (± SEM) number of PER2-ir cells (left panel) and photomicrographs depicting peak and trough expression (right panel) in the aPVN of DAS grass rats. Anatomical boundaries are based on Paxinos and Watson (1997), see Figure 1.1 Significant differences between ZTs are noted by different letters. Black bar indicates the dark phase of the cycle. Scale bar = 200μm. anterior PVN, aPVN; posterior PVN, pPVN; Period, PER; immunoreactive, ir; zeitgeber time, ZT; SEM, standard error of the mean

The two way ANOVA detected a significant ZT X chronotype interaction ($F_{5,72}$=4.268; p=0.002; Figure 1.3) in PER2 expression in the aPVN. Follow up analyses found a significant simple main effect of ZT in the DA group with peak expression at ZT10 ($F_{5,34}$=2.77; p=0.033) and a significant simple main effect of ZT in the NA group with a peak at ZT22 ($F_{5,38}$=4.114; p=0.004). For PER2 expressing cells in the pPVN, the ANOVA revealed no ZT x chronotype interaction, with no overall effects of ZT or group ($F_{5,71}$=1.028; p=0.408; data not shown).

Experiment 2

The two-way ANOVA of plasma melatonin revealed a significant ZT x group interaction ($F_{2,32}$=10.613; p=0.00;Figure 1.4). Tests of the simple main effect of ZT showed that for the three groups, night time values were significantly higher than day time ones ($ts_{10\text{-}11}$=10.50-13.270,ps=0.000). Within ZT, the simple main effect of group was statistically significant both at ZT6 ($F_{2,17}$=27.265; p=0.000) and at ZT18 ($F_{2,15}$=34.591; p=0.000). Individual group comparisons showed that for both ZTs, the groups differed significantly among themselves, with NA grass rats having the highest and DAS grass rats the lowest levels of melatonin.

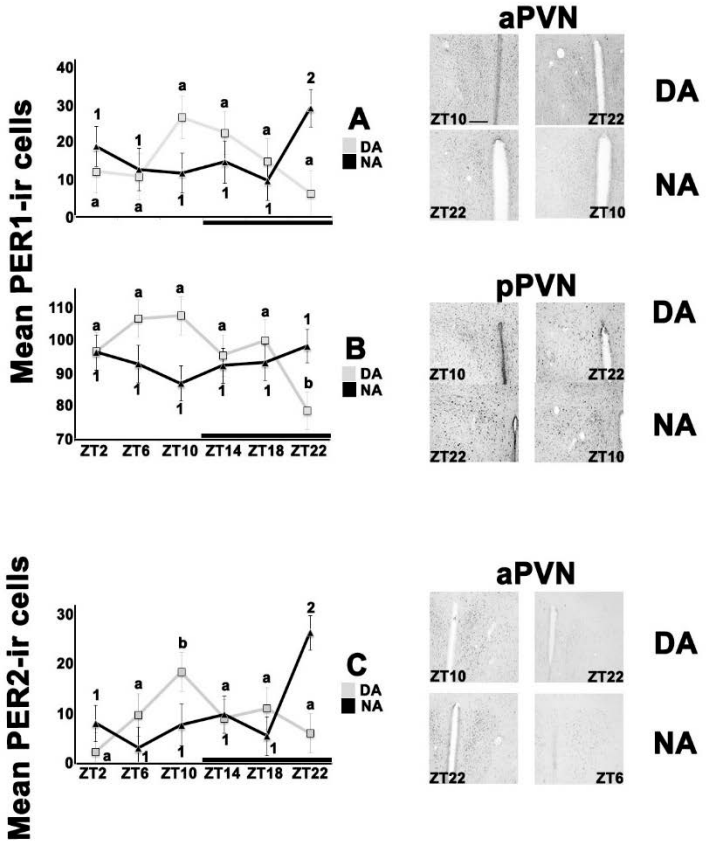

Figure 1.3: Chronotype and time of day differences in PER1 and 2 expression within the aPVN and pPVN of grass rats, There is an association between the preferred phase of activity and PVN PER rhythms, when present, in grass rats that have access to running wheels. The amplitude of PER1 rhythms are increased when diurnal grass rats have access to a running wheel. Left Upper Panels: line graphs showing the mean (± SEM) number of PER1 expressing cells for each ZT in the anterior (A) and posterior PVN (B) of DA and NA grass rats. Right Upper Panels: photomicrographs depicting peak and trough PER1 expression in the anterior (A) and posterior (B) PVN. Left Lower Panel (C): Line graphs showing the mean (± SEM) number of PER2 expressing cells for each ZT in the anterior PVN of DA and NA grass rats. Right Lower Panel(C): photomicrographs depicting peak and trough of PER2 expression in the anterior PVN. For all line graphs, significant differences between ZTs within chronotype are noted by different letters (DA) or different numbers (NA). Black bars indicate the dark phase of the cycle. For the photomicrographs, anatomical boundaries are based on Paxinos and Watson (1997), see Figure 1.1: scale bar = 200μm. anterior PVN, aPVN; posterior PVN, pPVN; Period, PER; zeitgeber time, ZT; immunoreactive, ir; day active, DA; night active, NA; SEM, standard error of the mean.

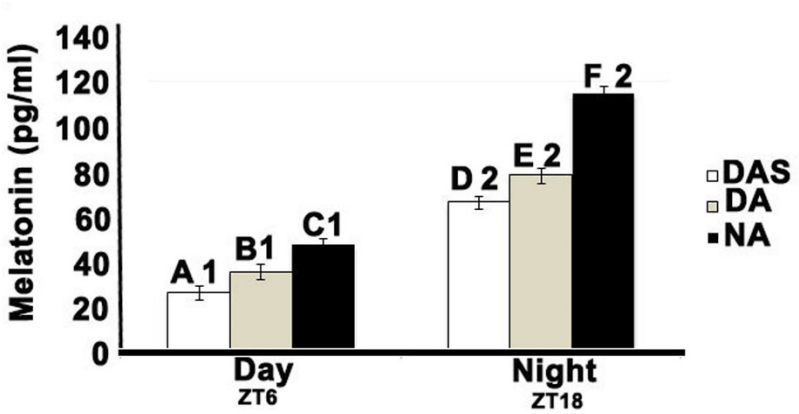

Figure 1.4: Day and Night differences in plasma melatonin levels in DAS, DA and NA grass rats, Nocturnal activity does not disrupt the nocturnal rise in plasma melatonin in the diurnal grass rat. Bar graph showing the mean (+ SEM) pg/ml of plasma melatonin for each group (DAS, DA and NA) and ZT (ZT6=Day and ZT18=Night). Significant differences between groups are denoted by the letters. Significant differences between ZTs are denoted by the numbers. See text for details of the statistical comparisons

Discussion

The major finding of Experiment 1A was that when a rhythm in PER2 protein expression was present in the PVN of grass rats, it featured a peak late in the light period (i.e., ZT 10), which is about 12 hours earlier than what has been reported for PER1 in nocturnal species (Asai et al., 2001, Minana-Solis et al., 2009, Dzirbikova et al., 2011). Since PER1 and 2 appear to have redundant roles in circadian regulation (for example see Maywood et al., 2014), these results serve to extend previous observations of phase differences in extra-SCN oscillators between grass rats and nocturnal rodents (Ramanathan et al., 2010b) to a hypothalamic nucleus with connections to autonomic neurons (Swanson and Kuypers, 1980, Swanson and Sawchenko, 1980, Swanson et al., 1980, Teclemariam-Mesbah et al., 1999) and to pre-autonomic brain sites (Stern, 2001, Kalsbeek et al., 2011). Species differences in the phase of the PVN oscillator may be responsible for the divergent phases of diurnal and nocturnal species with respect to rhythms that are controlled by autonomic outputs (Scheer et al., 1999, Scheer et al., 2001, Duarte et al., 2003, Scheer et al., 2003). Thus, these observations

support the hypothesis that the emergence of a diurnal profile in mammals depends, at least in part, upon a reversal of the phase of extra-SCN oscillators from that typical of nocturnal species with respect to the phase of the oscillator of the SCN (Ramanathan et al., 2010a, Ramanathan et al., 2010b) and the light-dark cycle.

The results of Experiment 1B show that access to running wheels increases the amplitude of the PER1 protein rhythm in the PVN of grass rats, and that the phase of that rhythm, as well as that of PER2 protein when present, is associated with the animals' phase preference for the display of activity. Thus, as grass rats adopt a nocturnal profile, the oscillator of the PVN shows a reversal in phase similar to what is seen in extra-SCN oscillators outside the hypothalamus (Ramanathan et al., 2010b). In contrast to the present results, the phase of the PER1 protein rhythm of the PVN is unaffected in nocturnal laboratory rats that become active during the day in response to a forced-activity paradigm that emulates the conditions of human shift workers (Salgado-Delgado et al., 2010). Although species differences may be responsible for these divergent outcomes, these observations nevertheless support the view that voluntary reversals of the phase of activity (present study) affect the brain and the circadian system in a fashion that differs from the effects of forced-activity paradigms (Karatsoreos et al., 2011, McDonald et al., 2013, Saderi et al., 2013, Hsieh et al., 2014). In agreement with this view are the results of direct comparisons of the effects on the brain of spontaneous and forced wakefulness (Castillo-Ruiz et al., 2010, Castillo-Ruiz and Nunez, 2011). Thus, in grass rats, voluntary nocturnal wakefulness results in increased neuronal activation, as indicated by Fos expression, in reward areas of the brain (i.e., horizontal diagonal band, ventral tegmental area, and supramammillary nuclei; Castillo-Ruiz et al.,2010), which is an observation not extended to studies using a forced-wakefulness paradigm with the same species (Castillo-Ruiz and Nunez, 2011). The NA grass rat appears to be an attractive model to understand the consequences

of activity during the rest phase in a diurnal species, without the effects of the stress associated with forced-wakefulness paradigms (Castillo-Ruiz and Nunez, 2011).

My observations about the reversal of the phase of the PVN oscillator in NA grass rats contrast with what has been seen in other extra-SCN hypothalamic sites in these animals. For example in the vSPZ, the switch to being active at night is not accompanied by a reversal in either neuronal activity as indicated by cFOS (Rose et al., 1999, Mahoney et al., 2000, Mahoney et al., 2001, Smale et al., 2001, Schwartz and Smale, 2005) or PER1 and 2 protein expression (Ramanathan et al., 2010b). A similar preservation of the diurnal like phase of PER1 and 2 rhythms, in spite of a reversal in the preferred phase for activity, is seen in the DMN and the dTMN of NA grass rats (Nunez et al., 2012). Thus, in the hypothalamus of the NA grass there is a mosaic of phases, and not the uniform phase reversal seen in extra-hypothalamic sites (Ramanathan et al., 2010b). This could signal an internal circadian desynchrony in areas of the brain that regulate important functions such as vigilance [i.e., the dTMN (Gerashchenko et al., 2004, Valdes et al., 2010)], or autonomic functions [i.e., the DMN(Chou et al., 2003) and PVN (Saper et al., 1976, Swanson and Sawchenko, 1980)], that desynchrony could be responsible for the sleep and metabolic pathologies of humans that show chronic enhanced activity at night (Asher and Schibler, 2011, Gamble and Young, 2013).

As expected given the results from many other diurnal species (Reiter, 1991b, a), I found in Experiment 2 that melatonin is elevated at night in grass rats. Combined with the observation of a phase difference in PER protein expression in the PVN of diurnal grass rats and nocturnal lab rats (Minana-Solis et al., 2009) it is possible that the phase of the local oscillator of the PVN does not modify the circadian signals from the SCN that dictate the phase of the melatonin rhythm. The results from comparisons of DA and NA grass rats also support this interpretation, since both chronotypes showed a nocturnal increase in melatonin, even though they had PER protein rhythms in the PVN that were 180^0 out of phase. Thus, it is possible that the inputs to the PVN from the

SCN that control the pineal gland in all mammalian species and chronotypes are directed to relay neurons of the PVN that lack rhythms in clock-gene expression. Alternatively, the PVN neurons that relay SCN information to the pineal may show a constant phase across species, and within-species chronotypes, even when the majority of the PVN neurons show phase reversals in clock-gene expression when diurnal and nocturnal species or chronotypes are compared.

The retention of a nocturnal elevation of circulating melatonin shown by NA grass rats is consistent with the results of studies with degus (Vivanco et al., 2007), another predominantly diurnal species in which individuals become night-active without losing the nocturnal elevation in circulating melatonin. In these cases, increased secretion of melatonin occurs during the active phase, which is the opposite of what diurnal species normally experience (Reiter, 1991b). NA grass rats, although they show substantial night-time wakefulness, they nevertheless display an increase in sleep-bout duration during the late night, between ZT16 and ZT20 (Schwartz and Smale, 2005), which includes a time of elevated melatonin levels in these animals (i.e., ZT18, present results). In many diurnal species such as birds, fish and non-human primates, melatonin acts as a powerful hypnotic (Mintz et al., 1998; Zhdanova, 2005; Rihel and Schier, 2013). Thus, it is possible that the increased sleep duration of NA grass rats in the late night is due to putative sleep promoting actions of melatonin in diurnal species. However, this possibility remains speculative, since in grass rats pinealectomy does not affect sleep or circadian rhythms (unpublished observations from Smale's laboratory). Interestingly degus that become night-active continue to show responses to melatonin, which include a drop in body temperature, typical of that predominantly diurnal species (Vivanco et al., 2007).

Finally, although the reversal of the phase of the PER protein rhythm of the PVN did not prevent the nocturnal elevation of melatonin in NA grass rats, melatonin levels were higher in these animals compared to those of the other day-active groups, both in the morning and at night. Since I

used only two sampling times, I am not able to rule out the possibility of a phase shift in the nevertheless nocturnal melatonin rhythm of the NA grass rats, as is the case for degus that become nocturnal (Otalora et al., 2010). However, since NA grass rats with access to wheels are significantly more active than diurnal grass rats (Blanchong et al., 1999), and since exercise results in elevated levels of melatonin (Escames et al., 2012), one viable alternative explanation is that the elevated levels of melatonin in the NA animals stem from their relatively high levels of activity. This explanation also fits the observation that DA grass rats with running wheels show melatonin levels that, although lower than those of NA grass rats, are significantly higher than those of diurnal sedentary grass rats.

Conclusion

In summary, I found that the phase of rhythms in PER protein expression in the PVN of diurnal grass rats is 180^0 out of phase to that of nocturnal species, thus providing a potential mechanism to explain species phase differences in rhythmic autonomic functions controlled by this nucleus. Since the phase of the melatonin rhythm is more or less the same for diurnal and nocturnal species, the phase of the PVN oscillator does not appear to influence the control of the pineal gland by the SCN. This assertion is supported by the observation that when grass rats adopt a nocturnal activity profile, the nocturnal elevation in melatonin production is retained, even though there is a phase reversal in the rhythm of PER protein expression in the PVN. Taking into account other observations about clock-gene rhythms in other hypothalamic nuclei in NA animals (Ramanathan et al., 2010b, Nunez et al., 2012), the reversal in the phase of the PER protein rhythm of the PVN of these animals may result in an internal circadian desynchrony affecting the control of sleep and autonomic functions. Thus, the NA grass rats presents itself as a useful model to study the causes of

human pathologies associated with the voluntary display of activity during the natural rest phase of diurnal species (Pietroiusti et al., 2010, Erren, 2013) .

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CHAPTER 3

Circadian Modulation of Memory and Plasticity Gene Products in a Diurnal Species

The work presented in this chapter has been published in manuscript form.
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Introduction

Endogenous rhythms with periods close to 24 hours are referred to as circadian rhythms (Ko and Takahashi, 2006). Many physiological and behavioral processes show circadian rhythms that are synchronized to environmental stimuli. In mammals, circadian rhythms in physiology and behavior depend upon the integrity of a master circadian oscillator residing within the hypothalamic suprachiasmatic nucleus (SCN)(Stephan and Zucker, 1972).The SCN receives inputs from specialized photoreceptive retinal ganglion cells via the retinohypothalmic tract, and this retinal projection entrains the SCN clock to the light-dark cycle (Welsh et al., 2010). Individual neurons within the SCN can generate circadian oscillations, but the expression of robust circadian rhythms depends upon the coupling of multiple cell autonomous oscillators within the SCN (Herzog et al., 1998; Herzog and Schwartz, 2002). Within neurons of the SCN, several genes are expressed in a circadian rhythmic fashion; some of them are called clock genes. Clock genes include Circadian Locomotor Output Cycles Kaput (CLOCK), brain and muscle aryl hydrocarbon receptor nuclear translocator like (BMAL1), Period (PER1-3) and Cryptochrome (CRY 1 and 2). Clock gene oscillation has been described as an autoregulatory transcriptional and translational feedback loop (Ko and Takahashi, 2006).

In addition to the rhythmic expression of clock genes in the SCN, there is evidence of local rhythmic expression of those same genes in areas of the brain outside of the SCN, and some of those areas, such as the hippocampus, are important for learning and memory (Amir and Stewart, 2009; Angeles-Castellanos et al., 2007; Duncan et al., 2013; Gilhooley et al., 2011; Ikeno et al., 2013; Li et al., 2013; Otalora et al., 2013; Ramanathan et al., 2010b; Roth and Sweatt, 2008). For example rhythmic expression of PER2 has been documented in the hippocampus (hipp), amygdala (amyg) and dorsal striatum (DSt) of nocturnal species (Amir and Stewart, 2009). These brain regions have been implicated in the mediation of relational learning, emotional learning and memory

consolidation, and procedural learning. Recently, using a diurnal animal model, the grass rat (*Arvicanthis niloticus*), Ramanathan et al 2010 described rhythms of PER1 and 2 expression in the hippocampus, amyg and DSt that were 180^0 out of phase from those reported for nocturnal rodents (Ramanathan et al., 2008a; Ramanathan et al., 2008b; Ramanathan et al., 2010a; Ramanathan et al., 2010b), although the phase of these rhythms is the same in the SCN of grass rats and nocturnal rodents ((Ramanathan et al., 2006) and as reviewed in (Smale et al., 2003) In the degu, another diurnal mammal, the peak of Per2 expression in the hippocampus is also out of phase with that of nocturnal species (Otalora et al., 2013). The results of work with diurnal rodents has been corroborated by evidence from post mortem human studies that report the same pattern of expression within the human hippocampus (Li et al., 2013; Otalora et al., 2013).

The circadian system modulates cognitive abilities (Daan, 2000; Gerstner et al., 2009a; Gerstner and Yin, 2010). For instance intervals of 24 hr from the time of initial learning are associated with optimal retention (Holloway and Wansley, 1973; Kamin, 1957; Kamin, 1963) and this phenomenon is eliminated by lesions (Stephan and Kovacevic, 1978) or disruptions (Ruby et al., 2008) of the SCN, thus suggesting a role for a circadian oscillator rather than dependence upon an interval timer. In aged mammals, weak circadian activity rhythms have been linked to poor performance on hippocampal dependent tasks, such as the Morris water maze (MWM) (Antoniadis et al., 2000; Haley et al., 2009), and advancing or delaying the onset of the illumination cycle during or after training (Fekete et al., 1985; Tapp and Holloway, 1981) can lead to deficits in learning and memory (Craig and McDonald, 2008; Devan et al., 2001; Fekete et al., 1985; Tapp and Holloway, 1981). These observations show the importance of robust and stable circadian rhythms for optimal acquisition and retention of a variety of tasks (Daan, 2000; Devan et al., 2001; Smarr et al., 2014; Winocur and Hasher, 2004), which are likely to depend upon multiple neural systems and processes (Packard and Knowlton, 2002).

There is evidence from both animal and human studies that time of training and or/testing can affect learning, retention and performance (Folkard et al., 1985; Folkard, 1990; Gerstner et al., 2009b). While there is no consensus on the optimal phase for performance across learning tasks, the trend is that both nocturnal and diurnal species perform best when trained and tested during their active phase and the poorest performance is observed when tested or trained during the inactive phase. But there are a few exceptions to this general finding in both human and animal studies. For example in human night-shift workers who have successfully adapted to working through the normal rest phase show a phase shift of peak performance to match the new nocturnal active phase (Folkard and Monk, 1985). Most animal studies have used only nocturnal species, and among those studies there is lack of consensus with respect to an optimal circadian phase for cognitive functions. One example of this lack of agreement comes from fear conditioning studies done using mice. Thus, for mice, there are two reports of superior contextual fear conditioning during the day (Chaudhury and Colwell, 2002; Eckel-Mahan et al., 2008), but there is also a report of the opposite effect of time of day (Valentinuzzi et al., 2001). One possible explanation for this discrepancy could be the use of the C57 mouse strain. Under natural conditions C57 mice show remarkable fluctuations in the distribution of activity across the day and night (Daan et al., 2011). Thus, C57 mice may have a more flexible optimal phase for learning due to their more plastic chronotype. In rats, the data support a positive nocturnal bias for the acquisition and retention of a signal-detection task and for retention of the MWM (Gritton et al., 2012), even when time of day differences in acquisition of the MWM task are absent (Valentinuzzi et al., 2004). Several studies with humans report the presence of performance rhythms that peak during the day and wane over the course of the afternoon, with the worst performance being observed during the evening hours (Furnham and Rawles, 1988; Oakhill, 1988; Payne, 1989). However, peaks in human performance are sensitive to the memory load of specific tasks, the individual's fluid intelligence, level of practice and age. The higher the memory

load, the earlier in the day the performance generally peaks (Folkard et al., 1983). Studies using non-human diurnal species also show optimal acquisition and retention during the light phase of the illumination cycle, which represents the opposite of what is shown by nocturnal rats (Haley et al., 2009; Lehmann et al., 2011; Valentinuzzi and Ferrari, 1997). With some exceptions (i.e., contextual fear conditioning in mice; see above), hippocampal dependent tasks in both rodent and human studies feature optimal performance when individuals are tested during the active phase of the species (Folkard and Monk, 1985; Furnham and Rawles, 1988; Gritton et al., 2012; Hoffmann, 1992; Payne, 1989; Testu, 1986; Valentinuzzi et al., 2004).

The circadian system could modulate cognitive functions via rhythmic clock- and/or plasticity-gene expression in the hippocampus. Thus, deletion of clock genes in the hippocampus results in deficits in hippocampal dependent learning and memory (De Bundel et al., 2013; Garcia et al., 2000; Jilg et al., 2010; Kondratova et al., 2010; Rawashdeh and Stehle, 2010), although exceptions exist (Mulder et al., 2013; Zueger et al., 2006), and some effects are task-specific (De Bundel et al., 2013). Rhythmic plasticity-gene expression in the hippocampus is also a potential mechanism for the circadian control of learning and memory. Gene products that may control different aspects of neural plasticity include, cyclic adenosine monophosphate (cAMP), activity-regulated cytoskeleton-associated (Arc), mitogen activated protein kinase (MAPK), phosphorylated cyclic adenosine monophosphate response element-binding protein (pCREB), phosphorylated Extracellular signal-regulated kinase (pERK), SCN circadian oscillatory protein (SCOP), brain derived neurotrophic factor (BDNF) and tyrosine kinase receptor (TrkB) (Berchtold et al., 1999; Bova et al., 1998; Cirelli and Tononi, 2000a; Cirelli and Tononi, 2000b; Dolci et al., 2003; Eckel-Mahan et al., 2008; Hamatake et al., 2011; Ikeno et al., 2013; Katoh-Semba et al., 2008; Roth and Sweatt, 2008; Selcher et al., 1999). Each of the above gene products is involved in signaling pathways

important for plasticity. For example, once the TrkB receptor is occupied by BDNF, a number of signaling cascades, including phosphatidylinositol 3-kinase [PI3-K], MAPK and phospholipase C-$_{\gamma}$[PLC$_{\gamma}$] as reviewed in Tapia-Arancibia (Tapia-Arancibia et al., 2004) are activated. Activation of these signaling cascades has been shown to be involved in many processes important for learning and neural plasticity, including hippocampal long-term potentiation (Hall et al., 2000; Tyler et al., 2002; Yamada and Nabeshima, 2003). Similar to the lack of consensus about optimal time of day for learning and retention, there is discordance in the evidence for the phase of peak expression of BDNF/Trkb in the hippocampus of nocturnal rodents, this is particularly true among reports that measure mRNA abundance across the day-night cycle (Berchtold et al., 1999; Bova et al., 1998; Golini et al., 2012; Ikeno et al., 2013; Schaaf et al., 2000). There are also some cases where there is a misalignment between mRNA and protein expression. In these cases an abundance of mRNA does not precede an abundance of protein expression (Bova et al., 1998; Cirelli and Tononi, 2000a; Dolci et al., 2003; Liang et al., 1998; Schaaf et al., 2000). The profile that emerges from the study of protein abundance is more consistent, showing peak hippocampal expression of BDNF/TrkB during the dark phase in adult nocturnal rats (Dolci et al., 2003; Hamatake et al., 2011; Katoh-Semba et al., 2008). Rhythmic expression of BDNF and TrkB may mediate circadian modulation of cognitive functions. However, knowledge about the rhythmic patterns of expression of these products is limited to the hippocampus of nocturnal species.

Here we used grass rats to investigate two aspects of the circadian regulation of hippocampal-dependent learning in a diurnal mammal. First, we used the MWM to determine if time of training affects performance and retention of a spatial navigation task in a diurnal species. The MWM is the gold standard for testing hippocampal dependent learning and memory in rodents, and does not

affect circadian entrainment (Glaser, 1910; Gritton et al., 2012; Morris, 1981). We used the reference memory version of the MWM to investigate time of day effects on the animals' ability and latency to find the hidden platform, and their swim path, swim velocity, and display of thigmotaxis. By analyzing these measures, we tested for time of day differences in cognitive performance, motor abilities, motivation and, thigmotaxis, which serves as a measure of fear or anxiety. Second, we determined expression patterns of BDNF and TrkB protein in the hippocampus of this diurnal species. There is evidence of functional heterogeneity and possible differential rhythmic expression of both plasticity and clock genes in the hippocampal formation (Berchtold et al., 1999; Bova et al., 1998; Cirelli and Tononi, 2000b; Ikeno et al., 2013; Schaaf et al., 2000). Therefore, we evaluated the patterns of protein expression in three distinct regions of the hippocampus, the CA1 area, the dorsal blade of the DG and the hilus. Finally we expanded the BDNF and TrkB anatomical work to include other regions important for learning and memory, i.e., the dorsal striatum (DSt) and baso-lateral amygdala (BLA).

Experimental Procedures

Animals

Male grass rats (3 – 5 months old; $n = 24$ for Experiment 1; $n = 36$ for Experiment 2) from our breeding colony at Michigan State University were housed individually in Plexiglas cages (34 × 28 × 17 cm), under a 12:12 h light/dark (LD) cycle, with lights on at Zeitgeber time (ZT) 0, with dim red lights on at all times 7lux) and *ab libitum* access to food (PMI Nutrition Prolab RMH 2000, Brentwood, MO, USA) and water. All experiments were performed in compliance with guidelines established by the Michigan State University All University Committee on Animal Use and Care, and the National Institute of Health guide for the Care and Use of Laboratory Animals.

Experiment 1:

Morris Water Maze

Handling

Grass rats, even after many generations in captivity, are not domesticated and are more reactive than common laboratory rodents. Therefore, the animals were gently handled daily for at least two weeks prior to the start of water maze training to reduce the effects of handling stress on learning. To transfer the animals to and from the water maze we used clear-plastic salad tongs as previously described (Walker, 2011) . The animals were habituated to contact with the plastic tongs during the two weeks of daily handling before the start of behavioral testing.

Testing Room Conditions and Apparatus

Behavioral testing took place in a room with the same LD cycle and illumination conditions of the animals' colony room. Black images (i.e. star, circle, square, and a triangle) were fixed to white walls to serve as high-contrast extra-maze cues. The location of these extra maze cues remained constant throughout the experiment. The testing apparatus was a circular pool, 140cm in diameter, which was filled with $22^0 \pm 5^0$ water. Pool wall height was 37.46 cm; the pool was filled with 19.05cm of water. The water was made opaque with nontoxic paint (ArtMinds ^{TM}). A clear 15.24 cm wide platform was placed in the center of the SW quadrant. The hidden platform was approximately 1.5cm below the surface of the water and invisible to grass rats.

Training

Animals were randomly assigned to either the AM or PM groups (n = 12/ZT) and trained for 6 consecutive days. The AM group was trained and tested at ZT4 while the PM group was tested and trained at ZT 16. Daily training sessions consisted of 4 trials. Each trial started when the grass

rat entered the water and ended when it found the platform or after 120 seconds had lapsed. Grass rats were placed in the water facing the walls of the pool at randomly assigned locations, except the location that that would later be used for the acquisition and retention probes (see below). Grass rats that failed to find the platform in 120 seconds were gently guided to the platform and allowed to rest for 15 seconds before the start of the next trial. At the end of the fourth trial, the animals were dried off manually with an absorbent towel and returned to their home cage.

Acquisition and Retention Probe Test

An acquisition probe test was given on the 7th day, following the 6 days of training. Grass rats were placed in the pool in a location, that had not been used during training and given 60 seconds to find the hidden platform. After the acquisition probe, grass rats were returned to their home cage. Fourteen days later the retention probe test was given in an identical manner to that of the acquisition probe.

Behavioral Quantification and Statistical Analysis

All training trials and probe tests were recorded and analyzed using the Noldus EthoVision-system (version 8.5; Noldus®). The proportion of grass rats to reach the platform, swim path lengths, swim velocity and latency to platform quadrant, were calculated. For the acquisition and retention probes, the proportion of animals showing thigmotaxis was also determined. In order to monitor thigmotaxic behavior, the pool was divided during analysis into two rings. Time spent swimming in the 10.5cm wide ring closest to the edge of the pool was quantified, and grass rats that spent more than 30secs swimming in the thigmotaxic zone were characterized as displaying thigmotaxis. The latencies to reach the platform were recorded, but were not used for group comparisons since not all animals reached the platform within the 2 minutes of each trial.

The Cochrans Q test was used to examine the effects of training days on the proportion of grass rats that found the platform over the course of the training. This analysis was performed for the AM and PM groups separately using both the average of the four trials per day or just for the first trial of each training day (Cochran and Cox, 1957; Conover, 1999). Chi squared tests were used to compare the AM and PM groups with respect to proportion of the animals reaching the platform (all trials) or showing thigmotaxic behavior during acquisition and retention probes. Two way analyses of variance (ANOVA) were used to examine the main effects of training day and time of training and the interaction of these two factors on swim path lengths, swim velocity and latency to platform quadrant during trial 1 of each training day and for the average of trials 1-4 for each training day. Student's t tests for independent samples were used to determine the effect of the time of training on swim path lengths, swim velocity and latency to platform quadrant, for both acquisition and retention probes. All statistical analyses for both experiments used SPSS version 17 software, and all differences were considered statistically significant when P was equal to or less than 0.05, using two-tailed probabilities when pertinent.

Experiment 2:

Tissue collection and immunocytochemistry

At 4-h intervals from ZT2 to ZT22, animals (n = 6/ZT) were deeply anesthetized with an intraperitoneal injection of 400mg/kg of sodium pentobarbital (Ovation Pharmaceutical, Deerfield, IL, USA) and perfused transcardially with 0.01 M phosphate buffered saline (PBS; pH 7.2) followed by 4% paraformaldehyde–lysine–sodium periodate (PLP) in 0.1 M phosphate buffer (Sigma, St Louis, MO USA). Brains were removed, post-fixed for 4-8 h and then transferred to 20% sucrose for 24h, before storing them in cryoprotectant at −20 °C. Sections (30 μm; coronal plane) were obtained using a freezing sliding microtome and stored in cryoprotectant until the

immunocytochemical (ICC) procedure for either BDNF or TrkB detection was performed on every other section.

Sections were taken out of cryoprotectant and stored overnight in 0.01M PBS at 4^0C. Just prior to undergoing ICC procedures, sections were rinsed three times (10 min/rinse) in fresh 0.01 M PBS. Unless otherwise noted, the sections were rinsed three times (10 min/rinse) in 0.01 M PBS between all steps of the ICC procedures. All steps were carried out at room temperature unless noted otherwise. Free-floating sections containing the hippocampus (CA1, hilus, and dorsal dentate blade) were rinsed in 0.01 M PBS, blocked for 1 h using 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) in PBS and incubated for 48 h in a rabbit anti-BDNF antibody at 4^0C (Chemicon/Millipore, Temecula, CA, USA; diluted 1:10,000 in PBS and 3% NGS). For detecting TrkB immunoreactivity, the same procedure was followed (rabbit polyclonal antibody against the carboxyl terminal domain of TrkB receptor ($trkB_{794}$) commercially available from Santa Cruz Biotechnologies, Santa Cruz, CA. U.S.A). All sections were then incubated for 1 h in a goat anti-rabbit biotinylated antibody (Vector Labs, Burlingame, CA, USA; diluted 1:200 in PBS and 3% NGS), and then for 1 h in avidin–biotin peroxidase complex (AB complex, Vector Laboratories, Burlingame, CA, USA; in PBS). After three rinses (10 min/rinse) in Acetate buffer (pH=7.2), the sections were reacted with 0.025% diaminobenzidine (DAB; Sigma-Aldrich) enhanced with 2.5% nickel sulfate (Sigma-Aldrich) in Tris buffer with 3% hydrogen peroxide (J.T. Baker, Phillipsburg, NJ, USA) for 12 min. The reaction was followed by three 10 min rinses in Acetate buffer (pH=7.2), All sections were mounted onto gelatin-coated slides, dehydrated, and coverslipped with Permount (Sigma-Aldrich). A set of control sections was selected and incubated in PBS and 3% NGS without anti-BDNF or anti-TrkB antibody at 4^0C. Immunoreactivity was undetectable in sections that were processed in the absence of primary antibody.

Quantitative and Statistical Analysis

A single section containing the three targeted hippocampal regions, i.e., CA1 and the hilus and dorsal blade of the DG, was identified for each animal using the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 2007). The approximate level of the sections was −2.80 mm from bregma. For each region, BDNF and TrkB labeled cells were counted bilaterally within an area defined by a 500×100 μm^2 counting box by an investigator unaware of the sampling time associated with each section. All BDNF counts were done using a light microscope (Leitz, Laborlux S, Wetzlar, Germany; 25X objective). Camera lucida drawings were made of visually identified labeled cells within the boxes in each area. TrkB counts were performed using photomicrographs that were taken of each region with a digital camera (MBF Bioscience Inc, 2007) attached to a Zeiss light microscope (Carl Zeiss, Göttingen, Germany). Photomicrographs were arranged in Photoshop CS5. For every region, TrkB labeled cells were counted within the region specific counting boxes. Bilateral cell counts were obtained using NIH Image J software (National Institute of Health, Bethesda, MD, USA). For each region counted for TrkB staining, circularity (0-10) and contrast intensity thresholds were used to distinguish immunoreactive labeled cells from background staining. Total bilateral counts of each area were subjected to one-way ANOVAs to assess the effect of ZT on BDNF and TrkB expression in each region. Significant *F* ratios were followed by individual comparisons using Fisher's Least Significant Difference (LSD) tests.

Experiment 3:

For counts of BDNF-immunoreactive or TrkB-immunoreactive cells, single sections containing each of the two brain regions were identified using the rat brain atlas of Paxinos and Watson (2007). For each region sampled, the approximate level of the section is given in reference to bregma from that atlas. To quantify BDNF or TrkB in these brain regions, immunoreactive

nuclear staining was counted bilaterally in the DSt (Bregma=0.70 mm), and BLA (Bregma=−2.12 mm). For each region, BDNF labeled cells were counted within a region defined by a counting box with the following dimensions: DSt (800×1000 μm^2) and BLA (800×1000 μm^2). All counts were done using the techniques described above in Experiment 2.

Total bilateral counts of each area were used for the statistical analysis. One-way analyses of variance (ANOVA) were used to assess the effect of ZT on BDNF and TrkB expression in each region. Significant *F* ratios were followed by comparisons between ZTs using Fisher's Least Significant Difference (LSD) tests. Differences were considered statistically significant when P was equal to or less than 0.05. The SPSS version 17 program was used to run all the analyses.

Results

Experiment 1:

Learning Curve

Figure 2.1 shows the proportion of animals that reached the platform on the first trial of each day of training (Panel A) and for the average of the four trials for each day of training (Panel B). For both the AM and PM groups, Cochran's Q analyses detected a significant effect of days of training, for the data shown on both panels A and B (See Figure 1 legend for statistical details). Comparisons between the AM and PM groups for each day of training found no significant differences for the data shown in panels A and B (all $\chi^2 s_{(1, N=24)}$ = 0.00- 3.22, all *ps*> 0.07). Table one summarizes the results of the 2X6 ANOVAs for the other three dependent variables using the averages of trials 1-4. Although there was a significant effect of training day for all of the dependent variables, there was not a significant effect of time of training and no significant interaction effects. The same results (data and analyses not shown) were obtained when the analysis was limited to the first trial of each training day.

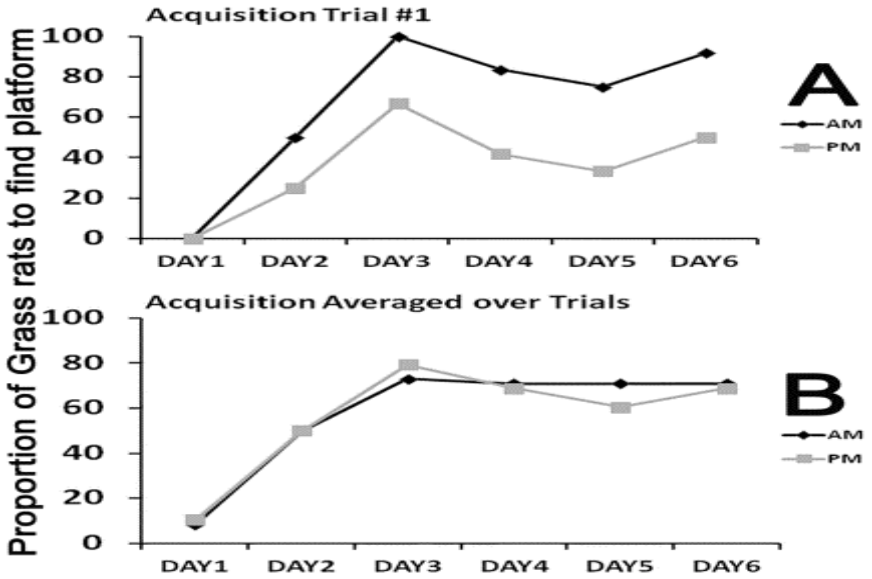

Figure 2.1 Time of training effects during acquisition trials, Cochran's Q analyses showed a significant effect of training days for both trial 1 data (panel A; AM group: Q(5)=31.957 and PM group: Q(5)=14.412, ps < .001) and trials 1-4 averaged data (panel B; AM group: Q(5)=69.985, and PM group: Q(5)=60.255,p <.001), but Chi squared analysis showed no effect of time of training on proportion of grass rats to find the platform each day for both data sets ($\chi^2_{(1,N=24)}$ = 0.00-3.227, all *ps*>0.07

Table1 Time of Day Effects over Acquisition Trials

Dependent Variable	Main effect of time of training	Main effect of days	Interaction
Swim Path	$F_{1,132}$=0.516,NS	$F_{5,132}$=7.357,p<0.05	NS
Swim Velocity	$F_{1,132}$=0.063,NS	$F_{5,132}$=6.655,p<0.05	NS
Platform Quadrant	$F_{1,132}$=0.564, NS	$F_{5,132}$=37.553,p<0.05	NS

Acquisition Probe

Figure 2.2 panel A shows the proportion of animals that reached the platform during the acquisition probe. Chi squared analysis found no significant difference between the AM and PM groups ($\chi 2_{(1, N=24)} = .202$, $p > 0.05$) (Figure 2.2A). Table two summarizes the analyses for the other four dependent variables. No significant differences between the AM and PM groups were detected by the t-tests for swim path, swim velocity, or latency to platform quadrant. Similarly the proportion of animals that displayed thigmotaxis did not differ between the AM and PM groups.

Table2 Time of Day Effects on Acquisition

Dependent Variable	T-test or $\chi 2$
Swim Path	Acquisition probe ($t_{(22)}=1.959$, $p>0.05$)
Swim Velocity	Acquisition probe ($t_{(22)}=1.473$, $p>0.05$)
Platform Quadrant	Acquisition probe ($t_{(22)}=0.627$, $p>0.05$)
Thigmotaxis	Acquisition probe ($\chi^2_{(1,N=24)} = 1.510$, $p > 0.05$)

Retention Probe

Figure 2.2 A and B shows the proportion of animals that reached the platform during the retention probe. Chi squared analysis showed a significant effect of time of training on this measure. The mean (+/- SEM) latency to reach the platform was 45.0 +/- 7.0 seconds for the AM group; the two animals from the PM group that reached the platform during the retention probe had latencies of 60 seconds. Figure 2.3 shows the data for the other four dependent variables. For swim path and latency to platform quadrant there was a significant effect of training time with superior performance

for the AM group. No significant differences were found for swim velocity, but thigmotaxis was significantly more frequent for the PM-trained animals. See Figure 2.3 legend for statistical details.

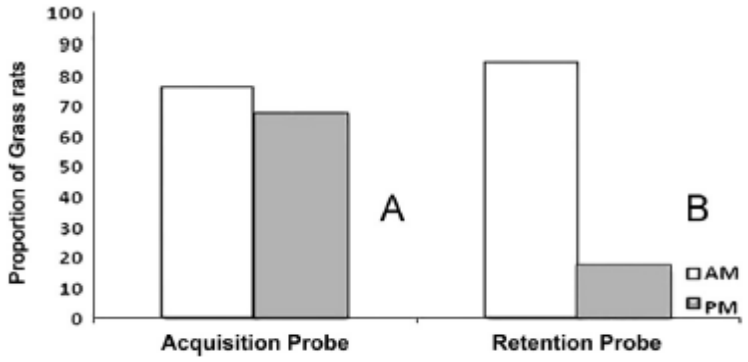

Figure 2.2 Differential effects of time of training on acquisition and retention probe test performance

Chi squared analyses revealed that although there was no effect of ZT on the proportion of grass rats to find the platform during the acquisition probe, ($\chi^2_{(1, N=24)}$= .202, $p > 0.05$) (panel A), during the retention probe (panel B) there was an effect of ZT ($\chi^2_{(1, N=24)}$=10.667, $p < 0.001$

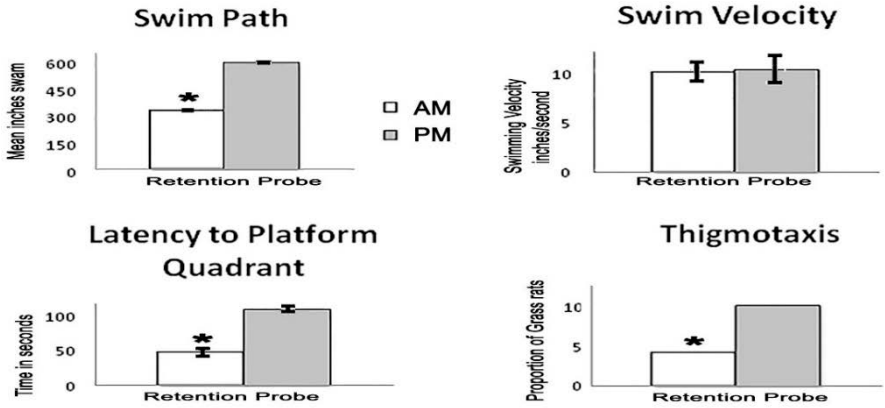

Figure 2.3 Time of day effects on retention probe

There was a significant effect of time of training on all measures except swim velocity, $t_{(22)}$ =.256, $p > 0.05$) during the retention probe. Significant time of training effects were seen in swim path, ($t_{(22)}$ =50.30, $p < 0.001$) and latency to platform quadrant, ($t_{(22)}$ =9.602, $p < 0.001$). AM trained grass rats were faster to the platform quadrant and had shorter swim paths than PM trained grass rats. Chi squared analysis revealed a significant effect of training time on the proportion of grass rats to engage in thigmotaxis ($\chi^2_{(1,N=24)}$ = 6.171, $p < 0.01$). Fewer AM trained grass rats engaged in thigmotaxic behavior than PM trained grass rats. Significant differences between AM and PM groups are denoted by asterisks.

Experiment 2:

For the number of cells expressing BDNF (Fig. 2.4 Center Panels) ANOVAs revealed a significant main effect of ZT in the CA1 ($F_{5,30}$=427.27,p<0.0001), hilus ($F_{5,30}$=161.140, p<0.0001), and dorsal blade of the DG ($F_{5,30}$=57.343,p<0.0001).In the CA1, BDNF expression peaked at ZT10. In the hilus and dorsal blade of the DG, BDNF expression was elevated at both ZT10 and ZT 14. For the number of cells expressing TrkB (Fig. 2.4 Right Panels), ANOVAs revealed a significant main effect of ZT in the CA1 ($F_{5,36}$=28.81,p<0.0001), hilus ($F_{5,26}$=7.21, p<0.0001), and the dorsal blade of the DG ($F_{5,31}$=1108.716, p<0.001). In the CA1, hilus and dorsal blade of the DG, TrkB expression peaked at ZT6. Fig 2.4. shows representative examples of BDNF (Center Panels) and of TrkB (Right Panels) staining in the three brain regions. For both BDNF/TrkB, cellular staining resembles what has been shown in nocturnal species (Cirelli and Tononi, 2000a; Dolci et al., 2003).

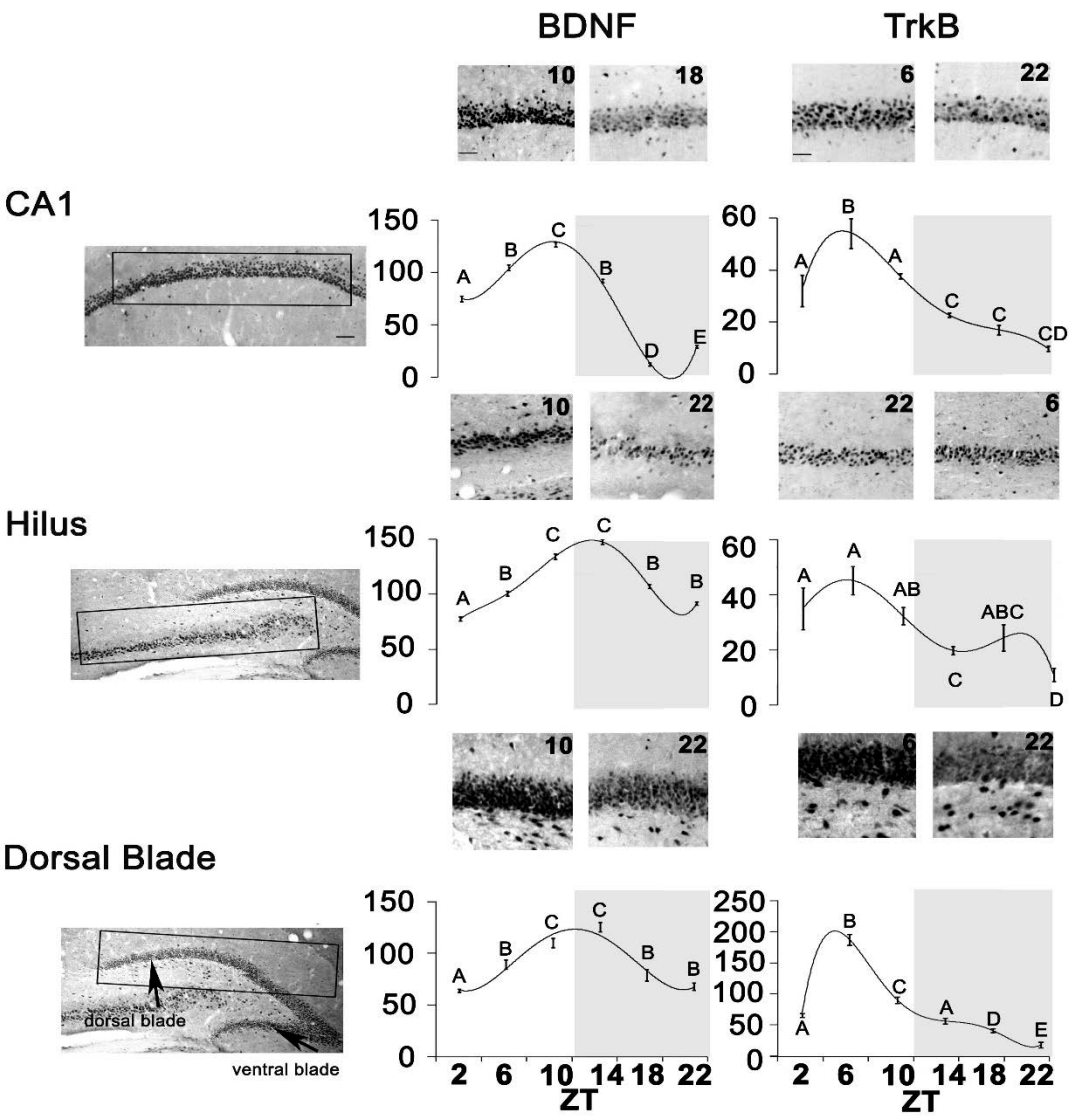

Figure 2.4 Rhythms in Plasticity gene products in the hippocampus
Left Panels: Sampling boxes(rectangles) were used for cell counts in of the CA1, dorsal blades of the DG, and hilus as described in Materials and Methods. Anatomical boundaries are based on Paxinos and Watson (1997). CA1, DG, dentate gyrus; ZT, Zeitgeber Time. Center and Right Panels: Photomicrographs depicting BDNF and TrkB expression at two different ZTs. Scale bar represent 200µm Line charts showing the mean (± SEM) number of BDNF (Center) and TrkB (Right) expressing cells for each ZT in the CA1, dorsal blade of the DG, and hilus of male grass rats. Significant differences between ZTs ($p<0.001$) are noted by different letters. Grey region on each chart indicates the dark phase of the cycle

Experiment 3:

BDNF/TrkB expression in extra-hippocampal regions associated with learning and memory

Fig. 2.5 shows representative examples of BDNF (right side of panel) and TrkB (left side of panel) staining in the DSt and the BLA. For the number of cells expressing BDNF one-way ANOVAS detected a significant main effect of ZT in the DSt ($F_{5,30}$=17.63,p<0.0001), and BLA ($F_{5,30}$=23.793, p<0.0001). For both the DSt, and BLA, BDNF expression peaked at ZT10.The BLA has an additional elevation seen during the late dark phase at ZT22. For the number of cells expressing TrkB, one-way ANOVAS detected a significant main effect of ZT in the DSt ($F_{5,25}$=4.416,p<0.005), and BLA ($F_{5,20}$=6.875, p<0.001).For the DSt, TrkB expression peaked at ZT6. For the BLA, TrkB expression was also elevated at ZT6 but the maximum expression was seen at ZT10.

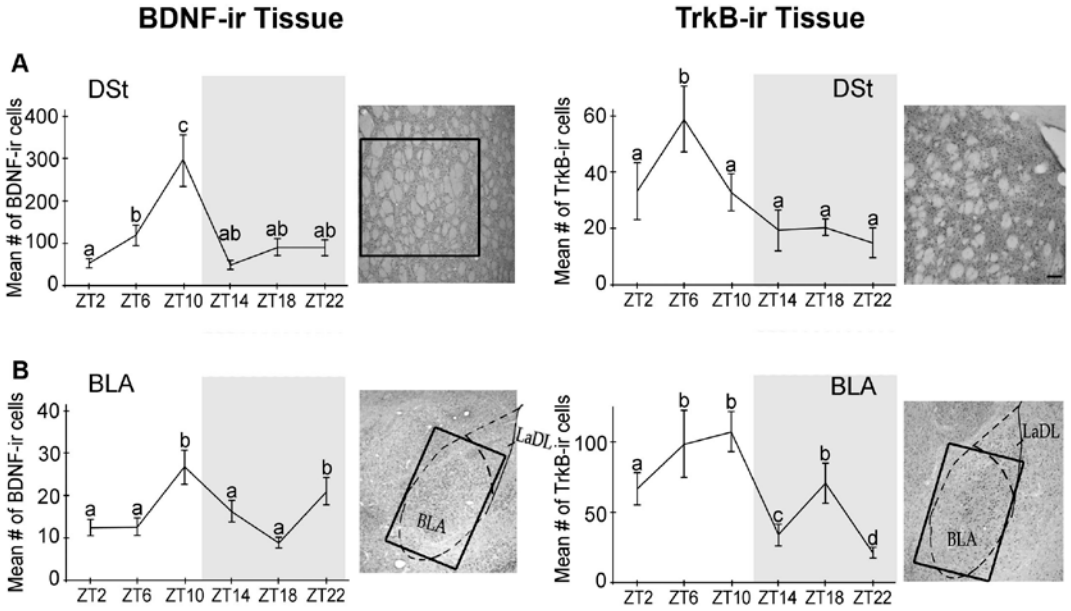

Figure 2.5. Rhythms in Plasticity gene products in the DSt and BLA Sampling boxes (black squares) were used for cell counts in of the DSt, and BLA as described in experimental procedures. Anatomical boundaries are based on Paxinos and Watson (1997). IMG:amygdaloid intramedullary gray; BLA: basolateral amygdale; LaDL,dorsolateral part of the lateral amygdaloid. DSt, dorsal striatum; ZT, zeitgeber. Photomicrographs depicting regions counted for Trk B (right side) expression. Representative Trk B expression seen here was similar to BDNF). Scale bar represent 200μm. Line charts showing the mean (+ SEM) number of BDNF (left panel) and TrkB (right panel) expressing cells for each ZT in the DSt and BLA of male grass rats. Significant differences between ZTs ($p<0.001$) are noted by different letters. Grey region on each chart indicates the dark cycle

Discussion

Our results provide evidence for an effect of time of training on the retention of a hippocampal dependent task and document the presence of rhythms in hippocampal protein expression of two plasticity gene products in a diurnal species. Our diurnal grass rats showed remarkably better retention of the MWM when trained and tested during the day, which is the opposite of what is seen in laboratory rats (Gritton et al., 2012); in that nocturnal species better retention is associated with night-time training and testing under similar conditions (Gritton et al., 2012). Thus, there is a positive bias for both nocturnal and diurnal species for optimal retention of

this task when the learning occurs during the active phase of the species. Interestingly, the rhythms of hippocampal BDNF and TrkB protein production of grass rats appear to be out of phase from those reported for laboratory rats (Cirelli and Tononi, 2000a; Dolci et al., 2003; Hamatake et al., 2011; Katoh-Semba et al., 2008). These data provide at least correlational evidence for the claim that that differences in optimal phase for retention of hippocampal dependent tasks between nocturnal and diurnal species stem from phase differences in the rhythmic expression of plasticity genes in the hippocampus. Information about differences in rhythmic expression of other plasticity genes, such as MAPK (Eckel-Mahan et al., 2008) between diurnal and nocturnal species would be of interests, however, direct manipulations of the rhythmic expression of these gene products are needed to establish a causal link between them and circadian regulation of learning and memory.

In grass rats there was no time of day difference in the acquisition of the MWM. Our animals showed no time of training differences in their ability to learn the location of the hidden platform and this insensitivity to time of training extended to all other measures that serve to assess motivation, motor functions (swim speed) and anxiety (thigmotaxis)(Clark et al., 2005a; D'Hooge and De Deyn, 2001; Jeltsch et al., 2001; Morris, 1984). Also, the behavior of the two groups was indistinguishable for the first trials of each training day, thus showing that all animals were equally able to remember over periods of 24 hours. These findings, and those previously reported for laboratory rats (Gritton et al., 2012), may be indicative of a selective role for the circadian system in affecting the long-term retention of hippocampal tasks. An explanation for the insensitivity to time of day for the acquisition of MWM task could be due to the moderate cognitive load of the MWM. Studies in both human and animals have shown that tasks that are not very cognitively demanding show little or no time of day differences during acquisition, whereas those that are highly demanding show more salient time of day differences (Folkard et al., 1983; Gritton et al., 2012). Work using more challenging hippocampal-dependent learning tasks (Eichenbaum et al., 1987; Eichenbaum et al., 1989;

Eichenbaum, 1992; Ergorul and Eichenbaum, 2006; Otto et al., 1991) could serve to clarify that issue.

Our results of time of training on long-term retention could be interpreted as a cognitive deficit stemming from an improper match between training/testing time and circadian rhythms in hippocampal neural plasticity, but other possibilities exist. For example on the retention probe, our PM animals displayed thigmotaxis, a measure of anxiety (Hostetter and Thomas, 1967; Huang et al., 2012; Simon et al., 1994; Treit and Fundytus, 1988), more frequently than the AM group. Thus, the poor performance of the animals tested at night could have been due to non-cognitive factors such as enhanced susceptibility to stress at that phase of the circadian cycle. However, counter to that interpretation is the fact that during all the other tests the PM animals did not differ from the AM ones in the display of thigmotaxis. Thus, it is possible that the apparent enhanced anxiety of the PM group during the retention probe was secondary to a memory deficit that made the situation appear less familiar to those animals. Our testing procedure resulted in different light intensities prevailing during the two testing times, raising the possibility that the poor performance of the PM group during the retention probe was caused by limited visual acuity. This is unlikely for two reasons. First, our PM animals did not show deficits during acquisition although the same lighting conditions were present. Second, there is evidence that rats and mice are able to navigate the MWM under both red light and complete darkness (Klapdor and VanderStaay, 1996; Valentinuzzi et al., 2004). Further, blind or visually impaired rodents are still able to learn and remember the location of the hidden platform in the MWM (Lindner et al., 1997; O'Steen et al., 1995; Spencer et al., 1995). Other non-cognitive deficits such as lack of motivation or diminished motor competence do not appear to be responsible for the differences seen in the retention probe, since swimming speed was not different between the two groups.

For experiment two, our main finding was the presence, in a diurnal mammal, of rhythmic expression of plasticity-gene products in three hippocampal regions known to play distinct roles in cognition (Clark et al., 2005a; Clark et al., 2005b; Dees and Kesner, 2013; Jeltsch et al., 2001; Kesner, 2013; McDonald and White, 1993; Morris et al., 2013; Morris et al., 1986; Packard and Knowlton, 2002; Sweatt, 2004). For all the hippocampal areas, the rhythms were about 180^0 out of phase with those reported in nocturnal laboratory rats. For nocturnal rodents, peaks in both BDNF and TrkB expression occur late in the dark period (Bova et al., 1998; Dolci et al., 2003), whereas, they occurred much earlier in our diurnal grass rats. Little is known about the relationship between the rhythms of BDNF and TrkB expression. Once the TrkB receptor is occupied by BDNF, a number of signaling cascades are activated (as reviewed in Tapia-Arancibia et al., 2004),which are involved in several processes important for learning and neural plasticity, including hippocampal long-term potentiation (Hall et al., 2000; Tyler et al., 2002; Yamada and Nabeshima, 2003). In our diurnal grass rats, the peak of TrkB expression within the hippocampal preceded that of BDNF by four hours, and a similar relationship between the two rhythms appears to exist for laboratory rats (Bova et al., 1998). Thus, the daily increase in receptor expression before the peak of BDNF may be a common feature of the mammalian hippocampal that results in optimal times of day for behavioral, physiological and morphological plasticity, which are reversed when nocturnal and diurnal species are compared.

The hippocampal data suggest that the phases of these rhythms correspond to the phase preference for the display of activity of each species, and thus may contribute to species differences in the circadian regulation of cognitive functions. However, for both nocturnal and diurnal species, we do not know if the daily fluctuations in the expression of plasticity genes are under the control of circadian oscillators, or are dependent upon either environmental influences or upon the prevalent

stage of vigilance of the organism at different times of day. Data on the rhythmic expression of these products obtained under constant environmental conditions and under experimental conditions that control for the influences of sleep and wakefulness are needed to test these alternative hypotheses.

The peak expression of BDNF/TrkB during the active phase of grass rats was not limited to the hipp. In Experiment 3, we also saw a distinct peak of expression of BDNF/TrkB during the active phase in the DSt. Unfortunately there are no studies of rhythms in BDNF or TrkB expression in the DSt of nocturnal animals, but increased striatal BDNF/TrkB expression has been linked to improvement in motor tasks (Cotman and Berchtold, 2002; Spires et al., 2004). Further, like in the hipp, the rhythms in plasticity-gene expression in the DSt were in phase with those for PER1 and 2 expression reported for this region (Ramanathan et al., 2010a). Thus, considering the role of the DSt in learning and memory, optimal acquisition or retention of motor or procedural tasks could be selectively facilitated by peak expression of BDNF/TrkB in the DSt during an animal's active phase.

While in grass rats rhythmic expression of BDNF/TrkB appears to have a distinct, single peak in the hipp and DSt, such is not the case for the BLA. There, the pattern of expression of both plasticity products features two separate peaks; one during the rest phase and one during the active phase (BDNF) and two elevations during the late light phase (TrkB), both with relatively low amplitude and relatively low overall abundance of labeled cells. Interestingly, rhythms of clock-gene expression, particularly those for PER1, are less robust in the BLA of grass rats, compared to those seen in other extra-SCN oscillators (Ramanathan et al., 2008a; Ramanathan et al., 2008b; Ramanathan et al., 2010a), a factor that may contribute to the low expression of plasticity-gene products seen here in that region. With respect to the bimodal aspect of the BDNF and TrkB rhythms of the BLA of our animals, it is possible that the elevation during the rest period represent a window for optimal acquisition of fear responses, whereas the diurnal peak may facilitate other

aspects of learning and memory that also involve the BLA. An argument for a facilitation of aversive conditioning during the rest phase of a species has been put forward using data from studies with mice (Chaudhury and Colwell, 2002; Eckel-Mahan et al., 2008) and flies (Gerstner et al., 2009a; Gerstner and Yin, 2010). This perspective remains very speculative, however, given the lack of substantial comparative data on the modulation of fear conditioning by time of day.

Future directions of this research with our diurnal model could include the examination of circadian regulation of hippocampal-independent memory that depend upon the integrity of other brain regions such as the amygdala and the DSt (McDonald and White, 1993), which also exhibit a phase reversal in PER1and 2 rhythms in grass rats compared to nocturnal mammals (Ramanathan et al., 2008a; Ramanathan et al., 2008b; Ramanathan et al., 2010a; Ramanathan et al., 2010b). Another direction for future studies could use grass rats to develop a diurnal animal model for understanding the cognitive deficits sometimes observed in human shift- and night-workers (Folkard, 1989; Gold et al., 1992). Interestingly, although strongly diurnal in the wild and under standard laboratory conditions (Blanchong et al., 1999; McElhinny et al., 1997), when given access to running wheels a subset of our grass rats voluntarily shifts to a predominantly nocturnal display of activity.(Blanchong et al., 1999). This remarkable shift in activity phase is accompanied by a phase reversal in the rhythmic expression of PER1 and 2 in many extra-SCN oscillators, including the hippocampus (Ramanathan et al., 2010b) . Understanding what effects these changes in the phase of activity rhythms and clock-gene rhythmic expression might have on rhythms of BDNF/TrkB expression and on the animals' cognitive functions could be instrumental in furthering our understanding of deficits seen in human night-shift workers and others who voluntary become active at night.(Barnard and Nolan, 2008; Czeisler, 2009). In summary, our results represent the first descriptions of time of day differences in hippocampal memory using a diurnal rodent model and of rhythms in the expression of plasticity-gene products in a diurnal brain. This information serves

to help us understand species differences in the circadian regulation of cognitive functions as well as time of day effects on human memory, and the cognitive deficits seen when circadian rhythms are disrupted, as in the case of human shift- or night- workers (Barnard and Nolan, 2008; Gold et al., 1992).

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CHAPTER 4

Cognitive Costs of Nocturnal Activity for a Diurnal Brain

Introduction

In Chapter 3, I established that training and testing in the morning results in better retention of a hippocampal dependent memory in the diurnal grass rats, and how these observations contrast with those from nocturnal laboratory rats (Gritton et al., 2012). I also established that the hippocampal expression of BDNF and its high-affinity receptor TrkB is rhythmic in grass rats and that the phase of those rhythms are apparently reversed from those of nocturnal laboratory rats (Hamatake et al., 2011; Katoh-Semba et al., 2008; Martin-Fairey and Nunez, 2014), as is the case for rhythms in hippocampal clock-gene expression (Ramanathan et al., 2010a).

In humans, optimal learning, retention and performance for most cognitive tasks is better in the morning than in the evening (Folkard et al., 1983; Folkard et al., 1985; Lipizzi et al., 2007; Preckel et al., 2013), and humans that engage in excessive nocturnal activity show deficits in cognitive functions (Randler and Frech, 2009) . Understanding of the neural mechanisms that support a day-time positive bias for cognitive functions in humans, or how those mechanisms are affected by nocturnal activity has been impeded by the lack of a convenient diurnal mammalian model. The data from Chapter 3 provide correlational data for the claim that optimal phase for learning a hippocampal-dependent task is dictated by the phase of rhythms in BDNF and TrkB in the hippocampus. In this chapter I extend that work to investigate the effects of nocturnal activity on the cognitive functions and hippocampal gene expression of the naturally diurnal grass rat.

The extension of the work of Chapter 3 presented here takes advantage of the "voluntary" adoption of a nocturnal activity that some grass rats display when they are able to run in wheels (Blanchong et al., 1999). As reviewed in Chapter 2, this reversal in activity is accompanied by a reversal of the phase of rhythms in PER1 and PER2 in the hippocampus, which then becomes similar to that of nocturnal species (Ramanathan et al., 2006; Ramanathan et al., 2008; Ramanathan et al., 2010b). One question addressed here using the DA and NA grass rats as models is whether the reversal in

both wheel running and hippocampal clock-gene rhythms affects the time for better retention of a hippocampal-dependent task identified by the results of Chapter 3. In humans, although nocturnal activity or "eveningness" results in cognitive deficits (Randler and Frech.,2009), there are also data showing that matching the time of testing to an individual's chronotype can prevent the deficit seen in humans that are predominantly active in the evening (Goldstein et al., 2007; Hahn et al., 2012). Thus, the present experimental design used two training and testing times (i.e., ZT 4 and ZT 16) to probe the possible interaction between chronotype and time of training/testing.

The results of the ICC experiments of Chapter 3 are consistent with the hypothesis that the phase of the rhythms in BDNF and TrkB influences the time for optimal retention in diurnal species. Thus, it is possible that when grass rats become NA, both the time of superior retention and the phase of rhythms of plasticity-gene expression in the hippocampus change to resemble those of nocturnal species, as it is the case for hippocampal rhythms in PER1 and PER2 (Ramanathan et al., 2010a; Ramanathan et al., 2010b). Alternatively, the profile of NA grass rats with respect to hippocampal rhythms may not universally resemble that of a nocturnal species, given the data reviewed in Chapter 2, which documents how NA animals appear to retain some diurnal features. That compromise between adoption of aspects of a nocturnal profile and retention of some diurnal features could result in cognitive deficits, rather than a simple phase reversal of the optimal time for learning and retention.

In addition to rhythmic expression of clock and plasticity genes, the hippocampus shows rhythms in neuronal activity reflected in the rhythmic expression of the immediate-early gene product cFOS. Thus, in both CA1 and the dentate gyrus of nocturnal laboratory rats cFOS expression is elevated at night (Angeles-Castellanos et al., 2007; Kononen et al., 1990). To compare the rhythmic patterns of hippocampal neuronal activity in DA and NA rats, and to probe further the possible circadian desynchrony of NA grass rats, I also monitored rhythms of cFOS expression in

the hippocampus of NA and DA grass rats. Thus in summary, the present study was designed to investigate whether the phase reversals in hippocampal clock gene expression of NA grass rats extends to both the optimal phase for acquisition and/or retention of a hippocampal-dependent task and to the rhythmic expression of gene products associated with neuronal activation and neural plasticity (e.g., c-FOS, BDNF and TrkB) in CA1, DG, and hilus.

Experimental Procedures

Animals

Animal housing and chronotype (day active: DA and night-active: NA) determination procedures were carried out as described in Chapter 2. Male grass rats from our breeding colony, 3 – 5 months old were used for all the experiments included in this chapter. All experiments were performed in compliance with guidelines established by the Michigan State University All University Committee on Animal Use and Care, and the National Institute of Health guide for the Care and Use of Laboratory Animals

Experiment 1:

Behavioral Quantification and Statistical Analysis

Behavioral data collection and quantification were carried out in the same manner as described in Chapter 3. However, different from the two-group design of Chapter 3, the present design included four groups, n = 12/group: DA animals trained and tested at either ZT 4 or ZT 16 (DA-AM and DA-PM respectively) and NA animals trained and tested at either ZT 4 or ZT 16 (NA-AM and NA-PM respectively). All training and probe trials were recorded and analyzed using the Noldus EthoVision-system (version 8.5; Noldus®). The proportion of grass rats to reach the platform, swim path lengths, swim velocity and latency to platform quadrant were calculated. For the

learning curve, acquisition and retention probes, the proportion of animals showing thigmotaxis was also determined. In order to monitor thigmotaxic behavior, the pool was divided during analysis into two rings. Time spent swimming in the 10.5cm wide ring closest to the edge of the pool was quantified, and grass rats that spent 50% or more of the trial period swimming in the thigmotaxic zone were characterized as displaying thigmotaxis. The latencies to reach the platform were recorded, but were not used for group comparisons since not all animals reached the platform within the 2 minutes of each trial. . All statistical analyses for all experiments used SPSS version 22 software, and all differences were considered statistically significant when P was equal to or less than 0.05, using two-tailed probabilities when pertinent.

Training: For the learning curve data, Cochran's Q tests (Cochran and Cox, 1957; Conover, 1999) were used to examine the effects of training days on the proportion of grass rats that found the platform over the course of the training. This within-group analysis was performed individually for the DA-AM, DA-PM, NA-AM and NA-PM groups using the average of the four trials per day, as well as using data from just trial one or just trial four of each training day. Comparisons between groups of the proportion of animals reaching the platform and showing thigmotaxis used Chi squared tests.

Two-way analyses of variance (ANOVAs) were used to examine the main effects of group (between group factor) and those of training day (within-group factor), as well as the interaction of these factors on swim path lengths, swim velocity and latency to platform quadrant. These ANOVAs were run for data averaged across all four daily trials. For all ANOVAs, significant interactions were followed by tests of the simple effects, and when appropriate comparisons of individual means using with Fisher's Least Significant Differences (LSD) *post hoc* tests.

Probes: For the acquisition and retention probes, separate overall Chi squared tests, followed when appropriate by individual group comparisons Chi squared tests, were used to evaluate the effects of group on the proportion of animals reaching the platform and showing thigmotaxic behavior. One-way ANOVAs for independent samples, followed when appropriate by Fisher's (LSD) *post hoc* tests, were used to determine the effects of groups on swim path lengths, swim velocity and latency to platform quadrant, for both the acquisition and retention probes.

Experiment 2:

Tissue collection and immunocytochemistry

Tissue collection and preparation for immunocytochemical staining for both BDNF and TrkB was carried out as described in Chapter 3, but now with separate groups of DA and NA grass rats. Animals (n = 4 -7/chronotype for BDNF staining and n = 5 – 8/chronotype for TrkB staining) were perfused at ZTs 2, 6, 10, 14, 18 and 22 and their brains processed for ICC. For TrkB ICC, the procedure was identical as the one described in Chapter 3. For the BDNF ICC, sections were taken out of cryoprotectant and stored overnight in 0.01M PBS at 4^0C. Just prior to undergoing ICC procedures, sections were rinsed three times (20 min/rinse) in fresh 0.01 M PBS. Unless otherwise noted, the sections were rinsed two times (20 min/rinse) in 0.01 M PBS between all steps of the ICC procedures. All steps were carried out at room temperature unless noted otherwise. Free-floating sections containing the hippocampus (CA1, hilus, and dorsal dentate blade) were rinsed in 0.01 M PBS, quenched in 3% H_2O_2, unmasked for 15min in 0.1M citric acid then blocked for 1 h using 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) in PBS and incubated overnight in a rabbit anti-BDNF antibody at 4^0C (Abcam, Temecula, CA, USA; diluted 1:2,000 in PBS and 5% NGS). All sections were then incubated for 1.5 h in a goat anti-rabbit biotinylated

antibody (Vector Labs, Burlingame, CA, USA; diluted 1:200 in PBS and 3% NGS), and then for 1 h in avidin–biotin peroxidase complex (AB complex, Vector Laboratories, Burlingame, CA, USA; in PBS). Sections were reacted with 0.025% diaminobenzidine (DAB; Vector) enhanced with nickel sulfate in Tris buffer with 3% hydrogen peroxide for 20 min. The reaction was followed by three 5 min rinses in PBS. All sections were mounted onto gelatin-coated slides, dehydrated, and coverslipped with DPX (Sigma-Aldrich). A set of control sections were selected and incubated in PBS and 5% NGS without anti-BDNF antibody at room temperature. Immunoreactivity was undetectable in sections that were processed in the absence of primary antibody.

Quantitative and Statistical Analysis

Quantification of BDNF-ir and TrkB-ir cells in all regions was carried out as described in Chapter 2. Total bilateral counts of each area were subjected to individual two-way ANOVAs to assess the effect of chronotype and ZT on BDNF and TrkB expression in each region. Significant interactions were followed by analyses of simple main effects and when appropriate by *post hoc* comparisons of individual means using Fisher's LSD tests. When the interactions were not significant, the main effects were interpreted without further probing.

Experiment 3:

Tissue collection and preparation for immunocytochemical staining for cFOS was carried out as described for plasticity gene products in Chapter 3. Animals (4 – 9/ chronotype) were perfused at ZTs 2, 6, 10, 14, 18 and 22, and their brains processed for ICC. The cFOS ICC procedure was carried out as previously described by Castillo-Ruiz et al 2010. Briefly, tissue was incubated in 5% NDS(Jackson,Westgate,PA) in PBS for 30mins, incubated for 24hrs in a 1:20,000 dilution of rabbit

anti-FOS antibody and 3% NDS (Santa Cruz) solution, followed by 1h incubations for both secondary antibody 3% NDS then ABC complex (Vector). Sections were reacted in DAB-nickel sulfate for 10mins then rinsed in PBS prior to being mounted on gelatin coated slides. A set of control sections were selected and incubated in PBS and 5% NGS without anti-cFOS antibody at room temperature. Immunoreactivity was undetectable in sections that were processed in the absence of primary antibody.

For counts of cFOS-immunoreactive cells, single sections containing each of the three hippocampal brain regions described in experiment 3 were identified using the rat brain atlas of Paxinos and Watson (2007). For each region, cFOS labeled cells were counted within each region defined by a counting box identical to that described in Chapter 3..Total bilateral counts of each area were used for the statistical analysis. Two-Way ANOVAs were used to assess the effect of chronotype and ZT on cFOS expression in each region. Significant interactions were followed by analyses of simple main effects and when appropriate *post hoc* comparisons of individual means using Fisher's LSD tests or *t*-tests. When the interactions were not significant, the main effects were interpreted without further probing.

Results

Experiment 1

Figure 3.1 shows the proportion of animals reaching the platform for each of the six training days using data for the four daily trials. Within-group analyses using Cochran's Q tests showed significant effects of days for all groups (Qs(5)=42.813-54.327,ps<0.001). Between-group comparisons using the Chi square test detected no group differences across the training days for the proportion of animals finding the platform ($\chi^2 s_{(3, N=48)}$= 0.00-0.457, all *ps*>0.05). Similarly, Chi square

tests failed to detect a difference between groups in the display of thigmotaxis ($\chi^2 s_{(3, N=48)}$= 0.00-1.524, all *ps*> 0.05).

ANOVAs (see Table Three) detected no interactions between groups and days of training for swim path, swim velocity and latency to platform quadrant and no main effects of group (data not shown). For all measures there was a significant main effect of days, with the performance of all groups improving across training days (data not shown). During data analyses Maulchy's test of sphericity detected a violation of the sphericity assumption of ANOVA. To insure accurate interpretation of the data, a Greenhouse-Geisser correction factor was used. The degrees of freedom reported in Table three have been multiplied by the appropriate Greenhouse-Geisser factor for each dependent variable.

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Table 3 Tests for Group Effects over Averaged Acquisition Trials

Dependent Variable	Main effect of group	Main effect of days	Interaction
Swim Path	$F_{1.084,11.919}$=2.136,NS	$F_{2.495,27.443}$=12.742,p<0.05	NS
Swim Velocity	$F_{1.036,11.400}$=1.326,NS	$F_{2.967,32.639}$=7.261,p<0.05	NS
Latency to Platform Quadrant	$F_{1.178,12.953}$=4.302,NS	$F_{2.680,29.485}$=49.849,p<0.05	NS

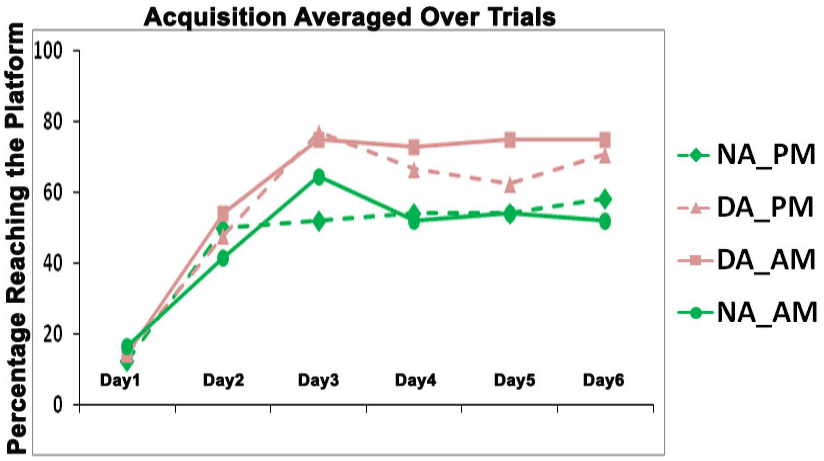

Figure 3.1 Significant effects of days of training with no group differences for the proportion of animals finding the platform using data from the four daily trials

Within-group comparisons using Cochran's Q tests found a significant effect of training days for all groups. Between-group comparisons using Chi squared tests detected no significant group differences. See text for statistical details.

Figure 3.2 depicts the results of analyses using data from either just trial 4 of each training day or just trial 1. As shown in Figure 3.2A, in trial 4 the performance of all groups was very similar across groups with no significant differences detected by Chi square tests (χ^2s $_{(3, N=48)}$ = 0.316-2.087, all *ps*> 0.05). In contrast, the analyses using Cochran's Q tests for just trial one of each training day (Panel B) found a significant effect of training days only for the two DA groups (Q(5)=15.00 and Q(5)=32.500, ps<0.001) , and not for the NA groups (Q(5)=3.33, Q(5)=6.364,ps>0.05).Further Chi square analysis of the proportion of animals reaching the platform on training day 6, found a significant difference between the DA-AM group and the two NA groups (χ^2s $_{(1, N=24)}$ = 8.40-10.741, all *ps*< 0.01); with the DA-PM group not different from the other three groups(Figure3.2, bottom panel).

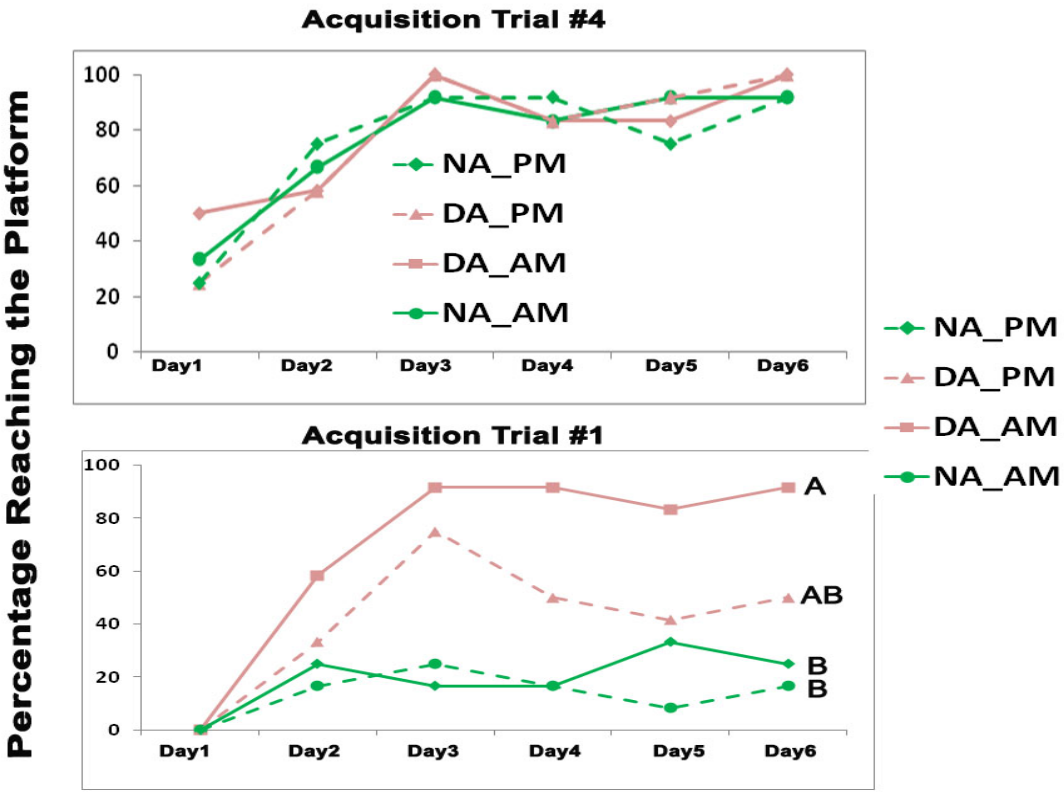

Figure 3.2 Significant group effects for the proportion of animals finding the platform for data from Trial 1 of each day, but not for trial 4

Panel A: Within group comparisons using Cochran's Q tests showed an effect of days for all groups with no between-group differences (Chi squared tests) when using the data for just trial 4 of each training day. Panel B: Within-group comparisons found a significant effect of training days only for the DA-AM and DA-PM groups and between-group comparisons detected significant differences for the proportion of animals finding the platform. For the between-group comparisons, groups with different letters are significantly different from each other

Figure 3.3 shows the proportion of animals to reach the platform for the acquisition (Panel A) and the retention probes (Panel B). Chi square analyses found an overall effect of groups ($\chi^2_{(3, N=48)} = 17.422$, all $p < 0.05$). Individual comparisons also using Chi square tests revealed superior performance for both DA groups compared to the two NA groups ($\chi^2_{(1, N=24)} = 8.711$, $ps = 0.003$). For the retention probe, Chi square analyses found and overall effect of groups ($\chi^2_{(3, N=48)} = 15.831$,

all $p<0.001$), individual comparisons detected a significant difference between DA-AM and all other groups ($\chi^2 s_{(1, N=24)}=0.003$-$10.741$, $ps<0.05$).

Figure 3.4 A presents acquisition probe data for swim path, ANOVA revealed a significant main effect ($F_{3,44}=9.534$,$p=0.000$) of group and post hoc tests showed that the two DA groups had significantly shorter swim paths when compared to both night active groups. For latency to platform quadrant (Panel B), ANOVA found a significant main effect of groups ($F_{3,44}=6.021$,$p=0.002$) and post hoc tests showed significantly shorter latencies for the two DA groups compared to the two NA groups. ANOVA found no significant main effect of group for swim velocity (Panel C; ($F_{3,44}=1.682$,$p=0.185$).Similarly Chi square analysis of the proportion of animals showing thigmotaxis detected no group differences (Panel D; $\chi^2_{(3, N=48)}= 0.750$, $p= 0.861$).

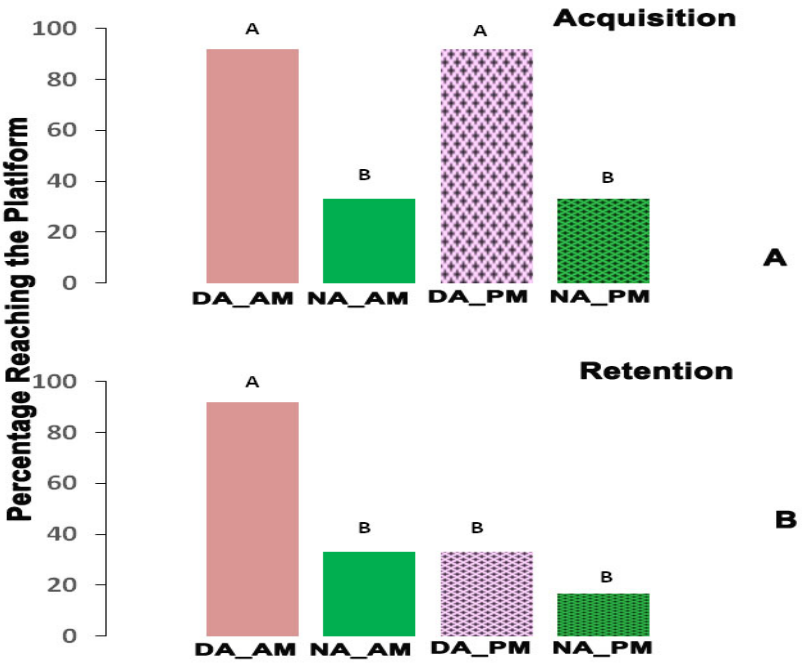

Figure 3.3 Superior performance of DA groups for the acquisition probe and of the DA-AM group for the retention probe

Panel A shows the proportion of animas finding the platform for the acquisition probe and Panel B shows the same data for the retention probe. For both panels, significant between-groups differences detected using Chi squared tests are indicated; groups with different letters are significantly different from each other

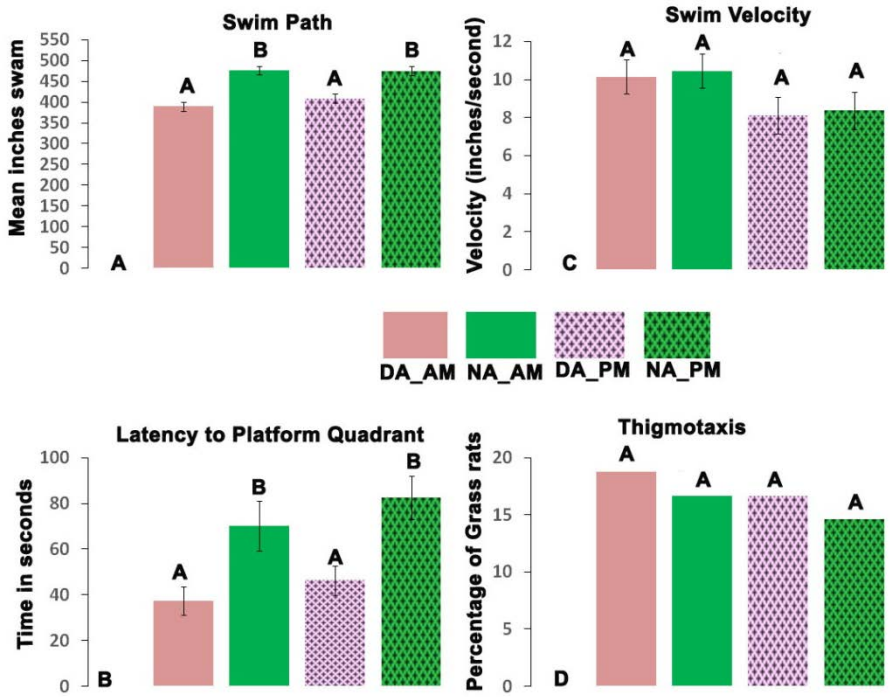

Figure 3.4 Superior performance of DA groups in the retention probe for swim path and latency to the platform quadrant, with no group differences in swim velocity or proportion of animals showing thigmotaxis

For the data shown in panel A (swim path) and B (latency to the platform quadrant) individual comparisons using Fisher's LSD tests found significant differences between the DA and the NA groups. The same test did not reveal differences between groups for swim velocity (panel C). Data shown in panels A – C are presented as means $\pm$ SEM. Chi squared analysis of the proportion of animals showing thigmotaxis (Panel D) found no group differences. For all panels, groups with different letters are significantly different from each other

Figure 3.5 A shows the average swim path of each group during the retention probe. ANOVA detected a significant main effect of group ($F_{3,44}$=1440.795,p=0.000) and post hoc tests found that the DA-AM group had a significantly shorter path than any of the other groups and that the path for the DA-PM was significantly shorter than those of the NA groups. The ANOVA for the latency to platform quadrant showed a significant main effect of groups ($F_{3,44}$=19.818,p=0.000), and post hoc tests found that the DA-AM group has a significantly shorter latency compared to all other groups. The latency for the DA-PM group was not significantly different that those of the NA groups.

A comparison between the two NA groups indicated that the NA-AM group had a shorter latency than the other NA group. For swim velocity (Panel C), the ANOVA found no significant main effect of group ($F_{3,44}$=0.047,p=0.986). Similarly groups did not differ significantly (Chi square analysis) with respect to proportion of animals showing thigmotaxic behavior (Panel D; $\chi^2_{(3, N=48)}$= 0.291,p> 0.05).

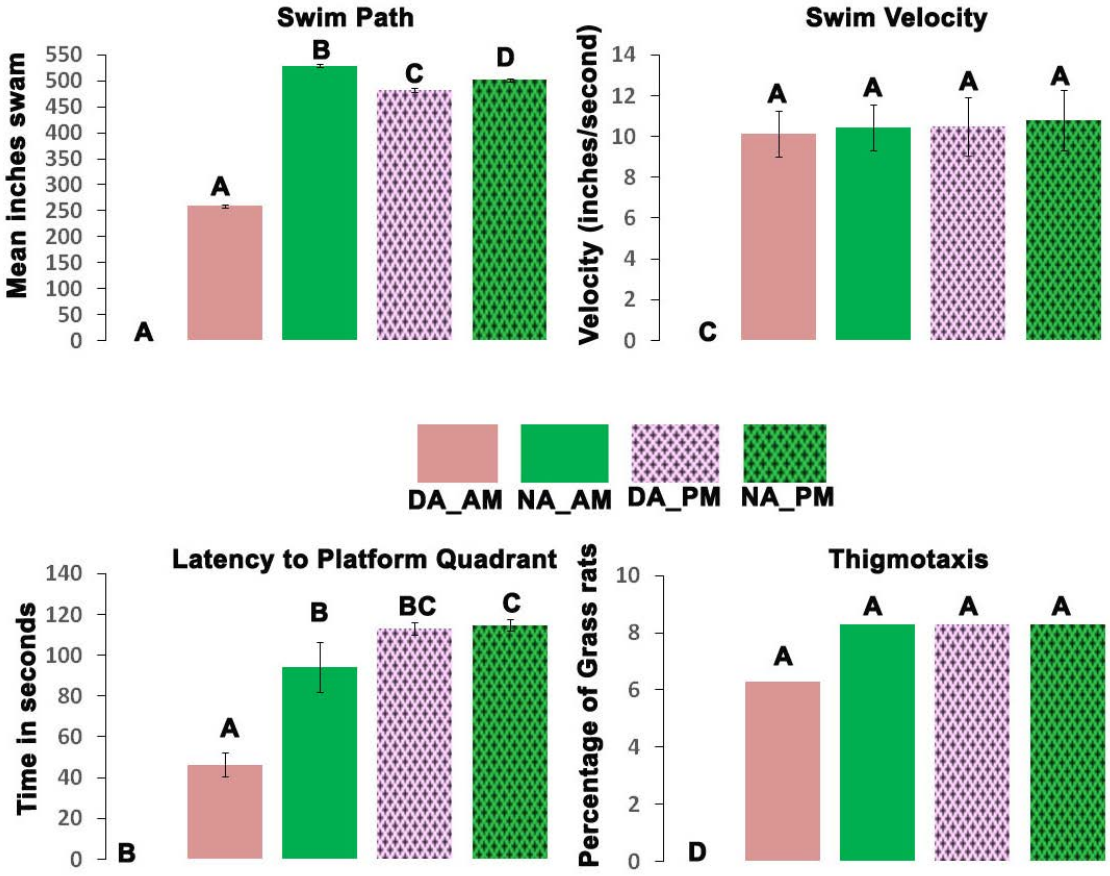

Figure 3.5 Superior performance of the DA-AM group in swim path and latency to the platform quadrant, with no group differences in swim velocity or proportion of animals showing thigmotaxis
For the data shown in panel A (swim path) and B (latency to the platform quadrant) individual comparisons using Fisher's LSD tests found significant differences between the DA-AM group and the other three groups. The same test did not reveal differences between groups for swim velocity (panel C). Data shown in panels A – C are presented as means ± SEM. Chi squared analysis of the proportion of animals showing thigmotaxis (Panel D) found no group differences. For all panels, groups with different letters are significantly different from each other

Experiment 2

Figure 3.6, left panels presents the data for BDNF rhythmic expression in CA1 (top), dorsal blade of the DG (Center) and hilus (Bottom). For all areas, two-way ANOVAs detected no significant interactions ($F_{5,1}$=0.622-2.193,*ps*>0.05) or main effects of chronotype ($F_{1,51}$=.513-1.767,*ps*>0.05). However, in every case there was a significant main effect of ZT (CA1:$F_{5,51}$=2.965, DG: $F_{5,51}$=3.725 and hilus: $F_{5,51}$=4.324,*ps*<0.05). The results of comparisons between individual means are described in Figure 3.6 A, B and C (left).

For the data on TrkB expression (Figure 3.6, right panels) two-way ANOVAs detected significant interaction effects for all three areas (CA1:$F_{5,59}$=63.368, DG: $F_{5,59}$=57.220 and hilus: $F_{5,59}$=61.369,*ps*=0.000). Analysis of the simple main effects of ZT within groups found significant effects for all areas (DA group CA1:$F_{5,28}$=14.214, DG: $F_{5,28}$=12.445,hilus: $F_{5,28}$=12.522 and NA group:CA1:$F_{5,31}$=583.751, DG: $F_{5,31}$=321.926,hilus: $F_{5,31}$=582.721,*ps*=0.000).The results of comparisons across ZTs using Fisher's LSD are presented in Figure A, B and C, right). Analyses of the simple main effects of chronotype within ZTs found significant differences between chronotypes at ZTs 2, 10 and 14 for all three areas (ZT2:*t(11)*=4.507,*p*<0.05, ZT10: *t(12)*=24.377,*p*<0.05, ZT14: *t(10)*=21.280,p<0.05), and in every case NA animals had significantly higher levels of TrkB.

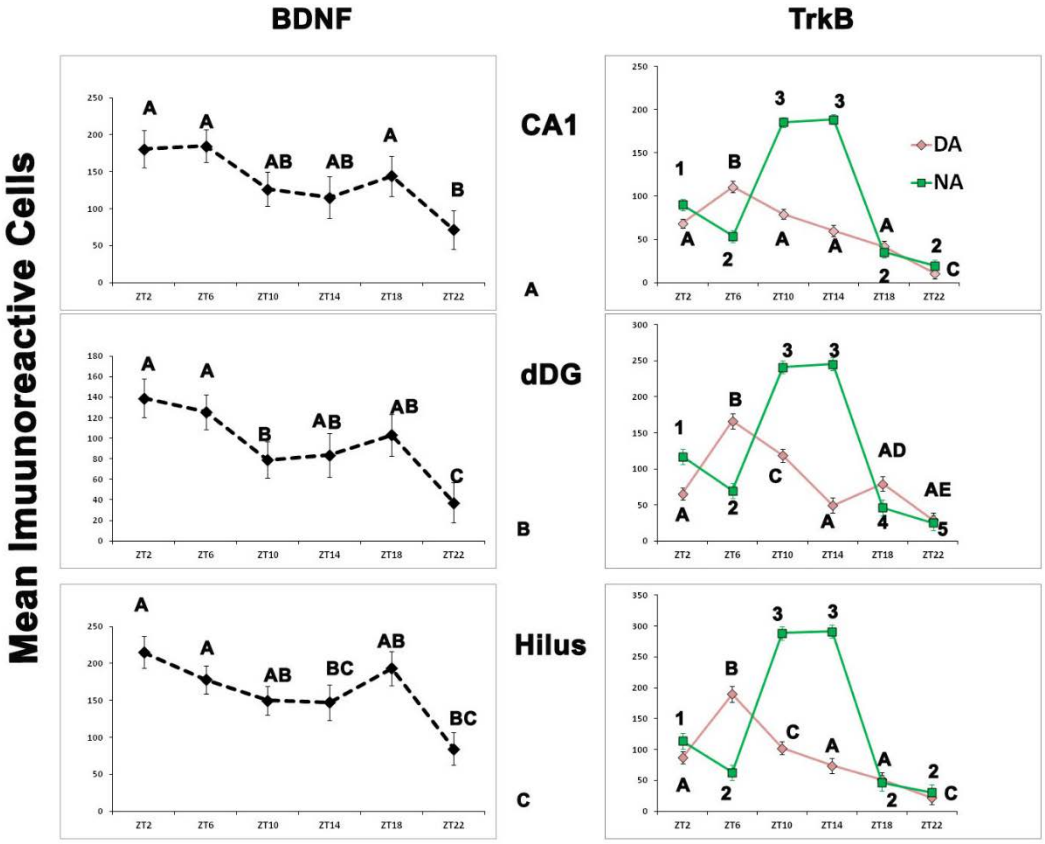

Figure 3.6 Chronotype differences in the rhythmic expression of TrkB, but not BDNF in the hippocampus

Line graphs showing the mean (± SEM) number of BDNF- (left) and TrkB- (right) expressing cells for each ZT in CA1, dorsal blade of the DG and hilus of male grass rats. ANOVAs for the BDNF data showed no effects of chronotype and no interactions, but a significant main effect of ZT. That main effect is shown combining data for DA and NA animals; significant differences between ZTs are indicated by letters, ZTs with different letters are significantly different from each other. For TrkB, the ANOVAs found a significant interaction and significant simple main effects of both Chronotype and ZT. Significant differences across ZTs are indicated by letters for the DA group and by numbers for the NA group; ZTs with different letters or numbers are significantly different from each other

Experiment 3

Figure 3.7 shows the data for rhythmic expression of cFOS in the three areas of the hippocampus. The data for CA1 is shown in the top panel, two-way ANOVA revealed a significant interaction ($F_{5,1}$=13.176,p=0.000). Analysis of the simple main effects of ZT found significant effects within each of the two chronotypes (DA group:$F_{5,33}$=15.212 and NA group:$F_{5,44}$=13.291, ps=0.000). The results of individual comparisons are presented in Figure 3.7 A. Peak expression occurred at ZT 10 in the DA group and at ZT 22 in the NA group. In contrast to the results for CA1, no significant interactions (dDG:$F_{5,1}$=0.260 and hilus group:$F_{5,1}$=0.742,ps>0.05) were found for the data from the dorsal blade of the DG or hilus. Similarly in the DG there was no effect of chronotype (dDG $F_{1,65}$=2.997,p>0.05). However, in the hilus due to a slightly higher amount of cFOS present in the NA group, an effect of group was detected (hilus $F_{1,65}$=6.892,p<0.05). For both of these areas there was a significant main effect of ZT, which is depicted in Figure seven B and C. The results of comparisons between individual ZTs are presented in Figure 3.7 B and C. For the dorsal blade of the DG peaks were associated with dawn and dusk, while the pattern for the hilus showed sustained levels across the day and early night that decreased between ZT 18 and 22.

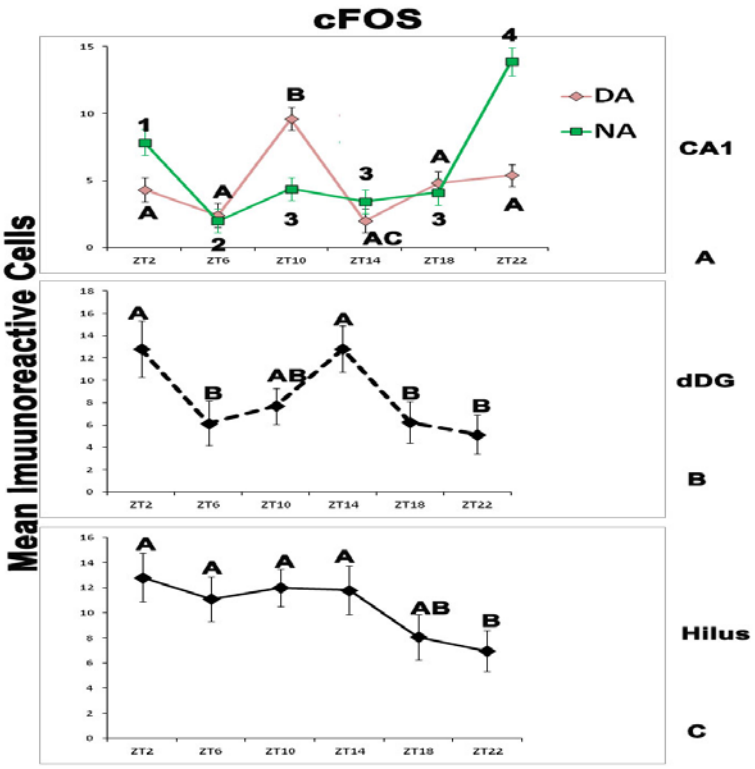

Figure 3.7:Chronotype differences in the rhythmic expression of cFOS in CA1, but not in the dorsal blade of the DG or in the hilus

Line graphs showing the mean (± SEM) number of cFOS-expressing cells for each ZT in CA1, dorsal blade of the DG and hilus of male grass. For CA1, there was a significant interaction between Chronotype and ZT, with significant simple main effects of ZT present for both Chronotypes. For CA1, significant differences across ZTs are indicated by letters for the DA group and by numbers for the NA group,; ZTs with different letters or numbers are significantly different from each other. For both the dorsal blade of the DG and for the hilus, there were no significant interactions, but the ANOVAs detected a significant main effect of ZT. Although there was no group effect detected in the dorsal blade of the DG, there was a significant effect of group detected in the hilus. For these two areas, the main effect of ZT is shown combining data for DA and NA animals; significant differences between ZTs are indicated by letters, ZTs with different letters are significantly different from each other

Discussion

Two major findings of experiment 1, as it was the case for Chapter 3, are that time of testing does not affect the shape of the standard learning/acquisition curve, and that for DA animals, training in the morning improves long-term retention. However, in experiment 1 there was also a dramatic difference between groups when using data from only the first trial of each training day. This group

difference was primarily due to the poor performance of the NA animals, which was evident regardless of time of testing; this difference was completely obscured when the analysis of the learning curve was based on all four daily trials, or on just data from trial 4. Thus, based on the analysis of just the first trial of each training day, the NA groups display evidence of the accelerated forgetting seen in aging, epileptic and partial hippocampal lesion models (Akers et al., 2014; Clark et al., 2005; Elliott et al., 2014; Evans et al., 2014; Mary et al., 2013). In those models there is diminished hippocampal function that results in apparent normal initial learning, but accompanied by very rapid forgetting. A similar partial loss of hippocampal function may be responsible for the deficits seen here in NA animals.

Similar to the results of Chapter 3, the performance of the DA-PM group did not differ significantly from that of the DA-AM group when trial 1 was used for the comparisons, although in both cases, the performance of the DA-PM animals was not as strong as that of their AM counterparts. Further, here the DA-PM animals' trial-1 data were not significantly different from those of the NA groups, thus suggesting that in DA animals, nocturnal training may have subtle detrimental effects on learning that may require higher statistical power in order to be detected.
Not surprisingly, given the results of the analyses of just trial-1 data, the performance of the NA groups was significantly poorer than that of the DA groups during the single acquisition probe that followed, after 24 hours, the completion of training. These results further document the apparent rapid forgetting displayed by the NA animals. For the retention probe, the poor performance of the NA animals was expected, given the evidence of a fast decay of memory in these animals obtained from the acquisition probe and from the analysis of the first trial of each training day. The retention-probe data for the DA groups confirmed the observations of Chapter 3, and those from studies with laboratory rats (Gritton et al., 2012); they support the claim that cognitive deficits associated with

training and testing at the natural rest phase of a species are manifested primarily as deficits in long-term retention.

In summary, I have presented evidence for distinct cognitive deficits that, in a diurnal species, are associated with either nocturnal activity or training and testing during the night. An alternative explanation for my findings could be that these results do not reflect cognitive deficits, but rather stem from non-cognitive factors such as group differences in motivation and anxiety that then influence performance in the MWM. Against that competing interpretation are data from my experiments showing lack of consistent group differences in swim velocity and thigmotaxic behavior, thus indicating no motivational or anxiety level differences between the groups over training days or during either probe test.

It is important to note the differences between my approach and those of human chronotype studies. Different from my findings there are reports of how a synchrony between an individual's chronotype and the time of testing can prevent the deficits seen when humans with an evening chronotype are tested early in the morning (Diaz-Morales and Escribano, 2013; Hahn et al., 2012; May, 1999). While there is a plethora of human work done to investigate time of day differences in learning and memory, as well as interactions between human chronotype and performance (Folkard et al., 1985; May, 1999; Natale and Lorenzetti, 1997; Preckel et al., 2013; Schmidt et al., 2007), many of the tasks used are not specifically hippocampal dependent (Schmidt et al., 2007). Further the testing times are never 12-hour out of phase, as it is the case with my experiments; humans are, as a rule, tested as early as 8am (morning) and normally no later than 4:30-6pm (evening hours) (Folkard and Monk, 1985; Schmidt et al., 2007; Wright et al., 2006). My study was designed to look selectively at hippocampal dependent learning and memory of both DA and NA grass rats at two times of day 12 hours apart from each other. Thus, my experimental approach fits nicely with the realities of night-shift workers and with the work done with that population (Folkard, 1989; Wright

et al., 2013), which contrasts with the approach of chronotype-synchrony studies using students and aged populations (Besoluk et al., 2011; Diaz-Morales et al., 2012; Escribano et al., 2012; Goldstein et al., 2007; Hasher et al., 2005; Preckel et al., 2013; Vollmer et al., 2013) .

Hippocampal circadian desynchrony may explain the cognitive deficits reported in night- and shift-workers (Gold et al., 1992; Wright et al., 2013) and those documented here for NA grass rats. In NA grass rats, the desynchrony of plasticity gene product rhythmic expression featured a pronounced delay in the phase of the TrkB receptor rhythm, from that expected for a diurnal species. That phase delay was not accompanied by a similar phase delay in the BDNF rhythm. Thus, the coupling of the rhythmic expression of BDNF and its high-affinity receptor appears to be disrupted, thus presenting a potential explanation for the cognitive deficits seen in NA grass rats (present data) and possibly in night- and shift-workers (Devore et al., 2013; Machi et al., 2012; Marquie et al., 2014). Interestingly, the overall production of hippocampal TrkB was elevated in NA grass rats (present data), which may indicate the presence of a compensatory mechanism triggered by the lack of coupling between the two rhythms of plasticity-gene products in the hippocampus of these animals. Alternatively, as discussed in chapter 2 in reference to melatonin production, the elevated levels of TrkB shown by NA grass rats may stem from their higher levels of wheel-running activity (Blanchong et al., 1999); at least in nocturnal rodents exercise has been associated with enhanced hippocampal TrkB production (Kim et al., 2005; Klintsova et al., 2004; M et al., 2013).

In Experiment 3, I found different patterns of neuronal activation in the three areas (CA1, dDG and hilus) of the hippocampus. In the CA1 regions there was a complete reversal of the time of peak cFOS expression in the NA grass rats compared to DA animals. In the CA1 NA grass rats showed peak expression during the dark phase, while DA grass rats showed peak expression during the light phase. While there was no chronotype difference detected in the dDG, there was a group effect detected in the hilus region with respect to the effects of sampling time on cFOS expression.

Thus, in addition to the apparent desynchrony between rhythms of plasticity-gene products that results from the adoption of a nocturnal activity profile, nocturnal activity has different effects on rhythms of neural activity across hippocampal regions. These observations add evidence to support the claim that the circadian harmony of the hippocampus is profoundly disrupted in NA grass rats, and potentially in night- and shift-workers.

Interestingly, for the NA grass rats peak cFOS expression in the hilus and dDG did not occur at the time expected for a nocturnal species (Angeles-Castellanos et al., 2007; Escobar et al., 2007), thus suggesting that in these areas, the hippocampal rhythm of cFOS expression represents another example of how diurnal features are retained in NA grass rats (see Chapter 2 for other examples). This interpretation is tentative, since data on cFOS rhythms in diurnal grass rats without wheels are not available, and there is evidence that wheel running can affect the patterns of cFOS expression, even when the animals with access to wheels do not adopt a nocturnal activity profile (Castillo-Ruiz et al., 2010).

Taken together, the data presented in this chapter argues against claims that the temporal niche of mammals is extremely plastic and adaptable to drastic phase-switching without major negative consequences (Hutt et al., 2002). Clearly, the disruption in both the circadian control of the hippocampus and hippocampal dependent memory indicates that when activity occurs during the rest phase of a diurnal species, not all functions adopt a nocturnal profile with negative consequences for the overall fitness of the individual. These negative consequences are likely to be most extreme in species, like ours, in which there is ample evidence of major neural adaptations to a diurnal life style. Thus, part of the broader impact of the results presented in this chapter is to alert educators and school administrators of the possibility that the nocturnal tendencies of many students may affect their brains and cognitive abilities in profound ways that are not reversed by changing the daily

schedule of school systems, just like changing the time of testing fails to remedy the memory deficits of NA grass rats.

The broader impacts of the data from this chapter relate to how they inform educational policy, particularly on the potential for failure of new interventions to delay the start of the school day in order to synchronize the sleep-wake cycle of children and adolescents to the school day (Pediatrics, 2014). If children shifting their activity patterns in ways similar to our NA grass rats, it could be the case that like the NA animals their brains have been altered in ways that would require interventions beyond just changing the current schedule, as most of the current human chronotype literature suggests.

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CHAPTER *5*: DISCUSSION

Summary, Conclusions, Future Directions and Impact

Internally, circadian rhythms serve to maintain optimal relationships among different aspects of the physiology and endocrinology of organisms, and when entrained to the day-night cycle, they prepare organisms to anticipate recurrent fluctuations of the external environment that result from the earth's rotation. Modern life and globalization have challenged the internal circadian organization of humans as well as the entrainment of endogenous rhythms to the day-night cycle. Examples of these challenges include those associated with night- or shift-work, non-circadian activity schedules and repeated traveling across time zones. These life styles are associated with a wide range of pathologies, including cognitive deficits (Folkard, 1989, Erren, 2013, Wright et al., 2013). In addition to work and travel demands that disrupt internal circadian organization and entrainment, a phenomenon labeled "eveningness" has been described as an additional challenge for humans. This condition refers to the display of activity and maintenance of wakefulness during the rest phase of diurnal species like ours. Similar to night- and shift-work, eveningness is associated with a number of health problems, as well as diminished cognitive performance (Lange and Randler, 2011, Prieto et al., 2012, Preckel et al., 2013, Diaz-Morales and Escribano, 2014). Progress in understanding the mechanisms responsible for pathologies and deficits associated with human nocturnal activity has been impeded by the lack of a diurnal mammalian model. Further, efforts to create laboratory conditions that emulate shift-work have, for the most part, involved the use of forced-wakefulness paradigms that generate substantial stress for the experimental animals (Salgado-Delgado et al., 2010, Hsieh et al., 2014).

General Approach:

In this dissertation, building on the work of previous members of the MSU-SYN Chronobiology group, I have used the grass rat to investigate the consequences and underlying

mechanisms associated with a change in phase preference for the display of activity. This work is innovative, not only because it uses a diurnal mammal as a model, but because the change in the distribution of activity does not involve the implementation of a forced-wakefulness paradigm. Previous work from our group (Castillo-Ruiz et al., 2010, Castillo-Ruiz and Nunez, 2011) shows that the "voluntary" phase reversal of the NA grass rats does not activate areas of the brain associated with stress, as it is the case when grass rats are forced to stay awake at night.

The Night-active grass rat and functions controlled by the hypothalamus:

In Chapter 2, I extended previous observations about the phase of extra-SCN oscillators, and its reversals in NA grass rats, to the PVN, which is a key component of the circuits by which the hypothalamus regulates autonomic outflow (Saper et al., 1976). Further, I showed that the phase reversal displayed by NA grass rats does not prevent the nocturnal rise in pineal melatonin. These data are important for several reasons, first they provide a mechanism to explain phase differences in the rhythmic control of autonomic functions between diurnal and nocturnal species and document how the phase of the oscillator of the PVN does not determine the phase of the melatonin rhythm across species. Second, taken together with the data about the phase of other hypothalamic extra-SCN oscillators (Ramanathan et al., 2006, Nunez et al., 2012) , these results suggest that there may be a serious autonomic desynchrony in NA animals. For this claim, consider the different responses to nocturnal activity observed for two hypothalamic nuclei. In the case of the PVN, the phase of the rhythms reverses (Chapter 2), but in the dorsomedial nucleus (DMN) the diurnal profile is maintained, with no phase differences between NA and DA grass rats(Nunez et al., 2012). Since both the PVN and the DMN project to autonomic and pre-autonomic sites (Saper et al., 1976, Dong et al., 2001) it is expected that this mosaic of phases in the hypothalamus would result in deficits in autonomic regulation and metabolic deficits. Consistent with this prediction are data showing a

mismatch between energy demands and liver glycogen mobilization in NA grass rats (Novak and Nunez unpublished observations). Third, the results of the melatonin measures indicate that while some rhythms may reverse phase in NA grass rats, others can maintain the same phase relationship with respect to the SCN and the light-dark cycle. For NA grass rats, this produces an atypical phase relationship between peak melatonin production and the active phase of these animals. Taken together, the work presented under Chapter 2 provides insights about the mechanisms responsible for the autonomic and metabolic deficits seen in humans that engage in chronic nocturnal activity (Pietroiusti et al., 2010, Erren, 2013). Further, they serve to document the utility of the NA grass rat as a model to study the consequences of nocturnal activity in a diurnal species, without introducing the stress associated with forced-wakefulness experimental paradigms.

Circadian regulation of cognitive functions and neural plasticity in a diurnal mammal:

In addition to their role in the regulation of physiological and endocrine functions, circadian influences are also evident in cognitive functions (Smarr et al., 2014). With some rare exceptions, learning as well as retention of what is learned are facilitated when the training and testing occurs during the active phase of the species. In the case of hippocampal dependent spatial learning, work with nocturnal laboratory rats has served to document the presence of superior retention when the animals are trained and tested during the night. In Chapter 3, I used the MWM to show that in the diurnal grass rat the bias for stronger retention is associated with daytime training and testing. In both laboratory rats and grass rats the circadian effects, at least for the MWM, are mostly limited to long-term retention, since no time-of-day effects are seen during acquisition, even though the performance of DA grass rats tends to be stronger when the testing takes place during the day (see Discussion of Chapter 4).

What may be responsible for the poor retention when the acquisition and probing occur during the rest phase of the species? This phenomenon may represent a deficit in memory consolidation, which refers to the process by which memories are stabilized in long-term storage via synaptic strengthening and circuit-level plasticity (McGaugh, 2000). For spatial memory, the processes of consolidation and retrieval are likely to engage the hippocampus, even over long delays between acquisition and retention probes (Nadel and Moscovitch, 1997). Thus, species differences in the phase of particular rhythms of the hippocampus may determine species-specific optimal time-of-day for retention of the MWM task.

Work by others from the MSU-SYN Chronobiology group (Ramanathan et al., 2010a) indicates that rhythms in clock-gene expression in the hippocampus are 180^0 out of phase between diurnal and nocturnal species. In Chapter 3, I describe rhythms in the expression of two gene products that have been shown to play a role in the process of memory consolidation, i.e., BDNF and its high affinity receptor TrkB (Yamada and Nabeshima, 2003). Consistent with the phase differences between species reported for clock genes (Ramanathan et al., 2010a), I found that the rhythms in abundance of these plasticity-gene products in the hippocampus of grass rats show consistently low levels late at night, which is the opposite of what is seen in nocturnal laboratory rats (Bova et al., 1998, Dolci et al., 2003, Katoh-Semba et al., 2008). Thus, although evidence for a causal link is missing, the data are consistent with the hypothesis that the phase of hypothalamic rhythms in BDNF and TrkB determines the phase for optimal retention for hippocampal dependent tasks. Interestingly, the same general pattern of rhythmic expression of BDNF and TrkB that were seen in other regions of the brain that have been associated with procedural [dorsal striatum; (McDonald and White, 1993)] and emotional [amygdala: (McDonald and White, 1993)] learning and memory. The role of these rhythms in mediating hippocampal independent memory

and species differences in their circadian control require additional behavioral data from grass rats and comparative data from nocturnal species.

Cognitive deficits in night-active grass rats:

In Chapter 2, I discussed the value of the NA grass rat as a model for understanding some of the health problems associated with nocturnal activity in humans (Saderi et al., 2013). In Chapter 4, I extended the analysis of these animals to possible effects of nocturnal activity to the domain of cognitive abilities, particularly hippocampal-dependent spatial learning. One possible outcome of this investigation was to find that the reversal in phase preference for the display of activity produces a similar reversal in the optimal time for the retention of the MWM (see Chapter 3). That expectation was anticipated by the observation that in humans, morning or night chronotype predicts optimal time for remembering (Natale and Lorenzetti, 1997, Randler and Frech, 2009, Preckel et al., 2013, Vollmer et al., 2013, Goldstein, et al. 2007, Hahn et al., 2012, Lara et al., 2014). That simple reversal is also consistent with theories that interpret the display of nocturnal or diurnal profiles as a very plastic aspect of the animals' circadian system, with switches easily accomplished in response to environmental changes in temperature and other energetic demands (Hutt et al., 2002). However, it is also evident that in the case of the NA grass rat, the multi-oscillatory circadian system appears to have only limited plasticity, thus creating the possibility for cognitive disruptions rather than a simple reversal of the phase for optimal learning.

The behavioral work of Chapter 4 clearly demonstrated that although overall the learning curves of NA and DA animals did not show any chronotype or time of training/testing effects, there was a clear deficit in the short term (24 hours) and the long term (14 days) retention of the MWM in NA grass rats, regardless of the time of testing. The rapid forgetting of NA grass rats is very similar

to that reported for humans with epilepsy; these patients show apparent normal acquisition and initial consolidation, followed by accelerating forgetting over hours or days (see Elliott, Isacc and Muhlert, 2014 for a recent critical review of this literature). The fact that the NA rats apparently relearn the task rather rapidly every day, but without savings over 24 hours, clearly indicates a distinction in the process of immediate consolidation and that of short and long-term retention; thus, the NA grass rat presents itself as an attractive model to study the neurobiology of those distinct phenomena. The fact that testing at night did not mitigate the memory deficits of NA grass rats points to a fundamental cognitive deficit that is not simply the result of a mismatch between the active phase of the individual and the time of testing. Thus the etiology of the memory deficits of NA grass rats are likely mediated by mechanisms different from those responsible for the milder retention deficits seen in DA animals tested at night (Chapters 3 and 4).

The hippocampus of the night-active grass rats:

The work of Ramanathan et al. 2010 shows how the phase reversal of the activity rhythms of NA grass rats is accompanied by a full reversal in the phase of hippocampal rhythms of clock gene expression. In Chapter 4, I report how that is not the case for rhythms in the expression of BDNF. A comparison of the temporal pattern of BDNF expression in the hippocampus of DA and NA grass rats resulted in a significant main effect of sampling time or ZT, but with no interactions between ZT and chronotype. So, rather than a reversal in that rhythm, the uniform pattern for all animals showed a consistent low point late at night, thus suggesting the presence of a diurnal profile in the NA animals. A completely different outcome was associated with the TrkB rhythm, which although short of showing a complete reversal, it featured a significant phase delay when the NA animals' pattern is compared to the one of the DA grass rats. The failure to see an effect of chronotype on the rhythmic expression of BDNF in the hippocampus, plus the lack of a full reversal

of the TrkB rhythms of these animals questions the claim that the phase of a local hippocampal oscillator drives the rhythmic expression of these two plasticity gene products; note that rhythms of PER1 and 2 are completely reversed in area CA1 and in the hilus of NA rats compared to DA ones (Ramanathan et al., 2010a). These data also indicate that the typical phase relationship between BDNF and TrkB abundance shown by DA grass rats with or without wheels is disrupted in the case of NA, thus pointing to a possible reason for the poor retention displayed by these animals when tested using the MWM (Chapter 4). Further evidence for a circadian disruption in the hippocampus of the NA grass rats comes from an analysis of patterns of neural activity as measured by FOS expression (Chapter 4). Here again, a phase reversal of the rhythm is not universally seen in all areas of the hippocampus.

Conclusions, future directions and impact:

Taken together, the results of this dissertation's research indicate that the display of nocturnal activity has serious negative consequences for a diurnal species. They include potential or evident internal desynchrony among rhythmic functions that regulate both homeostasis, via autonomic control, and cognitive functions, particularly those related to hippocampal dependent memory. The data also serve to argue against the claim that the mechanisms that determine whether an animal is day-active or night-active are completely plastic and respond with agility to energetic demands, without much of a functional cost (Hutt et al., 2002). Clearly for grass rats and perhaps for humans as well, nocturnal activity during the normal rest phase has deleterious effects that limit the ability to change successfully the phase preference of a particular species.

Future directions would include further analysis of the metabolic profile of NA rats to establish how the potential dissociation of rhythmic functions controlled by the hypothalamus results in metabolic pathologies similar to those seen in humans that are active at night (Gold et al., 1992, Pietroiusti et

al., 2010, Wright et al., 2013). The NA then could be further developed as a model to test the effects of interventions, such as scheduled feeding (Escobar et al., 2009, Minana-Solis et al., 2009) that may normalize metabolic rhythms by synchronizing hypothalamic and peripheral oscillators. The work on the cognitive deficits of NA grass rats should be followed up both at the behavioral level, to describe in detail the course of the remarkable accelerated forgetting of these animals, as well as by measuring additional endpoints in the hippocampus. For example models of epilepsy have linked that pathology to both rapid forgetting and to reduced hippocampal neurogenesis (Frankland et al., 2013, Elliott et al., 2014), thus measuring adult neurogenesis in the hippocampus of NA grass rats will serve to identify common physiological causes for the memory deficits of animals with apparently similar deficits, but that emerge from very different antecedent conditions, i.e. epileptic seizures and nocturnal activity. Additionally, manipulations of BDNF/TrkB expression within the hippocampal formation at specific times using techniques such as siRNA or conditional KO models could serve to help us better understand the role of rhythmic hippocampal BDNF/TrkB expression and cognition within a diurnal brain.

Finally, the overall impact of my research resides in the development of the grass rat to study "chronopathologies" that have been documented in humans. Current public concern with these chronopathologies is influencing policy; see for example the recommendation of the Pediatric Psychiatric Association regarding delaying the start of the school day to accommodate the eveningness tendencies of school children and teenagers (Pediatrics, 2014). That recommendation is based on data that document the phase of entrainment and distribution of sleep of these young individuals (Wright et al., 2006, Wolfson et al., 2007, Diaz-Morales et al., 2012, Preckel et al., 2013). However, my work provides a dose of pessimism about the success of that intervention and suggests that the deficits of a diurnal brain exposed to nocturnal activity are more fundamental than previously

assumed and the problems they present may not be resolved with a simple change in the daily academic scheduled.

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