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CIRCADIAN AND ENVIRONMENTAL MODULATION OF AROUSAL AND REWARD SYSTEMS OF A DIURNAL BRAIN

By

Alexandra Castillo-Ruiz

A DISSERTATION

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hypothesis that key functional determinants of a species chronotype reside downstream from the master clock of the SCN.

The data presented here also show that a voluntary change in the temporal distribution of activity in grass rats, namely a shift to a nocturnal bias triggered by access to a running wheel, results in changes in the brain that are clearly different from those produced by forced wakefulness during the natural rest phase of the species. These observations have important implications for the evaluation of studies with animal models that use forced wakefulness to emulate the conditions faced by human shift workers. Also important for the evaluation of previous research were results showing that the consequences of forced wakefulness, documented here in a diurnal species were saliently different from those reported in studies using nocturnal species. These differences included the particular wake-promoting regions that were affected as well as the effects of forced wakefulness on the master clock of the SCN.

In summary, the work presented here sheds new light on how wakefulness during the natural rest period of a diurnal species can influence brain function. It also highlights the need to diversify the current set of animal models used in chronobiological research, and in particular it urges the field to increase the representation of diurnal species in the study of circadian rhythms and how they are influenced by environmental demands.

This dissertation is dedicated to my parents for their love, support and encouragement; and to my advisor, Dr. Tony Nunez, for his invaluable guidance and friendship throughout the years.

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KEY TO ABBREVIATIONS

Abbreviation	Definition
3V	third ventricle
4V	fourth ventricle
5HT	serotonin
AB complex	avidin-biotin peroxidase complex
ac	anterior commissure
ACh	acetylcholine, cholinergic
AMPA	alpha-amino-3-hydroxy-5--4-propionic acid
ANOVA	analysis of variance
Aq	cerebral aqueduct
Arc	arcuate nucleus
aVTA	anterior ventral tegmental area
BF	basal forebrain
C	control
ChAT	choline acetyltransferase
cp	cerebral peduncle
CP	choroids plexus
CR	control recovery
CRH	corticotropin-releasing hormone
CSD	control sleep deprivation
CSF	cerebrospinal fluid
CTB	cholera toxin subunit beta

DA.....day-active grass rat

DAB.....diaminobenzidine

DR.....dorsal raphe

DTM.....dorsal tuberomammillary nucleus

DPX.....dibutyl phthalate xylene

EC.....ependymal cell layer

EDAC.....N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride

Fr.....fasciculus retroflexus

GABA.....gamma-amino-n-butyric acid

HA.....histamine

HDB.....horizontal diagonal band of Broca

HPC-θ.....hippocampal theta wave

ICC.....immunocytochemistry

IP.....interpeduncular nucleus

ir.....immunoreactive

LC.....locus coeruleus

LC1.....locus coeruleus level 1

LC2.....locus coeruleus level 2

LD.....light/dark

lDR.....lateral dorsal raphe

LH.....lateral hypothalamus

LV.....lateral ventricle

mDR.....medial dorsal raphe

mDR3.....medial dorsal raphe level 3

mDR4.....medial dorsal raphe level 4

mDR5.....medial dorsal raphe level 5

ME .....median eminence

mp.....mammillary peduncle

mPVN .....magnocellular paraventricular nucleus of the hypothalamus

MR .....median raphe

MS .....medial septum

mVTA.....medial ventral tegmental area

NA.....night-active grass rat

NAc.....nucleus accumbens

nACh.....non-cholinergic

NE.....norepinephrine

NDS .....normal donkey serum

NGS .....normal goat serum

NHS .....normal horse serum

NRS .....normal rabbit serum

NI .....nucleus incertus

nREM.....non-rapid eye movement sleep

nTH.....non-tyrosine hydroxylase

OB.....olfactory bulb

OX.....optic chiasm

OXA ..... orexin A

OXB ..... orexin B

PB ..... 0.1 M phosphate buffer

PBS ..... 0.01 M phosphate-buffered saline

PB-TX ..... 0.2% TX in 0.2 M phosphate buffer

Per ..... period

Pir ..... piriform cortex

PLP ..... paraformaldehyde-lysine-sodium periodate

pm ..... principal mammillary tract

Pn ..... pontine nuclei

PVN ..... paraventricular nucleus of the hypothalamus

pPVN ..... parvocellular paraventricular nucleus of the hypothalamus

pVTA ..... posterior ventral tegmental area

R ..... recovery

REM ..... rapid eye movement sleep

s.c. ..... subcutaneous injection

SCN ..... suprachiasmatic nucleus

SD ..... sleep deprivation

SEM ..... standard error of the mean

SUM ..... supramammillary nucleus

T ..... tegmentum

TBS.....tris-buffered saline

TH.....tyrosine hydroxylase

TMM.....tuberomammillary nucleus

TX.....Triton-X 100

VDB.....vertical diagonal band of Broca

VLPO.....ventrolateral preoptic area

vSPZ.....ventral subparaventricular zone

VTA.....ventral tegmental area

VTM.....ventral tuberomammmillary nucleus

ZT.....zeitgeber time

CHAPTER 1

Introduction

General introduction

The suprachiasmatic nucleus (SCN) of the hypothalamus orchestrates circadian rhythms in behavior, and in addition controls the physiological processes that support those rhythms (Moore and Eichler, 1972; Stephan and Zucker, 1972), all of which are entrained to the light dark cycle via direct retinal inputs to the SCN (reviewed in Rosenwasser, 2009). Under this circadian control, most mammalian species have evolved to make use of a particular phase of the solar day to be active. Humans, for instance, are active during the day and rest during the night. Although we are clearly a diurnal species, most of the research done in the field of chronobiology, has used nocturnal species as experimental subjects. In part, this has been due to the lack of suitable models of diurnality, and as a result of that practice, very little has been explored in diurnal species. The relatively few studies that have investigated the functioning of the SCN in diurnal species, have revealed that some aspects of the clock, such as clock-gene expression (Caldelas et al., 2003; Lambert et al., 2005) and metabolic activity (Schwartz et al., 1983), are similar to those seen in nocturnal species. But, others, have also revealed aspects that are different (Novak and Albers, 2004; Cuesta et al., 2007). This evidence indicates that the data gathered from nocturnal species might not necessarily apply to diurnal species, a group that includes human beings. Thus, it becomes imperative to investigate circadian rhythmicity using diurnal animal models.

The unstriped Nile grass rat, *Arvicanthis niloticus*, shows robust diurnal rhythms in both the lab and the field (Blanchong et al., 1999), and thus represents an ideal animal model for the exploration of mechanisms underlying circadian rhythmicity in diurnal species (Katona and Smale, 1997). In addition, when a running wheel becomes available, a subset of these animals shift to be predominantly active during the night (night-active, NA), whereas others remain active during the day (day-active, DA; Katona and Smale, 1997). Areas of the brain that regularly promote wakefulness and arousal during the active phase are likely to contribute to these behavioral shifts. Data obtained from the orexinergic arousal system of DA and NA grass rats support this hypothesis (Nixon and Smale, 2005).

Being active during the regular resting phase can have adverse consequences on a variety of physiological and behavioral processes, such as attention and memory (Rouch et al., 2005; Gritton et al., 2009), which are modulated by arousal system (Blandina et al., 2004; Gonzalez-Burgos and Feria-Velasco, 2008; Herrero et al., 2008). Interestingly, the circadian clock of the SCN is not affected by the temporal shifts in the distribution of activity shown by NA grass rats (Blanchong et al., 1999; Rose et al., 1999; Schwartz and Smale, 2005), Thus, it is plausible that whereas some wakefulness promoting areas remain faithful to circadian signals from the SCN, some others could dissociate from the circadian system and start responding to wakefulness *per se*, or to some other cue associated with running in a wheel. It is important to evaluate these possibilities given the widespread incidence of temporal shifts in activity of

human shift workers, and the adverse health consequences that are associated with shift-work practices, which include among others, an increase in the development of prostate and breast cancer (Davis et al., 2001; Schernhammer et al., 2001; Kubo et al., 2006). Therefore, in addition to serving as a diurnal animal model for chronobiological research in general, the grass rat can also be used as a model to study the physiological effects exerted by temporal shifts in activity.

In this introductory chapter, I provide an overview of wakefulness-promoting areas of the brain, emphasizing their role in circadian rhythmicity. I follow that overview with a discussion of our current understanding of how these regions respond to temporal shifts in activity in diurnal and nocturnal species. A short description of the use of Fos protein expression as a marker for neural activity is also provided, since it is the main tool I use to identify neural activation in wakefulness promoting areas. Finally I present a summary of the research questions addressed in each chapter.

Wakefulness-promoting systems and their role on circadian rhythmicity

Wakefulness-promoting neural populations are distributed throughout the mammalian brain. It is tempting to suggest that the apparent functional redundancy of these wakefulness-promoting areas reflects the action of strong selection for the evolution of brain mechanisms that keep organisms alert and engaged with the environment. This might have been to ensure the optimal occurrence of physiological processes and behaviors that promote fitness. Wakefulness-promoting areas of the brain include: noradrenergic neurons in the

locus coeruleus (LC), serotonergic neurons in the raphe nuclei, histaminergic neurons in the tuberomammillary nucleus of the hypothalamus (TMM), orexinergic neurons in the lateral hypothalamus (LH), and cholinergic neurons in the tegmentum (T) and basal forebrain (BF). From these different locations neurons send widespread projections to most of the brain. Wakefulness-promoting cellular groups increase their neural firing during the active phase of the organism, and with the exception of the cholinergic cells of the T and BF, they decrease firing during non-rapid eye movement (nREM) sleep and become silent during rapid eye movement (REM) sleep. Cholinergic cells of the T and BF cease their firing during nREM sleep, but in contrast to the other systems that support wakefulness, they increase their firing rate during REM sleep (reviewed in Jones, 2005). Fos expression in wakefulness-promoting areas appears to increase concurrently with increases in neural firing (e.g. Novak et al., 2000; Martinez et al., 2002; Ko et al., 2003). Thus, the neural activity of wakefulness-promoting areas follows a daily cycle, which suggests that these systems are under circadian control.

Anatomical evidence indicates that the SCN is likely to modulate the activity of wakefulness-promoting groups by sending direct as well as indirect projections to these areas. The orexin group of the LH, for example, receives direct projections from the SCN (Abrahamson et al., 2001), whereas other areas receive circadian information through relay sites, including the medial preoptic area, median preoptic nucleus, ventral subparaventricular zone, and dorsomedial hypothalamus (Deurveilher and Semba, 2005; Rosenwasser, 2009). The

communication between the SCN and the wakefulness groups, however, is not unidirectional since the wakefulness-promoting areas are anatomically well placed to influence SCN activity. Areas such as the TMM, BF, T, raphe nuclei, and LC send direct projections to the SCN (reviewed in Rosenwasser, 2009). Moreover, neural activity in the SCN appears to be modulated by these wakefulness promoting sites. For example, activity in the SCN is higher during the subjective day and during REM sleep in comparison to nREM sleep (Deboer et al., 2003). But these patterns can be modified if lab rats are deprived of REM or nREM sleep (Deboer et al., 2003). These findings suggest that arousal groups "talk back" to the SCN to possibly provide the SCN clock with information about the individual's vigilance state (Colwell and Michel, 2003). Intriguingly, these areas do not only seem to provide information about vigilance state to the SCN, but also appear to play a role in the entrainment of circadian rhythms (reviewed in Yannielli and Harrington, 2004; Rosenwasser, 2009). Most of the evidence about the functions of wakefulness-promoting brain systems discussed above has been obtained using nocturnal rodents, and very little about these systems has been explored in diurnal species. In what follows I discuss what is known about the role of wakefulness-promoting areas in circadian rhythmicity in nocturnal as well as diurnal species.

The cholinergic system

Acetylcholine (ACh) can affect the functioning of the circadian clock of the SCN in nocturnal species, but there is no consensus with respect to its

physiological role. Some of the available data suggest that ACh may facilitate responses to light (Zatz and Brownstein, 1979; Zatz and Herkenham, 1981; Earnest and Turek, 1983), but *in vitro* and *in vivo* direct cholinergic stimulation of the nucleus produces phase shifts that are only partially similar to the ones produced by light (Buchanan and Gillette, 2005). Additionally, there is evidence that ACh plays a role in the regulation of peptidergic systems in the SCN (Madeira et al., 2004). In diurnal species little is known about how ACh might participate in circadian rhythmicity. The available evidence suggests that, at least in the grass rat, this system might play a lesser role in circadian functions, since when compared to the lab rat, there is a reduction in the number of cholinergic fibers in the SCN of grass rats (Castillo-Ruiz and Nunez, 2007). Moreover, preliminary results indicate that after retrograde tracing injections to the SCN, the number of labeled neurons in the BF and T is reduced (Castillo-Ruiz et al., 2007) in grass rats, when compared to what is observed in lab rats (Bina et al., 1993). Together these results are in agreement with the claim of a reduction in cholinergic projections to the SCN in the diurnal grass rat and a lesser role for the cholinergic system in circadian functions in this species, and perhaps in other diurnal mammals.

The histaminergic system

In nocturnal rodents, histamine (HA) appears to play a role in light entrainment, since histaminergic stimulation of the SCN either *in vivo* or *in vitro* mimics the effects of light (i.e., produces phase delays early in the subjective

night and phase advances late in the subjective night; reviewed in Harrington et al., 2000). Additionally, if this system is inhibited, photic effects are attenuated (reviewed in Harrington et al., 2000). Very little is known about the role of HA in diurnal species. Preliminary data suggest that the SCN of grass rats receives histaminergic projections, but these projections do not appear to be as abundant as the ones present in lab rats (C. Alvarez-Barón, unpublished observations). No studies have addressed yet whether HA plays a role in entrainment in diurnal species.

The serotonergic system

The available evidence suggests that serotonin (5HT) plays a role in photic entrainment. In nocturnal rodents, direct application of serotonergic agonists into the SCN attenuates responses to light, whereas application of serotonergic antagonists exacerbates these responses (reviewed in Yannielli and Harrington, 2004). In diurnal species, however, the effects induced by 5HT might be different since at least for the Sudanese grass rat, *Arvicanthis ansorgei*, the responses to serotonergic agonists are only seen during the subjective night, which is different from what is seen in nocturnal rodents (Cuesta et al., 2007). Thus, the effects of 5HT on entrainment are likely to be arousal-dependent (Challet, 2007). Not surprisingly then, the effects of light and those of serotonergic agonists on the phase of circadian rhythms are antagonistic in nocturnal species and agonistic in diurnal animals (Cuesta et al., 2007).

In addition, 5HT appears to play a role in non-photic entrainment. Direct application of 5HT into the SCN results in phase shifting effects that are similar to the ones produced by non-photic stimuli in nocturnal rodents (i.e., phase shifting responses only during the subjective day), such as those produced by social interactions and handling (reviewed in Yannielli and Harrington, 2004); no study has determined if 5HT has non-photic effects in diurnal species.

The orexinergic system

After its discovery in 1998 (De Lecea et al., 1998; Sakurai et al., 1998), the orexinergic system, composed of orexin A (OXA, a.k.a. hypocretin-1), orexin B (OXB, a.k.a. hypocretin-2) and their receptors, has surfaced as an important regulator of arousal. If this system is impaired, due to either deficiency in its receptors or low production levels of orexins, human and non-human individuals show abrupt bouts of sleep that are not restricted to the rest phase (Jones, 2008). Moreover, it has been suggested that this system plays an important role in the regulation of other arousal systems through direct projections (reviewed in Saper et al., 2001) since direct application of orexinergic drugs enhances neural firing in places such as the BF, raphe nuclei, TMM, and LC (Eriksson et al., 2001; Brown et al., 2002; Wu et al., 2004; Gompf and Aston-Jones, 2008). In grass rats, activation of the orexinergic system is related to the display of activity (Nixon and Smale, 2005). Little is known, however, about orexinergic participation in the modulation of other arousal systems in diurnal species. In relation to potential circadian roles for orexin, an *in vitro* study demonstrated that OXA increases

neural firing of the SCN (Farkas et al., 2002). However, to my knowledge no study has evaluated yet whether the orexins have a resetting effect of SCN neural activity rhythms in either diurnal or in nocturnal species.

The noradrenergic system

In nocturnal rodents, noradrenergic drugs have effects on SCN functioning. The effects, however, differ depending on the duration of the administration of the drugs. While chronic treatment with the noradrenergic agonist drug clonidine attenuates light phase-shifting effects, acute treatment with the same agent mimics the phase shifting effects of light (Dwyer and Rosenwasser, 2000). Moreover, chronic administration of clonidine shortens the free running period both in constant light and constant darkness (Rosenwasser, 1996). Thus, norepinephrine (NE) appears to have a modulatory role on SCN functioning in nocturnal species. No study has explored the role of NE in diurnal species.

Wakefulness-promoting areas and temporal shifts in activity

Only a handful of studies, mainly done in nocturnal rodents, has evaluated the effects that temporal shifts in activity have on wakefulness-promoting areas of the brain. Most of these studies have used sleep deprivation techniques to induce wakefulness during the regular resting phase. The results of those studies suggest that these areas respond mainly to wakefulness *per se*, apparently overriding circadian regulation. For example, in cholinergic cells of the BF Fos expression is increased after 6 hours of sleep deprivation induced by gentle

handling (Greco et al., 2000). Also, after 3 hours of sleep deprivation following the same technique, Fos expression is increased in serotonergic as well as non-serotonergic cells of the caudal dorsal raphe (Webb et al., 2010), but not in cells of the LC (in and out of noradrenergic cells; Grossman et al., 2000). However, sleep deprivation induced by physical restraint during the same interval of time produces increased Fos expression in the LC (Grossman et al., 2000). In the diurnal grass rat, voluntary sleep deprivation during the regular resting phase induces an increase in Fos expression in OXA and OXB cells (Nixon and Smale, 2005) suggesting that this system is also responsive to wakefulness *per se* in a fashion that overshadows circadian regulation.

Given that humans who engage in temporal shifts in activity (e.g. night shift workers) show deficits in processes such as cognitive performance (Rouch et al., 2005), which are modulated by wakefulness-promoting areas, it is important to determine how these systems respond when wakefulness occurs during the rest phase of diurnal species. Furthermore, the paradigms that are commonly used to study the effects of shift work with animal models mostly rely on forcing wakefulness on the experimental animals (Murphy et al., 2003; Salgado-Delgado et al., 2008). Although these paradigms have shed some light on the physiological effects exerted by shift work, it is unlikely that the results are entirely applicable to human shift workers, since in contrast to animals that are forced to stay awake, most human situations involve the voluntary maintenance of wakefulness during the resting phase.

Fos expression as a marker of neuronal activity

The product of the immediate early gene *cfos*, i.e., Fos, has been used widely as a tool to assess neuronal activation induced by behavioral state, including sleep and wakefulness (Shiromani, 1998). Fos is a transcriptional regulator, therefore, when produced, it can modulate the expression of other genes. Although the presence of Fos in a cell does not necessarily imply enhanced neuronal firing (Shiromani, 1998), at least in wakefulness-promoting areas, that concordance exists; for those regions there is increased neuronal firing patterns (reviewed in Saper et al., 2001) as well as increased Fos expression during the active phase of diurnal and nocturnal rodents (Novak et al., 2000; Janusonis and Fite, 2001; Martinez et al., 2002; Ko et al., 2003; Kodama et al., 2005). Moreover, neurotransmitter release at the axon terminals of the cholinergic neurons of the BF in rats is increased at times when cholinergic cell bodies of this region show increased Fos expression (Greco et al., 1999; Greco et al., 2000). Thus, Fos appears to be a reliable marker of neural activity in wakefulness-promoting areas.

Overview of the chapters

In this dissertation I present experiments that aim to shed light on the relation that exists between the wakefulness-promoting systems of the brain and the circadian time keeping system in a diurnal brain. Particularly, the approach contrasts the patterns of neural activity associated with wakefulness during the regular active phase and during the inactive phase. For all experiments I used the male grass rat, *Arvicanthis niloticus*, as a diurnal animal model. One chapter

of this dissertation (Chapter 2) has been published with co-authors (Castillo-Ruiz et al., 2010), and for the sake of consistency, I elected to use first person plural rather than "I" in all the chapters that present data that are published or will be sent for peer review with colleagues as co-authors.

In Chapter 2, I investigated whether voluntary temporal shifts in activity in DA and NA grass rats were related to increase neural activation in the cholinergic system of the BF. I accomplished that aim by analyzing Fos expression during the day and during the night in several regions of the BF. In particular, I focused the analysis on three well characterized cholinergic groups: the medial septum (MS), the vertical diagonal band of Broca (VDB) and the horizontal band of Broca (HDB). In nocturnal species the orexinergic system appears to have a modulatory role in neural activity of the cholinergic system of the BF (Wu et al., 2004; Fadel and Frederick-Duus, 2008). For that reason, I evaluated the relationship between these two wakefulness-promoting systems by correlating Fos expression in the orexin cell group with Fos expression in the BF, and by looking at orexinergic axonal projections to cholinergic areas of the BF. Finally, given that running on a wheel might be self-reinforcing (reviewed in Sherwin, 1998), I investigated whether neural activity in the reward system is associated with wheel running at different phases of the light-dark cycle in DA and NA grass rats. To address this question, I focused on dopaminergic and non-dopaminergic cells of two components of the reward system: the ventral tegmental area (VTA) and supramammillary nucleus (SUM).

In Chapter 3, I followed up the results from Chapter 2 by investigating whether, in grass rats, induced wakefulness during the night elicits changes in neural activity in arousal and reward areas similar to those seen in grass rats that voluntarily become active at night. I evaluated this question by analyzing the BF, VTA, and SUM after 6 hours of induced wakefulness produced by gentle stimulation of the animals. The amount of time for this forced wakefulness was similar to the amount of time NA grass rats were voluntarily awake before they were euthanized for obtaining measures of Fos expression in their brains (Chapter 2). I also evaluated patterns of Fos expression in other wakefulness and sleep promoting areas - TMM, LC, LH, and VLPO among others-, circadian controlling areas- the SCN and ventral subparaventricular zone (sPVZ)-, as well as stress related areas – the parvocellular and magnocellular components of the paraventricular nucleus of the hypothalamus (pPVN and mPVN, respectively). In addition, I investigated whether 2 hours of sleep recovery, following the sleep deprivation episode, influenced neural activation in the same brain regions. Finally, I used correlational analyses to assess the relationships between Fos expression in areas that contain orexin cells and Fos expression in other brain areas related to wakefulness and sleep.

In Chapter 4, to characterize further the histaminergic and serotonergic regions of a diurnal brain, I analyzed patterns of Fos expression in the TMM and raphe nuclei. I also assessed whether these cellular groups have axonal projections that reach the cerebrospinal fluid (CSF). I did so by looking at patterns of cell labeling after injections of the retrograde tracer cholera toxin

subunit beta (CTB) to the third ventricle (3V). Finally, I investigated whether the dense cluster of retrogradely labeled cells found in the DR after the CTB injections was positive for 5HT.

In Chapter 5 I conclude this dissertation with a discussion of circadian patterns of neural activation in wakefulness-promoting areas in diurnal and nocturnal rodents. Then I discuss how voluntary and induced wakefulness during the inactive phase have differential effects on arousal and reward related areas in diurnal and nocturnal rodents, and I finish that chapter by discussing the implications of these findings for humans as well as non-human animals.

Chapter 2

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CHAPTER 2

Neural activation in arousal and reward areas of the brain in day-active and night-active grass rats

INTRODUCTION

Most of the research done in the field of circadian biology involves the use of nocturnal species as experimental subjects. In contrast, relatively little has been investigated in diurnal species, mainly due to the lack of suitable models of diurnality. The unstriped Nile grass rat, *Arvicanthis niloticus* (grass rat), shows robust diurnal rhythms in both the laboratory and in the field (Blanchong et al., 1999), and thus represents an ideal animal model for the study of mechanisms underlying circadian rhythmicity in diurnal species (Katona and Smale, 1997). Interestingly, when a running wheel becomes available, a subset of these diurnal animals shifts to be predominantly active during the night (night-active individuals, NA), whereas others remain active during the day (day-active individuals, DA; Blanchong et al., 1999). The shift in locomotor activity pattern seen in NA grass rats does not appear to reflect a change in the functioning of the master oscillator of the suprachiasmatic nucleus (SCN; Blanchong et al., 1999; Rose et al., 1999; Schwartz and Smale, 2005), but rather a change in the coupling between the SCN and the neural mechanisms that control activity or a complete dissociation of these mechanisms and the master pacemaker. Because voluntary activity during the resting phase is a phenomenon commonly seen in human shift workers, the grass rat also represents a useful model to study the physiological effects exerted by temporal shifts in activity. This model contrasts

with other animal models of shift work in which temporal shifts in activity (e.g. Murphy et al., 2003; Salgado-Delgado et al., 2008) are not produced voluntarily by the animal but induced by the researcher.

In sedentary grass rats (i.e., singly-housed with no wheels) wakefulness is associated with enhanced neural activity in the histaminergic (Novak et al., 2000) and orexinergic (Martinez et al., 2002) brain arousal systems, such that there is an increase in Fos expression in those cellular groups during the active phase as compared to the resting phase of the 24-hour cycle. Likewise, the orexinergic system of grass rats housed with wheels shows an increase in Fos expression at times when animals are actively running, regardless of the phase of the light-dark cycle where the locomotor activity occurs (Nixon and Smale, 2004). It is unclear, however, how other arousal systems of the brain might respond to this temporal segregation of activity. Of particular interest is the cholinergic (ACh) system of the basal forebrain (BF), since it participates in cortical arousal (reviewed in Jones, 2008). This system is involved in the generation of the hippocampal theta wave (HPC-θ), a rhythm that is displayed during wakefulness and paradoxical sleep (reviewed in Buzsaki, 2002) and has been associated with the execution of voluntary activity, such as wheel-running (Oddie et al., 1996). Also, when laboratory rats are stimulated to stay awake during their resting phase, ACh neurons of the BF show increased Fos expression (Greco et al., 2000) supporting the role of these neurons in arousal. In addition, neurons of the BF that secrete gamma-amino-n- butyric acid (GABA) and glutamate appear to play

a modulatory role in cortical activity, as well as in the HPC-θ (Leung and Shen, 2004; Yoder and Pang, 2005; Henny and Jones, 2008).

It is likely that neural activity in the ACh BF is influenced by arousal systems, such as the orexinergic system. This system, composed of neurons producing orexin A (OXA), orexin B (OXB) and their receptors, has been postulated to play an important role in the regulation of other arousal systems, including the ACh BF (Saper et al., 2001). In fact, orexinergic neurons project densely to the latter cellular group and these cells are responsive to orexin stimulation (Wu et al., 2004). Moreover, blockade of orexin receptors in ACh cells of the medial septum (MS), a component of the BF, attenuates the HPC-θ rhythm (Gerashchenko et al., 2001). Thus, the orexinergic system may play a role in the modulation of neural activity of the ACh BF in grass rats with access to wheels.

An additional system of the brain that might be stimulated by the temporal segregation of activity in DA and NA grass rats is the reward system. It has been suggested that wheel running is rewarding and possibly addictive (Eikelboom and Lattanzio, 2003), since animals not only work for wheel access, but also compromise vital activities such as food and water intake in order to engage in wheel running (reviewed in Sherwin, 1998). The reward system includes the ventral tegmental area (VTA) and the supramammillary nucleus of the hypothalamus (SUM). These areas show increased neural activation upon exposure to rewarding stimuli (e.g. Asmus and Newman, 1994; Balfour et al., 2004; Marcangione and Rompre, 2008), including acute access to a running wheel (Yanagita et al., 2007). The rewarding effects mediated by these systems

depend on direct (VTA; reviewed in Ikemoto and Panksepp, 1999) and indirect (SUM; Ikemoto et al., 2004) efferent projections to the nucleus accumbens (NAc), and require the release of dopamine in this nucleus (Yoshida et al., 1992; Schilstrom et al., 1998; Ikemoto et al., 2004). In addition to their role in reward, the VTA and SUM are also involved in the modulation of vigilance states. For example, there is increased activation of dopamine cells of the VTA during paradoxical sleep and during the execution of appetitive behaviors as compared to slow wave sleep and quiet wakefulness (Dahan et al., 2007). The SUM in turn is involved in the control of the HPC-θ rhythm, and thus may participate in learning and memory (reviewed in Pan and McNaughton, 2004). Additionally, the SUM contributes to emotional responses (Pan and McNaughton, 2004). Thus, given the roles of the VTA and SUM in motivation and arousal, it appears likely that neural activation of these regions is associated with wheel access in ways that may differ in animals that run at different times of day.

In the present study we investigated the relationship between neural activation in the BF, VTA, and SUM, and the voluntary shifts in locomotor activity seen in DA and NA grass rats. First, we evaluated whether neural activity in three major components of the ACh system of the grass rat BF – the MS, vertical diagonal band of Broca (VDB), and horizontal diagonal band of Broca (HDB) – is associated with temporal patterns of wheel running. Second, we evaluated the hypothesis that orexinergic cells might modulate neural activity of the grass rat BF by looking at whether activation of cells in these two systems is correlated and at whether orexin cells send direct axonal projections to the BF. Finally, we

investigated whether neural activity in the reward system is associated with wheel running at different phases of the light-dark cycle in DA and NA grass rats. To address this question, we focused on dopaminergic and non-dopaminergic cells of the VTA and SUM. Thus, the present study expands our current understanding of voluntary temporal shifts in activity and their associations with neural activation in arousal, as well as in brain reward areas in a diurnal mammal.

EXPERIMENTAL PROCEDURES

Animals

Thirty-six adult male grass rats were used in this study. Twenty-four had access to wheels (n = 12 DA, 12 NA), while the remaining 12 served as no-wheel controls (C). All animals were born in our laboratory and derived from a group brought from Kenya in 1993 (Katona and Smale, 1997). Animals were kept on a 12:12 light-dark cycle with a red light (< 5 lux) on at all times, and were provided with *ad libitum* access to water and food (Harlan Teklad 8640 rodent diet, Harlan Teklad Laboratory, Madison, WI, or PMI Nutrition Prolab RMH 2000, PMI Nutrition International, Brentwood, MO). The approach used to determine the chronotype of DA and NA individuals, as well as their housing conditions, were described previously, as these same animals were used in prior work from this lab (Nixon and Smale, 2004). Briefly, the animals were housed with a wheel (26 cm diameter, 8 cm width) in plexiglass cages (17 x 34 x 28 cm) and wheel-running rhythms were monitored using DSI Dataquest 3 system (MiniMitter, Sunriver, OR). After the rhythms were stable for at least one week, animals were

classified as DA if activity ceased within 2 hours after lights-out, or as NA if activity continued for more than 4 hours after lights-out. The third group of animals used in this study, the C group, consisted of singly-housed (plexiglass cages 17 x 34 x 28 cm) grass rats with no wheels. Under those conditions grass rats show patterns of sleep, general activity, and body temperature that are characteristic of diurnal species (McElhinny et al., 1997; Novak et al., 1999). All animals were kept under the aforementioned conditions for at least one month before sacrifice. All experiments were performed in compliance with guidelines established by the Michigan State University Institutional Animal Care and Use Committee, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tissue collection and preparation

Animals (n = 6 per group) were perfused at Zeitgeber times (ZT) 4 and 16. At the time of perfusion animals were deeply anesthetized with intraperitoneal injections of sodium pentobarbital (Ovation Pharmaceutical, Deerfield, IL). An aluminum hood was placed over the head of the animals euthanized during the dark phase to prevent exposure to light. Animals were perfused intracardially with 0.01 M phosphate-buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) with 75 mM lysine (Sigma-Aldrich) and 10 mM sodium periodate (Sigma-Aldrich; PLP). Brains were post-fixed in PLP for 4 – 8 h, and then transferred to 20% sucrose solution in 0.1 M PB overnight at 4°C. Then, coronal sections (30 µm) were cut on a freezing

sliding microtome, and alternate sections were collected in three series in cryoprotectant solution at -20°C until further processing.

Although the tissue from all groups was processed at the same time, the time elapsed between perfusion and immunocytochemical procedures was not the same for all groups. Whereas the brains of DAs and NAs were stored in cryoprotectant for over seven years, the brains of our Cs were stored in this solution only for one month. This difference in exposure to cryoprotectant, however, was not likely to have an effect on our results since tissue can be stored for many years in this solution without losing antigenicity (Hoffman and Le, 2004). Furthermore, our results were area and sample-time specific, and did not indicate reduced antigenicity in the tissue kept the longest in cryoprotectant. Thus, the data presented here are likely to reflect only neuronal activation associated with the conditions of the study pertinent to each group and not the effects of differential time since perfusion.

Experiment 1: Basal forebrain activation and its relation to orexinergic activation

1.1. Basal forebrain activation

Tissue was rinsed 3 times (10 min/rinse) in 0.01 M PBS between all steps of the immunocytochemical procedures and all steps were carried out at room temperature unless indicated otherwise. In addition, all incubations included 0.3% Triton X-100 (RPI, Elk Grove Village, IL). Free-floating sections containing the MS, VDB, and the HDB were rinsed (6 times, 10 min/rinse) in 0.01 M PBS, blocked for 1 h using 5% normal donkey serum (NDS; Jackson ImmunoResearch

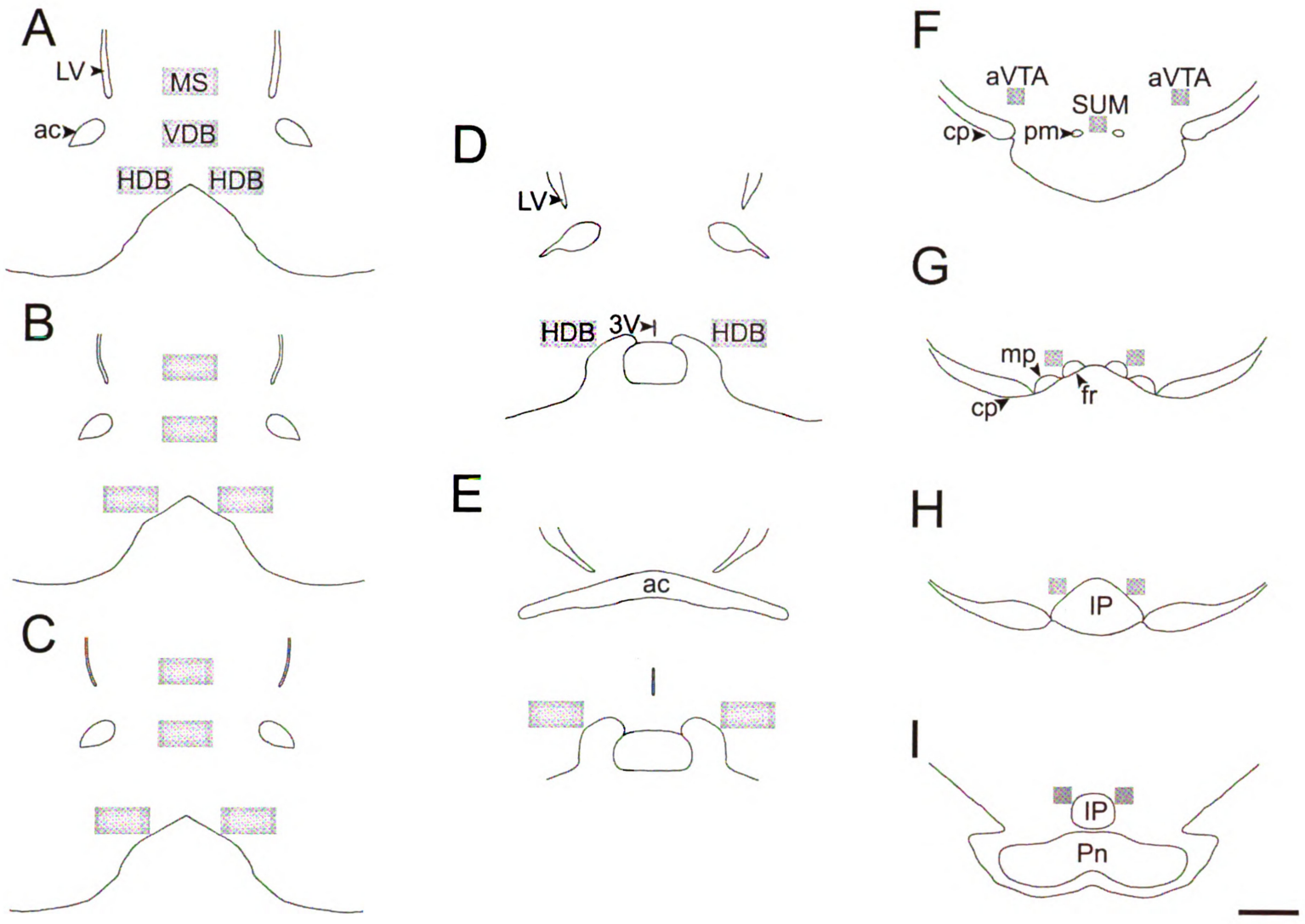
Laboratories, West Grove, PA) in PBS and incubated for 48 h in a rabbit anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:20,000 in PBS and 3% NDS). The sections were then incubated for 1 h 30 min in a donkey anti-rabbit biotinylated antibody (Jackson; diluted 1:200 in PBS and 3% NDS), and then for 1 h 30 min in avidin-biotin peroxidase complex (AB complex, Vector Laboratories, Burlingame, CA; in PBS). After 3 rinses (10 min/rinse) in Tris 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) for 7 min. This reaction was followed by four 10 min rinses in PBS. Then, sections were blocked for 1 h in 5% normal horse serum (NHS; Vector; in PBS) and incubated for 48 h in a goat anti-choline acetyltransferase (ChAT) antibody (Chemicon, Temecula, CA; diluted 1:10,000 in PBS and 3% NHS). Following primary incubation, the sections were incubated for 1 h 30 min in a horse anti-goat biotinylated antibody (Vector; diluted 1:200 in PBS and 3% NHS). Then, the tissue was incubated for 1 h 30 min with AB complex (Vector; in PBS). After 3 rinses (10 min/rinse) in Tris buffer, the sections were reacted with 0.02% DAB in the same buffer (with 0.35 µl 30% hydrogen peroxide/ml buffer) for 5 minutes, and then rinsed in 0.01 M PBS (4 times, 5 min/rinse). All sections were mounted onto gelatin-coated slides, dehydrated, and coverslipped with dibutyl phthalate xylene (Sigma-Aldrich).

In order to quantify Fos and ChAT expression, we selected three sections throughout the MS and VDB and five sections for the HDB. Sections for the MS and VDB corresponded approximately to plates 15 through 17 of the rat brain

atlas by Paxinos and Watson (1997), whereas sections for HDB corresponded to plates 15 through 20. For every section selected, cells expressing Fos, ChAT, and Fos+ChAT were counted within a region defined by a 600 μm (width) x 300 μm (height) box placed in the center of the studied areas (see Fig. 2.1A-2.1E). Stereo Investigator software (MBF Bioscience Inc, 2007) was used to place the box and perform counts. All counts were made by an investigator unaware of the source of the tissue. After the cell counts were completed, the total cell counts were divided by the area occupied by the box (0.18 mm^2) and that represented the data that were analyzed. Adjustments were done when small portions of the counting box fell outside the studied areas. For the HDB, we averaged data from the two sides of the brain per section. The MS and VDB did not require this procedure since they are located close to the midline (see Fig. 2.1A-2.1C).

To analyze the data obtained in the BF, we used three-way analysis of variance (ANOVA). These analyses had ZT (4 and 16) and group (DA, NA, and C) as between-subjects factors and level of the section (3 levels for MS and VDB, and 5 levels for HDB) as a within-subjects factor. The dependent variables analyzed per area were: (1) number of ACh cells/mm^2, (2) number of non-cholinergic (nACh) cells expressing Fos/mm^2, and (3) proportion of ACh cells expressing Fos. Because this latter variable was expressed as proportions, it was squared root transformed, and then, arcsine transformed to normalize its distribution. For all comparisons differences were considered significant when p was less than 0.05. Significant interaction effects were followed by analyses of

Figure 2.1. Rostro-caudal illustrations depicting representative sampling areas used to quantify Fos expression in ACh and nACh cells of the MS, VDB, and HDB (A-E), as well as Fos expression in nTH and TH cells of the VTA and SUM (F-I). Boxes used for cellular counts had the following dimensions: (A-E) 600 µm x 300 µm and (F-I) 160 µm x 160 µm. Scale bar= 1 mm.


A
LV
MS
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VDB
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HDB
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LV
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aVTA
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simple effects and post hoc comparisons using *t*- tests. The software used for the statistical analyses was SAS v 9.1 (SAS Institute, 2002).

1.2. BF activation and orexin activity in DA ans NA grass rats

To determine whether activation of orexin cells was correlated with BF activation, we used correlational analyses between Fos expression in OXA and OXB cells and Fos expression in ACh and nACh cells of the BF. Data for Fos in OXA and OXB cells in the same animals came from Nixon and Smale (2004; see Animals section above). We did not test whether orexin activation was related to BF activation in Cs since for this group we did not have data on the former variable. The program used to analyze the correlations was SPSS v. 15.0 (SPSS; SPSS, Chicago, IL).

To complement the correlational analyses, we evaluated whether the grass rat BF might receive orexinergic inputs. Particularly, we wanted to determine if orexin-containing fibers formed putative appositions with ACh cells. We limited the anatomical analyses to OXA only, since the distribution of orexin-containing fibers in the BF of grass rats is very similar for the two orexins (Nixon and Smale, 2007a). For this analysis, sections containing the BF from 2 animals per group (DA, NA, and C) were reacted for OXA, as described in Nixon and Smale (2004), and for ChAT. Briefly, tissue was blocked using NDS (Jackson), then reacted with goat anti-OXA (Santa Cruz; diluted 1:10,000), and donkey anti-goat secondary (Santa Cruz; diluted 1:500). Following PBS rinses after the OXA/nickel-DAB reaction (7 minutes), the tissue was blocked using NHS, and

then incubated in goat anti-ChAT primary following the same procedure described above for BF activation. Following DAB reaction (3 minutes), tissue was mounted and coverslipped with DPX. Putative contacts between OXA fibers and ACh cells were verified with a 100x objective.

Experiment 2: Activation of reward systems

Free- floating sections throughout the VTA were processed for Fos and tyrosine hydroxylase (TH) following procedures similar to the ones described above with the exceptions noted below. For the Fos staining, the tissue was incubated in 5% NDS (in PBS) for 30 min and with a rabbit anti-Fos antibody (Santa Cruz; diluted 1:20,000 in PBS and 3% NDS) for 24 h. The incubations with a donkey anti-rabbit biotinylated antibody (Jackson; diluted 1:200 in PBS and 3% NDS), as well as with the AB complex (in PBS) were for 1 h. The DAB-nickel sulfate reaction lasted 6.5 min. For TH staining, the tissue was incubated in 5% NHS (Vector; in PBS) for 30 minutes. Tissue was then incubated with mouse anti-TH (Immunostar, Hudson, WI; diluted 1:20,000 in PBS and 3% NHS) for 24 h. The biotinylated secondary antibody incubation with horse anti-mouse (Vector; diluted 1:200 in PBS and 3% NHS), as well as the AB complex incubation (in PBS) were for 1 h. The reaction time with the DAB solution lasted 2 min.

In order to quantify Fos and TH expression, we selected four sections throughout the VTA (rostral to caudal VTA). These sections corresponded approximately to plates 37 through 43 of the rat brain atlas by Paxinos and Watson (1997). For every section selected, cells expressing Fos, TH, and

Fos+TH were counted within a region defined by a 160 μm x 160 μm box that was placed lateral to the medial border of the VTA (see Fig. 2.1F-2.1I), in an area rich in TH staining. Counts were done bilaterally in the VTA and cell numbers were averaged per section. In addition, the counting box was placed in one section of the caudal SUM (see Fig. 2.1F). All counts were done using a 25x objective and performed by an investigator unaware of the source of the tissue.

To analyze the data obtained in the VTA, we could not use repeated measures ANOVA, because some sections were missing for a few animals. Instead, we used two-way ANOVAs per VTA region. The independent variables were ZT and group whereas the dependent variables analyzed were (1) number of Fos cells in non-TH (nTH) cells and (2) number of TH cells. Data on TH cells expressing Fos were not analyzed because double-labeled cells were very rare across all groups. Significant main effects were followed by post hoc comparisons using *t*-tests. The software used for the statistical analyses was SAS v 9.1 (SAS Institute, 2002).

RESULTS

Experiment 1: Basal forebrain activation and its relation to orexinergic activation

1.1. Basal forebrain activation

The total number of ACh cells in the MS, VDB, and HDB did not differ significantly for any comparison (all p values > 0.08). Also, the proportions of ACh cells that contained Fos in the MS and VDB, were not significantly affected

by time of day or by chronotype or by an interaction between these factors (all p values > 0.14; data not shown). The patterns of results for which ANOVAs revealed significant effects of our independent variables on cells within the BF are summarized in Figures 2.2-2.5.

HDB

Patterns of Fos expression in the HDB were not significantly affected by level of the HDB being analyzed nor by any of the interactions involving this factor ($p > 0.27$; data not shown). Thus, the data were averaged across sections and analyzed using two-way ANOVAs.

Patterns of Fos expression in ACh cells

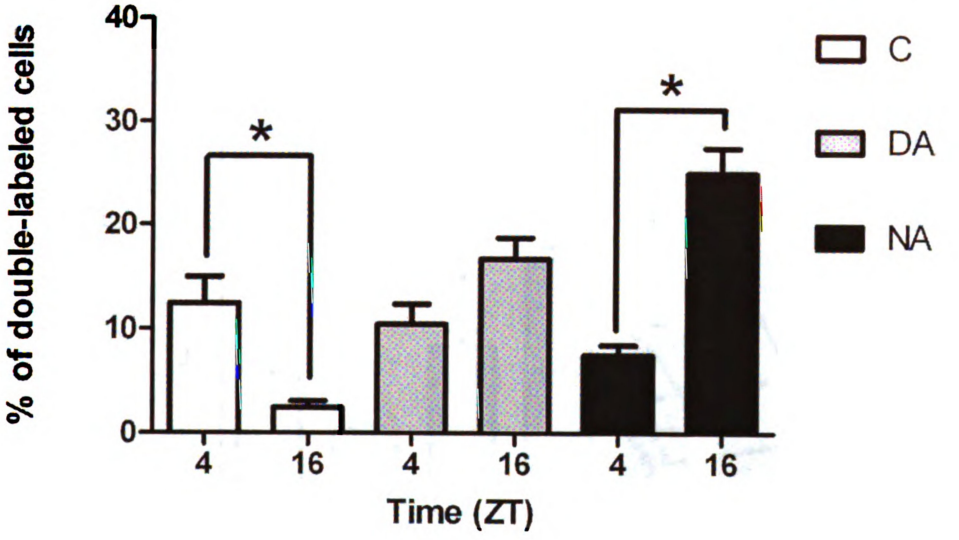
The interaction between ZT and group was significant [$F_{2, 27} = 6.76$, $p < 0.01$]. Analysis of the simple effect of ZT within group revealed that NAs had more double-labeled cells at ZT 16 than at ZT 4 [$F_{1, 27} = 8.80$, $p < 0.01$, Fig. 2.2A], whereas Cs had more double-labeled cells at ZT 4 than at ZT 16 [$F_{1, 27} = 4.63$, $p = 0.04$, Fig. 2.2A]. In contrast, there was no significant effect of ZT for DAs [$F_{1, 27} = 1.22$, $p = 0.28$, Fig. 2.2A]. Analysis of the simple effect of group detected no significant differences across groups at ZT 4 [$F_{2, 27} = 0.14$, $p = 0.87$], whereas a significant difference was observed at ZT 16 [$F_{2, 27} = 11.19$, $p < 0.01$]. At ZT 16, NAs and DAs had more double-labeled cells than Cs [$t_{27} = -4.62$, $p < 0.01$ and $t_{27} = -2.98$, $p < 0.01$, respectively, Fig. 2.2B). No differences were observed between DAs and NAs [$t_{27} = -1.16$, $p = 0.26$, Fig. 2.2B].

Figure 2.2. Patterns of Fos expression in ACh cells (A, B) and nACh cells (C, D) in the HDB. Panels A and C show significant ZT differences (asterisks) within groups, whereas panels B and D show significant group differences within ZT ($p < 0.05$). Note that group means with different letters are significantly different from each other, here as well as in Figures 3, 4, and 7.

HDB

Fos in ACh cells

A



B

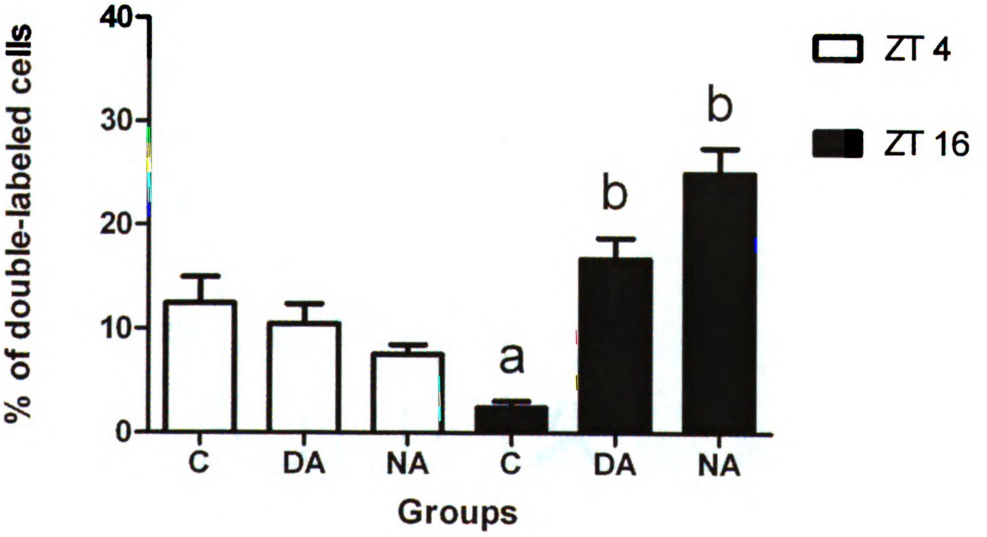
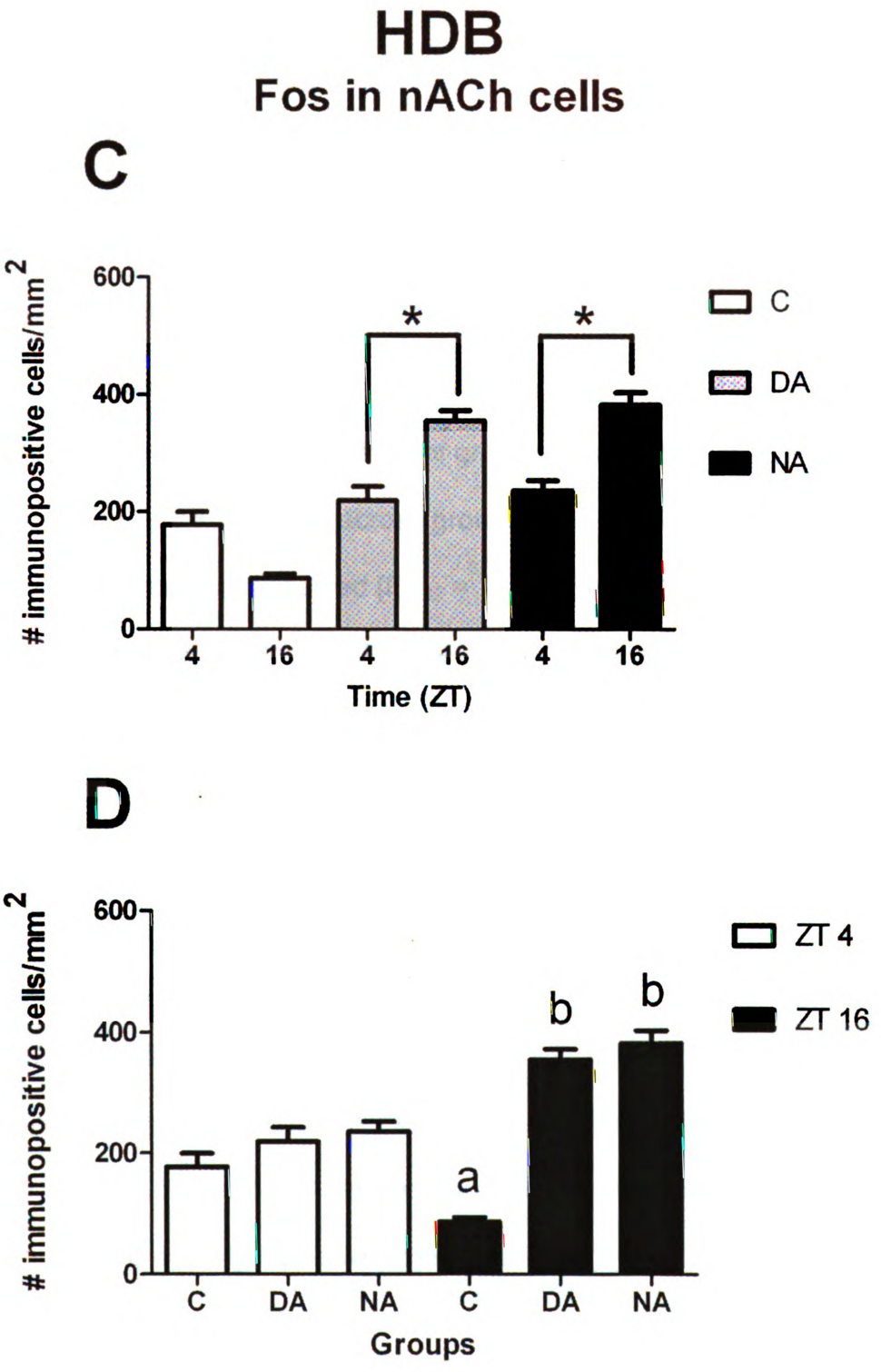


Figure 2.2. (cont'd)



Patterns of Fos expression in nACh cells

As the raw data were found to violate the normality assumption of the ANOVA, they were squared root transformed prior to analysis. The same transformation was done for all data sets involving nACh neurons. There was a significant interaction between ZT and group [$F_{2, 27} = 5.53$, $p < 0.01$]. Analysis of the simple effect of ZT revealed that DAs and NAs had more Fos expression at ZT 16 than at ZT 4 [$F_{1, 27} = 4.40$, $p = 0.05$ and $F_{1, 27} = 5.11$, $p = 0.03$, respectively, Fig. 2.2C], whereas no differences were observed for Cs [$F_{1, 27} = 3.36$, $p = 0.08$, Fig. 2.2C]. Analysis of the simple effect of group revealed that at ZT 4 there were no differences in Fos expression across groups [$F_{2, 27} = 0.99$, $p = 0.39$], whereas at ZT 16 differences were observed [$F_{2, 27} = 17.96$, $p < 0.01$]. At ZT 16, NAs and DAs had more Fos expression in nACh cells than Cs [$t_{27} = -5.48$, $p < 0.01$ and $t_{27} = -4.62$, $p < 0.01$, respectively, Fig. 2.2D]. No difference was observed between DAs and NAs [$t_{27} = -0.28$, $p = 0.78$, Fig. 2.2D].

MS

Patterns of Fos expression in nACh cells

The main effect of level was significant [$F_{2, 54} = 9.67$, $p < 0.01$], with the caudal level showing more Fos expression in nACh cells than the rostral level [$t_{54} = -4.39$, $p < 0.01$]. The middle level had more Fos expression than the rostral level [$t_{54} = -2.08$, $p = 0.04$], but lower expression than the caudal level [$t_{54} = -2.32$ $p = 0.02$]. These effects of level had no significant interactions with the effects of ZT or group. The interaction between ZT and group, however, was significant [$F_{2,}$

$_{27}$ = 5.38, p = 0.01]. Analysis of the simple effects of ZT revealed that NAs had more Fos expression in nACh cells at ZT 16 than at ZT 4 [$F_{1, 27}$ = 7.14, p = 0.01 Fig. 2.3A]. No differences were observed for Cs [$F_{1, 27}$ = 3.77, p = 0.06, Fig. 2.3A] or DAs [$F_{1, 27}$ = 0.01, p = 0.94, Fig. 2.3A]. Analysis of the simple effects of group revealed that at ZT 4 there were no statistical differences across groups [$F_{2, 27}$ = 0.37, p = 0.69], but at ZT 16 there was a significant effect of group [$F_{2, 27}$ = 7.22, p < 0.01]. Paired comparisons revealed that at ZT 16, NAs expressed more Fos in nACh cells than DAs and Cs [t_{27} = -2.18, p = 0.04 and t_{27} = -3.76, p < 0.01, respectively, Fig. 2.3B]. No differences were observed between DAs and Cs [t_{27} = -1.18, p = 0.25, Fig. 2.3B].

VDB

Patterns of Fos expression in nACh cells

There was a significant main effect of level [$F_{2, 54}$ = 4.60, p = 0.01], and t-tests revealed that the rostral level contained fewer nACh cells expressing Fos than the caudal level [t_{54} = -3.03, p < 0.01]. In contrast, no differences were observed between the middle and rostral [t_{54} = -1.48, p = 0.15] or the middle and caudal [t_{54} = -1.56 p = 0.12] levels. These effects of level had no significant interactions with the effects of ZT or group. In contrast, a significant interaction was observed between ZT and group [$F_{2, 27}$ = 3.82, p = 0.03]. Analysis of the simple effects of ZT revealed that none of the groups had differential expression of Fos at the two ZTs [C: $F_{1, 27}$ = 3.77, p = 0.06; DA: $F_{1, 27}$ = 0.26, p = 0.62; and

Figure 2.3. Patterns of Fos expression in nACh cells in the MS. Panel A shows significant ZT differences (asterisks) within groups, whereas panel B shows significant group differences (letters) within ZT ($p < 0.05$).

MS

Fos in nACh cells

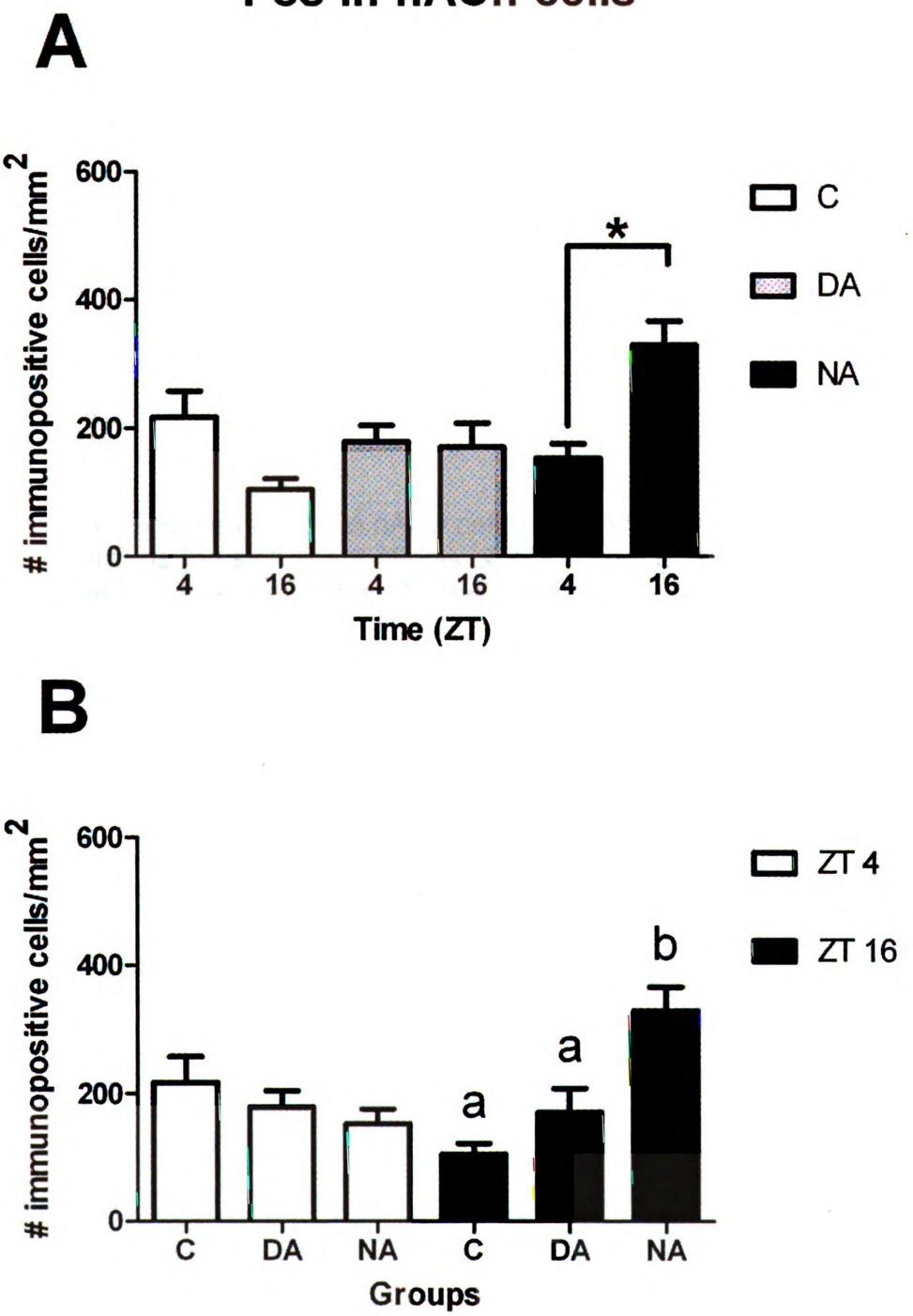
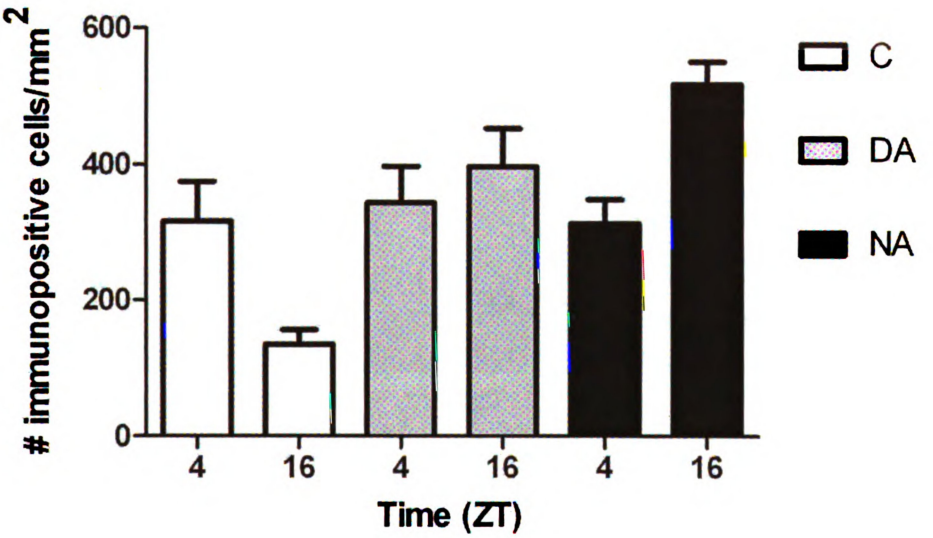


Figure 2.4. Patterns of Fos expression in nACh cells in the VDB. Panel A shows comparisons within groups (no significant effects of ZT). Panel B shows significant group differences (letters) within ZT ($p < 0.05$).

VDB

Fos in nACh cells

A



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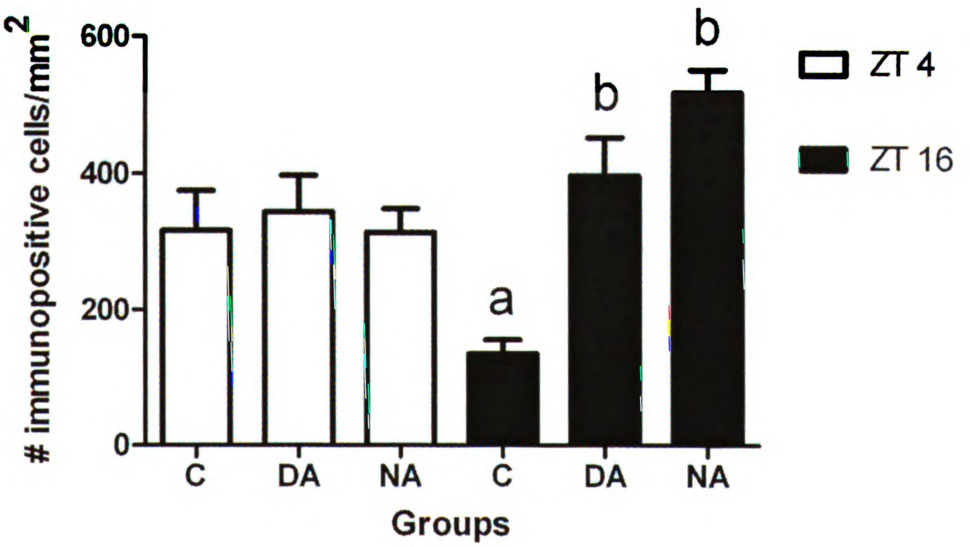


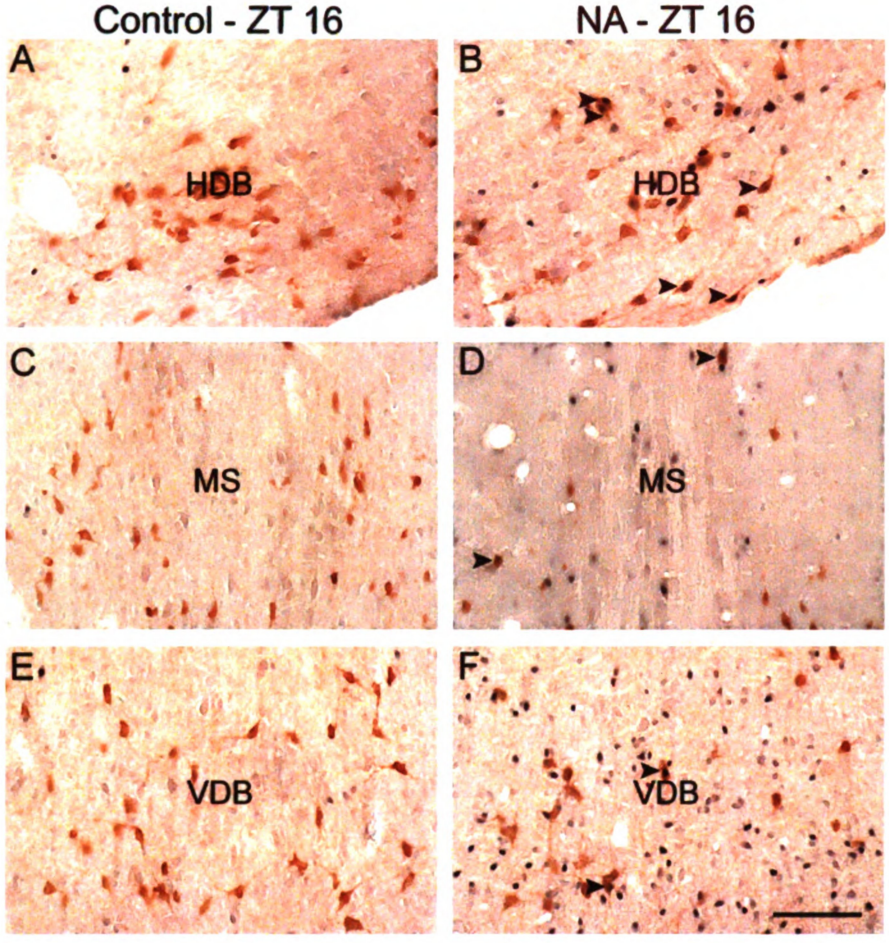


Figure 2.5. Photomicrographs of ACh cells (ChAT- positive) expressing Fos in the HDB (A, B), MS (C, D), and VDB (E, F) of a control (A, C, E) and a NA grass rat (B, D, F) euthanized at ZT 16. Arrowheads indicate some double-labeled cells. Fos: blue nuclear staining. ChAT: brown cell body staining. Scale bar= 100 µm.

NA $F_{1, 27} = 3.68$, $p = 0.07$, Fig. 2.4A]. Analysis of the simple effects of group, revealed that at ZT 4 there were no differences across groups [$F_{2, 27} = 0.12$, $p = 0.89$], whereas at ZT 16 significant differences were observed [$F_{2, 27} = 9.29$, $p < 0.01$]. At ZT 16, NAs and DAs had more Fos expression in nACh cells than Cs [$t_{27} = -4.21$, $p < 0.01$ and $t_{27} = -2.69$, $p = 0.01$, respectively, Fig. 2.4B], whereas no differences were observed between DAs and NAs [$t_{27} = -1.08$, $p = 0.29$, Fig. 2.4B].

1.2. BF activation and orexin activity in DA and NA grass rats

In the MS and VDB, Fos expression in ACh and nACh cells, was positively correlated with Fos expression in OXA cells, but only in NAs (Table 2.1). The results were identical for OXB (data not shown). In contrast, in the HDB no significant correlations between these measures were observed for either group. OXA fibers were visible in the MS, VDB, and HDB of all groups. Furthermore, the pattern and characteristics of OXA projections to those areas were similar across groups. In the three brain regions, OXA fibers were distributed sparsely and contained numerous varicosities. Those varicosities were observed in close proximity to ACh cells, as well as to nACh cells of the BF (Fig. 2.6).

Experiment 2: Activation of reward systems

The number of TH cells in the VTA ($p > 0.23$) and SUM ($p > 0.06$) was not significantly affected by any of the factors analyzed (data not shown). The pertinent results for the VTA and SUM are summarized in Figures 2.7 and 2.8.

Table 2.1. Correlations between Fos expression in MS, VDB, and HDB cells and Fos expression in OXA cells.

	DA	NA
MS		
Fos in nACh cells	*r*= .037	***r*= .758***
	p= .925	***p*= .004**
Fos in ACh cells	*r*= .307	***r*= .625***
	p= .421	***p*= .030**
Overall Fos expression	*r*= .080	***r*= .772***
	p= .839	***p*= .003**
VDB		
Fos in nACh cells	*r*= .295	***r*= .640***
	p= .441	***p*= .025**
Fos in ACh cells	*r*= .302	***r*= .782***
	p= .429	***p*= .003**
Overall Fos expression	*r*= .310	***r*= .664***
	p= .417	***p*= .018**
HDB		
Fos in nACh cells	*r*= -.132	*r*= .533
	p= .736	*p*= .074
Fos in ACh cells	*r*= .032	*r*= .558
	p= .935	*p*= .059
Overall Fos expression	*r*= -.104	*r*= .540
	p= .789	*p*= .070

(*) Significant correlation ($p < .05$).

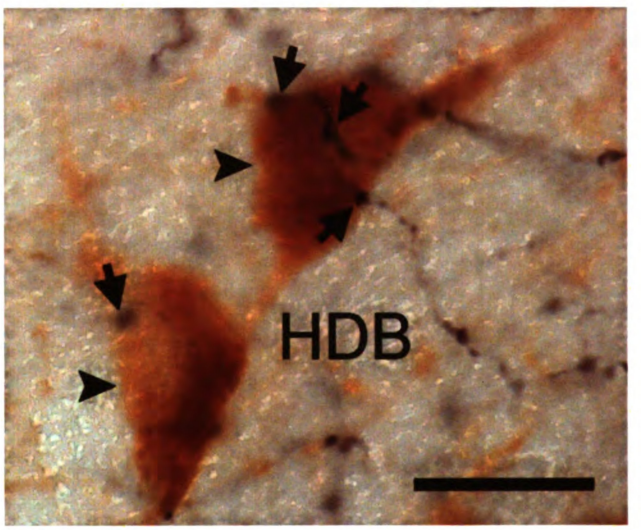


Figure 2.6. Photomicrograph of putative OXA fiber appositions (arrows) with ACh cells (arrowheads) in the HDB of a representative animal. Note that putative appositions are also observed in the vicinity of ACh cells. Scale bar= 30 µm.

VTA

In the most rostral level of the VTA (anterior VTA, aVTA) neither the effect of ZT [$F_{1, 29} = 0.11$, $p = 0.74$] nor the interaction between ZT and group [$F_{2, 29} = 0.36$, $p = 0.70$] were significant, whereas the main effect of group [$F_{2, 29} = 7.94$, $p < 0.01$] was significant, such that DAs and NAs expressed more Fos than Cs [$t_{29} = -2.86$, $p < 0.01$ and $t_{29} = -3.82$, $p < 0.01$, respectively, Fig. 2.7A and 2.7B]. No significant difference was observed between DAs and NAs [$t_{29} = -1.02$, $p = 0.32$, Fig. 2.7A and 2.7B]. For the rest of the VTA none of the factors analyzed or their interactions had a significant effect on Fos expression (data not shown).

SUM

Neither the main effect of ZT [$F_{1, 28} = 2.80$, $p = 0.11$] nor the interaction between ZT and group [$F_{2, 28} = 2.98$, $p = 0.07$] were significant. The main effect of group [$F_{2, 28} = 13.30$, $p < 0.01$], however, was significant, such that DAs and NAs expressed more Fos than Cs [$t_{28} = -4.97$, $p < 0.01$ and $t_{28} = -3.72$, $p < 0.01$, respectively, Fig. 2.7C and 2.7D]. No significant difference was observed between DAs and NAs [$t_{28} = -1.17$, $p = 0.25$, Fig. 2.7C and 2.7D].

DISCUSSION

Experiment 1: Basal forebrain activation and its relation to orexinergic activation

HDB

Figure 2.7. Patterns of Fos expression in nTH cells of the aVTA (A, B) and SUM (C, D). Panels A and C show the distribution of Fos expression in nTH cells for each group at each sampled time. Neither the effect of ZT nor the interaction between ZT and group were statistically significant. Panels B and D show the overall distribution of Fos expression in nTH cells for each group. The effect of group was statistically significant ($p < 0.05$).

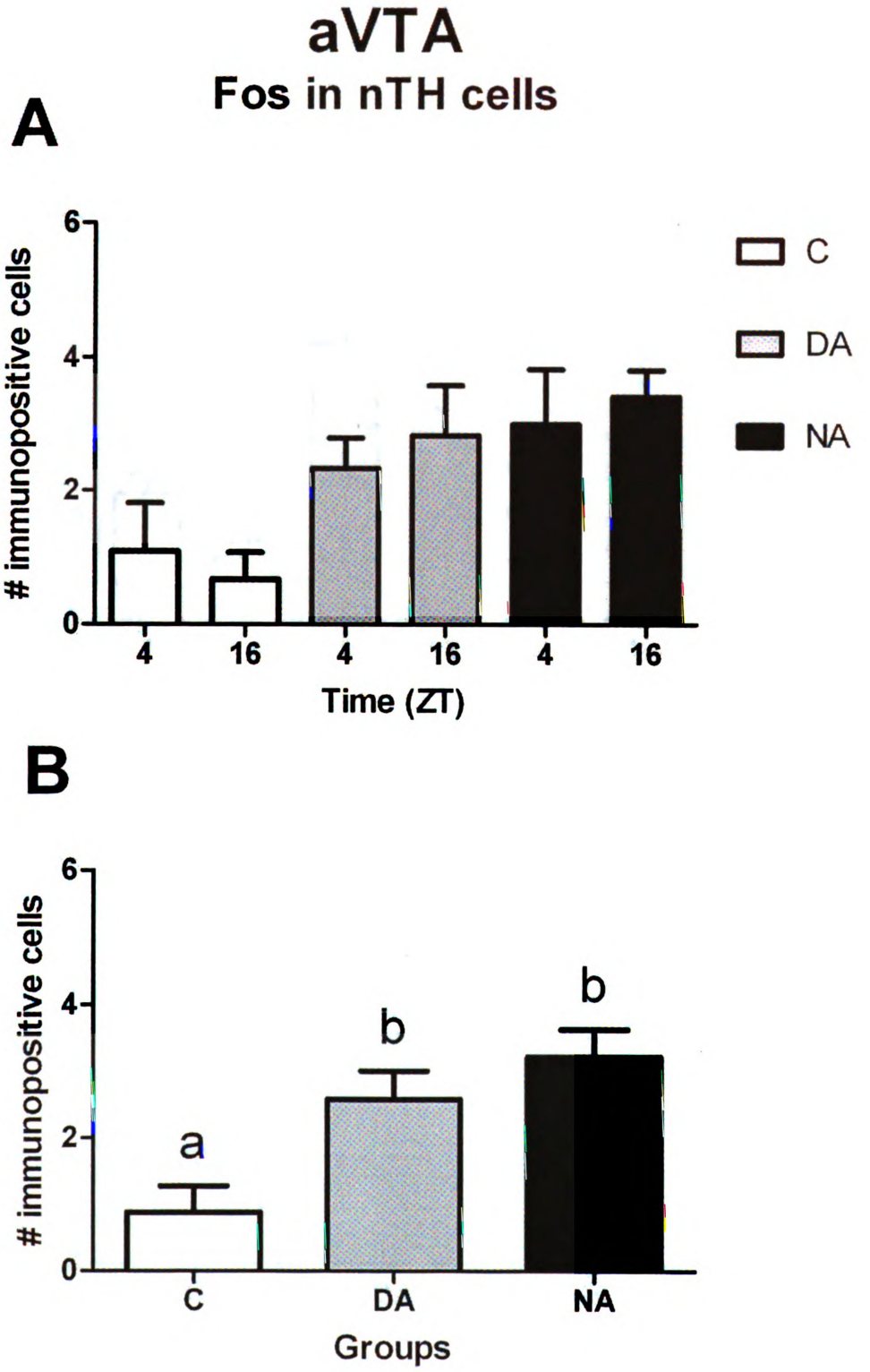
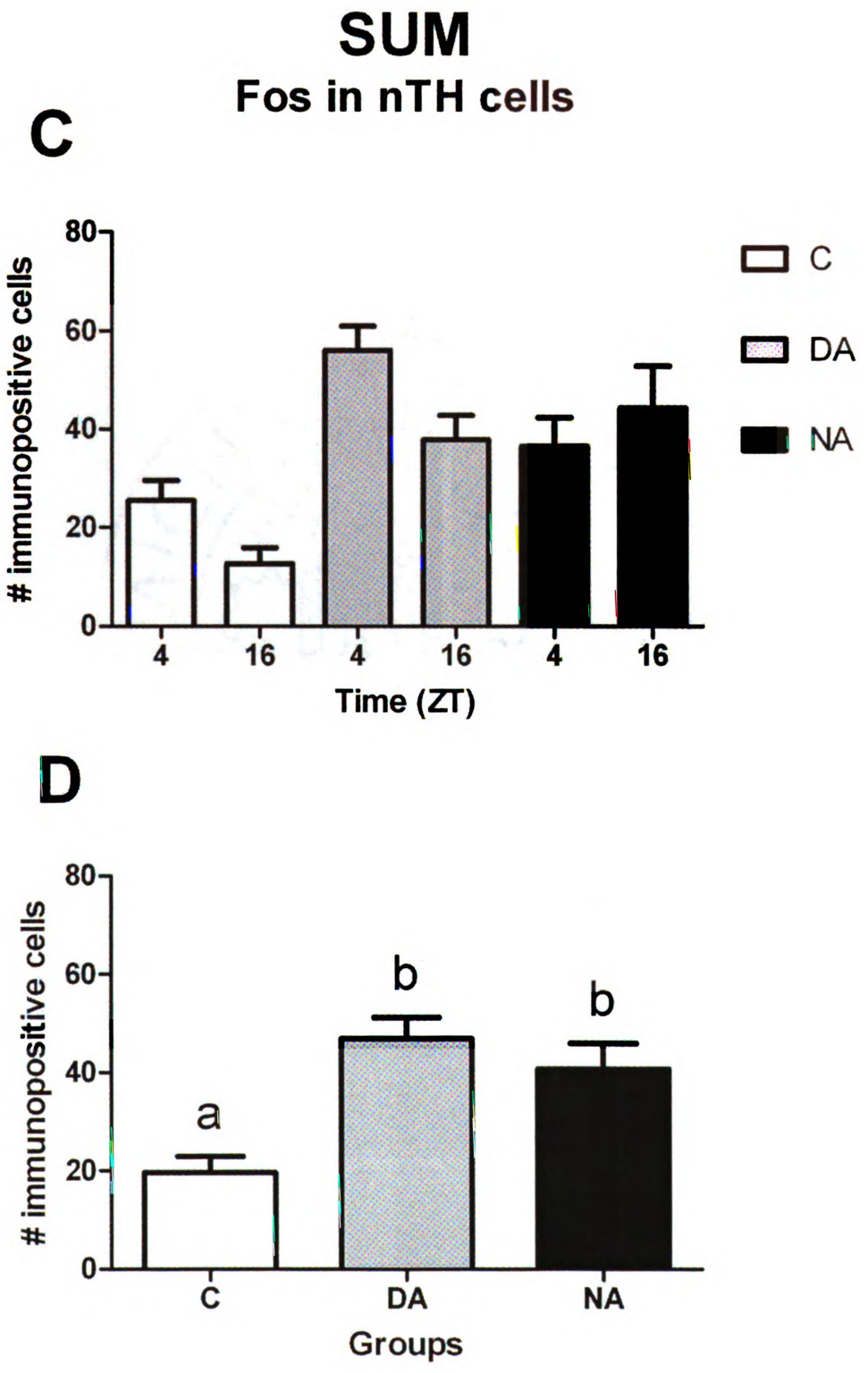

aVTA
Fos in nTH cells
A
immunopositive cells
6
4
2
0
4
16
4
16
4
16
Time (ZT)
C
DA
NA
B
immunopositive cells
6
4
2
0
a
b
b
C
DA
NA
Groups

Figure 2.7. (cont'd)



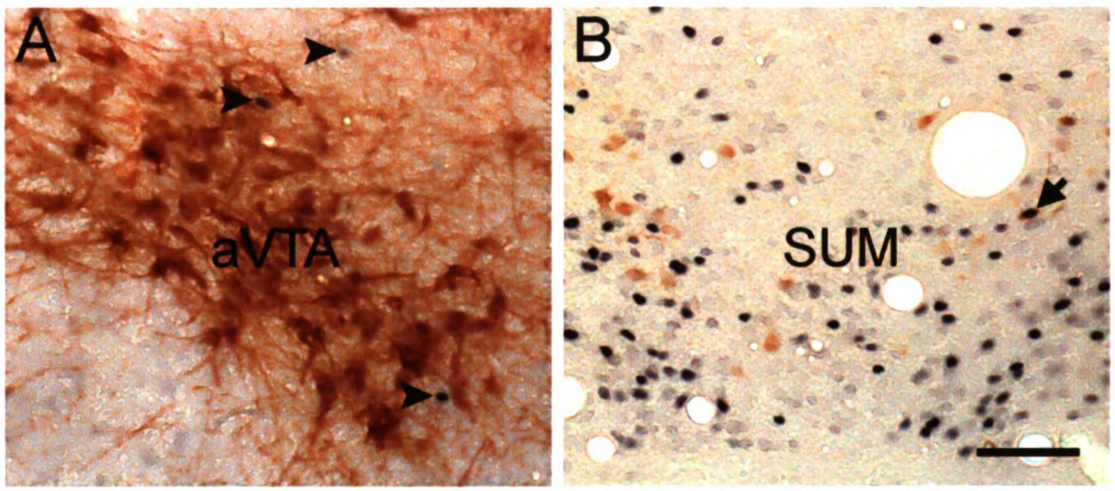


Figure 2.8. Photomicrographs of Fos and TH cells in the aVTA (A) and SUM (B). Double-labeled cells were scarce (arrow in B) or absent in both areas. In addition, note that TH cells were abundant in the aVTA. However, these cells were not positive for Fos, even though this protein was observed in the vicinity of those neurons (arrowheads). Fos: blue nuclear staining. TH: brown cell body staining. Scale bar= 100 µm.

In the HDB, we found that regardless of wheel access and chronotype Fos expression in ACh and nACh cells was similar across groups at ZT 4 (Fig. 2.2), even though NAs sleep more at this time (Schwartz and Smale, 2005). A likely explanation for these results is that a common experience (i.e., sleep) produces the return of neural activation to baseline levels in the BF. In fact, both DA and NA grass rats housed with wheels (Schwartz and Smale, 2005), as well as sedentary grass rats (Novak et al., 1999), increase the display of behavioral sleep late in the dark phase (between ZT 20 and 22). This universal display of sleep late at night could be the common experience that induces the return of neural activity to baseline levels in the grass rat BF, even if the sleep debt of NAs is not completely dissipated at ZT 4. Evidence for this claim comes from experiments using laboratory rats (Greco et al., 2000) and mice (Basheer et al., 1997). In both species, forced wakefulness produces an increase in Fos expression in the BF, but this pattern declines rapidly following 1-2 hours of recovery sleep. Furthermore, in laboratory rats, recovery sleep particularly decreases activation of ACh cells of the VDB and HDB (Greco et al., 2000). Thus, it is possible that at ZT 4 the similar pattern of Fos expression across groups in nACh and ACh cells of the HDB is a consequence of the sleep that occurs towards the end of the dark phase.

In sharp contrast to the results obtained during the day, during the night Fos expression in ACh and nACh cells in the HDB was higher in both groups of animals with wheels than in animals without them (Fig. 2.2 and 2.5). This was likely due to a decrease in Fos expression from day to night in the Cs, combined

with either an increase in Fos expression at night (i.e., in ACh and nACh cells of NAs and in the nACh cells of DA animals) or the maintenance of day time levels of expression when the animals were sampled at ZT 16 (i.e., in ACh cells of DAs). Thus, in general enhanced Fos expression in the HDB corresponded to the pattern of wakefulness or activity for the NAs and the Cs, but that relationship was absent in the DAs. This suggests that after many days of access to a running wheel the functioning of these cells becomes decoupled from wheel running.

The results of neuronal activation in the HDB of animals with wheel access contrast with those reported for the orexinergic system of these animals. In the orexigenic arousal system, expression of Fos is predominantly determined by the temporal display of activity: it is high at night for NAs and high during the day for DAs (Nixon and Smale, 2004). Thus, these results, together with the lack of significant correlations between Fos expression in the HDB and Fos expression in the orexin cells (Table 2.1), serve to argue against a prominent role for the orexinergic system in inducing Fos expression in the HDB of grass rats with access to wheels. This is in spite of anatomical evidence that orexinergic fibers appear to make contacts with ACh and nACh cell in the HDB of grass rats (Fig. 2.6). Considering the role of the orexins in the activation of serotonergic, histaminergic, and norepinephrinergic arousal systems (reviewed in Saper et al., 2001), our results raise the possibility that other arousal systems independent of orexinergic influences play a role in the activation of HDB cells seen here.

An outcome of enhanced neural activity at night in the HDB of both DAs and NAs could be neural changes in areas that receive input from the HDB. The olfactory bulbs (OBs) and piriform cortex (Pir), which are components of the olfactory system, receive ACh and GABAergic projections from the HDB (Luskin and Price, 1982; Mesulam et al., 1983; Zaborszky et al., 1986). In laboratory rats, Fos expression in ACh cells of the BF is associated with release of acetylcholine at their terminals (Greco et al., 1999; Greco et al., 2000). If this is also true for our animals with wheel access, it suggests that the OBs and Pir are being stimulated by the release of acetylcholine during the normal resting phase. Likewise, the GABAergic cellular group of DAs and NAs could be stimulated to release GABA at night in animals with wheel access, independently of the preferred phase of the day-night cycle for the display of activity. The release of these neurotransmitters in the OBs and Pir could alter functions modulated by these areas, such as olfactory processing in the case of the Pir and OBs (reviewed in Wilson et al., 2004), and thermoregulation, sexual behavior, and circadian rhythmicity in the case of the OBs (Brunjes, 1992). In laboratory rats, ACh cells of the BF also send projections to the main circadian pacemaker of the suprachiasmatic nucleus (SCN; Bina et al., 1993), and nocturnal release of ACh in the SCN can shift its functioning in a time dependent manner (i.e. phase advances throughout the night; Buchanan and Gillette, 2005). In grass rats, the shift to a nocturnal pattern of activity does not affect the phase of SCN rhythms (Blanchong et al., 1999; Rose et al., 1999; Schwartz and Smale, 2005), although it appears to activate at least some ACh cells of the BF (i.e., those of the HDB,

as shown here). A lack of a phase shift in SCN functioning may be due to the fact that in sharp contrast to laboratory rats, the SCN of grass rats receives only a few ACh projections (Castillo-Ruiz and Nunez, 2007), and these are not likely to originate in the BF (A. Castillo-Ruiz, unpublished observations).

MS and VDB

In the MS and VDB of all groups there was a rostro-caudal gradient of Fos expression in nACh cells, with more labeled cells present in the caudal sections. The BF, including the MS and VDB, is organized according to rostro-caudal bands that coil along the axis, and each band is proposed to contain sub-bands of neurons that produce specific neurotransmitters and peptides (Zaborszky and Duque, 2003). Therefore, our results may reflect that different cell groups across the rostro-caudal axis of the BF contribute differentially to neural activation of the BF, which may have in turn differential effects in the functioning of brain areas that receive inputs from these cellular groups.

Since in laboratory rats, ACh cells of the VDB, but not those of the MS, show increased Fos expression after prolonged waking (Greco et al., 2000), we expected to find that our NAs would show increased Fos expression in ACh cells of the VDB during their active time: the dark phase. However, we did not find effects of group or ZT on Fos expression in ACh cells in either the VDB or the MS. The discrepancy between Greco's results and ours in the VDB could be because the physiological effects produced in the VDB by voluntary activity maybe fundamentally different from those produced by forced wakefulness. That

is, forced wakefulness is likely to be more stressful and could induce more Fos expression in the BF than voluntary wakefulness during the normal resting phase. The MS and VDB of mice and laboratory rats contain the corticotropin-releasing hormone (CRH) receptor 1 (Radulovic et al., 1998), and in mice there is a high degree of colocalization of this receptor and ACh in neurons in both areas (Sauvage and Steckler, 2001). Furthermore, in laboratory rats CRH stimulates neuronal activity in the MS and VDB (Osada, 1997). Thus, experimental wakefulness paradigms may induce widespread Fos expression in ACh cells of the VDB due to the actions of CRH on these neurons, but only when stress responses are involved. Another possibility is that an acute episode of wakefulness during the resting phase activates more ACh neuronal groups than chronic exposure does. In Greco's study the animals were exposed to two hours of sleep deprivation, whereas in this study our NA animals were running in wheels and showing wheel running during the night for a month. In addition, our animals were not likely to be sleep deprived, due to their redistribution of sleep, which involves a compensatory increase in daytime sleep bouts (Schwartz and Smale, 2005).

In sharp contrast to our results for the ACh cells of the MS and VDB, we found statistically significant group differences in Fos expression in nACh cells in these areas, but only at night (Fig. 2.3-2.5). As discussed previously for the HDB, a potential explanation for the lack of group differences at ZT 4 is the resetting effect of the sleep that occurs late at night in all grass rats, and that may result in a return to baseline neural activity in the BF, regardless of group differences in

nocturnal locomotor activity. During the night, Fos expression in nACh cells of the VDB was higher in both groups of animals with wheels than in those without wheels, but in the MS nocturnal Fos expression was high only in NAs. As with the HDB, group differences in the VDB at ZT 16 appear to be due to a relative reduction in Fos expression between ZT 4 and ZT 16 for the Cs, combined with the opposite trend occurring during that time in NAs, and with the maintenance of daytime levels in DAs.

The MS and VDB project densely to the hippocampus, and ACh, GABAergic, as well as glutamatergic neurons of the MS and VDB modulate the HPC-θ (Leung and Shen, 2004; Yoder and Pang, 2005). The HPC-θ is seen when animals engage in voluntary behaviors, including wheel running , and in laboratory rats integrity of the MS connection to the hippocampus is needed not only to maintain the HPC-θ, but also to support wheel running (Oddie et al., 1996). These data suggest that the septal-hippocampal pathway is important for the modulation of wheel-running intensity. Interestingly, NA grass rats show substantially higher levels of wheel running when compared to DAs (Blanchong et al., 1999); and in the present study, that group had the highest levels of Fos expression in the MS. Thus, our results suggest that the high levels of running seen in NAs might be supported by this septal-hippocampal pathway.

In contrast to the results for the HDB, there appears to be a relationship between the expression of Fos in orexin producing cells and Fos expression in the ACh and nACh of the MS and VDB, but only for NAs (Table 2.1). Thus for these regions of the BF, the somewhat similar profiles of Fos expression seen in

NAs and DAs may in fact reflect the activation of different neural systems or that those neural systems are modulating BF neural activation in different ways. A role for the orexins in inducing Fos expression in the BF of NAs at ZT 16 is not surprising since at that time these animals are awake and very active (Blanchong et al., 1999). The DAs, on the other hand, show frequent bouts of sleep at night (Schwartz and Smale, 2005) and little Fos expression in orexin neurons at ZT 16 (Nixon and Smale, 2004). This is consistent with the view that neural systems independent of orexinergic activation drive Fos expression in the BF of the DAs. One possibility related to this idea is that at least for DAs Fos patterns may be induced by the activation of cells of the BF that play a role in paradoxical sleep. This is because cells of the BF are not only active during wakefulness, but also during this sleep stage (Lee et al., 2005).

Our anatomical data revealed that OXA containing fibers form putative appositions with ACh and nACh neurons in all of the areas studied, regardless of wheel access or chronotype, suggesting the potential for functional synaptic relationships between OXA-containing fibers and ACh or nACh neurons in these regions. It is possible, however, that differences between groups exist at the quantitative level, in that the number of synaptic contacts between OXA fibers and cell groups of the BF might differ. That possibility could explain differences in BF activation between DAs and NAs. However, in the present study we did not attempt to quantify the number of apparent contacts between OXA-containing fibers and ACh or nACh neurons.

Experiment 2: Activation of reward systems

We examined whether temporal patterns of wheel running were associated with patterns of Fos expression in TH cells of the VTA and SUM. However, none of the comparisons revealed significant differences. Furthermore, double-labeled cells were very rare (Fig. 2.8). While there are several potential explanations for the lack of double-labeled cells in the VTA and SUM of all groups, it is unlikely that the lack of double-labeling was due to a failure of the immunocytochemical procedure, since double-labeled cells were easily identified in the periaqueductal gray of the same sections (data not shown). One possible explanation for these results is that neural activation in dopaminergic cells of the reward system is only seen during the initial stage of running wheel access, and not after continuous access for several days or weeks. In laboratory rats, Fos expression in TH cells of the VTA is seen after acute exposure to voluntary wheel-running (Yanagita et al., 2007), as well as after acute exposure to other rewarding stimuli, especially in the rostral VTA (Asmus and Newman, 1994; Balfour et al., 2004). However, when animals are exposed repeatedly for several days to rewarding stimulus such as cocaine, there is a reduction in *c-fos* mRNA in the caudate putamen, an area which receives dopaminergic inputs (Ennulat et al., 1994). These data suggest that even though Fos expression in dopaminergic neurons is seen after acute reward presentations, repeated exposure to a rewarding stimulus can produce attenuation of the induction of Fos expression in dopamine cells of areas related to the reward system, which might be related to habituation to the stimulus. Thus, our results might reflect attenuation in Fos

expression in the dopaminergic reward system induced by chronic exposure to the wheel, after an initial increase when the wheels were first introduced, resulting in uniform low Fos expression in TH cells across groups. Finally, another potential explanation is that in dopaminergic cells, early genes other than Fos are expressed as a result of voluntary wheel running.

In contrast to our observations in TH cells, animals with wheels showed an increase in Fos expression in nTH cells of the aVTA, and did so regardless of chronotype (Fig. 2.7). This result is in agreement with those of other studies, which found that after acute exposure to a rewarding stimulus there is a region-specific expression of Fos in nTH cells of the VTA, with more Fos seen in rostral areas than in caudal areas (Hunt and McGregor, 1998; Balfour et al., 2004). Similarly in the SUM, we observed that DAs and NAs expressed more Fos in nTH cells than did the Cs (Fig. 2.7). These results are interesting, given the role that the SUM appears to play in the reward pathway. For example, administration of the glutamate receptor agonist alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) into the SUM, but not the VTA, produces rewarding effects (Ikemoto et al., 2004). Furthermore, they suggest that reward experienced over a relatively long interval may not involve activation of dopaminergic cell bodies. However, since some of the rewarding effects mediated by the SUM involve enhance release of dopamine from axonal terminals in the NAc (Ikemoto et al., 2004), we can not rule out a role for dopamine in the rewarding properties of long-term access to a running wheel.

Expected consequences of the neural activation of components of the reward system induced by wheel access are neural changes in regions that receive inputs from these areas. Components of the reward system, including the SUM and the VTA, send efferent projections to the BF (Gaykema and Zaborszky, 1996; Pan and McNaughton, 2004), and modulate the neural activity of that region. For example, stimulation of the VTA induces Fos expression in the MS and VDB (Sandner et al., 1992). Moreover, stimulation of the SUM is likely to have effects on the BF since it sends projections to cells of the MS and VDB (Pan and McNaughton, 2004). Thus, the patterns of Fos expression seen in some regions of the BF of grass rats with access to wheels may be in part due to a tonic increase in the activity of the SUM and aVTA. This contribution may be of particular significance with respect to Fos expression at night in the BF of DAs, which appears to be independent of the orexinergic system. In addition, changes in modulation of vigilance states are expected, given the participation of the VTA and SUM in this function.

SUMMARY AND CONCLUSIONS

One of the main findings of this study is that when diurnal grass rats shift to a night-active pattern of activity, the effects of that change are not uniform across arousal systems. Thus, although ACh cells of the HDB show a nocturnal increase in Fos expression in animals that are voluntarily active at night, that effect is not seen in other ACh cell groups of the BF known to contribute to the support of wakefulness (i.e., the MS and the VDB). An increase in nocturnal Fos expression in NA animals, similar to that seen in the orexinergic system (Nixon

and Smale, 2004) was almost universally seen in the nACh cells of all regions of the BF, but apparently only associated with activation of the orexinergic system for the MS and the VDB, thus providing further evidence for functional differences in how brain arousal systems respond to a shift in phase preference.

One unexpected observation of this study was that for some of the areas and cell types, but not all (see the data for the MS) the temporal patterns, and/or overall levels, of Fos expression of DA animals with wheel access appear similar to those of NAs. This may be due to an effect of voluntary exercise that is seen regardless of a shift in phase preference for the display of activity. These similarities in patterns of Fos expression between DA and NA animals may nevertheless stem from the activation of different neural systems, since Fos expression in the MS and VDB was only correlated with Fos expression in the orexinergic system in the case of NAs, and activation of the BF has been associated with the display of paradoxical sleep as well as wakefulness (Lee et al., 2005). Also common to both groups with wheels was an increase in Fos expression in two specific areas of the brain reward system that also participate in arousal, the SUM and aVTA. This effect appears to reflect a tonic upregulation of these brain regions, and was not affected by either ZT or by the display of day- or night-active patterns of activity.

Because the BF and the brain reward system project widely throughout the brain, the effects of altered neural activation in those regions are likely to be seen in a broad range of neural functions and behaviors. For example, since the MS, VDB and SUM project to the hippocampus, effects on learning and memory

processes may be expected. In addition, changes in sensory processes and motivation are likely to occur given the projections of the HDB and those of the reward system, respectively. The present study suggests these effects may also occur in other diurnal species, including humans, since voluntary exercise and voluntary display of activity during the night (e.g., shift work) are widespread practices around the world (Rajaratnam and Arendt, 2001). Therefore, the diurnal grass rat represents an interesting model to investigate how daily amount and temporal distribution of voluntary activity affects the functioning of brain systems that modulate a variety of important functions including vigilance states and motivation.

CHAPTER 3

Neural activation in arousal and reward areas of the brain in grass rats following induced wakefulness

INTRODUCTION

In modern human society, a significant part of the population is awake throughout the night due to, for example, job duties or social demands. This practice poses physiological challenges to the brain and body, since being a diurnal species, humans have evolved to be active during the day and to be resting during the night. A body of evidence shows that individuals who work the night-shift are prone to suffer from late-onset diabetes, gastrointestinal and cardiovascular disease (reviewed in Rajaratnam and Arendt, 2001), prostate (Kubo et al., 2006) and breast cancer (Davis et al., 2001; Schernhammer et al., 2001), among other health problems. Thus, being active during the night appears to have severe negative consequences for human health. How these consequences differ depending upon the conditions responsible for nocturnal activity, including motivational factors, remains to be elucidated.

Work with animal models has shed light on the etiology of some of the health problems associated with shift work (e.g. Penev et al., 1998; Martino et al., 2007; Martino et al., 2008; Preuss et al., 2008). However, the interpretation of the findings becomes problematic because the majority of these studies uses nocturnal rodents, and also, because the temporal shifts in activity are induced by the researcher, and not produced voluntarily by the animals. In the diurnal grass rat, *Arvicanthis niloticus*, access to a running wheel results in voluntary

shifts in the temporal distribution of activity in some individuals (night-active; NA), but not in others (day-active; DA; Blanchong et al., 1999). This makes the grass rat a suitable model to study the physiological consequences of being voluntarily active during the natural rest phase of a diurnal species.

In a previous study we found that grass rats that are actively running during the night show elevated Fos expression in areas of the brain related to reward and arousal, such as cholinergic (ACh) and non-cholinergic areas (nACh) of the basal forebrain (BF), and non-dopaminergic cells (as determined by lack of tyroxine hydroxylase; TH) of the supramammillary nucleus (SUM) and anterior VTA (aVTA; Castillo-Ruiz et al., 2010). Our observations about the expression of Fos in the BF of NA grass rats were not in agreement with those reported for lab rats that were stimulated to stay awake during their rest phase (Greco et al., 2000; McKenna et al., 2009). We reasoned that this discrepancy could be related to the fact that NA grass rats were voluntarily awake, rather than forced to be awake as it was the case in the experiments with lab rats (Greco et al., 2000; McKenna et al., 2009). Further, the elevated expression of Fos seen in rewards areas (i.e., SUM and aVTA) in grass rats with access to wheels suggest that enhanced voluntary exercise has rewarding properties, which are not likely shared with situations involving forced wakefulness.

In our previous study we also observed that in animals that were active during the night there were strong correlations between neural activity in orexin cells (orexin A and B; OXA and OXB, respectively) and neural activity in ACh and nACh cells of two major nuclei in the BF- the medial septum (MS) and vertical

diagonal band of Broca (VDB). We also identified putative appositions between OXA positive fibers and ACh and nACh cell bodies of the BF. These observations are in agreement with findings in nocturnal species that suggest that the orexinergic system modulates the activity of other arousal systems, including the ACh system (reviewed in Saper et al., 2001). Our previous results suggest that this might be also the case for diurnal species.

In the present study we evaluated whether in grass rats induced wakefulness during the night elicits changes in neural activity in arousal and reward areas similar to those seen in grass rats that voluntarily become active at night. To assess this question, we examined patterns of Fos expression in the BF, SUM and aVTA after 6 hours of induced wakefulness by gentle stimulation. We also included in our analyses other brain areas that are known to promote wakefulness or sleep –the ventro lateral preoptic area (VLPO), lateral hypothalamus (LH), tuberomammillary nuclei (TMM), raphe nuclei, locus coeruleus (LC), and nucleus incertus (NI)- areas of the brain involved in circadian control – suprachiasmatic nucleus (SCN) and ventral subparaventricular zone (vSPZ), as well as areas related to the stress response – parvocellular and magnocellular paraventricular nucleus of the hypothalamus (pPVN and mPVN, respectively). In addition, we investigated how 2 hours of sleep recovery, following the sleep deprivation episode, influenced neural activation in the same brain regions. Finally, we used correlational analyses to assess the relationships between Fos expression in the LH, which is an area rich in OXA and OXB cells, and Fos expression in other brain areas related to wakefulness and sleep.

EXPERIMENTAL PROCEDURES

Animals

Twenty-eight adult male grass rats bred in our laboratory were used in this study. All animals were housed individually in plexiglass cages (17 x 34 x 28 cm) for at least one month before the behavioral manipulation. The animals were kept on a 12:12 light-dark cycle with a red light (< 5 lux) on at all times, and were provided with *ad libitum* access to water and food (Harlan Teklad 8640 rodent diet, Harlan Teklad Laboratory, Madison, WI). All experiments were performed in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Michigan.

Induced wakefulness

Two groups of animals (n=7 per group) were stimulated to stay awake for 6 hours from Zeitgeber time (ZT; lights on at ZT 0) 10 to ZT 16. Wakefulness was induced by gently touching the animals with a Q-tip when we observed them showing signs of sleepiness, that is, when they were sitting curled up with their heads tucked into their bodies or when their eyes were closing as they laid on their side. After the period of induced wakefulness, one group of grass rats (sleep deprived group, SD) was perfused immediately; whereas the other group was left undisturbed for two hours (recovery, R), and perfused at ZT18. Two additional groups of undisturbed animals (n=7 per group) were perfused at ZT 16 and ZT 18 and were used as control groups for the SD and R groups (CSD and CR, respectively). Animals that were perfused during the dark phase were fit with an

aluminum foil hood over their heads to avoid exposure to light. At the time of perfusion, intraperitoneal injections of sodium pentobarbital (Ovation Pharmaceutical, Deerfield, IL) were used to deeply anesthetize the animals. Then, they were intracardially perfused with 0.01 M phosphate buffer saline (PBS), followed by 4% Paraformaldehyde (Sigma-Aldrich, St. Louis, MO) with 75 mM lysine (Sigma-Aldrich) and 10 mM sodium periodate (Sigma-Aldrich; PLP) in 0.1 M phosphate buffer (PB). Their brains were post-fixed for approximately 4 hours in PLP, and then they were transferred to 20% sucrose solution in 0.1 M PB overnight at 4°C. Coronal sections were cut on a freezing sliding microtome at 30 µm, and alternate sections were collected in three series in cryoprotectant solution at -20°C until further processing.

Immunocytochemistry (ICC)

Unless indicated otherwise, all ICC procedures were carried out at room temperature and all incubations involved gentle agitation. In addition, sections were rinsed 3 times (5min/rinse) in 0.01 M PBS between all the steps of the ICC protocol, and all incubations included 0.3% Triton X-100 (TX; RPI, Elk Grove Village, IL; TX).

Basal forebrain

Free-floating sections containing the forebrain were rinsed (6 times, 10 min/rinse) in 0.01 M PBS and blocked for 30 min in 0.01 M PBS with 3% hydrogen peroxide (J.T. Baker, Phillipsburg, NJ). Then, sections were rinsed in 0.01 M PBS (6 times, 10 min/rinse), blocked for 30 min using 5% normal donkey

serum (NDS; Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS, and incubated overnight in a rabbit anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:20,000 in PBS and 3% NDS) at 4° C. The sections were then incubated for 1 h in a donkey anti-rabbit biotinylated antibody (Jackson; diluted 1:200 in PBS and 3% NDS), and then for 1 h in avidin-biotin peroxidase complex (AB complex, 0.9% each avidin and biotin solutions; Vector Laboratories, Burlingame, CA; in PBS). After 3 rinses (10 min/rinse) in Tris buffer (pH 7.2), the sections were preincubated in 0.025% diaminobenzidine (DAB; Sigma-Aldrich) enhanced with 2.5% nickel sulfate (Sigma-Aldrich) in Tris buffer for 1 min, and then 3% hydrogen peroxide was added for the reaction. After a 2.5 min reaction, the tissue was rinsed 3 times in PBS with 0.03% TX (10 min/rinse), followed by one rinse in PBS (10 min). Then, sections were blocked for 30 min in 5% normal horse serum (NHS; Vector; in PBS) and incubated overnight in a goat anti-choline acetyltransferase (ChAT) antibody (Chemicon, Temecula, CA; diluted 1:2,000 in PBS and 3% NHS) at 4° C. Following primary incubation, the sections were incubated for 1 h in a horse anti-goat biotinylated antibody (Vector; diluted 1:200 in PBS and 3% NHS). Then, the tissue was incubated for 1 h with AB complex (0.9% each avidin and biotin solutions; Vector; in PBS). After 3 rinses (10 min/rinse) in Tris buffer, the sections were preincubated with 0.02% DAB in the same buffer for 1 min, and then hydrogen peroxide (0.35 µl 30% hydrogen peroxide/ml buffer) was added for the reaction. After a 1 min reaction, the tissue was rinsed in 0.01 M PBS (4 times, 5 min/rinse). Then, all sections

were mounted onto gelatin-coated slides, dehydrated, and later coverslipped with dibutyl phthalate xylene (Sigma-Aldrich; DPX).

Reward systems

Free- floating sections containing the caudal diencephalon and midbrain were processed for Fos and tyrosine hydroxylase (TH) following ICC procedures similar to the ones described above, but with the exceptions noted below. The reaction for Fos staining lasted 3 min. For TH staining, the tissue was blocked with 5% NHS (Vector; in PBS) for 30 minutes, and then incubated in a mouse anti-TH antibody (Immunostar, Hudson, WI; diluted 1: 150,000 in PBS and 3% NHS) for 24 h at 4° C. The biotinylated secondary antibody incubation with horse anti-mouse (Vector; diluted 1:200 in PBS and 3% NHS), as well as the AB complex incubation (in PBS), were both for 1 h. The pre-incubation step with the DAB solution was for 45 sec, and the reaction with hydrogen peroxide lasted 1.5 min.

ICC quantification

In order to quantify Fos and ChAT expression, we selected three sections through the medial septum (MS) and vertical diagonal band of Broca (VDB) and five sections that included the horizontal diagonal band of Broca (HDB). For every section selected, cells expressing Fos, ChAT, and Fos+ChAT were counted using a 600 µm (width) by 300 µm (height) sampling box placed at the center of the studied areas. To quantify Fos and TH expression, we selected three sections through the ventral tegmental area (VTA). These sections

corresponded to levels 1 to 3 described previously in (Castillo-Ruiz et al., 2010). Caudal regions of the VTA were not analyzed due to the scarce labeling seen in those areas. For every section selected, cells expressing Fos, TH, and Fos+TH were counted using 160 µm x 160 µm sampling box placed lateral to the medial border of the VTA, in an area rich in TH staining, following the criteria outlined in (Castillo-Ruiz et al., 2010).

We also quantified Fos expression in other brain areas involved in the regulation of vigilance stage. Sampling boxes were used to count Fos in one section through each of the following areas: 190 µm x 190 µm in the ventrolateral preoptic area (VLPO); 215 µm (width) x 160 µm (height), the ventral subparaventricular zone (vSPZ); 100 µm (width) x 200 µm (height), the magnocellular paraventricular nucleus of the hypothalamus (mPVN), the parvocellular PVN (pPVN); 1200 µm (width) x 700 µm (height), the lateral hypothalamus (LH; one hemisphere chosen at random was counted for each animal); 120 µm x 120 µm, the dorsal tuberomammillary (DTM); 150 µm x 150 µm, the ventral tuberomammillary (VTM); 160 µm x 160, the supramammillary nucleus (SUM); 150 µm (width) x 650 µm (height), the lateral dorsal raphe (lDR); 150 µm (width) x 700 µm (height), the median raphe (MR); and 200 µm x 200 µm, the nucleus incertus (NI). Additionally, we quantified Fos in one section of the medial suprachiasmatic nucleus (SCN). The borders of this nucleus were determined from Nissl–stained sections through the anterior hypothalamus of a representative animal. In addition, we counted Fos and/or TH positive cells in 2 sections containing the locus coeruleus (LC) and 3 sections containing the

median dorsal raphe (mDR). For the LC we used a 400 µm (width) x 700 µm (height) sampling box. For the mDR, the 3 sections analyzed corresponded to levels 3 through 5 of the mDR following the nomenclature of (Janusonis et al., 1999). Levels 1 and 2 of the mDR were not analyzed since Fos staining was rare in that region and because these levels in the grass rat do not show rhythmic Fos expression (A. Castillo-Ruiz, unpublished observations). The sampling boxes used for the analysis of the mDR had the following dimensions: 150 µm (width) x 650 µm (height) for levels 3 and 4; and 160 µm x 160 µm for level 5. Figure 3.1 shows the placement of sampling boxes in areas for which such placement has not been previously shown in published work from our group (Novak et al., 2000; Nixon and Smale, 2004; Schwartz and Smale, 2005; Castillo-Ruiz et al., 2010).

For areas located distal to the midline, cells were counted bilaterally and cell numbers were averaged per section. If more than one section was used for counts, cell numbers were averaged across sections, with the exception of the VTA and mDR, for which every section was treated as a different level. After the cell counts were completed, cell counts were divided by the area occupied by the box for the lDR, as well as for levels 3 and 4 of the mDR. These adjustments were necessary because for some sections part of the box fell outside the study area. The results for these areas are expressed as number of cells/mm^2. For all other study areas the results are expressed as number of cells within the sampling region. All counts were done at 25x, except for the counting of labeled cells in the LH, which was done at 10x. An investigator unaware of the source of the tissue collected all the anatomical data.

Figure 3.1. Rostro-caudal illustrations depicting the sampling areas used to quantify Fos expression in TH and nTH cells of the lDR, MR, mDR3 (A), mDR4 (B), mDR5 (C), LC (D and E); note that for this area the two sampling levels were averaged per section and level), as well as overall Fos expression in the NI (E) and PVN (F). See text for sampling box dimensions. The placement of boxes for the areas not depicted in the figure has been described previously (see text). (A-E) scale bar = 1 mm, (F) scale bar = 400 µm.

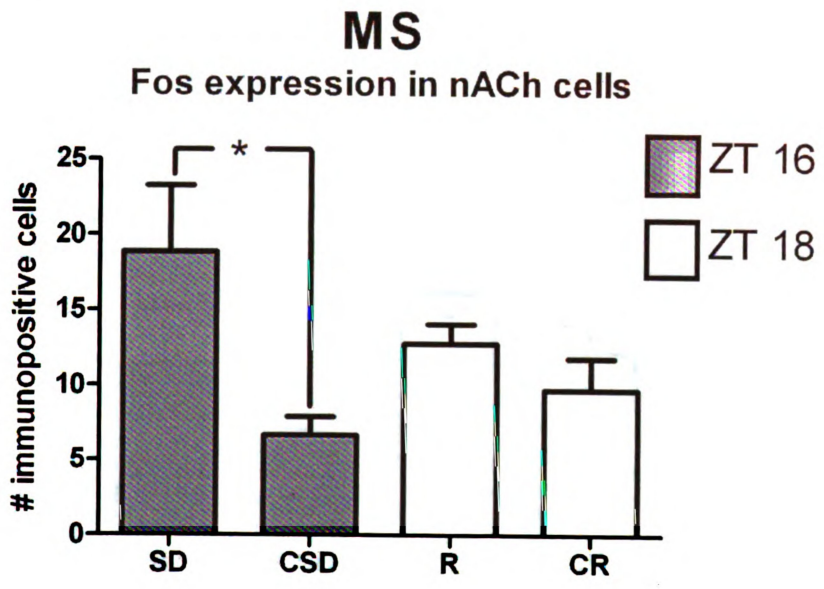
To analyze the effect of sleep deprivation and sleep recovery on neural activation, we compared each treatment group to their control separately (i.e., SD to CSD and R to CR). We also compared the control groups to each other to probe for circadian effects on neural activation. We, however, did not compare the two treatment groups to each other, because of the confounding effect of ZT. The data rarely met the assumptions of parametric statistical methods, even after transformations; therefore, all comparisons were done with non-parametric Mann-Whitney U tests. In addition, we used Pearson's r correlation tests to determine if neural activity of the LH was associated with neural activity in other brain regions. For all comparisons differences were considered significant when p was less than 0.05 (two-tailed tests). The software used for the statistical analyses was SPSS 17 (SPSS Inc., Chicago, IL, USA).

RESULTS

Abundant Fos, ChAT, and TH immunostaining was present in the tissue. While Fos immunoreactivity was observed throughout the brain, ChAT and TH immunoreactivity was limited to areas of the brain previously described as cholinergic or dopaminergic respectively in the grass rat, as well as in other species (Eckenstein and Sofroniew, 1983; Castillo-Ruiz and Nunez, 2007; Castillo-Ruiz et al., 2010). Significant differences in neural activation among treatments were seen only in some of the study areas, particularly in areas related to the modulation of wakefulness and sleep. Figure 3.2 shows, for each group, the patterns of Fos expression in the areas where significant effects and

Figure 3.2. Patterns of Fos expression for areas where significant effects and trends towards significance were observed across groups (A-J). The data are for overall Fos expression (C – E, G and H), Fos in nACh cells (A and B) and Fos in nTH cells (I and J). Single asterisks (*) represent significant differences within ZT ($p < 0.05$), whereas double asterisks (**) represent significant differences across ZTs for the control groups ($p < 0.05$).

A


MS
Fos expression in nACh cells
immunopositive cells
25
20
15
10
5
0
*
SD
CSD
R
CR
ZT 16
ZT 18

B

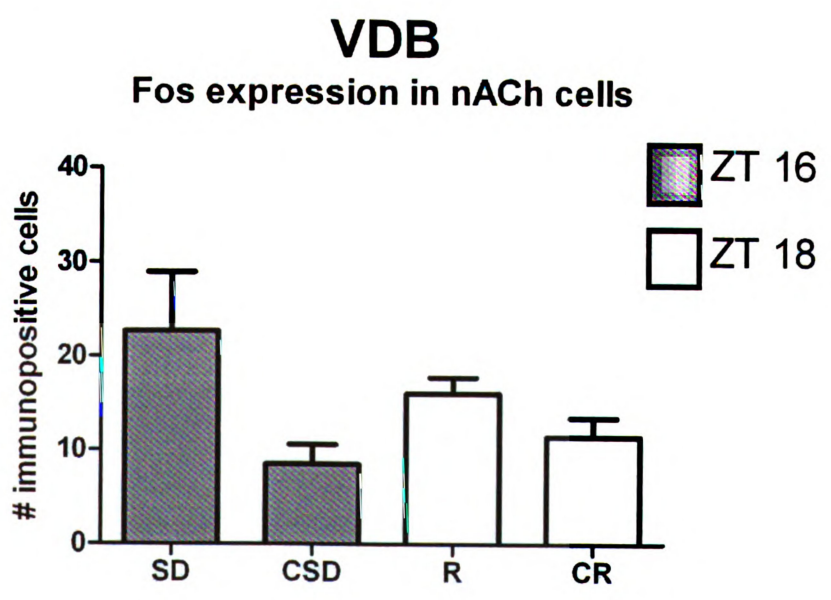
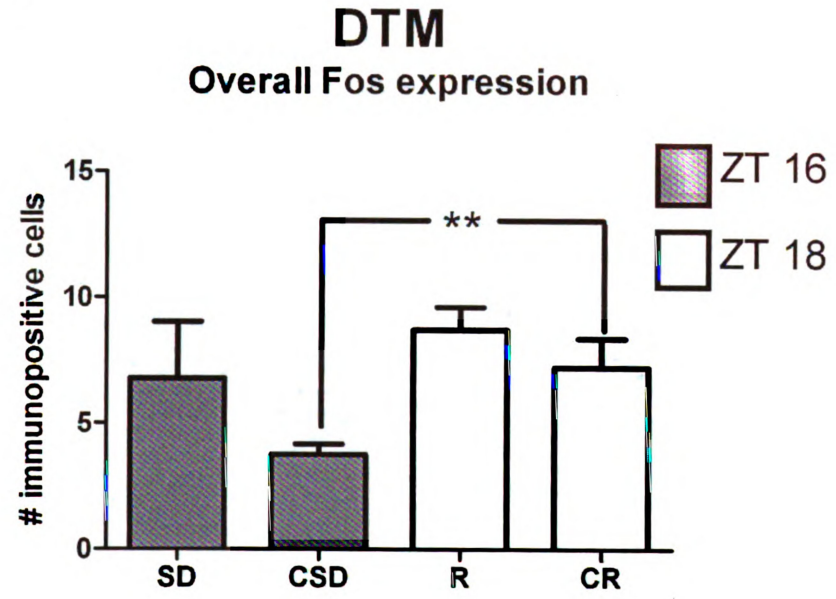

VDB
Fos expression in nACh cells
immunopositive cells
40
30
20
10
0
SD
CSD
R
CR
ZT 16
ZT 18

Figure 3.2. (cont'd)

C



D

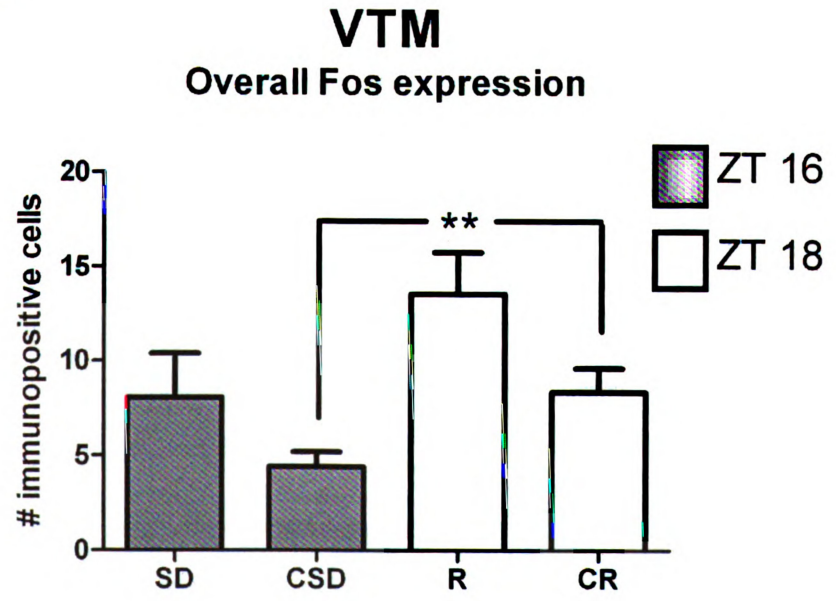
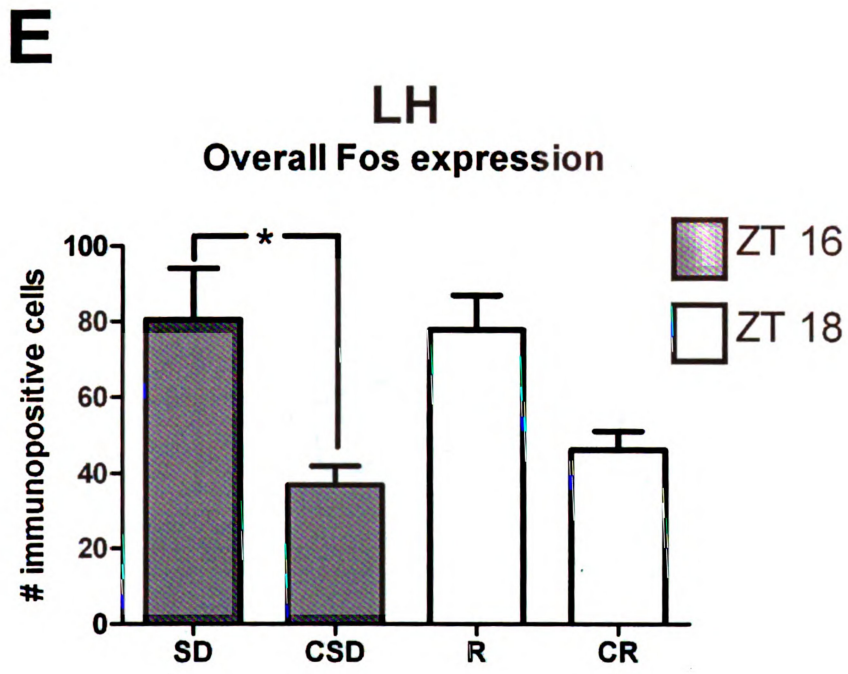


Figure 3.2. (cont'd)



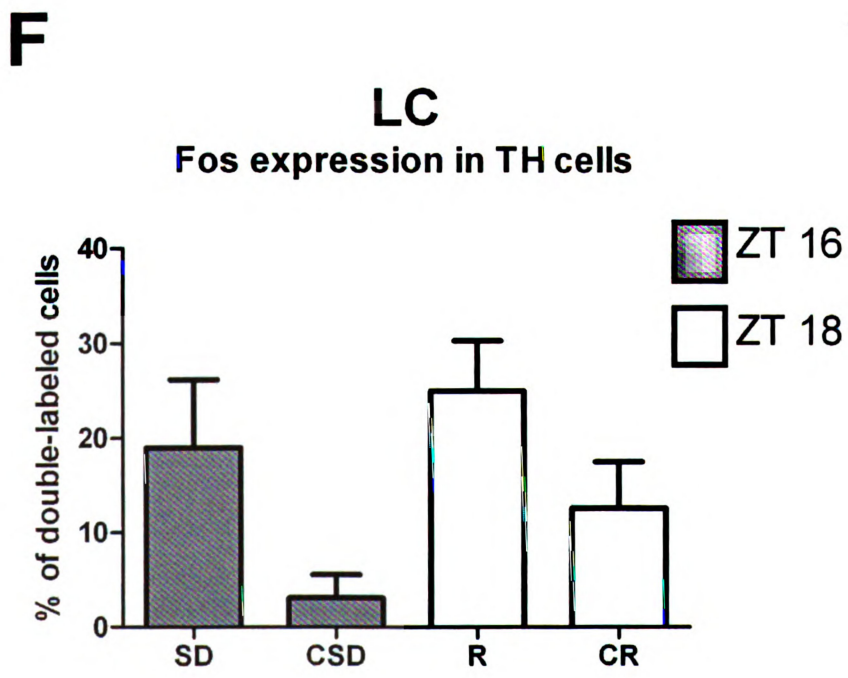
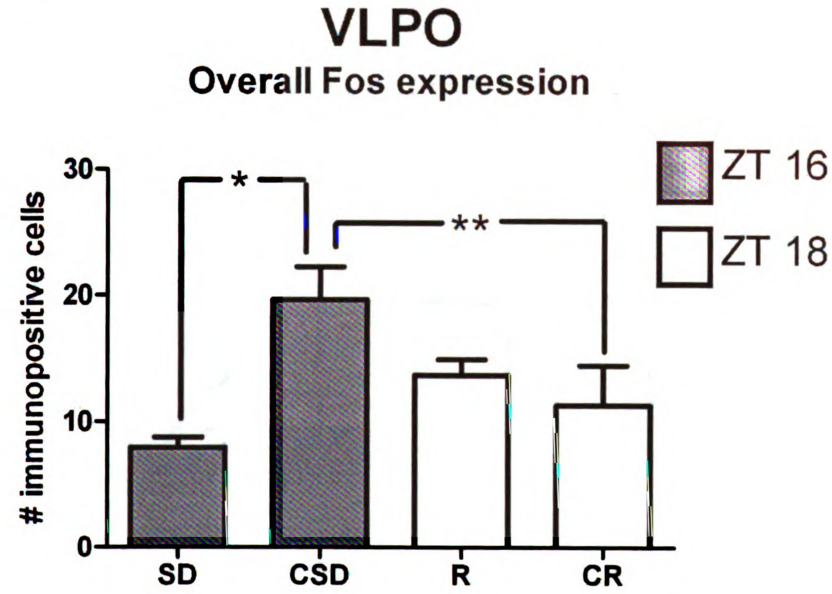


Figure 3.2. (cont'd)

G



H

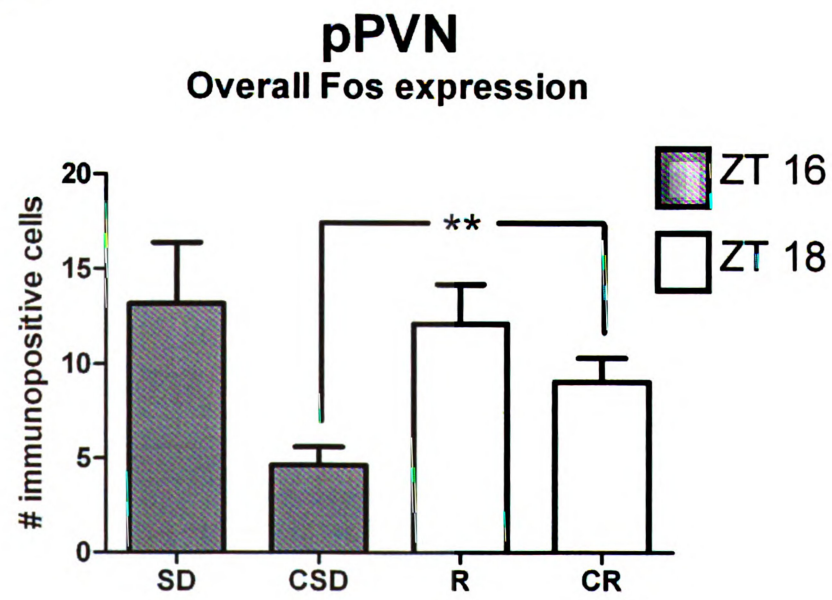
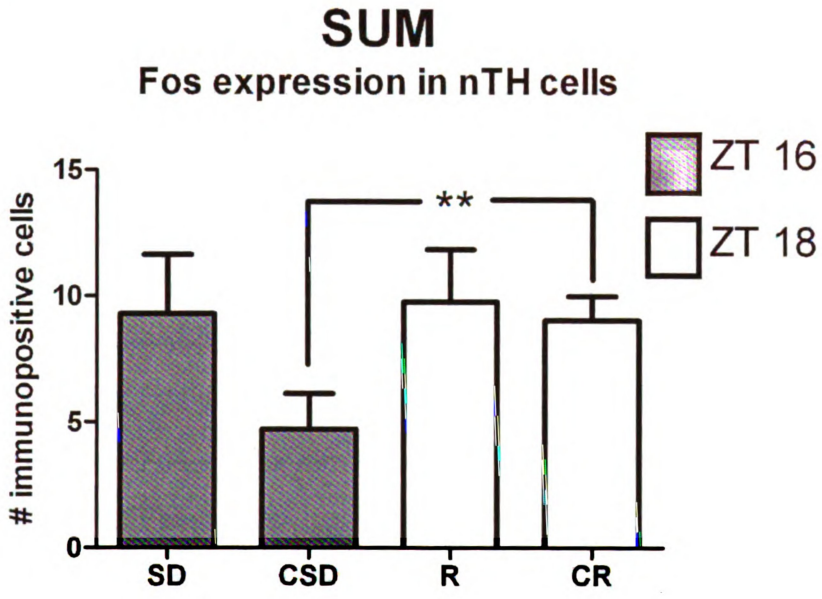


Figure 3.2. (cont'd)

I



J

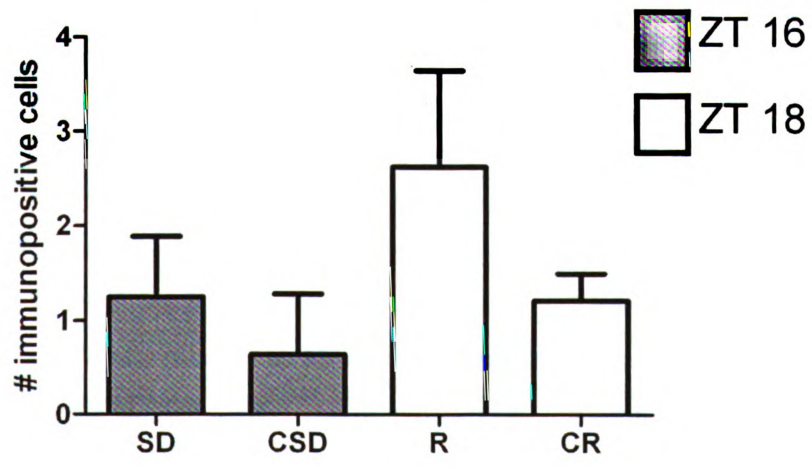


Table 3.1. Fos protein levels (Mean ± SEM) averaged across groups for the brain areas where significant effects were not observed. Fos-ir: Fos immunoreactivity, SEM: standard error of the mean.

		Mean ± SEM	p-value range
	Wakefulness controlling areas		
MS	% ACh cells containing Fos	1.23 ± 0.45	0.23 – 0.81
VDB	% ACh cells containing Fos	0.97 ± 0.24	0.26 – 0.81
HDB	% ACh cells containing Fos	1.06 ± 0.16	0.23 – 1.00
	# of nACh cells containing Fos	10.37 ± 0.91	0.21 - 0.71
mDR3	# of Fos-ir cells/ mm^2	139.65 ± 19.48	0.23 – 0.62
mDR4	# of Fos-ir cells/ mm^2	359.01 ± 48.10	0.08 – 0.41
mDR5	# of Fos-ir cells	5.00 ± 0.62	0.17 – 0.84
IDR	# of Fos-ir cells/ mm^2	221.12 ± 27.39	0.18 – 0.62
MR	# of Fos-ir cells	23.00 ± 2.59	0.62 – 1.00
LC	# of nTH cells containing Fos	14.51 ± 2.30	0.08 – 0.91
NI	# of Fos-ir cells	22.24 ± 1.76	0.46 – 1.00
	Reward related areas		
aVTA	% TH cells containing Fos	0.11 ± 0.08	0.46 – 1.00
mVTA	% TH cells containing Fos	0.05 ± 0.05	0.71 – 1.00
	# of nTH cells containing Fos	0.37 ± 0.08	0.23 – 0.46
pVTA	% TH cells containing Fos	0.93 ± 0.38	0.13 – 1.00
	# of nTH cells containing Fos	1.22 ± 0.16	0.10 – 0.71
	Circadian controlling areas		
SCN	# of Fos-ir cells	34.48 ± 2.45	0.54 – 1.00
vSPZ	# of Fos-ir cells	39.38 ± 2.37	0.41 – 0.63
	Areas related to stress responses		
mPVN	# of Fos-ir cells	13.46 ± 1.64	0.45 – 0.95

non-significant trends were seen, and Table 3.1 shows the patterns of Fos expression for all other areas.

Patterns of Fos expression in wakefulness and sleep promoting areas

The cholinergic BF: The total number of ACh cells in the MS, VDB, and HDB did not differ significantly for any comparison (all p values > 0.12). Also, the numbers of ACh cells that contained Fos in these areas, were not significantly affected by the treatments (all p values > 0.23). In the MS, however, there was a significant difference in the expression of Fos in nACh cells between the SD and CSD groups ($U = 7.50$, $p = 0.03$; Fig. 3.2A), with more Fos seen in the SD group. A non-significant trend for the same effect in nACh cells of these two treatment groups was observed in the VDB ($U = 10.00$, $p = 0.07$; Fig. 3.2B). All other comparisons in terms of Fos expression in the BF failed to reach statistical significance (all p values > 0.15).

TMM: In the VTM and DTM, ZT affected Fos expression in the control groups, with more cells expressing Fos at ZT 18 than at ZT 16 (both areas: $U = 5.50$, $p = 0.02$; Fig. 3.2C and 3.2D). In the VTM, there was a trend for more Fos expressed in R compared to CR animals ($U = 10.50$, $p = 0.07$; Fig. 3.2D). All other comparisons between SD and CSD in the VTM and DTM, as well as between R and CR in the DTM did not reach statistical significance (all p values > 0.23; Fig. 3.2C and 3.2D).

LH: For the LH more cells expressed Fos in the SD group than in the CSD group ($U = 3.00$, $p = 0.04$). In addition, there was a non-significant trend for higher Fos expression in the R group than in the CR animals ($U = 12.00$, $p =$

0.07; Fig. 3.2E). But no difference in Fos expression was seen between ZT 16 and ZT 18 for the control groups (U = 12.00, p = 0.13; Fig. 3.2E).

LC: In this region there was a non-significant trend for higher Fos expression in TH cells of SD in comparison to CSD animals (U = 2.00, p = 0.06; Fig. 3.2F). None of the other comparisons approached statistical significance (all p values > 0.08; Fig. 3.2F).

VLPO: In this area there was a significant difference between the SD and the CSD groups, with more cells expressing Fos in the latter group (U = 0.00, p < 0.01). Also, there was an effect of ZT in the expression of Fos in the control groups, with more cells expressing Fos at ZT 16 than at ZT 18 (U = 9.00, p = 0.05; Fig. 3.2G). There was, however, no difference between R and CR (U = 17.00, p = 0.23; Fig. 3.2G).

Raphe nuclei and NI: No significant differences were found in these brain areas among treatment groups (all p values > 0.08; Table 3.1).

Patterns of Fos expression in reward systems

SUM and VTA: None of the comparisons found significant effects in the total number of TH cells in the VTA and SUM (all p values > 0.23); or in the numbers of TH cells expressing Fos (all p values > 0.13) for any comparison. In the SUM, however, there was higher Fos expression in the nTH cells of control animals at ZT 18 than at ZT 16 (U = 8.50, p = 0.04; Fig. 3.2I). No significant differences were seen in Fos expression in nTH cells of the SUM between the SD and the CSD groups (U = 13.00, p = 0.17; Fig. 3.2I), or between the R and CR groups (U = 23.50, p = 0.61; Fig. 3.2I). In the aVTA of controls, there was a

non-significant trend for higher Fos expression in nTH cells at ZT 18 than at ZT 16 (U = 8.50, p = 0.07; Fig. 3.2J). However, inspection of the means and their standard errors suggests substantial overlap between the two sampling times. All other comparisons in terms of Fos expression in nTH cells in the medial and audal regions of the VTA failed to reached statistical significance (all p values > 0.09; Table 3.1).

Patterns of Fos expression in circadian controlling areas

SCN and vSPZ: There were no significant differences among treatment groups in these areas (all p values > 0.41; Table 3.1).

Patterns of Fos expression in stress related areas

PVN: In the mPVN, all comparisons revealed no significant effects among treatments on Fos expression (all p values > 0.46). In contrast, in the pPVN there was higher Fos expression at ZT 18 than at ZT 16 in the control groups (U = 4.50, p = 0.01; Fig. 3.2H). Also, there was a non-significant trend for higher Fos expression in SD than CSD animals (U = 8.00, p = 0.07; Fig. 3.2H). There was, however, no difference between the R and CR groups (U = 14.50, p = 0.23; Fig. 3.2H).

Neural activation in the LH in relation to other brain regions

In the MS and HDB, we found that Fos expression in both nACH and ACh cells, was positively correlated with Fos expression in LH cells; whereas in the VDB, Fos expression in only the nACh cell group was positively correlated with Fos expression in the LH (Table 3.2). In the LC, we detected a significant

Table 3.2. Correlations between Fos expression in LH cells and all other areas analyzed in this study.

		LH
MS	Fos in ACh cells	***r*= 0.393*; *p*= 0.047**
	Fos in nACh cells	***r*= 0.676*; *p*= 0.000**
VDB	Fos in ACh cells	*r*= 0.105; *p*= 0.610
	Fos in nACh cells	***r*= 0.721*; *p*= 0.000**
HDB	Fos in ACh cells	***r*= 0.534*; *p*= 0.005**
	Fos in nACh cells	***r*= 0.724*; *p*= 0.000**
VLPO	Overall Fos	*r*= -0.164; *p*= 0.422
SCN	Overall Fos	*r*= 0.133; *p*= 0.526
vSPZ	Overall Fos	*r*= 0.331; *p*= 0.106
pPVN	Overall Fos	***r*= 0.759*; *p*= 0.000**
mPVN	Overall Fos	***r*= 0.723*; *p*= 0.000**
DTM	Overall Fos	***r*= 0.620*; *p*= 0.001**
VTM	Overall Fos	***r*= 0.508*; *p*= 0.011**
SUM	Fos in nTH cells	***r*= 0.617*; *p*= 0.001**
LC	Fos in TH cells	***r*= 0.571*; *p*= 0.013**
	Fos in nTH cells	*r*= 0.437; *p*= 0.070
aVTA	Fos in nTH cells	*r*= 0.411*; *p*= 0.037
pVTA	Fos in nTH cells	*r*= 0.049; *p*= 0.815
mDR3	Overall Fos	***r*= 0.472*; *p*= 0.020**
mDR4	Overall Fos	***r*= 0.627*; *p*= 0.001**
mDR5	Overall Fos	***r*= 0.491*; *p*= 0.020**
IDR	Overall Fos	***r*= 0.602*; *p*= 0.002**
MR	Overall Fos	***r*= 0.438*; *p*= 0.032**
NI	Overall Fos	***r*= 0.158; *p*= 0.471**

(*) Significant correlation ($p < 0.05$)

positive association between Fos expression in TH cells and Fos expression in LH cells, and a non-significant trend for a positive correlation on that measure between nTH cells and LH cells (Table 3.2). For all other wakefulness promoting areas analyzed, overall patterns of Fos expression were positively correlated with Fos expression in the LH (Table 3.2). In addition, we observed positive correlations between Fos expression in LH cells and Fos expression in the pPVN, mPVN, SUM, and aVTA (Table 3.2). In contrast, patterns of Fos expression in the SCN, vSPZ, and VLPO were not correlated with Fos expression in LH cells (Table 3.2).

DISCUSSION

The results on neural activation in the BF, VTA, and SUM following induced wakefulness are discussed in relation to our previous findings on neural activation following voluntary wakefulness (Castillo-Ruiz et al., 2010). Overall, we found that induced and voluntary wakefulness have differential effects on areas of the brain that regulate arousal and reward.

Patterns of Fos expression in wakefulness and sleep promoting areas

MS, VDB, and HDB

In the MS we found that induced wakefulness elicits an increase in neural activation only in nACh cells (Fig. 3.2A). A trend for a similar effect was observed in the VDB, but this trend did not reach statistical significance (Fig. 3.2B). Interestingly, in our previous study we found that voluntary wakefulness during the night elicits increased Fos expression in nACh cells in the MS and VDB (Castillo-Ruiz et al., 2010). Taken together our previous and present results

suggest that nACh cells of the MS and VDB are responsive to wakefulness *per se*, regardless of the procedure that elicits it. It is likely that these nACh cells are GABAergic or glutamatergic since in nocturnal rodents these are the two other populations of cells present in the MS and VDB besides ACh cells (Leung and Shen, 2004; Yoder and Pang, 2005). Jointly these neural populations have as one of their main roles the regulation of HPC-θ (Leung and Shen, 2004; Yoder and Pang, 2005; Henny and Jones, 2008). Thus, it is likely that an outcome of the altered patterns of Fos expression seen in nACh cells of the MS and VDB is an altered HPC-θ, which in turn can have effects on learning and memory, processes that are modulated by this rhythm (reviewed in Buzsaki, 2002).

In sharp contrast with the data from the MS and VDB, we did not find evidence for induced wakefulness having an effect on neural activation in the HDB (Table 3.1). This result contrasts with our previous finding that in NA and DA grass rats there is elevated Fos expression in ACh and nACh cells of the HDB at night compared to Fos expression in those cell groups in sedentary animals (i.e., grass rats singly housed with no running wheels; Castillo-Ruiz et al., 2010). Hence, unlike the MS and VDB, neural activity in the HDB of grass rats does not seem to be responsive to wakefulness *per se*, but maybe regulated by amount of daily physical activity, independently of the phase of the light dark cycle in which the activity takes place. There is additional evidence for effects of physical activity in this region. In lab rats, for example, running in a wheel for 12 weeks is associated with an increase in the numbers of ACh cells in the HDB, but not in the MS or VDB (Ang et al., 2006). This may be the result of

either an increase in neurogenesis, or an increased survival of ACh neurons. Alternatively, it may be due to an up-regulation in the expression of the gene that codes for ChAT. Fos expression in the HDB may also be related to the rewarding effects of wheel running (reviewed in Sherwin, 1998; see below). We have evidence for differential neural activation in reward areas of the brains of DA and NA grass rats in comparison to sedentary animals (Castillo-Ruiz et al., 2010). Thus, neural activity in the HDB appears to be in part regulated by the reward system. This claim is supported by the finding that, at least in nocturnal rodents, reward areas of the brain project to the BF (Gaykema and Zaborszky, 1996).

A common finding with our previous study was that ACh neurons of the MS and VDB appear to be unresponsive to either induced or voluntary wakefulness (Castillo-Ruiz et al., 2010). These results in ACh cells of the MS and VDB in our previous and present studies contrast with those of reports on nocturnal rodents. For example, in lab rats, 6h of induced wakefulness during the day, following a procedure similar to the one used in here, elicits high Fos expression in ACh cells of the VDB and HDB, but not in the MS (Greco et al., 2000). The discrepancies between our results and the findings in nocturnal rodents could be related to species differences, or to lifestyle differences (i.e., being diurnal vs. being nocturnal). Another likely explanation is that the procedure used to induce wakefulness was more challenging for lab rats than for grass rats. This is because being nocturnal, lab rats restrict their activity to the dark phase of the light-dark (LD) cycle, whereas grass rats tend to be active during a longer interval of the LD cycle , as they show crepuscular bouts of

activity during the late and early night (McElhinny et al., 1997). Thus, it could be that for lab rats the sleep deprivation episode overlapped more fully with their inactive phase, than for grass rats, and hence resulted in more neuronal activation across the BF in the nocturnal species. Finally, another potential explanation is that there could be a combined effect of sleep deprivation and ZT. In our study we induced wakefulness between ZT 10 and ZT 16, whereas in Greco's study wakefulness was induced between ZT 6 and ZT 12 (Greco et al., 2000). This explanation, however, is unlikely since in lab rats only 2 h of sleep deprivation starting at ZT0, has effects that are similar to the ones reported by Greco (McKenna et al., 2009).

The neural activity elicited by induced wakefulness in the MS of our grass rats returns to baseline levels within 2 h after the animals are left undisturbed (Fig. 3.2A). In lab rats and mice 1-2 h of recovery sleep after 3-6 h of induced wakefulness produces the same effect, i.e., decreased Fos expression after sleep recovery, but in different areas of the brain (Basheer et al., 1997; Greco et al., 2000). Although electroencephalographic data were not collected in our study, it is likely that there was sleep displayed by the animals during the recovery period following forced wakefulness, since grass rats show increased bouts of sleep at this time, even without previous sleep deprivation (Novak et al., 1999). Sleep at this time is also seen in grass rats with wheel access, irrespective of their phase preference (Schwartz and Smale, 2005). Interestingly, we observed in our previous study that specific neural populations of the BF of DA, NA, and sedentary grass rats had similar patterns of Fos expression during

the day (Castillo-Ruiz et al., 2010). We argued that the similar patterns of Fos expression could be explained by the universal display of sleep observed between ZT 20 and ZT 22 in DA, NA and sedentary grass rats, which may return Fos expression to baseline levels regardless of presence or absence of wakefulness earlier in the night (Novak et al., 1999; Schwartz and Smale, 2005). Our current findings in the R group support that claim.

LH: neural activation and its relation to other wakefulness promoting areas

As observed in the MS, in the LH we found that neural activity was associated with wakefulness, since the animals that were induced to stay awake had higher levels of Fos expression than controls in that area (Fig. 3.2E). We also observed a trend for higher Fos expression in the R group than in the control group (Fig. 3.2E). Thus, it could be that at for the LH, 2 hours of recovery may not be sufficient to return neural activity to baseline levels. This contrasts with the observation that in the MS, neural activity is completely reset by 2 h of recovery (Fig. 3.2A). Together, these findings suggest that wakefulness-promoting areas of the brain respond differently to sleep deprivation, with some areas being able to recover faster than others.

In this study, we did not evaluate the phenotype of the LH neurons that express Fos. We, however, expect that the majority of these neurons are orexinergic. This is because in grass rats that are voluntarily awake during the rest phase there is a significant increase in Fos expression in the OXA and OXB cells of this region. Moreover, Fos expression in these cells is positively

correlated with neural activity in ACh and nACh cells of the MS and VDB, but only in animals that are voluntarily awake during the night (Castillo-Ruiz et al., 2010). Thus, given the role that orexin cells appear to play in the modulation of neural activity in other wakefulness promoting areas (reviewed in Saper et al., 2001), we predicted concordance between neural activation in orexin cells and neural activation of other wakefulness promoting areas. Using overall Fos expression in the LH as a proxy for orexin cell activation, we obtained strong positive correlations between Fos expression in all the wakefulness-promoting areas analyzed and Fos expression in the LH, with the exception of the NI and ACh cells of the VDB (Table 3.2). These observations are consistent with the view that the LH of grass rats modulates neural activity of other wakefulness promoting areas, as observed in nocturnal rodents (reviewed in Saper et al., 2001). Moreover, we found strong associations between Fos expression in the LH and Fos expression in other areas of the brain linked to arousal as well as reward, such as the SUM, aVTA, and pPVN (Table 3.2). The association between the LH and the pPVN is of particular interest given the effects that the orexins have on the stress axis (Kuru et al., 2000; Sakamoto et al., 2004). Taken together, these results suggest that in the diurnal grass rat there is a network of areas of the brain that is modulated by the orexins and regulates wakefulness and reward.

TMM nuclei

In the VTM and DTM, we did not find an effect of induced wakefulness or recovery on neural activation (Fig. 3.2C and 3.2D). The VTM and DTM show characteristic patterns of neural activation with higher Fos expression during the day than during the night (Novak et al., 2000; A. Castillo-Ruiz, unpublished observations), which are patterns that mirror vigilance states. This could suggest that these areas are in part responding passively to levels of vigilance. However, because we do not see a difference between the control groups and the SD and R group (Fig. 3. 2C and 3.2D), we suggest that instead these areas might be responding to circadian regulation. Recent observations from our group have shown that the DTM of grass rats has a daily rhythm in the expression of the clock genes *per 1* and *per 2*, with higher levels of Per 1 and Per 2 proteins during the day than during the night (C. Ramanathan, unpublished observation). Preliminary data suggest that Per 1 is also expressed in the VTM (C. Ramanathan, unpublished observations), but it remains to be determined whether the expression of this protein in this area has a circadian pattern. Future work needs to address the chemical identity of neurons of DTM and VTM that express clock genes in the grass rat, and whether they have a role in the circadian or environmental control of wakefulness.

Although, in the VTM and DTM, induced wakefulness did not affect neural activation, ZT had an effect, with Fos expression being higher at ZT 18 than at ZT 16 in both nuclei (Fig. 3.2C and 3.2D). This finding does not necessarily suggest that these changes in neural activity reflect an increase in wakefulness at ZT 18. In turn, they may reflect an increase in non-rapid eye movement

(nREM) sleep around this time. In nocturnal rodents, histaminergic cells of the TMM nuclei become almost silent during rapid eye movement (REM) sleep, whereas during nREM sleep they show some activity (reviewed in Saper et al., 2001). Interestingly, in the VLPO, an area that plays an important role in the onset of nREM sleep (reviewed in Saper et al., 2001), the pattern of neural activation observed in our animals was the opposite to the patterns observed in the TMM nuclei, i.e., higher Fos expression at ZT 16 than at ZT 18. We, however, did not see a significant association between neural activation in the VLPO and neural activation in the DTM and VTM (data not shown).

In this study we did not evaluate the phenotype of the neurons that expressed Fos in the TMM nuclei. In other rodents the DTM and VTM contain other neurotransmitters besides histamine, such as GABA and galanin (Airaksinen et al., 1992). In grass rats, we have confirmed that the VTM contains a cluster of histaminergic cells, but we have failed to identify histaminergic groups in the DTM (A. Castillo-Ruiz, unpublished observations). Future work needs to address the chemical identity of DTM neurons in the grass rat, and whether they have a role in the circadian or environmental control of wakefulness.

Raphe nuclei

The components of the raphe nuclei examined in this study, namely the 3 subdivisions of the DR and MR, did not show changes in neural activation due to either induced wakefulness or ZT (Table 3.1). In Syrian hamsters 3 hours of

sleep deprivation during the mid-rest period, following a procedure similar to the one used here, do not elicit changes in the DR or MR, except in serotonergic cells of the caudal DR (Webb et al., 2010). Similarly, voluntary activity during the same interval of time elicits increased Fos in n5HT cells of the caudal DR (Webb et al., 2010). In our case, we did not see this differential activation of specific regions of the DR (Table 3.1). In Syrian hamsters sleep deprivation, with or without wheel running, during the rest phase produces phase advances of the rhythm of locomotor activity (Antle and Mistlberger, 2000). The results of several studies suggest that serotonergic inputs from the raphe to the SCN may mediate these effects (Grossman et al., 2000). The finding that there is no change in neural activity in the raphe nuclei of grass rats forced to stay awake, could in part explain why in grass rats sleep deprivation with (Rose et al., 1999; Schwartz and Smale, 2005) and without (present results, Table 3.1) physical activity does not appear to have an effect on the functioning of the SCN pacemaker. It remains to be determined whether the discrepancies between our findings and the findings of studies with nocturnal species are due to general species and/or experimental procedural differences, or if they stem from fundamental differences between diurnal and nocturnal rodents.

Similarly to the TMM nuclei, the raphe nuclei appeared to be under tight circadian control. The levels of Fos expression detected in this study between ZT 16 and ZT 18 (Table 3.1) are similar to the levels of Fos expression seen inside and outside of 5HT cells in grass rats kept undisturbed under a 12-12 light-dark cycle (A. Castillo-Ruiz, unpublished observations). Future work needs to address

whether voluntary sleep deprivation has effects on the raphe nuclei not seen after induced wakefulness in grass rats.

LC

In the LC we also did not find a significant difference among the treatment groups (Table 3.1 and Fig. 3.2F). However, we observed a non-significant trend for higher Fos expression in TH cells of the LC in the SD group in comparison to its control group (Fig. 3.2F). In Syrian hamsters voluntary or forced wakefulness induced by gentle stimulation for 3h during the regular rest phase does not have an effect on Fos expression in the LC (Grossman et al., 2000). In contrast, physical restraint during the same interval of time produces increased Fos expression in the LC (Grossman et al., 2000). These results with hamsters are in agreement with the hypothesis that this region is involved in modulating attention to salient stimuli in the environment (Aston-Jones et al., 1994). Thus, the trend observed here in the level of Fos expression in TH cells of the LC could reflect an increase in the animals' vigilance. This is likely the case, since these animals, unlike lab rats or other laboratory rodents, do not seem at ease when humans are in the vicinity. The lack of statistical significance in TH cells of the LC was probably related to low statistical power.

NI

The NI was included in this study because of its role in the modulation of the HPC-Θ through its connections with areas of the brain previously studied in

grass rats, i.e., the SUM, MS, and VDB (Castillo-Ruiz et al., 2010). The NI is part of an ascending multisynaptic pathway that starts at the brainstem level and terminates in the hippocampus. In particular, the reticularis pontis oralis nucleus and the peduncolopontine tegmental nucleus appear to be among the areas of the brainstem that initiate the HPC-Θ (Nunez et al., 2006). These areas are connected to the NI, SUM, MR, MS and VDB through direct and indirect projections (Goto et al., 2001; Olucha-Bordonau et al., 2003). In addition, the NI may play a role in the display of activity, since relaxin-3, which is a neuropeptide produced solely in the NI, modulates the amplitude and distribution of circadian patterns of wheel running activity (Smith et al., 2009). We, however, did not a find an effect of induced wakefulness on the neural activity of this nucleus (Table 3.1). This is interesting giving that upstream components of the pathway that elicits the HPC-Θ, namely the MS, respond to induced wakefulness (present results). Thus, our results suggest that following induced wakefulness, functional dissociations exist among specific brain systems that regulate arousal and hippocampal activity.

VLPO

The VLPO is an area of the BF that modulates sleep stages; specifically it appears to play a role in the onset and maintenance of nREM sleep (reviewed in Saper et al., 2001). In agreement with this role in sleep regulation, we found that undisturbed animals had higher Fos expression in the VLPO than SD animals (Fig. 3.2G). We also found that in the VLPO, 2 h of recovery resulted in a return

of neuronal activity to baseline levels (Fig. 3.2G). Interestingly, in the VLPO, we saw a decrease in Fos expression in controls at ZT 18 as compared to ZT 16 (Fig. 3.2G). A similar phenomenon was observed when Fos expression in the VLPO of undisturbed grass rats was compared between ZT 23 and ZT 20 (Novak et al., 1999). In that study, grass rats had higher levels of Fos expression at ZT 20 than at ZT 23. Given the aforementioned role of the VLPO in nREM sleep onset (reviewed in Saper et al., 2001), the authors predicted higher levels of Fos expression in the VLPO in earlier hours of the inactive phase. Our results support that prediction.

Patterns of Fos expression in the reward system

There is a body of evidence suggesting that wheel running is rewarding. For example, in lab rats, acute access to a running wheel induces increase activation in dopaminergic cells of the VTA (Yanagita et al., 2007), an effect also seen after exposure to conventional rewarding stimuli (Asmus and Newman, 1994; Balfour et al., 2004). Moreover, lab rats not only compromise food and water intake in favor of wheel running, but also work in order to get access to a running wheel (reviewed in Sherwin, 1998). In our previous study we found that voluntary activity, regardless of the chronotype of the animals, elicits higher expression of Fos throughout the day in two specific components of the reward system: the SUM and aVTA (Castillo-Ruiz et al., 2010). We hypothesized that these results were likely to reflect the hedonic aspects of wheel running, and predicted that in comparison to grass rats that are voluntarily awake, grass rats forced to stay awake would not show differential patterns of neural activation in

reward areas. The lack of elevated Fos expression in reward areas seen here supports that prediction (Table 3.1). However, future work needs to evaluate whether the effect we previously observed in grass rats with access to wheels is in fact related to reward rather than to enhanced physical activity.

Patterns of Fos expression in circadian controlling areas

In Syrian hamsters, sleep deprivation induced by either physical activity or gentle handling induces phase shifts in the rhythm of Fos expression of the SCN (Antle and Mistlberger, 2000). In addition, in lab rats, sleep deprivation during the resting phase reduces the amplitude of the neuronal firing rhythm of the SCN (Deboer et al., 2007), and this effect persists even after 6-7 hours of sleep recovery (Deboer et al., 2007). Based on these observations, effects on Fos expression in the SCN of grass rats as a result of forced wakefulness would not have been surprising. However, we did not detect any changes in the SCN of sleep deprived grass rats (Table 3.1). This could be explained in part by the fact that the raphe nuclei, an area that appears to mediate the phase shifting effects of sleep deprivation in hamsters (Grossman et al., 2000), did not show changes in neural activation under our experimental conditions.

Another important brain area for circadian control is the vSPZ. This area receives a heavy projection from the SCN (Watts et al., 1987) and may play a role in the modulation of SCN output signals (Smale et al., 2008). The vSPZ regulates rhythms in locomotor activity in both grass rats (Schwartz et al., 2009) and lab rats (Lu et al., 2001). Moreover, in lab rats the vSPZ appears to play a role in the circadian regulation of sleep, although the available data are from only

a few post-surgical days following vSPZ lesions (Lu et al., 2001). Similar to the results for the SCN, forced sleep deprivation had no effects on the neural activity of the vSPZ of grass rats (Table 3.1). These observations for the SCN and the vSPZ are remarkably similar to those seen when grass rats voluntarily stay active at night (Rose et al., 1999; Schwartz and Smale, 2005), and suggest that key components of the circadian system of grass rats are refractory to the effects of sleep deprivation.

Cholinergic projections reach the SCN and vSPZ of grass rats (Castillo-Ruiz and Nunez, 2007). These projections may originate from the BF and tegmentum (Castillo-Ruiz et al., 2007), as seen in lab rats (Bina et al., 1993). But in comparison to nocturnal species, the SCN of grass rats receives fewer projections from those areas (Castillo-Ruiz et al., 2007). We do not know if this is also true for the vSPZ. However, that is likely to be the case, because overall the vSPZ of grass rats contains relatively few cholinergic fibers (Castillo-Ruiz and Nunez, 2007). The reduction in inputs to the SCN and vSPZ of grass rats from cholinergic areas that are active during wakefulness may explain the lack of responsiveness of these brain regions to forced or voluntary wakefulness during the normal rest phase of the species (reviewed in Rosenwasser, 2009).

Patterns of Fos expression in areas related to stress responses

In this study, we evaluated whether the procedure used to induce wakefulness was stressful to grass rats. To that aim, we analyzed the patterns of Fos expression in the PVN, since conditions that are considered challenging to an organism, such as exposure to a predator's odor or immobilization (Ceccatelli

et al., 1989; Dielenberg et al., 2001), elicit Fos expression in this nucleus. Two regions of the PVN - the pPVN and mPVN - were included in the analysis given that they both modulate a variety of physiological and behavioral variables that accompany the stress response (Jezova et al., 1995; Wotjak et al., 2001; Herman et al., 2003). Even though we did not find significant effects among the groups in the mPVN (Table 3.1), we saw a non-significant trend in the pPVN, with higher Fos expression in the SD group than in the control group (Fig. 3.2H). This is an indication that the procedure might have been stressful for grass rats.

We also observed an effect of ZT in the pPVN, with higher Fos expression at ZT 18 than at ZT16 (Fig. 3.2H). This finding is consistent with a previous report from our group (Nunez et al., 1999), in which a sharp increase in Fos expression was observed throughout the PVN between ZT 13 and ZT 17. The authors, however, did not evaluate whether the changes in Fos were restricted to the pPVN or the mPVN. Our present findings suggest that this increase in Fos expression was driven primarily by the pPVN. Evidence for a circadian modulation of the activity of this region is interesting, since there is evidence that the pPVN participates in the cascades of events that increases glucose availability before the beginning of the active phase (Leibowitz et al., 1988; Kalsbeek et al., 2004).

SUMMARY AND CONCLUSIONS

One of the main findings of this study was that in grass rats induced wakefulness during the resting phase does not produce uniform effects on neural activation in regions of the brain that promote wakefulness. Moreover, in these

animals the patterns of neural activation elicited by induced wakefulness in the BF, SUM, and VTA are strikingly different from the ones produced by voluntary wakefulness during the night (Castillo-Ruiz et al., 2010). Overall these results suggest that induced and voluntary wakefulness during the resting phase have different effects on neural systems involved in wakefulness and reward. Therefore, different behavioral and physiological outcomes are expected to occur depending upon the particular protocols used to keep animals awake during their rest phase. These observations are important for the evaluation of animal models of human shift work, since researchers use a variety of procedures to induce or facilitate wakefulness. These range from voluntary (e.g. wheel running in grass rats; Castillo-Ruiz et al., 2010) to forced wakefulness paradigms (e.g. forced wheel running in lab rats, Salgado-Delgado et al., 2008). Also, some models of shift work involve increased physical activity, whereas others demand attentional and/or cognitive effort (Lee et al., 2009). Most likely, the different approaches induce different changes in the nervous system that should be identified and taken into account when evaluating the ecological validity of these animal models of human night-shift work.

The second main finding was that neural activation observed after induced wakefulness returns to baseline levels within 2 h of undisturbed rest. In our previous study, we saw nocturnal elevation of Fos expression in the BF of grass rats with access to wheels compared to the sedentary controls, but in the morning, those differences were absent (Castillo-Ruiz et al., 2010). To explain these results, we proposed that the display of sleep towards the end of the dark

phase of the LD cycle, which is seen in grass rats regardless of housing conditions or chronotype, returns to baseline the neural activity in the BF of these animals (Castillo-Ruiz et al., 2010), thus abolishing group differences seen at night. Taken together our previous and present observations suggest that brief episodes of rest, which occur either acutely (in this study) or daily (in our previous study), can negate the effects of previous wakefulness on the neural activity of the BF.

We can not dismiss the possibility that the differential results of the current and previous study (Castillo-Ruiz et al., 2010) stem from features of the experimental conditions other than forced vs. spontaneous wakefulness. Those features include the length of exposure to the procedure and the degree of physical activity exerted by the animals. That is, in comparison to voluntary wheel–running, the procedure used to induce wakefulness was acute and only involved minor increases in general activity. Future work needs to address these issues. Also, it remains to be addressed whether voluntary and induced wakefulness has differential effects on neural activation in arousal and reward areas of the brain depending on age and/or sex. For example, sleep deprived female rats show enhanced Fos expression in arousal areas of the brain upon treatment with estradiol (Deurveilher et al., 2008).

Finally, we found that in the grass rat neural activity in most neural groups that promote wakefulness shows robust correlations with neural activity in the arousal system located in the LH. In nocturnal rodents, this system has been postulated to play an important role in the modulation of neural activity of other

wake-promoting neural groups (reviewed in Saper et al., 2001). The present study is the first to provide evidence for that hypothesis in a diurnal species.

In summary, our results expand the current understanding of the effects of wakefulness during the resting phase, followed by sleep recovery, on the neural substrates that support a wide array of behaviors and brain functions in a diurnal rodent. When compared to voluntary wakefulness, induced wakefulness produces different effects in arousal and reward areas of the brain, and in grass rats sleep deprivation produces results that contrast with those reported for nocturnal rodents (Basheer et al., 1997; Greco et al., 2000; McKenna et al., 2009). These observations strongly suggest that we must be very cautious when generalizing to situations involving human shift workers, the results of experiments with nocturnal animals that are forced to be awake.

CHAPTER 4

Rhythmic neural activation in monoaminergic areas of the brain with access to the cerebrospinal fluid in the diurnal grass rat

INTRODUCTION

Wakefulness is supported by several systems distributed throughout the mammalian brain, including the histaminergic tuberomammillary nucleus of the hypothalamus (TMM), and the serotonergic raphe nuclei (reviewed in Jones, 2005). Besides their role in arousal, these areas also have a role in photic and non-photic entrainment (reviewed in Yannielli and Harrington, 2004; Rosenwasser, 2009) of circadian rhythms. In agreement with their role in promoting wakefulness, histaminergic neurons of nocturnal species show increase activation during the active phase in comparison to the inactive phase of the animals' activity-rest cycle (Ko et al., 2003; Takahashi et al., 2006). In the diurnal grass rat, *Arvicanthis niloticus*, the ventral tuberomammillary nucleus (VTM) shows daily rhythms in the expression of Fos (Novak et al., 2000).The serotonergic system of crepuscular rodents also shows daily rhythms in Fos expression that mirror the wake/sleep cycle, but these rhythms only become evident at the caudal levels of the DR (Janusonis and Fite, 2001). To our knowledge, whether the raphe nuclei of diurnal species show daily patterns of neural activation has not been determined.

Given that neural activity in histaminergic and serotonergic areas appears to be regulated by circadian mechanisms, and that in other arousal systems

increased neural activity is accompanied by release of neurotransmitters at the terminal buttons (Greco et al., 1999; Greco et al., 2000); it is likely that the release of histamine (HA) and serotonin (5HT) into target areas also follows a circadian pattern. Of particular interest is the choroid plexus (CP) as a possible target for circadian modulation by histaminergic and/or serotonergic inputs. The CP is the site of production of the cerebrospinal fluid (CSF), which transports essential molecules throughout the nervous system. The CP contains receptors for both 5HT (Watson et al., 1995) and HA (Crook et al., 1986), and both neurotransmitters influence the metabolism of this area (Crook et al., 1984; Watson et al., 1995). Serotonin does not appear to exert its modulatory role through direct synaptic contacts with the CP, but rather, via its release into the CSF (Chan-Palay, 1976). For HA, details about its interactions with the CP have yet to be elucidated.

To characterize further the patterns of neural activation in histaminergic and serotonergic areas in a diurnal species, we measured Fos expression in the TMM and raphe nuclei in grass rats throughout the 24 h wake/sleep cycle. In particular, we analyzed Fos expression in the dorsal tuberomammillary (DTM), VTM, and two major nuclei in the raphe that have been implicated in entrainment as well as in the support of wakefulness – dorsal raphe (DR) and median raphe (MR). We also assessed whether, in this species, serotonergic and histaminergic areas have access to the CSF. We accomplished this by injecting the retrograde tracer cholera toxin subunit B (CTB) into the third ventricle (3V). Finally, we investigated whether the dense cluster of retrogradely labeled cells observed in

the DR after 3V injections of CTB was positive for 5HT. The results provide new insights on the functioning of monoaminergic brain areas in a diurnal mammal. These brain areas are known to not only participate in entrainment of rhythms and the support of wakefulness, but have also been linked to the etiology of behavioral disorders, such as depression and schizophrenia (Maes and Meltzer, 2000; Arrang, 2007). In addition to sampling these monoaminergic areas, we measured Fos expression in the supramammillary nucleus (SUM), which is of interest because of its role in reward and arousal (reviewed in Pan and McNaughton, 2004; Ikemoto, 2010).

EXPERIMENTAL PROCEDURES

Animals

Adult male grass rats were used in this study. These animals were born in our laboratory and derived from a group brought from Kenya in 1993 (Katona and Smale, 1997). Animals were kept on a 12:12 light-dark cycle (LD), and were provided with *ad libitum* access to water and food (Harlan Teklad 8640 rodent diet, Harlan Teklad Laboratory, Madison, WI). All experiments were performed in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Michigan State University Institutional Animal Care and Use Committee.

Experiment 1: Neural activation in monoaminergic areas

1.1. Histology

Animals (n = 6 per group) were perfused at Zeitgeber times (ZT) 2, 4, 10, 14, 18, and 22 (lights on at ZT 0). To prevent exposure to light animals that were perfused during the dark phase were fit with an aluminum foil hood over their heads. Intraperitoneal injections of sodium pentobarbital (Ovation Pharmaceutical, Deerfield, IL) were used to deeply anesthetize the animals, before they were intracardially perfused with 0.9% saline, followed by 4% N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; Sigma-Aldrich, St. Louis, MO; pH 7.4) in 0.1 M phosphate buffer (PB, pH 7.4), and then by 4% Paraformaldehyde (Sigma-Aldrich) with 75 mM lysine (Sigma-Aldrich) and 10 mM sodium periodate (Sigma-Aldrich; PLP) in 0.1 M PB. Their brains were post-fixed for approximately 4 hours in PLP and then transferred to 20% sucrose solution in 0.1 M phosphate buffer (PB; pH 7.4) overnight at 4°C. Coronal sections (30 µm) were cut on a freezing sliding microtome, and alternate sections were collected in three series in cryoprotectant solution at -20°C until further processing. One series was used for the co-localization of Fos and 5HT and the other for Fos and HA. Unless indicated otherwise, all immunocytochemical procedures were carried out at room temperature and all incubations involved gentle agitation. In addition, with the exception of experiment 2, sections were rinsed 3 times (5min/rinse) in 0.01 M PBS between all the immunocytochemical steps and all incubations included 0.3% Triton X-100 (TX; RPI, Elk Grove Village, IL; TX). Free-floating sections containing the TMM nuclei and the raphe nuclei were rinsed (6 times, 10 min/rinse) in 0.01 M PBS, blocked for 1 h using 5% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, West

Grove, PA) in PBS and incubated overnight in a rabbit anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:20,000 in PBS and 3% NDS) at 4° C. The sections were then incubated for 1 h min in a donkey anti-rabbit biotinylated antibody (Jackson; diluted 1:200 in PBS and 3% NDS), and then for 1 h in avidin-biotin peroxidase complex (AB complex, 0.9% each avidin and biotin solutions; Vector Laboratories, Burlingame, CA; in PBS). After 3 rinses (10 min/rinse) in Tris buffer (pH 7.2), the sections were preincubated in 0.025% diaminobenzidine (DAB; Sigma-Aldrich) enhanced with 2.5% nickel sulfate (Sigma-Aldrich) in Tris buffer for 1 min, and then 3% hydrogen peroxide (J.T. Baker, Phillipsburg, NJ) was added for the reaction. After a 5 min reaction, the tissue was rinsed in PBS (4 times, 10 min/rinse). Then, sections were blocked for 1 h in 5% normal goat serum (NGS; Vector; in PBS) and incubated overnight in a rabbit anti- 5HT antibody (Protos Biotech Corp., New York, NY; diluted 1:10,000 in PBS and 3% NGS) at 4°C. Following primary incubation, the sections were incubated for 1 h in a goat anti-rabbit biotinylated antibody (Vector; diluted 1:200 in PBS and 3% NGS). Then, the tissue was incubated for 1 h with AB complex (0.9% each avidin and biotin solutions, Vector; in PBS). After 3 rinses (10 min/rinse) in Tris buffer, the sections were preincubated with 0.02% DAB in the same buffer for 1 min, and then hydrogen peroxide (0.35 µl 30% hydrogen peroxide/ml buffer) was added for the reaction. After a 1 min reaction, the tissue was rinsed in 0.01 M PBS (3 times, 5 min/rinse). Then, all sections were mounted onto gelatin-coated slides, dehydrated, and later coverslipped with dibutyl phthalate xylene (Sigma-Aldrich; DPX).

1.2. Quantification of the Immunocytochemistry (ICC)

The ICC for Fos and HA did not produce adequate double labeling, therefore we limited our study to the analysis of Fos and 5HT in the DR and Fos in the TMM nuclei. In order to quantify Fos expression in the TMM nuclei, we selected one section that contained both the VTM and DTM. For the quantification of Fos and 5HT expression in the raphe nuclei, we selected three sections containing the medial subdivion of the dorsal raphe (mDR), one containing the lateral subdivision of this nucleus (lDR), and one of the MR. In addition, we quantified the expression of Fos in one section containing the SUM.

Sampling boxes of different dimensions were used to quantify Fos and/or 5HT in the studied areas. For the DTM, cells expressing Fos were counted within a region defined by a 120 µm x 120 µm box placed on either side of the 3V; whereas for the VTM, we used a 150 µm x 150 µm box placed on the edge of the brain. The placement of these boxes was done following the criteria used by (Novak et al., 2000). For the lDR, the sampling region was defined by a 150 µm (width) x 650 µm (height) box placed lateral to the mDR, immediately dorsal to the medial longitudinal fasciculus (Fig. 4.1A). For the mDR, the three sections analyzed corresponded to levels 3, 4 and 5 following the nomenclature of (Janusonis et al., 1999), and will be referred to as mDR3, mDR4, and mDR5, respectively. Levels 1 and 2 were not analyzed since Fos staining was rare in those regions. The sampling boxes used for mDR3 and mDR4 had the same dimensions as the box used for the lDR. These boxes were placed in midline, medial to the dorsal borders of the medial longitudinal fasciculi (Fig. 4.1). The

sampling box for mDR5 was 160 µm x 160 µm and was placed ventral to the cerebral aqueduct (Aq; Fig. 4.1C). For the MR, the sampling region was defined by a 150 µm (width) x 700 µm (height) box placed on midline in an area rich in 5HT staining (Fig. 4.1A). Finally, a 160 µm x 160 µm sampling box was placed in the caudal SUM as described previously in (Castillo-Ruiz et al., 2010).

Counts were done bilaterally in the DTM, VTM, and IDR, and cell numbers for these areas were averaged for each section. The mDR, MR, and SUM did not require this procedure since these regions are located close to the midline (see Fig. 4.1). In addition, for the IDR and all levels of the mDR cell counts were divided by the area occupied by their sampling box and the results are expressed as number of cells/mm^2. This procedure was necessary because for some sections the part of the sampling boxes fell outside the studied areas. All counts were done using a 25x objective and performed by an investigator unaware of the source of the tissue.

To analyze the effect of time on the expression of Fos in and out of 5HT cells, we used non-parametric Kruskal-Wallis tests. Significant effects of time were followed by Mann-Whitney U tests. For all comparisons differences were considered significant when p was less than 0.05. The software used for the statistical analyses was SPSS 17 (SPSS Inc., Chicago, IL, USA).

Experiment 2: Monoaminergic projections with access to the cerebrospinal fluid

2.1 Surgical procedures

Figure 4.1. Rostro-caudal illustrations depicting the sampling areas used to quantify Fos expression in TH and nTH cells of the lDR, MR, mDR3 (A), mDR4 (B), mDR5 (C). See text for sampling box dimensions. Scale bar = 1 mm.

Grass rats were deeply anesthetized with isoflurane (Abbott Laboratories, Abbott Park, Il) and their heads were shaved, disinfected with 10% povidone-iodine (Novation Inc., Irving, TX), and injected subcutaneously (s.c.) with lidocaine (Hospira Inc., Lake Forest, Il, 6 mg/kg). In addition, a s.c. injection of buprenex (PharmaForce, Inc., Hiliard, OH, 0.05 mg/kg) was given around the neck area. Then, the animals were placed in a sterotaxic apparatus (Stoelting Co., Wood Dale, Il) with the incisor bar placed 6 mm below the center of the ear bars. A small incision was made on their scalp and a Hamilton syringe (Hamilton Company, Reno, NV), filled with 20 nanoliters of CTB (10mg/ml; Sigma Aldrich, St. Louis, MO), was used to deliver the tracer. The syringe was angled at 10 degrees and the coordinates used to determine the injection site were 1.3 mm lateral and 1.4 mm anterior to bregma. Then, a small hole was drilled above the target area, and the syringe was lowered to injection depth with the coordinates obtained in relation to dura (i.e., 6.2 mm ventral to dura). The tracer was delivered over a 5 minute interval and the syringe was left in place for 15 minutes, and then drawn slowly. The incision was closed with autoclips and treated with novalsan antiseptic ointment (Fort Dodge Animal Heatlh, Fort Dodge, IA). To prevent dehydration, all animals received 2 cc of sterile saline (s.c; Hospira). The animals were left to recover on a heating pad and as post-operative care received either: (1) buprenex (s.c.) every 12 hours for the first day, and then ketoprofen (Fort Dodge Animal Heatlh; 5 mg/kg) every 24 hours for the following two days or (2) ketoprofen (s.c.; 5 mg/kg) during the first day, and then meloxicam (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO; 0.2

mg/kg) delivered in a piece of apple every 24 hours for the following two days. Seven days after the CTB injection animals were given an overdose of sodium pentobarbital (Ovation Pharmaceutical) and then were perfused transcardially with 0.01 M PBS, followed by PLP. Brains were post-fixed for 4 hours, and then transferred to 20% sucrose for approximately 48 hours. Then, brains were sectioned coronally at 30 um on a freezing microtome into 3 series and stored in cryoprotectant at -20°C.

2.2 ICC procedures

2.2.1. Distribution of retrogradelly labeled cells

Tissue was rinsed (6 times, 10 min/rinse) in tris-buffered saline (TBS) in 0.04% TX, blocked for 30 minutes using 5% normal rabbit serum (NRS; Vector), and rinsed once in 0.04% TX-TBS. Then, sections were incubated in a goat anti CTB antibody (List Biological Laboratories, Inc., Campbell, CA; diluted 1:5000 in 0.04% TX-TBS) for 60 hours at 4°C. After this step, the tissue was rinsed in 0.02% TX-TBS (3 times, 5 min/rinse) and incubated for 2 hours in a rabbit anti goat antibody (Vector; diluted 1:200 in 0.02% TX-TBS). Then, sections were rinsed in 0.02% TX-TBS (3 times, 5 min/rinse) and incubated in AB complex (0.9% each avidin and biotin solutions, Vector; in 0.02% TX-TBS) for 2 hr. After 3 rinses (5 min/rinse) in 0.02% TX-TBS and one rinse (5 min) in TBS, the tissue was preincubated in 0.01% DAB in 0.1 M PB for 1. 5 min, and then 0.3% hydrogen peroxide was added for the reaction. After a 20 min reaction, the tissue

was rinsed in Tris buffer (4 times, 5 min/rinse) and the sections were mounted, dehydrated, and later coverslipped with DPX.

2.2.2. Distribution of cells labeled for CTB and 5HT

Since CTB cells were absent in the VTM and rare in the DTM (Fig. 4.9B), and because the latter area is not likely to contain HA cells (A. Castillo-Ruiz, unpublished observations); we limited our double-labeling analysis to CTB and 5HT in mDR3 through mDR5, which were the only regions of the mDR containing CTB-positive cells. Free-floating sections containing levels 3-5 of the mDR were rinsed (6 times, 10 min/rinse) in 0.1% TX-PBS, blocked in a solution containing 5% NDS (Jackson) for 30 min, and incubated in a rabbit anti 5HT (Protos Biotech; diluted 1: 1000 in PBS and 0.03% TX) for 48 hours at 4°C. Then, sections were rinsed in 0.1% TX-PBS (3 times, 5 min/rinse) and incubated in a Cy2 donkey anti rabbit antibody (Jackson; diluted 1:100 in PBS and 0.03% TX) for 2 hours. From this point the tissue was protected from light by covering it with aluminum foil and by doing all tissue transfers away from strong sources of light. This was necessary due to the light sensitive nature of the secondary antibodies. Then, the tissue was rinsed in 0.1% TX-PBS (3 times, 5 min/rinse) and incubated in a goat anti CTB antibody (List; diluted 1:500 in PBS and 0.03% TX) for 48 hours at 4°C. After three rinses (5 min/rinse) in 0.1% TX-PBS, the sections were incubated in a Cy2 donkey anti goat antibody (Jackson; diluted 1:100 in PBS and 0.03% TX) for 2 hours and then rinsed in PBS (4 times, 5 min/rinse). Sections were mounted, dehydrated, and later coverslipped with DPX.

2.3 Tissue analysis

To examine CTB staining we used a light microscope (Leitz, Laborlux S, Wetzlar, Germany), whereas to examine colocalization of CTB and 5HT we used a fluorescent microscope (Zeiss Axioscop 2 plus, Carl Zeiss, Göttingen, Germany) equipped with green and red filters. Photomicrographs were taken at 40-100x magnification. For confirmation of colocalization of CTB and 5HT we combined red and green color channels using adobe Photoshop (Adobe Systems, San Jose, CA).

RESULTS

Experiment 1: Neural activation in monoaminergic areas

Serotonin and Fos immunoreactivity was evident following the ICC procedures (Fig. 4.2), and significant differences were observed in the expression of Fos in and outside of 5HT cells in several of the regions sampled. Figure 4.3 through 6 show the patterns of Fos expression across ZTs for all the areas where significant differences where found.

Patterns of Fos expression in serotonergic areas

The total number of 5HT cells in the raphe nuclei did not differ significantly for any comparison (all p values > 0.21). In addition, data on 5TH cells expressing Fos in the IDR, and mDR3 were not analyzed because double-labeled cells were very rare in those regions across all groups. Thus, for those areas only data on Fos expression in n5HT cells are presented, and their

analysis showed no significant effects of ZT (IDR: $X^2 = 9.93$, df = 5, p = 0.08; and mDR3: $X^2 = 8.17$, df = 5, p = 0.15).

For mDR4 and mDR5 there was a significant rhythm in Fos expression in 5HT cells ($X^2 = 16.45$, df = 5, $p < 0.01$; and $X^2 = 15.25$, df = 5, $p < 0.01$, respectively), with a drop in Fos expression between ZT 18 and ZT 22 (Fig. 4.3C and 4.3E). A rhythmic pattern of Fos expression was also evident in n5HT cells of mDR4 ($X^2 = 20.61$, df = 5, $p < 0.01$; Fig. 4.3B), with higher Fos expression between ZT 2 and ZT 14 than between ZT 18 and ZT 22. For mDR5 there was a non-significant trend for an effect of time on the expression of Fos in n5HT cells ($X^2 = 10.29$, df = 5, p = 0.07; Fig. 4.3D). Finally, in the MR, a rhythm in Fos expression was present in n5HT cells ($X^2 = 13.32$, df = 5, p = 0.02; Fig. 4.4A) but not in 5HT cells ($X^2 = 9.01$, df = 5, p = 0.11; Fig. 4.4B). The rhythm in n5HT cells of the MR featured higher Fos expression between ZT2 and ZT 14 than between ZT 18 and ZT 22 (Fig. 4.4A).

Patterns of Fos expression in the TMM nuclei and SUM

In the DTM, VTM, and SUM there was a significant effect of time on the expression of Fos ($X^2 = 19.46$, df = 5, p = 0.01; $X^2 = 11.92$, df = 5, p = 0.08; and $X^2 = 19.77$, df = 5, $p < 0.01$, respectively). For the VTM, paired comparisons revealed a decreased in Fos expression between ZT 18 and ZT 22, compared to all other ZTs, except ZT 14 (Fig. 4.5B). For the DTM, paired comparisons revealed a similar pattern to the VTM, with more cells expressing Fos during the light phase and early hours of the night than later in the night (Fig. 4.5A). Finally

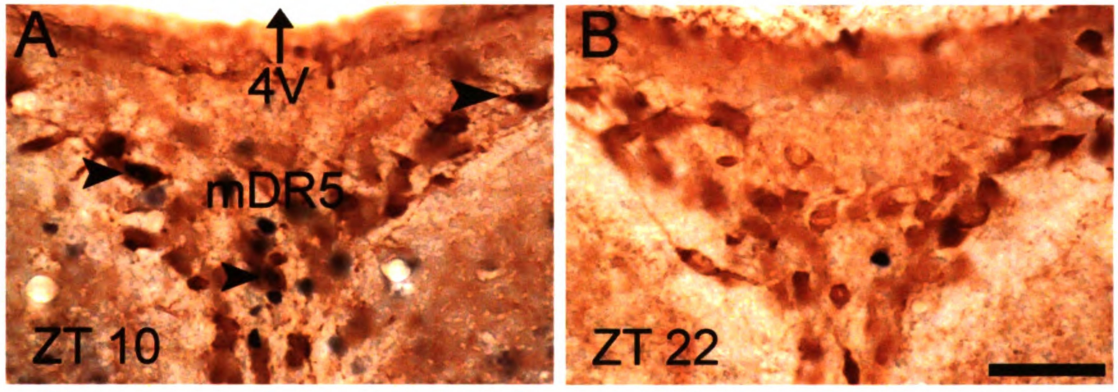
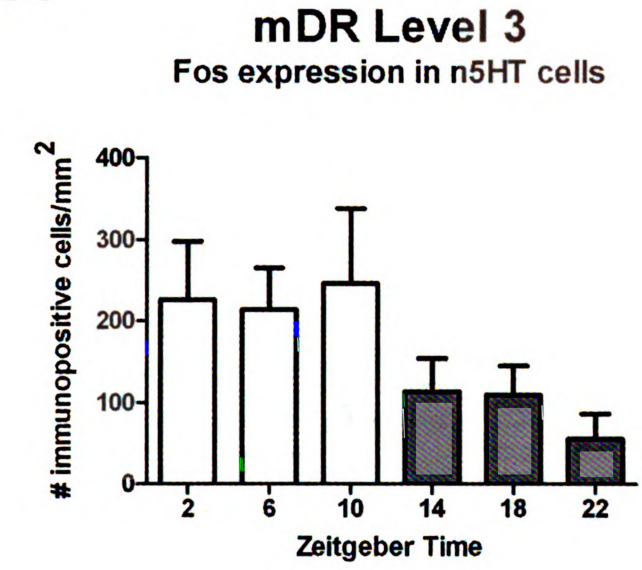


Figure 4.2. Photomicrographs of 5HT cells expressing Fos in the mDR5 at ZT 10 (A) and ZT 22(B). Arrowheads indicate some double-labeled cells. Fos: blue nuclear staining. 5HT: brown cell body staining. 4V: fourth ventricle. Scale bar= 50 μm.

Figure 4.3. Patterns of Fos expression in n5HT cells (A, B, and D) and 5HT cells (C and E) of the of the mDR3 (A), mDR4 (B and C), and mDR5 (D and E). Note that group means with different letters are significantly different from each other, here as well as in Figures 4, 5, and 6.

A


mDR Level 3
Fos expression in n5HT cells
immunopositive cells/mm2
400
300
200
100
0
2
6
10
14
18
22
Zeitgeber Time

B

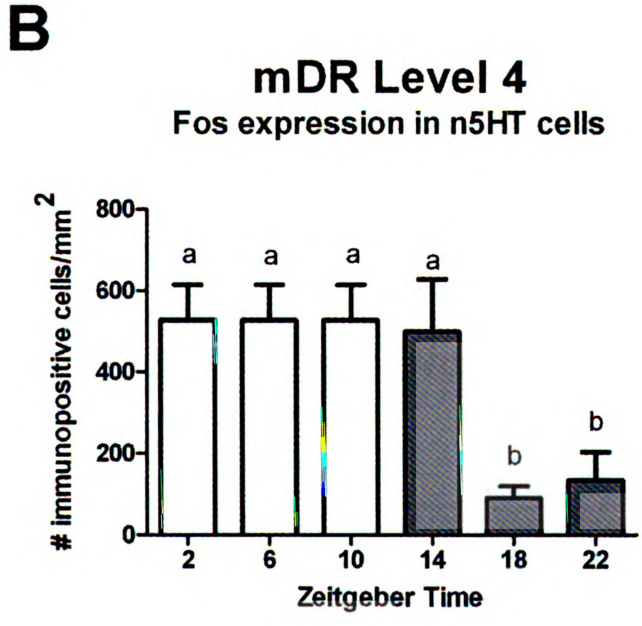
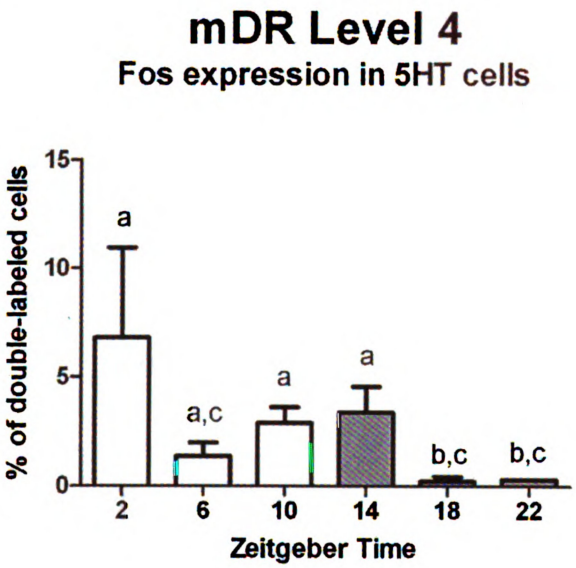

mDR Level 4
Fos expression in n5HT cells
immunopositive cells/mm2
800
600
400
200
0
a
a
a
a
b
b
2
6
10
14
18
22
Zeitgeber Time

Figure 4.3. (cont'd)

C



D

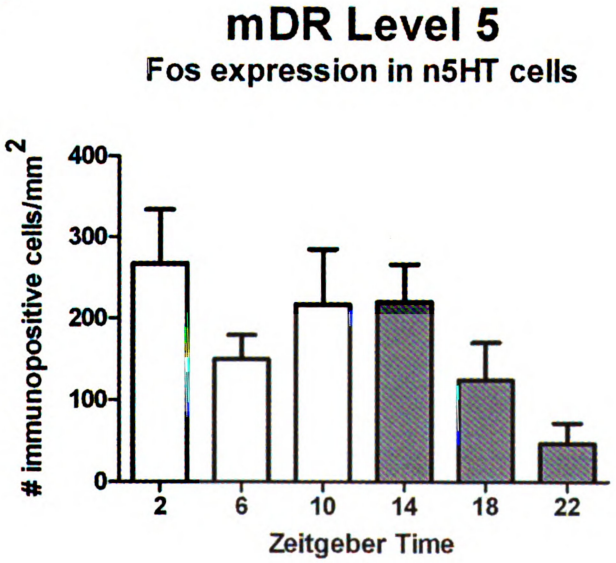
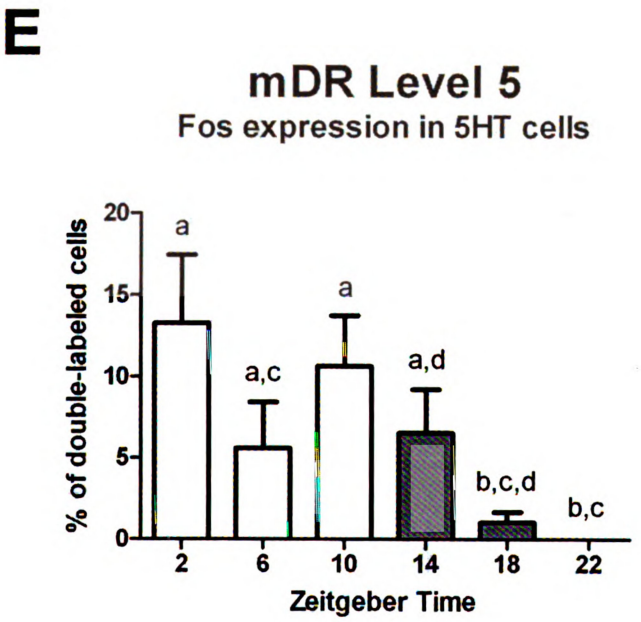
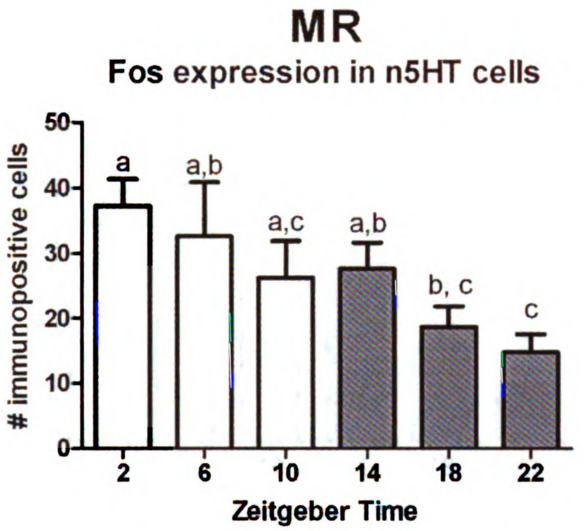


Figure 4.3. (cont'd)



A



B

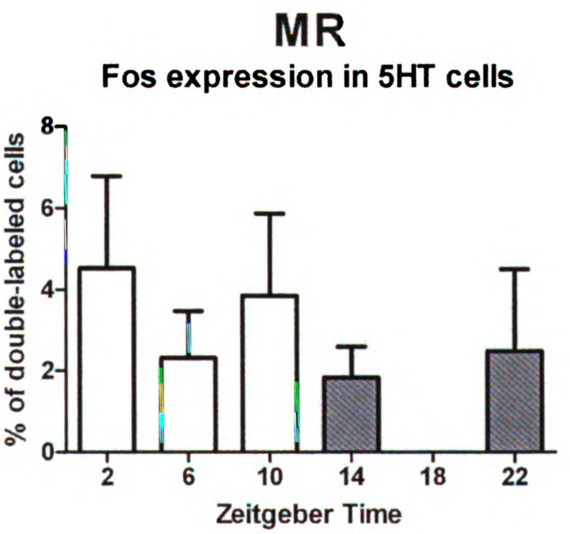


Figure 4.4. Patterns of Fos expression in n5HT cells (A) and 5HT cells (B) of the MR.

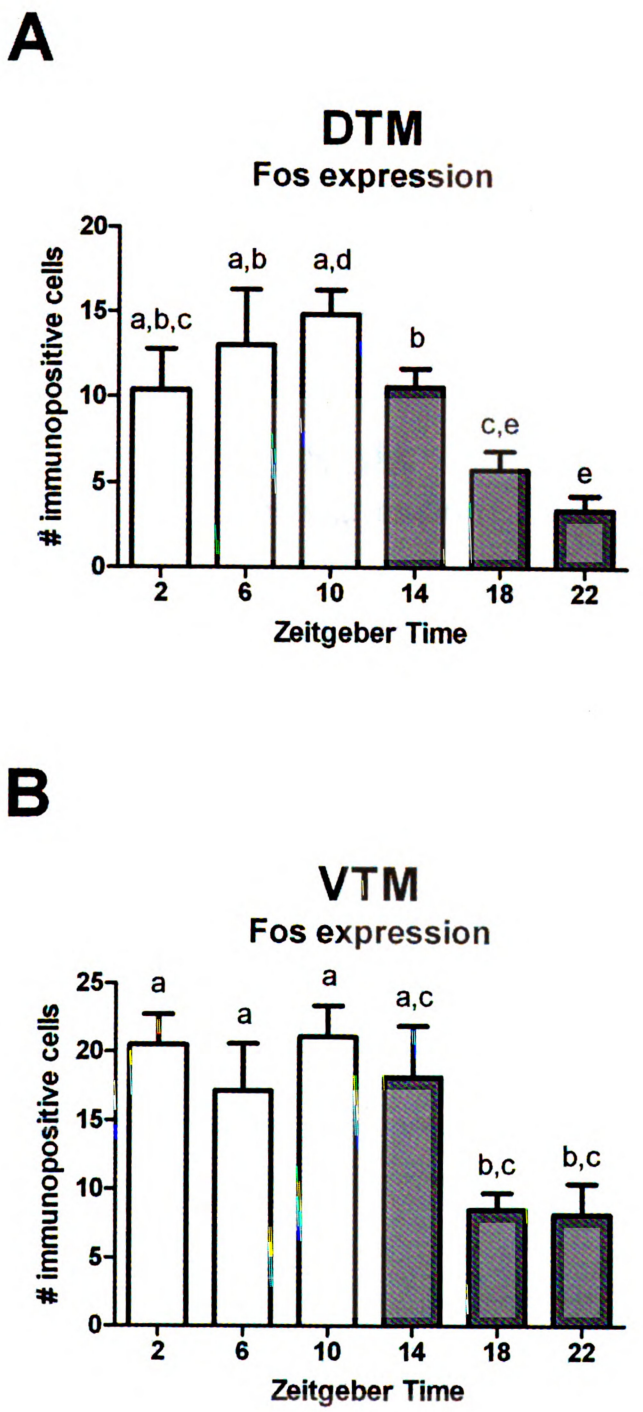


Figure 4.5. Patterns of Fos expression in the DTM (A) and VTM (B).

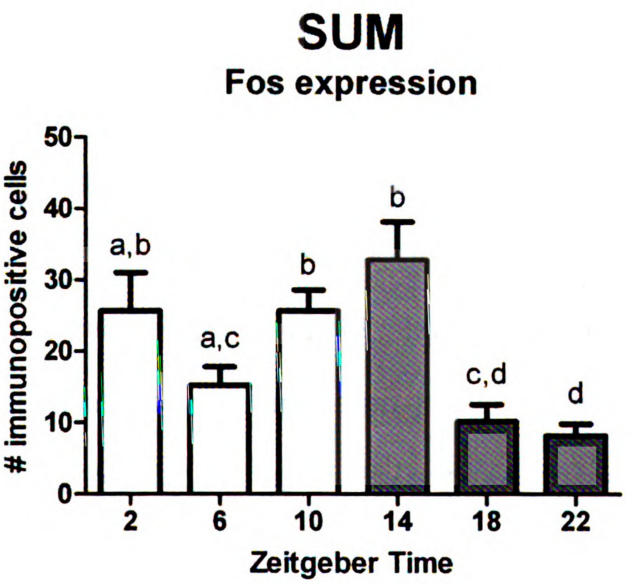


Figure 4.6. Patterns of Fos expression in the SUM.

for the SUM, paired comparisons revealed that Fos expression was lower between ZT 18 and ZT 22 compared to all other ZTs, except ZT 6 (Fig. 4.6).

Experiment 2: Monoaminergic projections with access to the cerebrospinal fluid

Retrograde tracing

The description of the retrograde tracing after 3V injections of CTB as well as the observations of colocalization of CTB and 5HT, are based on data from one representative animal. The injection of CTB into the 3V (Fig. 4.7) produced intense labeling in the ependymal cell layer surrounding the lateral ventricles, 3V, Aq, and fourth ventricle (4V). A dense cluster of labeled cells was observed in the caudal DR, specifically in caudal mDR3, and throughout the mDR4 and mDR5 (Fig. 4.9D). These labeled cells appeared first in the ventral part of the mDR3, and more caudally their location gradually migrated to the dorsal part of the DR. Labeled processes, that appeared to belong to these cells, were observable clearly approaching the ependymal cell layer of the Aq and 4V (Fig. 4.9D). Only a few scattered labeled cells were present in the MR. Outside of the raphe nuclei there was intense labeling of tanycytes in the median eminence (ME; Fig. 4.9C) and scattered cell labeling in the hypothalamus, including the suprachiasmatic nucleus (SCN; Fig. 4.8), ventral subparaventricular zone (vSPZ; Fig. 4.8), the paraventricular nucleus of the hypothalamus (PVN; Fig. 4.9A), DTM (Fig. 4.9B), and ME (Fig. 4.9C). In animals with injections that extended into the periventricular hypothalamic region (2 cases), the pattern of labeled cells and

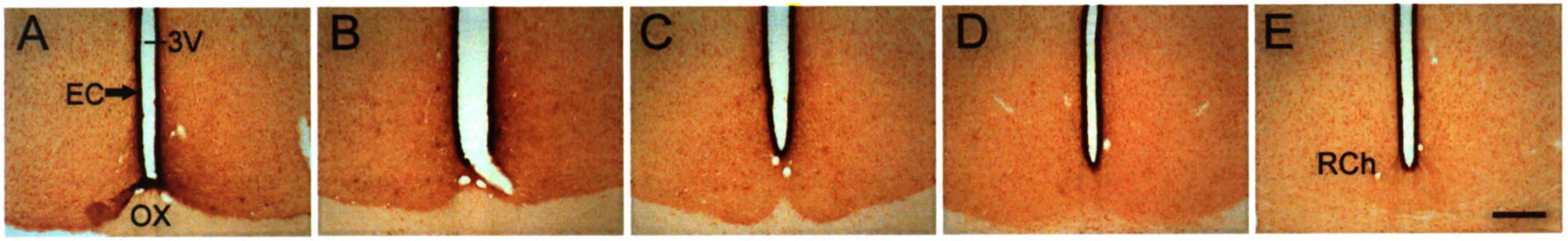


Figure 4.7. Rostro-caudal photomicrographs of the CTB injection site in the 3V (A-E). Note the strong cell labeling in the EC and the scattered cell labeling in the hypothalamus. Tanycytes are also observed in the RCh. EC: ependymal cell layer, OX: optic chiasm, RCh: retrochiasmatic area. Scale bar= 200 μm.

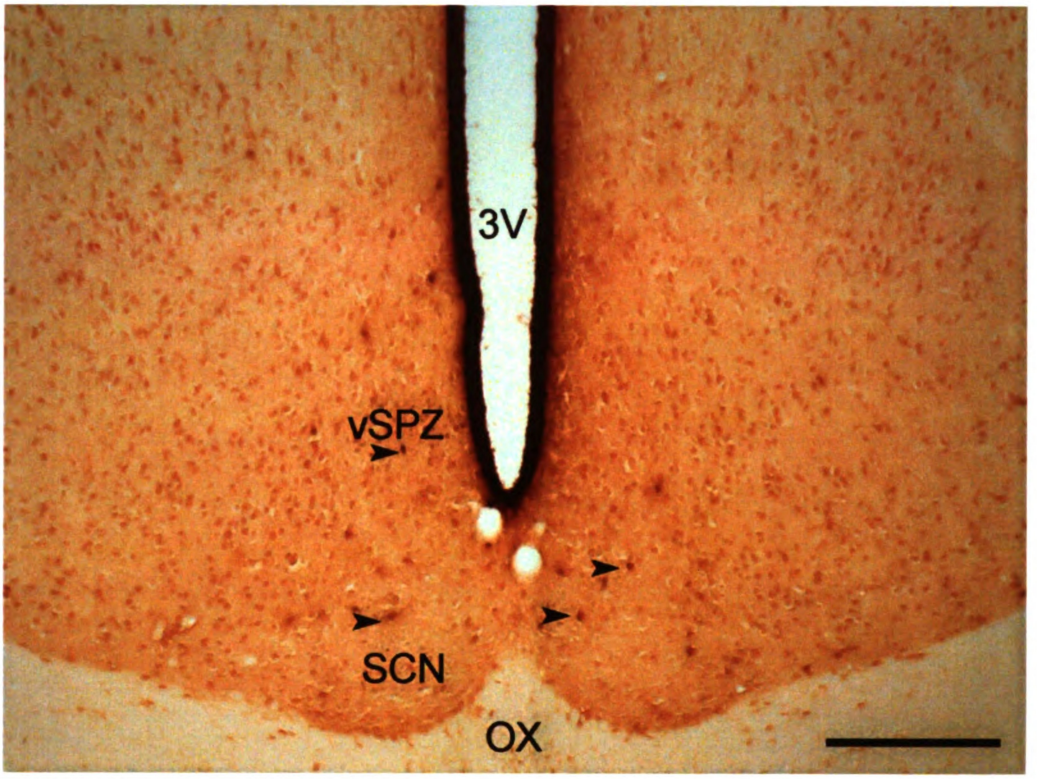


Figure 4.8. Photomicrograph of CTB-positive cells observed in the SCN and vSPZ after an injection to the 3V. Arrowheads indicate some CTB cells. OX: optic chiasm. Scale bar= 200 μm.

processes was similar to that produced by 3V injections, but in addition intense labeling was present throughout the hypothalamus, lateral septum, and amygdala (data not shown).

CTB and 5HT double-labeling

The distribution and abundance of neurons retrogradely labeled after the injection of CTB in the 3V were similar in brain sections processed with DAB or fluorescent ICC (Fig. 4.9D and Fig. 4.10C), with the characteristic rostro-caudal gradient of CTB positive cells extending from the caudal mDR3 to the end of the mDR5. Between 67 to 80% of the retrogradelly labeled cells of the mDR were immunopositive for 5HT, and 25-30% of 5HT cells were immunopositive for CTB in this region (Fig. 4.10B).

DISCUSSION

Patterns of Fos expression in the DR and MR

Serotonergic cells

In nocturnal animals, patterns of neuronal firing (Wu et al., 2003) in serotonergic cells of the raphe nuclei, as well as the release of 5HT from the raphe to its target areas, including the SCN (Barassin et al., 2002), are increased during the active phase of the animals. In agreement with these reports, in the Sudanese grass rat, a diurnal rodent related to our grass rat, release of 5HT in the SCN is increased during the day, in comparison to the night (Cuesta et al., 2007). However, that study did not address whether 5HT release occurred concurrently with increased neural activity in the raphe nuclei. In our grass rats,

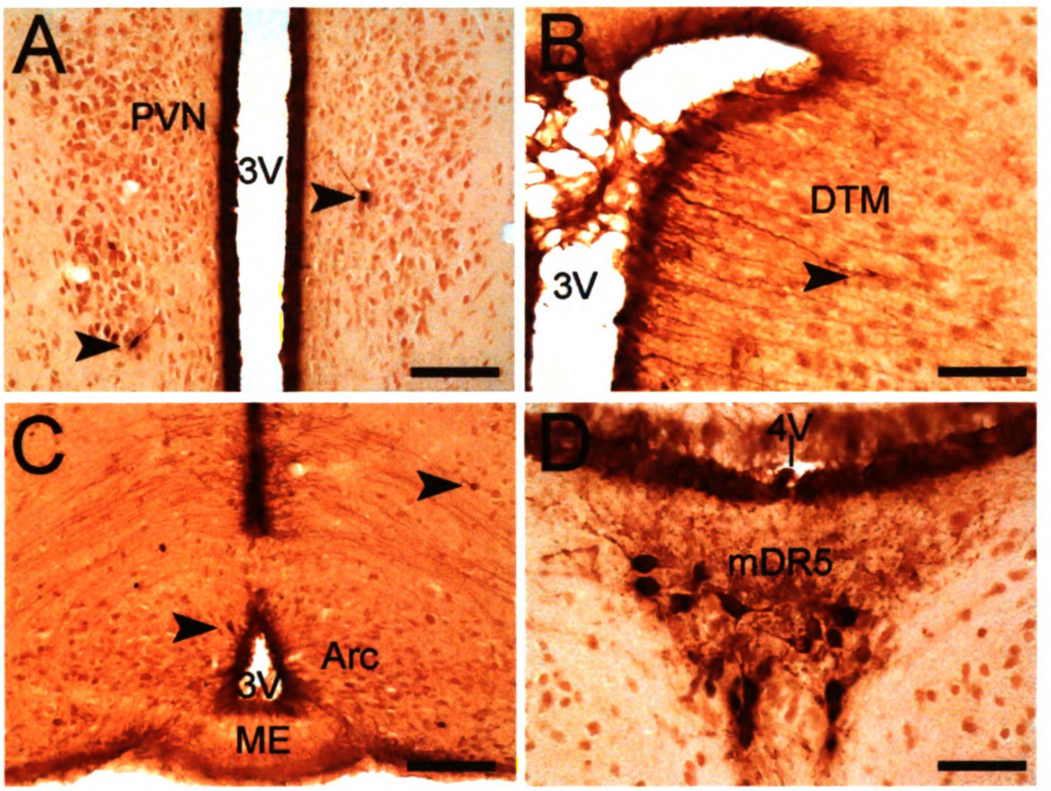


Figure 4.9. Photomicrographs of areas that contained CTB-labeled cells. Note the scattered cell labeling in the PVN (A), DTM (B), and Arc (C) in comparison to the dense cell labeling in the mDR5 (D). Arrow-heads indicate some CTB-labeled cells. (A and C) scale bar= 50 μm, (B and D) scale bar= 100 μm.

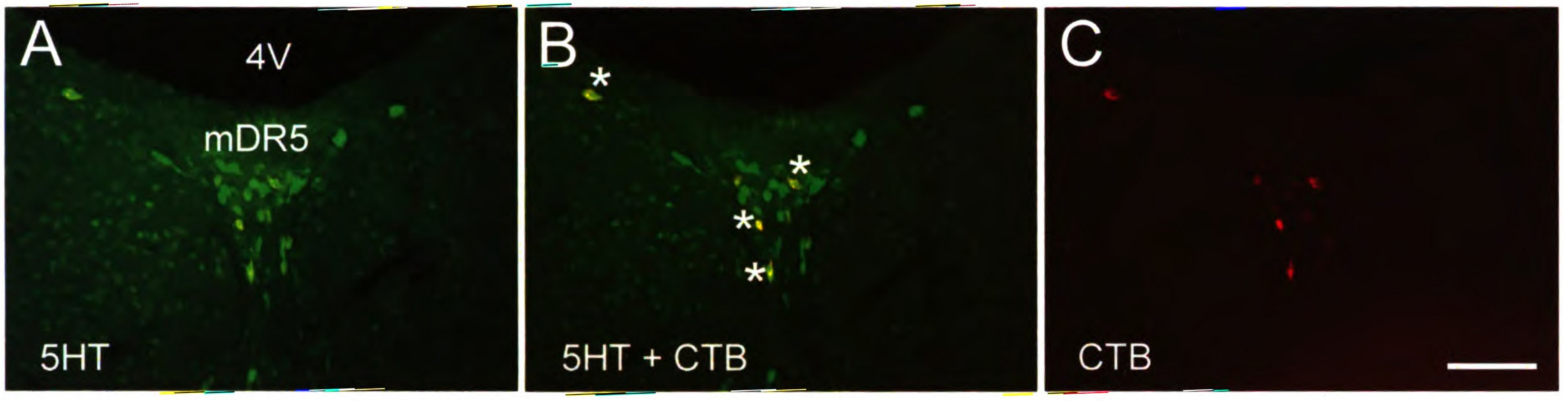


Figure 4.10. Photomicrographs depicting cells in the mDR5 after staining with Cy2 labeled 5HT (A, green fluorescence) and Cy3 labeled CTB (C, red fluorescence) in a representative animal that received a CTB injection directed to the 3V. Panel B is a composite image showing both labels. White asterisks indicate some double-labeled cells. Scale bar= 100 µm.

we observed increased Fos expression in specific regions of the raphe nuclei during the active phase (Fig. 4.3). Thus, our results complement those reported for the Sudanese grass rat. Taken together, these observations suggest that there is increased delivery of 5HT to target areas during the active phase of diurnal species.

Interestingly, the increase in Fos expression seen in serotonergic cells was not evident in all the regions of the raphe. The caudal mDR showed rhythmic expression of Fos in serotonergic cells (Fig. 4.3), but neither rostral areas of the mDR nor the MR (Fig. 4.4B) and lDR showed rhythmic Fos expression. Ours is not the first study to reports such rostro-caudal gradient in Fos expression in the mDR. For example, in the Mongolian gerbil, a crepuscular species, Fos expression increases during the transitions between lights on and off only in the caudal mDR (Janusonis and Fite, 2001). In this species, however, the pattern is evident only in non-serotonergic cells (Janusonis and Fite, 2001). In addition, subdivisions of the DR respond differently to stress. In the Syrian hamster, sleep deprivation induced by physical restraint increases Fos expression selectively in serotonergic cells of the caudal DR (Webb et al., 2010). In rats inescapable tail shock elicits Fos expression only in serotonergic cells of the mid-caudal mDR (Grahn et al., 1999). In contrast, in Syrian hamsters social defeat paradigms induces Fos expression in serotonergic as well as non-serotonergic cells of the rostral DR (Cooper et al., 2009). Finally, additional evidence for functional heterogeneity of DR subdivisions comes from human studies. In humans, binding of 5HT to $5HT_{1A}$ autoreceptors is higher in rostral regions of the DR than in

caudal regions, and the pattern is modified in depressed individuals who commit suicide (Boldrini et al., 2008).

In addition to its role in promoting wakefulness, 5HT is involved in a variety of physiological and behavioral functions including the regulation of neural activity in the hippocampus (Hajos et al., 2003), dynamics of the CSF (Hung, 93; see below), and circadian rhythmicity (reviewed in Yannielli and Harrington, 2004). Its role in circadian rhythmicity is of particular interest given that in nocturnal rodents it modulates circadian responses to non-photic stimuli (i.e., phase shifts only during the subjective day; reviewed in Yannielli and Harrington, 2004), such as phase shifts of activity rhythms produced by social interactions or handling. The DR and MR appear to mediate these phase shifts produced by non-photic stimuli (Meyer-Bernstein and Morin, 1999). The shifts seen in nocturnal animals are in sharp contrast to the ones observed in the diurnal Sudanese grass rat (Cuesta et al., 2007). In this diurnal rodent, the effects of 5HT on the phase of activity rhythms are seen only during the subjective night (Cuesta et al., 2007). Thus, in diurnal and nocturnal species the effects of 5HT on the circadian pacemaker seem to be restricted to the rest phase of the species (Challet, 2007). Interestingly, the phase shifting effects of light, which are similar in diurnal and nocturnal species (i.e., phase shifts throughout the night in both diurnal and nocturnal species, reviewed in Smale, 2008) are opposed by those produced by 5HT agonists in nocturnal rodents (reviewed in Yannielli and Harrington, 2004), but appear to be facilitated by 5HT agonists in the Sudanese grass rat (Cuesta et al., 2007). The implications of these observations are

important for understanding the differential role of 5HT in the circadian regulation of behavior in diurnal and nocturnal species. These data also inform translational initiatives, such as the development of drugs that counteract the effects of jet lag in humans (reviewed in Yannielli and Harrington, 2004).

Non-serotonergic cells

We also detected a rostro-caudal gradient in the activation of non-serotonergic neurons in the mDR. A clear rhythmic pattern was observed in the mDR4, with higher expression of Fos during the active phase (Fig. 4.3B). The mDR5 showed a similar pattern to that of the mDR4 (Fig. 4.3D). There, the lack of a significant rhythm was likely due to the relatively low power of the statistical tests. We did not identify the phenotype of these Fos-expressing cells, but in other species, the DR contains gamma-amino-n-butyric acid (GABA), glutamate, enkephalins, substance P, galanin, nitric oxide synthase, and dopamine in addition to 5HT (reviewed in Michelsen et al., 2007). Some of those molecules might be colocalized with 5HT (reviewed in Michelsen et al., 2007). In grass rats, it is unlikely that dopaminergic cells are the ones expressing the rhythm in Fos. That is because dopaminergic cells are located in the rostral DR (A. Castillo-Ruiz, unpublished observations), and, as seen in this study, there is little Fos expression in that region of the DR of grass rats. Additionally, those non-serotonergic rhythmic cells are not likely to be GABAergic, since neurons that contain GABA in the DR only show increased neural activity during sleep and not

during wakefulness (Yamuy et al., 1995; Maloney et al., 1999; Torterolo et al., 2000).

Non-serotonergic cells of the MR showed a rhythmic pattern of Fos expression that also mirrored the activity/rest cycle of grass rats, with higher levels of Fos expression during the active phase than during the inactive phase (Fig. 4.4A). Although we did not identify the phenotype of these non-serotonergic, some might be glutamatergic since in rats cells of that chemical phenotype are found in the MR (Jackson et al., 2009). Non-serotonergic cells of the MR, as well as serotonergic cells, have been implicated in the regulation of the hippocampal theta wave (HPC-Θ; Jackson et al., 2009). The HPC-Θ is a hippocampal rhythm that is observed during wakefulness and rapid eye movement sleep (reviewed in Buzsaki, 2002). During wakefulness this rhythm is present when animals are engaged in activities that involve alertness (reviewed in Buzsaki, 2002) and also appears to be important for spatial navigation (Hasselmo et al., 2002; Ekstrom et al., 2005). Thus, the increased Fos expression seen in the MR during the day could be related to its role in the modulation of hippocampal activity during wakefulness.

Patterns of Fos expression in the TMM and SUM

TMM

The TMM of several nocturnal species contains dense clusters of histaminergic neurons. These cells are distributed in the ventrolateral TMM (VTM group), the dorsomedial TMM (DTM group), and caudal TMM (Watanabe et al., 1984). Histaminergic cells of these areas show peak firing during wakefulness

(Takahashi et al., 2006), and they also show increase Fos expression at that times (Ko et al., 2003). Thus, Fos expression in the TMM, as in the case of the raphe nuclei, can also be used as a proxy for neural activity. Unfortunately, due to technical difficulties with the double-label ICC, in this study we could not determine specifically whether histaminergic cells of the TMM showed rhythmic Fos patterns. We, however, were able to identify overall rhythms of Fos expression in the VTM and DTM (Fig. 4.5).

In the TMM, we found that both the DTM and VTM had daily patterns of Fos expression with higher levels seen during the day than during the night (Fig. 4.5). The pattern observed in the VTM (Fig. 4.5B) is in agreement with a previous study with grass rats from our laboratory (Novak et al., 2000), whereas the pattern observed in the DTM (Fig. 4.5A) conflicts with the results of that report (Novak et al., 2000). In that experiment, however, only four time points were sampled. Thus, given that we saw a sharp decrease in Fos expression between ZT 18 and ZT 22, times that were not sampled in the aforementioned study, it is likely that the pattern of Fos expression in the DTM was previously missed.

The chemical identity of the neurons that express Fos rhythmically in the DTM and VTM remains to be elucidated. In other rodents these areas contain not only HA, but also GABA and galanin (Airaksinen et al., 1992). In grass rats, while we have been able to identify a cluster of histaminergic cells in the VTM, we have failed to do so in the DTM (A. Castillo-Ruiz, unpublished observations). Interestingly, the DTM of the grass rat shows a clear circadian pattern in the expression of the clock proteins Per 1 and Per 2 (C. Ramanathan, unpublished

observations) suggesting that even when this area lacks HA, it contains other molecules that are important for the regulation of circadian rhythmicity. Preliminary data suggests that Per 1 is also expressed in the VTM (C. Ramanathan, unpublished observations), although it remains to be determined whether its expression is circadian, and if it occurs in histaminergic neurons. Thus, the DTM and VTM neurons may contain extra-SCN oscillators that could participate in the circadian control of their anatomical targets such as the CP (see below). In nocturnal rodents histaminergic groups are known to project directly to the SCN (reviewed in Harrington et al., 2000), and in, the grass rat SCN there are histaminergic fibers (C. Alvarez-Barón, unpublished observations). If these histaminergic projections to the SCN are from cells that show rhythmic expression of clock genes (i.e., Per 1 and Per2), this circuit would provide a mechanism for feedback to the master oscillator of the SCN from an extra-SCN oscillator. Histaminergic projections to the SCN appear to have a role in modulating photic entrainment (reviewed in Harrington et al., 2000). But, no studies have addressed the role of HA in circadian rhythmicity in a diurnal species.

SUM

The SUM, like the MR, plays a role in the modulation of the HPC-Θ, through indirect, as well as direct projections to the hippocampus (reviewed in Pan and McNaughton, 2004). In addition, the SUM plays a role in reward; exposure to rewarding stimuli elicits neural activation in this area (e.g.

Marcangione and Rompre, 2008). Moreover, administration of excitatory glutamate agonists to the SUM induces a conditioned place preference (Ikemoto et al., 2004). Given that this area not only plays a role in the regulation of vigilance states, but also in reward, it was expected to show a circadian pattern of neural activity. Our data confirmed that expectation; neurons of the SUM displayed daily rhythms in Fos expression, with the highest values in the transitions between lights on and off, and the lowest values in the mid-day and night (Fig. 4.6). This pattern is similar to the locomotor activity patterns that grass rats show in the laboratory under a 12/12 light dark cycle (McElhinny et al., 1997). This concordance between patterns of Fos expression in the SUM and the display of activity by the animals is interesting given that a multisynaptic pathway, which includes the SUM, has been proposed as a modulator of physical activity, such as voluntary wheel running (Smith et al., 2009). Thus, in grass rats neural activity in the SUM could influence spontaneous locomotor activity patterns. Future work is needed to address this possibility.

Monoaminergic projections with access to the CSF

The CSF carries a myriad of molecules throughout the nervous system including 5HT and HA. Besides their role in the promotion of wakefulness and circadian rhythmicity (discussed above), 5HT and HA have also been linked to the etiology of behavioral disorders. For example, in schizophrenics there is an increase in the amount of HA metabolites in the CSF in comparison to healthy individuals (Prell et al., 1995). Also in patients with major depression with a history of suicidal attempts, there is a decrease in 5HT in the CSF in comparison

to non-suicidal patients (Hou et al., 2006). These phenomena are intriguing given that HA and 5HT are known to influence metabolism of the CP (Hung et al., 1993; Watson et al., 1995), the brain region that produces CSF. But, how are 5HT and HA reaching the CSF? The evidence suggests that a route may be the direct release of HA (Tillet et al., 1998) and 5HT (Chan-Palay, 1976) into the CSF from axonal terminals. However, it is unknown whether these cells could release their contents rhythmically into the CSF. In this study we addressed this question by analyzing whether CTB injected into the 3V of grass rats would result in the retrograde labeling of neurons in regions of the brain known to contain histaminergic and serotonergic cell bodies and to display rhythms in neural activity.

We found that after the 3V injections of CTB (Fig. 4.7), the caudal mDR showed strong cell labeling (Fig. 4.9D). Furthermore, labeled fibers were seen in close proximity to the 3V, which suggests that these cells could release their content directly into the CSF. These findings are in agreement with observations made in nocturnal lab rats (Mikkelsen et al., 1997; Simpson et al., 1998). In this species, CTB injections into the 3V produce a distribution of retrogradely labeled cells similar to the one seen here for grass rats (Fig. 4.8 and 9), with intense labeling in the caudal DR, and scattered cell labeling in areas such as the PVN, SCN, and MR (Mikkelsen et al., 1997). In contrast, animals with injections centered in the periventricular region had intense labeling throughout the entire hypothalamus, amygdala, and lateral septum. Our findings in the hypothalamus and amygdala are in agreement with previous observations in lab rats (Mikkelsen

et al., 1997). However, the lateral septum was not mentioned in the study with lab rats. The discrepancy may be due to species difference or a failure to report staining in that area.

The labeling of cells in the SCN after ventricular injections of CTB (Fig. 4.8) is interesting given that diffusible signals released from this nucleus have been proposed to control circadian activity rhythms (Schwartz and Reppert, 1985; Lehman et al., 1987; Silver et al., 1996). Our results suggest that a potential route for this control is through the CSF. We also observed a few labeled cells in the vSPZ (Fig. 4.8), an area that appears to participate in circadian rhythmicity in the grass rat and in nocturnal rodents (Lu et al., 2001; Schwartz et al., 2009). The DTM also contained some labeled cells (Fig. 4.9B). These cells, however, are unlikely to be histaminergic, since preliminary evidence suggests that the DTM of grass rats does not contain histaminergic neurons (A. Castillo-Ruiz, unpublished observations). It would be important to determine the phenotype of these cells given that in grass rats the same area that shows retrograde labeling also shows circadian expression of the clock genes Per 1 and Per 2 (C. Ramanathan, unpublished observations).

The majority of the retrogradely labeled neurons seen in the caudal DR appeared to be serotonergic (Fig. 4.10B). We found that approximately two thirds of the CTB labeled cells of the DR were positive for 5HT. This contrasts with the 50% reported in lab rats (Mikkelsen et al., 1997). The discrepancy may be due to species differences or related to the ICC procedure. Interestingly the double-labeled cells identified in this study were found in the same areas where we

detected a striking rhythm of Fos expression in 5HT cells (Fig. 4.2 and 4.3). These observations suggest that 5HT may be released into the CSF in a rhythmic fashion. Serotonin increases intracellular Ca^{2+} in the CP via activation of the $5HT_{2C}$ receptor (Watson et al., 1995) and influences the dynamics of Cl^- and K^+ channels in the CP at cellular locations where CSF is released (Hung et al., 1993). These results together with our anatomical and functional observations indicate that 5HT may play an important role in the control of CP functioning, including CSF release.

SUMMARY AND CONCLUSIONS

This study extends to a diurnal species the results of studies with nocturnal rodents of rhythms of neural activity in monoaminergic regions of the brain. In the diurnal grass rat, as previously reported for nocturnal rodents, rhythms in Fos expression in the TMM, and in specific subregions of the DR are related to vigilance states, with more Fos expression seen during the active phase than during the rest phase of the species. It is expected that the increase in Fos expression in these areas will be associated with increased HA and 5HT release at synaptic terminals, as is the case for the wakefulness promoting system of the cholinergic basal forebrain (Greco et al., 1999; Greco et al., 2000). Therefore, the maximal effects of HA and 5HT in target areas, including the main pacemaker of the SCN, are likely to be seen during the active phase. The effects that the 5HT and HA may have on SCN functioning is a subject that warrants further investigation in diurnal rodents.

Our observations also provide insights on the functional heterogeneity of the raphe nuclei in the diurnal grass rat. First, it describes a rostro-caudal gradient of neural activation in 5HT and n5HT cells of the mDR, with more activation in caudal areas. Second, it describes specific subpopulations of 5HT neurons in the caudal mDR that appear to have access to the CSF. Both features have been reported in other species, but never concurrently in a diurnal animal. Taken together our results suggest that rhythmic release of 5HT into the CSF may be involved in circadian control of various functions throughout the nervous system, including the production of CSF itself (see above). Understanding of the functional complexity of the raphe nuclei is important, since changes in the physiology of this nucleus have been linked to human mental health problems including suicide (Boldrini et al., 2008).

In addition to neurons from the caudal mDR, our anatomical results indicate that chemical signals from the SCN and the vSPZ could be release directly into the CSF. This avenue for the control of circadian rhythms has been proposed before for nocturnal species (Schwartz and Reppert, 1985; Lehman et al., 1987; Silver et al., 1996). Our study is the first to provide evidence for that hypothesis in a diurnal mammal.

CHAPTER 5

Summary and Conclusions

One-fifth of employees in the USA work evening or night shifts (Presser, 2004), and, in particular, night shifts are associated with impaired cognitive performance (e.g. Wyatt et al., 1999). For instance, as a result of workers' mistakes, there is a higher incidence of on-the-job injuries, such as falls and lacerations, during night shifts than during day-shifts (Fortson, 2004). The circadian misalignment of areas of the brain that promote wakefulness is potentially responsible for these deficits.

The consequences of misalignments among different wakefulness-promoting systems are expected to be seen in species other than humans. In the wild, for example, there are reports of temporal shifts in activity induced by predators or competitors (e.g. Fenn and Macdonald, 1995; Harrington et al., 2009). Humans can also indirectly induce temporal shifts in the activity of other animals. As an illustration, in urbanized areas, the black-backed jackal is a nocturnal creature, whereas these animals are diurnal in sites away from urban development (Fox, 1971). Moreover, humans can induce these temporal shifts directly by forcing animals to be awake during their regular resting time. Such is the case of some performing animals at circuses, animals at zoo exhibits, or even domestic animals. Thus, given the widespread incidence of these temporal shifts in activity in humans as well as non-humans, it is of extreme importance to understand the physiological effects exerted by this phenomenon.

In this dissertation work, I sought to shed light on those physiological effects. My focus was on wakefulness promoting areas of the brain, given their role in cognition and attention (e.g. Blandina et al., 2004; Gonzalez-Burgos and Feria-Velasco, 2008; Herrero et al., 2008). I evaluated this in the diurnal grass rat, by taking advantage of the fact that a subset of these animals shows a voluntary shift in activity when given access to running wheels (Blanchong et al., 1999). Using this model appears important because of the shortage of studies that address the problems associated with human shift work in diurnal species. The main aims of this work were: (i) to evaluate how arousal and reward areas of the brain respond to self-induced and forced temporal shifts (Chapters 2 and 3), and (ii) to characterize further the patterns of neural activation in wakefulness-promoting areas of a diurnal brain (Chapter 4).

In this final chapter, first I provide a discussion of circadian patterns of neural activation in wakefulness-promoting areas in the grass rat comparing them to those seen in nocturnal rodents. I also provide a discussion of how these rhythms might be modulated by environmental stimuli. Then I discuss how voluntary and induced wakefulness during the inactive phase induce differential effects in wakefulness-promoting areas in diurnal and nocturnal rodents. I conclude this chapter by discussing the implications of these findings for humans as well as non-human animals, and discussing recommendations for further work.

Neural activation in wakefulness-promoting areas of the grass rat

Neural rhythms in wakefulness-promoting areas of the brain of nocturnal species are well characterized. For instance, histaminergic, orexinergic, cholinergic, noradrenergic and serotonergic cellular populations show increased neural activation during the active phase as compared to the resting phase (reviewed in Saper et al., 2001; Jones, 2005). But very little is known about how these systems function in diurnal species. The work presented in this dissertation expands these findings to the diurnal grass rat.

In Chapter 4, I reported rhythmic Fos expression in the tuberomammillary nucleus (TMM) and in specific serotonergic and non-serotonergic areas of the raphe nuclei, with higher Fos expression during the active phase of grass rats. In Chapter 2, I described a similar pattern in specific cholinergic as well as non-cholinergic areas of the basal forebrain (BF; i.e., the patterns seen in the control groups). In Chapter 4, I also reported how Fos patterns in the DR could be associated with rhythmic release of serotonin (5HT) into the cerebrospinal fluid. Hence, providing a functional example of how neural patterns of Fos expression in wakefulness-promoting areas could be related to the physiology of areas that receive either their synaptic or humoral inputs. Together these findings indicate that in the grass rat wakefulness-promoting areas show circadian rhythms of Fos expression that track the activity-rest rhythm of the species, thus validating, in a diurnal model, what was previously documented only for nocturnal species. Future work should study other regions that promote wakefulness, but that were not included in this dissertation. For example, areas such as the nucleus basalis magnocellularis, which also mediates attention (e.g. Balducci et al., 2003; Harati

et al., 2008; Botly and De Rosa, 2009) or areas of the pons that control rapid eye movement (REM) sleep as well as wakefulness (reviewed in Jones, 2005).

In Chapter 3, I reported that rhythms in Fos expression in some but not all wakefulness-promoting areas are modified by forced temporal shifts in activity. These changes were observed without any apparent effects on the Fos patterns in the master oscillator of the suprachiasmatic nucleus (SCN), thus documenting how environmental changes can override the control of these areas by the SCN. Areas such as the medial septum (MS) and vertical diagonal band of Broca (VDB), for example, appear to respond to wakefulness *per se* (Chapters 2 and 3), whereas the TMM, and raphe appear to be faithful to circadian influences, even when the organism experiences wakefulness during the normal rest phase (Chapters 3 and 4). Taken together, the results indicate that under baseline conditions, the wakefulness-promoting areas of a diurnal brain are mainly responsive to circadian signals. But, when challenged by forced or voluntary shifts in the animal's activity pattern, they respond in different ways, in some cases deviating from their normal circadian pattern. This also appears to be the case for some wakefulness-promoting areas of nocturnal species (see below).

Several important questions are raised by these results. First: do these areas have the molecular machinery to drive Fos rhythms themselves? One way to evaluate this question is to examine whether wakefulness-promoting areas express clock genes. Preliminary evidence suggests that the dorsal tuberomammillary nucleus (DTM) of the grass rat expresses Per 1 and Per 2 rhythmically (C. Ramanathan, unpublished observations), in a similar manner to

the patterns of Fos expression described in Chapter 4. At this point, however, it is not known what is the role that Per rhythms play in the DTM, or if other wakefulness-promoting areas also express clock genes. Also, it is not clear whether wakefulness-promoting areas of nocturnal rodents express clock genes.

Another set of questions raised by my results relates to the entrainment of rhythms of neural activity in wake-promoting areas. For example: what are the stimuli that can entrain those rhythms in Fos expression? Are they entrained solely by circadian signals from the SCN and /or from local oscillators? Are these rhythms responsive to other cues, such as light or social stimulation? If so, which signals are more powerful? Are these signals synergistic or antagonistic? These are questions that future work needs to address. Especially because some wakefulness-promoting molecules, such as 5HT and histamine (HA), have been associated with the etiology of human psychological disorders (Maes and Meltzer, 2000; Arrang, 2007). Additionally, future work needs to address the role of the wakefulness-promoting molecules that reach the CSF. In Chapter 4, I reported that histaminergic and serotonergic areas appear to release their contents to the CSF. This finding together with that of rhythmic expression of Fos in those areas (Chapter 4) suggest that the released molecules could exert circadian effects throughout the brain via this route. My findings also suggest that the grass rat SCN could orchestrates circadian rhythmicity through its connections to the CSF (Chapter 4).

Finally, an interesting issue is raised by the findings presented in this dissertation. All the available data are consistent with the view that the molecular

clock of the SCN and its phase relation to the light-dark cycle are the same in diurnal and nocturnal rodents (Caldelas et al., 2003; Lambert et al., 2005; Ramanathan et al., 2006). Given that the arousal systems are more active during wakefulness in nocturnal (reviewed in Jones, 2005) and diurnal rodents (Novak et al., 2000; Martinez et al., 2002; as well as the results reported in this dissertation), any stimulation of the SCN associated with behavioral state should impact the SCN clock at opposite phases of its cycle, depending on whether an animal is diurnal or nocturnal. This is likely to result in different physiological and behavioral outcomes for diurnal and nocturnal species. This raises the question of how the circadian time keeping system of diurnal species respond to stimulation coming from these areas. Potential and not mutually exclusive answers are: (i) a change of the role of the projections, (ii) a change of mechanisms mediating the effects of the projections, and (iii) the purging or reduction of projections. At least for some wakefulness-promoting systems of the grass rat, the evidence appears to support the third hypothesis. This is because when the SCN of the grass rat is compared to that of the lab rat, it shows fewer cholinergic (Castillo-Ruiz and Nunez, 2007), orexinergic (Nixon and Smale, 2007b), as well as histaminergic projections (C. Alvarez-Barón, unpublished observations). More studies are needed to evaluate the hypotheses outlined above, and to shed light on the proximate mechanisms that were modified in the evolution of a diurnal brain from that of a nocturnal ancestor.

Wakefulness during the inactive phase and its effects on arousal and reward areas of the brain in diurnal and nocturnal rodents

In diurnal grass rats, I observed that neural activity in areas that support wakefulness is modified depending on the paradigm that is used to keep the animals awake. In Chapter 2, I found that voluntary wakefulness induces Fos expression in specific populations of cells of the BF of night-active (NA) grass rats, namely the non-cholinergic MS, VDB, and horizontal diagonal band of Broca (HDB); and the cholinergic HDB. In contrast, in Chapter 3, when grass rats were forced to stay awake only the non-cholinergic MS showed increase Fos expression. A small but non-significant increase in Fos expression was also seen in the non-cholinergic VDB. These results are in contrast to those from nocturnal rodents that are induced to stay awake during their normal resting phase (Greco et al., 2000).

In Chapter 3, I also measured how other wakefulness-promoting areas responded to forced wakefulness: the lateral hypothalamus (LH), the locus coeruleus (LC), TMM, and raphe nuclei. At this point I do not know whether these areas respond to voluntary wakefulness, but I can conclude that patterns of Fos expression in these areas do not appear to be modified by forced wakefulness, One possible exception being the LC where a trend towards higher Fos expression was observed in the sleep deprived (SD) group. In nocturnal rodents, sleep deprivation induced by a procedure similar to the one used in Chapter 3 produces comparable results in the LC and raphe (Webb et al., 2010). But, these patterns differ from those seen when animals are sleep deprived by physical restraint (Webb et al., 2010). In addition, in Chapter 3, I reported that the LH,

which contains orexinergic cells, had higher Fos expression in the SD group (Chapter 3). These findings are similar to those from experiments in which lab rats are induced to stay awake by gentle handling, novel wheel running, or physical restraint (Webb et al., 2008). Furthermore, they are in agreement with observations in the LH of grass rats that are voluntary awake (Nixon and Smale, 2005). Taken together the available data suggest that wakefulness-promoting areas under baseline conditions are synchronized with each other, and under tight circadian control. But, that they can decouple from each other and from circadian control when a challenge, such as sustained wakefulness, is presented during the resting phase. Evidence for the coupling among wakefulness-promoting areas was obtained in the correlational studies done in Chapters 2 and 3, which show that the orexinergic systems might play a big role in the modulation of other wakefulness systems. Additionally, my findings suggest that specific wake-promoting areas may respond in different ways to environmental challenges, such as forced wakefulness, depending upon whether the species is nocturnal or diurnal (Chapter 3). More work is needed to evaluate these possible species differences. In particular, future experiments should use the same procedures to induced wakefulness in nocturnal and diurnal animals.

Studies in nocturnal rodents have also shown that the SCN itself is responsive to temporal changes in activity (Antle and Mistlberger, 2000). The neural changes in the SCN are also likely to be contingent upon the experimental paradigm that is used to induce wakefulness. Thus, induction of wakefulness by gentle handling and exposure to a novel running wheel elicit phase advances,

but only when presented during the subjective day of nocturnal rodents (reviewed in Yannielli and Harrington, 2004), whereas inescapable stress, which also keeps the animals awake, does not elicit any shifts at any phase (Mistlberger et al., 2003). The phase resetting seen in the SCN of nocturnal rodents is likely due to the "talk back" from wakefulness-promoting areas to the SCN pacemaker. In fact, most wakefulness-promoting systems of nocturnal rodents are anatomically well positioned to have modulatory effects in the SCN via efferent projections to the pacemaker (reviewed in Rosenwasser, 2009 and Chapter 1). The SCN of grass rats receives efferent projections from wakefulness-promoting areas, however, these projections appear to be less prominent in grass rats compared to those of nocturnal rodents (C. Alvarez-Barón, unpublished observations; Castillo-Ruiz and Nunez, 2007; Nixon and Smale, 2007b).

In contrast to nocturnal rodents (Antle and Mistlberger, 2000), in grass rats forced wakefulness did not produce changes in Fos expression in the pacemaker or in the ventral subparaventricular zone (vSPZ; Chapter 3), an area that might play a role in circadian modulation in both diurnal (Schwartz et al., 2009) and nocturnal species (Lu et al., 2001; Abrahamson and Moore, 2006). Moreover, previous studies have shown that voluntary wakefulness in grass rats does not produce neural changes either in the SCN or vSPZ (Rose et al., 1999; Schwartz and Smale, 2005). This was the case even though forced and voluntary wakefulness in grass rats produced changes in areas that in nocturnal rodents are known to project to the SCN (i.e. the MS, VDB, and HDB; Bina et al., 1993) and affect its functioning (Abbott et al., 2003a; Abbott et al., 2003b; Buchanan

and Gillette, 2005). These findings suggest that the circadian system of grass rats is less sensitive to stimulation arising from wakefulness-promoting areas. This evidence supports the hypothesis of a lesser role for circadian modulation of wakefulness-promoting areas in a diurnal brain (Castillo-Ruiz and Nunez, 2007). One possible exception appears to be the influence of serotonergic on the functioning of the SCN in diurnal and nocturnal species. Thus in the diurnal Sudanese grass rat serotonergic stimulation of the SCN results in phase advances in the subjective night, whereas in lab rats the same stimulation also induces phase advances, but only during the subjective day (Cuesta et al., 2007). The interactions between the SCN and wakefulness-promoting regions in diurnal species is a relatively neglected area of research that deserves future attention.

In Chapters 2 and 3 I also evaluated whether reward areas of the brain were responsive to voluntary and induced wakefulness. In particular, I analyzed two main components of the reward system: the supramammillary nucleus (SUM), and the ventral tegmental area (VTA); areas that are also involved in arousal (Pan and McNaughton, 2004; Dahan et al., 2007). In Chapter 2, I found that voluntary wakefulness induced tonic upregulation of Fos in non-dopaminergic cells of the SUM and anterior VTA (aVTA) in animals with access to wheels, in comparison to control animals. Increased Fos expression in non-dopaminergic cells of the aVTA is also seen when nocturnal animals are exposed to a rewarding stimulus (Hunt and McGregor, 1998; Balfour et al., 2004). In contrast, in Chapter 3, I reported that forced wakefulness does not induce

differential Fos expression either in the SUM or aVTA. These findings provide evidence for rewarding effects of wheel access in grass rats. Intriguingly in Chapter 4, I reported rhythmic Fos expression in the SUM of grass rats that are housed with no wheels. The rhythm mirrored the rest/activity cycle of these animals. My results suggest that the rhythm in the SUM is disrupted when grass rats have continuous access to a rewarding stimulus. The consequences of these disruptions need to be evaluated given that the SUM is a part of a circuit that modulates learning and memory processes (reviewed in Pan and McNaughton, 2004).

A surprising finding of this dissertation was that neural activation in the BF, SUM, and aVTA of day-active (DA) grass rats was quite similar to that of NA grass rats, even though behaviorally these animals were no different from animals housed with no wheels: DA grass rats are more active during the day, than during the night (Blanchong et al., 1999), have more bouts of sleep during the night (Schwartz and Smale, 2005), and eat and drink more during the day (C. C. Ramanathan, unpublished observations). Furthermore, neural activation of the orexinergic system and the ventral lateral preoptic areas (VLPO), which promotes sleep, is similar to that of animals that have no access to wheels (Novak et al., 1999; Martinez et al., 2002; Nixon and Smale, 2004; Schwartz and Smale, 2005). However, my results suggest that DA grass rats have physiological characteristics that make them comparable to NA grass rats. *Ad libitum* access to a running wheel might be part of the explanation for this phenomenon. In Chapter 3, I tested that hypothesis by looking at patterns of Fos

expression in two components of the reward system of the brain that are known to project to the BF in nocturnal rats: the SUM (reviewed in Pan and McNaughton, 2004) and VTA (Gaykema and Zaborszky, 1996). The results appear to support my hypothesis. I found that these areas express more Fos during day and night in DA and NA grass rats than in controls, suggesting that these areas could be tonically influencing neural activation in the BF of both DA and NA grass rats. However, another potential explanation for the similarities between DA and NA grass rats is that the effects are the byproduct of the physical activity exerted by the animals. This hypothesis could be easily tested by stimulating groups of grass rats to run either during the day or during the night and then by investigating how reward and wakefulness-promoting areas respond to the stimulation.

Finally, this dissertation work provides evidence for a therapeutic effect of sleep on patterns of neural activation. In Chapter 2, I found that regardless of phase preference for activity, all experimental groups had similar levels of Fos expression in the BF during the day, even though the patterns were extremely different during the night. I hypothesized that the universal sleep that is seen in grass rats towards the end of the dark phase induced the return of Fos expression to baseline levels. In Chapter 3, I tested that hypothesis by leaving the animals free to sleep for 2 hours after a period of forced wakefulness. I found that this amount of time was sufficient to promote the return of Fos expression to baseline levels in the areas that were affected by the forced wakefulness. Interestingly, my results show that sleep induces these effects either after acute

(Chapter 3) or chronic (Chapter 2) exposure to wakefulness during the resting phase. My results could explain why after a brief nap during the night-shift humans score better at tasks that measure cognitive performance (Purnell et al., 2002).

Implications and recommendations

One of the main findings of this dissertation work is that voluntary and forced wakefulness have differential effects on regions of the brain that support wakefulness and reward. Thus, for example, working a night shift chosen voluntarily and a night of mandatory wakefulness are going have different physiological effects on the brain. A second main finding is that some areas of the brain that promote wakefulness appear to respond to challenges differently in diurnal vs. nocturnal species. This observation is worrisome given that mostly nocturnal species have been evaluated as models to understand the effects of temporal shifts in activity. Overall my results suggest that findings in nocturnal species should be interpreted with caution, especially if the results are to be applied to diurnal species. The findings of this dissertation work are relevant not only to humans but also to non-human animals, because the latter group also shows switches in their temporal patterns of activity (see introduction to this Chapter). This work, therefore, has relevance to a field that is often neglected by chronobiologists: the field of conservation biology.

A recommendation for the planning of future experiments is to adopt the use of more diurnal animal models to study the consequences of shifts in the distribution of daily activity. This is because in mammals, diurnality evolved

independently several times from nocturnal ancestors(Smale et al., 2003). Thus, the neural mechanisms that lead to convergent evolution of diurnality might have been different (i.e., grass rats when compared to the hominids). Another recommendation is that further work should address how age and/or sex might be factors modulating neural activation of wakefulness and reward related areas of diurnal species. This dissertation was done solely using adult male grass rats, but it is plausible that the effects observed in the study areas might have been enhanced or attenuated in aged grass rats or in females. For example, rhythms in Fos expression in the BF of pregnant rats are blunted when compared to non-pregnant females suggesting that these rhythms can also be modulated by pregnancy hormones (Schrader, 2009). Also, in female rats estradiol enhances Fos expression in wakefulness-promoting areas after sleep deprivation (Deurveilher et al., 2008).

In addition, future work needs to evaluate the effects of constant exposure to different levels of light intensity in diurnal models of temporal shifts. This is because, for example, night shift workers are likely to be exposed to light throughout the day. Research in nocturnal animals has shown that the constant exposure to light alters clock properties (reviewed in Pittendrigh and Daan, 1976). Furthermore, in mice even exposure to dim light during the night appears to alter SCN functioning (Shuboni and Yan, submitted). Given the associations between the functioning of the SCN and wakefulness-promoting areas (reviewed in Chapter 1), it is expected that the latter areas are also affected by continuous exposure to light. This has especial implications for non-human animals. This is

because the pollution of the environment by artificial night-light is likely to have a tremendous impact on the behavioral ecology of wild species (reviewed in Longcore and Rich, 2004).

In sum, this dissertation work provides new insights into the relationships between wake-promoting systems and the biological clock, as well as wake-promoting systems and rewarding systems in a diurnal species. Thus, this set of experiments is relevant not only to the field of chronobiology, but also to human and non-human health and mental health, as well as to the conservation biology field.

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