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CIRCADIAN REGULATION OF BRAIN AREAS REGULATING AROUSAL IN DIURNAL AND NOCTURNAL RODENTS

presented by

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has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Psychology

Major Professor's Signature

5/11/05

Date

CIRCADIAN REGULATION OF BRAIN AREAS REGULATING AROUSAL IN DIURNAL AND NOCTURNAL RODENTS

By

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A DISSERTATION

Submitted to

Michigan State University
In partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Psychology

2005

ABSTRACT

CIRCADIAN REGULATION OF BRAIN AREAS REGULATING AROUSAL IN DIURNAL AND NOCTURNAL RODENTS

By

Gladys Stella Martínez Martínez

The work of this dissertation focuses on the differential regulation of circadian rhythmicity by the central nervous systems of diurnal and nocturnal species. The experiments make use of a diurnal animal model *Arvicanthis niloticus* (or grass rat) and devote particular attention to the regions of the mammalian brain that are responsible for the support of wakefulness.

In grass rats, neurons expressing orexin (ORX) showed a significant daily endogenous rhythm in the expression of Fos (the product of the cfos gene) that correlated with the rhythm in sleep and wakefulness, and was reversed when compared to that seen in lab rats. In contrast to lab rats, orexinergic neurons in grass rats received substantial projections from vasoactive intestinal polypeptide (VIP) neurons of the suprachiasmatic nucleus (SCN), which suggests a direct control by the SCN on neurons expressing ORX in grass rats and an indirect regulation in lab rats. Histaminergic neurons in the ventral tuberomammillary nucleus (vTMN) instead lacked SCN projections in both lab and grass rats. In addition, lab and grass rats differed in the amount of retinal inputs to the lateral hypothalamic area (LHA) and the dorsal raphe nuclei (DRN), which suggests an important role of light in the regulation of wakefulness in the nocturnal lab rats but not in the diurnal grass rats.

Finally, different from what has been reported in several other species, PER1, a protein coded by one of the clock genes of the mammalian SCN, was detected in the

posterior part of the dorsomedial hypothalamus (DMH). Expression of PER1 in this area showed peaks every 8 hours, in a pattern that suggests an ultradian rather than circadian rhythm. It is still possible that the rhythm in this region reflects the activity of several populations of circadian neural oscillators within the DMH out of phase with each other. In contrast to the DMH, no clear rhythm was evident in the paraventricular nucleus of the thalamus (PVT), but differences were significant between different levels of the PVT (i.e., anterior, middle, and posterior), with higher levels in the anterior part.

In summary, these results are consistent with the hypothesis that differences between diurnal and nocturnal species are due to differences in the functions of targets of the SCN such as the ORX neurons and the DMH. In addition, differences in the distribution of retinal projections with respect to areas that control sleep and wakefulness may be responsible for species differences in the effects of light on vigilance and general activity.

Esta tesis está dedicada a mi familia que me ha dado todo el apoyo necesario para lograr este reto, aun sin entender las razones que me impulsaron a intentarlo. Especialmente, dedico esta tesis a mi querida Sofía, y a mis queridos Nicolás y Ricardo porque con el paso de los años se convirtieron en la mejor razón para terminar. También la dedico a esa otra parte de la familia, mi familia extendida, que siempre estuvo ahí recordándome lo que este logro significa para todos nosotros.

ACKNOWLEDGEMENTS

I wish to express my gratitude to all the members of my committee, Drs. Laura Smale, Lauren Harris, Lynwood Clemens, and Tony Nunez for their support along these years. I specially want to thank Tony for his guidance, suggestions, and most importantly, for his patience and direction, and for trying to make me feel at home, even in the coldest winter.

Thanks to Megan Mahoney for her friendship, support, patience to teach me everything I needed to know to work in the lab, and her disposition to introduce me to the American way. Thanks also to Josh and Mike for their readiness to help whenever I needed. Thanks to Chida for his friendship and valuable insights.

Special thanks to Julie Harris for her help during my initial training, and to the undergraduate students who were involved in these projects and who befriend me: Trisha Kulkanis, and Marie Sajewicz. Thanks also to Samantha Verberkmoes who helped me getting some information for the final part of my dissertation.

These studies were supported by NIMH MH53433 to LS, a Fulbright fellowship to GSM and several units at Michigan State University that provided me with funds: the Graduate School, the Department of Psychology, and the Office for International Students and Scholars.

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KEY TO ABBREVIATIONS

3V	third ventricle
ABC	avidin-biotin complex
AHA	anterior hypothalamic area
ANOVA	analysis of variance
AVPe	anteroventral periventricular nucleus
BDA	biotinylated dextran amine
BNST	bed nucleus of the stria terminalis
CCG	clock-controlled genes
CMT	centromedial thalamic nuclei
CSF	cerebrospinal fluid
DAB	Diaminobenzidine
DD	constant darkness
DMH	dorsomedial hypothalamus
DRN	dorsal raphe nuclei
EEG	Electroencephalogram
f	Fornix
GnRH	gonadotrophin-releasing hormone
GRP	gastrin-releasing peptide
ICC	Immunocytochemistry
ICV	Intracerebroventricular
IGL	intergeniculate leaflet
LC	locus coeruleus
LD	light-dark
LDT	laterodorsal tegmental nucleus
LHA	lateral hypothalamic area
LH	luteinizing hormone
LSD	least significant difference
MCH	melanin concentrating hormone
MPN	medial preoptic nucleus
mt	mammillothalamic tract
NDS	normal donkey serum
NHS	normal horse serum
nREM	non REM sleep
ORX	Orexin
PACAP	pituitary adenylyl cyclase-activating peptide
PBS	phosphate buffered saline
PK2	Prokineticin 2

PLP	4% paraformaldehyde mixed with 0.2% sodium periodate and 1.3% lysine
PLP	paraformaldehyde-sodium periodate-lysine
PPT	pedunculopontine tegmental nuclei
PVN	paraventricular hypothalamic nucleus
PVT	paraventricular nucleus of the thalamus
REM	rapid eye movements
RHT	retinohypothalamic tract
RN	raphe nuclei
SC	superior colliculus
SCN	suprachiasmatic nucleus
SEM	standard error mean
SON	supraoptic nucleus
sPVZ	subparaventricular zone of the hypothalamus
SWS	slow wave sleep
TGF-α	transforming growth factor-alpha
TMN	tuberomammillary nucleus
VIP	vasoactive intestinal polypeptide
VLPO	ventrolateral preoptic area
VMH	ventromedial hypothalamus
VP	arginine vasopressin
vTMN	ventral TMN
ZT	zeitgeber time

INTRODUCTION

The work of this dissertation focuses on the differential regulation of circadian rhythmicity by the central nervous systems of diurnal and nocturnal mammals. The experiments make use of a diurnal animal model *Arvicanthis niloticus* (or grass rat) and devote particular attention to the regions of the mammalian brain that are responsible for the support of wakefulness.

Most organisms, including humans, show daily rhythms of about 24 hours in physiology, hormonal function, and behavior. In mammals, these rhythms are controlled by an endogenous circadian pacemaker localized in the suprachiasmatic nucleus (SCN) of the hypothalamus that determines the temporal organization of several behaviors and physiological processes (Moore & Eichler, 1972; Stephan & Zucker, 1972). Evidence for the SCN as the circadian pacemaker comes from three main lines: (1) lesions of the SCN produce loss of nearly all circadian rhythms, although certain rhythms may remain (Rusak & Zucker, 1979; Satinoff & Prosser, 1988; Wachulec, Li, Tanaka, Peloso, & Satinoff, 1997); (2) in vivo or in vitro isolation of the SCN does not alter its ability to generate circadian signals (Green & Gillette, 1982; Inouye & Kawamura, 1979; Schwartz & Gainer, 1977; Shibata, Oomura, Kita, & Hattori, 1982); (3) SCN transplants can restore behavioral rhythms in arrhythmic animals with SCN lesions (Lehman et al., 1987), and the restored rhythm reflects the period of the donor (Ralph, Foster, Davis, & Menaker, 1990).

The clock is synchronized to environmental events through photic and non-photic signals or Zeitgebers. Light is the dominant Zeitgeber and resets the SCN through a direct projection from the retina, the retinohypothalamic tract (RHT) (Moore & Lenn, 1972); lesions of this pathway interfere with photic entrainment (Johnson, Moore, & Morin,

1988). Non-photic cues are provided by inputs to the SCN from serotonergic neurons in the raphe nuclei (RN) that modulate the magnitude of the phase shift induced by light (Rea, Glass, & Colwell, 1994). In addition, inputs from the intergeniculate leaflet (IGL) appear to mediate phase shifts produced by locomotor activity (Turek, 1989). A unique form of non-photic entrainment has been also shown in the fetus; information about time of day needed to entrain the fetal SCN is provided by the mother (Reppert & Schwartz, 1983).

The information received through these pathways as well as circadian signals generated in the SCN are conveyed to other regions through projections from the nucleus. The SCN sends projections to neuronal targets in the hypothalamus, including a dense projection to the subparaventricular zone of the hypothalamus (sPVZ), and projections to targets outside the hypothalamus such as the basal forebrain and the midline thalamus (Watts, 1991). Evidence for another type of output via diffusible signals has also been described (Silver, LeSauter, Tresco, & Lehman, 1996).

The SCN is not the only oscillator in mammals. Non-SCN oscillators have been found in the retina (Tosini & Menaker, 1996) and evidence suggests the presence of another extra-SCN oscillator entrained by feeding, but its location is still unknown (Stephan, Swann, & Sisk, 1979). Current data suggest that peripheral tissues contain damped oscillators that require intermittent input to sustain oscillation and that the SCN is involved in the process (Reppert & Weaver, 2001, 2002), but some evidence exists for non-SCN, self-sustained oscillators (see discussion of Exp. 5, p. 97)

Different mechanisms have been proposed to explain how the SCN conveys information to the rest of the brain. Studies in which the SCN was isolated through knife cuts showed loss of rhythmicity in behavior and suggested that axonal outputs from the SCN were the main route for transmission (Inouye & Kawamura, 1979). It is possible that

SCN axonal outputs encode information about the level of neural activity in the SCN with high levels during the day and low levels during the night and this information is transmitted to SCN targets. The most likely candidates for mediating axonal communication between the SCN and its targets are vasoactive intestinal polypeptide (VIP), arginine vasopressin (VP), and/or gamma aminobutyric acid GABA (Miller, 1993). In addition, studies in which SCN grafts of fetal tissue (tissue encapsulated in a membrane that prevented the formation of synaptic contacts) restored rhythmicity suggest that information from the SCN is transmitted through a diffusible factor and that this signal was sufficient to restore rhythms (Silver et al., 1996). Recent studies involving the infusion of substances into the third ventricle suggest that transforming growth factor-alpha (TGF-α) may act as an inhibitory SCN output signal that suppresses not only locomotor activity (Kramer et al., 2001) but also several other active behaviors such as grooming, exploring, and feeding (Snodgrass-Belt, Gilbert, & Davis, 2005). Prokineticin 2 (PK2), a cysteine-rich secreted protein, has been also proposed as an output signal from the SCN circadian clock (Cheng et al., 2002). However, other studies showed that SCN transplants that restore rhythms in locomotor activity fail to restore endocrine rhythms (Meyer-Bernstein et al., 1999). Thus, SCN axonal outputs are necessary for endocrine rhythms, but humoral outputs may be sufficient to generate some behavioral rhythms.

The molecular basis for the circadian oscillations involves autoregulatory transcriptional/translational negative and positive feedback loops. Two gene products, CLOCK and BMAL1 activate rhythmic transcription of *Cry and Per1-3* genes; information encoded by these genes is transcribed onto messenger RNA that leaves the nucleus and triggers the production of the proteins PER and CRY. If the concentration of these proteins is high enough, they form parts of negative factors that inhibit the transcription of the *Per*1

and *Per2* genes by interacting with CLOCK and/or BMAL1. The positive feedback loop is mediated by PER2, which positively regulates *Bmal1* transcription; BMAL1 then promotes heterodimerization of CLOCK:BMAIL1 so that the transcriptional cycles of *Per/Cry* may be restarted(Shearman et al., 2000). Recently, it has been suggested that the same kind of loop serves not only to produce the self-sustained circadian rhythmicity in clock genes but also to impose rhythmicity in the expression of clock-controlled genes (CCG) linked to the rhythmic production of proteins that may have some functions within the SCN but most importantly may have a role in the regulation of events outside the SCN; so far three CCG have been identified: the vasopressin prepropressophysin gene, the D-element binding protein (DBP) genes, and PK2 (Cheng et al., 2002; Reppert & Weaver, 2001).

Per genes (i.e., Per1, 2, and 3) are also widely expressed and rhythmic throughout the brain in non-SCN brain regions (Abe et al., 2002; Yamazaki et al., 2000). Some of the cells outside the SCN that express clock genes exhibit a rhythm in their expression that resembles rhythms in the SCN (Kriegsfeld, Korets, & Silver, 2003) but the precise role of these genes in these regions is still unknown. One possible explanation is that local clocks in areas being regulated by the SCN in turn regulate time-dependent sensitivity to signals from the SCN and that some type of redundancy is needed to anticipate these daily signals (Kriegsfeld et al., 2003). So, in addition to circadian regulation through the SCN, it is possible that activity in these non-SCN brain regions is regulated by the action of clock genes that provide a second signal and that these two signals when combined determine the activity pattern specific for that region.

The sleep-wake cycle

One of the several rhythms regulated by the SCN is the rhythm in sleep and wakefulness. Sleep and wakefulness involve several stages; these stages have been

characterized by using specific electrophysiological correlates. During a typical night, a person alternates between periods of rapid eye movements (REM) and non REM sleep (NREM; stages 1-4 are considered NREM sleep, and stages 3-4, are usually considered slow wave sleep or SWS). Each cycle is about 90-110 minutes long and within each cycle REM sleep occupies about 20 minutes. REM sleep is normally preceded by stage 4. Wakefulness is associated with increased muscle tone, beta waves in the electroencephalogram (EEG), and fast and low voltage EEG activity. During NREM sleep muscle tone decreases and the EEG shows periodic bursts or sleep spindles. This stage then leads to stage 3 of sleep characterized by high amplitude, slow waves (delta waves). After about an hour, small amplitude and high frequency waves appear and the pattern of activity looks similar to that seen during wakefulness, but the muscle tone is decreased. A specific EEG phenomenon is seen only during REM sleep, the PGO waves (PGO stands for pons, geniculate, and occipital cortex) that arise in the pons and reach the occipital cortex (as reviewed in Carlson, 2000).

Initial studies in which lesions in the reticular formation produced animals with constant sleep pattern in the EEG led to the conclusion that sleep is regulated by an arousal mechanism in the reticular formation, with arousal resulting from high levels of activity in the reticular formation, and with sleep resulting from low levels of activation (Moruzzi & Magoun, 1995). Experimental studies over the last 20 years seem to confirm that stimulation of this region produces the shift from sleep to EEG desynchrony and have identified components and neurotransmitters of the reticular activating system: this system sends projections from the brainstem and the posterior hypothalamus to the forebrain, including the thalamus and the hypothalamus. However, the view that sleep is simply a consequence of low levels of activity in circuits involved mainly in the maintenance of

arousal has been revisited; instead, it has been shown that during REM sleep several brain regions are activated and such activation is usually stimulated by the arousal system (Steriade, 1996). Also specific areas are active during sleep and are likely to be involved in inducing sleep (Novak & Nunez, 1998; Novak, Smale, & Nunez, 1999; Sherin, Shiromani, McCarley, & Saper, 1996).

Four systems of neurons seem to be important for different aspects of arousal: (1) noradrenergic neurons in the locus coeruleus (LC) exhibit a firing pattern related to the level of behavioral arousal: high activity during wakefulness and low activity during SWS and REM sleep (Hobson, McCarley, & Wyzinski, 1975); in addition, LC stimulation results in alertness and arousal signals in cortical and hippocampal EEG (Berridge & Foote, 1991); a feature of LC neurons is that they fire only if the animal is vigilant and paying attention to external stimuli (Aston-Jones, Rajkowski, Kubiak, & Alexinsky, 1994). (2) Stimulation of serotonergic neurons in the RN produces cortical arousal and inhibition of the synthesis of serotonin reduces this cortical arousal (Peck & Vanderwolf, 1991) Serotonergic neurons in the RN fire tonically at a slow rate during waking, considerably less during SWS, and cease to fire during REM sleep (Sakai & Crochet, 2001; Thakkar, Strecker, & McCarley, 1998).; in contrast with neurons in the LC, these neurons do not respond to external stimuli and seem to be important mostly for ongoing behaviors (Marrocco, Witte, & Davidson, 1994); (3) Acetylcholinergic neurons in the laterodorsal tegmental nucleus (LDT) and the pedunculopontine tegmental nuclei (PPT) also produce activation and cortical desynchrony (as shown in the EEG) after stimulation (Steriade, 1996); the activity of these neurons varies with behavioral state: high activity during wakefulness, decreased activity during SWS, and increased activity during REM sleep (Saper, Chou, & Scammell, 2001); (4) Whereas histamine administration produces an

arousing effect (Monnier, Sauer, & Hatt, 1970) inhibition of histamine synthesis, blockade of histamine receptors and lesions in the tuberomammillary nucleus (TMN) produce sleep, (Monti, 1993). Recently, another system involved in arousal has been discovered: Orexin (ORX) neurons in the lateral hypothalamic area (LHA) innervate all the components of the ascending arousal system (Peyron et al., 1998) and are predominantly active during wakefulness (Estabrooke et al., 2001; Torterolo, Yamuy, Sampogna, Morales, & Chase, 2001).

In addition to these four systems and the ORX neurons other areas in the thalamus such as the centromedial thalamic nuclei (CMT) and the paraventricular nucleus of the thalamus (PVT) may be also important in the sleep wake cycle. Activity in both the CMT and the PVT shows also an increase when the animal displays behaviors incompatible with sleep such as suckling (Allingham, von Saldern, Brennan, Distel, & Hudson, 1998) or if the animal is presented with stimuli that generate stress and anxiety (Chastrette, Pfaff, & Gibbs, 1991; Cullinan, Herman, Battaglia, Akil, & Watson, 1995; Duncan, Knapp, & Breese, 1996); a similar activation in these areas has also been reported in human subjects that are presented with tasks demanding attention (Kinomura, Larsson, Gulyas, & Roland, 1996). A role for the PVT in the control of some aspects of sleep and arousal has been suggested by studies showing an increase in Fos (the product of the *cfos* gene) expression during nighttime versus daytime in rats, a pattern that is related to their sleep-wake behavior (Peng, Grassi-Zucconi, & Bentivoglio, 1995). Studies with grass rats showed a daily rhythm in Fos in the PVT in a pattern resembling, at least in part, the activity pattern of this species, with a peak at Zeitgeber time (ZT) 1 (Novak, Smale, & Nunez, 2000). Further studies confirmed a projection from the SCN to the PVT in both grass and lab rats (Novak, Harris, Smale, & Nunez, 2000) and projections to this area from several areas also

involved in arousal (Novak, Harris et al., 2000), such as the LC and the RN (Morin, Goodless-Sanchez, Smale, & Moore, 1994; Otake, Ruggiero, & Nakamura, 1995).

The main system involved in SWS is located in the basal forebrain and more specifically the preoptic area and the adjacent hypothalamus, where neurons increase firing during NREM sleep (Alam, McGinty, & Szymusiak, 1996). Immunocytochemical studies with Fos identified sleep active neurons in the ventrolateral preoptic area (VLPO). These neurons contain GABA and galanin and send inhibitory projections to histaminergic cells in the TMN and to monoaminergic groups in the brainstem (Sherin, Elmquist, Torrealba, & Saper, 1998).

REM sleep is controlled by cholinergic neurons in the dorsolateral pons (LDT and PPT) that send projections to the pontine reticular formation and the thalamus (Steriade, Datta, Pare, Oakson, & Curro Dossi, 1990). Some neurons in these regions preferentially discharge just before and during REM sleep (REM-on neurons) (Steriade et al., 1990) and other neurons are active during wakefulness and REM sleep (Wake/REM-on neurons). Through the connections with the thalamus, neurons in this region control cortical arousal. Connections with the lateral geniculate nucleus make it possible for this region to control PGO waves (Steriade et al., 1990). Through projections to the tectum, neurons in this region control eye movements. Atonia typical of REM sleep is produced via a pathway connecting cholinergic neurons with the magnocellular nucleus of the medulla and finally with the spinal cord (Schenkel & Siegel, 1989). Projections to the PPT and the LDT arise mainly from a diffuse extension of the VLPO (extended VLPO), and lesions to the extended VLPO result in a reduction of REM sleep, which suggests that regulation of REM sleep is mediated by the VLPO (Saper et al., 2001)

Sleep is not an unitary phenomenon and instead is considered a two-process system that involves a homeostatic process that keeps track of time spent asleep and awake and circadian processes that determine when sleep and wakefulness must occur (Borbely, 1982; Borbely & Achermann, 1999). It is assumed that the propensity to be awake or to be sleep depends on the interaction between the sleep debt and signals from the circadian clock in the SCN (Dijk & Czeisler, 1994). In a variation of this model it has been proposed that the SCN sends a wake-promoting signal at the end of the active period that opposes the drive to initiate sleep so that sleep is consolidated during the rest period and wakefulness is consolidated during the active period (Edgar, Dement, & Fuller, 1993). Although it is considered that the timing and organization of sleep result from the interaction between these two systems there is still debate about the independent control by separate mechanisms of the two processes. The fact that they can be independently manipulated suggests that they must be controlled by separated mechanisms (Borbely, Dijk, Achermann, & Tobler, 2001). The assumption of independence between these two processes seems to be supported by studies with SCN lesions that produced no change in the total amount of sleep (Coindet, Chouvet, & Mouret, 1975) but instead reduced the amplitude of the circadian variation or abolished the circadian control of the sleep-wake cycle (Eastman, Mistlberger, & Rechtschaffen, 1984). In addition, when rats with SCN lesions were sleep-deprived, they showed the normal increase in sleep propensity, supporting the idea that the homeostatic system is not dependent upon the circadian clock (Tobler, Borbely, & Groos, 1983). However, conflicting results have been reported in studies with the diurnal squirrel monkey. In this species, SCN lesions disrupted the circadian variation in sleep and wakefulness, but they also produced an increase in the total amount of sleep and a reduced wake consolidation. A proposed explanation for the

differences in the relationship between these two processes in the squirrel monkey appeals to differences related to diurnality, but no additional evidence has been found to support that assumption (Edgar et al., 1993). More recently, studies with Per mutants seem to confirm the independence between homeostatic regulation of sleep and the circadian clock: mice with disruption of both *Per1* and *Per2* genes lost rhythmicity in sleep and wakefulness under free-run conditions but showed the same amount of sleep (Shiromani et al., 2004). However, a study with mouse CLOCK mutations showed a decrease in the total amount of sleep, suggesting a role for this clock gene in the homeostatic control of sleep, but the fact that CLOCK mRNA is expressed in several brain regions outside the SCN opens the possibility that those changes in the homeostatic processes are not directly related to the circadian clock (Naylor et al., 2000).

Differences between nocturnal and diurnal brains

Several lines of research with different animal models suggest that the SCN may function in a similar way across species and that the phase of its rhythms, with respect to the day-night cycle, is the same in diurnal and nocturnal mammals. For example, rates of glucose utilization and electrical activity in the SCN peak during the light phase in both nocturnal and diurnal species (Ruby & Heller, 1996; Schwartz, Reppert, Eagan, & Moore-Ede, 1983). The same is true for the expression of the immediate early gene product Fos; it is high during the light phase independently of whether the species is diurnal or nocturnal (Katona, Rose, & Smale, 1998; Kononen, Koistinaho, & Alho, 1990).

Despite these similarities, diurnal and nocturnal species differ in some SCN features: in both the nocturnal hamster and the diurnal chipmunk, photic stimulation induces Fos expression in the SCN if applied during the subjective night, whereas light pulses applied during the subjective day induce Fos expression in the chipmunk SCN but

not in the hamster's SCN (Abe, Honma, Shinohara, & Honma, 1995). Differences in Fos expression in SCN neurons containing VP have also been reported between the diurnal grass rat and the nocturnal lab rat: whereas little or no colocalization is found in the SCN of the lab rat, high colocalization of Fos and VP during the light period was found in the grass rat (Rose, Novak, Mahoney, Nunez, & Smale, 1999). It must be noted, however, that the species differences in the SCN do not segregate in a clear fashion with respect to the diurnal or nocturnal habits of the species studied so far.

Given the similarities in SCN organization in diurnal and nocturnal species, differences in behavioral rhythms in diurnal and nocturnal species may result from (1) differences in the responsiveness of brain regions receiving signals from the SCN (2) differences in activity in brain areas adjacent to the SCN that may modify SCN signals or (3) differences in connectivity between SCN subpopulations (Nunez, Bult, McElhinny, & Smale, 1999; Smale, Lee, & Nunez, 2003).

In the lab rat, brain areas involved in the onset and maintenance of sleep exhibit greater neural activity (i.e., enhanced Fos expression) during the light than during the dark phase of the cycle (Novak & Nunez, 1998; Peng et al., 1995; Sherin et al., 1996). In contrast, brain areas involved in the support of wakefulness and arousal show more activity during the dark phase of the cycle, when lab rats are active (Novak & Nunez, 1998; Novak, Smale et al., 2000). In the grass rat, a diurnal rodent, the sleep cycle is reversed. Unlike rats, these animals sleep during the dark phase of the cycle and are awake during the day with peaks of activity also at dawn and dusk (McElhinny, Smale, & Holekamp, 1997; Novak et al., 1999). Brain areas involved in sleep in grass rats also show rhythms of Fos expression concordant with their sleep pattern: Fos expression in the VLPO is high at Zeitgeber time (ZT) 17 (with ZT 0 at the beginning of the light phase of a 12:12 light dark

cycle) when these animal are likely to be sleeping, and is low at ZT 1 and ZT 13 when they are awake (Novak, Smale et al., 2000)

In the work presented here, I investigate the phase of rhythms in neural activity and gene expression in areas of the brain associated with wakefulness and look at the comparative anatomy of axonal projections from the SCN to these areas. In addition, some of the work examines the distribution of retinal inputs in areas of the brain that control wakefulness in the diurnal grass rat and in the nocturnal lab rat. Light not only serves as the primary entraining stimulus (Zeitgeber) for the wake-sleep cycle of mammals, but it can also influence the display of sleep or behavioral arousal independently of its entraining of the rhythm; these acute effects of light are often referred to as "masking" (Mrosovsky, 1999; Redlin, 2001; Smale et al., 2003). Light, particularly during the night, is expected to have very different effects on sleep and wakefulness depending upon whether the animal is diurnal or nocturnal. Therefore, it is likely that retinal inputs to areas of the brain that support wakefulness differ in fundamental ways between diurnal and nocturnal species.

GENERAL METHODS

Animals

All of the grass rats (*Arvicanthis niloticus*) used in the experiments came from the breeding colony established in this laboratory and derived from the original group caught in Kenya in 1993 (Katona & Smale, 1997). Animals were allowed free access to food (PMI Nutrition Prolab RMH 2000, Brentwood, MO, USA) and tap water and were housed in standard plastic Plexiglas cages (34X28X17 cm).

Sexually mature Long–Evans lab rats (*Rattus norvegicus*) were purchased from Harlan Sprague Dawley, Indiana. Upon arrival, these rats were housed in standard plastic cages (57×25×20 cm). Standard rat chow (Harlan Teklad 22/5 rodent diet 8640) and tap water were available ad libitum.

Unless otherwise noted, animals were kept in a 12:12-h light-dark (LD) cycle, with lights on at ZT 0, and with a dim red light (<5 lux) on at all time. Ambient temperature was kept at 22 °C and humidity at 48%.

Immunocytochemistry (ICC)

Animals were given an overdose of Nembutal (0.5cc) and were transcardially perfused with 0.01M phosphate buffered saline (PBS), pH 7.2, followed by 4% paraformaldehyde mixed with 0.2% sodium periodate and 1.3% lysine (PLP) in 0.1M phosphate buffer. Brains were removed and postfixed for about 8 hours in PLP before being transferred to a 20% sucrose solution in 0.1M PBS overnight. Brains were sectioned at 30μm thickness using a freezing microtome, divided in three alternate sets and stored into cryoprotectant in a -70°C freezer until ready to use.

Unless noted otherwise, incubations were done in 0.1M PBS with 3% Triton-X-100 (PBS-TX), at room temperature on a shaker. Free floating sections were rinsed in 0.1 M PBS (6X10) and preincubated in 5% normal serum for 1 h. Sections were then rinsed (1X10 min) and incubated in the primary antibody with 3% normal serum on a rotator for about 24 h at 4°C. Tissue was then washed in 0.1M PBS (3X10 min), placed in a biotinylated secondary with 3% normal serum for 1.5 h., rinsed again in 0.1M PBS (3X10 min), and incubated in avidin-biotin complex solution (ABC Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) for 1.5 h. After 3X10 min rinses in 0.1M PBS, tissue was pre-incubated in diaminobenzidine (DAB) in Trizma buffer for 5 min and reacted with 30% hydrogen peroxide (H_2O_2). Tissue was then rinsed, mounted onto gelatin-coated slides, dried, dehydrated, and coverslipped with DPX mounting medium.

For dual ICC the tissue was rinsed in 0.175 acetate buffer (ph 7.2) after the incubation in ABC. Tissue was then pre-incubated in DAB in acetate buffer with 2.5% nickel sulfate, reacted with 3% H_2O_2, rinsed in 0.1 M PBS-TX buffer (5X10 min) and then in 0.1M PBS (3X10 min). After this, tissue was incubated in normal serum, a second primary antibody and a biotinylated secondary antibody, with 3X10 min rinses between incubations. The second protein was visualized by reacting with DAB in Tris buffer and 30% H_2O_2, as before.

ICC controls

To verify specificity of each primary antibody, control sections were processed in the same way as described above, except that tissue was incubated without primary antibody. In the case of the ICC for PER1, specificity was also confirmed by blocking experiments in which the primary was pre-incubated with its antigen (1 μg/ml). Each one of these treatments eliminated staining.

EXPERIMENT 1: RHYTHMS IN FOS EXPRESSION IN A BRAIN REGION INVOLVED IN AROUSAL

ORX A and B, also known as hypocretin 1 and 2, have been implicated in the regulation of the sleep wake cycle (Kilduff & Peyron, 2000; Peyron et al., 1998). ORX-producing neurons send substantial projections to areas of the brain thought to play important roles in the control of vigilance and sleep. All the monoaminergic cell groups, which have wake-dependent activity and provide diffuse cortical activation (Sherin et al., 1998), receive projections from ORX-immunoreactive (ORX-ir) neurons; ORX-ir fibers terminate in the LC, the dorsal and medial raphe nuclei, and the TMN (Peyron et al., 1998). Cholinergic neurons in the LDT and the PPT, which mediate the cortical excitation characteristic of REM and also REM atonia (Jones, 1998), are among the targets of ORX-ir neurons (Peyron et al., 1998). Other brain regions thought to be involved in the support of wakefulness (e.g. the PVT) (Novak & Nunez, 1998) or in the facilitation of sleep (e.g. the lateral preoptic area) (Sherin et al., 1996) also receive substantial projections from ORX-ir neurons (Peyron et al., 1998).

Several studies have confirmed the role of ORX in the regulation of sleep and arousal. Studies in narcoleptic dogs showed that this condition is caused by the disruption of one of the two ORX receptors genes, the ORX_2 receptor (ORX_2R) gene (Lin et al., 1999). Behavioral and electroencephalographic studies found that preproorexin knockout mice exhibit a syndrome of sleep dysregulation similar to human and canine narcolepsy (Chemelli et al., 1999). Additionally, recent studies found deficiencies of ORX-A in the cerebrospinal fluid (CSF) in human narcolepsy (Nishino, Ripley, Overeem, Lammers, & Mignot, 2000). Further, ORX-A increases the firing rate of LC neurons in vitro (Hagan et

al., 1999; Horvath et al., 1999), decreases the amount of REM sleep (Bourgin et al., 2000), and strongly excites serotonergic neurons in the dorsal raphe nuclei (DRN) (Brown, Sergeeva, Eriksson, & Haas, 2001). Intracerebroventricular (ICV) administration of ORX-A at the onset of the normal sleep period increases the proportion of time spent awake and decreases the proportion of paradoxical sleep without a significant decrease in deep SWS (Hagan et al., 1999). ICV administration of ORX-A at the onset of the normal sleep period produces a dose-dependent increase in the proportion of time awake; high doses also produce a reduction in paradoxical sleep and deep SWS (Piper, Upton, Smith, & Hunter, 2000). ICV administration of ORX-A and -B induces expression of Fos in nuclei where ORX fibers project, including the LC (Date et al., 1999). Fos expression in ORX-A neurons correlates positively with wakefulness and negatively with non-REM and REM sleep in rats (Estabrooke et al., 2001) and in cats, Fos expression in orexinergic neurons peaks during active wakefulness (Torterolo et al., 2001).

Daily rhythms in the activity of ORX-A neurons were reported in a study with lab rats showing that Fos expression in these neurons peaked at night (Estabrooke et al., 2001). In addition, prepro-ORX mRNA and ORX-A in the pons and in the preoptic/anterior hypothalamic area increase during the dark hours and decrease during the day in lab rats, suggesting a role of ORX in the regulation of the sleep/wake cycle (Taheri et al., 2000).

To investigate variations in the activity of ORX-ir neurons during the light dark cycle in a diurnal rodent, I used double ICC staining for Fos and ORX-B. Animals were taken at five different times of day in order to obtain information about Fos expression in orexinergic neurons at the beginning and middle of the light and dark phases of the illumination cycle (ZT 1, ZT 5, ZT 13, ZT 17, and ZT 20).

Methods

Immunocytochemistry

Thirty-five single-housed adult male grass rats (n=7 per sampling time) were perfused at ZTs 1, 5, 13, 17, and 20 following the protocol described in General Methods. A light tight foil hood was placed over the heads of animals sacrificed during the dark phase to prevent acute effects of light on Fos expression.

To evaluate rhythms in Fos production in neurons expressing ORX, every third section was used for dual ICC as described before. Initially, sections were incubated in normal donkey serum (NDS, Jackson Immunoresearch Labs, CA), rabbit anti-cFos (Santa Cruz Biochemistry, Santa Cruz, CA, 1:25,000), and biotinylated donkey anti-rabbit (Jackson, 1:200). Fos was visualized with a DAB-nickel sulfate solution in acetate buffer, pH 7,2 and 3% H_2O2. Tissue was then processed for ORX by incubating first in normal horse serum (NHS, Vector) and then in the primary antibody goat anti-ORX B (Santa Cruz, 1:60,000) and the biotinylated secondary horse anti-goat (Vector, 1:200). ORX was visualized by reacting the tissue with DAB in Trizma buffer.

Data Analysis

Tissue was examined using a light microscope equipped with a drawing tube (Laborlux S; Leitz, Wetzlar, Germany). This tube was used to draw a horizontally-oriented (1200 μM X 700μm) rectangle that covered the LHA, with the fornix located in the central region of the rectangle just above the lower edge (see Figure 1a). Although this box does not include all areas of the grass rat brain containing ORX cells, examination of several animals revealed that most of ORX-ir cell bodies fall within the boundaries of the box. The rectangle was divided vertically in three regions of the same size in order to get the medial, central and lateral areas of this region. Sections were selected with the mammillothalamic

tract, the third ventricle, and the fornix as landmarks. This rectangle was used to make drawings from three sections from each animal.

For each section, drawings included Fos-ir cells, ORX-ir cells, and double-labeled cells. Cells were counted unilaterally by an individual blind to the time at which animals were perfused for each of the three areas, and counts were used to determine the total number of ORX-ir cells, the proportion of ORX-ir neurons containing Fos, and the number of Fos-ir cells not expressing ORX. Averages of the counts in the three sections per animal were used for data analysis

Two-way ANOVAs were used to evaluate the effects of ZT and area (medial, lateral, and central) on the percentage of ORX-ir cells containing Fos, the number of cells expressing Fos but no ORX, and the number of cells containing ORX.

Because of lack of homogeneity of variance, square root transformations were used for all three dependent variables. Comparisons among individual means used the Fisher's protected least significant difference (LSD) following the parametric ANOVAs. In addition, the percentage of ORX-ir cells expressing Fos was correlated with the number of cells expressing only Fos (Pearson Correlation Coefficient). In all analysis, differences were considered significant when $p<0.05$. Data are presented as mean ± standard error mean (S.E.M.).

Results

Representative examples of single and double-labeled ORX-ir cells in grass rats are shown in Figure 1b. No labeled cells were seen in the control sections.

Distribution of ORX-ir cells

A two-way analysis of variance (ANOVA) for the number of cells containing ORX (both single- and double-labeled) revealed a significant effect of area ($F=264.18$, $df = 2, 90$,

$p<0.001$) with no effect of ZT ($p=0.64$, see Figure 2a) or significant interaction ($p=0.92$). Pairwise comparison showed significant differences between the three areas, with the number of cells in the central area higher than the numbers in the lateral and medial areas, and the medial area with fewer ORX cells than the lateral area (Figure 2b).

Fos expression in ORX neurons

A two-way ANOVA for the proportion of ORX neurons expressing Fos revealed a significant main effect of ZT ($F=5.03$, df = 4, 90, $p=0.011$) and area ($F=4.57$, df = 2, 90, $p=0.01$) with no significant interaction ($p= 0.83$). Pair comparisons made to further probe the main effect of ZT (see Figure 3a) showed that the percentage of double labeled cells was significantly greater in animals perfused at ZTs 13 and 1 than in those perfused at ZTs 17 and 20. Animals perfused at ZT 13 also had a higher percentage than those perfused at ZT 5. Pair comparisons for the main effect of area (see Figure 3b) showed a higher percent of double labeled cells in the central area compared to both the medial and lateral areas.

Fos expression in non-ORX neurons

A two-way ANOVA performed to evaluate the effects of ZT and area on the number of cells expressing Fos but not containing ORX-ir revealed only a significant effect of area ($F=60.28$, df =2, 90, $p<0.001$), with no effect of ZT ($p<0.24$) or any interaction ($p<0.97$). Pair comparisons showed significant different between all the three areas, with the number of Fos cells not containing ORX being higher in the medial than in the central and in the lateral regions (Figure 4b).

Further analysis showed a significant positive correlation between cells expressing Fos in ORX-ir cells and cells not labeled for ORX ($r=0.30$, df=103; $p=0.001$)

Discussion

The distribution of ORX-ir cells is similar to what has been previously reported in grass rats (Novak & Albers, 2002). Under a light-dark cycle, orexinergic neurons in grass rats exhibited a daily rhythm in the expression of Fos in a pattern that corresponds to the activity patterns of this species. Although the animals activity was not recorded in this experiment, studies with grass rats have shown daily patterns of activity with two peaks, one around the time of lights-on and another around the time of lights-off (Blanchong & Smale, 2000). Fos expression in orexinergic neurons in grass rats followed this pattern: it was high at ZT 1, a time when these animals exhibit a peak on activity, and increased at ZT 13, a time when these animals show a second peak in activity. Fos expression decreased at ZT 17 and ZT 20, times in which these animals sleep (Novak, Smale et al., 2000).

Interestingly, the pattern of Fos expression in ORX-ir neurons is similar to what has been reported for another system involved in the support of wakefulness. In grass rats, Fos expression in histaminergic cells of the ventral TMN (vTMN) shows a rhythm that features a salient reduction at ZT 17 compared with the other ZTs sampled in that study (ZTs 1, 5, and 13) (Novak, Smale et al., 2000). Fos expression in both the ORX cells (present data) and the histaminergic group of the vTMN shows a pattern 180° out of phase with the rhythm seen in the VLPO of this diurnal species (Novak & Nunez, 1998; Novak et al., 1999; Novak, Smale et al., 2000). The VLPO becomes active when animals sleep and is likely to provide inhibitory inputs to the ORX and histaminergic cells.

In grass rats, the pattern of Fos expression typical of ORX-ir cells was absent among cells that do not express ORX-ir: no rhythm was present in neurons not labeled for ORX. An effect of ZT in the expression of Fos in this population with an increase at ZT 20 was seen in an earlier study using a single section per animal (Martinez, Smale, & Nunez,

2002) but was not confirmed with the more extensive sampling used here. Whereas double labeled cells were higher in the central area, cells expressing only Fos were found mainly in the medial area. Only a small (r =.30) positive correlation was found between the activity of cells not labeled for ORX and the activity of neurons expressing ORX, which suggest the presence of two relatively independent groups of neurons in this region at least with respect to Fos expression. The phenotype of these non ORX neurons remains unknown, but some of them may produce melanin concentrating hormone (MCH) (Bayer, Mairet-Coello, Risold, & Griffond, 2002; Elias et al., 1998).

The results from this experiment confirm the presence of daily rhythms in the expression of Fos in ORX-ir neurons in a diurnal species in a pattern that is related to the sleep wake cycle in this species and that is reversed with respect to that previously reported in lab rats (Estabrooke et al., 2001; Martinez et al., 2002): Fos expression in ORX-ir neurons is high when the animals are awake and low when the animals are asleep. These data support the claim that ORX neurons' activation is important for the promotion of wakefulness and also suggest a circadian modulation of activity in these neurons involved in the regulation of wakefulness. More studies are needed to confirm the role of the circadian clock in the control of these rhythms and the pathways that make possible such a regulation in both diurnal and nocturnal species.

Figure 1. (a) Rectangle used to count cells in each of three sections per animal. Cells were counted within an area defined using three landmarks: fornix (f), third ventricle (3V), and mammillothalamic tract (mt). Dark dots represent ORX-ir cells in the LHA of a grass rat (b) ORX-immunoreactivity and Fos immunoreactivity in grass rats as seen under high – power oil immersion lens. The image was edited with Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA, USA). Double arrow, Orexin-ir neurons; arrowhead, Fos-ir nucleus; single arrow, Fos-ir orx-ir neurons.

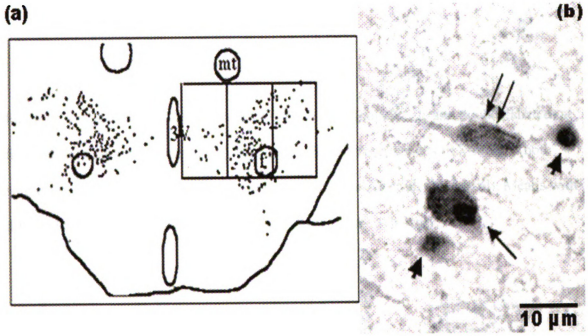
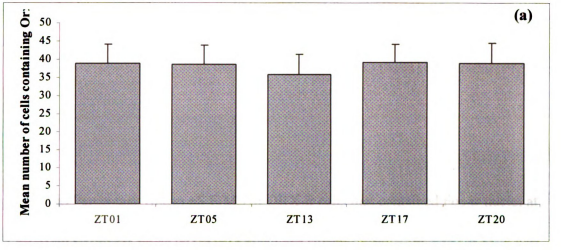

(a)
mt
3v
(b)
10 µm

Figure 2. (a) Mean (± S.E.M.) number of ORX-ir cells (both single and double labeled) at five different Zeitgeber times (ZTs). (b) Mean (±S.E.M.) number of ORX-ir cells (both single and double labeled) in three different areas (medial, central, and lateral). Means with different letters (a, b, or c) are significantly different from each other.


(a)
Mean number of cells containing Or
50
45
40
35
30
25
20
15
10
5
0
ZT01
ZT05
ZT13
ZT17
ZT20

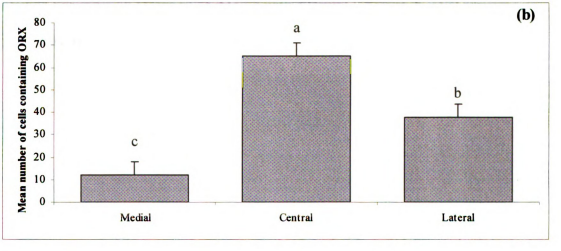
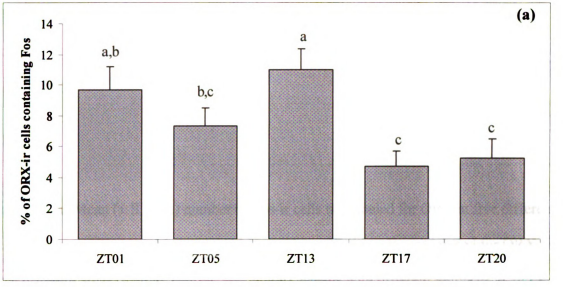

(b)
Mean number of cells containing ORX
80
70
60
50
40
30
20
10
0
a
b
c
Medial
Central
Lateral

Figure 3. (a) Percentage (mean ±S.E.M.) of ORX-ir cells that contained Fos-ir nuclei at five different Zeitgeber times (ZTs) (b) Percentage (mean ±S.E.M.) of ORX-ir cells that contained Fos-ir nuclei across areas. Means with different letters (a, b, or c) are significantly different from each other.


(a)
% of ORX-ir cells containing Fos
14
12
10
8
6
4
2
0
a,b
b,c
a
c
c
ZT01
ZT05
ZT13
ZT17
ZT20

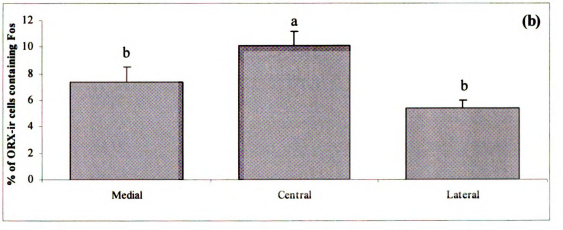
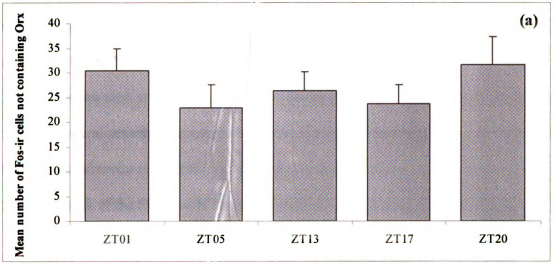
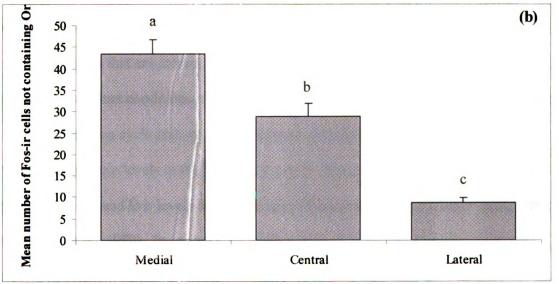

(b)
% of ORX-ir cells containing Fos
12
10
8
6
4
2
0
b
a
b
Medial
Central
Lateral

Figure 4. (a) Mean (± S.E.M.) number of Fos-ir cells not labeled for ORX at five different Zeitgeber times (ZTs) (b) Mean (±S.E.M.) number of Fos-ir cells not labeled for ORX in three different areas (medial, central, and lateral). Means with different letters (a, b, or c) are significantly different.


(a)
Mean number of Fos-ir cells not containing Orx
40
35
30
25
20
15
10
5
0
ZT01
ZT05
ZT13
ZT17
ZT20


(b)
Mean number of Fos-ir cells not containing Or
50
45
40
35
30
25
20
15
10
5
0
a
b
c
Medial
Central
Lateral

EXPERIMENT 2: RHYTHMS OF FOS EXPRESSION IN OREXINERGIC NEURONS IN CONSTANT DARKNESS

Data from Experiment One serve to confirm the presence of rhythmic activity across the day-night cycle in neurons expressing ORX in grass rats. The pattern of neural activity in the ORX system of grass rats is reversed, with respect to the day-night cycle, from the pattern seen in the nocturnal lab rat (Estabrooke et al., 2001). In lab rats, the rhythm of neural activity (i.e., expression of Fos) in ORX neurons persists in constant darkness (Estabrooke et al., 2001), which suggests that the rhythm is controlled by the circadian clock of the SCN. Additional evidence for circadian regulation of ORX in nocturnal species has been provided by recent experiments in which lesions to the SCN abolished circadian rhythms of ORX levels in the CSF (Zhang et al., 2004). Evidence for similar circadian control of the ORX system is lacking for diurnal species.

Rhythms that are present in LD may nevertheless disappear when the animals are exposed to constant conditions, thus indicating that those rhythms depend upon the effects of the illumination cycle and are not endogenous or circadian. For example, in rats, VIP mRNA and protein levels in the SCN show a daily rhythm under LD, with high levels during the night and low levels during the day (Albers et al., 1990; Okamoto et al., 1991; Takahashi et al., 1989). In contrast, no rhythms in both mRNA and protein levels are present in constant darkness (Isobe & Nishino, 1996; Shinohara, Funabashi, & Kimura, 1999; Takeuchi, Nagasaki, Shinohara, & Inouye, 1992). Experiment Two was designed to evaluate if the rhythm in Fos expression seen in grass rats kept under a LD cycle persists in constant darkness.

Methods

Activity monitoring

Grass rats were first put in a 12:12 LD cycle with infrared motion detectors over the cage to monitor rhythms in general activity. Data for activity rhythms were collected with the Dataquest 3 program (Data Sciences International, St Paul, MN, USA). After a 1-2 weeks period in LD, lights were turned off at 18:00 h and left off for 21-22 days. After this period of free running, actograms for each animal were independently examined by two investigators to determine activity onset. Actograms for each animal were used to determine activity onset and time of perfusions. One set of tissue of animals perfused at circadian time (CT) 5 (n=6), CT 13 (n=8), and CT 22 (n=7) was processed for dual ICC for Fos and ORX as described above. These times were chosen because actograms showed that grass rats are active at CTs 5 and 13, and less active (and apparently sleeping) at CT 22 (Figure 5).

Data Analysis

Counts were made on images of one section obtained at 10X magnification using a Zeiss AxioScop 2 plus microscope. A rectangle with the same characteristics as that used on animals kept in LD (Experiment 1) was placed over the pictures using the same landmarks as before. Labeling was confirmed by examining the tissue under 40X magnification. For each section, the number of cells containing ORX (both single and double labeled), double labeled cells, and cells expressing Fos but not labeled for ORX were unilaterally drawn and counted in each of the three areas (medial, central, and lateral). All counts were made without knowledge of the CT at which the animals were perfused.

A two-way ANOVA was used to evaluate the effects of CT and area on the number of cells expressing ORX. A square root transformation was used prior to this analysis. In

the case of the percentage of ORX cells expressing Fos a two-way ANOVA was not performed because of lack of homogeneity of the variance even after standard transformations were used. Instead, non parametric one-way ANOVAs (Kruskal–Wallis test) were used. The effects of CT and area on the number of cells expressing only Fos were evaluated by using a two-way ANOVA; a log transformation was performed on these data. Tukey's adjustment for multiple comparisons was used after the two-way ANOVAs to evaluate differences between means, and nonparametric multiple comparisons were used following the Kruskal–Wallis tests. Differences for all tests were considered significant when $p<0.05$. A correlational analysis was used to determine the relationship between Fos expression in and outside ORX neurons.

Results

Distribution of ORX-ir cells

A two-way ANOVA showed significant effects of area ($F=197,44$, df =2, 54, $p<0.001$) and no effect of CT ($p= 0.79$) or any interaction ($p= 0.67$; see Figures 6a and b). Pairwise comparisons showed significant differences between the three areas, with the number of ORX cells higher in the central area than in the lateral and medial areas and with significantly fewer ORX cells in the medial area compared to the other two.

Fos expression in ORX neurons

The one-way ANOVA on the percentage of ORX cells expressing Fos showed a significant effect of CT, with CT 13 being higher than CT 22 ($H=6.69$, df = 2, $p<0.03$) (Figure 7a). The same non parametric analysis showed a significant effect of area ($H=8.56$, df = 2, $p<0.01$), with the percentage of ORX cells expressing Fos being higher in the central area than in the medial area (Figure 7b).

Fos expression in non-ORX neurons

A similar rhythm in Fos expression to that found for ORX neurons was found for cells that did not contain ORX as indicated by a significant main effect of ZT (F=11,72, df=2, 62, p<0.001). The number of Fos cells not containing ORX was higher at CT 13 than at CTs 22 and 5. In these neurons there was also a significant effect of area (F=35.24, df=2, 62, p<0.001) and no interaction (F=0.19, df=4, 62, p<0.94). Pair comparisons showed significant differences between all three areas, with the number of Fos cells not containing ORX being higher in the medial than in the central and lateral regions (Figure 8). Further analysis revealed a significant positive correlation (r =.30, df=61,p=0.01) between cells expressing Fos, but not ORX and double labeled cells.

Discussion

The most important finding of this experiment is hat rhythms in Fos expression in the ORX cells of grass rats persist in constant conditions as expected of a true circadian rhythm. Although only 3 CTs were sampled , the temporal pattern of Fos expression in ORX neurons after 3 weeks or more in constant darkness (DD) is almost identical to what is seen under a LD cycle (Martinez et al., 2002; Experiment One). Thus, the rhythm of Fos expression in ORX cells is endogenous and therefore likely to be under the control of the SCN in both lab rats and grass rats (Estabrooke et al., 2001), even though the phase of the rhythm is completely reversed when the two species are compared.

Circadian control of the ORX system in diurnal and nocturnal species

Differences in the phase of the rhythm of Fos expression in the ORX system of diurnal and nocturnal species may result from differences along the pathways that communicate circadian information from SCN to ORX neurons. In lab rats, the densest projection from the SCN is to the sPVZ, including the lower subparaventricular area

(LSPV) (Watts, 1991). The SCN also provides inputs to the dorsomedial hypothalamus (DMH) (Watts, 1991). Both the LSPV and the DMH have been postulated to serve as important relay sites for SCN signals that control vigilance (Aston-Jones, Chen, Zhu, & Oshinsky, 2001; Chou et al., 2003), and in lab rats, lesions in the LSPV or in the DMH disrupt circadian rhythms of sleep (Chou et al., 2003; Lu et al., 2001). The DMH of lab rats receives direct inputs from the SCN and also indirect inputs via the LSPV (reviewed by Chou et al., 2003), and it sends glutamatergic, and thus excitatory, projections to ORX neurons (Chou et al., 2003). Thus, in the lab rat, the circadian regulation of activity in ORX neurons may involve an indirect route with the DMH as the primary relay nucleus of SCN signals to the ORX system.

Not much is known about the pathways from the SCN that could drive the rhythm of Fos expression in ORX neurons in grass rats and other diurnal species. If an indirect pathways involving the LSPV is also engaged in the regulation of the ORX system in grass rats, then the phase reversal of the rhythm in Fos expression seen in the diurnal species may be due to the fact that the LSPV shows very different patterns of Fos expression in lab rats and grass rats (Nunez et al., 1999; Schwartz, Nunez, & Smale, 2004); the LSPV may modulate SCN signals differently in diurnal and nocturnal species. Alternatively, in grass rats the circadian control of the ORX system may depend upon direct projections of the nucleus to ORX neurons. Studies of SCN efferent projections in grass rats have documented the presence of fibers of SCN origin in the region of the LHA where ORX neurons reside (Schwartz & Smale, 2004), but it is not known if these fibers in fact contact ORX neurons. The direct inputs of the SCN to areas that control arousal in grass rats and lab rats is the focus of Experiment Three.

Fos expression outside ORX neurons.

The anatomical distribution of ORX cells, double-labeled cells and cells expressing Fos but not ORX seen in DD was identical to those seen in LD (Experiment One). Also, the number of ORX cells was not affected by sampling time in both DD and LD (present data and Experiment One). But one remarkable difference between DD and LD was seen in the population of cells that shows Fos expression but no ORX labeling. Whereas in LD Fos expression outside ORX neurons was not rhythmic and/or did not match the activity patterns of grass rats (Experiment One; Martinez et al., 2002), in DD a rhythm was present with a pattern that not only resembled the pattern of activity of grass rats but also that of the rhythm of Fos expression in ORX neurons. One possible explanation for the difference between experiments may be that in Experiment Two the double ICC procedure was perhaps less effective in identifying double-labeled cells, so the rhythmic expression of Fos seen outside ORX neurons may reflect Fos expression in cells that express ORX but were missed by the double-label ICC of Experiment Two. What serves to argue against this interpretation is the fact that the distribution of cells that express Fos but not ORX was identical in LD and DD and different from that seen for ORX neurons. If expression of Fos outside ORX neurons in Experiment Two was due to Fos expression in "missed" ORX cells, then one would expect a preferential increase of cells expressing only Fos in the central area, where ORX neurons are abundant. Thus, the increase in Fos expression at CT 13 seen in DD but not in LD may represent an endogenous rhythm of neural activity in the non-ORX cells of the LHA that is masked by the light-dark cycle and only expressed in DD. It is unusual to encounter a circadian rhythm that is absent under LD but present in DD, but examples exist in the literature: in mice, the expression of mRNA for gastrin-

releasing peptide (GRP) in the SCN is not rhythmic in LD but shows a low amplitude circadian rhythm in DD (Dardente et al., 2004).

As in Experiment One, there was only a modest positive correlation between the expression of Fos in ORX neurons and in cells with no ORX labeling, thus the two populations appear to be relatively independent of each other under both LD and DD. The combined results of Experiments One and Two suggest the presence of a group of non-ORX cells in the LHA that is under circadian control, is affected by light, and exhibits a similar pattern of expression of Fos to that seen in ORX neurons, but only in DD. The specific phenotype of these cells remains unknown but may include neurons that express MCH. Neurons that express ORX or MCH are differentially regulated by adrenergic inputs to the LHA (Modirrousta, Mainville, & Jones, 2004).

Figure 5. Double plot actogram from a representative grass rats kept in constant darkness for 21 days. Grass rats are active at CTs 5 and 13, and less active (and apparently sleeping) at CT 22. Actograms for each animal were used to determine activity onset and time of perfusion. Each line represents a 48 hour period.

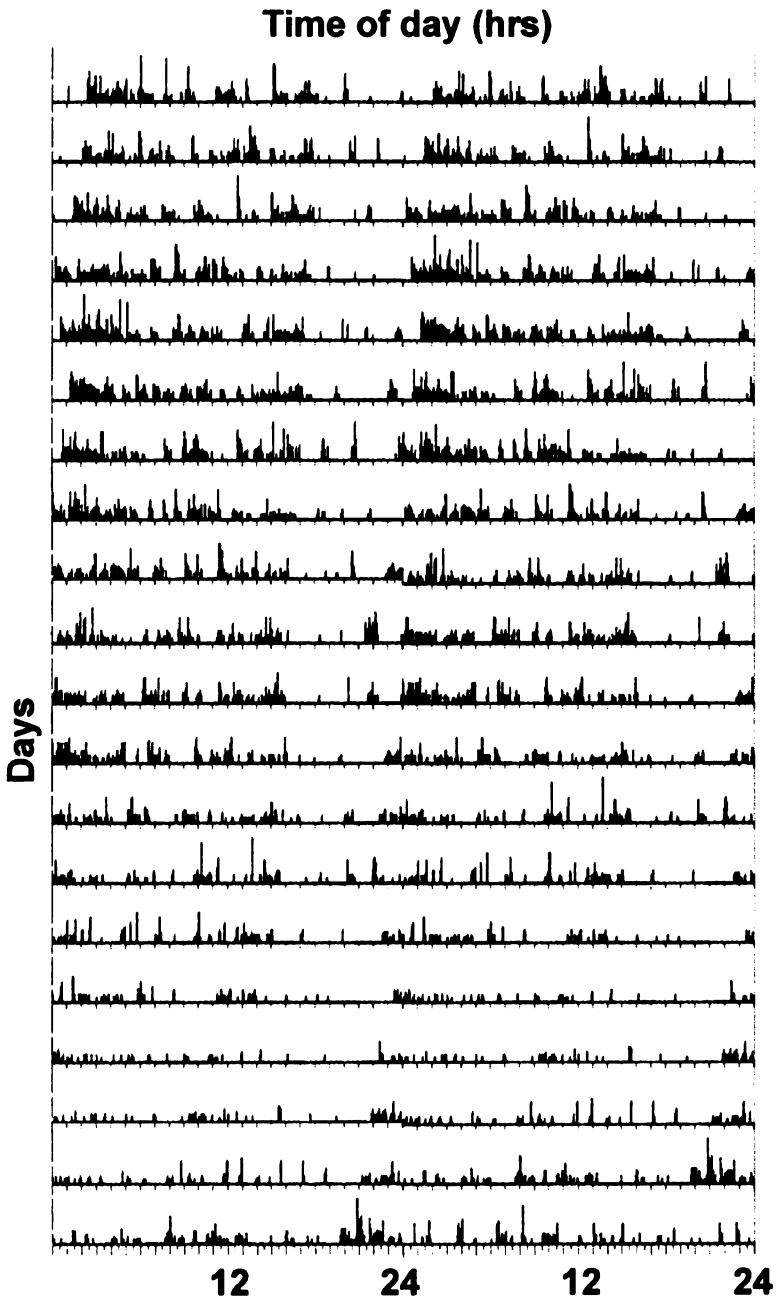
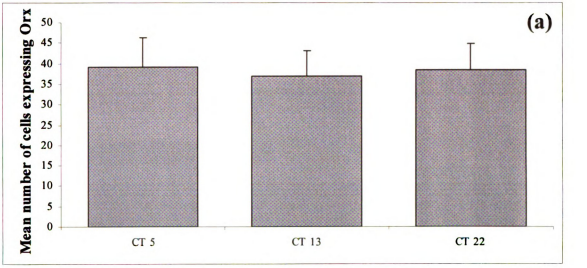

Time of day (hrs)
Days
12
24
12
24

Figure 6. (a) Mean (± S.E.M.) number of ORX-ir cells (both single and double labeled) at three different circadian times (CTs). (b) Mean (±S.E.M.) number of ORX-ir cells (both single and double labeled) in three different areas (medial, central, and lateral). Means with different letters (a, b, or c) are significantly different from each other.


(a)
Mean number of cells expressing Orx
50
45
40
35
30
25
20
15
10
5
0
CT 5
CT 13
CT 22

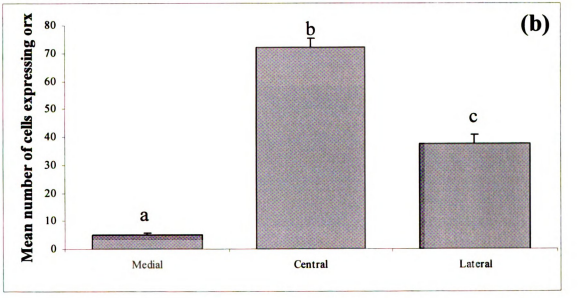
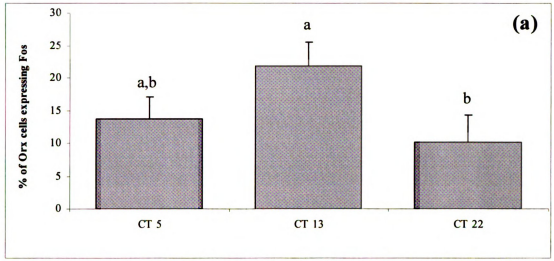

(b)
Mean number of cells expressing orx
80
70
60
50
40
30
20
10
0
a
b
c
Medial
Central
Lateral

Figure 7. (a) Percentage (mean ±S.E.M.) of ORX-ir cells that contained Fos-ir nuclei at three different circadian times (CTs). (b) Percentage (mean ±S.E.M.) of ORX-ir cells that contained Fos-ir nuclei across areas. Means with different letters (a, b, or c) are significantly different.


(a)
% of Orx cells expressing Fos
30
25
20
15
10
5
0
a,b
a
b
CT 5
CT 13
CT 22

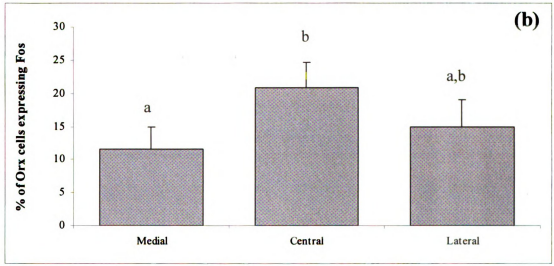
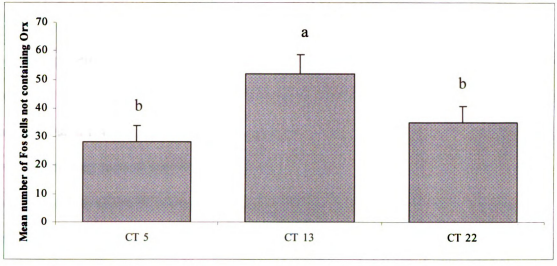
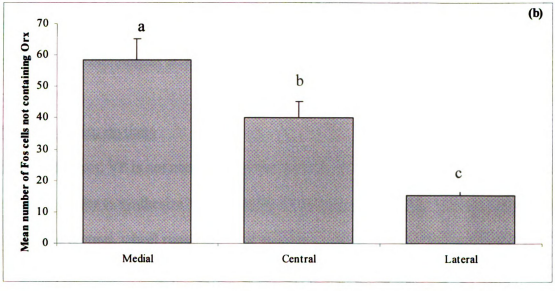

(b)
% of Orx cells expressing Fos
30
25
20
15
10
5
0
a
b
a,b
Medial
Central
Lateral

Figure 8. (a) Percentage (mean ±S.E.M) cells that contained only Fos-ir nuclei at three different circadian times (CTs); (b) Percentage (mean ±S.E.M.) of cells containing Fos-ir nuclei and not labeled for ORX across areas. Means with different letters (a, b, or c) are significantly different from each other.


Mean number of Fos cells not containing Orx
70
60
50
40
30
20
10
0
a
b
b
CT 5
CT 13
CT 22


(b)
Mean number of Fos cells not containing Orx
70
60
50
40
30
20
10
0
a
b
c
Medial
Central
Lateral

EXPERIMENT 3: COMPARATIVE ANALYSIS OF SCN INPUTS TO BRAIN AREAS INVOLVED IN THE REGULATION OF AROUSAL IN THE GRASS RAT AND THE LAB RAT

In Experiment One neural activity in an area regulating arousal in a diurnal species showed a pattern reversed from what has been reported in the nocturnal lab rat. Further, as previously shown for lab rats (Estabrooke et al., 2001), the results of Experiment 2 support the view that this rhythm is endogenous in grass rats. Interactions between the SCN and areas controlling arousal may be responsible for differences between diurnal and nocturnal species. Actions of the SCN on regions regulating arousal may be mediated by a direct neuronal connection between the SCN and neuronal systems that control vigilance. The SCN may transmit rhythmic information by releasing specific peptides into the target neurons. In lab rats, most neurons in the dorsomedial SCN synthesize VP, whereas neurons in the ventrolateral SCN synthesize VIP and/or GRP (van Esseveldt, Lehman, & Boer, 2000).

VP and circadian rhythms

In lab rats, VP is not necessary for the generation of circadian rhythms. Despite the lack of the ability to synthesize VP, rats of the Brattleboro strain exhibit circadian rhythms in sleep and arousal, wheel running, pineal serotonin, and drinking, either in LD or DD conditions, (Brown & Nunez, 1989; Groblewski, Nunez, & Gold, 1981; Peterson, Watkins, & Moore, 1980). These rhythms however, exhibit reduced circadian amplitude, which suggests that VP may serve to amplify or modulate rhythmicity (Ingram et al., 1998). Studies in vitro seem to support the idea of a modulatory effect. Thus, VP administration to slices of tissue from Brattleboro rats produces a dose dependent increase in activity in

VP sensitive neurons from the SCN (Ingram, Snowball, & Mihai, 1996) Although VP may not be essential for the expression of circadian rhythms within the SCN, there is evidence that the presence of the neurons expressing VP in the SCN affects the expression of circadian rhythms. Differences in daily activity level, time of peaks of activity, and period of free running rhythms are associated with differences in the number of VP-immunoreactive neurons in the SCN of mouse and rat strains selected on the basis of their nest-building behavior (Bult, Hiestand, Van der Zee, & Lynch, 1993; Wollnik & Bihler, 1996).

Independently of the role in generation or modulation of circadian rhythmicity within the SCN, it is clear that the release of VP plays an important role in the control of endocrine rhythms by the SCN. VP is involved in the regulation of daily rhythms of corticosterone (Kalsbeek, van Heerikhuize, Wortel, & Buijs, 1996). Infusions of a VP antagonist in the DMH increase levels of corticosterone (Kalsbeek et al., 1996), suggesting that vasopressinergic inputs to this area regulate the rhythm in adrenal corticosterone production that is abolished by SCN lesions (Moore & Eichler, 1972). Some evidence also suggests a excitatory role of VP in the preovulatory surge of luteinizing hormone (LH) if applied to the medial preoptic area during the second half of the light phase(Palm, Van Der Beek, Wiegant, Buijs, & Kalsbeek, 1999; Palm, van der Beek, Wiegant, Buijs, & Kalsbeek, 2001).

VIP and circadian rhythms

Four major VIP systems have been described in the brain: (1) an intracerebral cortical system, (2) one innervating the central amygdala and nucleus of the stria terminalis, (3) a pathway originating in the SCN of the hypothalamus and (4) another originating in the central grey of the midbrain (Sims, Hoffman, Said, & Zimmerman,

1980). Tract tracing studies have shown that after retrograde-tracer injections in the posterior hypothalamus, labeled cells co-localizing either VIP are not present in regions outside the SCN. This suggests that fibers containing VIP seen in the posterior hypothalamus represent direct inputs from the SCN to this region (Abrahamson, Leak, & Moore, 2001).

VIP neurons in the SCN receive input from the RHT and the IGL (Abrahamson & Moore, 2001a), innervate the entire SCN, and project to areas known to be SCN targets, such as the lateral septum, the sPVZ, the medial preoptic area, the DMH, the LHA, and the PVT (Abrahamson & Moore, 2001a; Watts, 1991).

Several studies suggest that VIP is required for normal expression of photic entrainment of the circadian clock and for conveying this entraining signal to the whole SCN (Piggins & Cutler, 2003): (1) VIP neurons in rats express Fos in response to light (Romijn, Sluiter, Pool, Wortel, & Buijs, 1996). (2) VIP alone or in combination with other peptides phase shifts circadian rhythms of wheel running activity in hamsters (Piggins, Antle, & Rusak, 1995). (3) A receptor for VIP and pituitary adenylyl cyclase-activating peptide (PACAP) is widely distributed within the SCN (Alberch & Gale, 1985; Piggins et al., 1995).

In addition to its role within the SCN, several studies suggest a role for VIP as an output signal of the SCN that controls endocrine rhythms: VIP neurons of the SCN project to gonadotropin-releasing hormone (GnRH) neurons in the preoptic area (Kriegsfeld, Silver, Gore, & Crews, 2002; Van der Beek, Horvath, Wiegant, Van den Hurk, & Buijs, 1997) and infusions of VIP antisense into SCN decrease the amplitude of the LH surge (Harney, Scarbrough, Rosewell, & Wise, 1996). Lesions of the SCN abolish VIP contacts on GnRH neurons (van der Beek, Wiegant, van der Donk, van den Hurk, & Buijs, 1993).

Together, these observations suggest a role for VIP in the regulation of the estrous cycle in the rat. VIP fibers, presumed to originate in the SCN, also project to neuroendocrine dopaminergic neurons, a pathway involved in regulation of prolactin secretion (Gerhold, Horvath, & Freeman, 2001). Further, infusion of VIP antisense abolishes rhythms of corticosterone secretion (Scarbrough, Harney, Rosewell, & Wise, 1996).

Differences between nocturnal and diurnal rodents

Although much is know about how the SCN controls circadian rhythms in nocturnal species, relatively little is known about how the SCN communicates circadian rhythmicity to brain regions that regulate specific behaviors in diurnal species. It is known for example, that the SCN projects to about the same regions in rats (Leak & Moore, 2001; Watts, 1991), hamsters (Kriegsfeld, Leak, Yackulic, LeSauter, & Silver, 2004; Morin et al., 1994), humans (Dai, Swaab, Van der Vliet, & Buijs, 1998; Dai, Van Der Vliet, Swaab, & Buijs, 1998), and more recently in the grass rat (Schwartz & Smale, 2004). It is also known that both VP and VIP are present in the SCN of almost all mammals (as reviewed in Smale & Boverhof, 1999). Despite these similarities, recent studies suggest that some differences between nocturnal and diurnal species may be related to differences in the connections between specific subpopulations of neurons in the SCN and other brain regions or on how targets of the SCN respond to common signals emanating from SCN neurons (Smale et al., 2003).

In diurnal and nocturnal species brain areas related to sleep and wakefulness show different patterns of activity over the light dark cycle and these patterns of activity exhibit different phase relationships with activity in the SCN (Martinez et al., 2002; Novak, Smale et al., 2000); Experiment 1 and 2). Differences between species may be related to

differences in signals that originate in the SCN or from relay cells that receive SCN inputs and project to effector systems that control behavior.

The studies described here investigated neural connections between the SCN and two areas controlling arousal (LHA and TMN) to determine if neurons that produce VIP or VP contribute to these connections in lab and grass rats

Methods

Animals.

Three adult female grass rats and three adult female lab rats (Long Evans) were used for this study.

Immunocytochemistry

Animals were perfused as described in General Methods, except that 4% paraformaldehyde was used instead of PLP. Tissue was then processed for dual ICC using the general procedure described earlier (p. 15). Reagents used for each reaction were as follows:

VIP/ORX

VIP: NDS (Jackson), guinea pig anti-VIP (Peninsula Laboratories, Belmont, CA; 1:1500 for lab rats, 1:1000 for grass rats), and biotinylated donkey anti-guinea pig (Jackson, 1:200). ORX: NHS (Vector), goat anti-ORX B (Santa Cruz, 1:60,000), and biotinylated horse anti-goat (Vector, 1:200).

VP/ORX

VP: NGS (Vector), guinea pig anti-VP (Peninsula, 1: 40,000), and biotinylated goat anti-guinea pig (Jackson, 1:200). ORX: NHS (Vector), goat anti-ORX B (Santa Cruz, 1:60,000), and biotinylated horse anti-goat (Vector, 1:200). .

VIP/HISTAMINE

VIP: NDS (Jackson), guinea pig anti-VIP (Peninsula Laboratories, Belmont, CA; 1:2000), and biotinylated donkey anti-guinea pig (Jackson, 1:200). Histamine: NGS (Vector), rabbit anti-histamine (Sigma, St Louis, MO; 1:2,000), and biotinylated goat anti-rabbit (Vector, 1:200).

VP/HISTAMINE

VP: NGS (Vector), guinea pig anti-VP (Peninsula, 1: 80,000), and biotinylated goat anti-guinea pig (Jackson, 1:200). Histamine: NDS (Jackson), rabbit anti-histamine (Sigma, St Louis, MO; 1:2,000), and biotinylated donkey anti-rabbit (Jackson, 1:200).

Data Analysis

High resolution photomicrographs of areas known to receive SCN projections in rats and mouse (Abrahamson et al., 2001)were taken using a digital camera (AxioCam MRc, Car Zeiss, Gottingen, Germany) attached to a Zeiss light microscope (AxioScop 2 plus) to confirm labeling for VIP or VP in both lab and grass rats. Image, contrast, and balance were adjusted using Zeiss Axiovision Software (Carl Zeiss Vision).

To examine the relationship between VIP or VP projections from the SCN and ORX-ir cells a picture of every section containing ORX neurons (average of 16 sections per animal) was taken at lower magnification (10X) to visualize the whole region of the hypothalamus with ORX+ cells. Counts of the total number of these neurons were made by evaluating the tissue at 40X magnification. After this, every neuron stained for ORX was evaluated at high magnification (100X, oil immersion lens) to determine if it was contacted by a VIP or VP labeled fiber; only axosomatic contacts of VIP or VP fibers on ORX neurons were considered. As proposed by Kriegsfeld et al. (2002) only those cases in which a VIP or VP bouton-like structure was observed in apposition with ORX neurons,

the bouton and the fiber were on the same focal plane, and the labeled bouton was a clear continuation of axon were scored as contacts (Figure 12). By following these criteria, those cases in which the bouton looked more like a granule than a fiber were not counted. The origin of VP fibers was determined by contrasting them with previous descriptions of these fibers in mice (Abrahamson & Moore, 2001b). Thus, VP fibers originating in the SCN are fine caliber and have irregularly spaced varicosities (Figure 10). In contrast, fibers originating outside the SCN are large caliber with very few varicosities (Abrahamson & Moore, 2001b)(Figure 10). With these data, the percentage of ORX neurons contacted by VIP or VP fibers per animal was determined. The non parametric Mann-Whitney U Test was used to test the differences in the number of fibers contacting Orx neurons between the two species

To evaluate SCN inputs to histaminergic neurons drawings of neurons containing histamine in the TMN were first made using a 5X lens. After this, each histaminergic neuron was observed under the microscopy using a 100X magnification lens, to determine if any labeled fiber was in close contact to it. Because very few labeled fibers of SCN origin were seen in the area that has histaminergic neurons, no statistical comparisons of the two species were made for this system.

Results

Overall distribution of VIP and VP

As it has been reported previously in lab rats, VIP fibers are dense in the sPVZ (Figure 9a), moderate in the PVT (Figure 9c), and less dense in the anterior hypothalamus (Figure 9b). Similar distribution is seen in grass rats but a clear difference was found at the level of the anterior hypothalamus where only few VIP fibers were seen for grass rats (Figure 9b).

In lab rats, VP fibers are abundant in the anteroventral periventricular nucleus (AVPe) and the AHA, moderate in the DMH and even less dense in the ventromedial hypothalamus (VMH). A similar distribution was seen in grass rats (Figure 11).

VP and VIP contacts on ORX neurons

Analysis of VP contacts showed very few VP/ORX contacts in both grass rats and lab rats, and in both cases those contacts seemed to come from neurons of the supraoptic nucleus (SON) and the paraventricular nucleus of the hypothalamus (PVN) rather than from the SCN; in contrast to fine caliber fibers from the SCN, fibers from the SON are large caliber and show few varicosities (Abrahamson & Moore, 2001b) (Figure 10).

Inspection of each animal's tissue under high magnification showed clear species differences in the abundance of VIP fibers approaching ORX neurons. Grass rats had proportionally more VIP contacts on ORX neurons compared to lab rats (7.9% and 1.6% respectively; U=0, p=0.05, one-tailed). See Figure 13

VP and VIP contacts on Histaminergic neurons

Given that histaminergic neurons in the dorsal TMN were sometimes difficult to differentiate from other neurons in this region, the analysis presented here was restricted to histaminergic neurons in the vTMN.

VIP fibers were seen in the vTMN, but contacts between VIP fibers and histaminergic neurons were rare in both grass and lab rats. The same was true for VP fibers in grass rats. Some VP fibers that seemed to originate in the SCN were seen in the vTMN but none in contact with histaminergic neurons.

Problems with background in lab rat tissue reacted for VP and histamine made it impossible to determine if histaminergic neurons were contacted by VP fibers from the

SCN. However, visual inspection of tissue single-labeled for VP showed, similar to grass rats, very few VP fibers from SCN origin in the vTMN (Figure 14).

Discussion

SCN inputs to neurons containing ORX

In grass rats there was evidence for a substantial contribution of VIP neurons of the SCN to the inputs received by ORX neurons. In contrast, very few contacts of this type were seen in lab rats. Differences in VIP projections from the SCN to ORX neurons may contribute to species differences in the regulation of the sleep wake cycle: whereas in grass rats regulation may be in part mediated by direct projections from the SCN to areas involved in arousal, in the nocturnal lab rat this regulation may involve an indirect pathway that connects the SCN to neurons in the lateral and posterior hypothalamus. Recent studies revealed several candidates in lab rats that may serve as relay centers between the SCN and areas that control vigilance including the medial preoptic area, the sPVZ, and the DMH (Aston-Jones et al., 2001; Deurveilher & Semba, 2005). Although it may be argued that differences between species seen here may be due to differences in the quality of the VIP staining, images taken at the level of the SCN contradict this possibility. In both species VIP fibers are readily identifiable in and above the SCN (Figure 9).

In a previous study with lab rats, biotinylated dextran amine (BDA) injections in the SCN resulted in a high number of labeled fibers in close proximity to ORX neurons, and injections of CTB in the posterior hypothalamus showed retrograde labeling of cells that contained VP or VIP in the SCN (Abrahamson et al., 2001). Although it was reported in that study that immunocytochemical analysis revealed frequent contacts between VP and VIP fibers with ORX neurons, no data supporting that statement were presented (Abrahamson et al., 2001). The present data show very few contacts between VIP fibers

and ORX neurons and no contact between VP fibers from SCN origin and ORX neurons in lab rats. Since no data were presented in the study by Abrahamson et al., (2001), it is difficult to account for the apparent discrepancies. It is possible that differences are related to differences in the type of contacts counted in each study. Whereas in the previous study both axosomatic and axodendritic putative contacts were considered, in the present study only axosomatic contacts were counted. Proximity to the trigger zone in axosomatic synapses allows for a faster and more profound effect compared to the more distal axodendritic synapses.

SCN inputs to histaminergic neurons in the TMN

Due to technical difficulties, data for the histaminergic system are available only for inputs to the ventral TMN, which is in fact the region that seems to be under circadian control, at least in the diurnal grass rat. Neurons in this particular region rhythmically express Fos in a pattern clearly correlated with the sleep wake cycle of the grass rat and opposite to the one seen in the VLPO and that is (Novak, Smale et al., 2000). In lab rats, instead, no rhythm in Fos expression has been observed in any part of the TMN (Novak & Nunez, 1998).

In contrast to what was seen in the neurons expressing ORX, no differences between species were found in SCN projections to histaminergic neurons. Although in both species VIP fibers were seen in the vicinity of neurons expressing histamine, these labeled fibers rarely contacted histaminergic cell bodies in either grass or lab rats. In grass rats contacts between VP fibers of SCN origin and histaminergic neurons were also very rare. Data for VP fibers from SCN origin are less conclusive for lab rats because of the presence of substantial background after double labeling with VP and histamine. However, by comparing data from the double-labeled tissue, with tissue labeled only for VP and

control tissue in which the primary for VP was omitted, it is clear that there are very few inputs from VP SCN neurons to the histaminergic system of both species

Previously it has been suggested that differences between diurnal and nocturnal species may involve differences in the strength of circadian regulation of brain regions that support wakefulness (Novak, Smale et al., 2000). The data for the ORX system are consistent with this interpretation since, at least with respect to VIP, more direct inputs of SCN origin to ORX neurons are evident in the diurnal species, thus suggesting stronger circadian regulation of this arousal system in grass rats.

The next experiment looks at possible differences in the regulation by light of these brain regions through direct retinal inputs.

Figure 9. Distribution of VIP fibers in lab and grass rats. Similar to lab rats, VIP fibers in grass rats are dense in the sPVZ (a) and moderate in the PVT (c) but, in contrast to lab rats, only very few VIP fibers are present in the AHA of grass rats (b). VIP, vasoactive intestinal polypeptide; sPVZ, subparaventricular zone of the hypothalamus; PVT, paraventricular nucleus of the thalamus; AHA, anterior hypothalamic area.

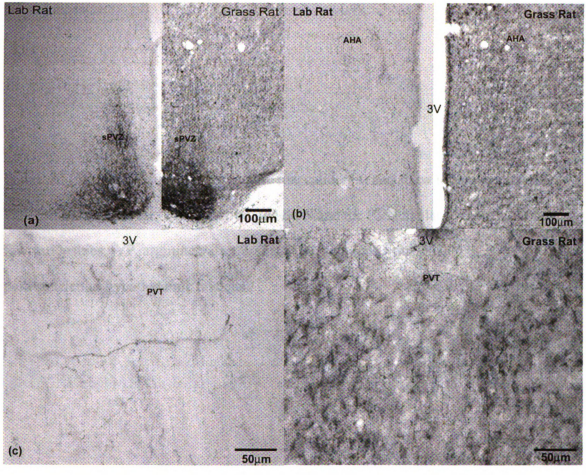

Lab Rat
Grass Rat
sPVZ
sPVZ
100μm
(a)
Lab Rat
Grass Rat
AHA
AHA
3V
100μm
(b)
3V
Lab Rat
PVT
(c)
50μm
3V
Grass Rat
PVT
50μm

Figure 10. VP Fibers from SCN origin are fine caliber (a) and show irregularly spaced varicosities. In contrast, VP fibers from the SON and the PVN are large caliber and show few varicosities. VP, arginine vasopressin; SON, supraoptic nucleus; PVN, paraventricular hypothalamic nucleus.

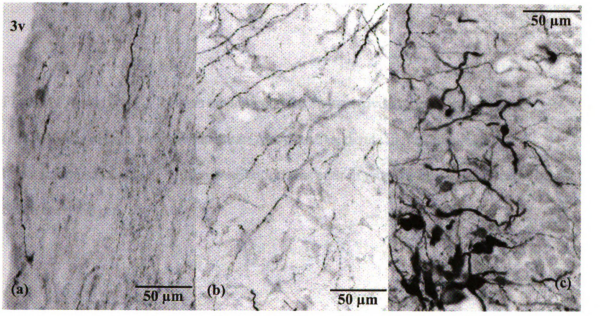

3v
(a)
50 µm
(b)
50 µm
50 µm
(c)

Figure 11. In lab rats, VP fibers are abundant in the anteroventral periventricular nucleus (AVPe) and the anterior hypothalamus (AHA) and moderate in the dorsomedial hypothalamus (DMH). A similar distribution was seen in grass rats. 3V, third ventricle. Scale bar = 50 μm

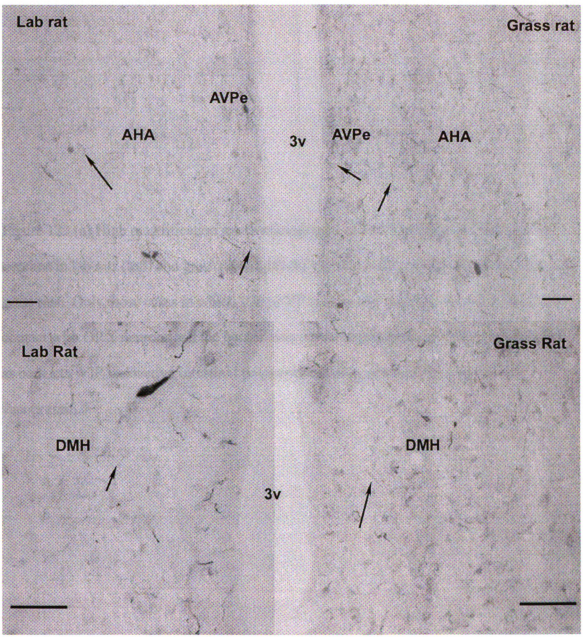

Lab rat
Grass rat
AVPe
AHA
3v
AVPe
AHA
Lab Rat
Grass Rat
DMH
3v
DMH

Figure 12. (a) High magnification micro photographs of VIP fibers approaching ORX neurons in lab rats (left) and grass rats (right) (b) VP fibers approaching an ORX neuron in grass rats. Only those cases in which VIP or VP bouton-like structure was observed in contact with ORX neurons and the bouton was a clear continuation of a fiber were scored as contacts. VIP vasoactive intestinal polypeptide; ORX, orexin; VP, arginine vasopressin.

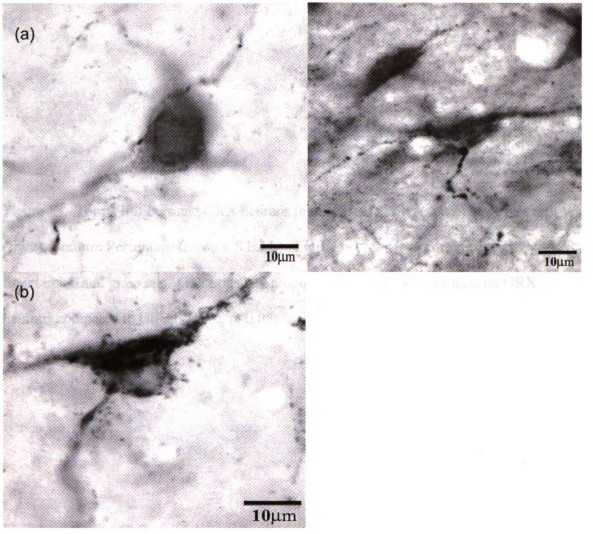
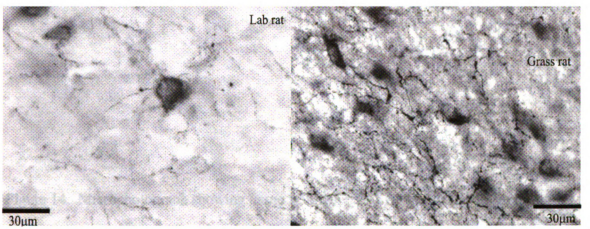

(a)
10μm
10μm
(b)
10μm

Figure 13. Top: VIP fibers and ORX neurons in the LHA in lab rats (left) and grass rats (right). Bottom: Percentage (mean ± S.E.M.) of ORX neurons in apparent contact with VIP fibers in lab and grass rats. Grass rats had proportionally more VIP contacts on ORX neurons compared to lab rats ($U=0$, $p<0.05$).


Lab rat
Grass rat
30µm
30µm

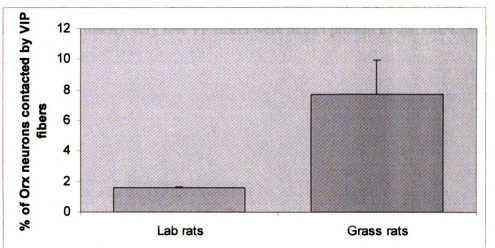
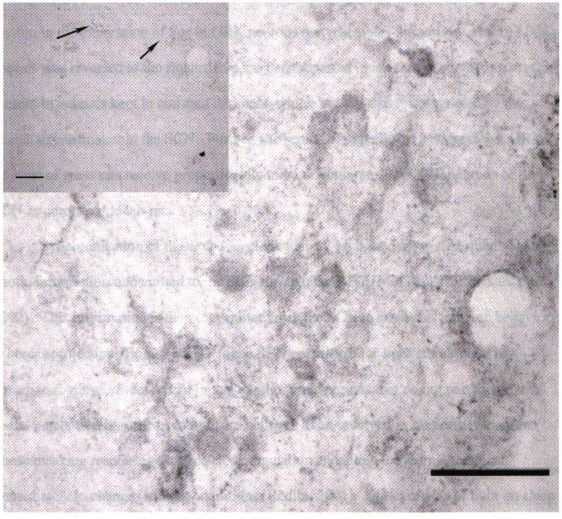

% of Orx neurons contacted by VIP fibers
12
10
8
6
4
2
0
Lab rats
Grass rats

Figure 14. Microphotograph showing tissue from lab rat single labeled for vasopressin (VP). Although VP fibers were clearly seen in regions dorsal to the tuberomammillary nucleus (TMN, see insert) very few VP fibers were in fact present in the TMN. Scale bar = 50 μm



EXPERIMENT 4: RETINAL INPUTS TO BRAIN REGIONS INVOLVED IN THE REGULATION OF AROUSAL

Data from the previous experiments suggest that there are clear differences in how the SCN regulates sleep and wakefulness in nocturnal and diurnal species. In grass rats a rhythm in the expression of Fos in ORX neurons that resembles the activity pattern of this species was reversed to the rhythm that has been reported in lab rats. This rhythm was also present in animals kept in constant darkness, which suggest that it is regulated by the circadian pacemaker in the SCN. Further, the results of experiment 3 suggest that ORX neurons of grass rats receive proportionally more direct inputs from VIP neurons of the SCN as compared to lab rats.

Synchronization of the circadian clock to the 24 h light dark cycle is mediated by photic information transmitted to the SCN through the RHT (Moore, 1973; Pickard et al., 2002). This information is then transmitted from the SCN to areas that regulate behavior. Whereas projections from the RHT to the SCN are critical for light entrainment of circadian rhythms, direct retinal inputs to other brain regions that do not participate in visual perception or reflexes to light are believed to be involved in masking responses. These masking responses override clock regulation and allow the animal to adjust immediately to changes in light conditions (Redlin, 2001). Direct effects of light on sleep vary depending on whether the animal is diurnal or nocturnal. In nocturnal species, light exposure for 1-3h will increase NREM sleep; this increase is independent of the circadian sleep wake cycle (Alfoldi, Franken, Tobler, & Borbely, 1991). Further, light pulses applied during the night, decrease activity in nocturnal animals (review in Mrosovsky, 1999). On

the other hand, light presented during the night increases alertness (Campbell & Dawson, 1990) and activity (Gander & Moore-Ede, 1983) in diurnal species.

Little is known about the pathways that mediate these masking effects, but direct retinal inputs to areas involved in the promotion of sleep and arousal have been described. Initial studies on rats and hamsters reported retinal inputs to the sPVZ and the LHA (Johnson, Morin, & Moore, 1988; Levine, Weiss, Rosenwasser, & Miselis, 1991). Several studies have also identified direct retinal projections to the DRN in rats (Shen & Semba, 1994), Mongolian gerbils (Fite, Janusonis, Foote, & Bengston, 1999), and the Chilean degus (Fite & Janusonis, 2001) and to the VLPO (Lu, Shiromani, & Saper, 1999) (Novak et al., 1999). In addition, recent studies with lab rats have shown that retinal ganglion cells containing melanopsin (the photopigment in retinal cells that project to the SCN) also project to the VLPO and the sPVZ (Gooley, Lu, Fischer, & Saper, 2003).

Differences in activity in brain regions regulating arousal in diurnal and nocturnal species may result not only from circadian modulation but also from differential effects of light on cells in those regions. Although very few studies have compared the magnitude of particular retinal projections in nocturnal and diurnal species, the evidence available suggests that retinal projections to areas regulating arousal, i.e. the DRN, are greater in diurnal species (Fite & Janusonis, 2001; Fite et al., 1999) than in lab rats (Shen & Semba, 1994), and retinal projections to areas that promote and support sleep (i.e., the VLPO) are instead more extensive in nocturnal (Gooley et al., 2003; Lu et al., 1999) than in diurnal species (Novak et al., 1999; Smale et al., 2003). These data suggest that as animals adopted a diurnal pattern of activity, retinal projections to areas that support wakefulness were enhanced and retinal inputs to areas that support sleep were instead reduced. If this is

the case, we can expect to find extensive retinal projections to the DRN and perhaps to other areas that support wakefulness in grass rats

The goal of the experiments presented below was to describe the retinal input to areas involved in the regulation of arousal, more specifically to the TMN, the DRN, and areas expressing ORX. In addition, it describes the distribution of serotonergic neurons in the DRN in the grass rat, a diurnal rodent.

Methods

Retinal injections

To determine retinal inputs to brain regions involved in the regulation of arousal, three grass rats and three lab rats received unilateral retinal injections of the anterograde tracer Cholera toxin subunit β (CTβ, List Biological Laboratories, Campbell, CA) diluted (1:1) with 4% dimethyl sulfoxide (DMSO) and 1.8% saline. While the animal was under metofane (Methoxyflurane, Pitman-Moore) anesthesia, 5µl of the CTB solution were injected behind the sclera into the posterior vitreous chamber of the eye using a 10µl Hamilton Syringe. The solution was injected over 30 seconds, and the needle was left in for about 3 minutes before being slowly removed. Animals were allowed 6 recovery days and then perfused.

Immunocytochemistry

Tissue was processed for single ICC for CTß using the following reagents: NHS (Vector), goat anti-CTß (List, 1:10,000), and horse anti-goat (Vector, 1:200). To identify retinal projections to the TMN and the DRN tissue from grass rats was processed for dual ICC with the following reagents: CTß: NHS (Vector), goat anti-CTß (List, 1:10,000), and horse anti-goat (Vector, 1:200). Histamine: NGS (Vector), rabbit anti-histamine (Sigma, 1:1,000), and goat anti-rabbit (Vector, 1:200). Serotonin: NDS (Jackson), rabbit anti-5HT

(1:50,000), and donkey anti-Rabbit (Jackson, 1:200). Tissue was then mounted, and coverslipped. Sections immunostained for CTB were analyzed under a Zeiss AxioScop 2 using at100X magnification lens to determine the presence of labeled fibers in the selected areas. An atlas of the rat brain was used to identify the nuclei and brain regions of interest (Paxinos & Watson, 1998).

Results

Comparative analysis of labeling in areas involved in the regulation of arousal in grass and lab rats showed clear differences. In grass rats, retinal fibers were absent in the LHA and the perifornical region, scattered in the TMN, and absent in the DRN (Figure 15). In lab rats, labeled fibers were instead moderate in the lateral hypothalamic area dorsal to the SON, also almost absent in the TMN, and moderate in the lateral "wings" of the DRN (Figure 16). No retinal fibers were seen in the PVT in either grass or lab rats.

As reported previously in lab rats, dense CTB labeled retinal fibers were prominent in the SON and the superior colliculus (SC) and moderate in the VLPO (Figure 17). In grass rats, very strong labeling was also seen in the SC and the SON whereas very sparse retinal fibers were seen in the VLPO as has been previously reported (Figure 18).

Distribution of serotonergic neurons in the DRN

The distribution of serotonergic neurons in the DRN was similar to that described previously in rats (Azmitia, 1978; Jacobs & Azmitia, 1992). Neurons in the DRN are clearly distributed in three main groups: a medial, a lateral, and a caudal component. Neurons in the medial component extend rostrally from the caudal border of the oculomotor nuclei and finally merge with the caudal component that lies dorsally to the MRN and behind the superior cerebellar decussation. A few neurons in this component are distributed in the dorsal part and most of them lie in the medial part of the ventral region.

The higher concentration of serotonergic neurons is in the lateral component (ventrolateral DRN), which extends also from the oculomotor nuclei. The caudal component is clearly seen below the fourth ventricle. At most rostral levels, serotonergic neurons are localized mainly in the medial region of the DRN. Moving caudally, serotonergic neurons organize in lateral, dorsal and ventral clusters. In the most caudal level of the DRN (at the level of the dorsal tegmental nucleus), serotonergic neurons concentrate mostly in the dorsal region (Figure 19)

Discussion

Different from what was expected for the grass rat, retinal projections were rare or absent in areas of the brain that support wakefulness in this diurnal species. In contrast, and consistent with other reports (Fite et al., 1999; Shen & Semba, 1994), evidence for a direct retinal projection to the DRN and to the perifornical LHA, the site of residency for ORX cells, was obtained for the nocturnal lab rat. In both species, the histaminergic cell groups of the TMN lacked any substantial retinal input. The results also confirmed previous observations of a substantial retinal input to the VLPO of lab rats (Gooley et al., 2003; Lu et al., 1999) and its relative absence in the VLPO of the diurnal grass rats (Novak et al., 1999; Smale et al., 2003). The species differences seen here and in other studies are not likely due to differences in the transport of the tracer from retina to brain in the two species, since robust labeling of retinal fibers was seen in several other regions of the brain in grass rats, including the SC and the dorsal border of the SON. CTB labeling in grass rats is also confirmed by the presence of fibers in the oculomotor nucleus in the midbrain.

The available data for grass and lab rats do not support the hypothesis that diurnality is associated with a reduction in retinal inputs to areas that promote sleep with a concurrent increase in retinal inputs to areas that promote wakefulness. Instead the data

suggest that in grass rats the development of a diurnal pattern of activity is associated with a general reduction in retinal input to areas that control vigilance. One prediction from these anatomical observations would be that the masking effects of light on sleep and arousal should be absent or diminished in grass rats, in contrast to was has been reported for nocturnal species and perhaps other diurnal animals as well. In this context it is interesting that when grass rats are kept in darkness and presented with light pulses of one hour at ZT 2 and ZT 13, they do not show any evidence of masking effects of light on their general activity, even though the same light exposure suppresses wheel running in these animals (Redlin & Mrosovsky, 2004). More comparative studies on the effects of masking are needed to evaluate the functional consequences of the anatomical differences in the retinal projections of lab and grass rats.

The results presented here differ from those of studies on retinal projections in two other diurnal species, the Chilean degus and the Mongolian gerbil. In both species, substantial retinal inputs to the DRN have been described using methods similar to those used here. However, it must be noted that even between these two species there are differences in the density of the retinal projections; although substantial projections from the retina are present in the DRN of the Chilean degus, the extent and density of these projections is not as pronounced as in Mongolian gerbils (Fite & Janusonis, 2001) Similar to grass rats, retinal projections to the DRN were not observed in the ground squirrel, another diurnal species (Major, Rodman, Libedinsky, & Karten, 2003). Differences between these diurnal animals may be related to changes that occurred in the independent transitions from nocturnality to diurnality of each one of these species (Smale et al., 2003)

In lab rats and perhaps in other species, direct projections from the retina to the DRN may mediate acute effects of light on serotonin synthesis and/or release. For

example, variations in serotonin content in several regions of the brain are due to direct changes in the environmental light and not to circadian factors; animals kept in constant conditions do not show diurnal variations in serotonin (Ferraro & Steger, 1990).

It is still unknown whether light has any effect on ORX release. Studies with SCN-lesioned animals showed that the rhythm of ORX in the CSF disappeared under LD conditions, but the possibility still exists that in those studies the retinal projections were also damaged by the lesions (Zhang et al., 2004). However, the persistence of the ORX rhythm under constant conditions argues for an endogenous regulation by the circadian clock rather that a regulation by changes in illumination. This lack of a direct role of light in the regulation of neural activity seems to be also true for the histaminergic system. Changes in activity of histaminergic neurons of the TMN of lab rats are related to the behavioral state rather than to changes in the light dark cycle: activity increases when the animals are awake and decreases when the animal sleeps, a change that occurs even if the animal is kept in constant darkness (Ko, Estabrooke, McCarthy, & Scammell, 2003; Mochizuki et al., 1992).

In addition to possibly mediating masking responses, retinal inputs to the DRN may modulate circadian functions in at least some species. In lab rats and hamsters, for example it has been shown that the ventrolateral region of the SCN receives dense serotonergic innervation (Bosler & Beaudet, 1985; Kawano, Decker, & Reuss, 1996; Moga & Moore, 1997). Serotonin terminals in the SCN have also been reported for the diurnal degus (Goel, Lee, & Smale, 1999). Although serotonin is not necessary for the normal expression of circadian rhythmicity, it alters the circadian system by inhibiting the effects of light (Medanic & Gillette, 1992; Morin & Blanchard, 1991; Pickard, Weber, Scott, Riberdy, & Rea, 1996; Prosser, Miller, & Heller, 1990; Smale, Michels, Moore, & Morin, 1990). The

involvement of the raphe, the primary source of serotonin, in this modulation of the circadian response to light is suggested by studies showing that electrical stimulation of the raphe reduces light-induced effects on Fos expression in the SCN and produces phases shifts in locomotor activity (Meyer-Bernstein & Morin, 1999). However, there is still insufficient information to conclude that this effect is mediated by the retina-DRN-SCN (or IGL) pathway. Some studies even suggest that the effect of serotonin involves the median and not the DRN (Meyer-Bernstein & Morin, 1998) or that neurons receiving the retinal inputs are not the same that project to either the SCN or the IGL (Kawano et al., 1996).

The presence of ORX fibers in the SCN suggests a role in the modulation of the circadian clock function (Mintz, van den Pol, Casano, & Albers, 2001; Nambu et al., 1999; Novak & Albers, 2002; Peyron et al., 1998). In addition, in vitro studies have shown a role of ORX in the regulation of the firing rate of the SCN (Farkas, Vilagi, & Detari, 2002). Evidence suggesting a role of histamine in the regulation of circadian rhythmicity has also been shown as follows: (1) the SCN receives histaminergic projections (Airaksinen & Panula, 1988; Inagaki et al., 1988; Panula, Pirvola, Auvinen, & Airaksinen, 1989); (2) histamine injections induce changes in the circadian phase of free-running rhythms in a manner similar to light: it causes phase delays if applied early in the subjective night and phase advances if applied later in the subjective night (Cote & Harrington, 1993; Itowi, Yamatodani, Nagai, Nakagawa, & Wada, 1990); but see Scott, Piggins, Semba, & Rusak, (1988); and (3) inhibition of histamine synthesis reduces light-induced phase shifts in circadian rhythms of wheel running (Eaton, Cote, & Harrington, 1995). Although in the present experiment I did not find retinal projections to the TMN, the main source of histaminergic neurons, the possibility still exists that these neurons receive photic information through indirect pathways.

The distribution of serotonergic neurons in the DRN was similar to what has been reported in lab rats but differs from Mongolian gerbils. Whereas in Mongolian gerbils serotonergic neurons in the more rostral DRN lie in the lateral regions (Janusonis & Fite, 2001), in grass rats these neurons are mostly restricted to the ventromedial part. The functional significance of these differences remains unknown but the differences are in agreement with the assumption of independent transitions from nocturnality to diurnality.

In summary, this experiment demonstrated differences in retinal projections to areas regulating arousal in the diurnal grass rat and the nocturnal lab rat. Whereas in lab rats retinal inputs reach both the LHA and the DRN, in grass rats inputs to these areas are rare. Different to these areas, the TMN, another area involved in the regulation of arousal, lacks substantial inputs in both diurnal and nocturnal species. Together, data from this and the previous experiment, suggest clear differences in the regulation of sleep and wakefulness in nocturnal and diurnal species. In addition, data from this experiment suggest a differential regulation by light of areas involved in the control of arousal.

The next experiment looks at the distribution of neurons that express PER1 in the grass rat brain and its profile in areas that regulate arousal.

Figure 15. Retinal fibers in areas regulating arousal in grass rats. Retinal fibers were totally absent in the LHA and the perifornical region, scattered in the TMN and absent in the DRN. LHA, lateral hypothalamic area; TMN, tuberomammillary nucleus; DRN, dorsal raphe nuclei.

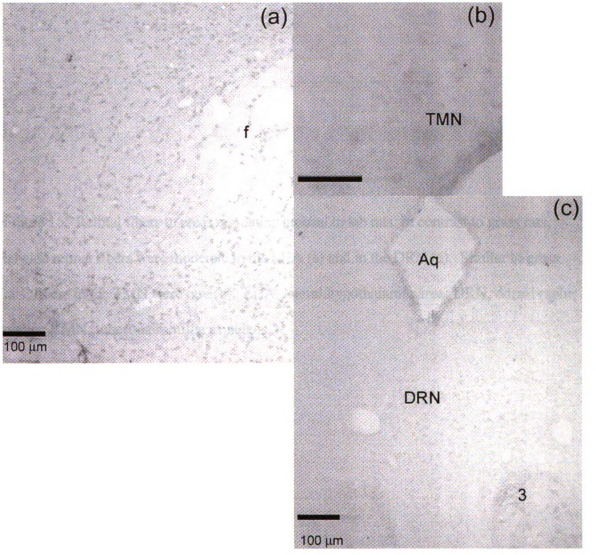

(a)
f
100 µm
(b)
TMN
(c)
Aq
DRN
3
100 µm

Figure 16. Retinal fibers to areas regulating arousal in lab rats. In contrast to grass rats, labeled retinal fibers were moderate in the LHA (a) and in the DRN (c). Similar to grass rats, fibers in the TMN were rare (b). LHA, lateral hypothalamic area; DRN, dorsal raphe nuclei; TMN, tuberomammillary nucleus.

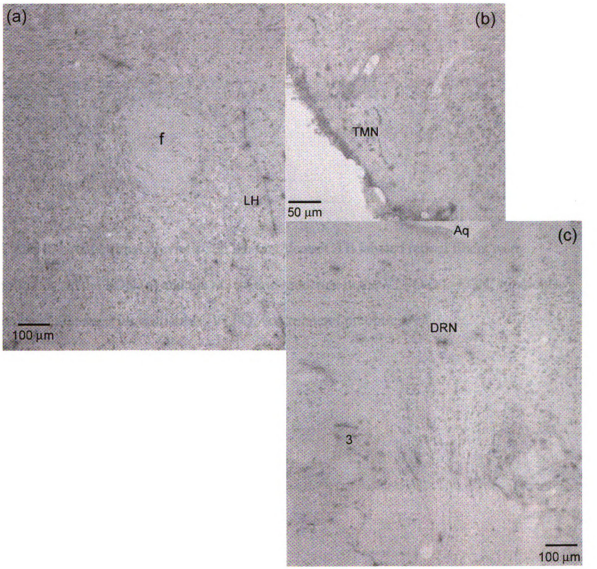

(a)
f
LH
100 µm
(b)
TMN
50 µm
Aq
(c)
DRN
3
100 µm

Figure 17. As reported previously in lab rats, dense CTB labeled retinal fibers were prominent in the SON (a) and the SC (c) and moderate in the VLPO (b). SON, supraoptic nucleus; SC, superior colliculus; VLPO, ventrolateral preoptic area.

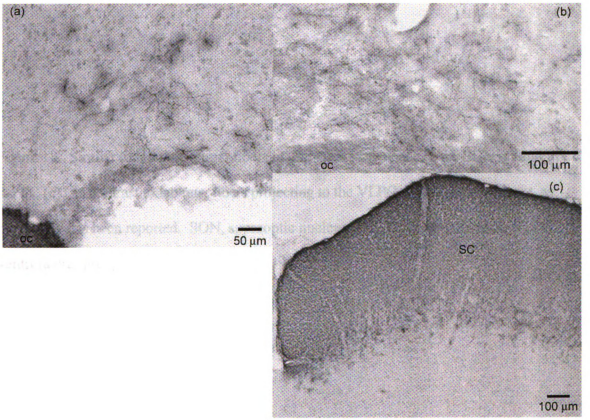

(a)
oc
50 µm
(b)
oc
100 µm
(c)
SC
100 µm

Figure 18. Similar to lab rats, retinal fibers in grass rats were dense in the SON (a) and in the SC (c). In contrast to lab rats, fibers projecting to the VLPO (b) are almost absent, as previously has been reported. SON, supraoptic nucleus; SC, superior colliculus; VLPO, ventrolateral preoptic area.

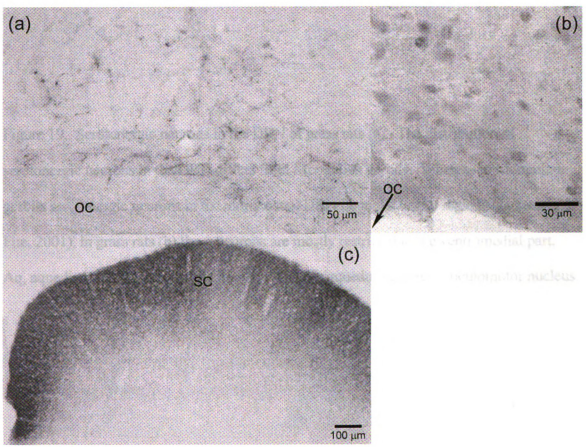
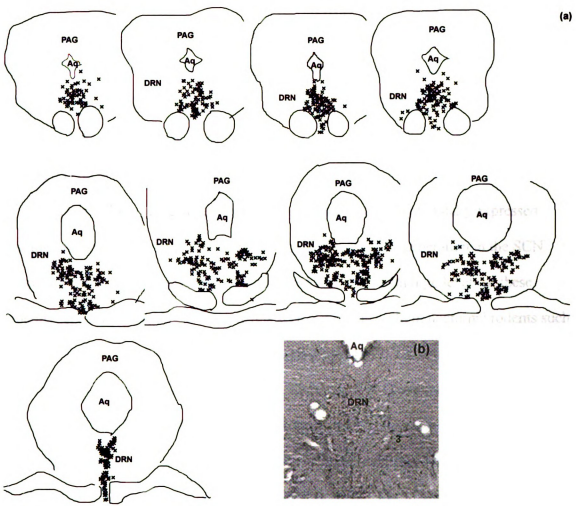
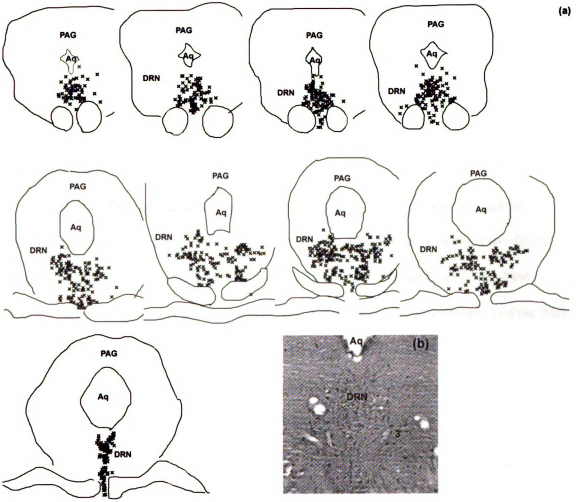

(a)
OC
50 µm
(b)
OC
30 µm
(c)
SC
100 µm

Figure 19. Serotonergic neurons in the DRN of grass rats (a). The distribution of serotonergic neurons in the DRN differs from Mongolian gerbils. Whereas in Mongolian gerbils serotonergic neurons in the more rostral DRN lie in the lateral regions (Janusonis & Fite, 2001), in grass rats (b) these neurons are mostly restricted to the ventromedial part. Aq, aqueduct; DRN, dorsal raphe nuclei; PAG, periaqueductal grey; 3, oculomotor nucleus.


(a)
PAG
Aq
DRN
(b)


(a)
PAG
Aq
DRN
(b)
3

EXPERIMENT 5: CLOCK GENES IN AREAS INVOLVED IN THE REGULATION OF AROUSAL

Circadian rhythms within the SCN are generated by an autoregulatory transcriptional/translational molecular feedback loop that involves different clock genes and their proteins (for a review of the literature on the molecular clock, see Reppert & Weaver, 2001). Whereas positive feedback within this loop is provided by the proteins CLOCK and BMAL1, negative feedback is mediated by PER (1-3) and CRY (1-2), the products of three *period* genes (*per* 1, *per* 2, and *per* 3) and two *cryptochrome* genes (*cry* 1 and *cry* 2) (Jin et al., 1999).

Several clock genes have been cloned and found to be rhythmically expressed within the SCN in a circadian fashion (Dunlap, 1999). Rhythm generation in the SCN seems to involve the circadian expression of Per1 and Per2, with high levels of these proteins seen during the day and low levels seen during the night in nocturnal rodents such as hamsters (Maywood, Mrosovsky, Field, & Hastings, 1999; Yamamoto et al., 2000), Wistar rats (Asai et al., 2001; Yan, Takekida, Shigeyoshi, & Okamura, 1999), and mice (Shearman, Zylka, Weaver, Kolakowski, & Reppert, 1997). These gene products also peak during the day in diurnal species such as *Arvicanthis ansorgei* (Caldelas, V.J, Sicard, Pevet, & Challet, 2003), ground squirrels (Mrosovsky, Edelstein, Hastings, & Maywood, 2001), and grass rats (unpublished data).

Per1 is also expressed rhythmically in non-SCN brain regions (Abe et al., 2002; Amir, Lamont, Robinson, & Stewart, 2004; Kriegsfeld et al., 2003; Masubuchi et al., 2000; Zylka, Shearman, Weaver, & Reppert, 1998), in peripheral tissues (Zylka et al., 1998), and in immortalized cell lines in culture (Yamazaki et al., 2000). Although most rhythms in

gene expression outside the SCN seem to be under SCN control and are abolished by SCN lesions (Sakamoto et al., 1998), there is evidence for the presence of autonomous oscillators in peripheral tissues, but their precise role is still unknown (Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001). It has been suggested that brain regions outside the SCN may express clock genes and may function as autonomous oscillators but still require a signal from the SCN in order to sustain circadian oscillations (Kriegsfeld et al., 2003). See however Granados-Fuentes, Prolo, Abraham, & Herzog (2004).

The SCN projects to brain regions that play a role in the regulation of sleep and arousal such as the DMH and the PVT (Novak, Harris et al., 2000; Watts, 1991). The DMH also receives projections from the sPVZ (Chou et al., 2003), the LHA and the TMN (Thompson & Swanson, 1998). In turn, it sends projections to the LHA and the VLPO (Chou et al., 2002). Different studies suggest a role for the DMH in the regulation of arousal, either as a relay nucleus between the SCN and the LC (Aston-Jones et al., 2001) or as a relay nucleus between the sPVZ and areas involved in the regulation of sleep and arousal (Lu et al., 2001).

The SCN also sends projections to the PVT (Novak, Harris et al., 2000), which in turn projects to areas involved in arousal such as the LC (Morin et al., 1994) and the raphe (Otake, Kin, & Nakamura, 2002). This area has been linked to behaviors that usually are incompatible with sleep (Allingham et al., 1998), including responses to stressors (Cullinan et al., 1995; Duncan et al., 1996).

The present experiment was undertaken to determine if areas involved in the regulation of sleep and arousal express PER1 protein in grass rats and, if so, to determine if the expression of this clock protein is rhythmic.

Methods

Male grass rats (n=30) kept in a 12:12 hours light dark cycle were perfused at six different ZTs: 2, 6,10,14,18, and 22 (with, lights on at ZT 0).

Immunocytochemistry

Every other section, extending from the lateral septal nucleus to the caudal end of the TMN, was processed for PER1 ICC with rabbit NDS (Jackson), anti PER1 as the primary antibody (PER1 #1177, a gift from Dr. David Weaver, University of Massachusetts, Worcester, MA, 1:20,000) and donkey anti rabbit (Jackson, 1:200) as the secondary. Tissue was reacted with DAB and nickel, mounted and coverslipped. Sections were examined under 5X magnification to determine the areas where the expression of PER1 was abundant.

ICC controls

Preadsorption of the primary antibody for PER1 with the blocking peptide (a gift from Dr. Weaver's Lab, 1.5µl of peptide with 1.5 ml of the primary antibody) and deletion of the primary antibody resulted in absence of stain.

Data Analysis

Rhythms in PER1 expression in the DMH

Bilateral counts of cells expressing PER1 were made in every section of the DMH that contained labeled neurons (average of 8 sections per animal), using a Zeiss AxioScop 2 plus microscope at 40X. One-way ANOVA was used to evaluate the effects of ZT on the expression of PER1 in this region. The Fisher's LSD test was used for pairwise comparisons.

Rhythms in PER1 expression in the PVT

The number of cells expressing PER1 was also counted in the PVT. The PVT was divided into anterior, middle, and posterior regions based on landmarks proposed by (Peng et al., 1995). Two sections per region were used to count the number of PER1-ir cells in each animal. Two-way ANOVA (area X ZT) was used to evaluate the effects of ZT and region in the expression of PER1. The Fisher's LSD test was used for pairwise comparisons. For both the DMH and the PVT, the counts were made without knowledge of the time of perfusion of the animals.

Results

Distribution of PER1 in grass rats

In general, the distribution of PER1 in grass rats is similar to that reported previously in lab rats (Hastings, Field, Maywood, Weaver, & Reppert, 1999) PER1 is expressed in several brain regions including the cerebral cortex, the bed nucleus of the stria terminalis (BNST) and the amygdala. In the basal ganglia, a moderate number of cells expressing PER1 were seen in the caudate putamen and nucleus accumbens. Within the thalamus, moderate expression was seen in the paratenial nucleus and the anterior PVT, and relatively fewer labeled cells were observed in the medial and posterior PVT and in the CMT. In the hypothalamus, in addition to the high level in the SCN and the LSPV, moderate levels were seen in the medial preoptic area, the VLPO, the AVPe, and the arcuate nucleus. A striking difference with what has been reported in several species was found in the DMH; no expression of PER1 has been reported in this nucleus for other species, but in grass rats intense immunoreactivity was seen in the posterior part of the DMH, also known as the "*pars compacta*" (Chou et al., 2003) (Figure 20a).

Rhythms in PER1 expression in the DMH

One-way ANOVA showed a rhythm in the expression of PER1 in the pars compacta of the DMH with a significant effect of ZT ($F=4.64$, $df = 5, 24$, $p<0.004$) and with ZT 2 and 10 being significantly different from ZTs 6, 14, and 22 (Figure 20b). Interestingly, this rhythm was not circadian but instead ultradian with cycles of apparently 8 hours and peaks at ZTs 2, 10, and 18.

Rhythms in the expression of PER 1 in the PVT

Two-way ANOVA showed a significant effect of ZT ($F=3.97$, $df = 5, 72$, $p=0.003$) and area ($F=18.52$, $df = 2, 24$, $p<0.001$) and no interaction ($p=0.99$) on the mean number of cells expressing PER1. The mean number of cells expressing PER1 at ZT 22 was significantly lower compared to ZTs 6, 10, and 18. Expression of PER1 at ZT 18 was also significantly higher than that seen at ZTs 2, and 14 ($F=3.97$ $df = 5, 72$, $p<0.003$) (Figure 21). A significant effect of area ($F=18.52$, $df = 2, 72$, $p<0.001$), with the number of cells expressing PER1 being higher in the anterior PVT than in the middle and posterior PVT. The middle region of the PVT also had higher number of cells expressing PER1 compared to the posterior PVT (Figure 21).

Discussion

Distribution of PER1 in grass rats

For the most part, the distribution of PER1 in the brain of grass rats was similar to what has been reported in lab rats and other nocturnal and diurnal species (Caldelas et al., 2003; Yamamoto et al., 2001) and confirms that PER1 is expressed not only in the SCN but also in several other brain regions. The significance of the expression of PER1 in brain regions other than the SCN remains to be determined.

Circadian oscillations in the expression of clock genes occur not only in the SCN but also in a variety of peripheral tissues, including the liver , skeletal muscle, kidney, and lung (Balsalobre, 2002; Stokkan et al., 2001; Yamazaki et al., 2000; Zylka et al., 1998). Several other neural tissues, including the retina, the olfactory bulb, as well as cultured fibroblasts also exhibit circadian expression of clock genes. Many of these rhythms can be entrained to the light cycle and are temperature compensated, which suggest that these tissues may also act as circadian oscillators (Granados-Fuentes et al., 2004; Izumo, Johnson, & Yamazaki, 2003; Tosini & Menaker, 1996). Although several non-SCN brain regions express clock genes, only some of them show intrinsic rhythmicity in vitro, and in contrast to the SCN, their peak of *Per1* expression occurs at night (Abe et al., 2002). Initial studies showing persistent circadian expression of clock genes in the SCN and dampened activity in non-SCN tissue when disconnected from the SCN suggested that the SCN acted as a master pacemaker able to generate its own self-sustained circadian rhythm and needed to sustain rhythms in damped peripheral oscillators (Balsalobre, 2002; Yamazaki et al., 2000). However, recent studies have challenged this view of the SCN by showing persistent circadian rhythms of PER2 in cultured peripheral tissue of mice, even after SCN lesions (Yoo et al., 2004) and cell-autonomous, self-sustained circadian oscillators in fibroblasts (Nagoshi et al., 2004). Unlike SCN neurons, which show coupling or synchrony among themselves (Liu, Weaver, Strogatz, & Reppert, 1997), cells in fibroblasts do not influence each other (Nagoshi et al., 2004). Further, SCN lesions disrupt phase synchrony in peripheral tissue (Yoo et al., 2004). Based on these observations it has been suggested that the actual role of the SCN is not to sustain dampened rhythms in peripheral tissue but to synchronize all the oscillators in the body (Yoo et al., 2004) and to keep them entrained to the light-dark cycle.

Evidence suggests similarities between diurnal and nocturnal species in the circadian oscillations of clock genes within the SCN (Dardente et al., 2004; Mrosovsky et al., 2001), but there is no information about their circadian expression in peripheral tissue in diurnal species. The present study shows, with one interesting exception (i.e., the DMH), a distribution of PER1 protein in non-SCN brain in a diurnal species that is similar to that reported for nocturnal animals. Since circadian oscillations of clock genes within the SCN seem to be similar between species that show divergent patterns of activity, it is likely that these behavioral differences are determined by local circuits of some SCN targets.

Profile of PER1 expression in the DMH

The expression of the clock protein PER1 in the posterior part of the DMH was high at ZTs 2, 10, and 18, and low at ZTs 6, 14, and 22, a pattern that suggests an ultradian rhythm that repeats every 8 hours. The functional significance of this apparent ultradian rhythm is unclear. In addition to a role in the control of vigilance, the DMH is believed to regulate ingestive behaviors (Bellinger & Bernardis, 2002; Chou et al., 2003). Analysis of the feeding and drinking patterns of grass rats revealed strong circadian rhythmicity but no evidence of ultradian patterns (unpublished data). However, the possibility exists for the emergence of ultradian components in the pattern of ingestive behaviors of grass rats lacking a strong circadian signal after SCN lesions. Disruption of circadian rhythms by constant bright lights (Eastman & Rechtschaffen, 1983), exposure to low temperatures (Gibbs, 1983), mutations, or lesions (Lu et al., 2001) often results in the expression of strong ultradian rhythms that may be generated by the DMH, at least in grass rats. However, studies with lab rats showing the persistence of 3-4 h ultradian cycles of sleep and wakefulness after DMH lesions (Chou et al., 2003) seem to rule out the possibility that

local circuits in the DMH generate ultradian rhythms at least in lab rats, but it may be the case that this is true only for nocturnal species.

Although some studies have shown SCN inputs to the DMH (Aston-Jones et al., 2001; Chou et al., 2003; Thompson & Swanson, 1998), it seems that those projections do not reach the posterior part of the nucleus(Watts, Swanson, & Sanchez-Watts, 1987), at least in the nocturnal lab rat. In contrast, data on SCN projections to the DMH in grass rats show dense projections to this posterior part (Schwartz, unpublished data). Previous studies on the distribution of PER1 in nocturnal species did not find neurons expressing this protein within the DMH, in contrast to what is seen in grass rats. This observation and the differential SCN input to the posterior part of the DMH in lab rats and grass rats (Watts, Swanson, & Sanchez-Watts, 1987) (Schwartz, unpublished data) suggest that the DMH plays different roles in the timing of behaviors in the two species.

Interestingly, the expression of PER1 within the DMH was restricted to the posterior part, an area cytoarchitectonically different from the rest of the DMH in that it does not project to the VLPO (Chou et al., 2002) and lacks thyrotropin-release hormone (TRH). Projections from the preoptic nuclei (except the parastrial nucleus), the anterior hypothalamus, the retrochiasmatic area, and the BNST tend to avoid the posterior region of the DMH. Inputs to this area are mostly from the parabrachial nucleus, the parastrial nucleus, and the nucleus of the solitary tract (NTS) (Thompson & Swanson, 1998).

Rather than reflecting an ultradian rhythm, the pattern of PER1 expression in the DMH of grass rats instead may reflect the activity of several populations of circadian neural oscillators within the DMH out of phase with each other. The DMH contains serotonin and dynorphin neurons and scattered NPY cells (Bernardis & Bellinger, 1998). Double labeling studies with these transmitters or modulators are needed to clarify the

circadian or ultradian profile of neurons expressing PER1 in this area, by tracking the patterns of gene expression in particular subpopulations of neurons in this area.

Profile of PER1 expression in the PVT

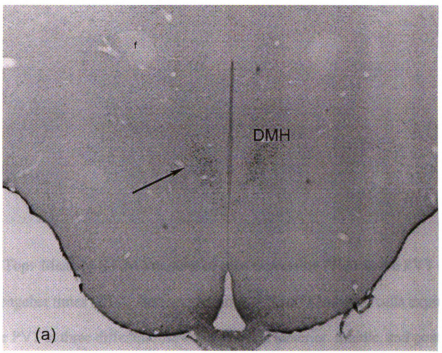
Although there was a significant effect of ZT on the expression of PER1 in the PVT, no clear rhythm was evident. Significant differences were observed between the three levels of the PVT, with the expression in the anterior region being higher than in the middle and posterior regions.

Topographical differences in the expression of PER1 may reflect functional differences of regions of the PVT. The PVT receives projections from histaminergic neurons in the TMN, dopaminergic neurons in the ventral tegmental area, noradrenergic neurons in the LC, and serotonergic input from the DRN (Van der Werf, Witter, & Groenewegen, 2002). The anterior PVT receives strong projections from the SCN and the sPVZ , which suggests an important role in the regulation of circadian rhythmicity (Moga, Weis, & Moore, 1995), and sends projections to several nuclei in the hypothalamus involved in the regulation of sleep and wakefulness including the SCN and the DMH (Moga et al., 1995). It also sends projections to areas regulating motivational and visceral information, including the VMH and the amygdala (Peng & Bentivoglio, 2004). The posterior PVT instead, receives a much less dense projection from the SCN (Peng & Bentivoglio, 2004) and projects to a more restricted area in the amygdala (the central, basomedial, and basolateral nuclei) and to other nuclei, including the nucleus accumbens and the anterior olfactory nucleus (Moga & Moore, 1997). Thus, the higher concentration of PER1 in the anterior PVT may be involved in the circadian regulation of behavior, but again no clear circadian pattern in gene expression was detected. Additional experiments

looking at the expression of other clock genes in these areas are warranted and should help discern the role of the PVT in circadian functions.

In lab rats, rhythmic activity in Per genes has been reported in cultured PVT tissue with peaks during the night (Abe et al., 2002). Rhythmic expression of Fos within the PVT has been reported in both grass and lab rats (Novak & Nunez, 1998; Novak, Smale et al., 2000) and both species receive dense projections from the SCN to the PVT (Kalsbeek, Teclemariam-Mesbah, & Pevet, 1993; Kawano, Krout , & Loewy, 2001; Novak, Harris et al., 2000; Watts & Swanson, 1987). It has been proposed that the absence of rhythmicity in gene expression in non-SCN brain regions may allow the SCN to have stronger control over the phase of the rhythms within those regions (Abe et al., 2002). In lab rats, rhythms in Fos expression in the PVT are for the most part out of phase with rhythms in the SCN (Novak & Nunez, 1998). The same seems to be true for the intrinsic *Per1* expression within this area (Abe et al., 2002). In grass rats, Fos expression in the PVT roughly resembles activity in the SCN (i.e., it is higher at ZT1 than at ZTs 5, 13, and 17). Absence of rhythmicity in PER1 expression and dense projections from the SCN in grass rats may allow for the strong phase relationship between the SCN and the PVT suggested by their similar patterns in Fos expression. In lab rats, instead, out of phase rhythms of Fos in the PVT (compared to those in the SCN) may be related to a less strong SCN control (and perhaps an indirect regulation through the sPVZ), which may be reflected in out of phase rhythms in PER1.

Figure 20. (a) Low power light photomicrograph showing the distribution of PER1-ir cells in the posterior part of the DMH (arrow). A striking difference with what has been reported in several species was found in the DMH; no expression of PER1 has been reported in this nucleus for other species, but in grass rats intense immunoreactivity was seen in the posterior part of the DMH, also known as the "*pars compacta*". (b) Mean (± S.E.M.) number of cells expressing PER1 in the pars compacta of the DMH. at six different Zeitgeber times (ZTs). Means with different letters (a, b, or c) are significantly different from each other. DMH, dorsomedial hypothalamus; f = fornix.


f
DMH
(a)

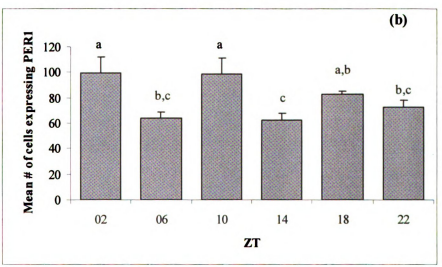
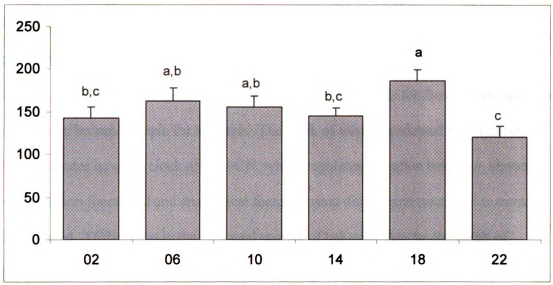
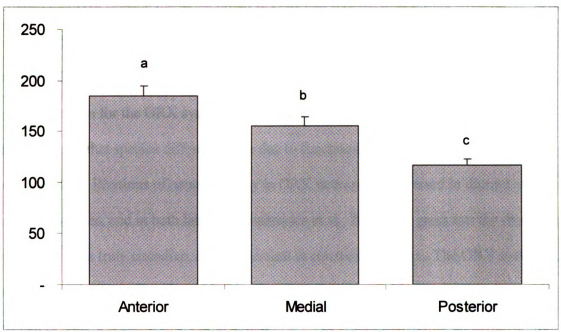

(b)
Mean # of cells expressing PER1
120
100
80
60
40
20
0
a
b,c
a
c
a,b
b,c
02
06
10
14
18
22
ZT

Figure 21. Top: Mean (± S.E.M.) number of cells expressing PER1 in the PVT at six different Zeitgeber times (ZTs). Bottom: Mean (± S.E.M.) number of cells expressing PER1 in the PVT at three different rostrocaudal levels (anterior, middle, and posterior). Means with different letters (a, b, or c) are significantly different.


250
200
150
100
50
0
b,c
a,b
a,b
b,c
a
c
02
06
10
14
18
22


250
200
150
100
50
-
a
b
c
Anterior
Medial
Posterior

GENERAL DISCUSSION

The results of the five experiments document at a functional- anatomical level differences and similarities in the neural systems that regulate arousal or wakefulness in diurnal and nocturnal rodents. Although there are differences in the timing of several behaviors between diurnal and nocturnal species, the neural basis for those differences has only recently become a topic for research. The work of others (reviewed by Smale et al., 2003) illustrates how the clock of the SCN, which regulates circadian behavior, shows many common functional and anatomical features when diurnal and nocturnal mammals are compared. Differences in the temporal control of behavior may be the result of differences either within the SCN (in clock cells, in output cells, or in the interactions between them) or outside the SCN, either in cells that receive direct input from the SCN or in intermediate areas that modulate SCN signals in a different way in diurnal and nocturnal species and then send this altered signal to brain targets.

The data for the ORX system reported in Experiments 1 and 2 are consistent with the hypothesis that species differences are due to functional neural differences downstream from the SCN. Rhythms of neural activity in ORX neurons are reversed in diurnal and nocturnal species, and in both lab rats (Estabrooke et al., 2001) and grass rats the rhythm in ORX neurons is truly circadian, since it persists in constant darkness. The ORX system can now be added to a list of areas that regulate sleep and wakefulness and that show a reversal or salient species differences in neural activity when diurnal and nocturnal animals are compared. This list includes the VLPO and the histaminergic system (Novak & Nunez, 1998; Novak, Smale et al., 2000).

There is evidence that SCN axonal projections reach areas that control wakefulness and that are reversed with respect to neural activity in diurnal and nocturnal species (Novak, Smale et al., 2000). However, until the work presented here in Experiment Three, there was no evidence of direct contacts between SCN axons and neurons that produce ORX. The data from Experiment Three indicate a monosynaptic pathway from the SCN to the ORX neuronal group of the LH. Since the SCN seems to function in a similar way across species, differences in rhythms of sleep and wakefulness may result from functional differences in these direct outputs from the SCN. Data from Experiment 3 also serve to identify differences in the contribution of neurons of the SCN to the inputs received by these ORX neurons in grass and lab rats. Whereas grass rats receive substantial inputs from VIP neurons of the SCN to neurons expressing ORX, lab rats receive very few VIP projections. Differences in VIP projections from the SCN to ORX neurons may contribute to species differences in the regulation of the sleep wake cycle: whereas in grass rats regulation may be in part mediated by direct projections from the SCN to areas involved in arousal, in the nocturnal lab rat this regulation may involve an indirect pathway that connects the SCN to neurons in the lateral and posterior hypothalamus. The DMH and the sPVZ have recently been proposed as relay centers between the SCN and areas regulating sleep and wakefulness in lab rats (Deurveilher & Semba, 2005; Aston-Jones et al., 2001). In contras to ORX neurons, histaminergic neurons in both lab and grass rats lack substantial VIP input from the SCN, which suggests an indirect regulation of their rhythmic activity or the involvement of populations of SCN output neurons other than VIP and VP.

In addition to their circadian regulation, areas controlling sleep and wakefulness may also be directly affected by light. Based on the observation that in grass rats very few retinal fibers reach the VLPO (Novak et al., 1999) and that in other diurnal species areas

associated with arousal and wakefulness had more retinal inputs than those seen in lab rats (Fite & Janusonis, 2001; Fite et al., 1999), it was proposed that the shift to diurnality was accompanied by a shift of retinal inputs away from sleep promoting areas in favor of areas that support wakefulness. But evidence presented in Experiment Four suggests that (at least in grass rats) the adaptation to a diurnal life style may have involved not just a redistribution of retinal inputs to sleep or wake promoting, but instead a reduction in the amount of retinal projections that reach areas that control vigilance. These anatomical features indicate a more general change in how light may affect behavior differently in different species: in grass rats, photic input to the areas involved in the regulation of sleep and wakefulness is either sparse (VLPO) or absent (ORX, TMN, and DRN). One prediction from these anatomical observations would be that the masking effects of light on sleep and arousal should be absent in grass rats, in contrast to what has been reported for nocturnal species. As discussed in Experiment Four, there are data to support that claim (Redlin & Mrosovsky, 2004): although light exposure suppresses wheel running in grass rats, the same treatment do not have masking effects in general activity. Another species that displays a very diurnal bias in the distribution of activity is the ground squirrel (Major et al., 2003), which, like grass rats, lack a retinal projection to the DRN. Additional studies combining data on the comparative anatomy of the retinal inputs to areas that control vigilance with data on the effects of masking of behavior by light exposure are needed to better understand the consequences of the anatomical differences seen across species. With respect to retinal inputs to the DNR, grass rats are like ground squirrels but different from degus and gerbils (Fite & Janusonis, 2001; Fite et al., 1999), species also classified as diurnal. As previously proposed (Smale et al., 2003), differences among diurnal mammals may be related to differences in how diurnality evolved.

Finally, Experiment Five addressed the issue of molecular rhythms in non-SCN brain regions, and more specifically the expression of PER1 in regions associated with arousal. Evidence suggests similarities between diurnal and nocturnal species in the circadian oscillations of clock genes, including PER 1, within the SCN (Dardente et al., 2004; Mrosovsky et al., 2001), but there is no information about their circadian expression in non-SCN brain regions in any diurnal species. For the most part, the distribution of PER1 in the brain of grass rats is similar to what has been reported in lab rats and other nocturnal and diurnal species (Caldelas et al., 2003; Yamamoto et al., 2001). But in contrast to what has been previously reported in other species, PER1 expression was also seen in the posterior region of the DMH. This region showed a pattern that suggests an ultradian rhythm in the expression of PER1 that repeats every 8 hours, rather than a circadian one. The functional significance of this ultradian rhythm is unclear and does not seem to be related to any patterns seen in the behavior of grass rats including meal patterns or patterns of water intake (unpublished data). More research on patterns of behavior and other functions in grass rats is needed to elucidate the function of this ultradian rhythm in PER 1 expression.

The pattern of PER1 expression in the DMH of grass rats may reflect not an ultradian rhythm but, alternatively, the outputs of circadian oscillators out of phase with each other. Little is known about the anatomy of the region of the DMH that expresses PER1 in grass rats, but it seems to differ in salient ways from the rest of the DMH and may contain neurons with different phenotypes and connections. One interesting observation is that whereas SCN axonal projections reach this area in grass rats, such inputs seem to be absent in lab rats. Differences in gene expression and connectivity in this area may be responsible for differences in circadian regulation in diurnal and nocturnal mammals.

Different from the DMH, no clear rhythm was seen in the expression of PER1 in the PVT, another region that has been involved in the regulation of wakefulness. However, significant differences in abundance of PER1 protein were clear across the three different anterior - posterior levels of the PVT, with more PER 1 expression in the anterior level. In contrast to the posterior PVT, the anterior PVT receives strong projections from the SCN and the sPVZ and sends projections to several nuclei involved in the regulation of sleep and wakefulness (Moga et al., 1995), which suggests an important role in the regulation of circadian rhythmicity. Further, the fact that the patterns of PER 1 expression in the PVT are different in lab rats and grass rats makes it possible for this area to respond differently to SCN inputs thus contributing to species differences in circadian behavior, even if the signal from the SCN is the same for both species.

In summary, experiments in this dissertation have confirmed rhythmic activity in ORX neurons in the LH, a brain region involved in the regulation of wakefulness. Similar to what has been reported for other brain regions neural activity in ORX neurons is reversed in the diurnal grass rat compared to the nocturnal lab rat. Although rhythms in these neurons are likely to be under the control of the circadian clock in both lab and grass rats, this regulation differs in that very few direct SCN projections from VIP neurons of the SCN are sent to the LH of lab rats and a substantial number of these VIP inputs are seen in grass rats. The results also show that species differences may also be the result of differences in the retinal projections to this and other areas regulating arousal and may therefore play an important role in the display of sleep and wakefulness across the light dark cycle. Finally, data on clock gene expression in the DMH suggest a differential role of this hypothalamic nucleus in the regulation of behavior in the grass and lab rats.

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