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Night and day: distinct retinohypothalamic innervation patterns predict the development of nocturnality and diurnality in two murid rodent species

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NIGHT AND DAY: DISTINCT RETINOHYPOTHALAMIC INNERVATION

PATTERNS PREDICT THE DEVELOPMENT OF NOCTURNALITY AND

DIURNALITY IN TWO MURID RODENT SPECIES

by

William David Todd III

<u>An Abstract</u>

Of a thesis submitted in partial fulfillment of the requirements for the Doctor of
Philosophy degree in Psychology in the Graduate College of The University of Iowa

May 2012

Thesis Supervisor: Professor Mark S. Blumberg

ABSTRACT

How does the brain develop differently to support nocturnality in some mammals, but diurnality in others? To answer this question, one might look to the suprachiasmatic nucleus (SCN), the pacemaker of the mammalian brain, which is required for all circadian biological and behavioral rhythms. Light arriving at the retina entrains the SCN to the daily light-dark cycle via the retinohypothalamic tract (RHT). However, in all mammals studied thus far, whether nocturnal or diurnal, the SCN exhibits a rhythm of increased activity during the day and decreased activity at night. Therefore, structures downstream of the SCN are likely to determine whether a species is nocturnal or diurnal.

From an evolutionary perspective, nocturnality appears to be the primitive condition in mammals, with diurnality having reemerged independently in some lineages. However, it is unclear what mechanisms underlie the development of one or the other circadian phase preference. In adult Norway rats (*Rattus norvegicus*), which are nocturnal, the RHT also projects to the ventral subparaventricular zone (vSPVZ), an adjacent region that expresses an *in-phase* pattern of SCN-vSPVZ neuronal activity (in other words, activity in the SCN and vSPVZ increase and decrease together). In contrast, in adult Nile grass rats (*Arvicanthis niloticus*), a diurnal species that is closely related to Norway rats, an *anti-phase* pattern of SCN-vSPVZ neuronal activity is expressed (in other words, activity in the SCN increases as activity in the vSPVZ decreases, and vice versa). We hypothesized that these species differences in activity pattern result in part from a weak or absent RHT→vSPVZ projection in grass rats.

Using a developmental comparative approach, we assessed differences in behavior, hypothalamic activity, and RHT and SCN connectivity to the vSPVZ between these two species. We report that a robust retina➔vSPVZ projection develops in Norway rats around the end of the second postnatal week when nocturnal wakefulness and the in-phase pattern of SCN-vSPVZ activity emerge. In grass rats, however, such a projection does not develop and the emergence of the anti-phase SCN-vSPVZ activity pattern during the second postnatal week is accompanied by increased diurnal wakefulness.

When considered within the context of previously published reports on RHT projections in a variety of other nocturnal and diurnal species, our current findings suggest that *how* and *when* the retina connects to the hypothalamus differentially shapes brain and behavior to produce animals that occupy opposing temporal niches.

Abstract Approved: _____
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The University of Iowa
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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To Mary

Night has always pushed up day

-Marcus Mumford

ACKNOWLEDGMENTS

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When considered within the context of previously published reports on RHT projections in a variety of other nocturnal and diurnal species, our current findings suggest that *how* and *when* the retina connects to the hypothalamus differentially shapes brain and behavior to produce animals that occupy opposing temporal niches.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance
CTB: Cholera toxin subunit B
E: Embryonic day
EEG: Electroencephalographic
EMG: Electromyographic
GABA: γ-aminobutyric acid
ot: Optic tract
P: Postnatal day
PAC1:   Pituitary adenylate cyclase-activating peptide type 1 receptor
PACAP: Pituitary adenylate cyclase-activating peptide
PBS: Phosphate buffered saline
PFA: Paraformaldehyde
SCN: Suprachiasmatic nucleus
SON: Supraoptic nucleus
SPVZ: Subparaventricular zone
VIP: Vasoactive intestinal peptide
vSPVZ: Ventral subparaventricular zone
3V: Third ventricle

CHAPTER 1

INTRODUCTION

The daily alternation of sun and shadow on the earth's surface imposes biological constraints that affect all animals. Because the earth consistently rotates on its axis at a period of about 24 hours, creating this predictable light-dark cycle, all animals have evolved adaptive behavioral and physiological processes that occur at specific times of the day. These 24-hour cycles, known as circadian rhythms, are primarily regulated in mammals by the suprachiasmatic nucleus (SCN) of the hypothalamus (Rusak and Zucker 1979). The functioning of the SCN is commonly conceptualized as a "clock" because its cells exhibit an endogenous rhythm of activity that oscillates at a period of about 24 h (Welsh, Logothetis et al. 1995). These rhythms persist even when the SCN is completely isolated in tissue culture. However, in order for an animal to take advantage of such a clock, the activity of the SCN must be reset each day so that it is synchronized to the daily light-dark cycle. This is accomplished because the SCN receives photic information from the retina, via the retinohypothalamic tract (RHT), and uses this information as an entraining stimulus (Rea 1998; Hannibal 2002).

Several aspects of mammalian behavior and physiology occur on a circadian rhythm, including body temperature, drinking, and heart rate (Stephan and Nunez 1977; Dark 1980; Scheer, Kalsbeek et al. 2003). Perhaps no behavior is more noticeably subject to a circadian rhythm than the sleep-wake cycle (Borbely, 1982). Since sleeping and waking occur predominately in different species during distinct times of the day, a given species can be classified as diurnal (day-active), nocturnal (night-active), or crepuscular (twilight-

active) based on its respective circadian phase preference. Interestingly, remarkably few differences have been found in the neural correlates underlying sleep-wake rhythms in mammals that occupy contrasting temporal niches. Therefore, how the brain differentially supports species-typical circadian preference is not known.

Although numerous studies have examined differences in the circadian control of behavior in species with varying circadian phase preferences (Smale, Lee et al. 2003; Mrosovsky and Hattar 2005; Challet 2007), almost all have limited their focus to adult subjects. Since species-typical behavior emerges from complex developmental interactions (Lehrman and Rosenblatt 1971; Gottlieb 1992; West-Eberhard 2003; Schwartz, Urbanski et al. 2011), studying the ontogeny of day-night differences in sleep and wakefulness should help tease apart the numerous factors contributing to the adult phenotype.

With this in mind, here we seek to better understand the mechanisms underlying circadian phase preference by employing a developmental comparative approach using nocturnal Norway rats (*Rattus norvegicus*) and diurnal Nile grass rats (*Arvicanthis niloticus*). Across 5 experiments, we examine developmental differences in behavior, hypothalamic activity, and RHT connectivity. Before describing these experiments in detail, more background information is needed. Therefore, the rest of this chapter discusses the evolutionary history of nocturnality and diurnality in mammals, the development of SCN function and circadian phase preference, as well as evidence guiding these experiments pertaining to the role of another neural area—the ventral subparaventricular zone (vSPVZ)—and the neurotransmitters that influence its functioning.

Evolutionary history of mammalian nocturnality and diurnality

Most existing mammalian species are nocturnal (Park 1940), and it has been argued that nocturnality is a primitive characteristic of Mammalia (Crompton, Taylor et al. 1978; Menaker, Moreira et al. 1997; Heesy and Hall 2010). Menaker and colleagues proposed that early mammals were squeezed through a "nocturnal bottleneck," a period in which it was evolutionary adaptive for species to rely less on daylight and be more active at night, perhaps in part to avoid diurnal reptilian predators. There is much evidence to support this theory. First, Menaker et al. explain that all existing vertebrates contain an ancient core "circadian axis" consisting of the retina, SCN, and pineal gland, each of which contributes to circadian rhythmicity in some way. However, whereas the pineal gland of some fish, lizards, and birds has been shown to contain endogenous circadian oscillators that are able to cycle rhythmically in vitro (like the mammalian SCN), no mammalian pineal has been found to have this property. Additionally, as shown in Table 1, mammals are the only existing class of vertebrate that lacks extraretinal photoceptors, while non-mammalian vertebrates exhibit multiple photoreceptors outside of the retina. As a result, enucleation in adult mammals completely abolishes all responses to light, including circadian entrainment (Nelson and Zucker 1981).

All vertebrate photoreceptors, whether in the retina or elsewhere, contain a broad class of intrinsically light-sensitive pigments called opsins (Peirson, Halford et al. 2009). One such photopigment, melanopsin, is particularly involved with the differences in extraretinal circadian photoreceptors between non-mammalian and mammalian vertebrate classes shown in Table 1. The expression of two melanopsin-encoding genes, *Opn4x* and

Opn4m, has been identified in present-day non-mammalian vertebrates in such tissues as the pineal gland, the parapineal organ, the retina, and neural areas associated with deep brain photoreceptors. Present-day mammalian species, however, have retained the *Opn4m* gene only in the retina, whereas the *Opn4x* gene has been "lost" altogether. Importantly, melanopsin is present in most of the mammalian retinal ganglion cells whose axons make up the RHT (Hannibal, Hindersson et al. 2002; Gooley, Lu et al. 2003). It is argued that the non-existence of extra-retinal photoreceptors in mammals, and the retention of melanopsin only in the retina, supports a nocturnal heritage because the scarce light under such conditions would have been unable to penetrate deeper tissues (leading to the disuse and eventual loss of such photoreceptors), while still being sufficient to entrain the SCN directly via the RHT (Foster and Soni 1998; Bellingham, Chaurasia et al. 2006; Heesy and Hall 2010).

Numerous other aspects of the present-day mammalian visual system also strongly support the nocturnal bottleneck view (Heesy and Hall 2010). Heesey and Hall explained that most mammals have retained an eye design that is generally only exhibited in low-light adapted amniotes (which include mammals, reptiles, and birds). Specifically, the larger corneal diameter relative to the axial length of the eye seen in most mammals is similar to that of nocturnal birds and lizards and enhances visual acuity by allowing more light to enter the eye. Additionally, Heesy and Hall argued that the higher incidence of the forward-facing orientation of the eyes in mammals, relative to birds and reptiles, reflects a nocturnal heritage because it creates a higher degree of binocular visual field overlap which serves to increase light capture in low-light environments. This is further supported by the

fact that the species of birds that are most dependent on binocular vision, including owls, are also mostly nocturnal.

Finally, evidence from the fossil record also suggests that early mammals may have benefited most from a nocturnal lifestyle. Fossilized mammals from the Mesozoic Era have strikingly increased brain size to body size ratios when compared with the most advanced mammal-like reptiles (Crompton, Taylor et al. 1978). This increase is perhaps due to increased sensory capabilities of smell and hearing compensating for the loss of visual information in a nocturnal niche. Additionally, such mammals seem to have lived in similar habitats as present-day nocturnal insectivores. Crompton et al. argued that it was the advent of homeothermy which allowed early mammals to invade the nocturnal niche, whereas diurnal reptiles are ectothermic and regulate their body temperature behaviorally using a wide range of ambient temperatures that sharply decrease when the sun sets.

Again, according to the nocturnal bottleneck view, diurnality is a derived character that has reemerged in some mammalian lineages. However, there is little agreement as to what developmental and evolutionary mechanisms played a role in this reemergence (Smale, Lee et al. 2003). As Smale et al. articulated, diurnality raises several important questions, across numerous disciplines, about origins and selective pressures, structure-function relationships, as well as the extent to which findings from nocturnal rodents can be generalized to diurnal humans.

As with mammals as a whole, it is generally agreed that the first rodents were nocturnal (Krubitzer, Campi et al. 2011), as depicted in Figure 1. However, there has been much debate over the primitive circadian phase preference of other mammalian orders—

namely primates (Heesy and Ross 2001; Ankel-Simons and Rasmussen 2008; Griffin, Matthews et al. 2012). It is possible that, within mammalian lineages, nocturnality and diurnality emerged or reemerged multiple times throughout evolution depending on the selective pressures and adaptability of a given species (Smale, Lee et al. 2003). The ramifications of this possibility for comparing systems seem daunting; however, the solution to this problem brings us back to development. By systematically comparing the ontogeny of distinct behavioral phenotypes in closely related species, one can assess which elements of the circadian system differentially emerge to support differences in behavior. Similar strategies have led to discoveries of the underlying neural differences associated with the development of monogamous and non-monogamous phenotypes in closely related species of vole (Wang, Liu et al. 1997; Wang and Young 1997; Wang, Young et al. 1997). Figure 1 also demonstrates that Norway rats and grass rats fit the criterion of closely related species, as they both belong to the Muridae family of rodents. Indeed, these species have been evolving separately for only about 10 million years. In contrast, other diurnal rodents, such as squirrels from the Sciuridae family, have been evolving independently from Murid rodents for as long as 70 million years (Huchon, Chevret et al. 2007).

The Murid subfamily, like Rodentia as a whole, is believed to have originated in Asia (Krinke 2000; Lecompte, Aplin et al. 2008). This is the case for Norway rats, which eventually migrated with humans to Europe and North America over perhaps thousands of years (Krinke 2000). Krinke explains that the Norway rat was the first animal to be domesticated for strictly scientific purposes and, since that time, inbred strains of this species have been used in an overwhelming majority of published experimental studies.

Grass rats, on the other hand, are indigenous to eastern Africa where they first appeared after the migration of their ancestors from Asia (Lecompte, Aplin et al. 2008). The grass rat colony used here was founded from breeders provided by Laura Smale at Michigan State University, who maintains a colony that originates from 29 individuals captured at the Masai Mara National Reserve in Kenya (Katona and Smale 1997).

<u>Development of SCN function and circadian phase preference</u>

The SCN of fetal Norway rats exhibits rhythms of glucose metabolism by embryonic day (E)19, and is entrained to the environmental light-dark cycle via the mother's circadian system (Reppert and Schwartz 1983; Reppert and Schwartz 1984; Reppert, Weaver et al. 1988). Additionally, melanopsin-containing retinal ganglion cells, whose axons comprise the RHT, are responsive to light and can drive SCN activity on the day of birth (Hannibal and Fahrenkrug 2004). By P8, the SCN transitions away from maternal entrainment and becomes synchronized to the light-dark cycle directly via photic information from the retina and RHT (Duncan, Banister et al. 1986).

Developmental investigations of sleep and wake behavior have previously been complicated by the fact that infants at the earliest ages do not show the same state-dependent electroencephalographic (EEG) activity as adults (Gramsbergen 1976; Seelke and Blumberg 2008). However, we now know that sleep and wakefulness can be reliably studied in infant rats using electromyographic (EMG) and behavioral criteria (Blumberg and Seelke 2010). Using these methods in Norway rats, the development of circadian phase preference was examined by measuring day-night differences in sleep and

wakefulness across the early postnatal period (Gall, Todd et al. 2008). In this species, day-night differences in sleep and wakefulness occur as early as P2; however, species-typical nocturnal wakefulness does not appear until around P15.

Although the findings of Gall et al. (2008) represent progress in our understanding of the ontogeny of circadian sleep-wake rhythms in nocturnal rats, we stand to learn even more about the factors contributing to circadian phase preference through comparisons with diurnal rodents. To this end, Chapter 2 presents a developmental analysis of day-night differences in sleep and wakefulness in grass rats, a subtropical species indigenous to eastern Africa that exhibits the behavioral and retinal hallmarks of diurnality (Blanchong, McElhinny et al. 1999; Gaillard, Bonfield et al. 2008). The circadian systems of grass rats and Norway rats have been compared during adulthood (Smale, Castleberry et al. 2001; Schwartz, Nunez et al. 2004; Nixon and Smale 2007), but this is the first time that developmental changes in sleep-wake rhythms have been compared in these two species.

<u>The ventral subparaventricular zone</u>

Interestingly, all mammals studied thus far—whether nocturnal or diurnal—exhibit a rhythm of increased SCN activity during the day (Fuller, Gooley et al. 2006; Challet 2007; Houben, Deboer et al. 2009). Consequently, interactions of the RHT and SCN with downstream structures are likely to account for species differences in phase preference (Smale, Lee et al. 2003). One candidate downstream structure, adjacent to the SCN, is the vSPVZ. In adult Norway rats and grass rats, lesions of the vSPVZ selectively disrupt circadian rhythms of sleep and wake behavior (Lu, Zhang et al. 2001; Schwartz, Nunez et

al. 2009). Also in both species, the vSPVZ receives the majority of the SCN's output and projects to the same downstream structures as the SCN (Deurveilher and Semba 2005; Schwartz, Urbanski et al. 2011), making it an ideal structure for mediating and modulating circadian information (Smale, Lee et al. 2003).

In adult Norway rats, the SCN and vSPVZ exhibit *in-phase* activity such that the SCN and vSPVZ are both more active during the day than during the night (Nunez, Bult et al. 1999; Schwartz, Nunez et al. 2004). In contrast, in adult grass rats, the SCN and vSPVZ exhibit *anti-phase* activity (Nunez, Bult et al. 1999; Schwartz, Nunez et al. 2004). To better understand the relationship between SCN-vSPVZ activity patterns and behavior, in Chapter 3 we measured day-night differences in Fos-immunoreactivity (Fos-ir; a marker of neuronal activation) in the SCN and vSPVZ in Norway rats and grass rats before and after major developmental changes in circadian wakefulness.

It is not known which features of the SCN, vSPVZ, or their downstream projections account for the different neural activity patterns in the adults of these two species (Schwartz, Urbanski et al. 2011). In addition to its projection to the SCN, the RHT sends a robust direct projection to the vSPVZ in adult Norway rats (Gooley, Lu et al. 2003; Hannibal and Fahrenkrug 2004). To determine whether developmental or species differences in RHT projections are associated with differences in hypothalamic activity and behavior, in Chapter 4 we performed retinal tracing in Norway rats and grass rats at various ages. Additionally, in Chapter 5, we assessed differences in functional connectivity between the retina and hypothalamus by measuring changes in Fos-ir after 2h of light exposure during the dark period in Norway rats and grass rats.

Neurotransmitters modulating the vSPVZ

The axon terminals that comprise the RHT co-store glutamate and pituitary-adenylate cylase (PACAP) (Hannibal, Moller et al. 2000; Hannibal 2002), both of which are required for proper species-typical photic entrainment (Hannibal 2006; Brown and Piggins 2007; Golombek and Rosenstein 2010). The excitatory actions of glutamate mediate photic entrainment of the SCN via NMDA receptor activation (Colwell, Ralph et al. 1990; Vindlacheruvu, Ebling et al. 1992), whereas PACAP modulates glutamatergic transmission (Kopp, Meissl et al. 2001; Michel, Itri et al. 2006). Michel et al. showed that exogenous application of PACAP increased the amplitude and frequency of excitatory postsynaptic potentials (EPSPs) in SCN neurons, while also enhancing AMPA-evoked and NMDA-evoked EPSPs through a mechanism mediated by PACAP type 1 (PAC1) receptors. The direct retinal projection to the vSPVZ has been suggested to play a role in negative masking (Kramer, Yang et al. 2001; Gooley, Lu et al. 2003)—the suppression of nighttime behavioral activity by light—in adult nocturnal rodents. Indeed, mice deficient in the PAC1 receptor, which is prevalent in both the SCN and vSPVZ (Bergstrom, Hannibal et al. 2003; Hannibal and Fahrenkrug 2004; Kalamatianos, Kallo et al. 2004), show significant impairments in negative masking and circadian entrainment (Hannibal, Brabet et al. 2008).

Vasoactive intestinal peptide (VIP)-synthesizing neurons in the SCN project to the vSPVZ in both Norway rats and grass rats (Card, Brecha et al. 1981; Moore, Speh et al. 2002; Mahoney, Ramanathan et al. 2007). γ-aminobutyric acid (GABA) is also co-localized within these neurons (Francois-Bellan, Kachidian et al. 1990; Buijs, Wortel et al. 1995;

Tanaka, Matsuda et al. 1997; Castel and Morris 2000; Belenky, Yarom et al. 2008). Indeed, in Norway rats, the direct innervation of the vSPVZ by the SCN is inhibitory and primarily mediated by $GABA_A$ receptors (Hermes, Kolaj et al. 2009). Moreover, Hermes et al. showed that VIP facilitates GABAergic inhibition of the vSPVZ via presynaptic VPAC2 receptors, even though binding to postsynaptic VPAC2 receptors has transient depolarizing effects. In order to compare developmental and species differences between the projections to the vSPVZ from the retina and SCN, in Chapter 6 we examined the developmental expression of presynaptic terminals expressing PACAP and VIP, respectively, in the vSPVZ of Norway rats and grass rats.

Summary and conclusion

Mammals exhibit sensory and thermoregulatory adaptations that are thought to have enhanced survival in a nocturnal niche, suggesting nocturnality is a primitive characteristic of Mammalia. Accordingly, diurnality has reemerged independently in several mammalian lineages. To better understand the underlying mechanisms contributing to opposing circadian phase preferences, we employed a developmental comparative approach in nocturnal Norway rats and diurnal grass rats, two closely related Murid rodents.

First, in Chapter 2, we analyzed the developmental trajectory of day-night differences in grass rats across the early postnatal period and compare our findings to a previous study in Norway rats at similar ages (Gall, Todd et al. 2008). We find that grass rats display diurnal wakefulness as early as P2; however, the most remarkable change seen in sleep-wake behavior over development is a dramatic increase in daytime wake bout

durations that emerges by P15. Therefore, both species experience significant changes in circadian phase preference between the end of the first and the end of the second postnatal weeks, indicating the emergence of nocturnal wakefulness in Norway rats and increased diurnal wakefulness in grass rats. These results suggest fundamental changes in the neural circuitry underlying the circadian control of wakefulness over this time period.

As described earlier, adult Norway rats and adult grass rats express different patterns of SCN-vSPVZ activity. In Chapter 3, we examine the developmental emergence of these species-typical activity patterns and their association with the behavioral changes seen in Chapter 2. We predicted that, for both Norway rats and grass rats, species-typical SCN-vSPVZ activity patterns would emerge at the end of the first postnatal week in association with the changes seen in circadian preference. Indeed, at P15 in both species, Norway rats first exhibit increased daytime Fos-ir in the vSPVZ whereas grass first exhibit increased nighttime Fos-ir in the vSPVZ, representing the development of the in-phase and anti-phase activity patterns, respectively. These results further support the notion that different SCN-vSPVZ activity patterns contribute to species differences in circadian wakefulness.

It has not been established what accounts for the development of the in-phase and anti-phase patterns of SCN-vSPVZ in Norway rats and grass rats, respectively. As mentioned previously, it is known that, in addition to its direct projection to the SCN, the RHT sends a significant projection to the vSPVZ in adult Norway rats. In Chapter 4, using retinal tracing, we tested the prediction that the development of this RHT→vSPVZ projection is associated with the emergence of the in-phase SCN-vSPVZ activity pattern in

Norway rats. We also predicted that the development of the anti-phase SCN-vSPVZ activity pattern in grass rats is associated with a weak or absent RHT→vSPVZ projection. This is exactly what we found.

In Chapter 5, we examined the functional connectivity of the RHT by measuring Fos-ir in the SCN and vSPVZ in P8 and P15 Norway rats and P15 grass rats after 2 h of light exposure during the dark period. As predicted, light exposure increased Fos-ir in the SCN in all subjects, whereas only P15 Norway rats exhibited increased Fos-ir in the vSPVZ. These results suggest that the RHT→vSPVZ projection becomes functional by P15 in Norway rats and further supports our hypothesis that it contributes to the emergence of the in-phase activity pattern that develops by this age.

Finally, in Chapter 6, we analyzed the developmental expression of PACAP- and VIP-expressing presynaptic terminals in the vSPVZ, which reflect projections from the RHT and SCN, respectively. We predicted that PACAP terminals, which co-release glutamate and have excitatory postsynaptic effects, would show a significant increase in the vSPVZ by P15 in Norway rats but not grass rats, further supporting the hypothesis that differences in retinal projections contribute to differences in vSPVZ activity at this age. We also predicted that VIP terminals, which co-release GABA and produce primarily inhibitory postsynaptic effects, would show a significant increase in the vSPVZ by P15, when the anti-phase activity pattern emerges in grass rats. Again, this is exactly what we found.

Overall, our findings, across 5 experiments, converge to suggest that circadian phase preference emerges through the developmental wiring of RHT afferents to

differentially affect interactions among the retina, SCN, and vSPVZ. This evidence highlights the importance of species differences in retinal projections to the vSPVZ and provides a framework for future experiments examining differences between nocturnal and diurnal species. Because humans are diurnal, and since most previous research on the circadian system comes from nocturnal species, this work may also have important translational implications that improve our understanding of circadian disruption and its associated health outcomes.

Table 1. Distributions of circadian photoreceptors within the vertebrate central nervous system.

Vertebrate class	Pineal	Parietal eye or parapineal organ	Retina	Deep brain photoreceptors
Cyclostome	Yes	Yes	Yes	Yes
Fish	Yes	?	Yes	Yes
Amphibian	Yes	Yes	Yes	Yes
Reptile	Yes	Yes	Yes	Yes
Bird	Yes	n/a	Yes	Yes
Mammal	No	n/a	Yes	No

From Menaker et al. 1997

?, not known

n/a, not applicable.
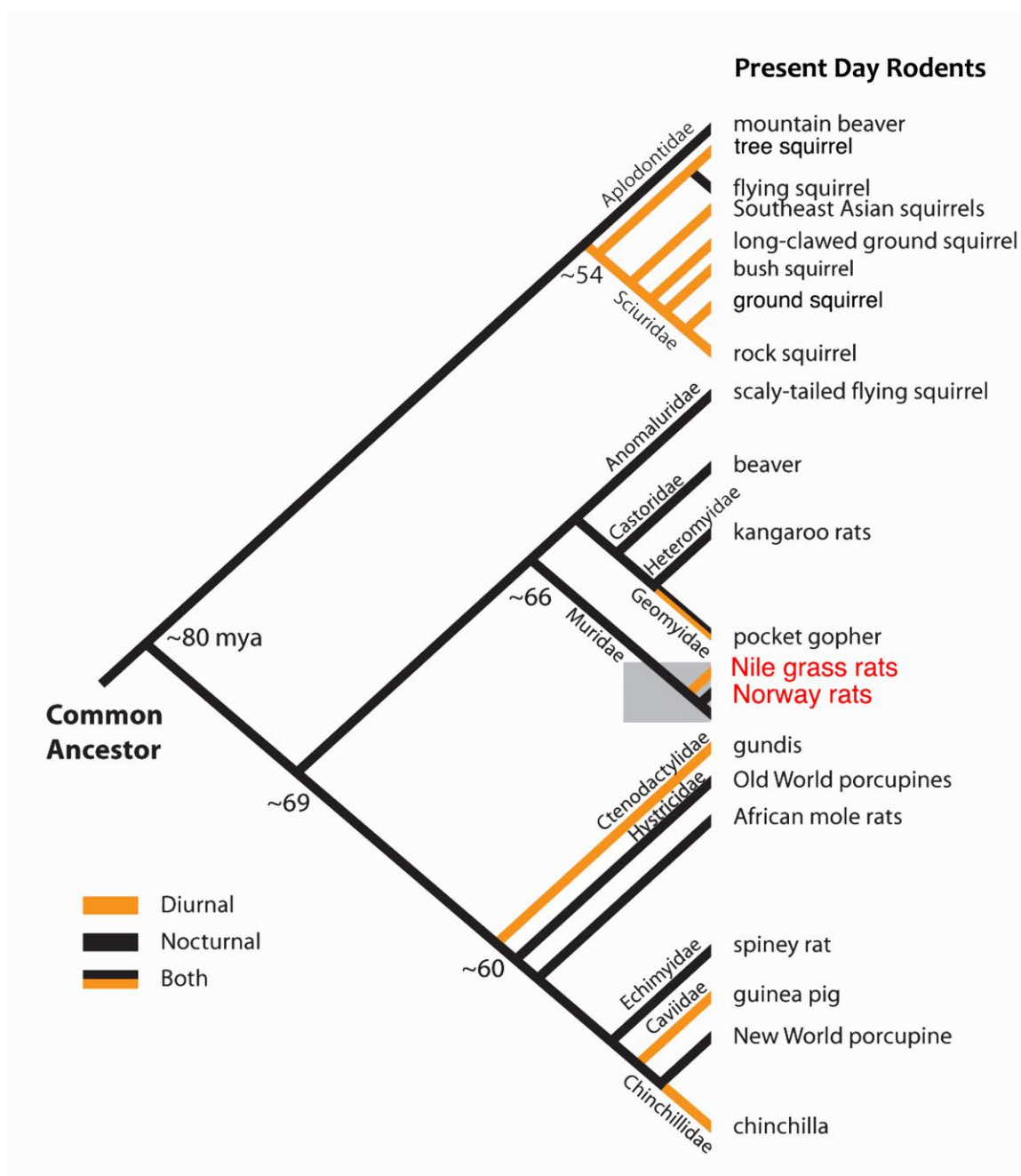
Figure 1. Phylogenetic tree of the order Rodentia depicting subfamilies comprised of nocturnal and diurnal species. Subfamilies are identified as comprising species with diurnal (orange bars), nocturnal (black bars), or both (orange and black bars) phase preferences. Column at right contains the names of representative species from each subfamily with Nile grass rats and Norway rats in larger red font. Numbers reference millions of years ago (mya) that groups diverged. Adapted from Krubitzer et al. (2011).

CHAPTER 2

DEVELOPMENTAL TRAJECTORIES OF DAY-NIGHT DIFFERENCES IN SLEEP

AND WAKEFULNESS IN NORWAY RATS AND GRASS RATS

Introduction

As described in Chapter 1, the SCN of Norway rats exhibits daily metabolic rhythms as early as E19 (Reppert and Schwartz 1983; Reppert and Schwartz 1984; Reppert, Weaver et al. 1988). Additionally, this species expresses day-night differences in sleep and wake bouts as early as P2 and nocturnal wakefulness first emerges between P8 and P15 (Gall, Todd et al. 2008). In the present study, we examined whether circadian phase preference develops similarly in a closely related diurnal species. Therefore, we recorded the developmental trajectory of sleep and wakefulness during the day and night in grass rats at P2-3 (P2), one day after eye opening at P5-7 (P6), and at P15-16 (P15). Our findings were then compared to previous measures of these behaviors in Norway rats at similar ages.

Methods

Subjects

A total of 36 P2 (n=12), P6 (n=12), and P15 (n=12) Nile grass rats were used. Males and females were equally distributed among all experimental groups. When littermates were used they were always assigned to different experimental groups. All pups were housed with their mothers in standard laboratory cages and received food and water *ad libitum*. All rats were maintained on a 12-hr light-dark cycle, with lights on at 0600.

Surgery

Twelve P2 (body weights: 3.3-5.3 g), 12 P6 (body weights: 6.4-10.7 g) and 12 P15 (body weights: 15.0-23.8 g) grass rats were used. Under isoflourane anesthesia, bipolar stainless steel hook electrodes (50 um diameter; California Fine Wire, Grover Beach, CA) were implanted bilaterally in the nuchal muscles and secured with flexible collodion. Surgeries and anesthesia typically lasted 5-7 min. A ground wire was looped through the back and secured with flexible collodion. The pup was then transferred to the testing chamber and allowed at least 45 min to recover and acclimate. Surgeries performed at night were illuminated using a dim 25W red bulb, with care being taken to ensure that pups were never exposed to white light.

Apparatus

The testing environment consisted of an electrically shielded, double-walled glass chamber (height = 17 cm, i.d. = 12.5 cm) with a Plexiglas lid. An access hole in the side of the chamber allowed for the passage of humidified air (flow rate: 300 ml/min) and EMG electrodes. Heated water circulated through the walls of the chamber to maintain air temperature at approximately 35.5°C for P2 grass rats, 35°C for P6 grass rats, and 33.5°C for P15 grass rats. In preliminary studies, these temperatures were determined to be within the thermoneutral range for grass rats at these ages using similar methods for studying thermoneutrality in developing Norway rats (Spiers and Adair 1986; Blumberg 2001).

Procedure

During either the day or night, freely moving pups were allowed to cycle undisturbed between sleep and wakefulness for 6 h while EMG data were recorded.

Nuchal electrodes were connected to differential amplifiers (A-M systems, Carlsbad, WA) and their signals were amplified (x10k) and filtered (300-5000 Hz). EMG signals were sampled at 1 kHz, integrated, and full-wave rectified using a data acquisition system (BioPac Systems, Inc., Santa Barbara, CA). Testing times were arranged so that the midpoint of each photoperiod coincided with the midpoint of the 6 h test. Accordingly, tests during the day occurred from 0900 to 1500 and tests during the night occurred from 2100 to 0300. Daytime and nighttime tests were counterbalanced.

Analysis

EMG data were analyzed as described previously (Karlsson, Gall et al. 2005). Briefly, the records were dichotomized into bouts of sleep and wakefulness as follows: For each rat, the amplitudes of 5 1-s segments of both atonia and high-tone were averaged and the midpoint between the two was determined. Periods of at least 1 s in which muscle tone was below this point were defined as atonia (indicative of sleep) while those above were defined as high tone (indicative of wakefulness). All data were scored by an experienced individual blind to experimental condition.

Sleep and wake bout durations for all pups were imported into JMP 5.0 (SAS Institute, Cary, NC). Mean bout durations for sleep and wakefulness were determined for each pup and percentage of time awake was found by dividing the mean wake bout duration by the sum of the mean sleep and wake bout durations and multiplying by 100. A 2-factor analysis of variance (ANOVA) was used to test for differences across age and time, and planned comparisons utilizing unpaired t tests served to reveal day-night differences within groups. Additionally, data were pooled for each pup in order to produce survivor

distributions of sleep and wakefulness, as described elsewhere (Blumberg, Seelke et al. 2005). These survivor distributions were analyzed using statistical methods as previously described (Gall, Todd et al. 2008). For all tests, alpha was set at .05.

<u>Results</u>

Figure 2 compares the developmental trajectory of day-night differences in wakefulness in grass rats to that found in a previous study using Norway rats at similar ages (Gall, Todd et al. 2008). Grass rats consistently express diurnal wakefulness as early as P2, whereas nocturnal wakefulness first emerges in Norway rats at P15. For percent time awake, ANOVA revealed significant main effects of age [$F(2,30) = 21.3$, $P < .0001$] and time [$F(1,30) = 33.7$, $P < .0001$], but not a significant age x time interaction [$F(2,30) = 0.1$]. Unpaired t tests revealed that mean percentage of time spent awake was higher during the day in grass rats regardless of age. For mean wake bout durations, ANOVA revealed significant main effects of age [$F(2,30) = 20.8$, $P < .0001$], time [$F(1,30) = 20.7$, $P < .0001$], and a significant age x time interaction [$F(2,30) = 7.5$, $P < .002$]. Unpaired t tests revealed that mean wake bout duration were higher during the day regardless of age.

Perhaps the most remarkable change in wake behavior in grass rats across development was a striking increase in diurnal wakefulness at P15. Unpaired t tests revealed that, at P15, daytime wake bout durations and percentage time spent awake during the day significantly increased compared to earlier ages, while these values did not differ significantly between P2 and P6.

Figure 2 also presents log-survivor distributions for pooled wake bout durations for Norway rats and grass rats. As demonstrated previously in Norway rats (Blumberg, Seelke et al. 2005; Gall, Todd et al. 2008), such plots can be used to reveal statistical distributions and developmental trajectories of sleep and wake bouts. Here we show that both species exhibit dramatic developmental changes in wakefulness between the first and second postnatal weeks. As mentioned above, grass rats exhibit a dramatic increase in diurnal wakefulness at P15, while nocturnal wakefulness first emerges at P15 in Norway rats.

Interestingly, mean sleep bout durations did not change significantly over development as ANOVA did not reveal significant main effects of age [$F(2,30)$ = 2.4] or time [$F(1,30)$ = 4.1], nor a significant age x time interaction [$F(2,30)$ = 1.0] (data not shown). Figure 3 presents survivor distributions for pooled sleep bout durations for Norway rats and grass rats. In addition to the lack of changes in mean sleep bout duration across development in grass rats, the overall distribution of sleep bouts did not noticeably change during this time. This is interesting since Norway rats show a marked increase in sleep bout consolidation between P8 and P15.

Discussion

The fact that grass rats exhibit diurnal wakefulness as early as P2, days before eyelid opening, may suggest that they were entrained to the circadian rhythmicity of the mother, as in infant and prenatal Norway rats (Reppert and Schwartz 1983; Reppert and Schwartz 1984; Reppert, Weaver et al. 1988). Alternatively, this day-active behavioral profile may suggest that the circadian system in P2 grass rats is developed enough to be entrained on its

own, by either diffuse light through the sealed eyelids or perhaps through the utilization of social zeitgebers. Regardless, the grass rats' wake behavior undergoes a dramatic change over the second postnatal week when wake bouts become substantially longer during the day. Interestingly, we showed previously that nocturnal wakefulness emerges over this same time period in Norway rats. We believe these behavioral changes reflect further development of the neural circuitry governing the circadian modulation of wakefulness.

Figure 2. Developmental trajectory of wakefulness during the day and night in Norway rats and grass rats. Log-survivor distributions of wake bouts (pooled across subjects; 2228-3325 points per plot) and mean percentage of time awake (insets) in Norway rats (left) and Nile grass rats (right). Recordings occurred during the day (red) and night (blue), at 3 postnatal ages (n=6 per group). * significant difference from nighttime value, $P < .05$. ** significant difference from nighttime value, $P < .005$. Norway rat data are from a previous study (Gall, Todd et al. 2008). Note the different y-axis scales in the insets for the Norway rats and grass rats. Means + standard errors.

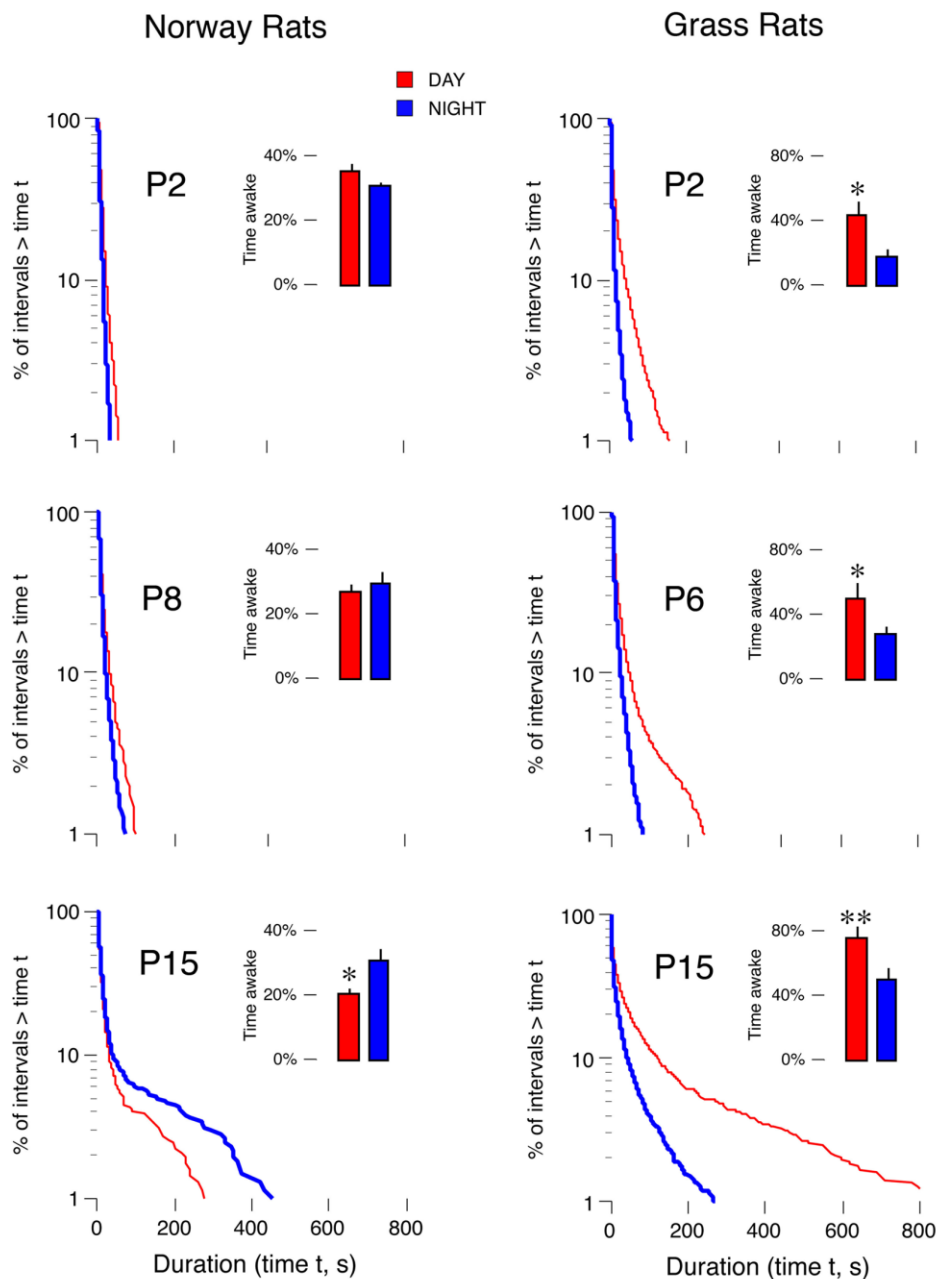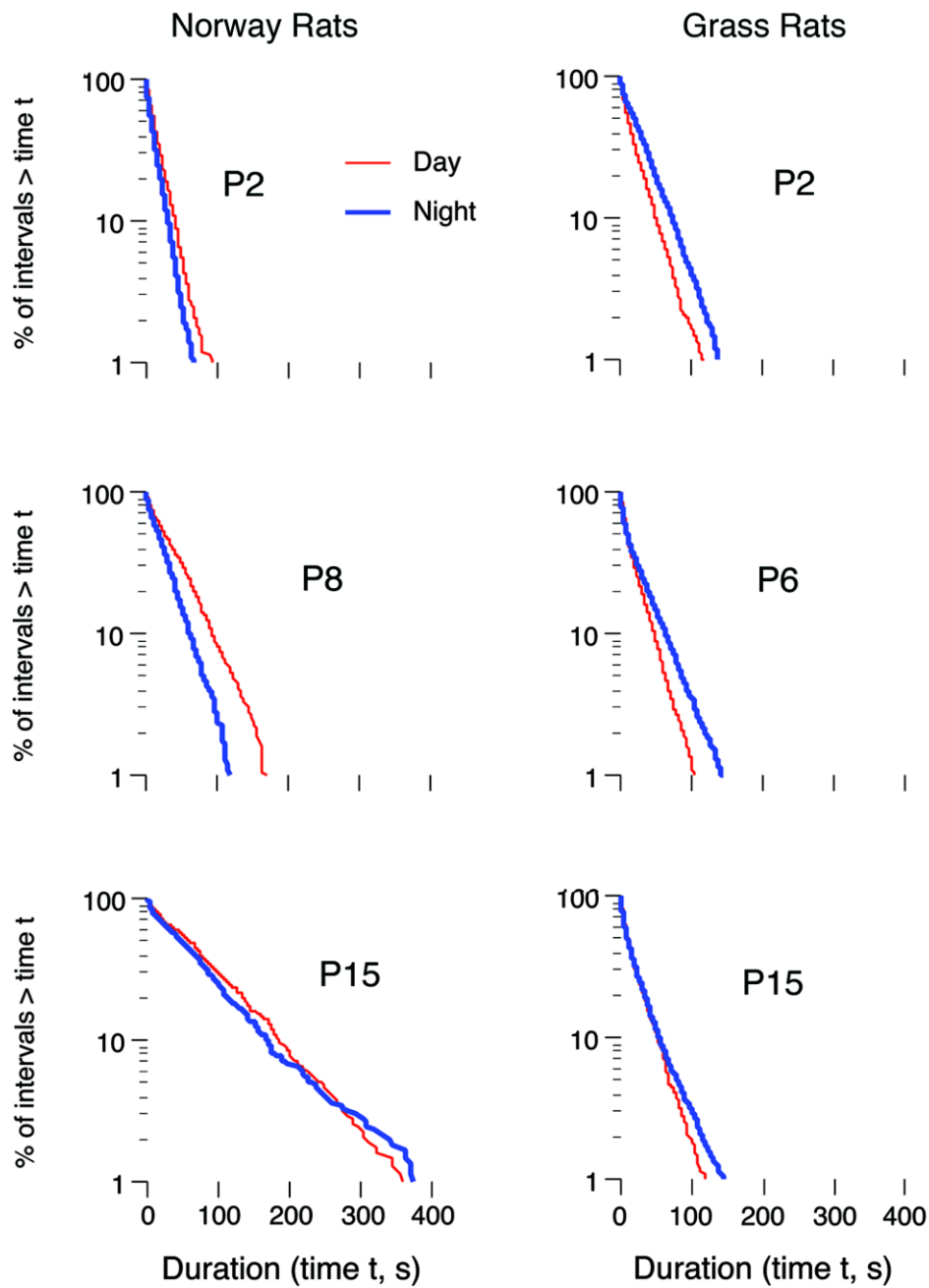Figure 3. Developmental trajectory of sleep bouts during the day and night in Norway rats and grass rats. Log-survivor distributions of sleep bouts (pooled across subjects; 2228-3325 points per plot) in Norway rats (left) and Nile grass rats (right). Recordings occurred during the day (red) and night (blue), at 3 postnatal ages (n=6 per group). Norway rat data are from a previous study (Gall, Todd et al. 2008).

CHAPTER 3

DEVELOPMENT OF SPECIES-TYPICAL PATTERNS OF HYPOTHALAMIC

ACTIVITY

Introduction

In Chapter 2, we showed that both Norway rats and grass rats exhibit profound developmental changes in circadian wake behavior between the end of the first and second postnatal weeks. Here we sought to investigate the neural mechanisms associated with these behavioral changes.

As described in Chapter 1, circadian rhythms of sleep and wakefulness are regulated by a system that includes the retina, SCN of the hypothalamus and their connection via the RHT (Hannibal, 2002a). Because the SCN is day-active in all mammals studied thus far, regardless of behavioral phenotype (Challet, 2007; Fuller et al., 2006; Houben et al., 2009), it alone is unlikely to determine circadian phase preference. Alternatively, in the vSPVZ, an area adjacent to the SCN that is also required for the expression of circadian sleep-wake rhythms (Lu et al., 2001; Schwartz et al., 2009), different patterns of neural activity are displayed in Norway rats and grass rats. Specifically, in adult Norway rats, the vSPVZ is more active during the day and, therefore, exhibits an in-phase pattern of activity with the SCN (Nunez et al., 1999; Schwartz et al., 2004). In adult grass rats, however, the vSPVZ is more active during the night and, thus, exhibits an anti-phase pattern of activity with the SCN (Nunez et al., 1999; Schwartz et al., 2004).

We hypothesized that the emergence of these species-typical patterns of SCN-vSPVZ activity contributes to the developmental changes in circadian phase preference shown in Chapter 2. To test this hypothesis, we examined day-night differences in Fos-ir in the SCN and vSPVZ of P8 and P15 Norway rats and P6 and P15 grass rats.

Methods

Subjects

A total of 24 P8 (n=12) and P15 (n=12) Sprague-Dawley Norway rats from 12 litters and a total of 24 (n=12) P6 and P15 (n=12) grass rats from 12 litters were used. Grass rats and Norway rats were maintained in separate rooms in the animal colony at the University of Iowa. All pups were housed with their mothers in standard laboratory cages and received food and water *ad libitum*. Litters of Norway rats were culled to 8 pups within 3 days of birth (day of birth = Day 0). Grass rat litters were not culled due to their relatively smaller litter size. Grass rat litters averaged about 6 pups and no litters comprising less than 4 pups were used. All rats were maintained on a 12-hr light-dark cycle, with lights on at 0600 for grass rats and at 0700 for Norway rats.

Tissue preparation

Twelve P8 (body weights: 17.7-25.8 g) and 12 P15 (body weights: 36.6-49.9 g) Norway rats and 12 P6 (body weights: 5.4-12.0 g) and 12 P15 (body weights: 19.3-30.5 g) grass rats were used. During the day or night, pups were removed from the litter and immediately sacrificed approximately 90 min after the midpoint of their respective photoperiod, allowing sufficient time for production of the Fos protein (Cirelli and

Tononi 2000). Accordingly, P8 and P15 Norway rats were sacrificed at 1430 or 0230 hrs and P6 and P15 grass rats were sacrificed at 1330 or 0130 hrs. At night, a dim red light was used for illumination and care was taken to ensure that these pups were never exposed to white light. Daytime and nighttime tests were counterbalanced. Animals were killed with an overdose of Nembutal and perfused transcardially with phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB; pH 7.4). Brains were removed and stored in 30% sucrose solution. After 5-10 days in sucrose solution, brains were placed on a freezing microtome and sliced in the coronal plane (40 μm sections).

<div align="center">Fos immunohistochemistry</div>

Immunohistochemistical procedures were performed as described previously (Todd, Gibson et al. 2010). All reactions were conducted at room temperature. Free-floating sections were pretreated with normal goat serum for 1 h, rinsed with PBS, and then incubated overnight with the primary antibody (1:2000; Santa Cruz Biotechnology, CA) in 0.01 M PBS and 0.3% Triton X. This rabbit polyclonal antibody was raised against amino acids 210-335 mapping at the C-terminus of human c-Fos and stained a single band of 55 kDa on Western blots from human carcinoma tissue (manufacturer's technical information). The next day, the sections were rinsed with PBS and then incubated in a biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 1 h in 0.01 M PBS and 0.3% Triton X. After additional PBS rinses, tissues were reacted with an avidin-biotin peroxidase complex (Vector Laboratories) for 1 h. The sections were again rinsed with PBS before reaction with diaminobenzidine solution

and hydrogen peroxide (Sigma-Aldrich, St. Louis, MO). The reaction was terminated with PBS. For sections serving as negative controls, primary antibodies were omitted. All sections were mounted, dehydrated with alcohol, and cleared with xylenes. All slides were then coverslipped with Depex.

Data Analysis

Images of 40 µm brain sections were captured using a Leica DM/LS microscope and imaging system with Firecam software (Leica Microsystems, Germany). Images were imported into ImageJ (National Institutes of Health) and adjusted to binary values. Using methods similar to those described elsewhere (Todd, Gibson et al. 2010), cells positive for Fos-ir were identified. Typically, cells were approximately 10 µm in diameter. For each subject, 2-3 sections containing the SCN and vSPVZ were selected for analysis. Using a counting box of known dimensions, profiles of Fos-positive cells were counted unilaterally by an experienced individual blind to experimental group. An Abercrombie correction was performed on the counts for each area within each section (Abercrombie 1946). The mean number of Fos-positive cells per section was then calculated for each pup.

The SCN and vSPVZ were identified using a stereotaxic atlas of the rat brain (Paxinos and Watson 1998), as well as previous publications describing these structures in adult grass rats and Norway rats (Lu, Zhang et al. 2001; Gooley, Lu et al. 2003; Schwartz, Nunez et al. 2004; Schwartz, Nunez et al. 2009). Although cell bodies were not counterstained, brain areas were determined on the basis of adjacent anatomical structures (e.g., third ventricle, optic chiasm). When needed, archival Nissl-stained sections from separate animals at similar ages were used to help delineate boundaries between areas. Data

were imported into JMP 5.0 and paired or unpaired t tests were used to reveal day-night differences within each brain area. Alpha was set at 0.05 and Bonferroni corrections were performed to correct for multiple comparisons.

Results

In Norway rats at both P8 and P15 (Fig. 4A, top), the mean number of Fos-positive cells in the SCN was greater during the day than during the night (P8: $t(10) = 7.5$, $P \leq 0.001$; P15: $t(10) = 5.8$, $P \leq 0.002$). In the vSPVZ at P8, Fos values did not differ between day and night ($t(10) = 1.0$), whereas higher values were found during the day at P15 ($t(10) = 6.3$, $P \leq 0.0001$). In grass rats at both P6 and P15 (Fig. 4B, top), Fos values in the SCN were again greater during the day than during the night (P6: $t(10) = 7.0$, $P \leq 0.0005$; P15: $t(10) = 13.8$, $P \leq 0.0001$). In the vSPVZ at P6, Fos values did not differ between day and night ($t(10) = 0.1$), whereas higher values were now found during the night at P15 ($t(10) = 9.7$, $P \leq 0.0001$). These patterns, at P15, of increased vSPVZ activity during periods of behavioral inactivity is typical of adults of these species (Schwartz, Nunez et al. 2004). Accordingly, this represents the development of the in-phase relationship between SCN and vSPVZ in Norway rats and the anti-phase relationship between these structures in grass rats.

Discussion

In contrast to the SCN, which displays a similar day-night pattern of activity in both species regardless of age, the vSPVZ may play a role in determining nocturnality or

diurnality since its activity increases during each species' inactive photoperiod. In fact, the vSPVZ has already been hypothesized to function as a "switch" in determining an animal's phase preference (Smale, Lee et al. 2003), since the activity and morphology of the SCN has been found to differ little across numerous diurnal and nocturnal species (Mrosovsky and Hattar 2005; Challet 2007). Indeed, in both Norway rats and grass rats, the vSPVZ receives the majority of SCN efferents, and both vSPVZ and SCN project to the same downstream structures (Schwartz, Urbanski et al. ; Silver and LeSauter 1993; Deurveilher and Semba 2005), further supporting the hypothesis that it interprets and modulates the SCN's signal. Finally, as previously mentioned, lesions of the vSPVZ selectively disrupt circadian rhythms of sleep and wakefulness in adult Norway rats and grass rats (Lu, Zhang et al. 2001).

Importantly, our results show that species differences in the pattern of SCN-vSPVZ activity first develop around the time that major changes appear in circadian wake behavior—the emergence of nocturnal wakefulness in Norway rats and a dramatic increase in diurnal wakefulness in grass rats. However, what causes the vSPVZ to develop different Fos-ir profiles in Norway rats and grass rats remains unknown.

Figure 4. Development of species-typical SCN-vSPVZ activity in Norway rats and grass rats. (A) Top: Mean number of Fos-positive cells per section in the suprachiasmatic nucleus (SCN) and ventral subparaventricular zone (vSPVZ) for P8 and P15 Norway rats. Bottom: Representative hypothalamic sections from P15 Norway rats depicting the in-phase Fos-ir activity profile in the SCN and vSPVZ. (B) Top: Mean number of Fos-positive cells per section in the SCN and vSPVZ for P6 and P15 Nile grass rats. Bottom: Representative hypothalamic sections from P15 grass rats depicting the anti-phase Fos-ir activity profile in the SCN and vSPVZ. Red bars represent rats sacrificed during the day and blue bars represent rats sacrificed at night. n=6 per group. * significant difference from nighttime group, $P < 0.005$. Means + standard errors. Scale bar: 100 µm.

CHAPTER 4

DEVELOPMENT OF RHT PROJECTIONS IN NORWAY RATS AND GRASS RATS

Introduction

In Chapter 3, we showed that the major developmental differences seen in behavior in Norway rats and grass rats are associated with the appearance of species-typical in-phase and anti-phase patterns of SCN-vSPVZ activity, respectively. Here we sought to investigate why these patterns of activity might differ between these two species.

It is known that the RHT projects to the SCN in both Norway rats and grass rats (Rea 1998; Smale and Boverhof 1999). Additionally, the RHT sends a robust direct projection to the vSPVZ in adult Norway rats (Gooley, Lu et al. 2003). This led us to wonder whether differences in RHT projections to the vSPVZ might account for species differences in SCN-vSPVZ activity.

Because the neurotransmitters released by the RHT have excitatory postsynaptic effects, as described in Chapter 1, we hypothesized that the development of this RHT→vSPVZ projection would be associated with the emergence of the in-phase SCN-vSPVZ activity pattern in Norway rats. Alternatively, we hypothesized that the development of the anti-phase SCN-vSPVZ activity pattern in grass rats would be associated with a weak or absent RHT→vSPVZ projection. Figure 5 presents a model depicting our prediction of species differences in RHT projections in Norway rats and grass rats. To test this prediction, we performed retinal tracing in Norway rats and grass rats at various ages.

<u>Methods</u>

Eye injection

Four P4 (body weights = 8.8-13.6 g), 4 P11 (body weights = 24.7-33.0 g), and 4 P17 (body weights = 49.0-54.2 g) Norway rats and 4 P2-3 (body weights = 4.7-8.3 g) and 4 P11 (body weights = 11.6-16.6 g) grass rats were used. Three additional adult grass rats (body weights = 90.0-135.0 g) were also used. Under isofluorane anesthesia, each rat received a unilateral intravitreal injection as follows: For P4 and P11 Norway rats, and P2 grass rats, a 30 gauge needle attached to a 10 µl Hamilton syringe was inserted through the skin between the sealed eyelids and into the orbit. For P17 Norway rats and P11 and adult grass rats, ages at which the eyelids have already opened, the procedure was the same except the needle was inserted directly into the orbit. Approximately 10 µl of Alexa Fluor 594 (Texas-Red)-conjugated CTB (1 mg/ml; Molecular Probes, Eugene OR, USA) in PBS was slowly injected into the right or left eye in a counterbalanced manner (Allen and Earnest 2005). For adult grass rats, 10-15 µl of CTB solution was injected. Following the injection, the needle remained in the eye for 15 s before removal.

Tissue Preparation and Histology

Four days after the injection, each rat was overdosed with Nembutal and perfused transcardially with PBS followed by 4% PFA. Brains were postfixed overnight in 4% PFA and then stored in 30% sucrose solution for 8-10 days. Using a freezing microtome, 40 µm sections were cut and collected. Every other section was mounted and coverslipped using Vectastain Hard Set (Vector) to preserve fluorescence. The remaining sections were

mounted and counterstained using cresyl violet. They were then dehydrated with alcohol, cleared with citrus clearing solution, and coverslipped using Depex.

Analysis

The SCN and vSPVZ were first identified by comparing counterstained sections to a stereotaxic atlas of the Norway rat brain (Paxinos and Watson 1998), as well as with previous work examining these structures in adult grass rats and Norway rats (Lu, Zhang et al. 2001; Gooley, Lu et al. 2003; Schwartz, Nunez et al. 2004; Schwartz, Nunez et al. 2009). Unstained sections were analyzed for the presence or absence of CTB labeling in the SCN and vSPVZ using a Leica DM/LS fluorescent microscope and imaging system. Light intensity and contrast levels were held constant for all sections across all subjects. Using a 20x objective, images of consecutive fluorescent and corresponding Nissl-stained sections were collected and then imported into Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA) to determine the locations of CTB labeling. To compare age differences in CTB labeling in the vSPVZ, 2 fluorescent sections were selected from each subject for further analysis. Images were scored by an individual blind to the age and species of each subject. Mean fluorescence intensity (MFI) in the red channel was quantified, contralateral to the injected eye, for each section using ImageJ software containing the RGB Measure plug-in (Bubier, Bennett et al. 2007). MFI per section was then calculated for each subject. Data were imported into Statview 5.0 (SAS Institute, Cary, NC). ANOVA was used to test for differences across ages and species, and Fisher's PLSD was used for post hoc comparisons. For all tests, alpha was set at 0.05.

<u>Results</u>

In P8 Norway rats, as expected, CTB labeling was detected in the SCN but only sparse and inconsistent labeling was found in the vSPVZ (Fig. 6A-C). In contrast, at P15, strong labeling was found in both the SCN and the vSPVZ (Fig. 6D-F). Finally, one week later at P21, this vSPVZ labeling was not noticeably denser (Fig. 7A-B). As shown in Figure 8, MFI in the vSPVZ of Norway rats increased progressively between P8, P15, and P21. ANOVA revealed a significant main effect of age on MFI [$F(2,9) = 7.8$, $P < 0.05$].

In grass rats, as predicted, we found intense CTB labeling of the SCN at P6 and P15 but little or no such labeling in the vSPVZ at those ages (Fig. 6G-I). We similarly found little or no labeling in the vSPVZ of the 3 adult grass rats (Fig. 7C-D). As shown in Figure 8, MFI in the vSPVZ of grass rats was very low at P6 and did not increase significantly by P15 or adulthood [$F(2,8) = 3.1$]. Finally, to ensure that our tracing method was reliable in this species to structures beyond the SCN, we confirmed labeling around the supraoptic nucleus (SON) in all subjects.

<u>Discussion</u>

We have identified a novel species difference in RHT projections that may have functional implications for circadian phase preference. Specifically, we have shown that Norway rats develop a robust RHT→vSPVZ projection between P8 and P15, whereas grass rats do not. As described in Chapter 1, the subset of retinal ganglion cells whose axons make up the RHT contain the photopigment melanopsin (Hannibal, Hindersson et al. 2002; Gooley, Lu et al. 2003), which has been implicated in circadian processing in

numerous species (Doyle and Menaker 2007; Hankins, Peirson et al. 2008; Paul, Saafir et al. 2009). Therefore, whether or not an animal's vSPVZ receives innervation from these cells likely has significant ramifications for vSPVZ activity and, in turn, behavior. Recall that in both grass rats and Norway rats, the major projection from the SCN innervates the vSPVZ, and both structures project to the same major sleep and wake nuclei (Schwartz, Urbanski et al.), thereby influencing the sleep-wake cycle. Indeed, we show here that the development of this direct projection is associated with the developmental emergence of nocturnal wakefulness in Norway rats.

These results confirm our model of predicted species differences in RHT projections presented in Figure 5. As also described in Chapter 1, the projections of the RHT, and their associated neurotransmitters PACAP and glutamate, have excitatory postsynaptic effects (Hannibal, Moller et al. 2000; Hannibal 2002). Therefore, light via the RHT can entrain the SCN and may also drive daytime vSPVZ activity in Norway rats, accounting for the species-typical in-phase pattern. In grass rats, which do not have an excitatory RHT→vSPVZ projection, an anti-phase pattern develops. This pattern is most likely due to the fact that the inhibitory projection from the SCN, which releases VIP and GABA (Hermes, Kolaj et al. 2009), can modulate vSPVZ activity without interference from such an excitatory RHT-vSPVZ projection. The predictions of this model will be tested further in subsequent chapters.

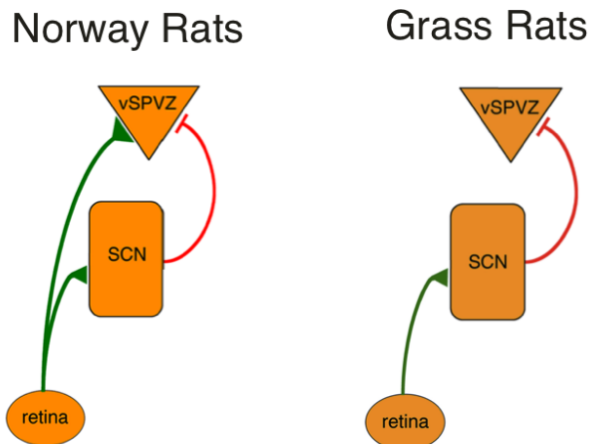Figure 5. Model of predicted species differences in RHT projections in Norway rats and grass rats. Green lines denote presumed excitatory connections releasing glutamate and pituitary adenylate cyclase (PACAP) and red lines denote presumed inhibitory connections releasing γ-aminobutyric acd (GABA) and vasoactive intestinal peptide (VIP). SCN, suprachiasmatic nucleus, vSPVZ, ventral subparaventricular zone.

Figure 6. CTB labeling following RHT tracing in a P8 and P15 Norway rat and a P15 grass rat. Fluorescent images from adjacent sections depicting CTB-labeled retinohypothalamic tract (RHT) projections to the suprachiasmatic nucleus (SCN) and ventral subparaventricular zone (vSPVZ) in representative P8 (A-C) and P15 (D-F) Norway rats and a representative P15 Nile grass rat (G-I). Sections are at the level of the rostral SCN (top), caudal SCN and rostral vSPVZ (middle), and caudal vSPVZ (bottom). Locations of landmarks, including the perimeter of the SCN (yellow dashed lines), were determined using corresponding Nissl-stained sections. Yellow arrows point to robust vSPVZ labeling in the P15 Norway rat. All images shown are contralateral to the injected eye. Sections are arranged, top to bottom, from rostral to caudal. 3V, third ventricle; ot, optic tract. Scale bar: 150 μm.
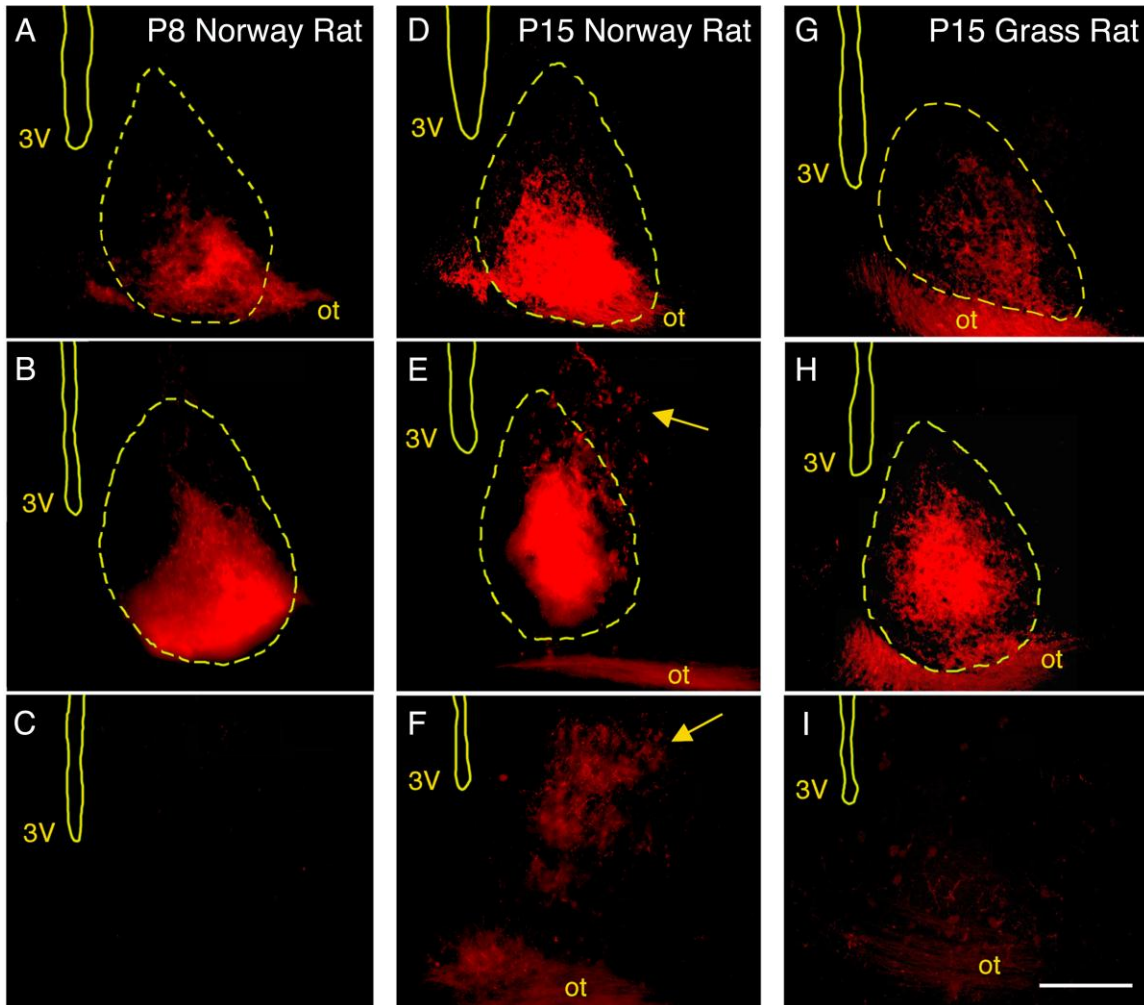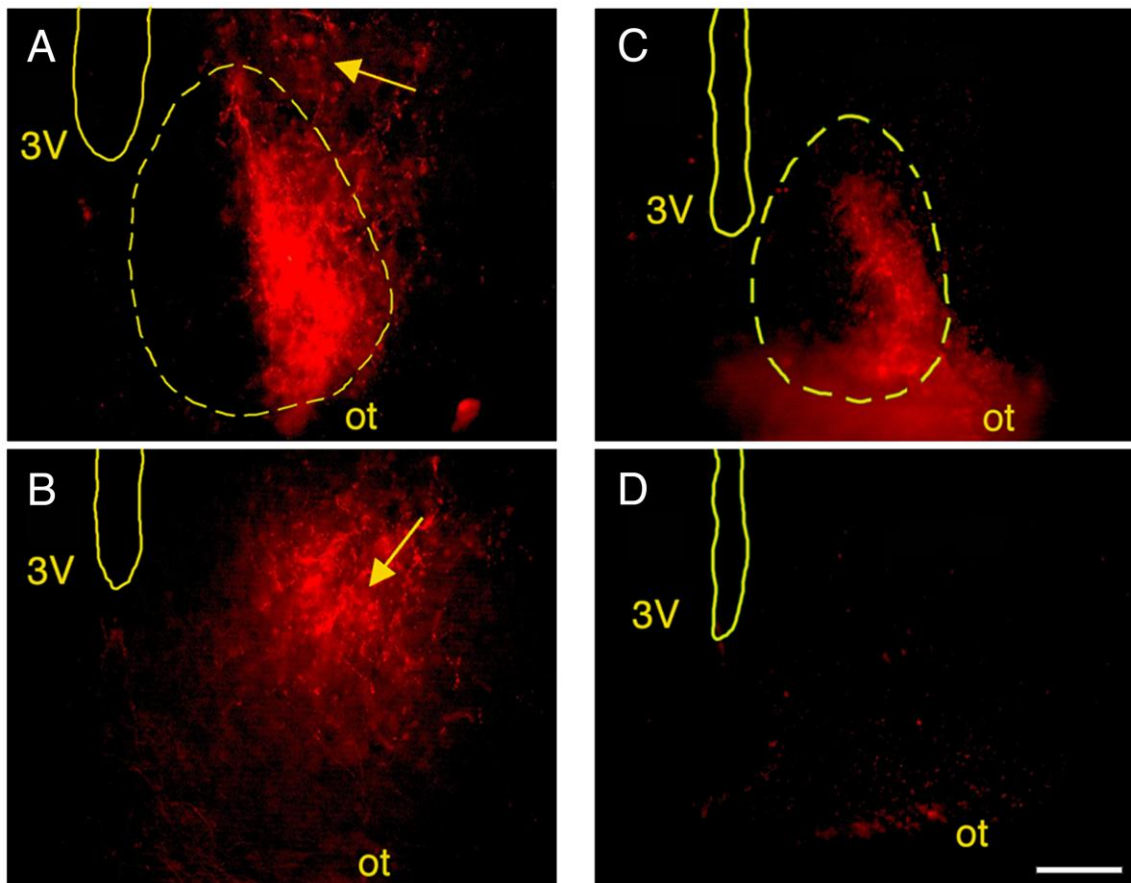
Figure 7. CTB labeling following RHT tracing in a P21 Norway rat and an adult grass rat. Fluorescent images from adjacent sections depicting CTB-labeled retinohypothalamic tract (RHT) projections to the suprachiasmatic nucleus (SCN) and ventral subparaventricular zone (vSPVZ) in a representative P21 Norway rat (A-B) and a representative adult Nile grass rat (C-D). Sections are at the level of the caudal SCN and rostral vSPVZ (top) and caudal vSPVZ (bottom). Locations of landmarks, including the perimeter of the SCN (yellow dashed lines), were determined using corresponding Nissl-stained sections. Yellow arrow points to robust vSPVZ labeling in the P21 Norway rat. All images are contralateral to the injected eye. Sections are arranged, top to bottom, from rostral to caudal. 3V, third ventricle; ot, optic tract. Scale bar: 150 μm.
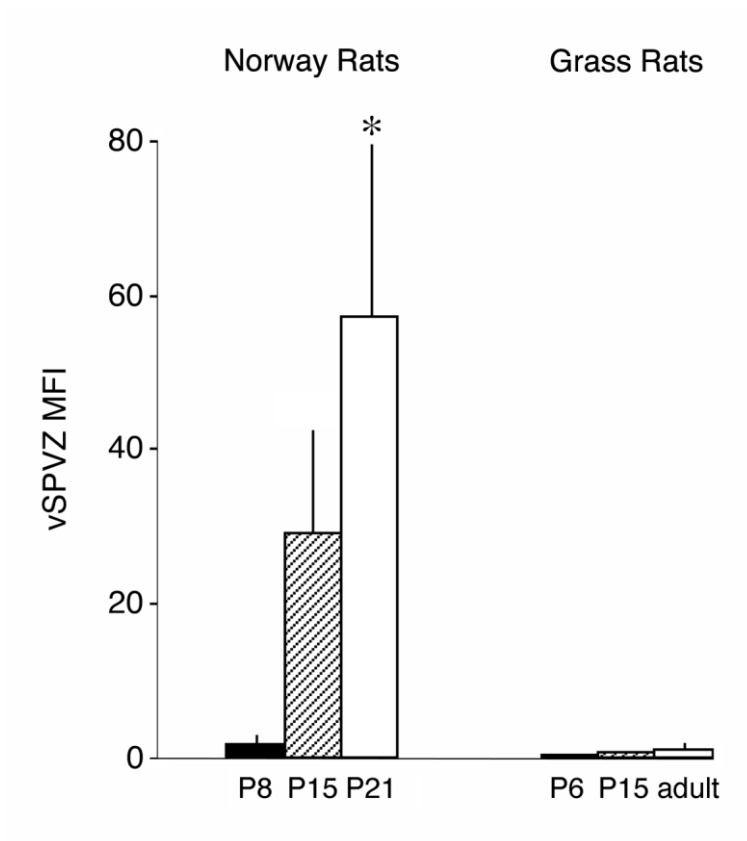
Figure 8. Mean fluorescent intensity of CTB labeling in the vSPVZ in Norway rats and grass rats across development. Developmental differences in mean fluorescence intensity (MFI) per section in the ventral subparaventricular zone (vSPVZ) following retinal tracing in P8, P15, and P21 Norway rats (left) and P6, P15, and adult Nile grass rats (right). n=4 per group except for adult grass rats (n=3). * significant difference from two other groups, $P < 0.05$. Means + standard errors.

CHAPTER 5

A FUNCTIONAL TEST OF RHT CONNECTIVITY

Introduction

In Chapter 4 we showed that the emergence of nocturnal wakefulness and the in-phase pattern of SCN-vSPVZ activity in Norway rats are associated with the development of robust RHT projections to the vSPVZ at P15. In contrast, in grass rats we found that the development of increased diurnal wakefulness and the anti-phase pattern at P15 were associated with little or no RHT projections to the vSPVZ. Here we examine whether retinal projections have measurable functional consequences on vSPVZ activity by P15.

In adult Norway rats and grass rats, light exposure increases Fos-ir in the SCN during the dark phase, when SCN activity is normally low (Earnest, Iadarola et al. 1990; Mahoney, Bult et al. 2001). As a functional test of our anatomical findings from Chapter 4, we measured changes in Fos-ir in the SCN and vSPVZ following 2 h of light exposure during the dark period in Norway rats at the end of the first and second postnatal week. Additionally, we measured Fos-ir in these areas following light exposure during the dark period in P15 grass rats. We predicted that light exposure would result in increased Fos-ir in the SCN in all subjects, but would result in increased Fos-ir in the vSPVZ only in Norway rats at P15, after the RHT→vSPVZ projection has developed. Determining that this excitatory projection is functional in P15 Norway rats would further support our hypothesis that it contributes to the in-phase SCN-vSPVZ activity pattern.

Methods

Procedure

Twelve P8 (body weights = 14.3-21.8 g) and 12 P15 (body weights = 45.7-53.0 g) Norway rats and 8 P15 grass rats (body weights = 12.1-17.1 g) were used. During the dark period, two littermates were removed and immediately placed—in separate rooms—into thermoneutral testing chambers identical to those described in Experiment 1. Chamber temperature was maintained at 35°C and 32°C for P8 and P15 rats, respectively, and 33.5°C for P15 grass rats. A dim red light was used for illumination in the dark. After 15 min of acclimation to the chamber, one littermate was maintained in the dark and the other was exposed to standard room lighting for 2 hrs. The midpoint of the 2-hr test was timed to occur during the midpoint of dark period, from 2400-0200.

Tissue preparation, immunohistochemistry, and histology

Immediately after the 2 h exposure period, all subjects were overdosed and perfused as in Experiment 2. Brains were postfixed, stored, and sliced as in Experiment 2. Every other section throughout the hypothalamus was collected and Fos-immunohistochemistry was performed as previously described. The remaining sections were collected and Nissl-stained as in Experiment 3. All sections were mounted, dehydrated and cleared, and coverslipped as described in Chapter 3.

Analysis

Sections processed for Fos-ir were compared to Nissl-stained sections. The locations of SCN and vSPVZ were determined as in previous experiments. In 3-4 sections per subject, Fos-postive cells in the SCN and vSPVZ were analyzed as in Chapter 3. Data were

imported into JMP 5.0 and paired *t* tests were used to reveal day-night differences within each brain area for each group. Alpha was set at 0.05 and Bonferroni corrections were performed to correct for multiple comparisons.

Results

Figure 9 presents the effects of 2 h of light exposure, during the dark period, on the mean number of Fos-positive cells in the SCN and vSPVZ in P8 and P15 Norway rats and P15 grass rats. As predicted, for Norway rats, light exposure produced significant increases in Fos values in the SCN at P8 ($t(10) = 6.4$, $P \leq 0.0001$) and P15 ($t(10) = 4.0$, $P \leq 0.005$). Also as predicted, light exposure in Norway rats did not produce a significant increase in Fos values in the vSPVZ at P8 ($t(10) = 1.1$) but did at P15 ($t(10) = 4.1$, $P \leq 0.005$). Alternatively, in P15 grass rats, light exposure increased Fos-ir in the SCN ($t(6) = 5.7$, $P \leq 0.005$) but not in the vSPVZ ($t(6) = 0.4$).

Discussion

For our functional test of RHT connectivity, we used Fos immunohistochemistry to measure neuronal activity changes in the SCN and vSPVZ in response to 2 h of light stimulation during the dark period. As predicted, whereas P8 and P15 Norway rats and P15 grass rats exhibited increased activity in the SCN, only P15 Norway rats exhibited increased activity in the vSPVZ. These results suggest that the direct RHT→vSPVZ projection, which we showed develops by P15 in Norway rats, is already functional by this age. Additionally, these results further support our hypothesis that the direct projection

contributes to the development of the in-phase pattern of SCN-vSPVZ activity as daily light exposure can not only excite and entrain the rhythm of daytime SCN activity, but can also now directly drive activity in the vSPVZ.

Although Fos immunohistochemistry is suited to detecting increases in neuronal activity, it is not suited to detecting short-term decreases because, once the Fos protein is produced, it takes several hours to degrade (Cirelli and Tononi 2000). This is important because, in P15 grass rats, Fos activity in the vSPVZ of control subjects was already very high (due to the anti-phase SCN-vSPVZ relationship in this species). A failure to detect an *increase* in vSPVZ activity could have been due to a ceiling effect. But we believe it is more likely that the failure to detect a *decrease* in vSPVZ activity resulted from insensitivity of the c-fos method for detecting short-term functional inhibition. It should be noted that the converse experiment—turning off the lights during the light period—would not resolve these interpretational difficulties. Direct measures of neurophysiological activity would be better able to detect the anti-phase relationship in grass rats under these conditions.

Because of the inherent limitations of this functional test, we determined that additional evidence was needed to test the model presented in Figure 5. This evidence is presented in Chapter 6.
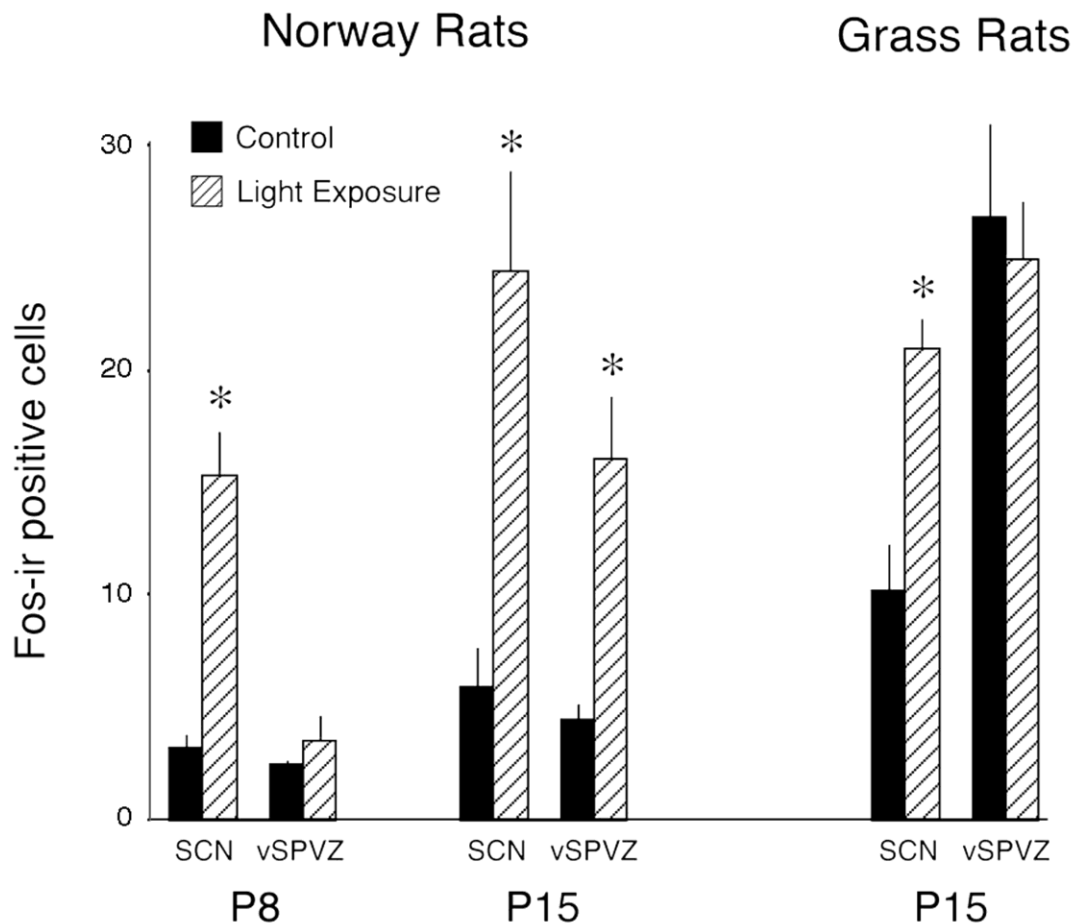
Figure 9. Effects of 2 h of light exposure during the dark period on SCN and vSPVZ activity in P8 and P15 Norway rats and P15 grass rats. Fos-positive cells per section in the suprachiasmatic nucleus (SCN) and ventral subparaventricular zone (vSPVZ) of P8 and P15 Norway rats (n = 6 per group) and P15 grass rats (n = 4 per group). Black bars represent control subjects maintained in the dark and hatched bars represent experimental subjects exposed to light. * significant difference from control group, $P < .005$. Means + standard errors.

CHAPTER 6

DEVELOPMENTAL EXPRESSION OF PRESYNAPTIC TERMINALS IN THE vSPVZ

IN NORWAY RATS AND GRASS RATS

Introduction

In the two previous chapters, we provided anatomical and functional evidence that differences in RHT→vSPVZ projections in Norway rats and grass rats underlie species differences in neural activity and behavior. As mentioned previously, the axon terminals of RHT projections to the SCN and vSPVZ co-store glutamate and PACAP (Hannibal, Moller et al. 2000; Hannibal 2002), which have excitatory postsynaptic effects (Michel, Itri et al. 2006). The projection from the SCN to the vSPVZ, however, is mainly composed of axon terminals that co-store GABA and VIP and is primarily inhibitory (Hermes, Kolaj et al. 2009). We believe that the development of excitatory (i.e., PACAP and glutamate) retinal projections to the vSPVZ contributes to the development of the in-phase Fos-ir relationship in Norway rats. Alternatively, we believe that developmental changes in the inhibitory input to the vSPVZ from the SCN (i.e., VIP and GABA), without interference from significant excitatory RHT→vSPVZ projections, accounts for the development of the anti-phase pattern in grass rats.

Although glutamate and GABA are prevalent throughout the brain, PACAP and VIP are present in relatively greater quantities in SCN and vSPVZ than in surrounding areas (Card, Brecha et al. 1981; Tanaka, Matsuda et al. 1997; Kalamatianos, Kallo et al. 2004). Therefore, it is possible to perform a detailed examination of the projections to the

vSPVZ from the retina or the SCN using immunohistochemical markers for PACAP and VIP, respectively (Hannibal and Fahrenkrug 2004; Kalamatianos, Kallo et al. 2004; Hermes, Kolaj et al. 2009). We employed this strategy here by examining the developmental expression of PACAP- and VIP-expressing presynaptic terminals in the vSPVZ in Norway rats and grass rats using immunohistochemistry and confocal microscopy. We predicted that Norway rats would show an increase in PACAP terminals in the vSPVZ by P15, when the RHT-vSPVZ projection and in-phase SCN-vSPVZ pattern have developed. Alternatively, we predicted grass rats would show little or no PACAP terminals in the vSPVZ at any age, and that the development of the anti-phase pattern by P15 would be accompanied by an increase in VIP terminals in the vSPVZ.

Methods

Subjects and tissue preparation

Six P8 (body weights = 19.4-23.8 g), 6 P11 (body weights = 22.8-30.8 g), 6 P15 (body weights = 36.0-48.1 g), and 6 P21 (body weights = 54.9-71.1 g) Norway rats and 6 P6 (body weights = 4.3-7.6 g), 6 P11 (body weights = 9.1-12.3 g), 6 P15 (body weights = 11.0-14.8 g), and 6 P21 (body weights = 22.8-25.4 g) grass rats were used. At the midpoint of their respective daytime photoperiods, pups were removed from the litter and immediately sacrificed. Accordingly, Norway rats were sacrificed at 1300 hrs and grass rats were sacrificed at 1200 hrs. Animals were killed with an overdose of Nembutal and perfused transcardially with PBS followed by 2% PFA and 0.2% picric acid in PB. Brains were removed and stored in 30% sucrose solution for 5-10 days. Brains were then immersed in

OCT compound (Sakura, Torrance, CA) and snap frozen in isopentane cooled in a dry ice/ethanol bath. Using a cryostat, 18 μm sections were cut in the coronal plane and thaw-mounted onto gelatin-subbed slides.

<div align="center">PACAP and VIP Immunohistochemistry</div>

Sections were blocked in 2.5% bovine serum albumin and 0.1% Triton X-100 in PBS for 1 h, followed by incubation with primary antibodies (anti-PACAP, 1:20, gift from J. Hannibal; anti-VIP, 1:200, Peninsula T-5030) at 4°C overnight in the same solution. The monoclonal antibody against PACAP was collected from mice immunized with rat PACAP-38 conjugated to ovalbumin by glutaraldehyde. This antibody displays equal affinity for PACAP-38 and PACAP-27, recognizing an epitope between amino acids 6-10, but has no affinity with VIP (Hannibal, Mikkelsen et al. 1995). Preabsorption with PACAP-38 or PACAP-27 blocks staining (Hannibal, Ding et al. 1997). This antibody has been previously used to detect PACAP in the RHT and stains a characteristic pattern in the SCN (Hannibal, Moller et al. 2000; Hannibal 2002).

The polyclonal antibody against VIP was collected from guinea pigs immunized with a synthetic peptide as the immunogen. Radioimmunoassay shows no cross reactivity with PHI-27, PHM-23, VIP (10-28), VIP (1-12), VIP guinea pig and chicken, PACAP-38, and substance P (manufacturer's technical information). This antibody stains a characteristic pattern in the SCN (Belenky, Yarom et al. 2008; Vida, Deli et al. 2010).

Sections were washed in PBS and secondary antibodies (Alexa Fluor 488- or 568-conjugated; 1:200; Molecular Probes) were applied in PBS for 1 h. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich) added to the final

wash. For sections serving as negative controls, the procedure was the same except primary antibodies were omitted. Slides were coverslipped with Fluorogel (Electron Microscopy Sciences, Hatfield, PA).

Confocal microscopy

Sections were imaged sequentially using a SP-5 Leica confocal laser scanning microscope (Leica Microsystems, Germany) with an argon 488 nm laser and a helium/neon 543 nm laser. Two sections containing the vSPVZ were selected from each animal at matching rostrocaudal levels across all groups. For each section, using an oil immersion 63x objective and a magnification factor of 4, a z-stack of optical images at 1 μm layer intervals was acquired and digitized into 512 X 512 pixel bitmaps. The area imaged corresponded to a 61.5 μm-wide square area within the vSPVZ. Laser settings were held constant across all subjects.

Analysis

To measure presynaptic terminals in the vSPVZ in Experiment 5, images of two confocal layers, evenly spaced within each z-stack, were extracted using Leica LAS AF software and imported into ImageJ. Data were scored by an experimenter blind to the age and species of each subject. Red- and green-fluorescent presynaptic terminals were automatically counted using the ImageJ Puncta Analyzer plugin as described elsewhere (Ippolito and Eroglu 2010). Mean values of PACAP and VIP terminals were calculated for each section and then means were calculated for each pup. Data were imported into Statview 5.0. ANOVA was used to test for differences across age within each species and

planned comparisons (unpaired t tests) were used to test developmental changes between P6/8 and P21. For all tests, alpha was set at 0.05.

<div align="center">Results</div>

As shown in Figure 10, the mean number of PACAP-expressing terminals in the vSPVZ of Norway rats increased progressively from P8 to P21. ANOVA revealed a significant main effect of age [$F(3,20) = 6.7$, $P < 0.005$]. In grass rats, the mean number of PACAP-expressing terminals in the vSPVZ was very low at P6 but, in contrast with Norway rats, did not increase significantly with age [$F(3,20) = 1.4$]. Planned comparisons revealed that the number of PACAP-expressing terminals in the vSPVZ increased significantly from P6/8 to P21 in Norway rats ($t(10) = 4.3$, $P \leq 0.01$), but not grass rats ($t(10) = 0.3$). Figure 11 depicts these species differences in a representative P21 Norway rat and a representative P21 grass rat.

As also shown in Figure 10, the mean number of VIP-expressing terminals in the vSPVZ in Norway rats increased between P8 and P21. ANOVA revealed a significant main effect of age [$F(3,20) = 6.2$, $P < 0.005$]. In grass rats, the mean number of VIP-expressing terminals in the vSPVZ also increased between P6 and P21; however, ANOVA did not reveal a significant main effect of age [$F(3,20) = 2.7$, $P = 0.07$]. Planned comparisons revealed that, for both species, VIP-expressing terminals in the vSPVZ increased significantly from P6/8 to P21 ($ts(10) \geq 3.1$, $P \leq 0.05$).

Discussion

Here we showed that, in Norway rats, a significant increase of PACAP-expressing terminals in the vSPVZ occurs by P15, reflecting the development of the direct retinal projection to vSPVZ, and in association with the emergence of in-phase SCN-vSPVZ activity. Additionally, we found that the development of the anti-phase pattern by P15 in grass rats is associated with an increase in VIP-expressing terminals in the vSPVZ, without significant increases in PACAP-expressing terminals.

Figure 12 presents a model, based on our results, depicting developmental and species differences in the retina-SCN-vSPVZ circuit. According to this model, the vSPVZ of Norway rats does not display day-night differences in neural activity at P8, and receives little or no input from the retina or SCN at this age. By P15, however, the direct excitatory RHT projection to the vSPVZ has developed, as has the inhibitory projection arriving from the SCN. Since light from the retina can now stimulate the vSPVZ directly, the excitatory actions of PACAP and glutamate may be strong enough to produce the in-phase Fos-ir relationship.

We therefore further hypothesize that this excitatory input from the RHT modulates vSPVZ activity in a way that outcompetes the inhibitory input from the day-active SCN, since VIP terminals also increase during this time. Evidence for such a mechanism may be found in the differences between PACAP and VIP receptors. As mentioned in Chapter 1, the vSPVZ in Norway rats expresses PACAP and VIP receptors, PAC1 and VPAC2, respectively (Hannibal and Fahrenkrug 2004; Kalamatianos, Kallo et al. 2004; Hermes, Kolaj et al. 2009). PACAP not only binds to PAC1 receptors, but also

competitively binds to VPAC2 receptors. VIP, however, does not competitively bind to PAC1 receptors. Therefore, PACAP released from the RHT may lead to excitation of vSPVZ neurons not only by facilitating glutamatergic excitation via PAC1 receptors, but also by preventing the inhibitory effects of VIP through competitive binding. To better understand the relative contributions of the retinal and SCN connections to the vSPVZ, we would ideally want to selectively disable these connections to observe their effects on hypothalamic activity. However, such selective manipulations are not currently feasible.

As also depicted in Figure 12, grass rats possess an excitatory RHT projection to the SCN, but they do not develop the robust RHT projection to the vSPVZ exhibited by Norway rats. At P6, the vSPVZ does not display day-night differences in neural activity, presumably due to the relative lack of input from the SCN and reflected by the low levels of VIP terminals in the vSPVZ at this age. By P15, however, the anti-phase pattern of SCN-vSPVZ activity develops and is associated with an increase in inhibitory VIP terminals in the vSPVZ. Since the SCN exhibits a rhythm of increased daytime activity, the strengthening of this inhibitory projection could explain why the vSPVZ becomes more active at night.
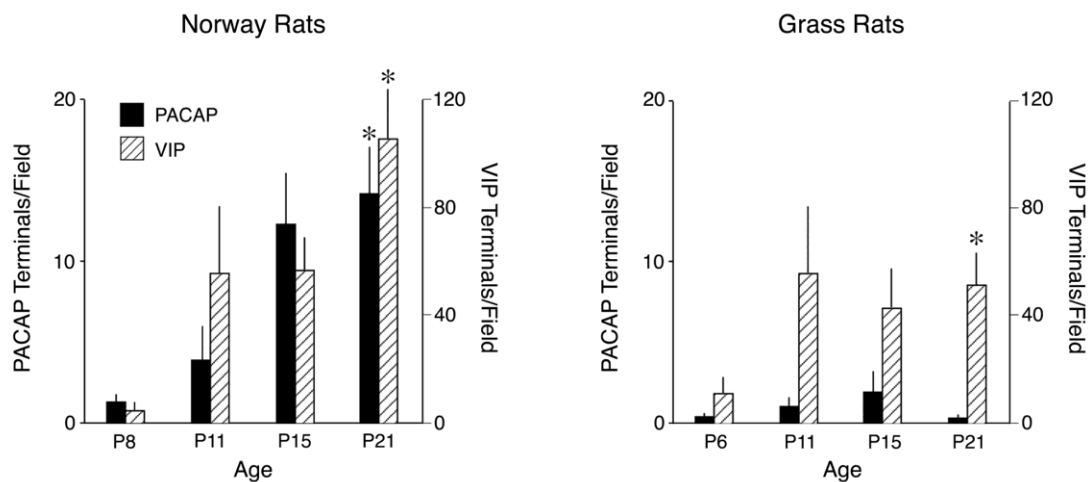
Figure 10. Developmental expression of PACAP- and VIP-expressing terminals in the vSPVZ in Norway rats and grass rats. Mean number of presynaptic terminals in the ventral subparaventricular zone (vSPVZ) in P8, P11, P15, and P21 Norway rats (left) and P6, P11, P15, and P21 Nile grass rats (right). n=6 per group. Black bars represent terminals expressing pituitary adenylate cyclase-activating peptide (PACAP) and hatched bars represent terminals expressing vasoactive intestinal peptide (VIP). * significant difference from P6/8, $P < 0.05$. Means + standard errors.
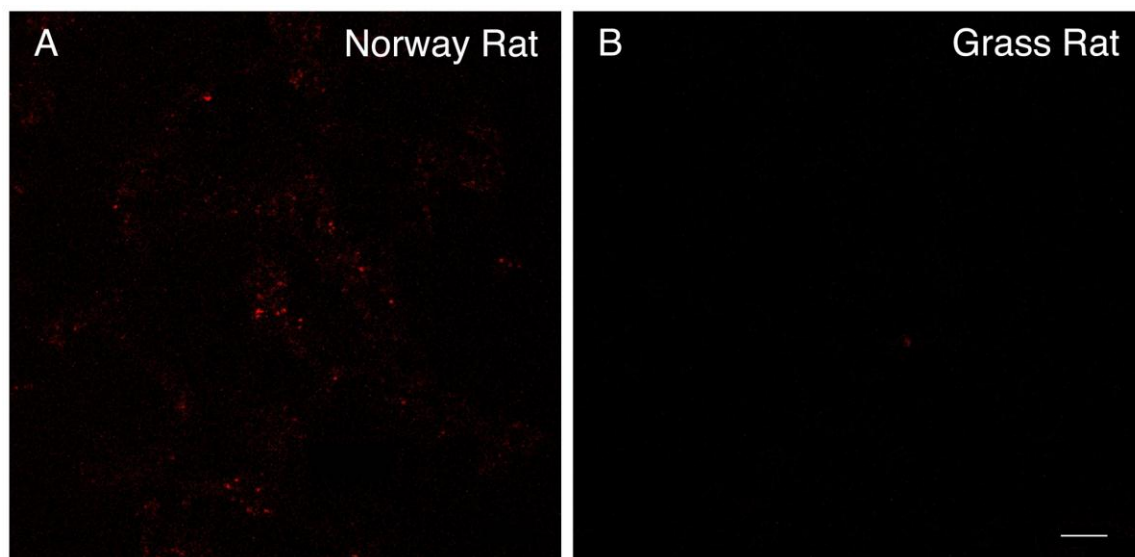
Figure 11. Species differences in PACAP-expressing terminals in the vSPVZ in a P21 Norway rat and a P21 grass rat. Representative images of terminals expressing pituitary adenylate cyclase-activating peptide (PACAP) in the ventral subparaventicular zone (vSPVZ) in a P21 Norway rat (A) and a P21 grass rat (B). Scale bar: 5 μm
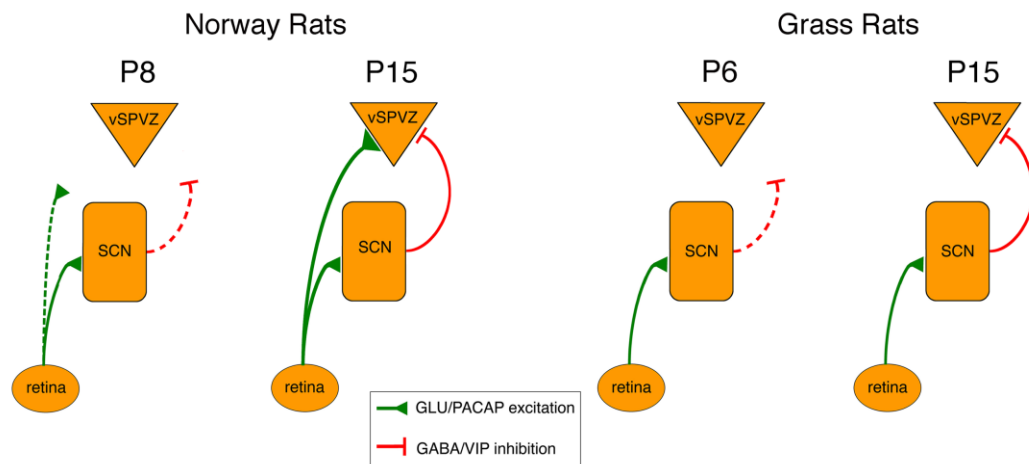
Figure 12. Model of developmental and species differences in neural connections among retina, SCN, and vSPVZ in Norway rats and grass rats. Green lines denote presumed excitatory connections releasing glutamate and pituitary adenylate cyclase (PACAP) and red lines denote presumed inhibitory connections releasing γ-aminobutyric acd (GABA) and vasoactive intestinal peptide (VIP). Dashed lines denote developing or relatively weak connections. SCN, suprachiasmatic nucleus, vSPVZ, ventral subparaventricular zone.

CHAPTER 7

OVERVIEW AND SIGNIFICANCE OF RESEARCH

We have shown here how developmental processes may differentially shape the brain and behavior to affect circadian phase preference in Norway rats and Nile grass rats, two closely related Murid rodents (Campi, Collins et al. 2011). First, nocturnal Norway rats and diurnal grass rats undergo profound developmental changes in circadian wake behavior between the first and second postnatal weeks. Second, these behavioral changes are associated with the emergence of an in-phase pattern of SCN and vSPVZ activity in Norway rats and an anti-phase pattern in grass rats. Third, Norway rats develop an abundance of RHT projections to the vSPVZ between P8 and P15, whereas grass rats do not. Fourth, in support of our anatomical evidence, 2 hours of light stimulation during the dark period in Norway rats activates the vSPVZ at P15 but not P8. Finally, Norway rats, but not grass rats, exhibit significant increases in PACAP-expressing presynaptic terminals in the vSPVZ during the second postnatal week, thereby providing additional evidence of species-differences in RHT connectivity. This converging evidence across 5 experiments suggests that the developmental pattern and timing of retinal projections to the hypothalamus contribute to the development of circadian phase preference in these two species.

Why might retinal projections to the vSPVZ differ between these two species? The fact that vSPVZ activity is strikingly higher in P6 grass rats than in P8 Norway rats (see Figure 4) suggests a possibility. Specifically, the higher vSPVZ activity in grass rats may

impede the development of the RHT connection with the vSPVZ; conversely, the lower vSPVZ activity in Norway rats may stimulate RHT connectivity. With regard to the first mechanism, it is interesting that ephrins repel developing axons in an activity-dependent manner (Klein 2009). A better understanding of the molecular factors underlying species differences in RHT development should help to reveal the ease with which the circadian system can evolve in changing environments.

To our knowledge, it has not previously been hypothesized that RHT connectivity to the vSPVZ helps determine circadian phase preference. Keeping in mind the need for caution when comparing data from multiple laboratories using different techniques, published reports suggest that this hypothesis is supported by a variety of other species. As shown in Table 2, nocturnal species including hamsters (Speh and Moore 1993; Morin, Blanchard et al. 2003), mink (Peytevin, Masson-Pevet et al. 1997), mice (Cassone, Speh et al. 1988; Hattar, Kumar et al. 2006), and opossums (Cassone, Speh et al. 1988) possess robust RHT projections extending beyond the SCN to the vSPVZ.

In contrast to nocturnal species and in addition to grass rats, RHT afferents do not significantly extend beyond the boundary of the SCN in such diurnal species as humans (Dai, Van der Vliet et al. 1998), *Cebus* monkeys (Pinato, Frazao et al. 2009), ground squirrels (Smale, Blanchard et al. 1991), and four-striped field mice (Schumann, Cooper et al. 2006).

Table 2 also shows two notable exceptions to the above pattern. Specifically, both sheep (Torrealba, Parraguez et al. 1993) and degus (Goel, Lee et al. 1999) are diurnal but exhibit robust RHT projections extending dorsally and caudally outside the SCN.

Interestingly, these two species are precocial in relation to all of the nocturnal species discussed above. Moreover, this direct projection develops prenatally in sheep (Torrealba, Parraguez et al. 1993), whereas it develops postnatally in rats (Speh and Moore 1993), hamsters (Speh and Moore 1993), and mink (Peytevin, Masson-Pevet et al. 1997). Thus, we hypothesize further that, for animals that develop the direct retina-to-vSPVZ connection, the precise timing of innervation may influence its subsequent effect on circadian phase preference. Although there are many behavioral and physiological factors that differ across the altricial-precocial spectrum that could potentially alter SCN function and connectivity, exposure to light may not be a critical factor since dark rearing of Norway rats has little or no effect on the development of RHT connectivity or circadian phase preference (Prichard, Fahy et al. 2004; Prichard, Armacanqui et al. 2007).

Finally, Table 2 indicates that RHT projections in guinea pigs and domestic cats are confined to the SCN (Cassone, Speh et al. 1988). However, substantial disagreement exists as to the circadian phase preferences of these two species (Bowersox, Baker et al. 1984; Jilge 1985; Kuwabara, Seki et al. 1986; Tobler and Scherschlicht 1990; Tobler, Franken et al. 1993; Akita, Ishii et al. 2001).

Interestingly, it has been reported in adult mice that the SCN and subparaventricular zone (SPVZ) exhibit an anti-phase pattern of multiunit activity (Nakamura, Yamazaki et al. 2008; Nakamura, Nakamura et al. 2011). Given that mice are nocturnal and possess a robust RHT→vSPVZ projection, this pattern of activity is opposite to what we would predict based on the present findings and those of others using Norway rats (Nunez, Bult et al. 1999; Schwartz, Nunez et al. 2004). However, in the one Nakamura

et al. paper where recording sites were indicated (Nakamura et al., 2008; supplemental data), some sites appear to be dorsal and rostral to the vSPVZ and none was confirmed as being within areas targeted by the RHT. These issues may be important because the SPVZ is a heterogeneous structure with dorsal and ventral subregions that make separable contributions to locomotion, sleep, and thermoregulation (Lu, Zhang et al. 2001).

In comparison to the existing literature in nocturnal and diurnal species, far less is known about the circadian systems of crepuscular mammals. Such species display a bimodal distribution of activity at dawn and dusk. Perhaps the only systematic study of the circadian system in a crepuscular species was carried out in the rock cavy (*Kerodon rupestris*) (Nascimento, Souza et al. 2010), a precocial Brazilian rodent from the Cavidiidae subfamily. Interestingly, the data presented showed that this species exhibits a robust RHT-vSPVZ projection. Additionally, Nascimento et al. reported that the only unique characteristic within the SCN of the rock cavy, as compared to other nocturnal and diurnal species, was a rostral-to-caudal distribution of vasopressin and VIP, respectively. It is unclear, however, how this might contribute to crepuscular behavior.

We believe that understanding the significance of retinal input, or the lack thereof, to the vSPVZ will have important translational implications since most of what we know about the mammalian circadian system comes from nocturnal rodents such as rats, hamsters, and mice—which have this projection—whereas humans are diurnal and do not. For instance, it is possible that differences in RHT→vSPVZ projections may underlie the opposing phase shifting effects of light that have been reported in nocturnal and diurnal species (Dijk and Archer 2009). Specifically, light exposure late in the day delays the onset

of activity in nocturnal animals while delaying the onset of inactivity in diurnal animals. Conversely, light exposure early in the day advances the onset of activity in diurnal animals, while advancing the onset of inactivity in nocturnal animals. Understanding the pathways by which circadian functioning is differentially modulated in nocturnal and diurnal species will provide a framework that could lead to a better understanding of the negative health outcomes associated with circadian disruption, whether due to natural variations in day length, which are most notably associated with seasonal affective disorder (Monteleone and Maj 2008), or from man-made causes like nighttime light pollution and shift work (Costa 1997; Navara and Nelson 2007). Such negative health outcomes associated with circadian disruption include depression, autoimmune suppression, cancer, and obesity (Klerman 2005; Navara and Nelson 2007; Turek 2007; Bass and Takahashi 2010; Gery and Koeffler 2010).

Overall, our findings suggest that species-typical circadian phase preference emerges through a developmental rewiring of the RHT to effect differential interactions among the retina, SCN, and vSPVZ. It bears noting that the mechanism identified here may be one of several; in other words, there may be multiple developmental paths to nocturnality and diurnality. Whatever the mechanism, once the system is established, most individuals will exhibit a species-typical bias toward one circadian phase preference (although it should be noted that this bias does not prevent individuals from shifting to a different temporal niche under certain ecological conditions; (Mrosovsky and Hattar 2005). All together, the present results indicate that a fuller understanding of the mechanisms underlying circadian

phase preference will require delineation, in a variety of species, of the developmental

relations among RHT connectivity, hypothalamic activity, and behavior.

Table 2. Relations among life-history variables and RHT connectivity to the vSPVZ in mammals.

Species	Circadian phase preference	RHT→vSPVZ connection?	Altricial/Precocial	RHT development
Norway rat (*R. norvegicus*)	nocturnal	yes[1,2]	Altricial	postnatal
Hamster (*M. auratus*)	nocturnal	yes[2,3]	Altricial	postnatal
Mink (*M. vison*)	nocturnal	yes[4]	Altricial	postnatal
Mouse (*M. musculus*)	nocturnal	yes[5,6]	Altricial	?
Opossums (*D. virginiana, M. domestica*)	nocturnal	yes[5]	Altricial	?
Nile grass rat (*A. niloticus*)	diurnal	no	Intermediate	n/a
Human (*H. sapien*)	diurnal	no[7]	Intermediate	n/a
Cebus monkey (*C. apella*)	diurnal	no[8]	Intermediate	n/a
Ground squirrel (*S. lateralis*)	diurnal	no[9]	Altricial	n/a
Four-striped grass mouse (*R. pumilio*)	diurnal	no[10]	Intermediate	n/a
Sheep (*O. aries*)	diurnal	yes[11]	Precocial	prenatal
Degu (*O. degus*)	diurnal	yes[12]	Precocial	?
Guinea pig (*C. porcellus*)	unclear[13,14,15]	no[5]	Precocial	n/a
Cat (*F. cattus*)	unclear[16,17,18]	no[5]	Altricial	n/a

[1]Gooley et al. 2003; [2]Speh and Moore 1993; [3]Morin et al. 2003; [4]Peytevin et al. 1997; [5]Cassone et al. 1988; [6]Hattar et al. 2006; [7]Dai et al. 1998; [8]Pinato et al. 2009; [9]Smale et al. 1991; [10]Schumann et al. 2006; [11]Torrealba et al. 1993; [12] Goel et al. 1999; [13]Jilge 1985; [14]Tobler et al. (1993); [15]Akita et al. 2001; [16]Bowersox et al. 1984; [17]Kuwabara et al. 1986; [18]Tobler and Scherschlicht 1990

?, unknown

n/a, not applicable

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