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The developmental emergence of a wake-promoting pathway regulating ultradian and circadian rhythms in infant rats

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THE DEVELOPMENTAL EMERGENCE OF A WAKE-PROMOTING PATHWAY REGULATING ULTRADIAN AND CIRCADIAN RHYTHMS IN INFANT RATS

by

Andrew Jason Gall

An Abstract

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

July 2011

Thesis Supervisor: Professor Mark S. Blumberg

ABSTRACT

In mammals, circadian rhythms are controlled by an endogenous clock located in the suprachiasmatic nucleus (SCN). The SCN is part of a wake-promoting pathway in adults involving the dorsomedial hypothalamus (DMH) and locus coeruleus (LC), but little is known about how this circuit develops. Therefore, we examined the neural mechanisms underlying the development of circadian and ultradian sleep-wake rhythms.

Circadian rhythms of sleep and wakefulness are exhibited by rats at postnatal day (P)2, but the influence of forebrain structures, including the SCN, has not been examined. In Experiment 1, although precollicular transections at P2 did not alter day-night differences in sleep and wakefulness, transections at P8 did eliminate these differences. In contrast, in Experiment 2, SCN lesions eliminated day-night differences in sleep and wakefulness at P2. These results suggest that the SCN exerts a humoral influence in newborns and gains neural control over brainstem structures over the first postnatal week.

Based on the results of Experiments 1 and 2, we hypothesized that neural connections among the SCN, DMH, and LC develop over the first postnatal week. In Experiment 3, we used fluorescent tracers to reveal that connections within this circuit are strengthened and elaborated—and also become bidirectional—between P2 and P8.

The results of Experiment 3 indicate that the SCN receives feedback from the LC. To explore the functional mechanisms by which the SCN receives this feedback, in Experiment 4, we deprived pups of sleep at P8 and used cFos to visualize brain areas that became active as a result of forced wakefulness. Our findings in intact pups and those injected with DSP-4, a neurotoxin that targets noradrenergic LC terminals, suggest that forced wakefulness activates the LC, which subsequently activates the DMH and SCN.

After connectivity among the SCN, DMH, and LC is established, we tested the functional role of each nucleus in the modulation of sleep and wakefulness. Infants cycle rapidly between states of sleep and wakefulness, resulting in fragmented bouts. Over development, these sleep and wake bouts consolidate and circadian rhythms become evident. Analyses of the statistical distributions of sleep and wake bouts have revealed dramatic changes in the dynamics of sleep-wake activity. Sleep bouts follow an exponential distribution throughout development. In contrast, wake bouts initially follow an exponential distribution, but transition to a power-law distribution around P15. In Experiments 5, 6, and 7, we explored the contributions of the LC, SCN, and DMH, respectively, to this developmental transition. We found that lesions of each area prevented the emergence of power-law wake behavior. Lesions of the SCN and DMH also prevented the expression of nocturnality.

Altogether, these findings reveal that neural connections between the SCN and brainstem develop over the first postnatal week. After this connectivity is established, the SCN-DMH-LC pathway is critical for the normal expression of power-law wake behavior and circadian rhythmicity. We suggest that the development of the SCN-DMH-LC circuit is critical for pups to regulate arousal and gain independence from the mother and littermates.

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CERTIFICATE OF APPROVAL

PH.D. THESIS

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To Heather, Addilyn, & Stella

The same stream of life that runs through my veins night and day runs through the world and dances in rhythmic measures.

Rabindranath Tagore, Stream of Life

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Based on the results of Experiments 1 and 2, we hypothesized that neural connections among the SCN, DMH, and LC develop over the first postnatal week. In Experiment 3, we used fluorescent tracers to reveal that, in fact, connections within this circuit are strengthened and elaborated—and also become bidirectional—between P2 and P8.

The results of Experiment 3 indicate that the SCN receives feedback from the LC. To explore the functional mechanisms by which the SCN receives this feedback, in Experiment 4, we deprived pups of sleep at P8 and used cFos to visualize brain areas that became active as a result of forced wakefulness. Our findings in intact pups and those

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LIST OF ABBREVIATIONS

AC:	Anterior commissure
AH:	Anterior hypothalamus
AHA:	Anterior hypothalamic area, anterior
AHC:	Anterior hypothalamic area, central
AHP:	Anterior hypothalamic area, posterior
AIC:	Akaike information criterion
ANOVA:	Analysis of variance
AP:	Anterior-posterior
AS:	Active sleep
Arc:	Arcuate nucleus
BF:	Basal forebrain
CTB:	Cholera toxin subunit B
DA:	Dopamine
DiI:	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMH:	Dorsomedial hypothalamus
DPX:	Depex
DR:	Dorsal raphe
DSP-4:	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
DTg:	Dorsal tegmental nucleus
DV:	Dorsal-ventral
EGFR:	Epidermal growth factor receptor
EMG:	Electromyogram
f:	Fornix
GABA:	Gamma-aminobutyric acid
HPLC:	High protein liquid chromatography
LC:	Locus coeruleus
LDT:	Laterodorsal tegmental nucleus
LH:	Lateral hypothalamus
LPO:	Lateral preoptic nucleus
LS:	Lateral septum
Me5:	Mesencephalic of 5
ML:	Medial-lateral
MnPO:	Median preoptic nucleus
MPA:	Medial preoptic area
MR:	Median raphe
N:	Normality
NA:	Noradrenaline
ox:	Optic chiasm
P:	Postnatal day
PaAP:	Anterior part of parvicellular nucleus
PBS:	Phosphate buffered saline
PeF:	Perifornical nucleus
PFA:	Paraformaldehyde
PH:	Posterior hypothalamus

PK2:	Prokineticin
PKR2:	Prokineticin receptor 2
PLSD:	Protected Least Significant Difference
PO:	Pontis oralis
PT:	Paratenial thalamus
PVN:	Paraventricular nucleus
QS:	Quiet sleep
SC:	Superior colliculus
SCN:	Suprachiasmatic nucleus
SE:	Standard error
SON:	Supraoptic nucleus
TFA:	Trifluoroacetic acid
TGF-alpha:	Transforming growth factor alpha
Th:	Thalamus
TMN:	Tuberomammillary nucleus
VLPO:	Ventrolateral preoptic nucleus
VMH:	Ventromedial hypothalamus
vSPVZ:	Ventral subparaventricular zone
VTg:	Ventral tegmental nucleus
3V:	Third ventricle
5-HT:	5-Hydroxytryptophan (Serotonin)

CHAPTER 1

INTRODUCTION

Circadian rhythms—daily cycles in biological activity—are exhibited by all living organisms from mammals, to plants, fungi, and even cyanobacteria. Circadian rhythms are adaptive because they allow animals to synchronize their behavioral and physiological processes with predictable changes in the environment—such as the light-dark cycle, daily temperature fluctuations, and daily prey availability (Sharma 2003).

Ultradian rhythms—cycles repeated throughout the day—are also expressed by all living organisms. Ultradian rhythms are diverse in period length (from milliseconds to hours), mechanisms, and functions. They are adaptive to organisms because they optimize energy usage during a specific phase of the circadian cycle (Gerkema, Groos et al. 1990). Examples of ultradian rhythms include hormone release, thermoregulation, appetite, and metabolism (Waterhouse, Minors et al. 1997).

The sleep-wake cycle follows both an ultradian and circadian rhythm. Sleep and wakefulness cycle repeatedly during a 24-hour period (i.e., ultradian), and within each sleep period, mammals cycle between various stages of slow-wave and Rapid Eye Movement (REM) sleep (Aserinsky and Kleitman 1953). These ultradian rhythms of sleep and wakefulness are also modulated by circadian rhythmicity. Circadian rhythms ensure that these bouts of sleep and wakefulness are expressed at the appropriate time of day. Nocturnal mammals exhibit more wakefulness at night, and diurnal mammals exhibit more wakefulness during the day.

All mammalian species exhibit periods of sleep and wakefulness (Zepelin and Rechtschaffen 1974; Siegel 1995; Shaw, Cirelli et al. 2000). Importantly, infants spend the majority of their time asleep (Jouvet-Mounier, Astic et al. 1970; Blumberg, Karlsson et al. 2005; Blumberg, Karlsson et al. 2007), which implies that sleep is particularly important for developing animals. Here we explore the mechanisms by which circadian and ultradian rhythms of sleep and wakefulness develop in infant rats.

In humans (Kleitman and Engelmann 1953), rats (Karlsson, Kreider et al. 2004), and mice (Blumberg, Coleman et al. 2007), newborns cycle rapidly between states of sleep and wakefulness throughout the day and night, resulting in very short bouts of each state. In other words, sleep and wakefulness at this early stage of development are fragmented. Importantly, during the first few weeks of life, this pattern of sleep-wake behavior changes dramatically. Specifically, one of the defining features of early sleep-wake development is the consolidation of individual sleep and wake bouts, as well as the circadian entrainment of the sleep-wake rhythm.

Using electromyographic (EMG) techniques, which allow for measurement of low and high muscle tone, indicative of sleep and wakefulness, respectively (Karlsson, Kreider et al. 2004), we have documented circadian changes in sleep and wakefulness over development (Gall, Todd et al. 2008). As shown in Figure 1, at postnatal day (P)2, day-night differences (i.e., circadian rhythms) in sleep and wakefulness are detectible. At this age, mean sleep and wake bout durations were generally shorter at night to result in approximately 50% more sleep-wake cycles at night as compared to the day. Although day-night differences in mean sleep and wake bout durations are small, it is important to emphasize that they are detectible before other standard measures of circadian

rhythmicity (e.g., locomotor activity, drinking behavior) can be used. The adult pattern of increased wakefulness at night—indicative of nocturnality—is not exhibited until P15. This nocturnal wakefulness becomes even more pronounced at P21.

Analyses of the statistical distributions of sleep and wake bouts revealed dramatic changes in the dynamics of sleep-wake cyclicity (Blumberg, Seelke et al. 2005). These distributions are vital for gaining a better understanding of the neural mechanisms that underlie ultradian and circadian rhythms of sleep and wakefulness. In all adult mammals studied thus far, sleep bouts follow an exponential distribution [$P(t) \sim \exp(-t/\tau)$], with a characteristic time scale τ (Lo, Amaral et al. 2002). Wake bouts in adults, in contrast, follow a power-law distribution [$P(t) \sim t^{-\alpha}$] with a scaling exponent α (Lo, Amaral et al. 2002).

We have shown previously (see Figure 2) that sleep bout durations throughout development fall along a straight line on semilog plots, indicative of exponential distributions. Importantly, exponential distributions signify randomness (Blumberg, Seelke et al. 2005). Wake bout durations, in contrast, initially follow an exponential distribution, but at P15 transition to a power-law distribution. These power-law distributions are characterized by a small percentage of wake bouts that are much longer than the others. Therefore, the longer an animal is awake, the more likely it is to stay awake. Because sleep and wake bouts distribute differentially from P15 to adulthood, it is likely that sleep and wakefulness are controlled by at least partially separate neural systems.

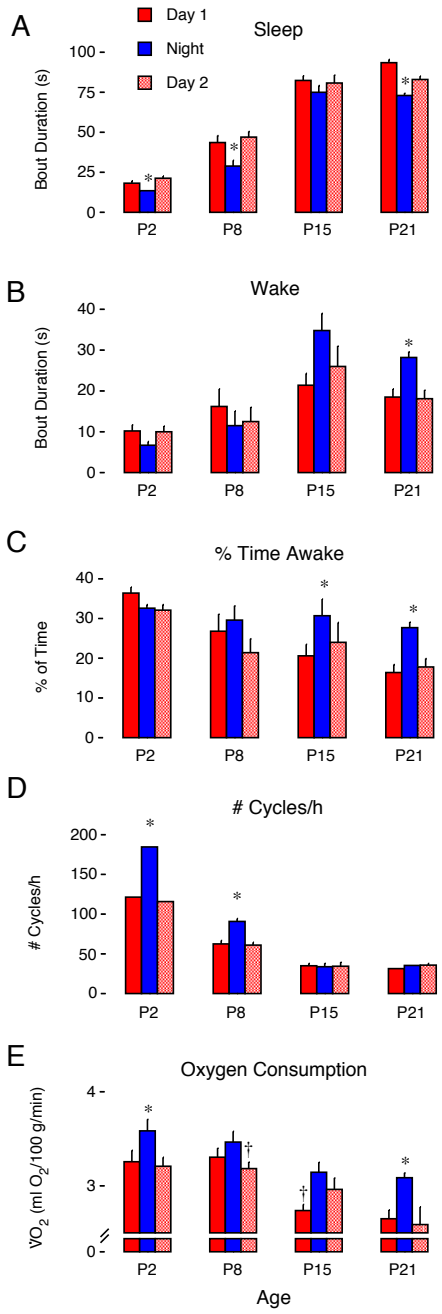

Figure 1. Day-night differences in sleep, wake, and metabolic parameters in infant rats. Mean sleep (A) and wake (B) bout durations during the day and night in P2, P8, P15, and P21 rats. (C) Mean percentage of time spent awake at each age. (D) Mean number of sleep-wake cycles per hour. (E) Mean oxygen consumption determined during periods of sleep. *Night value is significantly different from both day 1 and day 2. †Day 1 or day 2 value is significantly different from night. n = 6 subjects per group. Means + SE. (From Gall, Todd et al. 2008).

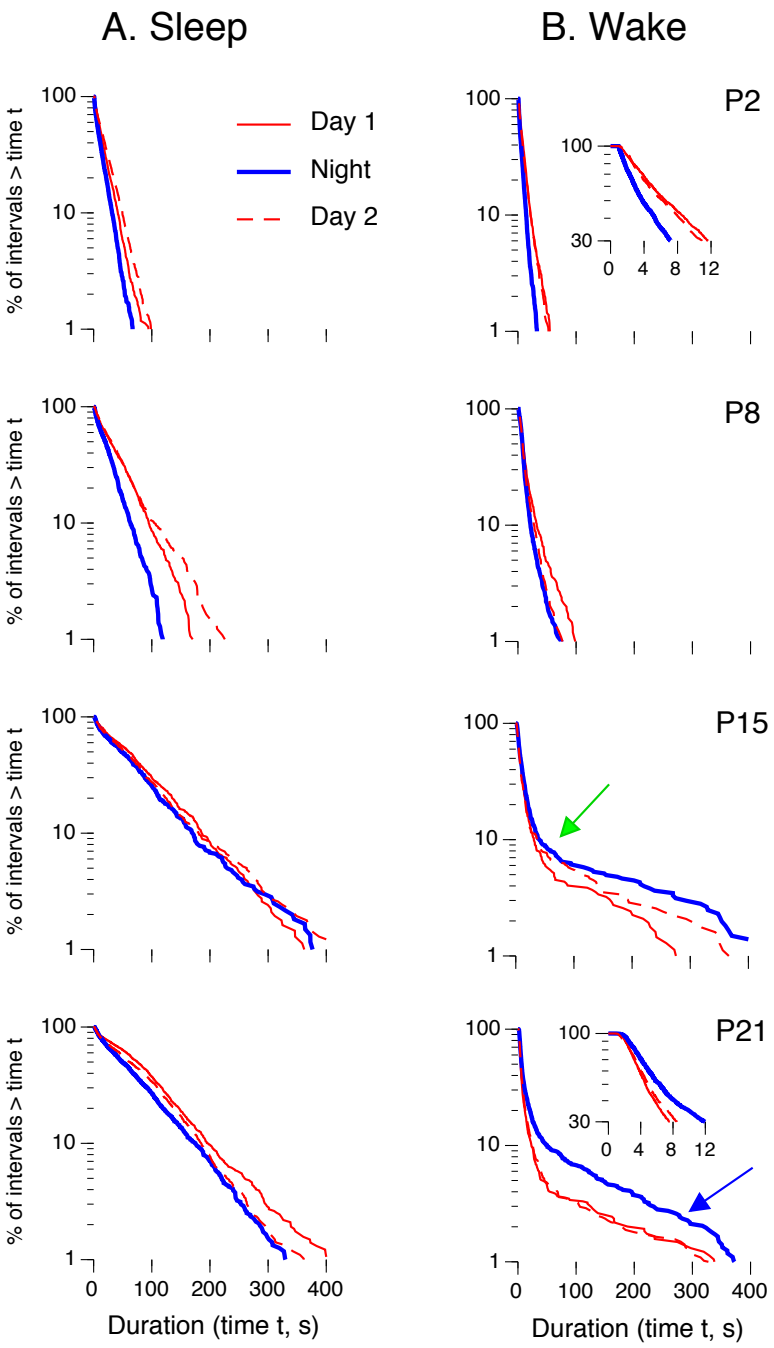

Figure 2. Log-survivor distributions of sleep and wake bout durations. Log-survivor plots of (A) sleep and (B) wake bout durations for rats at P2, P8, P15, and P21 on Day 1 (solid red line), at Night (solid blue line), and on Day 2 (dashed red line). Each plot is constructed from pooled data (620-2213 points per plot). Straight lines on these semi-log plots indicate that the data follow an exponential distribution. Insets provide magnified views at shorter bout durations. The green arrow indicates the development of power-law distributions of wakefulness. Although nocturnality begins developing at P15, it is more clearly established by P21 (blue arrow). (From Gall, Todd et al. 2008).

Consolidation of sleep and wake bout durations also occurs over development in rats (Blumberg, Seelke et al. 2005; Karlsson and Blumberg 2005; Gall, Todd et al. 2008; Gall, Joshi et al. 2009). As shown in Figure 2, at P2, the maximum length of sleep bout durations is 100 s, but by P21, this increases to 400 s. In spite of significant consolidation, sleep bout durations continue to express exponential distributions throughout development. Wake bout durations also elongate and consolidate over development. At P2, the maximum length of wake bout durations is 50 s, but by P21, this increases to 400 s.

The neurobiological mechanisms that underlie the developmental emergence of power-law distributions of wakefulness, consolidation of wake bouts, and nocturnality have not yet been explored. We hypothesize that the development of these processes is influenced by the suprachiasmatic nucleus (SCN) and downstream neural circuitry. Here we examine the mechanisms that underlie the development of circadian rhythms in infant rats, and explore the role of a wake-promoting pathway in the emerging development of nocturnality and power-law distributions of wakefulness in infant rats. Before describing these experiments, some background information is necessary. Therefore, the rest of this chapter examines the development of circadian rhythms of sleep and wakefulness and the role of the SCN.

Developmental emergence of circadian rhythms

The SCN, located near the midline of the anterior hypothalamus, is essential for the circadian timing of behavioral (e.g., sleep and wakefulness) and physiological (e.g., metabolic rate) rhythms in all mammals studied thus far (Moore 1972; Moore and Eichler

1972; Stephan and Zucker 1972). Specialized neurons within the SCN receive photic information from the retina via the retinohypothalamic tract (Welsh, Takahashi et al. 2010). Thus, the SCN is entrained to the light-dark cycle and transmits signals to the rest of the brain to produce circadian rhythmicity throughout the body (Rivkees and Hao 2000).

Electrolytic lesions of the SCN eliminate physiological and behavioral circadian rhythms in adult mammals (Stephan and Zucker 1972; Ibuka, Inouye et al. 1977). For example, SCN lesions in adult rats result in a fragmented pattern of sleep and wakefulness and an elimination of the sleep-wake circadian rhythm (Mosko and Moore 1978; Mouret, Coindet et al. 1978; Mistlberger, Bergmann et al. 1987). The SCN is capable of modulating these behavioral and physiological rhythms via neural efferents and diffusible signals (see Figure 3) (Silver and LeSauter 1993; Silver, LeSauter et al. 1996).

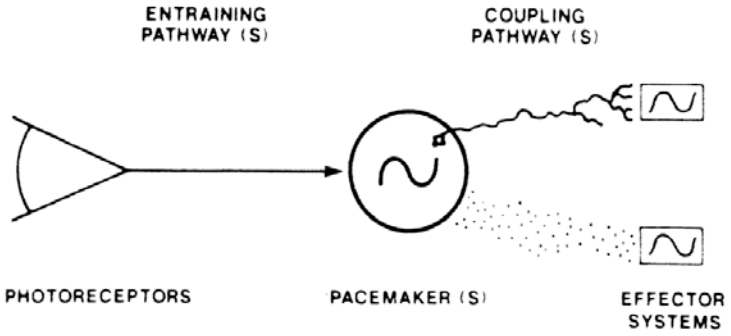

Figure 3. Schematic diagram illustrating an entraining pathway (e.g., light) to the pacemaker (e.g., SCN). The pacemaker is then capable of reaching effector systems (i.e., target nuclei) via 2 coupling pathways—neural efferents and diffusible signals. (From Silver, R., & LeSauter, J., *Journal of Biological Rhythms*, Vol. 8, 1993, S89-92).

SCN rhythms of metabolic activity are first detected prenatally at embryonic day (E)19 in rats (Reppert, Weaver et al. 1988). During the early postnatal period, physiological and behavioral circadian rhythms are detectable, including sleep and wakefulness (Gall, Todd et al. 2008), temperature (Imai-Matsumura, Kaul et al. 1995), metabolic activity (Spiers 1988), and hormonal secretions (Ellison, Weller et al. 1972).

In early infancy—before P8 in rats—the dam entrains circadian rhythms of her pups (Takahashi and Deguchi 1983; Duncan, Banister et al. 1986; Ohta, Honma et al. 2002). However, it is not known whether or not the SCN modulates the expression of circadian rhythms of sleep and wakefulness in pups at P2 independently of the dam. Therefore, we lesioned the SCN at P1 and tested pups at P2 using EMG methods to examine the influence of the SCN on circadian rhythms of sleep and wakefulness shortly after birth.

Projections to and from the SCN

Neurogenesis of the SCN in rats begins between gestational days 13 and 16, with synapses beginning to form on day 19 of gestation (Altman and Bayer 1978). At this early stage of development, the SCN exhibits a daily rhythm in glucose metabolism (Reppert and Schwartz 1983; Reppert and Schwartz 1984). Very few synapses from the SCN are present at birth (Moore and Bernstein 1989). Therefore, it has been suggested that the SCN expresses circadian function before neural connectivity has developed (Moore and Bernstein 1989). These authors "…offer the hypothesis that SCN neurons are born as individual oscillators that are initially coupled by non-neural signals among themselves and from the mother" (p. 2161). By P10, the number of axon terminals per

unit area of SCN increases substantially (Moore and Bernstein 1989). Synaptogenesis within the SCN is therefore mainly a postnatal event in altricial rodents (Speh and Moore 1993).

Developing neural connectivity of the SCN has been studied in hamsters (Muller and Torrealba 1998). At P0, no axons emerged from the SCN to surrounding regions. Starting at P1, SCN efferents began to invade target regions nearby to the SCN (e.g., dorsal and ventral lateral geniculate nuclei, paraventricular hypothalamic nucleus, anterior hypothalamic area, medial preoptic nucleus, intergeniculate leaflet), with slightly more distant targets being invaded by P3. Two targets in particular, the retinochiasmatic area and paraventricular nucleus of the hypothalamus—major targets of the SCN in adult hamsters and rats (Vrang, Mikkelsen et al. 1997; Muller and Torrealba 1998) —were not invaded by the SCN at P0–P3. Efferent SCN connections increased in density in the subsequent days in hamsters and rats (Moore and Bernstein 1989; Muller and Torrealba 1998). Therefore, developing neural circuitry may not begin modulating circadian rhythmicity until the end of the first postnatal week.

Major SCN efferent pathways (see Figure 4) in adult rats include projections to important sleep-wake nuclei in the brain (Miller, Morin et al. 1996; Lydic and Baghdoyan 1999). The SCN sends GABAergic and glutamatergic projections to the sleep-promoting ventrolateral preoptic nucleus (VLPO) (Sun, Whitefield et al. 2001). The lateral hypothalamus (LH), which contains orexin (also known as hypocretin), also receives projections from the SCN (Mochizuki and Scammell 2003). The ventral subparaventricular zone (vSPVZ) is a major target of the SCN, and exhibits differential cFos activity in diurnal grass rats as compared to nocturnal lab rats, suggesting its role in

circadian preference (Schwartz, Nunez et al. 2009). Finally, the SCN sends projections to the dorsomedial hypothalamus (DMH), which serves as a relay nucleus between the SCN and locus coeruleus (LC) (Aston-Jones, Chen et al. 2001).

In adult rats, the SCN receives feedback from many brain areas, some of which have been implicated in sleep and wakefulness. Five principal afferent projections to the SCN exist in adult mammals (see Figure 4). Specifically, inputs to the SCN arise from the retina (via the retinohypothalamic tract, RHT), from the intergeniculate leaflet (IGL) of the lateral geniculate nucleus, from the serotonergic midbrain raphe nuclei, from the lateral septum, and from the hypothalamus (Morin 1994; Lydic and Baghdoyan 1999). Of these inputs, serotonergic raphe and orexinergic hypothalamic nuclei have been shown to be critically involved in sleep and wakefulness (Monti and Monti 2007). The LC, a nucleus known to be important for arousal (Aston-Jones 2004), has extensive projections to hypothalamic and raphe nuclei (Barnes and Pompeiano 1991; Cedraz-Mercez, Mecawi et al. 2007). In addition, other brainstem structures known to be involved in sleep and wakefulness (e.g., laterodorsal tegmental nucleus, pedunculopontine tegmental nucleus) send projections to the SCN (Bina, Rusak et al. 1993; Krout, Kawano et al. 2002).

Afferent pathways to the SCN, similar to efferent pathways from the SCN, develop during the early postnatal period in rats. The development of synapses from the RHT occurs between P0 and P12 (Duncan, Banister et al. 1986; Speh and Moore 1993; Hannibal and Fahrenkrug 2004). Serotonergic innervation of the SCN develops between E22 and P21 (Ugrumov, Popov et al. 1994). The development of the geniculohypothalamic tract occurs between P4 and P11 (Kagotani, Hashimoto et al.

1989). On the basis of these findings, one can conclude that afferent projections to the SCN are developing during the first several postnatal weeks.

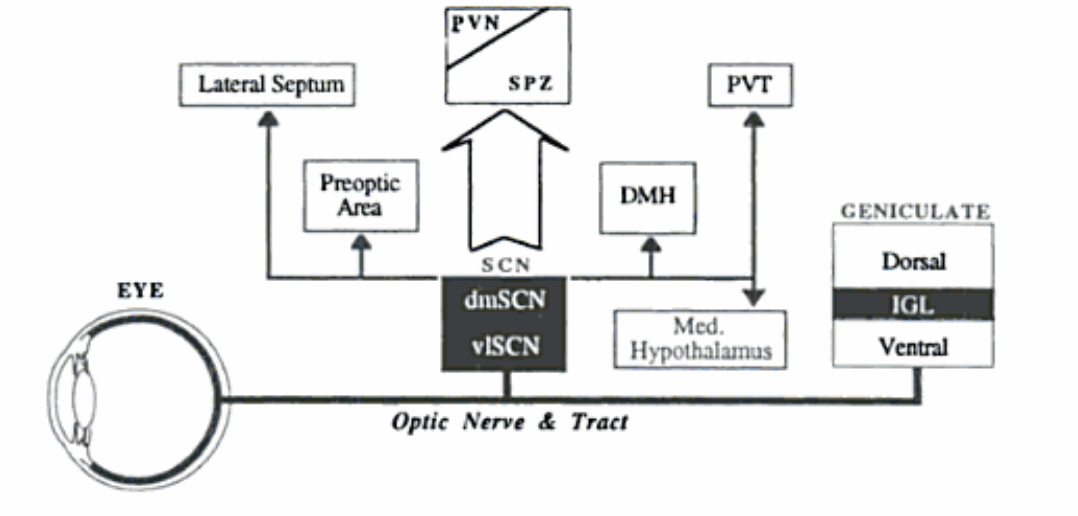

Figure 4. Schematic diagram illustrating the major SCN efferent pathways. (From Lydic, R. and H. A. Baghdoyan (1999). Handbook of behavioral state control: cellular and molecular mechanisms. Boca Raton, CRC Press).

Although much is known about afferents to and efferents from and from the SCN in adults (Miller, Morin et al. 1996; Lydic and Baghdoyan 1999), little is known about how these connections relate to behavior. In addition, many elements of the functional relationships between the SCN and downstream targets are still unknown. Here we explored the neural circuitry between the SCN and downstream structures at P2 and P8 to examine how neural circuitry within these areas changes over development and how these changes are related to behavior.

SCN-DMH-LC wake-promoting pathway

The neural elements that control the circadian aspect of arousal include the SCN, which projects to a nearby hypothalamic structure, the DMH, which in turn projects to a more distant brainstem structure, the LC. Aston-Jones et al. (2001) explored this neural pathway by utilizing several neurophysiological techniques. The LC, which releases noradrenaline (NA) throughout the brain, has been implicated in arousal functions in adult rats (Aston-Jones and Bloom 1981; Berridge and Waterhouse 2003). In order to explore the afferent neural connections to the LC, they microinjected Pseudorabies virus—a retrograde, transynaptic tracer—into the LC. Second, they recorded neural activity from the LC during the day and night to examine the circadian influence of LC neurons. Finally, they lesioned the DMH and performed neural recordings of the LC to explore the role of the DMH in the modulation of LC activity.

Aston-Jones and colleagues found that the SCN sends an indirect projection to the LC, with the DMH functioning as a relay between the two areas. Next, neural activity of the LC displayed a circadian rhythm in impulse activity. That is, neural activity within the LC was significantly higher at night, which was expected for a wake-promoting nucleus in nocturnal animals. Finally, lesioning the DMH resulted in the elimination of circadian changes in LC activity. Altogether, their results supported the notion that the SCN-DMH-LC circuit contributes to the circadian regulation of arousal.

Although it is widely accepted that the SCN is a circadian pacemaker, there is some debate regarding its role in homeostasis. According to one model, the timing of sleep and wakefulness are regulated by homeostatic (process "S") and circadian (process "C") processes. Process "C" is involved in patterning the timing of sleep and wakefulness

with respect to the 24-hour daily cycle, whereas process "S" builds up during waking and decreases during sleep, causing the need for sleep to be dependent upon prior amounts of wakefulness (Borbely and Achermann 1999; Borbely and Achermann 2000). Borbely and colleagues hypothesize that these two processes are independent. They also suggest that the SCN is only critical for process "C" and is not involved in process "S". In support of this notion, SCN lesions result in a disruption of circadian rhythms and fragmentation of locomotor activity, but do not affect total sleep time (Mouret, Coindet et al. 1978; Mistlberger, Bergmann et al. 1987).

Some studies, however, have challenged the notion that circadian and homeostatic systems are independent. SCN lesions in squirrel monkeys resulted in a disruption in circadian entrainment, but also resulted in a 4-hour reduction in total sleep time (Edgar, Dement et al. 1993). This finding led Edgar et al. (1993) to suggest, in opposition to Borbely and Achermann (1999), that circadian and homeostatic systems are dependent upon each other.

Others have examined the issue of whether or not the SCN is involved in both circadian and homeostatic processes by manipulating clock genes within the SCN. Specifically, Naylor and colleagues (2000) found that clock gene mutations in mice resulted in a disruption in circadian rhythmicity, but also affected total sleep time and percentage of REM sleep following sleep deprivation. Therefore, clock genes are capable of modulating circadian rhythmicity as well as sleep homeostasis (Naylor, Bergmann et al. 2000).

Deboer et al. (2003) were the first to provide physiological evidence that the vigilance state of an animal modulates neural activity in the SCN. Using rats, these

researchers combined recordings of SCN electrical activity with electroencephalogram (EEG) and EMG recordings. They showed convincingly that SCN electrical activity is significantly increased when an animal is in a state of REM sleep or wakefulness, in relation to Non-REM (NREM) sleep.

In a separate study, Deboer and colleagues extended these findings, showing that 6-hour sleep deprivation led to changes in SCN neuronal activity which were approximately 60% less than baseline levels (Deboer, Detari et al. 2007). All together, Deboer et al.'s findings, in addition to those cited above, provide converging evidence that the sleep homeostatic and circadian system *must* interact to regulate the timing, duration, and intensity of sleep.

Conclusion

Aston-Jones and colleagues (2001) identified the elements of a wake-promoting circuit involving the SCN, DMH, and LC in adult rats. This neural circuit provides a framework for this dissertation. Importantly, these authors did not examine the developmental influences of this circuit on the expression of sleep-wake behavior. As described earlier, over development, sleep and wakefulness consolidate dramatically and nocturnality begins to emerge. Therefore, we used transections, lesions, and tracing methods to examine how connectivity within this circuit changes over development to affect the expression of behavioral rhythms of sleep and wakefulness.

First, we performed transections and SCN lesions to identify the developmental influence of the SCN and other forebrain structures on the early expression of circadian rhythms of sleep and wakefulness. At P2, transections did not eliminate day-night

differences in sleep and wakefulness. In contrast, at P8, transections did result in an elimination of these day-night rhythms, suggesting that the SCN releases humoral factors at P2 to affect sleep and wakefulness. This suggestion was further supported by the finding that lesions at P2 eliminated circadian rhythms of sleep and wakefulness. These results suggest that the SCN exerts direct neural control over the brainstem between P2 and P8.

Because Experiments 1 and 2 suggested that neural influences from the SCN emerge over the first postnatal week, we predicted that connectivity among the SCN, DMH, and LC would emerge by P8. We examined this possibility in Experiment 3 by tracing these connections at P2 and P8. As predicted, we found that the SCN-DMH-LC circuit becomes intact and bidirectional at P8.

The functional consequences of this emerging circuit were tested in Experiment 4 using a sleep deprivation paradigm. Todd and colleagues (2010) showed that sleep deprivation in P2 rats led to heightened cFos activity in the LC and DMH, but not in the SCN. Based upon the findings in Experiment 3, we predicted that sleep deprivation would result in heightened activity in the SCN, DMH, and LC at P8. We also predicted that DSP-4, a neurotoxin that destroys LC terminals (Jonsson, Hallman et al. 1981; Jonsson, Hallman et al. 1982; Fritschy and Grzanna 1989; Fritschy and Grzanna 1991), would interfere with the activating effect of sleep deprivation on the SCN and DMH by blocking LC activity. In support of our predictions, in Experiment 4, we found heightened SCN, DMH, and LC activity in response to sleep deprivation at P8, and blockade of these effects in pups treated with DSP-4.

After the SCN-DMH-LC circuit has developed, consolidated wakefulness and nocturnality emerge around P15. In Experiments 5, 6, and 7, we tested the hypothesis that the SCN-DMH-LC circuit is involved in the developmental emergence of these processes. We lesioned each structure before the normal development of power-law distributions of wakefulness and nocturnality and tested pups after the normal development of these processes. Since the SCN and DMH play an integral role in a wake-promoting circuit with the LC, we hypothesized that all three nuclei would be critical for the expression of power-law distributions of wakefulness. Our results indicate that the SCN, DMH, and LC are critical for the expression of power-law distributions of wakefulness. In addition, the SCN and DMH are critical for nocturnality.

These dissertation experiments indicate that the SCN, DMH, and LC are critical for the maintenance of arousal. Understanding how the neural circuitry connecting these areas develops may allow researchers to identify their contributions to circadian rhythm disorders, problems associated with jet lag and shift work, or wake-related disorders such as narcolepsy.

Narcolepsy is a disorder that is associated with excessive daytime sleepiness and cataplexy (Taheri, Zeitzer et al. 2002). In other words, narcolepsy is characterized by difficulties maintaining arousal. Orexin, also known as hypocretin, is released from the DMH and the LH and plays a critical role in narcolepsy (Peyron, Tighe et al. 1998). Orexin knockout mice as well as dogs with a mutation of the orexin receptor exhibit a phenotype that is similar to narcoleptic humans (Chemelli, Willie et al. 1999; Lin, Faraco et al. 1999). In addition, dysregulation of the orexin system has been linked to human narcolepsy (Peyron, Faraco et al. 2000). Because the experiments described in this

dissertation indicate that the SCN and DMH are involved in wake bout consolidation, and the SCN, DMH, and LC are all critical for the expression of power-law distributions of wakefulness, we suggest that a better understanding of the development and function of the SCN-DMH-LC circuit will help us to better understand narcolepsy and related disorders of the sleep-wake system. Indeed, improved understanding of the SCN-DMH-LC circuit may prove vital for helping the 35 to 40% of the adult human population that suffers from some type of difficulty in maintaining sleep at night or arousal during the day (Hossain and Shapiro 2002).

CHAPTER 2

INFLUENCE OF FOREBRAIN STRUCTURES ON CIRCADIAN SLEEP-WAKE RHYTHMS IN EARLY INFANCY

Introduction

In adult mammals, the SCN modulates circadian rhythms of sleep and wakefulness, with the light-dark cycle being the predominant entraining stimulus (Ibuka, Inouye et al. 1977; Rusak and Zucker 1979; Moore and Card 1985). In infant mammals, however, circadian rhythms are entrained by maternal influences. Nonphotic cues from the pregnant or nursing mother entrain the SCN of her offspring (Ohta, Honma et al. 2002). Importantly, there is a sensitive period for the ability of nursing mothers to entrain circadian rhythms of their pups. Before P8, pups follow the rhythm of their mother, whereas after P8, the light-dark cycle becomes the predominant entraining stimulus in pups, just as it is in adults (Duncan, Banister et al. 1986). Although the mother entrains her pups' SCN, the expression of day-night differences in her pups may or may not be associated with functional SCN rhythmicity.

Reppert & Schwartz (1986) performed SCN lesions on pregnant dams on day 7 of gestation to determine whether pups are capable of expressing circadian rhythms in SCN activity independent of the mother. These authors found that dams with SCN lesions gave birth to pups that still exhibited circadian rhythms of SCN metabolic activity, but these rhythms were not synchronized among littermates or with the light-dark cycle (Reppert and Schwartz 1986). Thus, the dam is critical for entraining her pups' SCN activity, but she is not critical for her pups' expression of SCN rhythmicity.

As discussed in Chapter 1, day-night differences in sleep and wakefulness are present as early as P2 (Gall, Todd et al. 2008). But what brain mechanisms underlie these circadian differences in sleep and wakefulness? And are these circadian differences modulated independently by the pup? In infant rats, although it is known that the SCN expresses differential day-night metabolic activity (Reppert, Weaver et al. 1988), and the mother entrains these rhythms shortly after birth (Ohta, Honma et al. 2002), the functional role of the SCN in modulating circadian rhythms of sleep and wakefulness is not yet known.

In the present study, we hypothesized that the SCN contributes to the subtle but significant day-night differences in sleep and wakefulness that we have observed in infant rats. To test this hypothesis, we employed two different methods. In Experiment 1, we performed precollicular transections at P2 and P8 to remove neural influences from the forebrain, including the SCN, on downstream brainstem structures involved in sleep and wakefulness. In Experiment 2, we performed specific lesions of the SCN at P1 and tested one day later, at P2. We predicted that transections and SCN lesions would eliminate day-night differences. If they do, then we can conclude that circadian rhythms of sleep and wakefulness in early infancy are endogenously generated and dependent upon the SCN.

Methods

Subjects

All experiments were performed in accordance with National Institutes of Health guidelines for the care of animals in research and were approved by the Institutional Animal Care and Use Committee of the University of Iowa. All efforts were made to

minimize the number of animals used. For all experiments, males and females were equally represented and littermates were always assigned to different experimental groups. Litters were culled to 8 pups within 3 days of birth (day of birth = P0). Mothers and their litters were housed and raised in standard laboratory cages (48 x 20 x 26 cm) in the animal colony at the University of Iowa. Food and water were available *ad libitum*. All animals were maintained on a 12-h light-dark schedule with lights on at 0700 h.

In Experiment 1, a total of 48 P2 and P8 Sprague-Dawley rats from 12 litters (n = 6 at each age x 2 age groups x 2 test times x 2 conditions) were used. In Experiment 2, a total of 20 Sprague-Dawley P2-3 rats from 10 litters (n = 5 x 2 test times x 2 conditions) were used.

Procedure

Experiment 1: P2 and P8 Transections

On the day of testing, a P2 or P8 pup with a visible milk band was removed from the litter. Under isoflurane anesthesia, transections were made by drilling a small hole in the skull at approximately 3 mm caudal to lambda, and then manually inserting a blunted 25 g needle to the base of the brain and rotating the needle using a side-to-side motion (Karlsson, Kreider et al. 2004; Mohns, Karlsson et al. 2006). Sham littermates of the same age underwent anesthesia surgery and drilling, but the needle was not inserted. Vetbond was used to close the pup's scalp. Peanut oil was brushed lightly on the scalps of transected and sham pups to deter the mother from reopening the closed wound.

Next, still under isoflurane anesthesia, two bipolar stainless steel electrodes (50 μm diameter, California Fine Wire, Grover Beach, CA) were inserted bilaterally into the

nuchal muscles and secured with flexible collodion (Seelke, Karlsson et al. 2005). After surgery, pups recovered in a humidified incubator maintained at thermoneutrality (34-35˚C) for at least 1 h before testing. Skin temperatures were closely monitored for overheating (Mohns, Karlsson et al. 2006). If interscapular temperature exceeded 38.5˚C, the pup was injected subcutaneously with propranolol (Aldrich, Milwaukee, WI) to inhibit heat production (20 mg/kg; 1 µl/g body weight). In this experiment, 4 transected P8 pups were injected with propranolol. We have reported previously that propranolol has no effect on sleep or wake behavior in relation to untreated shams (Mohns, Karlsson et al. 2006).

P2 subjects were tested in an electrically shielded double-walled glass chamber sealed with a rubber lid during the day (1200 – 1400 h) and night (2400 – 0200 h), with order of test time counterbalanced. Two additional same-sex littermates were tested at P8. Air temperature in the testing chambers was regulated at thermoneutrality for each age (i.e., P2: 35.5˚C; P8: 35˚C). Nuchal electrodes were connected to differential amplifiers (A-M systems, Carlsborg, WA) and their signals were amplified (x10k) and filtered (300-5000 Hz). EMG data were acquired (1000 samples/s) and stored using a data acquisition system (BioPac Systems, Santa Barbara, CA).

Immediately after testing, all subjects were overdosed with sodium pentobarbital and perfused transcardially with phosphate-buffered saline followed by 3% formalin. Heads were post-fixed in a sucrose-formalin solution for at least 24 h. The rostrocaudal placement of the transection was determined using gross visual inspection and drawn onto a photomicrograph of a P2 and P8 sagittal section.

Experiment 2: SCN Lesions at P2

The same methodologies described in Experiment 1 were employed, with the exception that lesions were performed on same-sex littermates at P1 (in place of transections), with testing on P2-3 during the day and night.

On the day of testing, a P1 pup with a visible milk band was removed from the litter. Under isoflurane anesthesia, bilateral electrolytic SCN lesions were produced in P1 rats in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) using an insulated tungsten concentric bipolar electrode (1 MΩ, 3-4 μm at the tip, model TM33CCINS, World Precision Instruments, Sarasota, FL, USA). Electrolytic lesions, which ablate the SCN, have been shown to effectively eliminate circadian rhythms (Mosko and Moore 1978). Because SCN neurons are resistant to excitotoxicity (Bottum, Poon et al. 2010), electrolytic lesions of the SCN have become standard in the field and were used here. At 1000 h, an electrode was lowered bilaterally via 2 separate drops into the SCN of with the following coordinates: AP: -0.2 mm from bregma, ML: ± 0.1 mm, and DV: -4.5 mm ventral to the meningeal surface. Lesions were made using a stimulus generator (model SD9F; Grass, Quincy, MA, USA) and a linear stimulus isolator (model A395R; World Precision Instruments, Sarasota, FL, USA) to deliver 1.0 mA of DC current for 30 s. Vetbond was used to close the pup's scalp. Peanut oil was brushed lightly on the scalps of lesioned and sham pups to deter the mother from reopening the closed wound. The sham control group experienced the same procedures (e.g., 2 separate drops of the electrode under isoflurane anesthesia) except that current was not applied. EMG electrodes were implanted as described in Experiment 1.

After surgery, pups recovered for at least 2 h inside a humidified incubator maintained at 34-35˚C. Subjects then were placed back in the litter for testing the next day (1200 – 1400 h) or night (2400 – 0200 h), with order of test time counterbalanced. Pups were tested for sleep and wakefulness using EMG methods described above in Experiment 1.

At the conclusion of testing, pups were overdosed with sodium pentobarbital and perfused transcardially, as described in Experiment 1. Brains were sliced in 50 μm sections using a sliding microtome (Model SM 2000 R, Leica, Bensheim, Germany). Brain slices were stained using cresyl violet. The extent of the lesion was determined by examining sequential brain slices.

Data Analysis

EMG data were analyzed using AcqKnowledge software (BioPac Systems, Santa Barbara, CA). EMG signals were integrated and full-wave rectified, and dichotomized into high muscle tone and atonia (or wake and sleep, respectively), as described earlier (Karlsson, Kreider et al. 2004). Data were scored by an individual blind to experimental condition. Sleep and wake bout durations were imported into Statview 5.0 (SAS Institute, Cary, NC) for analysis. Mean sleep and wake bout durations were determined for each subject and across subjects (Mohns, Karlsson et al. 2006; Gall, Todd et al. 2008).

As described previously (Blumberg, Seelke et al. 2005), log-survivor distributions of sleep and wake bout durations were produced from individual and pooled data. These log-survivor distributions were not presented in Experiment 1, as they did not add anything significant to the mean data.

We used a second analytic technique to test whether bout duration data were better fit by a power-law or exponential model. Using pooled data, log-likelihood functions were calculated and maximized for each model (Burnham and Anderson 2002; Myung 2003; Clauset, Shalizi et al. 2007). The comparison was quantified by calculating Akaike weights, w_i (i = 1,2), for each model: $w_i = \exp(-\Delta_i/2)/(\exp(-\Delta_1/2) + \exp(-\Delta_2/2))$. Δ_i is a measure of each model relative to the better model; $\Delta_i = 0$ for the model with larger log-likelihood value. For the other model, $\Delta_i > 0$ is calculated as the difference of Akaike Information Criterion (AIC) values, $AIC_i = -2(\text{log-likelihood}_i) + 2k_i$, where k_i is the number of parameters in model i. The Akaike weight for a model estimates the probability that the model is the better of the two candidate models (Akaike 1974; Burnham and Anderson 2002). They give the probability of a certain model being a better fit among all the chosen candidate models. Therefore, the model with a weight of 1 is a better fit than the model with a weight of 0.

Analysis of variance (ANOVA) was used to test for differences across groups (i.e., lesion vs. control) and test times (i.e., day vs. night). If an interaction was significant, post hoc tests using Fisher PLSD were performed. Preplanned comparisons (i.e., unpaired *t* tests) were used to test day-night differences within groups.

Alpha was set at 0.05 with bonferroni correction. Means are presented with S.E.

Results

Experiment 1: Transections at P2 and P8

Figure 5 presents mean sleep and wake bout durations at P2 (Figure 5A) and P8 (Figure 5B) for shams and transected pups. Ranges of transections are shown in the far

right panel at each age. As shown in Figure 5A, mean sleep bout durations were significantly shorter at night than during the day for both shams and transected pups at P2. ANOVA revealed significant main effects for group ($F_{1,20}$ = 13.6, P < .005) and test time ($F_{1,20}$ = 22.4, P < .001), but not a significant group x test time interaction ($F_{1,20}$ = 1.9). Mean wake bout durations were also significantly shorter at night than during the day for both shams and transected pups at P2. ANOVA revealed a significant main effect for test time ($F_{1,20}$ = 9.5, P < .01), but not a significant main effect for group ($F_{1,20}$ = 2.9) and no significant group x test time interaction ($F_{1,20}$ = 0.04).

As shown in Figure 5B, mean sleep bout durations were significantly shorter at night for sham pups at P8, but no significant day-night differences were found for mean sleep bout durations in transected pups at this age. ANOVA revealed significant main effects for group ($F_{1,20}$ = 16.3, P < .001), but no significant effects for test time ($F_{1,20}$ = 2.9) and not a significant group x test time interaction ($F_{1,20}$ = 3.9). There were also no significant day-night differences found for mean wake bout durations in sham or transected pups when tested at P8. ANOVA revealed a significant main effect for group ($F_{1,20}$ = 14.9, P < .005), but no significant effect of test time ($F_{1,20}$ = 0.06) and no significant group x test time interaction ($F_{1,20}$ = 2.5).

Log-survivor distributions for pooled sleep and wake bout durations for transections and shams during the day and night (not presented here) were plotted on semi-log plots. Straight lines on these semi-log plots are indicative of exponential distributions (Blumberg, Seelke et al. 2005; Gall, Joshi et al. 2009). As expected for shams and transected animals during the day and night, both sleep and wake bouts

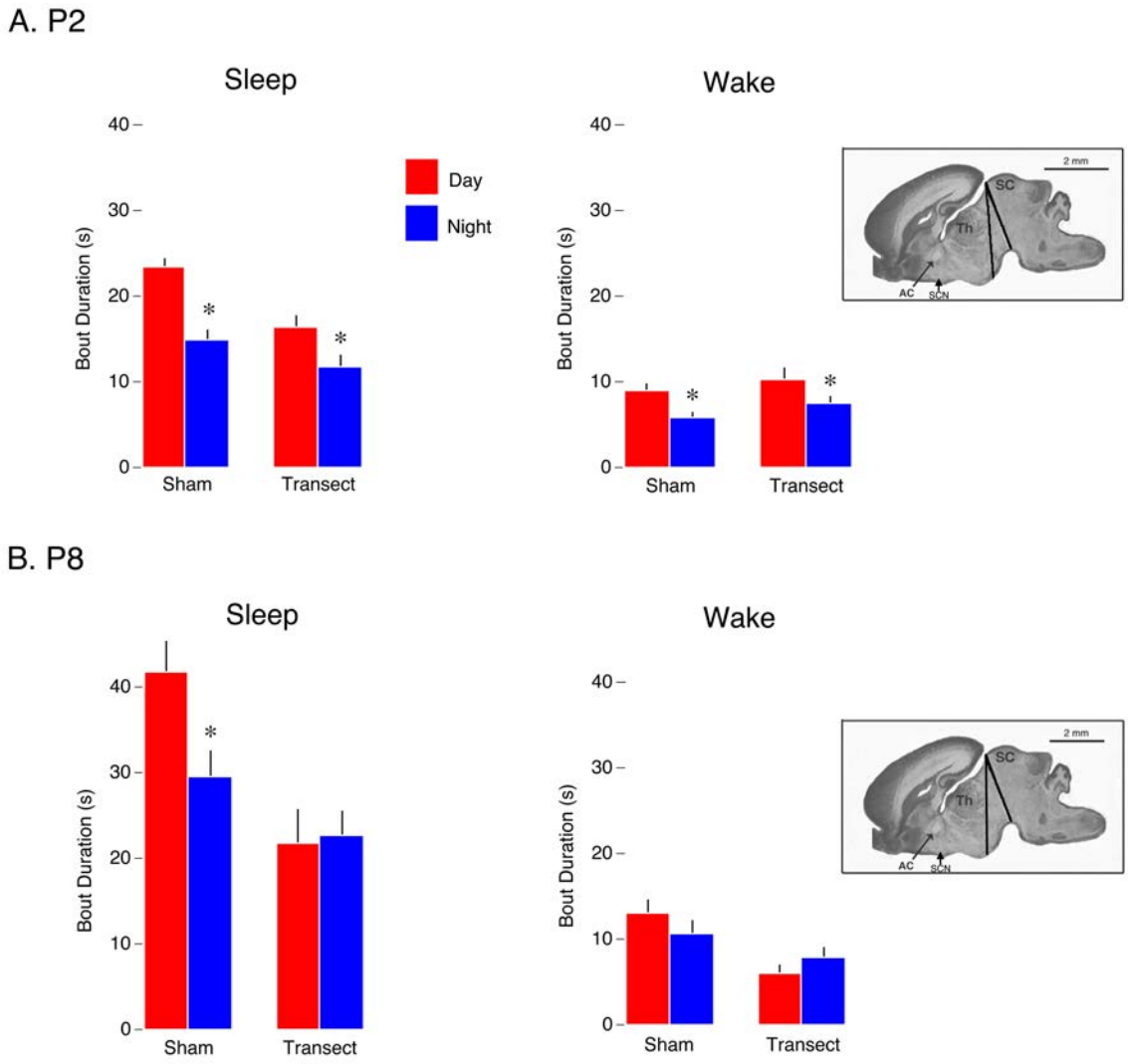

Figure 5. Day-night differences in mean sleep and wake bout durations in sham and transected pups at P2 and P8. Significant day-night differences in sleep and wakefulness in sham and transected pups are detectible at P2 in both sham and transected animals. In contrast, at P8, significant day-night differences in mean sleep bout durations are only detectible in shams; day-night differences in sleep are eliminated in transected pups at P8. Day-night differences in mean wake bout durations are not detectible at P8 in either group. *Significantly different from corresponding daytime value. n = 6 subjects per group. Means + SE. Abbreviations: AC: anterior commissure; SCN: suprachiasmatic nucleus; Th: thalamus; SC: superior colliculus.

distributed along a straight line on these semi-log plots. In support of visual inspection, for sleep and wake bouts in shams and transected animals during the day and night, Akaike weights for exponential distributions were equal to one and those for power-law distributions were equal to zero. Therefore, the bout durations for sleep and wakefulness in both groups and both test times were better fit by exponential distributions.

Experiment 2: SCN Lesions at P2

Figure 6 presents the effects of SCN lesions at P2 on sleep and wakefulness during the day and night. Log-survivor distributions for pooled sleep and wake bout durations for SCN lesioned pups and shams are presented in Figure 6A. As expected for shams during the day and night, sleep and wake bouts distributed along a straight line on these semi-log plots; therefore, exponential distributions were observed. Sleep and wake bouts for SCN lesioned pups during the day and night also distributed along a straight line on these semi-log plots; therefore, exponential distributions were observed. In support of visual inspection, Akaike weights for exponential distributions were equal to one, and Akaike weights for power-law distributions were equal to zero. Therefore, the sleep and wake bout durations in both shams and SCN lesioned pups were better fit by an exponential distribution. Finally, shams expressed shorter sleep and wake bout durations at night as compared to the day, whereas SCN lesioned pups did not express day-night differences in either measure. These results are highlighted by the insets in Figure 6A. Mean sleep bout durations for shams were significantly shorter at night than during the day. SCN lesioned pups, in contrast, did not express significant day-night differences in

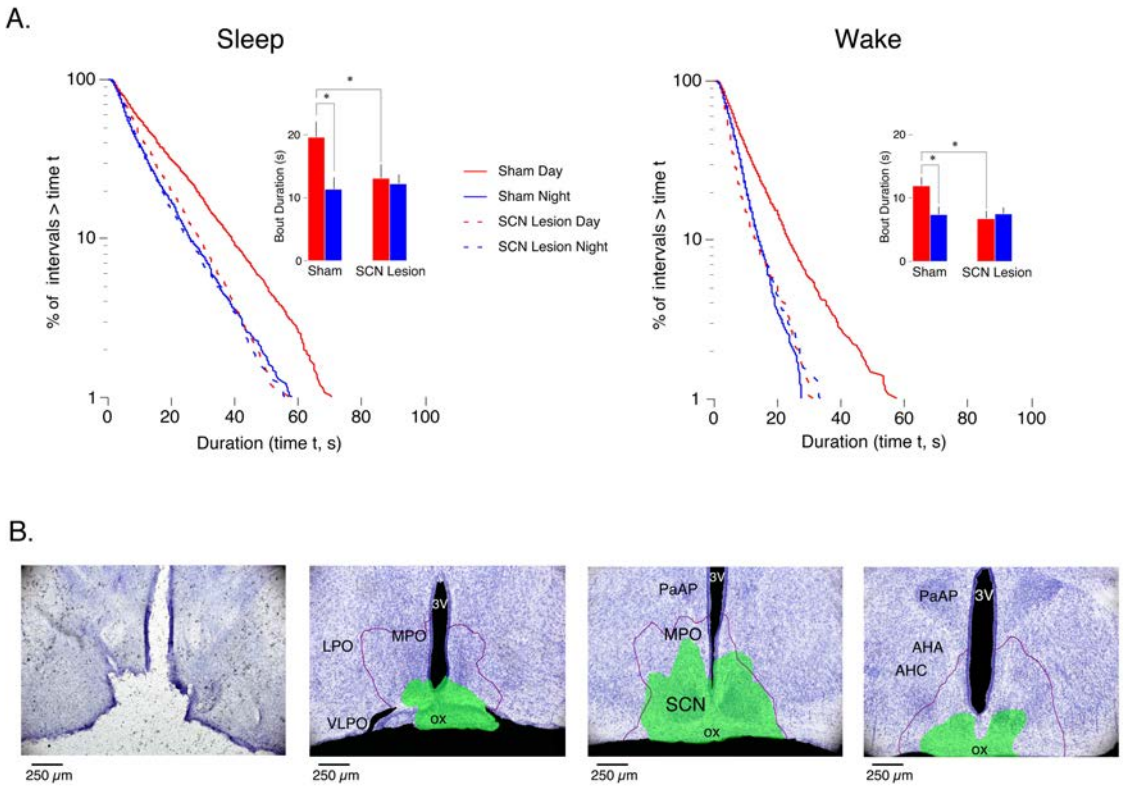

Figure 6. Effects of SCN lesions at P2 on sleep and wakefulness during the day and night. (A) Log-survivor plots of pooled sleep (left column) and pooled wake (right column) bout durations for subjects that experienced sham surgery or SCN lesions at P1 and tested at P2 (1169-1932 points per plot). Pups were tested during the day (red) and at night (blue) in shams (solid line) and SCN lesioned pups (dashed line). Insets provide mean sleep and wake bout durations for sham and lesioned pups during the day and at night. *Significantly different. n = 5 subjects per group. Means + SE. (B) Photograph of a representative bilateral electrolytic SCN lesion performed at P1 with testing occurring at P2 (left) followed by 3 sequential images (rostral to caudal) depicting the smallest (green filled area) and largest (purple outlined area) lesions per coronal section across all lesioned subjects. Sections are 500 μm apart. Abbreviations: SCN: suprachiasmatic nucleus; 3V: third ventricle; MPO: medial preoptic area; LPO: lateral preoptic area; VLPO: ventrolateral preoptic area; ox: optic chiasm; PaAP: Anterior part of parvicellular nucleus; AHA: anterior hypothalamic area, anterior; AHC: anterior hypothalamic area, central.

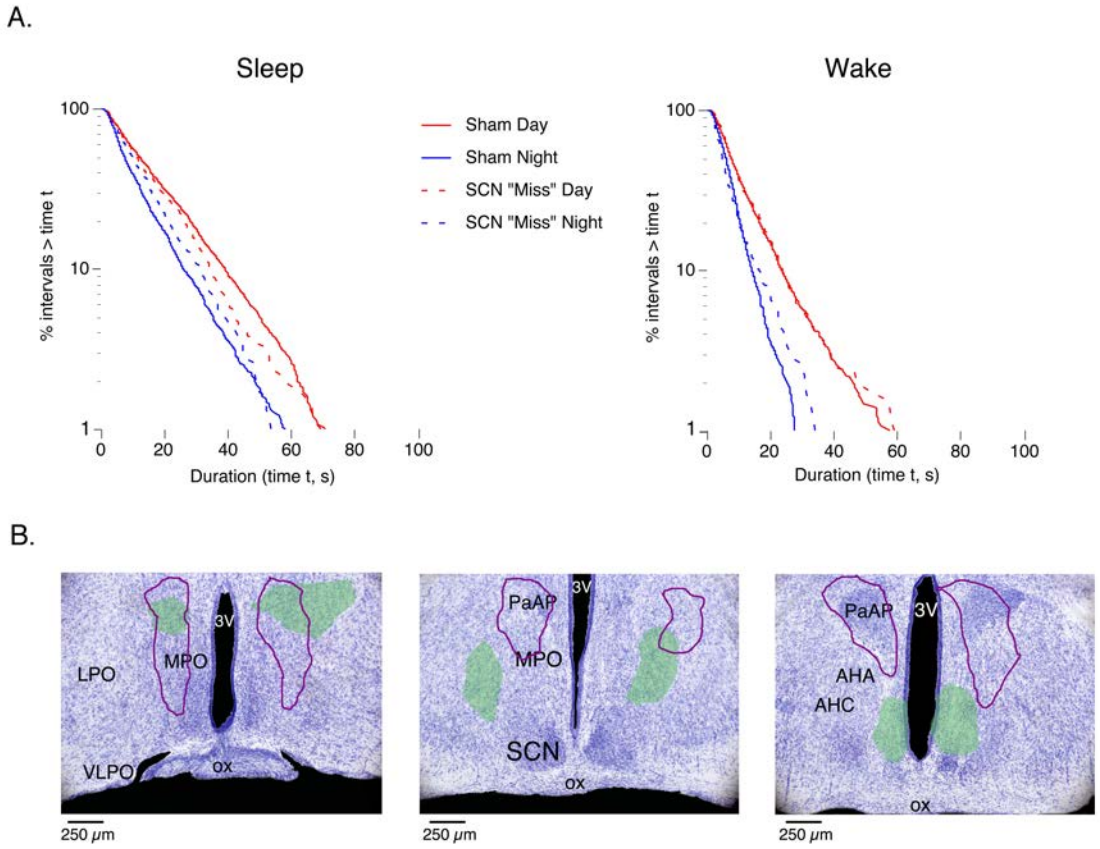

Figure 7. Effects of SCN "Misses" at P2 on sleep and wakefulness during the day and night. (A) Log-survivor plots of pooled sleep (left column) and pooled wake (right column) bout durations for subjects that experienced sham surgery or lesions that did not include the SCN at P1 and tested at P2 (469-1932 points per plot). Pups were tested during the day (red) and at night (blue) in shams (solid line) and lesioned pups (dashed line). (B) 3 sequential brain slices (rostral to caudal) depicting the smallest (green filled area) and largest (purple outlined area) lesions per coronal section across all lesioned subjects. Sections are 500 μm apart. Abbreviations are identical to Figure 6.

mean sleep bout durations. ANOVA revealed a significant main effect of test time ($F_{1,16}$ = 9.0, P < .01), and a significant group x test time interaction ($F_{1,16}$ = 5.7, P < .05), but not a significant main effect of group ($F_{1,16}$ = 3.5). Mean wake bout durations for shams were also significantly shorter at night than during the day. SCN lesioned pups did not express significant day-night differences in mean wake bout durations. ANOVA revealed a significant main effect of group ($F_{1,16}$ = 6.3, P < .05) and a group x test time interaction ($F_{1,16}$ = 6.8, P < .05), but not a significant main effect of test time ($F_{1,16}$ = 3.6). In addition, post hoc tests revealed that during the day, mean sleep and wake bout durations differed significantly between SCN lesioned and sham groups.

Figure 6B shows the extent of the lesions. The smallest (green filled) and largest (purple outline) lesion for each section is presented. Adjacent areas to the SCN were also affected by the electrolytic lesions in some pups, including parts of the optic chiasm (ox) and medial preoptic nucleus (MPO).

Six additional pups from 6 separate litters received electrolytic lesions that spared all cells within the SCN (i.e., SCN "Misses"). Log-survivor plots of sleep and wakefulness for SCN "Misses" and shams are presented in Figure 7A. All SCN "Misses" expressed day-night differences in sleep and wakefulness, similar to shams. Figure 7B shows the extent of the lesions. The smallest (green filled) and largest (purple outline) lesion for each section is presented. Note that all lesions spared cells within the SCN.

Discussion

In comparison to the vast literature on circadian rhythms of sleep and wakefulness in adults (Mistlberger 2005; Fuller, Gooley et al. 2006; Beersma and Gordijn 2007;

Rosenwasser 2009), very little is known about the emergence of such rhythms in newborns. We hypothesized that the SCN would be critical for the expression of circadian rhythms of sleep and wakefulness in infants, just as it is in adults (Ibuka, Inouye et al. 1977). Therefore, precollicular transections, which remove the neural influence of the SCN and other forebrain structures, were predicted to eliminate day-night differences in sleep and wakefulness. However, day-night differences in sleep and wakefulness were retained after performing precollicular transections at P2, suggesting that neural efferents from the SCN to downstream brainstem nuclei are not responsible for circadian differences at this age. In contrast, at P8, precollicular transections eliminated day-night differences in sleep and wakefulness, suggesting that neural outputs from the SCN have reached brainstem nuclei involved in sleep and wakefulness. These results suggest that the SCN exerts a humoral influence in newborns and gains neural control over the first postnatal week.

Diffusible factors have been shown to be released from the SCN to affect the expression of behavioral and physiological rhythms (Silver and LeSauter 1993). Evidence for diffusible factors being released from the SCN comes, in part, from transplantation studies. Transplantation of intact fetal SCN tissue into adult SCN lesioned animals restores circadian rhythms of locomotor activity. Therefore, neural efferents are not necessary for circadian rhythmicity; diffusible factors alone released from the SCN can account for these rhythms.

There are several possible diffusible factors released by the SCN that have been identified. For example, transforming growth factor alpha (TGF-alpha) is expressed in the SCN during early development (Seroogy, Lundgren et al. 1993; Kornblum, Hussain

et al. 1997). This growth factor suppresses circadian rhythms of locomotor activity and feeding when infused into the brain or administered systemically (Gilbert and Davis 2009). TGF-alpha binds to the epidermal growth factor receptor (EGFR), and both TGF-alpha and EGFRs are present at birth in infant rats (Seroogy, Gall et al. 1995).

A second diffusible signal, prokineticin 2 (PK2), also appears to modulate locomotor activity rhythms in rats. PK2 is robustly expressed in the SCN of infant and adult rats (Cheng, Bullock et al. 2002; Zhou and Cheng 2005; Vellani, Colucci et al. 2006). Infusion of PK2 into the lateral ventricle inhibits nocturnal wheel running rhythms in adult rats (Cheng, Bullock et al. 2002). PK2 mediates its effects upon the prokineticin receptor 2 (PKR2); this receptor has been detected in nuclei important for sleep-wake regulation, including the DMH (Zhou and Cheng 2005).

It is not known whether these diffusible factors are released from the core or shell of SCN, whether they are released by particular subtypes of SCN cells, or whether or not there are other candidate diffusible factors that play a role in sleep and wakefulness in rats. However, it is clear that diffusible factors are released by the SCN to affect locomotor activity rhythms in mammals (Silver, LeSauter et al. 1996).

If diffusible factors are responsible for day-night differences in sleep and wakefulness seen here at P2, we would expect that SCN lesions would eliminate these differences. This is exactly what we found; pups with complete SCN lesions no longer expressed day-night differences in sleep and wakefulness at P2. In contrast, shams and pups with lesions that spared the SCN (i.e., SCN "Misses") expressed the normal pattern of shorter sleep and wake bout durations at night. Therefore, the expression of circadian rhythmicity at P2 is attributable to the pup's SCN, rather than pups merely following the

mother's rhythmicity; SCN mediation of these rhythms may occur via diffusible factors at this age. By P8, however, our findings suggest that neural connectivity among the SCN and downstream structures develops. This possibility is tested in the next chapter.

CHAPTER 3

NEURAL TRACING OF THE SCN-DMH-LC PATHWAY OVER DEVELOPMENT

Introduction

As discussed in Chapter 1, emerging neural circuitry among the SCN and downstream structures develops over the first postnatal week (Moore and Bernstein 1989; Speh and Moore 1993). In Chapter 2, the effects of precollicular transections on sleep-wake activity suggested that the SCN makes direct neural connections with downstream structures involved in sleep and wakefulness by P8. Here, we seek to provide direct evidence for this developmental change in neural connectivity among the wake-promoting SCN-DMH-LC circuit (Aston-Jones, Chen et al. 2001).

In Experiment 3, we used fluorescent tracers placed within the SCN and DMH to reveal the developing neural circuitry among the SCN, DMH, and LC at P2 and P8. We placed DiI crystals in the SCN in fixed tissue at P2 and P8. This process was simple and effective, since the optic chiasm provides a distinct landmark at the base of the brain, and is just ventral to the SCN. Because DiI crystals cannot be inserted into the DMH, we instead injected cholera toxin subunit B (CTB) into this structure in vivo at P2 and P8. DiI crystals and CTB both produce anterograde and retrograde labeling of neurons, including axons, axon terminals, and cell bodies (Honig and Hume 1989; Honig and Hume 1989). Together, these methods allowed us to determine bidirectional developmental changes in connectivity among the SCN, DMH, and LC between P2 and P8.

Methods

DiI crystal in fixed tissue

Four P2 and four P8 pups were removed from the litter during the middle of the lights-on period (1430 h), killed, and perfused, as described in Experiment 1. Brains were extracted and allowed to soak in 4% paraformaldehyde (PFA) for at least 24 hours. Each brain was then removed from the PFA solution and placed on its dorsal surface under a dissecting microscope (Leica Instruments). Using forceps, one *FAST* DiI crystal (Molecular Probes, Eugene, OR), which travels at a rate of 0.2 – 0.6 mm/day (Friedland, Eden et al. 1996), was carefully inserted just dorsal to the optic chiasm unilaterally within the SCN of the perfused brain tissue. Each brain was then placed back in the PFA solution and allowed to rest for 6 weeks in a dark glass jar at 4°C (Friedland, Eden et al. 1996). Again using forceps, each crystal was removed from each brain. Using a freezing microtome, brains were sliced in 40 µm sections. Every other slice was used for fluorescence analysis by coverslipping and using Vectastain hard set (Vector Laboratories, Burlingame, CA). Remaining sections were Nissl stained using cresyl violet. These sections were rehydrated with distilled water, dehydrated with a set of alcohols, cleared with citrus clearing solution, and coverslipped using DPX.

Sections were analyzed for the presence or absence of DiI labeling using a fluorescent microscope and imaging system (Leica Instruments, Wetzlar, Germany). Fluorescent sections were directly compared to counterstained Nissl sections to identify nuclei in a stereotaxic atlas of the Norway rat brain (Paxinos and Watson 1998). Pictures of fluorescent sections and nissl sections were overlayed using Adobe Photoshop to determine the location of DiI labeling.

CTB in vivo

Four P2 and four P8 pups were injected with fluorescent CTB unilaterally into the DMH. Pups were placed into a sterotaxic instrument under isoflurane anesthesia. A small hole was drilled at the following coordinates: AP: -2.2 mm caudal to bregma, ML: 0.2 mm from midline, DV: -6.0 mm from the surface of the brain. A 30-gauge needle attached to a 1.0 µl Hamilton syringe was filled with Alexa Fluor 594 (Texas-Red)-conjugated CTB (1 mg/ml; Molecular Probes, Eugene, OR, USA). Once the target area was reached, the needle was left in place for 1 min. 0.1 µl of CTB was infused over 30 s. Following the injection, the needle remained in the brain for 1 min. The needle was removed from the brain slowly, Vetbond was used to close the pup's scalp, and peanut oil was brushed lightly on the scalps. The pup recovered for at least 2 h in a humidified incubator maintained at thermoneutrality and placed back in the litter. Three days later, pups were removed from the litter, killed, and perfused as described in Experiment 1. Data analysis was as described above for DiI crystals.

Results

As presented in Figure 8A, DiI crystal placed within the SCN at P2 reveals no fluorescence in regions surrounding the DMH. However, at P8, fluorescent cell bodies and terminals were revealed in the DMH, lateral hypothalamus (LH), and ventromedial hypothalamus (VMH). This suggests that at P2, these regions do not send or receive neural projections to or from the SCN, respectively. However, by P8, all 3 of these regions reciprocally communicate with the SCN, suggesting that neural connectivity between hypothalamic areas develops between P2 and P8.

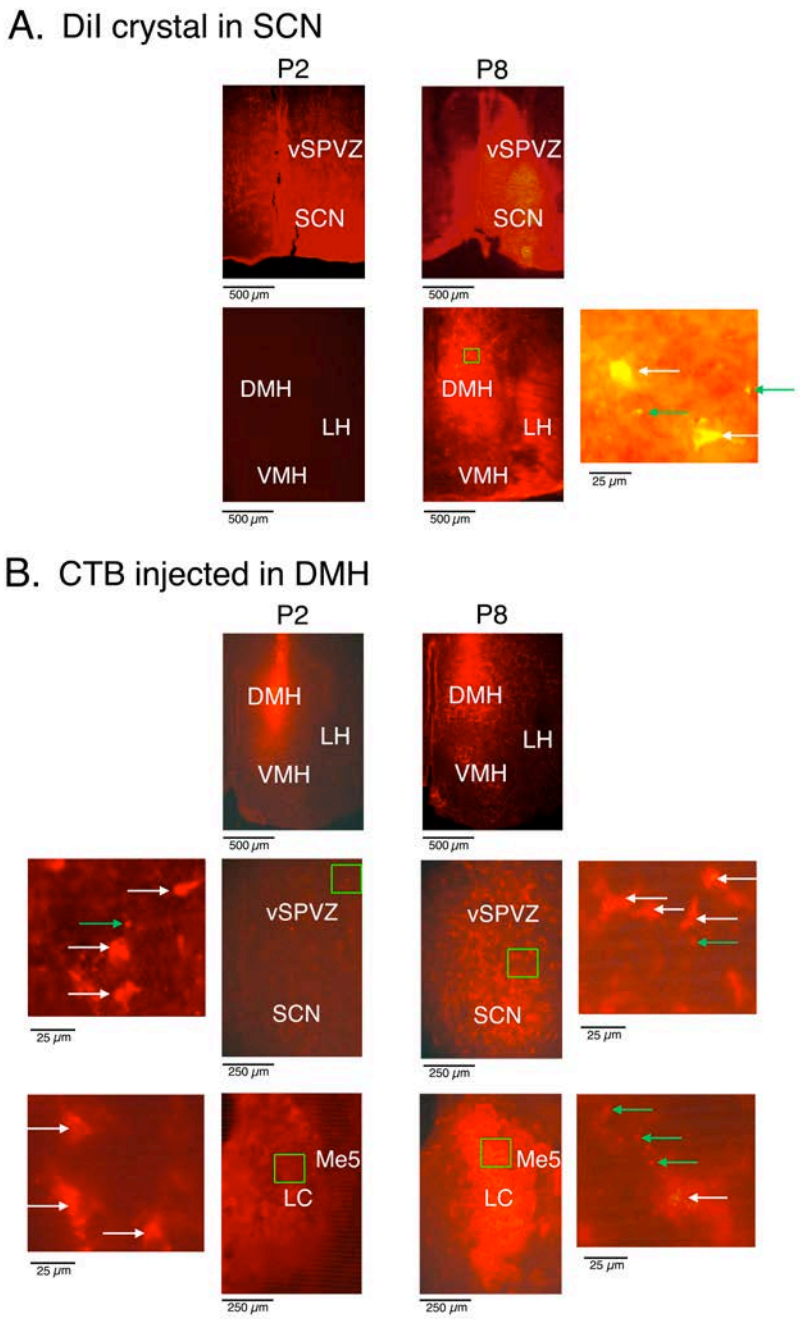

Figure 8. Neural tracing with DiI and CTB at P2 and P8 in the SCN and DMH. (A) One DiI crystal was placed in the SCN of fixed tissue at P2 (left) and P8 (right). Top row depicts placement of crystal. Bottom row depicts fluorescence in DMH, LH, and VMH. (B) 0.1 μL fluorescent CTB was injected unilaterally into the DMH at P2 (left) and P8 (right). Top row depicts injection site of CTB. Bottom 2 rows depict fluorescence in vSPVZ, SCN, LC, and Me5. Magnified insets (green squares) are provided to visualize cell bodies (white arrows) and axon terminals (green arrows) in each area of interest. n = 4 subjects per group. Abbreviations: DMH: dorsomedial hypothalamus; LH: lateral hypothalamus; VMH: ventromedial hypothalamus; vSPVZ: ventral subparaventricular zone; SCN: suprachiasmatic nucleus; Me5: Mesencephalic of 5; LC: locus coeruleus.

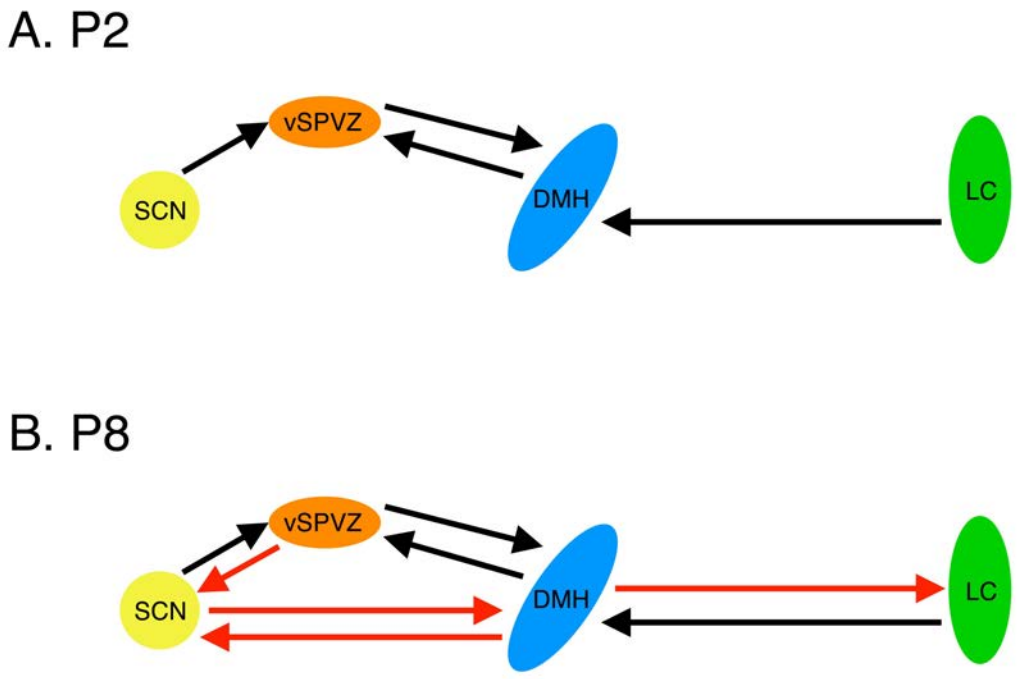

Figure 9. Model of SCN-DMH-LC developing circuitry. (A) Connectivity among the SCN, vSPVZ, DMH, and LC at P2 (black arrows). (B) Connectivity among the SCN, vSPVZ, DMH, and LC at P8. Red arrows indicate new connectivity that develops by P8. Note that the black arrows indicate connectivity at both P2 and P8. Abbreviations: SCN: suprachiasmatic nucleus; vSPVZ: ventral subparaventricular zone; DMH: dorsomedial hypothalamus; LC: locus coeruleus.

As presented in Figure 8B, unilateral injection of CTB into the DMH at P2 revealed cell bodies and terminals labeled within the vSPVZ, but not in the SCN. This suggests that at P2, bidirectional communication between the DMH and vSPVZ, but not between the DMH and SCN, is established. In addition, at P2, only cell bodies are evident within the LC, suggesting that the LC sends neural projections to the DMH. At P8, cell bodies and terminals were labeled within the vSPVZ and SCN, suggesting that bidirectional communication between the SCN and DMH develops by P8. In addition, at P8, cell bodies and terminals are evident in the LC, suggesting bidirectional communication between the LC and DMH by P8.

Two pups received injections of CTB that missed the DMH region (i.e., DMH "Miss"). In one pup at P2, the injection was in an undefined region dorsal to the DMH and ventral to the thalamic nucleus submedius. This resulted in no visible fluorescent labeling anywhere in the brain other than the injection site. In a second DMH "Miss" at P8, the CTB injection was within the medial tuberal nucleus, just lateral and dorsal to the DMH. This resulted in fluorescent axon terminals in the dorsal raphe nucleus, dorsal tegmental nucleus, and motor trigeminal nucleus. Importantly, both of these misses resulted in different patterns of fluorescence in comparison to DMH "Hits."

Figure 9 shows a schematic of established connectivity between the SCN, vSPVZ, DMH, and LC at P2 (Figure 9A) and P8 (Figure 9B) based upon the fluorescent data shown in Figure 8. Note that the black arrows in Figure 9A indicate connectivity at P2; the red arrows in Figure 9B indicate new connections that develop by P8.

Table 1 shows other regions where fluorescent labeling was consistently observed (in 100% of subjects with "Hits") following DiI crystal implantation into the SCN or

CTB injection into the DMH. In addition to these brain areas, we observed fluorescent axon terminals in the paraventricular thalamic nucleus in 2 brains with a DiI crystal inserted into the SCN at P2. In 1 brain with a DiI crystal inserted into the SCN at P2, fluorescent axon terminals were detected in the medial septal and paratenial thalamic nuclei. We observed fluorescent axon terminals in the IGL and VLPO in 2 brains after DiI was placed in the SCN at P8. Finally, in 2 brains injected with DMH at P8, fluorescent axon terminals within the basal forebrain were revealed.

Table 1. Locations of cell bodies and axon terminals detected in all subjects after DiI crystal placed in the SCN or CTB injected into the DMH at P2 or P8.

Tracer	Brain Area	Age	Cell Bodies	Axon Terminals
DiI	SCN	P2	--	LS, MPA, vSPVZ
DiI	SCN	P8	vSPVZ, DMH	LS, MPA, vSPVZ, DMH, VMH, SON, LH
CTB	DMH	P2	vSPVZ, LC	vSPVZ, PT, VMH
CTB	DMH	P8	vSPVZ, LC, SCN	vSPVZ, PT, VMH, SCN, LC, DR, DTg, VTg, LH, VLPO, PVN

Abbreviations: LS: lateral septum, MPA: medial preoptic area, vSPVZ: ventral subparaventricular zone, DMH: dorsomedial hypothalamus, VMH: ventromedial hypothalamus, SON: supraoptic nucleus, LH: lateral hypothalamus, LC: locus coeruleus, PT: paratenial thalamus, DR: dorsal raphe, DTg: dorsal tegmental nucleus, VTg: ventral tegmental nucleus, VLPO: ventrolateral preoptic nucleus, PVN: paraventricular nucleus.

Discussion

Based on the results in Chapter 2, we hypothesized that neural pathways from the SCN emerge during the first postnatal week. Therefore, we traced the developing neural circuitry among the SCN, DMH, and LC using fluorescent dyes (e.g., DiI, CTB) placed within the SCN and DMH at P2 and P8. We predicted that at P2, very few connections would be revealed, whereas by P8, neural connectivity within the SCN-DMH-LC circuit would be fully developed.

At P2, after implantation of a DiI crystal into the SCN, the SCN does not send or receive neural projections to or from hypothalamic areas such as the DMH, LH, or VMH. The SCN does, however, send projections to the nearby vSPVZ at P2. In contrast, at P8, the SCN sends and receives projections to and from all of these areas, suggesting that neural circuitry among the SCN and other hypothalamic areas including the DMH develops between P2 and P8. These data support the notion that neural connectivity among the SCN and downstream structures does not develop until the end of the first postnatal week, as we suggest here in Chapter 2.

We hypothesized that the neural connectivity to and from the DMH would also develop by the end of the first postnatal week. To test this hypothesis, we injected fluorescent CTB into the DMH at P2 and P8. At P2, we found evidence that the LC projects to the DMH. At P8, DMH connectivity to and from both the SCN and LC was revealed.

Therefore, by P8, the SCN-DMH-LC circuit is sufficiently developed such that bidirectional communication exists among the SCN, DMH, and LC. In addition, as shown in Table 1, many of the efferent projections from the SCN at P8 (e.g., LS, MPO,

vSPVZ, DMH, VMH, SON, LH) are areas that have been reported to be neural targets of the SCN in adult rats, as described in Chapter 1 (Miller, Morin et al. 1996; Lydic and Baghdoyan 1999).

Some methodological concerns remain. In fixed tissue, DiI diffuses within cell membranes that are exposed to high concentrations of the dye (Honig and Hume 1989). This produces both anterograde and retrograde labeling of neurons, including axons, axon terminals, and cell bodies (Honig and Hume 1989; Honig and Hume 1989). It is important that the crystal is small enough so that it is only inserted into the nucleus of interest. The delineation of the exact area in which the crystal was placed is critical for the interpretation of our data. It has been previously shown that the dye is incorporated into the cell membranes that are only in very close proximity to the implanted crystal (Godement, Vanselow et al. 1987). In addition, labeled cell bodies or axon terminals have been shown to be connected to structures that are located at the DiI implantation site, rather than the non-specific fluorescence that may surround this site (Balthazart, Dupiereux et al. 1994). Here we examined the placement of the crystal, and verified that it was placed only within the SCN. Therefore, we suggest that the cell bodies and axon terminals that fluoresce connect directly to the SCN.

Similar methodological concerns apply to CTB injections into the DMH in vivo. Similar to DiI, CTB also reveals anterograde and retrograde projections to and from nuclei of interest (Luppi, Fort et al. 1990). Again, we took care to examine the size of the fluorescent injection to include only the DMH, and not nearby structures. In addition, we allowed the needle to rest in the brain after the injection, to ensure that the tracer did not diffuse into other brain areas as the syringe was lifted out of the brain. Therefore, we are

confident that the results presented here are representative of the developing neural connectivity among the SCN and DMH.

We show here that the neural connectivity among the SCN and downstream hypothalamic and brainstem structures develops over the first postnatal week. These data suggest that wake-related areas such as the LC may provide *functional* feedback to the SCN. This hypothesis is tested in the next chapter using a sleep deprivation paradigm.

CHAPTER 4

EFFECT OF SLEEP DEPRIVATION ON WAKE-PROMOTING STRUCTURES

Introduction

Is the SCN capable of receiving feedback about vigilance state from wake-related structures such as the LC? Todd et al. (2010) sleep deprived rats at P2 and used cFos to visualize brain structures involved in forced wakefulness. These authors showed that the LC and DMH are significantly more active in response to sleep deprivation at P2, but the SCN is not (Todd, Gibson et al. 2010). This suggests that at P2, the SCN does not receive feedback about wakefulness from the LC.

In Chapter 3, we showed that bidirectional communication among the SCN, DMH, and LC emerges between P2 and P8. In light of these results, we predicted that, in contrast with Todd et al. (2010), sleep deprivation would result in SCN activation at P8. To test this prediction, in Experiment 4, we sleep deprived pups at P8 and used cFos to visualize brain structures involved in forced wakefulness.

In addition, to determine whether the LC is necessary for SCN activation during sleep deprivation, we also tested pups after pretreatment with DSP-4, a neurotoxin that selectively destroys noradrenergic LC terminals in infant (Jonsson, Hallman et al. 1982) and adult (Fritschy and Grzanna 1989; Fritschy and Grzanna 1992; Cirelli and Tononi 2004; Cirelli, Huber et al. 2005) rats. We predicted that DSP-4 deprived pups would result in markedly reduced LC activity, resulting in reduced DMH and SCN neural activity. These experiments reveal the effect of sleep deprivation on the SCN-DMH-LC pathway.

Methods

Subjects

A total of 24 rats from 6 litters (n = 6 x 4 conditions) were used.

Procedure

At P3, 2 pups with visible milk bands were removed from the litter and injected with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4; 50 mg/kg) and the other 2 pups were injected with saline. DSP-4 is a neurotoxin that selectively destroys noradrenergic LC terminals in infant and adult rats (Jonsson, Hallman et al. 1981; Jonsson, Hallman et al. 1982; Fritschy and Grzanna 1989). Pups were placed back in the litter until testing at P8. At P8, pups were implanted with EMGs in the nuchal muscle under isoflurane anesthesia, as described in Experiment 1. Each pup was assigned to one of four experimental groups: DSP-4 deprived, DSP-4 non-deprived, sham deprived, and sham non-deprived. Each pup was placed in a supine position and lightly restrained with soft pipe cleaners placed over the abdomen. The pup was then transferred into the testing chamber maintained at thermoneutrality (35°C) and allowed 1 h to recover and acclimate, as described in Experiment 1. Testing always occurred at the same time of day, during the middle of the pup's subjective day at 1300 h.

Each test consisted of 2 consecutive 30-min periods: a baseline period and a deprivation period. During the baseline period, all pups were allowed to cycle between sleep and wakefulness while EMG data was recorded with no manipulation to the animal. During the deprivation period, animals were deprived of sleep by applying a cold, metal spatula to the snout (Todd, Gibson et al. 2010). The stimulus was only applied when the

subject was asleep, as indicated by nuchal atonia, behavioral quiescence, or myoclonic twitching (Karlsson, Gall et al. 2005). Spatulas were kept in a beaker containing ice water to keep them cold. Each stimulus application was recorded by hitting a keystroke so that each application was recorded simultaneously with its occurrence. Same-sex littermates were prepared identically except they were allowed to cycle undisturbed between sleep and wakefulness throughout the 2 30-min periods.

After the deprivation procedure, pups remained in the chamber for 90 min following deprivation to prepare them for cFos immunohistochemistry.

Animals were then removed from the chamber, killed with an overdose of Nembutal, and perfused transcardially with PBS followed by 4% PFA. Brains were removed and post-fixed overnight in 4% PFA before being transferred to 30% sucrose solution. 40 μm coronal sections were cut using a microtome and placed in wells with PBS.

Sections were rinsed with PBS, placed in normal goat serum for 1 h, rinsed again with PBS, and incubated in a primary antibody solution (1:2000, sc-7202, in .01 M PBS and 0.3% Triton X; Santa Cruz Biotechnology, Santa Cruz, CA). After 24 hours, sections were rinsed with PBS and incubated in biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 1 h in .01 M PBS and .3% Triton X. Sections were rinsed with PBS and placed in an avidin-biotin peroxidase complex (Vector Laboratories) for 1 h. The sections were again rinsed with PBS before being placed in a .02% diaminobenzidine solution with 30% hydrogen peroxide. The sections were placed in PBS to stop the reaction. Sections were mounted and coverslipped with DPX.

Data analysis

Nuchal EMG data were analyzed as described for Experiments 1 and 2. The number of stimulus presentations was quantified for all 6 5-min segments during the 30-min deprivation period. A Leica microscope (Leica Instruments, Wetzlar, Germany) and imaging system were used to visualize brain sections at 20x magnification. Images were imported into ImageJ (National Institutes of Health) and adjusted to binary values.

For each subject, Fos-ir positive cells were counted unilaterally within a range of sections for each area from each subject. The number of cells within each section was divided by the surface area of a counting box to provide an estimate of the number of active cells per mm^2. The mean number of cells for each brain area was then calculated by summing the total number of active cells per mm^2 and dividing it by the total number of sections for each brain area. Brain nuclei were identified using a stereotaxic atlas of the rat brain (Paxinos and Watson 1998).

The LC, DMH, and SCN were selected for quantification based upon the known connectivity among these areas in adult rats (Aston-Jones, Chen et al. 2001) and P8 rats (see Chapter 3). The barrel cortex was selected for quantification because it receives somatosensory inputs from the whisker pad (Petersen 2007); therefore, this area was used as a positive control of our Fos-ir method's sensitivity for detecting whisker stimuation. The following wake-related brain areas and nuclei (Pompeiano, Cirelli et al. 1995; Ko, Estabrooke et al. 2003; Deurveilher and Semba 2005; Arrigoni, Mochizuki et al. 2009; Tsujino and Sakurai 2009; Hsieh, Gvilia et al. 2011) were selected for quantification: laterodorsal tegmentum (LDT), lateral hypothalamus (LH), basal forebrain (BF), ventral tegmental nucleus (VTg), dorsal tegmental nucleus (DTg), medial preoptic area (MPA),

perifornical area (PeF), and tuberomammillary nucleus (TMN). The following sleep-related brain areas and nuclei (Adell, Celada et al. 2002; Gall, Poremba et al. 2007; Hsieh, Gvilia et al. 2011) were selected for quantification: nucleus pontis oralis (PO), median preoptic nucleus (MnPO), ventrolateral preoptic nucleus (VLPO), dorsal raphe (DR), and median raphe (MR). Finally, c-fos activity within the ventral subparventricular zone (vSPVZ)—shown to be important for circadian rhythms of sleep and wakefulness (Schwartz, Nunez et al. 2009)—was quantified.

ANOVA was used to analyze group differences for each area sampled. Fisher's PLSD was used as a post hoc test. Alpha was set at .05 and a Bonferroni correction procedure was used to adjust alpha for multiple comparisons.

Results

Figure 10A (left column) presents cFos immunohistochemistry in deprived vs. non-deprived pups at P2 (data previously published) (Todd, Gibson et al. 2010). As reported previously, P2 pups express significantly more LC and DMH cFos activity in deprived pups as compared to non-deprived pups. Importantly, at this age, the SCN did not express differences in cFos activity in deprived pups as compared to non-deprived pups. Figure 10A (right column) presents cFos immunohistochemistry in deprived vs. non-deprived pups at P8 treated with DSP-4 or saline. Sham deprived pups expressed significantly more cFos activity in the LC, DMH, and SCN as compared to all other conditions. ANOVA revealed a significant main effect of group (i.e., sham vs. DSP-4) (Fs$_{1,20}$ > 23.5, Ps < .0001), a significant main effect of condition (i.e., deprived vs. non-deprived) (Fs$_{1,20}$ > 11.2, Ps < .005), and a significant group x condition interaction (Fs$_{1,20}$

> 19.9, Ps < .0005). Importantly, increased SCN cFos activity in sham deprived pups was shown to develop between P2 and P8. This increased SCN activity is directly attributable to increased LC activity, since DSP-4 was effective in blocking feedback from the LC to the SCN.

Representative photos of the LC, DMH, and SCN of sham deprived and sham non-deprived pups at P8 are presented in Figure 10B. The photographs show that LC, DMH, and SCN cFos activity is significantly heightened in sham deprived pups as compared to sham non-deprived pups.

Table 2 presents the mean number of Fos-ir positive cells per mm^2 in Sham Deprived, Sham Non-Deprived, DSP-4 Deprived, and DSP-4 Non-Deprived pups at P8. Sleep deprivation resulted in heightened activity within other wake related areas, such as the LDT, VTg, TMN, PeF, MPA, and BF. Sleep related areas (e.g., PnO, VLPO) did not express any significant differences in cFos activity in response to sleep deprivation. Barrel cortex expressed heightened cFos activity in Sham Deprived pups, indicating that the stimulation acted through the trigeminal system, as we have shown previously at P2 (Todd, Gibson et al. 2010).

Figure 11 presents the number of stimulations presented to the pup in order to maintain wakefulness in DSP-4 treated and sham pups. Similar to previous results in P2s and P8s (Todd, Gibson et al. 2010), significantly more stimulations were required to maintain wakefulness at the end of the deprivation period as compared to the beginning in shams. This was not the case, however, for DSP-4 treated pups. No significant

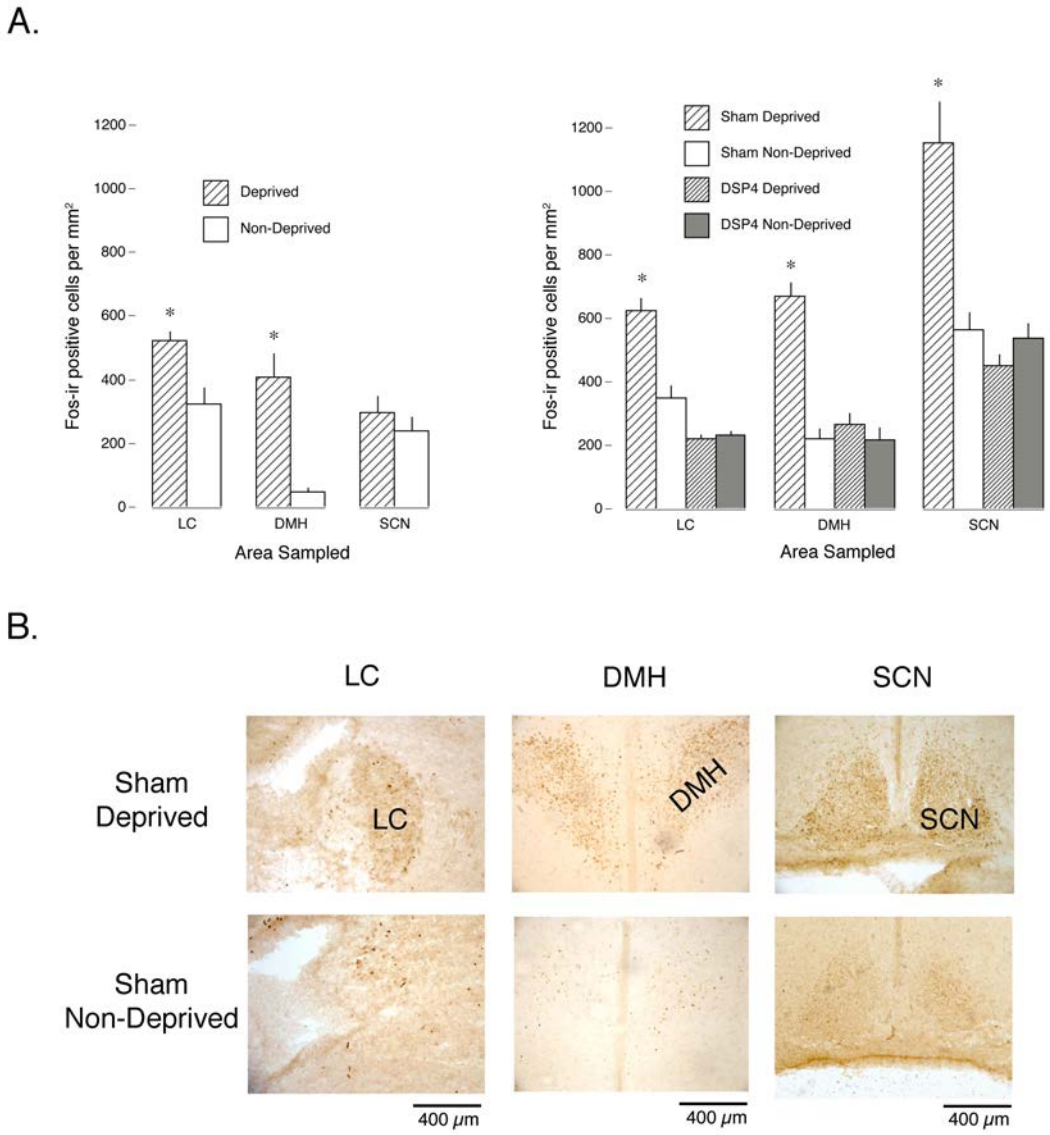

Figure 10. The effect of sleep deprivation at P2 and P8 on the SCN-DMH-LC wake-promoting circuit. (A) Mean number of Fos-ir positive cells per mm^2 for each area sampled in sleep deprived vs. non-deprived P2 rats (left panel; data previously published). Mean number of Fos-ir positive cells per mm^2 for each area sampled in P8 rats is presented in the right panel. At P8, 4 groups of pups were tested, including sham deprived, sham non-deprived, DSP4 deprived, and DSP4 non-deprived. *Significant difference from all other groups. Means + SE. (B) Photographs of the LC, DMH, and SCN in sham deprived (top row) and sham non-deprived (bottom row) pups at P8. Abbreviations: LC: locus coeruleus; DMH: dorsomedial hypothalamus; SCN: suprachiasmatic nucleus.

Table 2. Mean number of Fos-ir positive cells per mm^2 in Sham Deprived, Sham Non-Deprived, DSP-4 Deprived, and DSP-4 Non-Deprived pups at P8.

	Sham Deprived	Sham Non-Deprived	DSP-4 Deprived	DSP-4 Non-Deprived
LC	628.4 (36.8)*	351.8 (36.0)	221.5 (12.4)	235.1 (12.2)
DMH	670.2 (44.2)*	221.9 (32.5)	268.1 (34.1)	219.8 (39.1)
SCN	1154.1 (129.5)*	567.9 (50.2)	454.8 (32.6)	539.3 (46.5)
Barrel Cortex	609.9 (84.3)*	177.5 (16.9)	443.4 (32.5)	124.6 (21.0)
LDT	463.9 (33.3)†	172.1 (29.5)	369.7 (76.1)†	160.9 (15.6)
LH	227.2 (27.7)	158.9 (48.2)	210.5 (19.3)	147.7 (21.4)
BF	330.3 (35.7)†	215.5 (20.5)	331.1 (34.8)†	195.8 (27.2)
VTg	390.5 (29.9)*	173.5 (38.4)	260.5 (30.9)	191.1 (18.3)
DTg	281.0 (29.0)	298.7 (31.0)	279.9 (59.8)	202.2 (25.3)
MPA	358.1 (21.0)*	184.6 (34.9)	151.7 (24.4)	186.2 (19.7)
PeF	257.8 (52.6)*	154.1 (42.6)	128.2 (15.4)	122.6 (12.0)
TMN	425.5 (40.4)*	239.9 (17.7)	215.5 (16.2)	162.6 (4.8)
PO	103.5 (24.2)	91.4 (11.4)	116.7 (17.3)	106.2 (11.9)
MnPO	199.5 (40.2)	142.9 (22.3)	165.7 (25.8)	112.1 (22.6)
VLPO	153.8 (20.3)	156.6 (16.1)	130.2 (14.2)	150.1 (28.9)
DR	215.9 (39.8)	164.1 (35.2)	173.7 (25.2)	136.6 (10.2)
MR	273.1 (33.3)	229.2 (30.5)	262.0 (41.4)	186.5 (23.4)
vSPVZ	243.7 (18.9)	161.3 (25.5)	190.9 (23.3)	136.2 (5.5)

SE is presented in parentheses.

* Significantly different from all groups.

† Significantly different from Sham Non-Deprived and DSP-4 Non-Deprived.

Abbreviations: LC: locus coeruleus, DMH: dorsomedial hypothalamus, SCN: suprachiasmatic nucleus, LDT: laterodorsal tegmental nucleus, BF: basal forebrain, PeF: perifornical nucleus, TMN: tuberomammillary nucleus, VTg: ventral tegmental nucleus, MPA: medial preoptic area, vSPVZ: ventral subparaventricular zone, LH: lateral hypothalamus, VLPO: ventrolateral preoptic nucleus, MnPO: median preoptic nucleus, PO: pontis oralis, DR: dorsal raphe, MR: median raphe, DTg: dorsal tegmental nucleus.

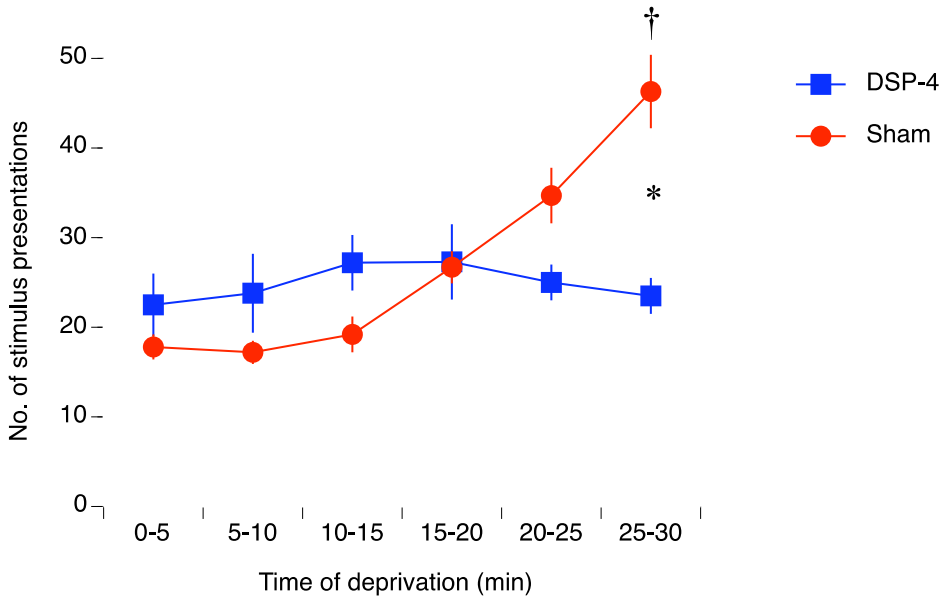

Figure 11. Number of presentations of cold spatula to DSP-4 vs. sham treated pups from the beginning of the experiment (0-5 minutes) to the end (25-30 minutes). Shams express significant sleep pressure from the beginning of the experiment as compared to the end. DSP-4 treated pups do not express significant sleep pressure. * Significant difference between groups. † Significant difference from beginning of the deprivation period to the end.

differences in number of stimulation presentations was found in DSP-4 pups between the beginning of the deprivation period to the end. In addition, significant differences between sham and DSP-4 treated pups were found in the last five minutes of the deprivation period. ANOVA revealed a significant effect of presentation epoch (e.g., 0-5 minutes) ($F_{1,60} = 6.9$, $P < .0001$) and a significant epoch x group interaction ($F_{1,60} = 7.1$, $P < .0001$), but not a significant group effect ($F_{1,60} = 3.9$).

Discussion

In Chapter 3, we showed that at P2, the LC projects to the DMH, but the DMH does not yet project to the SCN. As described previously, P2 rats expressed heightened LC and DMH activity in response to sleep deprivation, but the SCN did not (Todd, Gibson et al. 2010). Therefore, these results provide functional evidence that the LC sends projections to the DMH at P2, but the DMH does not send feedback to the SCN.

We have evidence from Chapter 3 that the SCN receives neural feedback from the LC via the DMH at P8. Therefore, we sleep deprived sham pups and DSP-4 injected pups at P8 and performed cFos immunohistochemistry to determine the functional role of the SCN in receiving feedback from the LC. We predicted that sleep deprivation in sham pups at P8 would lead to heightened cFos expression in the LC, DMH, and SCN. We also predicted that DSP-4 would result in reduced LC activity to thereby block SCN feedback.

Here we found that at P8, the LC and DMH exhibited significantly heightened cFos expression in response to sleep deprivation, similar to P2 animals (Todd, Gibson et al. 2010). Critically, the SCN also exhibited significantly increased cFos expression in

sham deprived pups at P8, thereby providing functional support for our observation of increased connectivity between the SCN and brainstem by P8.

Pups treated with DSP-4 exhibited significantly reduced cFos expression in the LC in response to sleep deprivation, which resulted in diminished cFos activity in both the DMH and SCN. These results indicate that DSP-4 treatment was effective in reducing overall activity within the LC, most likely via retrograde degeneration, as others have reported (Fritschy and Grzanna 1991).

As described in Chapter 1, the idea that circadian and homeostatic systems interact has been recently examined using electrophysiological methods. Electrical activity in the SCN has been recorded in vivo while monitoring sleep and wakefulness (Deboer, Vansteensel et al. 2003) to explore whether or not the SCN receives feedback about sleep and wake states in real time. Neuronal activity of the SCN was highly correlated with sleep and wake states of the animal. As expected, the electrical activity of the SCN showed a daily rhythm, with higher activity found during the day. Interestingly, regardless of time of day, SCN neurons fired at lower rates during quiet sleep (QS), and higher rates during active sleep (AS) and wakefulness. Therefore, information about vigilance state was transmitted to the SCN, possibly through brainstem nuclei involved in sleep and wakefulness.

Also as discussed in Chapter 1, converging evidence suggests that there is crosstalk between circadian and homeostatic processes. There is evidence in the literature that sleep deprivation can lead to changes in SCN activity. In one study, 6-hour sleep deprivations led to SCN neuronal activity which was approximately 60% less than baseline levels (Deboer, Detari et al. 2007). Although these results were in the opposite

direction as ours, these authors concluded that sleep deprivation influences sleep homeostatic mechanisms and SCN circadian amplitude—similar to the conclusions we reach here in our experiment. The method of deprivation that we used is an acute sleep deprivation paradigm, rather than long-term. Therefore, the effect of short-term deprivation on SCN activity is likely to differ mechanistically. Importantly, our results, indicating that the SCN receives functional feedback from the LC regarding vigilance state, also challenge the notion that the sleep homeostat and circadian clock are independent.

The LC may also play an important role in sleep homeostasis. As described in Chapter 1, sleep homeostasis (process "S") builds up during waking and decreases during sleep, causing the need for sleep to be dependent upon prior amounts of wakefulness (Borbely and Achermann 1999; Borbely and Achermann 2000). Traditionally, there are two dimensions that define sleep homeostasis: sleep deprivation leads to an increased need to fall asleep during the deprivation period (i.e., sleep pressure) and following sleep deprivation, a compensatory response is elicited, resulting in an increase in the amount of sleep (i.e., sleep rebound). We found evidence of a lack of sleep pressure in pups treated with DSP-4. This would suggest that the LC, or downstream structures from the LC, is critical for the maintenance of sleep pressure.

The LC maintains arousal, at least in part, by sending inhibitory outputs to GABAergic neurons in the VLPO, a sleep-promoting nucleus (Samuels and Szabadi 2008). However, recent results have shown that sleep pressure persists in P2 pups that are transected (Todd, Gibson et al. 2010). In other words, the brainstem alone is sufficient to produce sleep pressure. Therefore, we would expect that connections between the LC and

other brainstem structures, rather than connections to the VLPO, are responsible for sleep pressure.

The LC provides noradrenaline to many brainstem regions, including the wake-promoting dorsal raphe (DR) nucleus, and sleep- and wake-promoting pedunculopontine (PPT) and laterodorsal tegmental (LDT) nuclei (Samuels and Szabadi 2008). In our experiment, sleep deprivation led to heightened LDT activity, but not DR activity. The neurons of PPT and LDT nuclei are active during wakefulness and REM sleep, similar to neurons of the SCN (Kayama, Ohta et al. 1992; Kaur, Saxena et al. 2001; Deboer, Vansteensel et al. 2003; Jones 2005). In infant rats, LDT/PPT neurons are state-dependent with wakefulness and active sleep (Karlsson, Gall et al. 2005). These results suggest the possibility that neurons of the LDT/PPT, via direct connectivity with the LC, are involved in sleep pressure. This topic has not yet been explored, but would be interesting to focus on to better understand the role of brainstem nuclei on sleep pressure.

Altogether, our results indicate that the SCN receives functional feedback from the LC about wakefulness. But what is the functional significance of having an intact SCN-DMH-LC circuit on the consolidation of wakefulness and circadian rhythms of sleep and wakefulness? In the next two chapters, we test the hypothesis that this circuit is critical for maintaining arousal and nocturnality in developing rats.

CHAPTER 5

DEVELOPMENTAL EMERGENCE OF ULTRADIAN POWER-LAW WAKE BEHAVIOR DEPENDS UPON THE FUNCTIONAL INTEGRITY OF THE LOCUS COERULEUS

Introduction

The functional significance of having an intact SCN-DMH-LC bidirectional pathway has not yet been explored using sensitive measures of sleep and wakefulness. Muscle activity using EMGs allow for the examination of bout durations on a second-by-second basis, rather relying on epochs, to elucidate potential developmental changes in circadian rhythmicity and bout distributions of sleep and wakefulness.

As described in Chapter 1, sleep bout durations distribute exponentially in infant (Seelke and Blumberg 2005) and adult (Lo, Amaral et al. 2002; Lo, Chou et al. 2004) mammals. In contrast, wake bout durations distribute exponentially during the early postnatal period and then transition to a power-law distribution that, in rats, occurs around the time of eye opening at P15 (Blumberg, Seelke et al. 2005). What factors contribute to the developmental emergence of power-law wake behavior?

First, because the transition occurs around the time of eye opening, it is possible that some aspect of visual stimulation is involved. However, bilateral enucleation at P3 or P11 does not prevent the normal emergence of power-law wake behavior (Gall, Todd et al. 2008). Second, it is possible that orexinergic neurons, localized in the LH and DMH, contribute to the emergence of power-law wake behavior by activating wake-active monoaminergic and cholinergic neurons in the brainstem (Peyron, Tighe et al. 1998;

Nambu, Sakurai et al. 1999; Sakurai 2007). However, power-law wake behavior emerges in orexin knockout mice just as it does in wild-types (Blumberg, Coleman et al. 2007). A third possibility, examined here, is that functional changes in LC activity contribute to the development of power-law wake behavior.

In adult rats, activity levels of LC neurons increase during wakefulness, decrease their firing rate during quiet sleep, and are nearly silent during active sleep (Aston-Jones and Bloom 1981; Berridge and Waterhouse 2003). State-related changes in LC activity are detectable as early as P8 in rats (Karlsson, Gall et al. 2005). Increased discharge rates of LC neurons are associated with spontaneous or sensory-evoked interruptions of sleep (Foote, Aston-Jones et al. 1980; Aston-Jones and Bloom 1981). Finally, stimulation of LC activity promotes wakefulness (Frederickson and Hobson 1970; Kaitin, Bliwise et al. 1986), whereas inhibition of LC activity promotes sleep (Cespuglio, Gomez et al. 1982).

Here, in Experiment 5, to test the hypothesis that the LC contributes to the developmental emergence of power-law wake behavior, we administered DSP-4 to P7 rats and we examined their sleep-wake behavior 2 weeks later, at P21. We predicted that interfering with LC function in early infancy would prevent or dampen the developmental transition from exponential to power-law wake behavior.

Methods

Subjects

A total of 32 Sprague-Dawley rats from 8 litters were used (n = 8 x 2 test times x 2 conditions).

DSP-4 Treatment

At P7, four same-sex littermates were chosen as experimental subjects. Two of these pups were injected subcutaneously with 50 mg/kg of DSP-4 (Sigma, St. Louis, MO), and 2 control pups were injected with saline. Pups were then tattooed and placed back in the litter for testing 2 weeks later. The same procedure was used for the remaining 7 litters.

Surgery

On the day of testing at P21-22 (hereafter referred to as P21), two same-sex littermates, one previously injected with DSP-4 and one with saline, were implanted with nuchal EMG electrodes. Under isoflurane anesthesia, two bipolar stainless steel electrodes (50 μm diameter, California Fine Wire, Grover Beach, CA) were inserted bilaterally into the nuchal muscles and secured with flexible collodion (Seelke, Karlsson et al. 2005). After surgery, pups recovered in a humidified test chamber maintained at thermoneutrality. All surgeries took place at least 2 h before testing. For testing at night, surgery was performed under dim red light illumination, with care being taken to ensure that infants were not exposed to white light. After surgery, each pup was transferred to an individual test chamber maintained at thermoneutrality (i.e., 32˚C) where it recovered and acclimated for at least 2 h before testing.

Test Procedure

DSP-4 and saline control subjects were tested simultaneously but separately in two electrically shielded double-walled glass chambers (height, 18 cm; i.d., 12 cm). Air

temperature inside each chamber was maintained at thermoneutrality by regulating the temperature of the water that circulated through the walls of the chamber. Compressed, humidified air passed through the sealed chamber at the rate of 300 ml/min. A round platform constructed of polyethylene mesh was fitted inside each chamber, which allowed the pup to move freely on the platform's surface.

The nuchal electrodes were connected to differential amplifiers (A-M systems, Carlsborg, WA) and their signals were amplified (x10k) and filtered (300-5000 Hz). EMG data were acquired (1000 samples/s) and stored using a data acquisition system (Biopac Systems, Santa Barbara, CA).

During the day, data were acquired from 1100 to 1600 h. At night, the 2 remaining same-sex littermates were implanted with EMG electrodes (again, under red light illumination) and tested identically to those during the day, with data being acquired from 2300 to 0400 h. The order of daytime and nighttime tests was counterbalanced across litters.

HPLC Analysis of Noradrenaline (NA), Dopamine (DA), and Serotonin (5-HT) Concentrations

No more than 2 days after testing, each pup was anesthetized with isoflurane and decapitated. The brain was removed rapidly and placed on ice. The cerebral cortex was quickly dissected out, placed in 15 ml Sarstedt polypropylene tubes, and frozen in liquid nitrogen. Samples were stored at -80˚C until homogenization. The cerebral cortex of each rat was homogenized in 0.1 Normal (N) perchloric acid. Homogenate was centrifuged at 17,300 g for 10 min at 4˚C. The clear supernatant was filtered through a 0.22 µm Millex-

GP filter unit (Millipore, Bedford, MA) and analyzed for levels of NA. Analysis was performed using a Waters 2690 HPLC (Milford, MA) equipped with an ESA Coulochem III (Chemford, MA) with E1 and E2 potentials set to -150 mV and 200 mV, respectively. Eight µl of sample diluted 1:4 with water were injected into a Synergi Hydro 4 µm reversed phase column (Phenomenex, Torrance, CA) with a mobile phase containing 75 mM phosphoric acid, 25 mM citric acid, 1.8 mM sodium dodecyl sulfate, and 2% acetonitrile adjusted to pH 3.0.

A follow-up experiment was performed using 12 additional subjects from 6 litters. From each litter, 2 P7 littermates were injected with DSP-4 or saline as described earlier. At P21, the brain was removed, placed on ice, and the cortex was dissected out for analysis. In addition, the remaining brain tissue (including medulla, midbrain, and diencephalon, minus the cerebellum) was also saved. These cortical and non-cortical samples were prepared for HPLC analysis of NA, DA, and 5-HT concentrations. (These animals were not tested for sleep and wakefulness.) Samples were analyzed using an Agilent 1100 HPLC system (Santa Clara, CA) and an ESA Coulochem III electrochemical detector (settings as previously described). Separation was achieved by injecting 20 µl of undiluted sample onto a Thermo Aquasil C18 5 µm 150 x 2.1 mm column. DA and 5-HT were analyzed by an isocratic method using mobile phase containing 0.1% trifluoroacetic acid (TFA), 50mM citric acid, 1% methanol, and 1% acetonitrile adjusted to pH 3.0. NA analysis was carried out using a gradient program; the aqueous phase contained 0.2% trifluoroacetic acid, 50mM citric acid, and 2 mM sodium heptane sulfonate pH 3.0, and the organic phase contained acetonitrile 3-18% (3% at 0-8 min, linearly increasing to 18% by 19 min, and returning to 3% at 20 min).

Data Analysis

For all subjects, EMG data were analyzed off-line using AcqKnowledge software (Biopac Systems, Santa Barbara, CA). The EMG signal was integrated and full-wave rectified, and then dichotomized into periods of high muscle tone and atonia (or wakefulness and sleep, respectively), as described earlier (Karlsson, Kreider et al. 2004). An experienced individual blind to experimental condition scored the data. It should be stressed that EMG measures alone allow for accurate measurement of sleep and wake durations in infant rats before, during, and after the emergence of delta activity (Seelke and Blumberg 2008). Moreover, the statistical structures of sleep and wake bouts derived from EMG measures at P21 in rats (Blumberg, Seelke et al. 2005) and mice (Blumberg, Coleman et al. 2007) resemble those derived in adults using additional electrographic measures (Lo, Chou et al. 2004).

Sleep and wake bout durations were imported into Statview 5.0 (SAS Institute, Cary, NC) for analysis. Mean and median bout durations were determined for each subject and mean values were calculated across subjects. Mean percentage of time awake was calculated by dividing the mean wake bout duration by the sum of the mean sleep and wake bout durations, and multiplying by 100. The mean number of sleep-wake cycles per hour was also calculated. Two-factor analysis of variance (ANOVA) was used to test for differences across groups (i.e., DSP-4 vs. saline control) and test times (i.e., day vs. night).

NA, DA, and 5-HT concentrations were expressed as ng/ml tissue homogenate and reported as percentage change in DSP-4 subjects in relation to paired saline controls. Paired t tests were used to test group differences.

As described elsewhere (Blumberg, Seelke et al. 2005), log-survivor distributions of sleep and wake bouts were produced from individual and pooled data. Least-squares estimates (r^2) were calculated to assess the degree of fit to exponential and power-law distributions. Two-factor ANOVAs, performed separately for the day and night, were used to test for group differences (i.e., DSP-4 vs. saline control) and distribution (i.e., power-law vs. exponential). Planned comparisons (e.g., paired *t* tests) were used to assess within-group differences in r^2. As described in Chapter 2, we used Akaike weights to test whether bout durations better fit a power-law or exponential model.

For all tests, alpha was set at 0.05. Means are presented with their standard errors.

Results

Table 3 shows that mean sleep bout durations were not significantly affected by DSP-4 treatment during the day or night (group: $F_{1,28} = 0.7$; test time: $F_{1,28} = 1.5$; group x time interaction: $F_{1,28} = 0.0$); median sleep bout durations were significantly longer during the day ($F_{1,28} = 6.9$); there was no main effect of group ($F_{1,28} = 3.2$) and no group x time interaction ($F_{1,28} = 0.2$). Both mean and median wake bout durations were significantly longer in DSP-4 subjects ($F_{1,28}$s > 7.3, $p < 0.05$); there were no main effects of time ($F_{1,28}$s < 3.6) and no group x time interactions ($F_{1,28}$s < 1.0). Similarly, mean percentage time awake was significantly longer for DSP-4 subjects than saline controls ($F_{1,28} = 11.8$, $p < 0.005$), and ANOVA also revealed that both groups had significantly longer wake bout durations at night ($F_{1,28} = 7.0$, $p < 0.05$); the interaction was not significant ($F_{1,28} = 0.0$). Finally, the number of sleep-wake cycles per hour was not affected by DSP-4 treatment (group: $F_{1,28} = 0.2$; time: $F_{1,28} = 0.0$; interaction: $F_{1,28} = 0.1$).

Table 3. Sleep and wake bout durations, percentage of time awake, and number of sleep-wake cycles per hour in P21 rats treated on P7 with DSP-4 or saline.

Group	Sleep bout duration (s)		Wake bout duration (s)		Mean % of time awake	Mean no. of sleep-wake cycles per h
	Mean	Median	Mean	Median		
DSP-4						
Day	82.5 (8.2)	53.6 (7.4) [a]	40.9 (5.6) [b]	17.3 (2.7) [b]	33.3 (4.3) [a,b]	30.4 (2.4)
Night	73.4 (8.6)	32.9 (6.2)	55.2 (10.7) [b]	25.5 (7.2) [b]	41.4 (4.1) [b]	31.6 (4.5)
Saline						
Day	89.2 (7.0)	62.3 (6.9) [a]	25.4 (3.2)	6.5 (0.7)	21.9 (1.6) [a]	33.2 (3.8)
Night	79.8 (6.0)	48.0 (6.3)	35.7 (3.3)	7.1 (0.5)	30.8 (1.9)	32.1 (2.3)

Standard errors are in parentheses.

[a] significant main effect of test time (i.e., day vs. night)

[b] significant main effect of group (i.e., DSP-4 vs. saline)

Figure 12 presents pooled log-survivor distributions for P21 subjects treated at P7 with DSP-4 or saline. Sleep bout durations for both the DSP-4 and saline control P21 subjects during the day and night fall along a straight line, indicative of an exponential distribution (Figure 12A). As predicted, wake bout durations for DSP-4 subjects more closely follow an exponential distribution during the day and night (Figure 12B); in contrast, the wake bout durations for the saline control subjects follow a power-law distribution.

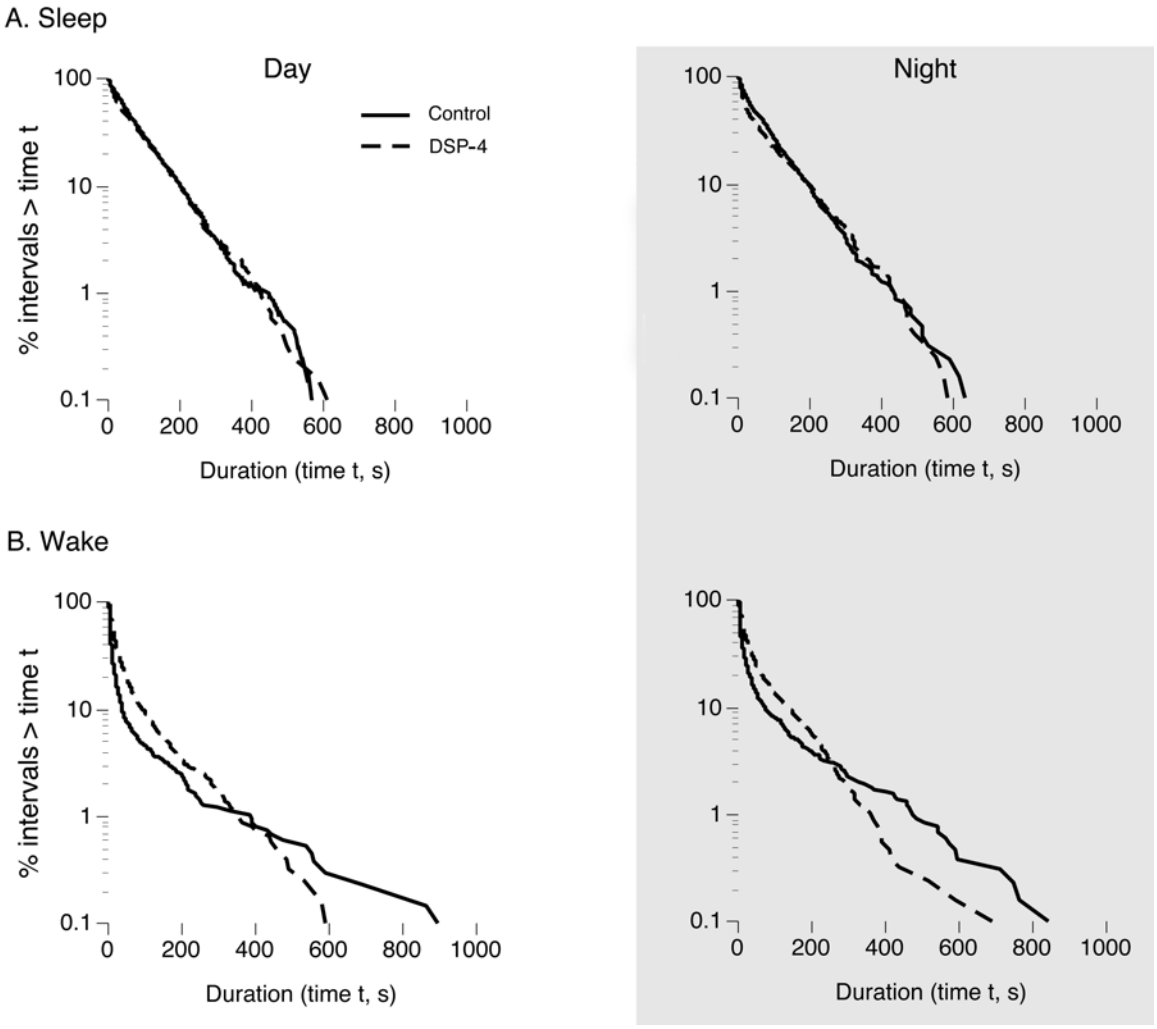

Figure 12. Log-survivor plots of sleep and wake bout durations in P21 rats treated with DSP-4 or saline. Each semi-log plot was constructed using pooled data (677-1180 data points). Straight lines on these plots indicate that the data follow an exponential distribution.

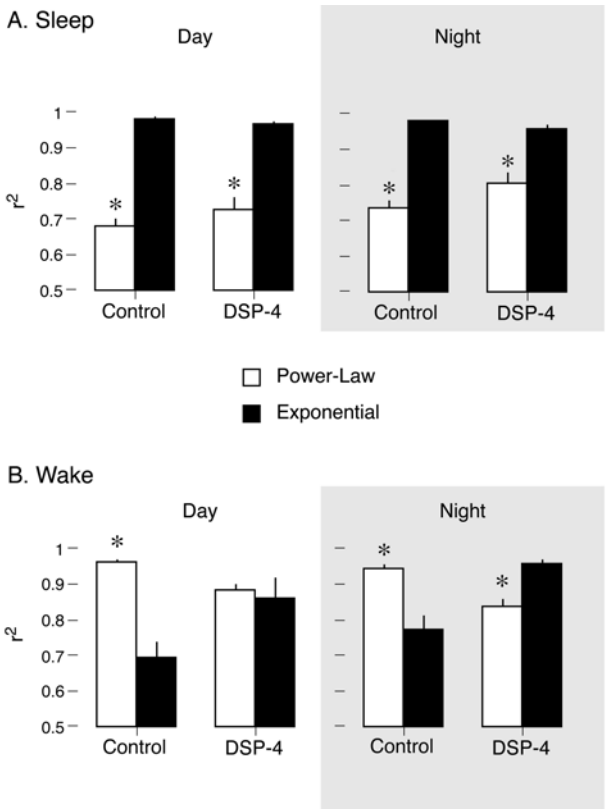

Figure 13. Values of r^2 using regression analysis of sleep and wake bout durations in DSP-4 and saline control P21 subjects. Sleep bout durations are better fit by exponential distributions during the day and night in subjects treated with DSP-4 or saline. Wake bout durations are better fit by power-law distributions in saline control subjects during the day and night. In contrast, wake bout durations in DSP-4 subjects are fit better by neither a power-law nor exponential distribution during the day, but are better fit by an exponential distribution at night. * Significant difference between exponential and power-law fits.

As shown in Figure 13A, sleep bout durations were always better fit by an exponential than a power-law distribution. ANOVAs performed separately for the day and night revealed significant main effects of distribution ($F_{1,28}$s > 139.5, ps < 0.0001), but there were no main effects of group ($F_{1,28}$s < 2.1). Also, the group x distribution interaction was significant at night ($F_{1,28}$ = 6.8, p < 0.05) but not during the day ($F_{1,28}$ = 2.6).

Figure 13B shows, as expected (Blumberg, Seelke et al. 2005), that wake bout durations for saline control subjects during the day and night were better fit by a power-law distribution. In contrast, for DSP-4 subjects at night, the better fit was to an exponential distribution. For DSP-4 subjects during the day, the fit to a power-law distribution was clearly reduced, although the fit to an exponential distribution was not increased. ANOVAs revealed a significant main effect of distribution during the day ($F_{1,28}$ = 16.4, p < 0.0005) but not at night ($F_{1,28}$ = 1.4). There were no main effects of group during the day or night ($F_{1,28}$s < 3.2), but the group x distribution interactions were significant ($F_{1,28}$s > 11.6, ps < 0.005).

As expected, analyses of pooled and individual Akaike weights indicated that sleep bout durations were more likely to fit an exponential model in both control and DSP-4 subjects during the day and night (Akaike weights equal to 1). Also as expected, wake bout durations for control subjects were more likely to fit a power-law model during both the day and night (Akaike weights equal to 1). In contrast, wake bout durations for DSP-4 subjects were more likely to fit an exponential model during both the day and night (Akaike weights equal to 1).

A follow-up analysis of individual data indicated that two DSP-4 subjects exhibited differences from the pooled results. Specifically, one DSP-4 subject's wake bout durations were more likely to fit a power-law model (Akaike weight equal to 1), and the other DSP-4 subject's wake bout durations distributed poorly to both exponential and power-law models.

Finally, to provide additional information regarding the effect of DSP-4 treatment on NA, DA, and 5-HT concentrations in cortical and non-cortical tissue, 12 additional subjects from 6 litters were tested identically to the initial set of subjects (with the exception that sleep-wake activity was not monitored). As shown in Table 4, animals treated with DSP-4 at P7 exhibited significant reductions at P21 in both cortical (t_{21} = 19.2, $p < 0.0001$) and non-cortical ($t_5 = 9.5$, $p < 0.0005$) NA concentration in relation to saline controls. Importantly, and as expected (Jonsson, Hallman et al. 1982), DSP-4 subjects did not exhibit significant changes in cortical or non-cortical DA or 5-HT concentration in relation to saline controls (t_5s > 0.1, *NS*).

Table 4. Mean percentage change in cortical and non-cortical noradrenaline (NA), dopamine (DA), and serotonin (5-HT) in P21 rats treated on P7 with DSP-4 in relation to saline controls.

	NA	DA	5-HT
Cortical tissue	-80.7 (4.2)*	-13.3 (23.0)	+4.5 (16.0)
Non-cortical tissue (excluding cerebellum)	-73.9 (7.8)*	-4.7 (37.1)	+23.6 (25.6)

Standard errors are in parentheses.

* significant difference from 0%

Discussion

As stated previously, the locus coeruleus is a major source of noradrenaline in the central nervous system that mediates spontaneous and evoked arousals (Foote, Aston-Jones et al. 1980; Aston-Jones and Bloom 1981). Here we used the neurotoxin DSP-4 to destroy NA terminals from the LC in order to assess the contributions of this structure to sleep and wake bout durations in P21 rats. We found no significant effect of DSP-4 on sleep bout durations, but wake bout durations were significantly increased, as was the percentage of time awake.

As expected, sleep bout durations uniformly followed exponential distributions that were unaffected by treatment with DSP-4. In contrast, whereas wake bout durations in saline control subjects were better fit by a power-law distribution, wake bout durations of DSP-4 subjects were better fit by an exponential distribution, as is the case in untreated younger rats (Blumberg, Seelke et al. 2005; Gall, Todd et al. 2008). These conclusions were supported using two analytic procedures: least-squares (r^2) and maximum likelihood estimation (Akaike 1974; Burnham and Anderson 2002; Myung 2003). Thus, it appears that developmental changes in LC function contribute to the development of power-law wake behavior during the third postnatal week.

Previous studies have examined the role of DSP-4 on sleep and wakefulness in adults using conventional measures (e.g., mean, median, total sleep and wake durations); these studies have yielded variable and inconsistent results (Monti, D'Angelo et al. 1988; Gonzalez, Debilly et al. 1998; Cirelli, Huber et al. 2005; Gonzalez and Aston-Jones 2006; Saponjic, Radulovacki et al. 2007). In these studies, sleep and wake bout distributions were not analyzed, thus making it difficult to make direct comparisons between the

results found in these earlier studies and those found here. Importantly, the current study is unique in that DSP-4 was injected in early infancy—in particular, *before* the development of power-law wake behavior. Thus, the timing of DSP-4 administration may critically determine its effects on wake bout distributions. It is also possible that analyses of sleep and wake bout distributions, rather than mere assessments of cumulative sleep and wake durations, provide a more reliable and sensitive indicator of sleep-wake dynamics and their underlying neural control (Blumberg, Seelke et al. 2005; Blumberg, Karlsson et al. 2007).

As discussed in Chapter 1, it has been suggested that arousal is maintained, at least in part, by an SCN-DMH-LC wake-active circuit (Aston-Jones, Chen et al. 2001). Orexinergic neurons within the DMH have been hypothesized to be critically involved in the maintenance of arousal (Aston-Jones, Chen et al. 2001) and provide dense projections to the LC (Peyron, Tighe et al. 1998). However, as stated earlier, power-law wake behavior develops normally in orexin knockout mice (Blumberg, Coleman et al. 2007) and is expressed in adult mice (Diniz Behn, Kopell et al. 2008). Thus, to the extent that the SCN-DMH-LC circuit is critically involved in arousal, it is possible that *non*-orexinergic afferents to the LC, possibly from the DMH or elsewhere (Aston-Jones, Chen et al. 2001), contribute to the development of power-law wake behavior in infant rats and mice. In addition, because the LC exerts inhibitory influences on the sleep-promoting VLPO (Gallopin, Fort et al. 2000), it is possible that DSP-4 diminished this inhibitory influence, thereby altering the reciprocal interactions among LC, VLPO, and other nuclei involved in the regulation of sleep and wakefulness (Saper, Scammell et al. 2005).

The effects of DSP-4 treatment on NA in specific brain areas depend upon the age at which DSP-4 is administered and the interval between DSP-4 administration and NA measurement (Jonsson, Hallman et al. 1982). Using similar methods to those used here, DSP-4 treatment (50 mg/kg) in week-old rats selectively and rapidly (i.e., within 1 day) depletes NA in cerebral cortex, brainstem, and spinal cord, and these effects persist into adulthood (Jonsson, Hallman et al. 1982). Thus, we chose to inject rats with DSP-4 at P7 and test them two weeks later with the expectation that NA would be depleted in any brain region to which the LC projects. In other words, NA concentration was used as a bioassay of the efficacy of the DSP-4 treatment. Our findings that DA and 5-HT concentrations were unaffected by DSP-4 treatment support previous findings that DSP-4 selectively reduces NA concentration in the brain (Jonsson, Hallman et al. 1982).

The expression of sleep and wakefulness throughout the day and night reflect the variable influences of circadian and homeostatic mechanisms (Borbely and Achermann 2000). However, sleep and wake durations accumulate one bout at a time, and we now know that these individual bouts follow discernible statistical rules (Lo, Amaral et al. 2002; Lo, Chou et al. 2004; Blumberg, Seelke et al. 2005; Gall, Todd et al. 2008). These rules, in turn, reflect the dynamic interactions among hypothalamic and brainstem nuclei involved in the expression of ultradian sleep-wake cycles.

Wake bouts follow a power-law distribution in humans, cats, and rats (Lo, Chou et al. 2004; Blumberg, Seelke et al. 2005; Simasko and Mukherjee 2009) and wild-type and orexin knockout mice (Lo, Chou et al. 2004; Blumberg, Coleman et al. 2007; Diniz Behn, Kopell et al. 2008). This power-law structure – in which a small percentage wake bouts is considerably longer than most other wake bouts – may contribute to the efficient

partitioning and budgeting of wake-related activities. Specifically, this power-law property may reflect the value for an animal of continuous and lengthy periods of wakefulness.

Among mathematical biologists, the significance of power-law distributions and the mechanisms that give rise to them are topics of vigorous discussion (Newman 2003; Newman 2006). In the present context, it is worth considering that a power-law distribution is the signature of so-called "scale-free" networks comprising centralized hubs from which a disproportionate number of connections to other nodes in the network is made (Barabasi and Oltvai 2004). In this regard, the neural system that produces wakefulness may be viewed as a complex network comprising a collection of hubs with varying degrees of connectivity (Amaral, Scala et al. 2000; Newman 2003). Interestingly, LC neurons differentiate very early in development (Lauder and Bloom 1974) and soon give rise to a complex network of dense connections that project widely throughout the central nervous system, including the cerebral cortex, hippocampus, thalamus, midbrain, brainstem, cerebellum, and spinal cord (Foote, Bloom et al. 1983; Marshall, Christie et al. 1991; Aston-Jones, Shipley et al. 1995; Maubecin and Williams 1999). The LC also receives dense projections from glutamatergic, GABAergic, orexinergic, serotonergic, and noradrenergic neurons, among others (Aston-Jones, Shipley et al. 1995). Accordingly, the LC, with its dense efferent and afferent projections, qualifies as a highly connected hub.

An interesting property of scale-free networks is that they are more robust (i.e., more resistant to failure) than are random networks (Albert, Jeong et al. 2000) in the face of random damage to the network. On the other hand, scale-free networks are more

vulnerable to failure when damage comes in the form of an attack targeted at a hub. Thus, we hypothesize that the neural circuitry underlying the power-law structure of wake bouts has evolved in part because of its enhanced robustness in the face of random attack and that the use here of DSP-4 can be considered a "targeted" (i.e., non-random) attack on the LC. If so, then the distinct statistical organization of sleep and wake bouts may provide clues to the relative susceptibility of sleep and wakefulness to pathological disruption (e.g., neural degeneration with aging). We caution, however, that the convincing application of network theory to sleep-wake organization will depend in part on the development of a process model of network behavior that can produce phenomena akin to sleep and wake bouts (Newman 2003).

The present results highlight how a structure like the LC exerts a subtle and complex influence that can be overlooked if sufficiently sensitive measures of sleep-wake cyclicity are not used. Thus, while we agree that the LC is likely more than simply a wake-active nucleus that modulates arousal (Aston-Jones and Cohen 2005), even our understanding of the role of the LC in the regulation of arousal is incomplete.

We suggest that the LC comprises one component of a system that serves to modify the temporal structure of wake bouts such that prolonged periods of spontaneous wakefulness are possible. We hypothesize that the SCN and DMH, each being part of a well established wake-promoting pathway in adults involving the LC (Aston-Jones, Chen et al. 2001), contribute to consolidated wakefulness as well as nocturnality. This hypothesis is specifically tested in the next chapter.

CHAPTER 6

EFFECT OF SCN AND DMH LESIONS ON CIRCADIAN RHYTHMS AND POWER-LAW DISTRIBUTIONS OF WAKEFULNESS

Introduction

We have shown in Chapters 3 and 4 that the SCN-DMH-LC circuit develops substantially between P2 and P8, thereby making possible bidirectional interactions between the LC and SCN. Here, we test the hypothesis that the SCN-DMH-LC circuit also contributes to the developmental emergence of power-law distributions of wakefulness and nocturnality, which are clearly evident by P15 (Gall, Todd et al. 2008).

SCN lesions in adult rats eliminate circadian sleep-wake rhythms and result in bout fragmentation (Ibuka, Inouye et al. 1977; Mosko and Moore 1978; Mouret, Coindet et al. 1978; Mistlberger, Bergmann et al. 1987). DMH lesions in adult rats also eliminate circadian rhythms of sleep and wakefulness, and reduce total daily wake time (Chou, Scammell et al. 2003). In these studies, sleep and wakefulness were measured in adults using epochs and locomotor activity. In the present study, we performed more careful analyses of data by scoring sleep and wakefulness on a second-by-second basis to reveal the neural mechanisms that underlie developmental changes in circadian rhythmicity and bout distributions of sleep and wakefulness.

Here, in Experiments 6 and 7, we lesioned the SCN and DMH at P8—*before* the normal development of power-law distributions of wakefulness and nocturnality—and tested pups at P21—*after* the normal development of these processes. We predicted that lesions of each in early infancy would prevent or dampen the developmental transition

from exponential to power-law wake behavior. We also predicted that lesions of the SCN and DMH would prevent or dampen the emergence of nocturnal wake behavior.

Methods

Subjects

In Experiment 6, for SCN lesions, a total of 24 P21 Sprague-Dawley rats from 12 litters (n = 6 x 2 test times x 2 conditions) were used. In Experiment 7, for DMH lesions, a total of 24 P21 Sprague-Dawley rats from 12 litters (n = 6 x 2 test times x 2 conditions) were used.

Procedure

Experiment 6: SCN Lesions at P21

The methodology for this experiment was identical to that in Experiment 2, with the exception that pups were lesioned at P8 and tested at P15 and P21. A pup with a visible milkband was removed from the litter, and underwent surgery for bilateral electrolytic SCN lesions via 2 separate drops at P8 under isoflurane anesthesia in a stereotaxic apparatus. A sham littermate experienced the same procedures (e.g., 2 separate drops of electrode under isoflurane anesthesia) except that current was not applied. As described in Chapter 2, electrolytic lesions were required because the SCN is resistant to excitotoxicity (Bottum, Poon et al. 2010). After lesions were performed, Vetbond was used to close the close the pup's scalp. Peanut oil was brushed lightly on the scalps of lesioned and sham pups to deter the mother from reopening the closed wound. Pups were placed back in the litter for subsequent testing at P15 and P21.

Coordinates for the SCN were: AP: -0.2 mm from bregma, ML: ± 0.1 mm, and DV: -5.5 mm ventral to the meningeal surface. One lesioned pup and one sham pup were tested at P15 during the day (1000 – 1600 h) or night (2200 – 0400 h), with test time counterbalanced, in a humidified thermoneutral chamber (described in Experiment 1) maintained at 32°C. Data for pups were scored the following day, using methods described in Experiment 1, to verify that power-law distributions of wake bouts had been abolished in the lesioned pup. If a pup's power-law distribution had been abolished (and therefore the lesion was inferred to have been successful), then the same pup was tested at P21 (at 29°C) during the opposing time of day (i.e., if the pup was tested at P15 during the day, it was tested at night at P21). If power-law was retained in a lesioned pup at P15, it was killed, perfused, and not run again at P21.

Six successful SCN lesioned pups were tested during the day at P15 and at night at P21, and an additional 6 successful hits were tested at night at P15 and during the day at P21. We used the same pups for testing at P15 and P21 in order to observe the development of circadian rhythms of sleep and wakefulness within an animal across age. However, data from P15 SCN lesioned "hits" are not presented here, as they provided redundant information to those tested at P21.

After testing, pups were killed and perfused as described in Experiment 1. Brains were fixed and sliced to observe the extent of the lesion, as described in Experiment 2.

Experiment 7: DMH Lesions at P21

The methodology for this experiment was identical to that in Experiment 2, with the exception that the DMH was lesioned at P8 using a 10% ibotenic acid solution

(Sigma, St. Louis, MO). Chemical lesions were used here because they do not destroy fibers of passage. We chose to use ibotenic acid, specifically, because it has been shown to be effective in lesioning the DMH of adult rats (Gooley, Schomer et al. 2006).

A pup with a visible milkband was removed from the litter and underwent surgery for bilateral DMH lesions at P8 under isoflurane anesthesia in a stereotaxic apparatus (an additional littermate served as a control). In lesioned animals, 0.1 µl of a 10% ibotenic solution was injected bilaterally via 2 separate drops into the DMH at P8. In control pups, 0.1 µl of saline was injected bilaterally via 2 separate drops into the DMH at P8. The rate of infusion was 0.1 µl injected in 30 s. Coordinates were the following: AP: -2.2 mm caudal to bregma, ML: ± 0.2 mm from midline, DV: -6.0 mm from the surface of the brain. After lesions were produced, Vetbond was used to close the pup's scalp. Peanut oil was brushed lightly on the scalps of lesioned and sham pups to deter the mother from reopening the closed wound. Pups were placed in an incubator for at least 2 h to recover, and then placed back in the litter for testing at P21.

On the day of testing, one lesioned pup and one control pup were placed in separate humidified chambers at thermoneutrality (29°C) for testing at P21. Each pup was tested only once for 6 h during the day (1000 – 1600 h) or for 6 h at night (2200 – 0400 h) with order of test time counterbalanced. After testing, pups were killed and perfused as described in Experiment 1. Brains were postfixed and sliced to observe the extent of the lesion, as described in Experiment 2.

Data analysis

Data analysis was identical to Experiments 1 and 2.

Results

Experiment 6: SCN Lesions at P21

Figure 14 presents the effects of SCN lesions at P8 on sleep and wakefulness in pups tested at P21 during the day and night. Log-survivor distributions for pooled sleep and wake bout durations for SCN lesioned pups and shams are presented in Figure 14A. As expected for shams during the day and night, sleep bouts distributed along a straight line on these semi-log plots; therefore, exponential distributions were observed. In support of visual inspection, for sleep bouts in shams during the day and night, Akaike weights for exponential distributions were equal to one and those for power-law distributions were equal to zero. Wake bouts for shams, in contrast, did not distribute along a straight line during the day or at night. In support of visual inspection, for wake bouts in shams during the day and night, Akaike weights for exponential distributions were equal to zero, and those for power-law distributions were equal to one. Therefore, wake bouts were better fit by power-law distributions in shams. In SCN lesioned animals, both sleep and wake bouts during the day and night distributed along a straight line on these semi-log plots; therefore, exponential distributions were observed. In support of visual inspection, for sleep and wake bouts in SCN lesioned pups during the day and night, Akaike weights for exponential distributions were equal to one and those for power-law distributions were equal to zero. Therefore, sleep and wake bouts were better fit by an exponential distribution for SCN lesioned animals.

These results are highlighted by the insets in Figure 14A. There were no significant day-night differences in mean sleep bout durations for sham or SCN lesioned pups. ANOVA revealed a significant main effect of group ($F_{1,20} = 4.9$, $P < .05$), but not a

significant main effect of test time ($F_{1,20}$ = 1.3) and no significant group x test time interaction ($F_{1,20}$ = 0.2). In contrast, mean wake bout durations for sham pups were significantly increased at night. However, these mean day-night differences in wakefulness in SCN lesioned pups were eliminated. ANOVA revealed a significant main effect of test time ($F_{1,20}$ = 4.8, P < .05), but not of group ($F_{1,20}$ = 3.7), and also not a significant group x test time interaction ($F_{1,20}$ = 2.3). In concordance with previous findings (Gall, Todd et al. 2008), percentage of time awake at night was significantly increased in shams tested at P21. SCN lesioned pups, however, did not express day-night differences in percentage of time awake. ANOVA revealed no main effect of group ($F_{1,20}$ = 0.1) or test time ($F_{1,20}$ = 4.1), and no significant interaction ($F_{1,20}$ = 1.5).

Figure 14B shows the extent of the lesions. The smallest (green filled) and largest (purple outline) for each section is presented. Other adjacent areas were also affected by the electrolytic lesions in some pups, including parts of the optic chiasm (ox) and medial preoptic nucleus (MPO).

Four additional pups from 4 separate litters received electrolytic lesions that spared all cells within the SCN (i.e., SCN "Misses"). Log-survivor plots of sleep and wakefulness for SCN "Misses" and shams are presented in Figure 15A. All SCN "Misses" expressed day-night differences in sleep and wakefulness along with power-law distributions of wakefulness, similar to shams. Figure 15B shows the extent of the lesions. Again, the smallest (green filled) and largest (purple outline) lesion for each section is presented. Note that all lesions spared cells within the SCN.

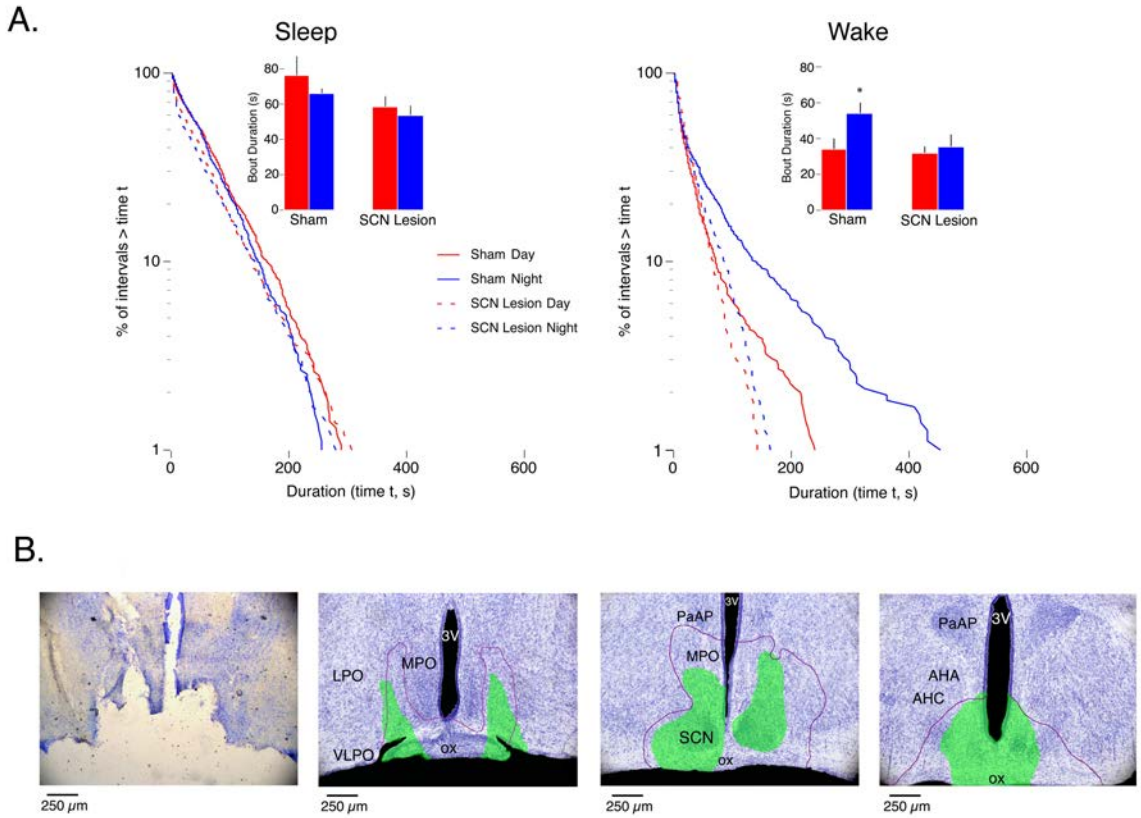

Figure 14. Effects of SCN lesions at P21 on sleep and wakefulness during the day and night. (A) Log-survivor plots of pooled sleep (left column) and pooled wake (right column) bout durations for subjects that experienced sham surgery or SCN lesions at P8 and tested at P21 (658 - 937 points per plot). Pups were tested during the day (red) and at night (blue) in shams (solid line) and SCN lesioned pups (dashed line). Insets provide mean sleep bout durations for sham and lesioned pups during the day and at night. *Significantly different from the corresponding daytime value. n = 6 subjects per group. Means + SE. (B) Photograph of a representative bilateral electrolytic SCN lesion performed at P8 with testing occurring at P21 (left) followed by 3 sequential images (rostral to caudal) depicting the smallest (green filled area) and largest (purple outlined area) lesions per coronal section across all lesioned subjects. Sections are 500 μm apart. Abbreviations are identical to Figure 6.

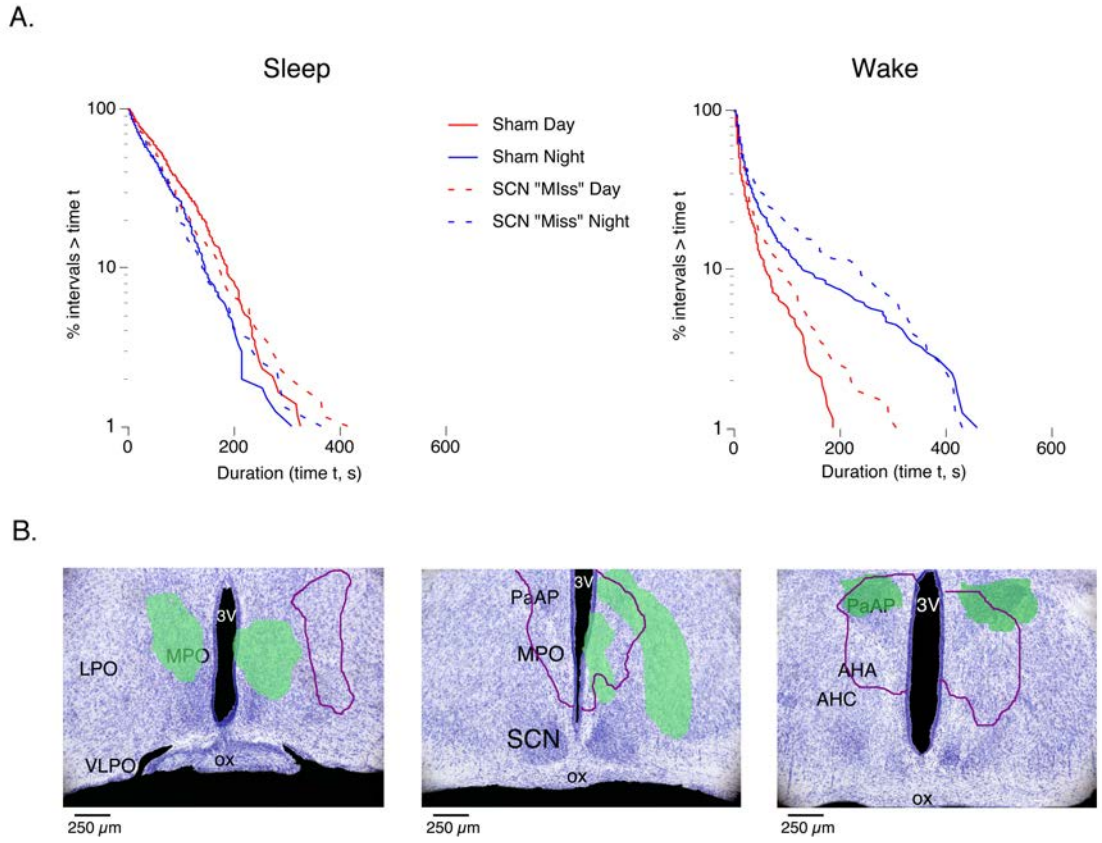

Figure 15. Effects of SCN "Misses" at P15 on sleep and wakefulness during the day and night. (A) Log-survivor plots of pooled sleep (left column) and pooled wake (right column) bout durations for subjects that experienced sham surgery or lesions that did not include the SCN at P8 and tested at P21 (222 - 420 points per plot). Pups were tested during the day (red) and at night (blue) in shams (solid line) and lesioned pups (dashed line). (B) 3 sequential brain slices (rostral to caudal) depicting the smallest (green filled area) and largest (purple outlined area) lesions per coronal section across all lesioned subjects. Sections are 500 μm apart. Abbreviations are identical to Figure 6.

Experiment 7: DMH Lesions at P21

Figure 16 presents the effects of DMH lesions at P8 on sleep and wakefulness in pups tested at P21 during the day and night. Log-survivor distributions for pooled sleep and wake bout durations for DMH lesioned pups and shams are presented in Figure 16A. As expected for shams during the day and night, sleep bouts distributed along a straight line on these semi-log plots; therefore, exponential distributions were observed. In support of visual inspection, for sleep bouts in shams during the day and night, Akaike weights for exponential distributions were equal to one and those for power-law distributions were equal to zero. Wake bouts for shams, in contrast, did not distribute along a straight line during the day or at night. In support of visual inspection, for wake bouts in shams during the day and night, Akaike weights for exponential distributions were equal to zero, and those for power-law distributions were equal to one. Therefore, for shams, wake bouts were better fit by power-law distributions. In DMH lesioned animals, both sleep and wake bouts during the day and night distributed along a straight line on these semi-log plots; therefore, exponential distributions were observed. In support of visual inspection, for sleep and wake bouts in DMH lesioned pups during the day and night, Akaike weights for exponential distributions were equal to one and those for power-law distributions were equal to zero. Therefore, for DMH lesioned animals, sleep and wake bouts were better fit by an exponential distribution.

These results are highlighted by the insets in Figure 16A. There were no significant day-night differences in mean sleep bout durations for sham or DMH lesioned pups. ANOVA revealed no significant main effects for group ($F_{1,20}$ = 2.2) or test time ($F_{1,20}$ = 0.002), and no significant group x test time interaction ($F_{1,20}$ = 3.1). In contrast,

mean wake bout durations for sham pups were significantly increased at night. However, day-night differences in wakefulness in DMH lesioned pups were eliminated. ANOVA revealed a significant main effect of group ($F_{1,20}$ = 14.5, P < .005) and test time ($F_{1,20}$ = 10.3, P < .005), and a significant group x test time interaction ($F_{1,20}$ = 12.1, P < .005). Post hoc tests revealed a significant difference between mean wake bout durations at night in DMH lesioned pups as compared to sham pups. In concordance with previous findings (Gall, Todd et al. 2008), percentage of time awake at night was significantly increased in shams tested at P21. In contrast, DMH lesioned pups did not express day-night differences in percentage of time awake. ANOVA revealed no main effect of group ($F_{1,20}$ = 3.2), but did reveal a significant main effect of test time ($F_{1,20}$ = 4.4, P < .05), and a significant interaction ($F_{1,20}$ = 11.2, P <.005).

Figure 16B shows the extent of the lesions. Other adjacent areas were also affected by DMH chemical lesions in some pups, including parts of the anterior hypothalamus (AHP) and posterior hypothalamus (PH).

Six additional pups from 6 separate litters received chemical lesions that spared all cells within the DMH (i.e., DMH "Misses"). Log-survivor plots of sleep and wakefulness for DMH "Misses" and shams are presented in Figure 17A. All DMH "Misses" expressed day-night differences in sleep and wakefulness along with power-law distributions of wakefulness, similar to shams. Figure 17B shows the extent of the lesions. Again, the smallest (green filled) and largest (purple outline) lesion for each section is presented. Note that all lesions spared cells within the DMH.

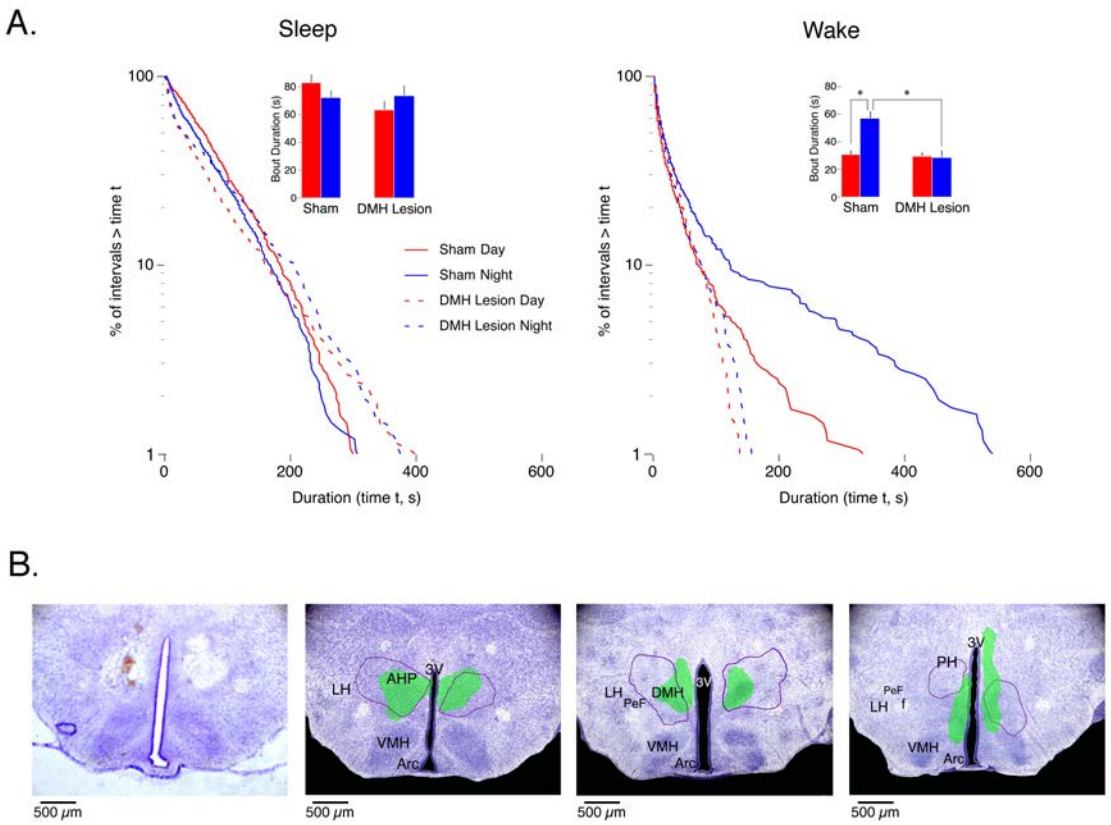

Figure 16. Effects of DMH lesions at P21 on sleep and wakefulness during the day and night. (A) Log-survivor plots of pooled sleep (left column) and pooled wake (right column) bout durations for subjects that experienced sham surgery or DMH lesions at P8 and tested at P21 (658 – 921 points per plot). Pups were tested during the day (red) and at night (blue) in shams (solid line) and DMH lesioned pups (dashed line). Insets provide mean sleep bout durations for sham and lesioned pups during the day and at night. * Significantly different. n = 6 subjects per group. Means + SE. (B) Photograph of a representative bilateral ibotenic acid lesion performed at P8 with testing occurring at P21 (left) followed by 3 sequential images (rostral to caudal) depicting the smallest (green filled area) and largest (purple outlined area) lesions per coronal section across all lesioned subjects. Sections are 500 μm apart. Abbreviations: DMH: dorsomedial hypothalamus; 3V: third ventricle; LH: lateral hypothalamus; AHP: anterior hypothalamic area, posterior; VMH: ventromedial hypothalamus; Arc: arcuate nucleus; PeF: perifornical nucleus; PH: posterior hypothalamus; f: fornix.

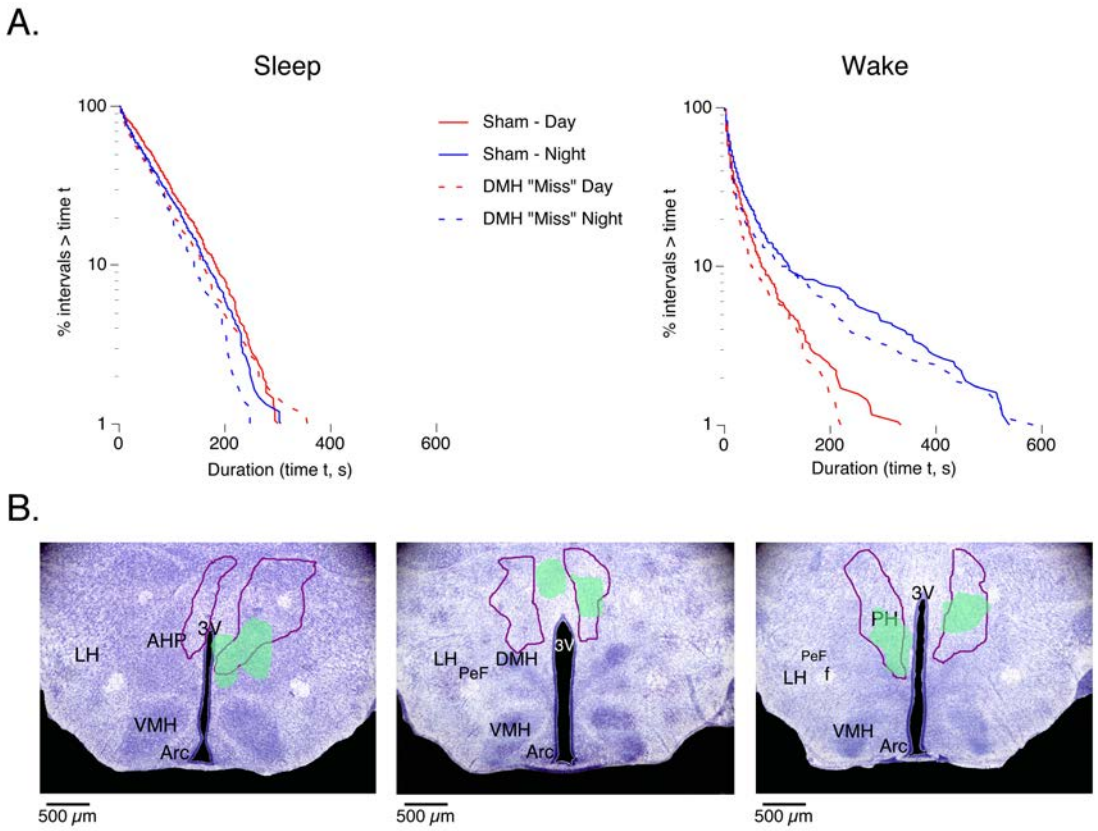

Figure 17. Effects of DMH "Misses" at P21 on sleep and wakefulness during the day and night. (A) Log-survivor plots of pooled sleep (left column) and pooled wake (right column) bout durations for subjects that experienced sham surgery or lesions that did not include the DMH at P8 and tested at P21 (560 – 749 points per plot). Pups were tested during the day (red) and at night (blue) in shams (solid line) and lesioned pups (dashed line). (B) 3 sequential brain slices (rostral to caudal) depicting the smallest (green filled area) and largest (purple outlined area) lesions per coronal section across all lesioned subjects. Sections are 500 μm apart. Abbreviations are identical to Figure 16.

Discussion

In this study we examined the functional significance of the SCN-DMH-LC pathway by lesioning the SCN and DMH at P8 and measuring EMG activity at P21 to analyze distributions of sleep and wakefulness. We show here that SCN and DMH lesions prevented the emergence of nocturnality and power-law distributions of wake bouts. In contrast, shams and pups with lesions that spared the SCN and DMH (i.e., SCN and DMH "Misses") expressed nocturnal and power-law wake behavior.

As highlighted by Figure 18, the different parts of the SCN-DMH-LC circuit contribute to differential behavioral effects of wakefulness. Lesions of the LC prevented the emergence of power-law wake behavior without fragmenting wakefulness. In contrast, SCN and DMH lesions not only prevented the emergence of power-law, but also fragmented wakefulness. Therefore, power-law distributions of wakefulness and consolidated wakefulness are likely to be controlled by separate neural mechanisms. Similar conclusions were drawn for orexin knockout mice, which exhibited power-law distributions of wakefulness with bout fragmentation relative to wild-types (Blumberg, Coleman et al. 2007).

There are at least two partly independent circuits governing the structure of sleep and wake bouts. Recent findings have suggested that forebrain areas such as the preoptic area (POA) and basal forebrain (BF) contribute to the consolidation of sleep in P9 rats (Mohns, Karlsson et al. 2006). These results provide evidence that forebrain structures other than the SCN and DMH contribute to the consolidation of sleep in infant rats.

The DMH contains orexin, a neurotransmitter involved in the consolidation of wakefulness (Peyron, Tighe et al. 1998; Sakurai 2007). Circadian rhythms of orexin are

eliminated after SCN lesions (Zhang, Zeitzer et al. 2004), indicating that inputs from the SCN directly influence orexinergic neuronal activity. However, as described in Chapter 5, orexin knockout mice still exhibit power-law distributions of wakefulness (Blumberg, Coleman et al. 2007). These authors hypothesized that non-orexinergic neurons contribute to the development of power-law wake behavior in mice. Because DMH lesions prevented the emergence of power-law distributions of wakefulness, the present results suggest that non-orexinergic neurons in the DMH contribute to power-law wake behavior in rats.

Although we focus here on sleep and wakefulness, future research should address the contributions of the SCN, DMH, and LC to include other behavioral and physiological rhythms in infant and adult mammals. In addition, there may be other nuclei that interact with the SCN-DMH-LC circuit to influence nocturnality and power-law distributions of wakefulness.

All together, lesions of the SCN or DMH prevent the emergence of power-law distributions of wakefulness and nocturnality, whereas lesions of the LC only prevent the emergence of power-law distributions of wakefulness. Therefore, the ability to stay awake hinges, at least in part, upon an intact SCN-DMH-LC circuit. We suggest here that the development of the SCN-DMH-LC circuit is critical for pups to regulate arousal and gain independence from the mother and littermates.

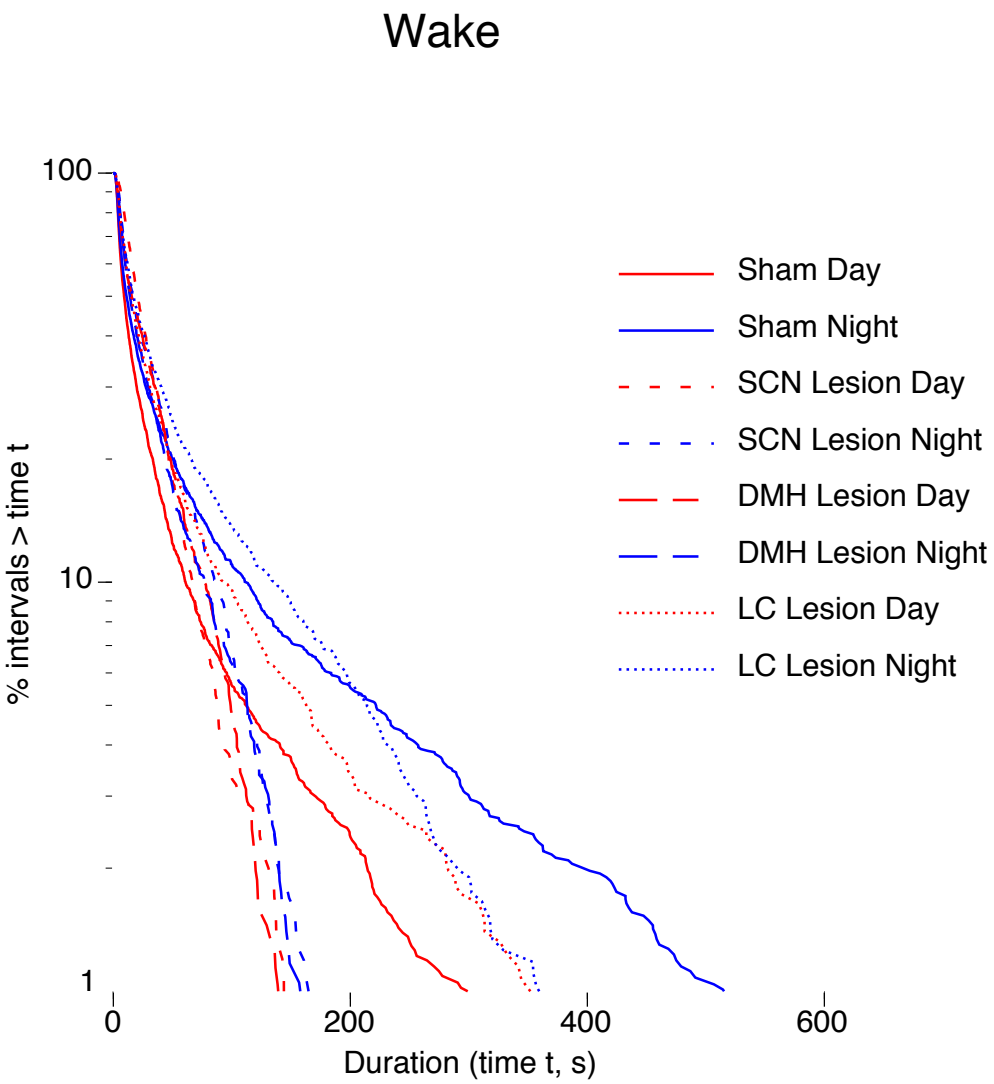

Figure 18. Effects of SCN, DMH, and LC lesions at P21 on wake bout distributions during the day and night. Log-survivor plots of pooled wake bout durations for subjects that experienced sham surgery or had SCN or DMH lesions at P8, or were injected with DSP-4 at P3 (i.e., LC lesions) and tested at P21 (805-1991 points per plot) are presented. Pups were tested during the day (red) and at night (blue) in shams (solid lines) and lesioned pups (dashed or dotted lines).

CHAPTER 7

OVERVIEW AND SIGNIFICANCE OF RESEARCH

Aston-Jones and colleagues (2001) identified the SCN-DMH-LC pathway that underlies arousal in adult rats. But how does neural connectivity within this circuit develop to contribute to ultradian and circadian sleep-wake activity? And what is the functional role of each nucleus in the developmental emergence of consolidated wakefulness, the emergence of power-law distributions of wakefulness, and nocturnality? Here we used transections, lesions, and tracing methods to elucidate the contributions of the SCN, DMH, and LC to the behavioral emergence of these ultradian and circadian sleep-wake processes.

In mammals, the SCN is critical for the expression of circadian rhythms of behavioral and physiological processes. In fetal rats, the SCN expresses circadian rhythms of metabolic activity and these rhythms are entrained by and synchronized to the mother (Reppert and Schwartz 1983). During the early postnatal period, behavioral (e.g., suckling) and physiological (e.g., temperature regulation) rhythms in infant rats are detectible (Ellison, Weller et al. 1972; Henning and Gisel 1980; Kittrell and Satinoff 1986; Nuesslein-Hildesheim, Imai-Matsumura et al. 1995). In fact, at P2, circadian sleep-wake rhythms are detectible (Gall, Todd et al. 2008). But is the emergence of circadian rhythmicity of sleep and wakefulness at P2 attributable to the pup's SCN?

The only way to specifically test the hypothesis that the SCN of infant rats is responsible for the expression circadian rhythms independently of the mother is to remove the influence of this nucleus in infants and measure circadian rhythmicity shortly

thereafter. Lesioning the SCN shortly after birth, however, is a technically challenging procedure, since the SCN is at the base of the brain and is a very small nucleus.

Therefore, we first performed a simple procedure to remove influences of the SCN on downstream sleep-wake circuitry. Precollicular transections were performed at P2 and P8 to effectively separate the forebrain, including the SCN, from downstream brainstem areas involved in sleep and wakefulness. We hypothesized that precollicular transections would eliminate day-night differences in sleep and wakefulness at both P2 and P8. Surprisingly, precollicular transections at P2 failed to eliminate day-night differences in sleep and wakefulness. Transections at P8, on the other hand, did eliminate them. These results suggest that the SCN exerts a humoral influence in newborns and gains neural control over brainstem structures over the first postnatal week.

Diffusible factors are released from the SCN, even in the absence of input from other brain areas. Accordingly, the SCN has a circadian rhythm of arginine vasopressin release, both in vivo (Majzoub, Robinson et al. 1991) and in vitro (Earnest and Sladek 1986). Fetal SCN grafts can restore the loss of circadian rhythms of drinking, locomotor activity, and temperature regulation in SCN lesioned adult animals (Hakim, DeBernardo et al. 1991; Lehman, Silver et al. 1991; Silver, LeSauter et al. 1996; Meyer-Bernstein, Jetton et al. 1999), indicating that diffusible factors are released from the SCN and are functional at restoring circadian rhythmicity.

If diffusible factors are responsible for day-night differences in sleep and wakefulness seen here at P2, we expected that SCN lesions—which remove neural and humoral influences of the SCN on downstream structures—would eliminate these differences. We were able to successfully lesion the SCN at P1 to show, for the first time,

that the SCN is *functional* at birth and modulates circadian rhythms of sleep and wakefulness. Specifically, SCN lesions eliminated day-night differences in sleep and wakefulness when tested at P2, indicating that pups modulate circadian rhythmicity independently via their SCN.

Between P2 and P8, the day-night differences in mean sleep and wake bout durations are small, but it is important to emphasize that they are detectible *before* EEG becomes state-dependent and *before* other standard measures of circadian rhythmicity (e.g., locomotor activity, drinking behavior) can be used. Therefore, using EMG measures alone, we are able to document the early onset of circadian rhythms of sleep and wakefulness in rats. These measures are an easy and efficient way to monitor circadian rhythms in early infancy, and provide a way for researchers to verify sleep-wake rhythms in infants well before other measures are feasible.

It is important that we understand the developmental precursors and mechanisms that lead to established, adult-like SCN function because environmental perturbations during early development have a profound effect upon the expression of circadian rhythms later in development. For example, we recently enucleated infant rats at P3 and P11, that is, before and after photic entrainment (Speh and Moore 1993; Hannibal and Fahrenkrug 2004). Whereas pups enucleated at P11 and tested at P21 exhibited increased wakefulness at night, similar to shams, pups enucleated at P3 and tested at P21 exhibited increased wakefulness during the day. Pups tested at P28 and P35 exhibited this same pattern of increased wakefulness during the day (Gall, Todd et al. 2008). These results indicate that the early influences of light and dark have long-term effects on circadian rhythms of sleep and wakefulness.

Because the SCN is more metabolically active during the day than the night in all species examined, regardless of whether they are diurnal or nocturnal (Smale, Lee et al. 2003), we do not expect that enucleation—or any other environmental manipulation—would alter the circadian timing of the SCN. Instead, we hypothesize that environmental factors induce changes in SCN interactions with downstream structures.

Maternal stress during the prenatal period is another example of an environmental perturbation that can affect the expression of circadian rhythms in offspring. In rats, high levels of maternal stress during the prenatal period induced a phase advance in circadian rhythmicity of locomotor activity when offspring were tested in adulthood (Maccari, Darnaudery et al. 2003). In addition, rats exposed to high levels of prenatal stress were slower to synchronize to a new light-dark rhythm than controls. These results suggest that early stressors, possibly via the release of corticosterone, play a role in modulating the circadian system.

Even drug use can have deleterious effects on the expression of circadian rhythms when animals are exposed during the prenatal period. Strother et al. (1998) discovered that cocaine administration by pregnant SCN-lesioned hamsters gave birth to offspring which, when tested on P21, showed a significant phase advance to a 1-hour light pulse compared to a phase delay in control pups (Strother, Vorhees et al. 1998). These results suggest that administration of cocaine affects the circadian circuit in early development to set up abnormalities in the circadian system throughout life.

All together, these studies reveal that there may be a sensitive period for the proper wiring of the circadian circuit early in development. Therefore, the importance of

understanding the developmental precursors that lead to established connectivity among the SCN and downstream structures cannot be underestimated.

Our results could also have important implications for how we provide light to premature infants in neonatal intensive care units (NICU). We have shown here that the SCN is functional shortly after birth for the expression of circadian rhythms of sleep and wakefulness in infant rats. Therefore, lighting in NICUs should be manipulated to match the lighting conditions in the natural environment so that the SCN stays in phase with the environment. At the very least, lights should be dimmed at night so that premature infants are not exposed to continuous light. It is possible that continuous light exposure to human infants can result in deleterious effects upon the circadian system, similar to results found in fetal lambs (Stark and Daniel 1989).

So far, we have demonstrated that the SCN is critical for the expression of circadian rhythms of sleep and wakefulness, and have suggested that neural influences from the SCN emerge over the first postnatal week. To examine the developing circuitry more specifically, we traced the SCN-DMH-LC circuit between P2 and P8. We hypothesized that neural connections among these structures would emerge during the first postnatal week. We found that neural connectivity among the SCN, DMH, and LC becomes strengthened and elaborated—and also becomes bidirectional—between P2 and P8.

What are the functional consequences of having a bidirectional and intact circuit? As shown previously by Todd et al. (2010), functional connectivity between the LC and DMH is present at P2, but the SCN does not receive feedback from these areas. At P8, however, we showed that functional connectivity among all three areas is present. As

discussed in Chapter 1, others have provided correlational evidence that SCN receives feedback about vigilance state in adult rats (Deboer, Vansteensel et al. 2003). Specifically, Deboer et al. (2003) showed that electrical activity of the SCN is elevated when an animal is in a state of wakefulness or REM sleep. Our results add to these findings, suggesting that the LC in particular is critical in providing feedback to the SCN at P8, but not at P2.

Therefore, because the SCN is capable of receiving feedback, and modulates its activity based upon vigilance state, we have also provided converging evidence that the SCN is involved in both circadian and homeostatic processes, as others have suggested (Edgar, Dement et al. 1993; Deboer, Overeem et al. 2004). These results have broad implications for the way we understand the functions of the SCN. Rather than the SCN being simply a circadian pacemaker, as one model predicts (Borbely and Achermann 1999), it is becoming increasingly clear that the SCN is intimately involved in both circadian and homeostatic aspects of sleep-wake regulation.

But what is the functional significance of having an intact SCN-DMH-LC circuit on the developmental emergence of ultradian and circadian rhythms of sleep and wakefulness? As described in Chapter 1, infant rats cycle very rapidly between states of sleep and wakefulness at birth, indicating that bouts were fragmented. Over development, these bouts consolidate dramatically. At P15, we observe a dramatic change in the distributions of wakefulness; they transition from exponential to power-law (Gall, Todd et al. 2008). Around the same time in development, nocturnality emerges. Because the SCN, DMH, and LC are part of an arousal promoting pathway (Aston-Jones, Chen et al. 2001), and this pathway is intact and bidirectional by P8, we predicted that lesioning each

nucleus before the normal expression of power-law wake behavior and nocturnality, and testing pups after their normal expression, would prevent the emergence of each.

We showed here that administration of DSP-4 at P3, which effectively destroys noradrenergic terminals from the LC (Jonsson, Hallman et al. 1981; Jonsson, Hallman et al. 1982; Fritschy and Grzanna 1989; Fritschy and Grzanna 1991), prevented the emergence of power-law distributions of wakefulness when pups were tested at P21. Also, performing SCN and DMH lesions at P8 also prevented the expression of power-law wake behavior when tested at P21. In addition, SCN and DMH lesions eliminated the expression of nocturnal wakefulness and resulted in fragmented wake bouts. Therefore, the SCN, DMH, and LC are functional by P21, and all contribute to the developmental emergence of power-law wake behavior.

Surprisingly, of the many SCN lesion studies in mammals (Ibuka, Inouye et al. 1977; Rusak and Zucker 1979; Mistlberger, Bergmann et al. 1983), very few have examined the effect of SCN lesions on sleep and wake bout durations. Using adult squirrel monkeys, Edgar et al. (1993) showed that SCN lesions produced a marked reduction in wake, but not in sleep, consolidation, relative to intact animals. In this study, the single longest wake bout was 462 minutes in intact animals, and only 51 minutes in SCN lesioned animals. Importantly, sleep bout durations were nearly identical in SCN lesioned animals as compared to controls. Edgar and colleagues were the first to suggest that the SCN is critically involved in wake, but not sleep, consolidation in adult animals.

In our study, although wake bout distributions were significantly affected by lesions of the SCN, DMH, and LC when pups were tested at P21, sleep bout distributions were not. These results indicate that the SCN-DMH-LC circuit preferentially modulates

arousal without affecting sleep by P21. The simple fact that wake bout distributions can be affected without affecting sleep bout distributions suggests that consolidation of sleep and wakefulness is controlled by distinct neural mechanisms. This study is the first to show that power-law distributions of wakefulness are significantly affected by lesions anywhere within the SCN-DMH-LC circuit.

Bout consolidation and power-law distributions of wakefulness are likely controlled by two partially independent mechanisms. LC lesions prevented the emergence of power-law distributions without affecting bout consolidation. SCN and DMH lesions also prevented the emergence of power-law distributions of wakefulness, but resulted in extreme bout fragmentation. Importantly, the loss of power-law in SCN and DMH lesioned pups may have been an incidental by-product of the extreme fragmentation. We conclude that there are two forms of consolidation. One form passes from the SCN to the DMH into the brainstem—and bypasses the LC—to result in wake bout consolidation. The other form of consolidation passes through the LC to result in power-law distributions of wakefulness.

The importance of the SCN-DMH-LC pathway is highlighted by the fact that behavioral aspects of attention, cognition, learning, and memory all depend upon our ability to maintain arousal (Devauges and Sara 1991; Johnson, Duffy et al. 1992; Usher, Cohen et al. 1999; Aston-Jones, Rajkowski et al. 2000; Aston-Jones, Chen et al. 2001). DSP-4 administration in adult rats and adult cats results in working memory deficits and reduced attention, suggesting that the LC is involved in these cognitive abilities (Delagrange, Tadjer et al. 1989; Sontag, Hauser et al. 2008). Also, SCN lesions in adult rats cause deficits in habituation and memory formation (Maruyama, Hara et al. 2007;

Holtfreter, Murphy et al. 2008). Although the specific role of the DMH on cognition, learning, and memory has not been tested, the fact that it is a relay nucleus between the SCN and LC suggests its potential role in these behavioral processes.

All together, the present results highlight how structures such as the SCN, DMH, and LC contribute to the circadian regulation of arousal in developing animals. We suggest that the SCN, DMH, and LC comprise components of a system that modify the distributions of wakefulness such that extended periods of wakefulness are possible. Given that power-law distributions of wakefulness and nocturnality emerge at the end of the second postnatal week, at a time when the weaning process is beginning (Thiels, Alberts et al. 1990), we suggest that the development of the SCN-DMH-LC circuit is critical for regulating arousal and gaining independence from the mother's care.

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