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of the requirements for the

Ph.D. degree in Zoology and Ecology, Evolutionary Biology and Behavior

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PLASTICITY IN THE CIRCADIAN SYSTEM: CHANGING RHYTHMS IN REPRODUCING FEMALE RODENTS

By

Jessica Anne Schrader

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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Zoology and Ecology, Evolutionary Biology, and Behavior

2009

ABSTRACT

PLASTICITY IN THE CIRCADIAN SYSTEM: CHANGING RHYTHMS IN REPRODUCING FEMALE RODENTS

By

Jessica Anne Schrader

Circadian rhythms in behavior and physiology are critical to ensure successful reproduction, but few studies have focused on how they may change with transitions in reproductive state. Those that have focused on pregnancy and lactation have revealed the emergence of new temporal patterns in a wide range of functions. The first part of this dissertation examines the hypothesis that these changes might be driven by modification of rhythms within the circadian system of the brain. I first characterized differences between non-pregnant and early pregnant laboratory rats in rhythms in protein expression of a neuronal activity marker (Fos) and in one component of the molecular oscillator (Per2) in the suprachiasmatic nucleus (SCN), which houses the primary pacemaker of the mammalian brain. In these studies I also examined the ventral subparaventricular zone (vSPZ), which is involved in modulating SCN output signals. Fos rhythms in one subregion of the SCN, the shell, and in the vSPZ underwent changes in waveform, and the peak of the Per2 rhythm in the whole SCN was phase-advanced during early pregnancy. To determine whether changes in Fos expression in the SCN shell and vSPZ were light-dependent, I next characterized differences in light-induced Fos expression in the SCN and vSPZ in non-pregnant and early pregnant rats housed in constant darkness. The rhythms of

light-responsivity of the SCN shell and vSPZ were altered in early pregnancy, but in a manner that indicates that changes in these areas in animals kept in a light/dark cycle were not light-dependent.

I next present evidence that rhythms in Per2 expression in some, but not all, extra-SCN oscillators within the brain also change in early pregnancy. This indicates that some aspect of early pregnancy induces modifications in the coupling of different elements of the circadian system. This could potentially cause rhythms in varying aspects of physiology and behavior to change in different ways. I also show that temporal patterns of Fos expression in some, but not all, non-circadian regulatory systems within the brain are modified in early pregnancy. Therefore, it appears that integration of concurrent changes in rhythms within and beyond the circadian system leads to a re-organization of rhythmicity within the brain. This might be part of the mechanism by which transitions from one reproductive state to another lead to the emergence of a diverse range of new rhythmic patterns in behavior and physiology.

In my final experiment, I characterized rhythms in activity and temperature in females of a diurnal rodent species, the Nile grass rat (*Arvicanthis niloticus*), as they progressed through a series of reproductive states. In contrast to nocturnal rodents, rhythms in females of this species underwent changes in amplitude, but not in phase, during pregnancy and lactation. This suggests that diurnal and nocturnal species may need to modulate their rhythms in different ways in order to reproduce successfully.

For every teacher and mentor that encouraged me to think critically.

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Acknowledgements

First, I would like to thank my advisor, Dr. Laura Smale, for her encouragement, support, advice, and critical comments throughout my graduate career. Thanks also to the other members of my guidance committee: Dr. Antonio Nuñez, who served as an unofficial co-advisor, Dr. Kay Holekamp, Dr. Joseph Lonstein, and Dr. Theresa Lee. Their advice and critiques of my research and writing have proved invaluable.

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I have been lucky to participate in seminars and activies of not only the Department of Zoology and Program in Ecology, Evolutionary Biology, and Behavior but also those of the Department of Psychology and Neuroscience Program. These communities have been exceptionally supportive and

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I have had the opportunity to teach many lab courses at MSU, and I am thankful to my teaching mentors and colleagues for shaping my development as an instructor. Special thanks are due to Dr. Barbara Lundrigan, Dr. April Harlan-Cognato, Sonya Lawrence, Dr. Carrie Scheele, Dr. Kevin Theis, Ryan Taylor, Emily Morrison, Emily Grman, and many fellow TAs from Bioscience 110. I also wish to thank my undergraduate teaching assistants: Diana Cerame, Melina Christofis, Jisoon Kim, Kari Mitsos, Jeremy Phan, Brittany Stanglewicz, Kayla Stomack, and Gabrielle Yezbick.

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Thanks to my family and friends who have supported me over the years, and thanks especially to my husband, Steve, for his love, support, and unblinking honesty.

TABLE OF CONTENTS

LIST OF TABLES x

LIST OF FIGURES xi

KEY TO ABBREVIATIONS xv

CHAPTER 1 Introduction 1

General introduction 1
The mammalian circadian timekeeping system 2
Female reproduction and circadian rhythms 5
The circadian system in early pregnancy 8
Overview of the chapters 12

CHAPTER 2 Changes in and dorsal to the rat suprachiasmatic nucleus during early pregnancy 15

INTRODUCTION 15
EXPERIMENTAL PROCEDURES 19
  Animals 19
  Determination of estrous cycle stages 19
  Tissue collection and immunocytochemistry 20
  Experiment 1: Fos and Per2 rhythms in LD 23
  Experiment 2: Light-induced Fos expression 24
  Quantitative and Statistical Analysis 27
RESULTS 29
  Experiment 1 29
  Experiment 2 33
DISCUSSION 36
  The SCN core 36
  The SCN shell 37
  The SCN core vs. shell 39
  Potential sex differences in the SCN 40
  Changes in the vSPZ during early pregnancy 41
  The role of the SCN and vSPZ during early pregnancy 44

CHAPTER 3 Differential changes in extra-SCN oscillators during early pregnancy in the rat 46

INTRODUCTION 46
EXPERIMENTAL PROCEDURES 48
  Animals 48
  Determination of estrous cycle stages 49
  Experimental Protocol 50
  Tissue collection and immunocytochemistry 50
  Quantitative and Statistical Analysis 52

C
c

D

CH
rep
nil
IN
M

R

DI
CO

CHA
Rn

RESULTS ... 55
Oval nucleus of the bed nucleus of the stria terminalis ... 55
Central and basolateral amygdala ... 57
A14 periventricular hypothalamic dopaminergic neurons ... 57
DISCUSSION ... 58

CHAPTER 4 Changing patterns of Fos expression in the rat brain during early pregnancy ... 64
INTRODUCTION ... 64
EXPERIMENTAL PROCEDURES ... 67
Animals ... 67
Determination of estrous cycle stages ... 67
Experimental Protocol ... 68
Tissue collection and immunocytochemistry ... 68
Quantitative and Statistical Analysis ... 70
RESULTS ... 72
The oval nucleus of the bed nucleus of the stria terminalis ... 72
The central and basolateral amygdala ... 74
The hypothalamic paraventricular nucleus ... 74
The medial preoptic area ... 75
The periventricular hypothalamus ... 75
The dorsomedial hypothalamus ... 75
The ventrolateral preoptic area ... 77
The basal forebrain ... 77
DISCUSSION ... 79

CHAPTER 5 Changing patterns of daily rhythmicity across reproductive states in diurnal female Nile grass rats (*Arvicanthis niloticus*) ... 86
INTRODUCTION ... 86
MATERIALS AND METHODS ... 88
Animals ... 88
Surgery ... 89
Experimental Manipulations ... 89
Activity and Temperature Data Analysis ... 91
Video Recordings and Behavioral Analysis of Nesting Females ... 93
RESULTS ... 94
Reproductive Output ... 94
Activity Rhythms ... 95
Temperature Rhythms ... 100
Behavioral Rhythms in Nesting Females ... 107
DISCUSSION ... 108
CONCLUSIONS AND IMPLICATIONS ... 116

CHAPTER 6 Conclusions ... 118
Rhythms in the brain in early pregnancy ... 118

F

Ir

Re

Rhythms and reproduction in diurnal versus nocturnal rodents 121
Implications of the present work 124

References 127

Ta
Te
Co
sig

LIST OF TABLES

Table 5.1. GMEM Results for Type III Tests of Fixed Effects for Activity and Temperature Rhythms. *CSH = Heterogeneous Compound Symmetry, CS = Compound Symmetry, see METHODS for model details. *n* = 5/group. NS = Not significant (P > 0.05). 97

LIST OF FIGURES

Images in this dissertation are presented in color.

Figure 1.1. Potential pathways of neural regulation of changes in overt rhythms in early pregnancy. (A) Some aspect of pregnancy may induce changes within the SCN (solid arrows), extra-SCN oscillators (dashed arrows), or non-oscillatory brain regions (dotted arrows) to promote changes in overt rhythms. (B) Effects of pregnancy may act at multiple levels in the brain in concert to lead to varied changes in different overt rhythms (combined arrows). Arrows indicate general pathways, not neural projections.......9

Figure 2.1. Photomicrograph of the SCN and vSPZ of a female lab rat stained for AVP-ir. The left SCN core and AVP-rich SCN shell are outlined, and the sampling region used for vSPZ counts is drawn to scale and placed above the left SCN. ocx, optic chiasm; 3v, third ventricle. Scale bar = 200 µm. 25

Figure 2.2. Representative photomicrographs of protein expression of Fos at ZT 2, 6, and 18 (A) and Per2 at ZT 2, 14, and 18 (B) in the middle SCN and vSPZ of diestrous (left) and pregnant rats (right) kept in a 12:12 LD cycle. Per2 is only expressed in the cell nucleus. Larger cells in (B) that are stained in the cytoplasm are TH-ir cells and are primarily located around the vSPZ and 3v. ocx, optic chiasm; 3v, third ventricle. Scale bar = 100 µm. 30

Figure 2.3. Expression of Fos (left column) and Per2 (right column) in the SCN core (A,B), SCN shell (C,D), and vSPZ (E,F) of diestrous and pregnant rats kept in a 12:12 LD cycle. Expression is measured as the percentage of the maximum individual value for each protein in each region. *indicates a time point where expression is significantly elevated relative to at least two other time points within the same reproductive state, and **indicates a time point at which expression significantly differs between reproductive states ($P<0.05$, post-hoc LSD (A,B,E,F) or Mann-Whitney U (C,D) tests).......31

Figure 2.4. Representative photomicrographs of protein expression of Fos in the middle SCN and vSPZ of control and light-pulsed diestrous (left) and pregnant rats (right) at CT 6 (top row) and CT 18 (bottom row). ocx, optic chiasm; 3v, third ventricle. Scale bar = 100 µm. 34

Figure 2.5. Expression of Fos in the SCN core (A), SCN shell (B), and vSPZ (C) of control and light-pulsed diestrous and pregnant rats kept in DD for two days. Expression is measured as the percentage of the maximum individual Fos-ir count within the region of interest. * indicates significantly higher Fos expression between light-pulsed and control females ($P<0.05$, post-hoc LSD (C) or Mann-Whitney U (A,B) tests). 35

Figure 3.1. Line drawings depicting location of the BnST-ov (A), PHDA (B), CEA, and BLA (C). Sampling boxes (black squares) were used for cell counts in the PHDA, CEA, and BLA. Anatomical boundaries are based on Paxinos and Watson (1997). 3v: third ventricle; AC: anterior commissure; CPu: caudate putamen; cx: cerebral cortex; ec: external capsule; f: fornix; LV: lateral ventricle; opt: optic tract; Pe: periventricular hypothalamic nucleus; RCh: retrochiasmatic area....54

Figure 3.2. Expression of Per2 in the BnST-ov (A,B), CEA (C,D), and BLA (E,F) of diestrous and pregnant rats kept in a 12:12 LD cycle. (A,C,E): * indicates a time point where Per2 expression is significantly elevated relative to at least one other time point within the same reproductive state, and ** indicates a time point at which expression significantly differs between reproductive states ($P<0.05$, post-hoc Mann-Whitney U tests). (B,D,F): Representative photomicrographs depicting between group differences in Per2 expression in the BnST-ov at ZT 6 (B), and within group variation at ZT 6 in the CEA (D) and at ZT 22 in the BLA (F). Scale bars= 200 µm. 56

Figure 3.3. Expression of Per2 in TH-expressing neurons in the PHDA of diestrous and pregnant rats kept in a 12:12 LD cycle (A). There was no significant interaction between reproductive state and ZT, and asterisks (*) indicate time points where Per2 expression in TH cells is significantly elevated relative to at least two other time points in the combined (pregnant and diestrous) rhythm. (B) Representative photomicrographs depicting Per2/TH-ir (left: purple/brown) and TH-ir (right: brown) cells in the PHDA in two different individuals (each row). Scale bars = 10 µm. 59

Figure 4.1. Line drawings depicting location of the sampling regions in the MS, VDB, and HDB (A); BnST-ov (B); VLPO and MPA (C); PVN and PeVN (D); CEA and BLA (E), and DMH (F). Sampling boxes (black squares) were used for cell counts in all regions except the BnST-ov (entire area in black). Anatomical boundaries are based on Paxinos and Watson (1997). 3v: third ventricle; AC: anterior commissure; ARC: arcuate nucleus; CPu: caudate putamen; cx: cerebral cortex; ec: external capsule; f: fornix; LV: lateral ventricle; ocx: optic chiasm; opt: optic tract; Pe: periventricular hypothalamic nucleus; RCh: retrochiasmatic area; VMPO: ventromedial preoptic area....71

Figure 4.2. Expression of Fos in the BnST-ov (A,B), CEA (C,D), and PVN (E,F) of diestrous and pregnant rats kept in a 12:12 LD cycle. (A,C,E): Main effects of reproductive state (RS) and ZT are indicated in text on each graph (A, E: two-way ANOVA; C: non-parametric tests). There was no significant interaction of ZT and reproductive state in the BnST-ov or PVN. NS: not significant ($P>0.05$). * indicates that Fos expression was significantly elevated relative to at least one other time point within the same reproductive state, and ** indicates a significant difference between reproductive states ($P<0.05$, post-hoc Mann-Whitney U tests). (B,D,F): Representative photomicrographs of Fos-labelled cells in each region at ZT 6. LV: lateral ventricle; 3v: third ventricle. Scale bars = 200 µm. 73

Figure 4
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Figure 4.3. Expression of Fos in the MPA (A,B), PeVN (C,D), and DMH (E,F) of diestrous and pregnant rats kept in a 12:12 LD cycle. (A,C,E): Main effects of reproductive state (RS) and ZT are indicated in text on each graph (A: non-parametric tests; C,E: two-way ANOVA). There was a significant interaction of ZT and reproductive state in the PeVN but not the DMH. Notation is the same as in Fig. 4.2 (post-hoc Mann-Whitney U tests (A) or LSD tests (C)). (B,D,F): Representative photomicrographs depicting between group differences in Fos expression in each region at ZT 18. 3v: third ventricle; f: fornix. Scale bars = 200 μm. 76

Figure 4.4. Expression of Fos in the VLPO (A,B), MS (C,D), and VDB (E,F) of diestrous and pregnant rats kept in a 12:12 LD cycle. (A,C,E): Main effects of reproductive state (RS) and ZT are indicated in text on each graph (A,E: non-parametric tests; C: two-way ANOVA). There was no significant interaction of ZT and reproductive state in the MS. Notation is the same as in Fig. 4.2 (post-hoc Mann-Whitney U tests). (B,D,F): Representative photomicrographs depicting between group differences in Fos expression in each region at ZT 22. ocx: optic chiasm. Scale bars = 200 μm 78

Figure 5.1. Averaged general locomotor activity rhythms in the reproductive (*left*) and control (*right*) groups (*n* = 5/group) under a 12h: 12h light (open bars)/dark (black bars) cycle for pregnancy, pregnancy plus lactation, and lactation (or equivalents). For each individual, the average activity of each half hour was standardized by dividing the raw value for that half hour on the day of interest by the average total daily activity of that female on the pre-reproductive day. Plotted values are means of these standardized values for each treatment group for each half hour. Standard errors were removed for ease of comparing the rhythms. 96

Figure 5.2. (*A*) Average daily activity across reproductive days (or equivalents) in the two treatment groups (*n* = 5/group). The average daily activity was standardized by dividing the average activity of a female for each day of interest by the average activity of that female on the pre-reproductive (or equivalent) day. Values are means ± SE. *Significant differences between the control and reproductive groups ($p < 0.05$; LSD test). The data for the reproductive group are re-plotted in (*B*) for comparisons of pregnancy and lactation to the combined state. ‡Significant differences between lactation and the combined (PL) state ($p < 0.05$; LSD test). 99

Figure 5.3. (*A*) Ratio of activity in the dark versus light phase across reproductive days (or equivalents) in the two treatment groups (*n* = 5/group). Values are means ± SE. Notation is the same as in Fig. 5.2A. The data for the reproductive group are re-plotted in (*B*) for comparisons of pregnancy and lactation to the combined state 101

Figure 5.4. Averaged T_b rhythms in the reproductive (*left*) and control (*right*) groups (*n* = 5/group) under a 12h: 12h light (open bars)/dark (black bars) cycle

for pregnancy, pregnancy plus lactation, and lactation (or equivalents: equiv.). Values are means for each treatment group for each half hour. Standard errors were removed for ease of comparing the rhythms. 102

Figure 5.5. (*A*) Daily amplitude of the T_b rhythm across reproductive days (or equivalents) in the two treatment groups (*n* = 5/group). Values are means ± SE. Notation is the same as in Fig. 5.2A. The data for the reproductive group are re-plotted in (*B*) for comparisons of pregnancy and lactation to the combined state. †Significant differences between pregnancy and the combined (PL) state ($p < 0.05$; LSD test). ‡Significant differences between lactation and the combined (PL) state ($p < 0.05$; LSD test). 104

Figure 5.6. (A and B) Average T_b during the midday (ZT 3 to 9, A) and midnight (ZT 15 to 21, B) periods across reproductive days (or equivalents) in the two treatment groups (n = 5/group). Values are means ± SE. Notation is the same as in Fig. 5.2A. The data for the reproductive group are re-plotted in (C) and (D) for comparisons of pregnancy and lactation to the combined state during the midday (C) and midnight (D) periods. Notation is the same as in Fig. 5.5B. 106

Figure 5.7. Frequencies of off-nest (*A*) and drinking (*B*) behaviors for each hour of the light (open bars, *top*): dark (black bar, *top*) cycle during lactation in a subset of the reproductive group (*n* = 3). Values are pooled means ± SE for days 2, 10, and 19 of lactation in 3 lactating dams. *Peaks and †troughs in each rhythm as explained in RESULTS 109

Figure 6.1. Proposed network model of the pathways (arrows) by which pregnancy affects rhythms in circadian and non-circadian systems of the brain to lead to changes in overt rhythms. Some aspect of pregnancy induces changes within the SCN and vSPZ (solid arrows), some extra-SCN oscillators (dashed arrows), and some non-oscillatory brain regions (dotted arrows) to promote changes in overt rhythms. These effects are likely to be integrated at multiple levels (converging arrows leading to combined arrows). In addition, rhythms in some brain regions are unaffected (*blocked arrows indicating resistance to upstream influences of pregnancy). Arrows indicate general pathways, not neural projections. 119

KEY TO ABBREVIATIONS

Abbreviation	Definition
3v	third ventricle
ABC	avidin-biotin peroxidase complex
AC	anterior commissure
ACTH	corticotropin
ANOVA	analysis of variance
ARC	arcuate nucleus
AVP	arginine vasopressin
BLA	basolateral amygdala
BnST-ov	oval nucleus of the bed nucleus of the stria terminalis
CEA	central amygdala
CPu	caudate putamen
CRF	corticotropin releasing factor
CS	compound symmetry
CSH	heterogeneous compound symmetry
CT	circadian time
cx	cerebral cortex
DAB	diaminobenzidine
DD	constant darkness
dm	dorsomedial
DMH	dorsomedial hypothalamus
ec	external capsule

Abbrevia

ER-β.......

f.............

FRA.......

GMEM....

GRP.......

HDB.......

ir.............

L. L#.......

LD..........

LSD........

LV..........

MPA.......

MS.........

NGS.......

NHS.......

NREMS..

NS..........

ocx.........

opt.........

OVX.......

Abbreviation	**Definition**
ER-β	estrogen-receptor beta
f	fornix
FRA	Fos-related antigens
GMEM	general mixed effects model
GRP	gastrin-releasing peptide
HDB	horizontal diagonal band of Broca
ir	immunoreactive
L, L#	lactation, lactation day #
LD	light/dark
LSD	least significant difference
LV	lateral ventricle
MPA	medial preoptic area
MS	medial septum
NGS	normal goat serum
NHS	normal horse serum
NREMS	non-rapid eye movement sleep
NS	not significant
ocx	optic chiasm
opt	optic tract
OVX	ovariectomized

Abbreviati

P, P#

PB

PBS

PB-TX

Pe, PeVN

Per

PHDA

PL, PL#

Post

Pre

PVN

RCh

REMS

RS

SCN

SE

SPZ

T_c

TH

THDA

Abbreviation	Definition
P, P#	pregnancy, pregnancy day #
PB	0.1 M phosphate buffer
PBS	0.01 M phosphate-buffered saline
PB-TX	0.2% TX in 0.2 M phosphate buffer
Pe, PeVN	periventricular hypothalamus
Per	period
PHDA	A14 periventricular hypothalamic dopaminergic neurons
PL, PL#	pregnancy plus lactation, pregnancy plus lactation day #
Post	post-reproductive
Pre	pre-reproductive
PVN	paraventricular hypothalamus
RCh	retrochiasmatic area
REMS	rapid eye movement sleep
RS	reproductive state
SCN	suprachiasmatic nucleus
SE	standard error
SPZ	subparaventricular zone
T_b	core body temperature
TH	tyrosine hydroxylase
THDA	A12 tuberohypophyseal dopaminergic neurons

Abbrevia

TIDA......

TX.........

VDB ......

VIP........

vl...........

VLPO.....

VMPO....

vSPZ......

ZT..........

Abbreviation	Definition
TIDA	A12 tuberoinfundibular dopaminergic neurons
TX	Triton-X 100
VDB	vertical diagonal band of Broca
VIP	vasoactive intestinal peptide
vl	ventrolateral
VLPO	ventrolateral preoptic area
VMPO	ventromedial preoptic area
vSPZ	ventral subparaventricular zone
ZT	zeitgeber time

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CHAPTER 1

Introduction

General introduction

Circadian rhythms in behavior and physiology, which have a period of roughly 24 hours and are generated endogenously, have been documented in most clades of life, including cyanobacteria, fungi, plants, and animals (Bell-Pedersen et al., 2005; Dunlap, 1999). When synchronized to the external environment, these rhythms allow organisms to anticipate predictable daily fluctuations in the environment and appropriately time behavioral and physiological events to anticipate those changes (Moore-Ede et al., 1982). The neural and molecular regulation of rhythms in mammals has been extensively studied, but this has been done primarily with adult males of nocturnal rodent species, particularly rats (*Rattus norvegicus*), hamsters (*Mesocricetus auratus*), and mice (*Mus musculus*). Few studies have addressed questions of whether or how transitions from one reproductive stage to the next influence rhythms in behavior and physiology or the underlying mechanisms that govern them. This leaves major gaps in our understanding of the circadian system, as all mammals, especially females, progress through a series of significant changes in their reproductive status. These transitions require plasticity in circadian systems that regulate a diversity of functions. In this introductory chapter, I will first review the neural and molecular mechanisms of the mammalian circadian timekeeping system. I will follow this with a discussion of our current understanding of how circadian rhythms change as female mammals transition from one reproductive

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state to another. I will then propose hypotheses that might account for how the circadian timekeeping system may be involved in promoting these changes. Finally I will present a summary of the research questions addressed in each chapter.

The mammalian circadian timekeeping system

Circadian rhythms, which each have a period that is close to, but not exactly, 24 hours, are revealed when animals are maintained in constant environmental conditions. However, they are ordinarily entrained by environmental stimuli, referred to as *zeitgebers*, or "time-givers" in German. The neural mechanisms controlling circadian rhythms in mammals have been well-characterized in males, and it is firmly established that the suprachiasmatic nucleus (SCN), located bilaterally in the anterior hypothalamus, houses the primary pacemaker (Moore and Eichler, 1972; Ralph et al., 1990; Rusak, 1977; Stephan and Zucker, 1972). This pacemaker is responsible for generating endogenous rhythms in constant conditions as well as entraining these rhythms to environmental signals. Light is the primary entraining agent, and photic information is conveyed to the SCN from the eyes directly via the retinohypothalamic tract (Johnson et al., 1988; Levine et al., 1991; Moore and Lenn, 1972) and indirectly via the geniculohypothalamic tract (Rusak et al., 1989).

The SCN is functionally and neurochemically heterogeneous, and it may be divided into two main subregions, referred to by some as the "core" and the "shell" and by others as the ventrolateral (vl-) and dorsomedial (dm-) SCN (Morin,

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2007). In this dissertation, I use the terms core and shell (as in Moore et al., 2002), even when referring to reports in which the authors used the terms vl-SCN and dm-SCN to differentiate between the two subregions (e.g. Lee et al., 1998; Cutrera et al., 2002). In laboratory rats, the retinohypothalamic tract innervates the core, which also receives non-photic information through other pathways (Johnson et al., 1988; Levine et al., 1991; Moore and Lenn, 1972; Moore et al., 2002). The shell relies on inputs from the core for photic information, and it receives non-photic information via the core as well as other brain regions (Antle and Silver, 2005; Daikoku et al., 1992; Ibata et al., 1993; Ibata et al., 1997; Moore et al., 2002). The SCN shell integrates these inputs with endogenous circadian processes and provides the majority of SCN output signals (Moore et al., 2002). Its primary target is the subparaventricular zone (SPZ; Leak and Moore, 2001; Watts and Swanson, 1987; Watts et al., 1987; Watts, 1991), which projects to many of the same targets as the SCN and may process circadian signals and homeostatic drive (Abrahamson and Moore, 2006; Kriegsfeld et al., 2004; Lu et al., 2001; Morin et al., 1994; Saper et al., 2005; Schwartz et al., 2004; Watts and Swanson, 1987; Watts et al., 1987). The ventral region of the SPZ (vSPZ), lies immediately dorsal to the SCN shell and is important in the control of locomotor activity rhythms (Abrahamson and Moore, 2006; Lu et al., 2001; Schwartz et al., 2009).

Although cells in both the core and shell of the rat SCN express the inhibitory neurotransmitter, GABA (Moore and Speh, 1993), the two subdivisions of the nucleus differ with respect to other peptides and transmitters. Cells in the

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SCN shell primarily express arginine vasopressin (AVP), whereas the primary core output signals are vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP; Antle and Silver, 2005; Moore et al., 2002; Romijn et al., 1998). VIP and GRP neurons in the core of the rat SCN have axonal projections that innervate the entire SCN and also project beyond the nucleus (Daikoku et al., 1992; Ibata et al., 1993; Ibata et al., 1997; Moore et al., 2002). Both subregions contain oscillator cells, each of which expresses a molecular transcription/translation loop that takes roughly 24 hours to complete. This "molecular oscillator" involves multiple "clock" proteins, including positive elements (Clock and BMAL) and negative elements (two cryptochrome and three period (Per) proteins), which also serve as transcription factors for other genes, called clock-controlled genes (Bell-Pedersen et al., 2005; Dunlap, 1999; Reppert and Weaver, 2002). Cells expressing this feedback loop are more prevalent in the SCN shell, where they co-express AVP, but some are in the core where they co-express VIP (Dardente et al., 2002).

The molecular oscillator is also expressed in cells outside the SCN. Populations of these cells, referred to as extra-SCN oscillators, have been found within a number of brain regions and throughout the body's periphery (Guilding and Piggins, 2007; Hastings et al., 2003; Reppert and Weaver, 2002; Weinert, 2005). The SCN is necessary to synchronize rhythms in most of these regions with one another, but there are conflicting data regarding the extent to which these oscillators require the SCN to maintain their rhythmicity, as some are less dependent on it than others (Yamazaki et al., 2000; Yoo et al., 2004). It has been

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proposed that the function of extra-SCN oscillators may be to integrate light-entrained circadian signals originating in the SCN with processes that vary among cell populations to meet site-specific needs (Guilding and Piggins, 2007; Herzog and Tosini, 2001; Weinert, 2005; Yoo et al., 2004).

Female reproduction and circadian rhythms

Circadian rhythms are especially critical to reproduction. In some mammals, including many rodents, the timing of ovulation is regulated by the SCN (for review, see de la Iglesia and Schwartz, 2006), and rhythms in mating behavior have been observed in multiple species (Beach and Levinson, 1949; Dobson and Michener, 1995; Gilbert et al., 1985; Hansen et al., 1979; Harlan et al., 1980; Mahoney and Smale, 2005). This probably optimizes the likelihood of successful fertilization. However, in all therian mammals, fertilization is followed by gestation (pregnancy) and lactation, which must also be carried out successfully. Circadian rhythms play important roles during these reproductive states as well, although significantly less work has focused on this.

The best-characterized animal model of circadian processes operating during pregnancy is the nocturnal lab rat. During early pregnancy in this species, locomotor activity generally decreases and becomes less rhythmic (Rosenwasser et al., 1987), whereas body temperature rhythms show an advance in their rising phase and a reduction in amplitude attributable to increases in the daily temperature minimum (Kittrell and Satinoff, 1988). Sleep patterns are altered, and the total amount of time spent in both non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) during the

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dark phase increases during early pregnancy, as does the number of REMS bouts in the dark phase (Kimura et al., 1996). Rhythms in hormone secretion also change. The amplitudes of the rhythms of circulating corticotropin (ACTH) and corticosterone decrease due to a reduction in the peak values, and the rising phase of the rhythm in ACTH is advanced (Atkinson and Waddell, 1995).

The most thoroughly-studied circadian rhythm of early pregnancy in the rat is that of prolactin secretion, which emerges after mating or vaginocervical stimulation and exhibits twice-daily surges around dawn and dusk (Butcher et al., 1972). This pattern promotes progesterone secretion by the corpora lutea, which is necessary for maintenance of the uterine endometrium to support pregnancy (for review, see Freeman et al., 2000). Similar rhythms have been found in other rodents (Edwards et al., 1995; McMillan and Wynne-Edwards, 1999; Talamantes et al., 1984), and this pattern can also be induced via cervical stimulation of intact or ovariectomized (OVX) females, a procedure that leads to pseudopregnancy (for review, see Erskine, 1995; Erskine et al., 2004). This rhythm is a true circadian one, as each prolactin surge free-runs with a period of approximately 24 hours in both blinded females and intact females in constant conditions (Bethea and Neill, 1979; Pieper and Gala, 1979; but see Yogev and Terkel, 1980).

Multiple lines of evidence support a role for the circadian system and the SCN in the regulation of the two prolactin surges during early pregnancy. First, the circadian phase at which they occur is unaffected by the timing of cervical stimulation that induces them (Smith and Neill, 1976). Second, both

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retrochiasmatic knife cuts and lesions of the SCN abolish the surges (Bethea and Neill, 1980; Freeman et al., 1974; Yogev and Terkel, 1980). Furthermore, transgenic mice lacking the *Clock* gene, which is a component of the molecular oscillator, exhibit higher rates of spontaneous abortion throughout gestation, display a decrease in mid-term progesterone secretion, and have shorter pseudopregnancies than wild-type females (Miller et al., 2004). This indicates that functional clock gene expression is vital for normal secretion of prolactin during early pregnancy and for successful gestation.

Hypothalamic dopamine serves as the primary regulator of prolactin release through its inhibitory effects on the pituitary gland (Ben-Jonathan, 1985), and an endogenous rhythm in dopamine secretion has been well-characterized in rats (for review, see Freeman et al., 2000). The prolactin surges of early pregnancy are also promoted by stimulation from various prolactin-releasing factors. VIP and serotonin may stimulate each peak by promoting oxytocin release, which is sufficient to induce the rhythm of prolactin secretion seen in early pregnancy (Arey and Freeman, 1989; Arey and Freeman, 1990; Arey and Freeman, 1992a; Arey and Freeman, 1992b; Egli et al., 2004; Egli et al., 2006; Kennett et al., 2008). VIP may also promote prolactin release by direct inhibition of dopaminergic neurons in the arcuate nucleus (Gerhold et al., 2001), since inhibition of transcription of *VIP* mRNA increases the expression of Fos-related antigens in these dopaminergic neurons (Gerhold et al., 2002).

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The circadian system in early pregnancy

Although changes in rhythms from a non-pregnant state to early pregnancy have been recognized for years, very little is known about how or whether the circadian system promotes them. One hypothesis is that the SCN is responsible (Fig. 1.1, solid arrow pathways). This could potentially occur via many mechanisms, such as a re-setting of the phase of the master pacemaker relative to the light/dark cycle or a change in coupling between the molecular oscillator network and output signals, such as VIP or AVP, within the SCN. Alternatively, changes within extra-SCN oscillators might be the primary cause of modifications in overt rhythms, and, for example, these extra-SCN oscillators might respond differently to the SCN (Fig. 1.1A, dashed arrow pathway). It is also possible that pregnancy acts at the level of both the SCN and downstream components of the circadian system (Fig. 1.1B). Another hypothesis is that the circadian system may not change at all, but its influence may be modulated by other physiological and behavioral systems that are modified as a female transitions from one reproductive condition to another (Fig. 1.1A, dotted arrow pathway). Finally, the physiological changes might originate within both circadian and non-circadian regulatory systems (Fig. 1.1B).

In order for any process, within or beyond the circadian system, to play a role in modifying rhythms during early pregnancy, it must be influenced by reproductive state. Presumably, some aspect of mating, fertilization, implantation, or fluctuations in reproductive hormones alter these systems. Considerable evidence indicates that circadian mechanisms in the brain are responsive to changes that accompany pregnancy. For example, in the SCN core, the

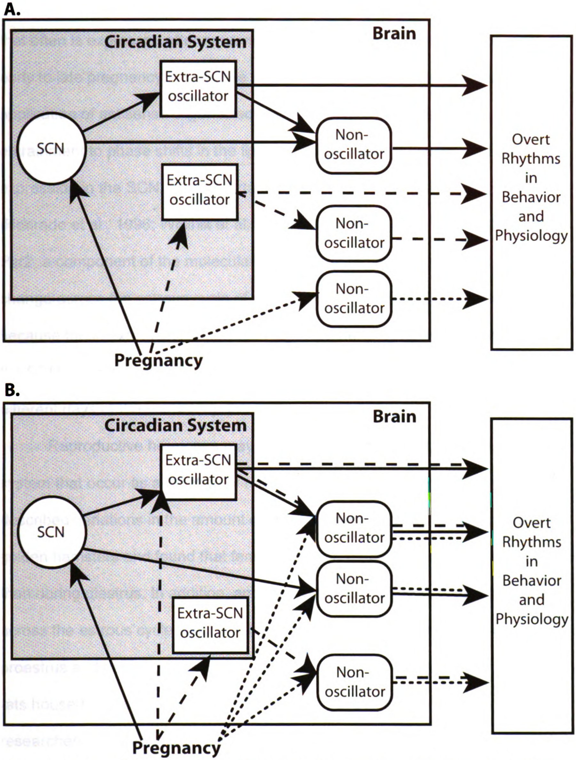

Figure 1.1. Potential pathways of neural regulation of changes in overt rhythms in early pregnancy. (A) Some aspect of pregnancy may induce changes within the SCN (solid arrows), extra-SCN oscillators (dashed arrows), or non-oscillatory brain regions (dotted arrows) to promote changes in overt rhythms. (B) Effects of pregnancy may act at multiple levels in the brain in concert to lead to varied changes in different overt rhythms (combined arrows). Arrows indicate general pathways, not neural projections.

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expression pattern of Fos, a protein encoded by the immediate early gene *cfos* that often is expressed after neuronal activation (Kovacs, 2008), changes from early to late pregnancy in rats (Lee et al., 1998). Knockout studies and application of antisense oligonucleotides to *cfos* mRNA prevent normal entrainment to phase shifts in the light/dark (LD) cycle, indicating that Fos expression in the SCN is an important component of circadian regulation (Honrado et al., 1996; Wollnik et al., 1995). In addition, rhythms in expression of Per2, a component of the molecular oscillator, in regions of the limbic forebrain change across the estrous cycle of rats (Perrin et al., 2006). This might occur because these extra-SCN oscillators respond differently to signals originating in the SCN or because the SCN emits these signals in a different manner on the different days of the estrous cycle.

Reproductive hormones may be responsible for changes in the circadian system that occur as a function of reproductive state. Richards (1966) first described variations in the amount of wheel running across the estrous cycle in golden hamsters and found that females are more active on proestrus and estrus than during diestrus. In addition, activity rhythms display a "scalloping" pattern across the estrous cycle due to a phase advance in activity onset during proestrus and estrus (Morin et al., 1977), and this "scalloping" pattern persists in rats housed in constant dim light (Albers et al., 1981). These data led researchers to hypothesize that changes in secretion of gonadal hormones influence endogenous rhythms. This was found to be the case, as estradiol treatment of OVX female golden hamsters shortens the periods of their free-running act

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running activity rhythms (Morin et al., 1977). Progesterone administration blocks this effect and decreases the amount of activity, whereas progesterone given alone to OVX females does not affect rhythms (Takahashi and Menaker, 1980). Similar responses to ovarian hormones have been demonstrated in rats (Albers, 1981; Gerall et al., 1973; Thomas and Armstrong, 1989).

Estrogenic effects on activity rhythms probably act via receptors within the SCN or via inputs to this nucleus. Estrogen receptor-containing neurons in multiple brain regions, particularly the preoptic area, project to the SCN in female hamsters (de la Iglesia et al., 1999). In both men and women, estrogen, progesterone, and androgen receptors have been found within the SCN itself (Kruijver and Swaab, 2002), and estrogen receptor- β (*ER-β*) is expressed in the SCN of female rats (Shughrue et al., 1997). Binding of hormones to these receptors may alter SCN function. This is suggested by the fact that, in OVX rats, the peak of the rhythm of mRNA for Per2 in the SCN is advanced by estradiol (Nakamura et al., 2005), and estradiol treatment can lead to increased Fos expression in the SCN in an LD cycle (Abizaid et al., 2004; Peterfi et al., 2004). Prolactin may also change the responsivity of the SCN to light. Pituitary grafts that increase circulating PRL ten-fold in male hamsters cause a heightened Fos response to light during the dark phase in both the SCN core and shell (Cutrera et al., 2002). These data indicate that pituitary and ovarian hormones may modulate how the SCN responds to light and that estradiol, at least, may reset the phase of the molecular oscillator. These changes could partially explain how

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new rhythms in behavior and physiology emerge as female mammals transition from one reproductive state to another.

Overview of the chapters

In this dissertation, I present experiments that were designed to extend our understanding of (1) how the circadian system might be involved in the modification of rhythms that occur as females progress through transitions in reproductive condition and (2) how such modifications might differ between nocturnal and diurnal species. To address the first issue, I sought to identify changes during early pregnancy in the mechanisms that regulate rhythmic functions (Chapters 2-4). In this series of studies, I compared patterns of change in protein expression throughout the day in circadian and non-circadian systems of the brain in laboratory rats (*Rattus norvegicus*) during diestrus and early pregnancy. This served as an ideal model for my research because the rat has been best characterized in terms of how circadian rhythms in various behaviors and physiological events change with reproductive state, particularly from a virgin state to early pregnancy.

In Chapter 2, I used the diestrus/early pregnant rat model system to evaluate the hypothesis that the primary pacemaker of the brain, which is housed in the SCN, might promote changes in overt rhythms in early pregnancy. Another question that I addressed is whether a major target of the SCN, the vSPZ, might be altered during early pregnancy, as signals from this region converge on other targets of the SCN and may therefore affect how they respond to SCN signals. For the purposes of this study, I compared rhythms between diestrus and early pregnant

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pregnant rats in two aspects of SCN and vSPZ function: neuronal activity (as measured by expression of Fos) and the molecular oscillator (as measured by expression of Per2). I also compared patterns of light-induced Fos expression in the SCN and vSPZ between diestrous and pregnant females housed in constant darkness.

In Chapter 3, I address the hypothesis that extra-SCN oscillators in the brain may be involved in driving changes in circadian rhythms during early pregnancy. If this hypothesis is correct, then rhythms in clock gene expression should differ between non-pregnant and early pregnant females. In this study, I used tissue from the same animals used in Chapter 2 to characterize rhythms in Per2 expression in extra-SCN oscillators within dopaminergic neurons that inhibit prolactin secretion and in portions of the extended amygdala, which regulate emotional state, anxiety, and the stress axis (Cardinal et al., 2002; Davis et al., 1997; Dong et al., 2001; Erb et al., 2001; Szafarczyk et al., 1983).

In Chapter 4, I examine how non-circadian regulatory systems of the brain might contribute to changes in temporal patterns of functions associated with early pregnancy. If they do contribute, then we should see changes in rhythms of their neuronal activity, which can often be assessed by examination of patterns of Fos expression. I therefore monitored Fos in regions of the brain involved in the regulation of sleep, arousal, thermal homeostasis, and hormone secretion. In this study, I used tissue from the same animals as in Chapters 2 and 3.

To address the second major issue of this dissertation, that of how diurnal and nocturnal species differ with respect to how overt rhythms are altered during

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transitions in reproductive state, I utilized a diurnal murid rodent model, the Nile grass rat (*Arvicanthis niloticus*), to determine how pregnancy, lactation, and the combination of the two affect daily rhythms in activity and core body temperature (Chapter 5). I compared these rhythms to those described in published reports of nocturnal models to determine whether multiple circadian strategies have evolved to cope with reproductive challenges in different chronotypes.

Finally, I conclude this dissertation with a discussion of how rhythms in the brain may be re-organized in early pregnancy to produce changes in daily rhythms, how this re-organization might differ in nocturnal and diurnal species, and the potential implications of these issues on mammalian conservation and biology (Chapter 6).

While I served as the primary investigator for the experiments presented in Chapters 2 through 5, these studies were collaborative efforts, and Chapter 5 has been published with co-authors. Therefore, in these chapters, I have elected to use the term "we" rather than "I" to indicate that this research was a collaborative effort.

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CHAPTER 2

Changes in and dorsal to the rat suprachiasmatic nucleus during early pregnancy

INTRODUCTION

Endogenously driven circadian rhythms that are entrained to external cues allow organisms to predict daily changes in the environment and appropriately time behavioral and physiological events to anticipate them (Moore-Ede et al., 1982). The circadian timekeeping system plays an important role in the coordination of a variety of physiological and behavioral processes essential for mammalian reproduction. Most of the research on these issues has focused on the timing of copulation and ovulation. However, in all therian mammals, fertilization is followed by gestation (pregnancy) and lactation, and the circadian system plays a critical role during these reproductive states as well.

During pregnancy, locomotor activity generally decreases and becomes less rhythmic in rats (Rosenwasser et al., 1987), while the peak of the body temperature rhythm advances (Kittrell and Satinoff, 1988). Pregnant rats also display more rapid eye movement (REMS) and non-rapid eye movement sleep (NREMS) during the dark phase than cycling females (Kimura et al., 1996). A novel prolactin rhythm, with peaks around dawn and dusk, emerges in early pregnancy and promotes progesterone secretion by the corpora lutea (Butcher et al., 1972; Freeman et al., 2000). Rhythms in the hypothalamic-pituitary-adrenal axis also change, with a reduction in the daily peak of secretion of both corticotropin (ACTH) and corticosterone (Atkinson and Waddell, 1995). While

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these changes have been established for some time, the neural mechanisms responsible for changes in all but the prolactin rhythm have received little attention.

The neural mechanisms controlling circadian rhythms in mammals have been well-characterized in males and nulliparous females, and it is firmly established that the suprachiasmatic nucleus (SCN), located bilaterally in the anterior hypothalamus, houses the primary pacemaker that generates these rhythms (Moore and Eichler, 1972; Ralph et al., 1990; Rusak, 1977; Stephan and Zucker, 1972). The SCN entrains to a light/dark (LD) cycle via the retinohypothalamic tract, which innervates a subregion of the SCN termed the “core” (Johnson et al., 1988; Levine et al., 1991; Moore and Lenn, 1972; Moore et al., 2002). This region also receives non-photic input, and it conveys the two kinds of information to the SCN “shell”, which also receives non-photic inputs from other brain regions (Antle and Silver, 2005; Daikoku et al., 1992; Ibata et al., 1993; Ibata et al., 1997; Moore et al., 2002). The SCN shell integrates these signals and provides the major output pathways from the nucleus to other brain regions (Moore et al., 2002). Its primary target is the subparaventricular zone (SPZ; Leak and Moore, 2001; Watts and Swanson, 1987; Watts et al., 1987; Watts, 1991). The ventral portion of the SPZ (vSPZ), immediately dorsal to the SCN shell, plays a role in the integration of circadian and homeostatic signals to regulate various functions, such as locomotor activity rhythms (Abrahamson and Moore, 2006; Lu et al., 2001; Saper et al., 2005; Schwartz et al., 2004; Schwartz et al., 2009).

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Although cells in both the core and shell of the rat SCN express the inhibitory neurotransmitter, GABA (Moore and Speh, 1993), the two subdivisions differ with respect to other peptides and transmitters they contain. Cells in the SCN shell primarily express arginine vasopressin (AVP), whereas the primary core output signals are vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP; Antle and Silver, 2005; Moore et al., 2002; Romijn et al., 1998). VIP and GRP neurons in the rat SCN core innervate the entire SCN and also project beyond the nucleus (Daikoku et al., 1992; Ibata et al., 1993; Ibata et al., 1997; Moore et al., 2002). Both subregions contain oscillator cells, which each have a molecular transcription/translation loop that takes roughly 24 hours to complete. This "molecular oscillator" involves a transcription-translation feedback loop comprised of multiple "clock" genes and their protein products. These include the period (Per) proteins, which are themselves transcription factors for other genes, referred to as clock-controlled genes (Bell-Pedersen et al., 2005; Dunlap, 1999; Reppert and Weaver, 2002). Cells expressing this feedback loop are more prevalent in the SCN shell, where they co-express AVP, but some are in the core and co-express VIP (Dardente et al., 2002). Populations of cells, called extra-SCN oscillators, express the same transcription-translation feedback loop. These populations have been found within the brain and throughout the periphery and, together with the SCN, comprise the circadian system (Guilding and Piggins, 2007; Hastings et al., 2003; Reppert and Weaver, 2002; Weinert, 2005).

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The various changes in rhythms that occur during early pregnancy raise the question of whether and how the circadian timekeeping system might be involved in promoting them. One possibility is that the SCN is the driving force behind these changes. If so, changes in SCN rhythms in peptide expression, clock gene oscillations, and/or neuronal firing should be apparent during this phase of reproduction. Alternatively, the SCN might remain the same, while extra-SCN oscillators and/or non-circadian systems are altered, perhaps in a site-specific manner. The purpose of this study was to examine the possibility that rhythms in the SCN, and its primary circadian target, the vSPZ, differ between non-pregnant and early pregnant rats. To do this, we characterized rhythms in Per2, a key component of the molecular oscillator, and Fos, the protein product of the immediate early gene *cfos* that often rises after neuronal activation (Kovacs, 2008), in pregnant and non-pregnant rats. The first group was examined on day 6 of pregnancy, as the changes in overt rhythms described above are all established by that time. Females on day 1 of diestrus, when circulating gonadal hormones are lowest, served as our non-pregnant controls. This initial experiment revealed differences in Fos expression in the SCN shell and vSPZ between the two groups of females in the mid-light and/or mid-dark phases of the LD cycle. We therefore conducted a second experiment to determine whether rhythms in light-induced Fos expression in the SCN and vSPZ could account for these patterns.

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EXPERIMENTAL PROCEDURES

Animals

Animals were adult female and male Sprague-Dawley laboratory rats obtained from Harlan Laboratories (Indianapolis, IN, USA). Males were housed in pairs or groups of three when not paired with females. Females were housed in groups of three or four for a habituation period of at least two weeks and were subsequently separated for single-housing. All subjects were housed in polypropylene cages (48x27x20 cm) with *ad libitum* access to food (Teklad 8640 rodent diet, Harlan) and water under a 12h:12h light/dark (LD) cycle (unless otherwise noted) with lights on at Zeitgeber Time (ZT) 0 and off at ZT 12. A dim red light (<5 lux) remained on constantly for animal care purposes. All experiments were performed in compliance with guidelines established by the Michigan State University Institutional Animal Care and Use Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to keep the number of animals used and their discomfort at a minimum.

Determination of estrous cycle stages

Once singly housed, females were subjected to daily vaginal smears between ZT 0 and ZT 3 to track their estrous cycles. A cotton-tipped applicator soaked in sterile physiological saline was inserted into the vaginal opening, gently rotated, and withdrawn. The applicator was immediately rolled onto a glass slide and allowed to air dry. The dried slides were soaked in 0.25% methylene blue dye for five minutes, rinsed with distilled water, and allowed to

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dry prior to examination under a light microscope for the presence of nucleated epithelial cells, cornified epithelial cells, leukocytes, and mucus. Estrous cycle stage was determined as follows: proestrus: predominantly nucleated epithelial cells; estrus: predominantly to exclusively cornified epithelial cells; diestrus (between 2 to 3 consecutive days): predominantly leukocytes, some cornified and/or nucleated epithelial cells and mucus present. At least three estrous cycles were established for each female prior to further experimental manipulations.

Tissue collection and immunocytochemistry

At the time of perfusion, animals were given an overdose of sodium pentobarbital and perfused transcardially with 0.01 M phosphate-buffered saline (PBS), pH 7.2, followed by 4% paraformaldehyde (Sigma, St. Louis, MO) in 0.1 M phosphate buffer, unless otherwise noted. Animals perfused during the dark phase or under constant darkness were fitted with a light-tight hood prior to perfusion to prevent acute exposure to light. Brains were post-fixed for 4 hours, transferred to 20% sucrose solution overnight, and stored in cryoprotectant at 4 °C overnight then at -20 °C until sectioning. Brains were sectioned coronally at 30 µm into three alternate series using either a freezing microtome or cryostat, and sections were placed in cryoprotectant for further storage at -20 °C.

For tissue series that were processed for immunocytochemical staining to detect Fos protein expression in the cell nucleus, free floating sections were rinsed in 0.01 M PBS and then incubated in 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) in PBS with 0.3% Triton X-100 (TX) for 1 h at room temperature. After a 10 minute rinse in PBS, sections were incubated

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with a primary anti-cFos antibody (made in rabbit, Santa Cruz Biochemistry, Santa Cruz, CA, USA; 1:25,000, unless otherwise noted) in 3% NGS and 0.3% TX in PBS on a rotator at 4°C for 48h, unless otherwise noted. Sections were then rinsed in PBS and incubated with a biotinylated secondary goat-anti-rabbit antibody (Vector; 1:200) in 3% NGS and 0.3% TX in PBS for 1 h at room temperature, rinsed again, and then incubated with an avidin-biotin peroxidase complex (0.9% each avidin and biotin solutions; ABC Vectastain kit; Vector) in 0.3% TX with PBS for 1 h at room temperature. Next, sections were rinsed in 0.125 M acetate buffer, pH 7.2, then reacted with diaminobenzidine (DAB; 0.25 mg/mL, Sigma), nickel sulfate (25 mg/mL, Sigma), and hydrogen peroxide (0.825 µL 0.3% H_2O_2 /mL buffer) to yield a blue-black reaction product.

The series of sections processed for Per2 was double labeled for detection of tyrosine hydroxylase (TH) for the analyses of extra-SCN oscillators described in Chapter 3. This tissue was processed in the same manner as for Fos except with a primary anti-Per2 antibody (mPER2 # 38, made in rabbit, 1:5,000, 24 h incubation; a generous gift from Dr. David Weaver, University of Massachusetts, MA, USA). After the reaction for Per2 staining, to process the tissue for TH expression, the same sections were rinsed first in 0.1 M phosphate buffer (PB) and then in 0.2% TX in 0.2 M PB (PB-TX), and they were then incubated in 5% normal horse serum (NHS; Vector) in PB-TX for 30 min at room temperature. After rinses in PB-TX, sections were incubated with a primary anti-TH antibody (made in mouse, Immunostar, Hudson, WI, USA; 1:20,000) in 3% NHS and PB-TX on a rotator at 4°C for 24 h. Sections were then rinsed in PB-TX and in

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and incubated with a biotinylated secondary horse-anti-mouse antibody (Vector; 1:200) in 3% NHS and PB-TX for 1 h at room temperature, rinsed again, and then incubated with an avidin-biotin peroxidase complex (0.9% each avidin and biotin solutions; ABC Vectastain kit; Vector) in PB-TX for 1 h at room temperature. Next, sections were rinsed in Trizma buffer (pH 7.2, Sigma) then reacted with DAB (0.2 mg/mL, Sigma) and hydrogen peroxide (0.35 µL 30% H_2O_2 /mL buffer) to yield a brown reaction product.

Some sections were stained for arginine vasopressin (AVP) in order to identify the boundary between the shell and core; these sections initially underwent processing for Fos, as described above, but that reaction failed due to a dilution error. After the Fos reaction, the tissue was processed in the same manner as for Fos with the following exceptions: (1) with a primary anti-vasopressin antibody (made in guinea pig, Peninsula Laboratories, Belmont, CA, USA; 1:30,000; 24 h incubation); (2) with a secondary goat-anti-guinea pig antibody (Vector; 1:200); and (3) after incubation with the avidin-biotin peroxidase complex, the tissue was rinsed in Trizma buffer (pH 7.2, Sigma) then reacted with DAB (0.2 mg/mL, Sigma) and hydrogen peroxide (0.35 µL 30% H_2O_2 /mL buffer) to yield a brown reaction product. Trial immunocytochemical reactions with the reagents used for each reaction above were performed with tissue from females that did not have easily determined estrous cycles or had mated but were not pregnant to ensure specificity of the primary antibodies (data not shown). All sections were mounted on clean slides, dehydrated, and coverslipped.

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Experiment 1: Fos and Per2 rhythms in LD

Animals were acclimated for at least two weeks to a 12h:12h LD cycle, and then females were singly housed, divided into one of two experimental groups, and subjected to daily vaginal smears to determine the estrous cycle of each. Rats in the first group were perfused at 4 h intervals (ZT 2, 6, 10, 14, 18, and 22) on day one of diestrus as described above, except that 1.3% lysine and 4% sodium periodate were added to the 4% paraformaldehyde. In the second group, a male was placed in the female's cage on the morning of proestrus (between ZT 0 and 3). Males were removed the next morning, and a vaginal smear was taken from the female to check for the presence of sperm to verify mating had occurred. If none were detected, the female was re-paired on the morning of her next proestrus period. Once mating had been verified, vaginal smears were discontinued, and the day of confirmation was designated as day 0 of pregnancy. Six days later, females were perfused in the same manner as the diestrous females. Pregnancy was confirmed by visual inspection of the uterus. Sections from one alternate series of tissue were processed for Fos immunoreactivity, and sections from another series were processed for Per2/TH immunoreactivity. The TH reaction was unnecessary for the present experiment, but it was included for use in Chapter 3.

Photographs of the SCN/vSPZ were taken from three sections, bilaterally, for each tissue series using a CCD video camera (CX900, MBF Bioscience, Williston, VT, USA) attached to a light microscope (Zeiss, Göttingen, Germany). These images were processed using Adobe Photoshop 7 and imported to Adobe Illustrator CS 4 (Adobe Systems, Mountain View, CA, USA) to draw boundaries

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for the SCN core and shell and the vSPZ. The borders of the SCN core and shell were based upon the distribution of AVP in the rat SCN (Moore et al., 2002). The vSPZ was sampled by counting cells within a 215 μm x 160 μm rectangle placed immediately dorsal to the SCN and lateral to the third ventricle (Fig. 2.1). Cells expressing Fos or Per2 were then counted bilaterally in these three regions for all three sections using the NIH ImageJ program (NIH, Bethesda, MD, USA). All counts were made by an investigator unaware of the reproductive state or time of perfusion of each animal.

Experiment 2: Light-induced Fos expression

Animals were acclimated for three weeks to a 12h:12h LD cycle with ZT 0 (lights on) at 1300 h. The females were then singly housed, divided into one of two experimental groups, and subjected to daily vaginal smears to determine the estrous cycle of each individual. Rats in the first group (diestrous) were moved to a separate room in constant darkness (DD) on the morning of proestrus (between ZT 0 and 3). Daily vaginal smears were taken from them in DD to ensure that they were still cycling. On the first day of diestrus (two days after entering DD), experimental females were transferred to a separate room for 30 min in DD and subsequently exposed to a one hour light pulse beginning at either circadian time (CT) 5 or CT 17. Animals were perfused immediately after the light pulse ended at CT 6 or CT 18. These circadian times were the equivalents of the ZTs from the previous LD cycle. In the second group, a male was placed in the female's cage on the morning of the last day of diestrus (between ZT 0 and 3). Males were removed the morning of estrus, and a vaginal

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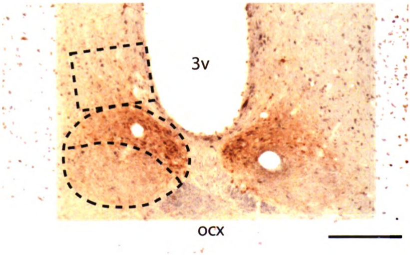

Figure 2.1. Photomicrograph of the SCN and vSPZ of a female lab rat stained for AVP-ir. The left SCN core and AVP-rich SCN shell are outlined, and the sampling region used for vSPZ counts is drawn to scale and placed above the left SCN. ocx, optic chiasm; 3v, third ventricle. Scale bar = 200 µm.

smear was taken from the female on both days of pairing to check for the presence of sperm to verify mating had occurred. We paired them one day earlier than in experiment 1 with the expectation that it would increase the likelihood of mating during the first pairing, which it did. If no sperm was detected, the female was re-paired as before during her next estrous cycle. Once mating had been verified, vaginal smears were discontinued, and the day of confirmation was designated as day 0 of pregnancy. Four days later, females were moved to DD, as above, and, after two days (i.e. day 6 of pregnancy), they were subjected to the same light pulse/perfusion paradigm as females in the diestrous group. Pregnancy was confirmed by visual inspection of the uterus. For both the pregnant and diestrous groups, control females were subjected to the same treatments but did not experience a light pulse in the hour prior to perfusion.

Sections from one series of tissue (every third section) were processed for Fos immunoreactivity (Fos primary concentration of 1:5,000; incubated for 24 h). We counted the number of Fos-positive cells in the SCN core, SCN shell, and vSPZ bilaterally in each section. This was done with a light microscope equipped with a drawing tube (Leitz, Laborlux S, Wetzlar, Germany), since this method was more sensitive to moderately-stained nuclei than the ImageJ method used in Experiment 1 and the Fos antibody used for this tissue (from a different lot) had a weaker affinity. The borders of the SCN core and shell were based upon the distribution of AVP in adjacent sections, and the vSPZ was delineated by a 215 μm x 160 μm rectangle dorsal to the SCN as described above (Fig. 2.1). All

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counts were made by an investigator unaware of the reproductive state, time of perfusion, or light treatment of each animal.

Quantitative and Statistical Analysis

In each experiment, three sections (one rostral, middle, and caudal) through the SCN and vSPZ were selected from each animal for analysis of protein expression. In Experiment 1, sections chosen for analysis of Per2 expression were adjacent to those selected for analysis of Fos. For each region of interest (SCN core, SCN shell, and vSPZ), counts of labeled cells from all sections from an individual were summed. We then converted that sum for each region in each individual to a percent of maximum value by dividing the sum by the value from the individual with the highest number of immunoreactive cells in the region in question. For all figures, these percentages, without further transformation, are presented as means ± SE.

The data for expression of each protein in each region were subjected to Levene's test for homogeneity of variances and then arcsine-transformed if they did not meet the criteria of that test. If arcsine transformation did not equalize the variances, then nonparametric tests (detailed below) were used to analyze the original percent of maximum values for that region/protein combination. All data analyses were conducted with SPSS 17 software (SPSS Inc., Chicago, IL, USA). All differences were considered significant when $P<0.05$. Statistical details are omitted from the text when they are presented in figure legends.

Experiment 1. A total of 57 females were used in the final analysis (n=28 diestrous, n=29 pregnant, n=4-6 per time point for each reproductive state). For

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data that met the homogeneity of variance criteria (vSPZ Fos, arcsine transformations of SCN core Fos and Per2 and vSPZ Per2), a 2-way analysis of variance (ANOVA) was performed, with reproductive state (pregnant or diestrous) and time of perfusion (ZT) as between-subjects factors. Relevant post-hoc least significant difference (LSD) tests were conducted with these data when any significant interactions were found. For data that did not meet the homogeneity of variance criteria even when transformed (SCN shell Fos and Per2), nonparametric analyses were conducted to compare reproductive states at each ZT (Mann-Whitney U tests) and to compare ZTs within each reproductive state (Kruskal-Wallis tests, followed by post-hoc pairwise Mann-Whitney U tests when significant effects were found).

Experiment 2. A total of 47 females were used in the final analysis (*n*=23 diestrous, *n*=24 pregnant, *n*=5-7 per light treatment/CT for diestrous rats and *n*=6 per light treatment/CT for pregnant rats). For data that met the homogeneity of variance criteria (vSPZ), a 3-way ANOVA was performed, with reproductive state (pregnant or diestrous), time of perfusion (CT), and light treatment (pulse or control) as between-subjects factors. Relevant post-hoc LSD tests were conducted with these data when any significant interactions were found. For data that did not meet the homogeneity of variance criteria even when transformed (SCN core and shell), nonparametric analyses were conducted to compare reproductive states (pulsed and control separately) and effects of light pulses (pregnant and diestrous separately and combined) at each CT (Mann-Whitney U tests

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tests) and to compare CTs within each reproductive state (pulsed and control separately, Mann-Whitney U tests).

RESULTS

Experiment 1

SCN core. Representative photomicrographs depicting patterns of Fos and Per2 expression are shown in Fig. 2.2. A two-way ANOVA of Fos expression in the core revealed a significant main effect of ZT, with peak expression at ZT 2 (Fig. 2.3A; F=27.034, *df*=5, $P<0.001$). A non-significant trend for higher Fos expression in pregnant than diestrous females was also found (F=3.623, *df*=1, $P<0.065$), but there was no interaction between reproductive state and ZT (F=0.808, *df*=5, $P>0.5$). A two-way ANOVA of Per2 expression revealed a significant interaction between reproductive state and ZT (F=3.316, *df*=5, $P<0.02$) as well as a significant main effect of ZT (F=4.146, *df*=5, $P<0.005$). In contrast to Fos expression in the SCN core, the rhythm of Per2 expression here differed as a function of reproductive state (Fig. 2.3B). The peak in diestrous females was narrow and occurred at ZT 18. In pregnant rats, the phase of elevated Per2 was longer, and expression was highest at ZT 14, at which point there was a significant difference between the two groups of females.

SCN shell. As in the core, Fos expression in the SCN shell peaked at ZT 2 and dropped to basal levels from ZT 6 through ZT 22 in the diestrous females (Fig. 2.3C; ZT: $X^2=11.918$, *df*=5, $P<0.04$, Kruskal-Wallis test). In pregnant rats, however, expression remained high throughout the light phase and did not reach significantly lower levels from ZT 2 or 6 until ZT 18 (ZT: $X^2=13.791$, *df*=5, $P<0.02$,

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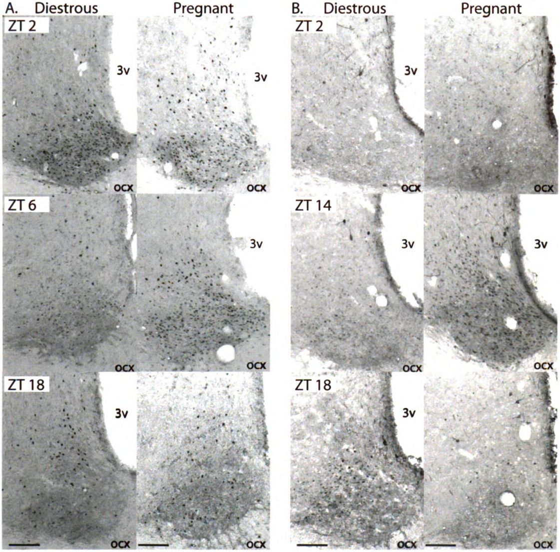
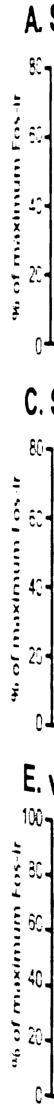


Figure 2.2. Representative photomicrographs of protein expression of Fos at ZT 2, 6, and 18 (A) and Per2 at ZT 2, 14, and 18 (B) in the middle SCN and vSPZ of diestrous (left) and pregnant rats (right) kept in a 12:12 LD cycle. Per2 is only expressed in the cell nucleus. Larger cells in (B) that are stained in the cytoplasm are TH-ir cells and are primarily located around the vSPZ and 3v. ocx, optic chiasm; 3v, third ventricle. Scale bar = 100 μm.



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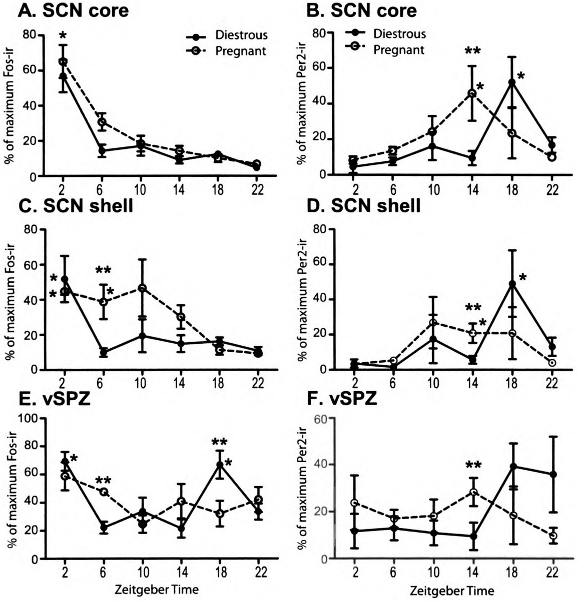

Figure 2.3. Expression of Fos (left column) and Per2 (right column) in the SCN core (A,B), SCN shell (C,D), and vSPZ (E,F) of diestrous and pregnant rats kept in a 12:12 LD cycle. Expression is measured as the percentage of the maximum individual value for each protein in each region. *indicates a time point where expression is significantly elevated relative to at least two other time points within the same reproductive state, and **indicates a time point at which expression significantly differs between reproductive states ($P<0.05$, post-hoc LSD (A,B,E,F) or Mann-Whitney U (C,D) tests).

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Kruskal-Wallis test). At ZT 6, expression was also significantly higher in pregnant than diestrous rats. As in the SCN core, the rhythm of Per2 expression in the SCN shell of diestrous rats peaked at ZT 18 (Fig. 2.3D; ZT: X^2=13.372, *df*=5, P≤0.02, Kruskal-Wallis test), but the rhythm in pregnant females was of a lower amplitude with a broader peak from ZT 10 to ZT 18 (ZT: X^2=12.420, *df*=5, P<0.03, Kruskal-Wallis test). Also, as in the core, expression in the shell of pregnant rats was higher than that of diestrous females at ZT 14. Although interactions cannot be directly assessed with nonparametric tests, these patterns suggest that an interaction between reproductive state and ZT may exist for both Fos and Per2 expression in the SCN shell.

vSPZ. A two-way ANOVA of Fos expression in the vSPZ revealed a significant interaction between reproductive state and ZT (F=3.459, *df*=5, P<0.02) as well as a significant main effect of ZT (F=5.203, *df*=5, P<0.002). In diestrous rats, there was a bimodal rhythm in Fos expression in the vSPZ, with peaks at ZT 2 and ZT 18 (Fig. 2.3E), but no rhythm existed in the vSPZ of pregnant rats (F=1.724, *df*=5, P>0.15, post-hoc one-way ANOVA for ZT effects). Expression in the vSPZ of the two groups differed at ZT 6 (more in pregnant females) and at ZT 18 (more in diestrous females). A two-way ANOVA on Per2 expression in the vSPZ revealed a significant interaction between reproductive state and ZT (F=2.822, *df*=5, P<0.03). There was no rhythm in Per2 expression in the vSPZ in pregnant rats (Fig. 2.3F; F=0.932, *df*=5,P>0.4, post-hoc one-way ANOVA), but a non-significant trend for a rhythm with highest expression in the mid to late dark phase was found in diestrous rats (F=2.191, *df*=5, P<0.095, post-hoc one-way

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ANOVA). Expression differed between the two reproductive states only at ZT 14, as pregnant females again had higher Per2 expression than diestrous females at this time.

Experiment 2

SCN core. Representative photomicrographs depicting patterns of light-induced Fos expression are shown in Fig. 2.4. Light-pulsed pregnant and diestrous rats expressed more Fos in the core than controls at both CT 6 and CT 18, with significantly more light-induced expression evident at CT 18 than at CT 6 in the core of pregnant rats (Fig. 2.5A; U=3,$P<0.02$, Mann-Whitney U test). A similar trend was seen in diestrous females (U=5.5,$P<0.085$, Mann-Whitney U test). There was no significant difference, however, between diestrous and pregnant rats in Fos responses to light in the SCN core at either CT 6 or CT 18 (U=17.5 and 8, $P>0.9$ and 0.2, respectively, Mann-Whitney U tests). Although we could not directly test interactions with nonparametric tests, these data suggest that neither light treatment nor time of perfusion interacted with reproductive state, although they did appear to interact with one another.

SCN shell. A light pulse at CT 6 increased Fos in the SCN shell of diestrous, but not pregnant, females (Fig. 2.5B; U=17, $P>0.9$, Mann-Whitney U test). At CT 18, however, a light pulse led to higher Fos expression in both groups of rats, and there was a trend for higher light-induced expression in pregnant than in diestrous females (U=5.5, $P<0.085$, Mann-Whitney U test). Although we could not directly test interactions with nonparametric tests, these

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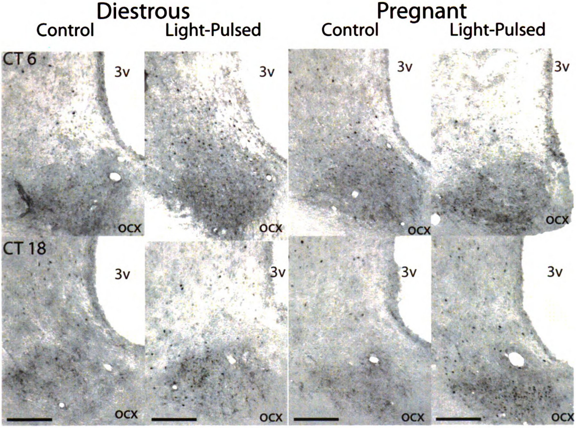

Figure 2.4. Representative photomicrographs of protein expression of Fos in the middle SCN and vSPZ of control and light-pulsed diestrous (left) and pregnant rats (right) at CT 6 (top row) and CT 18 (bottom row). ocx, optic chiasm; 3v, third ventricle. Scale bar = 100 μm.

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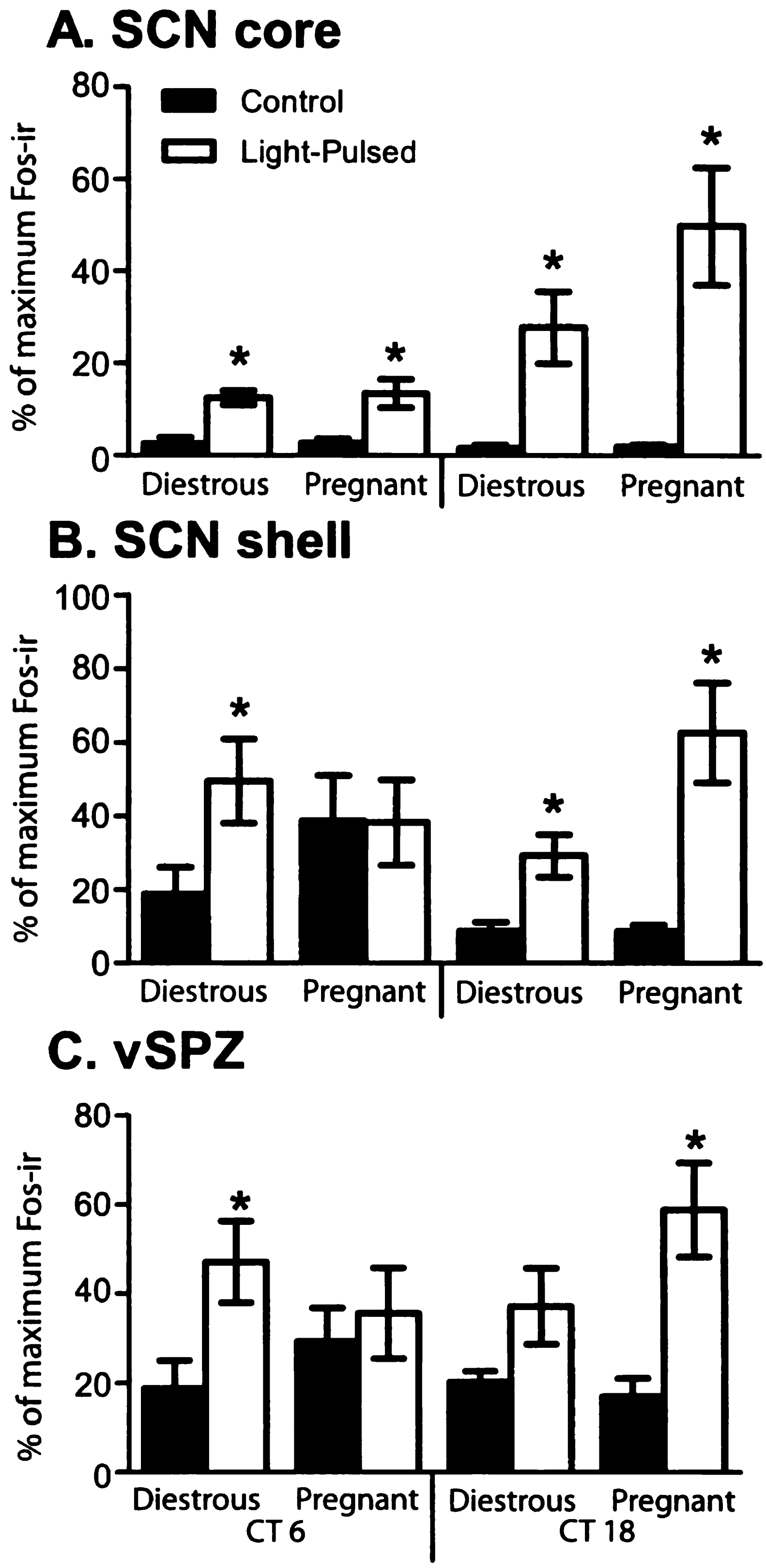

Figure 2.5. Expression of Fos in the SCN core (A), SCN shell (B), and vSPZ (C) of control and light-pulsed diestrous and pregnant rats kept in DD for two days. Expression is measured as the percentage of the maximum individual Fos-ir count within the region of interest. * indicates significantly higher Fos expression between light-pulsed and control females ($P<0.05$, post-hoc LSD (C) or Mann-Whitney U (A,B) tests).

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vSPZ. A three-way ANOVA revealed a significant three-way interaction (reproductive state x light treatment x CT, F=4.374, *df*=1, P<0.05) as well as a main effect of light treatment (F=17.252, *df*=1, P<0.001). Diestrous and pregnant rats exhibited responses to light at opposite times of day, with light-pulsed diestrous females expressing more Fos in the vSPZ than controls at CT 6 and light-pulsed pregnant rats expressing more Fos in the vSPZ than controls at CT 18 (Fig. 2.5C).

DISCUSSION

The patterns of results seen in these two studies reveal that functional properties of the SCN core, SCN shell, and vSPZ, and the relationships amongst these regions, are substantially re-organized during early pregnancy.

The SCN core

Expression of Fos in the SCN core did not differ between pregnant and diestrous rats. In both groups, Fos peaked in this region at ZT 2 and reached baseline levels by ZT 6, a pattern that mirrors those reported previously in rats (Guido et al., 1999; Jac et al., 2000; Schwartz et al., 2004; Schwartz et al., 1994; Sumova et al., 2000; Sumova and Illnerova, 2005). It is likely that the peak at ZT 2 reflects the induction of Fos by lights-on (Schwartz et al., 1994). Light-induced Fos expression in the SCN core, which occurred at both CT 6 and CT 18 but was higher at CT 18, was also the same in pregnant and diestrous rats.

Although no differences in Fos expression were seen in the SCN core, rhythms in Per2 in this region did vary as a function of reproductive state. In diestrous rats, Per2 peaked at ZT 18 and dropped by ZT 22. Previous reports have also shown peaks in Per2 expression in the dark phase in male and cycling female Wistar rats, although at an earlier time than in diestrous rats in this study (Amir et al., 2004; Perrin et al., 2006). In pregnant rats, however, the peak of the Per2 rhythm in the core occurred four hours earlier (Fig. 2.3B). This led to a new pattern of coupling between Fos and Per2 rhythms. This might reflect functionally distinct cell populations, or differences within cells. For example, transcriptional pathways driving Fos and Per2 expression may be differentially affected by pregnancy.

The SCN shell

In the SCN shell of animals kept in an LD cycle, the rhythm of Fos expression mirrored that in the core in diestrous females, with Fos rising sharply from ZT 22 to ZT 2 and then dropping by ZT 6. In pregnant rats, Fos also rose sharply from ZT 22 to ZT 2. However, in this case, high levels were maintained throughout the light phase. The heightened Fos expression in the SCN shell at ZT 6 was not due to an increase in responsivity to photic cues, as light did not induce Fos in pregnant rats at this time but did in diestrous ones (Fig. 2.5B).

Interestingly, at CT 18 there was a trend for a higher response to light in the SCN shell in pregnant females than in diestrous ones. This may be driven by elevated levels of circulating prolactin. The SCN expresses mRNA for the short form of the prolactin receptor on day 2 of pregnancy (Bakowska and Morrell, 2003), and it may do so throughout pregnancy, although no one has examined this. Furthermore, although the effects of prolactin on the SCN of rats are not known, in male hamsters, prolactin increases light-induced Fos in the SCN during the mid-dark phase of the LD cycle (Cutrera et al., 2002). Perhaps prolactin has a similar effect in the SCN shell in pregnant rats. It is unclear, however, whether effects of prolactin are different at mid-day than at mid-night. Circulating prolactin is nearing its peak at ZT 18 and reaches trough levels by ZT 6 on day 6 of pregnancy (Butcher et al., 1972; Freeman et al., 2000), which may be why the SCN shell is unresponsive at CT 6 and hyper-responsive at CT 18.

As was the case in the core of the SCN, the peak in the rhythm of Per2 expression in the shell advanced during early pregnancy. However, whereas the Per2 rhythm in the shell mirrored that in the core in diestrous rats, in pregnant rats, the phase of elevated Per2 in this region appeared to be broader, and levels were lower than in diestrous females. Although the Per2 peak appeared lower, the peak of Fos expression in the shell did not change in magnitude. However, the peaks of expression of both proteins were broadened in the shell of pregnant rats, and these trends could be linked. The shell is less heterogeneous in cell phenotype than the core, so perhaps Fos and Per2 are more often co-expressed in the shell, most likely in AVP cells (Dardente et al., 2002). This could explain why the changes their rhythms undergo in pregnancy are similar, as they may both reflect altered patterns of activity of AVPergic outputs from the shell.

The SCN core vs. shell

The difference between the SCN core and shell with respect to the plasticity of their rhythms in Fos expression during early pregnancy is intriguing. In a previous study, Lee et al. (1998) found that Fos rhythms in the SCN core, but not the shell, differed between early and late pregnant rats. Thus, whereas the transition from diestrus to early pregnancy is associated with changes in Fos rhythms in the shell but not the core (Fig. 2.3), the opposite is the case during the transition from early to late pregnancy. When we compare our data from early pregnancy to those of Lee et al. (1998) on the same day, we see similar trends in the two subregions. However, patterns in the core were somewhat different in the two studies because animals were not sampled at the same times of day. Lee et al. (1998), who did not sample between ZT 0 and ZT 4, may have missed the peak that occurred around ZT 2 (Schwartz et al., 1994). Given the similarities between the two studies on day 6 of pregnancy, it appears that, during late pregnancy, both subregions of the SCN should exhibit different Fos rhythms from those in a non-pregnant female, since changes in Fos expression in the shell during early pregnancy are conserved in late pregnancy, and the rhythm of Fos expression in the core changes from early to late pregnancy. Therefore, we cannot conclude that Fos expression in the core is resistant to changes in reproductive state in females, as might be deduced from our data alone, but rather that Fos expression is affected differently in the SCN core and shell at different stages of pregnancy.

Although Fos expression was differentially affected in the SCN core and shell during early pregnancy, the peaks of Per2 rhythms in both regions were

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advanced. These advances may be due to changes in ovarian hormone secretions. Nakamura et al. (2005) demonstrated that estradiol treatment of ovariectomized (OVX) rats phase-advances the peak of *Per2* mRNA expression from ZT 12 to ZT 8 and also reduces the amplitude of this rhythm in the SCN, but they did not examine the core and shell separately. Although progesterone and prolactin are the predominant reproductive hormones during early pregnancy, estrogens are also slightly elevated relative to diestrus. If estrogens can lead to a phase-advance in Per2 expression, this might explain how they advance the phase of behavioral rhythms of OVX females (Albers, 1981; Gerall et al., 1973; Morin et al., 1977; Thomas and Armstrong, 1989). This effect may not occur in early pregnancy, however, as progesterone counteracts the effects of estradiol on activity rhythms in OVX females. However, the physiological milieu of a pregnant rat is fundamentally different from that of an OVX, hormone-treated one, and individual hormones may induce different effects on the circadian system of females in the two reproductive states.

Potential sex differences in the SCN

Although male rats have repeatedly shown strong induction of Fos expression after a light pulse in the subjective night, it has long been thought that the SCN is non-responsive during the subjective day (Edelstein et al., 2000; Guido et al., 1999; Rusak et al., 1990; Schwartz et al., 1994; Schwartz et al., 2000). However, in our study, both diestrous and pregnant rats clearly expressed more Fos in the SCN core after a light pulse at CT 6, and the shell was also responsive in the diestrous rats at this time. These results therefore suggest that

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a sex difference may exist in how the SCN responds to light in this species. We do not know if this difference is dependent on gonadal hormones, but estradiol increases Fos expression in the SCN of female rats during the light phase (Abizaid et al., 2004; Peterfi et al., 2004). This action may be direct, since estrogen receptor- β (*ER-β*) is expressed in the SCN of female rats (Shughrue et al., 1997), and this may explain why the SCN in our females was responsive to light during the subjective day, while those of males in previous studies were not. Interestingly, the rhythm of SCN *VIP* mRNA transcription is out of phase in male and female rats, peaking in the dark phase in males and the light phase in females (Krajnak et al., 1998). Therefore, the high expression of *VIP* mRNA in the day may either lead to or be a result of our observed Fos responsivity in the core to light in the mid-subjective day in non-pregnant females.

Changes in the vSPZ during early pregnancy

Fos expression in the vSPZ was quite different in diestrous and pregnant females. The vSPZ of diestrous rats exhibited a clear bimodal rhythm of Fos expression, with peaks at ZT 2 and again at ZT 18. This pattern contrasts with that seen in the SCN shell, confirming that the vSPZ is not a functional extension of the shell. The second peak was lost during early pregnancy, when expression was highest from ZT 2 to ZT 6. The vSPZ of laboratory rats has not been thoroughly described, but, in males, there is a unimodal, light-dependant rhythm in this region, and it is very similar to that seen here in pregnant females (Schwartz et al., 2004). The disappearance of the rhythm in DD in the males of that study suggests that the peak at ZT 1 is stimulated by light onset (Schwartz et al. 2004). V

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al., 2004), which is also likely to be the case for the peak at ZT 2 in both groups of females in the current study. The second peak, at ZT 18, which is seen in diestrous but not pregnant females, is more difficult to explain. This rise has not been seen in male rats, although it is possible that it would if they had been sampled at ZT 18. This seems unlikely, however, as males have low levels of Fos in the vSPZ at both ZT 17 and ZT 20 (Schwartz et al., 2004). The causes of this potential difference between the sexes, and of the change from a diestrous to a pregnant state, are unclear but may be related to changes in communication between the SCN and the SPZ.

Under DD conditions, there were no time of day effects on Fos expression in the vSPZ in either group of females. However, in diestrous females, a light pulse induced Fos in the vSPZ at CT 6 but not CT 18, whereas in pregnant females, the opposite pattern was observed (Fig. 2.5C). Intriguingly, this parallels the differences in Fos expression at ZT 6 and ZT 18 in an LD cycle (Fig. 2.3E), in that rats of both groups exhibited a response to light at a time when Fos was low at the corresponding time in LD. In DD, however, no variation due to time of day or reproductive state was apparent in Fos expression in the absence of a light pulse (Fig. 2.5C). This suggests that the Fos rhythm seen in the vSPZ of female rats kept on an LD cycle may not be endogenous, which is also the case for males (Schwartz et al., 2004). However, the patterns of response to light pulses suggest that an endogenous rhythm in responsiveness to light may exist in the vSPZ and that this rhythm is very different in pregnant and diestrous females.

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No significant rhythm in Per2 expression was found in the vSPZ of our pregnant or diestrous rats, but there was a trend in the vSPZ of diestrous females ($P < 0.095$), with highest expression at ZT 18 and 22, when Per2 was low in the vSPZ of pregnant rats. Additionally, Per2 was higher in the vSPZ of pregnant rats at ZT 14, which coincided with peak Per2 expression in the SCN. This indicates that Per2 expression in the vSPZ, while not significantly rhythmic, is likely to be influenced either by the SCN or by the same mechanisms that influence Per2 in the SCN. Also, given that Fos expression is distinctly rhythmic in the vSPZ of diestrous females and that light can induce Fos expression in the vSPZ of both diestrous and pregnant rats, it is unlikely that Per2 expression in the vSPZ drives Fos expression in this region. Instead, inputs from the SCN may do so.

The vSPZ appears to serve as a modulator of SCN output signals and integrator of these with other internally driven processes and environmental cues (Saper et al., 2005; Schwartz et al., 2004; Schwartz et al., 2009). It is also necessary for the generation of activity rhythms in rats (Abrahamson and Moore, 2006; Lu et al., 2001). During early pregnancy, when locomotor rhythms are lost in DD (Rosenwasser et al., 1987), neither Fos nor Per2 expression in the vSPZ were rhythmic. This is especially intriguing because core body temperature, which is normally correlated with activity (Refinetti, 1997; Refinetti, 1999), remains robustly rhythmic (Kittrell and Satinoff, 1988). The loss of rhythms in the vSPZ reported here might therefore be responsible for this dissociation between body tempe

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The role of the SCN and vSPZ during early pregnancy

The changes in Fos and Per2 rhythms seen in this study may contribute to changes in physiological and behavioral rhythms that occur during this critical phase of pregnancy. This is supported by some comparisons between patterns seen in our data and those from previous reports of rhythms in early pregnancy, such as that noted above between the loss of locomotor activity and vSPZ rhythms. Similarly, the peak of the core body temperature rhythm is phase-advanced by a few hours (Kittrell and Satinoff, 1988), as is Per2 expression in the SCN, and the peaks of circulating ACTH and corticosterone rhythms are lowered (Atkinson and Waddell, 1995), as is the peak of Per2 expression in the SCN shell. Although there are no immediately apparent direct correlations between patterns seen in our data and the emergence of bimodal rhythms in prolactin or in the increase in nighttime sleep (Butcher et al., 1972; Kimura et al., 1996), it is still possible that the changes we observed contribute to these effects.

One important question raised by our results is how the changes in Fos and Per2 expression in the core, shell, and vSPZ associated with pregnancy are related to each other. One possibility is that the advance in phase of the Per2 rhythm in the core leads to changes in signals that it sends to the shell and to the vSPZ, where input from the core and shell converge (Antle and Silver, 2005; Daikoku et al., 1992; Ibata et al., 1993; Ibata et al., 1997; Leak and Moore, 2001; Moore et al., 2002; Watts and Swanson, 1987; Watts et al., 1987; Watts, 1991).

This model would require only a change in how the core communicates to the shell and vSPZ to account for the suite of changes in the rhythms that emerge during pregnancy. It predicts that pregnancy should be accompanied by changes in rhythms in core output signals, such as VIP and GRP, that are mirrored by altered rhythms in shell outputs, such as AVP, and output signals from the vSPZ, which have not yet been characterized.

Changes in processes seen here are likely to reflect only a few of the mechanisms responsible for the varied effects of pregnancy on overt rhythms. Other components of the circadian system, either within or beyond the SCN and vSPZ, and regulatory networks outside of the circadian system altogether are probably involved as well. Further examination of how these components change will be necessary to determine how overt rhythms are altered during early pregnancy.

CHAPTER 3

Differential changes in extra-SCN oscillators during early pregnancy in the rat

INTRODUCTION

Circadian rhythms allow organisms to predict changes in the environment and appropriately time behavioral and physiological events to anticipate them (Moore-Ede et al., 1982), and they are critical to the regulation of mammalian reproduction. During pregnancy, many overt rhythms in physiology and behavior are altered in various ways. For example, in laboratory rats, activity rhythms dampen while sleep increases during the dark phase (Kimura et al., 1996; Rosenwasser et al., 1987), and the peak of the body temperature rhythm advances (Kittrell and Satinoff, 1988). In these animals, a novel prolactin rhythm, with peaks around dawn and dusk, emerges in early pregnancy and promotes progesterone secretion by the corpora lutea (Butcher et al., 1972; Freeman et al., 2000). Rhythms in the hypothalamic-pituitary-adrenal axis also change, with a reduction in peak secretion of both corticotropin (ACTH) and corticosterone (Atkinson and Waddell, 1995). While the establishment of the prolactin rhythm has been well-studied (Erskine, 1995; Erskine et al., 2004; Freeman et al., 2000; Lee et al., 1998), the neural mechanisms mediating changes in other rhythms have received little attention.

Most daily rhythms are generated by endogenous circadian mechanisms in the brain, and changes in them during early pregnancy may be promoted by changes in these mechanisms. The suprachiasmatic nucleus (SCN), located bilaterally in the anterior hypothalamus, houses the primary pacemaker in mammals (Moore and Eichler, 1972; Ralph et al., 1990; Rusak, 1977; Stephan and Zucker, 1972). The SCN contains oscillator cells, which each express a molecular transcription/translation feedback loop that takes roughly 24 hours to complete. This "molecular oscillator" involves multiple "clock" proteins, including positive and negative elements, such as the period (Per) proteins, which are themselves transcription factors for other genes, called clock-controlled genes (Bell-Pedersen et al., 2005; Dunlap, 1999; Reppert and Weaver, 2002). Extra-SCN oscillators, in which the same molecular feedback loops are expressed, have been found within the brain and throughout the periphery and, together with the SCN, comprise the circadian system (Guilding and Piggins, 2007; Hastings et al., 2003; Reppert and Weaver, 2002; Weinert, 2005). The SCN is necessary to synchronize most of these regions with one another, but there are conflicting data on the extent to which extra-SCN oscillators within these regions require the SCN to maintain internal synchrony, with some being less dependent on it than others (Yamazaki et al., 2000; Yoo et al., 2004). It has been suggested that the function of extra-SCN oscillators may be to integrate non-photic stimuli with signals from the SCN to coordinate intracellular processes that vary from one brain region or peripheral tissue to another (Guilding and Piggins, 2007; Herzog and Tosini, 2001; Weinert, 2005; Yoo et al., 2004).

In Chapter 2, we demonstrated that both Fos and Per2 rhythms in the SCN are altered during early pregnancy and argued that this may be part of the mechanism driving changes in overt rhythms. However, other components of the circadian timekeeping system, such as extra-SCN oscillators, may also play a role. This seems particularly likely given that different overt rhythms are changing in varied ways. In this study, we sought to evaluate this hypothesis by examining Per2 expression patterns in oscillators within regions that regulate functions that change during early pregnancy. We evaluated rhythms in Per2 expression in diestrous and early pregnant rats first in three portions of the extended amygdala: the oval nucleus of the bed nucleus of the stria terminalis (BnST-ov), central amygdala (CEA), and basolateral amygdala (BLA). The extended amygdala is involved in regulating motivation and emotional state as well as stress responses (Cardinal et al., 2002; Davis et al., 1997; Dong et al., 2001; Erb et al., 2001; Szafarczyk et al., 1983), including insomnia (Cano et al., 2008). During early pregnancy, female rats may exhibit reduced levels of anxiety (Macbeth et al., 2008; but see Picazo and Fernandez-Guasti, 1993; Neumann et al., 1998), and the amplitudes of both the ACTH and cortisol rhythms are reduced (Atkinson and Waddell, 1995). We also examined patterns of Per2 expression in neuroendocrine dopaminergic cells that tonically inhibit prolactin secretion (Ben-Jonathan, 1985; Freeman et al., 2000), which exhibits a novel semicircadian rhythm in early pregnancy (Butcher et al., 1972).

EXPERIMENTAL PROCEDURES

Animals

Animals were the adult female and male Sprague-Dawley laboratory rats used in Chapter 2 and obtained from Harlan Laboratories (Indianapolis, IN, USA). Males were housed in pairs or groups of three when not paired with females. Females were housed in groups of three or four for a habituation period of at least two weeks and were subsequently separated for single-housing. All subjects were housed in polypropylene cages (48x27x20 cm) with *ad libitum* access to food (Teklad 8640 rodent diet, Harlan) and water under a 12h:12h light/dark (LD) cycle with lights on at Zeitgeber Time (ZT) 0 and off at ZT 12. A dim red light (<5 lux) remained on constantly for animal care purposes. All experiments were performed in compliance with guidelines established by the Michigan State University Institutional Animal Care and Use Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to keep the number of animals used and their discomfort at a minimum.

Determination of estrous cycle stages

Once singly housed, females were subjected to daily vaginal smears between ZT 0 and ZT 3 to track their estrous cycles. A cotton-tipped applicator soaked in sterile physiological saline was inserted into the vaginal opening, gently rotated, and withdrawn. The applicator was immediately rolled onto a glass slide and allowed to air dry. The dried slides were soaked in 0.25% methylene blue dye for five minutes, rinsed with distilled water, and allowed to dry prior to examination under a light microscope for the presence of nucleated epithelial cells, cornified epithelial cells, leukocytes, and mucus. Estrous cycle stage was determined as follows: proestrus: predominantly nucleated epithelial cells; estrus: predominantly to exclusively cornified epithelial cells; diestrus (between 2 to 3 consecutive days): predominantly leukocytes, some cornified and/or nucleated epithelial cells and mucus present. At least three estrous cycles were established for each female.

Experimental Protocol

One group of females was perfused (see below for details) on day one of diestrus, and another on day 6 of pregnancy. To obtain the second group, a male was placed in the female's cage on the morning of proestrus (between ZT 0 and 3). Males were removed the next morning, and a vaginal smear was taken to check for the presence of sperm. If none were detected, the female was re-paired on the morning of her next proestrus period. Once mating had been verified, vaginal smears were discontinued, and the day of confirmation was designated as day 0 of pregnancy. Females were perfused 6 days later, and pregnancy was confirmed by visual inspection of the uterus.

Tissue collection and immunocytochemistry

Tissue for this study was collected and processed in a previous experiment (Chapter 2). Groups of animals from each reproductive state were perfused at ZT 2, 6, 10, 14, 18, or 22. Each animal was given an overdose of sodium pentobarbital, and perfused transcardially with 0.01 M phosphate-buffered saline (PBS), pH 7.2, followed by 4% paraformaldehyde (Sigma, St. Louis, MO) in 0.1 M phosphate buffer with 1.3% lysine and 4% sodium periodate. Animals perfused during the dark phase were fitted with a light-tight hood prior to perfusion to prevent acute exposure to light. Brains were post-fixed for 4 hours, transferred to 20% sucrose solution overnight, and stored in cryoprotectant at 4 °C overnight then at -20 °C until sectioning. Brains were sectioned coronally at

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30 µm into three alternate series using a freezing microtome, and sections were placed in cryoprotectant for further storage at -20 °C.

One series of tissue was processed for immunocytochemical staining for double labeling of Per2 and tyrosine hydroxylase (TH). Free floating sections were rinsed in 0.01 M PBS and then incubated in 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) in PBS with 0.3% Triton X-100 (TX) for 1 h at room temperature. After a 10 minute rinse in PBS, sections were incubated with a primary anti-Per2 antibody (mPER2 # 38, made in rabbit, 1:5,000, 24 h incubation; a generous gift from Dr. David Weaver, University of Massachusetts, MA, USA) in 3% NGS and 0.3% TX in PBS on a rotator at 4°C for 48 h. Sections were then rinsed in PBS and incubated with a biotinylated secondary goat-anti-rabbit antibody (Vector; 1:200) in 3% NGS and 0.3% TX in PBS for 1 h at room temperature, rinsed again, and then incubated with an avidin-biotin peroxidase complex (0.9% each avidin and biotin solutions; ABC Vectastain kit; Vector) in 0.3% TX with PBS for 1 h at room temperature. Next, sections were rinsed in 0.125 M acetate buffer, pH 7.2, then reacted with diaminobenzidine (DAB; 0.25 mg/mL, Sigma), nickel sulfate (25 mg/mL, Sigma), and hydrogen peroxide (0.825 µL 0.3% H_2O_2 /mL buffer) to yield a blue-black reaction product. After the reaction for Per2 staining, to process the tissue for TH expression, the same sections were rinsed first in 0.1 M phosphate buffer (PB) and then in 0.2% TX in 0.2 M PB (PB-TX), and they were then incubated in 5% normal horse serum (NHS; Vector) in PB-TX for 30 min at room temperature. After rinses in PB-TX, sections were incubated with a primary anti-TH antibody (made in mouse, Immunostar, Hudson, WI, USA; 1:20,000) in 3% NHS and PB-TX on a rotator at 4°C for 24 h. Sections were then rinsed in PB-TX and incubated with a biotinylated secondary horse-anti-mouse antibody (Vector; 1:200) in 3% NHS and PB-TX for 1 h at room temperature, rinsed again, and then incubated with an avidin-biotin peroxidase complex (0.9% each avidin and biotin solutions; ABC Vectastain kit; Vector) in PB-TX for 1 h at room temperature. Next, sections were rinsed in Trizma buffer (pH 7.2, Sigma) then reacted with DAB (0.2 mg/mL, Sigma) and hydrogen peroxide (0.35 µL 30% H_2O_2 /mL buffer) to yield a brown reaction product. Trial immunocytochemical reactions with the reagents used for this reaction were performed with tissue from females that did not have easily determined estrous cycles or had mated but were not pregnant to ensure specificity of the primary antibodies (data not shown). All sections were mounted on clean slides, dehydrated, and coverslipped.

Quantitative and Statistical Analysis

For single-label Per2 counts in the BnST-ov, CEA, and BLA, bilateral images were taken from three sections of each region using a CCD video camera (CX900, MBF Bioscience, Williston, VT, USA) attached to a light microscope (Zeiss, Göttingen, Germany). These images were processed using Adobe Photoshop 7 (Adobe Systems, Mountain View, CA, USA). Per2-immunoreactive (-ir) cells were then counted in these images with the NIH ImageJ program (NIH, Bethesda, MD, USA). For the CEA and BLA, a 400 µm x

400 µm box was used to count Per2 cells, while the entire area of the BnST-ov was counted (Fig. 3.1).

Preliminary examination of the three neuroendocrine dopaminergic cell populations that inhibit prolactin secretion by the pituitary gland revealed that double-labeled staining in the A12 tuberoinfundibular (TIDA) and tuberohypophyseal (THDA) dopaminergic groups of the arcuate nucleus could not be reliably identified. However, this was not the case in the A14 dopaminergic cells of the periventricular hypothalamus (PHDA). Therefore, we examined co-expression only in these cells. PHDA neurons exhibit the same Per2 rhythm as the TIDA and THDA in ovariectomized (OVX) rats (Sellix et al., 2006) and the same rhythm in expression of Fos-related antigens (FRA) as the other two cell groups during pseudopregnancy in OVX females (Lerant et al., 1996). A 250 µm x 250 µm box was used to count the number of PHDA cells expressing TH and the number of cells co- expressing TH and Per2 bilaterally through three sections with a light microscope equipped with a drawing tube. These boxes were placed adjacent to the third ventricle, just ventral to the midpoint of its length (Fig. 3.1). All counts were made by an investigator unaware of the reproductive state or time of perfusion of each animal.

A total of 57 females were used in the final analysis (*n*=28 diestrous, *n*=29 pregnant, *n*=4-6 per time point for each reproductive state). A two-way analysis of variance (ANOVA) was performed on the number of Per2-ir cells in the CEA with reproductive state (pregnant or diestrous) and time of perfusion (ZT) as between-subjects factors. Nonparametric analyses were performed on data from

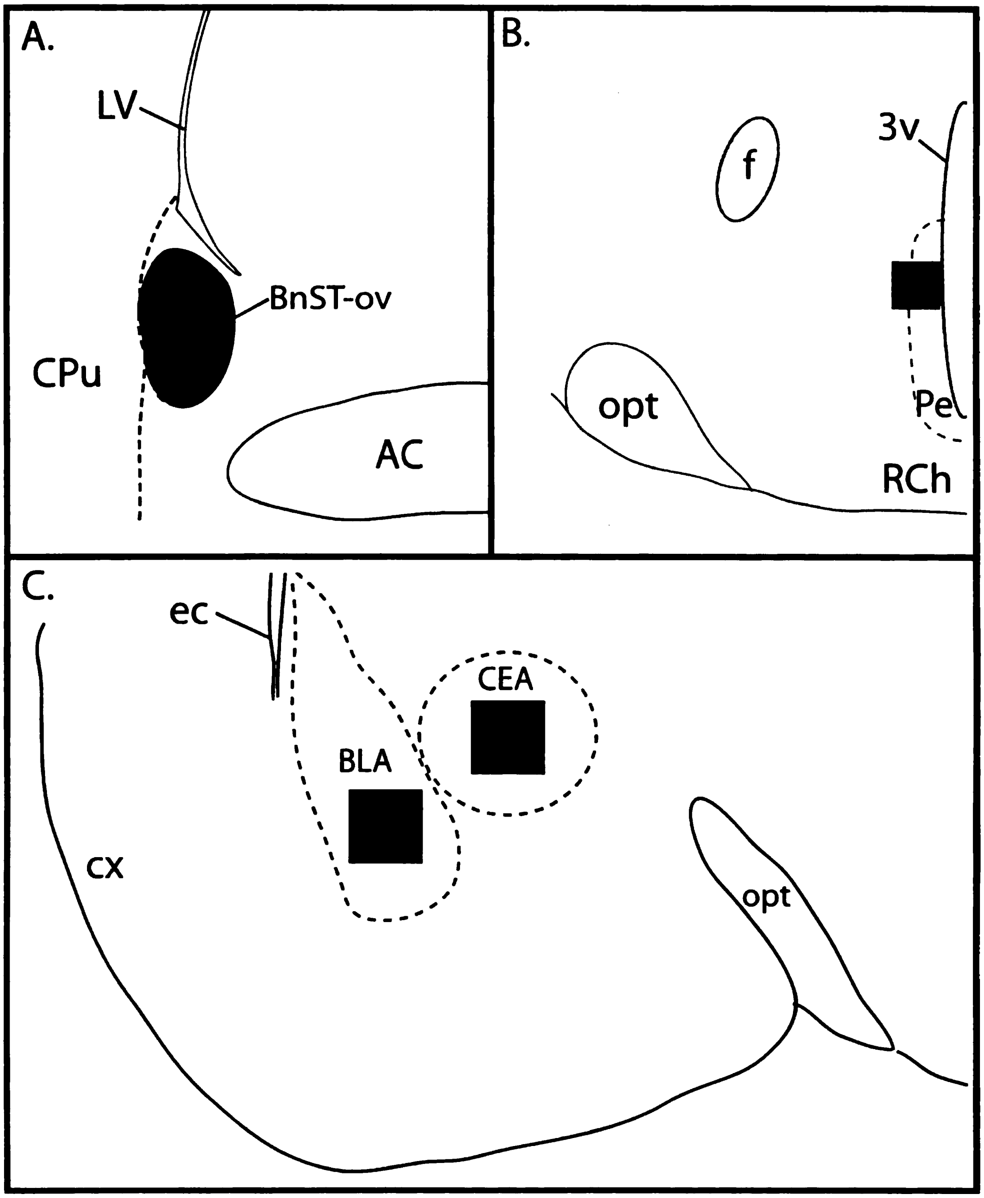

Figure 3.1. Line drawings depicting location of the BnST-ov (A), PHDA (B), CEA, and BLA (C). Sampling boxes (black squares) were used for cell counts in the PHDA, CEA, and BLA. Anatomical boundaries are based on Paxinos and Watson (1997). 3v: third ventricle; AC: anterior commissure; CPu: caudate putamen; cx: cerebral cortex; ec: external capsule; f: fornix; LV: lateral ventricle; opt: optic tract; Pe: periventricular hypothalamic nucleus; RCh: retrochiasmatic area.

the BnST-ov and BLA, as these datasets did not meet homogeneity of variance criteria, even with square-root transformation. We compared the effects of reproductive state at each ZT (Mann-Whitney U tests) and of ZT within each reproductive state (Kruskal-Wallis tests, followed by post-hoc pairwise Mann-Whitney U tests if significant effects were found). We also examined the main effects of ZT and reproductive state for the entire dataset using the same statistical tests. To examine whether rhythms in Per2 expression in TH-ir cells in the PHDA differed between diestrous and pregnant rats, we calculated the proportion of TH-ir cells that co-expressed Per2 for each female and subsequently arcsine-transformed these data. We then performed a two-way ANOVA on the transformed data with reproductive state and ZT as between-subjects factors. We performed a similar two-way ANOVA on the total number of TH-ir cells to determine whether this varied across time or between reproductive states. Post-hoc least significant differences (LSD) tests were conducted when significant main effects were found. All data analyses were conducted with SPSS 17 software (SPSS Inc., Chicago, IL, USA). All differences were considered significant when $P<0.05$. Statistical details are omitted from the text when they are presented in figure legends. Data are presented as means ± SE.

RESULTS

Oval nucleus of the bed nucleus of the stria terminalis

The expression of Per2 changed as a function of time in diestrous females (ZT: $X^2=11.832$, $df=5$, $P<0.04$, Kruskal-Wallis test), with peak values occurring from ZT 14 to 18, and trough values at ZT 22 (Fig. 3.2A). Per2 also changed as a

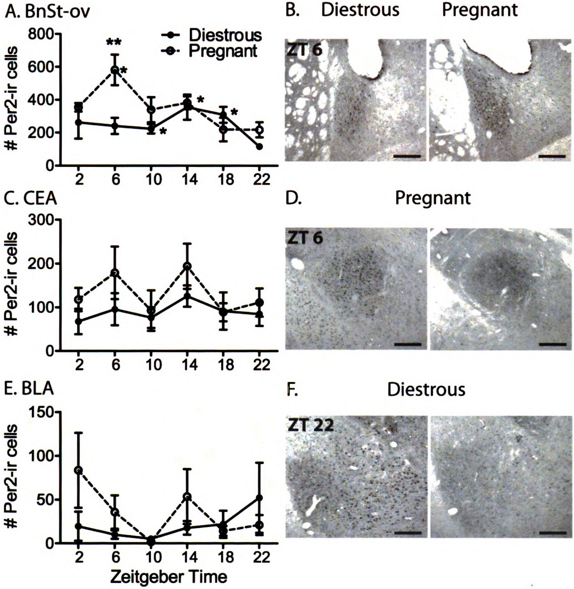

Figure 3.2. Expression of Per2 in the BnST-ov (A,B), CEA (C,D), and BLA (E,F) of diestrous and pregnant rats kept in a 12:12 LD cycle. (A,C,E): * indicates a time point where Per2 expression is significantly elevated relative to at least one other time point within the same reproductive state, and ** indicates a time point at which expression significantly differs between reproductive states (P<0.05, post-hoc Mann-Whitney U tests). (B,D,F): Representative photomicrographs depicting between group differences in Per2 expression in the BnST-ov at ZT 6 (B), and within group variation at ZT 6 in the CEA (D) and at ZT 22 in the BLA (F). Scale bars= 200 μm.

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function of time in pregnant females, but in this case, peak numbers of labeled cells were present at ZT 6, at which time levels were significantly higher than in diestrous females (Fig. 3.2A and B; ZT: X^2=13.391, *df*=5, P<0.02, Kruskal-Wallis test). Thus, although interactions cannot be directly assessed with nonparametric tests, these results reveal that rhythms in Per2 expression in the BnST-ov are quite different in pregnant and diestrous females.

Central and basolateral amygdala

A two-way ANOVA revealed no significant effect of ZT (F=1.274, *df*=5, P>0.2) and no interaction between ZT and reproductive state (F=0.362, *df*=5, P>0.8) on Per2 expression in the CEA (Fig. 3.2C). A non-significant trend for a main effect of reproductive state, with higher expression in pregnant than diestrous rats, was found (pregnant: 129.4±17.1 cells; diestrous: 89.4±12.5 cells; F=3.416, *df*=1, P<0.075), and expression in both groups was highly variable (Fig. 3.2D).

The density of Per2 labeled cells was much lower in the BLA than the CEA (Fig. 3.2E). Neither ZT nor reproductive state had any effect on the number of labeled cells in the BLA (ZT: X^2=9.338, *df*=5, P>0.09, Kruskal-Wallis test; reproductive state: U=356, P>0.15, Mann-Whitney U tests). As in the CEA, Per2 expression in both groups was highly variable (Fig. 3.2F).

A14 periventricular hypothalamic dopaminergic neurons

The total number of TH-ir neurons sampled in the PHDA varied from 31 to 81 cells (mean 50.9±1.3 cells), but the expression of TH did not differ between reproductive states and was not rhythmic (Reproductive state: F=0.009, *df*=1,

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P>0.9; ZT: F=0.572, *df*=5, P>0.7; Interaction: F=1.044, *df*=5, P>0.4, two-way ANOVA). The expression of Per2 in these neurons, however, was rhythmic and did differ as a function of reproductive state, although the effects of these variables did not significantly interact (Fig. 3.3; Reproductive state: F=5.508, *df*=1, P<0.025; ZT: F=2.917, *df*=5, P<0.025; Interaction: F=1.111, *df*=5, P>0.3, two-way ANOVA). Overall, pregnant rats expressed Per2 in a higher percentage of TH-ir cells (36.6±2.6%) than diestrous females (27.9±2.7%), and the rhythm in expression in the combined groups peaked from ZT 6 to ZT 10, with lowest expression occurring at ZT 18 and 22. While no significant interaction was found, examination of the data indicates that the peak at ZT 6 was primarily due to elevated Per2 expression in the pregnant rats, and a rise appears to have occurred between ZT 6 and ZT 10 in diestrous but not pregnant females (Fig. 3.3A).

DISCUSSION

The current data reveal the emergence of new patterns of relationships amongst extra-SCN oscillators during early pregnancy. The BnST-ov, CEA, and BLA are part of the extended amygdala and play critical roles in the regulation of fear, anxiety, and stress responses (Cardinal et al., 2002; Davis et al., 1997; Dong et al., 2001; Erb et al., 2001; Szafarczyk et al., 1983). In diestrous rats, Per2 expression was not rhythmic in either the CEA or BLA, and the rhythm of Per2 expression in the BnST-ov was blunted, with highest expression occurring in the early to mid-dark phase. The patterns in the CEA and BnST-ov seen here are similar to those reported by Perrin et al. (2006) from cycling rats on metestrus

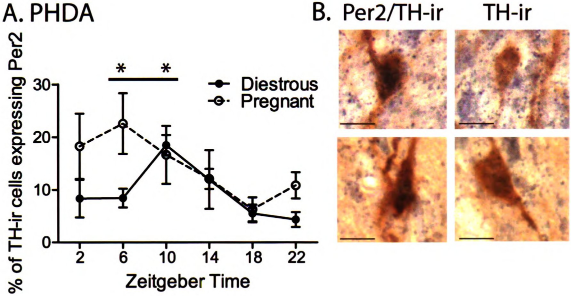

Figure 3.3. Expression of Per2 in TH-expressing neurons in the PHDA of diestrous and pregnant rats kept in a 12:12 LD cycle (A). There was no significant interaction between reproductive state and ZT, and asterisks (*) indicate time points where Per2 expression in TH cells is significantly elevated relative to at least two other time points in the combined (pregnant and diestrous) rhythm. (B) Representative photomicrographs depicting Per2/TH-ir (left: purple/brown) and TH-ir (right: brown) cells in the PHDA in two different individuals (each row). Scale bars = 10 μm.

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(the equivalent of day 1 of diestrus). However, Per2 expression in the BLA in that study was distinctly rhythmic, with peak expression occurring in the early light phase, which was not the case in our diestrous rats. The reasons for the discrepancy between their results and ours are unclear, but it may be due to the use of different Per2 antibodies.

Although Per2 expression in the CEA and BLA did not significantly change during early pregnancy, the peak of the rhythm of expression in the BnST-ov was higher and occurred roughly 8 to 12 hours earlier in pregnant rats than in diestrous ones. Specifically, expression peaked at ZT 6 in pregnant females, whereas in diestrous rats, it peaked from ZT 14 through 18 (Fig. 3.2A). A similar increase in peak expression of Per2 in the BnST-ov occurs from diestrus to proestrus and estrus, but on the latter days, it occurs around ZT 13 and is also evident in Per2 expression in the CEA (Perrin et al., 2006). The dissociation between Per2 expression in the CEA and BnST-ov has not, to our knowledge, been found under any of the many experimental conditions in which Per2 has been examined. Specifically, Per2 expression in these two nuclei responds in the same manner to fluctuations in various hormones, lesions of the SCN, removal of the adrenal glands, dopaminergic manipulation, and food restriction (Amir and Stewart, 2009a; Amir and Stewart, 2009b). The current data raise the question of what might cause such a dissociation to occur in early pregnancy. The CEA projects to the BnST-ov; thus it is possible that either signals sent from the CEA or responses to those signals in the BnST-ov are altered during early pregnancy.

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Corticotropin-releasing factor (CRF) and enkephalin are both expressed in cells within the BnST-ov and the CEA, including some that also contain neurotensin (Day et al., 1999; Dong et al., 2001; Honkaniemi et al., 1992). Per2 is expressed in the enkephalin-containing cells of the BnST-ov of male rats (Amir et al., 2004; Lamont et al., 2005), where the rhythm in its expression is influenced by the CRF neurons (Amir and Stewart, 2009a; Amir and Stewart, 2009b; Bhargava et al., 2004). We do not know whether CRF expression is different in the BnST-ov on day 6 of pregnancy. However, at this time, rhythms in ACTH and corticosterone secretion have reduced amplitudes, and ACTH secretion rises earlier and remains elevated longer than in diestrous females (Atkinson and Waddell, 1995). Although this advance is less dramatic than that in peak Per2 expression in the BnST-ov, it may also be, in part, a result of changes in CRF expression in the BnST-ov. Another possibility is that changes in Per2 expression in enkephalin cells could drive altered functioning of the CRF cells and lead to downstream effects on stress hormone secretion and behavioral responses to stress and anxiety.

Pregnancy is a reproductive state accompanied by a decline in stress hormone secretion and a potential reduction in anxiety (Atkinson and Waddell, 1995; Macbeth et al., 2008; but see Picazo and Fernandez-Guasti, 1993; Neumann et al., 1998), both of which are regulated by the BnST-ov (Davis et al., 1997; Dong et al., 2001; Erb et al., 2001; Szafarczyk et al., 1983). The inverse relationships between pregnancy-related changes in stress/anxiety and Per2 expression in the BnST-ov are most likely linked, as rhythmic expression of Per2 in this re

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in this region is dependent upon rhythmic exposure to glucocorticoids (Segall et al., 2006). Therefore, our observed increase in peak Per2 expression in the BnST-ov may reflect an increased sensitivity to feedback from adrenal hormones.

As in the BnST-ov, Per2 expression in the PHDA cells was elevated in pregnant relative to diestrous rats. This difference was driven by heightened expression in the early to mid-light phase in pregnant rats. Examination of the data (Fig. 3.3) suggests that the rising phase of the rhythm may have been advanced during early pregnancy. Changes in rhythms in neuroendocrine dopaminergic neurons may play a role in the emergence of the bimodal prolactin rhythm that occurs in this stage of pregnancy (Butcher et al., 1972; Freeman et al., 2000). In pseudopregnant rats, expression of FRA drops roughly 3 hours before each peak in the PHDA, as well as in the TIDA and THDA neurons, and dopamine concentrations in the PHDA follow this pattern of FRA expression (Lerant et al., 1996). In OVX rats, the temporal pattern of Per2 in the PHDA parallels that of dopamine turnover in the intermediate lobe of the pituitary gland (Sellix et al., 2006), to which the PHDA exclusively projects (Goudreau et al., 1995). We do not know if this relationship is maintained in early pregnancy, but the apparent advance in the rise of Per2 expression in the PHDA seen here may be necessary to allow FRA and dopamine synthesis in the PHDA to decline prior to the prolactin surge at ZT 12. It is unclear whether this is necessary for the establishment of the prolactin rhythm by vaginocervical stimulation. Prolactin inhibits its own secretion by activating these cells (Hentschel et al., 2000a;

Hentschel et al., 2000b; Moore et al., 1980), and changes in Per2 expression in them may therefore occur after the establishment of the prolactin rhythm and serve only to maintain it (Bertram et al., 2006; Helena et al., 2009).

Taken together, our data suggest that changes in rhythms in the SCN, such as those seen in Chapter 2, are not the only factors responsible for changes in physiological and behavioral rhythms that occur during early pregnancy. The fact that Per2 rhythms were modified in some extra-SCN oscillators, but not others, suggests that the larger circadian timekeeping system is re-organized during early pregnancy. The mechanisms responsible are uncertain, though vaginocervical stimulation during mating, or the prolactin surges induced by this stimulation, are very likely to play a role, perhaps influencing the SCN and extra-SCN oscillators in different ways. However, changes in the SCN may still contribute to those in extra-SCN oscillators. Rhythms in Per2 expression in these regions are dependent upon the SCN (Lamont et al., 2005; Sellix et al., 2006), and, although their magnitudes vary, phase advances in peak Per2 expression appear to occur in the SCN, BnST-ov, and potentially the PHDA. Overall, the data presented here support the hypothesis that extra-SCN oscillators serve to integrate information concerning reproductive state with photically entrained circadian signals from the SCN to differentially affect overt rhythms in a site-specific manner as females undergo natural transitions in reproductive state.

CHAPTER 4

Changing patterns of Fos expression in the rat brain during early pregnancy

INTRODUCTION

Circadian rhythms allow individuals to appropriately time behavioral and physiological events to predictable changes in the environment (Moore-Ede et al., 1982). These rhythms are essential to the regulation of mammalian reproduction and may be subject to change as individuals progress through various reproductive states. For example, during early pregnancy in laboratory rats, locomotor activity is reduced and arrhythmic (Rosenwasser et al., 1987), and sleep increases during the dark phase of the day (Kimura et al., 1996), a time when non-pregnant rats are most active. Whereas the sleep-wake cycle becomes less rhythmic, the core body temperature rhythm is robust but experiences an advance in the phase of its peak (Kittrell and Satinoff, 1988). Rhythms of both circulating corticotropin (ACTH) and corticosterone also persist, although their amplitude is reduced compared to the non-pregnant state (Atkinson and Waddell, 1995). Additionally, a new rhythm in prolactin secretion emerges during early pregnancy, with daily surges around dawn and dusk that serve to promote progesterone secretion by the corpora lutea (Butcher et al., 1972; Freeman et al., 2000). The neural mechanisms underlying the emergence of this rhythm have been partially identified (Erskine, 1995; Erskine et al., 2004; Freeman et al., 2000; Lee et al., 1998), but those leading to changes in other rhythms as a female progresses from a non-pregnant state to early pregnancy have not been examined.

The circadian system includes a primary circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore and Eichler, 1972; Ralph et al., 1990; Rusak, 1977; Stephan and Zucker, 1972), as well as various extra-SCN oscillators (Guilding and Piggins, 2007; Hastings et al., 2003; Reppert and Weaver, 2002; Weinert, 2005). This system undergoes some changes during early pregnancy (see Chapters 2 and 3), and these changes may drive altered functioning of non-circadian systems to lead to different rhythms in early pregnancy. However, non-circadian systems may also be influenced by transitions in reproductive state and respond to signals from the circadian system in different ways from one another to further modify overt rhythms. The fact that rhythms in different functions are modified in multiple ways suggests that changes in a more central circadian timekeeping system are unlikely to account entirely for the patterns that emerge in early pregnancy. In this study, we address this issue by comparing rhythms in expression of Fos in various brain regions that regulate these functions between early pregnant and diestrous rats. The Fos protein is encoded by the immediate early gene *cfos* that is often expressed after neuronal activation (Kovacs, 2008).

Of the brain regions we examined in this study, some regulate more than one behavioral or physiological system that is altered in early pregnancy, and others serve more specific functions. The medial preoptic area (MPA), for instance, plays a role in the regulation of core body temperature (Satinoff et al., 1982), the initiation of prolactin surges in pregnancy (Freeman and Banks, 1980; Gunnet and Freeman, 1984), and the maintenance of sleep, as well as sexual and parental behaviors (for reviews, see Boulant, 2000; Kumar et al., 2007; Numan, 2007; Sakuma, 2008). The paraventricular hypothalamus (PVN) and dorsomedial hypothalamus (DMH) have both been implicated in the regulation of the prolactin surges in early pregnancy and are also involved in the regulation of the stress axis (Arey and Freeman, 1992a; Arey and Freeman, 1992b; Bernardis and Bellinger, 1998; Freeman and Banks, 1980; Gunnet et al., 1981; Herman et al., 2003). In contrast, the ventrolateral preoptic area (VLPO) and cholinergic basal forebrain have more narrowly defined functions. The VLPO is a sleep-active brain region (Gaus et al., 2002; Sherin et al., 1996; Szymusiak et al., 1998), whereas wakefulness is promoted by parts of the basal forebrain: the medial septum (MS) and vertical and horizontal diagonal bands of Broca (VDB and HDB, respectively; Greco et al., 2000; Jones, 2008).

We also examined rhythms in Fos expression in regions that contain oscillator cells, such as the three regions of the extended amygdala studied in Chapter 3: the central and basolateral amygdala (CEA and BLA, respectively) and the oval nucleus of the bed nucleus of the stria terminalis (BnST-ov). These regions are involved in regulating motivation and emotional state as well as stress responses (Cardinal et al., 2002; Davis et al., 1997; Dong et al., 2001; Erb et al., 2001; Szafarczyk et al., 1983), including insomnia (Cano et al., 2008). We also characterized patterns of Fos expression in the periventricular hypothalamus (PeVN), which houses the periventricular dopaminergic neurons, studied in

Chapter 3, that inhibit prolactin secretion. This region also contains oxytocinergic neurons that may promote prolactin secretion (Kennett et al., 2008).

EXPERIMENTAL PROCEDURES

Animals

Animals were the adult female and male Sprague-Dawley laboratory rats used in Chapters 2 and 3 and obtained from Harlan Laboratories (Indianapolis, IN, USA). Males were housed in pairs or groups of three when not paired with females. Females were housed in groups of three or four for a habituation period of at least two weeks and were subsequently separated for single-housing. All subjects were housed in polypropylene cages (48x27x20 cm) with *ad libitum* access to food (Teklad 8640 rodent diet, Harlan) and water under a 12h:12h light/dark (LD) cycle with lights on at Zeitgeber Time (ZT) 0 and off at ZT 12. A dim red light (<5 lux) remained on constantly for animal care purposes. All experiments were performed in compliance with guidelines established by the Michigan State University Institutional Animal Care and Use Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to keep the number of animals used and their discomfort at a minimum.

Determination of estrous cycle stages

Once singly housed, females were subjected to daily vaginal smears between ZT 0 and ZT 3 to track their estrous cycles. A cotton-tipped applicator soaked in sterile physiological saline was inserted into the vaginal opening, gently rotated, and withdrawn. The applicator was immediately rolled onto a glass slide and allowed to air dry. The dried slides were soaked in 0.25% methylene blue dye for five minutes, rinsed with distilled water, and allowed to dry prior to examination under a light microscope for the presence of nucleated epithelial cells, cornified epithelial cells, leukocytes, and mucus. Estrous cycle stage was determined as follows: proestrus: predominantly nucleated epithelial cells; estrus: predominantly to exclusively cornified epithelial cells; diestrus (between 2 to 3 consecutive days): predominantly leukocytes, some cornified and/or nucleated epithelial cells and mucus present. At least three estrous cycles were established for each female prior to further experimental manipulations.

Experimental Protocol

One group of females was perfused (see below for details) on day one of diestrus, and another on day 6 of pregnancy. To obtain the second group, a male was placed in the female's cage on the morning of proestrus (between ZT 0 and 3). Males were removed the next morning, and a vaginal smear was taken to check for the presence of sperm. If none were detected, the female was re-paired on the morning of her next proestrus period. Once mating had been verified, vaginal smears were discontinued, and the day of confirmation was designated as day 0 of pregnancy. Females were perfused 6 days later, and pregnancy was confirmed by visual inspection of the uterus.

Tissue collection and immunocytochemistry

Tissue for this study was collected and processed in a previous experiment (Chapter 2). Groups of animals from each reproductive state were perfused at ZT 2, 6, 10, 14, 18, or 22. Each animal was given an overdose of

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sodium pentobarbital, and perfused transcardially with 0.01 M phosphate-buffered saline (PBS), pH 7.2, followed by 4% paraformaldehyde (Sigma, St. Louis, MO) in 0.1 M phosphate buffer with 1.3% lysine and 4% sodium periodate. Animals perfused during the dark phase were fitted with a light-tight hood prior to perfusion to prevent acute exposure to light. Brains were post-fixed for 4 hours, transferred to 20% sucrose solution overnight, and stored in cryoprotectant at 4 °C overnight then at -20 °C until sectioning. Brains were sectioned coronally at 30 μm into three alternate series using a freezing microtome, and sections were placed in cryoprotectant for further storage at -20 °C.

One series of tissue was processed for immunocytochemical staining to detect Fos protein expression in the cell nucleus. Free floating sections were rinsed in 0.01 M PBS and then incubated in 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) in PBS with 0.3% Triton X-100 (TX) for 1 h at room temperature. After a 10 minute rinse in PBS, sections were incubated with a primary anti-cFos antibody (made in rabbit, Santa Cruz Biochemistry, Santa Cruz, CA, USA; 1:25,000) in 3% NGS and 0.3% TX in PBS on a rotator at 4°C for 48h. Sections were then rinsed in PBS and incubated with a biotinylated secondary goat-anti-rabbit antibody (Vector; 1:200) in 3% NGS and 0.3% TX in PBS for 1 h at room temperature, rinsed again, and then incubated with an avidin-biotin peroxidase complex (0.9% each avidin and biotin solutions; ABC Vectastain kit; Vector) in 0.3% TX with PBS for 1 h at room temperature. Next, sections were rinsed in 0.125 M acetate buffer, pH 7.2, then reacted with diaminobenzidine (DAB; 0.25 mg/mL, Sigma), nickel sulfate (25 mg/mL, Sigma), and hydrogen peroxide (0.825 µL 0.3% H_2O_2/mL buffer) to yield a blue-black reaction product. Trial immunocytochemical reactions with the reagents used for this reaction were performed with tissue from females that did not have easily determined estrous cycles or had mated but were not pregnant to ensure specificity of the primary antibodies (data not shown). All sections were mounted on clean slides, dehydrated, and coverslipped.

Quantitative and Statistical Analysis

To count the number of Fos-immunoreactive (-ir) cells in each brain region, photographs were taken from three sections of each region, on both sides of the brain or at the midline (MS and VDB), using a CCD video camera (CX900, MBF Bioscience, Williston, VT, USA) attached to a light microscope (Zeiss, Göttingen, Germany). These images were processed using Adobe Photoshop 7 (Adobe Systems, Mountain View, CA, USA). Cells expressing Fos were then counted in the images with the NIH ImageJ program (NIH, Bethesda, MD, USA). The entire area of the BnST-ov was counted (Fig. 4.1), but sampling boxes of the following sizes were used to count Fos-ir cells in the other regions of interest: 400 µm x 400 µm in the CEA, BLA, MPA, and DMH; 300 µm x 300 µm in the VLPO and PVN; 300 µm x 600 µm in the MS, VDB, and HDB; 250 µm x 250 µm in the PeVN (see Fig. 4.1 for placement). All counts were made by an investigator unaware of the reproductive state or time of perfusion of each animal.

A total of 57 females were used in the final analysis (*n*=28 diestrous, *n*=29 pregnant, *n*=4-6 per time point for each reproductive state). A two-way analysis

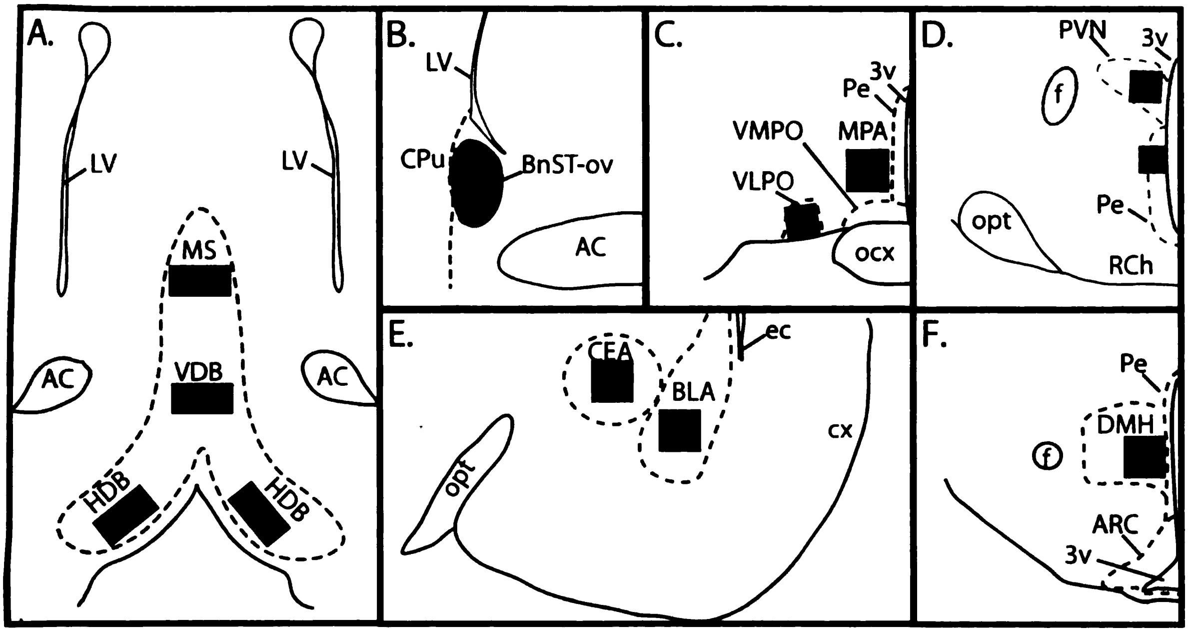

Figure 4.1. Line drawings depicting location of the sampling regions in the MS, VDB, and HDB (A); BnST-ov (B); VLPO and MPA (C); PVN and PeVN (D); CEA and BLA (E), and DMH (F). Sampling boxes (black squares) were used for cell counts in all regions except the BnST-ov (entire area in black). Anatomical boundaries are based on Paxinos and Watson (1997). 3v: third ventricle; AC: anterior commissure; ARC: arcuate nucleus; CPu: caudate putamen; cx: cerebral cortex; ec: external capsule; f: fornix; LV: lateral ventricle; ocx: optic chiasm; opt: optic tract; Pe: periventricular hypothalamic nucleus; RCh: retrochiasmatic area; VMPO: ventromedial preoptic area.

of variance (ANOVA), with reproductive state (pregnant or diestrous) and time of perfusion (ZT) as between-subjects factors, was performed on the number of Fos-ir cells in the PeVN, PVN, and DMH, and on square-root transformed counts in the BnST-ov and MS. These transformations were used to equalize variances amongst treatment groups. Post-hoc least significant differences (LSD) tests were conducted on these data when significant main effects were found. Nonparametric analyses were performed on data from regions in which square root transformations failed to equalize the variances: the CEA, BLA, VLPO, VDB, HDB, and MPA. In these cases, we compared the effects of reproductive state at each ZT and for all ZTs combined (Mann-Whitney U tests) and of ZT within each reproductive state and for the two reproductive states combined (Kruskal-Wallis tests, followed by post-hoc pairwise Mann-Whitney U tests if significant effects were found). All data analyses were conducted with SPSS 17 software (SPSS Inc., Chicago, IL, USA). All differences were considered significant when $P<0.05$. Details of statistics are omitted from the text when they are presented in figure legends. Data are presented as means ± SE.

RESULTS

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Fos expression in the BnST-ov was significantly affected by time (Fig. 4.2A; $F=8.853$, $df=5$, $P<0.001$, two-way ANOVA) and by reproductive state ($F=16.785$, $df=1$, $P<0.001$), but there was no interaction between these variables ($F=1.568$, $df=5$, $P> 0.15$). Pregnant females expressed more Fos in the BnST-ov

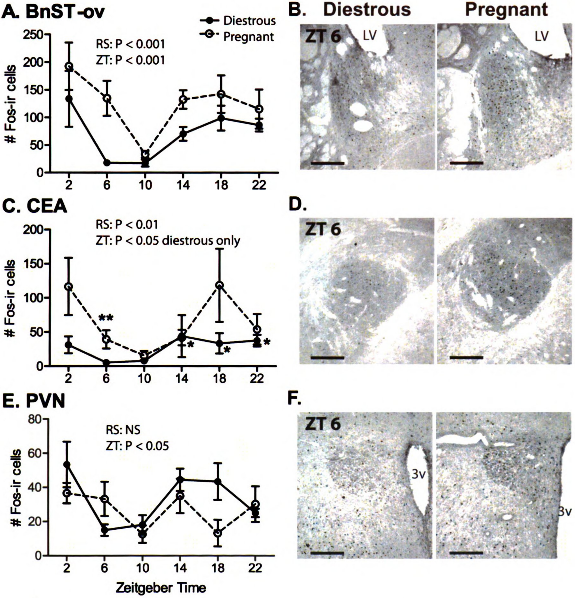

Figure 4.2. Expression of Fos in the BnST-ov (A,B), CEA (C,D), and PVN (E,F) of diestrous and pregnant rats kept in a 12:12 LD cycle. (A,C,E): Main effects of reproductive state (RS) and ZT are indicated in text on each graph (A, E: two-way ANOVA; C: non-parametric tests). There was no significant interaction of ZT and reproductive state in the BnST-ov or PVN. NS: not significant (P>0.05). * indicates that Fos expression was significantly elevated relative to at least one other time point within the same reproductive state, and ** indicates a significant difference between reproductive states (P<0.05, post-hoc Mann-Whitney U tests). (B,D,F): Representative photomicrographs of Fos-labelled cells in each region at ZT 6. LV: lateral ventricle; 3v: third ventricle. Scale bars = 200 μm.

than diestrous females, but the rhythm was the same in the two groups (Fig. 4.2A and B). It peaked at ZT 2 and reached trough values at ZT 10.

The central and basolateral amygdala

In the CEA, a low amplitude rhythm with a peak during the dark phase was seen in diestrous females (Fig. 4.2C; ZT: $X^2=11.304$, *df*=5, $P<0.05$, Kruskal-Wallis test), but there was no effect of time in pregnant females (ZT: $X^2=8.646$, *df*=5, $P>0.1$, Kruskal-Wallis test). In addition, Fos was higher in pregnant than in diestrous females (main effect: $U=243$, $P<0.01$, Mann-Whitney U-test), particularly at ZT 6 (Fig. 4.2C and D). Although interactions cannot be directly assessed with nonparametric tests, these patterns suggest that the transition to the pregnant state eliminates the rhythm in Fos within the CEA due to rises in its expression. In contrast, in the BLA, Fos expression was low (6.51±1.74 cells, all animals) and did not change over time among either pregnant or diestrous rats (pregnant ZT: $X^2=5.210$, *df*=5, $P>0.3$; diestrous ZT: $X^2=5.885$, *df*=5, $P>0.3$, Kruskal-Wallis tests), nor did it differ as a function of reproductive state ($U=329$, $P>0.2$, Mann-Whitney U test).

The hypothalamic paraventricular nucleus

Fos expression in the PVN was affected by time ($F=3.104$, *df*=5, $P<0.02$, two-way ANOVA) but not reproductive state ($F=1.652$, *df*=1, $P>0.2$) or an interaction between these variables (Fig. 4.2E and F; $F=1.872$; *df*=5, $P>0.15$). The pattern was bimodal with peak levels of expression occurring at ZT 2 and again at ZT 14 and troughs occurring in the mid to late light phase and again at ZT 22.

The medial preoptic area

In the MPA of diestrous rats, Fos expression changed as a function of time of day (Fig. 4.3A; X^2=14.434, *df*=5, P<0.02, Kruskal-Wallis test), and peak values occurred from the early dark phase through the early light phase, with significantly elevated expression at ZT 2 and 18. In pregnant rats, Fos expression in the MPA, which was tonically low, was arrhythmic (ZT: X^2=8.812, *df*=5, P>0.1, Kruskal-Wallis test). Fos was significantly lower in pregnant rats than in diestrous ones at ZT 2, 10, 18, and 22 (Fig. 4.3A and B). These patterns suggest that there is likely an interaction between the effects of reproductive state and ZT on Fos expression in this area.

The periventricular hypothalamus

Patterns of Fos expression in the PeVN were significantly affected by time of day (Fig. 4.3C; F=3.567, *df*=5, P<0.01, two-way ANOVA) and by an interaction between time of day and reproductive state (F=3.063, *df*=5, P<0.02). A rhythm was seen in diestrous females with peak values occurring at ZT 2 and again at ZT 18, but the effect of time on expression in the PeVN of pregnant females did not quite reach statistical significance (F=2.58, *df*=5, P<0.06, post-hoc one-way ANOVA). Expression was also significantly higher in the PeVN of diestrous rats at ZT 18 than in pregnant females (Fig. 4.3C and D).

The dorsomedial hypothalamus

In the DMH, Fos expression was affected by time (Fig. 4.3E; F=3.269; *df*=5, P<0.02, two-way ANOVA) but not by reproductive state (Fig. 4.3E and F; F=0.08, *df*=1, P>0.75), and there was no interaction between these variables

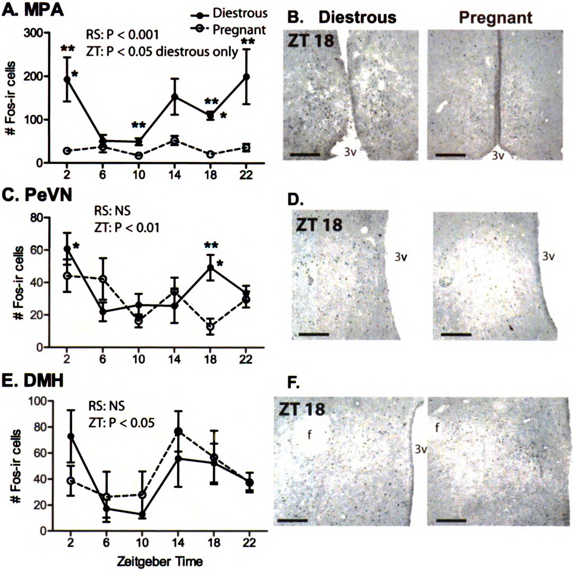

Figure 4.3. Expression of Fos in the MPA (A,B), PeVN (C,D), and DMH (E,F) of diestrous and pregnant rats kept in a 12:12 LD cycle. (A,C,E): Main effects of reproductive state (RS) and ZT are indicated in text on each graph (A: non-parametric tests; C,E: two-way ANOVA). There was a significant interaction of ZT and reproductive state in the PeVN but not the DMH. Notation is the same as in Fig. 4.2 (post-hoc Mann-Whitney U tests (A) or LSD tests (C)). (B,D,F): Representative photomicrographs depicting between group differences in Fos expression in each region at ZT 18. 3v: third ventricle; f: fornix. Scale bars = 200 μm.

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($F=0.91$, *df*=5, $P>0.45$). The rhythm peaked from the early dark phase through the early light phase, with highest expression at ZT 2, 14, and 18.

The ventrolateral preoptic area

Fos expression in the VLPO did not change as a function of time in diestrous rats (Fig. 4.4A; $X^2=6.399$, *df*=5, $P>0.25$, Kruskal-Wallis test), but it did in pregnant ones, in which peak values occurred from ZT 22 to 2 ($X^2=12.525$, *df*=5, $P<0.03$, Kruskal-Wallis test). Expression did not differ between the two reproductive states at any time point (Fig. 4.4A and B), but there was a trend for expression to be higher in the VLPO of diestrous females compared to pregnant ones (main effect: *U*=289.5, $P<0.1$, Mann-Whitney U-test). These patterns suggest that an interaction may exist between effects of reproductive state and ZT on Fos expression in this area.

The basal forebrain

Fos expression was higher in the MS in diestrous rats than pregnant females (Fig. 4.4C and D; $F=13.574$, *df*=1, $P<0.002$, two-way ANOVA), but it was not affected by time of day in either group ($F=1.638$, *df*=5, $P>0.15$). There was also no interaction between these variables ($F=0.948$, *df*=5, $P>0.45$). Patterns were somewhat similar in the VDB, but here a rhythm of Fos expression was detected in diestrous rats, in which Fos was elevated throughout the dark phase (Fig. 4.4E; $X^2=11.052$, *df*=5, $P<0.05$, Kruskal-Wallis test). In contrast, Fos expression in the VDB of pregnant rats was tonically low and was not affected by time of day ($X^2=4.929$, *df*=5, $P>0.4$, Kruskal-Wallis test). Expression was significantly higher in diestrous than pregnant females (main effect:

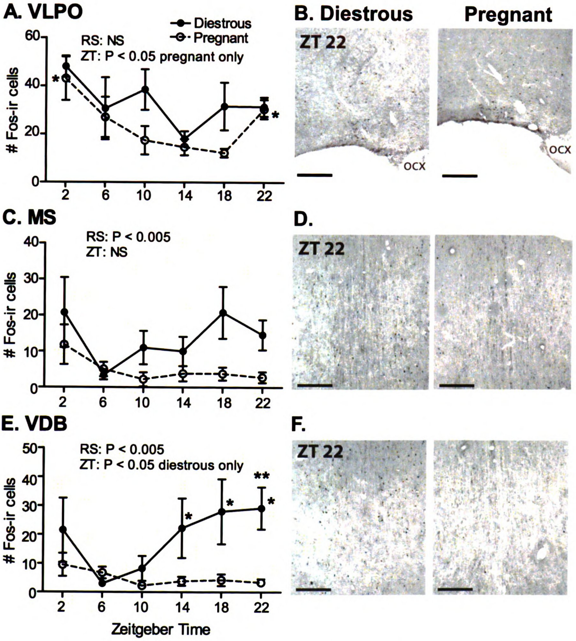

Figure 4.4. Expression of Fos in the VLPO (A,B), MS (C,D), and VDB (E,F) of diestrous and pregnant rats kept in a 12:12 LD cycle. (A,C,E): Main effects of reproductive state (RS) and ZT are indicated in text on each graph (A,E: non-parametric tests; C: two-way ANOVA). There was no significant interaction of ZT and reproductive state in the MS. Notation is the same as in Fig. 4.2 (post-hoc Mann-Whitney U tests). (B,D,F): Representative photomicrographs depicting between group differences in Fos expression in each region at ZT 22. ocx: optic chiasm. Scale bars = 200 μm.

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U=177.5, P<0.003, Mann-Whitney U-test), particularly at ZT 22 (Fig. 4.4E and F). These patterns suggest that there is likely an interaction between the effects of reproductive state and ZT on Fos expression in the VDB. In contrast, Fos expression in the HDB was low (8.64±1.36 cells, all animals) and did not differ as a function of reproductive state (*U*=290.5, P>0.1, Mann-Whitney U test). In this region, Fos was not affected by time of day in either pregnant or diestrous rats (pregnant ZT: X^2=2.437, *df*=5, P>0.75; diestrous ZT: X^2=4.442, *df*=5, P>0.45, Kruskal-Wallis tests).

DISCUSSION

The current data reveal that reproductive state affects light-entrained rhythms in Fos expression in various brain regions in site-specific ways. For instance, pregnancy led to increased expression of Fos in the BnST-ov and CEA and a decrease in Fos expression and lack of rhythmicity in the MPA, MS, and VDB, whereas there were no changes in the rhythms in the PVN or DMH. Additionally, pregnancy led to the emergence of a significant rhythm in Fos expression in the VLPO and the loss of a rhythm in the PeVN without affecting the total amount of Fos expressed in the day in either area. This variation indicates that the changes associated with pregnancy cannot be entirely explained by those in the SCN/vSPZ described in Chapter 2. For the same reason, all of these effects of reproductive state cannot be due entirely to modulation of the circadian system as a whole.

Some salient patterns seen in this study may reflect altered functioning of brain regions that could directly lead to changes in overt rhythms that occur during early pregnancy. For instance, pregnancy-induced modulation of the BnST-ov and CEA might alter stress hormone secretion and anxiety (Cardinal et al., 2002; Davis et al., 1997; Dong et al., 2001; Erb et al., 2001; Szafarczyk et al., 1983), both of which are reduced in early pregnancy in rats (Atkinson and Waddell, 1995; Macbeth et al., 2008; but see Picazo and Fernandez-Guasti, 1993; Neumann et al., 1998). The increase in Fos in these two brain regions, which contain glucocorticoid receptors (Honkaniemi et al., 1992; Lechner and Valentino, 1999), might reflect an increased sensitivity to stress hormone feedback. The lack of any effect on Fos expression in the PVN, however, which more directly regulates corticosterone and ACTH secretion, may indicate that changes in the BnST-ov and CEA are not directly influencing changes in the stress axis. Another possibility is that these changes are indicative of how pregnancy alters the regulation of anxiety by stress hormones (Roozendaal et al., 2009).

In the PeVN, which plays a role in the regulation of prolactin secretion (Ben-Jonathan, 1985; Freeman et al., 2000; Kennett et al., 2008), Fos expression was significantly lower in pregnant rats at ZT 18, a time when Per2 expression was also significantly lower within periventricular tyrosine hydroxylase (TH) cells of pregnant rats (Fig. 3.3A). The reduced Fos expression at ZT 18 in the PeVN occurs roughly two hours before the second daily surge in prolactin (Butcher et al., 1972; Freeman et al., 2000), a pattern suggesting that it might reflect activity of prolactin-inhibiting (dopaminergic) neurons. Intriguingly, the patterns of Fos expression in the PeVN of both diestrous and pregnant rats appear almost identical to those in Fos expression in the vSPZ (Fig. 2.3E), which lies adjacent to and projects sparsely to the PeVN (Kriegsfeld et al., 2004; Morin et al., 1994; Watts and Swanson, 1987; Watts et al., 1987). Perhaps a loss of rhythmicity in the vSPZ contributes to that occurring in the PeVN of pregnant females.

The MPA plays a role in the regulation of multiple behaviors and physiological parameters, including sexual and parental behaviors, sleep, and core body temperature (for reviews, see Boulant, 2000; Kumar et al., 2007; Numan, 2007; Sakuma, 2008). Fos expression in this region was strikingly different between pregnant and diestrous rats, being arrhythmic and tonically low in the former and high and rhythmic in the latter. These patterns contrast with those reported by Lee et al. (1998), who found a significant rhythm in Fos expression in the MPA on day 6 of pregnancy in the rat. The reason for the discrepancy is unclear, though perhaps it is due to the fact that Lee et al. (1998) sampled a more caudal portion of the MPA than we did. The change that our animals underwent from diestrus to pregnancy may be related to the absence of proceptive sexual behaviors, which are dependant on the MPA (Guarraci et al., 2004; Hoshina et al., 1994; Whitney, 1986; Yang and Clemens, 2000). The MPA also plays a critical role in the coordination of sleep and the associated decrease in core body temperature (Kumar et al., 2007). During early pregnancy, the sleep/wake cycle and rhythms in core body temperature are dissociated (Kimura et al., 1996; Kittrell and Satinoff, 1988; Rosenwasser et al., 1987). Therefore, the reduction of Fos expression in the MPA may reflect a dissociation between cells

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in this region that coordinate sleep/wake behavior and those that influence core body temperature.

Perhaps the most interesting findings in this study are those in the basal forebrain (the MS and VDB) and VLPO, which are wakefulness- and sleep-promoting regions of the brain, respectively (Gaus et al., 2002; Greco et al., 2000; Jones, 2008; Sherin et al., 1996; Szymusiak et al., 1998). Although we did not see a significant rhythm in Fos expression in the VLPO of diestrous females, the basic pattern of expression was similar to that in pregnant females despite the fact that sleep patterns are distinctly different between these two reproductive states. More specifically, the total amount of time spent in both non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) during the dark phase is significantly higher in early pregnancy, as is the number of REMS bouts in the dark phase (Kimura et al., 1996). Additionally, pregnant rats are less active throughout the day, especially during the dark phase (Rosenwasser et al., 1987). Our data indicate that these changes in sleep and activity may not be due to changes in the sleep-promoting cells in the VLPO but may, instead, be due to the loss of drive from arousal-promoting regions of the brain, such as the MS and VDB. A similar dissociation between activation of brain mechanisms that promote arousal and those leading to increases in sleep (VLPO) is also apparent during stress-induced insomnia in the rat (Cano et al., 2008). Together, these patterns suggest that plasticity in sleep/wake patterns is primarily driven by arousal systems of the brain. Further exploration of how activity in other sleep and arousal promoting regions change during early pregnancy would be useful in testing this hypothesis at a more general level. Patterns of activity in the arousal promoting orexinergic cells are of particular interest, as they project directly to the basal forebrain and modulate activity in this region (Saper et al., 2001; Wu et al., 2004).

The patterns of change in some, but not all, regions of the brain examined in this chapter highlight the fact that effects of pregnancy in these areas are time-of-day dependent. It is common practice to use Fos to examine effects of experimental manipulations on specific brain regions, but often these are done at only one, or perhaps two, time(s) of day. This could lead to a misunderstanding of the processes under consideration. For instance, if we compared Fos expression in the MPA of diestrous and pregnant rats at only ZT 6 (Fig. 4.4A), we would find no difference, which could lead to the erroneous conclusion that functions associated with Fos in this region are the same in the two reproductive states.

The mechanisms leading to the different effects of pregnancy on temporal patterns of Fos expression in various brain regions are unclear. Some effects may be driven by changes in the circadian system, such as those demonstrated in Chapters 2 and 3. However, others may be brought about more directly by changes in systems mediating aspects of physiology and behavior that are altered during early pregnancy. For example, the elimination of the rhythm in the VDB could be due to changes in inhibitory processes that do not originate in the circadian system. This issue is related to the question of which aspects of early pregnancy, such as modified patterns of hormone secretion or stimuli from mating. fertil

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mating, fertilization, or implantation, contribute to these changes and how this may vary amongst brain regions. Another important question raised be these data is how the different patterns of change in Fos expression in these myriad regions of the brain might contribute to modification of the temporal patterning of daily rhythms in behavior and physiology that occurs during early pregnancy.

Chapter 5

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CHAPTER 5

Changing patterns of daily rhythmicity across reproductive states in diurnal female Nile grass rats (*Arvicanthis niloticus*)

INTRODUCTION

Circadian rhythms allow organisms to predict fluctuations in the environment and appropriately time behavioral and physiological events to anticipate those changes (Moore-Ede et al., 1982). In mammals, the mechanisms governing these rhythms have been studied primarily in nocturnal rodents, particularly rats (*Rattus norvegicus*), hamsters (*Mesocricetus auratus*), and mice (*Mus musculus*), which exhibit behavioral and physiological rhythms that are roughly twelve hours out of phase with those of diurnal species (Refinetti, 2008). Circadian rhythms in females of these nocturnal species change across the estrous cycle (Morin et al., 1977) and throughout pregnancy and lactation (Kittrell and Satinoff, 1988; Scribner and Wynne-Edwards, 1994a). The influences of ovarian hormones responsible for changes in non-pregnant females have been examined extensively (Albers, 1981; Gerall et al., 1973; Labyak and Lee, 1995; Morin et al., 1977; Takahashi and Menaker, 1980; Thomas and Armstrong, 1989), but little is known about how pregnancy and lactation change circadian rhythms despite the fact that wild adult female mammals spend significantly more time in these states than they do undergoing estrous cycles.

To meet the demands of pregnancy and lactation, females must change many parameters of their behavior and physiology. Females of many species increase food intake or mobilize fat stores to sustain fetal and neonatal growth (Gittleman and Thompson, 1988; Speakman, 2008; Wade and Schneider, 1992), and hyperthermia during lactation is almost universal (Speakman, 2008). In many species, activity levels are low throughout pregnancy and lactation (Albers et al., 1981; Richards, 1966; Rosenwasser et al., 1987; Scribner and Wynne-Edwards 1994a; Speakman, 2008), and sleep disruption is more common (Kimura et al., 1996; Speakman, 2008). Although these general trends are well-established, relatively little is known about how circadian rhythms in these physiological and behavioral phenomena change (for an exception see Kittrell and Satinoff, 1988).

Most studies of the effects of pregnancy and lactation on circadian rhythms have focused on locomotor activity and core body temperature (T_b) in nocturnal rodents and have found changes in the phase and/or the amplitude of these rhythms. Lab rats and various hamster species are less active, and that activity is less rhythmic, when they are pregnant or pseudopregnant than when they are non-reproductive (Albers et al., 1981; Richards, 1966; Rosenwasser et al., 1987; Scribner and Wynne-Edwards 1994a). The peak of the T_b rhythm in lab rats is phase-advanced by several hours during both pregnancy and lactation, and the amplitude of the rhythm decreases during gestation and increases during lactation (Kittrell and Satinoff, 1988). We do not know whether any of these changes occur in diurnal rodents. One might expect them to be similar, as pregnancy and lactation impose major metabolic challenges regardless of an animal's temporal niche (Gittleman and Thompson, 1988; Speakman, 2008). However, animals that are active during the warmest time of the day may not be able to change their activity and T_b rhythms in the same manner as those that are most active during the coldest phase of the day-night cycle.

The current study used a diurnal species, the Nile grass rat (*Arvicanthis niloticus*), a murid rodent from sub-Saharan Africa (Rosevear, 1969), to examine two questions about how rhythms are modulated by reproductive state. The first was whether pregnancy and lactation affect circadian rhythms in activity and T_b in diurnal rodents in the same manner as they do in nocturnal ones. The second was whether the effects of pregnancy and lactation on circadian rhythms are additive or instead interact in more complex ways. To address these questions, we monitored T_b and activity of female grass rats as they progressed through a series of reproductive states: virgin, pregnant, pregnant and lactating, lactating only, and post-weaning.

MATERIALS AND METHODS

Animals

Animals were adult (>60 days) female and male Nile grass rats, *Arvicanthis niloticus*, obtained from a breeding colony at Michigan State University (Katona and Smale, 1997). All animals were housed in polycarbonate cages (48 x 28 x 16 cm) and given red transparent plastic huts (18 x 6 x 6 cm) for optional shelter. For singly housed animals and lactating females (see below), only one hut was provided, but mating couples were given two huts. Food (PMI Nutrition Prolab, RMH 2000, Brentwood, MD) and water were provided *ad libitum.* Animals were kept in a 12 h:12 h light/dark cycle (~250 lux during the light phase), with the lights on from Zeitgeber Time (ZT) 0 to ZT 12, and a red light (<5 lux) was on continuously to allow for visual observations and video recording during the dark phase. All experiments were performed in compliance with guidelines established by the Michigan State University Institutional Animal Care & Use Committee (04/03-053-00) and in accordance with the standard in the National Research Council Guide for Care and Use of Laboratory Animals (National Academy of Science).

Surgery

Calibrated temperature/activity transmitters (PDT ER-4000 G e-mitters, Minimitter, Bend, OR) were implanted intra-peritoneally into adult female grass rats under anesthesia (2-5 % isofluorane), and females were given pre-surgery subcutaneous injections of the analgesic, buprenorphine hydrochloride (Buprenex, 0.06 mg/kg of body weight). A single 1 cm incision was made along the midline of the abdomen and muscle wall, and a transmitter was inserted into the peritoneal cavity. The muscle wall was sutured with nylon sutures, and the skin was sutured subcutaneously with dissolvable chromic gut sutures, which were reinforced with autoclips. Animals were then given sterile saline (2.0 cc, 0.9% NaCl) subcutaneously, removed from anesthesia, and monitored until they regained normal motor capacity. They received injections of Buprenex (same dose as above) every 12 hours for the next two days.

Experimental Manipulations

Implanted females were housed singly in cages atop receiver bases (ER-4000, Minimitter, Bend, OR), which conveyed transmitter readings to a computer in an adjacent room that was equipped with Vitalview 4.0 software (Minimitter, Bend, OR). Gross motor activity and body temperature readings were collected and stored every five minutes, and the data file was exported once weekly, which required stopping the data collection for a five minute period. Once all the females had healed from surgery, recordings were taken for two weeks prior to splitting them into two treatment groups (*n* = 5 per group): reproductive females and age-matched virgin controls. Each female in the reproductive group was paired with an adult virgin male grass rat, while the controls were kept singly-housed throughout the experiment. Virgin females of this species do not exhibit a clear estrous cycle and appear to be in an anestrous state (McElhinny, 1996), but female grass rats do come into a reliable post-partum estrus (McElhinny et al., 1997). Therefore, we could not assess the onset of pregnancy until the first litter was born. The mating couples were checked daily for the presence of their first litter, and the day that litter was discovered was designated day 0 of pregnancy and lactation (PL0, beginning at ZT 12 prior to birth) since a post-partum mating had occurred. Gestation in this species lasts approximately 25 days (McElhinny et al., 1997), and the day of conception of the first litter (pregnancy day 0 = P0) was calculated as 25 days prior to day PL0. At 21 days of age (PL21), the first litter was separated from the mother, and the male was also removed. The female's cage was checked daily for the birth of the second litter, and the day of birth was designated as day 0 of lactation (L0). The second litter was separated from the mother at day L21, and recordings were taken from the female for at least two more weeks after weaning. Readings were taken from all of the control females until recording from the last reproductive female was complete.

Activity and Temperature Data Analysis

To determine how circadian rhythms in activity and body temperature varied among reproductive states, we sampled data from days 2, 10, and 19 of pregnancy (P), pregnancy plus lactation (PL), and lactation (L) from the reproductive females. These days were chosen because both pregnancy and lactation are dynamic states, and we wished to compare early, mid, and late days within these states without the confounds of other transitions, such as parturition (day P0), implantation (likely around P4 to P6), and weaning (L21). We also sampled on a pre-reproductive baseline day (Pre: four days before pairing) and a post-reproductive day (Post: 11 days after the second litter was weaned). Since the control females did not go through these reproductive states, we determined equivalent days for this group by sampling the data from each control female from the same calendar dates as we had sampled from a randomly pair-matched reproductive female so that five reproductive/control pairs of data were date-matched.

To examine how activity rhythms were altered by reproductive state, we generated actograms from the raw data in ClockLab v. 2.61 (Actimetrics, Wilmette, IL). For each day of interest, we used the program to determine both onset and offset of activity. Occasionally, the Clocklab algorithm designated the onset or offset to be at a time that was not consistent with visual inspection of the actogram. In these cases, the onset or offset was estimated instead by eye. To assess effects on the magnitude of general activity, we had to standardize the activity counts within each individual, since the activity outputs of the transmitters were not calibrated to one another. Therefore, to determine whether the total daily activity changed within individuals over the course of the study, we calculated the average daily activity for each reproductive day of interest (P2 through Post or control equivalent) as the ratio of the average daily activity to the average daily activity of the pre-reproductive day. We also calculated the dark: light ratio of total activity for each day of interest, including the pre-reproductive day.

The temperature readings were calibrated for each transmitter by Minimitter (Bend, OR), and these readings could be directly compared between individuals. For each day of interest, T_b measurements were averaged for each half hour. The amplitude of the daily rhythm was calculated as the difference between the maximum and minimum half hour T_b. To examine how the phase of the rhythm changed, the start of the half hour with the minimum T_b was determined. This served as the best phase marker as the timing of the daily maximum was much more variable amongst and within individuals (data not shown). A preliminary examination of the half hour averages revealed that the time periods exhibiting the most noticeable and consistent changes were the midday (ZT 3 to 9) and midnight (ZT 15 to 21) periods. The average T_b for each of these two six hour periods was therefore determined for each day of interest.

Since individual animals were randomly assigned to the two treatment groups and repeated measures were taken from each individual, a general mixed effects model (GMEM) was most appropriate to examine the influences of reproductive days on activity and T_b rhythms. For each dependent variable (rhythm parameter) described above, a GMEM was generated in SAS v. 9.1

(SAS Institute, Inc., Cary, NC) with reproductive day (or equivalent), treatment group, and the interaction between the two as fixed factors and reproductive day (or equivalent) as a repeated measure within the random effect of the individual within a group. In the GMEM, the covariance structure of the data had to be specified. To determine the best covariance structure for each dependent variable, the model was run with each of four different structures (compound symmetry, heterogeneous compound symmetry, autoregressive, and unstructured), and the Akaike's Information Criterion value for each model was used to determine which model best fit the data. Post-hoc least significant difference tests were performed for any significant factors identified by the GMEM with the best covariance structure for pair-wise comparisons of means between groups within a reproductive day and between days within a group.

Video Recordings and Behavioral Analysis of Nesting Females

In order to determine whether rhythms in nesting females might change across lactation and also to examine how core body temperatures might mediate nesting, we examined nesting and drinking rhythms in a subset of non-pregnant, lactating females (*n* = 3). Dams were videotaped with a low-light lens CCTV camera connected to a time-lapse video recorder that condensed a 24 hour period onto two hours of videotape. For each female, days 2, 10, and 19 of lactation were scored by two different individuals. All individuals involved in video scoring performed preliminary test scoring to ensure 94% or higher agreement between all scorers. This agreement threshold was defined as having 94% of all time bins scored the same by all scorers. Each video was scored for off-nest and drinking behaviors. Off-nest behavior was defined as the female leaving contact with the pups; drinking, eating, and general locomotion occurred during this time. Drinking behavior was defined as the female placing her tongue or snout against the ball bearing in the water bottle spout. It usually occurred during off-nest bouts, but some females could rear up while still on the nest and reach the water bottles. The twenty four hours of each video were split into five minute bins, and for each bin, each of the two scorers recorded in an all-or-none fashion which of the behaviors occurred. For each hour, the frequency for each behavior was calculated by dividing the number of bins scored positive for that behavior by both scorers by the total number of bins the scorers agreed upon within that hour (thus excluding any bins with discrepant scores).

For analysis of these frequencies, since all females were subjected to the same treatment, a two-way repeated measures ANOVA was performed for each behavior with the frequency for that behavior as the dependent variable and day of lactation (day 2, 10, or 19) and ZT (24 total hours) as fixed effects in SPSS v. 15 (SPSS, Inc., Chicago, IL). Least significant difference post-hoc tests were performed when significant effects were found. Differences were considered significant when $p < 0.05$. Data are given as means ± SE unless otherwise stated.

RESULTS

Reproductive Output

For all reproductive females, the first litters were born within 27 to 32 days of pairing with males (mean 29.8 ± 1.2 days). These initial litters ranged in size from 4 to 7 pups (mean 5.8 ± 0.5 pups), and their sex ratios varied from twice as many males as females to the opposite ratio (mean 1.05 ± 0.25 males/females). Second litters were born 25 to 26 days after births of first litters (mean 25.8 ± 0.2 days) and ranged in size from 3 to 9 pups (mean 5.6 ± 1.1 pups). Their sex ratios varied from no males to 3 males for every 2 females (mean 1.23 ± 0.52 males/females).

Activity Rhythms

As seen in previous laboratory work with this species (Katona and Smale, 1997; McElhinny et al., 1997), female grass rats in the current study exhibited diurnal activity rhythms with crepuscular tendencies (Fig. 5.1). This pattern is somewhat different from that seen in a natural setting where activity peaks in the middle of the day and there are no peaks around dawn or dusk (Blanchong and Smale, 2000). Although females were diurnal in the current study, the amount of activity and its distribution across the day varied among reproductive states. We measured the effects of the reproductive days (Pre through Post or equivalent days for the control group to account for the effects of age) on the phases of activity onset and offset, the average daily activity (relative to the pre-reproductive day), and the dark:light activity ratio. Table 5.1 contains a summary of the type III tests of fixed effects from the GMEM analyses for these variables. The phases of activity onset and offset were not significantly different across reproductive days (or equivalents) in either group, and they did not differ between groups (Table 5.1). Activity onsets occurred roughly 1.5 hours before lights on

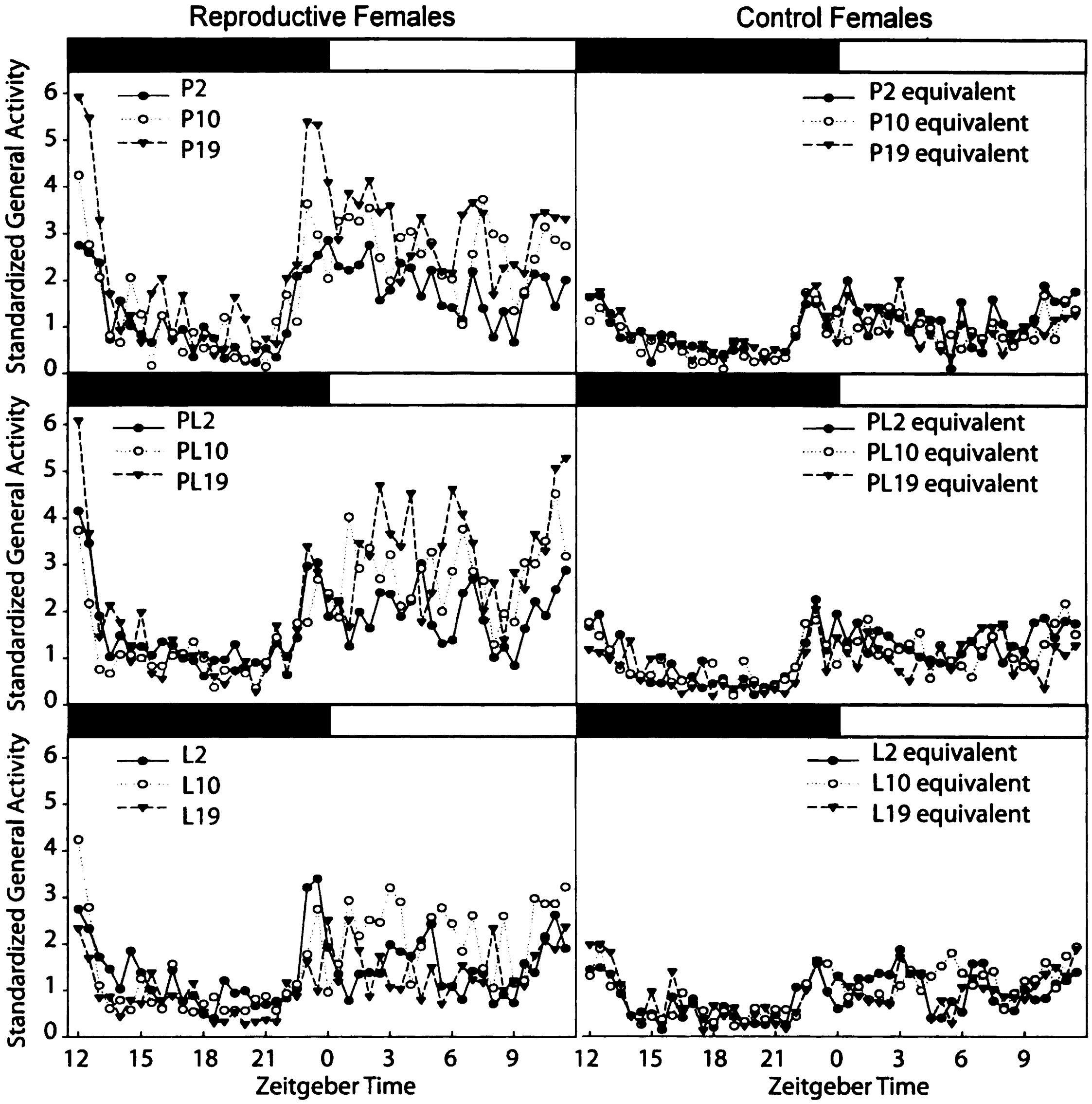

Figure 5.1. Averaged general locomotor activity rhythms in the reproductive (left) and control (right) groups (n = 5/group) under a 12h: 12h light (open bars)/dark (black bars) cycle for pregnancy, pregnancy plus lactation, and lactation (or equivalents). For each individual, the average activity of each half hour was standardized by dividing the raw value for that half hour on the day of interest by the average total daily activity of that female on the pre-reproductive day. Plotted values are means of these standardized values for each treatment group for each

Rhythm Parameter	Covariance Structure*	Reproductive Day (or Equivalent) Effect	Treatment Group Effect	Reproductive Day x Group Interaction
Phase of Activity Offset	CSH	$F(10, 26.6) = 1.25$ NS	$F(1, 7.94) = 0.76$ NS	$F(10, 26.6) = 1.24$ NS
Phase of Activity Onset	CSH	$F(10, 27.9) = 1.50$ NS	$F(1, 8.28) = 5.17$ NS	$F(10, 27.9) = 0.61$ NS
Average Daily Activity	CSH	$F(9, 27.6) = 3.51$ $P < 0.01$	$F(1, 8.01) = 5.11$ NS	$F(9, 27.6) = 2.74$ $P < 0.05$
Dark:Light Activity Ratio	CSH	$F(10, 25.7) = 2.08$ NS	$F(1, 8.08) = 0.26$ NS	$F(10, 25.7) = 2.67$ $P < 0.05$
Amplitude of Tb Rhythm	CS	$F(10, 80) = 10.60$ $P < 0.0001$	$F(1, 8) = 9.73$ $P < 0.05$	$F(10, 80) = 4.01$ $P < 0.0005$
Phase of Minimum Tb	CSH	$F(10, 26.3) = 1.27$ NS	$F(1, 10.4)= 6.69$ $P < 0.05$	$F(10, 26.3) = 0.90$ NS
Average Midday Tb	CS	$F(10, 80) = 12.90$ $P < 0.0001$	$F(1, 8) = 10.71$ $P < 0.05$	$F(10, 80) = 12.53$ $P < 0.0001$
Average Midnight Tb	CS	$F(10, 80) = 14.36$ $P < 0.0001$	$F(1, 8) = 18.73$ $P < 0.005$	$F(10, 80) = 14.79$ $P < 0.0001$

Table 5.1. GMEM Results for Type III Tests of Fixed Effects for Activity and Temperature Rhythms. *CSH = Heterogeneous Compound Symmetry, CS = Compound Symmetry, see METHODS for model details. n = 5/group. NS = Not significant ($P > 0.05$).

(ZT 22.58 ± 0.10 hours), and the offsets occurred around 1.75 hours after lights off (ZT 13.76 ± 0.17 hours).

Locomotor activity increased from early to late pregnancy, regardless of whether or not females were also lactating (Fig. 5.1). The average daily activity was significantly affected by the interaction between reproductive days (or equivalents) and treatment group and by the main effect of reproductive days (Table 5.1). In the reproductive group, the average daily activity was significantly higher than the post-reproductive day from day P19 through PL19 (Fig. 5.2A; $p < 0.05$; post-hoc pair-wise least significant difference (LSD) tests). Daily activity levels of the reproductive females appeared to return to pre-reproductive levels by the post-reproductive day (daily activity: 1.02 ± 0.23, with a value of 1 indicating that activity levels were the same as on the pre-reproductive day). Therefore, females were also more active on days P19 through PL19 than during the pre-reproductive period. Additionally, reproductive females were more active on days P19, PL10, and PL19 than controls on the equivalent days (Fig. 5.2A; $p < 0.05$; LSD tests). Within pregnancy, activity levels significantly increased from day 2 to day 19 (Fig. 5.2B; $p < 0.05$; LSD test). A similar trend was apparent during the period of pregnancy plus lactation, but there were no significant differences across days in this state (Fig. 5.2B). During lactation this trend was not seen, and females on day L19 had significantly lower levels of activity than on PL19 (Fig. 5.2B; $p < 0.05$; LSD test). The average daily activity did not vary significantly among any of the days of interest in the control group (Fig. 5.2A; mean for all days: 0.955 ± 0.25; $F(9, 7.73) = 0.44$, $p > 0.5$; post-hoc GMEM).

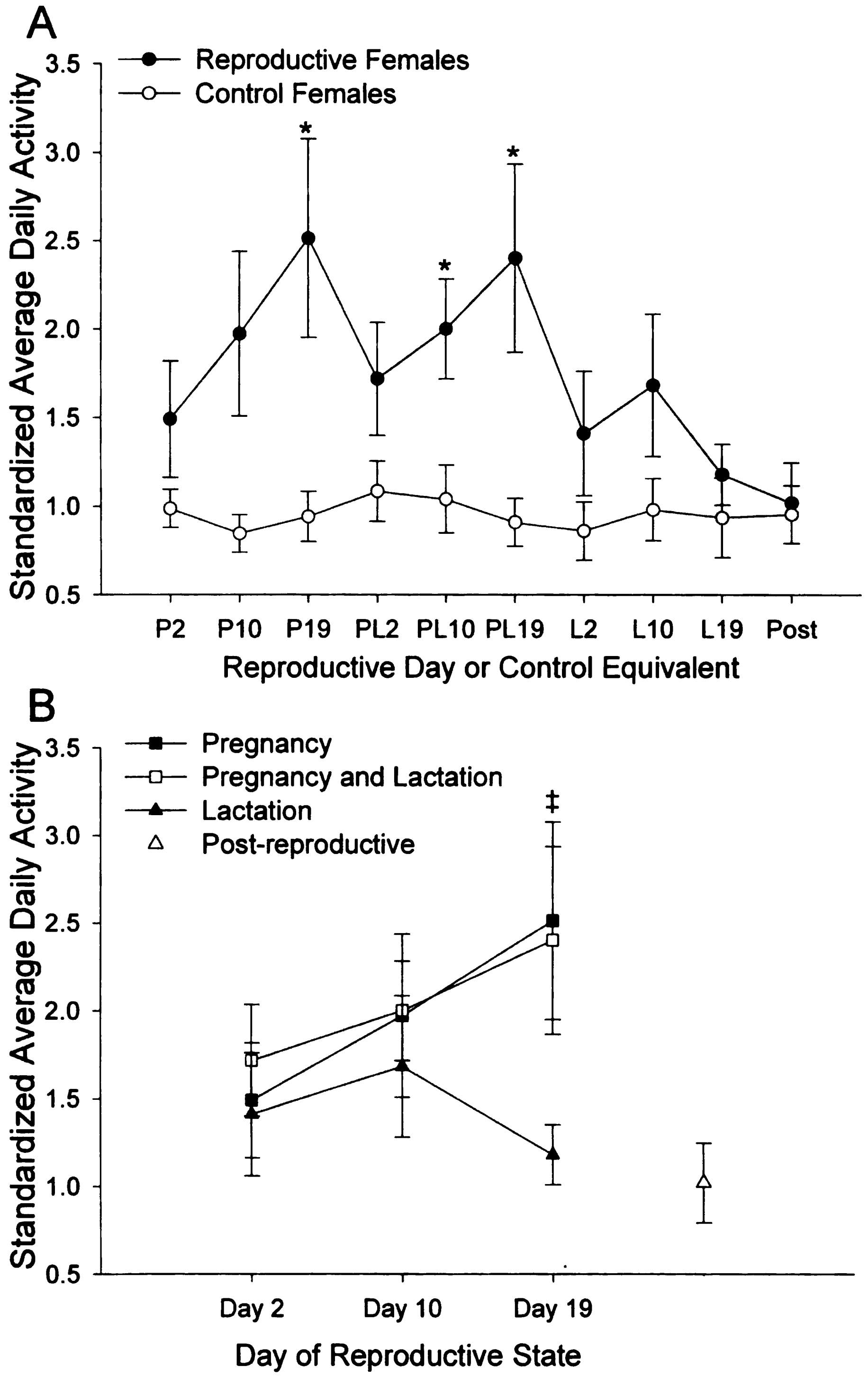

Figure 5.2. (A) Average daily activity across reproductive days (or equivalents) in the two treatment groups (n = 5/group). The average daily activity was standardized by dividing the average activity of a female for each day of interest by the average activity of that female on the pre-reproductive (or equivalent) day. Values are means ± SE. *Significant differences between the control and reproductive groups ($p < 0.05$; LSD test). The data for the reproductive group are re-plotted in (B) for comparisons of pregnancy and lactation to the combined state. ‡Significant differences between lactation and the combined (PL) state ($p < 0.05$; LSD test).

The distribution of activity across the light/dark cycle was highly labile (Fig. 5.3A), but this distribution was affected by the interaction between reproductive days (or equivalents) and treatment group (Table 5.1). The reproductive females had a significantly lower dark:light activity ratio on PL19 (more diurnal) and a significantly higher ratio on L2 (more nocturnal) than controls ($p < 0.05$; LSD tests), but the ratio did not differ significantly from the pre- or post-reproductive day for any day examined in either group (Fig. 5.3A). Lactating females were more nocturnal during early than mid-lactation, regardless of whether or not they were pregnant, and the ratio was significantly higher on day PL2 than PL10 or PL19 and on day L2 than on L10 (Fig. 5.3B; $p < 0.05$; LSD tests). Within the control group, the ratio of activity in the night versus the day did not change across the days of interest, and females were more active in the light than the dark (Fig. 5.3A; dark: light ratio: 0.714 ± 0.070; post-hoc GMEM; $F(10, 9.14) = 0.53$, $p > 0.8$).

Temperature Rhythms

Females were hyperthermic during much of pregnancy and lactation, with the most noticeable rises in T_b occurring during the mid-dark (major trough) and mid-light (minor trough) phases of the day, while rhythms in control females did not noticeably change over time (Fig. 5.4). Peaks occurred around the light/dark transitions. GMEM analysis of the amplitude of the T_b rhythms and midday and midnight T_b averages all revealed significant main effects of reproductive days (or equivalents) and treatment group and a significant interaction between the two (Table 5.1). However, reproductive state did not alter the phase of the rhythm as

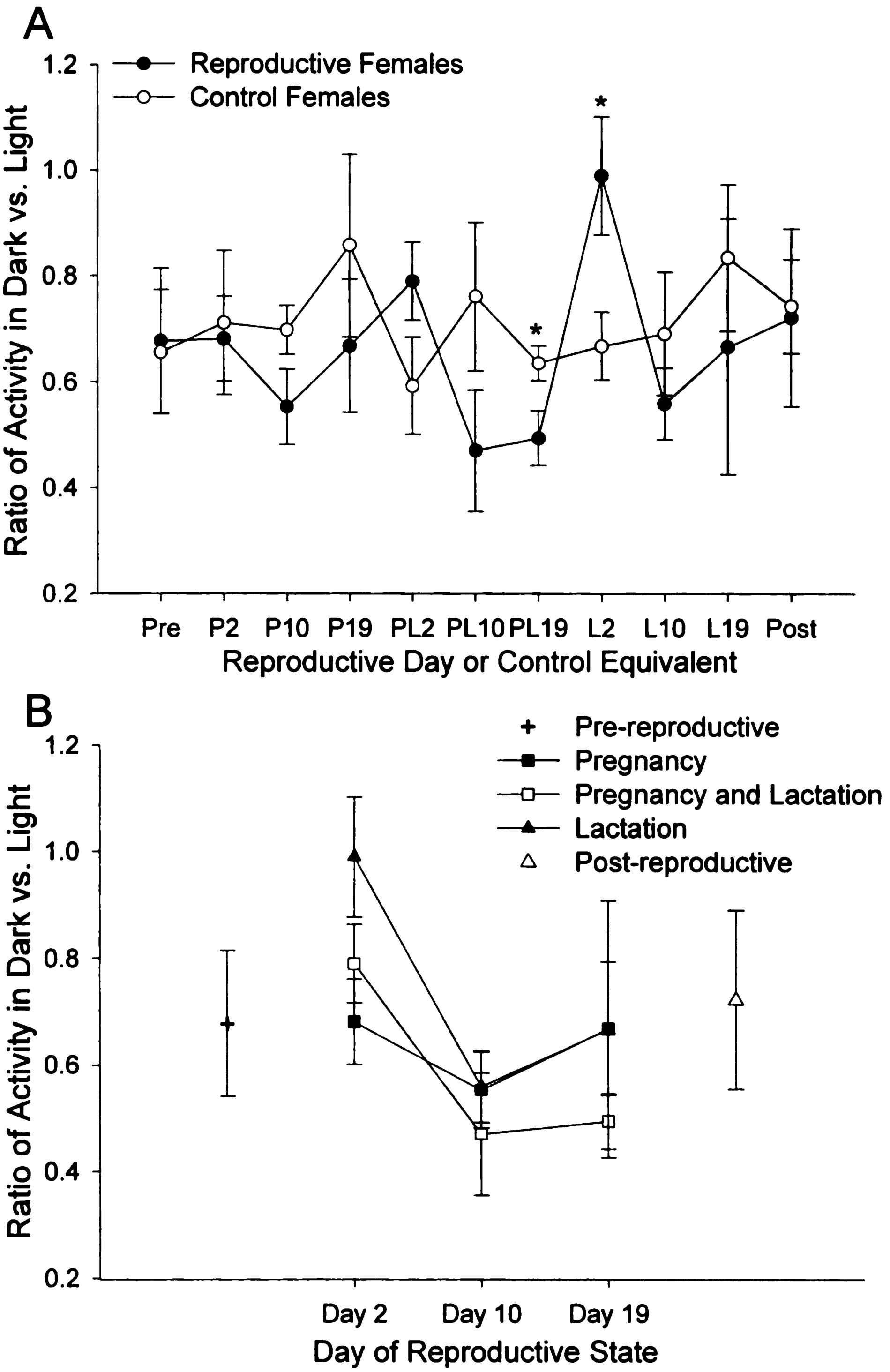

Figure 5.3. (A) Ratio of activity in the dark versus light phase across reproductive days (or equivalents) in the two treatment groups (n = 5/group). Values are means ± SE. Notation is the same as in Fig. 5.2A. The data for the reproductive group are re-plotted in (B) for comparisons of pregnancy and lactation to the combined state.

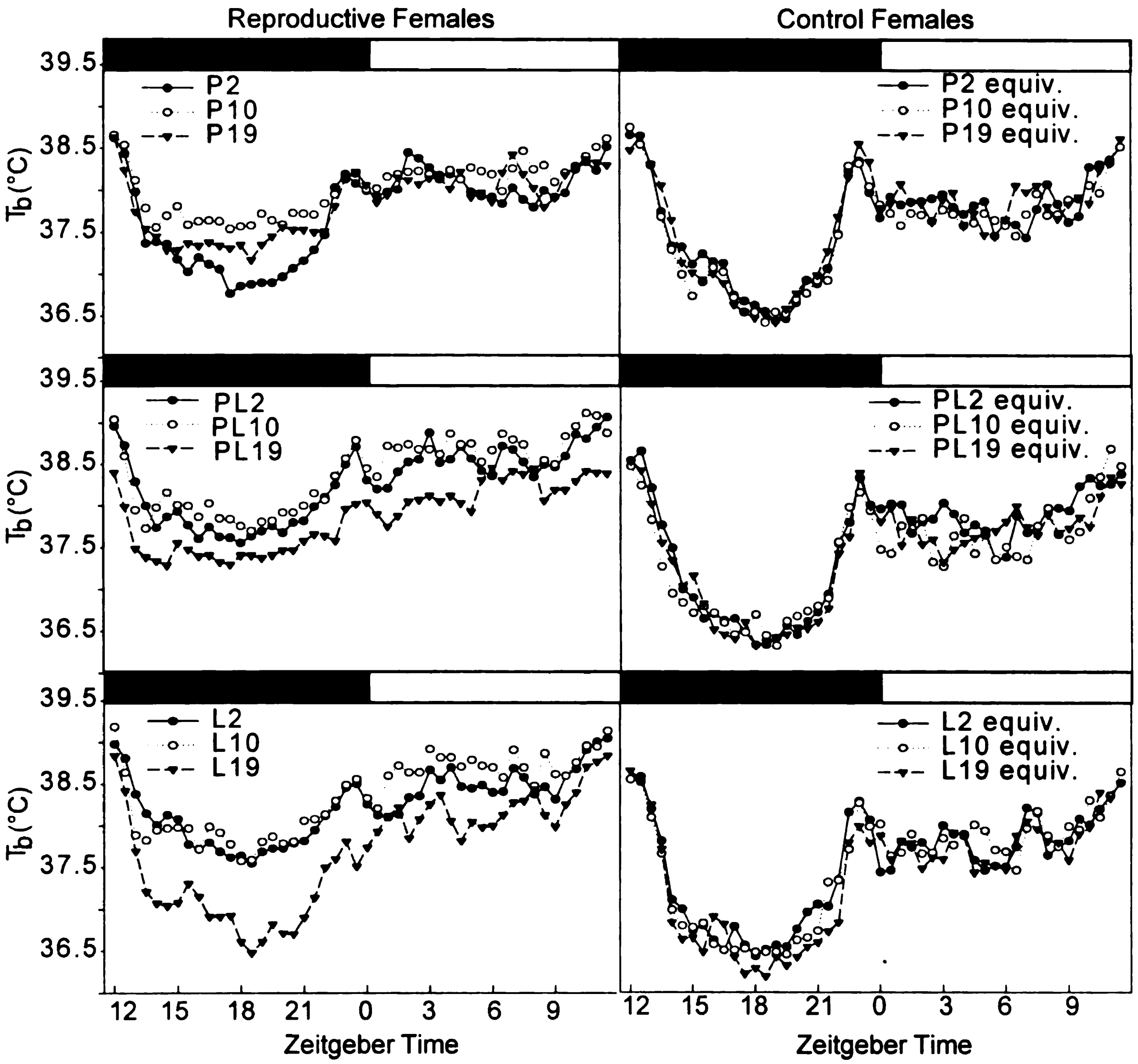

Figure 5.4. Averaged Tb rhythms in the reproductive (left) and control (right) groups (n = 5/group) under a 12h: 12h light (open bars)/dark (black bars) cycle for pregnancy, pregnancy plus lactation, and lactation (or equivalents: equiv.). Values are means for each treatment group for each half hour. Standard errors were removed for ease of comparing the rhythms.

indicated by the half-hour interval with the lowest average T_b (Table 5.1). There was a significant effect of treatment group, however, with control females exhibiting a slightly later minimum T_b (ZT 18.19 ± 0.34) than reproductive females (ZT 17.09 ± 0.34) throughout the study, including prior to pairing the reproductive females with their mates.

The amplitude of the T_b rhythm (based on half hour intervals) changed across time in both groups of animals but in different ways (Fig. 5.5A). Within the control group, the amplitude significantly increased toward the end of the study, from the PL19 and L2 equivalents to the post-reproductive equivalent (Fig. 5.5A; $p < 0.05$; LSD tests). In the reproductive group, the amplitude was also highest on the post-reproductive day, and it did not differ from that of controls at that point (Fig. 5.5A). These data indicate that amplitude increases with age. However, in the reproductive females, the amplitude of the T_b rhythm dropped from mid-pregnancy through mid-lactation and was significantly lower from days P10 through PL10 as well as on L2 and L10 than on the pre-reproductive day (Fig. 5.5A; $p < 0.05$; LSD tests). The amplitude from days P10 through PL10 was also significantly lower than in controls on the equivalent days (Fig. 5.5A; $p < 0.05$; LSD tests). Pregnancy and lactation influenced the rhythm amplitude differently during the combined state, with one masking the effects of the other on different days (Fig. 5.5B). The amplitude on PL10 was the same as on L10, but it was significantly higher than on P10 (Fig. 5.5B; $p < 0.01$; LSD test), suggesting that pregnancy has no effect on the rhythm amplitude in females that are 10 days into lactation. In contrast, the amplitude was the same on PL19 as

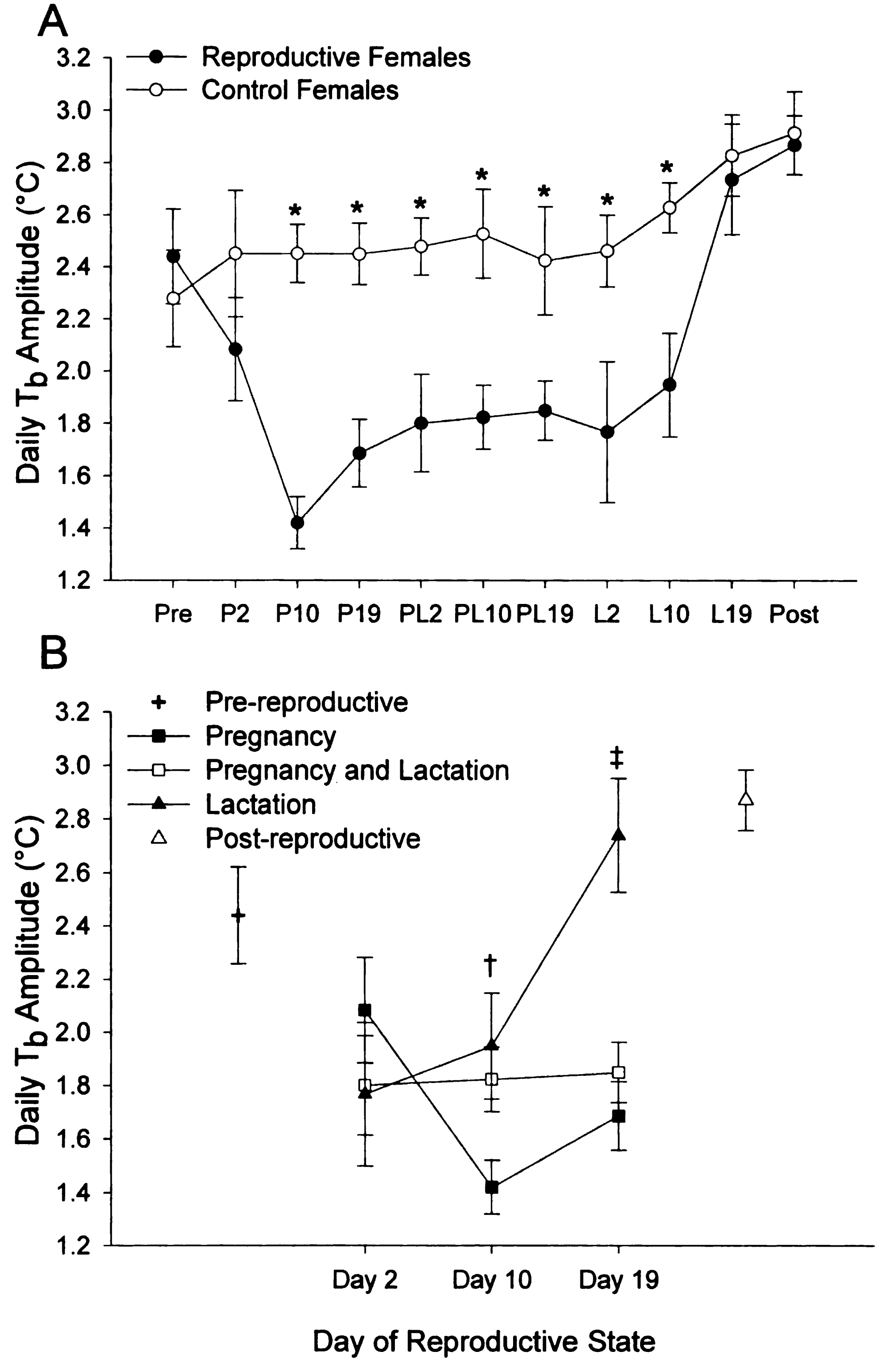

Figure 5.5. (A) Daily amplitude of the Tb rhythm across reproductive days (or equivalents) in the two treatment groups (n = 5/group). Values are means ± SE. Notation is the same as in Fig. 5.2A. The data for the reproductive group are re-plotted in (B) for comparisons of pregnancy and lactation to the combined state. †Significant differences between pregnancy and the combined (PL) state ($p < 0.05$; LSD test). ‡Significant differences between lactation and the combined (PL) state ($p < 0.05$; LSD test).

on P19 but was lower on PL19 than on L19 (Fig. 5.5B; $p < 0.01$: LSD test), indicating that by 19 days, lactation no longer had an effect on the rhythm amplitude, regardless of whether or not females were pregnant.

Drops in rhythm amplitude appear to be due to rises in the average body temperatures of females during the midnight period in all three reproductive states relative to the pre-reproductive period and to controls. Midday temperatures rose in a similar fashion (Fig. 5.6A and B). During the midday period, T_b values did not change in the control group (Fig. 5.6A; $F(10, 40) = 0.83$, $p > 0.5$; post-hoc GMEM), and during the midnight, T_b actually dropped in controls from the P19 equivalent to the post-reproductive equivalent day (Fig. 5.6B; $p < 0.05$; LSD test), which is the probable cause of the rise in rhythm amplitude seen at this time (Fig. 5.5A). In the reproductive group, however, the midday T_b averages increased from the pre-reproductive day for all days of pregnancy and lactation and, except on P19, were also higher on these days than on the post-reproductive day and than controls on the equivalent days (Fig. 5.6A; $p < 0.05$; LSD tests). Similar deviations from the pre- and post-reproductive days were seen for the midnight T_b averages (Fig. 5.6B). The mean midnight T_b was significantly higher than on the pre-reproductive day from P2 through L10 and than on the post-reproductive day from P10 through L10; reproductive females had significantly higher midnight T_b means than controls on these days as well (Fig. 5.6B; $p < 0.05$; LSD tests).

Midday and midnight T_b values were highest in early to mid lactation, whether or not females were also pregnant. During the midday period, the

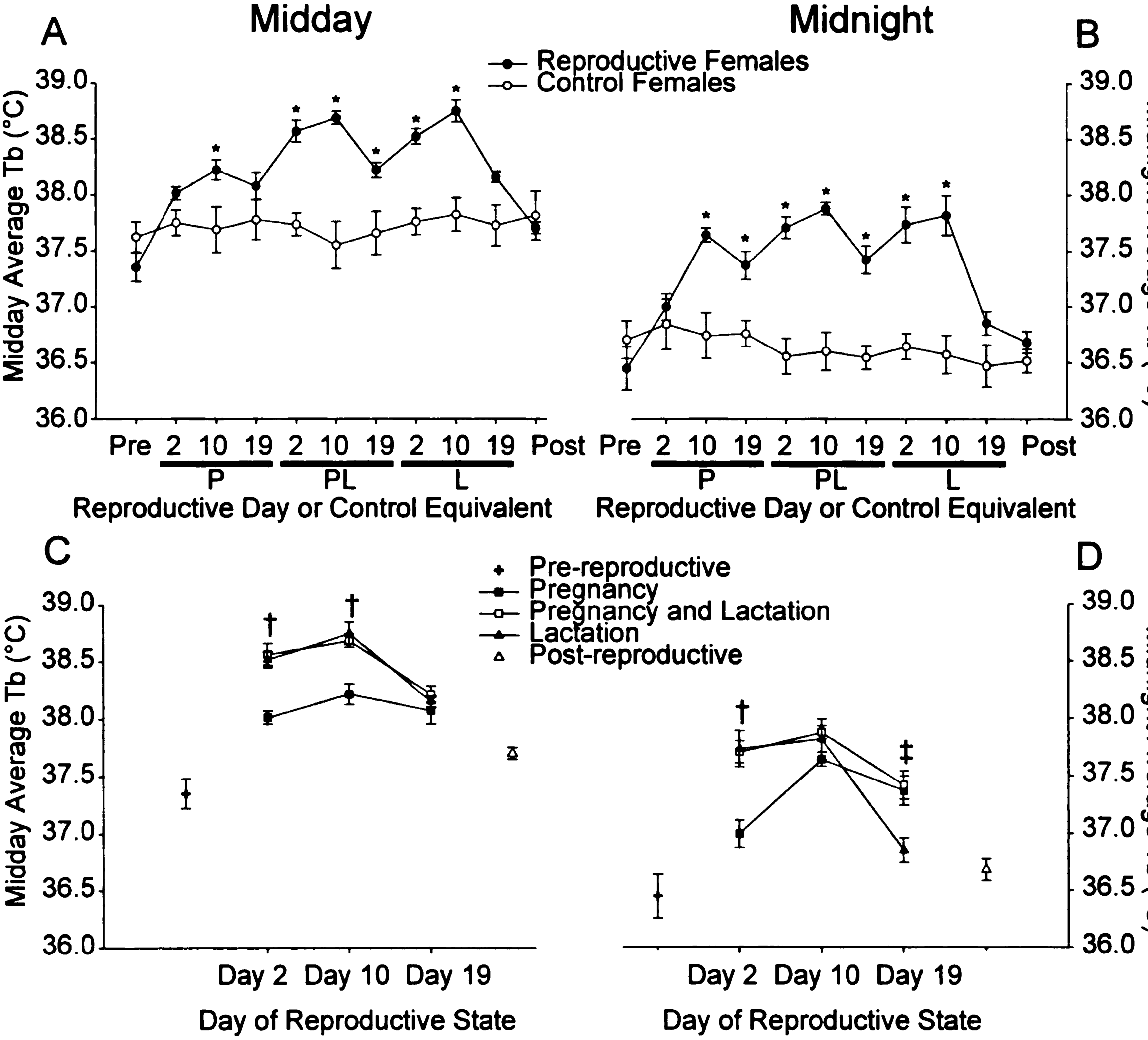

Figure 5.6. (A and B) Average Tb during the midday (ZT 3 to 9, A) and midnight (ZT 15 to 21, B) periods across reproductive days (or equivalents) in the two treatment groups (n = 5/group). Values are means ± SE. Notation is the same as in Fig. 5.2A. The data for the reproductive group are re-plotted in (C) and (D) for comparisons of pregnancy and lactation to the combined state during the midday (C) and midnight (D) periods. Notation is the same as in Fig. 5.5B.

average T_b values during days 2 and 10 of pregnancy were significantly lower than the corresponding days of the combined pregnant/lactating state (Fig. 5.6C; $p < 0.01$; LSD tests), and midday T_b did not change throughout pregnancy (Fig. 5.6C). However, the midday T_b significantly dropped from day 10 to 19 in both lactation and the combined pregnant/lactating state (Fig. 5.6C; $p < 0.005$; LSD tests). Similar trends were evident in the midnight period with some differences (Fig. 5.6D). The midnight T_b average was significantly lower on P2 than on PL2, and that on L19 was significantly lower than on PL19, again demonstrating differential effects of pregnancy and lactation during the combined state (Fig. 5.6D; $p < 0.05$; LSD tests). Midnight T_b means peaked on both P10 and PL10 and were significantly higher than on days 2 and 19 of the same states (Fig. 5.6D; $p < 0.05$; LSD tests). Similarly, the mean midnight T_b on L10 was significantly higher than on L19 ($p < 0.005$; LSD test) but did not differ from that on L2 (Fig. 5.6D).

Behavioral Rhythms in Nesting Females

Three of the females from the reproductive group were videotaped on days 2, 10, and 19 of lactation, and the tapes were scored to assess the proportion of five minute intervals each hour (beginning at the designated ZT) during which the lactating dam was off the nest and/or drinking. A two-way repeated measures ANOVA revealed no effects of day of lactation (Off-nest: $F(2, 4) = 0.459$, $p > 0.5$; Drinking: $F(2, 4) = 3.694$, $p > 0.1$) and no interaction between day of lactation and ZT for either behavior (Off-nest: $F(46, 92) = 1.047$, $p > 0.1$;

Drinking: $F(46, 92) = 0.948$, $p > 0.5$). However, there were significant effects of time of day (ZT) on each behavior (Off-nest: $F(23, 46) = 5.897$, $p < 0.001$; Drinking: $F(23, 46) = 2.227$, $p < 0.05$), indicating a daily rhythm in nesting and drinking that did not change across lactation. These rhythms reflected the crepuscular nature of activity in this species in captivity. Off-nest activity peaked near the light/dark transitions (ZT 1 and 12), and troughs occurred in the mid-light and mid-dark phases (ZT 7 and 20; $p < 0.05$: LSD tests between peaks and troughs), though off-nest behavior was significantly less frequent at ZT 20 than at ZT 7 (Fig. 5.7A; $p < 0.01$; LSD test). Drinking was less frequent than off-nest behavior, but the rhythm looks similar in shape, though not in amplitude, to that behavior (Fig. 5.7B). There was not one distinct peak for this rhythm, although the highest frequencies of drinking were around the light/dark transitions (ZT 2 and 11; Fig. 5.7B). The significant troughs of the rhythm occurred a few hours before lights-on (ZT 20 and 21) and again at lights-on (ZT 0; Fig. 5.7B; $p < 0.05$; LSD tests between each trough and ZT 1 and 2).

DISCUSSION

Many females spend most of their lives pregnant and/or lactating, which mandates major changes in various parameters of physiology and behavior that are rhythmic. In this study, we found that circadian strategies can be modulated during pregnancy and lactation in quite different ways in diurnal and nocturnal species. Additionally, we demonstrated that the effects of pregnancy and lactation are not simply additive during the combined state but rather that the effects of pregnancy are evident in certain parameters of activity and temperature

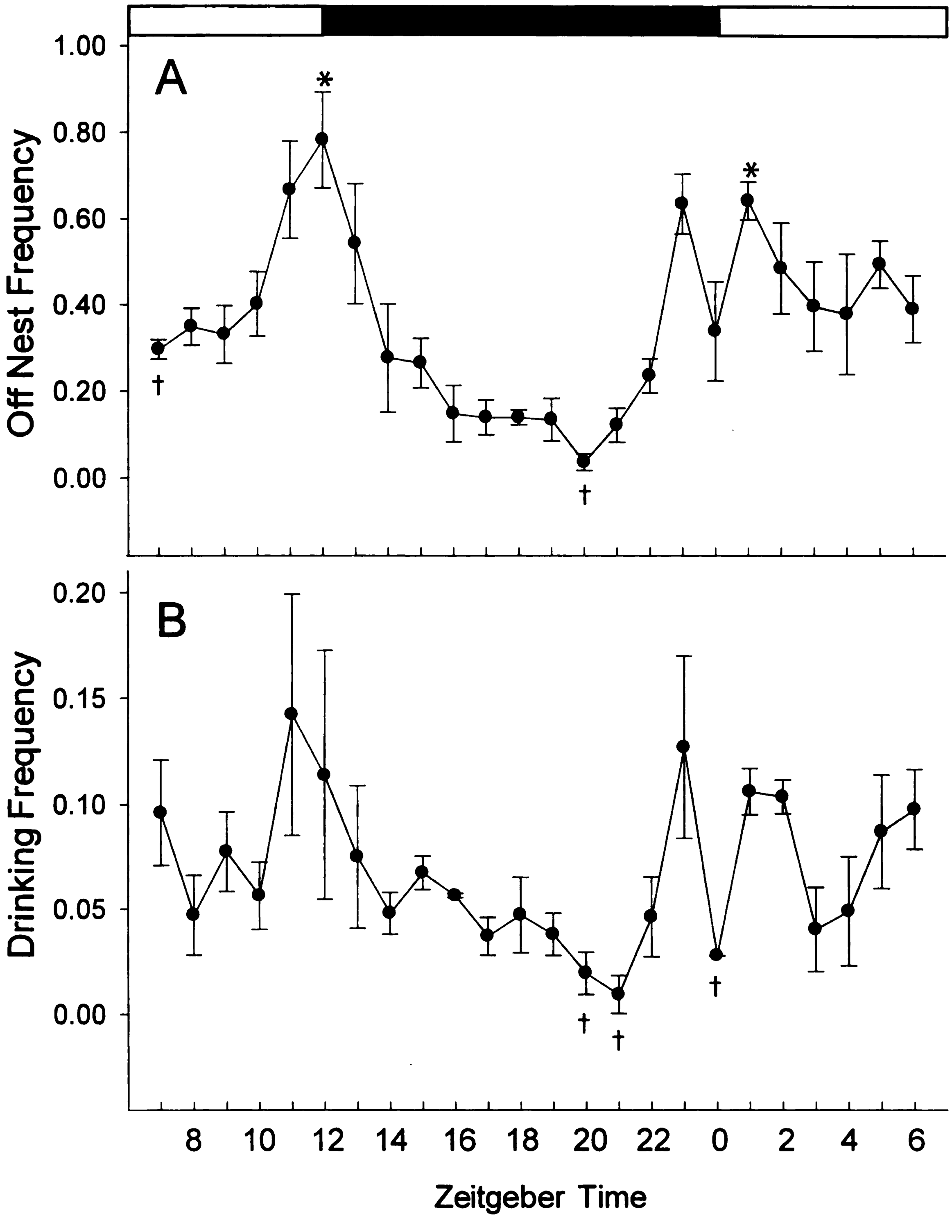

Figure 5.7. Frequencies of off-nest (A) and drinking (B) behaviors for each hour of the light (open bars, top): dark (black bar, top) cycle during lactation in a subset of the reproductive group (n = 3). Values are pooled means ± SE for days 2, 10, and 19 of lactation in 3 lactating dams. *Peaks and †troughs in each rhythm as explained in RESULTS.

rhythms while the effects of lactation are evident in others. We have also found that rhythms in maternal nesting of grass rats do not change over the first 19 days of pup development.

One important issue to consider when interpreting these data is that males remained with their females during both the pregnant and the combined pregnant/lactating states but were absent during the lactation-only state and among control females. Therefore, differences seen within females in the P/PL states compared to the L condition, as well as differences between P/PL females and controls, could theoretically be due to the presence/absence of the male. We were unable to include P and PL conditions without males because grass rats do not undergo spontaneous estrous cycles, and the postpartum estrus is the only one that can be reliably predicted. Effects of reproductive state can be evaluated independently of effects that males might have had on T_b or activity when we compare P with PL states (both of which occurred when the male was present) and when we compare L females with non-pregnant controls (neither of which involved the presence of a male). Both sets of comparisons reveal clear effects of reproductive state. Furthermore, changes occurring within any given state (P, PL and L) are likely to begin with changes in the female and/or the pups and cannot be due to changes in the presence/absence of males. It should also be noted that all of the comparisons we have made are likely to reflect what occurs in nature, where adult male and female grass rats live together in communal burrows with one or two generations of their offspring (Blanchong and Smale, 2000; Packer, 1983; Senzota, 1982) and where non-pregnant females (whether they are lactating or not) are unlikely to be living with males.

During pregnancy but not lactation, locomotor activity increased to roughly 2.5 times the level seen in virgin females (Fig. 5.1 and 5.2). However, there were no changes in the timing of the offset or onset of activity or in the relative amount of activity in the dark versus light portions of the day. This is entirely different from patterns observed in nocturnal rodent models, which become less rhythmic in their activity and show lower levels of both wheel-running and general activity (Albers et al., 1981; Richards, 1966; Rosenwasser et al., 1987; Scribner and Wynne-Edwards 1994a). In rats and hamsters, estradiol can consolidate, mildly phase-advance, and increase nighttime activity (Albers, 1981; Morin et al., 1977; Takahashi and Menaker, 1980), and, in hamsters, progesterone counteracts these effects (Takahashi and Menaker, 1980). Takahashi and Menaker (1980) argued that this might explain why pregnant rats, which have high levels of circulating progesterone relative to estrogens, are much less active and less rhythmic in their activity. Although we do not know the effects of these hormones on activity in grass rats, the patterns we see here suggest that responses to these hormones may be very different in this species compared to lab rats and hamsters.

The most dramatic effects of reproductive state on rhythms stemmed from rises in T_b that occurred during pregnancy, lactation, and when the two states were combined (Fig. 5.4). The increases were largest during the midday and midnight phases of the rhythm rather than at the light/dark transitions. This may reflect a physiological constraint limiting increases at those transitions, when T_b was high even in females that were not reproductive. The increases were also larger at night than during the day (Fig. 5.6), which resulted in a reduction in rhythm amplitude (Fig. 5.4). During pregnancy, the rises in T_b could have been partially driven by changes in activity, which, although not necessary for them, has modulatory effects on rhythms in T_b (Refinetti, 1997; Refinetti, 1999). However, it is unlikely that activity was solely responsible for the changes since it increased from early to late pregnancy. An increase in the midnight T_b did occur from day 2 to day 10 of pregnancy, but if increased activity were inducing hyperthermia, this would have also been evident in the midday, which it was not. Various other factors probably contributed to increasing T_b and changes in its temporal pattern during pregnancy. First, the increased secretion of certain hormones may have driven hyperthermia. Progesterone and prolactin increase T_b in rodents (Leon et al., 1978; Woodside et al., 1981), as do birth control pills, which contain progestagens, in humans (Baker et al., 2001a; Baker et al., 2001b; Wright and Badia, 1999). During the first half of pregnancy there is an endogenous circadian rhythm in prolactin secretion in many rodents (Butcher et al., 1972; Edwards et al., 1995; McMillan and Wynne-Edwards, 1999; Talamantes et al., 1984). The rhythmic release of this hormone might therefore influence the rhythmic rises in T_b. Other physiological processes associated with pregnancy could also contribute to rises in T_b and changes in its temporal patterning. Since this is an energetically expensive state (Gittleman and Thompson, 1988; Speakman, 2008), an increase in feeding or metabolic turnover, as has been seen in other rodent species (Biggerstaff and Mann, 1992; Cripps and Williams, 1975; Quek and Trayhurn, 1990; Wade et al., 1986), could account for the time-dependent changes in hyperthermia seen in grass rats.

Hyperthermia was even more pronounced in lactation than in pregnancy, especially during the energetically expensive early to mid-lactational period (Fig. 5.6). There are several possible explanations for this. Circulating hormones related to lactation and maternal care could contribute directly to increased T_b (Leon et al., 1978; Woodside et al., 1981), as may be the case with pregnancy. However, an increase in metabolic turnover causing hyperthermia is more consistent with our data. Hyperthermia during the mid-dark phase disappeared from mid to late lactation, when pups began to incorporate solid food into their diet, reducing energetic demands of the pups on their mother. Also, the fact that hyperthermia was more pronounced during lactation than pregnancy, which is less energetically expensive (Gittleman and Thompson, 1988; Speakman, 2008), lends support to this hypothesis. An alternative explanation was proposed by Croskerry et al. (1978) suggesting that contact with pups in the nest induces maternal hyperthermia and serves as a constraint on the amount of time a dam may spend on her nest. This hypothesis has received much attention, and evidence both for and against it has been gathered from various species (Scribner and Wynne-Edwards, 1994b; Walton and Wynne-Edwards, 1997; Woodside et al., 1980), but see (Bates et al., 1985; Stern and Lonstein, 1996; Stern and Azzara, 2002). Our data indicate that contact with pups did not correlate with peaks in T_b in lactating grass rats. The behavioral data show that females were off the nest least from ZT 15 to 22, when core body temperature had not yet peaked. If females were overheating on the nest, then they should have been warmest at these times. However, significant increases in T_b did occur during this mid-dark phase of the day, and maternal-pup thermal transfer may thus have contributed to some of the increase in T_b, though it certainly did not appear to constrain maternal care in grass rats. Kittrell and Satinoff (1988) argued that their data from lab rats were also inconsistent with the hypothesis of Croskerry et al. (1978). Their females exhibited the highest core body temperatures when they were most active off of the nest, not after long bouts on the nest. However, in grass rats, activity also could not account for lactational hyperthermia, as females were no more active than controls or than they themselves were before or after the period of reproduction. This would indicate that metabolic and/or hormonal processes were the most important factors driving hyperthermia in lactating grass rats.

Patterns of change in the phase of daily rhythms in T_b across reproductive states were different in grass rats from those seen in nocturnal lab rats. Specifically, whereas in lab rats, the phase of the peak of the T_b rhythm advances during both pregnancy and lactation (Kittrell and Satinoff, 1988), no changes in phase occurred in the rhythms of grass rats. This difference between the species may reflect their evolutionary histories in environments with different ecological constraints during the active phase. For instance, it may be that for a nocturnal animal, it would be more energetically expensive to increase T_b further during the cold night, when the T_b peak usually occurs, and in order to do so, the phase must be shifted so that it occurs under warmer ambient conditions. Another possibility is that the species difference is directly related to activity rhythms. When grass rats became pregnant, the amplitude of their activity rhythms remained high, and their phase did not change (Fig. 5.1). However, in pregnant lab rats, activity rhythms break down in constant darkness (Rosenwasser et al., 1987), and in hamsters, activity becomes less rhythmic under a light/dark cycle (Albers, 1981). Therefore, a lack of a stable activity rhythm in these nocturnal species may allow for a change in phase of the T_b rhythm whereas the presence of a stable activity rhythm may prevent such a change in grass rats.

When we focus directly on the rhythms and ask how they were affected by reproductive state, clear time-of-day effects on the nature of the interaction between pregnancy and lactation emerge. One example is apparent when considering reproductive females on day 10. Here, the midday T_b was identical in the two lactating conditions and significantly lower when females were just pregnant (Fig. 5.6C), but midnight T_b was indistinguishable amongst the three states (Fig. 5.6D). Thus, effects of lactation were considerably higher than those of pregnancy during the day, but not during the night. Another example is evident when considering day 19, at which point midday T_b was the same in the three reproductive states (Fig. 5.6C), but the midnight T_b was considerably lower when females were lactating but not pregnant (Fig. 5.6D). This pattern suggests that the return to patterns characteristic of the non-reproductive state, (evident at day 19 of lactation in non-pregnant females) occurs more rapidly for the nighttime than the daytime phase of the rhythm. In neither of these examples, at either time of day, were effects of pregnancy and lactation on rhythms simply additive. This could be due to time-of-day effects on some of the processes noted above. For example, in some cases (e.g. day 10) the non-additive effects could reflect a rhythm in a physiological ceiling on the maximum T_b (compare Fig. 5.6C and D). Although pregnancy and lactation are both energetically demanding, they are nonetheless physiologically different states. Perhaps it should not be surprising, therefore, that their effects are non-additive, but the intriguing part of the patterns are that they can be additive at some times of day but not others.

CONCLUSIONS AND IMPLICATIONS

The current data reveal clear patterns of change in daily rhythms of grass rats as they transition from one reproductive state to another and that these are not identical to the changes seen in nocturnal rodents. They also show interacting effects of lactation and pregnancy that could not have been predicted from either state alone and that these interactions themselves change as a function of time of day. The patterns observed here highlight the importance of considering the circadian timekeeping system as a dynamic one whose influence on behavioral and physiological processes can change in systematic ways with different reproductive states. These data raise the question of what neural and physiological processes might produce changes in temporal organization across these states. One possibility is that they are driven by modulation of the mechanisms generating circadian rhythms and transmitting temporal information from them to other regulatory systems. Alternatively, these basic circadian mechanisms could be buffered against changes in reproductive state, and the emergence of new patterns of rhythmicity could instead be brought about by more direct influences of reproductive processes on systems regulating T_b and activity. The most likely possibility, however, is that the modulation of rhythms from one reproductive state to another is driven by changes both within and beyond the circadian timekeeping system.

CHAPTER 6

Conclusions

Rhythms in the brain in early pregnancy

In the introductory chapter of this dissertation, I proposed three main hypotheses concerning whether and how the circadian system of the brain might promote changes in overt circadian rhythms during early pregnancy in the rat. The results presented in Chapters 2, 3, and 4 clearly indicate that some aspect of early pregnancy is influencing rhythms in the SCN, extra-SCN oscillators, and non-circadian regulatory areas of the brain. These influences are most likely acting at multiple levels of these neural networks (Fig. 6.1), given the variable effects in regions beyond the SCN (see Chapters 3 and 4).

The research presented in this dissertation provides a foundation for future research concerning the mechanisms that modulate rhythms as an individual progresses from one reproductive condition to another. Four main questions are raised by my data. First, it is unclear what aspect of early pregnancy leads to the effects seen in Chapters 2 through 4. Some of these might be caused by mating-related stimuli, such as auditory, tactile, or chemical cues associated with the male (e.g. pheromones or signals from the ejaculate). Other possibilities include fertilization- or implantation-related stimuli and endocrine changes associated with early pregnancy. It is possible that different stimuli induce changes in different regions of the brain. A serial process of elimination could be employed to identify which of the stimuli are necessary and/or sufficient to induce changes in temporal patterns of Fos and Per2

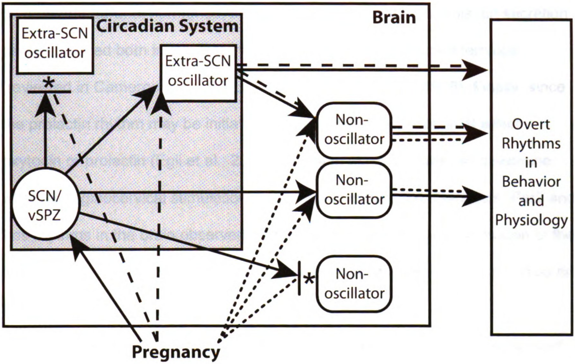

Figure 6.1. Proposed network model of the pathways (arrows) by which pregnancy affects rhythms in circadian and non-circadian systems of the brain to lead to changes in overt rhythms. Some aspect of pregnancy induces changes within the SCN and vSPZ (solid arrows), some extra-SCN oscillators (dashed arrows), and some non-oscillatory brain regions (dotted arrows) to promote changes in overt rhythms. These effects are likely to be integrated at multiple levels (converging arrows leading to combined arrows). In addition, rhythms in some brain regions are unaffected (*blocked arrows indicating resistance to upstream influences of pregnancy). Arrows indicate general pathways, not neural projections.

expression in each brain region of interest. The effects of non-mechanical mating stimuli, presence of the ejaculate, fertilization, and implantation could be discounted if the same changes seen here can be induced in a pseudopregnant model. This approach could also be used to determine the relative importance of ovarian and adrenal hormones, as a semicircadian rhythm in prolactin secretion can be induced both in ovariectomized and in adrenalectomized females (reviewed in Cameron et al., 2003; Freeman and Sterman, 1978). Finally, since the prolactin rhythm may be initiated with a single administration of either oxytocin or prolactin (Egli et al., 2006; Helena et al., 2009), we can determine whether vaginocervical stimulation is required to induce the changes in Per2 and Fos rhythms in the brain observed here. Additionally, a closer examination of the time courses of the post-copulation changes in rhythms within the brain could be informative.

The second major question raised by our data is how altered patterns of Fos and Per2 expression might lead to changes in overt rhythms. This is a difficult question to address, but some information bearing on it might be provided by determination of the phenotypes of the cells expressing Fos and Per2 and by examination of how rhythms in output signals within these cells, such as AVP in the SCN, change during early pregnancy.

A third question raised by the patterns observed here is whether and how these systems and interactions among them might change across other phases of an individual's reproductive life. We have demonstrated a major re-organization of rhythms within the circadian system and non-circadian regulatory systems on day 6 of pregnancy, but this is only one day of the development and care of a female's offspring. For a rat, gestation and lactation of one litter last almost two months, and rhythms in behavior and physiology are changing throughout this time (Atkinson and Waddell, 1995; Brunton et al., 2008; Freeman et al., 2000; Kimura et al., 1996; Speakman, 2008). Rhythms within several regions of the brain have been shown to differ between early and late pregnancy (Lee et al., 1998), and new patterns are likely to emerge as females progress beyond that state as well.

Finally, the changes in rhythms in the brain in early pregnancy were only characterized in one nocturnal rodent model in the studies presented in Chapters 2, 3, and 4. This raises the question of the ways in which the patterns seen here would, and would not, generalize to other species with different evolutionary histories or ecologies. The changes could be different, for example, in the brains of diurnal species or in species that exhibit paternal care. In the latter, it would be interesting to know if males undergo changes in their circadian systems similar to those of their mates. Studies of both neural and overt rhythms in a biparental model system may shed light on how fathers and mothers partition their time to optimize their reproductive output and whether circadian dysfunction in fathers might hamper the development of their offspring.

Rhythms and reproduction in diurnal versus nocturnal rodents

The Nile grass rat (*Arvicanthis niloticus*) is a diurnal murid rodent species closely related to laboratory rats and mice (Lecompte et al., 2008), and, at least in captivity, males of this species exhibit paternal care (personal observations). In

Chapter 5, I found two primary differences between this species and nocturnal models with respect to how activity and core body temperature (T_b) rhythms change as females progress through a series of reproductive states. First, robust activity rhythms persist throughout pregnancy and lactation in grass rats, with their amplitude actually increasing in the pregnant state. Second, the peak of the T_b rhythm is not advanced in phase as it is in lab rats.

The maintenance of a robust rhythm in locomotor activity in the pregnant grass rat contrasts with the loss of locomotor activity rhythms due to reduced activity in nocturnal models (Albers et al., 1981; Richards, 1966; Rosenwasser et al., 1987; Scribner and Wynne-Edwards, 1994a; Speakman, 2008). In rats, the loss of the locomotor activity rhythm in early pregnancy correlates with a lack of Fos and Per2 rhythms in the vSPZ (Fig. 2.3E and F) and in Fos rhythms in the MS and VDB (Fig. 4.4C and E), all regions involved in promoting activity and/or wakefulness (Abrahamson and Moore, 2006; Greco et al., 2000; Jones, 2008; Lu et al., 2001; Saper et al., 2005; Schwartz et al., 2004; Schwartz et al., 2009). Since activity is distinctly rhythmic in the pregnant grass rat, at least in a light/dark cycle (Fig. 5.1), we might expect to find no effect of pregnancy on rhythms in the vSPZ, MS, or VDB in this species or that they might even increase in amplitude, as does the rhythm in activity. We might also expect the maintenance of Fos rhythms in the MPA of grass rats, as this region is involved in integrating the sleep/wake cycle with T_b homeostasis (Kumar et al., 2007).

Studies comparing the circadian systems of nocturnal and diurnal species have found no major differences in the SCN of the two chronotypes (reviewed in

Smale et al., 2003; Smale et al., 2008). However, rhythms in Fos and Per1 expression in the vSPZ are different in male lab rats and grass rats (Ramanathan et al., 2006; Schwartz et al., 2004; Antonio Nunez, personal communication). Additionally, Fos rhythms in the vSPZ of male grass rats, but not lab rats, are endogenous and persist in constant darkness (Schwartz et al., 2004). These differences may contribute to the differential effects of pregnancy on activity rhythms in lab rats and grass rats. Since the vSPZ is endogenously rhythmic in the grass rat, its rhythms may persist during pregnancy and thus prevent the loss of the locomotor activity rhythm. In Chapter 5, I argue that the lack of a phase change in the T_b rhythm in pregnant grass rats might be due to the maintenance of the activity rhythm in this species. Therefore, this second major difference between lab rats and grass rats might also be due to differences in the vSPZ of these two species. However, Per2 rhythms in the SCN of lab rats advance in phase during early pregnancy, which also correlates with the advance in the peak of the T_b rhythm. Although Per2 rhythms in the SCN are similar in male lab rats and grass rats (Amir et al., 2004; Ramanathan et al., 2006), it is possible that, during early pregnancy, the Per2 rhythm differs between the two species. If no advance occurs in the SCN of grass rats, this might also explain why there is no change in phase in the T_b rhythm of pregnant grass rats.

Further work concerning how circadian rhythms change during pregnancy in diurnal versus nocturnal species, both in the brain and in overt rhythms, may shed light on how these two chronotypes acclimate to changes in reproductive state. However, it may also inform research concerning how diurnality is achieved in species that have evolved from nocturnal ancestors by exposing those aspects of the circadian system that are more or less plastic in diurnal and nocturnal species.

Implications of the present work

Female mammals spend the majority of their adult reproductive lives pregnant and/or lactating, states in which physiological and behavioral demands are changing in major, but predictable, ways. Many systems make substantial adjustments, including those that organize the daily patterning of integrative processes. However, virtually nothing is known about the ultimate reasons for, or the proximate causes of, the changes in circadian rhythms that pregnant and lactating females undergo. This dissertation describes the first studies designed to identify ways in which modulation of the mammalian circadian system might lead to the changes in rhythms that occur between one reproductive state and the next. It also provides an in-depth description of circadian rhythms in core body temperature and activity in grass rats during various reproductive states to demonstrate that diurnal and nocturnal mammals can adopt very different circadian strategies during pregnancy and lactation, which suggests that more than one strategy may be adaptive.

Aside from providing information about natural changes in circadian rhythms in adult mammals, the data from this dissertation indicate that this knowledge has implications for reproductive medicine and biology. Shift-working women encounter fertility and pregnancy complications (Boden and Kennaway, 2006). One reasonable assumption about the effect of shift-work is that it is mediated by stress, which can complicate a pregnancy. However, the data from Chapters 2 through 4 indicate that rhythms in protein expression in both circadian and non-circadian systems are dramatically re-organized in pregnancy. If these systems are desynchronized prior to copulation, as can result from shift work, the re-organization of these systems necessary to optimize pregnancy may not be properly achieved, resulting in reduced fertility and fecundity.

Circadian rhythms researchers often neglect the relevance of their work to mammalian conservation and reproduction. However, wild and captive mammals may experience circadian dysfunction, often due to anthropogenic effects. Captive zoo animals, domesticated pets, service animals, and animals used in entertainment, such as the circus, are subject to activity, enrichment, and feeding schedules determined by their owners and keepers. Certain enrichment items, such as running wheels (Blanchong et al., 1999; Eikelboom and Lattanzio, 2003), and food are powerful entraining agents (Mendoza, 2007), and they may lead to desynchrony amongst certain circadian oscillators (Mendoza, 2007; Chidambaram Ramanathan, personal communication). This could result in the inability of an individual to properly re-organize rhythms in the brain to promote altered overt rhythms during transitions in reproductive state. For those species which are more reliant upon circadian rhythms to reproduce, these entraining agents might thus reduce the success of captive breeding efforts. Additionally, anthropogenic effects on rhythms in wild mammals, which might occur via such entraining agents as introduced non-natural food items, non-native species, or artificial light, may similarly affect the reproductive success of these animals.

Applications of my dissertation to shift-working women are equally viable for these non-human "shift-workers." Given that diurnal and nocturnal species exhibit clear differences in how their overt rhythms are altered during pregnancy and lactation (Chapter 5), a more comparative approach to the interface between reproduction and circadian biology in the future may allow us to determine how reproduction in wild and captive mammals is affected by perturbations in circadian rhythmicity.

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