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SEX, SURGES AND CIRCADIAN RHYTHMS: THE TIMING OF REPRODUCTIVE EVENTS IN A DIURNAL RODENT

By

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ABSTRACT

SEX, SURGES AND CIRCADIAN RHYTHMS: THE TIMING OF REPRODUCTIVE EVENTS IN A DIURNAL RODENT

By

Megan M. Mahoney

Rhythms in the timing of reproductive events are reversed in diurnal and nocturnal rodents, but little is known about the neural mechanisms underlying these differences. I examined these issues by comparing the diurnal murid rodent *Arvicanthis niloticus* (grass rat) to nocturnal *Rattus norvegicus* (lab rat). In the first set of experiments, I examined the hypothesis that differences in the timing of estrous events in diurnal and nocturnal species are due to differences in rhythms in responsiveness to steroid hormones. I found that steroids were able to induce a rise in activity of neurons containing gonadotropin releasing hormone (GnRH) at only one time of day, which was 12 hours apart in grass rats and lab rats. These temporal patterns persisted in both species when they were housed in constant darkness for five days suggesting that an endogenous circadian clock drives the rhythms in responsiveness to hormones. Secondly, I determined whether cells within the suprachiasmatic nucleus (SCN), the site of the principle mammalian circadian clock, project to neuroendocrine and steroid sensitive cells in grass rats, and whether these pathways differed from those of lab rats. Anterograde tract-tracing in grass rats revealed that both GnRH and estrogen receptor (ER) containing cells appear to receive input from the SCN, as has been seen in nocturnal rodents. I then found that arginine vasopressin and vasoactive

intestinal polypeptide, two neuropeptides made in the SCN, were contained in axon terminals that contacted GnRH neurons, as is also the case in lab rats and hamsters. In the third set of experiments, I examined the hypothesis that inverted rhythms in the timing of estrus-related behaviors in diurnal and nocturnal rodents are due to differences in rhythms in sensitivity to steroid hormones. Pairs of males and hormone-primed females were tested for mating at four different times of day. I found that grass rats had a daily rhythm in sexual behavior that was 12 hours out of phase relative to that seen in nocturnal rodents. Specifically, both the lordosis quotient and rate of copulation were relatively low at zeitgeber time (ZT) 17 and then rose to a peak at ZT 23. I also observed a bimodal rhythm in male mounting behavior that peaked at ZT 11 and 23. Taken together, these results indicate that steroid-primed grass rats and lab rats are similar with respect to the temporal relationship among estrous-related events, but that the timing of these events relative to the light:dark cycle is dramatically different. These differences appear to be due to rhythms in responsiveness to steroid hormones whereas the structure of the neural pathways communicating temporal information from the SCN to cells within the reproductive axis appear to be the same. The mechanisms underlying the differences in rhythms of sensitivity to steroids might involve temporal patterns of signals emitted by the SCN and/or patterns of sensitivity of neural targets to these signals.

For Bill

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Key to abbreviations:

Abbreviation	Definition
3V	3rd ventricle
ac	anterior commisure
AHA	anterior hypothalamic area
ANOVA	Analysis of variance
ARC	arcuate nucleus
AVP	arginine vasopressin
AVPV	anteroventral portion of the periventricular nucleus
BDA	biotinylated dextran amine
BNST	bed nucleus of the stria terminalis
D3V	dorsal portion of 3rd ventricle
DAB	diaminobenzidine
DBB	diagonal band of Broca
DD	constant darkness
EB	estradiol benzoate
ER	estrogen receptor-α
f	fornix
GnRH	gonadotropin releasing hormone
hDBB	horizontal portion of DBB
HPG	hypothalamic-pituitary-gonadal
icv	intracerebroventriculary
im	intramuscularly
LH	luteinizing hormone
LPOA	lateral portion of POA
LQ	lordosis quotient
LS	lateral septum
LSPV	lower sub paraventricular zone
LSV	ventral portion of LS
LV	lateral ventricle
mPOA	medial portion of POA
MS	medial septum
NGS	normal goat serum
NHS	normal horse serum
oc	optic chiasm
ot	optic tract
OVLT	organum vasculosum of the lamina terminalis
P	progesterone
PBS	phosphate buffered saline
PeVN	periventricular nucleus
POA	preoptic area
PR	progestin receptors
PVN	paraventricular nucleus of the hypothalamus

Key to abbreviations:

Abbreviation	Definition
PVT	paraventricular thalamus
RCH	retrochiasmatic area
sc	subcutaneous
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SON	supraoptic nucleus
TX	triton-X
vDBB	vertical portion of DBB
VIP	vasoactive intestinal polypeptide
VLH	ventrolateral hypothalamus
VMH	ventromedial hypothalamus
VMPO	ventromedial portion of POA
ZT	zeitgeber time

Chapter 1

General Introduction

The research described in this dissertation is aimed at investigating the neuronal mechanisms that regulate the timing of reproductive events in a diurnal rodent, *Arvicanthis niloticus*. Specifically, I have focused on the timing of mating and of the ovulatory surge of luteinizing hormone (LH). In this introductory chapter I will provide 1) an overview of how the hypothalamic-pituitary-gonadal (HPG) axis functions to regulate the secretion of LH, 2) how the circadian timing system may interact with the HPG system to regulate the timing of reproductive events in female rodents, 3) a rationale for why I use a diurnal rodent model, and 4) the questions and experiments that will be addressed in each chapter of my dissertation.

The hypothalamic-pituitary-gonadal axis and the LH surge

Critical reproductive events associated with the mammalian estrous cycle such as mating, ovulation and preovulatory changes in hormone secretion depend upon the precise temporal coordination of functions occurring at different levels of the HPG axis (Figure 1.1). This integration requires: 1) a steroid-hormone sensitive system which responds to the increasing levels of estradiol released from the maturing follicles, 2) Gonadotropin releasing hormone (GnRH) neurons that stimulate the release of LH from the anterior pituitary, and 3) a precise timing mechanism which can coordinate the release of GnRH from a diffuse population of cells (van der Beek, 1996).

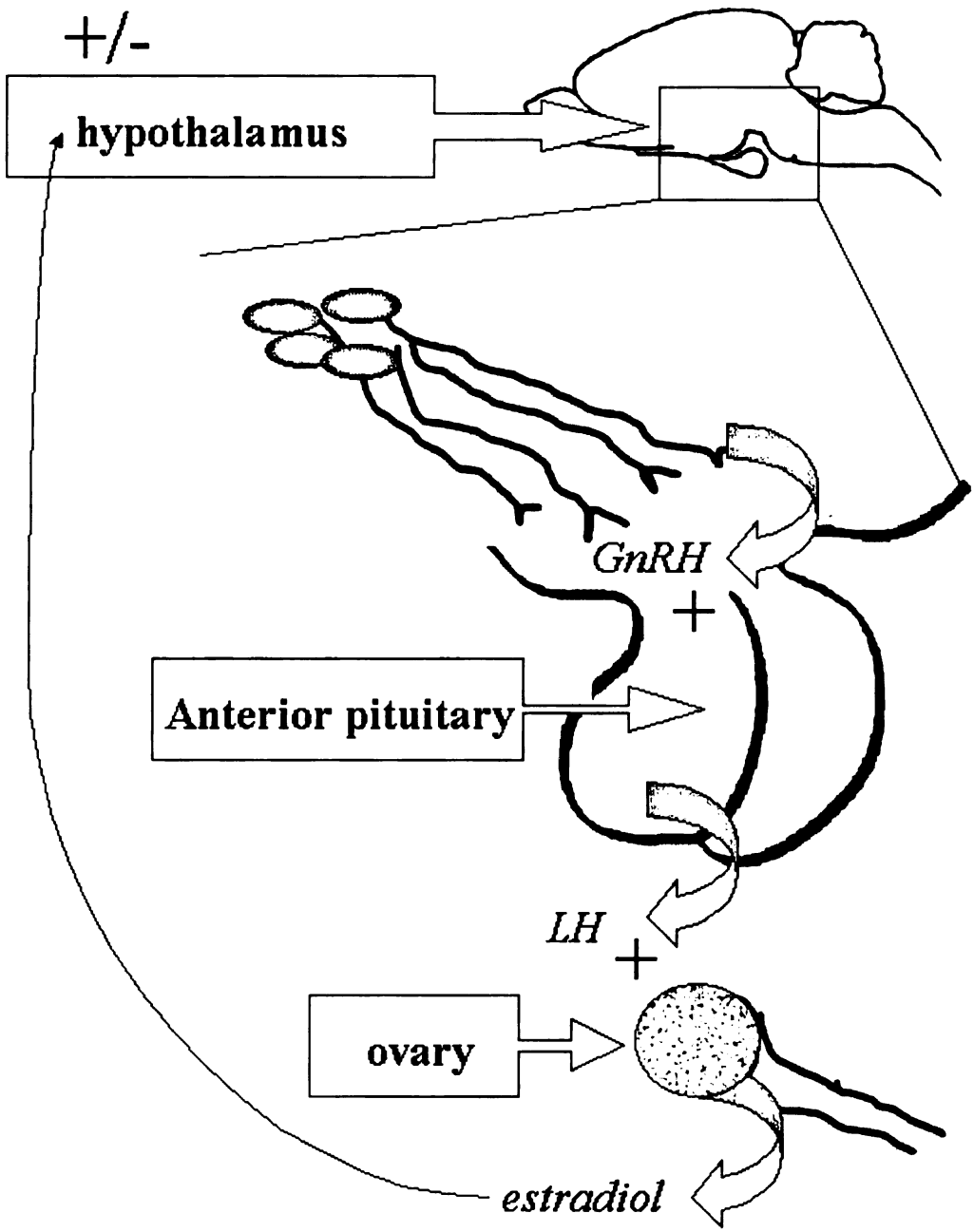

Figure 1.1. A schematic diagram of the hypothalamic-pituitary-gonadal axis. Immediately prior to the LH surge estradiol secreted from the ovaries acts as a signal that stimulates GnRH release into the portal blood system. From there, GnRH reaches the anterior pituitary and induces secretion of LH.

Much of our knowledge regarding the neuroendocrine changes associated with the estrous cycle is based on studies of laboratory rodents including *Rattus norvegicus* ("lab rats") and *Mesocricetus auratus* (golden hamsters). During the follicular phase of the estrous cycle, developing ovarian follicles release estradiol, which inhibits gonadotropin secretion through a negative feedback loop. At proestrous, estradiol levels eventually peak and this ovarian signal switches from an inhibitory to stimulatory factor. Estradiol acts on the hypothalamus, causing GnRH to be released into the portal circulatory system through which it reaches the anterior pituitary (reviewed in Freeman, 1994). GnRH then acts on gonadotropes in the pituitary to trigger LH release. Thus, during proestrous the surge in LH is driven by a surge in GnRH released from neurons within the hypothalamus. Further evidence from lab rats and hamsters indicates that the increase in steroid secretion from the ovaries induces a rise in GnRH mRNA (Levine and Ramirez, 1982; Petersen *et al.*, 1995; Porkka-Heiskanen *et al.*, 1994; Wang *et al.*, 1995) and a rise in the number of GnRH cells that contain Fos (Doan and Urbanski, 1994; Lee *et al.*, 1992; Lee *et al.*, 1990a; Lee *et al.*, 1990b). Fos, a protein product of the *c-fos* gene, has been found to be a reliable indicator of cellular activity (Hoffman *et al.*, 1994; Hoffman *et al.*, 1990; Wang *et al.*, 1995). Ovariectomized (OVXed) lab rats do not have a spontaneous LH surge due to the removal of their endogenous estrogens. However, administration (either by injections or capsules) of Estradiol (E) or a combination of E and progesterone in laboratory rodents (e.g. lab rats, mice) can stimulate an increase in GnRH, GnRH mRNA, GnRH neurons containing Fos, and LH secretion that resemble normal

proestrous events (Bronson and Vom Saal, 1979; Jimenez-Linan and Rubin, 2001; Lee *et al.*, 1990b; Legan *et al.*, 1975; Levine and Ramirez, 1982; Wu *et al.*, 1992). While coordination between estrogens and GnRH neurons is required to trigger the LH surge, a large body of evidence suggests that GnRH neurons lack estrogen receptor-α (ER-α; Herbison and Theodosis, 1992; Lehman and Karsch, 1993; Shivers *et al.*, 1983; Warembourg *et al.*, 1998). These data, from a variety of species, suggest that these cells do not receive information on ovarian status directly from circulating estrogens. Retrograde tracing in lab rats has revealed that ER-α positive neurons in the hypothalamus and caudal brainstem project to the vicinity of GnRH cells in the preoptic area, suggesting a possible anatomical substrate for integration of steroid signals with the GnRH cell system (Simonian *et al.*, 1999).

Recently, however, controversial reports have identified ER-α in both an immortalized GnRH cell line and in GnRH cells present in tissue slices of the lab rat preoptic area (Butler *et al.*, 1999; Roy *et al.*, 1999; Shen *et al.*, 1998; Skynner *et al.*, 1999). Additionally, ER-β, a second estrogen receptor isoform has recently been identified, and its mRNA and protein have been found in GnRH cells *in vivo* and *in vitro* (Hrabovszky *et al.*, 2000; Roy *et al.*, 1999; Skynner *et al.*, 1999). Although these data are intriguing, further investigations are needed to determine whether these steroid receptors in GnRH neurons play a role in mediating the LH surge.

The LH surge and the circadian system

In many rodents, the LH surge is regulated by the endogenous circadian system. The rhythm regulating the LH surge free-runs in constant conditions (Alleva *et al.*, 1971; Takeo, 1984), is phase-locked to the light:dark cycle (Fitzgerald and Zucker, 1976; Stetson and Gibson, 1977), and is delayed a full circadian cycle following a single injection of barbiturates (Everett and Sawyer, 1950). OVXed lab rats implanted with estradiol capsules have a daily proestrous-like surge, indicating that there is a daily signal for the initiation of LH release. This circadian signal appears to operate in conjunction with the high levels of circulating estradiol that are reached at proestrous to stimulate the LH surge once every 4 or 5 days in intact lab rats.

The SCN, the site of the primary circadian clock in mammals, plays an important role in the temporal coordination of these events in nocturnal laboratory rodents. Lab rats and hamsters with bilateral SCN lesions lack a preovulatory LH surge and a consistently functional estrous cycle, and do not exhibit an estradiol induced LH surge (Gray *et al.*, 1978; Kawakami *et al.*, 1980; Meyer-Bernstein *et al.*, 1999; Weigand and Terasawa, 1982). Fetal SCN transplants do not restore these endocrine rhythms, indicating that synaptic inputs are critical for mediating these estrous-related events (Meyer-Bernstein *et al.*, 1999). The SCN may communicate temporal information though direct neural connections to cells within the HPG system. Light and electron microscopy reveal SCN efferents on GnRH and ER-α containing cells in lab rats and hamsters (de la Iglesia *et al.*, 1995; van der Beek *et al.*, 1997; Watson *et al.*, 1995). Furthermore, these two

cell types also project back to cells contained within SCN and the region surrounding this nucleus (de la Iglesia *et al.*, 1999; van der Beek, Wiegant *et al.*, 1997). These data provide evidence for neural pathways that connect the circadian system, GnRH neurons, and ER-containing cells.

***Arvicanthis niloticus*: a diurnal murid rodent**

The rhythms regulating the timing of estrous related events are reversed in diurnal and nocturnal rodents. In lab rats, the preovulatory LH surge, GnRH cell activation, and the onset of sexual receptivity occur at the beginning of the active period, which is around the time of lights-off (reviewed in Silver and Bittman, 1984). In several diurnal rodents including ground squirrels and degus, mating behavior occurs at the beginning and throughout their active period, but in this case it occurs during the light phase of the light:dark cycle (Dobson and Michener, 1995; Michener, 1980; Labyak and Lee, 1995; Rossi and Lee personal communication). It is not clear how the neural mechanisms that regulate the timing of these events differ in diurnal and nocturnal species. This issue has been difficult to address in squirrels and degus for a variety of reasons.

The research in this thesis focuses on a diurnal rodent, the unstriped Nile grass rat, *Arvicanthis niloticus* ("grass rat"). This murid species is closely related to lab rats and hamsters, nocturnal animals often used for endocrine and chronobiology studies. Grass rats are diurnal with respect to patterns in general activity, body temperature, wheel-running, and activity in the field (Blanchong and Smale, 2000; McElhinny *et al.*, 1997). This species also exhibits a reversal in the rhythms controlling mating, the LH surge during the postpartum estrus period

(PPE), and GnRH cell activation during the PPE when compared to those of lab rats (McElhinny *et al.*, 1999). Much of the neuroanatomy of the SCN, as well as the GnRH cell distribution has been described in this species (Katona *et al.*, 1998; Mahoney *et al.*, 2001; McElhinny *et al.*, 1999; Smale and Boverhof, 1999). For these various reasons, the grass rat represents an ideal model with which to elucidate the regulation and timing of reproductive events in a diurnal mammal.

Overview of chapters

The goals of this dissertation research were to: 1) elucidate possible mechanisms underlying rhythms in the timing of reproductive events in *Arvicanthis niloticus* and 2) determine whether, and how, these mechanisms differ in grass rats compared to nocturnal rodent species. Specifically, in the work described in chapter 2 I sought to determine whether there is a daily pattern of change in responsiveness to steroid hormones in the diurnal grass rat that is reversed when compared to that of nocturnal rodents. To examine this issue, I first determined that OVXed grass rats have a surge of LH and an associated increase in GnRH cell activation following appropriate steroid treatment. Using immunocytochemistry I then examined whether grass rats and lab rats have a rhythm in the timing of GnRH cell activation, whether it is reversed in these species, and whether the rhythm is endogenous.

The experiments described in chapter 3 I characterized the pathways from the SCN to GnRH and ER-α cells in grass rats and compared them to those described earlier in nocturnal rodents. Anterograde tract-tracing was used to identify SCN efferents innervating these two cell types. I then used

immunocytochemistry to determine whether arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP), two peptides found in efferents of the SCN, contact GnRH cells in grass rats. A large body of evidence indicates that these peptides regulate the timing of the LH surge and sexual receptivity in female lab rats. I used immunocytochemistry to determine whether connections between GnRH neurons and VIP and/or AVP fibers differ between grass rats and lab rats.

In chapter 4 I sought to determine whether there is a rhythm in responsiveness of sexual behavior to steroid hormones in grass rats, and whether the rhythm is reversed compared to that seen in nocturnal rodents. To address this issue I analyzed the rates of sexual behaviors in paired grass rats tested at 4 different times of day. I then used immunocytochemistry to explore the possibility that the rhythm in behavioral responsiveness to steroids is associated with a rhythm in of progesterone receptors.

Chapter 2:

Circadian regulation of gonadotropin-releasing hormone neurons and the preovulatory surge in luteinizing hormone in diurnal and nocturnal rodents

Introduction

Reproductive events such as copulatory behavior, parturition, ovarian cyclicity, and the preovulatory surge in luteinizing hormone (LH) can occur at very different times of day in nocturnal and diurnal animals. Nocturnal female rats ("lab rats", *Rattus norvegicus*), mice (*Mus musculus*) and Syrian hamsters (*Mesocricetus auratus*) mate and have the LH surge in the late afternoon or early evening, around lights-off (Blake, 1976; Bronson and Vom Saal, 1979; Legan and Karsch, 1975; Seegal and Goldman, 1975; Sodersten, 1988; Stetson and Gibson, 1977; Wu *et al.*, 1992), whereas in the diurnal rodent, *Arvicanthis niloticus* ("grass rat"), sexual behavior and the LH surge occur very early in the morning, before lights-on (McElhinny *et al.*, 1999; McElhinny *et al.*, 1997). In addition grass rats and lab rats exhibit a rise in activity in gonadotropin releasing hormone (GnRH) cells at opposite times of day (Lee *et al.*, 1992; McElhinny *et al.*, 1999; Wang *et al.*, 1995). The release of GnRH from neurons in the hypothalamus induces LH secretion from the anterior pituitary. Most chronobiology research focuses on nocturnal lab rodents and it is not known what causes these estrus-related events to occur at different times of day in diurnal and nocturnal species.

Several lines of evidence have established that endogenous signals from the circadian system play a critical role in the coordination of reproductive rhythms in lab rats and hamsters. If the surge is suppressed by barbiturate treatment on the day of proestrous it does not occur immediately after recovery from the treatment, but is instead delayed a full circadian cycle (Everett and Sawyer, 1950; Stetson and Watson-Whitmyre, 1977). Furthermore, when these animals are housed in constant light they have free-running rhythms in the timing of the LH surge and in the onset of sexual receptivity that are "circa-quadridian". That is, these events occur at intervals that are four times as long as the period of the circadian rhythms (Alleva *et al.*, 1971; Fitzgerald and Zucker, 1976; Takeo, 1984). In lab rats and hamsters kept in a light:dark cycle both the LH surge and the onset of lordosis behavior are tightly coupled to the onset of activity (Alleva *et al.*, 1971; Moline *et al.*, 1981; Stetson and Gibson, 1977). When activity rhythms are phase shifted by pharmacological treatment, or when "splitting" of activity bouts occurs, the timing of the LH surge also changes such that the same temporal relationship is maintained between these functions (Fitzgerald and Zucker, 1976; Swann and Turek, 1982; Swann and Turek, 1985). A daily signal for this surge is also evident in ovariectomized lab rats exposed to constant levels of estradiol in which a preovulatory-like surge occurs at the same time each day (Legan *et al.*, 1975; Legan and Karsch, 1975).

The circadian signal that gates the timing of estrous-related events originates in the suprachiasmatic nucleus (SCN), the site of the primary circadian clock in mammals. Destruction of the SCN abolishes the preovulatory LH surge,

a consistently functional estrous cycle, and a behavioral rhythm in responsiveness to steroid hormones (Gray *et al.*, 1978; Kawakami *et al.*, 1980; Meyer-Bernstein *et al.*, 1999; Weigand and Terasawa, 1982). There is a direct pathway from the SCN to GnRH neurons in lab rats and hamsters, and in lab rats these contacts are clearly synaptic (de la Iglesia *et al.*, 1995; van der Beek *et al.*, 1997). It is not clear whether the SCN and circadian system are involved in the timing of estrous-related events in diurnal rodents as, until recently, there has not been a suitable model with which to investigate this question.

The unstriped Nile grass rat, *Arvicanthis niloticus*, has proven to be ideal for the elucidation of questions regarding the neural control of biological rhythms in diurnal mammals. This murid rodent exhibits a diurnal pattern of mating activity, body temperature, and above ground activity in the field (Blanchong and Smale, 2000; McElhinny *et al.*, 1997). These animals also have an LH surge during the post-partum estrus period that occurs nearly 12 hours out of phase with that of lab rats (McElhinny *et al.*, 1999). Previous studies of grass rats have focused on the post partum period because estrus at this time can be predicted with a high degree of certainty, which is not the case at other times (e.g. vaginal smears do not predict estrus).

The goals of this research were to investigate the neuroendocrine events associated with the LH surge in a diurnal rodent, and the timing of these events in nocturnal and diurnal rodents. More specifically I had three objectives: 1) to determine whether a surge could be induced in ovariectomized females primed with steroid hormones, 2) to evaluate the hypothesis that lab rats and grass rats

exhibit reversed temporal patterns of GnRH cell activity when given identical steroid hormone treatment and 3) to evaluate the hypothesis that the rhythms in GnRH cell activity are endogenous in these species. For the purposes of this paper, GnRH cell activity is used to refer to levels of Fos expression in GnRH neurons. The recruitment of GnRH neurons during the preovulatory LH surge of lab rats, guinea pigs, and hamsters has been clearly associated with the detection of the immediate early gene product Fos within these neuroendocrine cells (Doan and Urbanski, 1994; Hoffman *et al.*, 1990; King *et al.*, 1998; Lee *et al.*, 1992; Lee *et al.*, 1990a; Wang *et al.*, 1995; Wu *et al.*, 1992).

Materials and methods:

Animals

Adult female grass rats (>60 days) bred from laboratory stock and Sprague Dawley rats (Charles River) were housed in a 12:12 light:dark cycle and provided food (Teklad rodent chow 8640, Harlan Industries) and water *ad libitum*. A red light (<5 lux) was left on continuously in the animal rooms. Females were anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, <50 mg/kg sodium pentobarbital) and methoxyflurane (Metofane, Mallinckrodt Veterinary) and bilaterally ovariectomized. Incisions were closed with sutures (grass rats) or wound clips (lab rats) and treated with topical antibiotic (Nolvasan, Fort Dodge Animal Health). Following ovariectomy animals were given 1 cc 0.9% saline subcutaneously (sc) and 0.03 mg buprenorphine hydrochloride (intramuscularly; im, Buprenex, Reckitt & Coleman). I waited 7-14 days after ovariectomy before beginning each experiment. All experiments were performed

in compliance with Michigan State University All-University Committee on Animal Use and in accordance with the standard in the National Research Council *Guide for Care and Use of Laboratory Animals*. All efforts were made to minimize the suffering and the number of animals used in these experiments.

Immunocytochemistry

All animals were deeply anaesthetized with sodium pentobarbital and perfused transcardially with 0.01 M PBS (pH 7.2, 150-200 ml/animal) followed by 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer. Brains were post-fixed in paraformaldehyde for 4 hours, transferred to 20% sucrose in 0.1 M phosphate buffer overnight, then sectioned using a freezing microtome at 30 μm into 3 series, from the medial septum to the bed nucleus of the stria terminalis. Brains from animals in the first study, however, were cut into 2 series.

One series of brain sections from each animal was processed for the dual detection of GnRH and Fos immunoreactivity (+). Free floating tissue sections were incubated in (1) 5% normal goat serum (NGS, Vector Laboratories; in PBS with 0.3% triton-X; TX) for one hour at room temperature, then (2) rabbit anti-Fos primary antibody for 24 hours at 4°C (Santa Cruz Biotechnologies) in PBS with 0.3% TX and 3% NGS), followed by (3) biotinylated secondary antibody for one hour at room temperature (1:200, goat anti-rabbit in PBS with 0.3% TX and 3% NGS, Vector Laboratories) then (4) Avidin-biotin complex (ABC, Vectastain Elite Kit, Vector Laboratories) for one hour at room temperature. Fos was then visualized by incubating tissue in 0.175 M sodium acetate buffer containing diaminobenzidine (DAB, 0.5 mg/ml), 3% hydrogen peroxide (0.825 μl/ml buffer)

and 2.5% nickel sulfate. In between each step, tissue was rinsed three times for 10 minutes in PBS.

After Fos labeling, sections were processed for the detection of GnRH. The same immunocytochemistry procedure was used with the following exceptions: I used rabbit anti-GnRH primary antibody (1:5000, Chemicon International), normal donkey serum, and donkey anti-rabbit $F(ab)_2$ secondary antibody (Jackson ImmunoResearch Laboratories). Tissue was reacted in DAB (0.5 mg/ml, in Trizma buffer, pH 7.2) with 30% hydrogen peroxide (0.35 μl/ml buffer). Controls were done by repeating this dual label procedure but either Fos, or GnRH, or both antibodies were omitted. Following the immunocytochemical reactions, tissue was mounted, dehydrated, coverslipped and examined under a light microscope (Laborlux S, Leitz Wetzlar GBH). For each study, the numbers of GnRH+ cells with and without Fos+ were quantified.

Patterns of change in LH and in GnRH+ cells in grass rats

In this first study ovariectomized grass rats were primed with steroid hormones in order to induce a surge of LH. For two days, animals were injected (sc) with 10 μg 17-β estradiol benzoate suspended in sesame oil (EB) at zeitgeber time 19 (ZT; ZT 0 = lights on). On the third day, at the same ZT, females received 125 μg progesterone (P; sc). Following the P injection females were perfused at either ZT 20.5, 22, or 23.5 (n=5/timepoint). Another group of females was perfused at ZT 18.5, without receiving a P injection (n=5). At the time of perfusion, brains were collected, cardiac blood samples were taken and centrifuged, and the plasma was stored at -80°F. Brains were processed and the

numbers of GnRH+ cells with and without Fos+ were counted from 14 sections taken from each animal. Six of these contained the medial septum and diagonal and horizontal bands of Broca (MS/DBB), two contained the organum vasculosum of the lamina terminalis (OVLT) and six contained the anteroventral portion of the periventricular nucleus and preoptic area (AVPV/POA).

Plasma LH concentrations were measured by a double-antibody radioimmunoassay, which had previously been validated for use with grass rat plasma (McElhinny *et al.*, 1999). The primary antibody, mouse monoclonal anti-bovine LH (518B7, lot 4), was provided by Dr. Jan Roser (University of California, Davis). Dr. L. Reichert (Albany Medical College, NY) provided iodinated ovine LH LER 1056 C2 which was used as trace. Tubes for the standard curve were prepared with S26 ovine LH reference preparation obtained from NIDDK. LH values that fell below the lowest limit of detectability (0.5 ng) were rounded up to this value for statistical analysis.

GnRH+ and Fos+ in grass rats and lab rats in a light:dark cycle

In the second study, ovariectomized grass rats and lab rats received an injection of EB (sc; 50 μg/kg) at either ZT 7 or ZT 19 for two days. On the third day, at the same ZT, females were injected with P (sc; 2.5 mg/kg). Three hours following P injection animals were perfused at either ZT 10 or ZT 22 (n=7/group except grass rats at ZT 10; n=13). One series of tissue was processed for the immunocytochemical detection of GnRH+ and Fos+ as described above. In the first study I found that the highest percentage of GnRH+ cells containing Fos was in the OVLT and that GnRH+ cell activity in this region was representative of that

of the total GnRH+ cell population. Therefore, in this study I counted cells in two sections containing the OVLT.

GnRH+ and Fos in grass rats and lab rats in constant darkness

In the third study ovariectomized lab rats and grass rats were released into constant darkness (DD). On the third and fourth day of DD animals received injections of EB (sc, grass rats=10 μg/animal, lab rats =50 μg/kg) between either ZT 7 to 8 or ZT 19 and 20. On the fifth day in DD animals were injected with P (sc, grass rats=125 μg/animal, lab rats=2.5 mg/kg) then perfused three hours later between ZT 10 and 11 or ZT 22 and 23 (grass rats n=4/timepoint, lab rats n= 5/timepoint). One tissue series from each animal was processed for GnRH+ and Fos+, and two sections containing the OVLT were analyzed as described above.

Statistical analysis

Percentage data, which is nonparametric, were transformed for statistical analyses. In the first study I wanted to determine if hormone treatment was able to induce a rise in LH levels and/or percent of GnRH cells that contained Fos (log transformed). Data were plotted and examined to identify the timepoint when the "surge" occurred. Data from animals at this timepoint were then compared those of animals killed at ZT 18.5 using an unpaired t-test. These data were also examined with a two-sample t-test using P treatment as the independent variable. That is, animals perfused at ZT 18.5 were compared to the rest of the animals combined. I also used a chi-square analysis to compare plasma LH values in P treated (ZT 20.5, 22, 23.5) and untreated animals (ZT 18.5). I

predicted that both LH and GnRH+ cell activity would be higher at timepoints following P treatment, thus I used one-tailed tests in these analyses.

To determine whether sub-populations of GnRH+ neurons were different with respect to temporal patterns of activity, I used a two-way ANOVA. Time and region (MS/DBB, OVLT, and AVPV/POA) were the independent variables, and percent of GnRH+ cells that were active (sine transformed) was the dependent variable.

The percent of GnRH+ cells that contained Fos (not transformed) was correlated with the plasma LH concentration (ng/ml). Data from the second and third experiments were analyzed using a two-way ANOVA with species and time as independent factors and the log-corrected percent of GnRH+ cells that were active as the dependent variable. All analyses were done using Statview 5.0 and differences were considered significant when $p < 0.05$.

Results

Patterns of change in LH and in GnRH+ cells in grass rats

The objective of this first study was to create a model of the induction of the LH surge and associated neuroendocrine events in a diurnal rodent. Both LH levels and GnRH+ cell activity were highest at ZT 22 whereas animals at ZT 18.5 had virtually undetectable plasma LH or active GnRH+ cells (Figure 2.1, Figure 2.2). I therefore used data from ZT 22 to represent the surge in statistical analyses. In steroid-primed grass rats, the percent of GnRH+ cells that were active had a significant increase from ZT 18.5 to ZT 22 ($t=-7.12$, $df=8$, $p<0.0001$, Figure 2.1). Animals perfused at ZT 18.5 (n=5), before P treatment, had

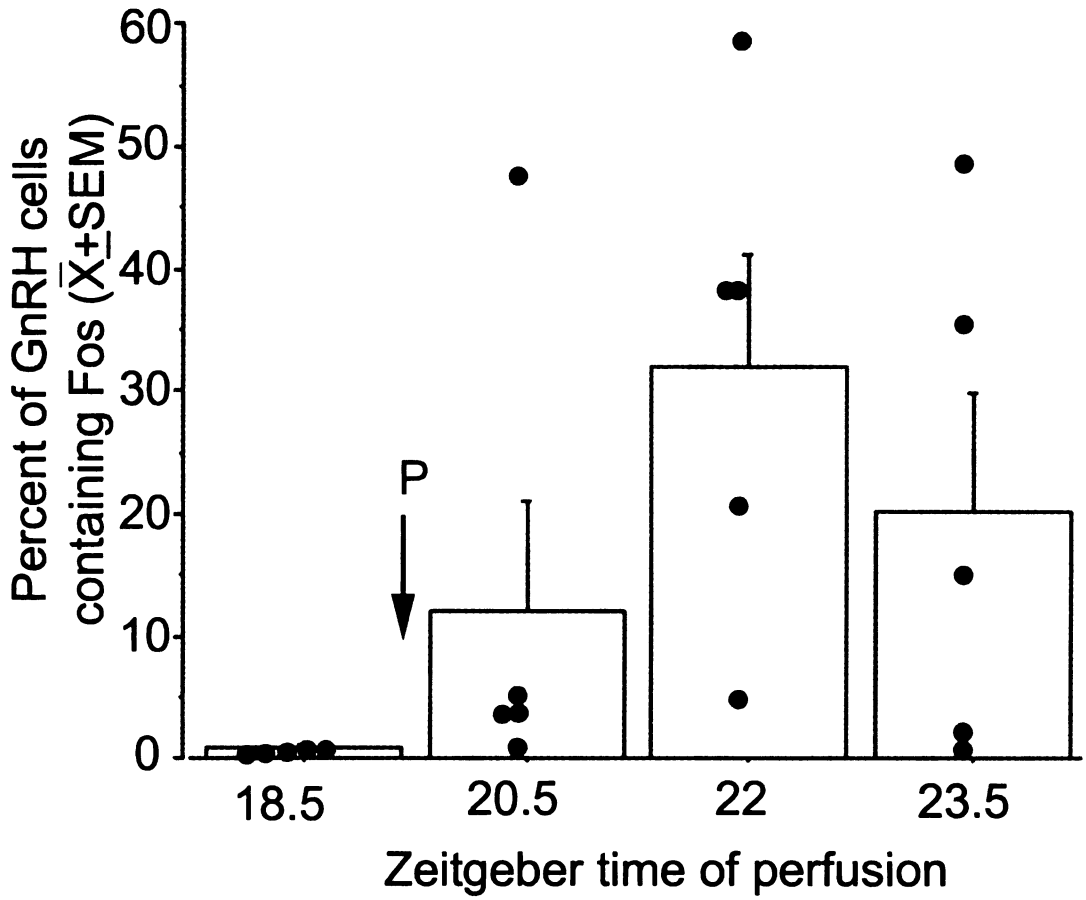

Figure 2.1. The average percent (±SEM) of GnRH cells that contained Fos in grass rats. Hormone injections occurred at ZT 19 (arrow). Dots represent the value for each individual (n=5/time point). Zeitgeber time 0 = lights-on.

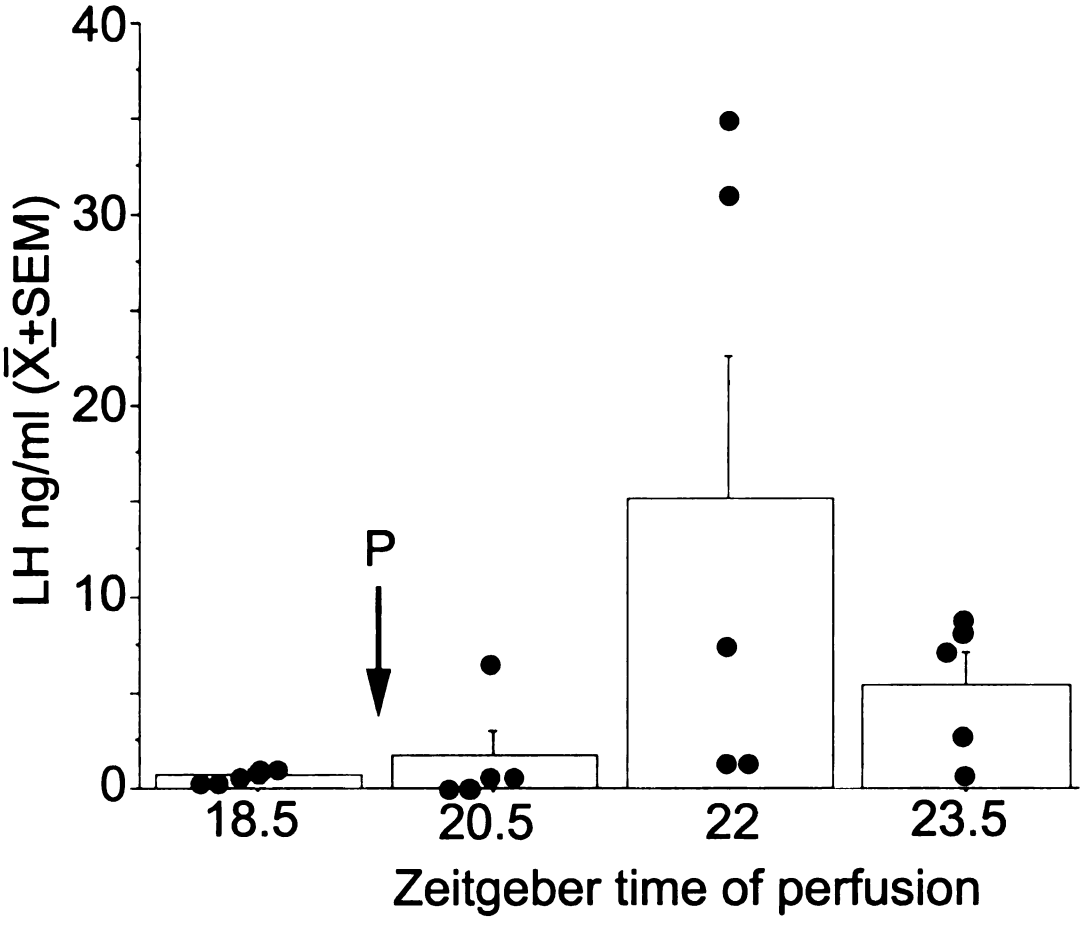

Figure 2.2. The average LH value (± SEM) in grass rats. See legend of Figure 2.1 for detail.

significantly lower GnRH+ cell activity than did animals killed at the three remaining timepoints (n=15, t = -3.44, df=18, p=0.001). The pattern of change in plasma LH over time resembled that of GnRH+ cell activity. That is, LH levels were significantly higher at ZT 22 compared to levels at ZT 18.5 (t=-1.96, df=8, p=0.04, Figure 2.2). No significant differences in plasma LH levels were detected between P treated animals and untreated animals. There were, however, more LH values over 1 ng/ml among the P treated animals (ZT 20.5, 22, 23.5) than among the untreated animals (ZT 18.5, X^2=6.67, df=1, p=0.009). LH values were significantly correlated with the percent of GnRH+ neurons that were active (r = 0.635, Z value=3.0, p= 0.002).

I detected a difference between sub-populations of GnRH+ cells with respect to temporal patterns of activity (time x sub-population, F=2.26, df=6, p=0.05). However, the GnRH+ cell population in each of the regions (MS/DBB, OVLT, and AVPV/POA) had peaks in activity at ZT 22 and this temporal pattern resembled the activity of GnRH+ cells from all regions combined (Figure 2.1).

GnRH+ and Fos+ in grass rats and lab rats in a light:dark cycle

In the second study, steroid primed lab rats and grass rats were perfused just before lights-on (ZT 22) or lights-off (ZT 10). Although hormone treatments were identical in the two groups, GnRH+ cell activity was higher in lab rats perfused at ZT 10 than at ZT 22, whereas grass rats had the inverse pattern (Figure 2.3). This interaction between species and time of day was significant (F=11.37, df=1, p<0.002).

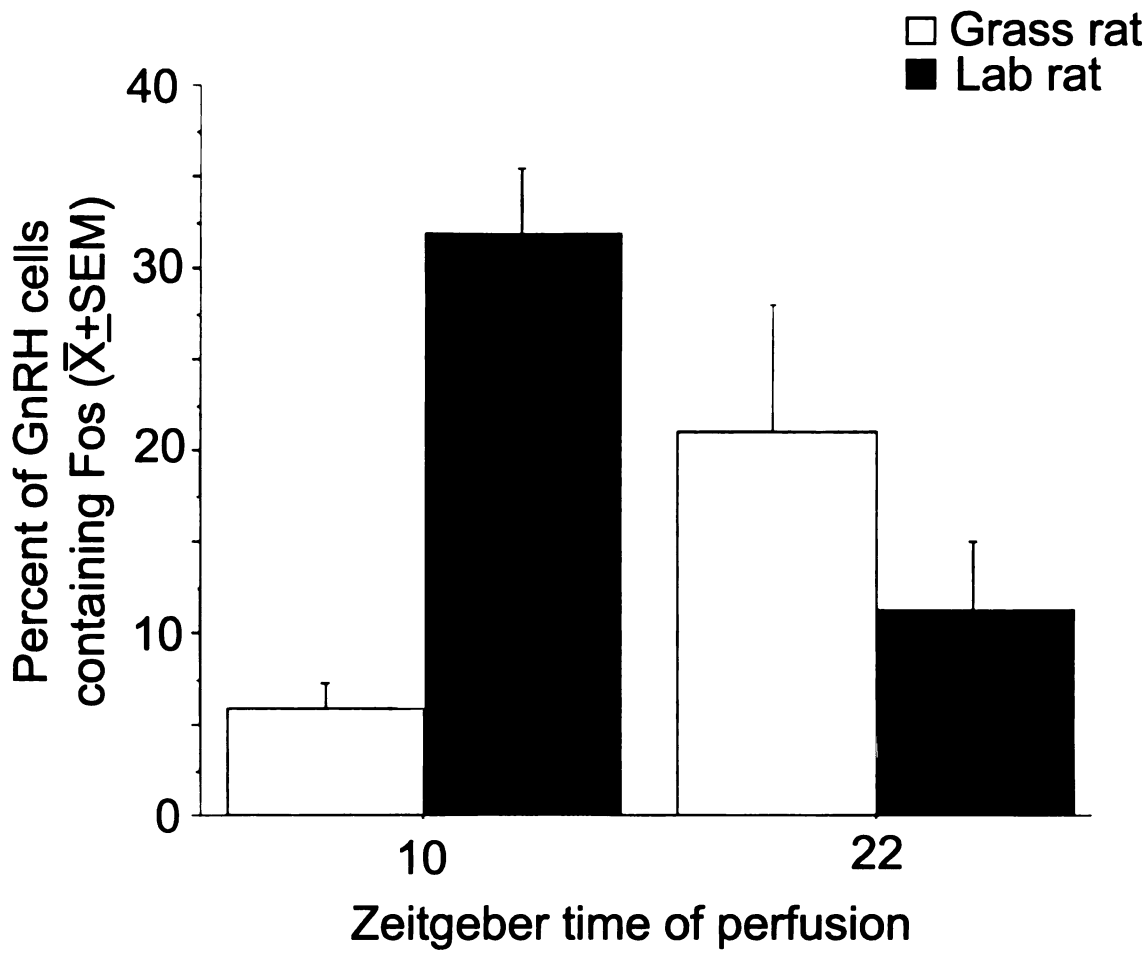

Figure 2.3. GnRH cell activity in steroid primed grass rats and lab rats housed in a 12:12 light:dark cycle. Zeitgeber time 0 = lights-on.

GnRH+ and Fos+ in grass rats and lab rats in constant darkness

Lab rats and grass rats kept in DD for 5 days had patterns of change in GnRH+ cell activity that were similar to those of animals kept in light:dark cycle (compare Figure 2.3 to 2.4). Specifically, the percent of GnRH+ cells that were active was higher in grass rats perfused at ZT 22-23 than at ZT 10-11 (Figure 2.4). Lab rats showed the reverse pattern, with a higher percentage of GnRH+ cells that were active at ZT 10-11 than at ZT 22-23. There was a significant interaction between species and time ($F=152.5$, $df=1$, $p<0.001$).

Discussion

In the first study, I established an effective diurnal animal model of neuroendocrine events associated with the estrous cycle. Grass rats treated with EB and P had a peak in both LH and GnRH+ cell activity two hours before lights-on (Figure 2.1, Figure 2.2). The second experiment established that both grass rats and lab rats have rhythms in sensitivity to steroid hormones with respect to GnRH+ neuron activity, and that these rhythms are temporally reversed in these two species (Figure 2.3). Data from the third experiment indicate that in these two species the temporal organization of responsiveness to steroid hormones is under circadian control (Figure 2.4).

In grass rats in the current study, the steroid induced LH surge and the activation of GnRH+ cells mirrored the patterns that occur in intact grass rats during the post partum estrous period, confirming the physiological relevance of this model (McElhinny *et al.*, 1999). The peaks in LH and GnRH+ cell activity also resembled those occurring in other proestrous or steroid primed laboratory

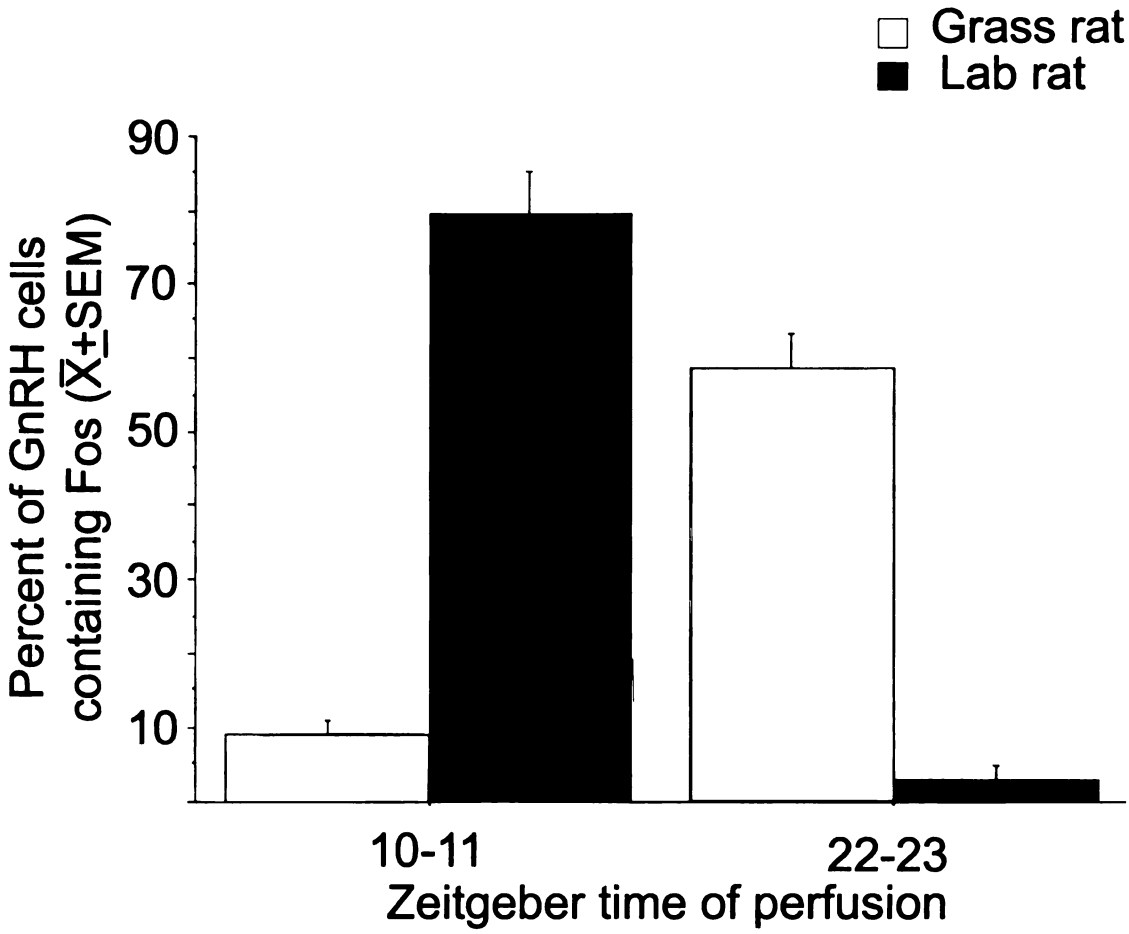

Figure 2.4. Percent of GnRH cells that were active in steroid primed grass rats and lab rats housed in constant darkness for 5 days before perfusion.

rodents (e.g. Bronson and Vom Saal, 1979; Doan and Urbanski, 1994; Finn *et al.*, 1998; Lee *et al.*, 1992; Rajendren, 2001; Wu *et al.*, 1992). Average LH values, even at ZT 22 (Figure 2.2), were somewhat lower than those typical of a surge produced by females during the post partum estrus (McElhinny *et al.*, 1999) and the variability was high. One explanation for this could be that the hormone treatments I utilized were only partially effective for the induction of the surge. Alternatively, the variability might be due to inter-individual differences with respect to the exact time of the surge. My sampling times may not have coincided with the peak in serum LH levels which may vary among individuals and may be brief, as is the case in lab rats and mice (Bronson and Vom Saal, 1979; Levine and Ramirez, 1982). However, at ZT 18.5 no individuals had an LH titer over 1 ng/ml whereas all five animals at ZT 22 and 4 of 5 animals at ZT 23.5 had values that were above 1 ng/ml, suggesting that the steroid treatment did cause LH levels to rise.

There was a tight correlation between the level of LH and the GnRH+ cell activity in the first study. This relationship has also been seen in other laboratory animals such as mice, lab rats and guinea pigs (King *et al.*, 1998; Lee *et al.*, 1992; van der Beek *et al.*, 1994; Wu *et al.*, 1992). In intact and steroid treated lab rats, the percent of GnRH cells that are active is highest during the ascending phase of the LH surge (Lee *et al.*, 1992; van der Beek *et al.*, 1994). In hamsters, the rise in GnRH cell activity follows the peak in plasma LH values and may be involved in the termination of the gonadotropin surge in this species (Doan and Urbanski, 1994). This is unlikely to be the case in hormone-primed grass rats

because both the LH values and GnRH+ cell activity were highest at ZT 22 and were quite low at ZT 20.5. If one event preceded the other it would have to occur within the same hour, a temporal pattern unlike that seen in hamsters.

Results from the current studies indicate that grass rats and lab rats have a daily rhythm in the responsiveness of GnRH+ neurons to the positive feedback effects of steroid hormones (Figure 2.3, Figure 2.4). Ovarian steroids induce a rise in the proportion of GnRH+ cells that contain Fos+ in ovariectomized lab rats and mice (Hoffman *et al.*, 1990; Lee *et al.*, 1990b; Wu *et al.*, 1992). These studies did not reveal a rhythm in responsiveness, as they did not look outside the time of day at which the proestrous LH surge normally occurs. Earlier evidence of an endogenous rhythm has come from organotypic cultures of the POA. In these cultures, GnRH is secreted in a circadian pattern but only when the tissue is incubated with estradiol (Funabashi *et al.*, 2000). To the best of my knowledge, the current results represent the first demonstration of a daily rhythm in the expression of Fos+ in GnRH+ neurons in any species.

Endogenous timing mechanisms appear to drive this rhythm in GnRH+ cell activity in both lab rats and grass rats (Figure 2.3, Figure 2.4). For both species, animals appeared to retain the rhythm after five days in constant darkness. That is, grass rats sacrificed before their subjective day had a dramatically higher percentage of GnRH+ cells that were active than those sacrificed 12 hours later; in lab rats this pattern was reversed (Figure 2.4). It remains possible that the rhythm in either species would have dampened out if animals had been left in constant darkness for a longer period of time. This is

unlikely however, because there was no hint that the rhythm was diminished after animals had been kept in DD for five days. Furthermore, in lab rats, the timing of the LH surge is driven by an endogenous circadian system and LH release is driven by the GnRH neurons (Alleva *et al.*, 1971; Fitzgerald and Zucker, 1976; Takeo, 1984). These data support the idea that the rhythms I observed in GnRH neurons are indeed endogenous. The neural mechanisms that regulate the rhythm in activity of GnRH neurons have not been established in either species but the SCN is likely to play a role though its projections to these neurons.

The SCN may communicate temporal information directly to cells responsible for coordination of estrous-related events. In lab rats and hamsters, tract-tracing studies suggest that the SCN projects to both estrogen receptor and GnRH containing cells, and lesions of the SCN eliminate the majority of contacts between the SCN and GnRH neurons (de la Iglesia *et al.*, 1995; van der Beek *et al.*, 1998; van der Beek *et al.*, 1993; Watson *et al.*, 1995). The temporal pattern of coupling observed here between GnRH cell activation and the LH surge was similar in lab rats and grass rats. The SCN may regulate the timing of these events, at least in part, through similar pathways in these two species. Preliminary evidence from my lab indicates that, in the grass rat, GnRH+ cells do in fact receive input from the SCN (Mahoney and Smale, 2000; chapter 3).

The current data raise interesting questions regarding the specific mechanisms responsible for the species differences in the timing of GnRH cell recruitment. Whereas proestrous and steroid-primed grass rats and lab rats are similar with respect to the temporal relationship between estrous-related events,

the timing of these events relative to the light:dark cycle is dramatically different. This difference is related to the animal's chronotype as both lab rats and grass rats have peaks in GnRH+ cell activity that occur around the onset of their activity periods. These differences in the timing of neuroendocrine events might be due to differences in the timing of SCN signals, differences in which neurochemical signals are emitted by the SCN, or differences in the responsiveness of GnRH and ER-containing cells to such signals. These closely related species provide a unique opportunity to evaluate these hypotheses and to determine how the neural mechanisms underlying circadian rhythms differ in diurnal and nocturnal species.

Chapter 3

Projections from the suprachiasmatic nucleus of grass rats to gonadotropin releasing hormone and estrogen receptor containing cells

Introduction

The mammalian suprachiasmatic nucleus (SCN) is the primary site for the generation and synchronization of circadian rhythms. Cells of the SCN have circadian rhythms in electrical activity, neurotransmitter secretion, and glucose uptake both *in vivo* and *in vitro* (Inouye and Kawamura, 1979; Murakami *et al.*, 1991; Schwartz *et al.*, 1983; Shinohara *et al.*, 1998; Sodersten *et al.*, 1985; Watanabe *et al.*, 1993; Welsh *et al.*, 1995; Yamazaki *et al.*, 1998). Destruction of this nucleus eliminates behavioral and hormonal rhythms and transplants of fetal SCN tissue into animals with bilateral lesions restore a number of these rhythms (LeSauter and Silver, 1999; Mahoney *et al.*, 2001; Moore and Eichler, 1972; Ralph *et al.*, 1990; Stephan and Zucker, 1972).

Several lines of evidence indicate that the circadian clock is critical for the timing of estrous-related events in some nocturnal rodents. In female lab rats and hamsters, rhythms in the timing of both the preovulatory surge of luteinizing hormone (LH) and the onset of sexual receptivity persist in the absence of environmental cues (Alleva *et al.*, 1971; Lucas *et al.*, 1999; Takeo, 1984). When these rhythms are phase shifted by changes in the light:dark cycle or by pharmacological treatments, the onset of behavioral estrus and the LH surge maintain a precise temporal relationship to other circadian rhythms such as the

onset of wheel-running activity (Fitzgerald and Zucker, 1976; Moline *et al.*, 1981; Swann and Turek, 1982). Lab rats and hamsters with bilateral SCN lesions fail to exhibit an LH surge, a consistently functional estrous cycle, or behavioral rhythms in responsiveness to steroid hormones (Gray *et al.*, 1978; Hansen *et al.*, 1979; Kawakami *et al.*, 1980; Meyer-Bernstein *et al.*, 1999; Weigand and Terasawa, 1982). Interestingly, SCN transplants that restore behavioral rhythms in lesioned animals do not restore estrous cyclicity, suggesting that synaptic contacts, rather than humoral signals, communicate temporal information from the SCN to cells that regulate reproductive functions (Meyer-Bernstein *et al.*, 1999).

Two cell types that are critical for mammalian reproductive function are gonadotropin releasing hormone (GnRH) and estrogen receptor-α containing (ER) neurons. ER knock-out mice do not exhibit the full suite of female sexual behaviors (Ogawa *et al.*, 1998; Rissman *et al.*, 1997) and hypogonadal mice that lack a functional gene for GnRH are infertile and have relatively low levels of gonadotropins (Gibson *et al.*, 1997). Tract-tracing studies of lab rats and hamsters have revealed that the SCN projects directly to both GnRH- and ER-containing cells. The majority of GnRH cells contacted by SCN efferents in these species are located in the organum vasculosum of the lamina terminalis (OVLT) and the preoptic area (POA) of lab rats, and in the diagonal band of Broca (DBB) and POA of hamsters (de la Iglesia *et al.*, 1995; van der Beek *et al.*, 1997). The SCN is also known to project to ER containing cells throughout many regions of the hypothalamus of hamsters (de la Iglesia *et al.*, 1995). Although a similar

systematic analysis has not been done in lab rats, SCN efferents are known to synapse on the soma of ER cells in the anteroventral portion of the periventricular nucleus (AVPV) in this species (Watson *et al.*, 1995). The AVPV is essential for the LH surge; animals with lesions of this nucleus fail to exhibit a rise in LH in response to steroid hormone treatment (Simerly, 1998; Weigand and Terasawa, 1982). These data suggest that one mechanism by which the circadian system mediates rhythms in the timing of reproductive events is via a direct pathway from the SCN to GnRH- and steroid-sensitive cells.

The region immediately dorsal to the SCN, the lower sub-paraventricular zone (LSPV), may also play an important role in the temporal organization of reproductive events. The LSPV receives a massive input from the SCN (Kalsbeek *et al.*, 1993; Leak *et al.*, 1999; Morin *et al.*, 1994; Watts *et al.*, 1987) and it appears to project to many of the same targets as the SCN, including GnRH- and ER-containing neurons (de la Iglesia *et al.*, 1995; van der Beek *et al.*, 1997; Watson *et al.*, 1995). Interestingly, these two kinds of cells also send efferents back to the LSPV and SCN (de la Iglesia *et al.*, 1999; van der Beek, Wiegant *et al.*, 1997). In ovariectomized lab rats, lesions of the LSPV result in an increase in tonic levels of LH (Docke *et al.*, 1982). Furthermore, knife cuts of the projection from the SCN to the LSPV result in an attenuation of the steroid-induced LH surge (Watts *et al.*, 1989). These data raise the possibility that SCN projections to the LSPV might also play a role in transmitting circadian signals that regulate estrous-related events.

Several lines of evidence indicate that the neuropeptides vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP) may mediate the effects of the SCN on the timing of the LH surge and GnRH cell activation. In lab rats and hamsters, VIP fibers appear to terminate on GnRH cells and in lab rats SCN lesions eliminate about 80% of these contacts (Horvath *et al.*, 1998; Krajnak *et al.*, 2001; Kriegsfeld *et al.*, 2002; van der Beek *et al.*, 1998; van der Beek *et al.*, 1993). Receptors for VIP are expressed on GnRH neurons *in vivo* and *in vitro* (Olcese *et al.*, 1997; Smith *et al.*, 2000), and female lab rats have more contacts between VIP boutons and GnRH cells, and more hypothalamic VIP protein than do males (Horvath *et al.*, 1998; Riskind *et al.*, 1989). Furthermore, the rhythm in VIP mRNA expression in female lab rats is 12 hours out of phase from that of males (Krajnak *et al.*, 1998). These differences between males and females may be causally related to sex differences in the ability to produce the preovulatory LH surge.

Although VIP treatment affects LH secretion in lab rats, conflicting evidence exists as to whether VIP provides an excitatory or stimulatory signal. One interpretation of the literature is that acute VIP treatments have an excitatory effect on LH release whereas chronic exposure to VIP is inhibitory. Short pulses (60 seconds) of VIP infused into the ventricles (icv) lead to an increase in plasma LH (Vijayan *et al.*, 1979). An icv microinjection of either antiserum or antisense oligonucleotides to VIP prior to the surge delays and inhibits the preovulatory or estradiol-induced peak of this hormone (Harney *et al.*, 1996; van der Beek *et al.*, 1999). On the other hand, lab rats administered VIP icv for 1.5 hours or more

have relatively low LH levels (Alexander *et al.*, 1985; Stobie and Weick, 1989; Weick and Stobie, 1992; Weick and Stobie, 1995) and this inhibitory effect can be blocked by treatment with a VIP receptor antagonist (Weick and Stobie, 1995).

AVP also appears to mediate the timing of estrous-related events such as LH secretion and sexual receptivity (Sodersten *et al.*, 1985; Sodersten *et al.*, 1983). However, as with VIP, the data are conflicting with respect to whether AVP inhibits or stimulates LH secretion. AVP administered icv to steroid-primed, ovariectomized female lab rats inhibits the LH surge (Salisbury *et al.*, 1980) whereas infusions of AVP into the medial preoptic region have a stimulatory effect (Palm *et al.*, 1999; Palm *et al.*, 2001). In intact lab rats, the proestrous LH surge can be blocked by an icv injection of an AVP receptor antagonist (Funabashi *et al.*, 1999). AVP may regulate GnRH neurons via direct input to them. AVP fibers contact GnRH neurons in the medial preoptic area of female lab rats and hamsters and in the supraoptic nucleus of female cynomolgus monkeys (Huhman and van Der Beek, 1998; Thind *et al.*, 1991; van der Beek *et al.*, 1998). SCN lesions in lab rats reduce the number of these contacts suggesting that at least some of these AVP fibers come from cells within the SCN (van der Beek *et al.*, 1998). Interestingly, in co-cultures of tissue containing the SCN and POA, circadian rhythms in AVP and GnRH release have identical periods and these differ from those of rhythms in VIP secretion. AVP administration to these cultures induces GnRH release, but VIP has no effect (Funabashi *et al.*, 2000).

Rhythms in the timing of estrous-related events such as mating, the preovulatory surge in LH, and GnRH cell activity are inverted in diurnal and nocturnal species (Bronson and Vom Saal, 1979; Dobson and Michener, 1995; Legan and Karsch, 1975; McElhinny *et al.*, 1999; Michener, 1980; Rood, 1970; Seegal and Goldman, 1975; Sodersten, 1988; Stetson and Gibson, 1977; Yeoman *et al.*, 1991; Chapters 2 and 4). These differences might be due to differences in the functional and/or anatomical relationships between SCN efferents and GnRH neurons. Specifically, nocturnal and diurnal species might differ with respect to the temporal pattern of transmitter release from SCN cells, which neurochemical signals are released from these cells (e.g. VIP or AVP), or - the sensitivity of target cells to SCN signals. Nocturnal and diurnal species may also differ with respect to patterns of connectivity between the SCN and GnRH- and/or ER-containing neurons, or some combination of these various factors.

The murid rodent *Arvicanthis niloticus* (grass rat) has proven to be an ideal model with which to investigate how the circadian system differs between diurnal and nocturnal species. This species exhibits a host of rhythms that are reversed relative to those of lab rats. These include rhythms in the timing of the LH surge, associated GnRH cell activation, and copulatory behavior (Blanchong *et al.*, 1999; McElhinny *et al.*, 1999; McElhinny *et al.*, 1997). Here, I used the grass rat to address the issue of whether the SCN might be anatomically positioned to regulate rhythms in estrous-related events in a diurnal species. My aims were: 1) to determine whether the SCN innervates GnRH- and/or ER-containing cells and 2) to determine whether AVP and VIP immunoreactive fibers

contact GnRH cells in grass rats. I also mapped the distribution of SCN efferents, AVP- and VIP-containing fibers, and ER-containing cells in grass rats. The hypothalamic distribution of GnRH cells has already been described for this species (McElhinny *et al.*, 1999).

Materials and methods

Animals

Adult female grass rats (>60 days) bred from laboratory stock and Sprague Dawley rats (Charles River) were singly housed in a 12:12 light:dark cycle and provided food (Teklad rodent chow 8640, Harlan Industries) and water *ad libitum*. A red light (< 5 lux) was left on continuously in each animal room. All experiments were performed in compliance with Michigan State University All-University Committee on Animal Use and in accordance with the standard in the National Research Council *Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize the suffering and the number of animals used in these experiments.

Surgeries

1. Tract-tracing

Animals (n=13) were anesthetized using sodium pentobarbital (Nembutal, <50 mg/kg, Abbott Laboratories) and supplemented with methoxyflurane (Metofane, Mallinckrodt Veterinary). The tops of their heads were shaved, cleaned with topical antiseptic (Betadine, Novaplus), and 2% lidocaine was subcutaneously (sc; 0.03 ml, Abbott Labs). Animals were then placed in a stereotaxic apparatus with the tooth bar set at –0.6 mm, the skull was exposed

and a hole was drilled (0.14 anterior and +0.13 mm lateral to bregma). A glass pipette (inner tip diameter = 10-15 µm) was set at a 10° angle (relative to the dorsal-ventral plane), filled with tracer, and then lowered to -0.61 mm ventral to dura. Animals received a unilateral injection of the anterograde tracer biotinylated dextran amine (BDA; 10,000 MW Sigma, 10% solution in H_2O (Coolen and Wood, 1998; Sequeira *et al.*, 2000)). BDA was delivered via iontophoresis over a ten-minute period with an alternating 5-microamp positive current (7 sec on/7 sec off). After surgery, animals received sterile saline (1 cc 0.9% sc) and the analgesic buprenorphine hydrochloride (0.03 mg, intramuscularly; im, Buprenex, Reckitt & Coleman). The incision was closed with wound clips and treated with topical antibiotic (Nolvasan, Fort Dodge Animal Health). Animals were perfused after a seven-day recovery period (Richardson, 2002; Veenman *et al.*, 1992).

2. Ovariectomy

In some studies grass rats were bilaterally ovariectomized while under Nembutal anesthesia (<50 mg/kg) supplemented with Metofane. Incisions were closed with sutures and treated with Nolvasan. Following ovariectomy animals were given saline (1 cc 0.9%, sc) and Buprenex (0.03 mg, im). Animals recovered for at least seven days prior to steroid hormone injections.

Tissue processing and analysis

1. General immunocytochemical procedure

Animals were deeply anaesthetized with Nembutal and perfused transcardially with 0.01 M phosphate buffered saline (PBS; pH 7.4, 150-200

ml/animal) followed by 4% paraformaldehyde (150-200 ml/animal, Sigma) in 0.1 M phosphate buffer. Brains were post-fixed in paraformaldehyde for four hours, transferred to 20% sucrose in 0.1 M phosphate buffer overnight, and then sectioned with a freezing microtome. Brains were cut into three series at 30 µm with the exception of tissue from steroid treated females labeled for VIP and GnRH, which was cut into two series at 40 µm. Brains from animals with BDA injections were sectioned through to the intergeniculate leaflet, and brains from all other animals were cut from the medial septum to the supraoptic nucleus.

All immunocytochemical labeling began with the following steps: free floating tissue sections were incubated in (1) 5% normal serum (in PBS with 0.3% triton-X; TX) for one hour at room temperature, followed by (2) primary antibody for 24 hours at 4°C (in PBS with 0.3% TX and 3% normal serum), followed by (3) biotinylated secondary antibody for one hour at room temperature (in PBS with 0.3% TX and 3% normal serum), followed by (4) avidin-biotin complex (Vectastain Elite Kit, Vector Laboratories, 0.9% of avidin and biotin) for one hour at room temperature.

For nickel-enhanced labeling (blue chromagen) the tissue was then rinsed in sodium acetate buffer (0.175 M, Sigma) three times for ten minutes and then reacted in a solution of diaminobenzidine (DAB, 0.25 mg/ml, Sigma), 3% hydrogen peroxide (0.825 µl/ml buffer) and 2.5% nickel sulfate in sodium acetate buffer. Tissue labeled with standard DAB chromagen went from step (4) into DAB (0.5 mg/ml, in Trizma buffer, pH 7.2) with 30% hydrogen peroxide (0.35 µl/ml buffer). Unless otherwise noted, tissue was rinsed three times for ten

minutes in PBS between each incubation step. Reagents used for immunocytochemistry are listed in Table 3.1. Following the labeling, tissue was mounted, dehydrated, coverslipped and examined under a light microscope (Laborlux S, Leitz Wetzlar GBH). In all cases, negative controls were done by performing the above procedures but with the omission of the primary antibody(s). No inappropriate staining was detected in control tissue.

2. ER+ cell distribution

Here I characterized the distribution of ER immunoreactive (+) cells in female grass rats. Ovariectomized animals (n=4) received an injection of estradiol benzoate (EB, in sesame oil, 10 µg/animal, sc) for two days. Twenty-four hours after the second EB injection, animals were perfused. One series of tissue was processed for the detection of ER using standard DAB as the chromagen (Table 3.1). A camera lucida was used to draw the distribution of ER+ cells in regions known to receive SCN input in the lab rat (Watts *et al.*, 1987). These images were then scanned into a drawing program (Photoshop 7.0) and retraced to produce pictures of ER+ cell distribution. Table 3.2 contains abbreviations for structures indicated in the drawings.

3. BDA labeled fibers

3a) Distribution of BDA labeled fibers

One series of tissue from BDA treated animals was processed to determine the site of injection. The tracer was already biotinylated, thus tissue was processed for nickel enhanced labeling by beginning with the AB-complex

Table 3.1. Antibodies and sera used for immunocytochemistry. Note that BDA was already biotinylated and thus did not require antibodies.

Primary	Dilution	Vendor	Biotinylated secondary	dilution	vendor	normal serum	vendor
Rabbit anti-**GnRH**	1:5000	Chemicon International	donkey anti-rabbit F(ab)2 fragment	1:200	Jackson Immuno-research	Donkey	Jackson Immuno-research
Guinea pig anti-**VIP**	1:2000	Peninsula Laboratories	Goat anti-guinea pig whole IgG	1:200	Vector Laboratories	Goat	Vector Laboratories
Guinea pig anti-**AVP**	1:10,000-1:30,000	Peninsula Laboratories	Goat anti-guinea pig whole IgG	1:200	Vector Laboratories	Goat	Vector Laboratories
Rat anti-**ER** (H222)	1:2000	Abbott Laboratories	Rabbit anti-rat whole IgG	1:200	Jackson Immuno-research	Rabbit	Jackson Immuno-research

Table 3.2. Definitions for abbreviations used in this report.

Abbreviation	Definition
3V	3rd ventricle
ac	anterior commisure
AHA	anterior hypothalamic area
AVP	arginine vasopressin
AVPV	anteroventral portion of the periventricular nucleus
BDA	biotinylated dextran amine
BNST	bed nucleus of the stria terminalis
D3V	dorsal portion of 3rd ventricle
DAB	diaminobenzidine
DBB	diagonal band of Broca
ER	estrogen receptor-α
f	fornix
GnRH	gonadotropin releasing hormone
hDBB	horizontal portion of DBB
icv	intracerebroventricular
im	intramuscularly
LH	luteinizing hormone
LPOA	lateral portion of POA
LS	lateral septum
LSPV	lower sub paraventricular zone
LSV	ventral portion of LS
LV	lateral ventricle
mPOA	medial portion of POA
MS	medial septum
oc	optic chiasm
ot	optic tract
OVLT	organum vasculosum of the lamina terminalis
PBS	phosphate buffered saline
PeVN	periventricular nucleus
POA	preoptic area
PVN	paraventricular nucleus of the hypothalamus
PVT	paraventricular thalamus
RCH	retrochiasmatic area
sc	subcutaneous
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SON	supraoptic nucleus
TX	Triton-X
vDBB	vertical portion of DBB
VIP	vasoactive intestinal polypeptide
VMPO	ventromedial portion of POA
ZT	zeitgeber time

incubation. Sections from animals in which BDA was effectively injected into the SCN (n=3) were compared with tissue from animals with misplaced injections. Using a light microscope digital photographs of sections were collected in a computer-imaging program. BDA labeled fibers were then retraced on top of the photographs to create drawings of SCN projections (Photoshop 7.0).

3b) BDA fibers and ER+/GnRH+ cell

To examine the relationship between SCN efferents and GnRH+ or ER+ cells, sections from BDA injected animals were first processed for BDA (nickel-enhanced DAB) then for ER or GnRH using brown DAB as the chromagen. For each of the three animals with a BDA injection in the SCN, 14 sections were selected for further analysis. Every GnRH+ cell was identified in six sections containing the medial septum (MS) and diagonal band of Broca (DBB), two sections containing the OVLT, two sections containing the AVPV, and 4 sections containing the POA/LSPV. Then each GnRH+ cell was reexamined using a high-powered oil immersion objective to determine whether it was contacted by a BDA labeled fiber. A GnRH+ neuron was considered "contacted" if 1) a blue-black swelling was observed abutting the cell body, 2) the bouton and the cell body were in the same plane of focus and 3) the BDA-labeled bouton appeared as a continuation of an axon. That is, it was not counted as a contact if the blue swelling resembled a granule rather than a fiber (Richardson, 2002).

4. AVP+ and VIP+ labeling

4a) Distribution of AVP+ and VIP+ fibers

Here I characterized the distribution of AVP+ (n=6 females) and VIP+ (n=6 females) fibers in the hypothalamus of the grass rat. Tissue was single labeled using either the standard or nickel-enhanced DAB as the chromagen (Table 3.1). For the detection of AVP I used concentrations ranging from 1:10,000 to 1:30,000. VIP+ fibers were visualized with 1:2000 concentration. A camera lucida was used to draw the distributions of AVP+ and VIP+ fibers from representative sections. These drawings were scanned into a drawing program and retraced to produce pictures (Photoshop 7.0).

4b) AVP+/VIP+ contacts on GnRH+ cells

Here I determined whether AVP+ and/or VIP+ fibers contact GnRH+ cells in grass rats. Tissue from intact female grass rats was processed for the detection of AVP (1:20,000, n=3) or VIP (1:2000; n=3) using nickel-enhanced DAB, then for GnRH using standard DAB as the chromagen (Table 3.1). For AVP/GnRH analysis I selected 14 sections that I divided into three groups: MS/DBB (six sections), OVLT (two sections), and AVPV/POA/LSPV (six sections). I excluded GnRH+ neurons from the population located dorsal and lateral to the supraoptic nucleus (SON), as these cells were likely receiving input from AVP+ fibers of non-SCN origin. Fewer sections were available for VIP/GnRH analysis; I examined GnRH+ cells in the MS (three sections), OVLT (two sections) and AVPV/POA/LSPV (four sections). Contacts on GnRH+ cells were identified using the same criteria outlined above for identifying BDA

contacts. The numbers of GnRH+ cells, with or without contacts, and the average numbers of boutons per contacted GnRH+ cell body were determined.

I then determined whether the number of VIP+ fibers in contact with GnRH+ neurons changed at the time of the steroid-induced LH surge in grass rats. I used tissue from two groups of females (n=5/group) that I had previously determined had relatively low and high titers of LH, respectively. Briefly, ovariectomized females received 10 µg estradiol benzoate (sc in sesame oil) at zeitgeber time 19 (ZT; ZT 0 = lights-on). On the third day, at the same time, animals received 125 µg progesterone (sc in sesame oil) and then they were perfused at either ZT 20.5 or ZT 22. Cardiac blood samples were taken at the time of perfusion, and LH levels were determined using a double antibody radioimmunoassay described elsewhere (McElhinny *et al.*, 1999). Females killed at ZT 20.5 had relatively low plasma hormone concentrations (1.68 $\pm$1.2 ng/ml SEM) compared to concentrations seen in animals killed at ZT 22 (15.15 $\pm$ 7 ng/ml SEM, Chapter 2).

Tissue from these animals was processed for the detection of VIP (Nickel-DAB) then GnRH (brown DAB; Table 3.1). I analyzed GnRH+ cells located in three anatomical regions: MS/DBB (six sections), OVLT (two sections), and AVPV/POA/LSPV (six sections). The percent of GnRH+ neurons contacted by VIP+ fibers was analyzed using a non-parametric Mann Whitney U with time as the independent variable (Statview 5.0). Differences were considered significant when $p < 0.05$.

Results

Single labeled BDA

BDA injections hit the SCN in 3 out of 13 animals (Figure 1A, B, C). In the remaining animals injections were rostral (n=5) or caudal to the SCN (n=2) or the tracer was injected into the 3rd ventricle or the optic chiasm and was not transported effectively (n=3). Within the injection site the neuropil was darkly stained such that fibers were difficult to discern, however they appeared to be shorter than BDA fibers elsewhere (Figure 3.1D). BDA fibers were abundant at the borders of the injection site. In all animals, fibers were distinct dark blue or black, and resembled those seen in golgi stained tissue (Figure 3.1E). BDA fibers were always more abundant in areas ipsilateral to the injection site. Retrogradely labeled cells were also observed (Figure 3.1F).

BDA fibers were seen in the same target regions in the three animals with SCN hits (Figure 3.2). In the most rostral sections, fibers were detected in the ventral portion of the lateral septal nucleus (LS) and in the DBB (Figs. 3.2A, 3.2B, 3.3A). Fibers coursed around the dorsal, ventral and medial sides of the anterior commisure (Figure 3.3B). In more caudal sections, the density of BDA fibers increased and they were wrapped around the anterior commisure and extended dorsally into the LS and the bed nucleus of the stria terminalis (BNST). At this level scattered fibers were also evident at the ventral edge of the rostral OVLT. In sections containing the central and caudal OVLT these fibers became more extensive and formed a dense plexus around the 3rd ventricle (Figure 3.2B, 3.3C).

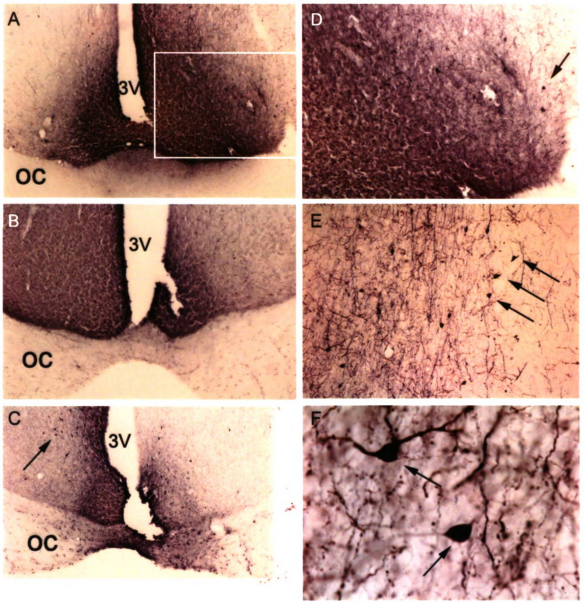

Figure 3.1. A-C) Photomicrographs depicting BDA injection sites in the SCN of 3 individual grass rats. The boxed area in A is enlarged in D. D) Note the dense staining within the injection site and that labeled fibers become visible at the borders of the site. E) An example of BDA labeled fibers in the LS taken at 200X magnification. F) An example of retrogradely labeled neurons in the LS. Arrows indicate retrogradely labeled cells. Abbreviations are found in table 3.2.

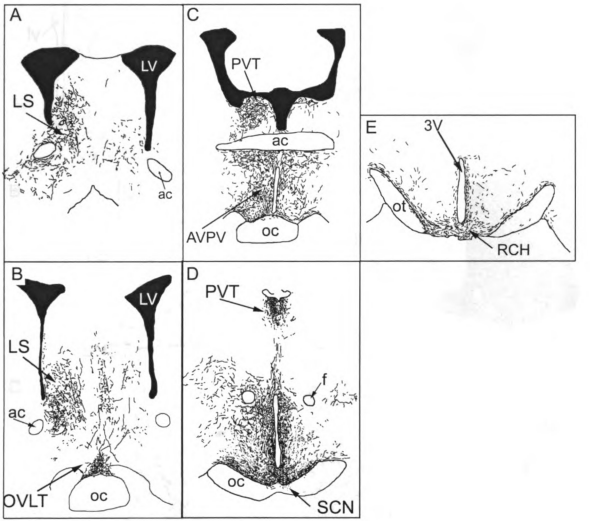

Figure 3.2. A series of line drawings based on 3 animals with BDA injections into the SCN. Black lines indicate BDA labeled fibers. In all pictures, the injection site was on the left side. Note that labeling was heavier on the side ipsilateral to the injection site. Abbreviations are noted in Table 3.2.

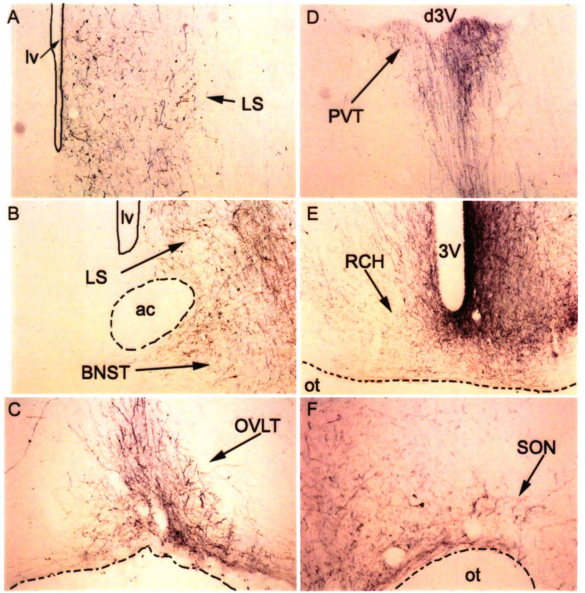

Figure 3.3. Photomicrographs depicting representative BDA labeled fibers in animals with an injection of BDA within the SCN. The dashed line in picture C indicates the ventral edge of the tissue. Dashed lines in B, E, and F outline structures. See Table 3.2 for abbreviations

Moderately dense BDA fibers were observed in all subdivisions of the POA, including the medial POA, medial preoptic nucleus, median preoptic nucleus, and anterodorsal preoptic nucleus. Numerous fibers were detected at the borders of the 3rd ventricle, in the periventricular nucleus (PeVN) and AVPV, and just dorsal to the optic chiasm, in the ventromedial and ventrolateral nuclei (Figure 3.2C, 3.2D). BDA fibers were also abundant in all subdivisions of the BNST, dorsal and ventral to the anterior commisure.

The most extensive BDA labeling was found in sections containing the rostral and central portions of the SCN. Dense fiber plexuses were detected in tissue both ipsilateral and contralateral to the injection site (Figure 3.2). Fibers were abundant in the PeVN and the preoptic nuclei adjacent to the 3rd ventricle then thinned out lateral to these regions and became relatively sparse in the lateral preoptic nucleus and horizontal limb of the DBB. At this level, fibers coursed laterally through the ventromarginal zone, along the dorsal surface of the optic chiasm, and sparse, scattered fibers terminated in the SON (Figure 3.3F). Fibers streamed vertically along the edges of the 3rd ventricle, passed through the paraventricular nucleus of the hypothalamus (PVN) and became sparser at the dorsal tip of the 3rd ventricle. Some fibers continued vertically along the midline to where they terminated in a dense plexus within the habenula and paraventricular thalamus (PVT, Figure 3.2D, 3.3D).

BDA fibers were less abundant in sections caudal to the SCN when compared to sections through the central portion of the nucleus. Here, relatively long BDA fibers extended laterally along the ventral edge of the tissue in the

retrochiasmatic area (RCH) and then along the dorsal border of the optic chiasm (Figure 3.2E, 3.3E). Numerous fibers were also detected in the PeVN, along the edges of the 3rd ventricle (Figure 3.3E). Scattered BDA fibers were seen in the PVN, PVT, and SON. In the most caudal sections examined, beyond the regions containing GnRH+ and ER+ cells, BDA fibers were present in the PVT, PeVN, PVN, arcuate nucleus, ventromedial hypothalamus, dorsomedial hypothalamus and intergeniculate leaflet.

ER+ cell distribution, BDA fibers and ER+ cells

Figure 3.4A depicts typical ER+ nuclear staining in a steroid-primed female grass rat. In rostral sections, scattered ER+ cells were seen in the MS and LS as well as the horizontal and vertical portions of the DBB (Figure 3.5). ER+ cells were extremely dense in the AVPV and PeVN, and then decreased in number further from the 3rd ventricle. That is, ER+ cells were most abundant in the AVPV > medial POA > lateral POA. In the BNST, ER+ cells were observed both dorsal and ventral to the anterior commisure, and this cell population increased in more caudal regions of the nucleus (Figure 3.5). At the level of the SCN and RCH, numerous ER+ cells were diffusely distributed through the anterior hypothalamus and BNST (Figure 3.5). ER+ cells were not detected in the SCN.

BDA fibers clearly projected to regions containing ER+ positive cells (Figure 3.4B, Figure 3.6). These labeled fibers wound around and between ER+ nuclei, and in many cases, appeared to contact the nuclei themselves (Figure 3.6C,D, E). ER+ cells were always detected in regions that contained BDA

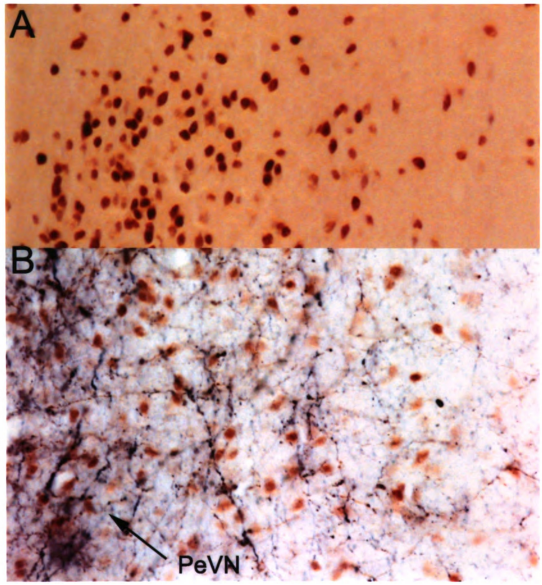

Figure 3.4. Photomicrographs depicting A) ER+ cells in the AVPV of an estradiol primed grass rat. B) Low power view of patterns of overlap between BDA labeled fibers (blue) and ER+ cells (brown) in the PeVN (left side of image). Note that as ER+ staining decreases so does the density of BDA staining. Abbreviations are found in Table 3.2.

Figure 3.5. A series of line drawings at 5 levels from rostral (1) to caudal (5) depicting the distribution of ER+ and GnRH+ cells and AVP+ and VIP+ fibers in the hypothalamus of grass rats. In figures of ER+ and GnRH+ each black dot represents 1 cell. Pictures of GnRH+ cell distribution are modified from McElhinny *et al.* (1999). See Table 3.2 for abbreviations.

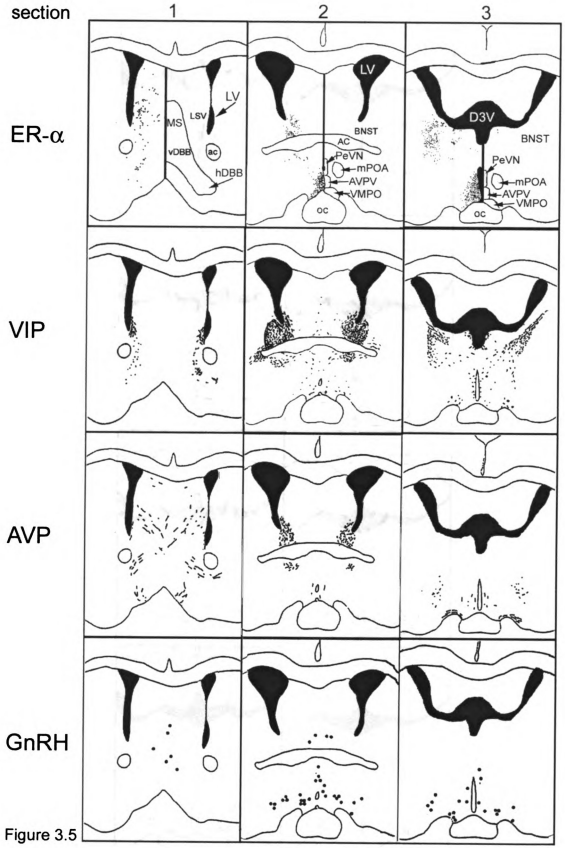
section
1
2
3
ER-α
VIP
AVP
GnRH
LV
LSV
MS
vDBB
ac
hDBB
BNST
AC
PeVN
mPOA
AVPV
VMPO
oc
D3V

Figure 3.5

ER-α

VIP

AVP

GnRH

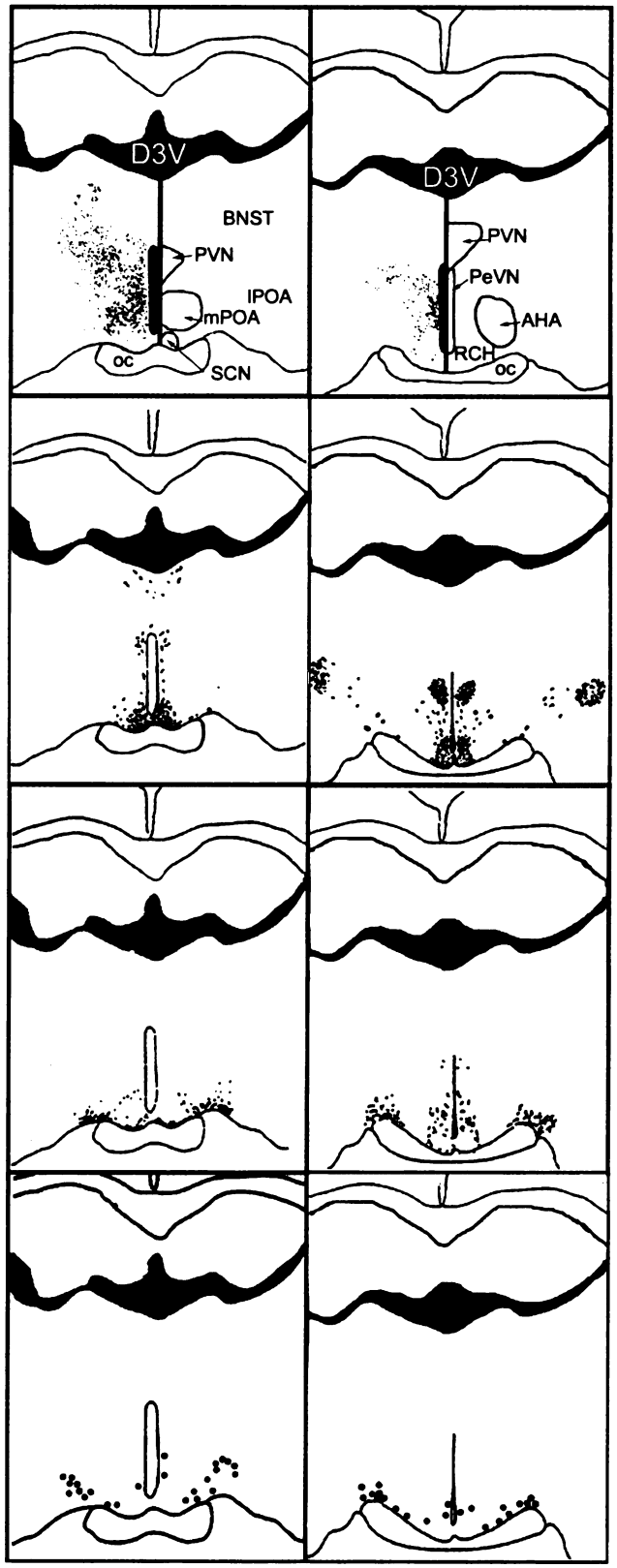

Figure 3.5 cont.

Figure 3.6. Photomicrographs depicting BDA fibers (blue staining) overlapping with ER+ cells (brown staining). Arrows indicate places where BDA fibers are in close apposition with ER+ cells.

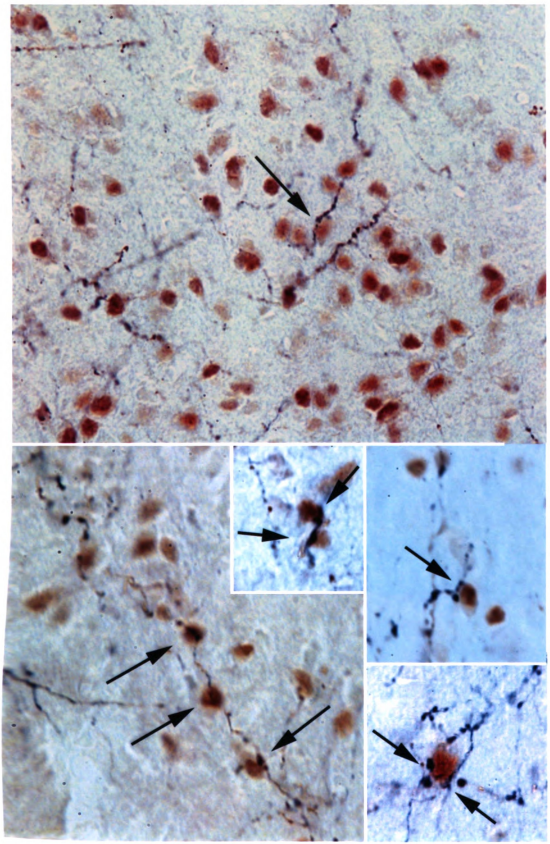

Figure 3.6

labeled fibers (compare Figure 3.2 with Figure 3.5). In comparison, BDA fibers were also detected in regions devoid of ER+ staining, however, fibers were less dense in these areas compared to regions of overlap between BDA and ER+ cells (Figure 3.4B).

BDA fibers and GnRH+ cells

Bipolar GnRH+ cells were visible as large, brown, oval-shaped soma that had long processes with relatively evenly spaced varicosities extending from them. As reported previously (McElhinny et al., 1999), GnRH+ cells and fibers were observed in the MS, the horizontal and vertical limbs of the DBB, OVLT, subdivisions within the POA, and in a region dorsal and lateral to the SON (Figure 3.5). GnRH+ cells were not found within the SCN, however, GnRH+ cells and fibers were detected within the LSPV and along the optic chiasm, just lateral to the nucleus.

BDA fibers were seen in apposition to GnRH+ cells in all regions examined (Figure 3.7C-G, Table 3.3). In particular, a dense plexus of BDA fibers surrounded GnRH+ cells that were located in the OVLT and AVPV. Interestingly, BDA and GnRH+ fibers also overlapped, particularly in these two regions (Figure 3.7A, B).

AVP+ and VIP+ fiber distribution

The distributions of AVP+ and VIP+ fibers in female grass rats are depicted in Figure 3.5. Both AVP+ and VIP+ fibers were relatively thin and fine, with irregular, small varicosities. In rostral sections, AVP+ fibers were present in the ventral and central portion of the LS, with scattered fibers found throughout

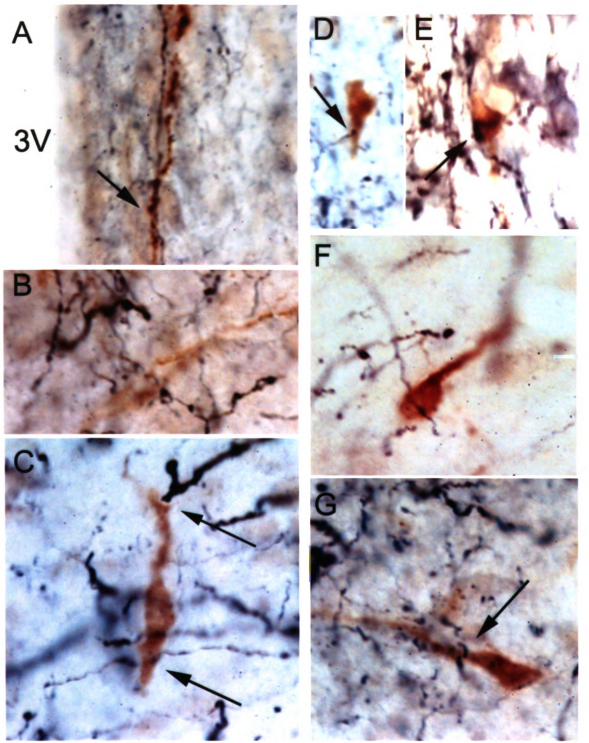

Figure 3.7. A, B) Photomicrographs depicting the pattern of overlap between BDA labeled fibers (blue) and GnRH+ fibers (brown) in female grass rats. C-G) BDA labeled fibers contacting GnRH+ cells. Arrows indicate putative contacts.

Table 3.3. Percentage of GnRH+ cells contacted by BDA labeled fibers. GnRH+ cells were analyzed in tissue from 3 individuals with iontophoretic BDA injections in the SCN. Abbreviations can be found in Table 3.2

GnRH+ cell location	% GnRH+ cells contacted by BDA fiber (±SEM)
MS/DBB	66 ± 0.08
OVLT	72 ± 0.09
AVPV	90 ± 0.10
POA/LSPV	67 ± 0.12

the MS. Fibers were also detected in the BNST, but were relatively sparse in the OVLT. Darkly stained AVP+ cells were concentrated in the dorsal portion of the SCN. Extensive AVP+ fibers extended from the SCN into the LSPV, and a moderate number of fibers continued into the PVN. Large magnocellular AVP+ cells were located in the SON and PVN. Labeled fibers originating in these nuclei were thicker than those found elsewhere.

The distribution of VIP+ fibers overlapped with that of AVP+ fibers in some regions (Figure 3.5). In the most rostral sections examined, VIP+ fibers were present in the ventral portion of the LS and within the BNST surrounding the anterior commisure. In sections containing the OVLT, a moderate number of short VIP+ fibers were scattered around the 3rd ventricle and optic chiasm. At this level, abundant VIP+ fibers were detected within the BNST. VIP+ cells were seen in the ventral portion of the SCN, along the dorsal edge of the optic chiasm, and VIP+ fibers were abundant throughout the rostrocaudal extent of the SCN. Numerous VIP+ fibers extended dorsally from the SCN into the LSPV and the PeVN.

AVP+ and VIP+ contacts on GnRH+ neurons

At the light microscope level, VIP+ and AVP+ contacts on GnRH+ cells were easily detected and were observed in all regions (Figure 3.8, Figure 3.9, Table 3.4). Typically, contacts were present as a dark blue bouton-like structure at the edge of the GnRH+ cell soma, although frequently a somewhat longer fiber was observed approaching and wrapping around the cell body and dendrite (Figure 3.8D).

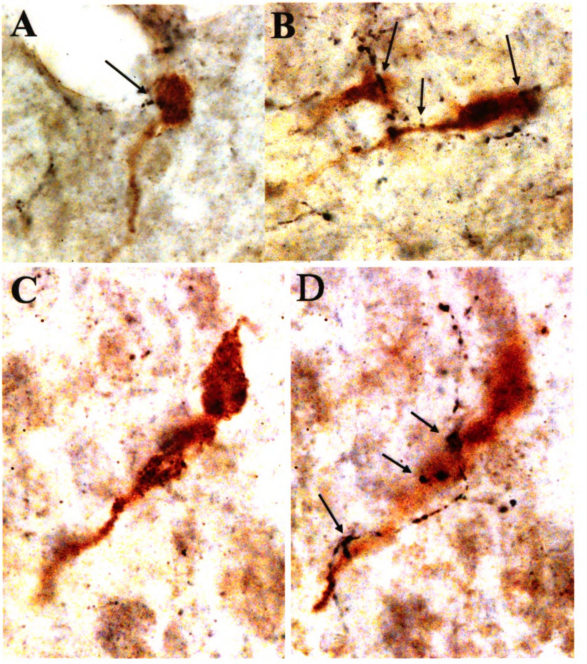

Figure 3.8. Photomicrographs depicting AVP+ fibers (blue) contacting GnRH+ cells (brown) in the hypothalamus of grass rats. Pictures in C and D are the same image taken at different planes of focus. Arrows indicate putative contacts.

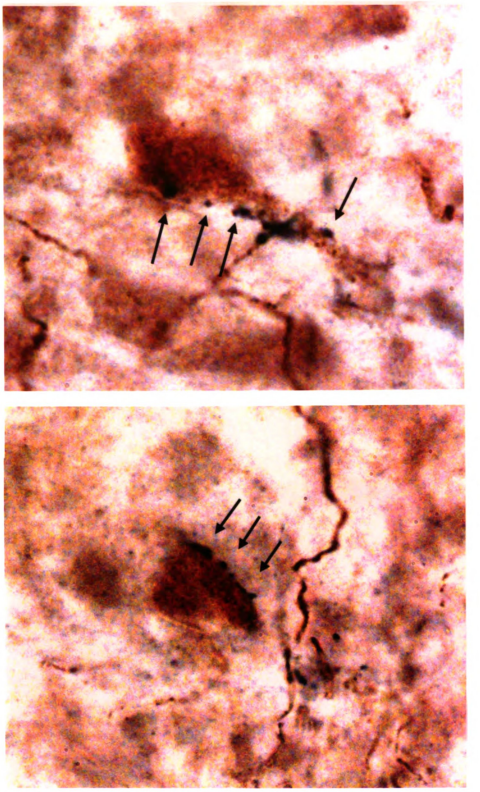

Figure 3.9. Photomicrographs of VIP+ fibers (blue) contacting GnRH+ cells (brown) in a steroid primed female grass rat. Arrows indicate putative contacts.

Table 3.4. Percent of GnRH+ neurons contacted by AVP+ and VIP+ fibers in the hypothalamus of grass rats (mean ±SEM)

Region	MS/DBB		OVLT		AVPV/POA/LSPV		Total	
	% GnRH+ cells contacted	# boutons/ GnRH+ cell	% GnRH+ cells contacted	# boutons/ GnRH+ cell	% GnRH+ cells contacted	# boutons/ GnRH+ cell	% GnRH+ cells contacted	# boutons/ GnRH+ cell
AVP (n=3 females)	14.4 ±9.1	0.67 ±0.33	20 ±10	0.79 ±0.41	34.3 ±14.5	1.71 ±0.4	24.4 ±3.4	1.08 ±0.12
VIP (n=3 intact females)	52 ±9	1.61 ±0.09	32.5 ±4.7	1.82 ± 0.25	31 ±11.2	1.42 ±0.25	35.4 ±9.2	1.67 ±0.05
VIP (n=10 steroid primed females)	14 ±2.1	1.75 ±0.16	18 ±9.5	2.13 ±0.5	17.6 ±3.3	2.4 ±0.31	14.12 ±2.65	2.23 ±0.14

AVP+ fibers contacted about 24% of GnRH+ cells (Table 3.4). The majority of these contacted cells were located in the AVPV and along the optic chiasm, lateral to the SCN. In intact female grass rats, VIP+ fibers contacted about 35% of the total population of GnRH+ neurons (Table 3.4). These cells were located predominantly in the MS and DBB; slightly more than half of the GnRH+ neurons present in these regions received VIP input. Generally, individual GnRH+ neurons had more contacts of VIP+ fibers than AVP+ (1.67 compared to 1.08 boutons/cell respectively). In the LSPV, VIP+ and AVP+ fibers overlapped with GnRH+ fibers.

In steroid-primed grass rats, as in intact female grass rats, VIP+ fibers contacted GnRH+ neurons. Whereas females killed at ZT 20.5 had relatively low LH titers (1.68 $\pm$1.2 ng/ml $\pm$SEM) when compared to those of animals killed at ZT 22 (15.15 $\pm$ 7 ng/ml $\pm$SEM), I did not detect a significant difference in the percent of GnRH+ cells contacted by VIP+ fibers between the two groups (Mann-Whitney U, p=0.17). I thus combined the data; VIP+ fibers contacted about 14% of the total GnRH+ cell population (Table 3.4). Although there were typically more GnRH+ neurons within the OVLT (data not shown), all regions contained about the same percentage of GnRH+ neurons contacted by VIP fibers (range 14-18%). The average percent of GnRH neurons contacted by VIP+ fibers was somewhat less in steroid-primed females than in intact females. Table 3.5 summarizes the data from all these studies.

Table 3.5. Summary of data indicating the location and density of BDA labeled fibers, VIP+ and AVP+ fibers, and ER+ and GnRH+ neurons in grass rats. Scale of density ranges from least dense (+) to most dense (++++). A dashed line indicates that labeling was not detected. Fiber and cell densities reflect the range for a given type of labeling and are not directly comparable across substances. See Table 3.2 for abbreviations.

	BDA	VIP+ fibers	AVP+ fibers	ER+ cells	GnRH+ cells
LS	+++	+	++	+	-
MS	+	-	++	-	+
BNST	++	+++	++	++	-
PeVN	+++	++	+	++++	+
OVLT	++	++	+	+	++++
AVPV	+++	+	+	++++	++
POA	++	+	+	++	++
SCN	++++	++	++	-	-
LSPV	++++	+++	+++	-	+
SON	+	-	+	-	++

Discussion

Results from this study indicate that grass rats are similar to nocturnal rodents with respect to projections of the SCN to the anterior hypothalamus. In grass rats, BDA injections that hit the SCN labeled fibers in nuclei known to receive SCN input in lab rats and hamsters (Table 3.5, Buijs *et al.*, 1993; Kalsbeek *et al.*, 1993; Morin *et al.*, 1994; Watts and Swanson, 1987; Watts *et al.*, 1987). The distributions of VIP+ and AVP+ fibers in grass rats also resembled those of other rodents (Figure 3.5; Abrahamson and Moore, 2001; Kalsbeek *et al.*, 1993; Morin *et al.*, 1994; Watts and Swanson, 1987).

The distribution of ER+ cells in the hypothalamus of grass rats also resembled that seen in a host of other mammals, including nocturnal lab rats and hamsters (Table 3.5; Figure 3.5; Blaustein, 1992; Cintra *et al.*, 1982; Don Carlos *et al.*, 1991; Fox *et al.*, 1991; Hnatczuk *et al.*, 1994; Li *et al.*, 1993; Tobet *et al.*, 1993). ER+ cells were all located in regions that received input from the SCN (Table 3.5). ER is typically expressed in the cell nucleus; thus, I was unable to establish definitely whether BDA-labeled fibers contacted ER+ cells. However, frequently ER+ cells were seen surrounded by a dense plexus of BDA fibers suggesting that these steroid sensitive cells receive information directly from the SCN in grass rats, as is the case in lab rats and hamsters (Figure 3.4, Figure 3.6; de la Iglesia *et al.*, 1995; Watson *et al.*, 1995).

As in McElhinny *et al.*, 1999, I found that the distribution of GnRH+ neurons in grass rats parallels that seen in other mammalian species. Additionally, I detected SCN input on GnRH+ cells in all regions that I analyzed

(Table 3.3, Table 3.5). However, I found that 60-90% of these neuroendocrine cells were contacted by BDA labeled fibers, an estimate that is much higher than that for lab rats (~2-20%; van der Beek *et al.*, 1997) or hamsters (11-13%; de la Iglesia *et al.*, 1995). These differences might be related to the tracers used (BDA vs. *Phaseolus vulgaris* leucoagglutinin), the locations and sizes of the injections, numbers of filled cells, efficacy of the transport, or the presence of retrogradely labeled fibers. BDA is predominantly transported in the anterograde direction, but some BDA contacts on GnRH+ perikarya might come from retrogradely labeled neurons (Rajakumar *et al.*, 1993; Veenman *et al.*, 1992).

I found that in grass rats, 24% of GnRH+ neurons were contacted by AVP+ fibers, a proportion similar to that reported for male lab rats (19%) but higher than that seen in females (10%; van der Beek *et al.*, 1998). It is quite possible that some AVP+ fibers contacting GnRH+ cells were not SCN efferents but rather originated from cells in the SON, PVN, or elsewhere. The largest proportion of GnRH+ cells contacted by AVP+ fibers was found in the AVPV/POA/LSPV. These regions are relatively close to the SCN so it is likely that at least these GnRH+ neurons were receiving input from AVP+ cells in the SCN. SCN lesions in female lab rats significantly reduced the percent of GnRH+ neurons contacted by AVP fibers in the POA (van der Beek *et al.*, 1998).

In intact female grass rats, about 35% of the GnRH+ cells were contacted by VIP+ fibers. These contacts probably came from cells in the SCN, where most of this peptide is produced. This is supported by studies of lab rats where unilateral SCN lesions decreased VIP innervation of GnRH cells on the side

ipsilateral to the lesion, compared to the contralateral side (van der Beek *et al.*, 1993). Previous studies on lab rats provide widely varying estimates of the percentage of GnRH cells that receive input from VIP cells. Some report that about 5% of GnRH neurons are contacted by VIP fibers (Kriegsfeld *et al.*, 2002), whereas others report figures of 34% (van der Beek *et al.*, 1994; Horvath *et al.*, 1998) or 45% (van der Beek *et al.*, 1993). In the current study I found that in intact grass rats GnRH neurons were more likely to be innervated by VIP+ fibers (35%) than were steroid-treated females (14%). I was unable to determine the endocrine state of intact grass rats, but undoubtedly they had lower estradiol levels than did steroid-primed females. These varied results from lab rats and grass rats may be due in part to: 1) varying levels of circulating steroid hormones, 2) differences in microscopy techniques (i.e. fluorescent labeling combined with confocal vs. DAB labeling combined with light), or 3) differences regarding whether contacts were counted on dendrites and soma or just on the soma.

By combining the data on anterograde tracing of SCN efferents, and immunocytochemistry for two known SCN peptides, I am able to make some inferences about which subsets of SCN cells may project to GnRH neurons in the grass rat (Table 3.5). The distribution of AVP+ fibers matched that of BDA labeled fibers in the SON and MS, regions where VIP+ fibers were not detected. On the other hand, the distribution of AVP+ and VIP+ fibers overlapped in some regions that received SCN input. Fibers containing these two peptides appeared to overlap to a similar extent with regions containing BDA labeled fibers in the

POA and LSPV. Within the lateral septum, VIP was contained in fibers in the ventral portion of the nucleus, whereas AVP+ fibers were present in the ventral and central portions. The SCN efferents in the BNST, PeVN, and OVLT overlapped predominantly with VIP+ fibers, but some AVP+ fibers were also detected in these regions. Interestingly, GnRH+ neurons were detected in the AVPV, a region that received extensive SCN input but contained relatively few VIP+ or AVP+ fibers.

These data suggest that the neurotransmitters AVP and VIP communicate circadian information to the reproductive axis in this diurnal species, as occurs in nocturnal rodents. It is still possible, however, that the inverted rhythms of estrous-related events in grass rats relative to nocturnal rodents are due to differences in communication between VIP and/or AVP cells in the SCN and GnRH+ cells. For example, the contacts I, or others, have observed may not reflect numbers of actual synapses; ultrastructural studies will be needed to evaluate that possibility. Furthermore, the numbers of contacts are not necessarily indicative of the overall intensity or pattern of signals. It is also important to consider the possibility that other transmitters such as GABA or glutamate are released from VIP and/or AVP terminals in a manner that differs between species.

The SCN may also influence GnRH+ neurons indirectly via intermediate cells that might modify the temporal pattern of the signal received by GnRH+ neurons. One such "relay station" might be the LSPV, which receives input from the SCN and projects to many of the same sites, including GnRH+ neurons (de la

Iglesia *et al.*, 1995; Morin *et al.*, 1994; van der Beek *et al.*, 1997; Watts and Swanson, 1987; Watts *et al.*, 1987). In lab rats, disruption of the projection from the SCN to the LSPV attenuates the steroid-induced LH surge (Watts *et al.*, 1989) and GnRH fibers synapse on structures in this region (van der Beek, Wiegant *et al.*, 1997). In the current study, I detected AVP+ and VIP+ fibers extending from the SCN to the LSPV and some of these fibers contacted GnRH+ cells located there. Together, these data raise the possibility that the LSPV modulates SCN signals relayed to the GnRH system. Unfortunately, I was unable to determine which BDA positive fibers emanated from cells in the SCN and which came from the LSPV.

The results of the current study suggest that the SCN may communicate temporal information directly to cells that regulate the timing of estrous-related events in a diurnal species, as they do in nocturnal ones. The similarity between diurnal and nocturnal rodents with respect to SCN targets suggests that SCN input to GnRH+, and perhaps ER+ neurons, might represent a common anatomical pathway for the regulation of reproductive events by the circadian system. This raises the possibility that the mechanisms underlying fundamental differences between nocturnal and diurnal species might reside within GnRH cells, SCN neurons projecting directly to them, or in intermediate structures such as the LSPV. Now that the anatomical groundwork has been laid, future studies may be directed at answering such functional questions.

Chapter 4:

A daily rhythm in mating behavior and progestin receptor expression in a diurnal murid rodent *Arvicanthis niloticus*

Introduction

The timing of many reproductive behaviors and associated physiological events in mammals is related to the activity patterns of the species. For example, diurnal sciurid (e.g. ground squirrels) and hystricomorph rodents (e.g. degus and cavies) mate during daylight hours (Rood, 1970; Dobson and Michener, 1995; Michener, 1980; Rossi and Lee, personal communication), whereas nocturnal rodents such as laboratory rats ("lab rats"), hamsters, and mice typically display more mating behaviors at the beginning and throughout their active period, during the dark portion of the light:dark cycle. Female lab rats for example, have higher lordosis quotients (LQ) and male rats have shorter latencies to first intromissions and ejaculations, and shorter intervals between these events, during the dark than the light phase of the day:night cycle (Beach and Levinson; Hansen *et al.*, 1979; Harlan *et al.*, 1980; Spinka, 1990; Stefanick, 1983).

In lab rats the daily rhythm in female sexual behaviors is due in part to rhythms in steroid hormone secretion but also to changes in responsiveness to these hormones (Hansen *et al.*, 1979; Hansen *et al.*, 1978; Sodersten *et al.*, 1981). Ovariectomized lab rats implanted with estradiol capsules and tested for sexual behavior 3 days later have peak LQ values during the dark and the lowest

LQ values during the light portion of the 24-hour cycle (Hansen *et al.*, 1979). Additionally, the response of ovariectomized lab rats to steroid hormone stimulation is phase-dependent. Specifically, when females are tested for sexual behavior 96 hours after receiving an estradiol capsule, they exhibit higher LQ values if the capsule was implanted 4 hours after lights-off than at other times of day (Hansen *et al.*, 1978). Interestingly, not all studies of lab rats have found daily rhythms in sexual behavior (Campbell and Baum, 1979; Erskine *et al.*, 1980; Harlan *et al.*, 1980). Erskine and colleagues (1980) suggest that the discrepancies may be due to differences in the strains of rats that were used in these studies (Wistar, Long Evans, Sprague-Dawley). That is, these strains may exhibit different thresholds of responsiveness to hormone treatment.

Rhythms in behavioral sensitivity to steroid hormones might theoretically be caused by rhythms in numbers of available hormone receptors. Progesting receptor-immunoreactive (PR+) cells that are critical for female sexual behavior are located in the ventromedial hypothalamus (VMH) of lab rats and hamsters, and in the ventrolateral hypothalamus (VLH) of guinea pigs (Blaustein and Brown, 1983; Blaustein *et al.*, 1994; DeBold *et al.*, 1982; Don Carlos *et al.*, 1989; Rainbow *et al.*, 1982; Sterner *et al.*, 1992; Young, 1969). Ovariectomized guinea pigs, lab rats, and hamsters implanted with intracranial estradiol-containing cannulae become sexually receptive to systemic progesterone (P) when the cannulae are placed bilaterally in the VMH/VLH region (Barfield *et al.*, 1983; Davis *et al.*, 1982; Delville and Blaustein, 1991; Rubin and Barfield, 1983). Furthermore, blocking the expression of PR in the VMH of lab rats with either

antisense oligonucleotides to PR mRNA, or by the administration of the P antagonist RU-486, inhibits female sexual behaviors (Etgen and Barfield, 1986; Mani *et al.*, 1994). It is therefore possible that the rhythm in female sexual behavior is promoted in part by a rhythm in the number of PRs in the VMH/VLH. To my knowledge, this issue has not been examined in any rodent species.

The timing of mating is reversed in the diurnal rodent, *Arvicanthis niloticus* ("grass rat") from that of the lab rat. These murid rodents are therefore ideal species for comparative studies that examine the mechanisms underlying the timing of reproductive events. Intact grass rats demonstrate diurnal patterns of rhythmicity with respect to the timing of copulation, the ovulatory surge in luteinizing hormone (LH), and activation of gonadotropin releasing hormone cells (Blanchong *et al.*, 1999; McElhinny, 1996; McElhinny *et al.*, 1999; McElhinny *et al.*, 1997). The reversal in rhythms of mating behaviors in diurnal compared to nocturnal species might be due to inverted rhythms in responsiveness to hormones, to altered patterns of hormone secretion, or both. In this study I examined the first of these hypotheses by pairing male and hormone-primed female grass rats and testing them for mating behavior at four different times of day. I then characterized rhythms in numbers of PR+ cells to determine whether numbers of these cells might account for rhythms in sexual receptivity in female grass rats.

Materials and methods:

Adult grass rats (>60 days) were singly housed in a 12:12 light:dark cycle and provided food and water *ad libitum*. A red light (<5 lux) was left on

continuously for the purposes of animal care and videotaping of behavioral tests. All experiments were performed in compliance with Michigan State University All-University Committee on Animal Use and in accordance with the standard in the National Research Council *Guide for Care and Use of Laboratory Animals*. All efforts were made to minimize the suffering and the number of animals used in these experiments.

Sexual behavior

In this first study I determined whether female grass rats have a rhythm in behavioral responsiveness to steroid hormones. Females were anesthetized with sodium pentobarbital (<50 mg/kg Nembutal; Abbott Laboratories) supplemented with methoxyflurane (Metofane, Mallinckrodt Veterinary) then bilaterally ovariectomized. Incisions were closed with sutures and treated with topical antibiotic (Nolvasan, Fort Dodge Animal Health). Animals were then given a subcutaneous (sc) injection of 1 cc 0.9% saline, and 0.03 mg buprenorphine hydrochloride (intramuscularly; im, Buprenex, Reckitt & Colman Pharmaceuticals Inc.). Seven to fourteen days after ovariectomy, females (n=16) were divided into four groups that received steroid hormone injections (sc) at different times of day. For two days, at zeitgeber time (ZT) 1, 7, 13, or 19 (ZT 0= lights-on), females were injected (sc) with10 µg 17-β estradiol benzoate in sesame oil (EB). On the third day, at the same ZT, females received injections (sc) of 125 µg P. Four hours following the P injection (at either ZT 5, 11, 17, or 23), females were placed in a clean, glass aquarium containing cedar shavings, food, and a water bottle and allowed to acclimate for 5 minutes. Then a sexually

experienced male was introduced and the pair was videotaped for 30 minutes. Each pair of animals was tested together at all 4 time points, in counter-balanced order. At least one week passed between the conclusion of one behavioral test and the start of the next series of hormone injections. Control females (n=4) were ovariectomized, injected with sesame oil (sc, 0.1 cc) and paired with the same male at each of two time points. These females received injections for 3 days at ZT 13 or 19 and were tested at ZT 17 or 23, respectively.

The initial 15 minutes of each behavioral test were analyzed. The scored behaviors were mounts, lordosis, and copulation. A mount was counted when the male placed his forepaws on the hindquarters of the female, and a lordosis was scored when a female adopted a stereotyped posture with her head and tail elevated and her back in concave flexion. The LQ was calculated from these scores as the number of female lordosis postures/number of male mounting attempts. A copulation was scored each time the female displayed lordosis as the male mounted her. Finally, I evaluated the longest interval between consecutive copulations as an indicator of mating intensity.

These behavioral variables were analyzed using repeated measure ANOVA and pairwise comparisons were analyzed with Fisher's PLSD post-hoc tests (Statview 5.0). Data were considered significant when $p<0.05$. One female was not receptive at any time point and all data from this animal were excluded from analysis.

Progesterone receptors

Here I determined whether the rhythm of sexual receptivity in female grass rats is associated with a rhythm in the number of PR+ cells. One week following ovariectomy, females were injected with 10 µg EB (sc) in sesame oil for two days at either ZT 13 or 19 (n=4/time point). On the third day at the same ZT, females were perfused. In this study, thus, animals received EB injections at times that steroid treatment led to peak and trough levels of female sex behaviors in the first study (ZT 19 and ZT 13, respectively), and the perfusions were done at the corresponding times at which P was injected in that study. Ovariectomized control females did not receive any injections and were perfused at either ZT 13 or ZT 17 (n=4/time point).

Because the results of the first study of PRs were unexpected I conducted a second study to determine whether those findings might be replicated, examine PR+ cell numbers in animals perfused at additional time points, and evaluate the efficacy of a different steroid priming protocol. In this study I used intact females implanted with EB-filled capsules because the procedure is less disruptive to the animals than gonadectomy followed by hormone injection. Capsules were prepared by cutting 20 mm lengths of silastic tubing (ID 0.04 in., Dow Corning), which were filled with EB in sesame oil (180 µg/ml, Krajnak *et al.*, 1998). The ends of the capsules were sealed with medical adhesive (silicone Type A, Dow Corning). Animals were anesthetized with metofane, capsules were implanted at the nape of neck (sc), and the incision closed with a wound clip. Ten days after

animals received the EB-capsule, they were perfused at either ZT 1, 5, 13, 17, or 20 (n= 6/time point except ZT 5; n=5).

All animals were deeply anaesthetized with sodium pentobarbital and perfused transcardially with 0.01 M PBS (pH 7.2, 150-200 ml/animal) followed by 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer. Brains were post-fixed in paraformaldehyde for four hours, transferred to 20% sucrose in 0.1 M phosphate buffer, and then sectioned at 30 µm using a freezing microtome.

Every third section from the medial septum to the VLH of each animal was processed for the immunocytochemical detection of PR as follows: free floating tissue was incubated in (1) 5% normal horse serum (NHS, Vector Laboratories) for one hour at room temperature (in PBS with 0.3% triton-X; PBS-TX), followed by (2) mouse anti-human PR primary antibody for 44 hours at 4°C (1:5000, Chemicon International, in PBS-TX and 3% NHS), followed by (3) biotinylated horse anti-mouse secondary antibody for one hour at room temperature (1:200, Vector Laboratories, in PBS- TX and 3% NHS), followed by (4) avidin-biotin complex for one hour at room temperature (in PBS-TX, Vectastain Elite Kit, Vector Laboratories). PR was visualized by incubating tissue in diaminobenzidine (0.5 mg/ml, in Trizma buffer, pH 7.2, Sigma) with 30% hydrogen peroxide (0.35 µl/ml buffer). A negative control was done by repeating the above procedures but omitting the PR antibody.

Tissue was mounted, dehydrated, coverslipped, and examined under a light microscope (Laborlux S, Leitz Wetzlar GBH). Sections containing the VLH were photographed using a black and white closed-circuit digital camera (Cohu)

and captured using Scion Image (v. 4.02, Scion Corp.). A box 200 x 400 μm was placed over the VLH region and the number of PR+ cells was counted in each of 6 hemi-sections (Figure 4.1). Data from EB-injected animals in the first study were analyzed using a two-way ANOVA with ZT and EB treatment as the independent variables and the total number of PR+ cells as the dependent variable. Pairwise comparisons were computed with Tukey Least Significant Difference post hoc test (Keppel, 1982). Data from animals with EB-capsules in the second study were log-transformed to correct for normality and two outlier samples were dropped, as they were more than 2 standard deviations away from the mean of their group. These data were analyzed using an ANOVA with the corrected PR value as the dependent variable and ZT as the independent variable. Post-hoc pairwise comparisons were done using Fisher's PLSD.

The numbers of PR+ cells were also determined for the anteroventral portion of the periventricular nucleus (AVPV) of EB-injected animals because this region has been implicated in the regulation of the LH surge (Simerly, 1998; Weigand and Terasawa, 1982). For each animal, one section containing the AVPV was selected and bilateral counts were performed. An ocular grid was placed over the densest staining, aligned with the edge of the 3rd ventricle, and the number of PR+ cells was counted in a region 260 μm wide X 360 μm long. These data were analyzed using a two-way ANOVA with ZT and EB treatment as the independent variables and the total number of PR+ cells as the dependent variable. In all statistical analyses differences were considered significant when $p<0.05$ (Statview 5.0).

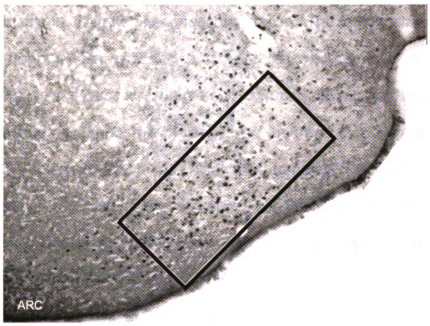

Figure 4.1. An example of PR+ cells in the ventrolateral hypothalamus in an estradiol-treated female grass rat. The box (200 x 400 um) in the figure indicates the region in which PR+ cells were counted. ARC = arcuate nucleus.

Results

Sexual behavior

In a typical mating sequence in grass rats the male approached the female and investigated her anogenital region. The female almost always moved away from the male after this contact and the male generally chased her in a tight circle for 1-2 seconds before copulation. Each mount was very brief lasting 1-2 seconds, and after the pair separated the male typically groomed his anogenital region. The female also groomed herself following most copulations and on many occasions she ran several centimeters away from the male before doing so. Grooming typically lasted about 1-2 seconds before the sequence of mating behaviors was repeated. Occasionally, following a copulation the pair would remain separated for several minutes before the male reinitiated contact with the female. Oil-treated control females always exhibited aggressive behaviors when the male approached (i.e. lunging, biting, rearing up) and no mating occurred in these pairs.

In male and female grass rats several indices of sexual behavior varied significantly as a function of time of day. Female sexual responsiveness, as indicated by the LQ, was affected by time ($F= 6.186$, $df=3$, $p=0.001$, Figure 4.2) and was significantly greater at ZT 23 than at either ZT 11 or 17 ($p<0.05$). When the LQs of individual females were plotted across the four time points the animals fell into two distinct groups (Figure 4.3). Approximately half (7/15) had a rhythm in sexual receptivity with a peak at ZT 23 and a trough at ZT 17 while the second

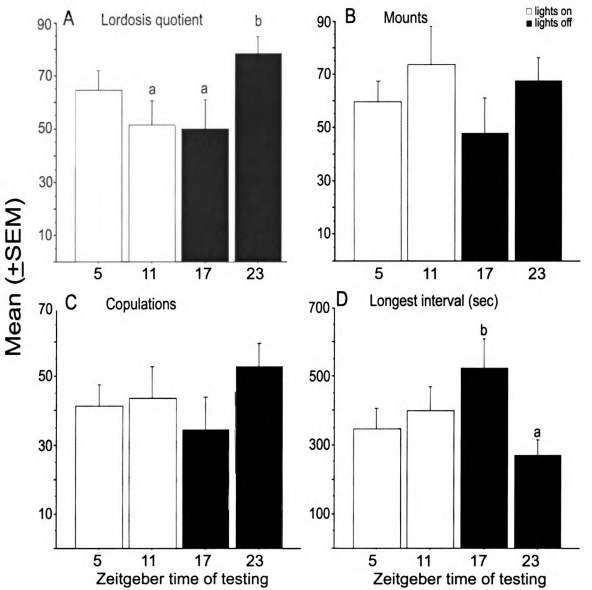

Figure 4.2. Average rates of sexual behaviors (± SEM) in 15 pairs of grass rats tested at 4 different times of day. Bars with different letters over them are significantly different from one another, $p<0.05$. Zeitgeber time 0=lights-on.

Figure 4.3. Lordosis quotient (number of lordosis responses/number of mounting attempts) for 15 individual grass rats. Animals were grouped into two types of sexual responsiveness on the basis of their behavior at ZT 17. Animals in the "low" group exhibited an LQ below 50% at ZT 17 (grey dots or bars); remaining individuals were placed in the "high" group (black dots or bars). Zeitgeber time 0=lights-on. A) LQ as a function of time for each individual. B) Average lordosis quotient (± SEM) for the two groups of responders.

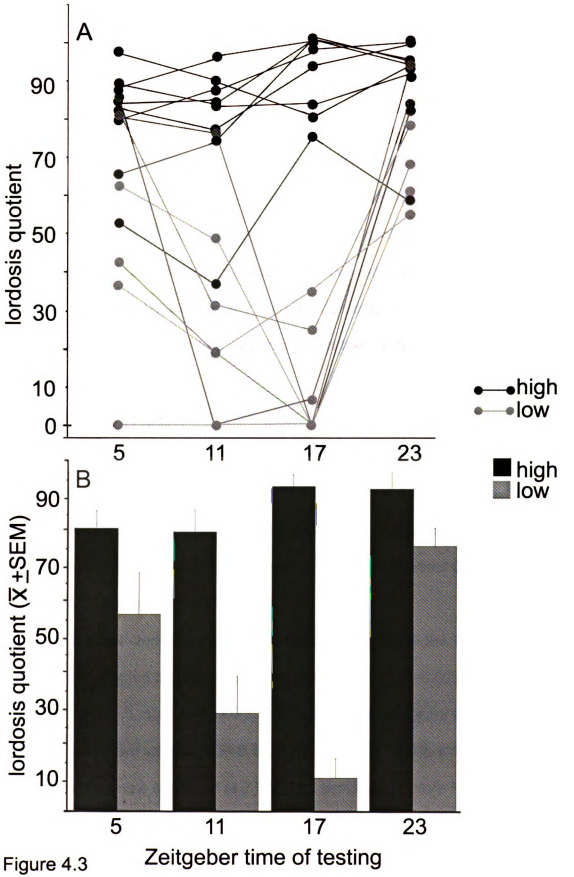
A
lordosis quotient
90
70
50
30
10
0
5
11
17
23
high
low
B
lordosis quotient (X̄ ±SEM)
90
70
50
30
10
5
11
17
23
Zeitgeber time of testing
high
low

Figure 4.3

group (8/15) displayed no rhythm at all and had high levels of receptivity at all timepoints.

Male sexual behavior, indicated by the rate of mounting attempts, also varied significantly as a function of time ($F= 3.97$, $df=3$, $p=0.01$, Figure 4.2). Unlike female grass rats however, males had high levels of sexual behavior at both lights-on (ZT 23) and lights-off (ZT 11). The total number of copulations varied significantly as a function of time ($F=4.023$, $df=3$, $p=0.012$, Figure 4.2). The longest interval between copulations also varied across the 4 time points ($F=5.58$, $df=3$, $p=0.002$, Figure 4.2) and the duration of this interval was shorter when animals were tested at ZT 23 than when they were tested at ZT 17 ($p<0.05$).

Progesterone receptors

All EB-injected females and all females implanted with EB-filled capsules had distinct and darkly stained PR+ cells that were most evident in the arcuate nucleus, AVPV, and the VLH. No PR staining was observed in tissue incubated without the primary antibody. In contrast to EB-treated animals, virtually no PR+ cells were observed in control females.

In the first study the number of PR+ cells present in the VLH was significantly affected by both time of day ($F=6.86$, $df=1$, $p=0.02$) and EB treatment ($F=15.0$, $df=1$, $p=0.002$), and there was a significant interaction between these two variables ($F=8.77$, $df=1$, $p=0.012$, Figure 4.4). Specifically, EB-injected animals sacrificed at ZT 13 had significantly more PR+ cells than did

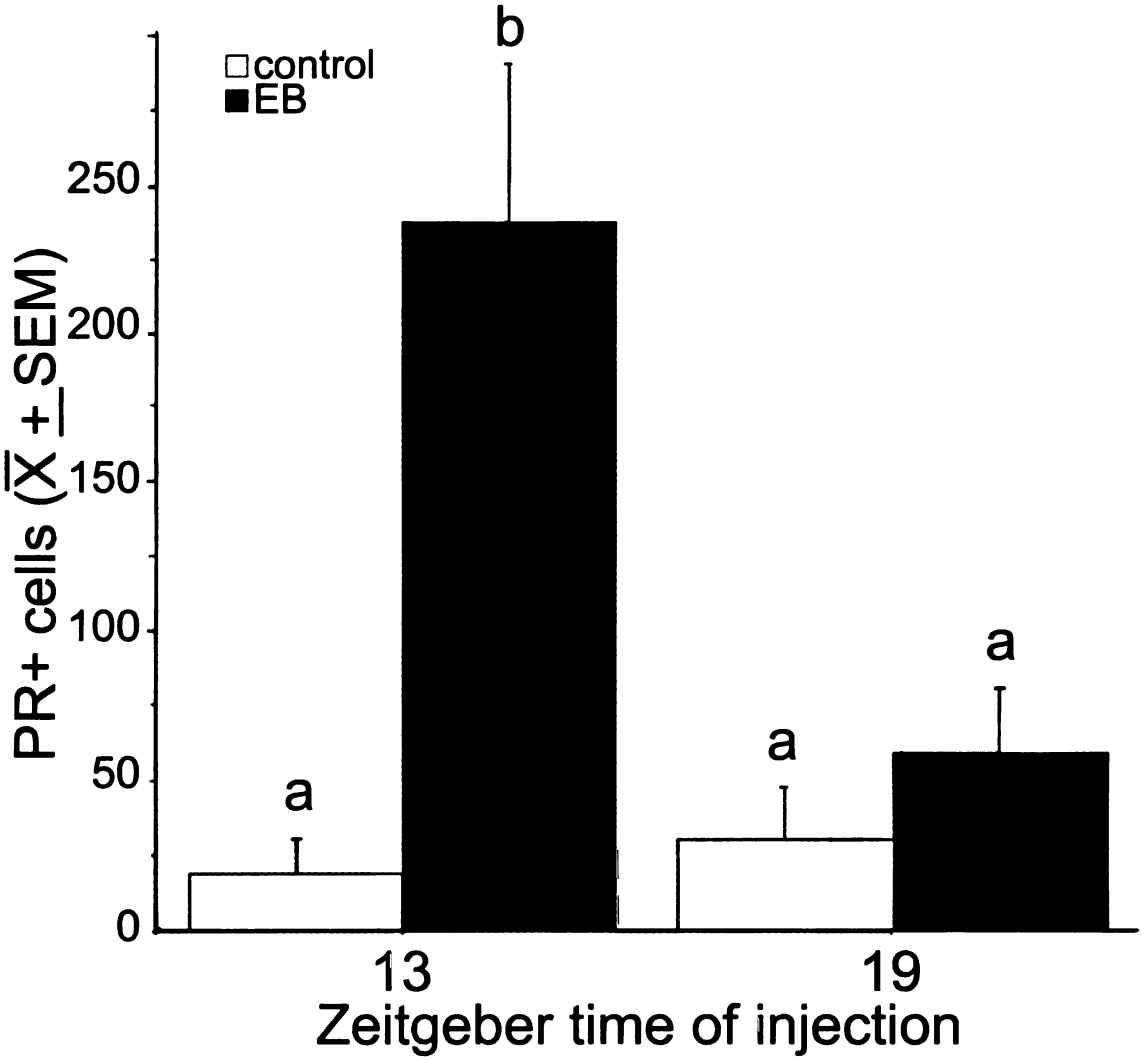

Figure 4.4. Average number (± SEM) of PR+ cells in the ventrolateral hypothalamus in female grass rats. Estradiol benzoate treated females received injections on days 1 and 2 and were sacrificed on day 3, at the same time. Bars with different letters over them are significantly different from one another, $p < 0.05$. ZT 0 = lights-on.

any other group of animals ($p<0.05$). The number of PR+ cells in the VLH was similar, and low, in EB-injected females sacrificed at ZT 19 and control females sacrificed at ZT 13 and 19. In EB-injected animals, no significant effects of time, hormone treatment, or an interaction of these variables was found on the number of PR+ cells present in the AVPV.

In the second study, the number of PR+ cells in the VLH of grass rats implanted with an EB-capsule also varied significantly as a function of time ($F=3.41$, $df=3$, $p=0.02$, Figure 4.5). Animals killed at ZT 20 had significantly fewer PR+ cells then did animals killed at ZT 13 or ZT 17 ($p< 0.05$). The drop in the number of PR+ cells from ZT 13 to ZT 20 mirrored that seen between ZT 13 and 19 in ovariectomized females injected with EB (compare Figure 4.4 and 4.5).

Discussion

These data show that grass rats have a rhythm in sexual behavior and suggest that it is due in part to changes in responsiveness of females to steroid hormones. Hormone-primed female grass rats exhibited the highest rates of sexual behaviors just before the lights came on (ZT 23, Figure 4.2, Figure 4.3) which is when intact grass rats mate (McElhinny, 1996; McElhinny *et al.*, 1997). Mating may occur around the beginning of the active time of day because heightened arousal at this time maximizes the chances of locating a mate, and the animals may be better able to avoid predators at this time.

The rhythm in behavioral responsiveness to hormones in female grass rats is presumably one factor regulating the timing of sexual activities. However,

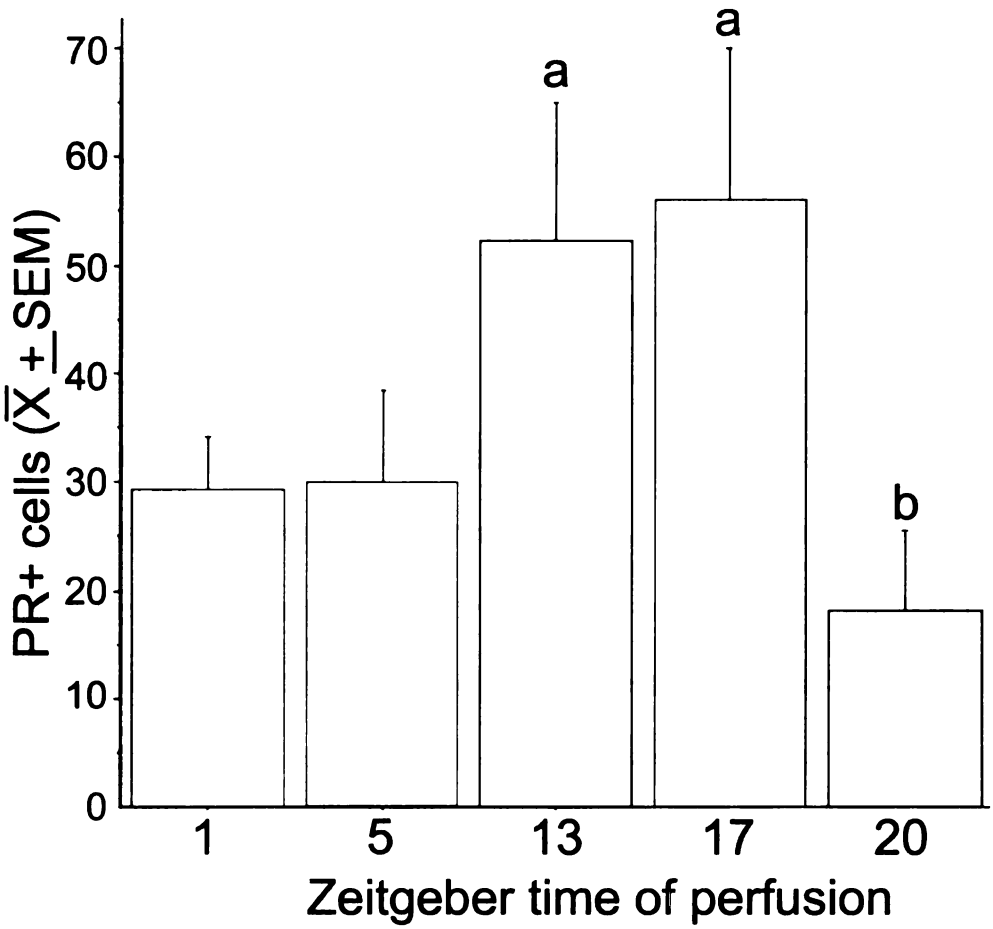

Figure 4.5. Average number (± SEM) of PR+ cells in the ventrolateral hypothalamus in female grass rats. Intact females were implanted with EB-containing silastic capsules. Bars with different letters over them are significantly different from one another, $p < 0.05$. ZT 0 = lights-on

it is probable that the timing of hormone secretion is also important. Intact grass rats mate almost exclusively around the time of lights-on (Blanchong *et al.*, 1999; McElhinny *et al.*, 1997) yet females are capable of exhibiting sexual behavior at other times of day when given appropriate hormonal treatment (Figure 4.2, Figure 4.3). This suggests that patterns in hormone secretion as well as the changes in responsiveness to these hormones, documented here, must regulate the expression of female sexual behavior in intact grass rats. I did not address the hypothesis that changes in hormone secretion are related to sexual behavior in this species because these animals do not undergo predictable estrous cycles and there is no clear outward indicator of when an intact female is in estrus.

Interestingly, the pattern of change in male sexual behavior did not parallel that of the females. Male sexual interest, as indicated by rates of mounting behavior, was highest when peaks in general activity are seen in grass rats in the lab, around lights-on (ZT 23) and lights-off (ZT 11) (McElhinny *et al.*, 1997). Rates of mounting behavior were lowest at ZT 17, when these animals are usually inactive (Blanchong *et al.*, 1999; Blanchong and Smale, 2000; Novak *et al.*, 1999). By contrast, the rhythm in lordosis, indicative of female receptivity, had a single peak at ZT 23 (Figure 4.2). This suggests that the rhythm in sexual behavior seen in intact grass rats reflects rhythms of the female rather than the male. The daily rhythm in sexual behavior of male grass rats may be mediated by a rhythm in general arousal whereas the rhythm in female sexual behavior may be more tightly coupled to changes in responsiveness to, and secretion of, hormones. It is also important to consider that lordosis is a reflexive action

whereas mounting behavior may be indicative of sexual motivation. Had I examined a similar reflex in males, such as ejaculation, I may have seen a single daily peak around lights-on. Conversely, a measure of female sexual motivation might have revealed a rhythm that more closely paralleled the rhythm in general activity.

The current data reveal considerable inter-individual variability with respect to responsiveness to hormones. Female grass rats administered identical doses of EB differed dramatically with respect to sexual receptivity, particularly at ZT 17, which was not the case at the other three time points examined. This raises the possibility that the hormone doses utilized in this study were close to a threshold level for the induction of sexual receptivity. Specifically, individuals with higher thresholds may have been unresponsive to hormone treatment at ZT 17, but as their behavioral responsiveness to progesterone changed across the day, the same hormone doses became sufficient to induce sexual receptivity. The LQ rhythm may have been more dramatic if all animals were treated with a lower concentration of hormones. Alternatively, these data might indicate individual variability with respect to whether or not animals have a rhythm in responsiveness to hormones.

Although this study was conducted in a 12:12 light:dark cycle, it is likely that a rhythm of sexual behavior in grass rats would also be seen in constant conditions, as it is in nocturnal rodents (Alleva *et al.*, 1971; Richter, 1970). In female lab rats and hamsters, rhythms in sexual receptivity and the timing of the LH surge are phase locked to the rhythm in activity onset and this temporal

relationship is maintained in animals free-running in constant conditions (Alleva *et al.*, 1971; Fitzgerald and Zucker, 1976; Moline *et al.*, 1981; Richter, 1970; Stetson and Gibson, 1977; Swann and Turek, 1982). The primary circadian clock in mammals, located in the suprachiasmatic nucleus (SCN), regulates the timing of mating behavior and estrous cyclicity in hamsters and lab rats. In these nocturnal rodents, SCN lesions eliminate estrous cyclicity, and behavioral rhythms in sensitivity to hormone treatment (Gray *et al.*, 1978; Hansen *et al.*, 1979; Hansen *et al.*, 1978; Meyer-Bernstein *et al.*, 1999). The SCN of grass rats and lab rats are similar with respect to rhythms in Fos expression, peptide distribution, and the effect of SCN lesions on locomotor rhythms (Katona *et al.*, 1998; Mahoney *et al.*, 2001; Nunez *et al.*, 1999; Rose *et al.*, 1999; Smale and Boverhof, 1999). Thus it is likely that the SCN of grass rats also contains a circadian clock, which regulates the timing of the rhythm in sexual behavior.

The inverse relationship between rhythms in sexual behavior and in the number of PR+ cells in the VLH was surprising. EB-injected females sacrificed at ZT 19 had *fewer* PR+ cells than did EB treated females sacrificed at ZT 13, despite the fact that females treated with P at ZT 19 had a significantly higher LQ at ZT 23 than at any other time of day. This pattern was confirmed in animals with EB-capsules, who also had significantly fewer PR+ cells at ZT 20 than at ZT 13. In other rodents estradiol treatment increases cytosolic PRs and increases behavioral sensitivity to P (Blaustein and Feder, 1979b; Don Carlos *et al.*, 1989; Fraile *et al.*, 1987; Parsons *et al.*, 1980; Parsons, Rainbow *et al.*, 1981). Furthermore, in lab rats, guinea pigs, and hamsters, sexual receptivity is

positively correlated with the number of PR+ cells (Blaustein and Feder, 1979b; Delville and Blaustein, 1991; Don Carlos *et al.*, 1989; Fraile *et al.*, 1987; Parsons *et al.*, 1980; Parsons, Rainbow *et al.*, 1981). Grass rats thus appear to be different from these other species with respect to the temporal relationship between mating behavior and PRs. One interpretation is that, although I did not see an increase in PR+ cell numbers from ZT 20 to ZT 1 in EB-capsule treated female grass rats, PR numbers might rise from ZT 19/20 to 23, and peak around the time when females have maximal sexual receptivity. Indeed, in guinea pigs, nuclear PR levels reach their peak two to four hours after a subcutaneous P injection, and in lab rats nuclear PR levels rise within 30 minutes of an intravenous P injection (Blaustein and Feder, 1980; McGinnis *et al.*, 1981).

An alternative hypothesis is that females with high levels of PR at ZT 13 are hypersensitive to refractory effects of P on sexual behavior, which might be responsible for the decrease in sexual behavior at ZT 17. Refractory effects of P have been observed in other rodents at the end of a period of sexual receptivity (Blaustein and Feder, 1979a; Morin, 1977; Parsons, McGinnis *et al.*, 1981; Zucker, 1968). This hypothesis is supported by the fact that grass rats with EB-capsules had high levels of PR+ cells at both ZT 13 and 17, perhaps reflecting a window of time during which P cannot induce sexual receptivity. Lastly, it is possible that changes in PR expression are not causally related to changes in sexual receptivity in this species.

Animals in this study had a daily rhythm in estrogen-induced PR expression (Figure 4.4, Figure 4.5). To the best of my knowledge, this is the first

report of which I am aware of that describes a daily rhythm in PRs in any species; although PRs have been found to change in other rhythmic contexts. PR protein and mRNA levels in the hypothalamus fluctuate over the course of the estrous cycle of lab rats (Guerra-Araiza *et al.*, 2000; Numan *et al.*, 1999; Siegel *et al.*, 1989; Simerly *et al.*, 1996), and female Syrian hamsters housed in short days (10L:14D) have significantly fewer estradiol-induced PRs than do females housed in long days (14L:10D; Mangels *et al.*, 1998). These patterns of change in PR expression are likely to be caused in part by changes in secretion of steroids from the ovary. Changes in circulating steroids are unlikely to account for this rhythm in PRs reported here because hormone treatments were standardized. It is not currently clear what causes the rhythm in PRs in grass rats, or whether time of day affects PR+ cell number in other rodents.

In summary, these findings demonstrate that diurnal grass rats have a rhythm in sexual behavior that is reversed from that of nocturnal lab rats, and that time of day influences PR expression of hormone-treated females. It will be interesting to determine whether, and how, these rhythms are functionally related, whether they are endogenous, and how the mechanisms underlying them differ between grass rats and nocturnal rodents.

Chapter 5

Conclusion

Chapter summary

Diurnal and nocturnal rodents differ dramatically with respect to rhythms in the timing of estrous-related events, but the mechanisms that underlie these rhythms are not known. In the second chapter I examined the hypothesis that differences in the timing of neuroendocrine events associated with estrous are due, in part, to rhythms in responsiveness to steroid hormones. I found that estradiol and progesterone treatment induced a rise in activity of gonadotropin releasing hormone-containing (GnRH+) neurons in both lab rats and grass rats. However, steroids were only able to increase GnRH+ cell activity at one time of day; in lab rats this was before lights-off, and in grass rats it was around lights-on. This rhythm in responsiveness to steroids appeared to be endogenous as it persisted in both species when they were housed in constant darkness for five days.

In the third chapter I examined the hypothesis that differences in the timing of reproductive events in diurnal and nocturnal rodents are due to differences in the pathways or connections from the suprachiasmatic nucleus (SCN) to GnRH+ and estrogen receptor (ER)+ neurons. Using anterograde tract-tracing in grass rats, I found that the SCN projects to both ER+ and GnRH+ neurons, as is the case in lab rats and hamsters (de la Iglesia *et al.*, 1995; van der Beek *et al.*, 1997). Furthermore, vasoactive intestinal polypeptide+ fibers

contacted about 34% of the GnRH+ neurons in grass rats and arginine vasopressin+ fibers contacted about 24% of these neuroendocrine cells.

In the fourth chapter I determined that grass rats had a daily rhythm in sensitivity of sexual behavior to steroid hormones. Ovariectomized female grass rats were primed with steroid hormones, paired with an intact male, and tested for mating activity at four different times of day. The lordosis quotient and rate of copulation rose from zeitgeber time (ZT) 17 to ZT 23, whereas rates of mounting behavior were relatively high at both ZT 23 and ZT 11. The work presented in this dissertation indicates that differences in the timing of reproductive events in diurnal and nocturnal rodents are due, in part, to a reversal in rhythms in responsiveness to steroid hormones.

What do these data mean? Where do I go from here?

Extensive evidence indicates that in lab rats, both the circadian system and ovarian hormones regulate estrous cyclicity (Herbison, 1998). Experiments described in this dissertation indicate that the same is true for grass rats; steroid hormones and time of day interact to regulate rhythms in mating behavior and GnRH+ cell activity (Chapters 2, 4). Furthermore, the SCN sends direct projections to GnRH+ and ER+ neurons in grass rats, as is the case in lab rats (Chapter 3; van der Beek *et al.*, 1997; Watson *et al.*, 1995). These anatomical data indicate a possible pathway, common to both lab rats and grass rats, by which the circadian system might mediate the timing of reproductive events. Diurnal and nocturnal murid rodents, and perhaps mammals in general, may be similar with respect to the neuroendocrine mechanisms that regulate

reproductive functions such as hormone surges, copulatory behaviors and parturition.

Future work determining the identity of, and rhythms in, signals emitted by the SCN, and responsiveness of neural targets to these signals, will further our understanding of how the LH surge and estrous behaviors are regulated, and how diurnal and nocturnal species may differ with respect to these regulatory mechanisms. In conclusion, although grass rats and lab rats are similar with respect to the temporal relationship between estrous-related events, the timing of these events relative to the light:dark cycle is dramatically different.

References

Abrahamson, E. E., and Moore, R. Y. (2001). Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. *Brain Research, 916*(1-2), 172-191.

Alexander, M. J., Clifton, D. K., and Steiner, R. A. (1985). Vasoactive intestinal polypeptide effects a central inhibition of pulsatile luteinizing hormone secretion in ovariectomized rats. *Endocrinology, 117*(5), 2134-2139.

Alleva, J. J., Waleski, M. V., and Alleva, F. R. (1971). A biological clock controlling the estrous cycle of the hamster. *Endocrinology, 88*(6), 1368-1379.

Barfield, R. J., Rubin, B. S., J. H., G., and Davis, P. G. (1983). Sites of action of ovarian hormones in the regulation of oestrous responsiveness in rats. In J. Balthazart and E. Prove and R. Gilles (Eds.), *Hormones and Behavior in Higher Vertebrates* (pp. 1-17). Berlin: Springer-Verlag.

Beach, F. A., and Levinson, G. (1949). Diurnal variations in the mating behavior of male rats. *Proceedings of the Society for Experimental Biology and Medicine, 72*, 78-80.

Blake, C. A. (1976). A detailed characterization of the proestrous luteinizing hormone surge. *Endocrinology, 98*(2), 445-450.

Blanchong, J. A., McElhinny, T. L., Mahoney, M. M., and Smale, L. (1999). Nocturnal and diurnal rhythms in the unstriped Nile Rat, *Arvicanthis niloticus. Journal of Biological Rhythms, 14*(5), 364-377.

Blanchong, J. A., and Smale, L. (2000). Temporal patterns of activity of the unstriped Nile rat, *Arvicanthis niloticus. Journal of Mammalogy, 81*(2), 595-599.

Blaustein, J. D. (1992). Cytoplasmic estrogen receptors in rat brain: immunocytochemical evidence using three antibodies with distinct epitopes. *Endocrinology, 131*(3), 1336-1342.

Blaustein, J. D., and Brown, T. J. (1983). Mechanisms of oestrogen-progestin interactions in the regulation of lordosis in female guinea pigs. In J. Balthazart and E. Prove and R. Gilles (Eds.), *Hormones and Behaviour in Higher Vertebrates*. Berlin: Springer-Verlag.

Blaustein, J. D., and Feder, H. H. (1979a). Cytoplasmic progestin receptors in female guinea pig brain and their relationship to refractoriness in expression of female sexual behavior. *Brain Research, 177*(3), 489-498.

Blaustein, J. D., and Feder, H. H. (1979b). Cytoplasmic progestin-receptors in guinea pig brain: characteristics and relationship to the induction of sexual behavior. *Brain Research, 169*(3), 481-497.

Blaustein, J. D., and Feder, H. H. (1980). Nuclear progestin receptors in guinea pig brain measured by an in vitro exchange assay after hormonal treatments that affect lordosis. *Endocrinology, 106*(4), 1061-1069.

Blaustein, J. D., Tetel, M. J., Ricciardi, K. H., Delville, Y., and Turcotte, J. C. (1994). Hypothalamic ovarian steroid hormone-sensitive neurons involved in female sexual behavior. *Psychoneuroendocrinology, 19*(5-7), 505-516.

Bronson, F. H., and Vom Saal, F. S. (1979). Control of the preovulatory release of luteinizing hormone by steroids in the mouse. *Endocrinology, 104*(5), 1247-1255.

Buijs, R. M., Markman, M., Nunes-Cardoso, B., Hou, Y. X., and Shinn, S. (1993). Projections of the suprachiasmatic nucleus to stress-related areas in the rat hypothalamus: a light and electron microscopic study. *Journal of Comparative Neurology, 335*(1), 42-54.

Butler, J. A., Sjoberg, M., and Coen, C. W. (1999). Evidence for oestrogen receptor alpha-immunoreactivity in gonadotrophin- releasing hormone-expressing neurones. *Journal of Neuroendocrinology, 11*(5), 331-335.

Campbell, C. S., and Baum, F. R. (1979). Long-term maintenance of receptivity by subcutaneous implants of estradiol. *Physiology & Behavior, 22*, 1073-1078.

Cintra, A., Fuxe, K., Harfstrand, A., Agnati, L. F., Miller, L. S., Greene, J. L., and Gustafsson, J. (1982). On the cellular localization and distribution of estrogen receptors in the rat tel- and diencephalon using monoclonal antibodies to human estrogen receptor. *Neurochemistry International, 8*(4), 587-595.

Coolen, L. M., and Wood, R. I. (1998). Bidirectional connections of the medial amygdaloid nucleus in the Syrian hamster brain: simultaneous anterograde and retrograde tract tracing. *Journal of Comparative Neurology, 399*(2), 189-209.

Davis, P. G., Krieger, M. S., Barfield, R. J., McEwen, B. S., and Pfaff, D. W. (1982). The site of action of intrahypothalamic estrogen implants in feminine sexual behavior: an autoradiographic analysis. *Endocrinology, 111*(5), 1581-1586.

de la Iglesia, H. O., Blaustein, J. D., and Bittman, E. L. (1995). The suprachiasmatic area in the female hamster projects to neurons containing estrogen receptors and GnRH. *NeuroReport, 6*(13), 1715-1722.

de la Iglesia, H. O., Blaustein, J. D., and Bittman, E. L. (1999). Oestrogen receptor-a-immunoreactive neurones project to the suprachiasmatic nucleus of the female Syrian hamster. *Journal of Neuroendocrinology, 11*, 481-490.

DeBold, J. F., Malsbury, C. W., Harris, V. S., and Malenka, R. (1982). Sexual receptivity: brain sites of estrogen action in female hamsters. *Physiology & Behavior, 29*(4), 589-593.

Delville, Y., and Blaustein, J. D. (1991). A site for estradiol priming of progesterone-facilitated sexual receptivity in the ventrolateral hypothalamus of female guinea pigs. *Brain Research, 559*(2), 191-199.

Doan, A., and Urbanski, H. F. (1994). Diurnal expression of Fos in luteinizing hormone-releasing hormone neurons of Syrian hamsters. *Biology of Reproduction, 50*(2), 301-308.

Dobson, F. S., and Michener, G. A. (1995). Maternal traits and reproduction in Richardson's ground squirrels. *Ecology, 76*(3), 851-862.

Docke, F., Lung, D. N., Rohde, W., and Dorner, G. (1982). Perisuprachiasmatic lesions enhance the increase of gonadotropin secretion in acutely ovariectomized rats. *Endokrinologie, 79*(2), 311-314.

Don Carlos, L. L., Greene, G. L., and Morrell, J. I. (1989). Estrogen plus progesterone increases progestin receptor immunoreactivity in the brain of ovariectomized guinea pigs. *Neuroendocrinology, 50*(6), 613-623.

Don Carlos, L. L., Monroy, E., and Morrell, J. I. (1991). Distribution of estrogen receptor-immunoreactive cells in the forebrain of the female guinea pig. *Journal of Comparative Neurology, 305*(4), 591-612.

Erskine, M. S., Marcus, J. I., and Baum, M. J. (1980). Absence of a diurnal rhythm in lordosis behaviour induced by oestrogen in gonadectomized rats. *Journal of Endocrinology, 86*(1), 127-134.

Etgen, A. M., and Barfield, R. J. (1986). Antagonism of female sexual behavior with intracerebral implants of antiprogestin RU 38486: correlation with binding to neural progestin receptors. *Endocrinology, 119*(4), 1610-1617.

Everett, J. W., and Sawyer, C. H. (1950). A 24-hour periodicity in the "LH-release apparatus" of female rats, disclosed by barbiturate sedation. *Endocrinology, 47*, 198-218.

Finn, P. D., Steiner, R. A., and Clifton, D. K. (1998). Temporal patterns of gonadotropin-releasing hormone (GnRH), c-fos, and galanin gene expression in GnRH neurons relative to the luteinizing hormone surge in the rat. *Journal of Neuroscience, 18*(2), 713-719.

Fitzgerald, K., and Zucker, I. (1976). Circadian organization of the estrous cycle of the golden hamster. *Proceedings of the National Academy of Science, USA, 73*(8), 2923-2927.

Fox, C. A., Ross, L. R., Handa, R. J., and Jacobson, C. D. (1991). Localization of cells containing estrogen receptor-like immunoreactivity in the Brazilian opossum brain. *Brain Research, 546*(1), 96-105.

Fraile, I. G., Pfaff, D. W., and McEwen, B. S. (1987). Progestin receptors with and without estrogen induction in male and female hamster brains. *Neuroendocrinology, 45*, 487-491.

Freeman, M. E. (1994). The neuroendocrine control of the ovarian cycle of the rat. In E. Knobil and J. D. Neill (Eds.), *The Physiology of Reproduction* (2nd ed., Vol. 2, pp. 613-658). New York: Raven Press.

Funabashi, T., Aiba, S., Sano, A., Shinohara, K., and Kimura, F. (1999). Intracerebroventricular injection of arginine-vasopressin V1 receptor antagonist attenuates the surge of luteinizing hormone and prolactin secretion in proestrous rats. *Neuroscience Letters, 260*(1), 37-40.

Funabashi, T., Shinohara, K., Mitsushima, D., and Kimura, F. (2000). Gonadotropin-releasing hormone exhibits circadian rhythm in phase with arginine-vasopressin in co-cultures of the female rat preoptic area and suprachiasmatic nucleus. *Journal of Neuroendocrinology, 12*(6), 521-528.

Gibson, M. J., Wu, T. J., Miller, G. M., and Silverman, A. J. (1997). What nature's knockout teaches us about GnRH activity: hypogonadal mice and neuronal grafts. *Hormones and Behavior, 31*(3), 212-220.

Gray, G. D., Sodersten, P., Tallentire, D., and Davidson, J. M. (1978). Effects of lesions in various structures of the suprachiasmatic-preoptic region on LH regulation and sexual behavior in female rats. *Neuroendocrinology, 25*, 174-191.

Guerra-Araiza, C., Cerbon, M. A., Morimoto, S., and Camacho-Arroyo, I. (2000). Progesterone receptor isoforms expression pattern in the rat brain during the estrous cycle. *Life Sciences, 66*(18), 1743-1752.

Hansen, S., Sodersten, P., Eneroth, P., Srebro, B., and Hole, K. (1979). A sexually dimorphic rhythm in oestradiol-activated lordosis behaviour in the rat. *Journal of Endocrinology, 83*(2), 267-274.

Hansen, S., Sodersten, P., and Srebro, B. (1978). A daily rhythm in the behavioural sensitivity of the female rat to oestradiol. *Journal of Endocrinology, 77*(3), 381-388.

Harlan, R. E., Shivers, B. D., Moss, R. L., Shryne, J. E., and Gorski, R. A. (1980). Sexual performance as a function of time of day in male and female rats. *Biology of Reproduction, 23*(1), 64-71.

Harney, J. P., Scarbrough, K., Rosewell, K. L., and Wise, P. M. (1996). *In vivo* antisense antagonism of vasoactive intestinal peptide in the suprachiasmatic nuclei causes aging-like changes in the estradiol-induced luteinizing hormone and prolactin surges. *Endocrinology, 137*(9), 3696-3701.

Herbison, A. E. (1998). Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. *Endocrine Reviews, 19*(3), 302-330.

Herbison, A. E., and Theodosis, D. T. (1992). Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat. *Neuroscience, 50*(2), 283-298.

Hnatczuk, O. C., Lisciotto, C. A., Don Carlos, L. L., Carter, C. S., and Morrell, J. I. (1994). Estrogen receptor immunoreactivity in specific brain areas of the prairie vole (Microtus ochrogaster) is altered by sexual receptivity and genetic sex. *Journal of Neuroendocrinology, 6*(1), 89-100.

Hoffman, G. E., Le, W. W., Abbud, R., Lee, W. S., and Smith, M. S. (1994). Use of Fos-related antigens (FRAs) as markers of neuronal activity: FRA changes in dopamine neurons during proestrus, pregnancy and lactation. *Brain Research, 654*(2), 207-215.

Hoffman, G. E., Lee, W. S., Attardi, B., Yann, V., and Fitzsimmons, M. D. (1990). Luteinizing hormone-releasing hormone neurons express c-fos antigen after steroid activation. *Endocrinology, 126*(3), 1736-1741.

Horvath, T. L., Cela, V., and van der Beek, E. M. (1998). Gender-specific apposition between vasoactive intestinal peptide- containing axons and gonadotrophin-releasing hormone-producing neurons in the rat. *Brain Research, 795*(1-2), 277-281.

Hrabovszky, E., Shughrue, P. J., Merchenthaler, I., Hajszan, T., Carpenter, C. D., Liposits, Z., and Petersen, S. L. (2000). Detection of estrogen receptor-beta messenger ribonucleic acid and 125I- estrogen binding sites in luteinizing hormone-releasing hormone neurons of the rat brain. *Endocrinology, 141*(9), 3506-3509.

Huhman, K. L., and van der Beek, E. M. (1998, November 16-21). *Peptidergic innervation of gonadotropin releasing hormone (GnRH) neurons in female Syrian hamsters.* Paper presented at the Society for Neuroscience, Washington D.C.

Inouye, S. T., and Kawamura, H. (1979). Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. *Proceedings of the National Academy of Science, USA, 76*(11), 5962-5966.

Jimenez-Linan, M., and Rubin, B. S. (2001). Dynamic changes in luteinizing hormone releasing hormone transcriptional activity are associated with the steroid-induced LH surge. *Brain Research, 922*(1), 71-79.

Kalsbeek, A., Teclemariam-Mesbah, R., and Pevet, P. (1993). Efferent projections of the suprachiasmatic nucleus in the golden hamster (Mesocricetus auratus). *Journal of Comparative Neurology, 332*(3), 293-314.

Katona, C., Rose, S., and Smale, L. (1998). The expression of Fos within the suprachiasmatic nucleus of the diurnal rodent *Arvicanthis niloticus. Brain Research, 791*, 27-34.

Kawakami, M., Arita, J., and Yoshioka, E. (1980). Loss of estrogen-induced daily surges of prolactin and gonadotropins by suprachiasmatic nucleus lesions in ovariectomized rats. *Endocrinology, 106*, 1987-1092.

Keppel, G. (1982). *Design and analysis : a researcher's handbook* (2nd ed.). Englewood Cliffs, N.J.: Prentice-Hall.

King, J. C., Liu, E., Ronsheim, P. M., Slonimski, M., and Rubin, B. S. (1998). Expression of Fos within luteinizing hormone-releasing hormone neurons, in relation to the steroid-induced luteinizing hormone surge in guinea pigs. *Biology of Reproduction, 58*(2), 316-322.

Krajnak, K., Kashon, M. L., Rosewell, K. L., and Wise, P. M. (1998). Sex differences in the daily rhythm of vasoactive intestinal polypeptide but not arginine vasopressin messenger ribonucleic acid in the suprachiasmatic nuclei. *Endocrinology, 139*(10), 4189-4196.

Krajnak, K., Rosewell, K. L., and Wise, P. M. (2001). Fos-induction in gonadotropin-releasing hormone neurons receiving vasoactive intestinal polypeptide innervation is reduced in middle-aged female rats. *Biology of Reproduction, 64*(4), 1160-1164.

Kriegsfeld, L. J., Silver, R., Gore, A. C., and Crews, D. (2002). Vasoactive intestinal polypeptide contacts on gonadotropin-releasing hormone neurones increase following puberty in female rats. *Journal of Neuroendocrinology, 14*(9), 685-690.

Labyak, S. E., and Lee, T. M. (1995). Estrus- and steroid-induced changes in circadian rhythms in a diurnal rodent, Octodon degus. *Physiology & Behavior, 58*(3), 573-585.

Leak, R. K., Card, J. P., and Moore, R. Y. (1999). Suprachiasmatic pacemaker organization analyzed by viral transynaptic transport. *Brain Research, 819*(1-2), 23-32.

Lee, W. S., Smith, M., and Hoffman, G. (1992). CFos activity identifies recruitment of luteinizing-hormone-releasing hormone neurons during the ascending phase of the proestrous luteinizing hormone surge. *Journal of Neuroendocrinology, 4*(2), 161-166.

Lee, W. S., Smith, M. S., and Hoffman, G. E. (1990a). Luteinizing hormone-releasing hormone neurons express Fos protein during the proestrous surge of luteinizing hormone. *Proceedings of the National Academy of Science, USA, 87*(13), 5163-5167.

Lee, W. S., Smith, M. S., and Hoffman, G. E. (1990b). Progesterone enhances the surge of luteinizing hormone by increasing the activation of luteinizing hormone-releasing hormone neurons. *Endocrinology, 127*(5), 2604-2606.

Legan, S. J., Coon, G. A., and Karsch, F. J. (1975). Role of estrogen as initiator of daily LH surges in the ovariectomized rat. *Endocrinology, 96*, 50-56.

Legan, S. J., and Karsch, F. J. (1975). A daily signal for the LH surge in the rat. *Endocrinology, 96*(1), 57-62.

Lehman, M. N., and Karsch, F. J. (1993). Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and beta- endorphin-immunoreactive neurons contain estrogen receptors? A double- label immunocytochemical study in the Suffolk ewe. *Endocrinology, 133*(2), 887-895.

LeSauter, J., and Silver, R. (1999). Localization of a suprachiasmatic nucleus subregion regulating locomotor rhythmicity. *Journal of Neuroscience, 19*(13), 5574-5585.

Levine, J. E., and Ramirez, V. D. (1982). Luteinizing hormone-releasing hormone release during the rat estrous cycle and after ovariectomy, as estimated with push-pull cannulae. *Endocrinology, 111*(5), 1439-1448.

Li, H. Y., Blaustein, J. D., De Vries, G. J., and Wade, G. N. (1993). Estrogen-receptor immunoreactivity in hamster brain: preoptic area, hypothalamus and amygdala. *Brain Research, 631*(2), 304-312.

Lucas, R. J., Stirland, J. A., Darrow, J. M., Menaker, M., and Loudon, A. S. (1999). Free running circadian rhythms of melatonin, luteinizing hormone, and cortisol in Syrian hamsters bearing the circadian tau mutation. *Endocrinology, 140*(2), 758-764.

Mahoney, M. M., Bult, A., and Smale, L. (2001). Phase response curve and light-induced Fos expression in the suprachiasmatic nucleus and adjacent hypothalamus of *Arvicanthis niloticus*. *Journal of Biological Rhythms, 16*(2), 149-162.

Mahoney, M. M., and Smale, L. (2000). *Vasoactive intestinal polypeptide and gonadotropin-releasing hormone immunoreactive cells in diurnal grass rats.* Paper presented at the Society for Behavioral Neuroendocrinology, Scottsdale, AZ.

Mangels, R. A., Powers, J. B., and Blaustein, J. D. (1998). Effect of photoperiod on neural estrogen and progestin receptor immunoreactivity in female Syrian hamsters. *Brain Research, 796*(1-2), 63-74.

Mani, S. K., Blaustein, J. D., Allen, J. M., Law, S. W., O'Malley, B. W., and Clark, J. H. (1994). Inhibition of rat sexual behavior by antisense oligonucleotides to the progesterone receptor. *Endocrinology, 135*(4), 1409-1414.

McElhinny, T. L. (1996). *Reproductive biology and biological rhythms in Arvicanthis niloticus.* Unpublished Masters of Science, Michigan State University, East Lansing, Michigan.

McElhinny, T. L., Sisk, C. L., Holekamp, K. E., and Smale, L. (1999). A morning surge in plasma luteinizing hormone coincides with elevated Fos expression in gonadotropin-releasing hormone-immunoreactive neurons in the diurnal rodent, *Arvicanthis niloticus*. *Biology of Reproduction, 61*(4), 1115-1122.

McElhinny, T. L., Smale, L., and Holekamp, K. E. (1997). Patterns in body temperature, activity and reproductive behavior in a tropical murid rodent, *Arvicanthis niloticus*. *Physiology and Behavior, 62*, 91-96.

McGinnis, M. Y., Parsons, B., Rainbow, T. C., Krey, L. C., and McEwen, B. S. (1981). Temporal relationship between cell nuclear progestin receptor levels and sexual receptivity following intravenous progesterone administration. *Brain Research, 218*(1-2), 365-371.

Meyer-Bernstein, E. L., Jetton, A. E., Matsumoto, S. I., Markuns, J. F., Lehman, M. N., and Bittman, E. L. (1999). Effects of suprachiasmatic transplants on circadian rhythms of neuroendocrine function in golden hamsters. *Endocrinology, 140*(1), 207-218.

Michener, G. A. (1980). Estrous and gestation periods in Richardson's ground squirrels. *Journal of Mammalogy, 61*(3), 531-534.

Moline, M. L., Albers, H. E., Todd, R. B., and Moore-Ede, M. C. (1981). Light-dark entrainment of proestrous LH surges and circadian locomotor activity in female hamsters. *Hormones and Behavior, 15*(4), 451-458.

Moore, R. Y., and Eichler, V. B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Research, 42*(1), 201-206.

Morin, L. P. (1977). Theoretical review. Progesterone: inhibition of rodent sexual behavior. *Physiology & Behavior, 18*(4), 701-715.

Morin, L. P., Goodless-Sanchez, N., Smale, L., and Moore, R. Y. (1994). Projections of the suprachiasmatic nuclei, subparaventricular zone and retrochiasmatic area in the golden hamster. *Neuroscience, 61*(2), 391-410.

Munch, I. C., Moller, M., Larsen, P. J., and Vrang, N. (2002). Light-induced c-Fos expression in suprachiasmatic nuclei neurons targeting the paraventricular nucleus of the hamster hypothalamus: phase dependence and immunochemical identification. *Journal of Comparative Neurology, 442*(1), 48-62.

Murakami, N., Takamure, M., Takahashi, K., Utunomiya, K., Kuroda, H., and Etoh, T. (1991). Long-term cultured neurons from rat suprachiasmatic nucleus retain the capacity for circadian oscillation of vasopressin release. *Brain Research, 545*(1-2), 347-350.

Novak, C. M., Smale, L., and Nunez, A. A. (1999). Fos expression in the sleep-active cell group of the ventrolateral preoptic area in the diurnal murid rodent, *Arvicanthis niloticus*. *Brain Research, 818*, 375-382.

Numan, M., Roach, J. K., del Cerro, M. C., Guillamon, A., Segovia, S., Sheehan, T. P., and Numan, M. J. (1999). Expression of intracellular progesterone receptors in rat brain during different reproductive states, and involvement in maternal behavior. *Brain Research, 830*(2), 358-371.

Nunez, A. A., Bult, A., McElhinny, T. L., and Smale, L. (1999). Daily rhythms of Fos expression in hypothalamic targets of the suprachiasmatic nucleus in diurnal and nocturnal rodents. *Journal of Biological Rhythms, 14*(4), 300-306.

Ogawa, S., Eng, V., Taylor, J., Lubahn, D. B., Korach, K. S., and Pfaff, D. W. (1998). Roles of estrogen receptor-alpha gene expression in reproduction-related behaviors in female mice. *Endocrinology, 139*(12), 5070-5081.

Olcese, J., McArdle, C. A., Middendorff, R., and Greenland, K. (1997). Pituitary adenylate cyclase-activating peptide and vasoactive intestinal peptide receptor expression in immortalized LHRH neurons. *Journal of Neuroendocrinology, 9*(12), 937-943.

Palm, I. F., van der Beek, E. M., Wiegant, V. M., Buijs, R. M., and Kalsbeek, A. (1999). Vasopressin induces a luteinizing hormone surge in ovariectomized, estradiol-treated rats with lesions of the suprachiasmatic nucleus. *Neuroscience, 93*(2), 659-666.

Palm, I. F., van der Beek, E. M., Wiegant, V. M., Buijs, R. M., and Kalsbeek, A. (2001). The stimulatory effect of vasopressin on the luteinizing hormone surge in ovariectomized, estradiol-treated rats is time-dependent. *Brain Research, 901*(1-2), 109-116.

Parsons, B., MacLusky, N. J., Krey, L., Pfaff, D. W., and McEwen, B. S. (1980). The temporal relationship between estrogen-inducible progestin receptors in the female rat brain and the time course of estrogen activation of mating behavior. *Endocrinology, 107*(3), 774-779.

Parsons, B., McGinnis, M. Y., and McEwen, B. S. (1981). Sequential inhibition by progesterone: effects on sexual receptivity and associated changes in brain cytosol progestin binding in the female rat. *Brain Research, 221*(1), 149-160.

Parsons, B., Rainbow, T. C., Pfaff, D. W., and McEwen, B. S. (1981). Oestradiol, sexual receptivity and cytosol progestin receptors in rat hypothalamus. *Nature, 292*(5818), 58-59.

Petersen, S. L., McCrone, S., Keller, M., and Shores, S. (1995). Effects of estrogen and progesterone on luteinizing hormone-releasing hormone messenger ribonucleic acid levels: consideration of temporal and neuroanatomical variables. *Endocrinology, 136*(8), 3604-3610.

Porkka-Heiskanen, T., Urban, J. H., Turek, F. W., and Levine, J. E. (1994). Gene expression in a subpopulation of luteinizing hormone-releasing hormone (LHRH) neurons prior to the preovulatory gonadotropin surge. *Journal of Neuroscience, 14*(9), 5548-5558.

Rainbow, T. C., Parsons, B., and McEwen, B. S. (1982). Sex differences in rat brain oestrogen and progestin receptors. *Nature, 300*(5893), 648-649.

Rajakumar, N., Elisevich, K., and Flumerfelt, B. A. (1993). Biotinylated dextran: a versatile anterograde and retrograde neuronal tracer. *Brain Research, 607*(1-2), 47-53.

Rajendren, G. (2001). Subsets of gonadotropin-releasing hormone (GnRH) neurons are activated during a steroid-induced luteinizing hormone surge and mating in mice: a combined retrograde tracing double immunohistochemical study. *Brain Research, 918*(1-2), 74-79.

Ralph, M. R., Foster, R. G., Davis, F. G., and Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. *Science, 247*, 975-978.

Richardson, H. N. (2002). *The gonadotropin releasing hormone (GnRH) system in male Syrian hamsters (Mesocricetus auratus): organization and regulation.* Unpublished Ph. D., Michigan State University, East Lansing.

Richter, C. P. (1970). Dependence of successful mating in rats on functioning of the 24-hour clocks of the male and female. *Communications in Behavioral Biology, 5*, 1-5.

Riskind, P. N., Allen, J. M., Gabriel, S. M., Koenig, J. I., and Audet-Arnold, J. (1989). Sex differences in vasoactive intestinal peptide (VIP) concentrations in the anterior pituitary and hypothalamus of rats. *Neuroscience Letters, 105*(1-2), 215-220.

Rissman, E. F., Early, A. H., Taylor, J. A., Korach, K. S., and Lubahn, D. B. (1997). Estrogen receptors are essential for female sexual receptivity. *Endocrinology, 138*(1), 507-510.

Rood, J. P. (1970). Ecology and social behavior of the desert cavy *(Microcavia australis). The American Midland Naturalist, 83*(2), 415-454.

Rose, S., Novak, C. M., Mahoney, M. M., Nunez, A. A., and Smale, L. (1999). Fos expression within vasopressin-containing neurons in the suprachiasmatic nucleus of diurnal compared to nocturnal rodents. *Journal of Biological Rhythms, 14*(1), 37-46.

Roy, D., Angelini, N. L., and Belsham, D. D. (1999). Estrogen directly represses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor-alpha (ERalpha)- and ERbeta-expressing GT1-7 GnRH neurons. *Endocrinology, 140*(11), 5045-5053.

Rubin, B. S., and Barfield, R. J. (1983). Induction of estrous behavior in ovariectomized rats by sequential replacement of estrogen and progesterone to the ventromedial hypothalamus. *Neuroendocrinology, 37*(3), 218-224.

Salisbury, R. L., Krieg, R. J., Jr., and Seibel, H. R. (1980). Effects of arginine vasotocin, oxytocin, and arginine vasopressin on steroid-induced surges of luteinizing hormone and prolactin in ovariectomized rats. *Acta Endocrinologica (Copenhagen), 94*(2), 166-173.

Schwartz, W. J., Reppert, S. M., Eagan, S. M., and Moore-Ede, M. C. (1983). In vivo metabolic activity of the suprachiasmatic nuclei: a comparative study. *Brain Research, 274*(1), 184-187.

Seegal, R. F., and Goldman, B. D. (1975). Effects of photoperiod on cyclicity and serum gonadotropins in the Syrian hamster. *Biology of Reproduction, 12*(2), 223-231.

Sequeira, H., Poulain, P., Ba-M'Hamed, S., and Viltart, O. (2000). Immunocytochemical detection of fos protein combined with anterograde tract-tracing using biotinylated dextran. *Brain Research Brain Research Protocols, 5*(1), 49-56.

Shen, E. S., Meade, E. H., Perez, M. C., Deecher, D. C., Negro-Vilar, A., and Lopez, F. J. (1998). Expression of functional estrogen receptors and galanin messenger ribonucleic acid in immortalized luteinizing hormone-releasing hormone neurons: estrogenic control of galanin gene expression. *Endocrinology, 139*(3), 939-948.

Shinohara, K., Honma, S., Katsuno, Y., Abe, H., and Honma, K. (1998). Circadian release of amino acids in the suprachiasmatic nucleus in vitro. *NeuroReport, 9*(1), 137-140.

Shivers, B. D., Harlan, R. E., Morrell, J. I., and Pfaff, D. W. (1983). Absence of oestradiol concentration in cell nuclei of LHRH- immunoreactive neurones. *Nature, 304*(5924), 345-347.

Siegel, H. I., Senatore, A., Rogers, S., and Ahdieh, H. B. (1989). Sexual receptivity in hamsters: brain nuclear estrogen and cytosolic progestin receptors after single and multiple steroid treatments and during the estrous cycle. *Hormones and Behavior, 23*(2), 173-184.

Silver, R., and Bittman, E. L. (1984). Reproductive mechanisms: interaction of circadian and interval timing. *Annals of the New York Academy of Science, 423*, 488-514.

Simerly, R. B. (1998). Organization and regulation of sexually dimorphic neuroendocrine pathways. *Behavior Brain Research, 92*(2), 195-203.

Simerly, R. B., Carr, A. M., Zee, M. C., and Lorang, D. (1996). Ovarian steroid regulation of estrogen and progesterone receptor messenger ribonucleic acid in the anteroventral periventricular nucleus of the rat. *Journal of Neuroendocrinology, 8*(1), 45-56.

Simonian, S. X., Spratt, D. P., and Herbison, A. E. (1999). Identification and characterization of estrogen receptor alpha- containing neurons projecting to the vicinity of the gonadotropin- releasing hormone perikarya in the rostral preoptic area of the rat. *Journal of Comparative Neurology, 411*(2), 346-358.

Skynner, M. J., Sim, J. A., and Herbison, A. E. (1999). Detection of estrogen receptor alpha and beta messenger ribonucleic acids in adult gonadotropin-releasing hormone neurons. *Endocrinology, 140*(11), 5195-5201.

Smale, L., and Boverhof, J. (1999). The suprachiasmatic nucleus and intergeniculate leaflet of *Arvicanthis niloticus*, a diurnal murid rodent from East Africa. *The Journal of Comparative Neurology, 403*(2), 190-208.

Smith, M. J., Jennes, L., and Wise, P. M. (2000). Localization of the VIP2 receptor protein on GnRH neurons in the female rat. *Endocrinology, 141*(11), 4317-4320.

Sodersten, P. (1988). Hormonal and behavioral rhythms related to reproduction, *Advances in Comparative and Environmental Physiology* (pp. 1-29).

Sodersten, P., De Vries, G. J., Buijs, R. M., and Melin, P. (1985). A daily rhythm in behavioral vasopressin sensitivity and brain vasopressin concentrations. *Neuroscience Letters, 58*(1), 37-41.

Sodersten, P., Eneroth, P., and Hansen, S. (1981). Neuroendocrine control of daily rhythms in rat reproductive behavior. In K. Fuxe and L. Wetterberg and J. A. Gustafsson (Eds.), *Steroid Hormonal Regulation of Brain* (pp. 301-315). New York: Pergamon.

Sodersten, P., Henning, M., Melin, P., and Ludin, S. (1983). Vasopressin alters female sexual behaviour by acting on the brain independently of alterations in blood pressure. *Nature, 301*(5901), 608-610.

Spinka, M. (1990). The effect of time of day on sperm competition and male reproductive success in laboratory rats. *Physiology & Behavior, 47*(3), 483-488.

Stefanick, M. L. (1983). The circadian patterns of spontaneous seminal emission, sexual activity and penile reflexes in the rat. *Physiology & Behavior, 31*(6), 737-743.

Stephan, F. K., and Zucker, I. (1972). Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proceedings of the National Academy of Science, USA, 69*(6), 1583-1586.

Sterner, M. R., Meisel, R. L., and Diekman, M. A. (1992). Forebrain sites of estradiol-17 beta action on sexual behavior and aggression in female Syrian hamsters. *Behavioral Neuroscience, 106*(1), 162-171.

Stetson, M. H., and Gibson, J. T. (1977). The estrous cycle in golden hamsters: a circadian pacemaker times preovulatory gonadotropin release. *Journal of Experimental Zoology, 201*, 289-294.

Stetson, M. H., and Watson-Whitmyre, M. (1977). The neural clock regulating estrous cyclicity in hamsters: gonadotropin release following barbiturate blockade. *Biology of Reproduction, 16*(4), 536-542.

Stobie, K. M., and Weick, R. F. (1989). Vasoactive intestinal peptide inhibits luteinizing hormone secretion: the inhibition is not mediated by dopamine. *Neuroendocrinology, 49*(6), 597-603.

Swann, J. M., and Turek, F. W. (1982). Cycle of lordosis behavior in female hamsters whose circadian activity rhythm has split into two components. *American Journal of Physiology (Regulatory Integrative Comparative Physiology), 243*, R112-R118.

Swann, J. M., and Turek, F. W. (1985). Multiple circadian oscillators regulate the timing of behavioral and endocrine rhythms in female golden hamsters. *Science, 228*, 898-900.

Takeo, Y. (1984). Influence of continuous illumination on estrous cycle of rats: time course of changes in levels of gonadotropins and ovarian steroids until occurrence of persistent estrus. *Neuroendocrinology, 39*, 97-104.

Thind, K. K., Boggan, J. E., and Goldsmith, P. C. (1991). Interactions between vasopressin- and gonadotropin-releasing-hormone- containing neuroendocrine neurons in the monkey supraoptic nucleus. *Neuroendocrinology, 53*(3), 287-297.

Tobet, S. A., Chickering, T. W., Fox, T. O., and Baum, M. J. (1993). Sex and regional differences in intracellular localization of estrogen receptor immunoreactivity in adult ferret forebrain. *Neuroendocrinology, 58*(3), 316-324.

van der Beek, E. M. (1996). Circadian control of reproduction in the female rat. *Progress in Brain Research, 111*, 295-320.

van der Beek, E. M., Horvath, T. L., Weigant, V. M., van den Hurk, R., and Buijs, R. (1997). Evidence for a direct neuronal pathway from the suprachiasmatic nucleus to the gonadotrophin-releasing hormone system: combined tracing and light and electron microscopic immunocytochemical studies. *Journal of Comparative Neurology, 384*(4), 569-579.

van der Beek, E. M., Oudheusden, H. J. C., Buijs, R. M., Van der Donk, H. A., van den Hurk, R., and Wiegant, V. M. (1994). Preferential induction of c-fos immunoreactivity in vasoactive intestinal polypeptide-innervated gonadotropin-releasing hormone neurons during a steroid induced LH surge in the female rat. *Endocrinology, 134*, 2636-2644.

van der Beek, E. M., Palm, I. F., Horvath, T. L., Kastelijn, J., and Wiegant, V. M. (1998, November 7-12). *Gender specific apposition of SCN-derived vasopressin containing axons on GnRH neurons in the preoptic area of adult rats.* Paper presented at the Society for Neuroscience, Los Angeles, CA.

van der Beek, E. M., Swarts, H. J., and Wiegant, V. M. (1999). Central administration of antiserum to vasoactive intestinal peptide delays and reduces luteinizing hormone and prolactin surges in ovariectomized, estrogen-treated rats. *Neuroendocrinology, 69*(4), 227-237.

van der Beek, E. M., Wiegant, V. M., van der Donk, H. A., van den Hurk, R., and Buijs, R. M. (1993). Lesions of the suprachiasmatic nucleus indicate the presence of a direct vasoactive intestinal polypeptide-containing projection to gonadotropin-releasing hormone neurons in the female rat. *Journal of Neuroendocrinology, 5*(2), 137-144.

van der Beek, E. M., Wiegant, V. M., van Oudheusden, H. J. C., van der Donk, H. A., van den Hurk, R., and Buijs, R. M. (1997). Synaptic contacts between gonadotropin-releasing hormone-containing fibers and neurons in the suprachiasmatic nucleus and the perichiasmatic area: an anatomical substrate for feedback regulation? *Brain Research, 755*(1), 101-111.

Veenman, C. L., Reiner, A., and Honig, M. G. (1992). Biotinylated dextran amine as an anterograde tracer for single- and double-labeling studies. *Journal of Neuroscience Methods, 41*(3), 239-254.

Vijayan, E., Samson, W. K., Said, S. I., and McCann, S. M. (1979). Vasoactive intestinal peptide: evidence for a hypothalamic site of action to release growth hormone, luteinizing hormone, and prolactin in conscious ovariectomized rats. *Endocrinology, 104*(1), 53-57.

Wang, H. J., Hoffman, G. E., and Smith, M. S. (1995). Increased GnRH mRNA in the GnRH neurons expressing cFos during the proestrous LH surge. *Endocrinology, 136*(8), 3673-3676.

Warembourg, M., Leroy, D., Peytevin, J., and Martinet, L. (1998). Estrogen receptor and progesterone receptor-immunoreactive cells are not co-localized with gonadotropin-releasing hormone in the brain of the female mink (Mustela vison). *Cell Tissue Research, 291*(1), 33-41.

Watanabe, K., Koibuchi, N., Ohtake, H., and Yamaoka, S. (1993). Circadian rhythms of vasopressin release in primary cultures of rat suprachiasmatic nucleus. *Brain Research, 624*(1-2), 115-120.

Watson, R. E., Jr., Langub, M. C., Jr., Engle, M. G., and Maley, B. E. (1995). Estrogen-receptive neurons in the anteroventral periventricular nucleus are synaptic targets of the suprachiasmatic nucleus and peri-suprachiasmatic region. *Brain Research, 689*(2), 254-264.

Watts, A. G., Sheward, W. J., Whale, D., and Fink, G. (1989). The effects of knife cuts in the sub-paraventricular zone of the female rat hypothalamus on oestrogen-induced diurnal surges of plasma prolactin and LH, and circadian wheel-running activity. *Journal of Endocrinology, 122*(2), 593-604.

Watts, A. G., and Swanson, L. W. (1987). Efferent projections of the suprachiasmatic nucleus: II. Studies using retrograde transport of fluorescent dyes and simultaneous peptide immunohistochemistry in the rat. *Journal of Comparative Neurology, 258*(2), 230-252.

Watts, A. G., Swanson, L. W., and Sanchez-Watts, G. (1987). Efferent projections of the suprachiasmatic nucleus: I. Studies using anterograde transport *of Phaseolus vulgaris* leucoagglutinin in the rat. *Journal of Comparative Neurology, 258*(2), 204-229.

Weick, R. F., and Stobie, K. M. (1992). Vasoactive intestinal peptide inhibits the steroid-induced LH surge in the ovariectomized rat. *Journal of Endocrinology, 133*(3), 433-437.

Weick, R. F., and Stobie, K. M. (1995). Role of VIP in the regulation of the LH secretion in the female rat. *Neuroscience and Biobehavioral Reviews, 19*(2), 251-259.

Weigand, S. J., and Terasawa, E. (1982). Discrete lesions reveal functional heterogeneity of suprachiasmatic structures in regulation of gonadotropin secretion in the female rat. *Neuroendocrinology, 34*(6), 395-404.

Welsh, D. K., Logothetis, D. E., Meister, M., and Reppert, S. M. (1995). Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron, 14*(4), 697-706.

Wu, T. J., Segal, A. Z., Miller, G. M., Gibson, M. J., and Silverman, A. J. (1992). FOS expression in gonadotropin-releasing hormone neurons: enhancement by steroid treatment and mating. *Endocrinology, 131*(5), 2045-2050.

Yamazaki, S., Kerbeshian, M. C., Hocker, C. G., Block, G. D., and Menaker, M. (1998). Rhythmic properties of the hamster suprachiasmatic nucleus in vivo. *Journal of Neuroscience, 18*(24), 10709-10723.

Yeoman, R. R., Williams, L. E., Aksel, S., and Abee, C. R. (1991). Mating-related estradiol fluctuations during the estrous cycle of the Bolivian squirrel monkey (Saimiri boliviensis boliviensis). *Biology of Reproduction, 44*(4), 640-647.

Young, W. C. (1969). Psychobiology of sexual behavior in the guinea pig. In D. S. Lehrman and R. A. Hinde and E. Shaw (Eds.), *Advances In the Study of Behavior* (Vol. II, pp. 1-110). London: Academic Press.

Zucker, I. (1968). Biphasic effects of progesterone on sexual receptivity in the female guinea pig. *Journal of Comparative Physiological Psychology, 65*(3), 472-478.