Circadian Regulation of Gonadotropin-Releasing Hormone Neurons and the Preovulatory Surge in Luteinizing Hormone in the Diurnal Rodent, Arvicanthis niloticus, and in a Nocturnal Rodent, Rattus norvegicus

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ABSTRACT

Daily rhythms in the timing of the preovulatory surge and the display of reproductive behavior are reversed in diurnal and nocturnal rodents, but little is known about the neural mechanisms underlying these differences. We examined this issue by comparing a diurnal murid rodent, Arvicanthis niloticus (the grass rat), to a nocturnal one, Rattus norvegicus (the lab rat). In the first study, we established that sequential estradiol and progesterone treatment induces a proestrous-like rise in LH secretion and in the percentage of GnRH neurons that express Fos in grass rats, as is the case in lab rats. Next, we tested the hypothesis that differences in the timing of estrus-related events in diurnal and nocturnal species are caused by differences in rhythms in responsiveness to steroid hormones. We found rhythms in GnRH neuron activity, as indicated by Fos, that were 12 hours out of phase in grass rats and lab rats. These patterns persisted in both species when animals were housed in constant darkness for 5 days, suggesting that they are driven by an endogenous circadian mechanism. These results indicate that steroid-primed grass rats and lab rats are similar with respect to the temporal relationship among estrus-related events, but that the timing of these events relative to the light-dark cycle is dramatically different and that this difference is caused by endogenous circadian mechanisms.

behavior, circadian rhythm, gonadotropin-releasing hormone, luteinizing hormone, neuroendocrinology

INTRODUCTION

Reproductive events, such as copulation, parturition, and the preovulatory surge in LH, can occur at very different times of day in nocturnal and diurnal animals. Nocturnal female lab rats (Rattus norvegicus), mice (Mus musculus), and Syrian hamsters (Mesocricetus auratus) mate and undergo an LH surge during the late afternoon or early evening, around lights-off [1–7], whereas in the diurnal rodent, Arvicanthis niloticus (the grass rat), sexual behavior and the LH surge occur very early in the morning, before lights-on [8, 9]. In GnRH neurons, which drive the surge, Fos expression rises along with LH, again, at opposite times of the day in lab rats and grass rats [8, 10, 11]. Most chronobiology research focuses on nocturnal lab rodents, and the mechanisms causing these estrus-related events to occur at different times of the day in diurnal and nocturnal species are not known.

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 proestrus, it does not occur immediately after recovery from the treatment but, instead, is delayed a full circadian cycle [12, 13]. Furthermore, when these animals are housed under constant light, they have free-running rhythms in the timing of the LH surge and in the onset of sexual receptivity that are “circa-quadradian.” That is, these events occur at intervals that are four times as long as the period of the circadian activity rhythms [14–16]. In lab rats and hamsters kept under a light-dark cycle, both the LH surge and the onset of lordosis behavior are tightly coupled to the onset of activity [4, 14, 17]. 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 [15, 18, 19]. A daily signal for the 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 [5, 20].

The circadian signal that gates the timing of estrus-related events originates in the suprachiasmatic nucleus (SCN), which is 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 [21–24]. A direct pathway extends from SCN cells to GnRH neurons in lab rats and hamsters, and in lab rats, these contacts are clearly synaptic [25, 26]. It is not clear, however, whether the SCN and circadian system are involved in the timing of estrus-related events in diurnal rodents, because until recently, a suitable model has not been available with which to investigate this question.

The unstriped Nile grass rat, A. 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 aboveground activity in the field [9, 27]. These animals also have an LH surge during the postpartum estrous period that occurs nearly 12 h out of phase with that of lab rats [8]. Previous studies of grass rats have

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focused on the postpartum 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 in this species).

The goals of the present study were to investigate the neuroendocrine events associated with the LH surge in a diurnal rodent and to explore how the timing of these events differs in nocturnal and diurnal species. More specifically, we had three objectives: 1) to confirm that ovariectomized grass rats primed with steroid hormones exhibit a surge in LH and GnRH cell activity, as in other laboratory rodents; 2) to evaluate the hypothesis that lab rats and grass rats exhibit reversed rhythms in GnRH cell activity when given the same steroid hormone treatment at different times of the day; and 3) to evaluate the hypothesis that the rhythms in GnRH cell activity are endogenous in these species. The increase in activation of GnRH neurons during the preovulatory LH surge of lab rats, guinea pigs, and hamsters has been clearly associated with detection of the immediate early gene product Fos within these neuroendocrine cells [3, 10, 11, 28–30], and for the purposes of the present study, we will use “GnRH cell activity” to refer to elevated Fos expression in GnRH neurons.

MATERIALS AND METHODS

Animals

Adult female grass rats (age, >60 days) bred from laboratory stock and Sprague-Dawley rats (Charles River, Cambridge, MA) were housed under a 12L:12D photoperiod and provided food and water ad libitum. A red light (<5 lux) was left on continuously in the animal rooms. Females were anesthetized with sodium pentobarbital (50 mg/kg) supplemented with methoxyflurane and then bilaterally ovariec tomized. Incisions were closed with Vutapatch (Vet-Med, TX) or wound clips (lab rats) and treated with a topical antibiotic (Nolvasan; Fort Dodge Animal Health, Overland Park, KS). Following ovariectomy, animals were given 1 ml of 0.9% saline s.c. and 0.03 mg of buprenorphine hydrochloride i.m. We waited 7–14 days after ovariec tomcy before beginning each experiment. All experiments were performed in compliance with the 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 (National Academy of Science). All efforts were made to minimize the suffering and number of animals used in these experiments.

Immunocytochemical Procedures

In each study, all animals were deeply anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M phosphate buffer overnight, and then sectioned (thick-tobarbital and perfused transcardially with 0.01 M PBS (pH 7.2, 150±200 mOsm) with 30% hydrogen peroxide (0.35 M/l buffer). Controls were done by performing this double-labeling procedure with the antibodies for Fos, for GnRH, or with both antibodies omitted. After the reaction, tissue was mounted, dehydrated, cover-slipped, and examined under a light microscope (Fig. 1). For each study, the numbers of GnRH+ cells with and without Fos+ were quantified. The GnRH+ cells, with and without Fos, could be readily distinguished from each other.

Patterns of Change in LH and in GnRH+ Cells in Grass Rats

In this first study, ovariectomized grass rats were injected (s.c.) for 2 days with 10 μg of 17β-estradiol benzoate (EB) suspended in sesame oil at zeitgeber time (ZT) 19 (ZT 0 = lights-on, ZT 12 = lights-off). On the third day, at the same ZT; three groups of females received 125 μg of progesterone (P; s.c.). Following the P injection, these animals were perfused at either ZT 20.5, 22, or 23.5 (n = 5 per time point). A fourth 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 taken and centrifuged, and the plasma stored at −80°C. Brains were processed, and the numbers of GnRH+ cells, with and without Fos+, were counted from 14 sections taken from each animal (Fig. 1). Six of these contained the medial septum and diagonal and horizontal bands of Broca (MS/DBB), two contained the rostral medial preoptic area (MPOA) adjacent to the organum vasculosum of the lamina terminalis, and six contained the anteromedial portion of the periventricular nucleus and preoptic area (AVPV/POA).

Plasma LH concentrations were measured by a double-antibody radioimmunoassay that has been previously validated for use with grass rats [8]. The primary antibody is a mouse monoclonal anti-bovine LH (518B7, lot 4), was provided by Dr. Jan Roser (University of California, Davis, CA). Dr. L. Reichert (Albany Medical College, Albany, NY) provided iodinated ovine LH LER 1056 C2, which was used as tracer. Tubes for the standard curve were prepared with S26 ovine LH reference preparation obtained from the NIDDK (Bethesda, MD). The LH values that fell below the lowest detectability (0.5 ng) were rounded up to this value for statistical analysis.

Patterns of GnRH+ and Fos+ in Grass Rats and Lab Rats under a Light-Dark Cycle

In the second study, ovariectomized grass rats and lab rats received an injection (s.c.) of EB (50 μg/kg) at either ZT 7 or ZT 19 for 2 days. On the third day, at the same ZT, females were injected (s.c.) with P (2.5 mg/kg). Three hours after P injection, animals were perfused; therefore, this was done at either ZT 10 or ZT 22 (n = 7 per group except for grass rats at ZT 10 [n = 11]). Tissue was processed for the immunocytochemical detection of GnRH+ and Fos+ as described above (Fig. 1). In the first study, we found that the highest percentage of GnRH+ cells containing Fos was in the MPA and that GnRH+ cell activity in this region was representative of that for the total GnRH+ cell population. Therefore, although Fos was induced in GnRH+ cells in many sections, we counted cells in only two, both of which contained the MPA.

Patterns of GnRH+ and Fos+ in Grass Rats and Lab Rats in Constant Darkness

In the third study, ovariectomized lab rats and grass rats were housed in constant darkness (DD). On the third and fourth days in DD, animals received injections (s.c.) of EB (10 μg/animal). When housed in DD, grass rats have a free-running period of approximately 23.8–24 h [31], and in lab rats, the period ranges between 23.8 and 24.2 h [32]. We used these estimates to determine times of hormone treatments and perfusions. Injections were given between circadian time (CT) 7 and 8 or between CT 19 and 20.5. On the fifth day in DD, animals were injected (s.c.) with P (grass rats, 125 μg/animal; lab rats, 2.5 mg/kg) and then perfused 3 h later, between CT 10 and 11.5 or between CT 22 and 23.5 (grass rats, n = 4 per group; lab rats, n = 5 per group). In grass rats housed in constant light, hormone injections given for 3 days do not induce phase shifts, and animals continue to exhibit free-running rhythms (unpublished results). Tissue was processed for GnRH+ and Fos+, and two sections containing the MPA were analyzed as described above (Fig. 1).
Statistical Analysis

In the first study, we determined if differences existed among the four groups using a one-way ANOVA with either LH values or the percentage of GnRH+ cells that were active (log transformed) as dependent variables. These data also were examined with a two-sample t-test to determine if animals with no P treatment (ZT 18.5) were different from animals with P treatment (all other animals combined). Additionally, we used a chi-square analysis to compare the frequency of plasma LH values greater or less than 1 ng/ml in P-treated (ZT 20.5, 22, and 23.5) and untreated animals (ZT 18.5). We predicted that both plasma LH and GnRH1 cell activity would be higher at time points following P treatment; thus, we used one-tailed tests in these analyses. To determine whether subpopulations of GnRH+ neurons were different with respect to temporal patterns of activity, we used a two-way ANOVA, with time and region (MS/DBB, MPA, and AVPV/POA) as the independent variables and the percentage of GnRH+ cells that were active (sine transformed) as the dependent variable [33]. The percentage of GnRH+ cells that contained Fos was correlated (Z test) 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 percentage of GnRH+ cells that were active (log transformed) as the dependent factor [33]. All analyses were done using Statview 5.0 (SAS Institute, Cary, NC).

RESULTS

Patterns of Change in LH and in GnRH+ Cells in Grass Rats

Levels of LH and GnRH+ cell activity were highest at ZT 22 and lowest at ZT 18.5, at which time LH was virtually undetectable and almost no GnRH+ cells expressed Fos (Figs. 1 and 2). In steroid-primed grass rats, the percentage of GnRH+ cells that were active was significantly affected by time ($F = 5.75$, $df = 3$, $P = 0.007$) (Figs. 1 and 2A). Animals perfused at ZT 18.5 ($n = 5$), before P treatment, had significantly lower GnRH+ cell activity compared to animals killed at the three remaining time points ($n = 15$, $t = -2.18$, $df = 18$, $P = 0.04$). The pattern of change in plasma LH levels over time resembled that of GnRH+ cell activity, and this effect approached significance ($F = 2.97$, $df = 3$, $P = 0.06$) (Fig. 1B). That is, LH levels were higher at ZT 22 than at ZT 18.5 (Fig. 2). No significant difference in average plasma LH levels of P-treated animals and untreated animals was detected, but more LH values greater than 1 ng/ml were found among the P-treated animals (ZT 20.5, 22, and 23.5) than among the untreated animals (ZT 18.5, $\chi^2 = 6.67$, $df = 1$, $P = 0.009$). The LH values were significantly correlated with the percentage of GnRH+ neurons that were active ($r = 0.635$, $Z = 3.0$, $P = 0.002$).

We found an interaction between subpopulations of GnRH+ cells and time ($F = 2.26$, $df = 6$, $P = 0.05$). The GnRH+ cell populations in all regions had peaks in activity at ZT 22 (Fig. 2), but the effect of time was greatest in the MPA, where the numbers of GnRH+ cells were substantially higher than in the other regions.

GnRH+ and Fos+ in Grass Rats and Lab Rats under 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 in those perfused at ZT 22, whereas grass rats had the inverse pattern (Fig. 3B). This interaction between species and time of day was significant ($F = 11.37, df = 1, P < 0.002$).

**GnRH+ and Fos+ in Grass Rats and Lab Rats in DD**

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 under a light-dark cycle (Fig. 3C). Specifically, the percentage of GnRH+ cells that were active was higher in grass rats perfused at CT 22–23.5 than at CT 10–11.5. Lab rats showed the reverse pattern, with a higher percentage of GnRH+ cells that were active at CT 10–11.5 than at CT 22–23.5. A significant interaction was observed between species and time ($F = 152.5, df = 1, P < 0.001$).

**DISCUSSION**

In the first study, we established an effective diurnal animal model of neuroendocrine events associated with the estrous cycle. Ovariectomized grass rats treated with EB and P had peaks in both LH and GnRH+ cell activity 2 h before lights-on (Fig. 2), which mirrors the pattern seen in intact grass rats sampled during the postpartum estrous period [8]. Average LH values, even at ZT 22 (Fig. 2), were somewhat lower here than those seen in postpartum females [8], and the variability was high. Perhaps the hormone treatments we used were only partially effective for induction of the surge such that either the peak values were lower or
some animals failed to respond altogether. The variability in LH values also could be caused by interindividual differences with respect to the exact time of the surge. That is, our sampling times may not have coincided with the peak in serum LH levels, which may vary among individuals and may be brief, as in lab rats and mice [7, 34]. However, at ZT 18.5, no individual had an LH titer greater than 1 ng/ml, whereas all five animals at ZT 22 and four of five animals at ZT 23.5 had values that were greater than 1 ng/ml.

A significant correlation was found between the levels of plasma LH and of GnRH+ cell activity, and these measures peaked at the same time. These relationships also have been seen in other laboratory mammals, such as mice, lab rats, guinea pigs, and ferrets [3, 11, 28, 35, 36]. In intact and steroid-treated lab rats, the percentage of GnRH cells that are active is highest during the ascending phase of the LH surge [11, 35]. In hamsters, the rise in GnRH cell activity follows the peak in plasma LH values and may be involved in termination of the gonadotropin surge in this species [29]. This is unlikely to be the case in hormone-primed grass rats, however, because both the LH values and GnRH+ cell activity were highest at ZT 22 and quite low at ZT 20.5. If one event preceded the other, then the second would have to occur within 1 h of the first, a pattern unlike that seen in hamsters.

The second experiment established that both grass rats and lab rats have rhythms in GnRH+ neuron activity and that these rhythms are temporally reversed in these two species (Fig. 3B and C), whereas the third experiment provides evidence that in both species the temporal organization of GnRH neuron function is under circadian control (Fig. 3C). Earlier studies have established that ovarian steroids induce a rise in the proportion of GnRH+ cells that contain Fos+ in lab rats and mice [3, 30, 37], but those studies did not look outside the time of day at which the proestrous LH surge normally occurs. Direct evidence of an endogenous rhythm in GnRH neurons has come from organotypic cultures of the POA in which GnRH is secreted in a circadian pattern when the tissue is incubated with estradiol [38]. The rhythms in GnRH neuron activity in animals maintained in DD (present study), or in GnRH secretion in vitro [38], could emerge from rhythmic processes intrinsic to these cells and/or from neurons that regulate GnRH cell function. To the best of our knowledge, the current results represent the first in vivo demonstration of a daily rhythm in the expression of Fos+ in GnRH+ neurons in any species.

Endogenous timing mechanisms likely drive this rhythm in GnRH+ cell activity in both lab rats and grass rats (Fig. 3C). For both species, animals retained the rhythm after 5 days in DD such that grass rats killed before their subjective day had a dramatically higher percentage of GnRH+ cells that were active than those killed 12 h later; in lab rats, this pattern was reversed (Fig. 3C). It remains possible that the rhythm in either species would have damped out if animals had been left in DD for a longer period of time. This is unlikely, however, because no hint was observed that the rhythm was diminished after animals had been kept in DD for 5 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 [14–16]. These data support the hypothesis that the rhythms we observed in GnRH neurons are, indeed, endogenous.

The neural mechanisms that regulate the rhythm in activity of GnRH neurons have not been clearly established in either species, but the SCN likely plays a key role [12, 13]. In lab rats and hamsters, tract-tracing studies suggest that the SCN projects directly to both estrogen receptor (ER)- and GnRH-containing cells [25, 39–41]. The SCN may regulate the timing of estrus-related events, at least in part, through similar pathways in grass rats. Preliminary anterograde tract-tracing experiments indicate that in the grass rat, GnRH+ cells do, in fact, receive input from the SCN [42].

The current data raise interesting questions regarding the specific mechanisms responsible for species differences in the timing of GnRH cell activation. Whereas proestrous and steroid-primed grass rats and lab rats are similar with respect to the temporal relationship between LH secretion and GnRH+ cell activation, the timing of these events relative to the light-dark cycle is dramatically different. Furthermore, grass rats exhibit rhythms in sexual behaviors such that females are behaviorally less sensitive to steroid treatment administered before lights-off when compared to steroid injections given before lights-on [42]. These differences are related to the animal’s chronotype, because both lab rats and grass rats have peaks in GnRH+ cell activity and sexual behavior that occur around the onset of their activity periods [42]. These differences in the timing of neuroendocrine events might be caused by 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. A more specific and intriguing version of the latter hypothesis suggests that GnRH neurons could contain a molecular clock [43] and that this coupling differs in nocturnal rodents. Comparisons between these closely related species now provide a unique opportunity to evaluate these hypotheses.

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