A circadian rhythm of luteinizing hormone secretion in ovariectomized rats

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24-HR PERIODICITY EXISTS in the neural mechanism responsible for release of the ovulation-inducing surge of luteinizing hormone (LH) in both immature gonadotropin-primed (13, 14, 25, 27) and mature (7) rats. The time of day at which this LH discharge occurs is set by the environmental light dark cycle (4, 6, 25). This cyclic LH discharge is eventually abolished if rats are exposed to an acyclic (constant light) regimen (3, 4, 10, 27). In spite of a daily period of neurogenic activation, the surge of LH is released from the pituitary only at proestrus, presumably because the high levels of ovarian steroids in the blood on that day synergize with the daily neurogenic stimulus (5, 15, 20, 21). Support for the view that ovarian steroids play a critical role in facilitating release of the ovulatory surge of LH is afforded by reports that steroid treatment induces LH release and ovulation in both immature (14) and mature (5, 7) rats, and that ovariectomy at 10 AM on the day before proestrus blocks the LH discharge of proestrus (21).

Gonadal steroids also regulate, by a negative feedback action (6, 23), the secretion of that follicle-stimulating hormone (FSH) and LH which is necessary for follicular growth and steroid secretion (11), and which is generally thought to be relatively constant or "tonic", in contrast to the ovulation-inducing surge of LH which is released during a short time period every 4th or 5th day. There is considerable evidence that the tonic gonadotropin secretion and the proestrus gonadotropin surge are controlled by two functionally and anatomically separate systems (1, 9, 23). The present study was undertaken to determine whether this so-called tonic LH secretion also exhibits a 24-hr periodicity. Ovariectomized, rather than cyclic, rats were used since the system regulating discharge of the cyclic ovulatory surge of LH does not appear to function in the absence of gonadal steroid feedbacks (21); tonic gonadotropin secretion continues following steroid withdrawal, although under such circumstances the basal level of such secretion becomes elevated. Five months after ovariectomy rats were autopsied at 4-hr intervals during a 24-hr time span, and the LH potency of pituitary and plasma samples collected at various times of day was assessed. Four separate experimental groups were studied: otherwise untreated rats, ovariectomized at either a) 32 or b) 71 days of age; rats ovariectomized at 32 days of age after treatment at 24 days of age with either c) pregnant mare's serum gonadotropin (PMSG) or d) human chorionic gonadotropin (HCG). These four groups were used to investigate possible differences in LH secretion between animals ovariectomized a) prepuberally and b) postpuberally, and also to assess the influence of c) PMSG and d) HCG pretreatment on the response to steroid withdrawal.

MATERIALS AND METHODS

Immature and mature rats, obtained from Sprague-Dawley, Inc. at 21 and 60 days of age, respectively, were...
were alternately killed during each of seven 1-hr
autopsies, within 6 months of collection. Whereas all of the
plasma samples were tested concurrently. The LH concentration of the plasma samples was
determined from the dose-response curves obtained with the LH standards.

Experiment 1. Animals which had received a single subcutaneous injection of either 50 IU of PMSG (Equinex, Ayerst) or 25 IU of HCG (Antuitrin "S", Parke, Davis) at 24 days of age were bilaterally ovariectomized under ether anesthesia at 32 days of age. Five months later they were bled from the dorsal aorta (22) and autopsied under ether anesthesia. Four rats from each treatment group were autopsied; the experimental design was identical to that employed in experiment 1, except that five rats from each group were autopsied at each of the seven time periods.

Measurement of LH. Pituitary (21) and plasma (22) LH were assayed using the ovarian ascorbic acid-depleting method (17) as described previously. Saline dilutions of the pituitaries, containing 1/128 and 1/32 pituitary equivalent/ml, were prepared on the day of assay; dilutions of a standard LH preparation (NIH-LH-S-4, corrected to NIH-LH-S-1 potency)—0.4 and 1.6 μg/ml—were tested simultaneously in a four-point assay (2). In the plasma LH assays, 2-ml samples of plasma were tested for their ovarian ascorbic acid-depleting activity, two doses of LH standard—0.05 and 0.2 μg/2 ml—were tested concurrently. The LH concentration of the plasma samples was determined from the dose-response curves obtained with the LH standards.

Analyses of variance. Pituitary and plasma LH data in the two experiments were analyzed by several two-way analyses of variance (24), first testing each variable within the two experiments, and then comparing data between the two experiments. Each two-way analysis permitted simultaneous assessment of the significance of a) differences in the level of the variable between the two groups in each experiment or between the two experiments, b) differences among times of autopsy, and c) possible interaction between the aforementioned factors. LH values obtained for pituitary and plasma samples collected during the second 10 AM to 11 AM autopsy period were omitted in all of the analyses of variance to preserve independence of the time of day effect.

RESULTS

1) Pituitary LH content over a 24-hr period. Five months after ovariectomy, the content of LH in pituitaries collected at each of the seven time periods from the PMSG- or HCG-treated ovariectomized rats (experiment 1, Fig. 1A) and from the rats ovariectomized either when immature or mature but otherwise untreated (experiment 2, Fig. 1B), was significantly greater than that of pituitaries of intact cyclic rats of the same strain (21); pituitary LH content in the latter reaches a maximum of approximately 9 μg at proestrus. Pituitary weights were similar in the four groups. Analyses of variance of the unweighted (2) assay data within each of the experiments did not reveal a) significant changes in pituitary LH content with time of collection, b) significant differences in pituitary LH content between the PMSG- and HCG-treated ovariectomized rats (experiment 1), or c) significant differences in LH content between pituitaries of rats ovariectomized when immature and the pituitaries of rats ovariectomized when mature (experiment 2).

Since there was no significant difference in pituitary LH content between the two experimental groups in experiment 1, the values obtained from assays of the four pituitaries collected at each of the seven times of day

![Graph](http://ajplegacy.physiology.org/)

**Fig. 1.** Pituitary LH content over a 24-hr period in chronically ovariectomized rats. Each point in A and B represents a single assay on a single pituitary. Each point in C represents the weighted mean, or combined, potency of the pituitaries assayed.
were combined. For the same reason, the values obtained in experiment 2 were combined similarly. An analysis of variance (between experiments 1 and 2), using these combined data, did not reveal any significant differences in pituitary LH content a) between the two experiments or b) at the different times of day in either experiment. Furthermore, there was no significant interaction between the experimental groups and time-of-day. The LH potencies of the eight pituitaries collected at each of the seven autopsy periods were then pooled; the resultant weighted mean (or combined) potencies and 95% confidence limits \((2)\) over the 24-hr period are illustrated in Fig. 1C.

2) Plasma LH concentration over a 24-hr period. Plasma LH concentration (Table 1) was consistently high, comparable to that found previously 1 week to 3 months after ovariectomy (22; Lawton, unpublished observations). These values exceed that found in plasma collected from cyclic rats during the proestrous critical period (mean of samples collected between 2:30 PM and 3:30 PM from 11 proestrous rats = 0.034 µg/2 ml, Lawton, unpublished observations). Analyses of variance of the plasma LH data (Table 1) disclosed findings which differed in some respects from those obtained for the pituitary LH data. In experiment 1, a significant difference \((P < 0.05)\) existed between the two groups, reflecting the tendency for a higher plasma LH concentration in the HCG-treated ovariectomized rats. In experiment 2, the LH potencies of the plasma samples from the two groups were not different and therefore were combined (Table 1). The fluctuation of plasma LH levels with time of day was of borderline significance \((P < 0.10)\) in experiment 1, but in experiment 2 plasma LH varied significantly with time \((P < 0.05)\) being higher in both groups during the 6 AM-2 PM collection periods than during the 6 PM-2 AM periods. In neither experiment did a significant interaction exist between the two groups and time-of-day, indicating that the significant time-of-day changes in the circulating levels of LH had a similar temporal pattern in the two experimental groups. Since the levels of LH in plasma from the PMSG-treated animals differed from those in plasma from the HCG-treated rats, the data could not be combined; therefore, each group was compared individually (again by an analysis of variance) with the combined experiment 2 data. Comparisons of the over-all PMSG and HCG results with those from experiment 2 in both instances yielded a highly significant difference \((P < 0.005)\), due to higher plasma LH levels in the experiment 2 rats. In both of these between-experiment analyses, time-of-day was a significant source of variation \((P < 0.005)\), but again the interaction between the experimental groups and time-of-day was negligible.

### Table 1. Plasma LH concentration over a 24-hr period in chronically ovariectomized rats

<table>
<thead>
<tr>
<th>Autopsy Time</th>
<th>Experiment 1 (µg/2 ml Plasma)</th>
<th>Experiment 2 (µg/2 ml Plasma)</th>
<th>Combined data (µg/2 ml Plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSG-treated</td>
<td>HCG-treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 AM</td>
<td>0.054±0.010 (4)</td>
<td>0.385±0.178 (3)</td>
<td>0.316±0.066 (10)</td>
</tr>
<tr>
<td>2 PM</td>
<td>0.035±0.007 (2)</td>
<td>0.142±0.047 (4)</td>
<td>0.350±0.087 (6)</td>
</tr>
<tr>
<td>6 PM</td>
<td>0.045±0.006 (3)</td>
<td>0.130±0.046 (3)</td>
<td>0.213±0.043 (6)</td>
</tr>
<tr>
<td>10 PM</td>
<td>0.079±0.013 (4)</td>
<td>0.128±0.080 (3)</td>
<td>0.213±0.079 (8)</td>
</tr>
<tr>
<td>2 AM</td>
<td>0.082±0.069 (2)</td>
<td>0.088±0.030 (2)</td>
<td>0.139±0.029 (7)</td>
</tr>
<tr>
<td>6 AM</td>
<td>0.317±0.153 (4)</td>
<td>0.287±0.084 (2)</td>
<td>0.392±0.061 (9)</td>
</tr>
<tr>
<td>10 AM</td>
<td>0.157±0.127 (3)</td>
<td>0.089±0.012 (3)</td>
<td>0.255±0.054 (9)</td>
</tr>
</tbody>
</table>

Values are given as mean ± se of the mean; figures in parentheses indicate no. of samples assayed. *Significant difference \((P < 0.05)\) between groups; †50 IU PMSG at 24 days of age, and ovariectomized at 32 days of age; ‡25 IU HCG at 24 days of age, and ovariectomized at 32 days of age; §Untreated; one group ovariectomized at 32 days of age, the other at 71 days of age.

This is consistent with the theory that gonadal steroids act, by a negative feedback, to hold tonic LH synthesis and release in check (8, 23). The findings with respect to plasma LH activity agree with those reported by Ramirez and McCann (18); plasma LH was high in animals ovariectomized either when immature or mature. Our results with respect to pituitary LH content (Fig. 1) do differ, however, from those of Ramirez and McCann, who found that pituitary LH content had increased significantly only in rats ovariectomized when mature. This difference between our findings and those of Ramirez and McCann suggests that the age at which the animals are autopsied, rather than the age at which the animals are ovariectomized, may be the critical factor, since those rats in our study which were gonadectomized when immature were considerably older when autopsied than were those of Ramirez and McCann. It is possible that neural loci within the brain and/or hypophysis must reach a certain level of maturation before they can act as "settings" or inputs to stimulate LH secretion manifested in the absence of negative steroid feedbacks.

From the elevated levels of pituitary and plasma LH obtained in experiment 1 (Fig. 1A, Table 1), it is clear that the PMSG- or HCG-treated rats were capable of responding to steroid withdrawal. The final levels of pituitary LH in these two groups did not differ significantly from those of the ovariectomized rats of experiment 2 (Fig. 1B), which had not received any preoperative treatment. Other investigators have reported a lowering of pituitary LH content in immature rats following PMSG and/or HCG priming (12, 16, 20), but these studies were acute in nature and the changes in LH content were accompanied by ovulation; thus, the drop in LH content apparently reflected release of an ovulatory surge of LH, rather than inhibition of tonic LH secretion. In the present study, however, LH in the plasma of the pre-treated
animals was significantly lower than that in plasma of the untreated animals (Table 1), suggesting a partial suppression of the increased rate of LH release normally seen following ovariectomy. This failure of the LH secretory mechanism to respond fully following withdrawal of the steroidal negative feedback in the gonadotropin-treated animals may have been mediated by a direct action upon the central nervous system of the exogenously administered gonadotropin (28) or of endogenous gonadotropin released acutely in response to such treatment (12-14, 20, 25, 27). Priming of immature rats with gonadotropins also induces a precocious secretion of ovarian steroids, as evidenced by pituitary (20) and uterine (27) hyper trophy; thus, a third, and seemingly more probable, possibility is that the effect may have been steroid mediated. This possibility is supported by our finding that plasma LH levels were significantly lower in the PMSG-treated animals than in the animals receiving HCG; PMSG, primarily FSH-like in activity, would perhaps be expected to be more effective than HCG, which is primarily LH-like, in stimulating ovarian steroid secretion. Despite the partial effects of gonadotropin pretreatment on the hypothalamic response to steroid withdrawal in the present study, however, the temporal pattern of LH synthesis and release was unaltered.

The significant changes in plasma LH concentration during the 24-hr light-dark cycle (Table 1) suggest the existence of a 24-hr rhythm of the heretofore designated tonic LH release. Nevertheless, we cannot at present rule out the possibility that such fluctuations may primarily reflect differences in the disappearance rate of LH from the plasma during the 24-hr period, although the existence of a circadian variation in the half-life of LH in the rat has not yet been investigated. It is also possible that the changes in the ovarian ascorbic acid-depleting activity of plasma observed over the 24-hr period may wholly, or in part, reflect changing levels of luteinizing hormone-releasing factor (LRF) in the plasma, since the recipient rats used in the plasma assays were not hypophysecomized (22). The rhythm was consistent in all experiments; no significant intergroup or interexperiment differences were noted in the temporal pattern of LH release (as assessed by the lack of significance of the interaction calculation). If the fluctuating plasma ovarian ascorbic acid-depleting activity reflects actual changes in LH release, then LH synthesis must also be changing in these animals, since pituitary LH content remained relatively constant; the failure to detect any alteration in pituitary LH content over the 24-hr period in any of the four experimental groups probably can be attributed to changing rates of LH synthesis which match the rates of LH release.

The apparent 24-hr periodicity in the LH-secreting system observed in these ovariectomized rats is obviously independent of fluctuating titers of ovarian steroids in the blood. It is also independent of any previous history of reproductive cyclicity, since the rhythm was apparent even in those animals which had been ovariectomized prior to reaching sexual maturity. Although such a periodicity may reflect circadian changes in the excitability of the neural system regulating such LH secretion, other possibilities are that this 24-hr periodicity in LH secretion may be induced indirectly by an internal feedback action of circulating plasma LH titers upon hypothalamic LH-RF secretion (19) or may reflect a 24-hr periodicity in adrenal cortical secretion (26). Although the ultimate origin of this circadian periodicity remains unknown, the results of the present study do suggest that the environmental light-dark cycle influences the phase timing of the secretory rhythm within this system in the ovariectomized rat, as it does within the system inducing ovulation in the cyclic rat (6, 10, 25, 27). Higher levels of LH were present in the plasma of these ovariectomized rats during the lights-on portion of the light-dark cycle than during the lights-off period (Table 1). The cyclic discharge of LH for ovulation also occurs during the lights-on portion of this cycle (7, 13, 22), although such release occurs during the latter half of light period whereas peak LH secretion in the ovariectomized rats occurred during the earlier portion of this period (Table 1). A recent study (9) of the timing of the gonadotropin secretion responsible for estrogen secretion on the day before proestrus in cyclic rats exposed to the same lighting regimen also suggests peak secretion during the earlier part of the lights-on period (before 3 PM). Thus it is tempting to speculate that the neural mechanisms regulating both types of gonadotropin secretion may be light-activated, but perhaps not at exactly the same time relative to the onset of the light period. The present findings are comparable to those obtained within the pituitary adrenal axis (26), where a peak in the pituitary adrenocorticotropic activity rhythm also occurs during the earlier segment of the lights-on period.

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