Effect of restraint stress on the preovulatory luteinizing hormone profile and ovulation in the rat

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Plasma profiles of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured during restraint stress on the day of pro-oestrus; these profiles were considered in relation to ovulation rate on the next day. Rats bearing a permanent jugular vein cannula were subjected to restraint, which was started 0, 1 or 2 h before the presumed onset of the LH surge and ended just before the beginning of the dark period. Exposure to restraint resulted in a suppression of the secretion of both gonadotrophins on the day of pro-oestrus. Suppression of the LH surge was virtually complete (plasma LH ≤ 0.2 ng/ml) in 15 out of 32 stressed rats, and the ovaries of these rats contained graafian follicles with oocytes in germinal vesicle stage. In these rats, the LH surge did not occur 24 h later. In the remaining 17 rats, restraint resulted in a considerable suppression of the LH surge. Of these rats, 5 had an ovulation rate of 100% and four ovulated partially. In unruptured follicles of the latter, the oocyte had not resumed meiosis and the follicle wall was not luteinized. In the remaining eight rats with a reduced LH surge, ovulations had not occurred and graafian follicles were unaffected. The results of this study indicate that during pro-oestrus restraint stress suppresses and does not delay the release of preovulatory gonadotrophins. Partial suppression of LH by restraint does not result in induction of meiotic resumption without subsequent ovulation or in luteinized unruptured follicles.

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Stress of physical or emotional origin may interfere with reproductive functions (1, 2). Stress can influence the release of gonadotrophin-releasing hormone (GnRH) (3) and also of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Most studies have been performed in male or in ovariectomized female animals (4–6). Little research has been done into the effect of stress on the pro-oestrous LH and FSH surge in intact female rats. In adult cyclic rats, unpredictable foot-shocks applied on pro-oestrus caused a partial inhibition of the LH surge and of subsequent ovulation (7). After exposure to immobilization stress on the day of pro-oestrus, ovulation was blocked and delayed for 1 day, implying also a delay of the LH surge by 1 day (8).

In the intact female rat, the LH surge on the day of pro-oestrus triggers ovulation. Luteinizing hormone also induces meiotic resumption of the oocyte and can induce premature luteinization in graafian follicles. In graafian follicles, meiotic resumption and luteinization have been shown to start after a dose of LH smaller than needed for ovulation (9, 10). If restraint stress partially suppresses the LH surge, it may conceivably induce either meiotic resumption without subsequent ovulation or luteinized unruptured follicles (LUF). In the present study, we investigated the effect of restraint stress on preovulatory surge profiles of gonadotrophins in rats. Different starting times of restraint on pro-oestrus were chosen with the aim of obtaining a variety of moderately to strongly reduced LH surge profiles. In these rats, we wanted to investigate whether a partially suppressed LH surge might induce meiotic resumption and/or luteinization without ovulation. To that end, the ovaries were collected for histology the day after restraint. Occurrence of ovulation, meiotic resumption without subsequent ovulation and LUF were studied in relation to the LH profiles.

Materials and methods

Rats, housing and surgery

The experiments were performed with female F1 hybrids (6–8 months of age, 200–250 g body weight) of two Wistar substrains (U-inbred males and R-inbred females) from the University breeding colony. They were maintained in a controlled temperature environment (22 ± 1 °C) with lights on from 00.00 to 14.00 h. With this light regimen these rats have 5-day cycles and the onset of the gonadotrophin surge is at around 09.00 h, i.e. 2 h after the middle of the light period (11).
The rats were housed individually and received standard food pellets and tapwater ad libitum. A dim light was left on during the dark period to facilitate blood sampling.

Rats were provided with a jugular vein cannula according to the method of Steffens (12), with some modifications (13) under ketamine (40–60 mg/kg intraperitoneal) and xylazine (210 µl sc of a 2% solution diluted with two volumes of saline) anaesthesia. Starting 1 day after surgery, the rats were handled daily to minimize stress during blood sampling. Oestrous cyclicity was assessed each day by inspection of vaginal smears and observation of lordosis behaviour induced after introduction of a male rat for a brief period in the cage. Rats that exhibited at least two consecutive 5-day cycles following surgery were used for experimentation.

Restraint procedure

After being connected to a blood sampling cannula, individual rats were placed in a perspex cylinder of 4.8 cm inner diameter. One end of the cylinder was cone-shaped and provided with perforations to facilitate air supply to the rat. The other end of the cylinder contained outlet drains for urine and an opening for the tail of the rat. A slit (0.7 cm wide) running along the length of the cylinder enabled fixation of a partition-wall to adjust the interior length of the cylinder to the size of the rat. The blood sampling cannula was exteriorized via the slit.

Experimental protocol

The experiments were conducted after approval by the University Committee on Animal Care and Use (DEC).

Rats (two to five rats at a time) were transferred to a novel experimental room and immediately placed in a restraint cylinder. The restraint was started at 0, 1 or 2 h before the onset of the LH surge, which was anticipated to begin at approximately 09.00 h. The rats were released and returned to their home cage shortly before the onset of the dark period at 14.00 h. After a recovery period of 2 h, oestrous behaviour (lordosis) was assessed by allowing a male into the cage. Blood samples were taken hourly from 09.00 h up to 17.00 h and also on the next day at 11.00, 12.00 and 13.00 h. After the 13.00 h sample the rats were anaesthetized by ether and killed by cervical dislocation. Ovaries were collected for histological examination. Controls were cannulated rats in pro-oestrus, of which blood samples were taken hourly in their home cage from 09.00 to 17.00 h and they were killed the next day at 13.00 h.

Radioimmunoassay

Blood samples (150 µl) were collected in ice-cooled heparinized tubes. After centrifugation, plasma samples (60 µl) were diluted with three volumes of phosphate-buffered saline (pH 7.5) containing 0.1% bovine serum albumin (Sigma RIA-grade) and stored at −20°C. Levels of LH and FSH were measured using kits provided by the NIDDK. Rat LH-RP-2 and FSH-RP-2 diluted in serum of hypophysectomized rats were used as reference materials. The second antibody was donkey anti-rabbit (Saccel, Wellcome Reagents, Beckenham, UK). Determinations were performed in triplicate. Quality control sera with low, medium and high LH or FSH concentrations were included in each RIA. Assay sensitivity at 90% B/B₀ was 0.05 ng/tube for LH and 0.4 ng/tube for FSH. In the diluted plasma samples, LH concentrations lower than 0.2 ng/ml and FSH concentrations lower than 1.6 ng/ml were considered as baseline levels. The intra-assay coefficients of variation for LH and FSH were 4.3% and 5.7%, respectively.

Histology

Ovaries were fixed in Bouin’s solution and embedded in paraffin wax. Serial sections (8–10 µm) were stained with hematoxylin (Gurr, UK) and eosin and mounted in DePeX (Gurr, UK). In both ovaries of each rat, graafian follicles with or without meiotic resumption, luteinized unruptured follicles and ruptured follicles were counted; the latter were compact structures containing numerous dark-staining nuclei and these structures could easily be distinguished from corpora lutea of preceding cycles (for details, see Ref. 9).

Statistical analysis

Peak values of LH and FSH were based on the value from zero to maximum. To compare the response curves of LH and FSH to restraint stress, the individual data of the surge between 09.00 and 17.00 h were expressed as the area under the curve (AUC). The AUC is the integrated area between the baseline and the LH/FSH response above the baseline. The AUC of LH and FSH, the peak values of LH and FSH and the ovulation rate were analysed by the Kruskal–Wallis test (one-way analysis of variance), with non-parametric comparisons made using the Mann–Whitney U-test when trends were found to be significant. For statistical analyses the Statistical Program System for Social Sciences (SPSS/PC+ V2.0, SPSS, Inc., Chicago, IL) was used. Significance was defined at the 0.05 level. Values are reported as means ± SEM.

Results

Figure 1 illustrates the effect of restraint on plasma LH during pro-oestrus. In individual control rats, the peak value of LH ranged between 7.5 and 10.5 ng/ml and was reached between 11.00 and 14.00 h. In restraint rats, the highest plasma LH levels ranged between basal and 4.3 ng/ml. Exposure to restraint resulted in a
significant inhibition of the LH response (peak value and AUC; both $p < 0.01$ compared to controls) in all experimental groups. No significant overall differences were observed between the three groups with different starting times of restraint.

A considerable variation of individual LH profiles was observed in all restraint groups. Restraint completely blocked the LH surge (plasma LH $\leq 0.2$ ng/ml) in 15 out of 32 rats. In 10 rats, maximum LH levels varied between 0.5 and 1.7 ng/ml. Maximum LH levels between 2.1 and 4.3 ng/ml were reached in seven rats. Figure 2 illustrates LH profiles of six individual rats in which restraint did not completely suppress the LH surge. In stressed rats, suppressed maximum LH levels were reached at any time point between 09.00 and 16.00 h. The rat in Fig. 2b shows an advanced onset of the LH surge. In all restraint-stressed rats the LH levels were low (0.2–0.4 ng/ml) on the next day at 11.00, 12.00 and 13.00 h. This indicates that the restraint had not delayed the surge of LH by 24 h.

As shown in Fig. 3, in control rats plasma FSH increased gradually on the day of pro-oestrus and restraint significantly suppressed this gradual FSH rise ($p < 0.01$). The FSH level was 2.9 ± 0.1 ng/ml at 11.00, 12.00 and 13.00 h the day after pro-oestrus. No correlation was observed between the peak and AUC values of LH and FSH. In Table 1 the data on the ovaries, peak LH concentration in the plasma, relative amount of LH released during LH surge (AUC) and ovulation rate are given for controls and stressed rats. Data for FSH are not given because no correlation was seen between ovulation rate and peak or AUC values of FSH. In control rats, virtually 100% ovulation had occurred. Ovulation rate was significantly reduced in the restraint groups ($p < 0.05$). No significant differences were found between the three restraint groups with regard to ovulation rate, so they were considered as one group. In the 15 rats that had LH levels $\leq 0.2$ ng/ml, the graafian follicles were unaffected, i.e. they contained an oocyte with germinal vesicle and showed no luteinization. In seven rats with a maximum LH level between 0.5 and 1.7 ng/ml and an AUC between 0.4 and 3.1, the graafian follicles were also apparently unaffected. Exceptions were two rats with a maximum LH level of 0.5 and 1.3 ng/ml and an AUC of 0.8 and 3.0 in which, respectively, 100% and 17% of the graafian follicles had ovulated. Rats with a peak LH level $> 2$ ng/ml or an AUC of $\geq 4.0$ generally showed a high ovulation rate, with the exception of one rat with a peak level of 2.8 ng/ml and an AUC of 4.0 that had not ovulated. In rats with partial ovulation, most of the unruptured graafian follicles were unaffected. Very few luteinized unruptured follicles were observed; they contained an oocyte in metaphase II stage.

A noteworthy trend was that the number of rats that had a relatively high LH peak, AUC and ovulation rate

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**Fig. 1.** Profiles of rat plasma LH during pro-oestrus: (□) controls (N = 8); (○) restraint 09.00–14.00 h (N = 10); (△) restraint 08.00–14.00 h (N = 11); (■) restraint 07.00–14.00 h (N = 11). Black horizontal bar represents dark period; data are given as means ± SEM.
was greater in the 6-h and 7-h restraint groups than in the 5-h restraint group.

On pro-oestrus all control rats and 70% of the stressed rats showed lordosis when a male was introduced in their home cage at 16.00 h.

Discussion

In male and ovariectomized female rats LH secretion is suppressed and the FSH secretion has been found not to be affected by various stressors (5, 14–17). In the present study, exposure of cyclic rats to restraint on the day of pro-oestrus resulted in a strong inhibition of both the LH and the FSH surge. Hulse and Coleman (7) observed a partial suppression of the LH surge in cyclic rats subjected to inescapable footshocks for 3 h during the surge on pro-oestrus. To the authors' knowledge, a suppressive effect of stress on the FSH surge of cyclic rats has not been reported before. A reduced FSH surge may conceivably cause a decrease of the number of ovulations in the next cycle.

Transferring the rats to a novel experimental room before restraint may be an additional stressor. With respect to restraint stress the effect of novel environment on the LH surge is probably small because we observed no effect of moving the rats to a novel experimental room in pilot studies.

Restraint totally blocked the LH surge in 15 rats. These rats did not have elevated LH and FSH levels the next day, indicating that the restraint had not delayed the surge of gonadotrophins by one day. The complete absence of ovulations and unruptured follicles containing an oocyte in metaphase indicates that in these rats no substantial gonadotrophin secretion had occurred between the period of blood sampling on pro-oestrus and the sampling period on the next day. This corroborates the view that in the rat the preovulatory surge of LH occurs in a fixed period of the day relative to the light/dark cycle (18).

In contrast to our results, Yonetani et al. (8) reported that forced immobilization for 4 h starting at the beginning of the LH surge delayed the LH surge by 1 day. These discrepant results may be due to a strain difference or may reflect a difference between the stress model used, i.e. restraint in a cylinder versus tying up in a supine position. The preovulatory LH surge is blocked and delayed by 24 h in 4- and 5-day cyclic rats after injection of Nembutal on the day of pro-oestrus (19, 20). After injection of Nembutal on pro-oestrus, LH and FSH are suppressed but oestradiol levels remain.

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**Fig. 2.** Release pattern of LH in six individual rats of which restraint suppressed the LH surge partially: (a, b) 5-h restraint; (c, d) 6-h restraint; (e, f) 7-h restraint. Black horizontal bar represents dark period.
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<td></td>
<td>N</td>
<td>Peak height (ng/ml)</td>
<td>Area under curve (arbitrary units)</td>
<td>Number of large follicles (GF + RF + LUF)</td>
<td>Number of graafian follicles (GF)</td>
<td>Number of ruptured follicles (RF)</td>
<td>Number of luteinized/unruptured follicles (LUF)</td>
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<td>Control</td>
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*Means ± sem are given for control rats and for stressed rats with completely suppressed LH surge; individual data are given for rats with partially suppressed LH surge. “Surge time” is the period of the day of pro-oestrus at which the LH surge was presumed to occur. Ovulation rate is the number of RF versus the number of large follicles.*

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Relatively high; apparently, oestradiol remains sufficiently high to evoke an LH surge on the day after pro-oestrus (21). In the present study, the absence of an LH surge on the day after restraint is hard to explain. It may have been caused by restraint-stimulated corticosterone secretion (22); increased corticosterone may inhibit FSH-induced aromatase activity and consequently oestrogen production in granulosa cells (23). However, the corticosterone increase during restraint is transient (24); it is unlikely that a relative a rise of corticosterone caused a lasting suppression of oestrogen synthesis. Stress-induced suppression of gonadotrophins might have caused atresia of follicles and so a decrease of oestrogen synthetic capacity. This is also unlikely because after Nembutal-induced suppression of gonadotrophins an LH and FSH surge occurred the next day (21).

Ovulation is initiated by a gonadotrophin surge on the day of pro-oestrus; the magnitude of the LH surge is considerably larger than needed to cause full ovulation. Greig and Weisz (25) reported that approximately 14% of the peak LH value is sufficient to induce ovulation, which agrees with the results of the present study. In the present study, it appears that also approximately 14% of the control AUC value is sufficient to trigger ovulation. The LH surge induces meiotic resumption of the oocyte in graafian follicles. In Nembutal-anesthetized rats, a dose-related effect of LH on meiotic resumption, luteinization and ovulation was reported (9, 10); injection of 1 μg of LH caused meiotic resumption but no ovulation; 2 μg of LH caused ovulation of some follicles and meiotic resumption plus luteinization but no ovulation of the other graafian follicles; 4 μg of LH caused more ovulations. Based on these data we expected that no ovulations but meiotic resumption with or without luteinization should have been induced in the rat with a small rise of LH during pro-oestrus. However, the present data show that after a considerably reduced LH surge either all graafian follicles ovulated or the graafian follicles appeared unaffected: they contained an oocyte in germinai vesicle stage and were not luteinized. The explanation of the discrepancy between Mattheij et al. (9, 10) and the present results may be that in the former studies injection of LH caused a steep and short-lasting (less than 60 min) increase of LH in the plasma, whereas in the present study the rats with a partially suppressed LH surge had a slightly increased LH level for several hours. The discrepancy may also be due to the fact that Mattheij et al. (9, 10) injected LH 8 h before the
presumed onset of the LH surge. Conceivably, at that time fewer LH receptors were present in the granulosa layer of the graafian follicles than at the beginning of the presumed onset of the LH surge (26).

In the present study, 70% of the stressed rats showed lordosis 2 h after the end of restraint while they had a reduced or totally suppressed LH surge. In the cyclic rat, oestradiol and progesterone (27, 28) together with GnRH (29) initiate and intensify oestrous behaviour. In 15 rats, restraint completely suppressed the LH surge. So an increase of the GnRH secretion in the hypophy¬seal portal system and a consequent increase of ovarian progesterone presumably did not occur. Yet 13 of these rats showed lordosis. The elevated plasma oestrogen on pro-oestrus together with a stress-induced increase of the production of adrenal progesterone (27) or other adrenal steroids (30) may have induced lordosis in these rats.

We conclude that restraint stress inhibited and did not delay gonadotrophin secretion on pro-oestrus. Partial inhibition of LH secretion by restraint is not followed by induction of meiotic resumption without subsequent ovulation or by luteinized unruptured follicles.

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References


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