EXPERIMENTAL STUDY

***In vitro* basal and GnRH-stimulated secretion of gonadotrophins reflects long-lasting modulatory effects, and peripheral levels are not predicted by pituitary responsiveness to GnRH**

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Abstract

Objective: Production of the appropriate pattern of gonadotrophin levels is crucial to proper functioning of the female reproductive system. We aimed to establish whether the pituitary has invariant secretory characteristics when isolated from *in vivo* controls. We aimed to obtain information during both the rising and declining phases of the gonadotrophin surge.

Design: This study investigated factors that are directed at the pituitary by isolating it from the acute influences of the *in vivo* environment and studying gonadotrophin secretion *in vitro*.

Methods: Pituitaries of adult female rats were collected at selected times during the day of pro-oestrus and incubated *in vitro*, and at the same time blood was collected. Peripheral levels of LH and FSH were measured over the whole day of pro-oestrus, basal *in vitro* secretions of LH and FSH from pituitaries were measured, GnRH-stimulated LH and FSH secretion were assessed, and the responsiveness of LH and FSH secretion to GnRH were calculated.

Results: Peripheral levels of LH peaked at 1800 h ($P<0.02$) followed by a subsequent decline. In contrast, although FSH had a peak at 1800 h ($P<0.01$), serum levels were also high at the end pro-oestrus. The profile of basal LH and FSH secretion from the pituitary *in vitro*, in the absence of added secretagogue, resembled that of the peripheral blood levels of each gonadotrophin. Pituitaries collected at 1800 h secreted most LH ($P<0.02$). FSH secretion was low early on the day of pro-oestrus and then increased to and was maintained at high levels in the last quarter of the day ($P<0.01$).

When the pituitaries were stimulated with GnRH the patterns of LH release and FSH release approximated those observed for basal release. Responsiveness of the pituitaries to GnRH was calculated by determining the ratio of GnRH-stimulated release to basal release. However, low levels of gonadotrophin were secreted even from pituitaries which were highly responsive as determined from consideration of percentage increase in secretion induced by GnRH.

Conclusions: The secretory activity was dependent on the time of day the pituitaries were collected. Since the secretion occurred after the tissue had been removed from the direct influence of the *in vivo* environment, the variations in secretion must reflect long-lasting components of the mechanism that regulate gonadotrophin concentrations. There were changes in both LH and FSH responsiveness to GnRH stimulation over the day of pro-oestrus. Delineation of the time courses and changing predominance of multiple processes is needed to assist understanding the mechanisms underlying the female reproductive cycle.

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Introduction

Circulating gonadotrophins are vital endocrine regulators of ovarian function. Follicles mature under the influence of follicle-stimulating hormone (FSH), and luteinising hormone (LH) provides the primary pituitary

signal for ovulation. The profiles of LH and FSH in the blood of rats during the 4 day oestrous cycle and on the day of pro-oestrus are well established (1–3). Coincident preovulatory peaks of the two gonadotrophins occur on the evening of pro-oestrus, followed by a decline in LH levels while FSH secretion remains high during late

pro-oestrus and early oestrus. Of less certainty is the relationship of peripheral levels to the changing cyclic behaviour of the pituitary gland. The levels of gonadotrophins are the result of a complex of signalling systems operating at the level of the anterior pituitary gland. Thus, the pituitary receives stimulatory signals from gonadotrophin-releasing hormone (GnRH) (4, 5), the primary hypothalamic LH/FSH-regulating peptide, as well as other peptides (6). The response to these signals is modulated by other factors, including ovarian steroids (7–9), inhibin, activin and follistatin (10–12). It is also known that under some circumstances GnRH will desensitise the pituitary and further exposure to GnRH will not be efficient in inducing gonadotrophin release (13–15). However the relevance of these factors to the LH and FSH patterns that are produced remains uncertain because there are still only sparse data on the time-dependent behaviour of the pituitary related to the progress of pro-oestrus.

When GnRH is administered to rats at a selected time during a day of the oestrous cycle, LH and FSH rise to an extent that is dependent on the stage of the cycle (16–18). In addition to that variation, there also is a time-dependent response of LH and FSH during the day of pro-oestrus (19). However, studies on the *in vivo* responses of the pituitary to GnRH have invariably been restricted to a limited number of time points. Therefore details of pituitary behaviour around and following the pro-oestrous gonadotrophin surge have remained unclear. Additionally, such studies are problematic since it is virtually impossible to control for all *in vivo* factors that can modulate the responses during the course of the study. *In vitro* studies have also demonstrated a change in response up to the time of the LH surge (20), but effects during the declining phase of the surge were not examined. Therefore, although alterations in responsiveness during the oestrous cycle and the day of pro-oestrus have been observed, the full details remain uncertain.

The present study provides information related to gonadotroph behaviour. First, an *in vitro* protocol was used to isolate pituitary tissue from multiple and changing *in vivo* factors. Secondly, the *in vitro* basal release of LH and FSH was examined at frequent intervals during the whole day of pro-oestrus, including after the gonadotrophin surge. Thirdly, responsiveness to GnRH was concurrently assessed by measuring gonadotrophin output after GnRH stimulation. Thus basal release, GnRH-stimulated release and circulating gonadotrophin levels were measured in parallel and directly compared. Also, differences in behaviour between LH and FSH were noted during pro-oestrus.

Methods

Adult female Wistar rats were housed in controlled lighting conditions, 14 h light:10 h darkness, lights on at 0500 h, off at 1900 h. The oestrous stage of the rats

was monitored by daily vaginal smears. Rats which had at least three 4-day oestrous cycles were used in this study. On the day of pro-oestrus, the rats were killed by exposure to a rising concentration of CO₂ gas and blood was collected by cardiac puncture. The serum was separated and stored at –20 °C until assayed by RIA for LH and FSH. The pituitary gland was collected and the posterior pituitary was discarded. Each anterior pituitary was halved and the two halves were transferred to 1000 µl prewarmed incubation medium in culture wells of a 24-well plate using standard methodology (21, 22). The incubation medium consisted of medium 199 with Hanks' salts, containing 1.4 g NaHCO₃/l, 5.95 g HEPES/l and 1 g BSA/l.

Incubations were carried out at 37 °C in 5 % CO₂ and 95 % air. After 30 min the medium was changed to fresh medium in each well, followed by a 120 min preincubation settling period, a time chosen following preliminary studies. The medium was changed after 60 min. Basal secretion of LH and FSH into fresh medium over a 60 min period was then evaluated. The medium was replaced and pituitaries were then exposed to 1 nmol/l GnRH for 60 min, and again the medium was collected, to determine GnRH-stimulated release. Medium was stored at –20 °C until assayed.

Blood and pituitaries were collected at 11 time points on the day of pro-oestrus; five or six rats were examined at each time. Two time points were in the morning dark phase (0000 h, 0400 h), eight were in the light phase (0800 h–1800 h) and three were in the evening dark phase (2000 h–2400 h). Basal pituitary secretion was evaluated at all time points, and GnRH-stimulated secretion was determined at all time points from 0800 h, after the morning dark phase. Serum was collected at all time points.

Radioimmunoassays for LH and FSH were performed using standard methods and reagents generously supplied by the National Hormone Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases and Dr A F Parlow, in a double antibody protocol. The results were calculated using NIDDK standard preparations as reference.

For statistical evaluation of differences in secretory activities at stages of the day of pro-oestrus, a range of time points was sometimes employed. In these cases, the individual levels or values were used in the analysis. Resulting *P* values are detailed in the text together with the groups that were used in the analysis. Graphs depict the separate groups. One way analysis of variance (ANOVA) was used to determine differences between groups; *P* < 0.05 was taken to indicate a significant difference between groups.

Results

The profile of mean LH levels in peripheral serum over the day of pro-oestrus showed a surge which peaked at 1800 h (*P* < 0.01; 1800 h vs 1000–1200 h). Levels rose

Figure 1 The levels of LH in serum of female rats at selected times during the day of pro-oestrus. A surge on the evening of pro-oestrus was observed. The means and S.E.M. are depicted.

rapidly and then declined to be low again by the end of the day (Fig. 1).

The pattern of release of LH from pituitaries incubated in the absence of any added peptide revealed a peak of secretion from tissue collected at 1800 h (Fig. 2) ($P < 0.02$; 1800 h vs 1000–1200 h). Thus the pituitaries had activity which had been incorporated *in vivo* into their secretory processes, and which influenced secretion *in vitro* without the presence of further secretagogue. The basal secretory activity changed over the day of pro-oestrus to produce the pattern observed. Thus basal secretion from the pituitary was dependent on the time of day that the tissue was removed from the *in vivo* environment.

The pituitaries which were all stimulated with the same concentration of GnRH secreted LH in a pattern

whereby those collected at 1800 h secreted more LH in response to GnRH than pituitaries collected at any other time ($P < 0.01$; 1800 h vs 1000–1200 h). Pituitaries collected after this time were found to have less secretory activity (Fig. 3).

Using these data, the percentage increase in LH response to GnRH (i.e. GnRH-stimulated secretion/basal secretion) was calculated at each time point, and the resulting ratio plotted. The ratio of stimulated LH secretion to basal LH secretion changed during the day of pro-oestrus. The increase began at 1600 h and exceeded values present earlier in pro-oestrus. Responsiveness remained high until the end of the day of pro-oestrus ($P < 0.01$; 1600–2400 h vs 0800–1400 h). Thus the pituitary was more sensitive to GnRH at the end of pro-oestrus than at the beginning (Fig. 4).

Figure 2 The levels of LH released from two hemipituitaries of female rats *in vitro* during a 1 h incubation. The pituitaries were collected from the rats at the times indicated on the day of pro-oestrus. Basal secretion of LH varied during the day, and a peak of secretion was observed in the evening. The means and S.E.M. are depicted.

Figure 3 The levels of LH released from two hemipituitaries of female rats *in vitro* during a 1 h incubation in the presence of 1nM GnRH. The pituitaries were collected from the rats at the times indicated on the day of pro-oestrus. The means and S.E.M. are depicted.

The profile of FSH levels in serum was somewhat different from that for LH. There was again a rise in the evening of pro-oestrus at 1800 h ($P < 0.01$; 1800 h vs 1000–1200 h) but the FSH level did not then return to baseline levels, but rather another peak developed. Thus, at the end of the day of pro-oestrus there were high levels of FSH in serum (Fig. 5).

The profile of the basal *in vitro* FSH secretion had an overall similarity to that of serum FSH levels; namely, low levels early in the day followed by a rise and continued high levels in the last quarter of the day (Fig. 6) ($P < 0.01$; 1800–2400 h vs 0800–1200 h).

When the pituitaries were stimulated with GnRH the pattern of FSH release *in vitro* was similar to that

observed for basal release (Fig. 7); again there was more FSH secreted later in the day ($P < 0.01$; 1800–2400 h vs 0800–1200 h).

When the FSH responsiveness of the pituitaries (GnRH-stimulated secretion/basal secretion) was calculated, it was found that the variations in the ratios and S.E.M. values were relatively large (Fig. 8). This suggested that in individual rats FSH responsiveness was not tightly tied to the time of day.

Discussion

The relationship between the events at the level of the pituitary and concentrations of LH and FSH that are

Figure 4 The responsiveness of LH secretion, to GnRH, from hemipituitaries of female rats *in vitro*. The tissue was incubated in control medium and then in the presence of 1nM GnRH; the ratio of GnRH-stimulated LH release to basal LH release was calculated. The pituitaries were collected from the rats at the times indicated on the day of pro-oestrus. Responsiveness was higher towards the end of the day than at the beginning. The means and S.E.M. are depicted.

Figure 5 The levels of FSH in serum of female rats at selected times during the day of pro-oestrus. There was a rise in FSH levels in the evening of pro-oestrus, and the levels remained high to the end of the day. The means and S.E.M. are depicted.

delivered to the ovary is a topic central to understanding reproductive function. The regulation of LH and that of FSH are at least partly distinct, and the peptides, including GnRH, which impinge on the pituitary seem to differentially affect LH and FSH (23–25). It is clear that a complex series of interrelated processes is involved in producing the peripheral profiles of FSH and LH. A number of these processes will be centred on the pituitary itself, and will vary with time in their effects on the dynamic, cyclic system. This study revealed time-dependent characteristics of secretion of gonadotrophins from the pituitary over the day of pro-oestrus.

Firstly, our study established a variation in basal secretion of LH from the pituitary when it is isolated from the hypothalamus at different times of the day. Basal release had a temporal pattern similar to that for

peripheral LH levels. GnRH increases have been observed in the portal blood in parallel with peripheral LH (26, 27) and it is believed that increased GnRH secretion is the important factor in eliciting the LH surge (28). In the present study, GnRH-stimulated release from the pituitary *in vitro* followed a similar pattern to basal release.

However, the *in vitro* incubation results did not exactly replicate the *in vivo* serum patterns that were concurrently determined in this study. The peak of *in vitro* secretion was proportionately less raised than the surge of serum levels. Factors present *in vivo*, such as GnRH self-priming (29–31), and other sensitising peptides, which include oxytocin (22, 32) and neuropeptide Y (33, 34), could account for the enhanced *in vivo* secretory activity as reflected by the serum

Figure 6 The levels of FSH released from two hemipituitaries of female rats *in vitro* during a 1 h incubation. The pituitaries were collected from the rats at the times indicated on the day of pro-oestrus. Basal secretion *in vitro* of FSH had a pattern similar to that of peripheral concentrations. The means and S.E.M. are depicted.

Figure 7 The levels of FSH released from two hemipituitaries of female rats *in vitro* during a 1 h incubation in the presence of 1nM GnRH. The pituitaries were collected from the rats at the times indicated on the day of pro-oestrus. The means and S.E.M. are depicted.

profile. These other influences were deliberately removed by using an *in vitro* protocol to study pituitary secretion. In this regard, our results were slightly different from those obtained in an earlier study using 2 h incubations, where a peak of LH secretion was not observed *in vitro* at 1800 h (35), and another which found a poor relationship between serum LH levels and basal secretion (36). It seems, therefore, that our protocol was more effective in providing an *in vitro* means of examining *in vivo* events.

The increase in LH serum levels in the evening of pro-oestrus may have resulted from a number of mechanisms other than a primary change in GnRH levels. Another possibility is that the increased secretion might have occurred via an increase in sensitivity of the

pituitary to a given amount of GnRH. Indeed we found that there was varied responsiveness during the day of pro-oestrus. However, the change in sensitivity did not follow the same pattern as either the levels of LH in the serum or LH secretion *in vitro*. During the day, the LH sensitivity of the pituitary to GnRH increased and remained elevated relative to the early times of pro-oestrus. Hence, it was notable that GnRH-stimulated secretion, using a constant concentration of GnRH, fell after 1800 h. This was in spite of the responsiveness index of the pituitary remaining high, suggesting that the pituitary could have secreted substantial amounts of LH if other factors had been favourable. There was, therefore, an important characteristic of pituitary behaviour observed in this study, namely that the

Figure 8 The responsiveness of FSH secretion, to GnRH, from hemipituitaries of female rats *in vitro*. The tissue was incubated in control medium and then in the presence of 1nM GnRH; the ratio of GnRH-stimulated FSH release to basal FSH release was calculated. The pituitaries were collected from the rats at the times indicated on the day of pro-oestrus. The means and S.E.M. are depicted.

absolute amount of GnRH-induced LH secretion declined at a time when the responsiveness (ratio of stimulated/basal) was high. This suggests either that stores of LH had been depleted or alternatively, or additionally, that factors other than GnRH were modulating the response to the primary secretagogue.

Changes in responsiveness of altered endocrinological environments have also been noted by other authors. In considering the full oestrous cycle, a change in LH responsiveness to GnRH from pro-oestrus to other stages of the cycle has been observed (16–18, 37). Our study considered in detail the day of pro-oestrus. Results were obtained that were at variance in some aspects with results obtained by others from *in vivo* studies. When studying the effect of administration of GnRH, it was observed that the ratio of postinjection levels of LH to preinjection levels (responsiveness) was actually less between 1600 h and 2100 h than earlier between 1000 h and 1500 h (19). Thus, in those *in vivo* studies, there was no increase in responsiveness during the time leading up to the LH surge. During extended incubations, the response *in vitro* to GnRH initially increased but later declined in pituitaries taken between 1000 h and 1800 h (38). However, no tissue was taken for study at times following the LH surge on pro-oestrus. There is indeed a paucity of experimental data on *in vitro* characteristics during the declining phase of the LH surge and in the presence of the correspondingly elevated levels of FSH. This is a time when the regulation of the two gonadotrophins has a clearly distinct component.

This present study was also designed to obtain data during the declining phase of the LH surge in contrast to most previous investigations. One possible component of the decline of the LH surge might be a lowered responsiveness to GnRH even in the continued presence of the secretagogue. In an investigation of the interval between the mornings of pro-oestrus and oestrus it seemed that lowered output of LH could be due to desensitisation (39) after the middle of the dark phase at the end of pro-oestrus, i.e. well after the LH decline. That our results did not detect desensitisation (i.e. reduced responsiveness) might be partly due to the chosen protocol. The incubation time was deliberately kept relatively short so that complications due to desensitisation might be reduced. In addition, pituitary pieces were employed which are likely to retain paracrine-regulated processes. Other authors, using *in vivo* protocols, have suggested that refractoriness plays only a minor role in the decline of the LH surge (40).

The results indicated that the basal levels secreted *in vitro*, in the absence of added GnRH, seem to be an important index of the amount of LH which is secreted *in vivo*, because responsiveness was not parallel to the serum profile. Our results point to an increase in responsiveness during the decline of the LH surge. The reduction in peripheral serum LH levels presumably implies that a reduction of GnRH-stimulated secretion

occurs. Thus, lower basal LH secretory activity (as observed after the LH peak), even accompanied by high responsiveness, is an impediment to high *in vivo* levels. There is some debate over the profile of GnRH during the declining phase of the LH surge, some noting an early decline after peak levels are reached (26), whereas others have observed a plateau of retained high GnRH levels (27). However, whatever the levels of GnRH which are delivered via the portal blood system to the pituitary, the control of basal secretory levels by the modulatory processes *in vivo* is apparently an important component of the regulation of the gonadotrophin serum profile. The ratio of mean LH serum level at 1800 h to that at 2400 h is bigger than the ratio of basal secretion levels, and also of *in vitro* GnRH-stimulated levels, at the same times (i.e. 1800 h and 2400 h). This also suggests that further factors, other than GnRH, are involved in determining serum concentrations; otherwise a tight relationship between these secretory parameters would be observed.

Alternatively or in addition to the effects detected *in vitro*, *in vivo* there might be other modulating factors which act at the pituitary level to reduce the stimulatory effectiveness of GnRH. Such factors can include desensitisation by endothelin (41), or inhibition by opioids acting on the pituitary (42, 43) or by substance P (44).

A different picture emerged from consideration of FSH profiles. The role of GnRH in the generation of FSH profiles has been debated for some time. The rise in serum FSH at the same time as that of LH is consistent with GnRH being primarily responsible for the first phase of the FSH rise (27, 45, 46). At the end of pro-oestrus, however, serum FSH was still raised, as previously observed in several studies (1–3), and in contrast to the fall in LH. Thus, the raised levels occurred in the apparent absence of raised GnRH levels (26). However, the second, late pro-oestrus increase of FSH does not take place as a result of an increase in responsiveness to GnRH. There were no alterations in responsiveness parallel to the changes in secretion during the day of pro-oestrus. There was a wide fluctuation of responsiveness of FSH to GnRH, implying that the responsiveness of FSH is less tightly associated in individual rats with time of day or basal level of hormone than responsiveness of LH. These results, therefore, provide further evidence for control of basal and of GnRH-stimulated FSH release via separate mechanisms. That there is a GnRH-independent component to FSH regulation, possibly involving activin and progesterone receptor, is established (47, 48). Modulation of gonadotrophin storage is also possible. *In vitro*, there was increased secretion, in the absence of added secretagogues, from pituitaries taken at the end of pro-oestrus. The results agree with those obtained by others who observed a similar pattern (35). A good relationship between serum FSH levels and basal secretion has been previously reported (36).

The variation in basal secretion, of both LH and FSH, from the pituitary over the day of pro-oestrus is observed some hours after the pituitary has been removed from the *in vivo* influences. Thus, the differences reflect long-lasting components of the regulatory mechanism, resulting in a peak of basal *in vitro* secretion from tissue taken at a time coincident with the *in vivo* peak. Of some interest in this regard is the suggestion that the duration of the effect of GnRH and other factors might extend beyond the time of their actual presence at the pituitary (49). Contributions from long-term activation of processes involving intrapituitary peptides might also be present (6).

In summary, with respect to the control of gonadotrophs, during the day of pro-oestrus, the amounts of LH and FSH which are induced by GnRH are dependent only partly on the responsiveness of cells to this secretagogue. There may be negative modulation to keep releasable stores low in late pro-oestrus or involvement of other factors which can contribute to the regulatory system *in vivo* (6, 9, 12). This current study reports on the direct relationship between measured peripheral levels and *in vitro* secretion from the pituitary source, and concludes that, even when isolated from the acute effect of the hypothalamus, the pituitary exhibits regulated activities. This implies that the temporal profile of modulating hypothalamic factors *in vivo* must take account of both the longevity of effect and also the residual effects of earlier controls.

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