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Progesterone Priming Is Essential for the Full Expression of the Positive Feedback Effect of Estradiol in Inducing the Preovulatory Gonadotropin-Releasing Hormone Surge in the Ewe

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ABSTRACT

The luteal phase elevation in circulating progesterone (P) powerfully inhibits GnRH and, consequently, LH release, thereby preventing premature preovulatory LH surges in the ewe. Whether luteal phase P modulates the response of the GnRH system to the positive feedback effect of estradiol is unknown. To investigate this possibility, two experiments were conducted during the anestrous season using an artificial model of the follicular phase in ovariectomized ewes bearing 10-mm sc 17β-estradiol SILASTIC brand implants (Dow Corning Corp.). In Exp 1, ewes (n = 10) were run through four successive artificial cycles during which a luteal phase level of P was either replaced (cycles 1 and 3) or not replaced (cycles 2 and 4). GnRH and LH secretions were monitored by sampling cerebrospinal fluid (CSF) and jugular blood from 10–35 h after four 30-mm 17β-estradiol SILASTIC implants were inserted sc. CSF could be collected from only four ewes over the four cycles. There was no P-dependent difference in the onset of the GnRH and LH surges, which may have been due to a progressive delay in the surge onsets over the four cycles (by ANOVA, $P < 0.05$). Due to this delay, it was not possible to obtain an accurate estimate of the duration of the GnRH and LH surges in all ewes, but the size of the GnRH surge was always greater when animals had been treated with P, resulting in a significant increase in the maximum ($P < 0.01$) and mean ($P < 0.05$) levels during the surge. In contrast, there was no effect on any parameter of LH secretion. In Exp 2, ewes (n = 10) were run through two artificial estrous cycles during which luteal phase P was either replaced or not replaced, using a cross-over experimental design. CSF was collected from seven ewes over the two cycles. GnRH and LH secretions were monitored from 10–53 h after estradiol administration. As in Exp 1, a clear significant increase in the maximal and mean GnRH levels ($P < 0.05$ for both) was observed during the surge when ewes had been pretreated with P. Again, no changes were observed in LH release during the surge. P priming did, however, delay the onsets of the GnRH ($P < 0.01$) and LH surges ($P < 0.01$). Our data show that the increase in P during the luteal phase of the estrous cycle is essential for the full expression of the positive feedback effect of estradiol in inducing the preovulatory GnRH surge in the ewe. (*Endocrinology* **140:** 165–170, 1999)

THE 16- TO 17-DAY estrous cycle of the ewe is characterized by a long luteal phase (13–14 days) followed by a short follicular phase (1). The change from one phase to another is mainly due to the sequential production of two major steroids by the ovary that exert positive and/or negative feedback on the hypothalamic GnRH pulse generator.

During most of the luteal phase, progesterone (P) produced by the corpus luteum is elevated and powerfully inhibits GnRH secretion (2). After luteolysis, P concentrations fall rapidly to undetectable levels the next day (1). In contrast to the rat (3) and monkey (4), P levels do not increase at the time of the preovulatory LH surge in the ewe (5, 6). Moreover, its continued presence during the follicular phase has been shown to prevent the occurrence of the estradiol-induced GnRH surge even when the quantity of estradiol administered is well above that needed for surge induction (7).

During the follicular phase, following the fall in P, gonadotropin secretion increases, which stimulates estradiol release. The rise in circulating estradiol induces the preovulatory gonadotropin surge, which results from a robust increase in GnRH secretion and pituitary responsiveness to GnRH. This positive feedback effect of estradiol on GnRH secretion has been well characterized in sheep due to the ability to collect hypophyseal portal blood from conscious animals (8). In ewes, both the spontaneous and estradiol-induced LH surges are accompanied by large and sustained increases in GnRH release that coincide with the LH and FSH increases but continue for many hours after gonadotropin levels have returned to baseline (9, 10).

Although the two phases of the ovine estrous cycle have been extensively studied, little attention has been given to the possible long term effect of P priming on the estradiol-dependent mechanisms during the follicular phase. We know, however, that ewes that have not been primed with P during an artificial estrous cycle exhibit a LH surge, but not estrous behavior, after estradiol administration (11). In this regard, the silent ovulation observed at the onset of the breeding season is thought to be due to the absence of a prior luteal phase. More recently, it has been shown that the presence and quantity of P given before estradiol administration delay, in a dose-dependent fashion, the onset of the LH surge (12). Nothing, however, is known about a possible role of P

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in the modulation of the response of the GnRH surge-generating system to estradiol.

Thus, in the present study we sought to determine whether P priming has any effect on the GnRH and LH responses to a surge-inducing estradiol treatment.

Materials and Methods

General

Experiments were performed on sexually mature Ile-de-France ewes that were housed in rooms with natural photoperiod, had free access to water and were fed hay, straw, and corn daily. Surgical implantation of guide tubes for collection of cerebrospinal fluid (CSF) from the third ventricle was performed aseptically under general anesthesia, according to procedures described in detail previously (13). In all experiments, ewes were restrained so that they could not turn around but were able to move forward and backward. To prevent the stress of social isolation, ewes were always in contact with other sheep.

CSF and blood collection

CSF collection was performed using a modification (14) of the procedure described by Skinner *et al.* (13). This method accurately relates GnRH changes occurring in hypophyseal portal blood at the time of the surge. CSF samples (~1.8 ml/h) were collected into tubes containing 3 ml methanol, and the total volume for each sample was recorded to calculate the concentration of GnRH per ml CSF for each ewe. After proteins were extracted by centrifugation, the supernatant was poured into a glass tube and dried in a vortex evaporator (Savant Instruments, Inc., Farmingdale, NY). Extracted samples were stored at −20 C until assayed for GnRH. To collect simultaneous iv blood samples, a catheter was inserted into the jugular vein. Samples were taken instantaneously at the end of a CSF-sampling period, centrifuged (20 min; 1500 × *g*; 4 C), and the plasma was stored at −20 C until assayed for LH.

Experimental design

Exp 1. This study was started at the beginning of the anestrous season (February). In mid-December, 10 ewes were implanted with guide cannula for collection of CSF. Two months later, these animals were ovariectomized and immediately run through artificial estrous cycles by manipulation of peripheral estradiol and P implants as previously described (15).

On the day of ovariectomy, animals were treated immediately with an intravaginal controlled internal drug P-releasing device (InterAg, Hamilton, New Zealand) and a 10-mm SILASTIC brand implant (Dow Corning Corp., Midland, MI) sc containing estradiol to simulate the steroidal milieu of the midluteal phase of the estrous cycle. After 12 days, the P implants were removed to simulate luteal regression, and 16 h later four 3-cm estradiol implants were inserted. This treatment raises circulating estradiol concentrations to a peak follicular phase level (16) and reliably induces a preovulatory-like surge of GnRH and LH in this model. CSF and blood samples were taken hourly for 25 h, starting 10 h after insertion of the four estradiol implants. Two days later, the four 3-cm estradiol implants were removed, and the ewe was run through three further 16-day cycles. However, in the second cycle, the P implants were not inserted, so that animals did not receive P during the 12-day period corresponding to the artificial luteal phase. The third cycle was identical to the first cycle, and the fourth cycle was identical to the second.

Exp 2. The first experiment revealed that P pretreatment induced higher concentrations of GnRH in the CSF after the estradiol challenge. However, as a progressive delay in the onset of the surges occurred over the 4 cycles and due to the length of the GnRH surge, only a portion of the GnRH surge could be analyzed during the observation period. To determine the effects of P on all parameters of the estradiol-induced GnRH surge, in a second experiment conducted during the second half of the anestrous season (May–June), 10 ewes were ovariectomized and immediately run through 2 artificial estrous cycles using a cross-over design. The 16-day artificial estrous cycles were similar to those described for Exp 1. P implants were inserted into 5 ewes during the artificial luteal phase of first cycle, and the other 5 ewew received no P implants. These treatments were reversed for the second cycle. After 12 days, the P implants were removed, and 16 h later, 4 3-cm estradiol implants were inserted. CSF and peripheral blood were collected hourly for 43 h, starting 10 h after insertion of the estradiol implants in both cycles.

Hormone assays

CSF samples were assayed for GnRH after extraction using the method of Caraty *et al.* (17). Buffer (500 μl) was added to the dried extract, from which two 100-μl aliquots were used to determine the GnRH concentration. All samples from an experiment were measured in duplicate in the same assay, and the intraassay coefficient of variation averaged 15% (six assays).

Blood samples were assayed for LH in duplicate 100-μl aliquots of plasma using the RIA method of Pelletier *et al.* (18) as modified by Montgomery *et al.* (19). All samples from an experiment were run in a single assay. The intraassay coefficient of variation averaged 9%, and assay sensitivity was 0.16 ± 0.05 ng/ml (four assays) standard 1051-CY-LH (*i.e.* 0.31 ng/ml NIH LH-S1).

Data analysis

Exp 1. The onset of the GnRH or LH surge was defined as the first LH or GnRH sample to exceed the presurge baseline by 3SD and remain elevated for at least 2 h, and is expressed relative to the time of estradiol insertion. The presurge baseline and SD were calculated from the samples collected for the first 4 h of the experiment. The amplitude of a surge was taken as the mean of the three consecutive highest values after surge onset. To obtain an estimate of the duration of the LH surge, the period between the onset and the time when a sample fell below half the surge amplitude was calculated. As the GnRH surge did not descend below half-maximal levels in several cycles, we could not make an accurate estimate of the duration of the GnRH surge. The mean LH concentration during the surge was estimated from between the onset of the surge and the half-surge amplitude. For GnRH, only the mean level of the first 5 h after the onset of the surge was calculated. In the event that no surge occurred during the collection period (observed for LH in one animal during one of the four cycles), the onset of the LH surge was set to hour 35 (the length of the collection period), the duration to 0, and the maximum value and mean level to 2.1 ng/ml (equivalent to the basal level). All surge parameters were statistically compared using two-factor (within = time, between = treatment), repeated measures ANOVA.

Exp 2. The onset and maximal concentration of the GnRH or LH surges were calculated as described for Exp 1. In this experiment, due to the longer sampling period, the duration of a surge was defined (for both GnRH and LH) as the period between surge onset and the time taken for hormone levels to fall by 75% from the surge maximum level. The mean concentration during the surge was estimated from the mean of all values over the duration. The time of onset and the duration of the surges were statistically compared using Student's paired *t* test. The mean and maximum levels of GnRH and LH data were statistically compared using the Wilcoxon signed rank test. A between-treatment coefficient of correlation was also determined for these parameters.

Results

Exp 1

Over the four cycles, CSF collection was completed successfully in only 4 of the 10 animals in this experiment. The absence of CSF flow at the opening of the guide cannula or irregular flow during the collection led us to discard some animals from the study (1 ewe was never sampled, 3 were sampled only once, 1 was sampled twice, and another was sampled 3 times). Thus, calculations reported here are for only 4 ewes. In 3 of these ewes, estradiol administration repeatedly induced a surge of GnRH and LH over the 4 cycles (Fig. 1). However, in 1 ewe (no. 080), no surge or increase in LH secretion was associated with a slight, but significant,

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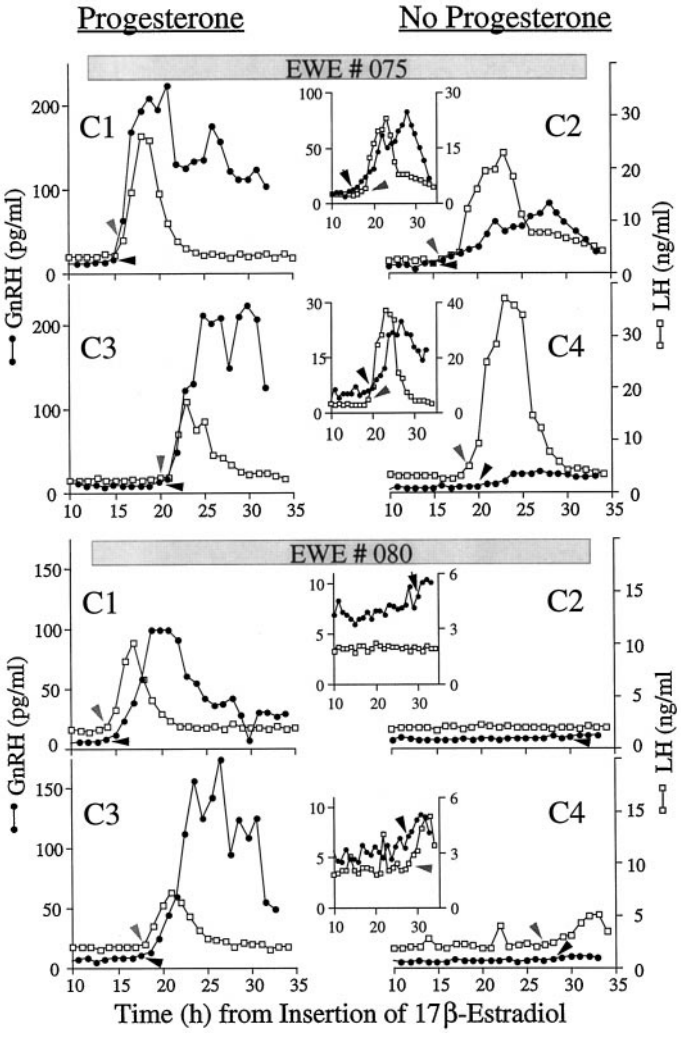

FIG. 1. Representative patterns of GnRH secretion in the CSF (*closed circles*) and LH secretion in jugular blood (*open squares*) for two ewes sampled over four successive artificial cycles (C1 to C4). Graphs on the *left* of the figure depict cycles in which P treatment was given, and graphs on the *right* are cycles in which no P was administered. The *insets* give GnRH and LH secretion profiles of cycles without P pretreatment, with an expanded *y*-axis. *Arrows* indicate the onset of the GnRH (*solid*) and LH (*shaded*) surges.

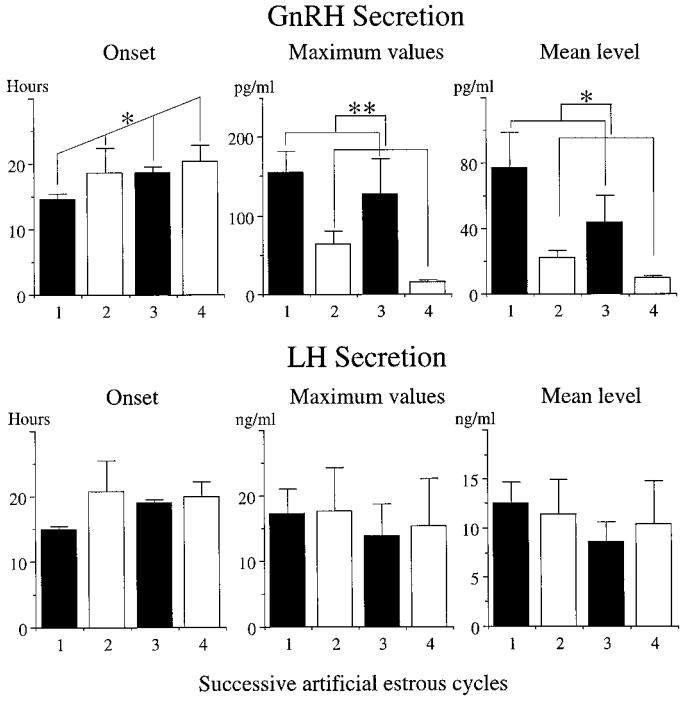

FIG. 2. Mean (±SEM; n = 4) values for the time of onset, maximum values, and level of hormone release during the estradiol-induced GnRH and LH surge with (*solid bars*) or without (*open bars*) P pretreatment during the four artificial estrous cycles. *, $P < 0.05$; **, $P < 0.01$.

increase in GnRH secretion observed at the end of the collection period of cycle 2.

The magnitude of the GnRH surge was always larger when animals had been pretreated with P (Fig. 1). This was associated with a significant increase in maximal ($P < 0.01$) and mean ($P < 0.05$) GnRH concentrations (Fig. 2). In contrast, there was no effect of P priming on any parameter of LH secretion. As illustrated (Fig. 1), when P priming was absent, a LH surge of full amplitude was induced even when the surge of GnRH was smaller. The *insets* with expanded scales in Fig. 1 show the coincident increase in GnRH and LH for these cycles. ANOVA revealed that the time of onset of the GnRH surge increased progressively ($P < 0.05$) among the four cycles (14.7, 18.5, 18.7, and 20.4 h for cycles 1–4, respectively). There was also a trend toward a progressive delay in the onset of the LH surge. If the onset value of the animal having no LH surge during cycle 2 (set at 35 h) is removed from the calculation, the mean LH onset was 15.3, 16, 19.3, and 20 h for cycles 1–4, respectively. The duration of the LH surges was not different among cycles (6.6 ± 1.2, 6.0 ± 2.1, 7.5 ± 1.3, and 8.0 ± 0.4 for cycles 1–4, respectively).

When data (n = 6) from the first two cycles were analyzed, using Student's paired *t* test, the increase in the magnitude of the GnRH surge when animals had been pretreated with P was even more significant [maximum GnRH, 130.6 ± 23.1 *vs.* 52.2 ± 16.4 pg/ml ($P < 0.001$); mean GnRH, 45.7 ± 14.0 *vs.* 15.98 ± 1.1 pg/ml ($P < 0.05$)]. Again, no effect of P priming on any parameter of LH secretion was evident.

Exp 2

From the 10 ewes in this experiment, CSF was collected successfully from 7 animals over the 2 cycles (1 not sampled and 2 sampled only once). As in Exp 1, a significant increase in the magnitude of the GnRH surge was observed when ewes had been pretreated with P (Fig. 3). This results in a significant increase in maximal (difference in maximum, 40.3 ± 9.6 pg/ml; $P < 0.05$) and mean (difference in mean, 31.2 ± 12.2 pg/ml; $P < 0.05$) concentrations of GnRH (Fig. 4), but not in the duration of the surge. P pretreatment significantly delayed the onsets of both the GnRH (difference in onset, 2.4 ± 0.6 h; $P < 0.01$) and LH (difference in onset, 2.9 ± 0.5 h; $P < 0.01$) surges. No differences were observed in either the maximal or mean LH concentrations during the surge. Between treatments, a significant intraanimal correlation was observed for the onset of the GnRH surge ($P < 0.05$), the onset of the LH surge ($P < 0.01$), the GnRH maximum values

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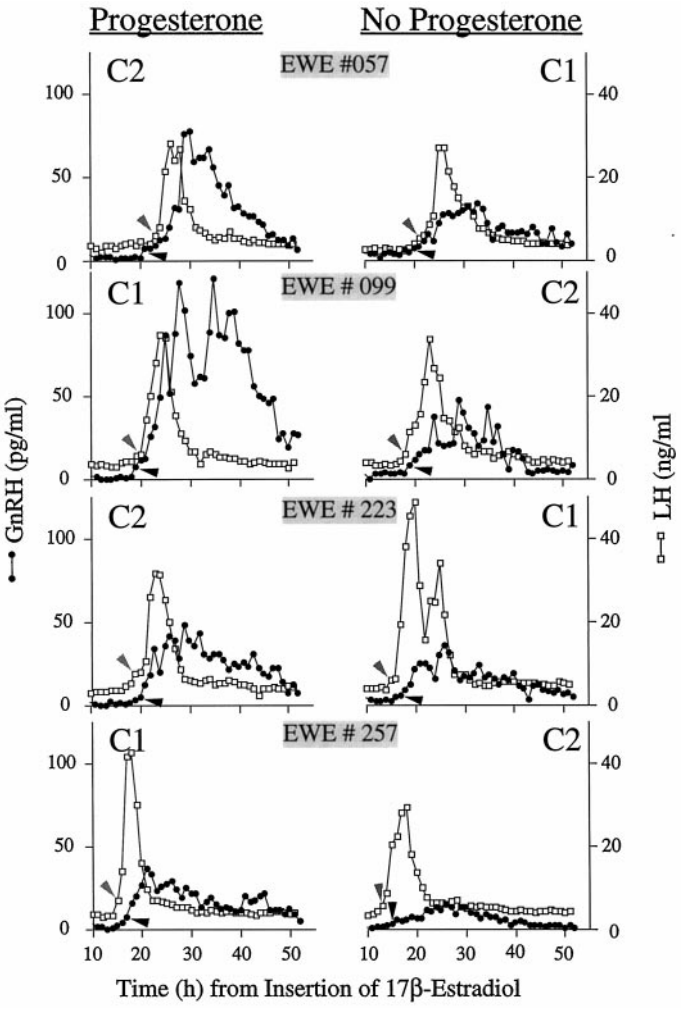

FIG. 3. Representative patterns of GnRH secretion in the CSF (*closed circles*) and LH secretion in jugular blood (*open squares*) for four ewes sampled over two successive artificial cycles (C1 and C2). Graphs on the *left* of the figure depict cycles in which P treatment was given, and graphs on the *right* are cycles in which no P was administered. *Arrows* indicate the onset of the GnRH (*solid*) and LH (*shaded*) surges.

($P < 0.01$), and the GnRH mean level ($P < 0.01$) during the surge.

Discussion

Our study shows for the first time that P priming is an important requirement for the full expression of the positive feedback action of estradiol on GnRH secretion in the ewe. Moreover, these data confirm recent suggestions that not all GnRH secreted during the GnRH surge is necessary to induce a full amplitude LH surge (20).

P priming greatly increased the size of the estradiol-induced GnRH surge in both experiments. Moreover, this increase in the magnitude of the GnRH surge occurs in the absence of any modification of its duration, suggesting that during a similar period, the neuronal GnRH system is able to release much more GnRH. A delay in the onset of both the GnRH and LH surges is also observed when animals have been pretreated with P. This concurs with several studies showing that the absence of P pretreatment causes an earlier

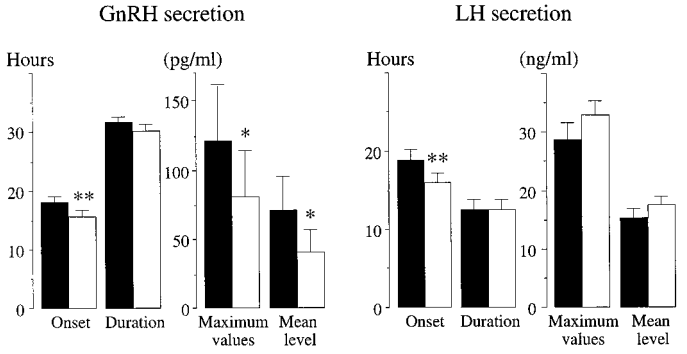

FIG. 4. Mean (±SEM; n = 7) values for the time of onset, duration, maximum values, and level of hormone release during the estradiol-induced GnRH and LH surge with (*solid bars*) or without (*open bars*) P pretreatment. *, $P < 0.05$; **, $P < 0.01$.

LH surge (11, 12). There was, however, no difference in the surge onsets in Exp 1, which may have been due to the progressive delay in the onset of the surges over the four cycles and because a cross-over experimental design had not been used. Moreover, as this experiment started at the transition period between the breeding season and anestrus, a seasonal change in sensitivity to estradiol could have had an effect. Indeed, a similar trend in the LH surge onsets after repeated artificial cycles has been reported during the early and late breeding seasons (15).

How does P induce a greater release of GnRH with a similar estradiol challenge? As P is cleared very quickly after removal of the P implants (21, 22) and because P is able to block the estradiol-induced GnRH surge (7), a direct effect of the steroid in augmenting the response of the GnRH system to estradiol is unlikely. Rather, it is probable that the differences found in our study are due to the long term exposure of neurons to P. In this regard, P reduces GnRH pulse frequency during the luteal phase of the cycle (23) and in ovariectomized ewes (24). Thus, the decreased amount of GnRH secreted during the luteal phase may contribute to an increase in the quantity of peptide available for release at the time of the surge. In support of this hypothesis, the content of GnRH in the preoptic area is highest during the luteal phase of the ovine estrous cycle (25), and luteal phase ewes appear to have higher levels of GnRH messenger RNA than anestrous animals (26). In the rat, the hypothalamic GnRH content is highest during diestrus (27), and P administration to ovariectomized female rats increases hypothalamic GnRH concentrations to levels found in intact animals (28). Alternatively, P pretreatment may affect the threshold of sensitivity to estradiol. In this respect, one ewe (no. 080) in Exp 1 is of particular interest. For the two cycles with P priming, a robust surge of GnRH was observed in the CSF after the estradiol challenge, whereas in the other two cycles, when P priming was absent, only a moderate or a very slight rise in GnRH was observed. Such an absence of response to the given estradiol challenge is surprising, as the estradiol level achieved by the sc implants is similar to those reported during the late follicular phase of the estrous cycle (22, 29) and reliably induces surges of GnRH and LH in a follicular phase model (10, 16). It is possible that this animal was relatively insensitive to estradiol. A large variability in GnRH

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pulsatility in response to a very low level of estradiol has been reported during anestrus (30), and thus, a similar range in sensitivity could exist between animals regarding the estradiol level required to induce a surge. Nevertheless, this clearly indicates that P priming reduces the threshold at which estradiol stimulates the GnRH surge system. One way in which P could affect the sensitivity to estradiol would be to increase the number of estradiol-responsive cells. Indeed, P treatment increases the number of estradiol receptors in the mediobasal hypothalamus (31). As the mediobasal hypothalamus is a major site of action of estradiol in inducing the preovulatory GnRH surge in the ewe (32), increasing the number of estrogen-responsive cells may augment the ability of the positive feedback system to respond to the estradiol signal. Thus, two mechanisms, which may not be mutually exclusive, could explain how P increases GnRH release: one increasing the peptide store in the GnRH neuron, and the other increasing the responsiveness of the system to estradiol.

What could be the role of this massive secretion of peptide in the CSF during a P-primed estradiol-induced GnRH surge? It is possible that this excess of GnRH has no particular function and merely represents a mirror image of GnRH release in portal blood; the quantity of GnRH secreted in a normal cycle is well above the quantity needed to induce a full amplitude LH surge as a safeguard to ensure that ovulation will occur at the right time (20). However, this excess of GnRH may have some function other than stimulating gonadotropin release. A possible role of CSF GnRH in regulating its own secretion is unlikely, because we have already shown that it does not modulate GnRH/LH release (33). CSF GnRH may be involved, however, in the regulation of sexual behavior. Although it is has been shown that the duration and intensity of estrous behavior are dependent on the concentration of estradiol (34), the silent ovulation that is observed at the onset of the breeding season is thought to be due to the absence of luteal phase P exposure. Furthermore, ewes that have not been primed with P before estradiol administration do not exhibit estrous behavior (11). It is also worth noting that during the natural estrous cycle, peripheral estradiol concentrations decrease before or coincident with the termination of the LH surge, while sexual receptivity continues for some hours (1). Interestingly, as shown here and in other studies (9, 10, 13), GnRH secretion outlasts the LH surge by several hours, and in ovariectomized ewes treated with P and estradiol, the period of estrous behavior coincides quite closely with the period of increased GnRH release (35). Certainly, the CSF provides a route through which GnRH may reach behavioral centers. In several species, sexual behavior can be evoked by the central administration of GnRH (36, 37), and GnRH receptors have been located in neural areas, such as the hippocampus (38), that have been implicated in the generation of sexual behavior. Thus, our observation that P significantly increases the amplitude of the estradiol-induced GnRH surge reopens the hypothesis that GnRH could participate in the expression of estrous behavior in the ewe; studies to test this hypothesis are in progress.

Although P has a strong effect on GnRH surge amplitude, it did not affect the generation of the LH surge. This is not surprising because previous experiments using an indirect approach indicate that only a portion of the GnRH surge is necessary to induce a full amplitude LH surge (20). Thus, LH surge amplitude does not appear to be a reliable index of the magnitude of the GnRH surge, and caution is necessary when extrapolating LH surge data to reflect GnRH changes. Our data may also explain why in some earlier studies, large increases in GnRH release during surges induced by estradiol alone were not reported in long term ovariectomized ewes (39).

Our result, indicating that only a small portion of the GnRH surge is needed for the full expression of the LH surge, is also interesting in terms of the question of whether the GnRH surge has a permissive or a deterministic role in the generation of the preovulatory surge of LH. These two theoretical models for how GnRH may participate in eliciting the preovulatory LH surge have been described in detail previously (40). The deterministic model holds that increased GnRH secretion is required to drive a LH surge, whereas the permissive model postulates that no changes in GnRH are needed, but that there is an increase in sensitivity to GnRH at the level of the pituitary. The large surge of GnRH observed during the natural estrous cycle favors the deterministic model in sheep (9). However, although the permissive and deterministic models differ in a fundamental sense, it has been shown using an indirect approach that only a small portion of the GnRH surge appears to be needed for a full LH surge (20), suggesting that the quantitative difference between the two models may be subtle. Our data suggest that this could exist in some physiological circumstances, such as when the luteal phase is missing at the onset of puberty or at the onset of the breeding season when a full LH surge may occur despite a possibly modest GnRH increase.

This demonstration of a role of P on the amplitude of the GnRH surge induced by estradiol has been possible by the use of the third ventricular CSF collection technique. The hypophyseal portal technique precludes such an investigation because not only is the GnRH surge amplitude highly variable between ewes (8–10), but portal blood cannot be collected from animals more than once, as levels decrease in successive collections (8). Although some limitations still remain as to whether animals can be sampled repeatedly with the CSF approach, animals can be used at least twice with reasonable success. Moreover, the high coefficient of correlation for GnRH values between the two cycles for each animal stresses the importance of using animals as their own controls.

In summary, our study demonstrates that P priming induces qualitative changes in the response of the positive feedback effect of estradiol on GnRH secretion by enhancing the magnitude of the GnRH surge. This effect, which is dependent on prior exposure to P, is not transduced to the pituitary, as no increase in the magnitude of the LH surge was observed. The role of this increase in GnRH release in the CSF as a possible modulator of estrous behavior in sheep remains to determined.

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