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Regulation of Amounts of mRNA for GnRH Receptors by Estradiol and Progesterone in Sheep

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Expression of GnRH receptors increases prior to the onset of the preovulatory surge of LH in sheep. Two experiments were conducted to investigate the interactions of progesterone (P) and estradiol (E) on amounts of mRNA for GnRH receptors and the number of receptors for GnRH. The first study was designed as a 2 × 2 factorial arrangement of treatments to investigate effects of removal of P and the presence of E. Ewes that had been ovariectomized (OVX) for at least 4 wk received one silastic implant containing E and two silastic implants containing P for 6 d to mimic concentrations of these steroids during the luteal phase of the estrous cycle. Anterior pituitary glands were collected (n = 4 animals/group):

1. Prior to implant removal and 12 h after removal of:
2. P only.
3. E only.
4. P and E.

Regardless of whether or not E was present, amounts of mRNA for GnRH receptors ($P = 0.87$) and number of GnRH receptors ($P = 0.43$) were not different within 12 h after removal of P. In the second experiment, ewes were OVX on d10–12 of the estrous cycle (d0 = estrus), and immediately received silastic implants containing E and P as described above. Anterior pituitary glands were collected on d12 of the estrous cycle ($n = 5$), prior to implant removal ($n = 5$), and from the remaining ewes 24 h after removal of P only ($n = 7$) or removal of P and E ($n = 6$). Relative amounts of mRNA for GnRH receptors and the number of GnRH receptors were similar ($P > 0.05$) on d12 of the estrous cycle and prior to implant removal. Removal of both P and E did not affect ($P > 0.05$) amounts of GnRH receptor mRNA or number of GnRH receptors. However, the removal of P and the presence of E increased ($P < 0.05$) amounts of mRNA for GnRH receptors, but did not affect ($P > 0.05$) the number of GnRH receptors. We conclude that increased amounts of GnRH receptor mRNA require the removal of P and the presence of E.

Key Words: Estradiol; GnRH receptors; mRNA; progesterone; sheep.

Introduction

The interaction of GnRH with its receptor in pituitary gonadotropes signals the stimulation of synthesis and secretion of LH and FSH. Endocrine regulation of expression of GnRH receptors may, therefore, be an important step in the control of reproduction. Using exogenous prostaglandin $F_{2\alpha}$ to regress the corpus luteum and synchronize the onset of the follicular phase, Crowder and Nett *(1)* reported that the number of GnRH receptors were relatively low during the luteal phase of the estrous cycle and increased prior to the onset of the preovulatory surge of LH. Increased numbers of GnRH receptors are thought to be required for manifestation of the preovulatory surge of LH. A subsequent study *(2)* with a similar animal model revealed that relative amounts of mRNA for GnRH receptors increased within 12 h and number of GnRH receptors increased within 24 h after injection of prostaglandin $F_{2\alpha}$ on d11 or 12 of the ovine estrous cycle. Endocrine changes in response to prostaglandin $F_{2\alpha}$ include decreased serum concentrations of progesterone (P) within 12 h *(2,3)*, and increased serum concentrations of estradiol (E) within approx 24 h *(2)*. Thus, increased expression of GnRH receptors prior to the preovulatory surge of LH could potentially be regulated:

1. Negatively by P.
2. Positively by E.
3. Negatively or positively by some other ovarian factor(s).

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There is evidence that P inhibits expression of GnRH receptors in sheep. Using primary cultures of ovine pituitaries, P decreased the sensitivity of gonadotropes to GnRH *(4)*, reduced the binding of GnRH *(5),* and decreased amounts of mRNA for GnRH receptors *(6)*. In contrast, chronic administration of P to ovariectomized (OVX) ewes did not alter the number of GnRH receptors compared to OVX *(7,8)* or ovary-intact ewes *(7)*. The effects of P on GnRH receptors are difficult to evaluate because responsiveness to P may be dependent on previous exposure to E *(9,10)*. In fact, E increased P binding and amounts of mRNA for P receptors in sheep pituitaries *(11,12)*.

During the follicular phase of the estrous cycle, ovarian follicles begin to grow and develop, resulting in increased secretion of E *(13)* and inhibin *(14)*. Effects of inhibin on expression of GnRH receptors in sheep are controversial. In primary cultures of ovine anterior pituitary glands, inhibin either inhibited the number of GnRH receptors *(15)* or stimulated the number of GnRH receptors *(5)* and expression of GnRH receptor mRNA *(6)*. In contrast, administration of bovine follicular fluid (as a source of inhibin) to OVX ewes did not affect amounts of GnRH receptor mRNA or number of GnRH receptors *(16)*.

Results from numerous studies indicate that E upregulates expression of GnRH receptors. E acts directly at the anterior pituitary gland to increase the number of GnRH receptors in vivo *(17)* and in vitro *(7,18,19)*. E also stimulated amounts of mRNA for GnRH receptors in vivo *(2,20,21)* through a direct action at the anterior pituitary gland *(22)* and in vitro *(6)*. Although high levels of exogenous E increased pituitary responsiveness to GnRH in anestrous ewes *(23)*, recent studies indicate that concentrations of E common to the follicular phase of the ovine estrous cycle (i.e., 3–6 pg/mL) are sufficient to enhance expression of GnRH receptors in ovariectomized ewes *(21)*.

To investigate further the interactions of E and P in regulating expression of GnRH receptors, two experiments were conducted to investigate the effects of removal of P with and without the presence of E. Our hypothesis was that the presence of low levels of E is necessary to upregulate expression of GnRH receptors following the removal of P.

Results

Experiment 1

Serum concentrations of P resulting from silastic implants averaged 1.9 ng/mL and were not different ($P > 0.05$) across treatments. Within 12 h after removal of P implants, serum concentrations of P were similar ($P > 0.05$) in ewes with (0.7 ± 0.1 ng/mL; mean ± SEM) or without (0.8 ± 0.2 ng/mL) E implants. Prior to removal of P and/or E, numbers of LH pulses and mean concentrations of LH were similar ($P > 0.05$) between treatments (Table 1). Removal of P resulted in increased ($P < 0.05$) frequency of LH pulses and mean concentrations of LH; however, removal of P implants for 12 h did not affect ($P > 0.05$) the amplitude of LH pulses. Removal of P implants increased ($P < 0.05$) mean concentrations of LH compared to that measured before P removal.

Northern blot analysis revealed four GnRH receptor transcripts in individual pituitaries. A representative Northern blot of GnRH receptor mRNA from one ewe in each treatment is shown in Fig. 1. There were no differences ($P > 0.05$) in transcript size or abundance between treatments. Amounts of mRNA for cyclophilin in the ovine pituitary gland were not affected ($P > 0.05$) by P alone or in combination with E (data not shown). In ewes that had been OVX for at least 4 wk and treated with ovarian steroids for 6 d, regardless of whether or not E was present, the relative amounts of GnRH receptor mRNA were not affected ($P = 0.87$) by removal of P (Fig. 2). There were also no effects ($P = 0.43$) of steroid removal on the number of GnRH receptors (Fig. 2).

Experiment 2

Data regarding the frequency and amplitude of LH pulses are shown in Table 2. Frequency and amplitute of LH pulses were similar ($P > 0.05$) on d10–12 of the estrous cycle (frequency = 0.1 pulses/h; amplitude = 0.9 ng/mL) and in OVX ewes treated with E and P for 48 h prior to implant removal (frequency = 0 pulses/h). Thus, data from d 10–12 of the estrous cycle and from ovariectomized ewes prior to implant removal were pooled and are presented as "control." Frequency and amplitude of LH pulses were similar ($P > 0.05$) in control animals and in OVX ewes treated with E and P implants prior to P removal. In addition, removal of P implants did not affect ($P > 0.05$) the frequency or amplitude of LH pulses or mean concentrations of LH compared to that measured before P removal.

Similar to experiment 1, Northern analysis of polyA+ RNA from individual sheep pituitaries revealed four GnRH receptor transcripts that did not differ in size or abundance across treatments (data not shown). Relative amounts of GnRH receptor mRNA were similar ($P > 0.05$) on d 10–12 of the estrous cycle (354.8 ± 70.8 arbitrary densitometric units) and in OVX ewes prior to implant removal (295.3 ± 66.0). In addition, the number of GnRH receptors was similar ($P = 0.39$) on d10–12 of the estrous cycle ($2.3 \pm 1.5 \times 10^{-16}$ mol receptor/μg protein) and in ovariectomized ewes prior to implant removal ($1.7 \pm 0.8 \times 10^{-16}$). Thus, these data were pooled and are presented as "controls." Relative amounts of GnRH receptor mRNA and number of GnRH receptors in controls and after ovariectomy during the luteal phase of the estrous cycle and steroid replacement are shown in Fig. 3. There were no differences ($P > 0.05$) in amounts of GnRH receptor mRNA between control animals and in OVX ewes in which both P and E implants were removed. However, removal of P and the presence of E resulted in increased ($P < 0.05$) amounts of GnRH receptor mRNA within 24 h after P removal (Fig. 3). There were no differences ($P > 0.05$) in number of GnRH receptors across treatments.

Table 1
Frequency and Amplitude of LH Pulses and Mean Concentrations of LH in Long-Term OVX Ewes Before and After Removal of Progesterone (P) and/or Estradiol (E) Implants

	Frequency, pulses/h		Amplitude, ng/mL		Mean, ng/mL	
Treatment	Prior to P removal	After P removal	Prior to P removal	After P removal	Prior to P removal	After P removal
+P+E	0	NA	0	NA	0.1	NA
–P+E	0	1.8[a]	0	1.0	0.2	1.1[a]
+P–E	0.6	NA	1.6	NA	1.0	NA
–P–E	0.8	2.5[a]	2.6	2.6	0.7	1.8[a]

[a]Different ($P < 0.05$) from values obtained prior to P removal.
NA = not applicable.

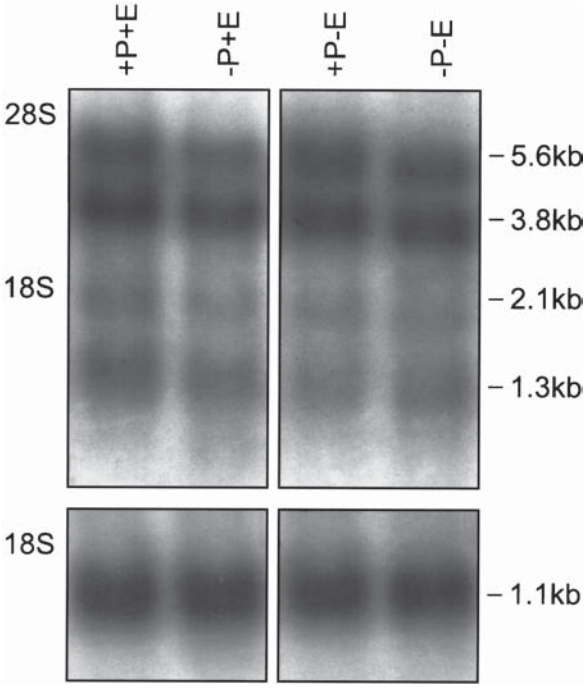

Fig. 1. Analysis of ovine pituitary poly A+ RNA by Northern blot. Five micrograms of polyA+ RNA from individual pituitaries of ewes that had been OVX for at least 4 wk and treated with estradiol (E) and/or progesterone (P) (*see text* for details) were hybridized to ovine GnRH receptor cDNA (top panel). The blot was stripped of radioactivity and reprobed with cyclophilin cDNA (bottom panel). The approximate sizes of GnRH receptor and cyclophilin transcripts are shown on the right and approximate location of 28S and 18S rRNA are indicated on the left.

Discussion

Two experimental models were used to investigate regulated expression of GnRH receptors by E and P. In long-term OVX ewes treated with P and E to mimic the luteal phase of the estrous cycle, removal of P implants to simulate the transition from the luteal to the follicular phase of the estrous cycle did not affect amounts of mRNA for GnRH receptors or number of GnRH receptors at 12 h after steroid removal. The lack of an effect of E and P on GnRH receptor expression in long-term OVX ewes was surprising, because administration of prostaglandin $F_{2\alpha}$ to ovary-intact ewes resulted in decreased serum concentrations of P, increased amounts of GnRH receptor mRNA within 12–48 h, and increased numbers of GnRH receptors within 24–48 h *(1,2,20,24)*. Other investigators *(7,8,25)*, however, were also unable to alter expression of GnRH receptors by chronic administration of P to long-term OVX ewes. One possible explanation for the inability of P to regulate expression of GnRH receptors in long-term OVX ewes is that expression of P receptors requires priming by E *(9,10,26,27)*. Recent studies indicate that E increases P binding sites in gonadotropes *(11)*. In the first study, long-term OVX ewes did not receive E priming prior to treatment with P, which may have resulted in unresponsiveness of the experimental model to P.

A second study was conducted using ewes that were OVX on d10–12 of the estrous cycle and immediately treated with E and P implants to mimic the luteal phase of the estrous cycle. The pattern of LH secretion and amounts of mRNA for GnRH receptors were similar on d10–12 of the estrous cycle and prior to implant removal indicating that the steroid hormone treatment protocol mimicked the luteal phase of the estrous cycle. Removal of P (to mimic luteolysis) accompanied by the presence of low levels of E (similar to those found during the early follicular phase) resulted in increased amounts of GnRH receptor mRNA compared to the luteal phase of the estrous cycle. These studies are the first to describe regulation of GnRH receptor mRNA by P and E, in the absence of other ovarian factors, in sheep.

Interestingly, removal of both P and E did not alter expression of GnRH receptor mRNA compared to the luteal phase of the estrous cycle. Previous studies demonstrated the inhibitory effects of P on expression of GnRH receptors in vitro *(5,6)*. If expression of GnRH receptors in vivo was only regulated in a negative manner by P, amounts of mRNA for GnRH receptors would be expected to increase following the removal of both P and E; this, however, was not the case. Increased expression of GnRH receptor mRNA

Table 2
Frequency and Amplitude of LH Pulses and Mean Concentrations of LH in Control Ewes and in Ewes OVX on d10–12 of the Estrous Cycle and Immediately Implanted with Estradiol (E) and Progesterone (P)

	Frequency, pulses/h		Amplitude, ng/mL		Mean, ng/mL	
Treatment	Prior to P removal	After P removal	Prior to P removal	After P removal	Prior to P removal	After P removal
Control	0.5	NA	0.5	NA	0.4	NA
–P+E	0.3	0.8	0.5	0.6	0.5	0.6
+P–E	0.3	0.8	0.2	0.2	0.4	0.5

NA = Not applicable.

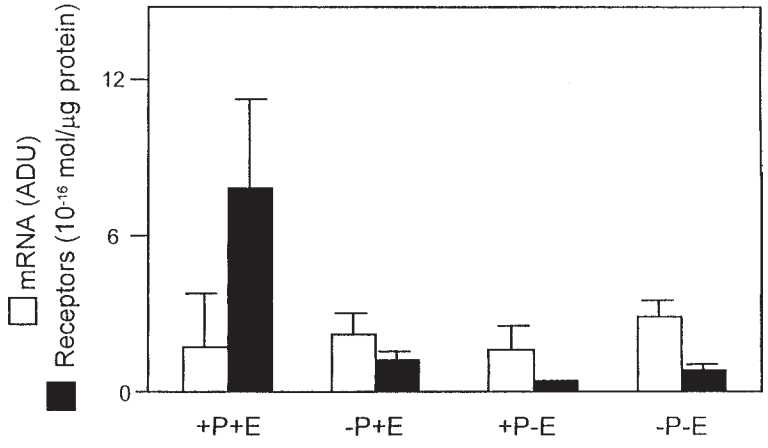

Fig. 2. Effect of E and P on expression of GnRH receptors in ewes that were OVX for at least 4 wk. Amounts of GnRH receptor mRNA are expressed relative to cyclophilin mRNA (arbitrary densitometric units; ADU). Values are mean ± SEM (n = 4/group).

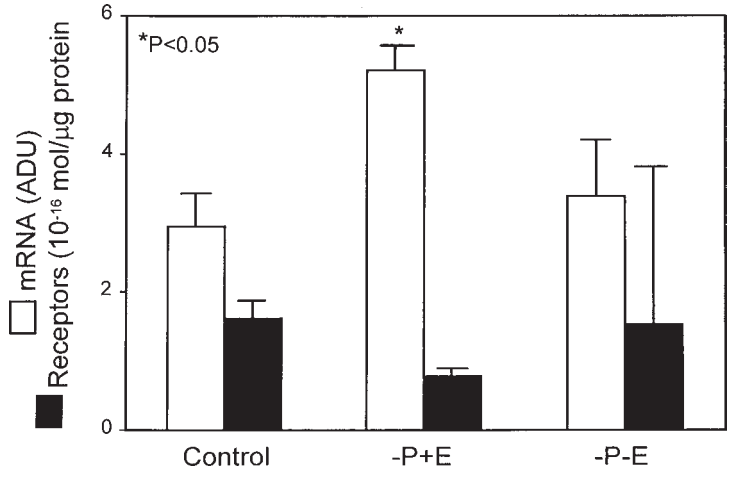

Fig. 3. Effect of E and P on expression of GnRH receptors following ovariectomy on d 10–12 of the estrous cycle (d0 = estrus) and treatment with ovarian steroids for 48 h. Amounts of GnRH receptor mRNA are expressed relative to cyclophilin mRNA (arbitrary densitometric units; ADU). Control (n = 10) consists of animals from d10–12 of the estrous cycle (n = 5) and 48 h of E and P treatment to ewes that were OVX on d10–12 of the estrous cycle (n = 5; *see text* for details). Values are mean ± SEM.

was only detected when P was removed and E was present. Thus, increased expression of GnRH receptors in vivo appears to require the removal of negative regulation by P and the stimulatory presence of E. Other ovarian factors do not appear to play a significant role in regulating expression of GnRH receptor mRNA during the follicular phase of the estrous cycle in sheep. These results are consistent with previous studies in which increased expression of GnRH receptors occurred when only E was replaced following ovariectomy on d 11–14 of the ovine estrous cycle

(20,21). Thus, it is concluded that E must be present to increase expression of GnRH receptors during the transition from the luteal to the follicular phase of the estrous cycle. The ability of E to increase expression of GnRH receptors through a direct action at the anterior pituitary gland in sheep has been consistently demonstrated in vivo *(17)* and in vitro *(18,19,28)*. Interestingly, the stimulatory effects of E were not able to override the inhibitory effects of P on expression of GnRH receptor mRNA in this study or in a previous study *(24)*.

After ovariectomy on d 10–12 of the estrous cycle, the number of GnRH receptors was not altered by insertion and removal of E and P implants to mimic endocrine changes associated with the transition from the luteal to the follicular phase of the ovine estrous cycle. These results were surprising, since previous studies *(1,2,20,29)* detected an increase in the number of GnRH receptors within 16–48 h after the removal of P by administration of prostaglandin $F_{2\alpha}$ or ovariectomy. A possible explanation for the discrepancy in these results is that animals in the current study received both P and E implants immediately after ovariectomy. The presence of P in combination with E after ovariectomy may have shifted the time-course for upregulation of GnRH receptors. In a previous study *(2)*, numbers of GnRH receptors increased approx 12 h after an increase in GnRH receptor mRNA, an interval that may reflect the time necessary for increased steady-state amounts of mRNA to be translated and functional GnRH receptors to be inserted into the membrane of gonadotropes. Expression of GnRH receptors appears to be regulated, at least in part, at the level of new protein synthesis. Actinomycin-D and cycloheximide prevented upregulation of GnRH receptors in primary cultures of ovine pituitary cells *(18)*. Because expression of GnRH receptors was only investigated at 24 h after removal of P and/or E, we speculate that pituitaries collected at a later time (i.e., 36 h) may have contained an increased number of GnRH receptors.

In summary, in long-term OVX ewes treated with E and P, amounts of GnRH receptor mRNA did not change within 12 h of steroid removal. However, when ovariectomy was performed on d 10–12 of the estrous cycle and E and P implants were immediately inserted to mimic the luteal phase, removal of P and the presence of E resulted in increased amounts of mRNA for GnRH receptors within 24 h after steroid removal. These results support the hypothesis that removal of P and the presence of E are necessary endocrine events to upregulate expression of GnRH receptors.

Materials and Methods

Animals

Experiment 1

All studies with animals were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Mature, white-faced ewes of mixed breeds common to the Western US, which had been OVX for at least 4 wk were utilized in this study. Silastic (Dow Corning Co., Midland, MI) implants containing crystalline P (Sigma Chemical Co., St. Louis, MO) or E 17-β (E; Sigma Chemical Co.) were constructed as described *(12,30)*. Ewes received two implants containing P and one implant containing E to mimic hormone concentrations found during the luteal phase of the estrous cycle (approx 2 ng P/mL serum and 3–6 pg E/mL serum). After 5–6 d of steroid treatment, ewes were then assigned at random to groups in a 2 × 2 factorial arrangement of treatments as follows:

1. No implant removal (+P+E).
2. Removal of P only (–P+E).
3. Removal of E only (+P–E).
4. Removal of both P and E (–P–E).

Blood samples were collected every 15 min for 4 h prior to removal of P and 4 h prior to tissue collection. Pituitaries were collected 12 h after implant removal, cut along the midsagittal plane and immediately frozen at –70°C.

Experiment 2

Ewes were synchronized to a common day of estrus using two injections of prostaglandin $F_{2\alpha}$ (Lutalyse; Upjohn, Kalamazoo, MI; 7.5 mg/injection; two injections given 4 h apart) at 11-day intervals. Estrus was detected with a vasectomized ram (d0 = estrus). Ewes were ovariectomized on d 10–12 of the estrous cycle and immediately implanted with silastic implants containing P and E to mimic luteal-phase concentrations of these hormones as described in experiment 1. All ovariectomized ewes received steroid implants for 48 h; P implants were then removed to simulate the end of the luteal phase. Anterior pituitary glands were collected:

1. From ovary-intact ewes on d10–12 of the estrous cycle (n = 5) and from ovariectomized ewes:
2. After 48 h of treatment with P and E, prior to implant removal (n = 5).
3. 24 h after removal of P only (-P+E; n = 7).
4. 24 h after removal of P and E (-P-E; n = 6).

Immediately prior to tissue collection, blood samples were collected at 15-min intervals for 8 h from ewes on d 10–12 of the estrous cycle and from ovariectomized ewes treated with E and P for 48 h (prior to implant removal). In the remaining animals, blood samples were collected every 15 min for 4 h prior to implant removal and for 24 h after implant removal.

mRNA Analysis

Total cellular RNA was isolated from half of each pituitary using a modification of the one-step method *(31)* and Trizol (Gibco-BRL, Gaithersburg, MD). Polyadenylated RNA (poly A+) was isolated from total cellular RNA using oligo dT cellulose chromatography (Qiagen, Santa Clarita, CA). Integrity of mRNA was determined by Northern blot analysis. Five micrograms of polyA+ RNA from individual

pituitaries were separated by electrophoresis through a 1.5% denaturing agarose gel, transferred to nylon filters (Duralon; Stratagene, LaJolla, CA), and cross-linked to the nylon filter by UV irradiation in a Stratalinker (Stratagene). Changes in GnRH receptor mRNA were quantitated by slot-blot analysis *(2,6,20)*. One microgram of polyA+ mRNA was denatured in 100 m*M* NaOH + 1 m*M* EDTA, applied to a nylon filter (Zeta Probe; Bio-Rad, Hercules, CA) on slot blots, and crosslinked to the nylon filter by UV irradiation as described above. RNA from animals in both experiments 1 and 2 were analyzed on the same slot blot. Using a ^{32}P-labeled ovine GnRH receptor cDNA probe *(2,20),* blots were hybridized overnight at 45°C and washed in a final solution of 0.1X SSC + 0.1% SDS at room temperature. Bands of [^{32}P]-cDNA:mRNA heteroduplexes were visualized and quantitated with a densitometer (Molecular Dynamics, Sunnyvale, CA). Blots were stripped of radioactivity by boiling for 30 min in a solution of 0.1X SSC + 0.1% SDS and rehybridized with a cDNA probe encoding cyclophilin *(32)* to correct for equal loading of RNA. Results are expressed as relative amounts of GnRH receptor mRNA per unit of cyclophilin mRNA (ADU GnRH receptor/ADU cyclophilin; *20*).

Radioimmunoassays

Serum concentrations of LH and P were determined by radioimmunoassay. The reference preparation for LH was NIADDK-ovine LH-1-3 as tracer and NIADDK-ovine LH-25 as standard. P concentrations in unextracted serum were determined with a DPC Coat-a-Count kit (Diagnostic Products, Inc., Los Angeles, CA; *33*). The intra-assay and interassay coefficients of variation were 11 and 12%, respectively, for LH. P samples were analyzed in a single assay with an intra-assay coefficient of variation of 16.9%.

GnRH Receptor Assays

Numbers of GnRH receptors were determined in a single standard curve radioreceptor assay using a partially purified membrane fraction prepared from ovine pituitaries *(34)*. D-Ala^6-Des- Gly^{10}-GnRH-ethylamide was obtained from Sigma Chemical Company. Protein was quantitated on half of each pituitary (Bio-Rad DC Protein Assay; Bio-Rad; *35*).

Statistical Analysis

Data were analyzed by analysis of variance and Duncan's New Multiple-Range test *(36)*. Pulses of LH were identified using the Cluster Analysis Program *(37)*. In experiment 2, data collected on d 10–12 of the estrous cycle and from OVX ewes prior to implant removal were not different ($P > 0.05$) by analysis of variance; therefore, data from these animals were pooled and are presented as "control."

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