Sexual Differentiation of the Surge Mode of Gonadotropin Secretion: Prenatal Androgens Abolish the Gonadotropin-Releasing Hormone Surge in the Sheep[1]

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Abstract

In sheep, the surge mode of gonadotropin secretion is sexually differentiated, i.e. the LH surge is present in the female, but not in the male. The present study tested the hypothesis that sexual differentiation of the LH surge mechanism reflects a sex difference in the pattern of GnRH, and that prenatal androgens abolish the surge mode of GnRH secretion. We monitored the pattern of GnRH secretion in pituitary portal blood after acute treatment with estradiol in gonadectomized postpubertal males (n = 6), females (n = 6), and androgenized females (exposed prenatally to testosterone from day 30–90 in gestation, n = 7). Four capsules, each containing a 30-mm column of estradiol were implanted s.c. into each lamb to produce high physiologic concentrations of the hormone. Beginning 7 h later, portal and peripheral blood samples were collected hourly for 48 h for measurement of GnRH and LH, respectively. All females exhibited a GnRH surge beginning 13.0 ± 0.4 h after estradiol treatment; this was accompanied by an LH surge. By contrast, only one male produced a small surge in GnRH (1.7 pg/min) with a latency of 32 h; a corresponding increase in LH occurred in this male. Likewise, among the androgenized females, only one exhibited GnRH and LH surges which began at about 22 h after estradiol treatment. Some of the androgenized females had sporadic increases in GnRH which were of lower amplitude than in the control females, and were unaccompanied by rises in LH. These findings provide the first direct evidence that the sex difference in the surge mode of LH secretion results from the sexual differentiation of the pattern of GnRH release. The study also suggests that androgens during prenatal development abolish the GnRH surge and subsequently, the generation of the LH surge.

Pituitary gonadotropins are released in both a tonic and a surge mode of secretion. Whereas the tonic mode is under the inhibitory control of gonadal steroids, the surge mode is activated by the stimulatory feedback action of estradiol. The tonic mode of secretion is operative in both sexes, but in some species including the rat (1, 2), guinea pig (3), hamster (4) and sheep (5), the surge is absent in males. This sex difference in the surge mode of secretion has been well-studied, particularly in the rat, and has been an area of considerable interest for some time. That testicular androgens in the neonatal rat organize both the neuroendocrine regulation of gonadotropin secretion and sexual behavior in the adult is firmly established (see 6, for review). When male rats are castrated within the first few days after birth, the surge mode of gonadotropin secretion is present (7) which can induce cyclical changes in vaginal grafts (8) and the formation of corpora lutea in ovarian grafts (8, 9). Neonatal testosterone treatment abolishes the surge mode of secretion in castrated males (9). Similarly in

females, exposure to androgens in the first few days of life also abolishes the surge (9–11).

Our previous study in the adult ram determined that the preovulatory surge mechanism is neither functional nor normally inducible by the administration of increased doses of estradiol (5) which are effective in the ewe. Early studies on sexual differentiation of neuroendocrine function in the sheep by Short (12) and Clarke *et al.* (13, 14) determined that the sex difference in the ability to generate the surge is due to the organizing action of testosterone during prenatal development. This finding has been extended by recent studies by our laboratory with prenatally androgenized female lambs (15–17).

The inability of the male to produce the LH surge may stem from the absence of a stimulatory feedback mechanism centrally. Hence, it is possible that in this sex, a GnRH surge does not occur to induce a massive discharge of LH from the pituitary.

Alternatively, it is possible that the GnRH surge mechanism is functional in the male, but the pituitary fails to respond. In the present study, we examined the former hypothesis by characterizing the pattern of GnRH secretion into the pituitary portal vasculature of males and females in response to acute estradiol stimulation. Methodology for the characterization of the pattern of GnRH secretion in sheep has been well developed (18), and we have recently adapted this for use in the smaller lamb (19). We also tested the hypothesis that a sex difference in the pattern of GnRH secretion during the surge is established by the action of testicular androgens during prenatal development. Our approach was to examine the pattern of GnRH in female lambs that have been exposed to androgens during a critical period of development *in utero* (15–17).

Results

Females

Fig. 1 illustrates GnRH and LH surge patterns in all six females; Table 1 summarizes characteristics of these surges. The GnRH surge began 13.0 ± 0.4 h (mean $\pm$ SEM) after insertion of estradiol implants, and all GnRH surges were accompanied by an LH surge beginning at the same time (12.5 ± 0.8 h, $P = 0.5$). The time to peak concentration from surge onset for the GnRH (5.8 ± 2.0 h)
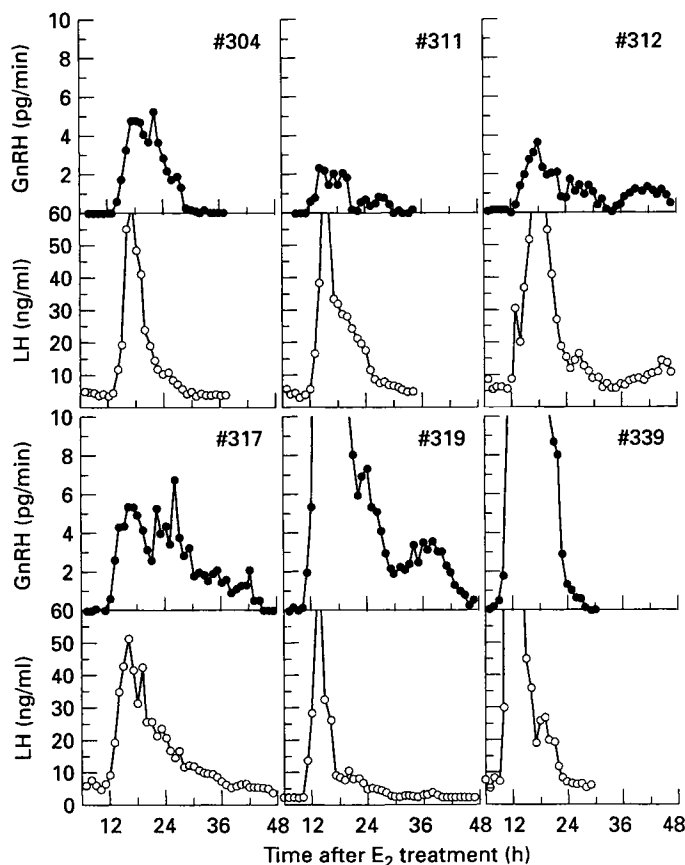

FIG. 1. GnRH (top) and LH (bottom) secretory patterns during the surge in females. All females ($n = 6$) exhibited a sustained increase in GnRH and LH in response to increased estradiol concentrations.

and LH (4.0 ± 0.6 h) surges was similar ($P = 1.1$). There was no difference between the average duration of the GnRH and LH surges ($P = 0.3$). The mean amplitude of the GnRH surge was 10.8 ± 4.5 pg/min; that of the LH surge was 67.8 ± 16.3 ng/ml. Characteristics of the LH surges were similar to those observed in the same females some five weeks earlier (17), before the installation of the portal sampling device.

Males

Fig. 2 illustrates patterns of GnRH and LH in all six males. In contrast to the females, only one male (#309) produced a GnRH surge which began much later (32 h) relative to that in the females (range: 12–14 h). This surge, albeit of very low amplitude (1.7 pg/min), was sufficient to elicit a corresponding LH surge. This LH surge began at the same time (32 h) as the GnRH surge, and was of a magnitude (101.8 ng/ml) comparable to that of LH surges in the females (range: 43–141 ng/ml). Surge characteristics in the male are presented in Table 1.

Androgenized females

All androgenized females had uniform, highly masculinized external genitalia (17). This consisted of a penis situated close to the navel, and an empty scrotum. Upon laparoscopic surgery at 3 weeks of age for ovariectomy, the appearance of the ovaries and uteri was similar to that of the normal females. These observations are consistent with those reported previously (15, 16, 20).

Fig. 3 illustrates patterns of GnRH and LH in all androgenized females. Most lambs did not produce a GnRH surge; rather, in 4 of 7 lambs, GnRH concentrations increased sporadically after estradiol treatment. Only 1 androgenized female (#392) had a sustained increase in GnRH (amplitude: 4.3 pg/min) which was accompanied by an LH surge (amplitude: 18.3 ng/ml). The surges in this animal occurred at approximately the same time (~22 h after estradiol treatment). Although statistical comparisons between the surges occurring in the androgenized female and the normal females were not possible, we noted that the timing of the surges tended to be later, and the magnitude of the LH surge to be smaller in the androgenized female compared to the normal females. There was no instance wherein LH increased in the absence of a sustained rise in GnRH. Surge characteristics in the androgenized female are presented in Table 1.

Discussion

By characterizing the pattern of GnRH in the pituitary portal circulation, the present study provides the first direct evidence that the sex differences in the generation of the gonadotropin surge originates centrally. In each female examined, there was an unambiguous rise in GnRH occurring approximately 13 h after treatment with estradiol; each surge of GnRH was accompanied by a concomitant rise in LH. This is in marked contrast to the males, wherein only one individual exhibited a sustained elevation in GnRH. Although the rise in GnRH in the single male was relatively small in comparison to those observed in the females, it was effective in eliciting a surge of LH. These observations lead to the conclusion that, with rare exception, the hypothalamic drive that stimulates the LH surge is generally not present in the male sheep.

Sexual differentiation of the mechanisms controlling the genera-

TABLE 1. GnRH and LH Surge Characteristics in Females, Males, and Androgenized Females.

	GnRH					
Surge parameter	Females	Males	Androgenized females	Females	Males	Androgenized females
Incidence*	100% (6/6)[a]	17% (1/6)[b]	14% (1/7)[b]	100% (6/6)[a]	17% (1/6)[b]	14% (1/7)[b]
Onset (h after E_2)	13.0±0.4	32	23	12.5±0.8	32	21
Duration (h)	16.2±3.3	6	14	11.2±0.7	not determined	11
Amplitude	10.8±4.5 pg/min	1.7 pg/min	4.3 pg/min	67.8±16.3 ng/ml	101.8 ng/ml	18.3 ng/ml
Time to Peak concentration (h)	5.8±2.0	3	11	4.2±0.6	6	6

Values are expressed as mean±SE. Different superscripts denote statistical significance. *Females vs males (P=0.015); Females vs androgenized females (P=0.004); Males vs androgenized females (P=1.000).
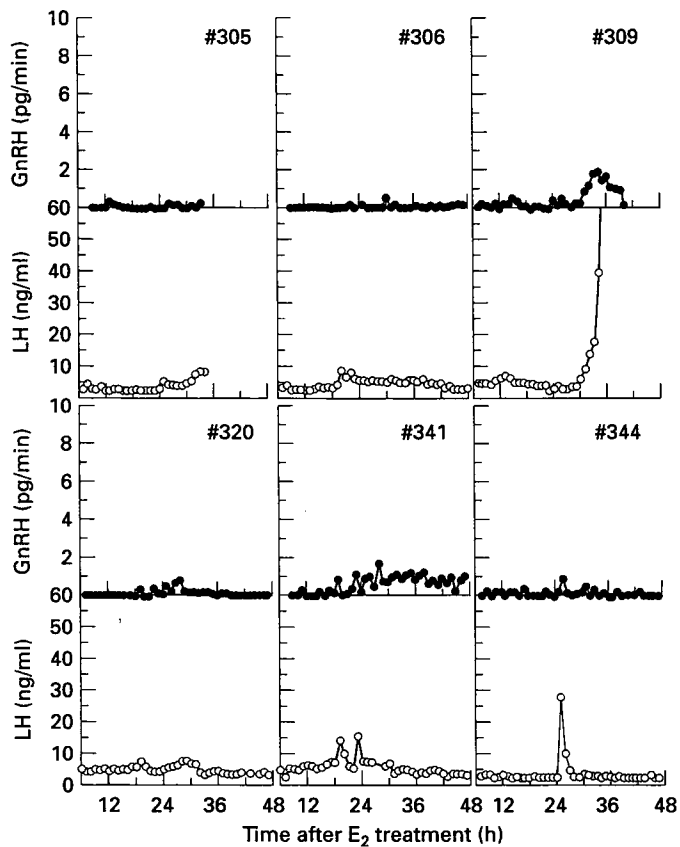

FIG. 2. GnRH (top) and LH (bottom) secretory patterns during the attempt to induce surges in males. Only one male (#309) exhibited a sustained increase in GnRH and LH in response to increased estradiol concentrations. Peak of the LH surge is off the scale.

tion of the gonadotropin surge in the sheep appears to be intermediate between the mechanisms present in the male rodent and monkey. In male rodents, the surge mode of gonadotropin secretion is completely eliminated (6, 9, 21–24), while in the castrate male monkey, the LH surge is inducible by exogenous estrogens (25–28). Although GnRH secretion during the surge has been characterized in the female rat using push-pull perfusion of the medial basal hypothalamus (29) or collection of pituitary stalk blood (30, 31), and in the female monkey using push-pull perfusion of the median eminence (32) or collection of cerebrospinal fluid obtained from the third ventricle (33), the pattern of

GnRH during an attempt to induce a surge in males of either of these species has not yet been determined. Thus, it is not known if the GnRH surge is completely obliterated in the case of the male rodent, or is generated in the male monkey. It is clear from the present study that at least in the male sheep, the positive feedback response can be activated occasionally. However, in general, males are largely incapable of producing a massive discharge of GnRH.

The present results provide evidence that the sex difference in the pattern of GnRH secretion during the surge is likely produced by the presence of testosterone before birth in the sheep. This was evident from the pattern of GnRH secretion during an attempt to induce a surge in females exposed to testosterone from the 30th–90th day of gestation, the period encompassing sexual differentiation of some neuroendocrine functions in sheep (12–14). We hypothesized that like the males, the GnRH surge in the androgenized females would be abolished by testosterone treatment. Indeed, in only one of seven individuals was a robust positive feedback response to increased estradiol observed. In some of the androgenized females, short-lived increases in GnRH occurred. We hypothesize that because the data generated in the study present cumulative GnRH release over the course of an hour, those increases may represent pooled sporadic and transient pulsatile releases of the hormone, and not sustained neuronal activity. Alternatively, the short-lived increases may be evidence of the existence of a rudimentary positive feedback response which persisted despite prenatal testosterone treatment. It may very well be that although our androgenization paradigm fully masculinized the pattern of tonic gonadotropin secretion in those females (17), complete obliteration of the surge mechanism requires a longer duration, higher levels, or a somewhat different period of exposure to androgens, or perhaps a combination of any of these. Nevertheless, it is important to note here that treatment with testosterone before birth clearly modified the pattern of GnRH secretion in all but one of the androgenized females to the extent that no LH surges were produced.

Our recent study outlined in the companion paper (34), supports our contention that the absence of the LH surge in male and androgenized female sheep results from the lack of sustained activity of GnRH neurons during acute estradiol stimulation. In that study, which examined the expression of c-fos as a marker of cellular activation, approximately 66% of GnRH immunoreactive neurons in the preoptic area and hypothalamus in females expressed Fos protein during the LH surge. In contrast, in the same region in males, as well as in androgenized females, very
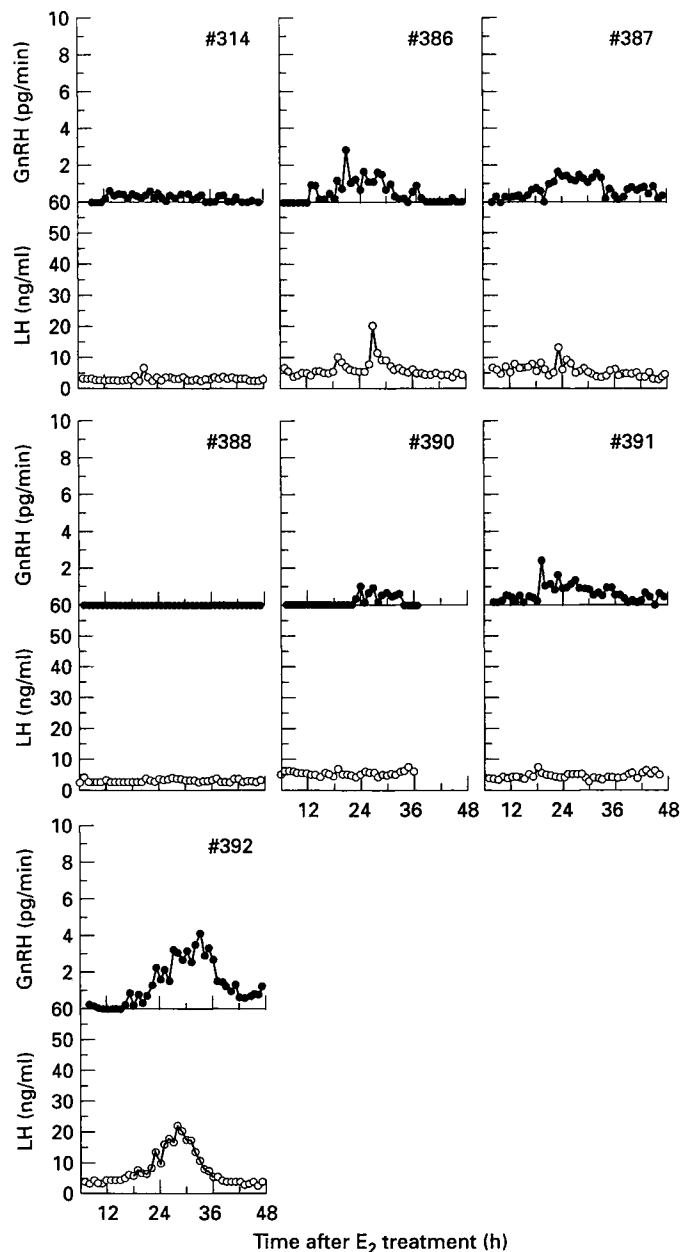
FIG. 3. GnRH (top) and LH (bottom) secretory patterns during the attempt to induce surges in androgenized females. Only one exhibited a sustained increase in both GnRH and LH in response to increased estradiol concentrations (#392). Short-lived elevations of GnRH above baseline were observed in some of the lambs.

few GnRH neurons expressed Fos, and these sheep did not produce an LH surge. It is possible that in view of the observations on the single male that produced an increase in GnRH in the present study (#309, Fig. 2), and the infrequent surges in males in other studies (5, 16), activation of GnRH neurons in males may occur occasionally, but after a longer latent period following estradiol stimulation.

Our limited data in the male also suggest that the pituitary response to GnRH is not sexually differentiated. The relatively small GnRH surge in the single male was effective in eliciting a

release in LH of comparable magnitude as those produced by normal females. Our understanding that the pituitary response to GnRH is not sexually differentiated is supported by past findings in the sheep (35), cattle (36) and monkey (37), that administration of the decapeptide in an appropriate pattern to the male can produce an LH response similar to that in females. While it has been shown that estrogen directly induces the LH surge in hypothalamo-pituitary disconnected adult female sheep (38), and that the LH surge is augmented by GnRH (39), similar studies in the male are lacking. It is therefore possible that the surge mechanism in males (and prenatally androgenized females) has a different threshold of response to steroids compared to females. This remains to be determined.

Currently, the neuronal mechanisms underlying the generation of the GnRH surge are poorly understood for any species, and therefore attempts to elucidate sex differences in those mechanisms are difficult. Because males can exhibit surges occasionally, we postulate that the basic neuronal circuitry for the surge is present in both sexes. Indeed, the GnRH neuron itself is not different between male and female sheep in terms of morphology, number, and distribution (40). However, it is possible that it is the environment of the GnRH neuron which is modified during sexual differentiation, resulting in a positive feedback mechanism that is more difficult to activate in the male. For example, it has been observed in the rhesus monkey that synaptic input to, and degree of glial ensheathment of GnRH neurons are subject to remodelling by gonadal steroids (41). Therefore various neurotransmitter inputs, both inhibitory (e.g. endogenous opioids, dopamine) and stimulatory (e.g. norepinephrine, neuropeptide-Y, excitatory amino acids) to GnRH secretion may be differentially organized by prenatal androgens in the sheep. In the male, this generally results in the uncoupling of GnRH neurons from the positive feedback effects of estradiol.

Materials and methods

Animals

Male (n = 6) and female (n = 6) lambs of predominantly Suffolk breeding were used. They were raised indoors under a natural simulate photoperiod (20), and were fed *ad libitum* with a diet consisting of alfalfa hay and a commercial pellet ration (Lamb 18, Kent Feed, Inc., Muscatine, IA, USA) containing 18% protein, supplemented with vitamins and minerals. The lambs grew rapidly and weighed approximately 50 kg at the time of the experiment, when they were about 41 weeks of age.

To determine if the GnRH surge is abolished by the organizing action of testicular androgens during fetal development, an additional group of female lambs (n = 7) was exposed prenatally to androgens. To produce the masculinized female lambs, five pregnant ewes were injected weekly from the 30th to the 90th day in gestation (term: $\sim$150 days) with testosterone cypionate (200 mg in 0.5 ml cottonseed oil, im; Sigma Chemical Co., St. Louis, MO, USA). During this broad period in fetal development, sexual differentiation of external genitalia and neuroendocrine function occur (12–16, 20, 42). The dose and duration of androgen treatment produced uniform, unequivocal, and complete masculinization of the external genitalia in these female lambs (17, 20). The androgenized female lambs resulting from this treatment, as well as the other male and female lambs, were all postpubertal at the time of the experiment, as evidenced by their patterns of circulating LH (17, 20).

Gonadectomy and induction of the GnRH/LH surge

The gonadectomized, steroid-replaced model was used. Testes were removed under local lidocaine anesthesia when males were approximately one week of age. Ovaries from normal and androgenized females were removed under acepromazine/ketamine anesthesia (0.2 mg/kg and 20 mg/kg body weight, respectively, i.m.) through a midline abdominal

incision at 3 weeks of age. To provide constant steroid feedback through-out development, an estradiol implant designed to produce physiologic concentrations of estradiol was inserted s.c. in each lamb at the time of gonadectomy. In this model, sexual maturity is defined by the sustained increase in LH secretion (43). After the attainment of puberty, the steroid implants were removed for 3 weeks before the surge induction experiment as described below.

To determine if there is a sex difference in the stimulatory feedback response of GnRH secretion to estradiol, we attempted to activate the LH surge mechanism in males, females, and androgenized females using a paradigm from our previous study (16). Briefly, 4 Silastic capsules containing estradiol were implanted s.c. in the axillary region of each lamb to provide high physiologic concentrations of the hormone (approximately 12 pg/ml). Each Silastic capsule (o.d. 0.46 cm, i.d. 0.34 cm; Dow Corning, Midland, MI, USA) contained a 30-mm column of packed crystalline estradiol 17-β (Sigma Chemical Co.) and was sealed with Silastic Adhesive Type A (Dow Corning). To prevent a postimplantation peak in steroid release, estradiol implants were preincubated in water overnight before insertion (25). Five weeks earlier, this paradigm was determined to be effective in inducing an LH surge in the same female lambs in the present experiment (17). In the current study, blood samples were collected beginning at about 7 h after the insertion of implants, using the procedure described below for the determination of patterns of GnRH and LH secretion.

Portal surgery and blood collection

When the lambs were approximately 42 weeks of age, a device designed for the collection of hypophyseal portal blood was installed into each lamb, using the technique described by Caraty et al. (18). The portal collection device was modified for use in the smaller lamb by reducing both the diameter of the collection cup which forms a seal around the portal blood vessels, and the overall length (19). One week after installa-tion of the device, blood was collected as described previously (18) from six lambs at a time; the lambs were kept unrestrained in separate stalls. In each stall, the lambs were free to behave normally, water and alfalfa hay were provided ad libitum. Each lamb was initially administered 25,000 U heparin (i.v.) every half-hour for 2 h. A maintenance dose of 16,000 U/h throughout the collection period was infused into a jugular vein via a peristaltic pump (Gilson Minipuls 3; Middleton, WI, USA). Portal vessels were lesioned by means of a stylette inserted into the upper guide tube of the collection device; the stylette was lowered a mm at a time by means of a custom-made threaded device (Turbine Tool and Gauge, Livonia, MI, USA) until adequate portal blood flow was estab-lished. Blood for the determination of GnRH was aspirated through the lower guide tube by a peristaltic pump. Portal blood was collected as hourly pools into chilled graduated cylinders containing 3 ml of bacitracin (3×10^{-3} M, Sigma) to reduce the degradation of GnRH. Jugular blood (5 ml) for the determination of LH was collected as a single hourly sample through a jugular catheter. Collection continued until 48 h after steroid implant insertion. Blood samples were centrifuged, and plasma was separated and rapidly frozen within one hour of collection. Portal and jugular blood hematocrits were monitored every 3–4 h to check for possible contamination of the portal blood samples with cerebrospinal fluid, and to assess the health status of each lamb. After the collection period, each lamb was euthanized using a barbiturate overdose (Beuthanasia-D Special, Schering-Plough Animal Health Corp., Kenilworth, NJ, USA). The placement of the portal blood collection device and the position of the lesion on the face of the pituitary were verified. All procedures were approved by the Committee for the Use and Care of Animals at the University of Michigan.

LH and GnRH radioimmunoassays

LH was measured in duplicate 25–200 μl aliquots of plasma using a modification (44, 45) of a radioimmunoassay developed by Niswender et al. (46). Assay sensitivity, defined as two standard deviations from maximum binding, averaged 0.62 ± 0.03 ng/ml (n = 5 assays) for 200 μl of plasma, expressed relative to NIH-LH-S12. Intraassay coefficient of variation (CV), determined from a serum pool that bound at 50%, averaged 8.15%; interassay CV averaged 9.12%.

GnRH was measured using a previously described radioimmunoassay (47, 48), which was modified for the present study with the use of the antiserum BDS 4/85, diluted at 1 : 400,000. Fig. 4 presents binding percent-ages of this antibody vs BDS 037 to a GnRH standard (1.8 ng/ml) as
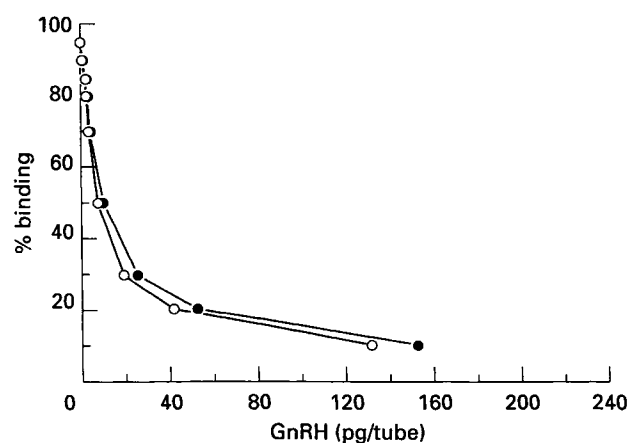

FIG. 4. Binding percentages of GnRH antibody BDS 4/85 vs BDS 037 against a GnRH standard (1.8 ng/ml). ● BDS 4/85; ○ BDS 037.

reported previously (47, 48). Briefly, portal blood samples (750 μl, containing ~600 μl of plasma and ~150 μl of bacitracin) were extracted with methanol and evaporated to dryness, then reconstituted in assay buffer [PBS (pH 7.4), containing 0.1% gelatin and 0.1% NaN_3]. Corrected plasma volume was 240 μl/assay tube based on the collection of a 2 ml portal blood sample with 0.5 ml bacitracin. Procedural blanks within assays consisted of extracts of peripheral samples taken into bacitracin (750 μl), bacitracin alone (750 μl), assay buffer (750 μl), and methanol (2 ml). Extracts were assayed in duplicate 50 μl aliquots, with all samples for each lamb measured in a single assay. Intraassay variation, based on the median variance ratio of assay replicates (49), averaged 0.06 ± 0.01. Assay sensitivity averaged 0.22 ± 0.02 pg/tube (n = 4 assays); displacement of $[^{125}I]$ GnRH to 50% of the buffer controls was 6.96 ± 0.73 pg/tube. To determine cross reactivity with peripheral plasma substances, 750 μl aliquots of 4 jugular plasma samples collected from each lamb at the beginning, middle, and end of the sampling series were processed as portal samples (i.e. extracted in methanol and reconstituted) and assayed simultaneously with the portal samples. There was minor activity detected in peripheral plasma of 75% of the lambs; in those, the average values for jugular samples (0.57 ± 0.11 pg/tube; n = 4 assays) were above the assay sensitivity (0.22 ± 0.02 pg/tube). The remaining 25% of the lambs did not exhibit any activity, thus the amount of activity differed between animals. The activity was not associated with the degree of heparinization nor the presence of bacitracin. Because there was no positive association of the level of activity with GnRH values in portal blood (high vs low), it is unlikely that the substance is GnRH. Therefore, to calculate the final value for GnRH, an average value for the interfering plasma activity was determined from the four estimates for each animal, and this plasma blank was subtracted from the measured value for GnRH.

The recovery rate was determined by adding a known quantity of GnRH (5 pg) to jugular plasma samples and then by assaying them at the same dilutions as plasma samples without GnRH. The recovery rates were usually 100%; if not, plasma concentrations were corrected for losses. GnRH concentrations are expressed as rate of collection (pg/min) rather than concentration (pg/ml) as justified previously (47). As discus-sed, this method corrects for three factors: first, for nonportal blood leaking into the collection area; second, for changes in sample collection rate that are independent of a change in GnRH secretion rate; third, for contamination of portal blood with cerebrospinal fluid (negligible in the present study).

Data analysis

GnRH and LH surges induced by increased estradiol concentrations were identified using the following method. Baseline amounts of GnRH and LH were determined from the average of the first three-hourly values obtained for each lamb. This average represented the minimum GnRH/LH concentration during inhibition by estradiol. Then, a surge was identified if amounts of GnRH or LH reached or exceeded three times the average baseline concentration, and remained elevated for at

least 6 h. The amplitude of the GnRH and LH surges were determined by subtracting the average pre-surge baseline amount from the peak value.

The incidence of GnRH and LH surges were compared among groups using Fisher's Exact test for 2×2 tables. Characteristics of the GnRH and LH surges (onset, duration, time to peak concentration) in the females were compared using a two-tailed Student's t-test, and Mann-Whitney U test. The end of the surge was defined as the first sample that decreased to below three times the average baseline concentration; this also determined the duration of the surge. If the last sample remained elevated, duration was not determined. It was not possible to make statistical comparisons of surge characteristics between groups because only 1 male and 1 androgenized female exhibited GnRH and LH surges. Values below assay sensitivity were assigned the limit of sensitivity of the assay.

Acknowledgements

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