Changes in LH secretion in response to an estradiol challenge in male- and female-oriented rams and in ewes

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Abstract

Two experiments were conducted to determine whether an estradiol challenge could cause a female-type LH surge in castrated male- and female-oriented rams (MORs and FORs). Administration of 17 β -estradiol to castrated MORs and FORs and ovariectomized ewes caused an initial reduction in LH secretion followed for 12–20 h by a surge release of LH in the ewes. No surge release of LH occurred in the MORs and FORs. The pattern of changes in LH secretion within rams and ewes did not differ between the breeding and nonbreeding seasons. Treatment failed to elicit female-typical receptive sexual behaviors in the rams but did stimulate increased sexual receptivity in the ewes as determined by the measures of responsiveness to the teaser ram. Overall, no differences were found in hypothalamic–hypophyseal function in response to exogenous estradiol between MORs and FORs. These data are interpreted to suggest that in contrast to sexual attraction, the neural mechanisms controlling the LH surge and female receptivity are defeminized in MORs.

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Introduction

It has been previously established that castration of rams causes a significant increase in systemic plasma concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH; Riggs & Malven 1974, Schanbacher & Ford 1977) and frequency of gonadotropin-releasing hormone pulses in hypophyseal portal blood (Caraty & Locatelli 1988). Administration of androgens and estrogens to castrated rams causes a marked decrease in plasma concentrations of the gonadotropins (Crim & Geschwind 1972, D'Occhio *et al.* 1982, Tilbrook *et al.* 1991). Although cells that express steroid receptors are widely distributed throughout the sheep hypothalamus, significant concentrations of androgen (ram) and estrogen (ram and ewe) receptors are located in the medial preoptic area, arcuate and ventromedial nuclei of the hypothalamus (Herbison 1995). The presence of steroid receptors in the hypothalamus and pituitary gland (Glass *et al.* 1984, Schanbacher *et al.* 1984) suggest that negative feedback by androgen or estrogen on gonadotropin secretion occurs at both the hypothalamus and pituitary.

Most rams readily court, mount, intromit, and ejaculate with estrous ewes and are classified as female-oriented rams (FORs). However, ~4–8% of rams express an exclusive sexual preference for other rams and are classified as male-oriented rams (MORs).

Castration of MORs and FORs results in the reduction of mounting behavior and ejaculations (Pinckard *et al.* 2000). Treatment of these rams with 17 β -estradiol (E₂) failed to restore sexual behaviors to precastration levels in both MORs and FORs. Whether the E₂ given to MORs may have affected their secretion of gonadotropins, especially LH, was not determined. Therefore, the present study was conducted to evaluate whether MORs are capable of responding to E₂ treatment with an LH surge and/or expression of female-typical sexual behavior.

Results

Treatment of ovariectomized ewes with E₂ during the nonbreeding season (Fig. 1A) induced a biphasic pattern of change in serum LH concentrations consisting of an initial depression (negative feedback) followed by an LH peak, similar to a preovulatory surge (positive feedback). A similar pattern of response to E₂ was observed during the breeding season when the ewes were treated with progestin preceding a bolus injection of E₂ (Fig. 2A). Rams also exhibited an initial negative feedback response to E₂ during both the nonbreeding and breeding seasons (Figs 1B and C and 2B and C). However, in contrast to ewes, this suppression of LH was not followed by a positive feedback-like release of LH, but rather by a prolonged recovery and overshoot to concentrations exceeding those prior to

Figure 1 Changes in LH secretion in (A) ovariectomized ewes, (B) male-oriented rams, and (C) female-oriented rams after injection (0 h) of 17 β -estradiol (E₂; 50 μ g i.m.) during the nonbreeding season. For each group, changes in LH concentrations were analyzed by one-way repeated-measures ANOVA followed by Fisher's least square difference test. Asterisk signifies significant difference compared with pretreatment LH concentrations.

E₂ administration (Figs 1B and C and 2B and C). Two-way ANOVA comparisons between MORs and FORs revealed no significant differences in their responses to the E₂ challenge. Injection of E₂ produced peak serum concentrations of E₂ of 100–150 pg/ml.

The characteristics of LH responses to E₂ in individual animals are shown in Table 1. Before estrogen stimulation, baseline concentrations of LH were similar in ewes, MORs, and FORs during both seasons. An LH surge was elicited in 50% of the ovariectomized ewes during the nonbreeding season and 100% of progesterone-suppressed ewes during the breeding season. The LH surge began ~14 h after estrogen exposure and lasted for 8–10 h during both tests. Mean surge levels of LH were three times higher during the nonbreeding season (76.5 ng/ml) than the breeding season (23.4 ± 9.8 S.E.M.). One MOR (#9351) showed LH levels that met the criterion for an LH surge in both experiments; however, the response latency and duration were longer and the peak levels substantially lower than these same parameters in the ewes, making it unlikely that this response constitutes a true female-typical surge response. One FOR (#5610) in experiment 2 also showed LH levels that met the criterion for an LH surge, but like the MOR the response was longer and more attenuated than that

Figure 2 Effect of exogenous 17 β -estradiol (E₂) on LH secretion in progesterin-suppressed (A) ovariectomized ewes, (B) male-oriented rams, and (C) female-oriented rams during the breeding season. Sheep were treated for 5 days with a progesterin implant to suppress LH, then given an i.m. injection of E₂ (50 μ g) 18 h after the implant was removed (0 h). Data were analyzed by one-way repeated-measures ANOVA followed by Fisher's least square difference test. Asterisk signifies significant difference compared with pretreatment LH concentration.

observed in the ewes. Composite results from two representative animals of each group from experiment 2 are shown in Fig. 3.

When individually paired with a ram, control ewes displayed both proceptive and receptive behaviors that distinguished them from both groups of rams (Fig. 4). All six ewes exhibited head-turning behavior (turns) in which they looked back at the ram while facing away. Only the ewes exhibited standing behavior and consequently received mounts (100% of ewes) and ejaculations (67% of ewes) from the teaser ram. None of the rams (MORs or FORs) showed female-type receptivity.

Discussion

It has previously been demonstrated that castration of rams and ovariectomy of ewes ultimately causes increased secretion of the gonadotropins (Tilbrook *et al.* 1991). This rise in secretion of the gonadotropins is presumably due to the absence of the negative feedback of gonadal steroids on the hypothalamic–hypophyseal axis. This is confirmed by the fact that administration of androgens and estrogens to castrated rams (Tilbrook *et al.* 1991) and ewes (Clarke & Cummins 1984) cause a comparatively rapid decrease in the blood concentrations of the gonadotropins.

The results of the present study demonstrate that treatment with E₂, whether alone or preceded by

Table 1 Characteristics of luteinizing hormone (LH) responses to 17 β -estradiol (E₂; 50 μ g) injections during the nonbreeding season (Exp. 1) and in progestin-suppressed sheep during the breeding season (Exp. 2).

	Fraction ^a responding	Baseline (ng/ml)	Latency ^b (h)	Duration ^c (h)	Peak ^d (ng/ml)
Ewes					
Exp. 1	2/4	4.8 $\pm$ 0.9	14.0	8.0	76.5
Exp. 2	6/6	2.0 $\pm$ 0.2	14.3 $\pm$ 1.0	9.0 $\pm$ 0.7	23.4 $\pm$ 9.8
MORs					
Exp. 1	1/4	7.0 $\pm$ 1.9	22.0	14.0	14.5
Exp. 2	1/6	3.4 $\pm$ 0.7	16.0	18.0	6.4
FORs					
Exp. 1	0/4	5.0 $\pm$ 0.9			
Exp. 2	1/6	2.3 $\pm$ 0.4	14.0	12.0	4.4

^aFraction responding represents the proportion of animals that exhibited an LH discharge that was twice the average pre-estradiol baseline for a minimum of 6 h. ^bLatency is the time of the first sample to meet the criterion for a surge. ^cDuration is the length of time the surge lasts. ^dPeak is the maximum LH concentration reached during a surge. Data are mean $\pm$ S.E.M. when $n > 2$.

progestin treatment, elicits a preovulatory-like surge of LH secretion in ovariectomized ewes, but not in castrated MORs or FORs. These data confirm earlier reports that the mechanism by which systemic E₂ induces an LH surge (i.e., the surge mechanism) in sheep is found only in females (Bolt 1971, Karsch & Foster 1974). A previous study by Perkins *et al.* (1995) reached a similar conclusion; however, it was not known whether the presence of the testes and the nature of circulating hormones in gonadally intact animals could have masked the differences in the responsiveness of MORs and FORs. The results of the present experiment using gonadectomized rams demonstrated that this is not the case and that both types of rams are unresponsive to the positive feedback effect of E₂. Our data also showed that while the regimen of progestin treatment, followed by E₂, elicited robust female-typical, sexual behavior in the ewes, it did not do so in MORs and FORs.

The lack of responsiveness to E₂ in MORs and FORs suggests that both groups of males were exposed to levels of testosterone during development that suppressed the LH surge mechanism and capacity for exhibiting female sexual behavior. Thus, these aspects of neuroendocrine function and behavior appear to be defeminized in MORs to the same extent as would be expected in FORs. Although earlier work in humans suggested that homosexual but not heterosexual men are responsive to estrogen feedback (Gladue *et al.* 1984, Dorner 1988), this observation was subsequently refuted (Gooren 2006). Rather, the surge mechanism in humans and nonhuman primates is equally competent in both sexes in contrast to rodents and sheep where it is permanently inhibited (i.e., defeminized) by perinatal exposure to androgens (Karsch *et al.* 1973, Karsch & Foster 1974).

The observation that MORs are attracted to males and not to females suggests the neural mechanisms that control sexual attraction are not defeminized. In female sheep, prenatal exposure to testosterone, but not to dihydrotestosterone, suppresses adult receptive behavior and the LH surge response to E₂, suggesting that these

Figure 3 Temporal relationship between serum levels of 17 β -estradiol (E₂) and serum concentrations of LH in two representative (A) ovariectomized ewes, (B) male-oriented rams, and (C) female-oriented rams. An LH surge was defined as LH values exceeding twice the average pre-estradiol baseline for a minimum of 6 h (three consecutive samples).

Figure 4 Percentage of ewes, MORs, and FORs that displayed specific components of female sexual behavior in response to surge-inducing doses of E_2 . Twenty-four hours after E_2 was injected, animals were individually paired with a stud male for 5 min and behaviors were recorded. Turn refers to head turn to rear; fan, tail wagging; stand, receptive female stance; sniff, genital sniffs. ^{a,b}Bars with different superscripts differed ($P < 0.05$).

features of behavioral and neuronal defeminization are accomplished prenatally and possibly involve aromatization (Wood & Foster 1998, Masek *et al.* 1999). Prenatal aromatization of testosterone also appears to be involved in behavioral masculinization in sheep (Masek *et al.* 1999, Roselli *et al.* 2006). By contrast, there is no evidence that prenatal aromatization is responsible for defeminization of sexual attraction in sheep (Roselli *et al.* 2006).

Research on several species indicates that defeminization of receptive responsiveness and sexual partner preference constitute separate processes (Wallen & Baum 2002). In pigs, it appears that defeminization of sexual partner preference is achieved by exposure to estrogenic metabolites of testosterone early after birth (Adkins-Regan *et al.* 1989), whereas defeminization of receptive responsiveness requires more prolonged pre- and postnatal exposure to testosterone (Ford & Christenson 1987). Male ferrets show no evidence of receptive defeminization, yet show a strong preference to approach the odors, sights, and sounds of opposite-sex versus same-sex stimulus ferrets (Baum 1979, Kelliher & Baum 2001). In view of these studies, it is evident that the critical period is not a single entity for all sexually differentiated brain functions. In fact, recent research has provided strong evidence that postnatal steroid exposure is needed to completely defeminize the estrogen-positive feedback mechanism in the sheep (Foster *et al.* 2006). Thus, further research is needed to determine the timing and hormonal requirements for behavioral sexual differentiation in the sheep.

Male sheep undergo seasonal changes in the secretion of LH and testosterone primarily because of alterations in the photoperiod (Schanbacher & Ford 1976). These

changes are most marked in ram breeds adapted to temperate climates. It has been found that concentrations of LH, frequency of LH pulses, and concentrations of FSH are greater during the breeding season than the nonbreeding season (Tilbrook & Clarke 2001). These seasonal variations in gonadotropin secretion are observed in castrated rams, indicating that photoperiod may act directly to affect the activity of the hypothalamic–hypophyseal unit, although photoperiod has also been shown to indirectly alter testosterone feedback (Tilbrook & Clarke 1995). In the current study, the administration of E_2 to ovariectomized ewes evoked the same pattern of LH secretion during both the nonbreeding and breeding seasons, i.e., an initial depression in LH secretion followed in ~18 h by a peak release of the gonadotropin. Similarly, during the nonbreeding and breeding seasons, administration of E_2 to castrated MORs and FORs caused an initial decrease in LH secretion but there was no surge of LH release as occurred in the ovariectomized ewes. Generally, these data suggest no differences in E_2 -induced LH secretion due to season. Although nuclear estrogen receptors exist in the anterior pituitary of the ram, the recent data of Arreguin-Arevalo & Nett (2006) suggest that the initial depression in LH secretion, which occurs in response to exogenous estrogen, is a consequence of the nongenomic action of this steroid.

In summary, MORs and FORs failed to respond to exogenous E_2 with a surge release of LH as detected in ovariectomized ewes. However, administration of E_2 to the rams and ewes did cause an almost immediate decrease in basal serum concentrations of LH. None of the rams responded to the exogenous E_2 with female-typical receptivity. These data suggest that in contrast to sexual attraction, the neural mechanisms controlling the LH surge and female receptivity are defeminized in MORs.

Materials and Methods

Animals

Eighteen adult sheep (~4 years old) of mixed Western breeds obtained from the United States Sheep Experiment Station in Dubois, Idaho, and reared as described previously (Resko *et al.* 1996) were used in this study. Ewes were ovariectomized and rams were gonadectomized by the veterinary surgeons at Oregon State University (OSU) at least 2 months before beginning the experiment. All procedures were performed in accordance with NIH guidelines and approved by the Institutional Animal Care and Use committee at OSU.

Ram classifications

At ~18 months of age, rams were given sexual behavior tests so that they could be classified according to their sexual preference. The complete testing procedure has been described previously (Resko *et al.* 1996, Stellflug & Berardinelli 2002, Roselli *et al.* 2004). Briefly, it consisted of 9 h of tests with estrous

ewes followed by four sexual partner preference tests in which the rams were allowed to choose between two estrous ewes and two unfamiliar rams that were all restrained. The sexual partner preference tests were conducted at the US Sheep Experiment Station over two consecutive years (two tests per year) in order to identify the rams that mated exclusively with males (i.e., MORs) and the rams that mated exclusively with females (i.e., FORs). All behavior tests were conducted during the breeding season (September to December). The numbers of genital sniffs, foreleg kicks, vocalizations, flehmen responses, mount attempts, mounts, and ejaculations were recorded during testing. The performance of the experimental rams in the sexual partner preference tests is summarized in Table 2. Age-matched experimental ewes were also obtained from the US Sheep Experiment Station. The rams and ewes were trucked to Oregon State University in the spring after testing was completed and behavioral assignments were made. They were housed in single-sex groups in larger fenced pastures with free access to water.

Experimental design

Two experiments were performed to evaluate the LH surge system in MORs, FORs, and control ewes.

Experiment 1: estradiol challenge

This experiment was conducted in July during the nonbreeding season. The sheep were given a single i.m. injection of 50 µg E₂. Blood samples (7 ml) were collected at 2 h intervals from an indwelling jugular vein catheter starting 2 h before and continuing for 48 h after the injection. Serum was harvested from the blood and stored at -20 °C until assayed for LH and E₂. An individual LH surge was defined as LH values exceeding twice the average pre-estradiol baseline concentration for a minimum of six consecutive hours i.e., three consecutive samples (Masek *et al.* 1999).

Experiment 2: progesterin suppression followed by estradiol challenge

This experiment was conducted in December during the breeding season. As described previously (Roselli *et al.* 2006), sheep were treated for 5 days with a progesterin (norgestomet) ear implant (Syncromate-B; Rhone Merieux Inc., Athens, GA, USA) to suppress serum LH concentrations. Eighteen hours after implant removal the sheep received an i.m. injection of 50 µg E₂. Venous blood samples (7 ml) were

collected from an indwelling catheter at 2-h intervals starting at 4 h before and continuing for 36 h after the injection. Serum was harvested and stored at -20 °C until assayed for LH. The LH surges were defined as described above. Progesterin priming simulates the elevated luteal progesterone levels during the estrous cycle of the ewe and has been shown to be essential for full expression of the positive feedback effect of E₂ (Caraty & Skinner 1999). All sheep were tested for female-typical sexual behavior at 24 h after E₂ injection.

Female sexual behavior

All rams and ewes were tested for female-typical sexual behavior during evaluation of the LH surge response. In adult females, sexual receptivity can be assessed 24 h after E₂ injection (Fabre-Nys & Martin 1991, Masek *et al.* 1999). To evaluate the quality of female sexual behavior after 24 h of E₂ stimulation, each experimental sheep was paired with a sexually vigorous stud ram for 5 min. Proceptive (sniff, head turns, tail fanning), receptive (standing), and agonistic behaviors (butts) were recorded by a single observer.

Hormone measurements

Serum concentrations of LH were determined in duplicate by RIA according to previously published methods (Fitzgerald & Stellflug 1991). The mean detection limit of the assays (2 s.d. from the buffer controls) was 0.3 ng/ml. The intra- and inter-assay coefficients of variation were 13 and 15% respectively. Serum concentrations of E₂ were measured by RIA after ether extraction and column chromatography fractionation as described previously (Resko *et al.* 1975). The recovery of E₂ was 81% and the intra- and interassay coefficients of variation were 7.0 and 11.5% respectively.

Statistical analysis

Within-group data on changes in serum concentrations of LH in response to E₂ injections were analyzed by one-way repeated-measures ANOVA after the data were log transformed to normalize variances. Data on the LH surge responses of MORs and FORs were analyzed by two-way repeated-measures ANOVA. When overall treatment effects were found, the *post hoc* Fisher's least square difference test was performed to determine significant differences among means.

Table 2 Averages ($\pm$ S.E.M.) of behaviors exhibited by female- and male-oriented rams during exposure to two estrous ewes and two rams in four separate 30-min partner preference tests.

Behaviors	Female-oriented rams (n=6)		Male-oriented rams (n=6)	
	Estrous stimulus ewe	Ram stimulus	Estrous stimulus ewe	Ram stimulus
Precopulatory behaviors ^a	33.4 $\pm$ 5.5	9.4 $\pm$ 1.9	2.6 $\pm$ 0.5	37.0 $\pm$ 10.4
Mount attempts ^b	0.4 $\pm$ 0.2	0.1 $\pm$ 0.1	0	0.4 $\pm$ 0.1
Mounts	9.2 $\pm$ 1.2	0.5 $\pm$ 0.3	0	11.1 $\pm$ 3.3
Ejaculations	2.8 $\pm$ 0.3	0	0	0.6 $\pm$ 0.3

Before partner preference tests, rams were given performance tests with estrous ewes for a total of 9 h. Male-oriented rams did not mount ewes in any test. ^aPrecopulatory behaviors include the sum of genital sniffs, foreleg kicks, vocalizations, and flehmen responses (lip curls). ^bMount attempts signify unsuccessful mounts in which both front feet left the ground but the ram did not become firmly positioned on the ewes stimulus animals rump.

Behavioral data were analyzed by Fisher's exact probability tests, adjusted for multiple comparisons. In all cases, differences between groups were considered to be significant at $P < 0.05$.

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