Endocrine, *vol. 7, no. 2, 235–243, October 1997* 0969–711X/97/7:235–243/$10.25

Gonadotrophin and Prolactin Secretion in Castrated Male Sheep Following Subcutaneous or Intracranial Treatment with Testicular Hormones

Dominique Blache,[1] Soedjiharti Tjondronegoro,[1,2] Margaret A. Blackberry,[1] Stephen T. Anderson,[3] Jonathan D. Curlewis,[3] and Graeme B. Martin[1]

[1]*Faculty of Agriculture (Animal Science), University of Western Australia, Nedlands, Australia 6907;*
[2]*Present address: Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia;*
and [3]*Department of Physiology and Pharmacology, University of Queensland, Queensland 4072, Australia*

Interactions between testosterone, estradiol, and inhibin in the control of gonadotrophin secretion in males are poorly understood. Castrated rams were treated with steroid-free bovine follicular fluid (bFF), testosterone, or estradiol and for 7 d (2 × 2 × 2 factorial design). Given independently, none of the exogenous hormones affected follicle-stimulating hormone (FSH) concentrations, but the combination of one or both steroids with bFF reduced FSH secretion. Testosterone and estradiol reduced luteinizing hormone (LH) pulse frequency (there was no synergism), and bFF had no effect. Plasma prolactin concentrations were not affected by any treatment. To locate the central sites of steroid action, castrated rams were bilaterally implanted in the preoptic area (POA), ventromedial nucleus (VMH), or arcuate nucleus (ARC). These implants did not affect FSH or prolactin concentrations, or LH pulse amplitude. The frequency of the LH pulses was not affected by testosterone in any site. Estradiol located in the ARC, but not the POA or VMH, decreased LH pulse frequency. In summary, FSH secretion is controlled by synergistic interactions between inhibin and estradiol or testosterone, whereas GnRH/LH pulse frequency is controlled by testicular steroids. Estradiol acts partly, at least, in the ARC, but the central site of action, testosterone remains unknown.

Key Words: Sheep; male; sex steroid; gonadotrophin; CNS.

Introduction

The pulsatile pattern of gonadotrophin releasing hormone (GnRH) secretion from the brain controls the secretion of the gonadotrophins, LH, and FSH, by the pituitary gland. In female sheep, the control of the secretion of GnRH and gonadotrophins by gonadal hormones has been intensively studied, and we know that synergistic interactions between estrogen and progesterone, acting at the brain level, control GnRH (and thus LH) pulse frequency (review in *1*), whereas the synergistic interactions between estrogen and inhibin, acting at the pituitary level, control FSH secretion *(2)*. Studies based on implantation of steroids *(3,4)* and placement of lesions within the brain *(5)* have suggested that the most likely sites of action of negative feedback by steroids are the ventromedial hypothalamus and the preoptic area.

Similarly, for male sheep, we know that LH secretion is inhibited by testosterone and estradiol, and that FSH secretion is inhibited by both testicular steroids and inhibin *(6–16)*. By contrast with the situation in the ewe, little is known concerning the possible synergistic interactions between these testicular hormones in the ram. This was therefore the subject of the first part of this study.

For male sheep, the central sites of action of negative feedback by steroids have not been detailed either. Early studies showed that testosterone reduces the responsiveness of the pituitary cells to GnRH, and suggested actions at both central and pituitary levels *(17–21)*. More recent work, however, has made it clear that testosterone acts predominantly in the hypothalamus to inhibit GnRH secretion *(22–24)*. In the male rat, implantations of testosterone or estradiol inhibit LH secretion when they are located in the arcuate nucleus or the preoptic suprachiasmatic area *(25)*. Similar studies have not been done in the ram, but we do know that the GnRH cells and fibers are distributed throughout the preoptico-hypothalamic continuum, as are the cells containing androgen and estrogen receptors (*see* review in *26*). However, steroid receptors and GnRH are not colocalized in the same neurons *(27)*, so the overlap in distribution does not precisely define the sites of negative feedback. In the second part of this study, we therefore investigated short-term effects of testosterone and estradiol implants in the preoptic area (POA), ventromedial nucleus of the hypo-

Received March 31, 1997; Revised June 25, 1997; Accepted June 25, 1997.
Author to whom all correspondence and reprint requests should be addressed: Dr. Dominique Blache, Faculty of Agriculture (Animal Science), University of Western Australia, Nedlands 6907 Australia. E-mail: dblache@agric.uwa.edu.au

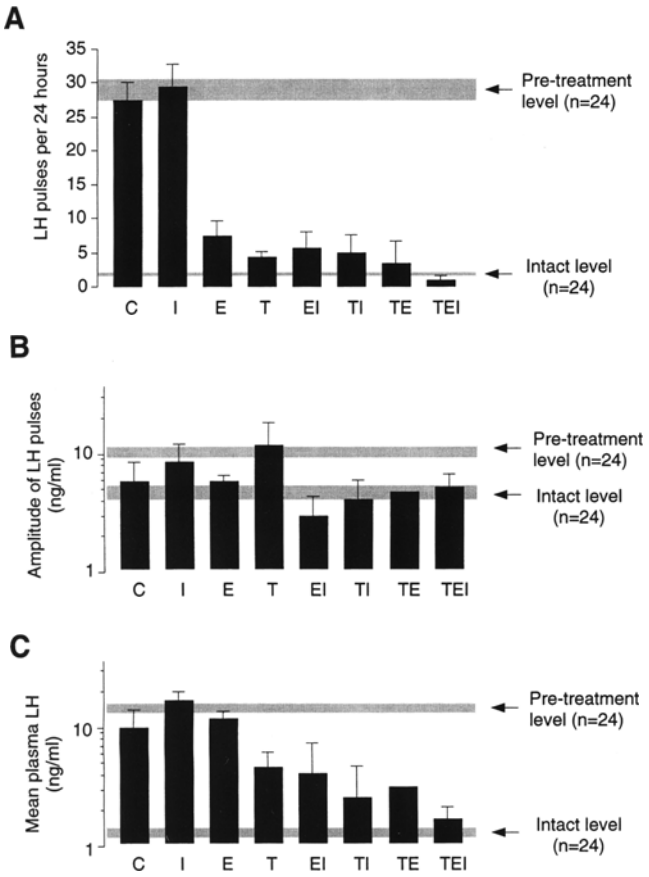

Fig. 1. The effect of peripheral gonadal hormone treatments on **(A)** the number of LH pulses/24 h, **(B)** the amplitude of LH pulses (mean ± SEM ng/mL), and **(C)** the mean concentrations of LH (mean ± SEM ng/mL) in castrated Merino rams. C: no treatment (control), I: inhibin (bFF), E: estradiol-17β, T: testosterone, EI: estradiol-17β + bFF, TI: testosterone + bFF, TE: testosterone + estradiol-17β, TEI: testosterone + estradiol-17β + bFF ($n = 3$). The shaded bars represent the values (mean ± SEM ng/mL) observed in the same rams ($n = 24$) before and after castration. Note the logarithmic scale on the *Y*-axis.

thalamus (VMH), or arcuate nucleus (ARC) of mature rams. Inhibin acts at pituitary level *(28)*, so it was not tested in these experiments. In both experiments, the effects of treatment with gonadal hormones on plasma prolactin concentrations was also determined as an indication of the specificity of the treatment on GnRH and gonadotrophin secretion.

In the present study, we report that in the male sheep, FSH secretion is controlled by synergistic interactions between inhibin and estradiol or testosterone, whereas GnRH/LH pulse frequency is testicular steroids, and estradiol acts partly, at least, in the ARC, but the central site of action of testosterone to reduce GnRH pulse frequency is not known.

Results

Part A: Synergism Between Steroids and Inhibin

The plasma concentrations of estradiol-17β and testosterone before implantation in the castrated rams were 3.22 ± 0.36 pg/mL and 0.15 ± 0.02 ng/mL, respectively. The mean daily concentrations of estradiol-17β and testosterone after implantation during the 7-d treatment were 5.53 ± 0.65 pg/mL and 3.1 ± 0.22 ng/mL, respectively.

Table 1
Effects of bFF, Estradiol-17β (E_2), and Testosterone (T) on FSH Concentrations in Castrated Rams, as Revealed by Analysis of Orthogonal Polynomial Coefficients

	F-test	*P* value
Control vs bFF alone	0.005	NS
Control vs E_2 alone	0.164	NS
Control vs T alone	0.191	NS
bFF vs bFF + steroid(s)	13.88	<0.005
E_2 or T vs E_2 + T	2.526	NS
bFF or E_2 or T vs bFF + E_2 + T	15.65	<0.005
bFF + E_2 or bFF + T vs bFF + E_2 + T	0.534	NS

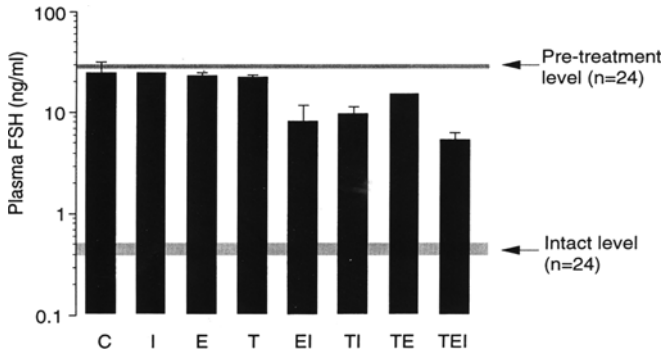

Fig. 2. The effect of peripheral gonadal hormone treatment on the concentrations of FSH (mean ± SEM ng/mL) in castrated Merino rams. *See* Fig. 1 for abbreviations. Note the logarithmic scale on the *Y*-axis.

A three-factor analysis of variance of the FSH data indicated that the main effects of testosterone ($P = 0.024$), estradiol-17β ($P = 0.014$), and bFF ($P = 0.004$) were significant. Testosterone, estradiol-17β, or bFF given alone did not reduce FSH concentrations. The concentrations of FSH were significantly reduced only when the rams were given a combination of one of the two steroids with bFF or all three hormones, but even then the levels remained above those seen before castration (Fig. 1). The specific hypotheses tested using orthogonal polynomial coefficients with 7° of freedom are presented in Table 1. Compared with the control group, treatment with bFF, estradiol-17β, or testosterone alone did not reduce FSH secretion, whereas treatments using a combination of bFF and steroid(s) were more effective in inhibiting FSH secretion than treatment with any of the three hormones separately. The effectiveness of a combination of two steroids (estradiol-17β and testosterone) did not differ from that of either steroid alone. Treatments with a combination of bFF, and either steroid

Table 2
Effects of bFF, Estradiol-17β (E_2), and Testosterone (T) on the Amplitude (ng/mL) of the LH Response 10 min after the GnRH Injection, and on Area Under the Response Curve (Arbitrary Units) in Castrated Merino Rams[a]

		Response amplitude		Area under response curve	
		–T	+T	–T	+T
$-E_2$	–bFF	85.5 ± 22.8	65.9 ± 30.5	292.5 ± 35.8	247.4 ± 59.5
	+bFF	38.9 ± 6.5	72.0 ± 7.3	179.9 ± 40.9	316.0 ± 55.3
$+E_2$	–bFF	62.2 ± 1.5	52.0 ± 9.5	325.1 ± 37.1	312.4 ± 44.9
	+bFF	61.8 ± 24.5	65.2 ± 26.1	326.9 ± 156.1	243.7 ± 72.2

[a]All values are mean ± SEM (n = 3).

significantly reduced the concentrations of FSH, but the combination of bFF and both steroids did not seem to be more effective than the combination of bFF and either steroid (Fig. 1).

Treatment with bFF did not reduce LH pulse frequency, pulse amplitude, and mean LH concentration when compared to the control group or from the pretreatment levels. Treatment with testosterone, estradiol-17β, and the combination of both steroids significantly reduced the frequency in the castrated rams (Fig. 2). Only one animal in the group treated with combined testosterone and estradiol-17β had any pulses during the observation period. A three-factor analysis of variance also indicated that testosterone and estradiol-17β, but not bFF, had a significant effect on the frequency of LH pulses (p = 0.0001), and the interaction between testosterone and estradiol-17β was also significant (p = 0.0001). Since bFF did not affect LH pulse frequency, the data were pooled, based on testosterone and estradiol-17β treatments. Treatment with steroids reduced the frequency of LH pulses in castrated rams, but there was no significant difference between the steroid-treated groups. Compared to the control values (28.3 ± 1.8 pulses/24 h), treatment with testosterone reduced the frequency by 83.5% (to 4.7 ± 1.1 pulses/24 h), treatment with estradiol-17β reduced it by 77.1% (to 6.5 ± 1.4 pulses/24 h), and the combination of testosterone and estradiol-17β reduced it by 92.3% (to 2.2 ± 1.5 pulses/24 h).

The mean amplitude of LH pulses in testosterone or bFF-treated animals did not differ from that in the control group or pretreatment levels. Treatment with estradiol-17β alone or any combination with estradiol-17β appeared to reduce the amplitude to intact levels, but analysis of variance showed no significant effect of any treatment. A three-factor analysis of variance, with or without logarithmic transformation of the data, did not reveal any significant effects of testosterone, estradiol-17β, or bFF on the LH response to exogenous GnRH. This applied to both the response amplitude and the sum of all values after the injection (Table 2). Treatment with estradiol-17β or bFF did not reduce the mean concentration of LH compared with the control or pretreatment levels, but testosterone was strongly inhibitory (P = 0.001). There were no interactions between any of the gonadal hormones, but treatment with a combination of all three reduced mean LH concentrations to levels similar to those observed in the intact ram.

Table 3
Effects of bFF, Estradiol-17β (E_2), and Testosterone (T) on the Concentrations of Prolactin (mean ± SEM ng/mL) in Castrated Merino Rams (n = 3) 7 D after the Start of Treatment

		–T, n = 3	+T, n = 3
$-E_2$	–bFF (n = 3)	40.6 ± 13.5	47.7 ± 13.4
	+bFF (n = 3)	31.0 ± 11.6	30.6 ± 9.8
$+E_2$	–bFF (n = 3)	49.1 ± 11.0	51.0 ± 8.7
	+bFF (n = 3)	60.3 ± 19.0	35.7 ± 4.3

The mean concentration of prolactin before treatment was 27.08 ± 1.87 ng/mL (n = 24). Following treatments with gonadal hormones, the differences in the concentrations of prolactin between groups were not significant (Table 3), and the three-factor analysis of variance did not reveal significant effects of any treatments.

Part B: Central Site of Action of Testosterone and Estradiol

The implants were distributed in the three different areas targeted: the POA, VMH, and ARC (Fig. 3). Plasma concentrations of estradiol and testosterone before and after central implantation were always under the limit of detection of the assays. LH pulse frequency in the five control animals was constant during the 7 wk of the experiment (3.8 ± 1.1 pulses/5 h). Bilateral implantation of cholesterol in any location did not affect LH pulse frequency or amplitude, or the mean LH concentration (Figs. 4 and 5).

Bilateral estradiol implants located in the ARC significantly decreased LH pulse frequency, but those in the POA or VMH did not (Figs. 4 and 5). The amplitude of LH pulses (overall mean ± SEM: 11.8 ± 0.6 ng/mL) was not significantly affected by any of the treatments when compared to the 5-h preimplantation period.

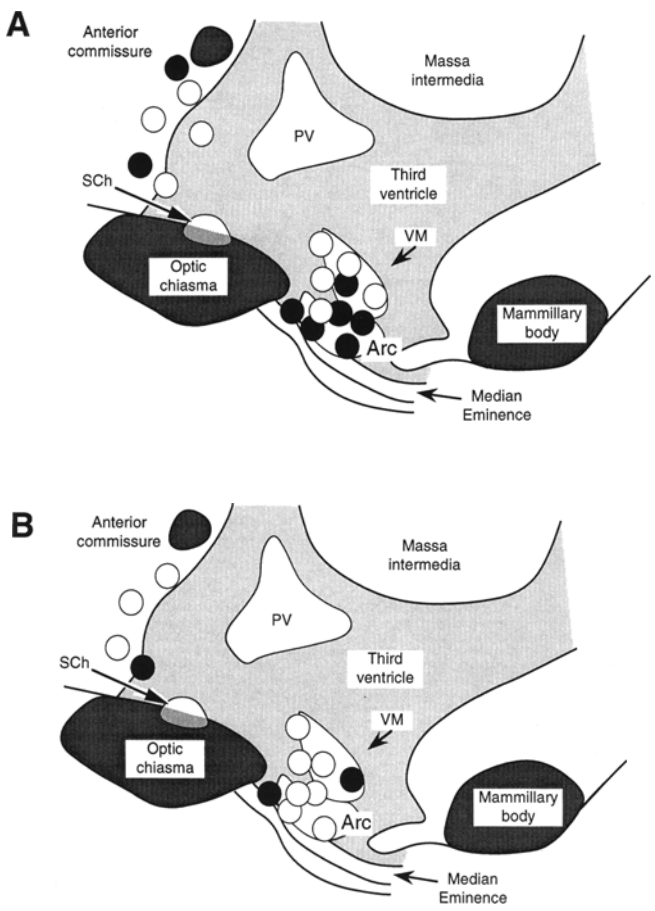

Fig. 3. Localization of the implantation sites and effect on LH pulse frequency; **(A)** bilateral implantation of estradiol and **(B)** bilateral implantation of testosterone. Open circles: no effect on LH pulse frequency, closed circle: decrease of at least 2 LH pulses/5 h compared to the control period.

Bilateral implantation of testosterone, in any of the three areas studied, did not significantly change LH pulse frequency or amplitude (Figs. 4 and 5). Central implants of estradiol, testosterone, or cholesterol did not affect the concentrations of FSH (overall mean ± SEM: 23.6 ± 0.5 ng/mL) or prolactin (overall mean ± SEM: 12.6 ± 1.1 ng/mL).

Discussion

Peripheral treatment of castrated rams with testosterone and estradiol-17β, but not inhibin, appears equally effective at inhibiting the episodic release of LH, and there appears to be a synergistic interaction between testosterone and estradiol-17β. The central implantation study suggests that estradiol exerts this negative feedback effect at the ARC. In contrast, the secretion of FSH is inhibited by a synergistic interaction between inhibin and testosterone or estrogen, exerted at the pituitary gland *(28)*. Prolactin secretion does not appear to be affected by testosterone, estradiol, or inhibin, either alone or in combination.

Exogenous peripheral treatment with testosterone and estradiol-17β both reduced LH pulse frequency in castrated rams. The effectiveness of estrogen agrees with previous findings *(6,8,13,29)*. It is important to point out that the concentration of estradiol-17β in the present study was only 5.5 pg/mL, whereas that for testosterone was 3.1 ng/mL. Both values fall within the range normally seen in intact Merino rams in our laboratory. Moreover, the estradiol concentrations are similar to those observed by other laboratories in intact rams, and in estradiol-implanted castrates in which gonadotrophin secretion has been reduced *(21,30)*. Thus, on a molar basis, estradiol-17β is far more potent than testosterone as a component of the negative feedback loop.

This high potency of estradiol is also illustrated by our central implantation study. The same-sized implant induced negative feedback when it was filled with estradiol, but not when it was filled with testosterone. Other studies have suggested that both testosterone and estradiol act on the hypothalamus *(23,25)*, and our data do not necessarily negate this hypothesis, but they could be explained by the ARC being more sensitive to estradiol than to testosterone. Thus, our intracerebral implants of testosterone may not have suppressed LH pulse frequency simply because they did not release enough testosterone into the brain tissue.

The peripheral implantation study suggested that either testosterone or estradiol alone would be sufficient to account entirely for testicular negative feedback on LH secretion, and that additive or synergistic effects are not necessary. There was a tendency for LH pulse frequency to be reduced more when the two steroids were given simultaneously than when they were given independently. However, we are prevented from drawing a solid conclusion here because of limitations of the experimental model, especially the fact that at the doses used, we had already established very low pulse frequencies and left no room for further significant reductions. This issue can only be resolved with smaller doses of steroid to exert more subtle effects, or with longer sampling periods for more accurate determination of low-pulse frequencies. The potential importance of interactions between the steroids is illustrated by data showing that in male guinea pigs, a combination of testosterone and estradiol is more effective than either steroid alone *(31)*, and that in the male rhesus monkey, both estradiol and testosterone are needed to explain fully negative feedback *(32)*. In female sheep during the breeding season, synergistic interactions between estrogen and progesterone are central to the control of LH secretion (review in *1*). Arguably, the synergism with progesterone allows the expression of ovulatory cycles during the breeding season, and the specificity of this phenomenon to the female might mean that a similar control system might not have evolved in the male.

The amplitude of LH pulses was not affected by peripheral treatment with testosterone, estradiol-17β, or inhibin, and the LH responses to GnRH injection did not differ between treatment groups in that study. Also, in the central implant study, LH pulse amplitude was not modified by

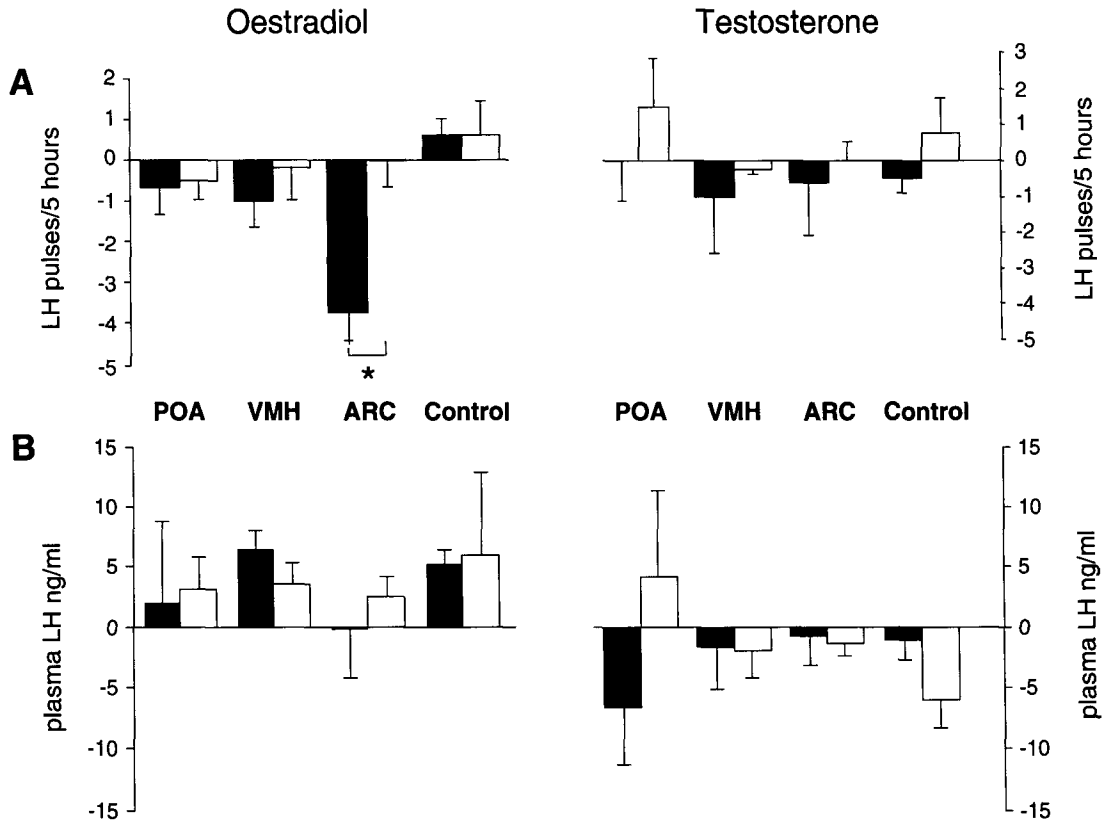

Fig. 4. Change in **(A)** the number of LH pulses/5 h, **(B)** the amplitude of LH pulses (mean ± SEM ng/mL) induced by bilateral implantation of estradiol (black bars, left panel), testosterone (black bars, right panel), or cholesterol (open bars) on in short-term castrated rams. POA: preoptic area; VMH: ventromedial hypothalamus; ARC: arcuate nucleus. $*p < 0.05$.

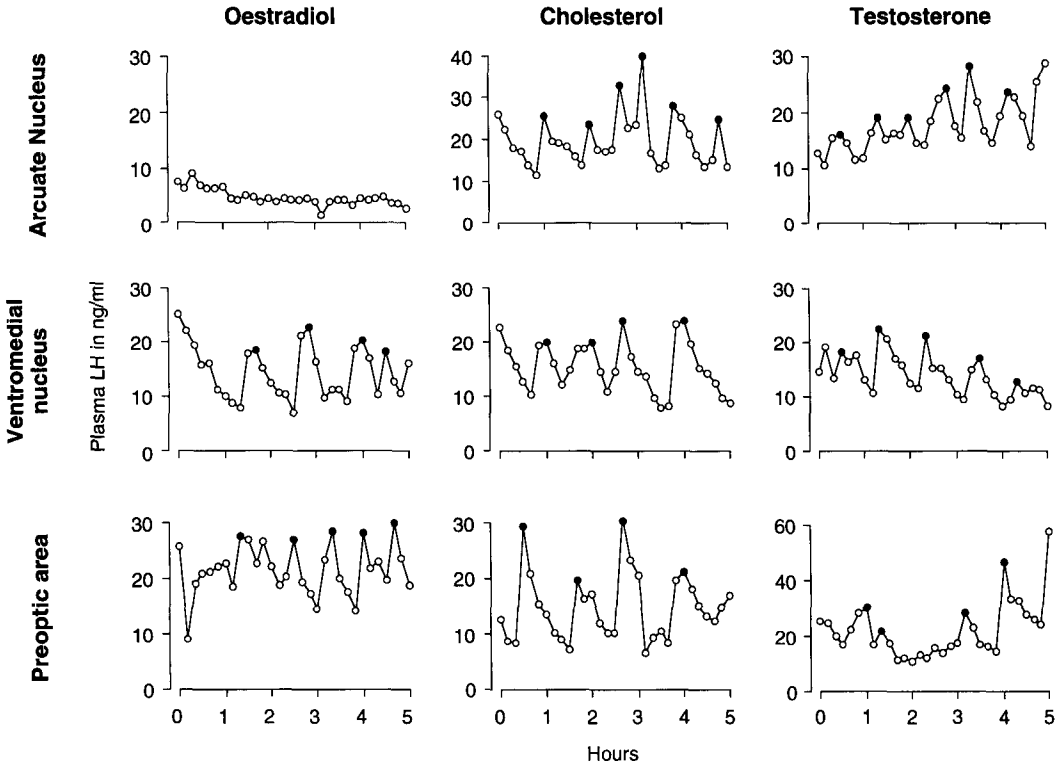

Fig. 5. Examples of LH profiles after central implantation of estradiol, testosterone, or cholesterol in different brain loci in short-term castrated rams. POA: preoptic area.

any of the steroid treatments, arguably no surprise with the restricted diffusion and low amount of steroid released by these implants. Nevertheless, taken together, these observations strongly suggest that negative feedback by sex steroids is exclusively exerted at the level of hypothalamus, where it reduces the frequency of GnRH pulses *(16,23,24)*.

An action of estradiol in the ARC has also been observed in male rats *(33)*, and it is supported by studies showing numerous cells containing estrogen receptors in this area in female sheep *(34,35)*. It is noticeable that the estradiol implants located in the lowest part of the VMH also induced a negative feedback effect (*see* Fig. 1), perhaps reflecting diffusion from those implants into the ARC. When these implants are used in sheep nervous tissue, steroids diffuse to a radius of about 1.5 mm from the tip of the implants *(3)*, similar to the distance between the ventral VMH and the ARC. On the other hand, the ventromedial VMH does contain estrogen receptors in the female sheep, so this region might also be involved in negative feedback *(35)*. The inhibition of LH secretion after implantation with estradiol was not the result of the physical lesion, because the cholesterol implants did not change LH pulse frequency and because the LH pulse frequency returned to normal after implantation (i.e., within a week).

We did not observe a significant decrease in LH pulse frequency after intracerebral implantation of testosterone. These results disagree with numerous reports that testosterone, administered either by peripheral or intracerebral routes, acts on LH secretion at the hypothalamic level in the male to reduce GnRH and gonadotrophin secretion *(23,25,36–39)*. In most of these studies, and as discussed above with respect to our own work, inhibition of gonadotrophin secretion in castrates required far more testosterone than estradiol because of differences between the steroids in their potency *(21,40)*. In the present study, the intracerebral implants were the same size for each steroid, and so the amount delivered would have been similar. It is therefore most likely that testosterone treatment did not affect gonadotrophin secretion, because the quantity released was too low. The low quantity released by the implants allows us to target accurately the most sensitive areas of the brain, but excludes study of sites in which androgen-responsive neurons are widely dispersed.

Even with the combination of bFF and steroid(s), the concentrations of FSH remained above the values observed when the rams were intact. Since the testosterone and estradiol-17β implants used in this study produced physiological concentrations of the two steroids, it seems unlikely that the steroid component of the negative feedback signal is deficient compared with intact rams. In contrast, however, it is known that the bFF treatment we used gives inhibin concentrations that are about 60% of those in intact rams *(41)*, and it is also possible that the inhibin in bFF could have lower biological activity in male sheep than in female sheep and cattle where it has been extensively tested. It seems likely, therefore, that the level of inhibin feedback achieved in the present study is less than that in intact rams. Notwithstanding this minor problem, the most effective inhibition of FSH secretion was observed with inhibin plus either estradiol or testosterone. The orthogonal comparisons suggested that FSH secretion is inhibited by a synergistic interaction between inhibin plus sex steroid(s), as has been reported for the ewe *(2,42)*. On the other hand, testosterone and estrogen alone were not very effective, and there was no suggestion of synergism between them, so it seems likely that in the absence of inhibin, feedback by the steroids is insufficient to control FSH secretion intact rams. Thus, inhibin is an essential component of gonadal feedback on FSH secretion in the ram, and as long as it is present, either one of the steroids is adequate to effect the synergism.

The central implants of steroid had no effect on FSH secretion. The delay between the implantation in the central structures and the sampling period was very short and much less than the 8–10 d that are required for a change in GnRH pulse frequency to stimulate FSH secretion *(43)*. In any case, the rams in this study were not treated with inhibin during the period of intracranial treatment, so an effect on FSH secretion, say by diffusion from the implantation site to the pituitary gland, is not likely.

In the male rat, estrogen reduces pituitary responsiveness to dopamine, which in turn increases the release of prolactin from the anterior pituitary gland *(44,45)*. In the present studies with castrated rams, prolactin concentrations before and after treatment with gonadal hormones did not differ, suggesting that testosterone, estradiol, and inhibin do not play a role in the control of prolactin secretion in the ram. This may reflect differences between species. The lack of effect of the intracranial implants on the plasma level of prolactin indicates the specificity of the effect on LH and also suggests that we did not interfere with that part of the dopaminergic system in the basal hypothalamus that is involved in controlling prolactin secretion *(46)*.

In summary, our experiments show that in the male sheep: (1) LH (and therefore GnRH) pulse frequency is controlled by an interplay (possibly synergistic) between the testicular steroids, testosterone and estradiol-17β; (2) FSH secretion is controlled by synergistic interactions between these steroids and inhibin. The site at which testosterone acts within the central nervous system to reduce GnRH pulse frequency is not known, but estradiol acts within the hypothalamus, mainly in the arcuate nucleus.

Materials and Methods

Animals

Adult Merino rams were housed in individual indoor pens separated from females, under natural photoperiod (at latitude 31°56'S, the range is 10L:14D in winter to 14L:10D in summer). They were fed 1 kg/head/d of wheaten chaff

with 10% lupin seed, sufficient to maintain constant body weight. The experiment was conducted in the breeding season (Feb.–March), but Merino rams are sexually active all year round in our environment *(1)*.

Part A: Synergism Between Steroids and Inhibin

Forty days after castration, 24 rams were assigned to one of eight treatments in a $2 \times 2 \times 2$ factorial design to receive two levels (+, –) of estradiol-17β, of testosterone, and of inhibin. The steroids were incorporated into subcutaneous implants made using silastic products from Dow Corning (Midland, MI) and estradiol-17β and testosterone from Sigma Chemical Company (St. Louis, MO). All implants were preincubated in distilled water at 37°C for several days to establish a constant rate of release, and then were washed and sterilized in 70% ethanol before insertion. The estradiol was tubular and 12 mm in length with an OD of 4.65 mm and an ID of 3.38 mm. The testosterone implants were made from 1-mm thick silastic sheeting (500-1), formed into an envelope with a surface area of either 20 cm^2 (2×5 cm) or 40 cm^2 filled with 1–2 g of testosterone (4-androsten-17β-ol-3-one) and sealed with silastic adhesive (732 RTV). One testosterone implant of each size and one estradiol implant were placed subcutaneously in the axillary region. Bovine follicular fluid (bFF) was used as a source of inhibin. It was aspirated from the large, noncystic follicles in ovaries collected from the local abattoir, pooled, extracted with charcoal to remove about 98% of the steroid, as described by Wallace and McNeilly *(47)*, and stored in small aliquots at –20°C until required. The steroid concentrations in the bFF were below the limits of detection of our assays, and previous work has demonstrated clearly that this preparation can be considered as inhibin *(42,48)*. The bFF was injected subcutaneously (2 mL) every 8 h for 7 d, and blood was sampled immediately before each injection. Blood was also sampled every 10 min for 12 h from animals with no steroid implants (high LH pulse frequency expected) and every 20 min for 24 h from the same animals before castration and 7 d after hormonal replacement (low LH pulse frequency expected). After the last serial sampling (day 7), pituitary responsiveness was tested by injecting all animals intravenously with GnRH in saline (4 ng/kg body wt; Gonadorelin, Ayerst Laboratories Pty Ltd, Parramatta, NSW, Australia). Blood was sampled from the time of injection of GnRH until 2 h later; the first three samples were taken at 10-min intervals, and the remainder every 20 min. The concentrations of estradiol-17β and testosterone were measured in daily samples. The concentrations of FSH and prolactin were measured in 8-h samples, and LH concentrations were measured in all serial samples.

Part B: Central Site of Action of Testosterone and Estradiol

Cerebral implants made of stainless-steel tubing (ID 0.45 mm, OD 0.7 mm) were filled with crystalline estradiol-17β, testosterone, or cholesterol. The outer surface was then carefully cleaned with ethanol, and the success of the procedure was verified under a dissecting microscope.

Guide-cannula were implanted bilaterally into the VMH, ARC, or POA using the technique described by Fabre-Nys et al. *(49)*. About 2 wk later, the rams were castrated and, after a further 2 wk, each animal received bilateral implants containing either estradiol or cholesterol in a crossover design with 1 wk for recovery between each implantation. The crossover experiment was then repeated, but with implants containing testosterone or cholesterol. During each period of steroid treatment, blood was sampled every 10 min for two periods of 5 h, the first starting 5 h before intracerebral implantation and the second starting 16 h after implantation. Another group of five noncannulated adult Merino rams (control) was castrated, but did not undergo brain surgery and they were sampled on all occasions to control for seasonal effects. Any ram that showed even the slightest sign of illness during the experiment was euthanized, so the number of rams per treatment varied slightly.

At the end of the experiment, animals were euthanized, the heads were perfused with 10% paraformaldehyde solution, the brains were removed, and the sites of implantation were verified on frozen coronal sections stained with thionine. All of the implants were considered to be bilateral, because the two implants on each side of the brain of each ram were within 1 mm of the same anatomical region. Pools of plasma were made from aliquots of hourly samples taken during each bleeding session, and used to determine mean concentrations of estradiol, testosterone, FSH, and prolactin, before and after central implantation of steroids. All serial samples were assayed for LH.

Hormone Assays

Plasma LH from part A was assayed using the radioimmunoassay as described by Tjondronegoro et al. *(41)*. The preparation CNRS-M3 (biopotency 1.8 IU NIH-LH-S1/mg) used for reference was kindly supplied by M. Jutisz (Collège de France, Paris, France). The tracer was prepared using NIDDK-oLH-I-3 donated by the National Institute of Diabetes, Digestive and Kidney Disease (Baltimore, MD). The anti-LH serum R1 was raised in a rabbit in our laboratory and had the following crossreactions; 100% with NIH-LH-S1, 97% with NIH-LH-S20, 18% with NIAMDD-oFSH-RP1, 0.93% with NIH-FSH-S12, 8.2% with oGH and 5% with NIH-TSH-S8.

Plasma LH from part B of the study was measured using the same assay system, but modified to run over 2 d instead of 5. Plasma and standards were mixed with anti-LH serum, and incubated for 2 h at 30°C before addition of tracer. Tubes were incubated overnight at 30°C before the secondary antibody was added. The tubes were incubated for 1 h at 30°C, and 1 mL of 6% of polyethylene glycol 4000 in water was added before centrifugation. The limit of detection of the standard curve was 1 ng/mL for all assays. The

intraassay coefficients of variation were estimated using five pooled samples containing 1 ng/mL (28.2%), 3 ng/mL (15.5%), 5 ng/mL (10.4%), 10 ng/mL (5.8%), and 20 ng/mL (11%). The interassay coefficients of variation were 42, 22, 17, 6, and 12%, respectively. The pool with very low LH concentrations (near the limit of detection) is used to refine pulse analysis around the baseline. All the samples from the same animals were measured in the same assay to avoid the effects of between-assay variation on pulse analysis.

Plasma FSH was measured in duplicate by double-antibody radioimmunoassay (RIA) using ovine FSH for standards and tracer (NIAMDD-oFSH-RP-1, with biopotency 75×NIH-FSH-S1, reference # AFP-5678C) and NIADDK-anti-oFSH-1 serum raised in rabbits. The samples were assayed as duplicate 100-μL aliquots, and the limit of detection was 0.12 ng/mL. Included in the assay were six replicates of three pooled samples containing 0.7, 4.3, and 6.5 ng/mL. They were used to estimate the intraassay coefficients of variation (mean ± SEM) of 9.5 ± 2.9, 12.3 ± 1.1, and 9.9 ± 0.6%.

Plasma prolactin was measured in duplicate by double-antibody homologous RIA. The standard was NIADDK-oPrl-I-2 (reference # AFP-7150B) and the antiserum (R 160, raised in rabbits) was kindly donated by J. A. Avenell (CSIRO Division of Animal Production, NSW, Australia). Crossreactions of <1% were found with oFSH (NIDDK-I-1), oLH (NIDDK-23), oTSH (AFP-4017C), oGH (AFP-5285C), and hACTH (L61-14). The samples were assayed as duplicate 10-μL aliquots, and the limit of detection was 0.66 ng/mL. Included in the assay were six replicates of two pooled samples containing 0.6 and 7.1 ng/mL. They were used to estimate the intraassay coefficients of variation (mean ± SEM) of 5.3 ± 1.9 and 10.4 ± 0.9%.

Plasma testosterone was measured in 25-μL duplicates of plasma using a non-extraction radioimmunoassay *(50)*. The limit of detection of the assay was 0.12 ng/mL, and the within-assay coefficients of variation (mean ± SEM) were 7.85% ± 0.4, 7.25% ± 0.75, and 9.3% ± 0.2 for quality controls containing 5.15, 2.05, and 0.9 ng/mL, respectively.

Plasma estradiol concentrations were measured in duplicate using a double-antibody RIA following affinity chromatography based on the method described by Webb et al. *(51)*. The antisera for the affinity chromatography and the RIA were kindly donated by R. Webb (AFRC, Edinburgh). The primary antibody (rabbit antiserum R48) was raised against 17β-estradiol-11β-succinyl-BSA. The steroid standard or resuspended samples were dispensed in duplicate and made up to a constant volume of 500 μL with assay buffer. Iodinated 17β-estradiol-11β-succinyl-tyrosine-methylester was then added, immediately followed by antiestradiol serum. The tubes were incubated at room temperature for 4 h before addition of normal rabbit serum and donkey antirabbit serum. The assay was incubated for 36 h at 4°C, and 2 mL ice-cold 0.1% gelatine in phosphate buffer were added before centrifugation, and the tubes centrifuged at 2000*g* for 30 min at 4°C. Major crossreactions were 16% with estrone, 1.2% with testosterone, 3% with estriol, and 0.1% with 5α-dihydrotestosterone. The limit of detection of the assay was 0.4 pg/mL and the within-assay coefficients of variation (mean ± SEM) were 4.9% ± 0.4, 4.2% ± 0.75, and 3.6% ± 0.2 for quality controls containing 2.45, 4.65, and 19.2 pg/mL respectively.

Data Analysis

The serial samples were analyzed with a modified version of the "Pulsar" algorithm developed by Merriam and Wachter *(52)* and modified for Apple Macintosh computer ("Munro," Zaristow Software, West Morham, Haddington, East Lothian, UK). The G parameters (the number of standard deviations by which a peak must exceed the baseline in order to be accepted) were 3.98, 2.4, 1.68, 1.24, and 0.93 for G1-G5, these being the requirements for pulses composed of one to five samples that exceed the baseline, respectively. The Baxter parameters describing the parabolic relationship between the concentration of a hormone in a sample and the standard deviation (assay variation) about that concentration were 0.30853 (b_1, the y-intercept), 0.00213 (b_2, the x-coefficient), and 0.00268 (b_3, x^2-coefficient). The variance of the data for pulse interval, amplitude, nadir, and mean LH tended to increase in proportion to the mean so the data were log-transformed before analysis (untransformed values are presented). The differences between treatment groups in LH, FSH, and prolactin secretion, and in LH response to GnRH injection was tested using analysis of variance. The LH response to GnRH injection was evaluated first by subtracting the concentration at time 0 (baseline) from all subsequent values, and then by comparing these adjusted LH concentrations at 10 min after the injection (response amplitude) and the sum of all concentrations after injection (equivalent to the area under the response curve). Some specific hypotheses in part A were tested using orthogonal polynomial coefficients *(53)*.

The data from the central implant study were analyzed in groups formulated retrospectively according to the histological localization of the implants. An "effective negative feedback response" was recorded when an animal secreted two less pulses in the second than in the first of the successive 5-h period of sampling. Proportions of animals responding in this way were analyzed by the Fisher's exact probability test (independent groups) and the McNemar test (dependent groups).

Acknowledgments

This work was funded by the Australian Research Council and the CSIRO Division of Animal Production. For their assistance during these studies, we would like to thank the Animal Science Group of the University of Western Australia, particularly S. R. D. Sutherland, R. Boukhliq, and D. W. Miller. For supplying the FSH and prolactin assay

reagents used in these studies, we would like to thank the National Institute of Diabetes, Digestive and Kidney Disease, the Center for Population Research of the National Institute of Child Health, and the Agricultural Research Service of the US Department of Agriculture, as well as the University of Maryland School of Medicine.

References

1. Martin, G. B. (1984). *Biol. Rev.* **59,** 1–87.
2. Martin, G. B., Price, C. A., Thiéry, J.-C., and Webb, R. (1988). *J. Reprod. Fertil.* **82,** 319–328.
3. Blache, D., Fabre-Nys, C., and Venier, G. (1991). *Brain Res.* **546,** 241–249.
4. Blache, D., Fabre-Nys, C., and Venier, G. (1996). *Brain Res.* **741,** 117–122.
5. Thiéry, J. C., Pelletier, J., and Signoret, J. P. (1978). *Ann. Biol. Anim. Biochem. Biophys.* **18,** 1413–1426.
6. Bolt, D. J. (1971). *J. Reprod. Fertil.* **24,** 435–438.
7. Crim, L. W. and Geschwind, I. I. (1972). *Biol. Reprod.* **7,** 47–54.
8. Riggs, B. L. and Malven, P. V. (1974). *J. Anim. Sci.* **38,** 1239–1244.
9. Blanc, M. R. and Dacheux, J. L. (1976). *IRCS Med. Sci.* **4,** 460.
10. Keogh, E. J., Lee, V. W. K., Rennie, G. C., Burger, H. G., Hudson, B., and De Krester, D. M. (1976). *Endocrinology* **98,** 997–1004.
11. Schanbacher, B. D. and Ford, J. J. (1977). *Endocrinology* **100,** 387–393.
12. Main, S. J., Davies, R. V., and Setchell, B. P. (1979). *J. Reprod. Fertil.* **Suppl. 26,** 3–14.
13. Parrott, R. T. and Davies, R. V. (1979). *J. Reprod. Fertil.* **56,** 543–548.
14. Schanbacher, B. D. (1979). *J. Reprod. Fertil.* **Suppl. 26,** 15–16.
15. D'Occhio, M. J., Schanbacher, B. D., and Kinder, J. E. (1983). *Acta Encdocrinologica* **102,** 499–504.
16. Schanbacher, B. D. (1984). *Endocrinology* **115,** 944–950.
17. Pelletier, J. (1974). *J. Reprod. Fertil.* **41,** 397–402.
18. Galloway, D. B. and Pelletier, J. (1975). *J. Endocrinol.* **64,** 7–16.
19. Garnier, D. H., Terqui, M., and Pelletier, J. (1977). *J. Reprod. Fertil.* **49,** 359–361.
20. Bremner, W. J., Findlay, J. K., Lee, V. W. K., De Krestser, D. M., and Cumming, I. A. (1980). *Endocrinology* **106,** 329–336.
21. D'Occhio, M. J., Schanbacher, B. D., and Kinder, J. E. (1982). *Endocrinology* **110,** 1547–1554.
22. Schanbacher, B. D. and D'Occhio, M. J. (1984). *J. Reprod. Fertil.* **72,** 537–542.
23. Tilbrook, A. J., DeKretser, D. M., Cummins, J. T., and Clarke, I. J. (1991). *Endocrinology* **129,** 3080–3092.
24. Caraty, A., Bouchard, P., and Blanc, M. (1992). In: Modes of Action of GnRH and GnRH Analogs. Crowley, W. F. and Conn, P. M. (eds.) Springer-Verlag, Berlin, pp. 193–209.
25. Danguy, A., Ectors, F., Pasteels, J. L. (1977). *J. Endocrinol.* **73,** 105–114.
26. Herbison, A. E. (1995). *J. Reprod. Fertil.* **Suppl. 49,** 271–283.
27. Lehman, M. N. and Karsch, F. J. (1993). *Endocrinology* **133,** 887–895.
28. Tilbrook, A. J., De Kretser, D. M., and Clarke, I. J. (1993). *J. Endocrinol.* **138,** 181–189.
29. D'Occhio, M. J., Schanbacher, B. D., and Kinder, J. E. (1983). *J. Androl.* **4,** 210–215.
30. Schanbacher, B. D. and Ford, J. J. (1976). *Endocrinology* **99,** 752–757.
31. Buhl, A. E. and Cornette, J. C. (1982). *Biol. Reprod.* **26,** 404–412.
32. Resko, J. A., Quadri, S. K., and Spies, H. G. (1977). *Endocrinology* **101,** 215–224.
33. Danguy, A., Pasteels, J. L., Ectors, F. (1977). *J. Endocrinol.* **74,** 315–322.
34. Lehman, M. N., Ebling, J. P., Moenter, S. M., and Karsch, F. J. (1993). *Endocrinology* **133,** 876–886.
35. Blache, D., Batailler, M., and Fabre-Nys, C. (1994). *J. Neuroendocrinol.* **6,** 329–339.
36. Kalra, P. S. and Kalra, S. P. (1980). *Endocrinology* **106,** 390–397.
37. Kalra, P. S. and Kalra, S. P. (1980). *Endocrinology* **111,** 24–29.
38. Jackson, G. L., Kuehl, D., and Rhim, T. J. (1991). *Biol. Reprod.* **45,** 188–194.
39. Tang, Y. P. and Sisk, C. L. (1988). *J. Endocrinol.* **117,** 461–466.
40. Verjans, H. L., Eik-Nes, K. B., Aafjes, J. H., Vels, F. J., and van der Molen, H. J. (1974). *Acta Endocrinol.* **77,** 643–654.
41. Tjondronegoro, S., Martin, G. B., Sutherland, S. R. D., and Boukhliq, R. (1996). *Reprod. Fertil. Devel.* **8,** 855–862.
42. Findlay, J. K., Robertson, D. M., Clarke, I. J., Klein, R., Doughton, B. W., Xiao, S., et al. (1992). *Anim. Reprod. Sci.* **28,** 319–328.
43. Lincoln, G. A. (1979). *J. Endocrinol.* **80,** 133–140.
44. Haug, E. and Gautvik, K. M. (1976). *Endocrinology* **99,** 1482–1489.
45. Raymond, V., Beaulieu, M., Labrie, F., and Boissier, J. (1978). *Science* **200,** 1173–1175.
46. Moore, K. E. (1987). *Biol. Reprod.* **36,** 47–58.
47. Wallace, J. M. and McNeilly, A. S. (1985). *J. Reprod. Fertil.* **73,** 505–515.
48. Knight, P. G., Wrathall, J. H. M., Glencross, R. G., and McLeod, B. J. (1991). *J. Endocrinol.* **128,** 403–410.
49. Fabre-Nys, C., Blache, D., Lavenet, C. (1991). In: *Neuroendocrine Research Methods: Implantation and Transfection Procedures.* Greenstein, B. D. (ed.) Harwood Academic Publishers: Chur, pp. 295–314.
50. Hötzel, M. J., Walkden-Brown, S. W., Blackberry M. A., and Martin, G. B. (1995). *J. Endocrinol.* **147,** 75–85.
51. Webb, R., Baxter, D., McBride, D., Nordblom, G. D., and Shaw, M. P. K. (1985). *J. Steroid Biochem.* **23,** 1043–1051.
52. Merriam, G. R. and Wachter, K. W. (1982). *Am. J. Physiol.* **243,** E310–E318.
53. Steele, R. G. D. and Torrie, J. H. (1980). *Principles and Procedures of Statistics. A Biometric Approach.* McGraw-Hill, New York.