

Progesterone and testosterone in combination act in the hypothalamus of castrated rams to regulate the secretion of LH

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Abstract

We tested the hypotheses that progesterone enhances the negative feedback actions of testosterone in rams and that this occurs through actions at the hypothalamus. In the first part of this study, blood samples were collected every 10 min for 12 h before and after 7 days of treatment (i.m.) of castrated Romney Marsh rams ($n=5$ per group) with vehicle, progesterone (4 mg/12 h), testosterone (4 mg/12 h) or a combination of progesterone (4 mg/12 h) and testosterone (4 mg/12 h). In the second part of this study the brains of four gonad-intact Romney Marsh rams were collected, the hypothalamus was sectioned and *in situ* hybridisation of mRNA for progesterone receptors conducted. After 7 days of treatment with vehicle or progesterone or testosterone alone, there were no changes in the secretion of LH. In contrast, treatment with a combination

of progesterone and testosterone resulted in a significant ($P<0.01$, repeated measures ANOVA) decrease in mean plasma concentrations of LH, the number of LH pulses per hour and the pre-LH pulse nadir and a significant ($P<0.01$) increase in the inter-LH pulse interval. We found cells containing mRNA for progesterone receptors throughout the hypothalamus, including the preoptic area (where most GnRH neurons are located in sheep), the periventricular, ventromedial and arcuate nuclei and the bed nucleus of the stria terminalis. This study shows that progesterone is capable of acting centrally with testosterone to suppress the secretion of LH in castrated rams and that cells containing mRNA for progesterone receptors are located in the hypothalamus of rams in the vicinity of GnRH neurons.

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Introduction

Progestins used in combination with androgens are showing potential as a male contraceptive (Meriggiola & Bremner 1997, Wu *et al.* 1999). The inclusion of a progestin treatment has been found to produce a more effective suppression of sperm production than androgen alone (Bebb *et al.* 1996, Handelsman *et al.* 1996, Meriggiola *et al.* 1998) without the problems associated with an excess of androgens. The action of this combined treatment is predominantly through a reduction in the secretion of the gonadotrophins (Meriggiola *et al.* 1998, Wu *et al.* 1999). Nevertheless, little is known about the mechanism by which progestins reduce gonadotrophin secretion in males or the physiological importance of such actions. Male progesterone receptor knock-out mice show elevated circulating concentrations of luteinising hormone (LH) relative to wild-type mice (Schneider *et al.* 1999) suggesting a physiological role for negative feedback by progesterone on LH in males, at least in this species.

The sheep has proven to be a good model in which to study the feedback regulation of gonadotrophin secretion and the patterns of secretion and relationships between

the hormones of the reproductive axis have been well characterised in the ram (Tilbrook & Clarke 1995). While many studies have illustrated that testosterone negatively regulates the secretion of LH by actions in the hypothalamus (for reviews see Tilbrook & Clarke 1995, Hileman & Jackson 1999), only a few studies have considered a role for progesterone and none have investigated the combined actions of testosterone and progesterone. Furthermore, the results of studies that have investigated the effect of progesterone alone on LH secretion in male sheep have been contradictory. Plasma concentrations of LH were suppressed following a large single injection of progesterone in adult rams (Bolt 1971) or after a subcutaneous implant of progesterone in ram lambs (Echternkamp & Lunstra 1984). In another study (Edgerton & Baile 1977), LH was not affected by infusion of progesterone in wethers and recently, Van Lier *et al.* (1999) reported that subcutaneous progesterone implants did not alter the pattern of pulsatile LH release in short-term castrated rams. In this latter study, the circulating progesterone concentrations achieved by these implants were low (~ 0.7 ng/ml). It would, therefore, seem reasonable to expect that, in males, a combination of

testosterone and progesterone would exert a greater negative effect on the secretion of LH than testosterone alone.

The site at which progesterone acts to regulate the secretion of LH in males is also unknown. While it has been suggested that these actions may be due to a reduction in gonadotrophin-releasing hormone (GnRH) receptors in the pituitary gland (Sakurai *et al.* 1997), cells containing progesterone receptors have been described in several hypothalamic nuclei of male rats using *in situ* hybridisation (Lauber *et al.* 1991), and in male guinea pigs using immunocytochemistry (Dufourny *et al.* 1997). The existence of cells containing progesterone receptors in the brain of male sheep is unknown.

In this study, we measured the secretion of LH before and after treatment of castrated rams with a combined treatment of testosterone and progesterone or with either steroid alone and we also used *in situ* hybridisation to determine whether mRNA for progesterone receptors is expressed in the hypothalamus of rams. Using these two approaches, we tested the hypothesis that progesterone acts with testosterone in the hypothalamus of castrated rams to suppress the secretion of LH.

Materials and Methods

This work was conducted in accordance with the Australian Prevention of Cruelty to Animals Act 1986 and the 'Australian Code of Practice for the Care and Use of Animals for Scientific Purposes' and was approved in advance by the Animal Ethics Committee of the Victorian Institute of Animal Science.

Experiment 1: the effect of treatment with progesterone and testosterone on the secretion of LH in castrated rams

This experiment was conducted during the early part of the non-breeding season using adult Romney Marsh rams ($n=20$) that were 4–5 years old and had been castrated after puberty but at least 18 months before the commencement of the experiment. These animals were housed in individual pens for the duration of the experiment. They were fed a maintenance ration of alfalfa hay and water and allowed to feed *ad libitum*. Previous work in this laboratory (Tilbrook *et al.* 1999) has demonstrated that the negative feedback actions of testosterone on LH secretion are not influenced by season in this breed.

Blood samples were collected from the rams ($n=5$ per group) every 10 min for 12 h before and after 7 days of treatment with vehicle, progesterone (4 mg/12 h; Progestin, Intervet International B.V., Boxmeer, Holland), testosterone (4 mg/12 h; testosterone propionate, Sigma Chemical Co., St Louis, MO, USA) or a combination of progesterone (4 mg/12 h) and testosterone (4 mg/12 h). The dose of testosterone was chosen because it is half the

dose required to significantly reduce plasma concentrations of LH in castrated rams of this breed (Tilbrook *et al.* 1991). On the day prior to the experiment, all sheep had an indwelling catheter inserted into one jugular vein to facilitate blood sampling. This catheter was flushed twice daily with saline containing 100 IU/ml heparin to maintain patency for the duration of the experiment. Concentrations of LH, progesterone and testosterone were measured in plasma harvested from blood samples.

Measurement of plasma concentrations of progesterone and testosterone

Plasma concentrations of progesterone were measured using a RIA kit that is available commercially (ICN, Costa Mesa, CA, USA). All samples were analysed in one assay for which the sensitivity was 0.15 ng/ml. Plasma concentrations of testosterone were also measured using a commercial kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA). One assay was conducted and the sensitivity was 0.04 ng/ml.

Measurement of plasma concentrations of LH

The plasma concentrations of LH were determined using a RIA described by Lee *et al.* (1976) using NIH LH S18 as standard. Eleven assays were conducted with a mean (\pm S.E.M.) assay sensitivity of 0.47 ± 0.13 ng/ml. Samples that fell below assay sensitivity were assayed again using a larger volume of sample. The intra-assay coefficient of variation was 4% at 6.4 ng/ml, 3% at 9.2 ng/ml and 8% at 23.5 ng/ml. The inter-assay coefficient of variation was 13% at 6.7 ng/ml, 13% at 10.1 ng/ml and 11% at 25.5 ng/ml.

Parameters of LH secretion

Pulses of LH were defined according to Karsch *et al.* (1987) as abrupt increases that were greater than the assay sensitivity, that exceeded the previous value by at least three times the standard deviation of that previous value, and that were followed by a progressive decline at a rate consistent with the reported half life for LH in sheep of 29 min (Geschwind & Dewey 1968). Mean LH was calculated as the mean of all values of LH during 12 h of sampling. The pre-LH pulse nadir was calculated as the mean of all of the lowest values that immediately preceded pulses. LH pulse amplitude was calculated as the mean of the peak concentration of LH minus the concentration of LH at the preceding nadir. The number of LH pulses per hour was calculated as the number of pulses that occurred during 12 h of sampling divided by 12. The inter-pulse interval was calculated as the mean of the duration from the peak of each pulse to the peak of the subsequent pulse.

Statistical analyses

Plasma concentrations of progesterone, testosterone and each of the parameters of LH secretion were compared

Table 1 Mean (\pm S.E.M.) plasma concentrations of testosterone (ng/ml) and progesterone (ng/ml) in castrated rams treated twice daily for 7 days with vehicle, progesterone alone, testosterone alone or progesterone and testosterone in combination

	Plasma testosterone		Plasma progesterone	
	Before treatment	After 7 days of treatment	Before treatment	After 7 days of treatment
Vehicle	0.08 \pm 0.03	0.07 \pm 0.01	0.21 \pm 0.03	0.15 \pm 0
Progesterone	0.07 \pm 0.02	0.14 \pm 0.06	0.15 \pm 0	0.62 \pm 0.21*
Testosterone	0.06 \pm 0.01	2.31 \pm 0.18**	0.16 \pm 0.01	0.15 \pm 0
Progesterone/ Testosterone	0.11 \pm 0.03	2.46 \pm 0.14**	0.18 \pm 0.03	0.86 \pm 0.25**

* $P < 0.05$ compared with before treatment.

** $P < 0.01$ compared with before treatment.

within and between treatments using repeated measures analysis of variance where day was the within subject factor and treatment was the between subject factor. Within-subject and between-subject *post hoc* multiple comparisons were made using least significant differences. The variance of all parameters except the inter-LH pulse interval was homogeneous. Thus, the inter-LH pulse interval was subjected to square root transformation prior to analysis and this rectified the lack of homogeneity of variance.

Experiment 2: in situ hybridisation of mRNA for progesterone receptors in the hypothalamus of rams

Brain tissue was obtained from gonad-intact adult Romney Marsh rams ($n=4$) during the breeding season for this breed (Bremner *et al.* 1984). Rams were killed by an overdose of sodium pentobarbital (Lethabarb, Virbac, Peakhurst, NSW, Australia). The head was removed and perfused through both carotid arteries with 2 litres of normal saline containing 12.5 IU/ml heparin, followed by 3 litres 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), the final litre containing 20% sucrose. The brain was then removed, the hypothalamus collected and post-fixed at 4 °C in fixative containing 30% sucrose for 7 days. Cryostat sections (20 μ m) were cut, placed into cryoprotectant (Simmons *et al.* 1989) containing 2% paraformaldehyde, and stored at -20 °C. I

In situ hybridisation was conducted according to the protocol of Simmons *et al.* (1989) using a riboprobe generated from a 894 base pair fragment of cDNA from the ligand binding domain of the ovine progesterone receptor, cloned by Ing *et al.* (1996). The procedures used for ³⁵S-labelling of the probe, hybridisation and pre/post hybridisation washes have been described in detail previously (Scott *et al.* 2000). Following completion of the post-hybridisation treatments, the sections were exposed to Kodak BMR film for 3–5 days, then dipped in Ilford K5 photographic emulsion and exposed for 2 weeks. The dipped slides were then photographically processed, lightly

counterstained with Cresyl Violet and covered with a cover-slip. Sections at intervals of 360 μ m were analysed using a Nikon Eclipse microscope.

Results

Experiment 1: the effect of treatment with progesterone and testosterone on the secretion of LH in castrated rams

Plasma concentrations of testosterone and progesterone Following 7 days of treatment of rams with testosterone alone or with testosterone and progesterone in combination, plasma concentrations of testosterone were significantly ($P < 0.01$) higher than pre-treatment concentrations (Table 1). There was no significant difference between the testosterone alone and testosterone and progesterone in combination treatments in the plasma concentrations of testosterone following 7 days of treatment. Similarly, following 7 days of treatment with progesterone alone or with progesterone and testosterone in combination, plasma concentrations of progesterone were significantly ($P < 0.05$) higher than pre-treatment concentrations (Table 1). There was no significant difference between the progesterone alone and testosterone and progesterone in combination treatments in the plasma concentrations of progesterone following 7 days of treatment. Treatment with vehicle or progesterone did not significantly influence plasma concentrations of testosterone and treatment with vehicle or testosterone did not significantly influence plasma concentrations of progesterone (Table 1).

Plasma concentrations of LH Treatment with vehicle, testosterone alone or progesterone alone did not significantly influence any parameters of LH secretion (Table 2). In contrast, following treatment with testosterone and progesterone in combination for 7 days, mean LH, the number of LH pulses per hour and the pre-LH pulse nadir were significantly ($P < 0.01$) decreased and inter-LH pulse interval was significantly ($P < 0.01$) increased (Table 2).

Table 2 Parameters of LH secretion (mean \pm S.E.M.) for castrated rams treated twice daily for 7 days with vehicle, progesterone alone, testosterone alone or progesterone and testosterone in combination. The parameters of LH secretion that were measured were the mean concentrations of LH (ng/ml), the number of pulses per hour, the inter-LH pulse interval (minutes), the amplitude of LH pulses (ng/ml) and the pre-LH pulse nadir (ng/ml)

	Mean LH		Pulses per hour		Inter-pulse interval		LH pulse amplitude		Pre-LH pulse nadir	
	Before treatment	After 7 days of treatment	Before treatment	After 7 days of treatment	Before treatment	After 7 days of treatment	Before treatment	After 7 days of treatment	Before treatment	After 7 days of treatment
Vehicle	5.5 \pm 1.1	4.7 \pm 1.3	1.1 \pm 0.3	1.1 \pm 0.2	72 \pm 25	67 \pm 18	2.4 \pm 0.5	2.6 \pm 0.5	4.0 \pm 0.7	3.9 \pm 1.2
Progesterone	8.3 \pm 2.0	8.1 \pm 2.1	1.4 \pm 0.1	1.1 \pm 0.1	45 \pm 4	55 \pm 7	3.6 \pm 0.8	3.9 \pm 0.8	7.1 \pm 1.9	7.3 \pm 2.4
Testosterone	7.8 \pm 2.0	7.7 \pm 1.7	1.2 \pm 0.2	1.1 \pm 0.2	48 \pm 8	59 \pm 14	3.0 \pm 0.9	3.2 \pm 0.6	6.8 \pm 1.5	6.3 \pm 1.5
Progesterone/ Testosterone	7.9 \pm 1.6	4.3 \pm 1.3**	1.2 \pm 0.2	0.5 \pm 0.2**	48 \pm 4	156 \pm 45**	2.5 \pm 0.6	3.7 \pm 1.4	7.0 \pm 1.3	3.4 \pm 1.1**

** $P < 0.01$ compared with before treatment.

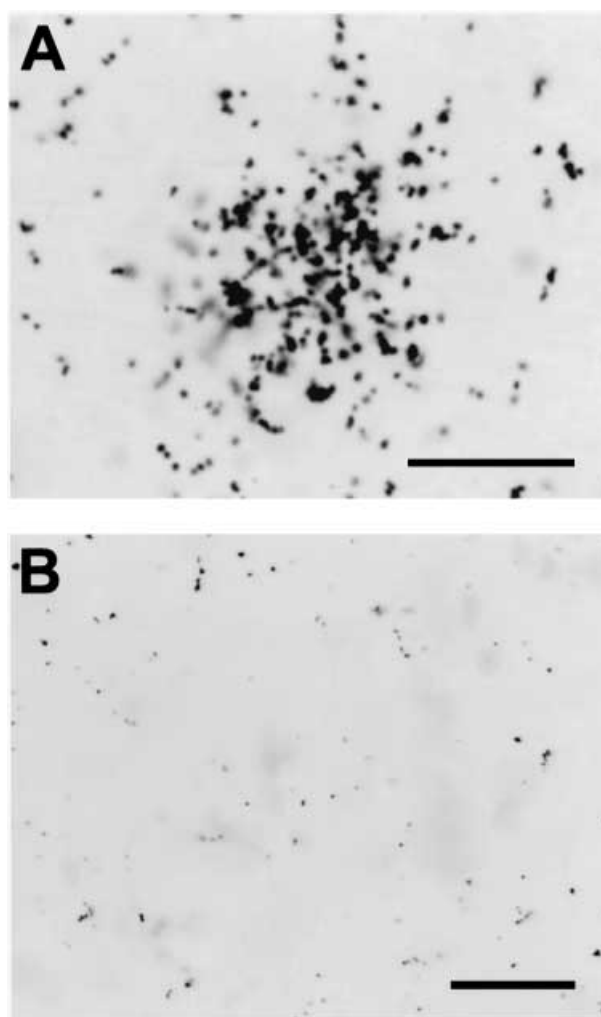


Figure 1 High power photomicrographs of the arcuate nucleus from a representative ram showing tissue hybridised with either (A) antisense or (B) sense mRNA for ovine progesterone receptor. Clusters of silver grains indicate the location of progesterone receptors. Scale bar = 10 μ m in (A) and 20 μ m in (B).

The amplitude of LH pulses was not significantly influenced by the combined testosterone and progesterone treatment.

Experiment 2: in situ hybridisation of mRNA for progesterone receptors in the hypothalamus of rams

Cells that produce mRNA for progesterone receptors were identified as clusters of silver grains over single neurons (Fig. 1A). This accumulation of silver grains was absent when sections were hybridised with the sense strand, indicating the specificity of this labelling (Fig. 1B). The distribution of cells in the preoptic area/hypothalamus that contain mRNA for progesterone receptors is shown in a series of film autoradiograms from a representative ram (Fig. 2).

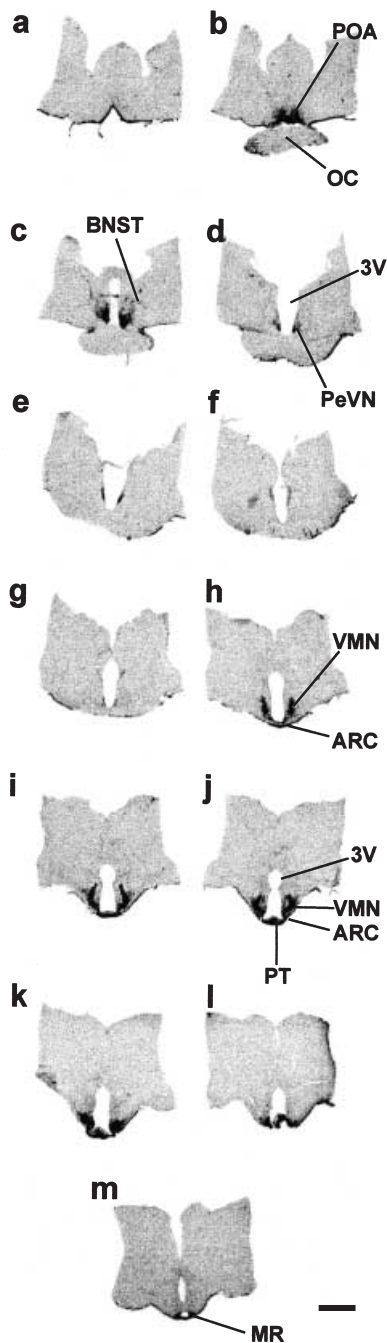


Figure 2 A series of film autoradiograms showing the distribution of cells containing mRNA for progesterone receptors in a representative ram. Panels a–m show 20 μm sections at 360 μm intervals where 'a' is the most rostral section and 'm' is the most caudal section. Scale bar=5 mm. Abbreviations: POA=preoptic area, OC=optic chiasm, BNST=bed nucleus of the stria terminalis, 3V=third cerebral ventricle, PeVN=periventricular nucleus, VMN=ventromedial nucleus, ARC=arcuate nucleus, PT=pars tuberalis, MR=mamillary recess of the third cerebral ventricle.

Scattered cells containing mRNA for progesterone receptors were observed in the diagonal band of Broca. In addition, cells containing mRNA for progesterone receptors were found throughout the entire preoptic area (Fig. 3A), with particularly strong labelling in the ventral part, adjacent to the organum vasculosum of the lamina terminalis, and extending in a lateral and dorsal direction. The organum vasculosum of the lamina terminalis itself contained several labelled cells. A small number of strongly labelled cells were also found in the bed nucleus of the stria terminalis (Fig. 3B), both in the dorsolateral and ventral subdivisions. Strongly labelled cells were also found throughout the periventricular nucleus, with the number of labelled cells decreasing steadily towards the caudal end of the nucleus. A dense population of strongly labelled neurons was found in both the ventromedial and arcuate nuclei (Fig. 3C and D). In the rostral parts of the arcuate nucleus, labelling was predominantly in the lateral regions and, in the caudal part, labelling was predominantly in the ventral regions, below the mamillary recess of the third ventricle.

Discussion

The decrease in frequency of LH pulses that followed the treatment of castrated rams with a combination of progesterone and testosterone indicates that these steroids reduced the secretion of LH by acting directly within the hypothalamus to decrease the synthesis and/or secretion of GnRH. Furthermore, the demonstration of progesterone receptors within the hypothalamus of the ram, in the vicinity of the GnRH neurons involved in regulating the secretion of LH, provides a path by which progesterone may act to suppress the secretion of LH. Thus, we have provided support for our hypothesis that, in combination with testosterone, progesterone can act in the hypothalamus of castrated rams to suppress the secretion of LH. Hypothalamic actions of testosterone were expected as these have been found previously by a number of researchers (Jackson *et al.* 1991, Tilbrook *et al.* 1991, Lubbers & Jackson 1993). Indeed, the hypothalamus has been found to be the primary site for the negative feedback actions of testosterone (Jackson *et al.* 1991, Tilbrook *et al.* 1991). In contrast, the site of action of progesterone in males has not previously been investigated. The lack of change in the secretion of LH following treatment with testosterone alone was an intentional outcome in this study. The dose of testosterone used in this study was half that required to suppress the secretion of LH (Tilbrook *et al.* 1991). There are two possible methods by which the combined treatment with progesterone and testosterone influenced the secretion of LH. First, the total amount of steroid administered may have been sufficient to affect the secretion of LH. The equivalent total dose of testosterone alone suppresses the secretion of LH (Tilbrook *et al.* 1991).

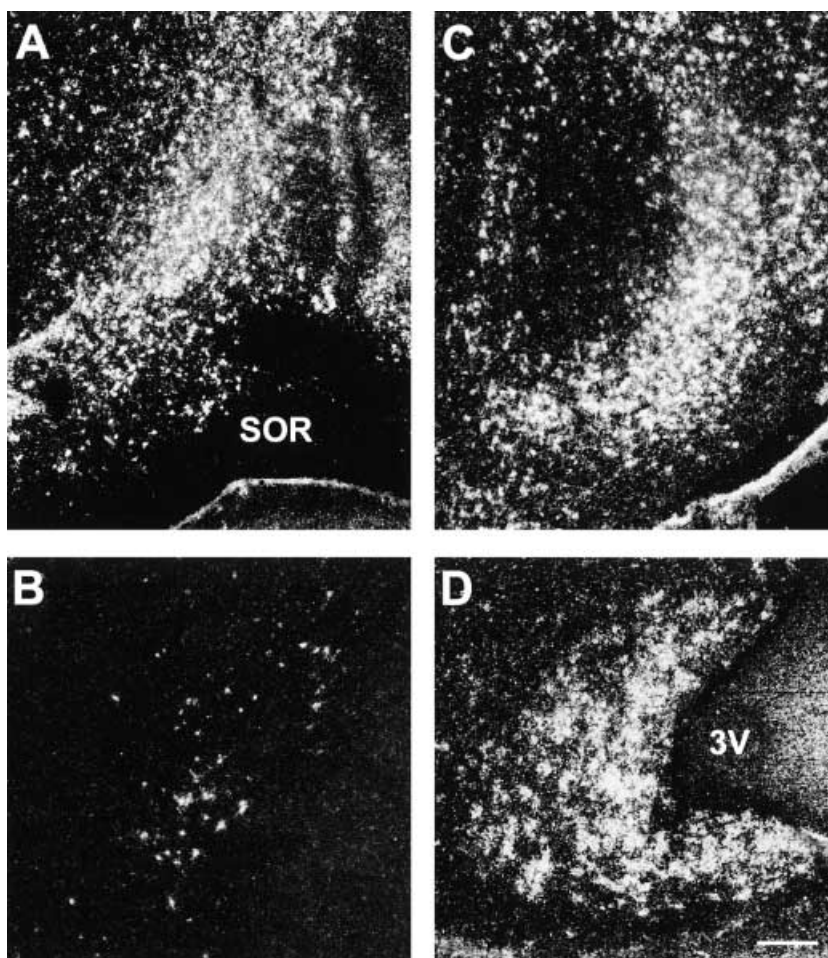


Figure 3 Low power dark field photomicrographs of hybridisation with antisense mRNA for ovine progesterone receptor in the preoptic area (A), the bed nucleus of the stria terminalis (B), the ventromedial nucleus (C) and the arcuate nucleus (D) of a representative ram. Scale bar=200 μ m. Abbreviations: SOR=supraoptic recess of the third cerebral ventricle, 3V=third cerebral ventricle.

Alternatively, the presence of progesterone may have enhanced the negative feedback actions of testosterone. From the results of the current study, it is not possible to distinguish between these two possible mechanisms. Furthermore, notwithstanding the suppressive effects of progesterone found in this study, the relevance of such actions of progesterone to a 'physiological' setting are not clear.

As the plasma concentrations of progesterone and testosterone in rams given the combined treatment were no greater than those in rams given each steroid alone, it seems likely that each steroid acted in its own right. This may have occurred by progesterone binding to androgen receptors since studies into the use of oral contraceptives in women have shown that this can occur (Collins 1994, Carr 1997). Nevertheless, we also showed that progesterone receptors are located throughout the preoptic area and

hypothalamus being well placed for progesterone to act through association with its own receptors. Alternatively then, the two steroids might have interacted to impair the secretion of LH by one of the steroids inducing an increase in the number of receptors for the other steroid in the hypothalamus. We previously found an interaction of this nature in ewes where oestrogen increased the number of progesterone receptors in the ventromedial nucleus and arcuate nucleus in the ewe (Scott *et al.* 2000). Oestrogen had a similar action on hypothalamic progesterone receptors in male guinea pigs (Dufourny *et al.* 1997) but such interactions have not been studied in rams. Nevertheless, it seems feasible that, in our study, testosterone may have acted directly, or following aromatisation to oestrogen, to increase the number of progesterone receptors in the hypothalamus, thereby enhancing any suppressive effects that progesterone may have on the secretion of LH.

Conversely, it is also feasible that progesterone may have induced an increase in the number of androgen receptors to enhance the negative feedback effects of testosterone. Our inference that a change in the frequency of LH pulses indicates central actions of our treatment, is based on the finding that there is a high level of concordance between the pulsatile secretion of GnRH and that of LH, with each pulse of LH being immediately preceded by a pulse of GnRH (Clarke & Cummins 1982, Jackson *et al.* 1991, Tilbrook *et al.* 1991). Nevertheless, the hypothalamus might not be the only site at which progesterone acts in the male to regulate the secretion of LH as treatment with progesterone was shown to decrease the concentration of GnRH receptor in the pituitary gland of wethers (Sakurai *et al.* 1997).

Our study is the first to describe progesterone receptors in the preoptic area and hypothalamus of rams. The distribution of cells containing progesterone receptors in the ram is similar to that previously described in the male rat (Lauber *et al.* 1991) and guinea pig (Olster & Blaustein 1990, Dufourny & Warembourg 1996, Dufourny *et al.* 1997). Also, the distribution of these cells was, with the exception of the bed nucleus of the stria terminalis, similar to that which we have previously described in the hypothalamus of the ewe (Scott *et al.* 2000). Studies in rats (Lauber *et al.* 1991) and guinea pigs (Olster & Blaustein 1990, Dufourny & Warembourg 1996) have reported a similarity between the sexes in the distribution of cells in the hypothalamus that contain progesterone receptors. Thus, differences between the sexes in the gonadotrophin response to progesterone in these species are unlikely to be due to the number of cells in the hypothalamus containing progesterone receptors. Nevertheless, it is possible that there are sex differences in the affinity for the receptor, as suggested by binding studies in guinea pigs (Blaustein *et al.* 1980, Ryer & Feder 1984, Brown *et al.* 1996). Binding studies in rats, however, suggest that there is no sex difference in this regard (Etgen 1981, Brown *et al.* 1987) and this issue remains unresolved. We observed a small number of strongly labelled cells in the bed nucleus of the stria terminalis of the ram although none were detected in this nucleus in the ewe (Scott *et al.* 2000). The functional implications of this sex difference are unknown.

Thus, through a multi-faceted approach, our study provides evidence that progesterone, in combination with testosterone, is capable of acting in the hypothalamus of rams to regulate the secretion of LH. Nevertheless, the physiological relevance of such actions of progesterone in males is currently not clear.

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