Alterations in pituitary gland sensitivity in ram lambs to physiological doses of gonadotrophin-releasing hormone (GnRH), after divergent selection based on the luteinizing hormone response to a pharmacological GnRH challenge

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Summary. Divergent selection in 10-week-old Finn-Dorset ram lambs was based on the luteinizing hormone (LH) response to a pharmacological dose of GnRH (5 µg). After eight generations of selection, the LH responses of the two lines (low and high) to GnRH differed by a factor of five. This study investigates the pituitary sensitivity of the two lines to exogenous GnRH. Initially, two pilot studies were performed: one to determine the range of doses of GnRH which would stimulate LH pulses of similar amplitude to those seen endogenously, and the other to confirm that sodium pentobarbitone prevents pulsatile LH secretion in prepubertal ram lambs. The results indicated that barbiturate anaesthesia suppressed pulsatile LH secretion in castrated and intact ram lambs. A model system was therefore constructed in 18 10-week-old intact ram lambs (high $n = 7$, low $n = 11$), whereby endogenous pulsatile LH secretion was prevented by sodium pentobarbitone anaesthesia and the amplitudes of LH pulses produced in response to different doses of exogenous GnRH could be measured. The GnRH dose-response curves demonstrated that there was a five-fold difference in the sensitivity of the pituitary glands of the two lines to stimulation with GnRH. The projected minimum concentration of GnRH required to produce a measurable pulse of LH was 4.75 ng for the high-line animals and 26.6 ng for the low-line animals. The results indicated that the low-line animals required five times more GnRH than the high-line lambs to stimulate LH pulses of similar amplitude (high line 43.67 ng; low line 206.55 ng).

These results demonstrate that selection has produced two lines of sheep which differ in the control of LH secretion at the level of the hypothalamus–pituitary gland.

Keywords: pituitary sensitivity; GnRH; sodium pentobarbitone; ram lambs

Introduction

Luteinizing hormone (LH) is secreted in a pulsatile fashion, pulses being observed in response to discrete pulses of gonadotrophin-releasing hormone (GnRH) released from the hypothalamus (Clarke & Cummins, 1982; Levine *et al.*, 1982; Caraty & Locatelli, 1988). The quantity of LH released is dependent on the amount of GnRH secreted by the hypothalamus and the sensitivity of the pituitary gland to GnRH. These characteristics are regulated endogenously by a series of feedback systems, which are either localized within the hypothalamo–pituitary complex or require

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input from peripheral organs such as the testes or the ovaries. In prepubertal ram lambs, the mean peripheral LH concentration increases between ~4 and 13 weeks of age, as a result of a gradual change in the pattern of LH secretion from low-frequency, high-amplitude LH pulses at 4 weeks of age, to high-frequency, low-amplitude pulses at 13 weeks (Yarney & Sanford, 1985a, b; Olster & Foster, 1986). These changes in the pattern of gonadotrophin secretion indicate the increasing maturity of the hypothalamus and the increasing sensitivity of the hypothalamo-pituitary gland complex to GnRH, and the increasing effects of gonadal negative feedback.

Sodium pentobarbitone and sodium phenobarbitone anaesthesia, which reduce neuronal activity, have a profound effect on gonadotrophin and steroid secretion in anaesthetized sheep. Anaesthesia during the breeding season prevents endogenous pulsatile LH secretion in intact (Radford, 1966; Dobson & Ward, 1977; Radford *et al.*, 1978; Webb *et al.*, 1981; Goodman & Meyer, 1984) and in ovariectomized ewes after a transient period of high LH concentrations (Goodman & Meyer, 1984), whilst anaesthesia of seasonally anoestrous ewes causes an increase in LH pulse frequency (Goodman & Meyer, 1984). The pituitary gland is not directly affected by anaesthesia, as administration of exogenous GnRH results in the stimulation of LH secretion (Radford & Wallace, 1974; Blake, 1974, 1976; Webb *et al.*, 1981). Therefore, barbiturate anaesthesia provides a means of constructing a model system in which the regulation of LH secretion can be studied, in the absence of endogenous GnRH.

Two lines of Finn-Dorset sheep maintained at the AFRC Institute of Animal Physiology and Genetics Research, Roslin, differ in their ability to respond to a pharmacological dose of GnRH. The lines were formed by divergent selection in 10-week-old ram lambs, based on their LH response to a 5 µg dose of GnRH. After eight generations of selection, there is a 5.5-fold difference in the LH response between the two lines (Lee & Land, 1985; Haley *et al.*, 1989). A previous study (Evans *et al.*, 1991), using castrated and intact ram lambs from the two selection lines, has demonstrated that this difference between the two lines appears to exist at the level of either the hypothalamus or the pituitary gland, and is not due to differences in steroid negative feedback.

This study (i) developed a model system that allowed the construction of GnRH dose-response curves for each line, in the absence of endogenous GnRH secretion; (ii) used the dose-response curves to investigate whether differences exist in the sensitivity of the pituitary glands of the two lines to GnRH, pituitary sensitivity being defined as the minimum dose of GnRH required to stimulate the release of LH from the pituitary gland, producing a significant increase in the concentration of LH in the peripheral circulation; and (iii) used the GnRH dose-response curves to obtain an indirect measure of the amounts of GnRH required to produce an endogenous LH pulse in the two selection lines.

Materials and Methods

Animals. The two GnRH selection lines are maintained at the Farm of the AFRC Institute of Animal Physiology and Genetics Research in southern Scotland. Ewes were mated within lines and the experimental animals were born indoors during the first 2 weeks of April. The ewes and lambs were turned out on the day following birth and were maintained at pasture under normal husbandry conditions. Castrated lambs were produced by the normal commercial practice of the application of a rubber ring at the base of the scrotum soon after birth; ram lambs received no treatment. All lambs were housed during the experimental periods.

Experiment 1. The aim of this experiment was to determine the doses of GnRH required to stimulate the secretion of pulses of LH of physiological amplitude. Five entire, 6-week-old ram lambs (± 3 days) (high $n = 2$, low $n = 3$), were fitted with indwelling jugular cannulae (16G: B Braun, Dunlop Veterinary suppliers). The lambs were challenged with 5 bolus intravenous injections of GnRH (HRF: Ayerst Laboratories Ltd, Andover, Hants, UK) at 90-min intervals (25, 50, 100, 250 and 500 ng) in an ascending order. Blood samples were collected at 15-min intervals, starting from the time of injection, and centrifuged at 1500 g for 20 min; the resulting plasma was frozen at -20°C until assayed for LH and testosterone.

Experiment 2. The aim of this experiment was to establish an animal model in which endogenous LH pulses were abolished, but the ability of the pituitary to respond to exogenous GnRH was not compromised. Castrated animals were used, as the removal of gonadal feedback leads to increased hypothalamic activity with a resulting increase in LH

pulse frequency (Crim & Geschwind, 1972; Riggs & Malven, 1974). Six-week-old castrated ram lambs, one from each selection line, were fitted with indwelling jugular cannulae and anaesthetized for 3.5 h using sodium pentobarbitone (Sagatal, May & Baker). Blood was sampled from the lambs at 10-min intervals during anaesthesia and processed as described in Expt 1.

Experiment 3. Eighteen 10-week-old ram lambs (± 3 days) from the high ($n = 7$) and low ($n = 11$) GnRH selection lines were fitted with bilateral, indwelling jugular cannulae. One cannula was used for the collection of blood samples and the other for the administration of GnRH and sodium pentobarbitone. On Day 1, serial blood samples at 15-min intervals for 3 h, were taken from each animal to estimate endogenous LH pulse amplitude. On Day 2, anaesthesia was induced with sodium pentobarbitone at ~ 30 mg/kg, injected in two stages: half the volume was injected rapidly and the remainder was given slowly to effect. The animals were then intubated and maintained under light anaesthesia with incremental sodium pentobarbitone. The lambs were restrained in sternal recumbency with head raised to prevent reflux and inhalation of oesophageal fluid. The animals were turned at regular intervals and their condition and temperature monitored closely for the duration of anaesthesia. Supplementary oxygen was available.

The results from Expt 1 indicated that 50, 100, 250 and 500 ng of GnRH, administered as a 2-ml bolus injection, would enable the construction of an LH dose-response curve which should cover the physiological range of LH pulse amplitudes. The results of Expt 2 indicated that the animals should be anaesthetized for 2 h before the initiation of GnRH treatment.

After the induction of anaesthesia, blood was sampled from the animals every 15 min for 2 h (period A). Each animal then received each of the four doses of GnRH, in a random order, at 90-min intervals. After each GnRH injection, blood samples were collected at 5-min intervals for 30 min and every 10 min thereafter until the next GnRH injection (period B). At the end of the experiment, the animals regained consciousness and were treated with antibiotics before being returned to the flock.

Hormone assays. The concentration of LH in plasma samples was measured by radioimmunoassay (Martensz *et al.*, 1976; Webb *et al.*, 1985a). All samples from each animal were measured in duplicate in a single assay to reduce interassay variation. The detection limit for the LH assay was 0.17 ng NIH-LH-S18/ml, and the inter- and intra-assay coefficients of variation were 9.52 and 11.59%, respectively. Testosterone was measured following solvent extraction of the samples (recovery efficiency 83%) in a radioimmunoassay developed in this laboratory and described previously (Webb *et al.*, 1985b). The mean detection limit of the assay was 5.8 pg/tube and the intra- and interassay coefficients of variation were 6.25 and 0.54%, respectively.

Statistical analysis. During Expt 3, the peripheral LH concentrations in some of the animals from both selection lines, after the 250 and 500 ng doses of GnRH, did not return to baseline before the next injection. The results were therefore analysed by fitting separate exponential models for each animal, assuming an LH peak at the third sample following GnRH treatment. The model gives estimates for the pulse amplitude and the rates of rise and fall of LH for each dose of GnRH. The fitted equation for the post-injection rise in the LH concentration is:

$$y = \alpha \exp\{(\chi - 3)/\beta\}$$

where α is pulse amplitude, β is the rate of rise and χ is the number of samples. The equation for the decay in the LH concentration after the peak is:

$$y = \alpha \exp\{(\chi - 3)/\gamma\}$$

where α and χ are as previously defined and γ is the rate of decay. At the peak, when $\chi = 3$, both equations have the same value, namely α , the pulse amplitude. Thus the peak LH concentration for each animal and each dose of GnRH can be characterized by the three parameters (α , β , γ) and the values of these parameters for different animals and different doses can be evaluated (A. J. Springbett, unpublished).

Results

Experiment 1

During the 7.5-h experimental period a number of endogenous LH pulses were observed. Irrespective of line, when LH secretion was stimulated by exogenous GnRH, the concentration of LH measured in the peripheral circulation increased in the first sample taken after GnRH administration (+15 min). The maximum concentration of LH occurred in the first sample in all animals and the concentration of LH in the peripheral circulation decayed exponentially thereafter. The maximum LH concentration measured was taken as the LH response. On average, the 25 and 50 ng doses of GnRH failed to induce a significant LH response in the low-line animals (Fig. 1). The high-line animals all released a measurable pulse of LH in response to the 50 ng dose of GnRH, but, as with the low line, the average LH response after 25 ng of GnRH was not significantly greater than baseline. The LH responses of the animals of both lines to the higher doses of GnRH were much

greater than those seen endogenously in 10-week-old ram lambs from the two selection lines (high: 3–45 ng/ml; low: 2–28 ng/ml; Evans, 1991). It was concluded that the lowest dose of GnRH to be used in the dose–response study (Expt 3) should be 50 ng and the maximum dose should be 500 ng.

Fig. 1. Individual luteinizing hormone (LH) responses in two lambs of lines selected for low (■) and 3 selected for high (□) response to gonadotrophin-releasing hormone (GnRH), to five doses of GnRH.

Experiment 2

Initially, LH concentrations were high in both animals, but LH concentration decreased to 'baseline' within 120 min and no further increases were seen in the peripheral LH concentrations of the two lambs (Fig. 2). Because of the small sample number, no conclusions can be drawn from this initial increase in LH secretion, but the high-line animal released approximately four times more LH than the low-line animal.

Fig. 2. Effect of anaesthesia on the peripheral luteinizing hormone (LH) concentration in individual lambs from the lines selected for high (□) and low (◆) response to gonadotrophin-releasing hormone.

This study therefore demonstrates that sodium pentobarbital anaesthesia inhibits the secretion of endogenous pulses of LH from the pituitary glands of 6-week-old ram lambs, presumably by the suppression of GnRH secretion, and confirms the suitability of barbiturate anaesthesia for the construction of a model system to study pituitary sensitivity of GnRH stimulation and thereby to obtain an indirect measurement of endogenous GnRH secretion.

Experiment 3

Period A. During the 2-h pre-GnRH treatment period, two of the 18 animals exhibited high plasma LH concentrations at the start of the sampling period. In both animals, the LH concentration decayed to baseline before the administration of GnRH. LH pulses were abolished by anaesthesia in all but one animal, which released a single LH pulse during this period (Fig. 3).

Fig. 3. Profiles of luteinizing hormone (LH) from a representative lamb from lines selected for (a) high and (b) low response to gonadotrophin-releasing hormone (GnRH) during period A when the lambs were maintained anaesthetized with sodium pentobarbitone and period B when anaesthesia was maintained and the lambs were challenged with GnRH.

Period B. Throughout period B (6 h), endogenous LH pulses were abolished in all animals. An immediate release of LH was detected in the peripheral plasma of all the lambs in response to the injections of GnRH. A normal exponential pattern of decay was seen after each LH pulse (Table 1).

In both lines, the magnitude of the LH responses measured were correlated to the GnRH doses received. Mean LH responses increased significantly in accordance with increasing doses of GnRH in the animals from both lines. The relationship between the dose of GnRH given and the LH response was linear at low doses of GnRH, but at higher doses of GnRH the relationship changed,

Table 1. Mean luteinizing hormone (LH) responses of ram lambs from lines selected for high and low response to gonadotrophin-releasing hormone (GnRH) after the administration of various doses of GnRH

GnRH dose (ng)	Mean LH response (ng/ml $\pm$ s.e.m.)	
	High line ($n = 7$)	Low line ($n = 11$)
50	10.1 $\pm$ 2.8 ^{a,w}	1.8 $\pm$ 0.8 ^{b,w}
100	27.6 $\pm$ 2.9 ^{a,x}	5.1 $\pm$ 0.7 ^{b,x}
250	65.9 $\pm$ 3.2 ^{a,y}	13.9 $\pm$ 0.8 ^{b,y}
500	113.9 $\pm$ 3.1 ^{a,z}	24.6 $\pm$ 0.8 ^{b,z}

Within columns (w, x, y, z) and within rows (a, b), different superscripts indicate significant differences ($P < 0.05$).

as the responses to the different doses were less marked (Table 1). When individual animals from both lines were studied, increased LH concentrations were detected in the peripheral circulation of all animals in response to the 100, 250 and 500-ng doses of GnRH. The 50-ng dose of GnRH produced significant releases of LH in all of the high-line animals (10.1 $\pm$ 2.8 ng/ml), but only four of the low-line animals responded, these animals releasing significantly less ($P < 0.05$) LH (1.8 $\pm$ 0.8 ng/ml) than the high line.

A statistically significant interaction existed between the dose of GnRH and the line of origin of the lambs. This effect was removed by transformation of the data onto the log scale, and regression lines were fitted (Fig. 4). Extrapolation of the dose-response curves enabled the pituitary gland sensitivity to be assessed. The interception of the dose-response curves with the x axis indicates the

Fig. 4. Regression analysis for the log gonadotrophin-releasing hormone (GnRH) dose-response curves for lambs from the lines selected for high (●, $n = 7$) and low (○, $n = 11$) response to GnRH. Pituitary gland sensitivity was estimated for the two lines as the dose of GnRH where the regression lines cross the x axis. Indirect measurements of the amplitudes of the endogenous GnRH pulses were also obtained for the high (—) and low (---) lines.

theoretical dose of GnRH in each line that must be exceeded for an LH response to be measurable in the peripheral circulation. The regression lines intercepted the x axis at the equivalent of 26.6 ng and 4.75 ng of GnRH for the low and high lines, respectively, thus demonstrating a 5.5-fold difference in the sensitivity of the pituitary glands of the two lines to GnRH stimulation.

The endogenous LH pulse amplitudes of both selection lines were estimated from the results of the window bleed on the first day of Expt 3; because of the low LH pulse frequency in lambs of this age, not all of the lambs secreted endogenous LH pulses during the 3-h sampling period. The amplitudes of the LH pulses observed tended to be greater in the high line than in the low line (10.13 ± 4.65 ng/ml and 9.99 ± 1.58 ng/ml for the high and low lines, respectively), but great variation was observed within each line. The mean endogenous LH pulse amplitudes were transposed onto the dose-response curves to obtain an indirect estimate of the quantity of GnRH needed to reproduce an endogenous LH pulse. The dose of GnRH needed was estimated to be 43.67 ng for the high line and 206.55 ng for the low line. The mean testosterone concentrations were assessed for each lamb in five samples taken during the Day-1 serial bleed. The mean testosterone concentrations in the high and low lines were 74.17 ± 16.00 pg/ml and 58.36 ± 12.33 pg/ml, respectively; because of the wide variation observed within and between lambs the between-line difference was not, however, statistically significant.

Discussion

The results demonstrate that (i) sodium pentobarbitone anaesthesia of intact prepubertal ram lambs blocked endogenous GnRH secretion, thereby providing a suitable model for studying pituitary gland sensitivity to GnRH in the absence of endogenous GnRH; (ii) the high-line animals are five times more sensitive to GnRH stimulation than the low line; and (iii) when assessed indirectly, the amplitude of the endogenous GnRH pulses in the high-line ram lambs was five times less than that observed in the low-line lambs.

The effects of barbiturate anaesthesia on gonadotrophin secretion have been described in adult ewes in a wide range of physiological conditions (Radford, 1966; Ellicott *et al.*, 1975; Dobson & Ward, 1977; Radford *et al.*, 1978; Webb *et al.*, 1981; Goodman & Meyer, 1984), but, as far as we are aware, the effects of sodium pentobarbitone anaesthesia have not been reported in prepubertal ram lambs. The results of Expts 2 and 3 demonstrate that endogenous pulsatile LH secretion is abolished in castrated and intact ram lambs during sodium pentobarbitone anaesthesia. At the start of anaesthesia, both lambs in Expt 2 and two of those in Expt 3 had high LH concentrations, which then declined to baseline and remained low for the duration of anaesthesia; this result is similar to the changes observed in LH secretion in ovariectomized ewes anaesthetized during the breeding season (Goodman & Meyer, 1984). As this transient increase in LH secretion was seen in only a small proportion of the animals, no conclusions can be drawn from the data; but the magnitude of this LH release differed between the animals from the two selection lines, the magnitude of the between-line difference being similar to the difference in the LH response to a GnRH challenge (Haley *et al.*, 1989; Evans *et al.*, 1991). The reason for these high LH concentrations is not known, but LH may be released in response to stress, or the phenomenon may be due to an additional neurological effect at the induction of anaesthesia.

In the present study, during anaesthesia, endogenous pulsatile LH secretion was abolished in all of the lambs, irrespective of line. However, pulses of LH secretion were seen in most of the animals in response to exogenous GnRH. Therefore, as in previous studies (Radford & Wallace, 1974; Blake, 1974, 1976; Webb *et al.*, 1981), it can be concluded that the inhibitory effect of barbiturate anaesthesia on LH secretion is not at the level of the pituitary gland, but occurs at the level of the hypothalamus. It has been suggested that gonadotrophin secretion (Goodman & Meyer, 1984) is under the control of two neuronal systems, a stimulatory system (the 'GnRH pulse generator') and

an inhibitory oestradiol-sensitive system. Barbiturate anaesthesia has been demonstrated to cause a reduction in the neuronal activity of the hypothalamus (Cross & Dyer, 1971; Barker & Ransom, 1978) and it was therefore concluded that the effects of anaesthesia on gonadotrophin secretion are a result of alterations in GnRH secretion by the hypothalamus.

The GnRH dose-response curves obtained in this experiment (Fig. 4) were extrapolated to obtain a measure of the sensitivity of the pituitary glands of the two lines to GnRH stimulation; the mean sensitivity of the pituitary glands of the ram lambs from the two selection lines differed by a factor of five. As far as we are aware, this study provides the first documented evidence of two strains of sheep, derived from a single breed, differing in the sensitivity of their pituitary glands to GnRH stimulation. Following the administration of all of the doses of GnRH used in this study, the high-line lambs secreted approximately five times more LH than the low-line lambs. The magnitude of the between-line difference in the LH response to a pharmacological dose (5 µg) of GnRH, which has previously been reported for 10- and 20-week-old ram lambs, also differs by approximately five times (Haley *et al.*, 1989). Therefore, the 5-fold between-line difference in the ability of the lambs to secrete LH in response to GnRH is maintained at a constant magnitude over both physiological and pharmacological doses of GnRH.

The mean endogenous LH pulse amplitudes of the two lines were calculated from the LH concentrations measured in the serial blood samples collected on Day 1 of Expt 3. The doses of GnRH which must be administered intravenously to simulate endogenous pulses were calculated for each line from the dose-response curves and were 44 ng for the high line and 207 ng for the low line. The results indicate that low-line animals require approximately five times more GnRH to produce LH pulses of a given amplitude than high-line animals. The concentration of GnRH in the peripheral circulation and presumably the hypothalamo-pituitary portal blood following the intravenous administration of GnRH has been measured in adult ewes and estimated to range between 0.01 and 0.027% of the dose of GnRH given (Clarke *et al.*, 1984). If the same realized concentration of GnRH in the hypophyseal portal blood stream was to hold for the lambs in this study, the concentration of GnRH required to stimulate LH pulses of similar amplitude to those produced endogenously in the high line and the low line lambs would be 4.4–11.9 and 20.7–55.9 pg GnRH/ml, respectively. Therefore, although the accuracy of these estimates is limited by the indirect method of assessment, the range observed is similar to the endogenous GnRH concentrations measured in hypophyseal portal blood after direct sampling in ovariectomized (Clarke & Cummins, 1982; Levine *et al.*, 1982; Clarke *et al.*, 1987; Karsch *et al.*, 1987) and intact cyclic ewes (Clarke *et al.*, 1987) and intact and castrated ram lambs (Caraty & Locatelli, 1988). This indicates that the hypothalami of the low-line lambs are required to secrete more GnRH than those in the high-line lambs to stimulate the release of endogenous LH pulses of similar amplitude.

The results of this study demonstrate that the model constructed is suitable for studying the regulation of LH secretion by the hypothalamo-pituitary gland complex and that the pituitary glands of the high-line lambs were five times more sensitive to GnRH stimulation than those of the low line. Pituitary gland sensitivity, however, as defined here, is a comprehensive term encompassing variables such as the number of gonadotrophs, the number of available GnRH receptors, the amount of stored LH and the rate at which the pituitary gland is able to transcribe and translate DNA into active protein. Further studies are in progress to study individual aspects of 'pituitary sensitivity' in animals from the two selection lines. As the endogenous LH pulse amplitudes were the same in the high- and low-line lambs the results of this experiment suggest that the low-line lambs endogenously secrete five times more GnRH than the high-line lambs.

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