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Ovarian steroid hormone involvement in endogenous opioid modulation of LH secretion in mature ewes during the breeding and non-breeding seasons*

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Summary. The opioid antagonist WIN-44441-3 (WIN-3, Sterling-Winthrop) caused significant increases in LH secretion in ovariectomized ewes treated with progesterone but not in ovariectomized animals treated with oestradiol-17β. In the non-breeding season, plasma LH concentrations in ovariectomized ewes without steroid therapy, given oestradiol-17β or oestradiol-17β and progesterone together were not affected by treatment with WIN-3 on Day 6 after ovariectomy (there was a significant increase in LH as a result of WIN-3 treatment 13 days after ovariectomy in sheep given no steroid therapy). However, WIN-3 treatment of ovariectomized sheep given progesterone resulted in a significant increase in plasma LH. WIN-3 was ineffective when given to intact ewes treated with progesterone during the non-breeding season. With ovariectomized sheep during the breeding season there was again no response to WIN-3 at 6 days after ovariectomy in sheep given oestradiol-17β, but significant LH elevations in animals given no steroid, those given progesterone and those given progesterone + oestradiol-17β. The lack of an LH response to WIN-3 in ovariectomized sheep treated with oestradiol-17β did not result from a reduced pituitary response to GnRH since such animals responded normally to exogenous GnRH treatment. Overall, these results are consistent with the idea that, irrespective of the time of year, progesterone exerts negative feedback upon LH release at least in part through an opioidergic mechanism, whereas oestradiol-17β exerts negative feedback through steps unlikely to involve opioids. Progesterone can override the effect of oestradiol-17β during the breeding season only. Further, there appears to be a steroid-independent opioid involvement in LH suppression, operating at both times of year.

Keywords: opioids; LH; oestradiol-17β; progesterone; ewes

Introduction

There is now much evidence that endogenous opioids are involved in the modulation of LH secretion in a variety of species and they generally have an inhibitory action (for review, see Meites, 1984; Brooks *et al.*, 1986a). Furthermore, the degree of opioid inhibition adjudged by responses of animals to the opioid antagonist, naloxone, seems to vary, depending upon the degree of gonadal steroid production at the time of naloxone administration (see Ebling & Lincoln, 1985; Brooks

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et al., 1986b). Most of the studies support the concept that gonadal steroid hormones exert their negative feedback effects upon LH secretion via an opioidergic mechanism (Kalra & Kalra, 1984). In ewes, however, the situation is complicated by the seasonality of breeding. For instance, Brooks *et al.* (1986b) have demonstrated that, in mature ewes, naloxone treatment is ineffective during seasonal anoestrus, but enhances LH secretion during both the luteal and follicular phases of the cycle. This suggests (1) that the lower concentrations of oestrogens found during the non-breeding season may exert their powerful negative feedback effects upon LH secretion by routes not involving opioid peptides, and (2) that, during the cycle, progesterone and possibly oestrogen also may exert feedback action through opioidergic mechanisms. In support of these ideas, Brooks *et al.* (1986c) have shown that during the non-breeding season naloxone does not alter LH secretion when given to ovariectomized sheep implanted with oestrogen, but causes marked elevations in LH when administered to ovariectomized sheep given progesterone. This experiment does not, however, give any indication (1) as to whether administration of progesterone would allow opioid antagonists to be effective in the presence of the powerful negative feedback of oestradiol-17β found in sheep during the non-breeding season; (2) or how progesterone exerts negative feedback when it is normally present during the breeding season; or (3) how oestradiol-17β may exert its feedback effects during the time of year when cycles normally occur. Neither is it possible, from the experiments carried out on intact sheep during the follicular phase, to draw any conclusions about oestradiol-17β. Since the follicular phase of the sheep is short, there may be residual progesterone effects and in previous work we did not study the action of naloxone in ovariectomized sheep given oestradiol-17β and progesterone together. Therefore this paper reports experiments designed to overcome these deficiencies by investigating, during the breeding and non-breeding season, the effects of an opioid antagonist in ovariectomized sheep treated with oestradiol-17β or progesterone alone or in combination. The effect of administering progesterone to intact sheep on the response to opioid antagonists during the non-breeding season was also determined. Because the opioid antagonist WIN 44441-3 (see Ward *et al.*, 1983) was used, rather than naloxone, preliminary studies with this compound are included. Part of the work has already been presented in abstract form (Brooks *et al.*, 1985a,b).

Materials and Methods

Animals and general protocols. Mature Suffolk-cross ewes weighing 69·6 ± 1·5 kg were used. They were kept at pasture and given a supplementary diet of standard ewe concentrates until the start of the experimental periods when they were housed indoors under a natural light regimen and given free access to water, hay and the same concentrate ration. A cannula was placed in a jugular vein using aseptic procedures 24 h before the start of blood sampling for LH determinations. Blood (3 ml) was taken at 15-min intervals from the cannula for various periods of time depending upon the particular experiment. The blood was centrifuged immediately, and the plasma removed and stored at −15°C until required for LH assay. Ovariectomies and laparoscopies were performed under pentobarbitone-induced anaesthesia. WIN 44441-3 (WIN-3) and its (+)enantiomer, WIN 44441-2 (WIN-2), both supplied by Sterling-Winthrop (Onslow St, Guildford, Surrey, U.K.), were administered through the jugular venous cannula over about a 10-sec period, at an appropriate dose dissolved in 3 ml sterile 5% (w/v) dextrose solution. In Exps 1 and 2, oestrous cycles were synchronized by giving two intramuscular injections of 150 μg of the synthetic prostaglandin analogue cloprostenol (Estrumate, Coopers Animal Health Ltd, Crewe, Cheshire, U.K.), separated by an interval of 10 days.

For simplicity of explanation the protocols for Exps 2 and 3 are described as if all animals in each experiment were used together. In fact, because of practical considerations, they were treated as two sub-sets of 12 (Exp. 2) or 10 (Exp. 3) with two animals from each group per sub-set. Synchronization of oestrus, ovariectomy and steroid replacement, and blood sampling of each sub-set were carried out 24 h apart and the results were then combined.

Experiment 1: efficacy and specificity of WIN-3. This experiment was designed to test the efficacy and specificity of the opioid antagonist WIN-3 in regard to the LH response. Twelve ewes were treated with 2 injections of cloprostenol given 10 days apart in November. They were assumed to be in the luteal phase of the cycle 10 days after the second cloprostenol injection and at this time they were subjected to the following procedure. Blood samples were collected for 18 h starting at 08:00 h. At 14:00 h and 16:00 h 4 animals were given 12·5 mg WIN-3, 4 were given 25 mg WIN-3 and 4 were given 25 mg WIN-2.

Experiment 2 (breeding season): ovariectomy, steroid replacement and WIN-3. The aim of this experiment was to determine the effect of ovariectomy and steroid replacement during the breeding season on the LH response to an

opioid antagonist. The oestrous cycles of 24 ewes were synchronized with cloprostenol in January and the ewes were then randomly divided into 6 equal groups. The 20 animals (Groups 1b–5b) were ovariectomized about 18 h after the second cloprostenol injection when in the early follicular phase of the oestrous cycle. The remaining 4 ewes (Group 6b) were to serve as intact controls. Immediately after ovariectomy the animals were treated as follows: Groups 1b and 2b, no steroid hormone treatment; Group 3b received an oestradiol-17β implant (made and applied as described previously by Brooks *et al.* (1986c)); Group 4b had a pessary containing 500 mg progesterone (Fabwerthe Hoeschst A.G., Frankfurt, West Germany) placed in the vagina; and Group 5b received simultaneously both an oestradiol-17β implant and a progesterone pessary. Starting at 08:00 h on Day 1 after ovariectomy (Groups 1b and 2b only) and Day 6 after ovariectomy (Groups 1b–5b) blood samples were taken for a 24-h period. During each sampling period Group 1b received 8 injections of dextrose and Groups 2b–5b 4 injections of 3 ml dextrose followed by 4 injections of 12·5 mg WIN-3, all at 2-h intervals starting at 08:00 h. Group 6b received dextrose followed by WIN-3 as above during a 24-h blood sampling period starting 18 h (early follicular phase) and 5 days (early luteal phase) after the second cloprostenol injection.

Experiment 3 (non-breeding season): ovariectomy, steroid replacement and WIN-3. This was essentially a repeat of Exp. 2 but carried out during the non-breeding season. Twenty ewes were confirmed as anoestrous by laparoscopy in late June and ovariectomized in early July. They were allocated to equal groups, 1n–5n corresponding to Groups 1b–5b (Exp. 2) and treated similarly with steroid hormones (one animal in Group 4n (ovariectomized and progesterone treated) died 24 h after ovariectomy reducing the group size to 3). The protocol for Exp. 2 was then carried out with the following additions: Groups 3n, 4n and 5n as well as Groups 1n and 2n were blood sampled and given WIN-3 on Day 1. At 12 days after ovariectomy the oestradiol-17β implants and progesterone pessaries were removed and the animals were blood sampled and given dextrose (Group 1n) or dextrose followed by WIN-3 (all other groups) on Day 13 to examine the short-term effect of steroid withdrawal.

Experiment 4 (non-breeding season): intact sheep given progesterone and WIN-3. This experiment was to determine whether treatment with an opioid antagonist would evoke an LH response in intact sheep treated with progesterone during the non-breeding season, and for how long a response might be found after removal of progesterone. In June, 8 sheep were given an intravaginal progesterone pessary as in Exp. 2. After 12 days, blood samples were collected from all animals for 6 h (Period 1), the pessaries were removed and blood sampling continued for 12 h (considered as Periods 2 and 3 each of 6 h for analysis purposes), then for further 6-h periods at 24 h (Period 4), 48 h (Period 5) and 72 h (Period 6) after pessary removal. Starting at the withdrawal of the first blood sample, WIN-3 (12·5 mg/dose) was administered every 2 h throughout each bleeding period in 4 sheep. The remaining 4 sheep were treated similarly but received the dextrose vehicle only.

Experiment 5: LH response to GnRH in ovariectomized ewes given oestradiol-17β. This experiment was designed to establish the ability of the pituitary gland to release LH 6 days after ovariectomy and oestradiol-17β therapy.

In June, 10 animals were ovariectomized and given oestradiol-17β implants as in Exp. 2: 5 others were kept as intact controls. Blood samples were taken from jugular venous cannulae every 2 h for 48 h after ovariectomy. Starting at 08:00 h on Day 6 after ovariectomy, blood samples were collected at 15-min intervals for 16 h. After the first 6 h, 5 ovariectomized and 5 intact animals received 5 injections of GnRH (300 ng Lutal, Fabwerke Hoechst A.G., Frankfurt, West Germany) at 2-h intervals given as a bolus through the blood sampling cannulae and the other 5 ovariectomized animals received 3 injections of WIN-3 (12·5 mg/injection) at 2-h intervals.

Hormone assays. All plasma samples were analysed for LH concentrations by the specific double-antibody radioimmunoassay as described by McLeod *et al.* (1982). The limit of sensitivity was 0·13 ng NIH-LH-S24 equiv./ml plasma. Intra- and inter-assay coefficients of variation were 8·1% and 13·2% respectively. Progesterone was measured by the method of Haresign *et al.* (1975). The limit of sensitivity was 0·1 ng/ml plasma and intra- and inter-assay coefficients of variation were $< 12\%$.

Statistical analysis. An LH episode was defined according to the criteria of Goodman & Karsch (1980), namely: (1) the peak concentration associated with the episode occurred within two samples of the previous nadir; (2) the amplitude of the episode exceeded the sensitivity of the assay; and (3) the LH concentration at the peak exceeded the 95% confidence limits of the LH concentration at the preceding and subsequent nadirs.

LH episode amplitude was taken as the concentration at the height of the episode minus the concentration at the previous nadir.

Mean LH concentrations and episode amplitude were examined by a split-plot analysis of variance.

Analysis of deviance using a Poisson distribution was adopted to compare discrete data that did not conform to a normal distribution, i.e. episode frequency (Nelder & Wedderburn, 1972).

Results

Experiment 1: efficacy and specificity of WIN-3

Representative LH profiles are shown in Fig. 1, and mean data for LH concentrations, LH episode frequency and episode amplitude are given in Table 1. There were behavioural changes in

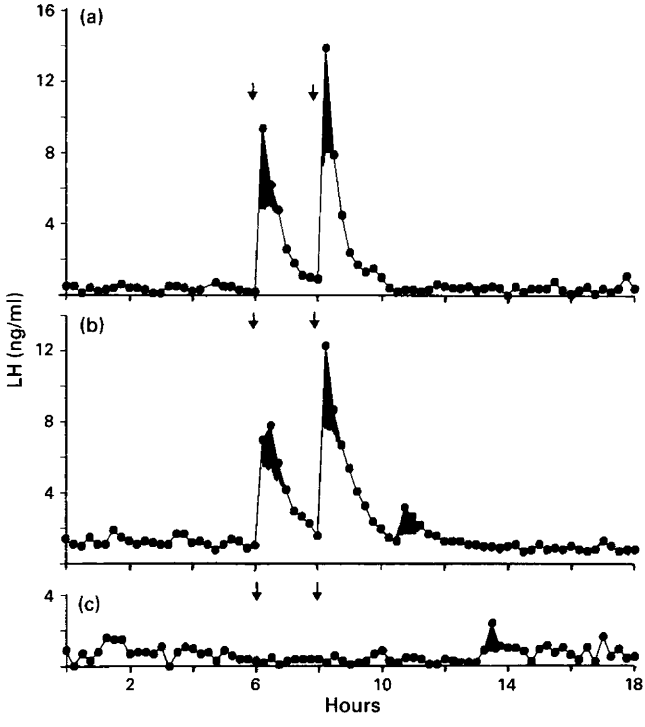

Fig. 1. Representative LH profiles for 3 luteal-phase ewes given (arrows) (a) 12·5 mg WIN-3 per dose, (b) 25 mg WIN-3 per dose, and (c) 25 mg WIN-2 per dose. LH episodes as defined in the text are indicated by black shading.

Table 1. Mean (±s.e.m.) plasma LH concentration, episode frequency (range in parentheses) and episode amplitude for sheep (N) treated with WIN-3 or WIN-2 during the mid-luteal phase of the cycle

	Control period (6 h)	Treatment period (6 h)	Run-off period (6 h)
WIN-3 (12·5 mg/dose) (N = 4)			
Mean LH conc. (ng/ml)	1·09 ± 0·23[a]	4·98 ± 0·51[d]	0·91 ± 0·20[a]
Mean LH episode frequency/6 h	0·75 (0–1)[a]	2·00 (2)[c]	0·33 (0–1)[a]
Mean LH episode amplitude (ng/ml)	4·65 ± 0·76[a]	9·86 ± 0·69[d]	3·39 ± 0·72[a]
WIN-3 (25 mg/dose) (N = 3)			
Mean LH conc. (ng/ml)	0·78 ± 0·23[a]	4·91 ± 0·91[d]	1·00 ± 0·29[a]
Mean LH episode frequency/6 h	0·00 (0)[a]	2·33 (2–3)[c]	0·33 (0–1)[a]
Mean LH episode amplitude (ng/ml)	6·32 ± 0·95[a]	10·37 ± 0·92[d]	3·47 ± 0·95[a]
WIN-2 (25 mg/dose) (N = 4)			
Mean LH conc. (ng/ml)	0·96 ± 0·15	1·24 ± 0·31	1·50 ± 0·41
Mean LH episode frequency/6 h	0·50 (0–1)	0·50 (0–1)	1·25 (1–2)
Mean LH episode amplitude (ng/ml)	4·62 ± 1·30	2·34 ± 1·46	5·32 ± 1·55

Across rows: a *vs* c, $P < 0{\cdot}01$; a *vs* d, $P < 0{\cdot}001$.

sheep receiving the 25 mg dose of WIN-3. These were similar to those reported by Sharman & Stephens (1974) for cattle and sheep given apomorphine and included excitement, circular movements round the pen and intense chewing at their food bowls, each other's skins and the person taking blood samples. One sheep collapsed and died 15 min after the second WIN-3 injection; in the

others the behavioural symptoms had disappeared some 3 h later. No behavioural changes were observed in any animals given the 12·5 mg dose in this experiment or any subsequent studies. WIN-3 caused significant increases in all measures of LH secretion examined and there were no significant differences between doses. Hence the 12·5 mg dose was chosen for use in further experiments. WIN-2 was ineffective in stimulating LH release, and did not affect behaviour.

Ovariectomy, steroid replacement and WIN-3

Breeding season (Exp. 2). Mean data for this experiment are shown in Fig. 2. Individual LH profiles for sheep in Groups 2b and 4b are depicted in Fig. 4. There were no significant changes in LH measures after dextrose treatment of Group 1b (no steroid therapy). In Group 2b, also given no steroids, WIN-3 treatment resulted in increases in LH concentration and pulse frequency at 1 day and 6 days after ovariectomy. LH measures in the control periods for Groups 1b and 2b were characteristic of those normally seen in ovariectomized animals. In Group 3b, oestradiol-17β implants maintained LH concentrations at low levels (0·70 mg/ml compared to 5·01 mg/ml in Group 1b) and there was no effect of WIN-3 upon LH secretion. Progesterone treatment (Group 4b) resulted in a mean control period LH concentration between that found in Groups 1b and 3b. In Group 4b, WIN-3 treatment resulted in significant increases in mean LH concentration, episode frequency and episode amplitude on Day 6. In Group 5b, combined oestradiol-17β and progesterone treatment resulted in mean LH concentrations similar to those of Group 3b (oestradiol-17β alone) and WIN-3 treatment gave rise to small but significant increases in all measures of LH secretion. WIN-3 was also effective in significantly increasing mean LH concentration and episode frequency, but not episode amplitude in the Group 6b during the follicular and early luteal phases of the cycle.

Non-breeding season (Exp. 3). Mean data are given in Fig. 3 and individual LH profiles for sheep in Groups 2n and 4n at 6 days after ovariectomy are shown in Fig. 4. There were no changes in LH as a result of dextrose treatment in Group 1n. In Group 2n, WIN-3 resulted in significant increases in LH concentration on Day 13 only, in episode frequency on Day 6 and Day 13, and there were no significant effects on episode amplitude. In Group 3n, results obtained 1 day after ovariectomy showed that all animals had a preovulatory type LH surge starting some 12 h after oestradiol-17β implant insertion, with a range of peak LH from 50 to 70 ng/ml. It was not possible, therefore, to interpret the effects of WIN-3 at this time. On Day 6 there were low LH concentrations and no effects of WIN-3 (or during Day 13, 24 h after implant removal). In Group 4n, progesterone treatment was ineffective in producing significant LH responses to WIN-3 on Day 1 after ovariectomy but caused significant increases in mean LH concentrations, episode frequency and episode amplitude in response to WIN-3 on Day 6. On Day 13 (24 h after steroid removal) there was a response in terms of mean LH concentration only. In Group 5n, receiving oestradiol-17β and progesterone there were no significant responses to WIN-3.

Experiment 4 (non-breeding season): intact sheep given progesterone and WIN-3

Mean data are shown in Table 2. For analysis each period of WIN-3 treatment was compared with the equivalent period in control animals. There were no significant LH responses to WIN-3 in any treatment period.

Experiment 5: LH response to GnRH in ovariectomized ewes given oestradiol-17β

Treatment with WIN-3 had no effect on LH concentrations (Table 3). GnRH treatment significantly enhanced all measures of LH secretion in both groups of animals. In addition, mean LH concentrations as a result of GnRH treatment were significantly higher ($P < 0·05$) in the ovariectomized oestradiol-17β treated group compared to intact animals.

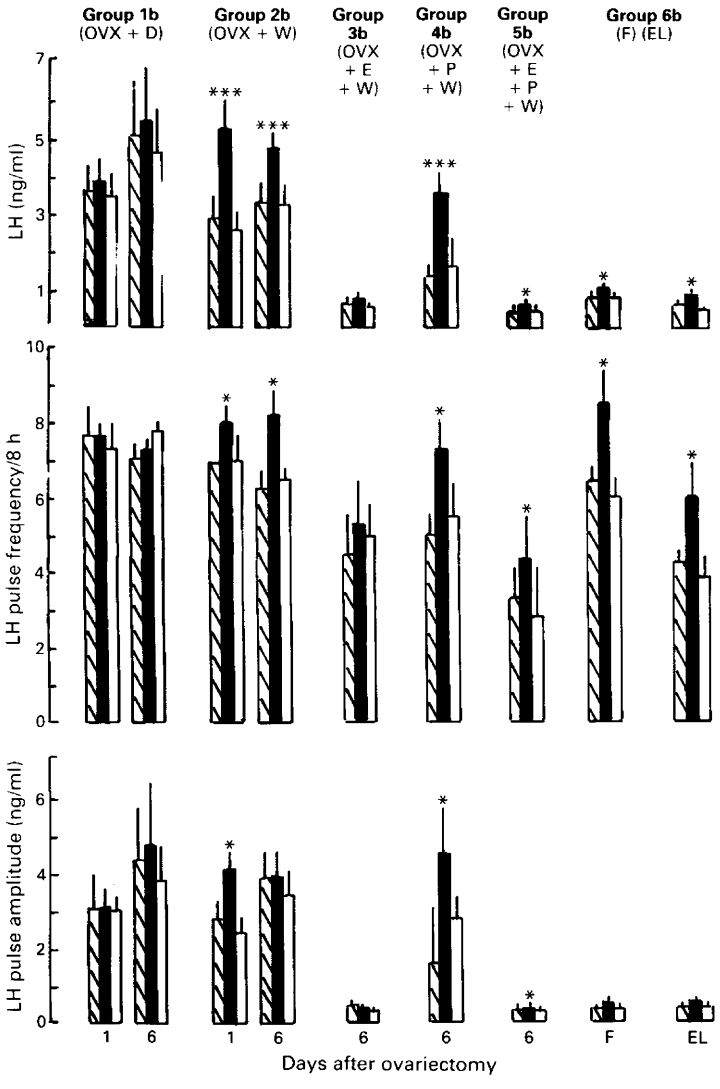

Fig. 2. Mean (±s.e.m.) LH concentrations, episode frequency and amplitude for sheep in Groups 1b–6b (Exp. 2; ewes treated during the breeding season) during control (▨), treatment (■) and run-off (□) periods. OVX = ovariectomized; D = dextrose treatment; W = WIN-3 treatment; P = progesterone treatment; E = oestradiol-17β treatment; F = follicular phase; EL = early luteal phase. $***P < 0{\cdot}001$, $*P < 0{\cdot}05$ for treatment and control periods.

Discussion

The preliminary studies with WIN-3 demonstrated that this opioid antagonist enhanced LH secretion in a manner similar to naloxone when given to ewes during the luteal phase of the cycle. Moreover, the ineffectiveness of the (+)enantiomer WIN-2 provides evidence that the LH response to WIN-3 is specifically mediated through opioid receptors.

In accord with our previous studies using naloxone (Brooks *et al.*, 1986b) WIN-3 treatment during the non-breeding season was effective in increasing LH secretion in ewes which were

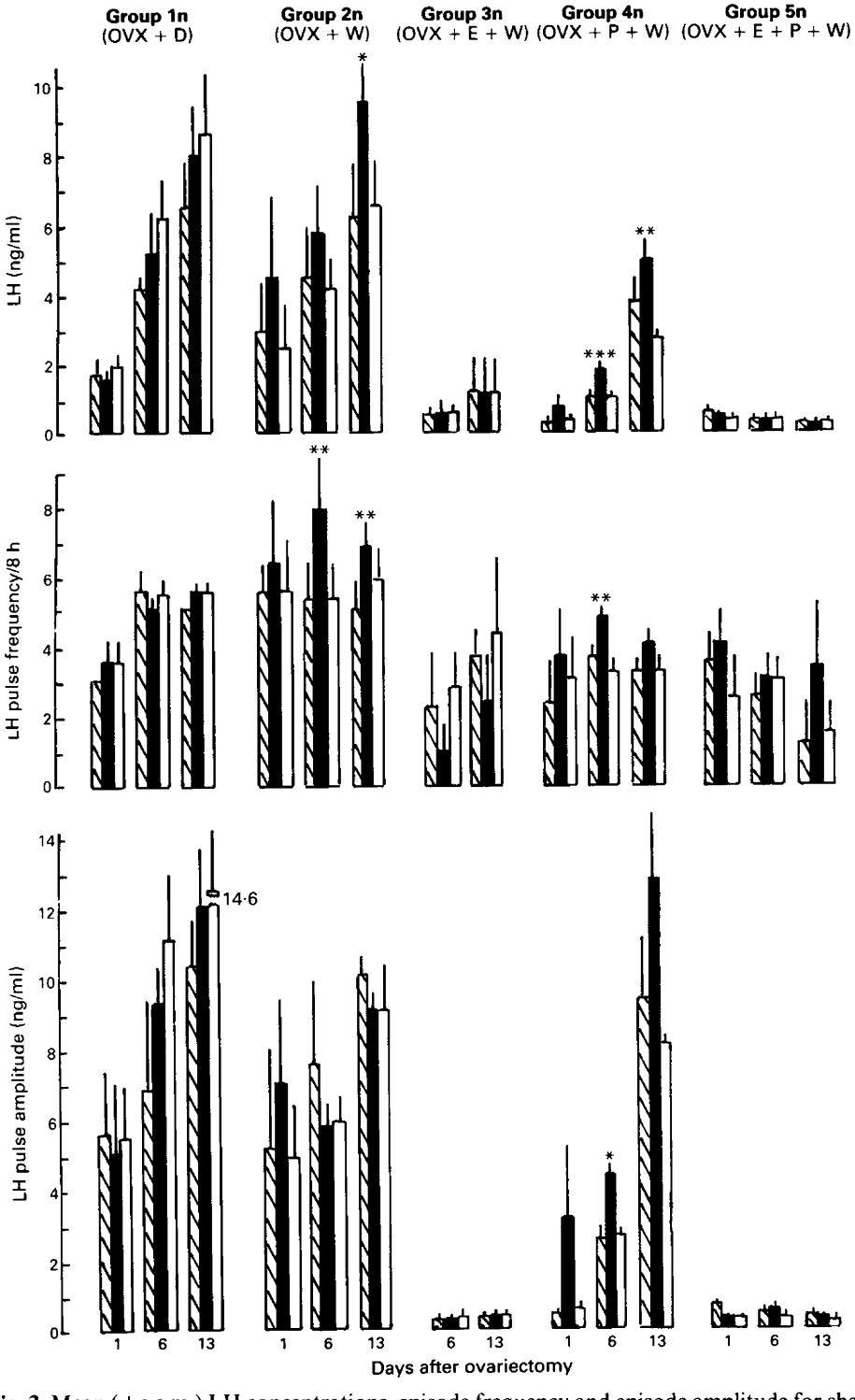

Fig. 3. Mean (±s.e.m.) LH concentrations, episode frequency and episode amplitude for sheep in Groups 1n–5n, (Exp. 3; ewes treated during the non-breeding season) during control (▨), treatment (■) and run-off (□) periods. OVX = ovariectomized; D = dextrose treatment; W = WIN-3 treatment; P = progesterone treatment; E = oestradiol-17β treatment. ***$P < 0{\cdot}001$, **$P < 0{\cdot}01$, *$P < 0{\cdot}05$ for treatment and control periods.

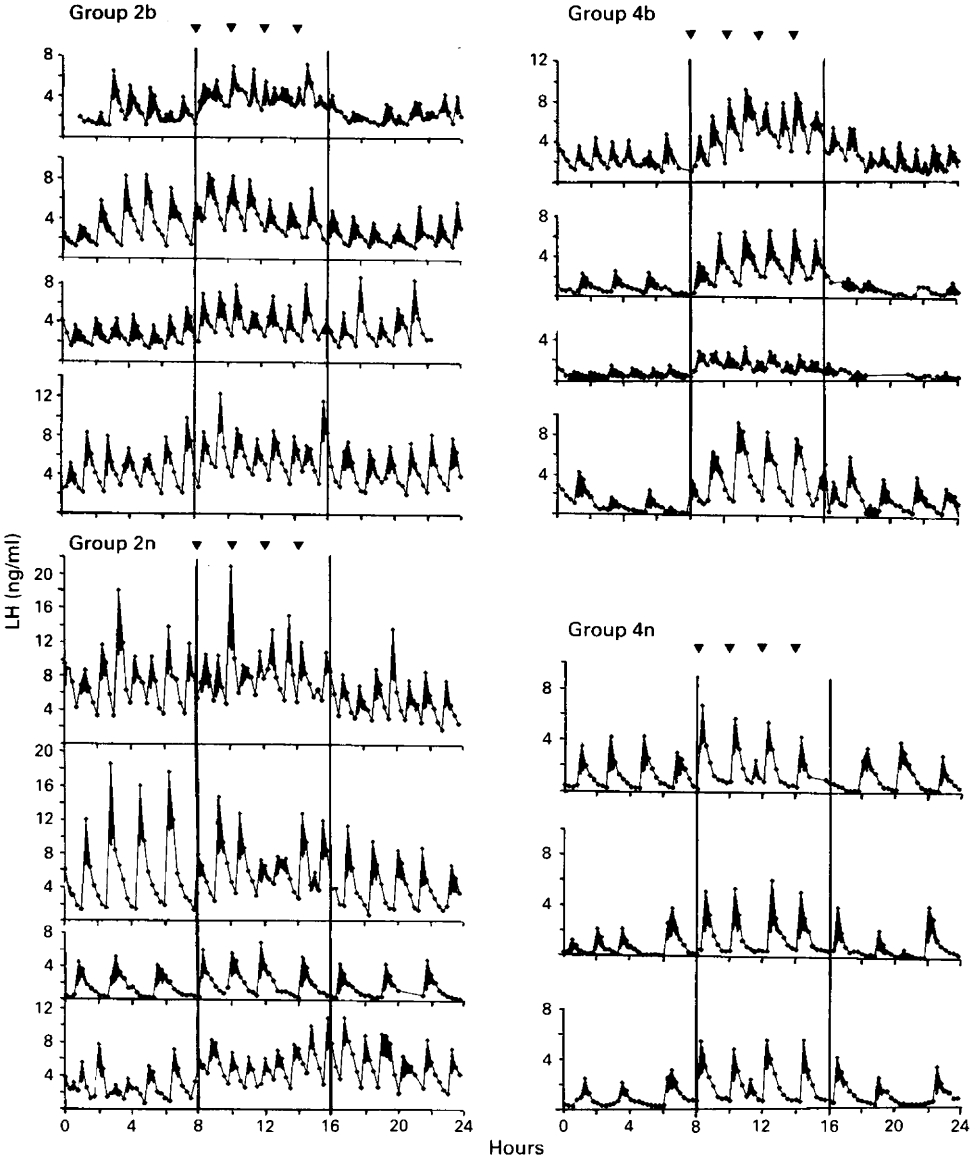

Fig. 4. Individual LH profiles for sheep treated with WIN-3 (▼) 6 days after ovariectomy with no steroid hormone therapy (Group 2b, breeding season and Group 2n, non-breeding season) and 6 days after ovariectomy with progesterone treatment (Group 4b, breeding season, and Group 4n, non-breeding season). LH episodes as defined in the text are indicated by black shading.

ovariectomized and given progesterone 6 days before WIN-3, but was not effective in animals ovariectomized and implanted with oestradiol-17β. During the breeding season, the results were equivalent, namely no demonstrable LH response to WIN-3 treatment at Day 6 after ovariectomy in oestradiol-17β-treated animals and a significant response in those given progesterone. Hence, the two hormones behave similarly under different photoperiodic conditions and this is at variance with the findings for testosterone in the male hamster. In this species, naloxone caused a marked

Table 2. Mean (±s.e.m.) plasma LH concentration, episode frequency (range in parentheses) and episode amplitude for intact sheep (4/group) treated with progesterone and WIN-3 during the non-breeding season

Sampling period	Mean LH conc. (ng/ml)		Mean LH episode amplitude (ng/ml)		Mean LH episode frequency/6 h	
	WIN-3	Control	WIN-3	Control	WIN-3	Control
1	0·31 ± 0·1	0·27 ± 0·03	0·79 ± 0·26	0·32 ± 0·10	1·3 (0–2)	1·3 (1–2)
2 (0 h)	0·63 ± 0·1	0·51 ± 0·14	3·76 ± 0·30	1·42 ± 0·51	1·0 (1)	2·0 (1–3)
3 (6 h)	0·26 ± 0·07	0·47 ± 0·19	0·30 ± 0·04	0·43 ± 0·22	1·8 (1–3)	2·0 (0–6)
4 (24 h)	0·50 ± 0·18	0·28 ± 0·06	2·04 ± 1·12	0·80 ± 0·46	1·5 (1–2)	1·5 (1–2)
5 (48 h)	0·38 ± 0·13	0·33 ± 0·08	0·89 ± 0·32	0·75 ± 0·36	1·8 (1–2)	1·8 (1–2)
6 (72 h)	0·30 ± 0·08	0·33 ± 0·11	0·55 ± 0·13	0·98 ± 0·39	1·8 (1–3)	1·5 (1–2)

*A progesterone pessary was *in situ* during sampling period 1. Figures in parentheses indicate the number of hours elapsed after progesterone withdrawal to the start of the particular sampling period.
There were no significant differences in any measures of LH between WIN-3 and Control data during any period.

Table 3. Mean (±s.e.m.) plasma LH concentration, episode frequency (range in parentheses) and episode amplitude for sheep (N) ovariectomized, given oestradiol-17β implants and treated with GnRH at Day 6 after ovariectomy and intact sheep treated with GnRH

	Control period (8 h)	GnRH treatment period (8 h)
Ovariectomy + oestradiol (N = 4)		
Mean LH conc. (ng/ml)	0·34[a] ± 0·03	4·45[d]† ± 1·08
Mean LH episode frequency/8 h	0·40[a] (0–1)	5·60[d] (5–6)
Mean LH episode amplitude (ng/ml)	0·38[a] ± 0·08	5·13[d] ± 0·94
Intact (N = 4)		
Mean LH conc. (ng/ml)	0·67[a] ± 0·17	2·73[c] ± 0·33
Mean LH episode frequency/8 h	1·80[a] (1–3)	5·40[c] (5–6)
Mean LH episode amplitude (ng/ml)	1·67[a] ± 0·39	4·50[d] ± 0·41

Across rows: a *vs* c, $P < 0·01$; a *vs* d, $P < 0·001$.
†LH concentration after GnRH in this group was significantly greater ($P < 0·05$) than that after GnRH treatment of the intact group.

increase in LH in castrated, testosterone-implanted males when given during a long stimulatory photoperiod but not during a short, suppressive photoperiod (Roberts *et al.*, 1985). The results also demonstrate a difference between female and male sheep. In rams, an opioidergic mechanism operating upon LH secretion, as demonstrated by naloxone and morphine treatment, was much more pronounced during the breeding season when gonadal steroid concentrations in plasma are high, than during the non-breeding season when levels are low (Ebling & Lincoln, 1985). This is a similar situation to that found in intact ewes (Brooks *et al.*, 1986c). On the other hand, testosterone therapy in castrated rams restored a response to naloxone in animals maintained under short photoperiod but was ineffective in animals under long photoperiod (Lincoln, Ebling & Martin, 1987). In ovariectomized ewes given a combined treatment of progesterone + oestradiol-17β there was a difference between the times of year, a small but significant LH response to WIN-3 treatment only being observed during the breeding season. Presumably the less powerful negative feedback effects of oestradiol-17β upon the LH generating system at this time (Karsch *et al.*, 1984) allows an opioidergic mechanism of LH control to manifest itself in the presence of progesterone. The fact that an opioidergic mechanism of LH control cannot be demonstrated when the two steroid

hormones are present at the same time during the non-breeding season, in treated castrates or in entire animals given progesterone raises the question as to whether potent oestradiol-17β activity at this time actively prevents an opioidergic system from operating or whether oestradiol-17β action is so powerful that it merely passively overrides an existing system such that it cannot be demonstrated by antagonist challenge. This question is not answered by the current experiments. The occurrence of an ovulatory type LH surge at Day 1 after ovariectomy in sheep implanted with oestradiol-17β was of concern since the lack of a response to WIN-3 at Day 6 after ovariectomy could be explained on the basis that a previously depleted pituitary had not recovered sufficiently to respond to WIN-3-induced GnRH release. The fact that such animals respond normally to a GnRH challenge (Exp. 5), however, precludes this possibility.

It was considered that an examination for the presence or absence of a response to WIN-3 soon after the application and withdrawal of a steroid could provide information as to whether the steroid, for example progesterone, was merely activating existing receptor systems in which case a rapid response could be expected, or the chronic presence of steroid is required for receptor development. The lack of response in progesterone-treated animals at Day 1 after ovariectomy, however, is not open to interpretation in terms of progesterone alone since LH concentrations in the ovariectomized ewes given no steroids were still low during the comparable period, presumably because of residual negative feedback effects of oestradiol-17β. This assumption is supported by the observation that control period LH values had also not risen in animals 24 h after removal of oestradiol-17β implants.

During the breeding season, a significant increase in LH concentration and pulse frequency occurred after WIN-3 treatment at 6 days after ovariectomy in animals given no steroid. Hence there appears to be a steroid-independent opioid mechanism operating in the ewe at this time of year. This was different from the results obtained with castrates given no steroids during the non-breeding season using either naloxone, which produced no significant effects on LH secretion (Brooks *et al.*, 1986c), or WIN-3 to which there was only a significant response in pulse frequency. However, examination of individual ewe data for the non-breeding season WIN-3 group (see Fig. 4, Group 2n) revealed an increase in LH concentration in 3/4 animals during WIN-3 treatment but overall significance was probably confounded by the wide variety in LH patterns between animals. Moreover, WIN-3 treatment did result in a significant increase in LH concentration in this group when given at 13 days after ovariectomy and hence a steroid-independent opioidergic involvement in LH probably also exists during the non-breeding season.

A progesterone milieu is clearly not a prerequisite in ewes for the demonstration of an opioidergic involvement in LH control. These findings accord with those of Schillo *et al.* (1985) and Schanbacher (1985) who reported a significant naloxone-induced stimulation of LH secretion in ovariectomized ewes and castrated rams respectively. Furthermore, whilst such findings do not negate the hypothesis that an opioid system is involved in the negative feedback effects of progesterone, they allow for an alternative explanation, namely that progesterone could exert negative feedback upon LH secretion partly through a mechanism not involving opioids; this would be complementary to a steroid-independent opioid system also depressing LH, with progesterone being merely permissive in allowing this to be demonstrated by use of the antagonist WIN-3. Such a possibility has been suggested for the rat (Spencer & Whitehead, 1986). The original hypothesis that progesterone negative feedback is mediated, at least in part, through an opioidergic mechanism is, however, still favoured on two counts. Firstly, notwithstanding the fact that no obvious explanation for the difference between the naloxone studies of Brooks *et al.* (1986b) and WIN-3 studies here is forthcoming, the lack of response in untreated ovariectomized ewes given naloxone compared to the dramatic response in progesterone-treated animals given the same antagonist was clearcut. Secondly, the fact that during the breeding season a small but significant increase in LH secretion after administration of WIN-3 was found in ovariectomized animals given a combined treatment with oestradiol-17β and progesterone, but not in animals given oestradiol-17β alone, is difficult to explain through a merely permissive action of progesterone.

Overall, these studies with WIN-3 during the breeding and non-breeding seasons remain consistent with the idea that progesterone exerts its negative feedback upon LH secretions through an opioidergic mechanism, and this can be demonstrated in the ewe at both times of year. They support previous findings that some suppression of LH by opioids exists in the absence of ovarian steroids and, in contrast to progesterone, there is no evidence for involvement of opioids in oestradiol-17β-mediated negative feedback, whatever the season.

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