Initiation of the oestradiol-induced inhibition of pulsatile LH secretion in ewes under long days: comparison of peripheral versus central treatment and neurochemical correlates

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Abstract

In the ewe, the inhibition of pulsatile LH secretion by oestradiol during long days depends on dopaminergic activity and could involve amino acid transmitters. In the first experiment of the present study we observed the changes in LH secretion in ovariectomised ewes under long days immediately after subcutaneous implantation of oestradiol (peripheral treatment). In the second experiment, in order to identify the site of action of oestradiol, we observed the LH changes following intracerebral infusion of oestradiol through a microdialysis membrane (central treatment) within the preoptic area, the medio-basal hypothalamus (MBH) or the retrochiasmatic area (RCh) and measured amino acids and catecholaminergic transmitters and metabolites within the dialysates. With peripheral treatment, the amplitude, the nadir and the area under the LH pulse curve decreased within 4 to 8 h of the insertion of a subcutaneous oestradiol implant. After 18 h, the amplitude and the area under the pulses increased, as well as the intervals between pulses (from 49.9 ± 1.4 min to 75.6 ± 5.9 min). With central oestradiol treatment, LH changes were similar whatever the site of oestradiol infusion, suggesting either multiple sites of action or diffusion between structures. Twenty hours after the beginning of intracerebral oestradiol treatment, the amplitude and the area under the pulses increased, as did the interval between LH pulses (from 49.5 ± 4.1 min to 73.2 ± 14.2 min). Comparison of peripheral with central oestradiol treatment suggested that the long-lasting decrease in the nadir, as well as the transitory decrease in the amplitude and area, before 18 h in experiment 1 are reflections of hypophysial effects. In contrast, the increases in amplitude and area under the LH pulse curve seen 18–20 h after oestradiol in the two experiments could be due to the higher amplitude of LHRH pulses, as a result of an early stimulatory effect of oestradiol. After central oestradiol infusion, there was a decline in the concentration in the dialysate of two metabolites of dopamine, 3,4-dihydroxyphenylacetic acid and homovanillic acid in the RCh, suggesting an early inhibition of monoamine oxidase by the steroid. During the inhibition of LH pulsatility the concentration of γ-aminobutyric acid in the dialysate from the RCh and the MBH increased, suggesting the participation of this transmitter in the changes induced by oestradiol under long days.

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Introduction

Ovine females from temperate latitudes show seasonal variations in reproductive activity, exhibiting a complete cessation of oestrus during spring and summer. Changes in daylength around the year are responsible for this alteration in reproductive activity via effects on secretion of gonadotrophins, particularly luteinizing hormone (LH). Ovariectomised ewes bearing subcutaneous oestradiol implants will exhibit decreased LH secretion during the normal time of anoestrus in intact animals (Goodman et al. 1982, Martin et al. 1983), and this same effect can be induced under artificial long-day treatment (Karsch et al. 1986). In such an experimental situation the decrease in LH pulsatility is usually only taken into consideration a few days after the implantation of the steroid. The changes in the secretion of LH reflect variations in the frequency of LH pulses, which in turn are controlled by similar modifications in the frequency of gonadotrophin-releasing hormone (GnRH) emission within the hypothalamo-hypophysial portal vessels (Barrell et al. 1992, Viguié et al. 1995b). Thus, seasonal inhibition in reproductive activity in the female sheep is primarily controlled by the strengthening of the inhibitory effect of oestradiol on the frequency of GnRH pulses.

Previous studies (Thiéry et al. 1989) suggest that the dopaminergic A15 nucleus from the retrochiasmatic area of the hypothalamus (RCh) and the A14 from the
posterior preoptic area (POA) (Havern et al. 1994) are involved in this inhibition. Additionally, the involvement of excitatory amino acids (Viguié et al. 1995b), and γ-aminobutyric acid (GABA), an inhibitory amino acid (Scott & Clarke 1993), has also been suggested in the inhibition of gonadotrophin secretion under long days. However, the site of action of oestradiol remains unknown. Thus, identification of the area(s) containing the oestradiol-sensitive neurones involved in the inhibition of LH under long days is required for further analysis of the regulation.

Localisation of oestradiol receptors in the brain of sheep using histochemistry has been performed in several studies. The POA contains oestradiol receptors (Hershson et al. 1993), making this anatomical structure a putative site for the effect of the steroid. Additionally, oestradiol receptors have also been found in both the arcuate nucleus and the ventromedial nucleus, making the mediobasal hypothalamus (MBH) an alternative candidate for the site of action of oestradiol (Batailler et al. 1992, Blache et al. 1994, Hershson 1995). In contrast, oestradiol receptors have not been found either in the A15 or the A14 nuclei (Lehman et al. 1993, Blache et al. 1994), although a direct action of oestradiol on dopaminergic structures is possible (Pasqualini et al. 1995).

In the first experiment of the present study we observed the changes in LH secretion in ewes under long days immediately after subcutaneous implantation of oestradiol in order to determine the minimal delay required for measuring a significant modification in the frequency of pulsatile LH release. This information was needed for further neurophysiological studies of the phenomenon conducted in the second experiment where we tried to identify the site of action of oestradiol. To this purpose, we used microdialysis probes to bring the steroid within the POA, the MBH or the RCh. This method allowed us to control the exact amount of oestradiol released and to study its effect on LH secretion when infused centrally. Microdialysis also allowed us to collect from the extracellular milieu in which we measured the changes in amino acids and catecholaminergic transmitters or metabolites which accompanied the effect of oestradiol.

Materials and Methods

Animals

Two experiments were performed during an artificial anoestrous induced by long days in 15 adult Romanov ewes ovariectomised at least 1 month earlier. For this purpose, ewes were first maintained in a light-sealed room under an artificial short photoperiod (8 h of light:16 h of darkness; 8L:16D) for at least 70 days. The ewes were then subjected to a long inhibitory photoperiod (16L:8D) for 60–150 days so that gonadotrophin secretion was inhibited (Robinson & Karsch 1987).

![Figure 1 Percentage of oestradiol diffusing from four pairs of dialysis probes of 2 mm membrane, at a perfusion rate of 1·5 µl/min. Tritiated oestradiol tracer (1·3 × 10⁻³ c.p.m. in 200 µl) was added to 10 ml of a 3 µg/ml solution of oestradiol in Ringer lactate. Each pair of probes was dipped separately in 0·5 ml Ringer lactate contained in a test tube kept at 37 °C in a thermostatic bath. The test tubes were changed every hour for 7 h. From the eighth hour Ringer lactate without oestradiol was perfused in order to observe the kinetics of oestradiol elimination from the circuitry.](image)

Oestradiol treatments

Experiment 1 The oestradiol implants were made from Silastic tubing (Dow Corning, Sigma Medical France, Nanterre, France; inside diameter 3·35 mm; outside diameter 4·5 mm; length 2 cm (Karsch et al. 1973)). These implants maintain plasma oestradiol concentrations of 5–8 pg/ml, similar to those found in the same breed from day 2–14 of the oestrous cycle (Cahill et al. 1981). Prior to being used, implants were inserted in two alien ewes and removed 30 min before use in the experiment. This procedure prevents an excessive release of the oestradiol which could take place after implantation and could trigger a preovulatory-like surge of LH, thus severely disturbing our experiment.

Experiment 2 In vitro measurement of the rates of oestradiol release through the microdialysis membrane perfused at a rate of 1·5 µl/min were determined using tritiated oestradiol and were 8% for the two 2 mm dialysis probes together (Fig. 1). Thus, in vivo we used a concentration of 5 µg oestradiol/ml (5% ethanol) in the dialysis medium giving an amount of 1·3 × 10⁻¹⁰ mol oestradiol released per hour of dialysis. Additionally we performed in vitro measurement of the release from intracranial implants of crystallised oestradiol having the same characteristics to those previously described in sheep (Blache et al. 1991). To this purpose, three intracranial implants of crystallised non-radioactive oestradiol were dipped in 500 ml of a solution of Ringer lactate maintained at 37 °C
under continuous agitation. Samples of 200 µl were taken at 0 h (just before dipping the oestradiol implants), and at 3, 12, 24, 48, 72, 96, 120 and 144 h after oestradiol. Oestradiol was assayed in the samples by RIA (intra-assay coefficient of variation was 7-7%), according to Terqui (1978). Under these conditions the amount released per implant was $2 \times 10^{-10}$ mol/h during the first 24 h of incubation. Ethanol was not added to the dialysate medium during the control period but the dose of ethanol that we used is in the same range as that which has been shown to be inactive in modifying LH-releasing hormone (LHRH) secretion when infused in the median eminence of the female monkey (Pau et al. 1990). Furthermore, this dose is even lower considering the recovery rate of the dialysis membrane.

**Surgery**

General anaesthesia was induced by injection of barbiturate (Nesdonal; 8 mg/kg) and maintained with halothane (4% in oxygen). Cannulae were implanted under radiographic control using an especially adapted stereotaxic frame (Gayard et al. 1992). At least 1 week before the dialysis session, bilateral stainless steel guide cannulae (length 43 mm; outer diameter 1.2 mm; inner diameter 0.8 mm) were fitted to the POA, the RCh (length 48 mm) and the MBH (length 53 mm), in such a way that the ends of the cannulae were situated 6 mm above the sites of interest. The stereotaxic position of the POA (six animals) was 33 mm anterior to the interaural line (AP=33), 1.5 mm lateral to the mid-sagittal plane (L=1.5) and within 0.5 mm of the base of the third ventricle (D=0.5). The stereotaxic positions of the RCh (four animals) and the MBH (five animals) were AP=29.5, L=2.5, D=0.5 and AP=28, L=2, D=1.5 mm respectively.

**Microdialysis**

Microdialysis was performed on four ewes at a time. Ewes were placed in their usual pen among similarly treated ewes but isolated in individual boxes where they could stand or lie down but always facing the experimenter. Experiments were carried out with pairs of microdialysis probes (the membranes were 2 mm long and 0.23 mm in diameter) constructed according to the method of Robinson & Whishaw (1988). The dialysis medium consisted of a solution of ringer lactate (130 mmol Na$^+$/l; 2.7 mmol Ca$^{2+}$/l; 4 mmol K$^+$/l; 115.7 mmol Cl$^-$/l; 27.7 mmol lactate/l; pH=6.7) perfused at a rate of 1.5 µl/min. Dialysates from the two probes were collected into the same tube containing 2 µl 0.1% perchloric acid. Tubes were immediately frozen at −20 °C, kept at this temperature until the end of dialysis and then stored at −80 °C until analysis for neurotransmitters.

**Experimental design**

**Experiment 1** The 15 ewes underwent surgical implantation of intracerebral cannulae before the first experiment. After a 5-day recovery period, they were pretreated with 2 cm subcutaneous oestradiol implants for 2 weeks. The implants were removed 10 days prior to the experiment proper. The day before the sampling session, ewes were fitted with indwelling jugular cannulae from which serial samples were taken every 10 min throughout the five 4-h long sampling sessions. On the day of blood sampling, ewes were bled from 1000 to 1400 h (control period, P1). Subcutaneous oestradiol implants were then administered in the inguinal region under local xylocaine anaesthesia and two successive sampling periods followed immediately; from 1400 to 1800 h (P2) and from 1800 to 2200 h (P3). The fourth sampling period (P4), 18 to 22 h after subcutaneous implantation of oestradiol, started in the next morning after the lights went on at 0800 h. The final period of sampling took place from 42 to 46 h after implantation (P5).

**Experiment 2** One animal from each of the RCh and the MBH groups died between the two experiments; thus we performed the dialysis of experiment 2 on six animals in the POA, three in the RCh and four in the MBH groups. Microdialysis probes and indwelling jugular cannulae were implanted at 1600 h. On the subsequent day, perfusion of microdialysis probes started at 0900 h. Blood sampling (every 10 min) was performed from 1000 to 1400 h (P1). At 1400 h oestradiol was added to the ringer lactate solution and the dialysis continued until 2200 h (P2 and P3). Blood sampling was performed again from 1000 to 1400 h the subsequent day (P4), i.e. 20 to 24 h after the start of the oestradiol treatment. Dialysates were collected every hour during the four periods.

**Histological localisation of the probes**

Animals were killed by decapitation. The heads were immediately perfused with 4% paraformaldehyde in phosphate buffer via the carotid arteries. Brains were removed and stored in the same fixative for a day and then transferrered to 15% sucrose in PBS.

A cryomicrotome was used to prepare 40 µm thick frontal sections which were stained with cresyl violet. The antero-posterior position was identified by reference to the anterior commissure (A32, according to the Richard (1969) stereotaxic atlas) and depth with reference to the bottom of the brain and laterality to the distance from the midline.

**Neurotransmitter measurements**

**Catecholamine analysis** Noradrenaline (NA), 3-methoxy-4-hydroxyphenylglycol (MHPG), 3,4-dihydroxy-phenylacetic acid (DOPAC), homovanillic acid (HVA) and
Table 1 Effect of subcutaneous implants of oestradiol on the pulsatile secretion of LH. Values are means ± S.E.M.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
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<tr>
<td>Interval (min)</td>
<td>49.9 ± 1.4*</td>
<td>49.3 ± 3.6*</td>
<td>68.9 ± 12.9ab</td>
<td>75.6 ± 5.9b</td>
<td>122.7 ± 19.4c</td>
</tr>
<tr>
<td>Nadir (ng/ml)</td>
<td>8.0 ± 0.4*</td>
<td>7.7 ± 0.4*</td>
<td>6.0 ± 0.4ab</td>
<td>5.9 ± 0.4bc</td>
<td>5.4 ± 0.4c</td>
</tr>
<tr>
<td>Amplitude (ng/ml)</td>
<td>4.8 ± 0.6*</td>
<td>3.2 ± 0.4bc</td>
<td>2.1 ± 0.3c</td>
<td>3.8 ± 0.3ab</td>
<td>3.8 ± 0.8bc</td>
</tr>
<tr>
<td>Area under the pulses</td>
<td>85.5 ± 12.1ac</td>
<td>58.4 ± 7.5ab</td>
<td>45.1 ± 5.9b</td>
<td>103.9 ± 13.7c</td>
<td>86.8 ± 15.6ac</td>
</tr>
</tbody>
</table>

Means without common letters are significantly different (P<0.01, except for amplitude at P2, P<0.04 and at P4, P<0.02).

dopamine (DA) were quantified using HPLC (Pump Spectra Physics SP8810; autosampler CMA200) and electrochemical detection (Waters model M460). The mobile phase consisted of KH$_2$PO$_4$ (85 g/l), sodium octane sulphonate (0.093 g/l), EDTA (0.08 g/l) and MeOH (5%) in 1 litre distilled water adjusted to pH 4.2. The flow rate was 1.2 ml/min. The limits of detection for each sample were defined as the quantity giving a peak with an amplitude equal to twice the amplitude of the baseline noise. The values were 3.4 nm/l (NA), 10.9 nm/l (MHPG), 2.7 nm/l (DOPAC), 4.0 nm/l (DA) and 15.3 nm/l (HVA).

Amino acid analysis The concentrations of aspartate, glutamate and GABA in dialysate samples were determined by HPLC with fluorescence detection (Waters scanning fluorescence detector model 474, using 334 nm excitation and 425 nm emission filters) following automatic precolumn derivatisation with o-phthalaldehyde. The HPLC system consisted of a Gradient Waters 600E Powerline pump, a Waters 717plus autosampler and a reverse phase column Resolve C18 (150 × 3.9 mm, 5 µm). Amino acids were derivatised automatically by mixing 15 µl dialysate sample with 25 µl derivatization reagent for 2 min, injecting it immediately onto the column and separating with the gradient elution of solvents A and B. Solvent A consisted of MeOH:THF:H$_2$O, 2:2:96 containing 0.05 M Na$_2$HPO$_4$ and 0.05 M NaOAc adjusted to pH 7.5 with acetic acid. Solvent B consisted of a mixture of MeOH:H$_2$O, 65:35. Elution was started with 100% solvent A at a flow rate of 1.5 ml/min. At 12 min, the gradient was adjusted to 50% solvent B, then increased linearly to 100% for 9 min. Finally the gradient was returned to the initial mobile phase (100% solvent A) at 26 min. The quantification of sample peaks was accomplished by comparing peak areas with those of known concentrations of standards. The limits of detection were 6.5 nm/l for glutamate and aspartate and 7.5 nm/l for GABA.

LH assays

LH was measured using RIA as described previously (Pelletier et al. 1982). The limit of detection (two standard deviations from buffer controls) was 0.1 ng/ml and the intra- and interassay coefficients of variation were 10% and 12% respectively.

Analysis of the data

LH secretion The profile from each individual was analysed according to the five (experiment 1) or four periods (experiment 2) of 4 h. Mathematical analysis was conducted using the 'Monroe' algorithm according to the method of Moncrieff & Wachtler (1982). Thus we took into consideration the interval between pulses of LH, the nadir, the amplitude and the area under the pulse. These four parameters were subjected to multiple ANOVA to search for differences between periods (experiment 1), or first for interactions between periods and brain structure groups and secondly for differences between periods (experiment 2).

Transmitters and metabolites The values of neurotransmitter or metabolite concentrations in the dialysates were subjected to multiple ANOVA to search first for interactions between the periods and the three structure groups and secondly for differences between periods. Given evidence of interactions they were also searched for between two groups or between periods within one given group. Multiple analysis of variance was performed using the program 'Super Anova'.

All means presented in the results are given with the S.E.M.

Results

Experiment 1

The results from experiment 1 are shown in Table 1. The initial mean interval between LH pulses in our group of 15 ovariectomised ewes was 49.9 ± 1.4 min. Following insertion of subcutaneous oestradiol implants, we observed an increase in the interval between pulses of LH in a few individuals during P3 (Fig. 2). However, the effect was only significant for the whole group (P<0.01) during P4 (75.6 ± 5.9 min) and P5 (122.7 ± 19.4 min). While some
individuals showed a rapid decrease in the nadir during P2, the first statistically significant change after oestriadiol treatment was a decrease in the amplitude of the LH pulses during P2. Subsequently, the nadir and area under the pulses decreased during P3. At P4, i.e. 18 h after the insertion of the oestriadiol implant, the amplitude and the area under the curve were significantly increased. These values then stabilised (amplitude) or recovered the preimplantation ones (area).

Experiment 2

The traces of the microdialysis membranes are shown in Fig. 3.

Effects of oestradiol infusion on LH secretion Statistical analysis revealed no differences in the effect of oestriadiol on LH secretion in relation to the position of the probe in the brain. We therefore analysed the effect of oestriadiol in the whole group of animals, independently of the structures (Table 2). The initial mean interval in our group of 13 ovariectomised ewes was 49.5 ± 4.1 min. Following the intracerebral oestriadiol treatment, the interval between LH pulses became increased significantly (73.2 ± 14.2 min) above that of the control period during P4, 20 h later (Fig. 2). Except for a weak increase in the mean nadir during P3, already observed in some individuals during P2, none of the other parameters used to estimate LH secretion was statistically different during P2 or P3. Thereafter, at P4, amplitude and area under the pulses increased.

When we compared the effects of peripheral versus central treatments of oestriadiol on LH pulsatile secretion, there was no difference in terms of interval between
pulses. In contrast, the other parameters of LH secretion differed during P3 and P4. Comparison of the LH secretion between the two experiments are summarised in Table 3.

Effects of oestradiol infusion on catecholamines

Only metabolites and not primary catecholamines were routinely detected (Fig. 4). During the experiment DOPAC concentrations changed differentially between structures (interaction time × structures, P<0.03). The two by two comparison between groups revealed that DOPAC levels changed differentially in the POA and the RCh groups (P<0.02). The MBH group was not different from either of these two groups. ANOVA did not reveal any proper effect of time. HVA levels decreased over time (P<0.03) but the changes in HVA levels were not statistically significant between groups (interaction time × structures, P>0.05). In the four animals from the

Figure 3  Schematic drawing of the hypothalamus of the ewe in lateral view. Anterior planes A32 through the anterior commissure and A29 tangential to the medial thalamus are used for radiographic and histological landmarks. The black bars indicate the size of the membrane of dialysis probes and show the mean histological localisation in the three sites (according to Richard 1969) while the hatched area around them represents the S.E.M. of these positions. IIIV, Third ventricle (shaded area); MT, medial thalamus; AC, anterior commissure; OCh, optic chiasma; POA, preoptic area; A15, dopaminergic A15 nucleus; VMH, ventromedial nucleus; MB, mammillary bodies; ME, median eminence; PIT, pituitary gland.

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Table 2 Effect of intracerebral infusion of oestradiol on the pulsatile secretion of LH from the three groups of brain structures and after gathering all groups independently of the structure (All groups). Values are means ± S.E.M.

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>P3</th>
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<td>POA</td>
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<td>A15</td>
<td>45·5 ± 5·46</td>
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<td>44·17 ± 0·85</td>
<td>61·39 ± 7·14</td>
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<td>MBH</td>
<td>60·75 ± 10·4</td>
<td>40·08 ± 4·49</td>
<td>59·21 ± 14·97</td>
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<td>All groups</td>
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<td>48·2 ± 3·6a</td>
<td>52·0 ± 4·6a</td>
<td>73·2 ± 14·2b</td>
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<tr>
<td>Nadir (ng/ml)</td>
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<tr>
<td>POA</td>
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<td>9·75 ± 0·75</td>
<td>10·21 ± 0·63</td>
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<td>Amplitude (ng/ml)</td>
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<tr>
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<td>3·23 ± 0·05</td>
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<td>MBH</td>
<td>3·94 ± 0·59</td>
<td>3·82 ± 0·86</td>
<td>3·39 ± 0·48</td>
<td>5·55 ± 1·13</td>
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<td>All groups</td>
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<td>4·3 ± 0·7a</td>
<td>4·4 ± 0·7a</td>
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<td>Area under the pulses</td>
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<tr>
<td>POA</td>
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<td>112·18 ± 34·04</td>
<td>160·57 ± 41·72</td>
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<td>A15</td>
<td>65·45 ± 10·64</td>
<td>58·2 ± 11·34</td>
<td>68·29 ± 6·6</td>
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<td>MBH</td>
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<td>73·84 ± 20·76</td>
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<td>All groups</td>
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<td>79·4 ± 13·3a</td>
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<td>148·7 ± 22·2b</td>
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</table>

Multiple ANOVA of the values revealed no significant difference between periods in relation to the three structures for the four parameters studied. Within all groups, means without common letters are statistically different (P<0·01, except for nadir at P3, P<0·02).

MBH group, we detected DA in only one sample before oestradiol infusion (P1), while after 20 h (P4), we detected DA in six samples from two individuals.

Effects of oestradiol infusion on amino acid neurotransmitters GABA, aspartate and glutamate were routinely detected (Fig. 5). GABA levels increased over time (P<0·01) and these changes were different between structures (interaction time × structures, P>0·02). GABA levels during P4 were higher than during P1, P2 and P3 only in the RCh and MBH groups (P<0·001), not in the POA. Aspartate levels did not change over time.

Glutamate levels did not change over time but were different between structures; they were higher in the RCh than in the two other structures. (P<0·01).

Discussion

The results from the first experiment showed that the interval between pulses of LH was significantly greater 18 h after peripheral implantation of oestradiol (P4), while the further increase at P5 reinforced the observed effect, showing that the modifications effectively represented the initiation of the negative feedback of oestradiol usually described in this model (Goodman et al. 1982, Martin et al. 1983). That the first effect on LH secretion was observed between 0 and 4 h after implantation indicated a very rapid subcutaneous release of oestradiol. Consequently, the delay observed in the increase of the interval between pulses of LH appears to be due not to methodological but to physiological effects. Thus, oestradiol requires more than 8 h and less than 18 h to initiate a statistically significant, although limited decrease in the frequency of LH pulses under long days in ovariectomised ewes.

Our results showed that the time-lag between the implantation of oestradiol and its effect is short enough to

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Table 3 Comparison of the effect of peripheral versus central oestradiol treatment on the pulsatile secretion of LH

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>P3</th>
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<td>Nadir</td>
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<td>Area</td>
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</table>

Each number shows the P values when comparing the same period between the first and the second experiment after gathering the animals from the three groups of structures (All groups from Table 2).
Figure 4 Changes in the mean ± S.E.M. extracellular DOPAC and HVA in the dialysates from the POA, RCh and MBH groups before (P1), from 4 to 8 h (P2 and P3) and from 20 to 24 h (P4) following the addition of oestradiol to the dialysate milieu. For these histograms the four values from each period for individuals were averaged and the mean considered as an individual variable for the period.

be compatible with the use of microdialysis in sheep (Kendrick et al. 1989, Gayrard et al. 1992, 1994). As differential effects of oestradiol on LH secretion in relation to the sites of dialysis were not obtained, we have not yet succeeded in the identification of the site of action of the steroid. The small number of individuals in the RCh and MBH groups could account for this, but the absence of difference between structures does not support this assumption. Also, the similarities between the effects of oestradiol on LH secretion in the three groups allowed us to eliminate possible destruction of the critical site by the dialysis probes. Until now, two explanations could be put forward. First, a true effect on all three sites is possible,

Figure 5 Changes in the mean ± S.E.M. extracellular GABA, glutamate and aspartate in the dialysates from the POA, RCh and MBH groups before (P1), from 4 to 8 h (P2 and P3) and from 20 to 24 h (P4) following the addition of oestradiol to the dialysate milieu. For these histograms the four values from each period for individuals were averaged and the mean considered as an individual variable for the period.
though, as far as we know, there is no other evidence for this in the literature. Alternatively, diffusion of the steroid could be suspected. Such a phenomenon could involve either the nervous tissue or the third ventricle, but the latter is unlikely as the three sites are located at various distances from the ventricle while the effects on LH secretion are identical. Although our in vitro measurements provide us with a comparison of the dialysis probes versus crystalline implants rather than a real estimation of in vivo release, the results at least suggested that the diffusion was not especially related to our choice of method. In female rats (Akema et al. 1983) and sheep (Blache et al. 1991), crystalline implants of oestradiol induced modifications in the pulsatile or preovulatory-like release of LH respectively. In these two studies, diffusion of the steroid between structures does not seem to be involved. However, these effects observed on LH depend on systems which require more oestradiol than the negative feedback system operating in ewes under long days, since the increased sensitivity is the key factor in this period of reproductive life. Thus, it is conceivable that the eventual diffusion could have attained the relatively low threshold required. If this is the case, an alternate strategy may be necessary to identify the site of action of oestradiol.

The lack of differences in the effect of oestradiol on LH secretion as a function of structures encourages us to consider the POA, MBH and RCh groups as a single one, and allows us to compare the effect of peripheral versus central treatment in the same group of animals. The observed differences between the two experiments concern the nadir, amplitude and area under the LH pulse curves but not the intervals. Nadir is linked to both the amplitude and frequency of the pulses of LH. Amplitude and area under the pulses are linked together and depend on the secretion of the hormone during the pulses. The absence of a decrease in the nadir and the amplitude observed when oestradiol is administered directly to the brain is an unexpected, striking difference from the effects of peripheral oestradiol treatment usually seen in most data in the literature. Thus it is likely that the long-lasting decrease in the nadir as well as the short-lasting decrease in the amplitude seen in subcutaneously treated animals are reflections of hypophysial effects. The increase in LH pulse amplitude and area observed after 18 h with peripheral oestradiol treatments is more easily evidenced with central treatment. This strongly suggests a higher amplitude of LHRH pulses, in accordance with previous observations in ewes under long days (Barrell et al. 1992). Our experiment thus suggests that such an increase in LHRH secretion could result from an early stimulatory effect of oestradiol rather than a simple inverted relationship between the amplitude and the frequency.

Whatever the site of action of oestradiol is, the local neurochemical changes observed at the initiation of the inhibition should be taken into consideration, even if they may depend on the remote action of the steroid since, under normal physiological situations, oestradiol reaches all these structures where such changes must take place. Analysis of the data reveals a decrease in DOPAC within the RCh versus POA groups, and also a general decrease in HVA levels within the three sites. Such data are at variance with the usually observed increase in the metabolites of DA in the A15 nucleus or tyrosine hydroxylase activity (Gayrard et al. 1992, 1994) evidenced after 6 to 10 days of oestradiol treatment. The present results could suggest an early inhibition of monoamine oxidase, a positive effect of oestradiol on dopaminergic tone already demonstrated in the hypothalamus of the rat (Luine & Hearns 1990). The weak increase in detectable values of DA in the MBH when the LH pulsatility decreases could be in accordance with such a hypothesis.

GABA showed anatomically differential changes with an increase in the whole ventral hypothalamus, i.e. the MBH and the RCh. It can be noted that in male rats, GABA was shown to interact with DA at the level of the median eminence (Wagner et al. 1994). Until now, GABAergic neurones, some of them bearing oestradiol receptors, have been identified in the POA of sheep, but not in the ventral hypothalamus (Herbison et al. 1993). An increase in extracellular GABA was observed in the POA close to the LHRH cell bodies in ewes after progesterone treatment (Robinson & Kendrick 1992) while a decrease in GABA preceded the preovulatory release of LH (Robinson et al. 1991). Thus, GABA could be involved in the inhibition of LHRH and the level of the cell bodies and the median eminence, according to various endocrine situations. Indeed, further investigations are required to estimate the possible involvement of this inhibitory amino acid since modifications of the balance between different categories of its receptors have also been shown to occur between anoestrum and the breeding season in female sheep (Scott & Clarke 1993).

In conclusion, our data show that about 20 h are necessary to evidence the oestradiol inhibition of LH pulsatility in ewes under long days. Despite this, we were not able to identify the exact site of action of oestradiol in our experiments. The direct infusion of oestradiol within the brain has allowed us to unmask the effect of the pituitary gland and suggests an early positive effect of this steroid on the amplitude of the LH pulses. Initial action of oestradiol on LH secretion is accompanied by time-related changes in extracellular DA metabolites and GABA. The significance of these remains to be determined as do the putative anatomical and physiological links with the steroid-sensitive cells on one hand, and with the A14 and A15 nuclei on the other.

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