Implication of D2-like dopaminergic receptors in the median eminence during the establishment of long-day inhibition of LH secretion in the ewe

F Bertrand, J-C Thiery, S Picard and B Malpaux

Institut National de la Recherche Agronomique, URA Centre National de la Recherche Scientifique 1291, Laboratoire de Neuroendocrinologie Sexuelle, 37380 Nouzilly, France

(Requests for offprints should be addressed to B Malpaux, INRA-PRMD, Laboratoire de Neuroendocrinologie Sexuelle, 37380 Nouzilly, France; Email: malpaux@tours.inra.fr)

Abstract

In ewes, photoperiod modulates LH release and dopaminergic terminals in the median eminence (ME) have a critical role in the establishment of long-day inhibition of LH secretion. This study was undertaken to determine the type of dopaminergic receptors, D1-like or D2-like, that mediate the action of dopamine on LH secretion at the ME level in this situation. This was assessed, in ovariectomized and estradiol-treated ewes, with the use of reverse microdialysis in the ME in three experiments: first, when LH secretion was stimulated by short days, by determining the response to three doses (0.01, 0.1 or 1 mg/ml) of a D1-like (SKF38393) and a D2-like (quinpirole) agonist; secondly, during early long-day inhibition of LH secretion, by determining the ability of SKF38393 and quinpirole (1 mg/ml) to mimic the inhibitory effects of dopamine, after a blockade of its synthesis with α -methyl-paratyrosine (α MPT; 2 mg/ml); and thirdly, during early long-day inhibition of LH secretion, by determining the response to three doses (0.009, 0.09 or 0.9 mg/ml) of a D1-like (SCH23390) and a D2-like (sulpiride) antagonist.

In none of the conditions was effect of the D1-like analogs on LH secretion found, compared with the control treatments. In contrast, the D2-like analogs caused changes in LH secretion. First, with short days, quinpirole in the highest dose significantly reduced mean LH concentration ($P < 0.05$) and LH pulse frequency ($P < 0.01$). Secondly, with long days, addition of quinpirole to α MPT caused a significant decrease in LH secretion relative to α MPT alone ($P < 0.05$). Thirdly, with long days, sulpiride at the highest dose significantly increased mean LH concentration (during the first 3 h of treatment, $P < 0.05$) and LH pulse frequency ($P < 0.05$). Prolactin secretion was also determined in these experiments, and D2-like agonist and antagonist caused an inhibition and a stimulation of prolactin secretion, respectively. These results demonstrate that, in the ME, inhibitory action of dopamine on LH secretion, critical for the initiation of long-day-induced inhibition, is mediated by D2-like, not D1-like, dopaminergic receptors.

Journal of Endocrinology (1999) **163**, 243–254

Introduction

In ewes, photoperiod controls reproductive activity by regulating the pulsatile secretion of luteinizing hormone (LH) (Karsch *et al.* 1984). In ovariectomized and estradiol-treated (OVX+E) ewes, long days inhibit luteinizing hormone (LH) secretion after 20–30 days of exposure, whereas short days stimulate it after 40–60 days. These photoperiodically mediated changes in LH secretion reflect a shift in response of the LH-releasing hormone (LHRH) pulse generator to estradiol negative feedback, which constitutes a primary neuroendocrine mechanism for seasonal changes in ovarian cyclicity (Legan & Karsch 1980). Photoperiodic information is transduced to the brain through the circadian rhythm of melatonin secretion. The neuroendocrine mechanism lying downstream from

melatonin action and leading to regulation of LHRH and, consequently, of LH secretion, appears to involve several dopaminergic structures of the hypothalamus, such as A14 and A15 nuclei of the lateral retrochiasmatic area (Thiéry *et al.* 1989, Havern *et al.* 1994, Vigié *et al.* 1996), or the arcuate nucleus–median eminence (ME) region (Anderson *et al.* 1997, Bertrand *et al.* 1998, Vigié *et al.* 1998).

The ME dopaminergic terminals, which are located in the external layer of the ME (Tillet & Thibault 1989), establish synaptic contacts with LHRH terminals, suggesting the possibility of a presynaptic control of LHRH secretion by dopamine (Kuljis & Advis 1989). Several arguments suggest that these terminals are implicated in one of the final steps of the long-day-induced inhibition of LH secretion. Indeed, ME dopamine content and tyrosine hydroxylase (rate-limiting enzyme of the catecholamine

biosynthesis) activity are lower under short days (high LH secretion) than under long days (low LH secretion; Thiéry 1991, Vigié *et al.* 1996, 1997). Importantly, blockade of dopamine synthesis in the ME, by local infusion of α -methyl-para-tyrosine (α MPT, a competitive tyrosine hydroxylase inhibitor) induces a stimulation of LH secretion in long day-inhibited OVX+E ewes (Vigié *et al.* 1998). It is noteworthy that this effect of α MPT is observed during short-term long-day inhibition (30–40 days), but is no longer found in the longer term (70 days), strongly suggesting that an increased tyrosine hydroxylase activity in the ME is critical only during the establishment of the response (Bertrand *et al.* 1998).

An important step in the unraveling of the mechanisms of dopamine action during the establishment of the long-day inhibition of LH secretion is the identification of the type of receptors mediating the action of dopamine, and thus the possibility of locating them at a cellular level. The action of dopamine can be mediated by two major types of dopaminergic receptors, D1-like and D2-like (Kebabian & Calne 1979). Although the implication of D2-like receptors in the control of LH secretion has been shown in several physiological conditions in sheep (Meyer & Goodman 1985, Curlewis *et al.* 1991, Havern *et al.* 1991, Anderson *et al.* 1997), the nature of the receptors involved in the ME is not known. Indeed, effects on LH secretion of D2-like receptor analogs delivered in the ME were observed, but dose-dependent comparisons of the effects of specific analogs of each type of receptor have not been performed in this site. In addition, because a high tyrosine hydroxylase activity is critical only during the establishment of the long-day inhibition, dopaminergic-specific neural mechanisms may be involved only during this stage of the response (Bertrand *et al.* 1998). It is therefore important to identify the type of receptors functionally important in the ME, and it is critical to perform this identification during the establishment of the long-day response. For this, the effects on LH secretion, used as an index of LHRH secretion, of D1-like and D2-like agonists (after blockade of dopamine synthesis) and antagonists infused locally in the ME were studied in OVX+E ewes exposed to 30–50 long days, when ME dopamine has been shown to be critical for the inhibition of LH secretion (Bertrand *et al.* 1998). Because prolactin (PRL) secretion is strongly regulated by photoperiod (Thimonier *et al.* 1978) and dopamine is involved in this regulation (Curlewis 1992), the effects of the different treatments on PRL secretion were also assessed.

Materials and Methods

General

These studies were performed at the Institut National de la Recherche Agronomique Research Center of Nouzilly, France. They were conducted on adult Ile de France ewes,

maintained in photoperiod-controlled rooms under an artificial light regimen, alternating periods of constant long day (16 h light : 8 h darkness) and constant short day (8 h light : 16 h darkness). Animals were fed with a standard diet of concentrate and straw and had free access to water. All animal procedures and care were performed in accordance with Authorization A37801 of the French Ministry of Agriculture.

The experiments consisted in assessing the effects of D1-like and D2-like specific dopaminergic analogs on LHRH secretion, estimated by LH secretion, using reverse microdialysis in the ME, in animals at the beginning of the long-day inhibition (30–50 days of long-day exposure). Two approaches were used. The aim of Experiment 1b was to block dopamine synthesis in the ME by infusion of α MPT and to test the ability of a D1-like or D2-like agonist to restore the effects of dopamine on LH secretion. As it was difficult to determine the appropriate dose of agonist with such a procedure, a preliminary experiment (Experiment 1a) was carried out on short-day-stimulated ewes, to assess which agonist and what dose was able to inhibit LH secretion. The principle of the second experiment (Experiment 2) was to infuse a D1-like or D2-like antagonist in three doses to determine which was able to stimulate LH secretion. All the drugs were purchased from RBI (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France).

Animal model

The animal model we used was the OVX+E ewe, bearing subcutaneous estradiol Silastic implants as described previously (Legan *et al.* 1977). For each experiment, ewes were ovariectomized at least 6 weeks before the microdialysis sessions and at the same time implanted with two subcutaneous Silastic estradiol implants of 0.5 and 1.5 cm length. This dose of estradiol was maintained before the microdialysis sessions, during the photoperiodic preparation of the animals. For the pharmacological challenges, the dose of estradiol was reduced by removing the 1.5 cm implant 72 h before microdialysis session, leaving the 0.5 cm implant in place. This model has been discussed previously (Bertrand *et al.* 1998).

Design of experiment 1a

Seven OVX+E ewes (Nos 1–7) were placed in a photoperiod-controlled room on September 8th. They were exposed to 84 long days followed by 78 short days before the beginning of the experiment, to stimulate LH secretion (Fig. 1). At this time, on three consecutive days, animals were infused in the ME with SKF38393 (specific D1-like agonist), quinpirole (specific D2-like agonist) or vehicle (Ringer's lactate). SKF38393 was dissolved in Ringer's lactate and quinpirole was dissolved first in HCl 0.1 N, then diluted nine times in Ringer's lactate and pH

Figure 1 Experimental designs. Left panels: Photoperiodic treatments and theoretical time-course of mean LH concentration in OVX+E ewes. Arrows indicate the beginning of the reverse microdialysis sessions. Right panels: Reverse microdialysis sessions designs. For each experiment, the sessions were performed on three consecutive days, with the order of the treatments randomized for each animal. Blood samples were collected every 10 min. For Experiment 1a and Experiment 2, the dose of dopaminergic analogs was increased 10 times at the end of period 1 and period 2. (See text for details.)

was finally adjusted by adding NaOH 0.1 N. The ewes received one treatment each day, and the order of the three treatments was chosen according to a latin square design, in order to control experiment-duration and treatment-order effects. On each day, reverse microdialysis was performed for 15 h (beginning at 0930 h), divided for both D1-like and D2-like agonist treatments in three periods of 5 h, during which increasing successive doses were infused (0.01, 0.1 and 1 mg/ml). On the control day, animals were infused with Ringer's lactate for 15 h (Fig. 1).

Design of experiment 1b

Nine OVX+E ewes (Nos 11–19) were placed in a photoperiod-controlled room on July 1st and were exposed to 88 short days. Three additional animals (Nos 8–10) were placed in a photoperiod-controlled room on November 15th and were exposed to 70 long days, followed by 68 short days. After short-day exposure, all the animals were submitted to 33 long days before the start of the experiment (Fig. 1). At this time, they were

submitted, over three consecutive days, to three pharmacological treatments: infusion (in the ME) of αMPT (2 mg/ml) alone, αMPT+SKF38393, or αMPT+quinpirole. The dose for the agonists was 1 mg/ml, as this dose of quinpirole was shown to be effective in Experiment 1a. αMPT and αMPT+SKF38393 were dissolved in Ringer's lactate and αMPT+quinpirole were dissolved in the same way as quinpirole in Experiment 1a. The ewes received one treatment each day, and the order of the three treatments was chosen according to a latin square design, in order to control experiment-duration and treatment-order effects. Microdialysis sessions lasted 10 h (beginning at 0900 h), with a 5-h control period (infusion of vehicle Ringer's lactate), followed by 5 h of pharmacological treatment (Fig. 1).

Design of experiment 2

Six ewes (Nos 20–25) were placed in a photoperiod-controlled room on December 24th and were exposed to 75 long days, followed by 76 short days. Animals were then exposed to 49 long days before the experiment

(Fig. 1). At this time, they were submitted to the same design as in Experiment 1a, but with two antagonists, SCH23390 (specific D1-like antagonist) and sulpiride (specific D2-like antagonist) at the doses of 0.009, 0.09 and 0.9 mg/ml (Fig. 1). SCH23390 was dissolved in Ringer's lactate and sulpiride was dissolved in the same way as quinpirole in Experiment 1a.

Surgical access to the ME and microdialysis sessions

At least 1 month before microdialysis, animals were implanted with a stainless-steel guide cannula (external diameter 1.2 mm; internal diameter 0.8 mm; length 55 mm) aimed toward the ME according to a surgical procedure described previously (Viguié *et al.* 1998). The site targeted was located 1 mm in front of the tip of the infundibular recess, 1 mm below the third ventricle floor and 0.8–1 mm from the midline. The cannula was implanted with an angle of 6° with respect to the sagittal plane, in order to avoid crossing the sagittal sinus and perfusing the drugs within the third ventricle. The tip of the guide cannula was 6 mm above the site. At the end of the surgery, a stainless-steel stylet of the length of the cannula was placed in the cannula; it was removed at the time of insertion of the probe, before the beginning of the microdialysis session.

The day before microdialysis, animals were placed in individual pens, and a polyethylene catheter was inserted in the jugular vein. At least 12 h before the beginning of the pharmacological challenge, the microdialysis probe was inserted in the guide cannula and infused with Ringer's lactate at a slow rate (0.5 µl/min) overnight. The probes were built according to a procedure described previously (Robinson & Whishaw 1988), with an active membrane length of 3 mm.

About 1 h before the first blood sample on the first day of the experiment, the flow rate was increased to 1 µl/min. Between two days of dialysis, probes were left in place and infused overnight with Ringer's lactate. Drugs were dissolved just before the beginning of the experiments. Blood samples (10 min) were collected into heparinized tubes, centrifuged, and the plasma removed for later RIA to assess the effect of the drugs on LH and PRL secretion.

Histological processing of the brains

Ewes were killed, with their probes left in place, one day after the end of microdialysis treatment. Brains were processed according to a procedure described previously, to locate the position of the microdialysis probes within the tissue (Robinson *et al.* 1991). Coronal sections (40 µm thick) of the hypothalamic block containing ME were collected and stained with cresyl violet acetate as described previously (Klüver & Barrera 1953).

Hormonal assays

All the samples from one experiment were assayed in the same RIA. LH was assayed in duplicate 100 µl aliquots of plasma using the RIA of Pelletier *et al.* (1968) modified by Montgomery *et al.* (1985). Sensitivity (2 s.d. from buffer controls) was 0.1 ng/ml 1051-CY-LH (0.2 ng/ml NIH-LH-S1). The intra-assay coefficient of variation (CV) for three plasma pools averaged 16%. PRL was assayed in duplicate 10 µl aliquots of plasma using the RIA of Kann (1971). Sensitivity was 8.5 ng/ml NIDDK-oPRL. The intra-assay CV for three plasma pools averaged 21%.

Data analysis

Concentrations of LH and PRL below the limit of detection were assigned the value of assay sensitivity for data analysis and plotting. LH pulse detection was performed by means of the Munro software (Zaristow software, West Morham, Haddington, East Lothian EH41 4PD, Scotland), according to the method of Merriam & Wachter (1982) (G parameters: G1=3.78; G2=2.20; G3=1.60; G4=1.24; G5=0.93; Baxter parameters: b1=0.07997; b2=0.03; b3=0.003). LH pulse frequency was evaluated by the inter-pulse interval (IPI).

The effects of the different pharmacological treatments on mean LH and PRL concentrations, and on LH pulsatility parameters (IPI and amplitude), were analyzed by repeated measure analysis of variance with Super-ANOVA software (Abacus Concepts, Berkeley, CA, USA). Data from Experiment 1a and Experiment 2 were analyzed by a one-way ANOVA (within factor: dialysis periods) followed by least square means test (two-by-two comparisons). For Experiment 1b, data were analyzed by a two-way ANOVA (within factors: treatment (αMPT alone, αMPT+SKF38393, αMPT+quinpirole) and dialysis period (control, drug infusion)); because each treatment was preceded by a 5-h control period of Ringer perfusion, the effects of the analogs were tested through an interaction between treatment and dialysis period. In order to standardize variances, statistical analysis was performed after a log transformation of IPI and mean PRL concentration values.

Results

Location of dialysis probes

Histological examination of the probe location at the end of the experiments revealed that for Experiment 1a, five of seven ewes (Nos 1–5) had their probe accurately located within the ME (Fig. 2). The ewe No. 6 had its probe placed in the plane A28 of Richard stereotaxic atlas (Richard 1967), above the ME in the arcuate nucleus region, and ewe No. 7 had its probe located in the optic

Figure 2 Location of the microdialysis probes within the median eminence. Top panels: Schematic sections of the mediobasal hypothalamus in four consecutive antero-posterior planes (A26–29), adapted from Richard (1967). fx, fornix; ic, internal capsule; mtt, mammillo-thalamic tract; ot, optic tract; pt, pars tuberalis; v, third ventricle. Bottom panels: Location of the dialysis probes (magnified view of the areas in top panels delimited with dotted lines). Numbered circles depict the location of the middle part of the 3 mm active membrane of the probe for each animal (with an angle of 6° with respect to the sagittal plane). Black circles correspond to Experiment 1a (Nos 1–5), white circles to Experiment 1b (Nos 9–19) and white squares to Experiment 2 (Nos 20–25).

chiasma. For Experiment 1b, 11 of 12 ewes (Nos 9–19) had their probes accurately placed within the ME (Fig. 2). The remaining ewe (No. 8) had its probe located in plane A30 of Richard stereotaxic atlas, above the ME, in the area of the rostral part of the arcuate nucleus. For Experiment 2, all the six ewes had their probe accurately located in the ME (Fig. 2).

The three animals with their probes located outside ME were excluded from statistical analysis; consequently, analysis was for five animals for Experiment 1a, eleven for Experiment 1b, and six for Experiment 2.

Effects of the pharmacological treatments on LH secretion

Experiment 1a Individual profiles of LH secretion are presented in Fig. 3. Infusion of SKF38393 did not significantly affect mean LH concentrations and IPI compared with vehicle treatment, regardless of the dose (Fig. 4). Conversely, infusion of quinpirole significantly reduced mean LH concentration and increased IPI compared with vehicle and SKF38393. This effect depended on the dose, as the difference became significant only in period 3, corresponding to the highest dose of agonist ($P < 0.01$ for IPI and $P < 0.05$ for mean LH concentration). No effect on pulse amplitude was found. In contrast to these five ewes with probes accurately located in the ME, which all showed an inhibition of LH secretion during treatment with the highest dose of quinpirole, no inhibition of LH secretion was found in the other two ewes with misplaced probes, particularly ewe No. 6 in which the

probe was in the arcuate nucleus area (mean plasma LH during period 3: ewe No. 6, 6.0–8.7–6.4 ng/ml; ewe No. 7, 7.4–8.8–7.8 ng/ml, Ringer-SKF38393–quinpirole, respectively).

Experiment 1b Individual profiles of LH secretion are presented in Fig. 5. A global interaction between treatment and period of dialysis was found ($P < 0.01$ for IPI and $P = 0.001$ for mean LH concentration; Fig. 6). In two-by-two comparisons, no treatment $\times$ dialysis period interaction was found between α MPT alone and α MPT+SKF38393 treatments. Conversely, a significant treatment $\times$ dialysis period interaction was observed between α MPT+quinpirole treatment and the other two treatments ($P < 0.05$ for both mean LH concentration and IPI). No effect on pulse amplitude was found (analysis on 10 animals, as one animal did not present any pulse during the control period). Because of the small relative amplitude of the effects of the different treatments and of their inter-individual variability in these 11 animals with probes placed correctly in the ME, the data of ewe No. 8, with misplaced probe, could not provide any pertinent insight about the relationship between probe placement and LH response.

Experiment 2 Individual profiles of LH secretion are presented in Fig. 7. Infusion of SCH23390 did not significantly affect mean LH concentration and IPI compared with vehicle treatment, regardless of the dose (Fig. 8). Conversely, infusion of sulpiride significantly

Figure 3 Experiment 1a. LH secretory profiles of ewe No. 3. Blood was collected every 10 min during the 15 h of dialysis. During the periods 0–5 h, 5–10 h and 10–15 h, the doses of dopaminergic agonists (D1, SKF38393; D2, quinpirole) were 0.01, 0.1 and 1 mg/ml, respectively. ●, Pulses identified by Munro algorithm.

decreased IPI compared with vehicle and SKF38393 treatments in the same period. This effect depended on the dose, as it became significant only in period 3 (highest dose; $P < 0.05$ for IPI). Surprisingly, no difference was found in mean LH concentration. Careful observation of the individual LH profiles revealed that, during infusion of the highest dose of sulpiride, a biphasic response was observed: mean LH concentrations tended to increase, but this effect did not last and LH concentrations returned to baseline after about 3 h (Fig. 7). On the basis of this observation, analysis was also performed on mean LH concentration during the first 3 h of each treatment period. No difference was then found between vehicle and SCH23390 treatments, but, in contrast, sulpiride induced a significant increase in mean LH concentration at the highest dose ($P < 0.05$; Fig. 8). Analysis of pulse amplitude was not performed because two animals did not present any pulse during at least one period.

Effects of pharmacological treatments on PRL concentrations

Experiment 1a Infusion of SKF38393 did not significantly affect mean PRL concentration compared with vehicle treatment, regardless of the dose (Table 1). Conversely, infusion of quinpirole significantly reduced mean PRL concentration compared with vehicle and SKF38393. This effect depended on the dose, as the effect

Figure 4 Experiment 1a. Effects of infusion in the ME of Ringer's lactate (vehicle), SKF38393 (D1 agonist) and quinpirole (D2 agonist) on mean ($\pm$ S.E.M.) LH inter-pulse interval and concentration in OVX+E ewes after a 78 short-days exposure. At the end of periods 1 and 2, the dose of dopaminergic agonists was increased (0.01, 0.1 and 1 mg/ml for periods 1, 2 and 3, respectively). * $P < 0.05$, ** $P < 0.01$; $n = 5$.

became significant in period 2 ($-47 \pm 5\%$ compared with Ringer's lactate, $P < 0.05$; $n = 5$) and in period 3 ($-54 \pm 6\%$, $P < 0.01$). In contrast to all other ewes, in ewe No. 6 (probe located in the arcuate nucleus region), SKF38393 induced a strong increase in mean PRL concentration (+181% compared with Ringer's lactate, period 3) and quinpirole did not seem to induce a decrease (+13%). In ewe No. 7 (probe located in the optic chiasma), no obvious effect of the different treatments was observed.

Experiment 1b Analysis of mean PRL concentration revealed an interaction between treatment and period of dialysis ($P < 0.05$; Table 1). The effect of the treatment with α MPT+SKF38393 was significantly different from treatment with α MPT alone (interaction treatment $\times$ dialysis period, $P < 0.05$), with a decrease in PRL concentration (Table 1). The effect of treatment with α MPT+quinpirole was significantly different from the other two treatments (interaction treatment $\times$ dialysis

Figure 5 Experiment 1b. LH secretory profiles of ewe No. 10. Blood was collected every 10 min during the 10 h of dialysis. During the period 0–5 h, only vehicle (Ringer's lactate) was infused. During the period 5–10 h, α MPT alone (2 mg/ml), α MPT+SKF38393 (1 mg/ml) or α MPT+quinpirole (1 mg/ml) were infused. ●, Pulses identified by Munro algorithm.

period, $P < 0.01$ compared with both other treatments), with a important decrease in PRL concentration (Table 1). In ewe No. 8 (probe located in the rostral part of the arcuate nucleus region), a decrease in PRL concentrations was observed regardless of the treatment (α MPT alone, –32%; α MPT+SKF38393, –64%; α MPT+quinpirole, –41% compared with control period).

Experiment 2 Infusion of SCH23390 did not significantly affect mean PRL concentration compared with vehicle treatment, whatever the dose (Table 1). Conversely, infusion of sulpiride significantly increased mean PRL concentration compared with vehicle and SCH23390. This effect depended on the dose, as it became significant in period 2 ($+64 \pm 23\%$ compared with Ringer's lactate, $P < 0.05$; $n = 6$), and was dramatically expressed in period 3 ($+197 \pm 50\%$, $P < 0.001$), corresponding to the highest dose of sulpiride.

Discussion

The present study, carried out in conditions in which the critical role of the ME dopaminergic terminals has been shown (Bertrand *et al.* 1998), demonstrates that infusion of a D2-like specific agonist in the ME of long-day exposed

Figure 6 Experiment 1b. Comparison of the effects of infusion in the ME of α MPT alone (2 mg/ml), α MPT+SKF38393 (1 mg/ml) and α MPT+quinpirole (1 mg/ml) on mean ($\pm$ S.E.M.) LH inter-pulse interval and concentration in OVX+E ewes after a 33 long-days exposure. Significant interaction between treatment and dialysis period: * $P < 0.05$, ** $P < 0.01$; $n = 11$.

OVX+E ewes can restore the inhibition of LH secretion removed by blockade of dopamine synthesis. Furthermore, it shows that infusion of a D2-like specific antagonist can disinhibit LH secretion. The D2-like analogs always induced a change in LH pulse frequency, suggesting that they acted at the ME level on LHRH pulsatile secretion, rather than directly at the pituitary level. The same treatments with D1-like specific analogs did not produce any of these effects. Altogether, these results strongly suggest that the initiation of long-day-induced inhibition of LHRH (and consequently LH) secretion by dopamine in the ME occurs through D2-like dopaminergic receptors, not through D1-like ones.

Our results confirm and extend previous experiments in sheep suggesting that dopamine acts through D2-like dopaminergic receptors for the inhibition of LH secretion. For example, i.v. injection of pimozide, a D2-like antagonist, stimulated LH secretion in intact and OVX+E ewes during the anestrus season (Meyer & Goodman 1985, 1986). In the ram, s.c. injection of sulpiride during long days stimulated LH secretion, whereas s.c. injection of bromocriptine, a D2-like agonist, reduced LH secretion

Figure 7 Experiment 2. LH secretory profiles of ewe No. 24. Blood was collected every 10 min during the 15 h of dialysis. During the periods 0–5 h, 5–10 h and 10–15 h, the doses of dopaminergic antagonists (D1, SCH23390; D2, sulpiride) were 0.009, 0.09 and 0.9 mg/ml, respectively. ●, Pulses identified by Munro algorithm.

under short days (Tortones & Lincoln 1994). Intracerebroventricular administration of quinpirole reduced LH pulse frequency in OVX+E ewes during the breeding season, whereas the same treatment with SKF38393 did not have any effect on LH secretion (Curlewis *et al.* 1991). Conversely, i.v. injection of pimozide tends to increase LH pulse frequency in OVX+E ewes during anestrus, while SCH23390 has no effect (Curlewis *et al.* 1991). Moreover, placement of pimozide microimplants in the ME in anestrus ewes stimulated LH secretion (Havern *et al.* 1991), whereas local injection of quinpirole in the ME–arcuate nucleus region inhibited LH secretion in OVX ewes (Anderson *et al.* 1997). Our study complements these results, by providing the first comparison of the effects of local delivery of D1-like and D2-like analogs in the ME. Furthermore, the role of dopamine in the ME may vary during the time course of the long-day-induced inhibition of LH secretion (Bertrand *et al.* 1998). Importantly, our two main experiments (Experiment 1b and 2) were carried out under photoperiodic conditions (exposure to 30–50 long days) under which it has been shown that an increased activity of ME dopaminergic terminals is critical for the inhibition of LH secretion (Bertrand *et al.* 1998, Vigué *et al.* 1998). Our results therefore demonstrate that the initiation of long-day-

Figure 8 Experiment 2. Effects of infusion in the ME of Ringer's lactate (vehicle), SCH23390 (D1 antagonist) and sulpiride (D2 antagonist) on mean ($\pm$ S.E.M.) LH inter-pulse interval and concentration in OVX+E ewes after a 49 long-days exposure. At the end of periods 1 and 2, the dose of dopaminergic antagonists was increased (0.009, 0.09 and 0.9 mg/ml for periods 1, 2 and 3, respectively). Mean LH concentration was calculated on the first 3 h of each period (see Results for explanation). * $P < 0.05$; $n = 6$.

induced inhibition of LHRH, and consequently of LH, secretion by dopamine in the ME is mediated by D2-like, not D1-like, dopaminergic receptors.

In Experiment 2, infusion of sulpiride in the highest dose induced an increase in LH secretion, but this effect did not last throughout the 5-h infusion period. A biphasic response characterized by a rapid increase in LH pulse frequency and mean levels, and by a return to basal levels, was observed. As LHRH secretion was not measured directly in this experiment, it is difficult to know whether the observed changes in LH secretion reflect those in LHRH secretion. It is possible that the effect of sulpiride on LHRH secretion was characterized by the induction of a short-lasting high-frequency release, followed by a return to baseline. Alternatively, the stimulation of LHRH release may have been maintained for a longer time, but have led to a decrease of the pituitary responsiveness to LHRH. Indeed, pituitary desensitization was described *in vivo* and *in vitro* after continuous infusion or high-frequency delivery (more than 3 pulses/h) of LHRH

Table 1 Effects of D1-like and D2-like analogs on PRL secretion. For the Pilot experiment and Experiment 2, from period 1 to 3, increasing doses of drugs were infused (0.01, 0.1 and 1 mg/ml; 0.009, 0.09 and 0.9 mg/ml, respectively). For Experiment 1, Ringer's lactate was infused during the control period and drugs were infused during the treatment period (α MPT 2 mg/ml; SKF38393 and quinpirole 1 mg/ml)

	PRL concentration (ng/ml)		
	Period 1	Period 2	Period 3
Experiment 1a (n=5)			
Ringer's lactate	33 $\pm$ 7 ^a	34 $\pm$ 6 ^a	34 $\pm$ 5 ^a
SKF38393	39 $\pm$ 5 ^a	33 $\pm$ 5 ^a	36 $\pm$ 6 ^a
Quinpirole	30 $\pm$ 3 ^a	17 $\pm$ 3 ^b	15 $\pm$ 1 ^b
	Control period	Treatment period	Difference (%)
Experiment 1b (n=11)			
α MPT alone	147 $\pm$ 17	144 $\pm$ 17	+3 $\pm$ 10 ^a
α MPT+SKF38393	170 $\pm$ 18	128 $\pm$ 13	-18 $\pm$ 9 ^b
α MPT+Quinpirole	143 $\pm$ 13	79 $\pm$ 13	-39 $\pm$ 14 ^c
	Period 1	Period 2	Period 3
Experiment 2 (n=6)			
Ringer's lactate	111 $\pm$ 16 ^a	135 $\pm$ 39 ^a	120 $\pm$ 24 ^a
SCH23390	91 $\pm$ 11 ^a	102 $\pm$ 10 ^a	111 $\pm$ 15 ^a
Sulpiride	129 $\pm$ 19 ^{ab}	205 $\pm$ 46 ^b	341 $\pm$ 66 ^b

In each experiment, significant differences are indicated by differing superscript letters.

(McIntosh & McIntosh 1983, Baird *et al.* 1984, Kamel *et al.* 1987). For instance, treatment of perfused pituitary cells with LHRH, as given one 2-min pulse every 16 min, caused an initial important release of LH, followed by a decrease within 2–3 h (McIntosh & McIntosh 1983). It is to be noted that, in Experiment 2 during the initial 2–3 h of treatment with the highest dose of sulpiride, high concentrations of LH with low-amplitude pulses suggestive of a high LHRH frequency were found, and it is likely that LH pulses were poorly detected in this situation. Regardless of the explanation for this biphasic effect, the initial stimulation of LH secretion during this period, indicative of a stimulation of LHRH secretion, which did not occur with the SCH23390, is in favor of the implication of D2-like receptors.

In our studies, large effects of D2-like analogs were found on PRL secretion. These effects have been described previously (Lincoln & Tortonese 1995, Anderson *et al.* 1997) and may account for a local effect on neurons located in the ME region, or for a transport of the drugs by hypophysial portal blood towards the anterior pituitary, where dopamine can act on lactotrophs through D2-like receptors to inhibit PRL secretion (Ben-Jonathan 1985, Anderson *et al.* 1997). However, it is noteworthy that, in ewes with probes located accurately in the ME, no stimulatory effect of SKF38393 nor inhibitory effect of SCH23390 on PRL secretion were found. These results contrast with the demonstration that a local infusion of the same D1-like antagonist SCH23390, in the ventromedial

hypothalamic nucleus inhibited PRL secretion (Curlewis *et al.* 1995a). This nucleus, in which a high density of D1-like receptors is found (Colthorpe & Curlewis 1996), is believed to be involved in a stimulatory pathway of PRL secretion in sheep (Curlewis *et al.* 1995b). Interestingly, in Experiment 1a, a strong stimulation of PRL secretion was observed for ewe No. 6, after infusion of the D1-like agonist, SKF38393, in the arcuate nucleus region. This result is not surprising, because of the proximity of the arcuate nucleus to the ventromedial hypothalamic nucleus. However, in Experiment 1b, no effect of SKF38393 on PRL secretion was found for ewe No. 8, in which the probe was also located in the arcuate nucleus region, but much more rostrally than that in ewe No. 6. This result is consistent with those of Curlewis *et al.* (1995a), who found SCH23390 to be ineffective to inhibit PRL secretion when delivered in the rostral part of the ventromedial hypothalamic nucleus. Taking into account the results on PRL secretion of ewes No. 6 and No. 8, and because of the proximity of the ventromedial hypothalamic nucleus to the ME and to the third ventricle, the absence of stimulatory and inhibitory effects of D1-like agonist and antagonist, respectively, indicates that diffusion of the drugs to other hypothalamic areas was very limited in our experiments. It also indicates that, despite the proximity of the ME to the third ventricle, diffusion toward this cavity was limited, and that the dilution of the drugs in the cerebrospinal fluid prevented them reaching target sites in other hypothalamic areas close to the

ventricle in amounts sufficient to produce any effect. These conclusions are reinforced by observations of LH secretion made in animals with misplaced probes. In these animals, infusion of the D2-like analogs in the arcuate nucleus region (ewe No. 6 and No. 8) or in the optic chiasma (ewe No. 7) produced effects on LH secretion different from those observed when infusion was made in the ME. Therefore, the changes in LH secretion caused by the D2-like analogs most probably resulted from an action limited to the ME region.

As strong effects of the D2-like analogs on PRL secretion were observed concomitantly to the effects on LH secretion, it could be hypothesized that the changes in LH secretion were the consequence of those in PRL secretion. This hypothesis is very unlikely to be valid. Indeed, it has been shown in several animal species that hyperprolactinemia was responsible for inhibition of LH secretion (Evans *et al.* 1982) whereas, in our experiments, changes in LH and PRL secretion were positively correlated after the D2-like analog treatments. Moreover, findings from several experiments in the sheep suggest that PRL is not a modulator of LH secretion. For example, active immunization against PRL, which results in an important decrease in plasma PRL concentrations, does not affect plasma LH concentration in the ram (Ohlson *et al.* 1981). Also, increased plasma PRL concentrations as a result of hypothalamo–neurohypophysial tract disruption did not change plasma LH concentrations in the ewe (Thomas *et al.* 1989). Moreover, the inverse relationship between PRL concentrations and reproductive activity normally seen during the year is not critical to the changes in seasonal reproductive activity (Worthy & Haresign 1983, Worthy *et al.* 1985).

No data exist in the literature regarding the presence of D2-like receptors in the ME of the ewe. A binding study by Cronin & Weiner (1979) suggested the presence of putative dopaminergic receptors in the stalk–ME of the ewe. However, this study must be interpreted cautiously, as it was carried out with [³H]spiperone, a ligand which has been demonstrated, since then, also to bind serotonergic receptors (Leysen 1981). Concerning D1-like receptors, a binding study with [¹²⁵I]SCH23982, a D1-like specific ligand, did not demonstrate any specific binding in the ME and the arcuate nucleus, in contrast to the ventromedial hypothalamic nucleus (Colthorpe & Curlewis 1996). Therefore, the results of our study raise the question of the presence of D2-like dopaminergic receptors in the ME. This could be resolved by binding or autoradiographic studies with an appropriate ligand, such as [¹²⁵I]iodosulpride. The question also remains whether dopamine in the ME acts directly on LHRH terminals, or if there exist intermediate neurons relaying dopamine action. No evidence exists for the presence of dopamine receptors on LHRH neurons, so the implication of an interneuronal system cannot be excluded. Nevertheless, a direct action of dopamine on LHRH terminals is suggested

by the observation of axo–axonic synaptic contacts between dopaminergic and LHRH terminals within the ME (Kuljis & Advis 1989). Such organization is also found in rats (Ajika 1979, Ugrumov *et al.* 1989) and in hens (Contijoch *et al.* 1992). The ability of dopamine to act directly on LHRH neurons could be determined, for instance, by assessing the presence of D2-like receptors on LHRH neurons by immunocytochemistry at the electron microscopic level, or by testing the action of dopamine *in vitro* on cultured LHRH neurons.

In conclusion, this study provides the first comparison of the effects on LH secretion of D1-like and D2-like dopaminergic analogs in the ME, where a critical step for the establishment of long-day-induced inhibition of LH secretion takes place. Our results strongly suggest that the dopamine inhibition of LHRH secretion in the ME, critical for the establishment of the long-day-inhibitory effects on LH secretion, is mediated by D2-like, not D1-like, dopaminergic receptors. As a possible interaction between dopamine and LHRH terminals in the ME has been suggested on the basis of anatomical data (Kuljis & Advis 1989), our study enables the use of adequate probes to resolve the question of a direct regulation of LHRH secretion by dopamine in this structure.

Acknowledgements

The authors thank Drs P Chemineau and D C Skinner for comments on the manuscript; Ms F Maurice-Mandon, K Courvoisier, A Daveau, Mr C Gauthier, G Duarte and Dr L Zarazaga for assistance with the experimentation; Mr G Durand, F Dupont and F Paulmier for animal care.

F Bertrand was supported by a PhD grant from the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie.

Findings of this study were presented in preliminary form at the 'XXVeme Colloque de la Société de Neuroendocrinologie Expérimentale', Strasbourg, France, 27–28 September 1996 and '3eme Colloque de la Société des Neurosciences', Bordeaux, France, 25–28 May 1997.

References

- Ajika K 1979 Simultaneous localization of LHRH and catecholamines in rat hypothalamus. *Journal of Anatomy* **128** 331–347.
- Anderson ST, Sawangjaroen K & Curlewis JD 1997 A method for drug infusion into the lateral median eminence and arcuate nucleus of sheep. *Journal of Neuroscience Methods* **71** 169–176.
- Baird CJ, Tharandt L & Tamarkin L 1984 Regulation of luteinizing hormone release by pulsatile and continuous administration of gonadotropin-releasing hormone to superfused rat and hamster pituitary cells. *Endocrinology* **114** 1041–1047.
- Ben-Jonathan N 1985 Dopamine: a prolactin-inhibiting hormone. *Endocrine Reviews* **6** 564–589.
- Bertrand F, Viguié C, Picard S & Malpoux B 1998 Median eminence dopaminergic activation is critical for the early long-day inhibition of luteinizing hormone secretion in the ewe. *Endocrinology* **139** 5094–5102.

- Colthorpe KL & Curlewis JD 1996 Localization and characterization of dopamine D1 receptors in sheep hypothalamus and striatum. *Journal of Neuroendocrinology* **8** 561–568.
- Contiouch AM, Gonzalez C, Singh HN, Malamed S, Troncoso S & Advis JP 1992 Dopaminergic regulation of luteinizing hormone-releasing hormone release at the median eminence level: immunocytochemical and physiological evidence in hens. *Neuroendocrinology* **55** 290–300.
- Cronin MJ & Weiner RI 1979 [^3H]Spiroperidol (spiperone) binding to a putative dopamine receptor in sheep and steer pituitary and stalk median eminence. *Endocrinology* **104** 307–312.
- Curlewis JD 1992 Seasonal prolactin secretion and its role in seasonal reproduction: a review. *Reproduction Fertility and Development* **4** 1–23.
- Curlewis JD, Naylor AM & McNeilly AS 1991 Evaluation of a possible role of dopamine D1 and D2 receptors in the steroid-dependent suppression of luteinizing hormone secretion in the seasonally anoestrus ewe. *Journal of Neuroendocrinology* **3** 387–391.
- Curlewis JD, Thiéry JC & Malpoux B 1995a Effect of hypothalamic infusion of a dopamine D1 receptor antagonist on prolactin secretion in the ewe. *Brain Research* **697** 48–52.
- Curlewis JD, Thiéry JC & Malpoux B 1995b Evidence for dopamine D1 receptor-mediated stimulation of prolactin secretion in ewes under long day length. *Journal of Reproduction and Fertility Supplement* **49** 539–543.
- Evans WS, Cronin MJ & Thorner MO 1982 Hypogonadism in hyperprolactinemia: proposed mechanisms. In *Frontiers in Neuroendocrinology*, vol 7, pp 77–122. Eds WF Ganong & L Martini. New York: Raven Press.
- Havern RL, Whisnant CS & Goodman RL 1991 Hypothalamic sites of catecholamine inhibition of luteinizing hormone in the anoestrus ewe. *Biology of Reproduction* **44** 476–482.
- Havern RL, Whisnant CS & Goodman RL 1994 Dopaminergic structures in the ovine hypothalamus mediating estradiol negative feed-back in anoestrus ewes. *Endocrinology* **134** 1905–1914.
- Kamel F, Balz JA, Kubajak CL & Schneider VA 1987 Effects of luteinizing hormone (LH)-releasing hormone pulse amplitude and frequency on LH secretion by perfused rat anterior pituitary cells. *Endocrinology* **120** 1644–1650.
- Kann G 1971 Dosage radioimmunologique de la prolactine plasmatique chez les ovins. *Comptes Rendus de l'Académie des Sciences III* **272** 2808–2811.
- Karsch FJ, Bittman EL, Foster DL, Goodman RL, Legan SJ & Robinson JE 1984 Neuroendocrine basis of seasonal reproduction. *Recent Progress in Hormone Research* **40** 185–232.
- Kebabian JW & Calne DB 1979 Multiple receptors for dopamine. *Nature* **277** 93–96.
- Klüver A & Barrera E 1953 A method of combining staining of cells and fibers in the nervous system. *Journal of Neuropathology and Clinical Neurology* **12** 400–403.
- Kuljis RO & Advis JP 1989 Immunocytochemical and physiological evidence of a synapse between dopamine- and luteinizing hormone releasing hormone-containing neurons in the ewe median eminence. *Endocrinology* **124** 1579–1581.
- Legan SJ & Karsch FJ 1980 Photoperiodic control of seasonal breeding in ewes: modulation of the negative feedback action of estradiol. *Biology of Reproduction* **23** 1061–1068.
- Legan SJ, Karsch FJ & Foster DL 1977 The endocrine control of seasonal reproductive function in the ewe: a marked change in response to the negative feedback action of estradiol on luteinizing hormone secretion. *Endocrinology* **101** 818–824.
- Leysen JE 1981 Serotonergic receptors in brain tissue: properties and identification of various 3H-ligand binding sites *in vitro*. *Journal of Physiology* **77** 351–362.
- Lincoln GA & Tortorese DJ 1995 Does melatonin act on dopaminergic pathways in the mediobasal hypothalamus to mediate effects of photoperiod on prolactin secretion in the ram? *Neuroendocrinology* **62** 425–433.
- McIntosh RP & McIntosh JE 1983 Influence of the characteristics of pulses of gonadotrophin releasing hormone on the dynamics of luteinizing hormone release from perfused sheep pituitary cells. *Journal of Endocrinology* **98** 411–421.
- Merriam GR & Wachter KW 1982 Algorithms for the study of episodic hormone secretion. *American Journal of Physiology* **243** E310–E318.
- Meyer SL & Goodman RL 1985 Neurotransmitters involved in mediating the steroid-dependent suppression of pulsatile luteinizing hormone secretion in anoestrus ewes: effects of receptor antagonists. *Endocrinology* **116** 2054–2061.
- Meyer SL & Goodman RL 1986 Separate neural systems mediate the steroid-dependent and steroid-independent suppression of tonic luteinizing hormone secretion in the anoestrus ewe. *Biology of Reproduction* **35** 562–571.
- Montgomery GW, Martin GB & Pelletier J 1985 Changes in pulsatile LH secretion after ovariectomy in Ile-de-France ewes in two seasons. *Journal of Reproduction and Fertility* **73** 173–183.
- Ohlson DL, Spicer LJ & Davis SL 1981 Use of active immunization against prolactin to study the influence of prolactin on growth and reproduction in the ram. *Journal of Animal Science* **52** 1350–1359.
- Pelletier J, Kann J, Dolais J & Rosselin G 1968 Dosage radioimmunologique de l'hormone lutéinisante plasmatique chez le mouton. Mise au point de la technique de dosage. *Comptes Rendus de l'Académie des Sciences III* **266** 2291–2294.
- Richard P 1967 *Atlas Stéréotaxique du Cerveau de Brebis*, pp 84. Paris, France: INRA Editions.
- Robinson TE & Whishaw IQ 1988 Normalization of extracellular dopamine in striatum following recovery from a partial 6-OHDA lesion of the substantia nigra: a microdialysis study in freely moving rats. *Brain Research* **450** 209–224.
- Robinson JE, Kendrick KM & Lambart CE 1991 Changes in the release of GABA and catecholamines in the preoptic/septal prior to and during the preovulatory surge of luteinizing hormone in the ewe. *Journal of Neuroendocrinology* **3** 393–399.
- Thiéry JC 1991 Monoamine content of the stalk-median eminence and hypothalamus in adult female sheep as affected by daylength. *Journal of Neuroendocrinology* **3** 407–411.
- Thiéry JC, Martin GB, Tillet Y, Caldani M, Quentin M, Jamain C & Ravault JP 1989 Role of hypothalamic catecholamines in the regulation of luteinizing hormone and prolactin secretion in the ewe during seasonal anoestrus. *Neuroendocrinology* **49** 80–87.
- Thimonier J, Ravault JP & Ortavant R 1978 Plasma prolactin variations and cyclic ovarian activity in ewes submitted to different light regimens. *Annales de Biologie Animale Biochimie Biophysique* **18** 1229–1235.
- Thomas GB, Cummins JT, Doughton BW, Griffin N, Smythe GA, Gleeson RM & Clarke IJ 1989 Direct pituitary inhibition of prolactin secretion by dopamine and noradrenaline in sheep. *Journal of Endocrinology* **123** 393–402.
- Tillet Y & Thibault J 1989 Catecholamine-containing neurons in the sheep brainstem and diencephalon: immunohistochemical study with tyrosine hydroxylase TH and dopamine- β -hydroxylase DBH antibodies. *Journal of Comparative Neurology* **290** 69–104.
- Tortorese DJ & Lincoln GA 1994 Photoperiodic modulation of the dopaminergic control of pulsatile LH secretion in sheep. *Journal of Endocrinology* **143** 25–32.
- Ugrumov M, Hisano S & Daikoku S 1989 Topographic relations between tyrosine hydroxylase- and luteinizing hormone-releasing hormone-immunoreactive fibers in the median eminence of adult rats. *Neuroscience Letters* **102** 159–164.
- Viguié C, Thibault J, Thiéry JC, Tillet Y & Malpoux B 1996 Photoperiodic modulation of monoamines and amino-acids involved in the control of prolactin and LH secretion in the ewe: evidence for a regulation of tyrosine hydroxylase activity. *Journal of Neuroendocrinology* **8** 465–474.
- Viguié C, Thibault J, Thiéry JC, Tillet Y & Malpoux B 1997 Characterization of short day-induced decrease in median eminence

- tyrosine hydroxylase activity in the ewe: temporal relationship to the changes in luteinizing hormone and prolactin secretion and short-day like effect of melatonin. *Endocrinology* **138** 499–506.
- Viguié C, Picard S, Thiéry JC & Malpoux B 1998 Blockade of tyrosine hydroxylase activity in the median eminence partially reverses the long day-induced inhibition of pulsatile LH secretion in the ewe. *Journal of Neuroendocrinology* **10** 551–558.
- Worthy K & Haresign W 1983 Evidence that the onset of seasonal anoestrus in the ewe may be independent of increasing prolactin concentrations and daylength. *Journal of Reproduction and Fertility* **69** 41–48.
- Worthy K, Haresign W, Dodson S, McLeod BJ, Foxcroft GR & Haynes NB 1985 Evidence that the onset of the breeding season in the ewe may be independent of decreasing plasma prolactin concentrations. *Journal of Reproduction and Fertility* **75** 237–246.

Received 8 March 1999

Accepted 23 June 1999