Effects of DPDPE (a specific \( \delta \)-opioid receptor agonist) and naloxone on hypothalamic monoamine concentrations during the pre-ovulatory LH surge in the rat

Bayram Yilmaz, Desmond P Gilmore and Catherine A Wilson

Institute of Biomedical and Life Sciences, West Medical Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK

(Correspondence should be addressed to B Yilmaz who is now at Firat Universitesi, TIP Fakultesi, Fizyoloji Anabilim Dali, 23169 Elazig, Turkey)
(C Wilson is now at Department of Obstetrics and Gynaecology, St George’s Hospital Medical School, London SW17 0RE, UK)

Abstract
We have investigated the inter-relationship between the opioid and aminergic systems in the control of secretion of the pro-oestrous LH surge and the involvement of \( \delta \)-opioid receptor subtypes in this process. Conscious female rats bearing a cannula in the femoral artery were injected i.p. with a selective \( \delta \)-opioid receptor agonist (DPDPE) either alone or with the opioid antagonist (naloxone) at 1300 h on the day of pro-oestrus. Blood samples were collected hourly between 1500 h and 1900 h, and plasma LH levels were measured by RIA. At the end of this period (1900 h), the animals were autopsied and the concentrations of the amines (noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5HT)) and their metabolites (dihydroxyphenolglycol (DHPG) and 5-hydroxyindoleacetic acid (5HIAA), metabolites of NA and 5HT respectively) were determined by HPLC with electrochemical detection in the medial preoptic area, suprachiasmatic nucleus, median eminence and arcuate nucleus.

DPDPE abolished the LH surge and concomitantly decreased hypothalamic NA and DHPG concentrations in all the areas examined. The levels of DA, 5HT and 5HIAA were also reduced in all hypothalamic regions studied, except DA and 5HIAA in the suprachiasmatic nucleus. Naloxone reversed these inhibitory effects of the \( \delta \)-agonist.

We conclude that activation of \( \delta \)-opioid receptors may exert an inhibitory effect on LH release. The effect is probably an indirect one mediated by the monoaminergic systems, as they are suppressed by DPDPE in nearly all the hypothalamic regions studied.

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Introduction
The control of gonadotrophin-releasing hormone (GnRH) activity and hence luteinising hormone (LH) release involves a multiplicity of brain neurotransmitter systems including the monoamines and opioids that modulate GnRH neurones within the hypothalamus (1, 2). The opioid system exerts a physiological tonic inhibitory effect on GnRH neurones as revealed by the enhancement of LH release after treatment with the opioid antagonist, naloxone (3), and Allen & Kalra (4) have proposed that a reduction in the endogenous opioid tone before the onset of the pre-ovulatory LH surge may be the initial neural stimulus for the generation of the LH surge.

Although direct synaptic connections exist between opioid peptidergic nerve terminals and GnRH neurones (5), there is some evidence that the opioids can affect GnRH release directly (6). There is also a bulk of evidence indicating that the opioids also act indirectly by influencing the monoaminergic systems (7–10).

The effects of the opioids are mediated by three main receptor subtypes, designated \( \mu \), \( \kappa \), and \( \delta \). The effects of these opioid subtypes employing selective agonists (to date there is only one \( \sigma \)-agonist; see ref (11)) on the secretion of LH have been investigated previously by several groups, and \( \mu \)- and \( \kappa \)-agonist activity has been shown to exert an inhibitory effect on the GnRH system (12–14). However, the role of \( \delta \)-opioid receptors is less well understood. Thus the \( \delta \)-agonist ([\( \text{D}-\text{Pen}^{2,5}\text{D}-\text{Pen}^{5}\text{]}\text{enkephalin; DPDPE}) was seen to inhibit LH release in females (15), but a specific \( \delta \)-antagonist (ICI 154, 129) did not affect LH levels in males (16). The \( \delta \)-receptors have a more restricted distribution than the \( \mu \)- and \( \kappa \)-receptors in the brain, and their density has been described as low to moderate in the rat hypothalamus with the highest levels located in the medial preoptic area (MPOA) and suprachiasmatic nucleus (SCN) (17, 18). The \( \delta \)-opioid receptors are thought to mediate the actions of the enkephalins, and both Met- and Leu-enkephalin perikarya are present in most hypothalamic nuclei (19).

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In the present study, we have investigated the modulating effects of a specific δ-receptor agonist, DPDPE (20), either alone or when co-administered with the opioid antagonist (naloxone) on the release of LH and at the same time on concentrations of monoamines and some of their metabolites in specific regions of the rat hypothalamus. The investigation was confined to the modulation of the pre-ovulatory LH surge in conscious intact female rats on the afternoon of pro-oestrus.

**Materials and methods**

**Animals**

Adult female Sprague–Dawley rats (Harlan UK Ltd, Oxon, UK) weighing 220–300 g were maintained under controlled temperature (21 ± 1 °C) and light conditions (lights on from 0700 h to 1900 h). Food and water were provided *ad libitum*. Vaginal smearing was performed each morning (0900 h-1000 h), and the morphology of the cells present used to identify the different stages of the oestrous cycle. Only those animals that had exhibited at least three consecutive 4-day oestrous cycles were selected for experimentation.

On the late morning of pro-oestrus, a plastic cannula (Portex Ltd, Kent, UK; outside diameter 0.63 mm) combined with vinyl tubing (internal diameter 0.55 mm) was inserted into the right femoral artery under halothane anaesthesia (complete cessation of the hind limb flexor withdrawal reflex). With the aid of a stainless-steel guide cannula, the vinyl tubing was fed under the dorsal skin to emerge at the back of the neck. The animals were allowed to recover and then in the early afternoon, just before the onset of the pre-ovulatory LH surge, injected i.p. with either DPDPE (1 mg/kg; National Institute On Drug Abuse, Rockville, MD, USA; *n* = 10) or DPDPE plus naloxone (10 mg/kg; Sigma Chemical Corp., Poole, Dorset, UK; *n* = 8). Controls received saline alone (1 ml/kg; *n* = 16). Blood samples (200 μl) were withdrawn through the heparinised (sodium heparin, 10 units/ml; Leo Laboratories Ltd, Bucks, UK) cannula at hourly intervals from the freely moving conscious animals throughout the afternoon of pro-oestrus, commencing at 1300 h. Drug administrations were carried out under light halothane anaesthesia.

The blood samples collected were centrifuged at 3000 r.p.m. at 4 °C for 10 min. The plasma was then transferred into fresh tubes and stored at −20 °C until assayed for LH by RIA. At 1900 h the animals were decapitated, and the brains rapidly removed and frozen on dry ice. Coronal brain slices (500μm) were cut. The MPOA, SCN, median eminence (ME) and arcuate nucleus (ARN) were micropunctured using modified stainless-steel hypodermic needles (0.7 and 0.3 mm internal diameter) under a dissection microscope, using the stereotaxic atlas of the rat brain for guidance (21). Experiments were carried out under the guidelines of the UK Home Office.

**Chromatography**

The specific hypothalamic areas collected were kept at −80 °C before analysis. HCl (100 μl; 0.1 mol/l) was added to each sample, along with 50 μl 3,4-dihydroxybenzylamine (1 ng) as an internal standard. The samples were homogenised and then centrifuged at 3000 r.p.m. for 10 min at 4 °C. Aliquots of supernatant (10 μl) were injected on to a reverse-phase HPLC column (S5ODS2–250A; 5 μm; 4.6 mm internal diameter × 25 cm; Hichrom, Anachem Ltd, Luton, UK) coupled to an electrochemical detector (model 141; Gilson, Villiers-le-Bel, France). The monoamine content of the hypothalamic regions was simultaneously detected.

The mobile phase consisted of 6.74 g citric acid, 4.81 g sodium citrate, 47 mg EDTA, 200 mg heptasulphonic acid, 1.15 ml glacial acetic acid, 3 ml tetrahydrofuran and 25 ml HPLC-grade methanol. It was made up to 1 litre with HPLC-grade water and then brought to pH 4.9 using 10 mol/l NaOH. The flow rate was set at 1 ml/min. The minimum detectable quantity of each of the biogenic amines was 0.4 pg/μg supernatant protein, depending on the noise level of the system. The method has been described previously (14).

**LH assay**

Plasma LH levels were measured by RIA at St George’s Hospital Medical School in London. The immunoreactivity of LH was used to determine its relative abundance in each of the serum samples using a homologous double antibody. The samples were assayed in duplicate and the standard curve in triplicate. The latter involved an eight point curve representing concentrations that ranged from 6.25 pg/10 μl to 800 pg/10 μl which was provided by a stock solution of LH (100 ng/ml). The standard used was NIADDK-rLH-RP-3 and the antibody NIADDK-anti-rLH S10. RIA reagents were obtained from the National Hormone and Pituitary Program (Baltimore, MD, USA). The inter- and intra-assay coefficients of variation were 8.0 and 9.5% respectively. The sensitivity of the assay was 10 pg/tube (1 ng/ml).

**Protein determination**

Protein was determined by the modified method of Lowry et al. (22). The method is detailed in our previous report (14).

**Statistics**

One-way ANOVA (MINITAB for Windows, 10) was performed on the hypothalamic monoamine results. When the F-test was significant it was followed by the Kruskal–Wallis non-parametric test in groups where standard deviations between means were large.
Results

Effect of DPDPE on LH release on the afternoon of pro-oestrus

Plasma samples were obtained at hourly intervals between 1500 h and 1900 h on the afternoon of pro-oestrus. The plasma LH concentrations rose significantly to a peak at 1800 h with a slight non-significant fall at 1900 h in 12 of 16 animals (mean ± s.e.m. LH levels (ng/ml): at 1500 h 3.1 ± 1.3; at 1600 h 11.5 ± 4.1; at 1700 h 22.3 ± 7.9; at 1800 h 27.3 ± 9.4; at 1900 h 23.6 ± 6.3). Intraperitoneal administration of 1 mg/kg DPDPE at 1300 h completely suppressed the plasma LH surge throughout the afternoon period of sampling in eight of ten rats, with two animals showing a rise to peak concentrations of 18.0 and 19.0 ng/ml each. This inhibitory effect of DPDPE was reversed in five of eight rats given 10 mg/kg naloxone concomitantly with the δ-agonist, such that the peak LH levels in this combined treatment group were no longer different from the controls (Fig. 1).

Effect of DPDPE on hypothalamic monoamine concentrations

Table 1 shows the concentrations of the three neurotransmitters, noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5HT) and the NA metabolite, 3,4-dihydroxyphenylglycol (DHPG) and the 5HT metabolite, 5-hydroxyindoleacetic acid (5HIAA) in the four hypothalamic areas, MPOA, SCN, ME and ARN.

When DPDPE was administered to rats at 1300 h on the day of pro-oestrus, there were significant reductions in the concentrations of the three neurotransmitters and two metabolites in all the hypothalamic regions examined (except for DA and 5HIAA in the SCN) at 1900 h.

In the DPDPE-treated group, NA activity (as assessed by the DHPG/NA ratio) was significantly decreased in the MPOA (P < 0.001), SCN (P < 0.001) and ARN (P < 0.05) compared with those values seen in the saline-treated animals; δ-agonist, such that the peak LH levels in this combined treatment group were no longer different from the controls (Fig. 1).

Table 1. Monoamine concentrations in pg/μg protein (mean ± s.e.m.) in the MPOA, SCN, ME and ARN of the rat hypothalamus at 1900 h on pro-oestrus after administration of saline or DPDPE or co-administration of DPDPE with the opioid antagonist, naloxone, at 1300 h on the same day.

<table>
<thead>
<tr>
<th>Area</th>
<th>NA</th>
<th>DHPG</th>
<th>DA</th>
<th>SHT</th>
<th>5HIAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 16)</td>
<td>(n = 10)</td>
<td>(n = 8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPOA</td>
<td>34.5 ± 1.5</td>
<td>15.8 ± 1.9</td>
<td>6.1 ± 0.8</td>
<td>8.7 ± 1.3</td>
<td>7.2 ± 0.9</td>
</tr>
<tr>
<td>SCN</td>
<td>35.1 ± 2.5</td>
<td>20.0 ± 1.5</td>
<td>6.2 ± 1.1</td>
<td>6.2 ± 0.7</td>
<td>7.2 ± 0.9</td>
</tr>
<tr>
<td>ME</td>
<td>33.2 ± 1.7</td>
<td>14.4 ± 1.6</td>
<td>19.1 ± 1.6</td>
<td>7.7 ± 0.6</td>
<td>7.8 ± 1.1</td>
</tr>
<tr>
<td>ARN</td>
<td>32.5 ± 1.6</td>
<td>16.0 ± 2.0</td>
<td>6.2 ± 1.0</td>
<td>5.9 ± 0.8</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>MPOA</td>
<td>20.1 ± 1.6a</td>
<td>3.9 ± 0.4a</td>
<td>2.3 ± 0.2a</td>
<td>3.7 ± 0.7b</td>
<td>2.9 ± 0.5c</td>
</tr>
<tr>
<td>SCN</td>
<td>25.3 ± 1.6b</td>
<td>6.9 ± 1.4a</td>
<td>4.0 ± 0.6</td>
<td>4.2 ± 0.5c</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>ME</td>
<td>18.2 ± 1.8a</td>
<td>5.3 ± 0.9a</td>
<td>11.2 ± 1.8b</td>
<td>3.2 ± 0.5a</td>
<td>3.2 ± 0.6a</td>
</tr>
<tr>
<td>ARN</td>
<td>18.9 ± 1.7a</td>
<td>4.4 ± 0.7a</td>
<td>3.3 ± 0.6b</td>
<td>3.0 ± 0.8b</td>
<td>2.6 ± 0.3b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Area</th>
<th>NA</th>
<th>DHPG</th>
<th>DA</th>
<th>SHT</th>
<th>5HIAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPOA</td>
<td>46.3 ± 3.6d</td>
<td>11.5 ± 2.7a</td>
<td>5.7 ± 0.5d</td>
<td>17.8 ± 1.1d</td>
<td>14.8 ± 1.3d</td>
</tr>
<tr>
<td>SCN</td>
<td>51.9 ± 5.8d</td>
<td>14.7 ± 1.9d</td>
<td>7.0 ± 0.8</td>
<td>9.4 ± 2.6f</td>
<td>12.2 ± 2.5f</td>
</tr>
<tr>
<td>ME</td>
<td>46.1 ± 4.2d</td>
<td>14.3 ± 3.2a</td>
<td>35.9 ± 4.8d</td>
<td>13.6 ± 2.7d</td>
<td>17.8 ± 3.1f</td>
</tr>
<tr>
<td>ARN</td>
<td>41.3 ± 3.9d</td>
<td>11.1 ± 3.1f</td>
<td>5.2 ± 0.4f</td>
<td>12.1 ± 2.6e</td>
<td>15.6 ± 2.4e</td>
</tr>
</tbody>
</table>

α P < 0.001, β P < 0.01, γ P < 0.05 compared with the saline-treated animals; δ P < 0.001, ε P < 0.01, ι P < 0.05 compared with the DPDPE-treated animals, using one-way ANOVA and the Kruskal–Wallis non-parametric test.
control group. 5HT activity (as assessed by the 5HIAA/5HT ratio) was not significantly altered by the δ-agonist in any of the hypothalamic regions examined. Unfortunately, dihydroxyphenylacetic acid (DOPAC), a major metabolite of DA, could not be detected and so an equivalent assessment could not be made for DA.

**Effect of naloxone on the action of DPDPE on hypothalamic neurotransmitter activity**

Naloxone is a non-specific opioid antagonist (23) and has been used in our previous experiments to antagonise μ- and κ-agonist activity. In those experiments, when it was given i.p. alone at 10 mg/kg, it induced a 2.5-fold \( P < 0.05 \) increase in LH levels at 1800 h on pro-oestrus. It did not significantly effect 5HT or DA release. A 2.5-fold \( P < 0.05 \) increase in LH levels at 1800 h could be seen in the MPOA: control 8.7 ± 1.3; naloxone 19.9 ± 4.0 (\( P < 0.05 \)); ME: control 5.1 ± 0.8; naloxone 11.9 ± 1.8 (\( P < 0.05 \)); (9).

In the present experiment, when naloxone was co-administered with DPDPE, the inhibitory effects of the δ-receptor agonist on all the amines and metabolites in all the hypothalamic regions examined were significantly reversed (except DA in the SCN) and levels either returned to those seen in the controls or in many cases were raised to values significantly higher than the controls (Table 1). Combination of naloxone with DPDPE had no significant effect on the ratio of DHPG/NA or 5HIAA/5HT in any of the hypothalamic areas examined.

**Discussion**

There are several mechanisms by which endogenous opioids might act to modulate LH release. They could act at the pituitary level, but this is unlikely as in vitro experiments have shown that they have no direct effect on LH secretion and in addition the anterior pituitary possesses a low level of opioid receptors (1, 24). It is more likely that the opioids act at the hypothalamic level to modulate GnRH neuronal activity. They could act directly on these neurones, as synaptic contacts between opioid peptidergic and GnRH neurones have been demonstrated (5). However, an indirect effect via a cross-communication between them might occur (40). It is not inconceivable that activation of the δ-receptors would allosterically influence μ-receptor activity (41). It is therefore possible that the DPDPE action may be mediated, at least in part, indirectly via μ-subtypes. Naloxone would reverse the inhibitory effects of DPDPE whether they were due to μ- or δ-receptor activation.

In summary, we have shown that, in addition to the other opioid receptors, activation of the δ-opioid receptors may exert an inhibitory influence on GnRH and LH release, as the selective δ-agonist, DPDPE, markedly inhibits the pre-ovulatory LH surge in a naloxone-dependent manner. The effect may be an indirect one mediated by the hypothalamic catechol- and indole-amnergic neurotransmitter systems, as they are suppressed by DPDPE in all the nuclear areas of the hypothalamus investigated.

In this report we have investigated the involvement of δ-opioid subtypes in LH release in more detail, using a specific δ-receptor agonist, DPDPE. We have shown that this agent has a potent suppressant effect on plasma LH levels at the time of the pre-ovulatory LH surge. It also inhibits NA and 5HT activity as seen by parallel reduction in concentrations of not only the neurotransmitters themselves, but also their main metabolites. DA concentrations were also reduced. This indicates that, along with the μ- and κ-opioid receptors, δ-subtypes have an inhibitory effect on LH release and that this involves an indirect action through reduction of the activity of the catecholamine and indolamine systems. NA, DA and 5HT all have dual effects on gonadotrophin release. Their stimulatory effects are thought to be mediated in the MPOA in particular (31–34). NA may also be stimulatory in the ME and ARN (32) and 5HT in the ME (35). Our own findings support the stimulatory effects of 5HT in the MPOA and ME and NA in the ARN, since the ratio of metabolite to neurotransmitter was greater in those animals showing an LH surge than in those where the LH surge was absent. There is abundant evidence that DA exerts an inhibitory effect on LH in the ME (36). In fact, others (37) have shown that DPDPE increased tuberoinfundibular DA neuronal activity, although in our hands the levels of the DA in the ARN and ME were reduced: perhaps analysis of DOPAC levels may have resolved this conflict and shown that the reduction in the DA concentration was due to an increase in release. In a previous study, it was shown that morphine treatment resulted in significant increases in DOPAC concentrations in the tuberoinfundibular DA neurones where DA itself was not detected (38).

The effects of DPDPE on both LH release and monoamine levels were reversed by naloxone, which is a non-specific opioid antagonist acting predominantly at μ- and also to some extent at κ-opioid receptors (see ref (23)). In the rat brain, the μ- and δ-receptors can coexist on the same neurone and are physically associated (39). So, a cross-communication between them might occur (40). It is not inconceivable that activation of the δ-receptors would allosterically influence μ-receptor activity (41). It is therefore possible that the DPDPE action may be mediated, at least in part, indirectly via μ-subtypes. Naloxone would reverse the inhibitory effects of DPDPE whether they were due to μ- or δ-receptor activation.
Acknowledgements
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