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ORIGINAL ARTICLE

Opioid modulation of hypothalamic catecholaminergic neurotransmission and the pre-ovulatory LH surge in the rat

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Abstract

We have investigated the inter-relationship between the opioid and catecholaminergic systems in the control of LH secretion, and the involvement of μ- and κ-opioid subtypes in this process. Conscious female rats were intraperitoneally injected with either μ- (diamorphine) or κ-opioid agonists (U-50488H) alone or with their respective antagonists (naloxone and MR2266) before the critical period on proestrus. Hypothalamic catecholamine and plasma LH levels were determined by HPLC-ECD and RIA, respectively. Both μ- and κ-agonists significantly decreased concentrations of noradrenaline and its metabolite (DHPG) in all the hypothalamic regions examined concomitant with inhibition of the LH surge. Dopamine levels were selectively reduced only by the μ–agonist in the MPOA. The inhibitory effects of both opioid agonists were mostly reversed following their co-administration with naloxone and MR2266 (except the κ–antagonist on LH). These results indicate that both the μ– and κ–opioid subtypes may be involved in the inhibition of the LH surge by altering the hypothalamic noradrenaline content.

Introduction

The control of gonadotrophin-releasing hormone (GnRH) activity and hence luteinizing hormone (LH) release involves a multiplicity of brain neurotransmitter systems including the monoamines and the opioids which modulate GnRH neurones within the hypothalamus (Bicknell 1985; Barra-clough 1994; Kalra *et al.* 1997). 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 (Piva *et al.* 1985; Brown *et al.* 1994). Conversely, administration of opioid agonists, just before the critical period on the day of pro-estrus, inhibits release of the pre-ovulatory LH surge and hence ovulation (Grossman and Dyer 1989; Kalra *et al.* 1989). It has been 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 (Allen and Kalra 1986; Lieberman *et al.* 1998). Several different classes of opioid receptor subtypes exist in the hypothalamus (Mansour *et al.* 1988; Desjardins *et al.* 1990).

Direct synaptic connections exist between opioid peptidergic nerve terminals and GnRH neurones in the medial preoptic area (MPOA) and median eminence (ME) (Leranth *et al.* 1988) and there is some evidence that the opioids can affect GnRH release directly (Mehmanesh *et al.* 1988). There is also a bulk of evidence indicating that the opioids act indirectly by influencing the monoaminergic systems (Diez-Guerra et al. 1987; Nishihara *et al.* 1991; Moyse *et al.* 1997).

In the present study, we have further investigated the modulating effects of diamorphine (μ-agonist) and U-50488H (κ-agonist), either alone or when co-administered with their receptor antagonists (naloxone and MR2266, respectively) on the release of LH and at the same time on concentrations of catecholamines in specific regions of the rat hypothalamus. The investigation has been confined to the modulation of the pre-ovulatory LH surge in conscious female rats on the afternoon of pro-estrus.

Materials and methods

Animals:

Adult female Sprague-Dawley rats (Harlan UK Ltd., Oxon, England) weighing 220-300 g were maintained under controlled temperatures (21±1°C) and light conditions (lights on from 07.00h to 19.00h). Food and water were provided *ad libitum*. Vaginal smearing was performed each morning (09.00h-10.00h) and the morphology of the cells present used to identify the different stages of the oestrus cycle. Only those animals which had exhibited at least three consecutive four-day estrus cycles were selected for experimentation.

On the late morning of pro-estrus, a plastic cannula (Portex, o.d. 0.63 mm) combined with vinyl tubing (i.d. 0.55 mm) was inserted into the right femoral artery under halothane anesthesia (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, intraperitoneally (IP) injected either with diamorphine (3 mg/kg, n=10; Napp Laboratories Ltd, Cambridge UK), U-50488H (10 mg/kg, n=8; Boehringer Ingelheim, Germany), diamorphine plus naloxone (15 mg/kg, n=13; Sigma Chemicals Corporation, Poole, Dorset, UK) or U-50488H plus MR2266 (10 mg/kg, n=9; Boehringer Ingelheim, Germany). Controls received saline alone (1 ml/kg; n=16). Blood samples (200μl) were withdrawn through the heparinized cannula at hourly intervals from the freely-moving conscious animals throughout the afternoon of pro-estrus, commencing at 15.00h. Drug administrations were carried out under light halothane anesthesia.

The blood samples collected were centrifuged at 4°C for 10 mins at 3000 rpm. The plasma was then transferred into fresh tubes and stored at -20°C until assayed for LH determination by radio-immunassay (RIA). At 19.00h the animals were decapitated, and the brains rapidly removed and frozen on dry ice. 500μm coronal brain slices were cut. The MPOA, suprachiasmatic nucleus (SCN), ME and arcuate nucleus (ARN) were micropunched using modified stainless steel hypodermic needles (0.7 and 0.3 mm internal diameters) under a dissection microscope, according to the Stereotaxic Atlas of the Rat Brain (Paxinos and Watson 1986).

Chromatography:

The specific hypothalamic areas collected were kept at -80°C prior to the analysis. 100μl of 0.1M HCl was added to each sample, along with 50μl of 3.4-dihydroxybenzylamine: 1ng) as an internal standard. The samples were homogenized and then centrifuged at 4°C for 10 mins at 3000 rpm. 10μl aliquots of supernatant were injected on to a reverse phase high performance liquid chromatographic (HPLC) column (S5ODS2-250A, 5μm, 4.6 mm i.d.x25 cm, HICHROM) coupled to an electrochemical detector (ECD, Model 141, GILSON). The monoamine content of the hypothalamic regions was simultaneously detected. The method has been previously described (Yilmaz *et al.* 1996).

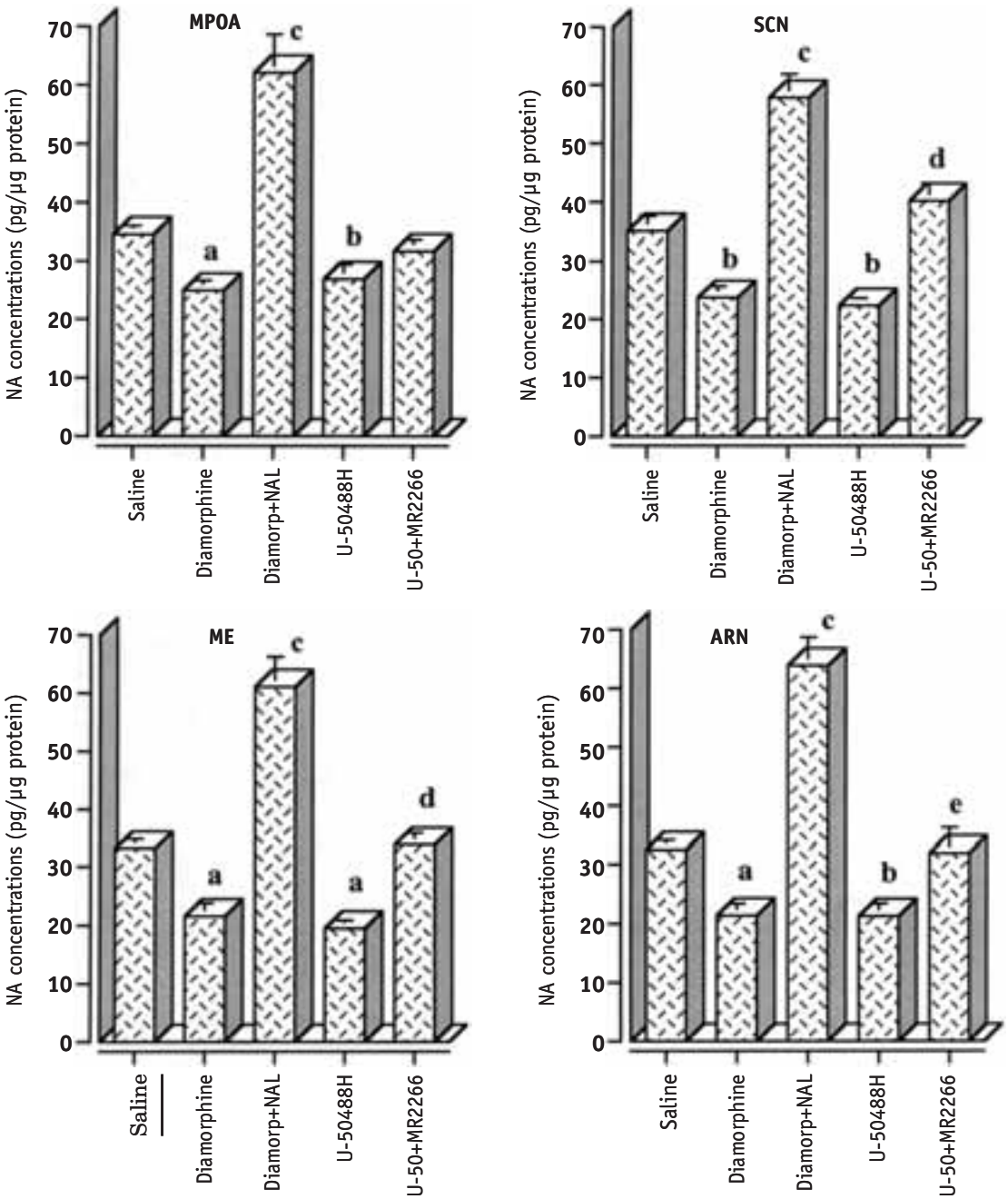

Figure 1. NA concentrations (pg/µg protein± SEM) in the MPOA, SCN, ME and ARN of the rat hypothalamus at 19.00h on pro-estrus following administration or co-administration of µ- and κ-opioid agonists and antagonists at 13.00h on the same day. **a:** p<0.001, **b:** p<0.01 compared to the saline-treated animals, **c:** p<0.001 compared to the diamorphine-treated animals, **d**: p<0.001, **e:** p<0.05 compared to the U-50488H-treated animals, using ANOVA and the Kruskal-Wallis non-parametric test.

LH Assay:

Plasma LH levels were measured by RIA. 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, Maryland, USA). The inter- and intra-assay co-efficients of variation were 8.0% and 9.5%, respectively. The sensitivity of the assay was 10 pg/tube (1 ng/ml).

Protein Estimation:

Protein estimations were made according to the modified method of Lowry et al. (1951). The method is detailed in our previous report (Yilmaz *et al.* 1996).

Statistics:

One-way analysis of variance (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 were large between means.

Results

1. Effect of opioid agonists and antagonists on hypothalamic catecholamine concentrations:

Noradrenaline (NA) and dihydroxyphenylglycol (DHPG) results are summarized as histograms in Figures 1 and 2. In the controls, NA concentrations (values in pg/µg protein, mean±SEM) were high in all the hypothalamic regions examined. They were significantly reduced by both diamorphine (MPOA, ME, ARN p<0.001 and SCN p<0.01) and U-50488H (SCN, ME, ARN p<0.001 and MPOA p<0.05). The

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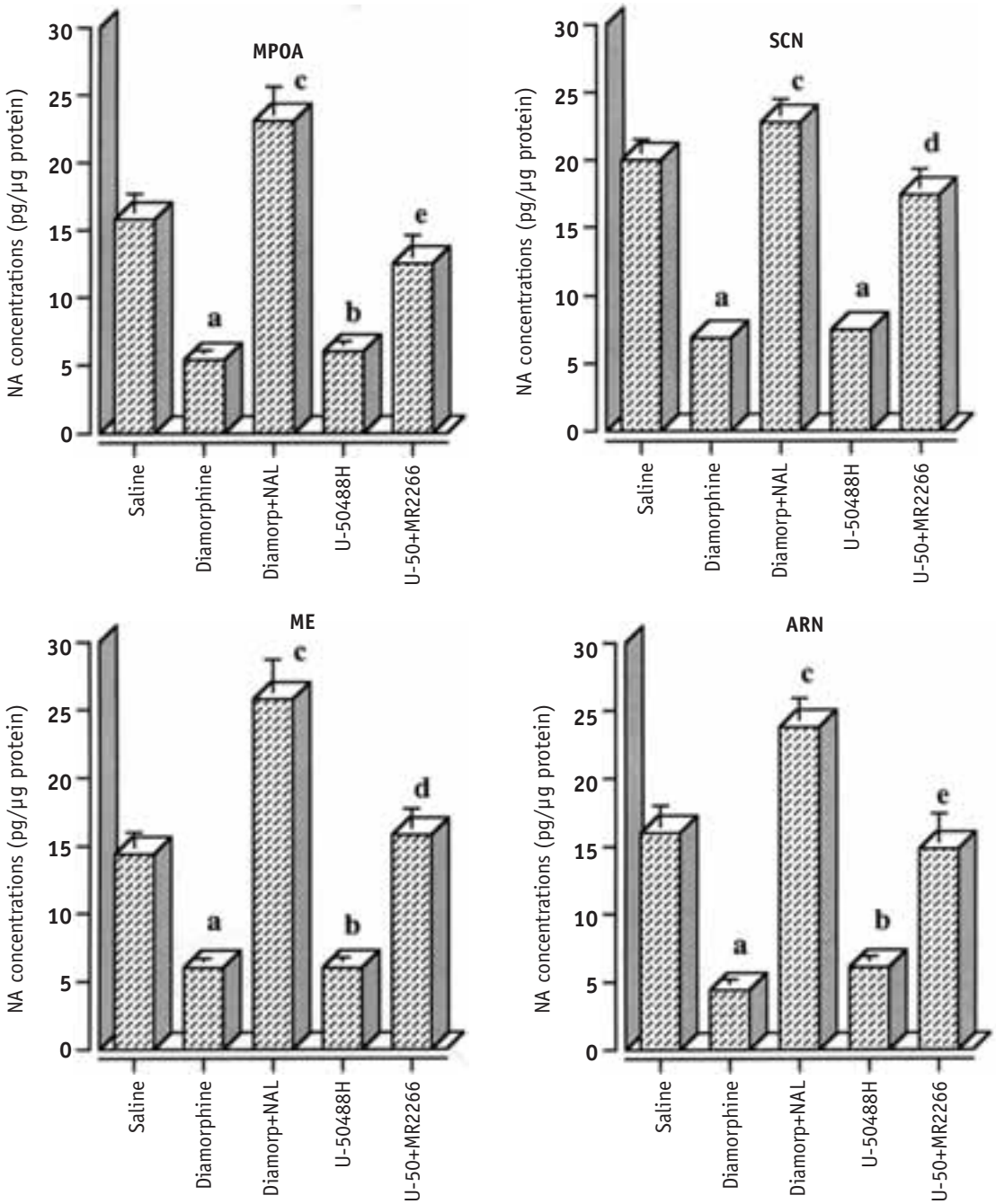


Figure 2. DHPG concentrations (pg/µg protein± SEM) in the MPOA, SCN, ME and ARN of the rat hypothalamus at 19.00h on pro-estrus following administration or co-administration of µ- and κ-opioid agonists and antagonists at 13.00h on the same day. **a:** p<0.001, **b:** p<0.005 compared to the saline-treated animals, **c:** p<0.001 compared to the diamorphine-treated animals, **d**: p<0.001, **e:** p<0.01 compared to the U-50488H-treated animals, using ANOVA and the Kruskal-Wallis nonparametric test.

inhibitory effects of these µ- and κ-agonists on NA levels were prevented in all four hypothalamic areas (except U-50488H in the MPOA) following their co-administration with naloxone ($p<0.001$) and MR2266 ($p<0.01$), respectively. Diamorphine ($p<0.001$) and U-50488H ($p<0.005$) also decreased DHPG levels in all the hypothalamic regions examined. These inhibitory effects were negated in the MPOA ($p<0.01$), SCN ($p<0.001$), ME ($p<0.001$) and ARN ($p<0.01$) following concomitant administration of diamor-phine and U-50488H with their respective antagonists, naloxone and MR2266.

Dopamine (DA) concentrations (Table 1) were reduced by the µ-agonist; however, these reductions were found to be significant only in the MPOA ($p<0.01$), but not in the SCN, ME and ARN. Naloxone significantly elevated DA levels in the MPOA ($p<0.05$), SCN, ME and ARN ($p<0.001$) compared to those receiving diamorphine alone. The κ-agonist had no significant effect on DA levels in any of the hypothalamic regions examined. They were significantly increased only in the ME ($p<0.05$) following co-administration of U-50488H with MR2266.

Diamorphine group results were subdivided with respect to plasma LH levels (given below). NA concentrations were significantly higher in the MPOA, ME and ARN of the µ-agonist-treated rats showing an LH surge than those did not (Table 2). However, naloxone induced significant increases in NA levels compared to both sub-groups. DHPG and DA results did not significantly differ between the diamorphine sub-groups in any of the hypothalamic areas examined.

Table 1. Dopamine concentrations (pg amine/μg protein ± SEM) in the MPOA, SCN, ME and ARN of the rat hypothalamus at 19.00h on pro-estrus after administration or co-administration of m- and k-opioid agonists and antagonists at 13.00h on the same day. **a:** p<0.01 compared to the saline-, **b:** p<0.001; **c**: p<0.005 compared to the diamorphine-, **d**: p<0.05 compared to the U-50488H-treated animals. ANOVA and Kruskal-Wallis non-parametric testis were utilized to examine the data.

Area	Saline (n=16)	Diamorphine (n=10)	Diamor+NAL (n=13)	U-50488H (n=8)	U-50+MR2266 (n=9)
MPOA	6.1±0.8	3.5±0.3 **a**	8.1±0.9 **b**	5.8±0.7	5.2±0.6
SCN	6.2±1.1	4.7±0.5	7.9±0.6 **c**	5.8±0.9	6.9±0.6
ME	19.1±1.6	17.5±2.0	30.2±3.1 **c**	16.6±2.7	26.0±2.9 **d**
ARN	6.2±1.0	4.2±0.5	8.3±0.9 **b**	5.9±1.1	7.0±0.7

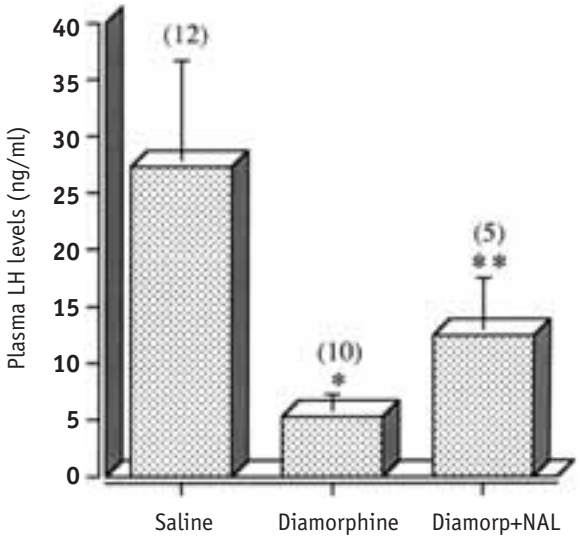
Table 2. Noradrenaline concentrations (pg amine/μg protein ± SEM) in the MPOA, SCN, ME and ARN of the rats with or without an LH surge at 19.00h on pro-estrus after administration of diamorphine (m-agonist) at 13.00h on the same day. **a:** p<0.01, **b:** p<0.05 compared to those animals showing LH surge (students' t-test).

Area	Diamorphine (LH surge; n=5)	Diamorphine (no LH surge; n=5)
MPOA	28.5 ± 2.4	21.2 ± 1.5 **a**
SCN	24.8 ± 2.2	22.9 ± 3.2
ME	25.5 ± 3.0	17.7 ± 2.5 **a**
ARN	26.5 ± 2.0	16.2 ± 1.5 **b**

2. Effect of opioid agonists and antagonists on LH release on the afternoon of pro-estrus:

Plasma samples were obtained at hourly intervals between 15.00h and 19.00h on the afternoon of pro-estrus and the LH concentrations were seen to rise significantly to a peak at 18.00h with a slight non-significant fall at 19.00h in 12 out of 16 animals (LH levels ng/ml ± SEM: at 15.00h = 3.1±1.3; 16.00h = 11.5 ± 4.1; 17.00h = 22.3 ± 7.9; 18.00h = 27.3 ± 9.4; 19.00h = 23.6 ± 6.3). These LH results are consistent with those reported by Dow *et al*. (1994) using the same experimental technique. Administration of U-50488H at 13.00h completely suppressed plasma LH levels throughout the afternoon of pro-estrus. The κ-antagonist, MR2266, failed to prevent the inhibitory effects of the κ-agonist on LH secretion in 8/9 rats with one animal showing a rise to peak LH levels of 19.5 ng/ml at 19.00h. Administration of diamorphine suppressed plasma LH levels in 5/10 rats with five animals showing a rise to peak concentrations of 18.5, 12.8, 10.9, 10.7 and 5.5 ng/ml each at 18.00h and 19.00h sampling intervals. This inhibitory effect of diamorphine was reversed in 5/6 rats which were given 15 mg/kg naloxone concomitantly with the μ-agonist. Peak concentrations of LH over the afternoon of pro-estrus are shown in Figure 3.

Figure 3. Peak concentrations of LH over the afternoon of pro-estrus in conscious rats treated with saline, diamorphine alone or with naloxone. Numbers in each group are given in brackets. *p<0.01 compared to saline-treated animals; **p<0.05 compared to the diamorphine-treated animals using One-Way ANOVA.



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Discussion

It has been proposed that the noradrenergic system in the brain is the trigger for initiation of the pre-ovulatory LH surge in the presence of circulating estrogen. Blockade of the α-adrenoreceptors in the preoptic/anterior hypothalamic area, ARN and ME as well as systemic application of the catecholamine synthesis inhibitor, diethyl dithiocarbamate, inhibits the LH surge and also basal LH pulses (Estes *et al.* 1982; Jarry *et al.* 1990). Noradrenergic nerve terminals make synaptic contacts with the GnRH neurones in the MPOA and to a lesser extent with GnRH nerve terminals in the ARN and ME (Watanabe and Nakai 1987). Noradrenergic terminals within the hypothalamus possess a mixed population of μ- and κ-opioid receptors, and stimulation of μ- and κ-opioid activity inhibits GnRH release. The ability of morphine (a μ-agonist) to suppress LH secretion has been demonstrated in a variety of experimental conditions (see Kalra *et al.* 1989) and the effect is attributed to an inhibition of release of NA from its nerve terminals (Akabori and Barraclough 1986). The results of the present study support this hypothesis, as we have shown that the μ-opioid receptor agonist, diamorphine, also reduces the secretion of LH on the afternoon of pro-estrus and concomitantly decreases hypothalamic NA and DHPG levels. When diamorphine was co-administered with naloxone, the opioid inhibition of LH release was prevented. This suggests that increased LH release results from an antagonism at post-synaptic μ-opioid receptors. Also, the inhibitory opioidergic tone, although decreased, is not totally eliminated during the critical period. Naloxone also negated the inhibitory influence of diamorphine on concentrations of NA and its metabolite in all the hypothalamic regions examined. In some cases, in the diamorphine plus naloxone-treated group, amine levels were found to be even higher than those seen in the controls. Administration of naloxone alone has been shown to enhance the amplitude of the pre-ovulatory LH surge and have stimulatory action on NA release and/or turnover within the hypothalamus (Akabori and Barraclough 1986).

It is interesting to note that NA concentrations were higher in the rats showing rises in plasma LH levels than those with totally inhibited LH surge. This observation further confirms the crucial role of central noradrenergic neurotransmission in the control of LH release. DHPG levels did not significantly differ between the two sub-groups.

There are conflicting reports on the involvement of κ-opioid receptors in the regulation of LH secretion. Inhibition of LH release occurs after administration of specific κ-opioid agonists (Leadem and Yeganova 1987; Gopalan *et al.* 1989). However, the specificity of the κ-opioid effect has been questioned (Pfeiffer *et al.* 1987). In the present study, the LH surge was completely abolished by a selective κ-agonist throughout the afternoon of pro-estrus. The κ-opioid action on LH release is believed to be mediated at the level of the hypothalamus in view of the finding that κ-agonists inhibit GnRH release *in vitro* (Leposavic *et al.* 1991). Furthermore, in our study the effect is thought to be exerted via the central noradrenergic system since U-50488H brought about significant decreases in both NA and DHPG levels in all the hypothalamic regions examined. When MR2266 was co-administered with the κ-agonist, the falls in both amine and metabolite concentrations were prevented in the SCN, ME and ARN, but not in the MPOA. The lack of effect of the κ-antagonist was unexpected because κ-opioid receptors are found in the MPOA as well as in the other hypothalamic sites (Mansour *et al.* 1988). MR2266 also failed to reverse the suppressive effects of U-50488H on plasma LH levels. During the pre-ovulatory LH surge, all GnRH neurones synchronize to initiate synthesis of this neuropeptide (Silverman and Witkin 1994). The simultaneous firing of many, if not all, GnRH neurones is required for the pulsatile discharge of GnRH (Wetsel *et al.* 1992). Perhaps the lack of an essential stimulatory noradrenergic component to the GnRH neurones in the MPOA accounts for the failure of MR2266 to elevate plasma LH levels. Although the present results imply participation of κ-opioid receptors in the LH secretory systems, further studies using more selective antagonists may reveal additional information.

Endogenous opioid peptides alter NA neurotransmission by their action at pre-synaptic nerve terminals. Reduction of the amount of neurotransmitter released is a common feature of opioid action (Grossman and Dyer 1989). It is thought that diamorphine and U-50488H lower NA synthesis by inhibiting its release from the nerve terminals, as both NA and DHPG concentrations were reduced in parallel. Prevention of the falls in the amine levels by naloxone and MR2266 would point to specific μ- and κ-opioid action, respectively, in discrete hypothalamic areas.

The role of DA in the central regulation of LH release is less clear-cut, as it has both stimulatory and inhibitory effects on LH secretion depending on the site of action (MacKenzie *et al.* 1988; Kordon *et al.* 1994; Chandolia *et al.* 1997). There were isolated area-dependent effects in the rats treated with diamorphine or U-50488H, but these were not consistent and difficult to interpret. Diamorphine reduced DA levels selectively in the MPOA. Naloxone elevated DA concentrations in all four hypothalamic areas following its co-administration with μ-agonist.

Others have indicated in the MPOA that DA has a stimulatory effect on LH release (Kawakami *et al.* 1975), perhaps μ-opioid, but not κ-opioid receptors have a modulatory effect on dopaminergic activity in this area.

In summary, activation of the μ- and κ-opioid receptors may exert an inhibitory influence LH release, since both diamorphine and U-50488H inhibit the preovulatory LH surge. The effect is probably an indirect one mediated by the hypothalamic catecholeaminergic (especially noradrenergic) neurotransmitter systems since they are suppressed by diamorphine and U-50488H in the all nuclear areas of the hypothalamus investigated.

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