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Neuropeptide FF receptors control morphine-induced analgesia in the parafascicular nucleus and the dorsal raphe nucleus

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Abstract

The ability of (1DMe)Y8Fa (D.Tyr–Leu–(NMe)Phe–Gln–Pro–Gln–Arg–Phe–NH_2), a selective neuropeptide FF analog resistant to enzymatic degradation, to control morphine-induced analgesia was investigated in rat after microinfusion into the dorsal raphe nucleus and the nucleus parafascicularis of the thalamus. Infusion of (1DMe)Y8Fa (2.5 nmol) in the nucleus raphe dorsalis did not modify the animal response in the tail-immersion test but significantly reversed analgesia induced by coinjected morphine (27 nmol). Similarly, (1DMe)Y8Fa (5 nmol) inhibited morphine effects in the hot-plate test after co-injection into the parafascicular nucleus. Furthermore, (1DMe)Y8Fa injected into the parafascicular nucleus attenuated analgesia induced by morphine injected into the nucleus raphe dorsalis and similarly, the neuropeptide FF analog in the nucleus raphe dorsalis decreased the effects of 27 nmol morphine injected in the parafascicular nucleus. The density of neuropeptide FF receptors did not decrease in the nucleus raphe dorsalis after lesion of serotonergic neurons by 5,7-dihydroxytryptamine. However, after this lesion, (1DMe)Y8Fa injected in the nucleus raphe dorsalis was no longer able to modify analgesic effects of morphine in hot-plate and tail-immersion tests. Similarly, the serotonin (5-HT) depletion induced by a systemic administration of *para*-chlorophenylalanine did not modify morphine analgesia microinjected into the nucleus raphe dorsalis and the parafascicular nucleus but blocked the ability of (1DMe)Y8Fa to reverse morphine effects in both nuclei. These data show that neuropeptide FF exerts anti-opioid effects directly into both the nucleus raphe dorsalis and the parafascicular nucleus and acts also at distance on opioid functions. Furthermore, anti-opioid effects of neuropeptide FF require functional serotonergic neurons although neuropeptide FF receptors are not carried on these neurons. © 1997 Elsevier Science B.V.

Keywords: Neuropeptide FF; Receptors; Morphine-analgesia; Rat; Dorsal raphe nucleus; Parafascicular nucleus; Serotonin

1. Introduction

Neuropeptide FF (Phe–Leu–Phe–Gln–Pro–Gln–Arg–Phe–NH_2) originally isolated from bovine brain (Yang et al., 1985) was identified in the central nervous system of several mammals including humans (Panula et al., 1987; Kivipelto et al., 1989; Majane et al., 1988). Neuropeptide FF is believed to act as a neurotransmitter since neuropeptide FF-immunoreactivity was restricted to vesicles in axonal profiles in the spinal cord (Allard et al., 1991) and could be released by depolarization from nerve terminals to stimulate specific neuropeptide FF receptors (Allard et al., 1989; Zhu et al., 1992; Devillers et al., 1994).

Neuropeptide FF generates its pharmacological effects by interacting in the central nervous system with specific binding sites localized in high concentrations in the thalamus, brain stem and spinal cord areas implicated in the control of nociception (Allard et al., 1989; Allard et al., 1992; Dupouy et al., 1996; Dupouy and Zajac, 1996). Several pharmacological results indicate that neuropeptide FF is involved in opioid control of nociceptive information (Yang et al., 1985; Gicquel et al., 1992) and could play a role in opioid tolerance and dependence (Tang et al., 1984; Malin et al., 1990a,b; Stinus et al., 1995). Neuropeptide FF antagonizes opioid analgesia after intracerebroventricular injection in rat (Yang et al., 1985; Millon et al., 1993) and mouse (Kavaliers and Yang, 1989; Gicquel et al., 1992). Third ventricle injection of neuropeptide FF in non-dependent rats induced a morphine-withdrawal-like behavioral syndrome (Malin et al., 1990a; Gicquel et al., 1994). Furthermore, highly selective antiserum against neuropeptide FF attenuated chronic morphine tolerance (Lake et al., 1991) and neuropeptide FF precipitated withdrawal in morphine-dependent rats (Malin et al., 1990a).

However, neuropeptide FF exerts complex pharmaco-

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logical actions (Desprat and Zajac, 1994) since intrathecal infusion of neuropeptide FF in rats produces analgesia (Gouardères et al., 1993; Gouardères et al., 1996). Neuropeptide FF analogs, similarly to opiates, inhibit intestinal transit after i.c.v. injection in mice (Gicquel et al., 1993) and reduced the postprandial changes in intestinal motility in rats (Millon et al., 1993).

All these observations revealed a possible neuromodulatory role of neuropeptide FF on opioid functions but at this time the molecular and cellular mechanisms implied in these modulations (Rebeyrolles et al., 1996; Roumy and Zajac, 1996) as well as the anatomical sites involved in functional interactions between opioids and neuropeptide FF are largely unknown (Dupouy, 1995). We have recently demonstrated that in the nucleus raphe dorsalis, identified by stimulation-produced-analgesia studies as a 'pure analgesia region' (Fardin et al., 1984), microinfusion of neuropeptide FF analogs reversed morphine analgesia (Dupouy, 1995). One other likely site for pain modulation in the forebrain could be in the intralaminar thalamic nuclei, the parafascicular thalami nucleus. This region is especially responsive to noxious stimuli (Peshanski et al., 1981) and receives serotonergic projections from the nucleus raphe dorsalis (Moore et al., 1978; Clements et al., 1985; Chen et al., 1992). In the medial thalamic nuclei, the cells responding exclusively to the noxious stimuli are concentrated within the parafascicular nucleus (Andersen and Dafny, 1983). Dorsal raphe stimulation modulates the spontaneous activity and the noxious-evoked responses of parafascicular nucleus neurons (Reyes-Vazquez et al., 1989). On the other hand, parafascicular nucleus stimulation activates neurons of periaqueductal gray sensitive to peripheral nociceptive stimuli (Sakata et al., 1988). Thus, it has been proposed that the nucleus raphe dorsalis–periaqueductal gray matter and parafascicular nucleus area play a role in endogenous antinociceptive control mechanisms and pain modulation systems in rodents. One hypothesis is that neuropeptide FF modulates opioid activity in the dorsal raphe nucleus and parafascicular nucleus.

The aim of this study was to determine the modulatory role of neuropeptide FF receptor activation on opioid analgesia in the nucleus raphe dorsalis and the parafascicular nucleus by using (1DMe)Y8Fa (D.Tyr–Leu–(NMe)Phe–Gln–Pro–Gln–Arg–Phe–NH_2), a neuropeptide FF receptor agonist resistant to enzymatic degradation (Gicquel et al., 1992) and to investigate the participation of serotonin in the anti-opioid effects of neuropeptide FF.

2. Materials and methods

2.1. Chemicals

Morphine hydrochloride was obtained from Francopia and naloxone from Sigma. (1DMe)Y8Fa (D.Tyr–Leu–(NMe)Phe–Gln–Pro–Gln–Arg–Phe–NH_2) was synthesized as previously described (Gicquel et al., 1992).

2.2. Microinfusion of animals

Animals were tested in accordance with the recommendations of the International Association for Study of Pain.

Experiments were performed on male Sprague–Dawley rats weighing 300 ± 30 g at the time of surgery. Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and stereotaxically implanted with a stainless-steel guide cannula (0.45 mm outer diameter) located 2 mm above the infusion site.

The stereotaxic coordinates of infusion sites according to the atlas of Paxinos and Watson (1986) were for the nucleus raphe dorsalis (mm): A–P = −7.8 from bregma, M–L = 0 from medial suture, D–V = 6.9 down from the top of the skull. Infusion was performed in the nucleus parafascicularis of the thalamus using the following stereotaxic coordinates (mm): A–P = −4.2, M–L = ±1.5, D–V = 6.9.

Animals were tested seven days after cannula implantation.

Microinfusion was performed through an infusion cannula in a volume of 0.5 μl (0.25 μl/min). Morphine was dissolved in isotonic saline solution, (1DMe)Y8Fa in acid solution and neutralized with 1 M NaOH.

Upon completion of the experiments, all animals were sacrificed and the location of injection site in the nucleus raphe dorsalis and the parafascicular nucleus was verified histologically after microinfusion of methylene blue (Fig. 1).

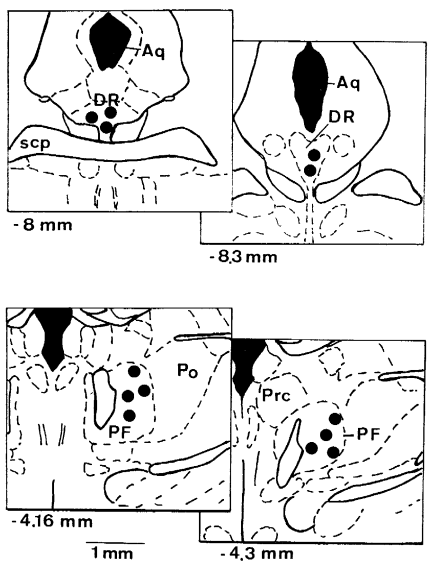

Fig. 1. Histological verification of cannula placements. Brain sites at which morphine and (1DMe)Y8Fa were administered are shown in rat coronal sections. Closed circles indicate the different injection sites obtained for each animals group used (8 to 15 animals). The number adopted from the atlas of Paxinos and Watson (1986) indicate the anterioposterior coordinates from the Bregma.

2.3. Analgesia

2.3.1. Tail immersion

The nociceptive responses were assessed by the tail-immersion test, using water at 56°C. Baseline tail withdrawal latencies, measured 45, 30 and 15 min before infusion, were between 1.8 and 2.2 s. In the absence of a response, tests were terminated after 7 s. Groups of 8–13 rats were injected with morphine hydrochloride and/or 1DMe(Y8Fa) (0.5 μl) into the nucleus raphe dorsalis or the parafascicular nucleus. The tail immersion responses were measured 10, 20, 30, 45, 60, 90, 120, 150 and 180 min after infusion.

2.3.2. Hot plate

The hot plate used a copper surface heated to 55°C by a waterbath. The behavioral endpoint was the licking of the hindpaw. Baseline withdrawal latencies were between 5 and 15 s. In order to prevent tissue damage, the hot plate was terminated after 30 s in the absence of a response.

The responses were measured 45, 30 and 15 min before infusion and 15, 30, 45, 60, 90 and 120 min after infusion.

2.4. Lesion of serotonergic neurons

2.4.1. Para-chlorophenylalanine depletion

Depletion of 5-HT (serotonin) in serotonergic neurons was accomplished by systemic administration of *para*-chlorophenylalanine (350 mg/kg 24 h before), an amino acid which competes with tryptophan, the substrate of tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of indolamines.

2.4.2. 5,7-dihydroxytryptamine lesions

Rats were pre-treated with desipramine (10 mg/kg, i.p.) to prevent non-specific damage to catecholaminergic neurons and anesthetized with pentobarbital (50 mg/kg, i.p.). Rats were treated with antibiotics (Totapen, 1 mg/kg i.p.) for 48 h after surgery.

Microinjections of 4 mg 5,7-dihydroxytryptamine base (Sigma) in 2 μl were made at 0.25 μl/min into eight sites around the nucleus raphe dorsalis and the dorsal raphe magnus. Stereotaxic coordinates were according to the atlas of Paxinos and Watson (1986):

A–P: −7.2 mm from bregma; D–V: −6.0 mm from the skull; M–L: ±0.5 mm from midline

A–P: −8.4 mm; D–V: −6.6 mm; M–L: ±0.5 mm

A–P: −7.2 mm; D–V: −8.5 mm; M–L: ±0.5 mm

A–P: −8.4 mm; D–V: −8.6 mm; M–L: ±0.5 mm

Control animals underwent the same procedure except for the microinjection. Confirmation of the location of lesions was made by macroscopic histological examination of serial coronal sections through the brainstem after the test.

2.5. Autoradiographic procedures

1DMe(Y8Fa) ([D.Tyr^1, (NMe)Phe^3]NPFF) was iodinated as previously described (Gicquel et al., 1992). Slide-mounted brain sections were incubated during 180 min at 25°C with 0.05 nM [^{125}I][D.Tyr^1, (NMe)Phe^3]NPFF in 50 mM Tris–HCl buffer pH 7.4 containing 120 mM NaCl and 0.1% bovine serum albumin. Non-specific binding of [^{125}I][D.Tyr^1, (NMe)Phe^3]NPFF was determined in adjacent sections by adding 1 mM of unlabelled [D.Tyr^1, (NMe)Phe^3]NPFF to the incubation medium. Slides were transferred sequentially through four rinses (5 min each) in ice-cold 50 mM Tris-HCl buffer pH 7.4 and a final dipping in ice-cold distilled water and exposed for 4–6 days at room temperature in contact with tritium-sensitive film (Hyperfilm, Amersham).

The autoradiograms were analyzed by a computerized image analysis system RAG200 (Biocom, France). The average optical density was transformed to pCi/unit area by reference to iodinated standards (Dupouy and Zajac, 1996) co-exposed with the tissue sections.

2.6. Data analysis

Changes of latency responses were converted to maximum percentage effect for each animal, calculated as:

$$100 \times \frac{(\text{post} - \text{infusion latency} - \text{baseline latency}}{\text{cut} - \text{off value} - \text{baseline latency})}$$

The basal tail-withdrawal latency time for an animal was obtained as the mean of three determinations 45, 30 and 15 min before infusion. The baseline latency time used in the calculation of maximum percentage effect for a rat correspond to the mean of basal latency time of the same animal.

The area under the maximum percentage effect versus time curve (AUC) was computed by trapezoidal approximation of the AUC for each rat between 0 and 120 min for the hot plate and 0 to 180 min for the tail immersion test.

The data were compared using the rank order Mann–Whitney U-non-parametric test (Statwiew program, Macintosh). Mean values were compared to that of control animals. *P* values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Single site injections

Microinjection of 0.008 to 8.4 nmol (1DMe)Y8Fa alone (0.25 μl/min) in the parafascicular nucleus and the nucleus raphe dorsalis did not modify significantly latencies in the hot-plate and tail-immersion tests (data not shown).

Table 1
Reduction of antinociceptive effects of morphine by 1DMe(Y8Fa) microinjected into the dorsal raphe nucleus

Pretreatment	Treatment	Tail immersion	Hot plate
None	morphine	4738 ± 795	10507 ± 257
None	morphine + 1DMe(Y8Fa) 2.5 nmol	2343 ± 682 [a]	7216 ± 1552 [a]
NaCl	morphine	3890 ± 734	10400 ± 200
pCPA	morphine	3353 ± 505	11346 ± 118
pCPA	morphine + 1DMe(Y8Fa) 2.5 nmol	2903 ± 770	9585 ± 2321
5,7 DHT	morphine	3824 ± 1047	6671 ± 1790
5,7 DHT	morphine + 1DMe(Y8Fa) 2.5 nmol	3404 ± 427	5843 ± 1309
5,7 DHT	morphine + 1DMe(Y8Fa) 5 nmol	4549 ± 800	7956 ± 1264

Animals were pretreated by *para*-chlorophenylalanine (pCPA) or lesioned by microinjection of 5,7-dihydroxytryptamine (5,7 DHT). Morphine was injected into the nucleus raphe dorsalis at a dose of 27 nmol and antinociception measured in the tail immersion and hot plate tests.
Data are means (± S.E.M.) of AUC from 8 to 13 animals.
[a] $P < 0.05$ significantly different versus morphine alone.

Table 2
Antinociceptive effects of morphine and 1DMe(Y8Fa) microinjected into the nucleus parafascicularis of the thalamus

Pretreatment	Treatment	AUC
None	morphine 27 nmol	5190 ± 1042
None	morphine + 1DMe(Y8Fa) 2.5 nmol	2056 ± 1005
None	morphine + 1DMe(Y8Fa) 5 nmol	2080 ± 666 [a]
NaCl	morphine 10 nmol	6754 ± 1134
pCPA	morphine 10 nmol	9246 ± 608
pCPA	morphine + 1DMe(Y8Fa) 5 nmol	5973 ± 1255

Animals were pretreated by *para*-chlorophenylalanine (300 mg/kg 24 h before) (pCPA) or NaCl. The effects of morphine injected into the parafascicular nucleus was measured in the hot plate test. 1DMe(Y8Fa) was injected 10 min after morphine in the parafascicular nucleus.
Data are means (± S.E.M.) of AUC from 8 to 13 animals.
[a] $P < 0.05$ significantly different versus morphine alone.

3.1.1. Effects of (1DMe)Y8Fa on morphine analgesia in the dorsal raphe nucleus

As previously demonstrated (Dupouy and Zajac, 1995), the microinjection of morphine into the nucleus raphe dorsalis produced a dose-dependent analgesic response as evidenced by the prolongation of the tail-immersion latency (Fig. 2A). The maximal effect of 27 nmol morphine occurred 20 min after injection and the tail-flick latencies returned to base-line values by 120 min.

(1DMe)Y8Fa (2.5 nmol) coinjected into the nucleus raphe dorsalis reversed significantly (60% at peak effect) the analgesia induced by morphine (Fig. 2A). Comparison of the mean areas under the curve indicated that 2.5 nmol (1DMe)Y8Fa decreased significantly morphine analgesia in hot plate (−31%) and tail immersion (−51%) tests (Table 1). Similarly, coinjected naloxone (2.5 nmol) inhibited completely morphine analgesia in both tests (data not shown).

3.1.2. Effects of (1DMe)Y8Fa on morphine analgesia in the parafascicular nucleus

Morphine (27 nmol) injected in the parafascicular nucleus did not modify the tail-flick latency but produced a clear increase of the paw-lick latency which peaked at 60 min (Fig. 2B). (1DMe)Y8Fa (2.5 and 5 nmol) injected 10 min after morphine decreased significantly the morphine response (Fig. 2, Table 2). Comparison of the mean areas under the curve with and without 5 nmol (1DMe)Y8Fa shows a significant reduction (60%) of morphine analgesia (Table 2). Naloxone (2.5 nmol) preinjected 10 min before morphine into the parafascicular nucleus blocked more than 70% of the opioid effects (not shown).

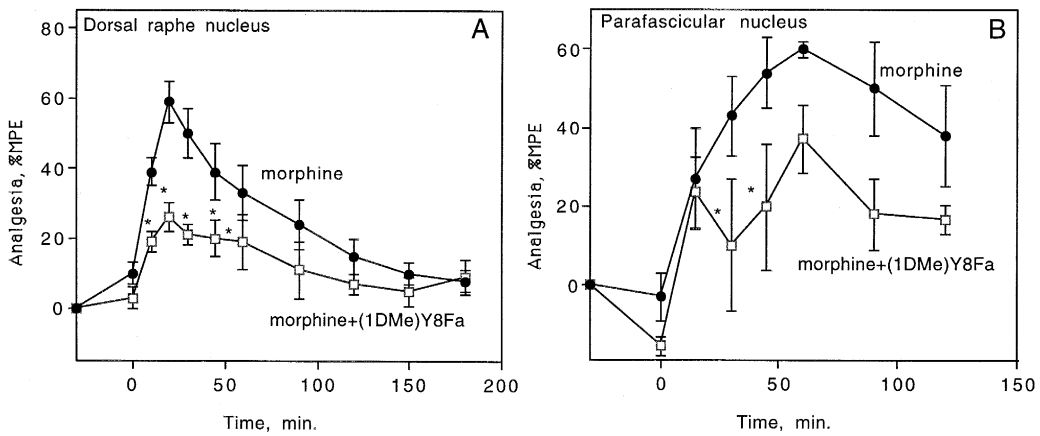

Fig. 2. Effects of (1DMe)Y8Fa in the nucleus raphe dorsalis and the parafascicular nucleus on the antinociceptive effects of morphine injected in the nucleus raphe dorsalis or the parafascicular nucleus. (A) Effects of 2.5 nmol (1DMe)Y8Fa on the time-course analgesia induced by a coinjection of 27 nmol morphine into the nucleus raphe dorsalis, in the tail immersion test. (B) Effects of 5 nmol (1DMe)Y8Fa on the time-course antinociception induced by 27 nmol morphine injected 10 min before into the parafascicular nucleus, in the hot plate test. Data are means (± S.E.M.) from 8 to 13 rats. [a] $P < 0.05$ significantly different from morphine treated group.

Table 3
Control of morphine analgesia by 1DMe(Y8Fa) injected into the nucleus raphe dorsalis and the parafascicular nucleus

	AUC
Tail-immersion test	
Morphine in the nucleus raphe dorsalis	3852 ± 859
+ 1DMe(Y8Fa) 2.5 nmol in the parafascicular nucleus	454 ± 1443
+ 1DMe(Y8Fa) 5 nmol in the parafascicular nucleus	853 ± 772 [a]
Hot plate test	
Morphine in the parafascicular nucleus	5629 ± 1464
+ 1DMe(Y8Fa) 2.5 nmol in the nucleus raphe dorsalis	3112 ± 2112
+ 1DMe(Y8Fa) 5 nmol in the nucleus raphe dorsalis	783 ± 637 [a]

Animals were microinjected with morphine 27 nmol in the nucleus raphe dorsalis or the nucleus parafascicularis. The effects of 1DMe(Y8Fa) injected 10 min before in the parafascicular nucleus and the nucleus raphe dorsalis was measured in the tail immersion and in the hot plate test. Data are means (± S.E.M.) of AUC from 8 to 11 animals.
[a] $P < 0.05$ significantly different versus morphine alone.

3.2. Distant control of neuropeptide FF receptors on morphine analgesia

The analgesic activity of 27 nmol morphine microinjected into the nucleus raphe dorsalis was significantly attenuated in the tail-immersion test when (1DMe)Y8Fa (2.5 and 5 nmol) was injected 10 min before into the parafascicular nucleus (Fig. 3A, Table 3). The AUC between 0 and 180 min in animals treated with 5 nmol (1DMe)Y8Fa was significantly less (Table 3) than the AUC in morphine treated animals in the tail-flick (−78%) and the hot plate tests (−83%).

Similarly, although (1DMe)Y8Fa alone did not modify the hot plate latency, this peptide (2.5 and 5 nmol) injected into the nucleus raphe dorsalis decreased analgesia produced by 27 nmol morphine microinjected into the parafascicular nucleus 10 min later (Fig. 3B). The effect of 5 nmol of (1DMe)Y8Fa was significant from 60 to 120 min. The AUC shows that only 5 nmol (1DMe)Y8Fa into the nucleus raphe dorsalis reversed significantly morphine analgesia (−86%) produced by microinjection into the parafascicular nucleus (Table 3).

3.3. Effects of serotonin depletion and lesion

We have studied the effects on neuropeptide FF activity of a reduction in intracerebral serotonin content produced by the systemic administration of *para*-chlorophenylalanine or by the lesion of serotonin-containing terminals with 5,7-dihydroxytryptamine. In a separate group of identically treated rats, 5-HT (serotonin) levels in all brain areas considered decreased at least 95% and 5-hydroxyindoleacetic acid levels were reduced by 95% after the lesions (Allard et al., 1996).

3.3.1. Effects of para-chlorophenylalanine

Para-chlorophenylalanine (350 mg/kg), a serotonin synthesis inhibitor, injected 24 h before testing, did not reduce the tail-immersion and hot plate baseline latencies. Furthermore, under our experimental protocol, *para*-chlorophenylalanine treatment did not modify analgesia induced by morphine (27 nmol) microinjected into the nucleus raphe dorsalis or the parafascicular nucleus (Tables 1 and 2). Furthermore, morphine effects in the parafascicular nucleus increased in *para*-chlorophenylalanine treated rats but only in a non-significant manner (Table 2).

(1DMe)Y8Fa injected into the nucleus raphe dorsalis was unable to modify significantly analgesia induced by morphine injected into the same nucleus (Table 1, Fig. 4B).

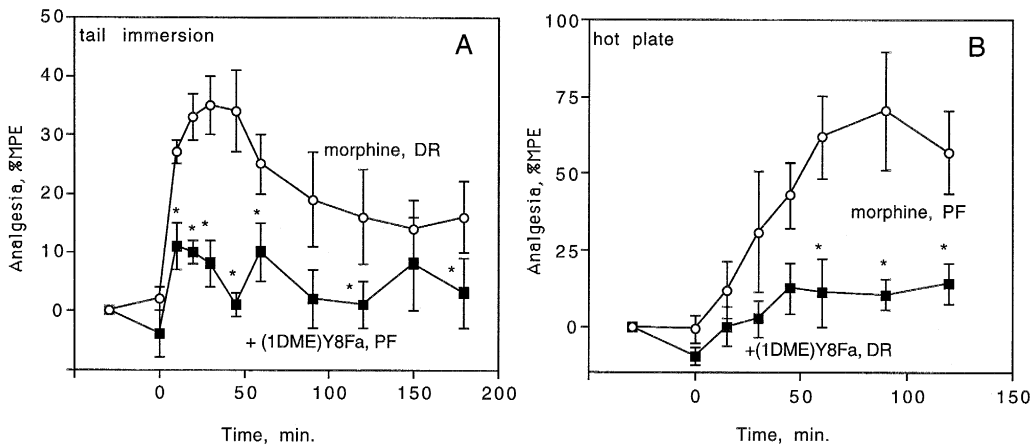

Fig. 3. Time course of the antinociceptive responses induced by morphine into the nucleus raphe dorsalis and the parafascicular nucleus after administration of 1DMe(Y8Fa) into the parafascicular nucleus and the nucleus raphe dorsalis. (A) 1DMe(Y8Fa) (5 nmol) was injected into the parafascicular nucleus (PF) 10 min before 27 nmol morphine into the nucleus raphe dorsalis (DR) and the antinociception was measured in the tail immersion test. (B) 5 nmol 1DMe(Y8Fa) was injected into the nucleus raphe dorsalis (DR) 10 min before 27 nmol morphine into the parafascicular nucleus (PF) and the antinociception was measured in the hot plate test. Data are means (± S.E.M.) from 8 to 13 rats. [a] $P < 0.05$ significantly different from morphine alone.

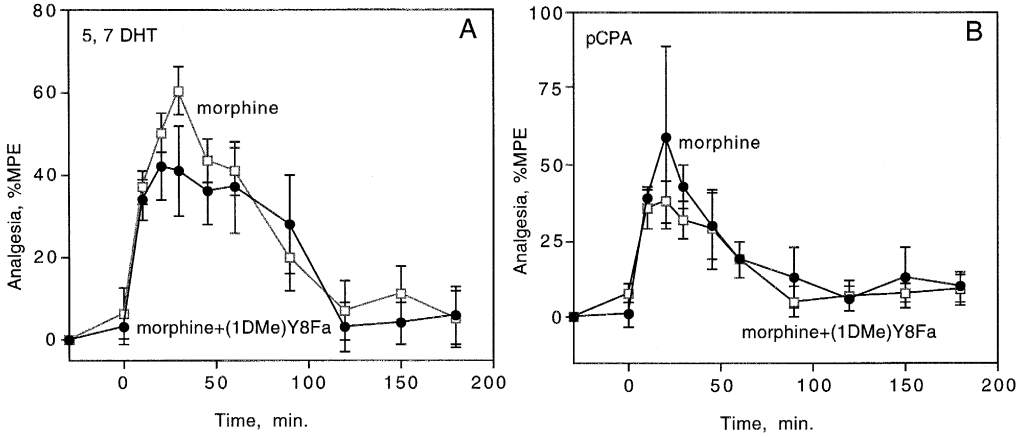

Fig. 4. Lesion (A) and depletion (B) of serotonergic neurons on the effects of morphine and 1DMe(Y8Fa). (A) Time-course of morphine antinociception (27 nmol) and morphine plus 2.5 nmol 1DMe(Y8Fa) co-injected into the nucleus raphe dorsalis in the tail immersion test in animals treated by 5,7-dihydroxytryptamine (5,7 DHT). (B) Effects of 27 nmol morphine and morphine plus 5 nmol 1DMe(Y8Fa) injected into the nucleus raphe dorsalis in the tail immersion test in animals treated with *para*-chlorophenylalanine (pCPA). Data are means (±S.E.M.) from 10 animals.

Similarly, in *para*-chlorophenylalanine treated rats, (1DMe)Y8Fa injected in the parafascicular nucleus did not change significantly the hot-plate latency increase induced by morphine microinjected in the parafascicular nucleus 10 min (Table 2).

3.3.2. Effects of 5,7-dihydroxytryptamine

Eleven days after the lesion of serotonin neurons by 5,7-dihydroxytryptamine, microinjection of morphine in the nucleus raphe dorsalis induced an analgesia, measured in the hot plate test, identical to that recorded in control animals (Table 1). However, in 5,7-dihydroxytryptamine treated rats (1DMe)Y8Fa (2.5 and 5 nmol) co-injected into the nucleus raphe dorsalis was no longer able to modify significantly the analgesic effects of morphine injected in the nucleus raphe dorsalis (Table 1, Fig. 4A).

Table 4
Neuropeptide FF receptors in several brain areas 11 days after serotonergic lesions by 5,7-dihydroxytryptamine

	Neuropeptide FF receptors density		
	Control	Lesioned	% Change
Caudate putamen	159±12	155±13	−2.5
Lateral septal nucleus, intermediate part	276±26	267±42	−3.3
Lateral septal nucleus, dorsal part	82±8	97±17	+18.4
Lateral hypothalamic area	619±76	692±84	+11.8
Laterodorsal thalamic nucleus	460±51	479±43	+4.1
Parafascicular thalamic nucleus	765±62	903±64	+18.1
Periacqueductal gray matter, ventrolateral	179±8	228±16	+27.7
Dorsal raphe nucleus	164±7	252±25	+53.3 [a]
Lateral parabrachial nucleus	131±11	140±9	+6.2
Solitary tract	275±27	356±22	+29.3 [a]
Gracile nucleus	682±49	651±36	−4.6
Spinal trigeminal tract	546±84	529±63	−3

Changes in [^{125}I]1DMe(Y8Fa) binding produced by lesions with 5,7-dihydroxytryptamine were measured by quantitative autoradiography. Values (pCi/unit of area) are means (±S.E.M.) of 18 measurements obtained in three control animals and three treated rats.
[a] Significantly different ($P < 0.05$) from control values in Mann-Whitney test.

3.4. Effects of 5,7-dihydroxytryptamine lesions on neuropeptide FF receptor density

Table 4 summarizes the density of [^{125}I](1DMe)Y8Fa binding sites (0.05 nM) in several brain areas in control and in 5,7-dihydroxytryptamine treated animals. The selective lesions of the 5-HT pathways did not result in a decrease in the neuropeptide FF binding density 11 days after lesioning. In contrast, a significant increase in neuropeptide FF receptor density could be observed in the nucleus raphe dorsalis and the solitary tract.

4. Discussion

The physiological role of the neuropeptide FF system has been essentially explored in pharmacological experiments by using intracerebroventricular or intrathecal injections (Yang et al., 1985; Gouardères et al., 1993). Such routes of administration could recruit receptors acting on complex networks involving several brain areas simultaneously. In contrast, microinjections in discrete brain nuclei would help to define the anatomical connections between neuropeptide FF and opioid systems and the exact role of neuropeptide FF receptors in the control of nociceptive information.

The single site study confirms the analgesic activity of morphine after microinjection in both the dorsal raphe nucleus and the parafascicular nucleus (Sharpe et al., 1974; Reyes-Vazquez et al., 1989; Bandler and Shipley, 1994). Our experiments have been performed by injection in the

parafascicular nucleus and the nucleus raphe dorsalis of a morphine dose able to produce about 50% analgesia in order to reveal slight modifications in antinociceptive responses. Although microinjections of (1DMe)Y8Fa alone are inactive given into the nucleus raphe dorsalis or the parafascicular nucleus, they significantly reversed analgesia elicited by coinjection with morphine. These data indicate that neuropeptide FF receptors could modulate opioid control on nociceptive informations in several brain nuclei. The fact that (1DMe)Y8Fa induced similar quantitative effects in both nuclei demonstrates that neuropeptide FF receptors in the nucleus raphe dorsalis were as efficient as those present in the parafascicular nucleus to modulate opioid analgesia.

No pro-opioid effects of (1DMe)Y8Fa alone and no potentiation of morphine effects have been observed even at high doses (8 nmol) in contrast to that observed after i.c.v. administration in young mice (Desprat and Zajac, 1994) or i.t. injections in rats (Gouardères et al., 1996) indicating that only anti-opioid effects of neuropeptide FF could be observed after microinjection into the nucleus raphe dorsalis and the parafascicular nucleus. Although intracerebroventricular injection of neuropeptide FF caused hyperalgesia in rat (Yang et al., 1985; Oberling et al., 1993) this was not observed after microinjection into the nucleus raphe dorsalis or the parafascicular nucleus suggesting that these nuclei could only be responsible for anti-opioid effects of neuropeptide FF.

The double-site study reveals that neuropeptide FF receptor activation could block morphine antinociception produced at distance, since (1DMe)Y8Fa injected into the nucleus raphe dorsalis and the parafascicular nucleus reversed analgesia induced by morphine microinjected into the parafascicular nucleus and the nucleus raphe dorsalis, respectively. From a methodological point of view, it is assumed that the locally-administered compounds produced their effects on anatomically-discrete populations of receptors in the vicinity of the microinjection site. It is unlikely that neuropeptide FF injected in the nucleus raphe dorsalis had diffused to the parafascicular nucleus through the aqueduct of Sylvius since these area are 3.8 mm apart and an equal volume of methylene blue or radioactive morphine and (1DMe)Y8Fa did not reach these structures (data not shown).

The most abundant neurotransmitter in the nucleus raphe dorsalis is serotonin (Descarries et al., 1982), since in the rat a third of the neurons of this nucleus contains serotonin. The disappearance of (1DMe)Y8Fa effects in the nucleus raphe dorsalis on morphine analgesia after a reduction in intracerebral serotoninergic activity or a destruction of serotonin-containing neurons reveals an important role of serotonergic neurons in neuropeptide FF activity.

Neuropeptide FF receptors are not located on 5-HT neurons since a serotoninergic lesion induced an increase in the number of (1DMe)Y8Fa binding. This modification represents rather a denervation supersensitivity of neuropeptide FF sites carried on cells postsynaptic to serotonergic neurons.

Central networks of 5-HT have traditionally been thought to play a role in antinociception. Serotonergic pathways from midbrain raphe nuclei project to the periaqueductal gray, thalamus and other structures involved in antinociceptive mechanisms, whereas there is a major descending serotoninergic pathway from medullary raphe nuclei to the dorsal horn of the spinal cord, the site of primary processing of afferent nociceptive information (Besson and Chaouch, 1987). Thus, neuropeptide FF receptors in the nucleus raphe dorsalis could stimulate the 5-HT neurons described in the descending pain inhibition path via the magnus raphe nucleus to influence the noxious sensitive neurons in the dorsal horn of the spinal cord (Besson and Chaouch, 1987). In contrast to the antinociceptive function, some neurons in the nucleus raphe dorsalis are also nociceptive-responsive (Sanders et al., 1980). Such cells could also be activated by neuropeptide FF and could inhibit antinociceptive effects although the activity of neuropeptide FF alone was not observed.

Microinjections of 5,6-dihydroxytryptamine into the dorsal raphe nucleus had no effect on morphine analgesia (Deakin and Dostrovsky, 1978) while lesions of the spinal cord 5-HT pathways reduced morphine analgesia in the tail flick test. Administration of 5,6-dihydroxytryptamine by intrathecal route suppressed the antinociceptive effects of systemically given morphine in the hot plate test (Kuraishi et al., 1983) but not in the tail-flick test.

Our pharmacological experiments using the tail-immersion test and hot plate tests suggest, however, that (1DMe)Y8Fa after injection in the nucleus raphe dorsalis was unable to reverse the morphine effect after lesion or depletion of 5-HT neurons in the nucleus raphe dorsalis. Since the density of neuropeptide FF binding sites increased after lesioning, it is very unlikely that the lack of the neuropeptide FF effect was due to a disappearance of neuropeptide FF sites.

In the nucleus raphe dorsalis, neuropeptide FF and opioids probably did not act on the same cells since 5-HT depletion modify the neuropeptide FF effect but not morphine action. Furthermore, it has been demonstrated, in contrast to neuropeptide FF receptors, that some opioid receptors are located on 5-HT neurons in the nucleus raphe dorsalis (Allen et al., 1993). Opioid actions could result from the depression of the amplitude of synaptic potentials mediated by γ-aminobutyric acid (GABA) since in this nucleus, GABAergic terminals were presynaptic to the 5-HT cell perikarya. Such synapses have been found to be inhibitory in some physiological studies (Nishikawa and Scatton, 1983) and thus GABA must have an opposite action against the 5-HT neurons. The enkephalinergic axon terminals make synapses with GABAergic elements and the GABAergic axon terminals in turn make synapses with the 5-HT elements (Wang and Nakai, 1993). Furthermore, a direct synaptic relation of the enkephalinergic neurons

and the 5-HT neurons (Field et al., 1991; Wang et al., 1992) has been described. Taking into account these anatomical data, neuropeptide FF receptors could inhibit the 5-HT neurons via GABA or enkephalinergic neurons.

Injected into the parafascicular nucleus, morphine (27 or 54 nmol) did not modify significantly the latency in the tail immersion test while these doses effectively increased the response in the hot-plate test. This suggests that opioid receptors present in this nucleus do not influence spinal reflexes but control an ascending pain modulation pathway (Morgan et al., 1989) using in part serotonin. The neurotransmitter involved in the nucleus raphe dorsalis pathway to the parafascicular nucleus is also likely to be 5-HT (Reyes-Vazquez et al., 1989).

Recently, many authors have mentioned a direct ascending pain modulation projection from the nucleus raphe dorsalis (Andersen and Dafny, 1983; Qiao and Dafny, 1988; Dong et al., 1991). Demonstration of the convergence of inputs from the nucleus raphe dorsalis and the spinal tract of the trigeminal nerve to the parafascicular nucleus further confirmed the same idea (McCluny and Dafny, 1980). Reyes (Reyes-Vazquez et al., 1989) found that the effects of nucleus raphe dorsalis stimulation on pain-sensitive neurons in the parafascicular nucleus were similar to that of the direct iontophoresis of 5-HT locally in the parafascicular nucleus.

It has also been suggested that 5-HT and opioids make up two different systems with antinociceptive functions in the parafascicular nucleus (Wang and Nakai, 1994). Our data suggest that neuropeptide FF receptors are not located on serotoninergic neurons originating from the nucleus raphe dorsalis since their densities in binding sites were not modified by lesion of the 5-HT neurons.

The depletion of 5-HT is sufficient to block the inhibitory effect of (1DMe)Y8Fa on morphine analgesia. Since the depletion could not abolish serotoninergic neuronal activity, this probably indicates that a low activity of 5-HT neurons could not be sufficient to preserve neuropeptide FF effects.

In summary, our data indicate that neuropeptide FF exerts anti-opioid effects directly into the nucleus raphe dorsalis and the parafascicular nucleus and acts also at distance on opioid functions. Furthermore, anti-opioid effects of neuropeptide FF require functional 5-HT neurons although neuropeptide FF receptors are not carried on serotonergic neurons.

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References

Allard, M., Geoffre, S., Legendre, P., Vincent, J.D., Simonnet, G., 1989. Characterization of rat spinal cord receptors to FLFQPQRFamide, a mammalian morphine modulating peptide: A binding study. Brain Res. 500, 169–176.

Allard, M., Theodosis, D.T., Rousselot, P., Lombard, M.C., Simonnet, G., 1991. Characterization and localization of a putative morphine-modulating peptide, FLFQPQRFamide, in the rat spinal cord: Biochemical and immunocytochemical studies. Neuroscience 40, 81–92.

Allard, M., Zajac, J.-M., Simonnet, G., 1992. Autoradiographic distribution of receptors to FLFQPQRFamide, a morphine-modulating peptide, in rat central nervous system. Neuroscience 49, 101–116.

Allard, M., Rivot, J.P., Stinus, L., Delort-Laval, S., Bertrand, A., Weil-Fugazza, J., 1996. In: Abstracts 8th World Congress on Pain.

Allen, D.L., Johnson, A.E., Tempel, A., Zukin, R.S., Luine, V.N., McEwen, B.S., 1993. Serotoninergic lesions decrease μ- and δ-opiate receptor binding in discrete areas of the hypothalamus and in the midbrain central gray. Brain Res. 625, 269–275.

Andersen, E., Dafny, N., 1983. An ascending serotoninergic pain modulation pathway from dorsal raphe nucleus to the parafascicularis nucleus of the thalamus. Brain Res. 269, 57–67.

Bandler, R., Shipley, M.T., 1994. Columnar organization in the midbrain periaqueductal gray: Modules for emotional expression?. Trends Neurosci. 17, 379–389.

Besson, J.-M., Chaouch, A., 1987. Peripheral and spinal mechanisms of nociception. Physiol. Rev. 67, 67–186.

Chen, J., Zeng, S.-L., Rao, Z.-R., Shi, J.-W., 1992. Serotoninergic projections from the midbrain periaqueductal gray and nucleus raphe dorsalis to the nucleus parafascicularis of the thalamus. Brain Res. 584, 294–298.

Clements, J.R., Beitz, A.J., Fletcher, T.F., Mullett, M.A., 1985. Immunocytochemical localization of serotonin in the rat periaqueductal gray: A quantitative light and electron microscopic study. J. Comp. Neurol. 236, 60–70.

Deakin, J.F.W., Dostrovsky, J.O., 1978. Involvement of the periaqueductal grey matter and spinal 5-hydroxytryptaminergic pathways in morphine analgesia: Effects of lesions and 5-hydroxytryptamine depletion. Br. J. Pharmacol. 63, 159–165.

Descarries, L., Watkins, K.C., Garcia, S., Beaudet, A., 1982. The serotonin neurons in nucleus raphe dorsalis of adult rat: A light and electron microscope radioautographic study. J. Comp. Neurol. 207, 239–254.

Desprat, C., Zajac, J.-M., 1994. Ontogeny of neuropeptide FF pharmacology and receptors in mouse brain. Dev. Brain Res. 82, 118–126.

Devillers, J.-P., Mazarguil, H., Allard, M., Dickenson, A.H., Zajac, J.-M., Simonnet, G., 1994. Characterization of a potent agonist for NPFF receptors: Binding study in rat spinal cord membranes. Neuropharmacology 33, 661–669.

Dong, W.-Q., Qiao, J.-T., Skolnick, M., Dafny, M., 1991. Focal dorsal raphe stimulation and pinnal electrical stimulation modulate spontaneous and noxious evoked responses in thalamic neurons. Int. J. Neurosci. 57, 123–140.

Dupouy, V., 1995. Neuroanatomie Fonctionnelle du Neuropeptide FF, Relation avec les Systèmes Opioïdes. Université Paul Sabatier, Toulouse III.

Dupouy, V., Zajac, J.-M., 1995. Effects of neuropeptide FF analogs on morphine analgesia in the nucleus raphe dorsalis. Regul. Pept. 59, 349–356.

Dupouy, V., Zajac, J.-M., 1996. Neuropeptide FF receptors in rat brain: A quantitative light microscopic autoradiographic study using [^{125}I] [D.Tyr^{1}, (NMe)Phe^{3}] NPFF. Synapse 24, 282–296.

Dupouy, V., Puget, A., Eschalier, A., Zajac, J.-M., 1996. Species differences in the localization of neuropeptide FF receptors in rodents and lagomorph brain and spinal cord. Peptides 17, 399–405.

Fardin, V., Oliveras, J.-L., Besson, J.-M., 1984. A reinvestigation of the analgesic effects induced by stimulation of the periaqueductal gray matter in the rat. I. The production of behavioral side effects together with analgesia. Brain Res. 306, 105–123.

Field, H.L., Heinricher, M.M., Mason, P., 1991. Neurotransmitters in nociceptive modulatory circuits. Annu. Rev. Neurosci. 14, 219–245.

Gicquel, S., Mazarguil, H., Allard, M., Simonnet, G., Zajac, J.-M., 1992. Analogues of F8Famide resistant to degradation, with high affinity and in vivo effects. Eur. J. Pharmacol. 222, 61–67.

Gicquel, S., Fioramonti, J., Bueno, L., Zajac, J.-M., 1993. Effects of F8Famide analogs on intestinal transit in mice. Peptides 14, 749–753.

Gicquel, S., Mazarguil, H., Desprat, C., Allard, M., Devillers, J.-P., Simonnet, G., Zajac, J.-M., 1994. Structure-activity study of Neuropeptide FF: Contribution of N-terminal regions to affinity and activity. J. Med. Chem. 37, 3477–3481.

Gouardères, C., Sutak, M., Zajac, J.-M., Jhamandas, K., 1993. Antinociceptive effects of intrathecally administered F8Famide and FMRFamide in the rat. Eur. J. Pharmacol. 237, 73–81.

Gouardères, C., Jhamandas, K., Sutak, M., Zajac, J.-M., 1996. Role of opioid receptors in the spinal antinociceptive effects of neuropeptide FF analogues. Br. J. Pharmacol. 117, 493–501.

Kavaliers, M., Yang, H.-Y.T., 1989. IgG from antiserum against endogenous mammalian FMRF-NH2-related peptides augments morphine- and stress-induced analgesia in mice. Peptides 10, 741–745.

Kivipelto, L., Majane, E.A., Yang, H.-Y.T., Panula, P., 1989. Immunohistochemical distribution and partial characterization of FLFQPQRFamide-like peptides in the central nervous system of rats. J. Comp. Neurol. 286, 269–287.

Kuraishi, Y., Harada, Y., Aratani, S., Satoh, M., Takagi, H., 1983. Separate involvement of the spinal noradrenergic and serotoninergic systems in morphine analgesia: The differences in mechanical and thermal algesic tests. Brain Res. 273, 245–252.

Lake, J.R., Hammond, M.V., Shaddox, R.C., Hunsicker, L.M., Yang, H.-Y.T., Malin, D.H., 1991. IgG from neuropeptide FF antiserum reverses morphine tolerance in the rat. Neurosci. Lett. 132, 29–32.

Majane, E.A., Casanova, M.F., Yang, H.-Y.T., 1988. Biochemical characterization of FMRFNH2-like peptide in spinal cord of various mammalian species using specific radioimmunoassays. Peptides 9, 1137–1144.

Malin, D.H., Lake, J.R., Fowler, D.E., Hammond, M.V., Brown, S.L., Leyva, J.E., Prasco, P.E., Dougherty, T.M., 1990a. FMRF-NH2-like mammalian peptide precipitates opiate-withdrawal syndrome in the rat. Peptides 11, 277–280.

Malin, D.H., Lake, J.R., Hammond, M.V., Fowler, D.E., Rogillio, R.B., Brown, S.L., Sims, J.L., Leecraft, B.M., Yang, H.-Y.T., 1990b. FMRF-NH2-like mammalian octapeptide: Possible role in opiate dependence and abstinence. Peptides 11, 969–972.

McCluny, R.E., Dafny, N., 1980. The parafascicular nucleus of the thalamus exhibits convergence input from the dorsal raphe and the spinal tract of the trigeminal nerve. Brain Res. 197, 525–531.

Millon, M., Fioramonti, J., Gicquel, S., Zajac, J.-M., Bueno, L., 1993. Comparative action of Phe–Leu–Phe–Gln–Pro–Gln–Arg–Phe–NH2 analogs on intestinal motility and nociception in rats. J. Pharm. Exp. Ther. 265, 96–102.

Moore, R.Y., Halaris, A.E., Jones, B.E., 1978. Serotonin neurons of the midbrain raphe: Ascending projections. J. Comp. Neurol. 180, 417–438.

Morgan, M.M., Sohn, J.-H., Liebeskind, C., 1989. Stimulation of the periaqueductal gray matter inhibits nociception at the supraspinal as well as spinal level. Brain Res. 502, 61–66.

Nishikawa, T., Scatton, B., 1983. Evidence for a GABAergic inhibitory influence on serotonergic neurons originating from the dorsal raphe. Brain Res. 279, 325–329.

Oberling, P., Stinus, L., Le Moal, M., Simonnet, G., 1993. Biphasic effect on nociception and antiopiate activity of Neuropeptide FF (FLFQPQRFamide) in the rat. Peptides 14, 919–924.

Panula, P., Kivipelto, L., Nieminen, O., Majane, E.A., Yang, H.-Y.T., 1987. Neuroanatomy of morphine-modulating peptides. Med. Biol. 65, 127–135.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates. Academic Press Australia, Sydney.

Peshanski, M., Guilbaud, G.G., Gautron, M., 1981. Posterior intralaminar nuclei in the rat neuronal responses to noxious and non-noxious cutaneous stimuli. Exp. Neurol. 72, 226–238.

Qiao, J.T., Dafny, N., 1988. Dorsal raphe stimulation modulates nociceptive responses in thalamic parafascicular neurons via an ascending pathway. Pain 34, 65–74.

Rebeyrolles, S., Zajac, J.-M., Roumy, M., 1996. Neuropeptide FF reverses μ-opioid effect on $Ca2+$ channels in rat spinal ganglion neurons. NeuroReport 7, 2979–2981.

Reyes-Vazquez, C., Qiao, J.-T., Dafny, N., 1989. Nociceptive responses in nucleus parafascicularis thalami are modulated by dorsal raphe stimulation and microiontophoretic application of morphine and serotonin. Brain Res. Bull. 23, 405–411.

Roumy, M., Zajac, J.-M., 1996. Effects of Neuropeptide FF on intracellular calcium in mouse spinal ganglion neurones. Eur. J. Pharmacol. 306, 291–295.

Sakata, S., Shima, F., Kato, M., Fukui, M., 1988. Effects of thalamic parafascicular stimulation on the periaqueductal gray and adjacent reticular formation neurons. A possible contribution to pain control mechanisms. Brain Res. 451, 85–96.

Sanders, K.H., Klein, C.E., Mayer, T.E., Heym, C., Handwerker, H.O., 1980. Differential effects of noxious and nonnoxious imput on neurones according to location in ventral periaqueductal grey or dorsal raphe nucleus. Brain Res. 186, 83–97.

Sharpe, L.G., Garnett, J.E., Cicero, T.J., 1974. Analgesia and hyperreactivity produced by intracranial microinjections of morphine into the periaqueductal gray matter of the rat. Behav. Biol. 11, 303–313.

Stinus, L., Allard, M., Gold, L., Simonnet, G., 1995. Change in CNS Neuropeptide FF-like material, pain sensitivity, and opiate dependence following chronic morphine treatment. Peptides 16, 1235–1241.

Tang, J., Yang, H.-Y.T., Costa, E., 1984. Inhibition of spontaneous and opiate-modified nociception by an endogenous neuropeptide with Phe–Met–Arg–Phe–NH2-like immunoreactivity. Proc. Natl. Acad. Sci. 81, 5002–5005.

Wang, Q.-P., Nakai, Y., 1993. Enkephalinergic innervation of GABAergic neurons in the dorsal raphe nucleus of the rat. Brain Res. Bull. 32, 315–320.

Wang, Q.P., Nakai, Y., 1994. The dorsal raphe: An important nucleus in pain modulation. Brain Res. Bull. 34, 575–585.

Wang, Q.-P., Ochiai, H., Nakai, Y., 1992. GABAergic innervation of serotonergic neurons in the dorsal raphe nucleus of the rat studied by electron microscopy double immunostaining. Brain Res. Bull. 29, 943–948.

Yang, H.-Y.T., Fratta, W., Majane, E.A., Costa, E., 1985. Isolation, sequencing, synthesis, and pharmacological characterization of the two brain neuropeptides that modulate the action of morphine. Proc. Natl. Acad. Sci. 82, 7757–7761.

Zhu, J., Jhamandas, K., Yang, H.-Y.T., 1992. Release of neuropeptide FF ($FLFQPQRF\text{-}NH_2$) from rat spinal cord. Brain Res. 592, 326–332.