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Role of opioid receptors in the spinal antinociceptive effects of neuropeptide FF analogues

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1 Neuropeptide FF (NPFF) has been shown to produce antinociceptive effects and enhance morphine-induced antinociception after intrathecal (i.t.) injection. In this study, the spinal effects of two NPFF analogues, [D-Tyr[1],(NMe)Phe[3]]NPFF (1DMe) and [D-Tyr[1],D-Leu[2],D-Phe[3]]NPFF (3D), which are resistant to degradation and exhibit a high affinity for NPFF binding sites, were examined in tests of thermal and mechanical nociception.

2 1DMe and 3D produced potent dose-dependent spinal antinociception in the tail-flick test. On a molar basis, 1DMe was 20 and 50 times more potent than 3D and morphine, respectively, and high doses of 1DMe and 3D produced a sustained antinociceptive effect without visible signs of motor impairment.

3 Spinal antinociceptive effects produced by 1DMe (0.86 nmol) or 3D (8.6 nmol) were significantly reduced by i.t. co-administration of naloxone (11 nmol) or i.t. pre-administration of D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP, 9.25 nmol) or β-funaltrexamine (β-FNA, 2 nmol) or naltrindole (2.2 nmol). The doses of the μ-antagonists (CTOP and β-FNA) or the δ-antagonist (naltrindole) used in 1DMe and 3D experiments blocked the antinociceptive effects of μ-or δ-receptor-selective agonists.

4 When administered in combination with antinociceptive doses of the μ-receptor agonist, morphine (13.2 nmol) or the δ-receptor agonist, [D-Ala[2]]deltorphin I (20 nmol), sub-effective dose of 1DMe or 3D (0.009 nmol) enhanced and prolonged the spinal effects of these opioid agonists.

5 The results of this study show that spinal μ-and δ-opioid receptors play a role in antinociception produced by NPFF analogues. These results also suggest a role for NPFF in modulation of nociceptive signals at the spinal level.

Keywords: Neuropeptide FF; μ- and δ-opioids; nociception; intrathecal; rat spinal cord

Introduction

Neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2, NPFF) was first isolated from bovine brain and shown to antagonize morphine-induced supraspinal antinociception (Yang *et al.*, 1985). NPFF is widely distributed in the rat central nervous system (Majane *et al.*, 1989; Kivipelto *et al.*, 1989; 1991; Kivipelto & Panula, 1991a, b; Allard *et al.*, 1991) and in porcine spinal cord (Wasowicz & Panula, 1994). This neuropeptide binds with high affinity to specific sites (Allard *et al.*, 1989; Payza *et al.*, 1993; Devillers *et al.*, 1994) that are localized in the superficial laminae of the rat (Allard *et al.*, 1992), mouse (Desprat & Zajac, 1994) and human spinal cord (Allard *et al.*, 1994).

In rodents, NPFF exerts a modulatory action on a variety of behavioural and physiological responses (Roth *et al.*, 1987; Guzman *et al.*, 1989; Magnuson *et al.*, 1990; Sullivan *et al.*, 1991; Kavaliers & Colwell, 1993; Demichel *et al.*, 1993). Pharmacological studies have shown that intracerebroventricular (i.c.v.) administration of NPFF attenuates morphine-induced analgesia in rats (Yang *et al.*, 1985; Million *et al.*, 1993; Oberling *et al.*, 1993) and mice (Kavaliers, 1990; Gicquel *et al.*, 1992; Desprat & Zajac, 1994). Injection of IgG directed against NPFF potentiates analgesia induced by morphine or stress (Kavaliers & Yang, 1989). Furthermore, NPFF has been implicated in opioid tolerance and dependence (Malin *et al.*, 1990). These findings have led to the suggestion that NPFF may function as an endogenous anti-opioid agent (Yang *et al.*, 1985; Malin *et al.*, 1991; 1993; Lake *et al.*, 1992; Rothman, 1992).

However, in addition to its potential anti-opioid actions, NPFF has also been shown to possess pro-opioid effects. Like morphine, NPFF decreased colonic bead expulsion in mice (Raffa & Jacoby, 1989; 1990). In rats, intrathecal NPFF injection elicited a long-lasting spinal antinociception that was partly attenuated by naloxone (Gouardères *et al.*, 1993c).

Recently, two analogues of NPFF, [D-Tyr[1], (NMe) Phe[3]] NPFF (1DMe) and [D-Tyr[1], D-Leu[2],D-Phe[3]]NPFF (3D) which are partially protected against enzymatic breakdown, especially against aminopeptidase activity (Gicquel *et al.*, 1992), were reported both to inhibit as well as mimic the action of opioids after i.c.v. injection in mice (Gicquel *et al.*, 1992; 1993; Desprat & Zajac, 1994) or in rats (Million *et al.*, 1993). Both 1DMe and 3D were shown to bind with a high affinity to rat spinal cord NPFF binding sites (Gicquel *et al.*, 1992; Devillers *et al.*, 1994). Therefore, to gain additional insight into the role of NPFF in spinal nociception, we have investigated the effects of intrathecally administered NPFF analogues, 1DMe and 3D, using the tail-flick and paw-pressure tests. The interaction of these peptides with μ-(morphine) and δ-([D-Ala[2]]deltorphin I or DPDPE) opioid agonists, and with μ-(D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) or β-funaltrexamine (β-FNA)) and δ-(naltrindole) opioid antagonists have been examined to determine the relative role of spinal opioid receptor types in the antinociception elicited by spinally administered peptides.

Methods

Intrathecal cannulation and drug injection

Male Sprague-Dawley rats (250-300 g, Charles River Inc., St. Constant, Que., Canada) were anaesthetized with halothane

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and intrathecal polyethylene catheters (PE 10, 7.5–8 cm length) were inserted through the cisterna magna to lie at the rostral edge of the lumbar enlargement (Yaksh & Rudy, 1976). The catheters were then flushed with 10 μl of physiological saline (0.9% NaCl). Following surgery, the animals were allowed to recover and housed in individual cages for a four-day period. Animals showing neurological deficits (flexion of hindlimbs, rigidity or paralysis) were excluded from experiments.

Agents injected intrathecally alone or in combination with another agent were dissolved in saline to deliver the dose under study in a 10 μl volume. The injections were made through the indwelling intrathecal catheter via a 50 μl Hamilton syringe. Each drug injection was followed by an additional injection of 10 μl of saline to flush the catheter. Control animals received 10 μl intrathecal saline followed by a flush of 10 μl saline. In the antagonism experiments, the antagonist under study was administered intrathecally in 8 μl volume followed by 8 μl saline flush 15 min or 24 h (β-FNA) prior to the agonist. The latter was delivered in an 8 μl volume followed by a flush with 8 μl saline. Other animals received intrathecal injections by the same procedure: 8 μl saline + 8 μl saline flush 15 min or 24 h prior to 8 μl saline followed by 8 μl saline flush to determine the effects of multiple injections on the nociceptive responses. Saline injections did not influence these responses.

Nociceptive assays

The nociceptive responses were assessed by the tail-flick (D'Amour & Smith, 1941) and paw-pressure (Loomis *et al.*, 1987) tests. In the tail-flick test, the baseline latency was set at 2–3 s and the cut-off time was set at 10 s to minimize tissue damage (Loomis *et al.*, 1985). In the paw-pressure test, mechanical pressure was applied with a pressurized syringe to the upper surface of the hind paw until a withdrawal response was elicited. The pressure producing a withdrawal response was 90–110 mmHg, and the maximum pressure applied (cut-off) was 300 mmHg. The rats were handled and tested in groups of six in both tests and each animal was used once only. The nociception tests were used in tandem, with the tail-flick test preceding the paw-pressure test. The effects of peptides were evaluated at 10 min intervals for the first 60 min and every 30 min thereafter up to 180 min. Additionally, the tail-flick and paw-pressure responses were assessed 24 and 48 h or longer after the intrathecal injection to determine the reversibility of the observed effects. All experiments were performed in the morning between 09 h 00 min–12 h 00 min.

Following the end of nociceptive tests, the placement of catheters in the lumbar space was confirmed by intrathecal dye injection. Animals in which catheters were not located in the lumbar region were excluded from the study.

Data analysis

All data of response latency(s) or threshold response pressure (mmHg) were converted to maximum percentage effect (MPE) calculated as:

MPE = 100 × [(post-drug response − baseline response)/(cut-off response − baseline response)].

The results of experiments are presented as the entire time-course of the MPE.

To determine the dose-response curves for the intrathecal effects of drugs, the area under the curve (AUC), depicting total MPE vs. time, was computed by trapezoidal approximation over the period 10–180 min and was expressed as a percentage of the maximum possible AUC that could be obtained if the animal displayed a 100% MPE at each time point in the experimental period.

Data were analyzed by one-way analysis of variance (ANOVA). *Post-hoc* comparisons were made with Dunnett's *t* test or Newman-Keuls test for multiple comparisons. The level of significance was set at $P<0.05$.

Chemicals

1DMe ([D-Tyr^1,(NMe)Phe^3]NPFF) and 3D ([D-Tyr^1, D-Leu^2,D-Phe^3]NPFF) were synthesized by solid phase methods as described by Gicquel *et al.* (1992). [D-Ala^2]deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) was synthesized as described previously (Dupin *et al.*, 1991). CTOP hydrochloride (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) was purchased from Peninsula Laboratories. Naloxone hydrochloride was obtained from Endo Laboratories (U.S.A.) and morphine sulphate from BDH Pharmaceuticals (Canada). β-FNA (β-funaltrexamine), DPDPE ([D-Pen^2,D-Pen^5]enkephalin) and naltrindole were obtained from RBI (France).

Results

Behavioural effects of peptides

The intrathecal (i.t.) injection of 1DMe (0.009–6.4 nmol) or 3D (0.009–25.8 nmol) had no apparent effects on the general behaviour of the rat or on normal motor functions as assessed by the methods of Faden & Jacobs (1984) and Rivlin & Tator (1977) (data not shown). Intrathecal doses of 1DMe higher than 6.4 nmol tended to produce an impairment of hindlimb function in some animals, and therefore such doses were not included in this study. The i.t. injection of morphine (6.6–52.8 nmol) and [D-Ala^2]deltorphin I (5–20 nmol) did not induce any detectable effect on righting reflexes or hind-paw placing and stepping reflexes. However, the highest dose of [D-Ala^2]deltorphin I (40 nmol) produced suppression of spontaneous activity in animals and was excluded from the study.

Antinociceptive effects of peptides

The comparative effects of different doses of intrathecally administered 1DMe and 3D in the tail-flick and paw-pressure tests are illustrated in Figure 1. Spinal antinociceptive effects of morphine, a μ-agonist, and [D-Ala^2]deltorphin I, a δ-agonist, are shown in Figure 2. Both NPFF analogues produced long lasting antinociceptive effects (Figure 1). The effect of 1DMe in both nociception tests was characterized by a rapid onset (10–20 min) and was long lasting, 30–90% effects in the tail-flick test being still observable at 180 min post-injection (Figure 1a). The increase of tail-flick latency peaked at 30–40 min for 3D and declined progressively thereafter (Figure 1c). Both μ- and δ-opioid agonists produced similar effects (Figure 2). However, the 25.8 nmol dose of 3D produced significant antinociception 24 h after the i.t. injection, while the maximal doses of the two opioid agonists were ineffective at this time period (Table 1). In both nociception tests, the effect of the highest dose of 1DMe (6.4 nmol) was still apparent 24 and 48 h post-injection (Table 1). The elevated latencies or thresholds induced by both NPFF analogues returned to baseline levels between 24 and 72 h and none of the animals showed visible motor deficits.

Both 1DMe and 3D produced dose-dependent increases in the tail-flick antinociceptive effect expressed as % area under the curve (% AUC) during the 10 to 180 min period following the i.t. injection with ED_{50} values approximating to 1 and 20 nmol, respectively (data not shown). In contrast to the marked effects seen in the tail-flick test, the magnitude of effects of the two NPFF analogues was lower in the paw-pressure test, as evinced by shallow dose-response curves. Morphine was equipotent in both tests with a value for ED_{50} of approximately 50 nmol. [D-Ala^2]deltorphin I displayed antinociceptive activity in both tests, but as the highest dose of the δ-agonist was limited to 20 nmol, since higher doses tended to suppress motor activity, the value of apparent ED_{50} was not derived.

Effects of naloxone on the spinal antinociceptive action of NPFF analogues and morphine

The effect of intrathecal naloxone (11 nmol) on the spinal effects of 1DMe, 3D and morphine, at doses producing comparable antinociceptive responses in the two nociceptive tests, are shown in Figure 3. Intrathecal naloxone alone had no effect on tail-flick latency or paw-withdrawal response (Figure 3a and b). However, naloxone administration significantly attenuated the effect of 1DMe in both tests during the time period 10–60 min (Figure 3a and b). Naloxone also attenuated the antinociceptive response to 3D but this was evident only in the tail-flick test (Figure 3c). The dose of intrathecal naloxone used here significantly reduced the antinociceptive action of morphine in both tests (Figure 3e and f).

Effects of CTOP and β-FNA on the spinal antinociceptive action of NPFF analogues and morphine

As shown in Figure 4 (left panel), CTOP (9.25 nmol) injected intrathecally 15 min prior to 1DMe (Figure 4a) or 3D (Figure 4c) or morphine (Figure 4e) significantly attenuated the first phase (10–60 min) of the thermal antinociceptive responses produced by these compounds. The antinociceptive responses produced by 1DMe or 3D 60–180 min post-injection, however, were significantly higher than those obtained with the peptides in the absence of CTOP (Figure 4a and c). This rebound response was not seen in similar experiments with morphine (Figure 4e). Thus, CTOP treatment enhanced the second phase of antinociception produced by the two peptides but not morphine in the tail-flick test. As illustrated in Figure 4 (right panel), CTOP pretreatment (9.25 nmol) attenuated the antinociceptive effects elicited in the paw-pressure test by 1DMe (Figure 4b) during the first 60 min period after injection of the peptide. The subsequent response to 1DMe was enhanced in CTOP-treated animals (Figure 4b). The antagonistic effect of CTOP was not evident in experiments with 3D (Figure 4d) but it was apparent in experiments with morphine (Figure 4f).

As shown in Figure 5 (left panel), β-FNA (2 nmol), injected i.t. 24 h prior to the two NPFF analogues, effectively blocked both the early and delayed antinociceptive responses produced

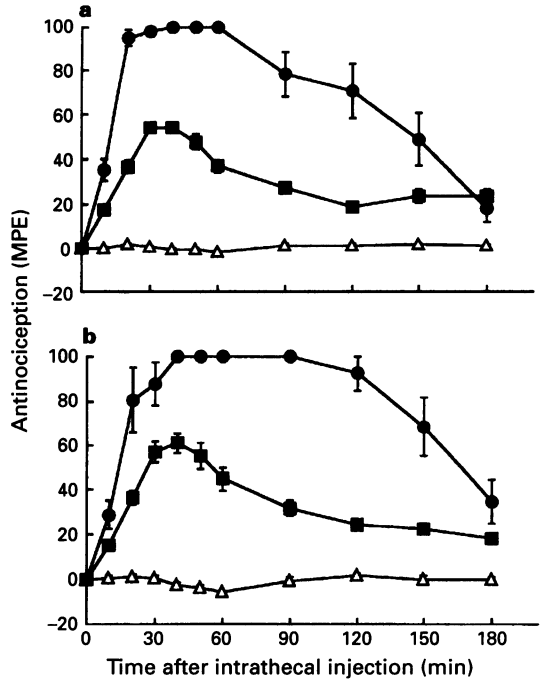

Figure 2 Time-course of the antinociceptive responses induced by intrathecally administered saline (△), 52.8 nmol morphine (●) and 20 nmol [D-Ala^2]deltorphin I (■) in the tail-flick test (a) and paw-pressure test (b). The doses used were the maximal doses that did not produce visible signs of motor disturbance in rats. Data are means ± s.e.mean of MPE from 6 to 15 rats.

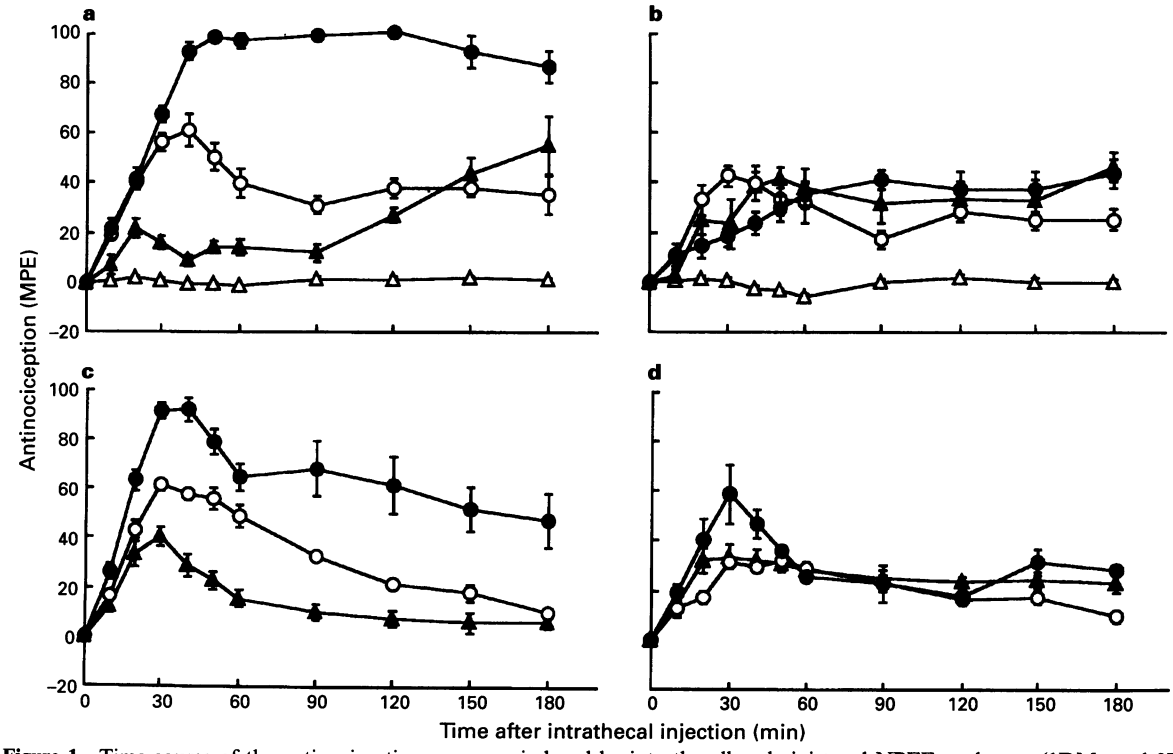

Figure 1 Time-course of the antinociceptive responses induced by intrathecally administered NPFF analogues (1DMe and 3D) in the tail-flick test (a, c) and paw-pressure test (b, d). Data are means ± s.e.mean of MPE from 6 to 14 rats. Saline (△); 1DMe (a, b): 0.086 nmol (▲), 0.86 nmol (○), 6.4 nmol (●); 3D (c, d): 4.3 nmol (▲), 8.6 nmol (○), 25.8 nmol (●). The absence of an error bar indicates that the value of the s.e.mean is smaller than the size of the symbol.

Table 1 Antinociceptive responses observed 24, 48 and 72 h after a single intrathecal injection of NPFF analogues and opioid agonists in the tail-flick (TF) and paw-pressure (PP) tests in rat

Treatment	24 h		48 h		72 h	
	TF	PP	TF	PP	TF	PP
Saline	0.6±0.6	1.2±1.3	0.6±0.6	1.3±1.0	1.2±1.1	1.4±0.8
1DMe (6.4 nmol)	48.4±7.2**	42.4±4.2**	23.2±3.3**	25.7±6.3**	7.5±1.4	7.0±1.9
3D (25.8 nmol)	14.6±2.0*	10.7±3.2*	5.6±1.4	3.6±1.9	2.6±1.7	1.8±0.8
Morphine (52.8 nmol)	1.2±0.5	1.1±0.9	0.4±0.3	1.1±0.6	0.3±0.4	0.4±0.3
[D-Ala^2]deltorphin I (20 nmol)	7.5±1.5	8.5±1.8	3.9±1.0	5.3±1.1	2.9±1.9	3.9±3.2

These doses were the highest used which did not induce any motor dysfunction.
Data are means±s.e.mean of MPE from at least 6 rats per treatment.
Differences are significantly different from saline. *$P<0.05$; **$P<0.01$.

by these peptides in the tail-flick test (Figure 5a and c). It also abolished the effect of morphine (Figure 5e). β-FNA treatment alone produced a small but significant antinociceptive action (MPE=20%) (Figure 5a). Interestingly, the dose of β-FNA (2 nmol) used here did not influence the antinociceptive effect of 40 nmol intrathecal DPDPE, a δ-selective opioid agonist (Figure 5g). In the paw-pressure test (Figure 5, right panel), β-FNA decreased the antinociceptive effects of both 1DMe (Figure 5b) and morphine (Figure 5f) during the 10–40 min period, but enhanced the effects induced by 3D during the same period (Figure 5d). In contrast, β-FNA failed to influence the antinociception induced by the selective δ-agonist DPDPE (Figure 5h).

Effects of naltrindole on the spinal antinociceptive action of NPFF analogues and DPDPE

The administration of the δ-receptor antagonist, naltrindole (2.2 nmol), injected intrathecally 15 min prior to 1DMe or 3D, significantly attenuated the antinociceptive responses produced by either peptide during the initial 60 min period in the tail-flick and paw-pressure tests (Figure 6a, b, c and d). However, in the following period, the combination of naltrindole with 1DMe or 3D produced greater antinociceptive responses than those produced by the peptides alone in either test (Figure 6a, b, c and d). The dose of naltrindole used in these experiments was effective in blocking the action of DPDPE (40 nmol) in both nociception tests (Figure 6e and f).

Action of 1DMe or 3D on morphine-induced antinociception

The effects of a sub-effective intrathecal dose of 1DMe or 3D on the spinal antinociceptive action of morphine in the tail-flick and paw-pressure tests are shown in Figure 7. In both tests, morphine (13.2 nmol) produced a monophasic response that peaked between 30–40 min and returned to baseline by 60–90 min. When morphine was injected along with a sub-effective dose of 1DMe (0.009 nmol), the action of morphine

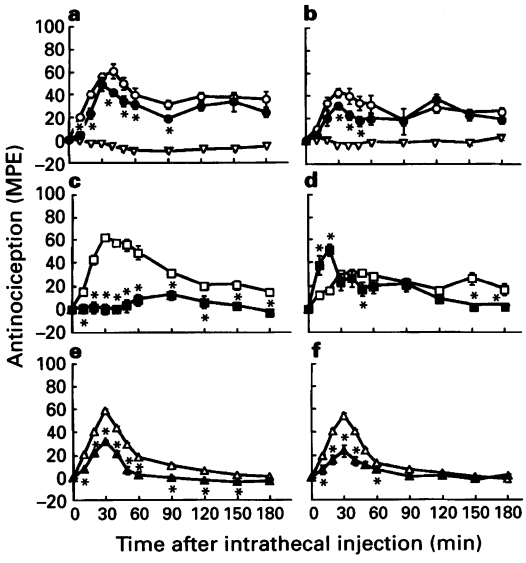

Figure 3 Effects of intrathecal naloxone (11 nmol) on the time-course of spinal antinociception induced by 1DMe (a, b), 3D (c, d) or morphine (e, f) in the tail-flick test (a, c, e) and paw-pressure test (b, d, f). 1DMe (0.86 nmol, ○, ●), 3D (8.6 nmol, □, ■) or morphine (13.2 nmol, △, ▲) were injected intrathecally alone (open symbols) or co-injected with naloxone (solid symbols). Data are means± s.e.mean of MPE from at least 6 rats. *Significant differences from the action of agonist alone ($P<0.05$). At these doses, no antinociceptive effect was observed 24 h after the spinal administration of agonists used alone or in combination with naloxone. Intrathecal naloxone alone (▽) did not influence baseline nociceptive responses. The absence of an error bar indicates that the value of the s.e.mean is smaller than the size of the symbol.

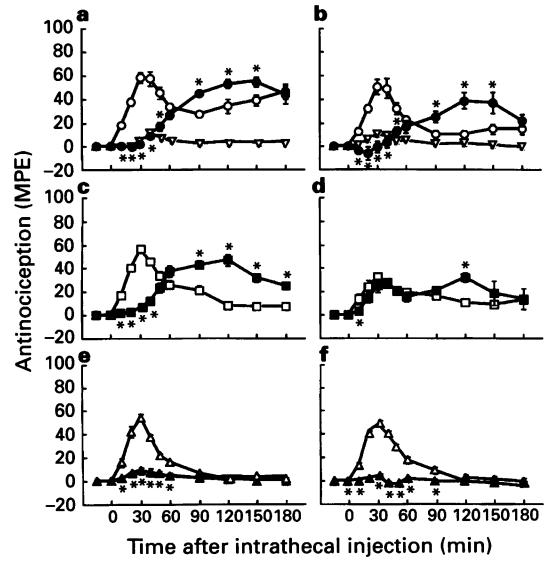

Figure 4 Effects of the μ-opioid receptor-selective antagonist CTOP on the time-course of spinal antinociception induced by 1DMe (a, b), 3D (c, d) or morphine (e, f) in the tail-flick test (a, c, e) and paw-pressure test (b, d, f). CTOP (9.25 nmol) was injected intrathecally alone (▽) or 15 min prior to 1DMe (0.86 nmol, ●), 3D (8.6 nmol, ■) or morphine (13.2 nmol, ▲). Separate animals were injected intrathecally with 1DMe (○), 3D (□) or morphine (△) alone. Data are means±s.e.mean of MPE from at least 6 rats. *Significant differences from the action of the agonist alone ($P<0.05$).

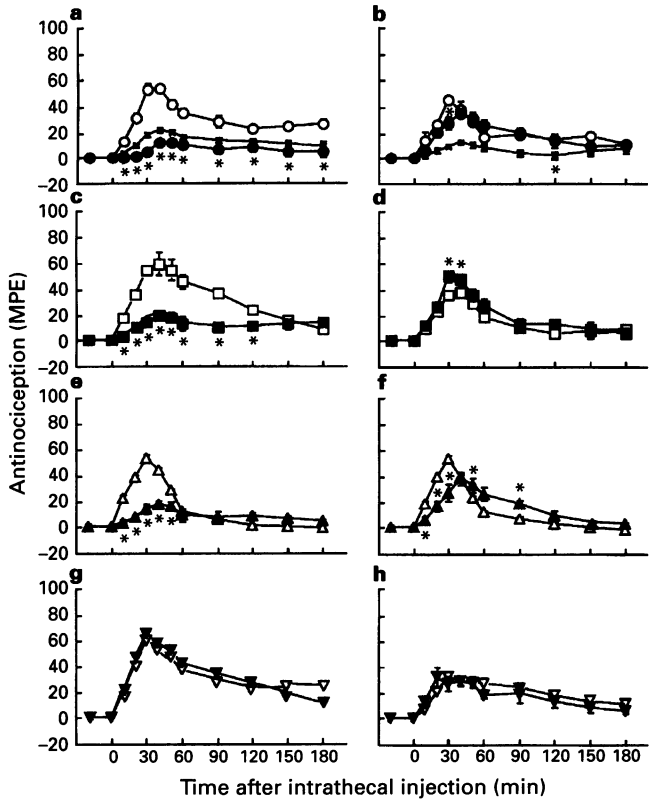

Figure 5 Effects of the μ-opioid receptor-selective antagonist β-FNA on the time-course of spinal antinociception induced by 1DMe (a, b), 3D (c, d), morphine (e, f) or DPDPE (g, h) in the tail-flick test (a, c, e, g) and paw-pressure test (b, d, f, h). β-FNA (2 nmol) was injected intrathecally alone (×) or 24 h prior to 1DMe (0.86 nmol, ●), 3D (8.6 nmol, ■), morphine (13.2 nmol, ▲) or DPDPE (40 nmol, ▼). Separate animals were injected intrathecally with 1DMe (○), 3D (□), morphine (△) or DPDPE (▽) alone. Data are means ± s.e.mean of MPE from at least 6 rats *Significant differences from the action of the agonist alone ($P<0.05$). The absence of an error bar indicates that the value of the s.e.mean is smaller than the size of the symbol.

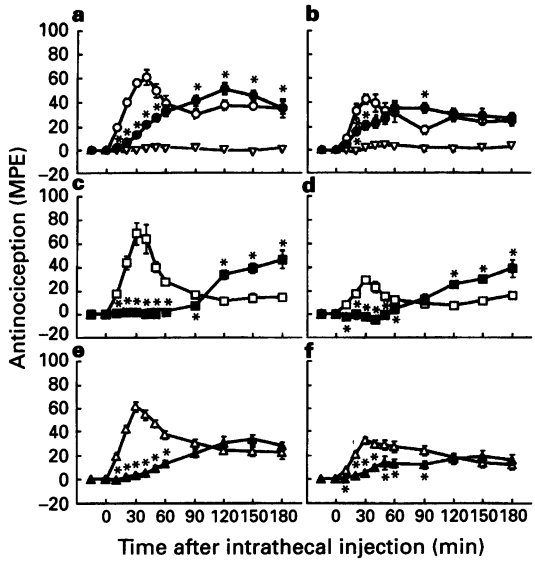

Figure 6 Effects of the δ-opioid receptor-selective antagonist, naltrindole, on the time-course of spinal antinociception induced by 1DMe (a, b), 3D (c, d) or DPDPE (e, f) in the tail-flick test (a, c, e) and paw-pressure test (b, d, f). Naltrindole (2.2 nmol) was injected intrathecally alone (▽) or 15 min prior to 1DMe (0.86 nmol, ●), 3D (8.6 nmol, ■) or DPDPE (40 nmol, ▲). Separate animals were injected intrathecally with 1DMe (○), 3D (□) or DPDPE (△) alone. Data are means ± s.e.mean of MPE from at least 6 rats. *Significant differences from the action of the agonist alone ($P<0.05$).

was attenuated during the first 20 min period but markedly enhanced and prolonged during the subsequent time period (40 – 180 min) (Figure 7a and b). Co-injection of morphine and a sub-effective dose of 3D (0.009 nmol) also resulted in a prolongation and enhancement of the effect of morphine in both tests in the same time period (Figure 7c and d). To determine if the actions of the combination of peptide and morphine were reversible, the antinociceptive actions were evaluated in both tests at 24 h post injection. The antinociceptive responses (MPE) of the 1DMe or 3D and morphine combination 24 h after injection was MPE = 0.1 ± 0.4 ($n=11$) and 3.6 ± 1.8 ($n=12$) (tail-flick test) and 3.3 ± 1.2 ($n=11$) and 5.2 ± 3.1 ($n=12$) (paw-pressure test), respectively. These responses were not significantly different from the baseline values in either test. Thus, both 1DMe and 3D reversibly enhanced the antinociceptive actions of morphine in the two tests.

Effects of 1DMe or 3D on [D-Ala²]deltorphin I-induced antinociception

[D-Ala²]deltorphin I (20 nmol, i.t.) produced a prolonged monophasic antinociceptive effect that reached a peak at 30 min and slowly returned to baseline in both tests (Figure 8). Intrathecal co-injection of a sub-effective dose (0.009 nmol) of 1DMe or 3D produced a marked and long lasting significant increase in the effect of [D-Ala²]deltorphin I in the tail-flick test over the 10 min to 48 h period for 1DMe (Figure 8a) and over the 40 min to 24 h period for 3D (Figure 8c). The responses returned to baseline values by 72 h post-injection for 1DMe (data not shown) and by 48 h for 3D (Figure 8c). In the paw-pressure test, 1DMe also enhanced the action of [D-Ala²]deltorphin I but the increase was not statistically significant (Figure 8b). Similarly, 3D also tended to enhance the antinociceptive responses produced by the δ-opioid agonist but this effect was significant only at 90 and 120 min after coinjection (Figure 8d).

Discussion

The results of the present study demonstrate that the intrathecal injection of 1DMe and 3D, two relatively peptidase-resistant NPFF analogues (Gicquel *et al.*, 1992), produces antinociception in the tail-flick and paw-pressure tests in rats. This confirms our previous observations showing that NPFF exerts an antinociceptive effect at the spinal level (Gouardères *et al.*, 1993c). Like NPFF, the spinal antinociceptive action of 1DMe and 3D was dose-related in the tail-flick but not the in paw-pressure test. This difference may be consistent with findings showing that different neuromodulatory systems participate in thermal and mechanical nociception (Kuraishi *et al.*, 1983; 1985a,b). Thus, neural mechanisms involved in thermal nociception may be more sensitive to the action of these peptides. Interestingly, on a molar basis, 1DMe appears to be 10 to 20 times more potent than both NPFF and 3D in the thermal antinociception test.

Like NPFF (Gouardères *et al.*, 1993c), its analogues produced sustained antinociception. This prolonged effect is unlikely to result from the action of intact peptide since NPFF analogues are degraded by mouse brain homogenates within 20 min in the absence of peptidase inhibitors (Gicquel *et al.*, 1992). Thus, their prolonged effect could be due to formation of active metabolites or other factors such as sustained release of endogenous opioids.

NPFF analogues, μ- and δ-opioids or their combination, at doses tested in the current study did not produce any visible signs of motor disturbances. The fact that NPFF analogues in

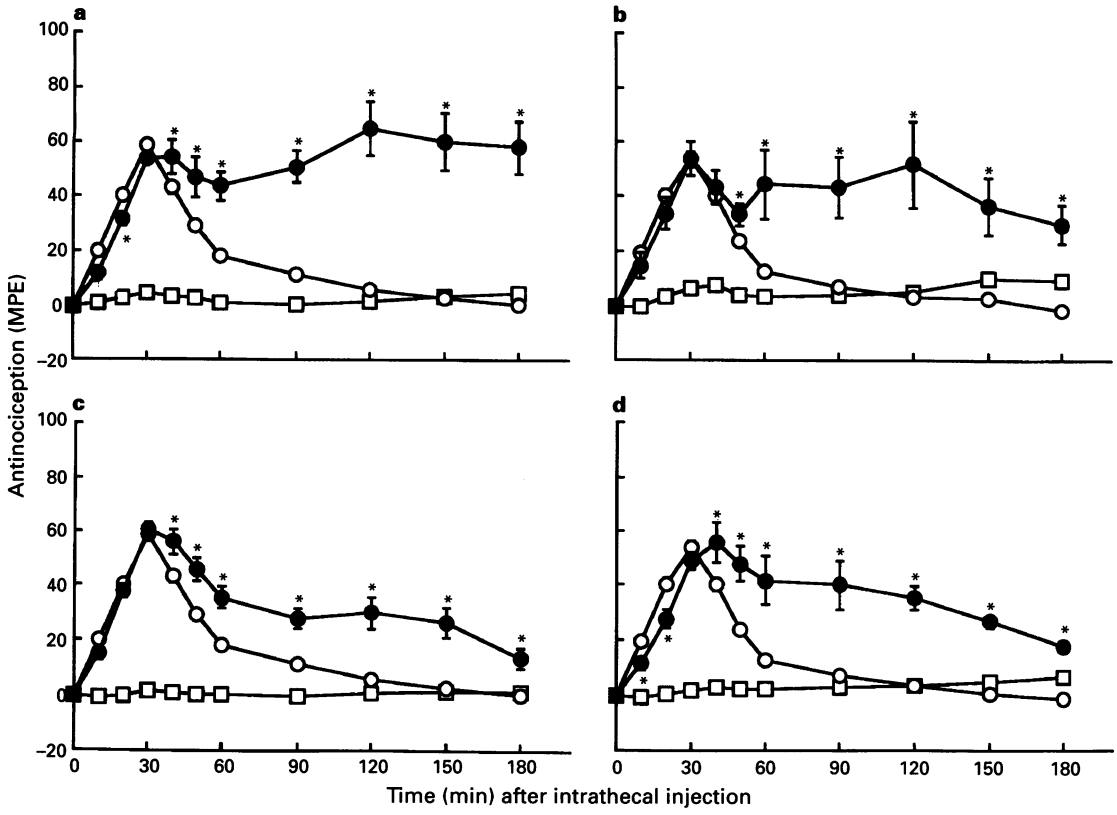

Figure 7 Effects of sub-effective doses of 1DMe (a, b) and 3D (c, d) on the time-course of the antinociception induced by spinal morphine in the tail-flick test (a, c) and paw-pressure test (b, d). A dose of 0.009 nmol 1DMe or 3D was co-injected intrathecally with 13.2 nmol morphine (●). Separate animals were injected intrathecally with the peptides (□) or the morphine (○) alone. Data are means ± s.e.mean of MPE from 11 to 21 animals. *Significant differences from the action of morphine alone ($P<0.05$). The responses from the combination returned to baseline values by 24 h post-injection without any signs of motor impairment.

spinal cord may be involved more in sensory rather than in motor function seems to be in accord with the finding that the content of endogenous NPFF in the dorsal spinal cord is much higher than that in the ventral cord (Majane *et al.*, 1989; Allard *et al.*, 1991). In addition, the dorsal cord contains a larger portion of the spinal NPFF binding sites (Allard *et al.*, 1992). A spinal site for the observed antinociceptive effects is suggested by results of previous studies in which i.c.v. administration of a similar concentration of peptides failed to induce an antinociceptive effect in mice or rats (Gicquel *et al.*, 1992; Million *et al.*, 1993; Desprat & Zajac, 1994; Dupouy & Zajac, 1995). Thus, the ability of 1DMe and 3D to produce antinociception following their intrathecal injection is not due to an effect at a supraspinal site following diffusion to that site. Additionally, diffusion of peptides from the spinal subarachnoid space to supraspinal sites is minimized by use of a small injection volume and by focusing drug application to caudal (lumbosacral) areas of the spinal cord. These considerations strongly suggest that the observed antinociceptive effect of intrathecal NPFF analogues results from a direct action at the spinal level. It is possible that sites targeted by intrathecal NPFF analogues form part of an endogenous mechanism modulating nociceptive transmission at the spinal level. The presence of a high density of NPFF immunoreactivity (Majane *et al.*, 1989; Allard *et al.*, 1991; Kivipelto *et al.*, 1991; Kivipelto & Panula, 1991a, b) as well as binding sites (Allard *et al.*, 1992; 1994; Devillers *et al.*, 1994), that are partly localized on terminals of the primary afferents (Gouardères *et al.*, 1993b), supports this notion. Furthermore, in cultured spinal neurones, NPFF has been found to produce both depolarizing and hyperpolarizing responses (Guzman *et al.*, 1989). NPFF was also reported to decrease the inhibitory action of a μ-opioid agonist (Magnuson *et al.*, 1990) or an α_2-adrenoceptor agonist (Sullivan *et al.*, 1991) on evoked C-fibre firing in the rat spinal cord. In addition, Zhu *et al.* (1992) have shown a release of NPFF from rat spinal cord following stimulation by potassium or substance P. These findings suggest that NPFF may be involved in modulation of nociception at the spinal level.

The spinal antinociceptive response produced by the two NPFF analogues was partially but significantly attenuated by intrathecal naloxone at a dose that was effective against intrathecal morphine. Naloxone was previously shown to attenuate spinal NPFF effects suggesting involvement of opioid systems in its action (Gouardères *et al.*, 1993c). In the rat spinal cord, NPFF receptors (Allard *et al.*, 1992; Devillers *et al.*, 1994) and opioid receptors (Gouardères *et al.*, 1985; 1991; 1993d) are similarly distributed. However, spinal NPFF binding sites exhibited a very low affinity for opioid agonists (Allard *et al.*, 1989; Devillers *et al.*, 1994; Gouardères *et al.*, unpublished results) and NPFF and its analogues do not bind to opioid receptors *in vitro* (Raffa *et al.*, 1994; Gouardères & Zajac, unpublished data). Therefore, the effect of naloxone on the antinociceptive effect of NPFF analogues suggests their indirect action in releasing endogenous opioid peptides in the spinal cord, the mechanism of which is not known.

Since naloxone can interact with μ-, δ- and κ-opioid receptors that are present in the rat spinal cord (Gouardères *et al.*, 1985; 1991; 1993d), the action of μ-receptor-selective (CTOP, β-FNA) and δ-receptor-selective (naltrindole) antagonists on the peptide-induced effects was evaluated. The μ-selective antagonists, CTOP (Gulya *et al.*, 1988) and β-FNA (Zimmerman *et al.*, 1987; Mjanger & Yaksh, 1991) pre-injected intrathecally partially attenuated the antinociceptive effects of 1DMe and 3D at doses that were ineffective alone but antagonized the actions of morphine. Indeed, the dose of β-FNA used here did not influence the antinociceptive effects of the opioid δ-agonist DPDPE (as was shown previously by Mjanger & Yaksh, 1991) suggesting that the action of β-FNA on antinociception induced by the two peptides involved interac-

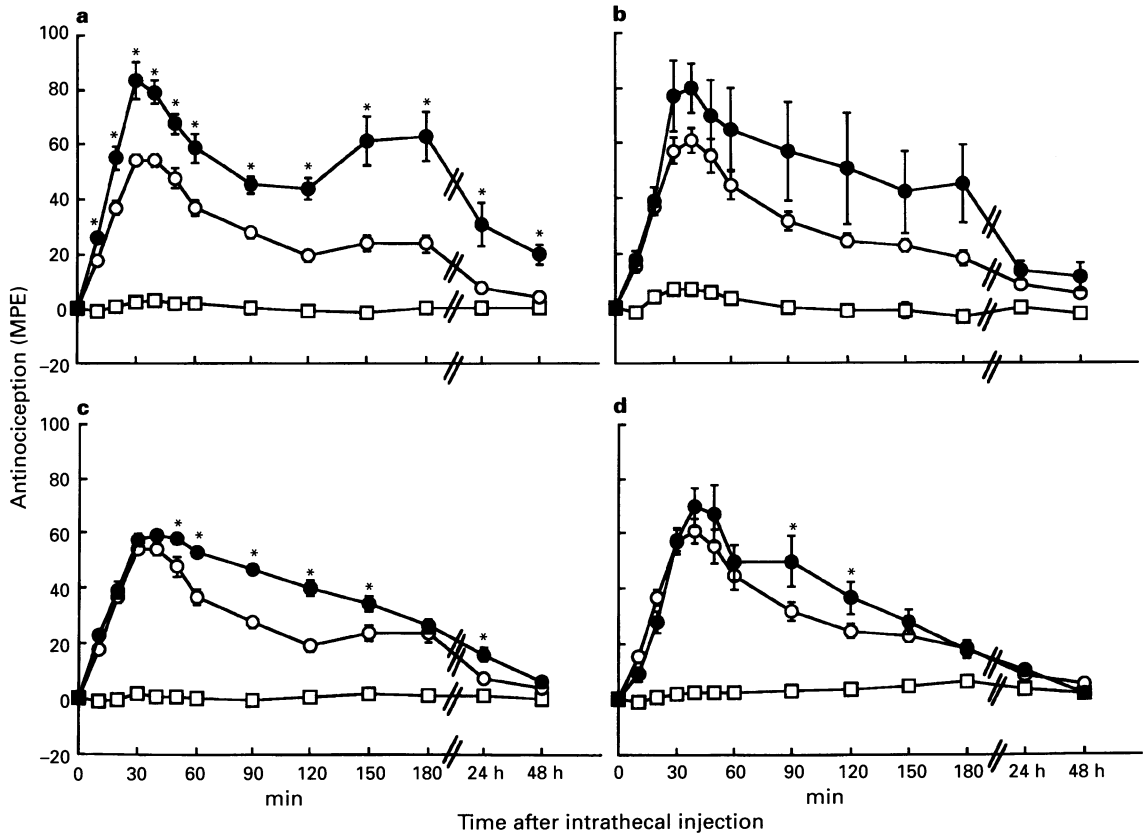

Figure 8 Effects of sub-effective doses of 1DMe (a, b) and 3D (c, d) on the time-course of the antinociception induced by spinal [D-Ala^2]deltorphin I in the tail-flick test (a, c) and paw-pressure test (b, d). A dose of 0.009 nmol 1DMe or 3D was co-injected intrathecally with 20 nmol [D-Ala^2]deltorphin I (●). Separate animals were injected intrathecally with the peptides (□) or the [D-Ala^2]deltorphin I (○) alone. Data are means ± s.e.mean of MPE from 5 to 15 animals. *Significant differences from the action of [D-Ala^2]deltorphin I alone ($P<0.05$). The responses from the combination returned to baseline values by 72 h post-injection without any signs of motor impairment.

tion with spinal μ-receptors. Similarly, the δ-selective antagonist, naltrindole (Portoghese *et al.*, 1988; Calcagnetti & Holtzman, 1991; Drower *et al.*, 1991; Tiseo & Yaksh, 1993) attenuated the spinal antinociceptive effects of NPFF analogues at a dose that blocked the action of DPDPE. This suggests that the naltrindole-sensitive antinociceptive effect of NPFF analogues involves interaction with δ-receptors via an indirect mechanism since NPFF-like agonists do not bind to δ-opioid receptors in rat spinal cord sections (Gouardères & Zajac, unpublished results). Unlike β-FNA, both CTOP and naltrindole affected only the first phase of the peptide-induced antinociceptive response. It is likely that these antagonists were not present in sufficient amounts to influence the second phase of response sensitive to β-FNA.

At a low dose (0.009 nmol) inactive on its own in antinociception tests, both NPFF analogues produced a remarkable enhancement of morphine antinociception in both tests, as was observed previously with NPFF (Gouardères *et al.*, 1993c). Similarly, co-injection of this sub-effective dose of 1DMe or 3D with [D-Ala^2]deltorphin I both enhanced and prolonged the effect of this δ-opioid agonist. The fact that NPFF-like drugs induced an increase of the spinal antinociceptive effects of morphine and [D-Ala^2]deltorphin I, opioid agonists that preferentially activate μ- and δ-opioid receptors, respectively, in the spinal cord (Tung & Yaksh, 1982; Omote *et al.*, 1990; 1991; Mattia *et al.*, 1992) suggests that one of the functions of endogenous NPFF in the spinal cord may be to modulate the action of endogenous opioids such as enkephalins or β-endorphin that normally interact with μ- and δ-receptors. The interactions of these peptides with endogenous opioids are under study.

The enhancement of morphine and [D-Ala^2]deltorphin I effects observed here in unanaesthetized animals contrasts with the antagonism by NPFF of the effects of DAMGO (Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol, a μ-opioid agonist) in an electrophysiological study of C-fibre driven spinal nociceptive neurones in anaesthetized rats (Magnuson *et al.*, 1990). However, in that study the activity of DSTBULET (Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr), a δ-opioid agonist, was not blocked by NPFF. In contrast, the results of the present study show that NPFF analogues can enhance the effects of a δ-receptor-selective agonist, [D-Ala^2]deltorphin I. The reason for this discrepancy is not clear but factors such as anaesthesia, nociceptive stimulus modality, peptide dose and period of observation may be contributing factors in differences between behavioural and electrophysiological experiments.

The interaction between NPFF analogues and morphine or [D-Ala^2]deltorphin I may have some practical implications. Since only a minimal activation of NPFF receptors and μ- or δ-receptors is required to produce a substantial antinociception, one would anticipate a reduction in the development of tolerance for analgesia when a combination of an NPFF analogue and an opioid agonist is administered.

The prolonged enhancement of μ- and δ-opioid antinociceptive action by agents previously thought to have opposite roles in the mediation of nociceptive processes is reminescent of the potentiation of morphine analgesia in the spinal cord by 10 to 100 pmol substance P (without any effect on tail-flick thresholds alone) (Kream *et al.*, 1993). Another interesting finding in our study is the sharp contrast between brain and spinal cord for NPFF/opioid interaction. While NPFF and NPFF analogues clearly enhanced morphine antinociception in the spinal cord (Gouardères *et al.*, 1993c; this study), they have been reported to inhibit morphine antinociception following brain injections (see Introduction). However, the doses used for combination studies in the brain are 250–2500 times higher than the dose of NPFF analogues used in the spinal study. Indeed, the amount of NPFF or

NPFF analogues required for the negative modulation of opioid action is relatively large, approximately 2–10 nmol in the rat (Million *et al.*, 1993; Dupouy & Zajac, 1995) and 9–22 nmol in the mouse (Gicquel *et al.*, 1992; Desprat & Zajac, 1994). These supraspinal doses were reported to have no antinociceptive effects when used alone. Their actions on nociception were seen only in combination with opioid agonists (anti-opioid action). However, in the spinal cord, these doses of NPFF analogues (this study) and NPFF (Gouardères *et al.*, 1993c) were clearly effective in producing antinociceptive effects.

NPFF analogues, as well NPFF, may influence supraspinal and spinal nociception by different mechanisms. In the brain, it is suggested that NPFF acts as an endogenous opioid antagonist. The evidence for this is largely provided by studies on morphine-dependent animals in which NPFF has a profile of activity that corresponds to that of an antagonist (Malin *et al.*, 1990; 1991; 1993). However, Rothman *et al.* (1993) have recently shown that chronic i.c.v. infusion of NPFF down-regulates μ-opioid binding sites in brain, an effect that is inconsistent with that of naloxone which has been shown to upregulate μ-receptors (Gouardères *et al.*, 1993a; Yoburn *et al.*, 1993). In the spinal cord, the nociception tests involving use of NPFF and its two analogues have yielded a profile of activity that suggests an agonist-like role, although electrophysiological studies have shown an antagonist-like action against μ- but not δ-agonists (Magnuson *et al.*, 1990). Thus, the role of NPFF-like peptides in spinal and supraspinal structures remains to be determined.

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