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Role of adenosine in the spinal antinociceptive and morphine modulatory actions of neuropeptide FF analogs

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Abstract

The neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2) and its synthetic analogs bind to specific receptors in the spinal cord to produce antinociceptive effects that are partially attenuated by opioid antagonists, and at sub-effective doses neuropeptide FF receptor agonists augment spinal opioid antinociception. Since adenosine plays an intermediary role in the production of spinal opioid antinociception, this study investigated whether this purine has a similar role in the expression of spinal effects produced by neuropeptide FF receptor agonists. In rats bearing indwelling spinal catheters, injection of adenosine receptor agonists, N6-cyclohexyladenosine (CHA, 1.72 nmol) and *N*-ethylcarboxiamidoadenosine (NECA, 1.95 nmol), as well as morphine (13.2 nmol) elicited antinociception in the tail-flick and paw-pressure tests. Pretreatment with intrathecal 8-phenyltheophylline (5.9 and 11.7 nmol), an adenosine receptor antagonist, blocked the effect of all three agents without influencing baseline responses. Administration of two synthetic neuropeptide FF (NPFF) analogs, [D-Tyr^1,(NMe)Phe^3]NPFF (1DMe, 0.86 nmol) and [D-Tyr^1,D-leu^2,D-Phe^3]NPFF (3D, 8.6 nmol) produced sustained thermal and mechanical antinociception. Pretreatment with doses of intrathecal 8-phenyltheophylline (5.9, 11.7 and 23.5 nmol), producing adenosine receptor blockade, significantly inhibited the antinociceptive effects of 1DMe or 3D. Injection of a sub-antinociceptive dose of 1DMe (0.009 nmol) significantly augmented the antinociceptive effect of intrathecal morphine (13.2 nmol) in the tail-flick and paw-pressure tests. Intrathecal 8-phenyltheophylline (11.7 nmol) reduced the effect of this combination. Administration of low dose of 1DMe (0.009 nmol) or 3D (0.009 nmol) very markedly potentiated the antinociceptive actions of the adenosine receptor agonist, N6-cyclohexyladenosine (0.43, 0.86 and 1.72 nmol) in the tail-flick and paw-pressure tests 50 min after injection. The results suggest that the antinociceptive and morphine modulatory effects resulting from activation of spinal NPFF receptors could be due to an increase in the actions or availability of adenosine.

Keywords: Neuropeptide FF; Morphine; Adenosine; Spinal cord; Antinociception

1. Introduction

In Yang et al. (1985) using antisera against the molluscan neuropeptide FMRFamide, isolated a mammalian octapeptide, neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2). On the basis of their ability to influence analgesia produced by opioid agonists such as morphine, neuropeptide FF and related peptides have been designated as opioid modulatory peptides. neuropeptide FF exerts its effects by interacting specifically with binding sites (Allard et al., 1989; Desprat and Zajac, 1994) localized to specific brain and spinal cord areas (Allard et al., 1992; Dupouy and Zajac, 1996; Dupouy et al., 1996). Synthetic analogs of neuropeptide FF showing greater resistance to metabolism have been developed (Gicquel et al., 1992) but currently neuropeptide FF antagonists are not available. The physiological role of neuropeptide FF in the nervous system is not clear but activity of neuropeptide FF receptors has been implicated in modulation of autonomic, somatic and endocrine functions and also in the control of pain transmission (see Panula et al., 1996; Roumy and Zajac, 1998)

Although the functional significance of neuropeptide FF remains to be determined, evidence from pharmacological studies, utilizing this peptide or synthetic analogs, suggests that this peptide may modulate transmission of nociceptive

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signals at the spinal cord level. Specific neuropeptide FF binding sites and peptide immunoreactivity are enriched in the dorsal horn (Majane et al., 1989; Allard et al., 1991, 1992, 1994; Kivipelto and Panula, 1991; Dupouy et al., 1996; Gouardères et al., 2000), a region that plays an important role in the processing of nociceptive signals. neuropeptide FF-like immunoreactivity is released from the isolated spinal cord in response to depolarizing stimuli, and this response shows dependency on the presence of external calcium ions (Zhu et al., 1992). A portion of the spinal neuropeptide FF binding site is localized to capsaicin-sensitive primary afferents, a neuronal population that is also known to transmit noxious input and to bear presynaptic opioid receptors (Gouardères et al., 1996a,b). However, an important population of the neuropeptide FF binding sites is located on spinal intrinsic neurons (Gouardères et al., 2000). Administered intrathecally at low doses lacking intrinsic activity, neuropeptide FF or its synthetic analogs can markedly enhance the antinociceptive effects produced by similar administration of mu or delta opioid receptor agonists (Gouardères et al., 1993, 1996a,b; Kontinen and Kalso, 1995). Administered alone, higher intrathecal doses of these peptides elicit sustained thermal and mechanical antinociception that is significantly reduced by treatment with intrathecal mu or delta opioid receptor antagonists (Gouardères et al., 1996a,b). A recent study showed that a synthetic neuropeptide FF analog provokes enkephalin release from the intact rat spinal cord (Ballet et al., 1999). These and other studies suggest that the activity of neuropeptide FF receptors influences pain transmission in the dorsal horn partly by promoting opioid activity.

The mechanisms by which neuropeptide FF and related peptides influence spinal nociception are poorly understood. However, considering the existence of an opioid link in their antinociceptive activity, and that adenosine mediates the antinociceptive action of spinal opioids (see below), it is likely that the effects of neuropeptide FF and its analogs are expressed through a purinergic mechanism. Previous neurochemical and pharmacological studies have provided strong experimental evidence (see Sawynok, 1998) favouring adenosine as a mediator of spinal opioid analgesia: activation of opioid receptors in the dorsal horn releases adenosine, spinal administration of adenosine receptor agonists produces antinociception, and pharmacological blockade of adenosine receptors effectively inhibits spinal opioid antinociception (Sawynok et al., 1986; Sweeney et al., 1989; DeLander and Keil, 1994). Thus, adenosine could contribute to antinociceptive effects resulting from activity of the spinal neuropeptide FF receptors. However, this possibility has not been examined previously. The present study thus investigated whether the intrinsic antinociception of higher doses and the opioid modulatory effects of lower doses of neuropeptide FF peptides at spinal sites involve a purinergic mechanism. This objective was addressed by examining the interaction of synthetic peptides that activate neuropeptide FF sites but have a greater metabolic stability (Gicquel et al., 1992) with agents that influence activity of adenosine receptors using a well established model of spinal analgesia.

2. Materials and methods

Experiments reported in this study were conducted in accordance with the guidelines of the Canadian Council on Animal Care using protocol approved by the University Animal Care Committee. All experiments were performed on male Sprague–Dawley rats (Charles River, St. Constant, Que, Canada) weighing between 250 and 300 g. Animals were given free access to food and water, and acclimatized to the environment 3 to 4 days before surgery. The room temperature was maintained at 21°C under a 12 h light/12 h dark cycle.

2.1. Intrathecal cannulation and drug injection

The animals were anaesthetised with halothane, placed in a stereotaxic frame, and the cisterna magna exposed for insertion of an intrathecal catheter. The overlying dura was punctured by a sharp needle tip and a polyethylene catheter (PE 10, 7.5–8.0 cm length) was inserted through the opening to rest at the rostral edge of the lumbar enlargement, as described previously by Yaksh and Rudy (1976). The catheter was then flushed with 10 μl 0.9% NaCl. The muscle and skin overlying the cisternal opening was closed and the catheter exteriorized to protrude through an opening in the skin on top of the skull. After surgery, the animals were allowed to recover for a 4-day period during which they were given a daily injection of 0.9% saline in addition to unrestricted access to food and water. Animals showing neurological deficits such as hindlimb or forelimb flexion, rigidity, paralysis or persistent allodynia were excluded from experiments.

Intrathecal injections of the agents under study were made using a 50-μl Hamilton syringe through the catheter in a 10-μl volume followed by a 10-μl flush with 0.9% saline. All drugs were freshly prepared and dissolved in saline. In antagonism experiments, the agent under study was administered in an 8-μl volume followed by an 8-μl flush given 15 min prior to injection of the agonist. The latter was delivered in an 8-μl injection volume and followed by an 8-μl saline flush. Injection of saline alone using this protocol of multiple injections in a previous study (Gouardères et al., 1996b) was found not to influence the baseline nociceptive responses.

2.2. Assessment of antinociception

Antinociceptive effects were evaluated using a modification of the tail-flick test (D'Amour and Smith, 1941) and paw-pressure test (Loomis et al., 1987). In the tail-flick test, the thermal stimulus was applied to the base of the tail

using an analgesic meter (Owen et al., 1981) and the time latency for the tail-flick response was recorded. The baseline latency was set at 2–3 s and the cut-off time was set at 10 s to prevent tissue damage. In the paw-pressure test, increasing level of pressure was applied to the upper surface of the hind paw using an inverted air-filled syringe connected to a pressure gauge (see Loomis et al., 1987). The pressure was gradually increased until a withdrawal response was elicited and the pressure value (mm Hg) recorded. In the majority of animals, the baseline pressure eliciting the response was 90–110 mm Hg and the maximal pressure (cut-off value) was set at 300 mm Hg. All animals undergoing antinociception testing were handled prior to the procedure and the tests were performed in tandem, with the tail-flick test preceding the paw-pressure test. The effects of drugs were evaluated every 10 min for the first 60-min period, and every 30 min thereafter for 180 min. In some experiments, the responses in both tests were monitored at 24 and 48 h to determine reversibility of the elicited drug effect. All experiments were performed between 9:00 a.m. and 1:00 p.m.

At the completion of experiments, the placement of the catheters in the lumbar region was confirmed by spinal exposure, and animals in which the catheters were located outside the lumbar region were excluded from the study. Generally, over 90% of animals exhibited correct placement of the intrathecal catheter.

2.3. Data analysis

The response latency (seconds) or threshold response pressure values (mm Hg) were converted to maximum percentage effect (MPE) to facilitate comparisons between the effects of different treatments:

$$\text{MPE} = 100[\text{post-drug response} - \text{baseline response}] / [\text{cut-off response} - \text{baseline response}]$$

Data were analysed by two-way analysis of variance (ANOVA) followed by post-hoc multiple comparisons made with Newman–Keuls or Dunnett's tests. The level of significance was set at $P < 0.05$.

2.4. Chemicals

1DMe([D-Tyr^1,(NMe)Phe^3]NPFF (1DMe) and [D-Tyr^1,D-leu^2,D-Phe^3]NPFF (3D) were synthesized by solid phase methodology as described by Gicquel et al. (1992). 8-phenyltheophylline, N6-cyclohexyladenosine (CHA), and

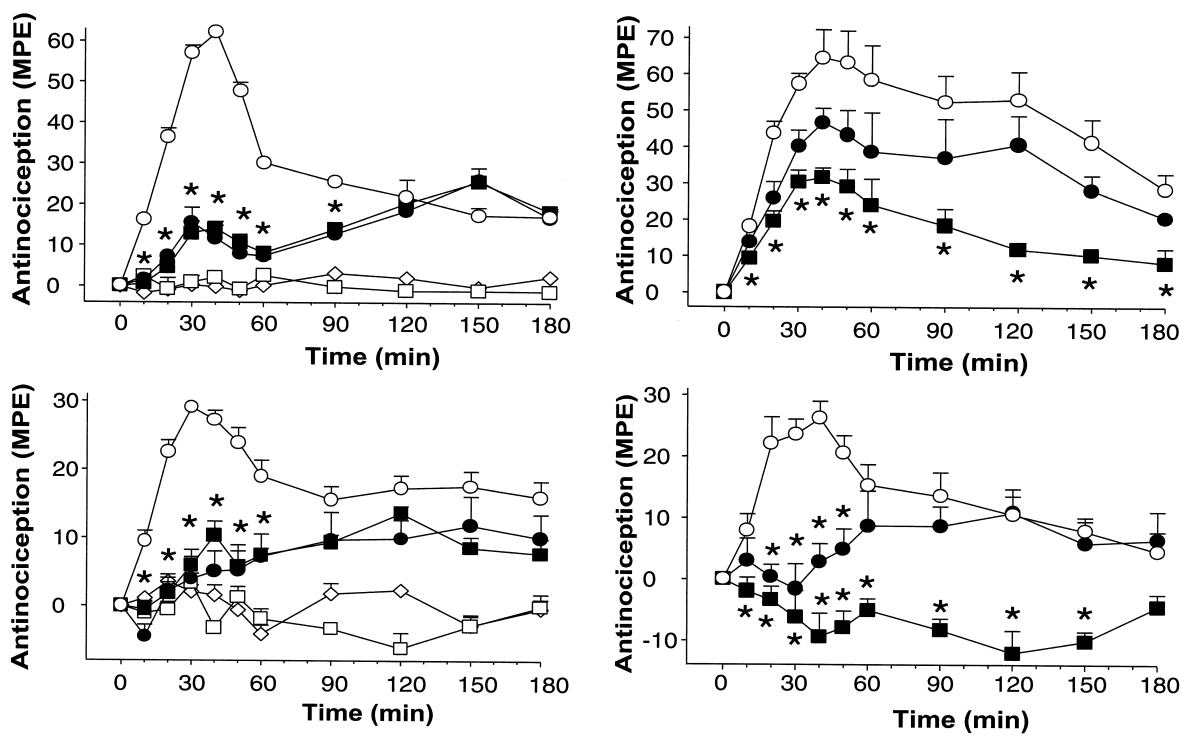

Fig. 1. Effects of the adenosine receptor antagonist 8-phenyltheophylline on the spinal antinociceptive action of the adenosine receptor agonists N6-cyclohexyladenosine (left side) and *N*-ethylcarboxiamidoadenosine (right side) in the tail-flick (upper panel) and paw-pressure (lower panel) tests. 8-phenyltheophylline doses (5.9 nmol, ◇, ●; 11.7 nmol, □, ■) were administered intrathecally alone (open symbols) or 15 min prior to the intrathecal injection of N6-cyclohexyladenosine (1.72 nmol) or *N*-ethylcarboxiamidoadenosine (1.95 nmol) (solid symbols). Separate animals were injected intrathecally with N6-cyclohexyladenosine or *N*-ethylcarboxiamidoadenosine alone (○). Data are means ± S.E.M. of MPE from 5 to 12 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.M. is smaller than the size of the symbol. All responses returned to baseline values by 24 h post injection. Intrathecal 8-phenyltheophylline alone did not influence baseline nociceptive responses.

N-ethylcarboxiamidoadenosine (NECA) were purchased from RBI Natick, MA, USA. Morphine sulfate was obtained from BDH Pharmaceuticals (Canada).

3. Results

The action of intrathecal 8-phenyltheophylline, an adenosine receptor antagonist, was examined on the spinal antinociceptive actions of two neuropeptide FF analogs, 1DMe and 3D. Antagonist dose was determined on the basis of its ability to inhibit the antinociception produced by the adenosine receptor agonists, N6-cyclohexyladenosine and *N*-ethylcarboxiamidoadenosine, and by morphine in the tail-flick and the paw-pressure tests.

3.1. Effects of 8-phenyltheophylline on spinal antinociception produced by adenosine agonists

Fig. 1 shows results of experiments involving the interaction of 8-phenyltheophylline with N6-cyclohexyladenosine and *N*-ethylcarboxiamidoadenosine. Intrathecal N6-cyclohexyladenosine (1.72 nmol) and *N*-ethylcarboxiamidoadenosine (1.95 nmol) produced submaximal antinociception of comparable magnitude in both tests. Both agonists produced an initial response that peaked at 30 min post injection and a delayed response, which was still observable at 180 min post-drug injection. Animals showed full recovery 24 h post injection (not shown). Injection of 8-phenyltheophylline (5.9 and 11.7 nmol) significantly reduced the peak effects of N6-cyclohexyladenosine in both the tail-flick and paw-pressure tests (Fig. 1). This agent also inhibited the effect of *N*-ethylcarboxiamidoadenosine in both tests. At the doses used, 8-phenyltheophylline (5.9, 11.7 nmol) itself did not significantly affect nociception in either test.

3.2. Effects of 8-phenyltheophylline on spinal antinociception produced by morphine

Fig. 2 shows effects of 8-phenyltheophylline on the action of spinal morphine in the tail-flick and paw-pressure tests. Administration of morphine (13.2 nmol) produced an antinociceptive response comparable in the size to that produced by the two adenosine agonists, especially in the tail-flick test. However, morphine produced a monophasic response, which peaked at 30 min and terminated at 90 min. An injection of 11.7 nmol 8-phenyltheophylline, which previously inhibited the effects of N6-cyclohexyladenosine and *N*-ethylcarboxiamidoadenosine (Fig. 1), significantly attenuated the effect of morphine in both tests. However, in the 8-phenyltheophylline-treated group, the antinociceptive response during the 90–180 min time period was significantly greater than that observed in the morphine group. The responses returned towards the pre-drug baseline levels beyond 48 h, reflecting full recovery from the drug effects. Intrathecal injection of 8-phenyltheophylline (11.7 nmol) alone did not produce significant antinociception in either test.

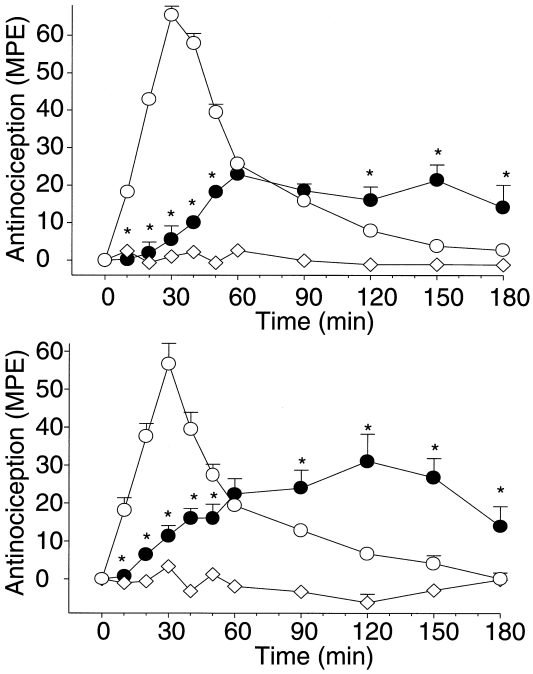

Fig. 2. Effects of the adenosine receptor antagonist 8-phenyltheophylline on the spinal antinociceptive action of morphine in the tail-flick (upper panel) and paw-pressure (lower panel) tests. 8-phenyltheophylline (11.7 nmol) was administered intrathecally alone (◇) or 15 min prior to the injection of morphine (13.2 nmol, ●). Separate animals were injected intrathecally with morphine alone (○). Data are means ± S.E.M. of MPE from 5 to 6 rats. * Significant differences from the action of the morphine alone ($P < 0.05$). The absence of an error bar indicates that the value of the S.E.M. is smaller than the size of the symbol. At 24 h following its intrathecal injection, the combination of 8-phenyltheophylline with morphine produced significantly ($P < 0.05$) greater antinociceptive responses than those produced by the morphine alone: MPE = 12.2 ± 5.6 and 9.8 ± 3.2, in tail-flick and paw-pressure tests, respectively. Full recovery was observed 48 h post injection. Intrathecal 8-phenyltheophylline (data obtained from Fig. 1) alone did not influence baseline nociceptive responses.

3.3. Effects of 8-phenyltheophylline on spinal antinociception produced by neuropeptide FF analogs

Fig. 3 and Table 1 show the effects of 8-phenyltheophylline on the antinociceptive effects produced by injection of the two neuropeptide FF analogs, 1DMe and 3D. Intrathecal injection of 1DMe (0.86 nmol) resulted in a sustained biphasic antinociceptive response: an initial rapid response elicited over a 60-min period, and a slower response apparent between 90 min and 24 h after the drug injection (Fig. 3, left panel). Pretreatment with 8-phenyltheophylline (5.9, 11.7 and 23.4 nmol i.t.) produced a dose-related attenuation of the first phase of the 1DMe-induced response in both tests, and, depending on the dose used, 8-phenyltheophylline augmented or depressed the

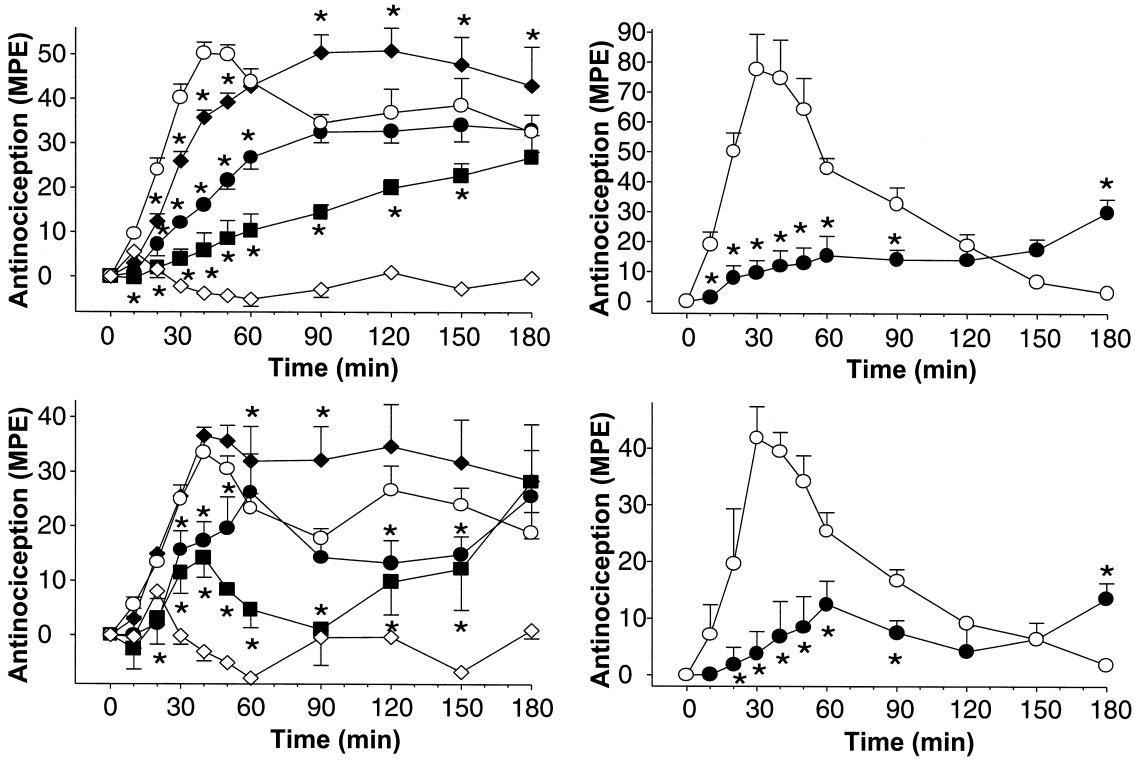

Fig. 3. Effects of the adenosine receptor antagonist 8-phenyltheophylline on the spinal antinociceptive action of the neuropeptide FF analogs 1DMe (left side) and 3D (right side) in the tail-flick (upper panel) and paw-pressure (lower panel) tests. 8-phenyltheophylline doses (5.9 nmol, ◆; 11.7 nmol, ●; 23.4 nmol, ■) were administered intrathecally 15 min prior to the intrathecal injection of 1DMe (0.86 nmol) or 3D (8.6 nmol). Separate animals were injected intrathecally with 8-phenyltheophylline (23.4 nmol, ◇), 1DMe or 3D (○) alone. Data are means ± S.E.M. of MPE from 5 to 8 rats. * Significant differences from the action of the peptide alone ($P < 0.05$). The absence of an error bar indicates that the value of the S.E.M. is smaller than the size of the symbol. An antinociceptive effect was observed 24 h after the spinal administration of 1DMe used alone or in combination with 8-phenyltheophylline in both tests (see Table 1). Responses returned to baseline values at 48 h. This delayed effect (24 h) was not observed with 3D. Intrathecal 8-phenyltheophylline alone did not influence baseline nociceptive responses (this graph and Fig. 1).

second phase of this response. The lowest 8-phenyltheophylline dose (5.9 nmol) augmented the second phase of 1DMe-induced antinociception, an effect similar to that observed in the preceding morphine experiments (Fig. 2). The middle dose (11.7 nmol) had no significant influence while the highest 8-phenyltheophylline dose (23.4 nmol) reduced second phase of the antinociceptive response and augmented it at 24 h post injection. In contrast to 1DMe, spinal injection of 3D (8.6 nmol) produced a monophasic antinociceptive effect (Fig. 3, right panel) similar to that

Table 1
Antinociceptive responses observed 24 and 48 h after the intrathecal injection of neuropeptide FF analogs in rats pretreated 15 min before with saline or the adenosine receptor antagonist 8-phenyltheophylline in the tail-flick (TF) and paw-pressure (PP) tests

Treatment	24 h		48 h	
	TF	PP	TF	PP
Saline + saline (5)	1.4 ± 1.1	0.6 ± 1.4	1.3 ± 0.2	1.5 ± 1.8
Saline + 1DMe (8)	15.5 ± 3.7[a]	13.7 ± 5.2[a]	3.0 ± 1.3	2.2 ± 2.8
5.9 nmol 8-PT + 1DMe (5)	16.8 ± 5.8[a]	13.2 ± 5.3[a]	0.1 ± 1.8	2.8 ± 2.0
11.7 nmol 8-PT + 1DMe (5)	19.9 ± 4.2[a]	12.5 ± 4.5[a]	0.8 ± 0.8	3.7 ± 3.5
23.4 nmol 8-PT + 1DMe (5)	25.5 ± 3.8[a,b]	27.5 ± 3.4[a,b]	4.2 ± 3.0	3.9 ± 2.0
Saline + 3D (5)	2.1 ± 1.3	0.6 ± 2.6	4.2 ± 1.5	0.9 ± 0.5
11.7 nmol 8-PT + 3D (5)	6.2 ± 4.9	40 ± 4.6	2.0 ± 3.2	0.3 ± 2.6

The doses of 1DMe (0.86 nmol) or 3D (8.6 nmol) were used. Data are means ± S.E.M. of MPE from *n* rats per treatment. Intrathecal 8-phenyltheophylline alone did not influence baseline nociceptive responses at any time following the injection.

[a] Values significantly different ($P < 0.05$) from control saline.

[b] Values significantly different ($P < 0.05$) from 1DMe.

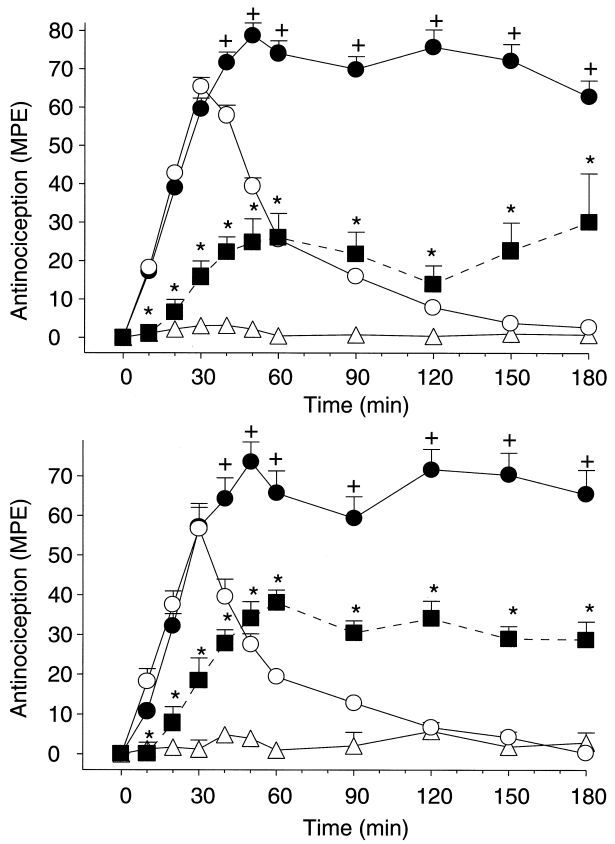

Fig. 4. The action of a sub-effective dose of 1DMe on the spinal antinociceptive effect of morphine, and the influence of 8-phenyltheophylline on this interaction in the tail-flick (upper panel) and paw-pressure (lower panel) tests. 1DMe (0.009 nmol) was co-injected intrathecally with 13.2 nmol of morphine (●). 8-phenyltheophylline (11.7 nmol) was administered 15 min prior to the combination (■). Separate animals were injected intrathecally with 1DMe (△) or morphine (○) alone. Data are means ± S.E.M. of MPE from 5 to 14 rats. Values significantly different ($P < 0.05$) from the action of morphine alone (+) and morphine combined with 1DMe (*). The absence of an error bar indicates that the value of the S.E.M. is smaller than the size of the symbol. An antinociceptive effect was observed 24 h after the spinal administration of (1DMe/morphine) combination, even in rats pretreated with 8-phenyltheophylline in the tail-flick test (see Table 2). Responses returned to baseline values at 48 h. Intrathecal 1DMe (this graph) or 8-phenyltheophylline (Fig. 2) alone did not influence baseline nociceptive responses.

produced by morphine (Fig. 2). Pretreatment with 8-phenyltheophylline (11.7 nmol) effectively inhibited this response in both the tail-flick and paw-pressure tests (Fig. 3). In this group, there was also a delayed slight augmentation of the antinociceptive response (at 180 min post injection). Thus, in these experiments, pretreatment with the adenosine receptor antagonist significantly reduced the antinociceptive effect produced by the two neuropeptide FF related peptides in both tests. As was observed earlier, 8-phenyltheophylline alone did not show significant activity.

3.4. Effects of 8-phenyltheophylline on the potentiation of spinal morphine antinociception by 1DME

A sub-analgesic dose (0.009 nmol) of 1DMe or 3D has previously been reported to produce a very significant augmentation of antinociception produced by spinal opioid agonists, including morphine (Gouardères et al., 1996b). To examine potential involvement of adenosine in this opioid modulatory effect, the effect of 8-phenyltheophylline on antinociception produced by the 1DMe/morphine combination was investigated. The results of these experiments are presented in Fig. 4. Intrathecal morphine (13.2 nmol) elicited a submaximal analgesic response, which peaked at 30 min and terminated at 90 min post injection. An injection of a low 1DMe dose (0.009 nmol) failed to produce an effect in the two tests. However, combined administration of the two agents produced a robust and sustained antinociceptive response in both the tail-flick and paw-pressure tests (Fig. 4). This antinociceptive effect persisted over a 24-h period (MPE = 9.6 ± 2.7 and 24.7 ± 2.5 in the tail-flick and paw-pressure tests, respectively) and full recovery followed at 48 h post injection (Table 2). When the 1DMe/morphine combination was administered to the animals pretreated with 8-phenyltheophylline (11.7 nmol), the antinociceptive response was significantly reduced. The animals in the 8-phenyltheophylline pretreated group, however, continued to exhibit a sustained antinociceptive effect but its magnitude was significantly lower

Table 2
Antinociceptive responses observed 24 and 48 h after the intrathecal co-injection of 1DMe with morphine in rats pretreated 15 min before with saline or the adenosine receptor antagonist 8-phenyltheophylline in the tail-flick (TF) and paw-pressure (PP) tests

Treatment	24 h		48 h	
	TF	PP	TF	PP
Saline + saline (5)	1.2 ± 1.7	1.5 ± 1.8	1.0 ± 2.5	0.5 ± 1.1
Saline + 13.2 nmol morphine (6)	0.5 ± 0.8	1.4 ± 1.4	0.5 ± 0.6	0.6 ± 1.2
Saline + (1DMe with morphine) (14)	9.6 ± 2.7[a,b]	24.7 ± 2.5[a,b]	7.1 ± 1.7	9.3 ± 3.5
11.7 nmol 8-PT + (1DMe with morphine) (5)	14.0 ± 7.7[a,b]	4.6 ± 11.8[c]	6.4 ± 3.3	0.5 ± 5.8

A sub-effective dose (0.009 nmol) of 1DMe was used. Data are means ± S.E.M. of MPE from *n* rats per treatment. Intrathecal 1DMe or 8-phenyltheophylline alone did not influence baseline nociceptive responses at any time following the injection.

[a] Values significantly different ($P < 0.05$) from control saline.

[b] Values significantly different ($P < 0.05$) from morphine.

[c] Values significantly different ($P < 0.05$) from 1DMe/morphine combination.

than that observed in the group not receiving 8-phenyltheophylline pretreatment. Thus, the adenosine receptor antagonist reduced the potentiating action of 1DMe on morphine-induced antinociception.

3.5. Effects of neuropeptide FF analogs on antinociception produced by N6-cyclohexyladenosine

To determine if 1DMe augmented morphine effect by increasing sensitivity of spinal adenosine receptors mediating the opioid antinociception, its action and that of 3D on the antinociceptive activity of N6-cyclohexyladenosine was examined. A fixed sub-effective dose (0.009 nmol) of 1DMe or 3D was combined with each of three different doses of N6-cyclohexyladenosine (0.43, 0.86 and 1.72 nmol). Intrathecal N6-cyclohexyladenosine produced a dose-related antinociceptive effect in the 50–180-min period in both the tail-flick and paw-pressure tests (Fig. 5). The administration of 1DMe significantly enhanced the antinociceptive effect of all three N6-cyclohexyladenosine doses in the tail-flick and paw-pressure tests (Fig. 5), the potentiating effect being especially prominent in experiments involving the low or middle dose of N6-cyclohexyladenosine. In experiments with the lowest N6-cyclohexyladenosine dose (0.43 nmol), 1DMe increased both the peak effect and duration of antinociception, the effect being still apparent at 180 min post injection. In experiments with middle and high N6-cyclohexyladenosine doses, 1DMe slightly decreased the peak effect at 30–45 min but it prolonged the duration of the antinociceptive effect. Thus, while the response induced by 0.86 nmol N6-cyclohexyladenosine recovered towards baseline levels at 60 min post injection, that induced by the combination was still observable at 150 min. In experiments

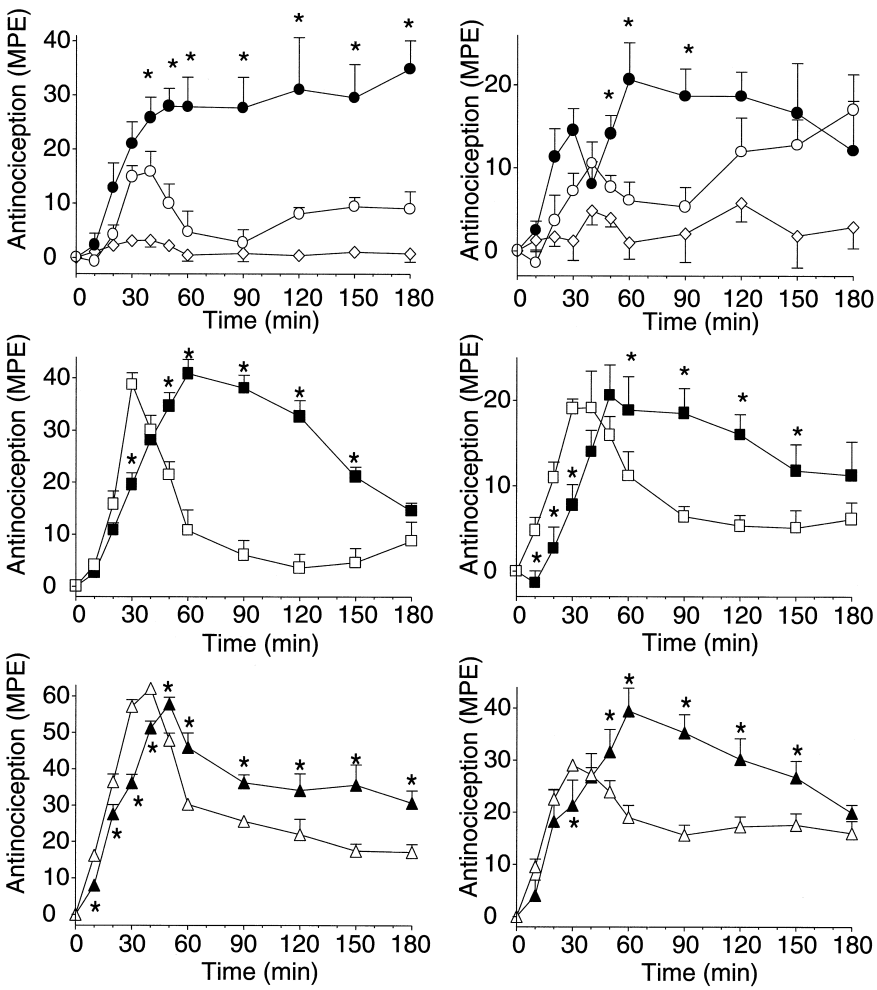

Fig. 5. The effect of a sub-effective dose of 1DMe on the spinal antinociceptive effects produced by three different doses of the adenosine receptor agonist N6-cyclohexyladenosine in the tail-flick (left side) and the paw-pressure (right side) tests. 1DMe (0.009 nmol) was co-injected intrathecally with 0.43 nmol (○, ●), 0.86 nmol (□, ■) and 1.72 nmol (△, ▲) N6-cyclohexyladenosine. Separate animals were injected intrathecally with 1DMe (◇) or different doses of N6-cyclohexyladenosine (open symbols) alone. Data are means ± S.E.M. of MPE from 5 to 10 rats. * Significant differences from the action of N6-cyclohexyladenosine alone ($P < 0.05$). The absence of an error bar indicates that the value of the S.E.M. is smaller than the size of the symbol. The responses from N6-cyclohexyladenosine alone or in combination with 1DMe returned to baseline values by 24 h post injection. Intrathecal 1DMe alone did not influence baseline nociceptive responses.

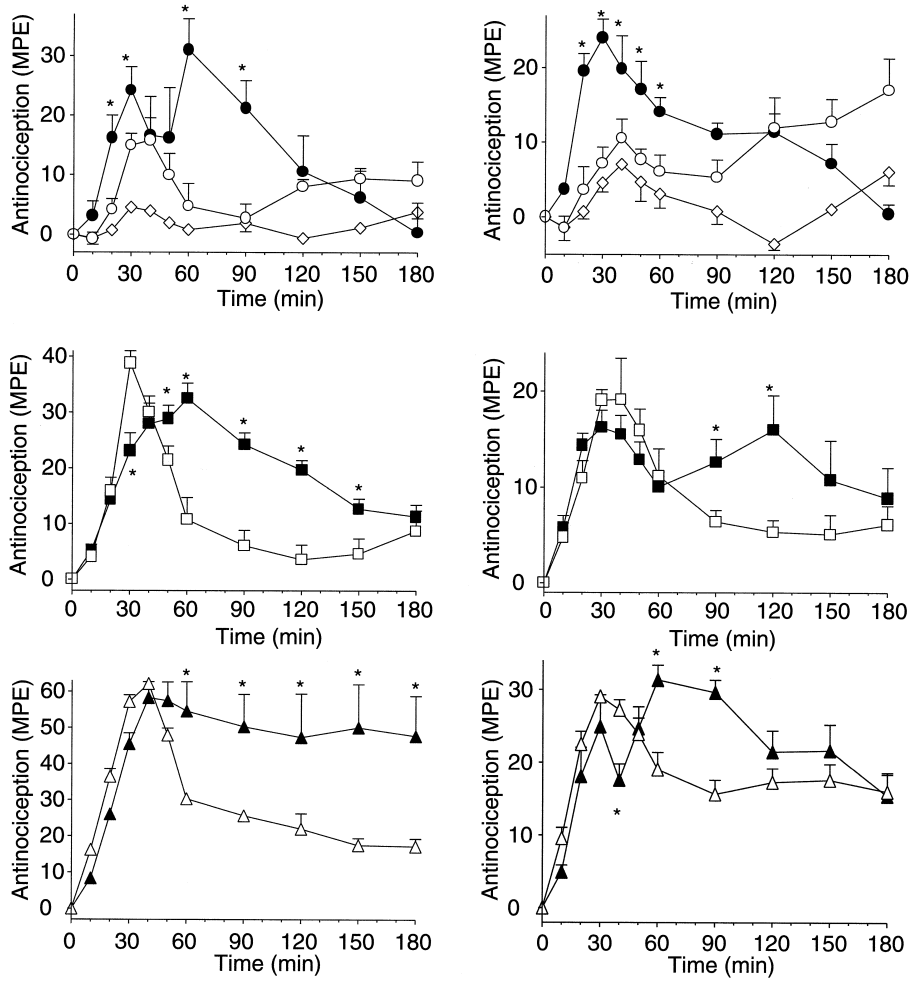

Fig. 6. The effect of a sub-effective dose of 3D on the spinal antinociceptive effects produced by three different doses of the adenosine receptor agonist N6-cyclohexyladenosine in the tail-flick (left side) and the paw-pressure (right side) tests. 3D (0.009 nmol) was co-injected intrathecally with 0.43 nmol (○, ●), 0.86 nmol (□, ■) and 1.72 nmol (△, ▲) N6-cyclohexyladenosine. Separate animals were injected intrathecally with 3D (◇) or the different doses of N6-cyclohexyladenosine (open symbols) alone. Data are means ± S.E.M. of MPE from 5 to 10 rats. * Significant differences from the action of N6-cyclohexyladenosine alone ($P < 0.05$). The absence of an error bar indicates that the value of the S.E.M. is smaller than the size of the symbol. The responses from N6-cyclohexyladenosine alone or in combination with 3D returned to baseline values by 24 h post injection. Intrathecal 3D alone did not influence baseline nociceptive responses.

involving the highest N6-cyclohexyladenosine dose (1.72 nmol), the adenosine receptor agonist itself produced a sustained antinociception. Addition of 1DMe to this dose augmented the response in both tests. Administration of 3D also augmented the effects of all three N6-cyclohexyladenosine doses from 50 min post injection (Fig. 6), the profile of responses in the tail-flick test being similar to that observed in experiments with 1DMe (Fig. 5). As noted earlier, the morphine-potentiating action of 3D on the N6-cyclohexyladenosine-induced antinociception was more marked in experiments involving the lowest dose of the adenosine receptor agonist. The augmentation of response produced by the other two doses was more modest. Thus, in these experiments, administration of the two neuropeptide FF-like peptides, at a low dose lacking intrinsic activity, augmented the spinal antinociception produced by activation of spinal adenosine receptors.

At doses used in the experiments reported here, none of the drug treatments when administered alone or in combination produced visible signs of motor impairment. In experiments involving interaction of 1DMe or 3D with morphine or the two adenosine receptor agonists, the sustained antinociceptive responses exhibited full recovery between 24–48 h.

4. Discussion

The present study investigated whether adenosine, which plays an intermediary role in the production of spinal opioid antinociception, is involved in the expression of spinal antinociceptive effects produced by neuropeptide FF receptor agonists. In previous studies, intrathecal administration of two such agonists, 1DMe and 3D, was reported

to produce a sustained thermal and mechanical antinociception through indirect activation of spinal opioid receptors (Gouardères et al., 1993, 1996b; Xu et al., 1999). At sub-analgesic doses, these peptides and neuropeptide FF were found to produce a marked enhancement of the antinociceptive effects produced by spinal injection of opioid receptor agonists (Gouardères et al., 1993, 1996b; Kontinen and Kalso, 1995; Xu et al., 1999). The results of this study show that blockade of spinal adenosine receptors inhibits both the intrinsic antinociceptive activity and opioid-potentiating effect of neuropeptide FF-like peptides. Additionally, antinociceptive activity produced by adenosine receptor agonists is delayed and increased by these peptides. Thus, neuropeptide agonists could exert their actions by releasing adenosine and/or increasing the sensitivity of adenosine receptors mediating antinociception at the spinal level.

The thermal and mechanical antinociception produced by intrathecal administration of neuropeptide FF peptides such as 1DMe and 3D likely results from the release of adenosine due to indirect activation of opioid receptors in the dorsal horn. Previous pharmacological and neurochemical studies have shown that adenosine receptor blockade inhibits the antinociceptive effects of spinal opioids (Sweeney et al., 1989), opioid receptor agonists stimulate release of adenosine from synaptosomes prepared from the dorsal spinal cord (Sweeney et al., 1989; Cahill et al., 1995), and intrathecal injections of adenosine receptors agonists elicit antinociceptive effects (Sawynok et al., 1986; DeLander and Keil, 1994). These and related observations (see Sawynok, 1998) have suggested that the activity of opioid receptors in the dorsal horn mobilizes adenosine and the latter mediates spinal opioid analgesia. In view of the existence of an opioid link in the effects of neuropeptide FF receptor agonists (see below), these effects may be mediated by adenosine released at the spinal level. The antagonism of 1DMe-induced antinociception by 8-phenyltheophylline, which blocks adenosine receptors, provides support in favour of this mechanism of neuropeptide FF action. However, it is unlikely that 1DMe or 3D release adenosine by a direct stimulation of spinal opioid receptors since receptor binding studies reveal that these peptides display a very low affinity for mu, delta or kappa opioid receptors in rat spinal cord (Gouardères et al., 1997, 1998). Recently, exposure to 1DMe has been shown to release methionine enkephalin-like immunoreactivity from the perfused rat spinal cord in vivo (Ballet et al., 1999). The released endogenous peptide could activate opioid receptors, mobilise adenosine from neurons in the dorsal horn and produce antinociception. This model of neuropeptide FF antinociception is consistent with the finding that both the opioid (Gouardères et al., 1993, 1996b; Xu et al., 1999) and adenosine receptor antagonists, at doses producing appropriate receptor blockade, can inhibit such antinociception. The effects of neuropeptide FF and related peptides on spinal adenosine release have not been examined, but in view of the present findings, these clearly merit attention in future studies.

The opioid-potentiating action of neuropeptide FF receptor agonists also appear to be based on a purinergic mechanism. As was observed previously (Gouardères et al., 1996b; Xu et al., 1999), a low intrathecal dose of 1DMe markedly enhanced the antinociception produced by spinal morphine, and 3D exerted a similar effect. Pretreatment with a dose of 8-phenyltheophylline, that produced a blockade of the effect of N6-cyclohexyladenosine, an adenosine receptor agonist, significantly reduced the morphine potentiating action of 1DMe, suggesting dependency of this action on adenosine. Thus, 1DMe may exert its effect partly by augmenting the stimulatory action of morphine on the spinal adenosine release. However, an alternate possibility is that 1DMe increases the sensitivity of adenosine receptors mediating antinociception at the spinal level. This possibility was examined in the present study by evaluating the action of both 1DMe and 3D on the effect produced by intrathecal N6-cyclohexyladenosine. Interestingly, both peptides augmented effects of N6-cyclohexyladenosine in the tail-flick and paw-pressure tests especially at low doses. The 1DMe/N6-cyclohexyladenosine combination produced antinociceptive effects reminiscent of those produced by the 1DMe/morphine combination; both elicited highly sustained but reversible antinociceptive responses in the two tests. Thus, one interpretation of the opioid-potentiating effects is that neuropeptide FF related peptides increase the sensitivity of spinal adenosine receptors mediating spinal antinociception.

Adenosine is known to activate multiple receptor types. The particular type whose activity is influenced by neuropeptide FF activity is not known, although the selectivity of N6-cyclohexyladenosine action points to the A_1 subtype as a likely candidate. Evidence from pharmacological experiments implicates G protein-coupled A_1 adenosine receptor subtype, whose stimulation inhibits adenylyl cyclase activity, in the production of spinal opioid antinociception (Sawynok et al., 1986). A population of adenosine receptors in the spinal cord is thought to be presynaptic on sensory afferent terminals and activity of these receptors inhibits the release of nociceptive neuropeptide transmitters (Santicioli et al., 1993). There is recent evidence that a portion of the spinal neuropeptide FF binding sites in the spinal cord is also presynaptic (Gouardères et al., 2000). It would therefore appear that neuropeptide FF sites in the spinal cord are strategically localized to influence function of adenosine receptors. Thus, activity of neuropeptide FF receptors could augment the presynaptic inhibitory action of adenosine on release of nociceptive transmitters, and the adenosine released in response to morphine would be more effective in the presence of neuropeptide FF related peptides.

The effects of neuropeptide FF activity on spinal nociception can be considered in terms of a model involving

two adenosine-based mechanisms. Thus, low doses of the neuropeptide FF peptides facilitate opioid antinociception by increasing sensitivity of the spinal adenosine receptors whose activity inhibits presynaptic release of nociceptive transmitters, while higher doses alone produce antinociception partly by releasing an endogenous opioid that interacts with spinal opioid receptors to release adenosine, and this in turn stimulates purinergic receptors and inhibits pain transmission. The unusually long duration of the neuropeptide antinociception may be explained by the fact that neuropeptide FF activity additionally increases adenosine receptor sensitivity.

Although results provide data in support of a purinergic basis of neuropeptide FF activity producing antinociception, the potential role of other mechanisms also contributing to such activity cannot be excluded. The effects of neuropeptide FF peptides were not completely blocked by opioid antagonists in a previous study (Gouardères et al., 1996b) or by 8-phenyltheophylline in this study. Indeed, under certain conditions, 8-phenyltheophylline augmented antinociception in both morphine and neuropeptide FF peptide experiments. Thus, additional mechanisms clearly also play a role in the expression of the spinal effects produced by activity neuropeptide FF receptors. The nature of such mechanisms remains to be explored in future studies. The unusual effects of neuropeptide FF peptides on spinal nociception could involve a synergy between different mechanisms.

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