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# Neuropeptide FF attenuates allodynia in models of chronic inflammation and neuropathy following intrathecal or intracerebroventricular administration

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**Abstract**

Experiments were conducted to explore the effects of Neuropeptide FF acting at spinal and supraspinal sites in models of chronic inflammatory or neuropathic pain and of acute pain. Neuropeptide FF was administered intrathecally (i.t.; 10.0, 25.0 and 50.0 nmol) or intracerebroventricularly (i.c.v.; 10.0, 12.5 and 15.0 nmol) either 24 h after inflammation-inducing injections of Freund's Complete Adjuvant in one hind paw or 7 days after unilateral sciatic nerve constriction. Evoked pain was assessed by measuring the withdrawal response threshold (in grams of pressure) to a mechanical stimulus applied to the plantar surface of the injured paw. Neuropeptide FF dose-dependently attenuated the allodynic response (i.e., withdrawal from a normally innocuous stimulus) to mechanical stimulation in the inflammatory and neuropathic model following i.t. ($ED_{50}$ = 20.86 nmol and $ED_{50}$ = 18.91 nmol, respectively) and i.c.v. ($ED_{50}$ = 12.31 nmol and $ED_{50}$ = 11.68 nmol, respectively) administration. Pretreatment with naloxone (2.0 mg/kg; s.c.) attenuated the anti-allodynic effect of i.t. or i.c.v. Neuropeptide FF in rats experiencing inflammatory, but not neuropathic pain. In contrast, Neuropeptide FF administered i.t. (10.0, 25.0 and 50.0 nmol) or i.c.v. (10.0, 12.5 and 15.0 nmol) had no effect on the response to acute thermal or mechanical stimulation. Neuropeptide FF injected i.t. or i.c.v. in inflamed or neuropathic rats did not produce any sign of motor dysfunction. These results suggest that Neuropeptide FF acting at spinal and supraspinal sites plays a role in modulating chronic, but not acute pain. Furthermore, the results suggest that the anti-allodynic effect of Neuropeptide FF is mediated indirectly by naloxone-sensitive opioid mechanisms in rats subjected to inflammatory, but not neuropathic pain. 

*Keywords:* Analgesia; Antinociception; Pain; Neuropeptide FF; Spinal cord; Supraspinal

## 1. Introduction

Several lines of evidence suggest that the mammalian octapeptide Neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-$NH_2$) displays anti-opioid properties when acting in the central nervous system. For instance, Neuropeptide FF attenuates the antinociceptive effect of morphine following intracerebroventricular (i.c.v.) administration (Kavaliers, 1990; Tang et al., 1984; Yang et al., 1985), whereas injection at this site of a Neuropeptide FF antibody potentiates the antinociceptive effect of morphine (Kavaliers and Yang, 1989). Neuropeptide FF has been suggested to play a role in the development of tolerance to the antinociceptive effects of chronic opioid administration (Lake et al., 1991; Malin et al., 1990a,b). Interestingly, Neuropeptide FF receptors are different from opioid receptors and Neuropeptide FF does not bind to opioid receptors, but rather stimulates specific Neuropeptide FF receptors (Allard et al., 1989, 1992). Thus, Neuropeptide FF appears to induce its anti-opioid effects by modulating indirectly the activity of endogeneous opioids. Neuropeptide FF receptors are localized throughout the central nervous system (e.g., Allard et al., 1989, 1992, 1994; Dupouy et al., 1996; Dupouy and Zajac, 1996; Lombard et al., 1995; Marco et al., 1995; Panula et al., 1996; Roumy and Zajac, 1998). A high density of these receptors is localized in the dorsal horns of the spinal cord where pain

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signals coming from the periphery are transmitted (Allard et al., 1992). At supraspinal sites, these receptors are present in various nuclei of the pons and medulla, and in several structures of the mesencephalon and diencephalon (Allard et al., 1992).

In recent years, it has been discovered that Neuropeptide FF also displays opioid-like properties. For instance, it has been shown that Neuropeptide FF administered intrathecally (i.t.) elicits potent, dose-dependent, and long-lasting antinociception mediated indirectly by the release of endogeneous opioids (Gouardères et al., 1993). Similarly, the Neuropeptide FF analogues, [D-Tyr$^1$,(NMe)Phe$^3$] Neuropeptide FF (1DMe) and [D-Tyr$^1$,D-Leu$^2$,D-Phe$^3$] Neuropeptide FF (3D), which are resistant to enzymatic degradation and are highly selective for Neuropeptide FF receptors (Gicquel et al., 1992; Devillers et al., 1994), induce dose-dependent, sustained, and naloxone-sensitive antinociception (Gouardères et al., 1996; Xu et al., 1999). The antinociceptive effects of these Neuropeptide FF analogues are attenuated by the μ-opioid receptor antagonist, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$ (CTOP), and the δ-opioid receptor antagonist, naltrindole, administered i.t., suggesting that μ- and δ-opioid receptor subtypes play a role in mediating the antinociceptive effects of Neuropeptide FF acting spinally (Gouardères et al., 1996). In addition, [D-Tyr$^1$,(NMe)Phe$^3$] Neuropeptide FF (1DMe) and [D-Tyr$^1$,D-Leu$^2$,D-Phe$^3$] Neuropeptide FF (3D) administered i.t. potentiate the antinociceptive effects of both i.t. morphine and the δ-opioid receptor agonist, [D-Ala$^2$]deltorphin I spinally (Gouardères et al., 1996; Xu et al., 1999).

The studies described above examined the role of Neuropeptide FF in the modulation of pain using tests which assay acute pain, such as the tail-flick test. The pain assayed in such tests is transient, short lasting, rapidly rising, well localized, and escapable. Furthermore, it is usually elicited by non-damaging stimuli and the withdrawal response is organized at the level of the spinal cord, as it is elicited in spinally transected rats (Irwin et al., 1951). Thus, the findings derived from studies employing models of acute pain may provide limited information about the effectiveness of Neuropeptide FF on inescapable chronic pain of pathological origin.

A few recent studies explored the effect of Neuropeptide FF acting spinally or supraspinally in clinically relevant models of chronic pain and the results suggest that this peptide is also effective at alleviating this type of pain. For instance, Courteix et al. (1999) showed that the Neuropeptide FF analogue, [D-Tyr$^1$,(NMe)Phe$^3$] Neuropeptide FF (1DMe), administered i.t. increases paw pressure-induced vocalization thresholds in rats with a neuropathy (i.e., nerve injury) due to chronic ligature of the sciatic nerve or to diabetes (Courteix et al., 1999). These effects were blocked by the μ-opioid receptor antagonist, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$ (CTOP), and the δ-opioid receptor antagonist, naltrindole, suggesting that the antinociceptive effects of spinal Neuropeptide FF occur through an interaction with μ and δ receptors. The pro-opioid effects of [D-Tyr$^1$,(NMe)Phe$^3$] Neuropeptide FF (1DMe) were also evidenced by the superadditive effects of the Neuropeptide FF analogue combined with morphine in the diabetes-induced neuropathic model. Similarly, Xu et al. (1999) found that the i.t. administration of [D-Tyr$^1$,(NMe)Phe$^3$] Neuropeptide FF (1DMe) attenuates mechanical and cold allodynia (i.e., increased reactivity to an innocuous stimulus) in a model of neuropathy resulting from spinal nerve ligation. They also showed that the Neuropeptide FF analogue decreases mechanical allodynia and thermal hyperalgesia in a model of carrageenan-induced inflammation and that morphine potentiates the anti-allodynic effect of [D-Tyr$^1$,(NMe)Phe$^3$] Neuropeptide FF (1DMe). The antinociceptive effect of Neuropeptide FF has also been evidenced following supraspinal administration (Wei et al., 1998). More specifically, this study found that Neuropeptide FF administered directly into the periaqueductal gray dose-dependently attenuates mechanical allodynia in rats with a spinal nerve ligation. Neuropeptide FF administered at this site did not, however, affect mechanical or thermal hyperalgesia in neuropathic rats.

The present study further explored the effects of Neuropeptide FF in clinically relevant animal models of chronic pain. More specifically, the study examined the effects of Neuropeptide FF administered i.t. or i.c.v. on the allodynic response to mechanical stimulation in an inflammatory and neuropathic model. Given the evidence that Neuropeptide FF induces its effects indirectly through endogeneous opioid mechanisms, the study also examined the role of opioids in the effects of Neuropeptide FF observed on inflammatory and neuropathic pain. For comparison, experiments were conducted to assess the effects of Neuropeptide FF on the response to acute thermal and mechanical stimulation. Finally, to assess the possibility that the effects of Neuropeptide FF on pain are due to secondary motor disturbances, experiments were designed to explore the effect of Neuropeptide FF administered i.t. or i.c.v. on spontaneous locomotor activity.

## 2. Materials and methods

### *2.1. Subjects, housing, and habituation*

Naive male Sprague–Dawley rats (Charles River Canada, St. Constant, Québec) weighing 150–175 g were used. Experimental protocols were approved by the Astra Animal Care Committee. Upon arrival, animals were housed three per cage in standard clear plastic cages with wire tops. They had continuous access to food and water and were maintained on a 12 h light–dark cycle (lights on at 0630 and off at 1830). All testing took place during the light portion of the cycle. Animals were allowed to habituate to the test room for 30 min before testing began.

### 2.2. Apparatus

To test the response to a mechanical stimulus applied to an injured paw, animals were placed in cubicles ($14 \times 14 \times 23.5$ cm) made from clear plexiglas mounted on a grid floor with rods arranged to form $1.2 \times 1.2$ cm$^2$. The surface ($55.5 \times 19.5$ in.) of the grid floor could accommodate a total of nine cubicles per side. Mechanical stimulation was applied by a steel cannula constructed by cutting 5 mm off the two extremities of a 22-gauge 1 1/2 needle. The cannula was then attached to the tip of a differential pressure transducer, which was connected to a Sensel ab 4-channel amplifier (Somedic Sales, Farsta, Sweden). The differential pressure transducer recorded changes in pressure from the moment the cannula was applied to an injured paw until the animal withdrew its paw. These changes were automatically recorded in the Polyview Data Acquisition and Analysis System (Grass Instrument Division, Astro Med, West Warwick, RI) and were subsequently analyzed by the system to determine the response threshold (in grams of pressure) for each tested animal. The tail-flick and paw-pressure apparatus were commercially available from Life Science International, IITC, Woodlands Hills, CA. Locomotor activity was measured in $44 \times 27 \times 24$-cm plexiglas boxes surrounded by an activity monitor (AM1051, Benwick Electronics, Life Science International, IITC). The activity monitor measured three types of motor behaviors via infrared light beams displayed on an upper and lower panel and arranged on either a 'fine' $12 \times 7$ beam matrix on a 1′ grid or a 'coarse' $2 \times 3$ beam matrix on a 4′ grid: mobile activity (i.e., horizontal crossing) counts, static activity (i.e., stereotypy) counts, and rearing activity (i.e., vertical crossing) counts.

### 2.3. Induction of inflammation

Inflammatory pain was induced by injecting Freund's Complete Adjuvant (Sigma, St. Louis, MO) in the plantar surface of the left hind paw of animals anesthetized under AErrane (isoflurane; Janssen, North York, Ontario). A total volume of 20.0 μl of Freund's Complete Adjuvant was injected in the hind paw, with 10.0 μl injected in the dorsal surface, and 10.0 μl injected in the plantar surface. We preferred to inject a volume of 20.0 μl, which is lower than that typically used in the literature. In a previous pilot study, we found that 20.0 μl of Freund's Complete Adjuvant injected in the dorsal and plantar surface of the paw (10.0 μl in each surface) yields a homogeneous inflammation of the paw and significant allodynia. Rats assigned to the sham condition were anesthetized only. We found previously that there are no significant differences in the response to mechanical stimulation between anesthetized sham rats injected with or without the vehicle in the hind paw. Thus, sham rats in the present experiment were not injected with the vehicle. Following the injections, rats were housed three per cage in clean standard plastic cages with wire tops until testing began 24 h later.

### 2.4. Induction of neuropathy

Neuropathic pain was induced by the surgical constriction of the left sciatic nerve. This procedure was adapted from Mosconi and Kruger (1996). Rats were anesthetized with a solution containing both Ketamine HCl and Xylazine HCl (80 and 12 mg/kg, respectively; i.p.; RBI, Natick, MA). The left sciatic nerve was isolated and exposed following dissection through biceps femoris of the thigh. One 2-mm-long cuff made from polyethylene tubing (PE 20) slit longitudinally was fitted around the sciatic nerve. The muscles and skin were then sutured. The same procedure was followed to prepare the rats assigned to the sham condition, except that no cuff was applied to the sciatic nerve. Upon recovery on a heat pad, animals were housed three per cage in clean standard plastic cages and allowed a 7-day recovery period before testing began.

### 2.5. Drugs

Neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-$NH_2$; Bachem) was dissolved in saline and injected either i.t. (10.0, 25.0, and 50.0 nmol/10.0 μl) or i.c.v. (10.0, 12.5, or 15.0 nmol/10.0 μl). The range of doses used for i.c.v. injections was lower than that used for i.t. injections because doses higher than 15.0 nmol/10.0 μl produce 'barrel wheel-rotation' side effects in some rats following i.c.v. injections. Naloxone HCl (RBI) was dissolved in saline and injected s.c. using a dose of 2.0 mg/kg. This dose of naloxone was selected because we found in previous studies that it is effective at blocking the anti-allodynic effects of morphine and selective opioid receptor agonists in our models of inflammatory and neuropathic pain. All solutions were prepared immediately prior to testing.

### 2.6. Experimental design and procedure: chronic pain

Twenty-four hours following the Freund's Complete Adjuvant injections, or 7 days following chronic nerve constriction, animals were transferred to the test room and were left there for 30 min to habituate to the environment. Animals were then lightly anesthetized with isoflurane, and received an i.t. or i.c.v. injection (see injection procedures below) of either saline (10.0 μl) or Neuropeptide FF (10.0, 25.0, 50.0, and 10.0, 12.5, 15.0 nmol/10.0 μl, respectively). Thus, using a counterbalanced between-subjects design, there were five conditions per experiment: Sham-Saline, Freund's Complete Adjuvant-Saline or Chronic Nerve Constriction-Saline, Freund's Complete Adjuvant-Neuropeptide FF (dose 1) or Chronic Nerve Constriction-Neuropeptide FF (dose 1), Freund's Complete Adjuvant-Neuropeptide FF (dose 2) or Chronic Nerve Constriction-Neuropeptide FF (dose 2), etc. In order to

verify the replicability of the findings, each experiment was conducted three times, using naive rats for each experiment. For example, the experiment on the effect of i.t. Neuropeptide FF on the allodynic response to mechanical stimulation in the inflammatory model was conducted three times. In another set of experiments, using the same design and procedure, rats were pretreated with either naloxone (2.0 mg/kg, s.c.) or saline 10 min prior to receiving an i.t. or i.c.v. injection of either Neuropeptide FF (one dose) or saline. Thus, the following five conditions were tested : Sham-Saline + Saline, Freund's Complete Adjuvant or Chronic Nerve Constriction-Saline + Saline, Freund's Complete Adjuvant or Chronic Nerve Constriction-Saline + Neuropeptide FF, Freund's Complete Adjuvant or Chronic Nerve Constriction-Naloxone + Neuropeptide FF, Freund's Complete Adjuvant or Chronic Nerve Constriction-Naloxone + Saline. Baseline measurements were not taken before chronic pain induction because the design of each experiment included a control group, which provided information about how animals responded in the absence of inflammatory and neuropathic pain (i.e., Sham-Saline conditions).

Rats which failed to show an inflammatory response to the left paw or an abnormal posture due to sciatic nerve constriction to the left thigh were not tested. Immediately following the injections, rats were placed in a test cubicle and were tested for their response to mechanical stimulation after a 30-min period of adaptation. Evoked pain was assessed by measuring the paw withdrawal response threshold, in grams of pressure, to a mechanical stimulus applied with escalating pressure (approximately 5 g/s) to the inflamed or neuropathic paw. We observed previously that paw withdrawal response thresholds were more variable upon the first than second application of the mechanical stimulus to the paw and were stable between the second and third application of this type of stimulus to the paw. Therefore, each animal was assessed for its response to mechanical stimulation twice, at a 10-s interval. Animals were not tested while grooming or moving.

### 2.7. Experimental design and procedure: acute pain

The response to acute thermal pain was assessed 30 min, 60 min, and 3 h after i.t. or i.c.v. administration of saline or Neuropeptide FF (50.0 and 15.0 nmol/10.0 μl, respectively) using a counterbalanced between-subjects design. A test trial consisted of hand-holding a rat on the platform and placing the distal 5–10 cm of the tail in the groove directly under the source of the beam of light. The latency for the animal to flick its tail was recorded. Using the same design and procedure, a separate group of animals was assessed in the paw pressure test. A test trial consisted of hand-holding a rat and placing its right hind paw on a platform and placing an inverted moveable cone over the dorsal surface of the paw. Upon the activation of a foot-switch connected to the paw pressure apparatus, slowly increasing pressure was automatically applied to the paw by the cone. The latency for the animal to withdraw its paw was recorded.

### 2.8. Experimental design and procedure: locomotor activity

Animals were tested 24 h after inflammation-inducing injections of Freund's Complete Adjuvant in one hind paw or 7 days after sciatic nerve constriction in one paw. Animals were placed, four at a time, into the activity boxes and, after a 5-min period of habituation to the boxes, baseline activity counts were recorded for 1 min. After 1 min, animals were immediately taken out of the boxes and received i.t. or i.c.v. injections of either saline or Neuropeptide FF (50.0 nmol and 15.0 nmol/10.0 μl, respectively) under isoflurane anesthesia. They were replaced in their holding cages following the injections and were reintroduced into the activity boxes 15 min later. After a 1-min habituation period, activity counts were recorded for 1 min, following which animals were replaced into their cages. The same procedure was repeated 30 and 45 min after the injection. Animals were allocated relatively little time to habituate to the activity boxes before or after the injections because we found in pilot studies that animals become inactive when given more time, thus preventing us from examining whether Neuropeptide FF induces hypoactive in addition to hyperactive effects.

### 2.9. i.t. and i.c.v. injections

The i.t. injections were performed according to Mestre et al. (1994). Briefly, a 26-gauge 5/8 needle connected to a 100-μl Hamilton syringe was inserted transcutaneously into the intervertebral space between $L_5$ and $L_6$ in anesthetized and unrestrained animals. The injection was successful when insertion of the needle elicited a sudden tail-flick reflex. Immediately following this motor reflex, the solution was administered over 10 s. The needle remained in place for an additional 10 s to allow diffusion of the compound around the site of injection.

The procedure employed for i.c.v. injections was adapted from the technique developed by Bouchard et al. (1996). Under isoflurane anesthesia, an incision was made longitudinally through the skin of the skull, with precaution taken to avoid damaging the membrane covering the skull. A 5-mm-long 26 gauge 3/8 needle connected via polyethylene tubing (PE 20) to a 100-μl Hamilton syringe was implanted into the left ventricle, using the following coordinates: −0.8 mm posterior from bregma, +1.5 mm lateral from midline, −4.0 ventral from skull surface (Paxinos and Watson, 1986). The 5-mm long 26 gauge 3/8 needle was constructed by inserting a 4-mm-long stopper made from a 20-gauge needle, and securing their connection with silicone glue applied around the area.

### 2.10. Statistics

Data from the tail-flick and paw pressure tests were analyzed using two-way analyses of variance (ANOVA) with Treatment (two levels) as a between-subjects variable and Time (three levels) as a within-subjects variable. If appropriate, the analyses were followed by post hoc tests. All statistical analyses and graphical representations of data obtained from tests in which pain was evoked by mechanical stimulation of an injured paw were based on the paw withdrawal response thresholds elicited by the second mechanical stimulus application (as mentioned previously, rats were tested twice for their response to mechanical stimulation). These data were analyzed by one-way ANOVAs with Treatment (five levels) as a between-subjects variable, followed by Tukey's post-hoc test for overall differences between conditions. Linear regression was performed to calculate the half-maximal effective dose ($ED_{50}$). Percent of Anti-Allodynia was calculated as follows: $100 \times [(\text{post-drug threshold} - \text{control threshold})/(\text{maximum threshold} - \text{control threshold})]$, where 'maximum threshold' was the threshold for Sham rats and 'control threshold' was the threshold for Freund's Complete Adjuvant or Chronic Nerve Constriction rats that received saline. Dose–response curves were produced by plotting the mean percentage of anti-allodynia (+S.E.M.) of three experiments against log dose. Mobile, static, and rearing activity data were analyzed by separate two-way ANOVAs with Treatment (saline vs. Neuropeptide FF) as a between-subjects variable and Time (three post-injection time points) as a within-subjects variable. If appropriate, the analyses were followed by post hoc tests. Mobile, static, and rearing activity data at baseline were analyzed by separate t tests for independent (i.e., unpaired) samples.

## 3. Results

### 3.1. Effect of i.t. or i.c.v. Neuropeptide FF on acute pain

Neuropeptide FF administered i.t. (50.0 nmol/10.0 μl) was without effect on tail-flick latencies at any of the time-points tested, $F_{(1,97)} = 2.42$, $P > 0.07$ (data not shown). Similarly, i.t. Neuropeptide FF (50.0 nmol/10.0 μl) had no effect on the response to acute mechanical stimulation in the paw-pressure test, $F_{(1,81)} = 1.66$, $P = 0.18$ (data not shown). Neuropeptide FF administered i.c.v. (15.0 nmol/10.0 μl) was without effect in the tail-flick and paw-pressure tests (data not shown), $F_{(1,75)} = 2.36$, $P = 0.08$ and $F_{(1,84)} = 0.36$, $P = 0.34$, respectively.

### 3.2. Effect of i.t. Neuropeptide FF in chronic pain models

Fig. 1 shows the effect of Neuropeptide FF on the response to mechanical stimulation in the inflammatory and neuropathic models following i.t. administration of escalating doses (10.0, 25.0, and 50.0 nmol/10.0 μl). Each experiment was conducted three times. In the inflammatory pain model, all three experiments yielded significant treatment effects, $F_{(4,\ 35)} = 11.56$, $P < 0.0001$; $F_{(4,40)} = 8.91$, $P < 0.0001$; $F_{(4,40)} = 11.33$, $P < 0.0001$. Similarly, there were significant treatment effects in all experiments conducted in the neuropathic pain model, $F_{(4,34)} = 14.04$, $P < 0.0001$; $F_{(4,35)} = 4.56$, $P < 0.005$; $F_{(4,35)} = 9.66$, $P < 0.0001$. Mechanical pressure applied to an inflamed or neuropathic paw caused significant allodynia ($P$s $< 0.05$). Neuropeptide FF administered i.t. dose-dependently attenuated allodynia in the inflammatory model ($P$s $< 0.05$), causing 90.37% + 1.71 anti-allodynia at the highest dose tested ($ED_{50} = 20.86 + 1.31$). Similarly, i.t. Neuropeptide FF significantly attenuated allodynia in the neuropathic pain model ($P < 0.05$), causing

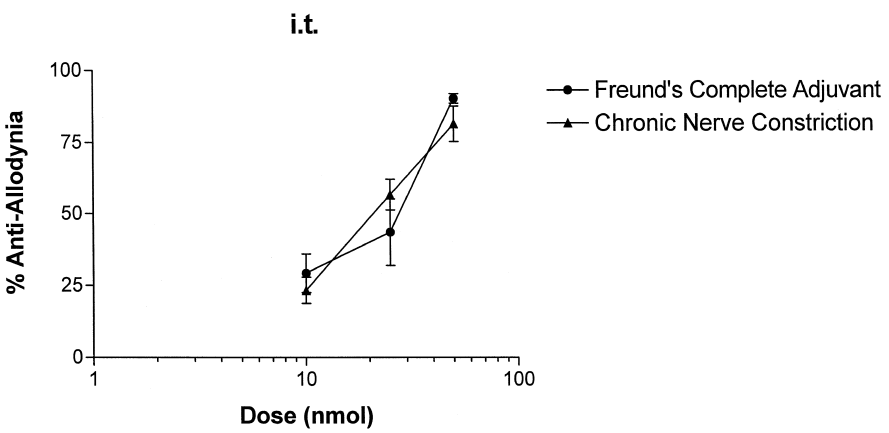


Fig. 1. Mean percentage of anti-allodynia (±S.E.M.) 30 min following escalating doses of Neuropeptide FF (10.0, 25.0, and 50.0 nmol/10.0 μl) administered i.t. in a model of inflammatory (Freund's Complete Adjuvant; closed circles) or neuropathic (Chronic Nerve Constriction; closed triangles) pain. There were eight to nine rats per dose in each experiment. Each point represents the average (±S.E.M.) of the mean percentage of anti-allodynia (±S.E.M.) of three experiments.

Table 1
$ED_{50}$ values (in nmol) of Neuropeptide FF in the two chronic pain models as a function of the administration route. Values represent the means (±S.E.M.) of three experiments

| Pain model | Route of administration | |
|---|---|---|
| | i.t. | i.c.v. |
| Inflammatory | 20.86 (±1.31) | 12.31 (±0.66) |
| Neuropathic | 18.91 (±1.32) | 11.68 (±0.48) |

81.57% + 6.18 anti-allodynia at the highest dose tested ($ED_{50}$ = 18.91 + 1.32). Table 1 summarizes the $ED_{50}$ values of i.t. Neuropeptide FF in the inflammatory and neuropathic models. Four rats did not show an inflammatory response and were therefore not tested. One rat subjected to sciatic nerve constriction failed to show an abnormal posture and thus was excluded from testing.

### 3.3. Effect of i.c.v. Neuropeptide FF in chronic pain models

Fig. 2 shows the effect of escalating doses of Neuropeptide FF (10.0, 12.5, and 15.0 nmol/10.0 μl) administered i.c.v. on the response to inflammatory or neuropathic pain. Each experiment was conducted three times. There were significant differences in response thresholds between conditions in the inflammatory model, $F_{(4,34)} = 7.21$, $P < 0.001$; $F_{(4,35)} = 11.54$, $P < 0.001$; $F_{(4,35)} = 13.24$, $P < 0.0001$, and neuropathic model, $F_{(4,40)} = 6.81$, $P < 0.001$; $F_{(4,\ 40)} = 15.01$, $P < 0.0001$; $F_{(4,\ 35)} = 6.85$, $P < 0.001$. Animals subjected to inflammatory or neuropathic pain displayed significant allodynia ($P$s < 0.001) in response to mechanical stimulation applied to the injured paw. Neuropeptide FF administered i.c.v. dose-dependently attenuated allodynia in the inflammatory model, causing 90.5% + 5.8 anti-allodynia at 15.0 nmol ($ED_{50}$ = 12.31 + 0.66). In the neuropathic pain model, i.c.v. Neuropeptide FF similarly attenuated allodynia in a dose-dependent way, causing 87.03% + 3.25 anti-allodynia ($ED_{50}$ = 11.68 + 0.48). Table 1 presents a summary of the $ED_{50}$ values of i.c.v. Neuropeptide FF in the two pain models. Two rats failed to show an inflammatory response and two rats subjected to sciatic nerve constriction did not show an abnormal posture. These rats' data were therefore excluded from the analyses.

### 3.4. Effect of naloxone on i.t. Neuropeptide FF-induced anti-allodynia

Fig. 3 shows the effect of pretreatment with naloxone (2.0 mg/kg, s.c.) on the anti-allodynic response induced by Neuropeptide FF (50.0 nmol/10.0 μl) administered i.t. in Freund's Complete Adjuvant-treated rats. There were significant differences in response thresholds between conditions, $F_{(4,39)} = 10.19$, $P < 0.0001$. The allodynic response to mechanical pressure applied to an inflamed paw ($P < 0.001$) was significantly (62.76%) attenuated by Neuropeptide FF ($P < 0.05$), as seen by comparing the thresholds from Freund's Complete Adjuvant conditions Saline–Saline vs. Saline–Neuropeptide FF. Pretreatment with naloxone completely prevented this anti-allodynic effect of Neuropeptide FF ($P < 0.05$). Naloxone pretreatment alone had no effect on response thresholds ($P > 0.05$). One rat was not tested because it failed to show an inflammatory response.

Fig. 4 shows the effect of blocking opioid receptors on the anti-allodynic response to Neuropeptide FF (50.0 nmol/10.0 μl) administered i.t. in rats experiencing neuropathic pain. There was a significant treatment effect,

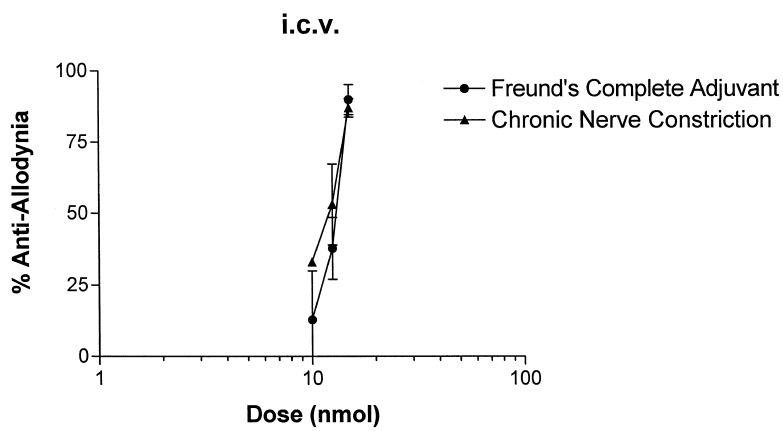


Fig. 2. Mean percentage of anti-allodynia (±S.E.M.) 30 min following escalating doses of Neuropeptide FF (10.0, 12.5, and 15.0 nmol/10.0 μl) administered i.c.v. in a model of inflammatory (Freund's Complete Adjuvant; closed circles) or neuropathic (Chronic Nerve Constriction; closed triangles) pain. There were eight to nine rats per dose in each experiment. Each point represents the average (±S.E.M.) of the mean percentage of anti-allodynia (±S.E.M.) of three experiments.

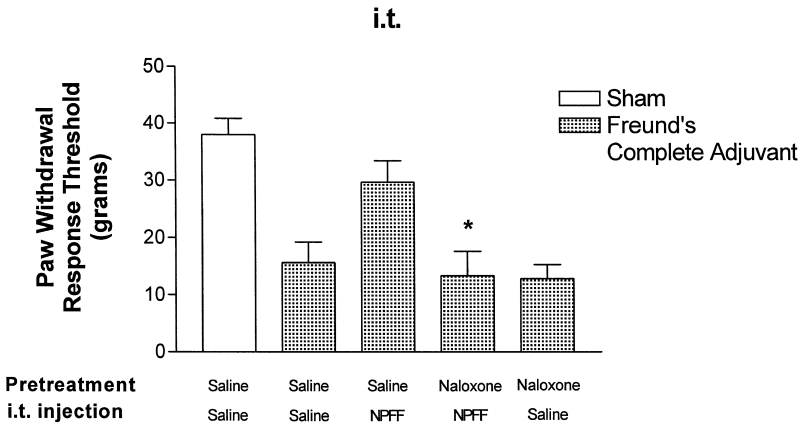


Fig. 3. Mean paw withdrawal response thresholds (±S.E.M.) to mechanical stimulation applied to either an inflamed (Freund's Complete Adjuvant; closed bars) or a non-inflamed (Sham; open bar) paw 30 min following the i.t. administration of either Neuropeptide FF (NPFF; 50.0 nmol/10.0 μl) or saline. All rats (eight to nine per condition) were pretreated with a s.c. injection of either the opioid receptor antagonist, naloxone (2.0 mg/kg), or saline. Significantly different from Freund's Complete Adjuvant condition Saline–NPFF: $^{*}P < 0.05$.

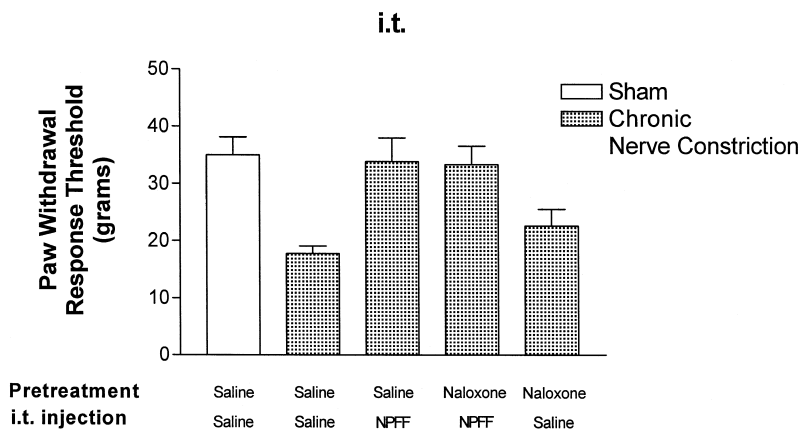


Fig. 4. Mean paw withdrawal response thresholds (±S.E.M.) to mechanical stimulation applied to a chronic nerve-constricted paw (Chronic Nerve Constriction; closed bars) or a sham-operated paw (Sham; open bar) 30 min following Neuropeptide FF (NPFF; 15.0 nmol/10.0 μl) or saline administered i.t. All rats (eight to nine per condition) were pretreated with a s.c. injection of either the opioid receptor antagonist, naloxone (2.0 mg/kg), or saline.

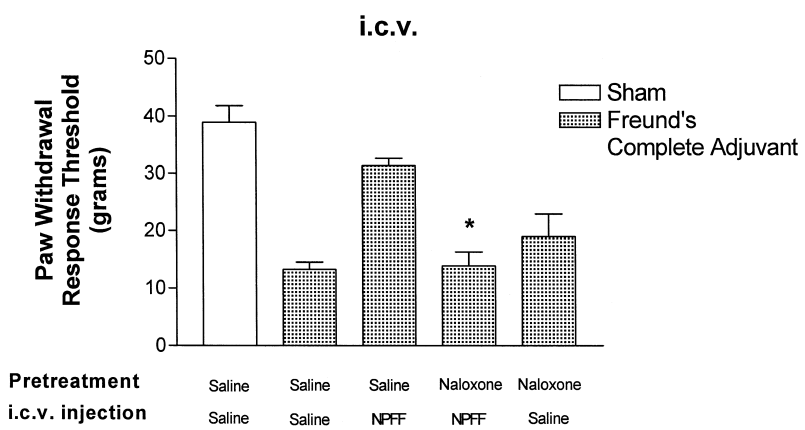


Fig. 5. Mean paw withdrawal response thresholds (±S.E.M.) to mechanical stimulation applied to either an inflamed (Freund's Complete Adjuvant; closed bars) or a non-inflamed (Sham; open bar) paw 30 min following the i.c.v. administration of either Neuropeptide FF (NPFF; 15.0 nmol/10.0 μl) or saline. All rats (seven to eight per condition) were pretreated with a s.c. injection of either the opioid receptor antagonist, naloxone (2.0 mg/kg), or saline. Significantly different from Freund's Complete Adjuvant condition Saline–NPFF: $^{*}P < 0.001$.

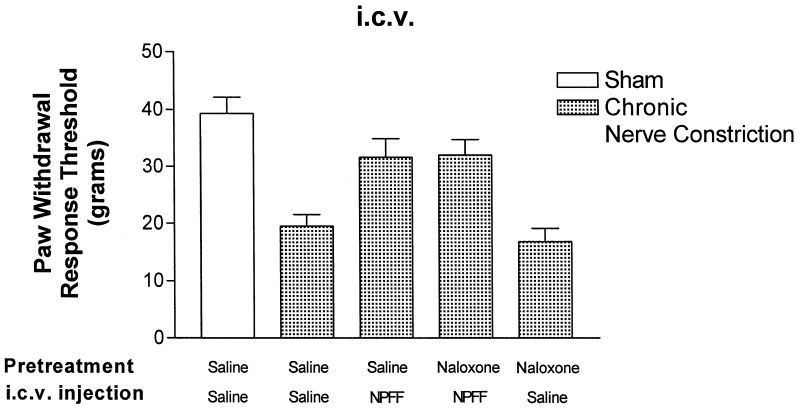


Fig. 6. Mean paw withdrawal response thresholds (±S.E.M.) to mechanical stimulation applied to a chronic nerve-constricted paw (Chronic Nerve Constriction; closed bars) or to a sham-operated paw (Sham; open bar) 30 min following Neuropeptide FF (NPFF; 15.0 nmol/10.0 μl) or saline administered i.c.v. All rats (seven to eight per condition) were pretreated with a s.c. injection of either the opioid receptor antagonist, naloxone (2.0 mg/kg), or saline.

$F_{(4,38)} = 6.78$, $P < 0.0005$. Neuropeptide FF caused potent (93.48%) anti-allodynia, as seen by comparing the thresholds from Chronic Nerve Constriction conditions Saline–Saline vs. Saline–Neuropeptide FF ($P < 0.01$). The anti-allodynic effect of i.t. Neuropeptide FF was not attenuated by naloxone (2.0 mg/kg, s.c.) pretreatment ($P > 0.05$). Naloxone administered alone was without effect on the response to neuropathic pain ($P > 0.05$). All rats subjected to sciatic nerve constriction were tested as they all showed an abnormal posture.

### 3.5. Effect of naloxone on i.c.v. Neuropeptide FF-induced anti-allodynia

Fig. 5 shows the effect of naloxone on the anti-allodynic effect of Neuropeptide FF (15.0 nmol/10.0 μl) administered i.c.v. to rats experiencing inflammatory pain. The ANOVA revealed that treatment conditions were significantly different, $F_{(4,33)} = 18.54$, $P < 0.0001$. Animals subjected to inflammatory pain displayed significant allodynia ($P < 0.001$), and i.c.v. Neuropeptide FF significantly (70.52%) suppressed this response ($P < 0.001$). Pretreatment with naloxone (2.0 mg/kg, s.c.) completely prevented the anti-allodynic effect of i.c.v. Neuropeptide FF ($P < 0.001$). Naloxone administered alone was without effect on response thresholds to neuropathic pain ($P > 0.05$).

Fig. 6 shows the effect of Neuropeptide FF (15.0 nmol/10.0 μl) administered i.c.v. on allodynia in the neuropathic model in rats pretreated with naloxone (2.0 mg/kg, s.c.). Response thresholds were significantly different between conditions, $F_{(4,33)} = 11.79$, $P < 0.0001$. Neuropeptide FF significantly (61.27%) attenuated the response to neuropathic pain ($P < 0.05$), but this effect was not attenuated by naloxone ($P > 0.05$). Naloxone itself had no effect on the response to neuropathic pain ($P > 0.05$).

### 3.6. Effect of i.t. or i.c.v. Neuropeptide FF on locomotor activity

Experiments were designed to study the time-course of the effect of Neuropeptide FF administered i.t. or i.c.v. (50.0 and 15.0 nmol, respectively) on locomotor activity in neuropathic or inflamed animals (data not shown). Neuropeptide FF was without effect on mobile activity counts in neuropathic and inflamed animals following i.t. [$F_{(1,42)} = 0.61$, $P = 0.44$ and $F_{(1,45)} = 0.06$, $P = 0.8$, respectively] or i.c.v. [$F_{(1,45)} = 0.09$, $P = 0.76$ and $F_{(1,42)} = 0.13$, $P = 0.72$, respectively] administration. In terms of static activity (i.e., stereotypy) counts, there were no significant differences between saline and Neuropeptide FF conditions in neuropathic and inflamed rats at any time point following the i.t. [$F_{(1,42)} = 0.64$, $P = 0.43$, and $F_{(1,42)} = 0.17$, $P = 0.69$, respectively] or i.c.v. [$F_{(1,42)} = 0.44$, $P = 0.51$, and $F_{(1,41)} = 0.27$, $P = 0.61$, respectively] injections. With respect to rearing activity counts, Neuropeptide FF did not affect rearing activity counts of neuropathic and inflamed rats at any time point after i.t. [$F_{(1,42)} = 0.34$, $P = 0.56$ and $F_{(1,45)} = 0.13$, $P = 0.72$, respectively] or i.c.v. [$F_{(1,42)} = 0.32$, $P = 0.57$ and $F_{(1,41)} = 0.21$, $P = 0.65$, respectively] injections.

## 4. Discussion

The present findings indicate that, at the doses tested, Neuropeptide FF administered i.t. or i.c.v. does not affect the withdrawal responses in the tail-flick or paw pressure test. These findings suggest that, at the doses tested, Neuropeptide FF acting in the central nervous system does not play a role in the modulation of acute thermal and

mechanical pain. The finding that i.t. Neuropeptide FF was without effect on acute pain is inconsistent with that of Gouardères et al. (1993) and Xu et al (1999) who found that i.t. Neuropeptide FF induces potent antinociceptive and long-lasting effects in the same acute pain tests. It is unclear why different effects were observed. Differences in the doses administered might account for these inconsistent findings. For instance, in our study, Neuropeptide FF was administered using a dose of 50.0 nmol whereas, in the study by Gouardères et al. (1993), doses ranging between 0.44 and 17.5 nmol induced antinociceptive effects. Perhaps, the antinociceptive effect of Neuropeptide FF in acute pain tests is lost using doses higher than 17.5 nmol. It is also possible that differences in the method of injection and/or exact injection site explain the different findings. We administered Neuropeptide FF via a direct transcutaneous injection into the intrathecal space between $L_5$ and $L_6$, whereas Gouardères et al. (1993) and Xu et al. (1999) administered the peptide i.t. at the rostral edge of the lumbar enlargement using chronic intrathecal cannulae. With respect to the supraspinal effects of Neuropeptide FF on acute pain, our finding that i.c.v. Neuropeptide FF (15.0 nmol) was ineffective is consistent with that of Oberling et al. (1993). In the latter study, Neuropeptide FF administered i.c.v. (0.009–9.25 nmol) during the light phase of the light–dark cycle had no effect on tail-flick latencies at the same time points (30 and 60 min post-injection) that were tested in our experiment.

Interestingly, whereas Neuropeptide FF was without effect on acute pain in normal animals, it decreased the threshold of the withdrawal response to a mechanical stimulus in models of chronic inflammatory and neuropathic pain. Indeed, Neuropeptide FF administered directly into either the intrathecal space between $L_5$ and $L_6$ or into the cerebral ventricles dose-dependently attenuated the allodynic response to mechanical stimulation applied to either an inflamed paw or a paw subjected to sciatic nerve constriction. It is unlikely that the anti-allodynic effects of Neuropeptide FF acting spinally and supraspinally were due to secondary motor effects because we found that Neuropeptide FF injected i.t. or i.c.v. in inflamed and neuropathic rats did not produce any sign of motor dysfunction at the time (i.e., 30 min post-injection) at which the anti-allodynic effects were measured. Xu et al. (1999) similarly concluded that the antinociceptive effects of i.t. Neuropeptide FF they observed in models of inflammation and neuropathy were unlikely to be confounded by secondary motor disturbances.

With respect to the effects of i.t. Neuropeptide FF in neuropathic rats, the present results are consistent with those of Courteix et al. (1999) and Xu et al (1999) who found that the Neuropeptide FF analogue, [D-Tyr$^1$,-(NMe)Phe$^3$] Neuropeptide FF (1DMe), induces dose-dependant antinociception in rats with a neuropathy induced by either a nerve injury or diabetes. As well, our finding that i.t. Neuropeptide FF attenuates allodynia in a model of inflammation parallels that of Xu et al. (1999) who showed that [D-Tyr$^1$,(NMe)Phe$^3$] Neuropeptide FF (1DMe) induces anti-allodynia and anti-hyperalgesia in inflamed rats. In the case of nerve injury-induced neuropathic pain, our findings as well as those of Courteix et al. (1999) and Xu et al (1999) indicate that the antinociceptive effects of i.t. Neuropeptide FF are present 30 min following the injection. Unfortunately, it was beyond the scope of the present investigation to examine the time-course of the effects of Neuropeptide FF in models of chronic pain, as did Courteix et al. (1999) and Xu et al. (1999) who showed that the effects persisted until 120 and 45 min, respectively. Preliminary findings from our laboratory, however, suggest that the anti-allodynic effects of i.t. Neuropeptide FF in the inflammatory pain model are long-lasting, persisting until 24 h after its administration. This long-lasting effect of i.t. Neuropeptide FF is reminiscent of that found by Gouardères et al. (1993) using a model of acute pain. In further comparing the present results with those of Courteix et al. (1999) and Xu et al. (1999), it is interesting to note that i.t. Neuropeptide FF induces antinociception in models of neuropathy whether evoked pain behavior is measured in terms of a motor withdrawal response or a vocalization threshold. These findings suggest that Neuropeptide FF may modulate not only the sensory but also the affective dimension of chronic pain. Finally, with respect to the effects of i.c.v. Neuropeptide FF on neuropathic pain, our findings agree with those of Wei et al. (1998) who found that Neuropeptide FF administered supraspinally into the periaqueductal gray is antinociceptive in a model of neuropathic pain. This previous finding suggests that the anti-allodynic effects of i.c.v. observed here could be mediated by the stimulation of Neuropeptide FF receptors in the periaqueductal gray. The antinociceptive effects of i.c.v. Neuropeptide FF could also be mediated by the stimulation of Neuropeptide FF receptors in the ventral tegmental area of the midbrain, where these receptors are present (Marco et al., 1995) and where the activation of dopamine neurons at this site inhibits chronic pain in rats (Altier and Stewart, 1999).

Pretreatment with the opioid receptor antagonist, naloxone, blocked the anti-allodynic effect of Neuropeptide FF injected i.t. or i.c.v. in the inflammatory, but not the neuropathic pain model. These findings suggest that the anti-allodynic effects of Neuropeptide FF are mediated indirectly by naloxone-sensitive opioid mechanisms in the former, but not the latter model. It is not clear why Neuropeptide FF causes an apparent opioid vs. non-opioid anti-allodynic effect depending on the type of chronic painful stimulus. Perhaps the non-opioid nature of Neuropeptide FF's antinociceptive effect in neuropathic rats is related to the finding that neuropathic pain in animal models is unresponsive to opioids (e.g., Kontinen et al., 1998; Wegert et al., 1997), although this issue remains unclear as there are numerous inconsistencies in the literature. It could be argued that a dose of naloxone higher than

the one used in the present studies (i.e., 2.0 mg/kg) might have been effective at blocking the anti-allodynic effects of i.t. and i.c.v. Neuropeptide FF in the neuropathic model. Using a higher dose of naloxone, however, will not change the interpretation of the finding that there is a dissociation in the effect of naloxone on Neuropeptide FF-induced anti-allodynia depending on the type of pain experienced, using a dose that is known to be effective at blocking naloxone-sensitive opioid receptors. Nevertheless, to verify the replicability of the present findings, future studies should explore in more detail the role of opioids in the anti-allodynic effects of spinal and supraspinal Neuropeptide FF in models of chronic pain, using a large range of doses of opioid receptor antagonists.

The finding that Neuropeptide FF induces antinociception through non-opioid mechanisms in the neuropathic pain model is consistent with the results of Wei et al. (1998). Indeed, they similarly found that naloxone injected systemically (as well as into the periaqueductal gray) failed to block the anti-allodynic effect of Neuropeptide FF administered into the periaqueductal gray. Our findings do not, however, agree with those of Courteix et al. (1999) who showed that the μ receptor antagonist, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$ (CTOP), and the δ receptor antagonist, naltrindole, block the antinociceptive effects of [D-$Tyr^1$,(NMe)$Phe^3$] Neuropeptide FF (1DMe). These contradictory results are difficult to explain. They could be due to differences in the methods used between the two studies such as the route of administration of the opioid receptor antagonists (s.c. vs. i.t.), the drugs and doses administered (e.g., naloxone vs. D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$ and naltrindole), the response measured (allodynia vs. vocalization) or the extent of the physiological changes caused by the nerve injury (e.g., loss of pre-synaptic opioid receptors). Future studies should clarify the inconsistencies observed concerning the neurochemical nature of the antinociceptive effects of Neuropeptide FF in models of neuropathic pain.

In summary, the present findings indicate that Neuropeptide FF acting in the spinal cord and at supraspinal levels dose-dependently attenuates mechanical allodynia in models of chronic inflammatory and neuropathic pain without inducing any motor side effect. The anti-allodynic effects of Neuropeptide FF appear to be mediated indirectly by naloxone-sensitive endogeneous opioids in rats experiencing inflammatory, but not neuropathic pain. In contrast, Neuropeptide FF was without effects on acute pain in the tail-flick and paw pressure tests. These findings suggest that Neuropeptide FF plays an important role in modulating chronic, but not acute pain and that its effects on chronic pain occur through an interaction with opioid and/or non-opioid mechanisms, depending on the nature of the chronic painful stimulus. These results also suggest that Neuropeptide FF may represent a promising pharmacological tool for the treatment of persistent pain.

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