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Spinal μ-, δ- and κ-opioid receptors mediate intense stimulation-elicited inhibition of a nociceptive reflex in the rat

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Abstract

Intense electrical stimulation of meridian points in the rat inhibits the nociceptive tail withdrawal reflex. The objective of the present study was to determine whether spinal opioid receptors mediate this inhibition. Electrical stimulation was applied with 2 ms square pulses, at 4 Hz for 20 min at 20 times the threshold, to previously defined meridian points in the hindlimb. Threshold was the minimum current required to elicit muscle twitch. In lightly anaesthetized intact rats ($n = 8$) stimulation inhibited tail withdrawal during and for greater than one hour after the end of stimulation. In unanaesthetized spinal rats ($n = 12$) this inhibition was less and the post-stimulation effect lasted for 15 min. In control anaesthetized intact ($n = 28$) and unanaesthetized spinal rats ($n = 14$) placement of electrodes without stimulation had no effect. In spinal rats, preadministration of naloxone (25 mg/kg, i.p.) blocked the evoked inhibition ($n = 11$). In intact animals both naloxone ($n = 8$) and the μ-opioid receptor antagonist, β-funaltrexamine (10 nmol; $n = 9$), given via a chronic intrathecal catheter, attenuated inhibitions during and after the end of stimulation by 50–60%. The δ-opioid receptor antagonist H–Tyr–ticψ[CH_2NH]Phe–Phe–OH (TIPP[ψ]; 10 nmol; $n = 7$) and the κ-opioid receptor antagonist nor-binaltorphimine (10 nmol; $n = 13$) given by lumbar puncture attenuated the inhibition during the stimulation by 30% and 56%, respectively; both antagonists blocked the post-stimulation effect and even facilitated the withdrawal. The data suggest that spinal μ-, δ- and κ-opioid receptors each contribute to the evoked inhibition.

Keywords: Nociception; Antinociception; Pain; Analgesia; Spinal reflex; Opioid receptor; Antagonist; Naloxone; β-Funaltrexamine; H–Tyr–ticψ[CH_2NH]Phe–Phe–OH, TIPPψ; Nor-binaltorphimine; Electrical stimulation; (Rat)

1. Introduction

A brief noxious stimulation applied cutaneously inhibits responses of convergent dorsal horn neurones to C fibre input (Le Bars and Villanueva, 1988; Ness and Gebhart, 1991) as well as nociceptive withdrawal reflexes in the rat (Morgan et al., 1994; Ness and Gebhart, 1991; Pitcher et al., 1995b). On the other hand, prolonged intense electrical stimulation of a peripheral nerve or applied cutaneously produces a long-lasting depression of the flexor reflex or of the sural–gastrocnemius reflex in the spinal and decerebrated cat (Chung et al., 1983) and the spinal rabbit (Taylor et al., 1990), and intense stimulation of a peripheral nerve or hindlimb meridian points produces a long-lasting inhibition of the jaw-opening reflex in lightly anaesthetized rats (Kawakita and Funakoshi, 1982).

High intensity stimulation of different somatotopic sites evokes antinociceptive effects which are different in their profile. In lightly anaesthetized rats (Toda and Ichioka, 1978) and in cats (Fung and Chan, 1976) intense stimulation of meridian points evokes antinociceptive effects greater than those in response to stimulation of non-meridian points. Furthermore, we have recently shown that high intensity stimulation of meridian points depresses the tail withdrawal reflex during the stimulation and for greater than 1 h after the end of stimulation and stimulation of non-meridian points evokes only a short lasting inhibition (Romita and Henry, 1996).

Antinociceptive effects, evoked by either intense, brief (Le Bars and Villanueva, 1988) or prolonged stimulation (Chung et al., 1983) or intense stimulation of cutaneous structures (Taylor et al., 1990) are antagonized by naloxone, suggesting that the evoked effect is mediated via activation of opioid receptors. Although opiates have been used for the treatment of pain dating back to the times of

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ancient Greece and Rome, it was not until the early 1970's that opiates such as morphine were implicated in the modulation of sensory transmission at the level of the first synapse in spinal cord (Dostrovsky and Pomeranz, 1973; Calvillo et al., 1974). Since then, μ-, δ- and κ-opioid receptors (Besse et al., 1991; Stevens et al., 1991) have been identified in dorsal horn of the spinal cord and implicated in antinociceptive mechanisms (Fields and Basbaum, 1989). The objective of the present study was to determine whether activation of spinal μ-, δ- and κ-opioid receptors are involved in the inhibition of the tail withdrawal reflex evoked by intense acupuncture-like stimulation of meridian points in the rat. Preliminary data have appeared in abstract form (Romita and Henry, 1994).

2. Materials and methods

In all cases, the guidelines described in The Care and Use of Experimental Animals, of the Canadian Council of Animal Care, Vols. I and II, Second Edition, were strictly followed. The experimental protocols were also reviewed and approved by the McGill University Animal Care Committee.

Male Sprague Dawley rats (300–400 g) were used.

2.1. Intrathecal injection

In initial experiments, intrathecal catheters were used to deliver drug or vehicle and therefore necessitated prior implantation of catheters using the following procedure. Rats were anaesthetized with chloral hydrate (300 mg/kg, i.p.; Fisher Scientific, Montreal, Quebec, Canada). The muscle at the base of the skull was retracted until the occipital membrane was exposed. An incision was made through the membrane and the dura lying underneath. A polyethylene catheter (Intramedic PE-10; Fisher Scientific) of predetermined length was inserted through the incision at the atlanto-occipital junction and gently advanced under the dura until the tip of the catheter lay at the level of the sixth lumbar vertebra. The outer portion of the catheter was fixed with dental cement to a stainless steel cranial screw embedded in the skull. The antibiotic, Tribrissen 24% (0.02 ml/100 g; trimethoprim and sulfodiazine) was injected subcutaneously 3 h prior to and 3 h after the surgery. Animals were left to recover for 2–3 weeks prior to testing, provided no neurological deficit was apparent. Position and patency of the catheter were determined by intrathecal injection of 20 μl of a 2% solution of lidocaine HCl (Astra Pharma, Mississauga, Ontario, Canada) followed by 10 to 15 μl of artificial cerebrospinal fluid (aCSF; aqueous solution of 128.6 mM NaCl, 2.6 mM KCl, 2.0 mM $MgCl_2$ and 1.4 mM $CaCl_2$; phosphate buffered, pH 7.33) into the catheter 1–2 days prior to experimentation; this produced motor deficits such as dragging of the hindlimbs and sensory deficits manifested as lack of withdrawal of the hindlimb and lack of vocalization in response to a noxious pinch of the tail or hindlimb. Only those animals that showed reversible sensory and motor deficits after injection of the local anaesthetic were used in subsequent experiments. The position of the tip of the catheter was confirmed by post-mortem verification.

In other experiments drugs were administered spinally by lumbar puncture at the level of the sixth lumbar vertebra in lightly anaesthetized rats. This method of drug delivery was chosen because the surgical stress resulting from implantation of intrathecal catheters rendered 50% of rats tested unresponsive to electrical stimulation of meridian points. In animals injected by lumbar puncture less than 10% of the rats were unresponsive to electrical stimulation of meridian points. Briefly, a 27 gauge needle was inserted between the fifth and sixth lumbar vertebrae. Upon penetration of the dura, the tail would flick and this was taken as an indication of a successful penetration. Twenty μl of vehicle or drug was delivered using a 50 μl Hamilton syringe.

2.2. Spinal transection

In some animals the spinal cord was transected to determine whether the effects observed in intact rats could be elicited after spinal section and whether these effects could be blocked using naloxone. Rats were anaesthetized with chloral hydrate (300 mg/kg, i.p.; Fisher Scientific). Tribrissen was given as above. The spinal cord was exposed at the 6th and 7th thoracic segments. After making a slit in the dorsal part of the dura mater the cord was transected and aspirated by suction approximately 2 mm caudal and rostral to the level of transection. Gelfoam and/or bone wax was placed into the empty vertebral column to reduce bleeding and to seal the empty cavity. Spinalized rats were maintained on their regular diet and their bladders were voided 3 to 4 times daily for the first 8 days following surgery; after this time bladder function had returned in all animals. Animals were used experimentally 21 days after spinal transection. Animals were selected for study on the basis of the following criteria: (1) all were healthy in general appearance; (2) normal eating and drinking were maintained and there was no long-term weight loss; (3) normal voiding and defecation were sustained after the 8 day period of maintenance; (4) all animals showed normal grooming behaviour; (5) all animals were mobile despite hindlimb paralysis and hindlimb pinch produced a withdrawal reaction. In fact, all animals thus selected exhibited a consistent baseline reaction time throughout the recovery period. Therefore, no animals were omitted for inconsistency of responding.

2.3. Tail withdrawal test

To measure the tail withdrawal latency, a portion of the tip of the tail was blackened to facilitate the absorption of

heat. The blackened tip was positioned above a focused projector bulb which heated the skin surface and provoked the tail withdrawal reflex. Withdrawal of the tail exposed the light beam to a photodetector which stopped a timer measuring reaction time to 0.01 s (Isabel et al., 1981). The intensity of the bulb was set so that the baseline reaction time was 4–6 s. Trials were terminated automatically if the withdrawal did not occur within 12 s, the so-called cut-off time.

At each sample time two readings were taken, separated by 40–50 s, at two different sites within the 2 cm blackened segment of the tail. Thus, the withdrawal latency was never measured twice from the same site within a three to five min interval. The average of the two readings was calculated and the value expressed as a percent of the maximum possible inhibition (MPI) according to a modification of the formula of Yaksh and Rudy (1977).

$$\text{MPI} = \frac{(\text{post-treatment latency} - \text{pre-treatment latency})}{(\text{cut-off time} - \text{pre-treatment latency})} \times 100$$

To measure the withdrawal latency during stimulation, the stimulator was temporarily turned off just long enough for the reading to be taken; this was necessary because the stimulus was above the threshold to elicit a direct contraction of muscles.

2.4. Experimental protocol

In experiments with intact rats, at the beginning of the experiment a rat was lightly anaesthetized with an initial intraperitoneal injection of a freshly prepared mixture of sodium pentobarbital (20 mg/kg, Abbott Laboratories) and chloral hydrate (120 mg/kg, Fisher Scientific). The final solution contained 50% propylene glycol (Baker Chemical, Phillipsburg, NJ, USA) and 30% physiological saline (0.9% NaCl). To maintain a light state of anaesthesia throughout the full duration of the experiment, subsequent injections of the mixture were given. Thus, 1/3 of the initial dose of anaesthetic was given 35.5 min after the first injection; this second injection was timed to occur 1.5 min prior to the beginning of electrical stimulation. Thereafter, subsequent injections of 1/3, 1/4 and 1/6 the initial dose were given at 30 min intervals. The level of anaesthesia was sufficient to prevent any overt sign of discomfort to the rat during experimentation, yet stable withdrawal latencies were obtained for greater than 1.5 h after the second supplementary dose.

The lightly anaesthetized rat was placed in a plastic restrainer on the apparatus used to measure the withdrawal latency (Isabel et al., 1981), needles were inserted and threshold determined. Before testing was started the animals were allowed to stabilize for 30 min, then three baseline readings of the withdrawal latency were taken at 3 min intervals. Stimulation was then applied starting 1 min after the last baseline reading and withdrawal latency was recorded at 2 and 5 min after the onset of stimulation, and at 5 min intervals for up to 95 min.

In spinal transected rats, experiments were performed 21 days after spinal transection. Unanaesthetized rats were placed in plastic restrainers so that only the tail and one hindlimb protruded. Animals were unanaesthetized because in pilot studies the anaesthetic produced inconsistent withdrawal latencies at the doses used in intact rats. The restrainer was covered with a black cloth to minimize visual stimuli. Rats were introduced and habituated to the restrainer for 60–90 min 1 or 2 days prior to experimentation. The protocol was otherwise followed as described above for the lightly anaesthetized preparation.

2.5. Placement of stimulation needles

Two pairs of stainless steel insect pins were inserted into meridian points *fengshi* (GB-31), *femur-futu* (ST-32) and *zusanli* (ST-36) as defined by other groups (Lovenberg and Miller, 1990; Pomeranz and Cheng, 1979; Pomeranz, 1987; Ulett, 1982). To stimulate *femur-futu* (ST-32) the cathode was placed along the medial side of the knee and the anode was inserted through *femur-futu* and placed along the lateral side of the knee so that it lay across *zusanli* (ST-36). To stimulate *fengshi* the electrodes were placed under the femur midway between the hip and the knee. Fig. 1 schematically illustrates the position of hindlimb meridian points with respect to skeletal structures.

2.6. Parameters of electrical stimulation

Needles were connected to coupled Grass stimulators (SD9 and SD5) which passed a train of monophasic square wave pulses to stimulate the four meridian points simultaneously. The standard parameters were 2 ms pulses at 4 Hz for a duration of 20 min and applied at 20 times the

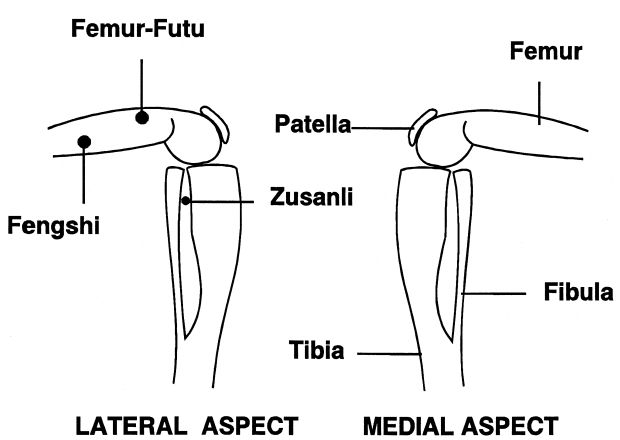

Fig. 1. Schematic location of meridian points of the hindlimb with respect to skeletal structures; medial and lateral aspects.

threshold (20–30 mA). Threshold was the lowest intensity which just produced muscle contraction. Control groups were implanted with stimulating electrodes but received no electrical stimulation.

2.7. Drug preparation

The wide spectrum opiate antagonist, naloxone (Endo Laboratories, NJ, USA; 25 mg/kg i.p.), was dissolved in physiological saline (0.9%) to yield 2.5 mg of antagonist/100 μl saline and administered via intraperitoneal injection 30 min prior to electrical stimulation in intact and spinal animals. The selective μ-opioid receptor antagonist β-funaltrexamine (β-FNA; Research Biochemicals International, Natick, MA, USA) was dissolved in aCSF to yield 10 nmol of antagonist/10 μl of aCSF and was given intrathecally. This solution was made immediately prior to administration, which occurred 24 h prior to the experiment to ensure covalent binding to μ-opioid receptors (Mjanger and Yaksh, 1991). H–Tyr–ticψ-[CH_2NH]Phe–Phe–OH (TIPP[ψ]) (Schiller et al., 1993), a stable pseudopeptide δ-opioid receptor antagonist, was first dissolved in DMSO (dimethyl sulfoxide; Fisher Scientific) and then diluted with aCSF. The final solution contained 10 nmol of the antagonist/20 μl of aCSF and 7.4% of DMSO. Nor-binaltorphimine (nor-BNI; Research Biochemicals International), a selective κ-opioid receptor antagonist, was dissolved in aCSF to yield 10 nmol of the antagonist/20 μl of aCSF. This solution was made immediately prior to the experiment. Twenty μl of TIPP[ψ], nor-binaltorphimine or vehicle was administered via lumbar puncture 1.5 min prior to electrical stimulation.

2.8. Statistical analysis

Results are expressed in two ways either as the net mean increase in tail withdrawal latency expressed as the net mean maximum possible inhibition during each time point or as the average mean maximum possible inhibition calculated for the period of stimulation and for subsequent post-stimulation periods. In lightly anaesthetized rats, the net mean maximum possible inhibition was obtained by subtracting the mean maximum possible inhibition at each time point in the control group from the respective mean maximum possible inhibition in the test group, to eliminate the influence of supplemental doses of anaesthetic. The average mean maximum possible inhibition for the period during the 20 min of stimulation (period 1) and for the three subsequent 25 min periods following the end of stimulation (periods 2–4), was calculated by averaging the net mean maximum possible inhibition obtained from the five readings taken during each period. Mean values calculated for each period were expressed as histograms. All values are expressed as the mean ± standard error of the mean (S.E.M.). Data from treated rats which received electrical stimulation and control animals which received no stimulation were analyzed using two way analysis of variance (ANOVA). Stimulation vs no stimulation or drug treated vs. vehicle was taken as the between subject factor and time was taken as the within subject factor. Tukey's Wholly Significant Difference Test was used to make post hoc comparisons between means. One way repeated measures analysis of variance, non-pair-wise multiple comparisons (Student–Newman–Keuls method) and non-paired Student's *t*-test for single pair-wise comparisons were used to compare the net mean increase in withdrawal latency in periods 1–4, between treatment groups.

3. Results

3.1. Effect of electrical stimulation on withdrawal reflex in intact rats

In control animals treated with an i.p. injection of 0.9% NaCl ($n = 8$), electrical stimulation of hindlimb meridian points inhibited the withdrawal reflex. The mean inhibition during the period of stimulation was 51.7 ± 8.44% of the maximum possible inhibition. After the stimulation ended this inhibition persisted for at least 1 h. Two-way ANOVA showed that this effect during the stimulation and the post-stimulation inhibition was significant ($P \leq 0.01$) when compared to the unstimulated control group ($n = 12$).

3.2. Effect of naloxone on response to electrical stimulation in intact rats

In contrast to vehicle-treated rats, the evoked inhibition in naloxone-treated rats ($n = 8$) was less. The mean inhibition during this period was 35.1 ± 3.56% of the maximum possible inhibition. A low-amplitude post-stimulation effect was observed which lasted only 15 min after the end of stimulation. Two-way ANOVA showed that both the inhibition during electrical stimulation and the persistent post-stimulation inhibition were greater than those in the unstimulated control group and less than those in the stimulated group receiving an i.p. injection of saline ($p \leq 0.01$), respectively. Fig. 2 illustrates the data as histograms representing the net mean inhibition calculated for the period during the stimulation and for the following post-stimulation periods for both the vehicle and naloxone treated groups. Non-paired Student's *t*-tests show that the attenuation produced by naloxone was significant for all periods ($P \leq 0.05$). In Fig. 6, the percent inhibition of the evoked response for each of the respective periods and by each of the opioid antagonists is summarized. Naloxone attenuated the evoked response by 32.0% and 56.7% in periods 1 and 2, respectively. For periods 3 and 4 the inhibition of the effect was approximately 61% of that in the control group.

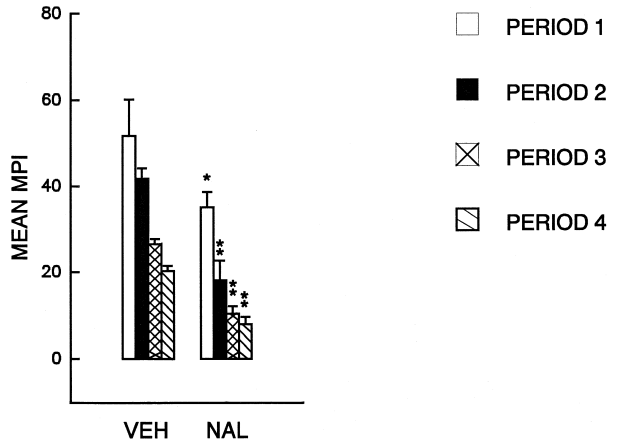

Fig. 2. Effect of intense electrical stimulation of hindlimb meridian points *femur-futu* (GB-31), *fengshi* (ST-32) and *zusanli* (ST-36) on tail withdrawal latency in vehicle ($n = 8$) and naloxone (25 mg/kg; i.p; $n = 8$) treated anaesthetized intact rats. Standard parameters of stimulation were 2 ms square wave pulses, at 2 Hz for 20 min at 20 × threshold to just evoke muscle twitch. The net mean inhibition of the tail withdrawal was calculated for the first 20 min period during stimulation and for subsequent 25 min periods after the end of stimulation. Data are expressed as the mean % maximum possible inhibition (MPI). The open bar represents period 1 (0–20 min) during the stimulation, the closed bar represents period 2 (25–45 min) after the stimulation, the cross hatched bar represents period 3 (50–70 min), and the diagonal filled bar represents the final period of observation, period 4 (75 – 95 min). $*$ $P < 0.05$; $**$ $P < 0.01$.

3.3. Effect of electrical stimulation in spinalized rats

In unanaesthetized rats, three weeks after spinal transection electrical stimulation produced a small but significant inhibition both during and after the end of stimulation ($n = 12$). The evoked inhibition reached a value of 23.1 ± 4.81 and 22.0 ± 3.09% of the maximum possible inhibition at 10 and 20 min after the onset of stimulation, respectively. For 15 min after the end of stimulation the inhibition remained slightly elevated at about 8% of the maximum possible inhibition. A two-way ANOVA showed this effect to be statistically different ($P < 0.01$) from the unstimulated control group ($n = 14$). Spinalization attenuated the amplitude and shortened the duration of the evoked antinociception when compared to the stimulated intact rats ($n = 8$). The data are illustrated in Fig. 3.

3.4. Effect of naloxone on response to electrical stimulation in spinalized rats

Pretreatment with naloxone blocked the stimulation-evoked inhibition of the withdrawal reflex ($n = 11$). The data are illustrated in Fig. 3. A two-way ANOVA showed this antagonism to be significant ($P < 0.01$) when compared to the vehicle-treated rats ($n = 12$) during stimulation. Although the values of the mean inhibition for each time taken during the first 15 min after the end of stimulation were lower in the naloxone treated group than the

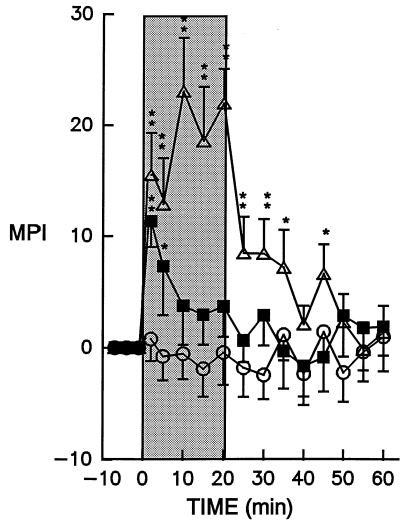

Fig. 3. Effect of naloxone on stimulation-evoked inhibition in unanaesthetized spinalized rats three weeks after transection. Transection was at the T6/T7 spinal level. △: vehicle ($n = 12$), ■: naloxone (25 mg/kg; i.p.; $n = 11$) and ○: needles only ($n = 14$). Parameters of stimulation are as in Fig. 2. $**$ $P < 0.01$.

those obtained from the vehicle-treated group, no difference was found when these two groups were compared statistically. Also, no significant difference between the naloxone treated group and the unstimulated control group was found.

3.5. Effect of spinal administration of vehicle on the stimulation-evoked inhibition in intact rats

Fig. 4 illustrates the brief and the long-lasting inhibition of the tail withdrawal reflex provoked by intense electrical

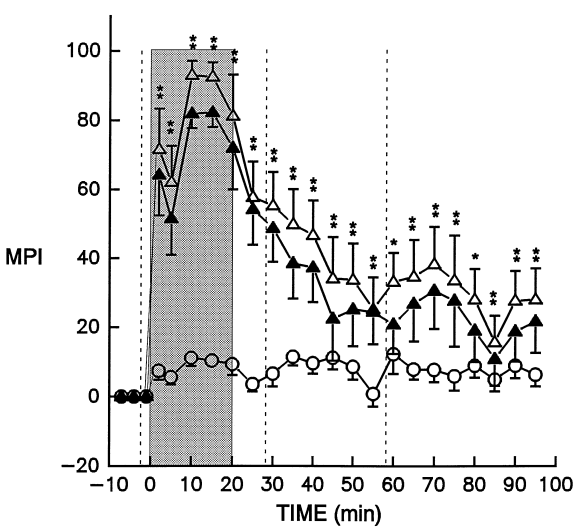

Fig. 4. Effect of spinal administration of vehicle on the stimulation-evoked inhibition in intact rats. Vertical dotted lines indicate times of administration of anaesthetic. Inhibition of the withdrawal reflex is expressed as a % of the maximum possible inhibition. △ stimulated group ($n = 8$); ○ needles only ($n = 16$); ▲ difference between the two groups. $**$ $P < 0.01$.

stimulation in rats given vehicle by lumbar puncture ($n = 8$). During the stimulation the effect reached 82.2 ± 4.17% of the maximum possible inhibition. The mean effect calculated during stimulation was 70.3 ± 5.79% of the maximum possible inhibition. This inhibitory effect on the withdrawal reflex persisted for greater than one hour after the end of stimulation. A two-way ANOVA showed that the inhibition was significant ($P \leq 0.01$) when compared to the unstimulated control group ($n = 16$). Both the test group and the unstimulated control group received spinal administration of aCSF or aCSF containing DMSO, by lumbar puncture. There was no difference in the evoked inhibition between groups treated with aCSF ($n = 4$) or aCSF-DMSO ($n = 4$) by lumbar puncture nor was there any difference between the two unstimulated control groups ($n = 8$ in each group) treated with aCSF or aCSF containing DMSO. Therefore, the data were pooled. The ANOVA also failed to show any statistical difference in the evoked inhibition between rats in which vehicle was given intraperitoneally ($n = 8$), intrathecally ($n = 10$) or by lumbar puncture ($n = 8$).

3.6. Effect of β-funaltrexamine on stimulation-evoked inhibition in intact rats

Intrathecal administration of β-funaltrexamine 24 h before testing attenuated the inhibition during stimulation and blocked the post-stimulation antinociception ($n = 9$). The mean effect was 34.3 ± 5.93% of the maximum possible inhibition during the period of stimulation. A two-way ANOVA showed that this effect was significantly different ($P \leq 0.05$) when compared to the unstimulated control group ($n = 8$). No statistical difference was seen after the end of stimulation between these two groups. By comparison, in rats treated with an intrathecal injection of aCSF ($n = 10$), stimulation produced a mean inhibition of 72.3 ± 4.8% of the maximum possible inhibition during the stimulation. This effect lasted beyond 1 h after the end of stimulation. The evoked inhibition in this group of rats was statistically greater ($P \leq 0.01$) than the inhibition evoked in the β-funaltrexamine treated group. Fig. 5A illustrates the net mean inhibition elicited by stimulation for periods 1 to 4 for both the vehicle and β-funaltrexamine treated rats. Non-paired Student's *t*-tests showed that the attenuation produced by the μ-opioid receptor antagonist was significant for all periods ($P \leq 0.01$). β-funaltrexamine produced a 53% inhibition of the evoked response during period 1, an 85.2% inhibition during period 2 and a 50–60% inhibition during periods 3 and 4 (Fig. 6).

3.7. Effect of TIPP[ψ] on stimulation-evoked inhibition in intact rats

TIPP[ψ] given via lumbar puncture slightly attenuated the inhibition produced during the stimulation but completely blocked the post-stimulation effect ($n = 7$). During the stimulation the mean inhibition was 48.9 ± 4.70% of the maximum possible inhibition. This effect was statistically significant ($P \leq 0.01$) when compared to the unstimulated control group ($n = 16$) using two-way ANOVA. No significant difference for the post-stimulation values was

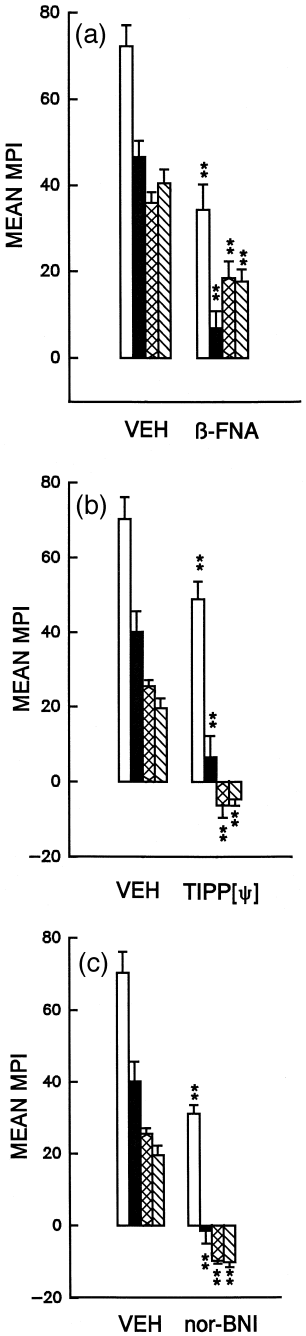

Fig. 5. Comparison of effects of spinal administration of vehicle and of selective opioid receptor antagonists on stimulation-evoked inhibition in anaesthetized intact rats. (A) β-funaltrexamine (10 nmol; $n = 9$) or vehicle ($n = 10$) was given to the lower lumbar level 24 h prior to stimulation. (B) TIPP[ψ] (10 nmol; $n = 7$), (C) Nor-binaltorphimine (10 nmol; $n = 13$) or vehicle ($n = 8$) was given to the lower lumbar level 1.5 min prior to stimulation. Details are as in Fig. 2.

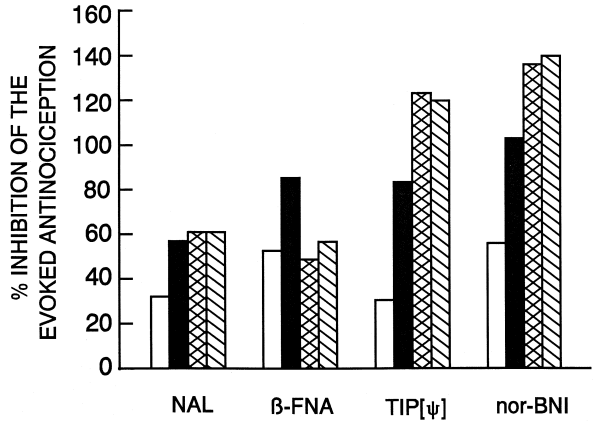

Fig. 6. Percent inhibition of stimulation-evoked antinociception for periods 1 through 4 by naloxone, β-funaltrexamine, TIPP[ψ] and nor-binaltorphimine. The horizontal dotted line represents 100% inhibition of the evoked antinociception. Values above this line represent facilitation of the withdrawal reflex.

seen between these two groups. The inhibition produced in rats treated with TIPP[ψ] was also significantly less ($P \leq 0.01$) than that produced in the stimulated group receiving only vehicle. In the vehicle treated rats, the stimulation evoked a mean inhibition of about 72% of the maximum possible inhibition during the stimulation and this effect persisted for more than 1 h after the end of stimulation ($n = 8$) as shown in Fig. 4. Illustrated in Fig. 5B and 6 is the effect of TIPP[ψ] on the evoked response. The evoked antinociception during periods 1 and 2 was attenuated by 30.4% and 83.3%, respectively. During periods 3 and 4, TIPP[ψ] produced a reversal (i.e. facilitation of the tail withdrawal reflex); the net mean maximum possible inhibition was reversed to approximately 125% of the control. Non-paired Student's *t*-tests showed the attenuation and reversal by TIPP[ψ] to be significant ($P \leq 0.01$).

3.8. Effect of nor-binaltorphimine on stimulation-evoked inhibition

Nor-binaltorphimine given by lumbar puncture also attenuated the evoked inhibition during the stimulation and blocked the post-stimulation effect ($n = 13$). During the stimulation, the mean inhibition calculated for this period was $31.2 \pm 2.41\%$ of the maximum possible inhibition. This response was statistically different ($P < 0.01$) when compared to the vehicle-treated, unstimulated control group ($n = 16$) using two-way ANOVA. The post-stimulation values were not statistically different between these two groups. The mean inhibition at each time point was also significantly less than the evoked inhibition in the stimulated vehicle group ($0.05 \geq P \leq 0.01$). Fig. 5C illustrates the average net mean inhibition calculated for periods 1 to 4. Fig. 6 shows that the κ-opioid receptor antagonist decreased the evoked inhibition for period 1 by approximately 56%. During period 2 the inhibition was completely blocked and during periods 3 and 4 there was a net mean facilitation in the withdrawal reflex; responses during periods 3 and 4 were increased to 136% and 140% of the control values. Non-paired Student's *t*-tests demonstrated that the attenuation during the stimulation, and the block and reversal were statistically significant ($P < 0.01$).

4. Discussion

These data show that spinal μ-, δ- and κ-opioid receptors are involved in mediation of the decreased activity in nociceptive pathways provoked by sustained intense peripheral electrical stimulation. In our paradigm this decrease was reflected as an inhibition of the thermally evoked tail withdrawal reflex. This inhibition occurred during the conditioning stimulation and persisted for more than one hour after the end of the stimulation. Furthermore, it appears that the three opioid receptors differentially contribute to the evoked inhibition of this reflex. During the stimulation μ-, κ- and, to a lesser extent, δ-opioid receptors mediate at least part of the inhibition, while δ-, κ- and, to a lesser extent, μ-opioid receptors mediate the post-stimulation inhibition.

4.1. Naloxone antagonism implicates opioid receptors in stimulation-evoked inhibition of the tail withdrawal reflex

In the pilot study done to determine if an opioid mechanism might be involved, a high dose of naloxone attenuated the evoked inhibition in intact animals and completely blocked inhibition of the tail withdrawal in chronic spinal transected rats. This suggested that the evoked inhibition was mediated at least partly by activation of μ- and probably also by activation of other opioid receptors. In vitro studies show that naloxone is predominantly a μ-opioid receptor antagonist but at higher concentrations it can act as a wide spectrum antagonist at δ- and κ-receptors (Lord et al., 1977). Furthermore, in animal studies a high dose of naloxone given systemically has been used to block effects believed to be mediated via multiple opioid receptors (Berkowitz et al., 1977). Systemic doses up to 40 mg/kg have been administered to block opioid mediated inhibition of the tail withdrawal reflex (Berkowitz et al., 1977) in the rat. In light of this evidence, we chose to give 25 mg/kg of naloxone to ensure maximum opioid receptor block without compromising the tail withdrawal reflex; 30 mg/kg of naloxone given systemically does not affect the tail withdrawal reflex per se (Berkowitz et al., 1977).

Naloxone blocked the evoked inhibition during the stimulation to the same degree as did the other three antagonists. However, naloxone did not affect the post-stimulation inhibition to the same extent. For example, the mean evoked inhibition during the post-stimulation periods

were completely blocked and reversed by TIPP[ψ] or nor-binaltorphimine. Therefore, even at a dose of 25 mg/kg, naloxone antagonism of the post-stimulation effect appears to be limited. This limitation may be related to the pharmacokinetic properties of naloxone when administered subcutaneously or intraperitoneally, the half life being only 0.4 h (Misra et al., 1976). Furthermore, δ- and κ-opioid receptors have 10 and 30 times less affinity for naloxone binding, respectively, as compared to the μ-opioid receptor (Chang et al., 1980). Therefore, at post-stimulation periods monitored more than one hour after administration of naloxone, levels of the antagonist in the central nervous system may have been inadequate for full block of all opioid receptors.

Our findings are consistent with reports of naloxone antagonism of antinociceptive effects evoked by intense peripheral stimulation. Both cutaneously evoked and acupuncture evoked inhibition of dorsal horn sensory neurones were partially antagonized by naloxone (Bing et al., 1990).

In chronically spinal transected rats, studies have shown that intense peripheral stimulation also produces a naloxone-reversible antinociception in the rat (Watkins et al., 1992a; Stein et al., 1992; Vaccarino et al., 1992; Watkins et al., 1992b). Even in the decerebrated and spinalized cat stimulation of Aδ and C fibres in the common tibial or peroneal nerve evokes a long-lasting depression of the flexion reflex recorded electrophysiologically from the ventral roots (Chung et al., 1983) and in the rabbit prolonged intense electrical stimulation applied to the toes depresses the sural–gastrocnemius reflex (Taylor et al., 1990). In both studies the evoked inhibition was completely reversed by naloxone. It appears from our data and from those reported in the literature, that stimulation-evoked inhibition in the intact animal is only partially reversed by naloxone while almost complete reversal is seen in spinal preparations. Not surprisingly, it appears that spinal antinociceptive mechanisms are opioid mediated while in the spinally intact preparation non-opioid mechanisms may participate also (Fields and Basbaum, 1989).

4.2. β-Funaltrexamine antagonism implicates μ-opioid receptors

Twenty-four hour pretreatment with spinally administered β-funaltrexamine attenuated the stimulus-evoked inhibition. β-funaltrexamine is a non-competitive μ-opioid receptor antagonist which binds covalently and irreversibly to the μ-opioid receptor complex, and has little activity with δ- and κ-opioid receptors (Ward et al., 1982). In the hot plate test, 24 h pretreatment with intrathecally administered β-funaltrexamine produced a concentration-dependent rightward shift of dose response curves for antinociceptive effects produced by spinal administration of several μ-opioid receptor agonists (Mjanger and Yaksh, 1991). In experiments where intense somatic stimulation evokes antinociceptive effects, spinal application of μ-opioid receptor antagonists blocks this effect (Pitcher et al., 1995a). Our results suggest that intense peripheral stimulation of the hindlimb activates short and long-lasting inhibitory mechanisms in the central nervous system which depress transmission of sensory information in the spinal cord, at least in part via activation of μ-opioid receptors.

4.3. Role of μ-opioid receptors in sensory processing in the spinal cord

Our data are consistent with the role of μ-opioid receptor involvement in spinal sensory mechanisms. In the dorsal horn the majority of opioid receptors are of the μ-type (Stevens et al., 1991), located in laminae I and II (Besse et al., 1991; Ding et al., 1996; Honda and Arvidsson, 1995), predominantly presynaptically on primary afferent terminals (Cheng et al., 1996). Activation of presynaptic μ-opioid receptors has been shown to inhibit the release of aspartate and glutamate (Kangrga and Randic, 1991), as well as substance P and CGRP (Bourgoin et al., 1994; Collin et al., 1993) from the spinal cord. Activation of μ-opioid receptors in the spinal slice depresses mEPSCs in dorsal horn neurones but has no effect on responses to exogenous glutamate, suggesting a presynaptic location of μ-opioid receptors (Hori et al., 1992). m-RNA for the μ-opioid receptor has been found in dorsal horn neurones as well as in dorsal root ganglion cells (Mansour et al., 1994). Thus, in addition to a presynaptic site, there is evidence to suggest postsynaptic modulation of sensory transmission via activation of the μ-opioid receptor (Arvidsson et al., 1995; Cheng et al., 1996; Grudt and Williams, 1994; Rusin and Randic, 1991).

It is proposed that intense stimulation of meridian points in the hindlimb of the rat evokes the release of opioids in the spinal cord (Bing et al., 1991; Han et al., 1984) which, through activation of μ-opioid receptors, may attenuate the release of neurotransmitter and/or attenuate postsynaptic responses of neurones in nociceptive pathways. This is supported by evidence demonstrating that substance P-containing boutons of nociceptive neurones synapse onto enkephalin-containing dorsal horn nociceptive neurones (Ribeiro-da-Silva et al., 1992) and, in another study, enkephalin-containing axons oppose μ-opioid receptor-containing dorsal horn neurones (Arvidsson et al., 1995).

4.4. TIPP[ψ] implicates δ-opioid receptors

TIPP[ψ] had a smaller effect on the response during the stimulation than did β-funaltrexamine, yet for the post-stimulation periods this antagonism was greater and the tail withdrawal reflex was even facilitated. TIPP[ψ] is a stable pseudotetrapeptide reported to be a pure δ-opioid

receptor antagonist with selectivity orders of magnitude higher than other such δ-opioid receptor antagonists, both in the central nervous system (Visconti et al., 1994) and in the periphery (Schiller et al., 1993). Ten nmol was given because doses of δ-opioid receptor antagonists in this range have been shown to block the depression of the tail withdrawal reflex elicited by spinal administration of DPDPE, a δ_1-opioid receptor agonist (Drower et al., 1991) and by noxious thermal cutaneous stimulation (Pitcher et al., 1995a) in the rat. In the present study, the antagonism of the evoked effect by TIPP[ψ] suggests that δ-opioid receptors also participate in the stimulation-evoked inhibition.

4.5. Role of δ-opioid receptors in processing of sensory information in the spinal cord

While a smaller portion of the opioid receptors in the dorsal horn are of the of the δ-type (Stevens et al., 1991), there is, nevertheless, evidence that they play a role in spinal nociceptive mechanisms. Antinociception induced by noxious peripheral stimulation is depressed by block of spinal δ-opioid receptors (Pitcher et al., 1995a) and a δ-opioid receptor antagonist increase nociceptive scores in the formalin test (Ossipov et al., 1996). Spinal administration of δ-opioid receptor agonists (Shah et al., 1994; Sofuoglu et al., 1991) depresses the nociceptive tail withdrawal reflex evoked by noxious thermal stimulation. δ-Opioid receptors are found mainly in laminae I and II (Besse et al., 1991; Dado et al., 1993; Honda and Arvidsson, 1995; Zerari et al., 1994), predominantly presynaptically on primary afferent terminals (Le Bars and Villanueva, 1988), although δ-opioid receptor mRNA has been found in both dorsal horn neurones and dorsal root ganglion cells (Mansour et al., 1994). Activation of δ-opioid receptors (Bourgoin et al., 1994; Kangrga and Randic, 1991) inhibits the release of substance P and CGRP from primary afferent terminals (Bourgoin et al., 1994; Collin et al., 1991) and electrophysiological data show that δ-opioid receptor agonists depress synaptically elicited activity in dorsal horn neurones, predominantly via a presynaptic effect (Glaum et al., 1994; Omote et al., 1991; Villanueva et al., 1991). Thus, our data are consistent with the idea that intense stimulation of meridian points in the rat evokes the release of opioids in the spinal cord which, through activation of δ-opioid receptors, may attenuate the release of transmitters and/or attenuate postsynaptic responses of neurones in nociceptive pathways.

4.6. Nor-binaltorphimine implicates κ-opioid receptors

Nor-binaltorphimine antagonized the evoked effect during the stimulation and blocked, and even reversed, the post-stimulation inhibition. Nor-binaltorphimine is a highly potent and selective κ-opioid receptor antagonist with little activity at μ- or δ-opioid receptors and blocks κ-opioid receptor agonist-evoked antinociception in the acetic acid-induced writhing test in mice (Takemori et al., 1988). Nor-binaltorphimine has been given intrathecally to block evoked antinociceptive responses in other experimental paradigms; the effective doses ranged from 6.5 nmol (Pitcher et al., 1995a) and 12 nmol (Chen and Han, 1992) to a high of 39 nmol (Guirimand et al., 1994). On the basis of these studies a dose of 10 nmol was chosen. Antagonism by nor-binaltorphimine of the stimulation-evoked inhibition of the tail withdrawal reflex in our study implicates κ-opioid receptors and suggests a predominant role in mediation of the post-stimulation response.

4.7. Role of κ-opioid receptors in processing of sensory information in the spinal cord

κ-Opioid receptors are sparse in the adult rat spinal cord (Stevens et al., 1991), but there is some binding in the superficial layers of the dorsal horn (Besse et al., 1991). κ-Opioid receptor mRNA is observed in dorsal horn neurones as well as in dorsal root ganglion cells (Mansour et al., 1994), indicating a pre- and postsynaptic location of these receptors. Functional studies have implicated κ-opioid receptors in spinal nociceptive mechanisms. Inhibition of the tail withdrawal reflex by noxious peripheral stimulation is inhibited by intrathecal administration of nor-binaltorphimine (Pitcher et al., 1995a) and inhibition of this reflex has also been shown to occur by a G-protein linked, κ-opioid receptor mechanism (Dawson-Basoa and Gintzler, 1996). κ-Opioid receptor activation also depresses nociceptive responses in the formalin test (Ossipov et al., 1996). Thus, our data are also consistent with the involvement of κ-opioid receptors, either via pre- or postsynaptic mechanisms.

4.8. Evoked antinociception may involve interaction of multiple opioid receptors

We propose that the intense, electrical conditioning stimulation used in our study provokes the release of endogenous opioids which may depress the tail withdrawal reflex by acting on presynaptic and/or postsynaptic opioid receptors in the spinal cord.

Evidence from the literature suggests a functional interaction of opioid receptors. Activation of δ-opioid receptors potentiates the antinociception achieved by activation of μ-opioid receptors (Malmberg and Yaksh, 1992). Concomitant administration of μ- and κ-opioid receptor agonists produces a greater and longer lasting antinociception than either alone (Furst, 1991; Sutters et al., 1990). These synergistic effects could be manifested by either a direct physical/functional coupling of μ- and δ-opioid receptors as shown in rat brain (Vaught et al., 1982) or by activation of common second messenger pathways (Welch and Dunlow, 1993; Zhang et al., 1990). This interactive or syner-

gistic effect may explain our results in that at least in our paradigm there may be a differential contribution by the three opioid receptors with δ- and κ-opioid receptors having a greater role in mediation of the post-stimulation effects. Therefore, activation of μ-opioid receptors may be important in mediation of inhibition during the stimulation and post-stimulation periods, but in addition, activation of δ- and κ-opioid receptors are required to produce responses necessary for the full expression of the post-stimulation antinociception.

4.9. Summary and conclusion

Prolonged intense, low frequency electrical stimulation of specific peripheral sites in the rat hindlimb evokes a long-lasting inhibition of the thermally evoked tail withdrawal reflex. On the basis of antagonism by selective μ-, δ- and κ-opioid receptor antagonists we conclude that activation of all three opioid receptors in the spinal cord is important for the expression of the antinociception. Furthermore, the data suggest that activation of these receptors differentially contributes to the evoked inhibition, in that the antinociception elicited during the stimulation appears to be partially mediated by activation of μ-, κ- and to a lesser extent by δ-opioid receptors. The post-stimulation antinociception appears to be dependent on δ- and κ-opioid receptor activation while μ-opioid receptors playing a lesser role. These data may shed some light on the neurochemical mediation of the long-lasting and general analgesic effects produced by intense electrical stimulation for the clinical treatment of pain.

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References

Arvidsson, U., Riedl, M., Chakrabarti, S., Lee, J.H., Nakano, A.H., Dado, R.J., Loh, H.H., Law, P.Y., Wessendorf, M.W., Elde, R., 1995. Distribution and targeting of a μ-opioid receptor (MOR1) in brain and spinal cord. J. Neurosci. 15, 3328–3341.

Berkowitz, B.A., Finck, A.D., Ngai, S.H., 1977. Nitrous oxide analgesia: reversal by naloxone and development of tolerance. J. Pharmacol. Exp. Ther. 203, 539–547.

Besse, D., Lombard, M.C., Besson, J.M., 1991. Autoradiographic distribution of μ, δ and k opioid binding sites in the superficial dorsal horn, over the rostrocaudal axis of the rat spinal cord. Brain Res. 548, 287–291.

Bing, Z., Villanueva, L., Le Bars, D., 1990. Acupuncture and diffuse noxious inhibitory controls: Naloxone-reversible depression of activities of trigeminal convergent neurons. Neuroscience 37, 809–818.

Bing, Z., Cesselin, F., Bourgoin, S., Clot, A.M., Hamon, M., Le Bars, D., 1991. Acupuncture-like stimulation induces a heterosegmental release of Met-enkephalin-like material in the rat spinal cord. Pain 47, 71–77.

Bourgoin, S., Benoliel, J.J., Collin, E., Mauborgne, A., Pohl, M., Hamon, M., Cesselin, F., 1994. Opioidergic control of the spinal release of neuropeptides. Possible significance for the analgesic effects of opioids. Fundam. Clin. Pharmacol. 8, 307–321.

Calvillo, O., Henry, J.L., Neuman, R.S., 1974. Effects of morphine and naloxone on dorsal horn neurones in the cat. Can. J. Physiol. Pharmacol. 52, 1207–1211.

Chang, K.-J., Hazum, E., Cuatrecasas, P., 1980. Multiple opiate receptors. Trends Neurosci. 3, 160–162.

Chen, X.-H., Han, J.-S., 1992. All three types of opioid receptors in the spinal cord are important for 2/15 Hz electroacupuncture analgesia. Eur. J. Pharmacol. 211, 203–210.

Cheng, P.Y., Moriwaki, A., Wang, J.B., Uhl, G.R., Pickel, V.M., 1996. Ultrastructural localization of μ-opioid receptors in the superficial layers of the rat cervical spinal cord: extrasynaptic localization and proximity to Leu^5-enkephalin. Brain Res. 731, 141–154.

Chung, J.M., Fang, Z.R., Cargill, C.L., Willis, W.D., 1983. Prolonged, naloxone-reversible inhibition of the flexion reflex in the cat. Pain 15, 35–53.

Collin, E., Mauborgne, A., Bourgoin, S., Chantrel, D., Hamon, M., Cesselin, F., 1991. In vivo tonic inhibition of spinal substance P (-like material) release by endogenous opioid(s) acting at δ receptors. Neuroscience 44, 725–731.

Collin, E., Mantelet, S., Frechilla, D., Pohl, M., Bourgoin, S., Hamon, M., Cesselin, F., 1993. Increased in vivo release of calcitonin gene-related peptide-like material from the spinal cord in arthritic rats. Pain 54, 203–211.

Dado, R.J., Law, P.Y., Loh, H.H., Elde, R., 1993. Immunofluorescent identification of a (δ)-opioid receptor on primary afferent nerve terminals. NeuroReport 5, 341–344.

Dawson-Basoa, M.E., Gintzler, A.R., 1996. Estrogen and progesterone activate spinal kappa-opiate receptor analgesic mechanisms. Pain 64, 169–177.

Ding, Y.Q., Kaneko, T., Nomura, S., Mizuno, N., 1996. Immunohistochemical localization of μ-opioid receptors in the central nervous system of the rat. J. Comp. Neurol. 367, 375–402.

Dostrovsky, J., Pomeranz, B., 1973. Morphine blockade of amino acid putative transmitters on cat spinal cord sensory interneurones. Nature 246, 222–224.

Drower, E.J., Stapelfeld, A., Rafferty, M.F., De Costa, B.R., Rice, K.C., Hammond, D.L., 1991. Selective antagonism by naltrindole of the antinociceptive effects of the delta opioid agonist cyclic[D-penicillamine2-D-penicillamine5]enkephalin in the rat. J. Pharmacol. Exp. Ther. 259, 725–731.

Fields, H.L., Basbaum, A.I., 1989. Endogenous pain control mechanisms. In: Wall, P.D., Melzack, R. (Eds.), Textbook of Pain, Vol. 2, Churchill Livingston, Edinburgh, p. 206.

Fung, S.J., Chan, S.H.H., 1976. Primary afferent depolarization evoked by electroacupuncture in the lumbar cord of the cat. Exp. Neurol. 52, 168–176.

Furst, S., 1991. Pharmacological interaction of opiates with various classes of centrally acting dopaminergic drugs. Drug Metab. Drug Inter. 9, 77–102.

Glaum, S.R., Miller, R.J., Hammond, D.L., 1994. Inhibitory actions of δ 1-, δ 2-, and μ-opioid receptor agonists on excitatory transmission in lamina II neurons of adult rat spinal cord. J. Neurosci. 14, 4965–4971.

Grudt, T.J., Williams, J.T., 1994. μ-Opioid agonists inhibit spinal trigeminal substantia gelatinosa neurons in guinea pig and rat. J. Neurosci. 14, 1646–1654.

Guirimand, F., Strimbu-Gozariu, M., Willer, J.C., Le Bars, D., 1994. Effects of *mu*, *delta* and *kappa* opioid antagonists on the depression of a C-fiber reflex by intrathecal morphine and DAGO in the rat. J. Pharmacol. Exp. Ther. 269, 1007–1020.

Han, J.S., Xie, G.X., Zhou, Z.F., Folkesson, R., Terenius, L., 1984.

Acupuncture mechanisms in rabbits studied with microinjection of antibodies against beta-endorphin, enkephalin and substance P. Neuropharmacology 23, 1–5.

Honda, C.N., Arvidsson, U., 1995. Immunohistochemical localization of delta- and mu-opioid receptors in primate spinal cord. NeuroReport 6, 1025–1028.

Hori, Y., Endo, K., Takahashi, T., 1992. Presynaptic inhibitory action of enkephalin on excitatory transmission in superficial dorsal horn of rat spinal cord. J. Physiol. (London) 450, 673–685.

Isabel, G., Wright, D.M., Henry, J.L., 1981. Design for an inexpensive unit for measuring tail flick latencies. J. Pharmacol. Methods 5, 241–247.

Kangrga, I., Randic, M., 1991. Outflow of endogenous aspartate and glutamate from the rat spinal dorsal horn in vitro by activation of low- and high-threshold primary afferent fibers. Modulation by μ-opioids. Brain Res. 553, 347–352.

Kawakita, K., Funakoshi, M., 1982. Suppression of the jaw-opening reflex by conditioning A-delta fiber stimulation and electroacupuncture in the rat. Exp. Neurol. 78, 461–465.

Le Bars, D., Villanueva, L., 1988. Electrophysiological evidence for the activation of descending inhibitory controls by nociceptive afferent pathways. Prog. Brain Res. 77, 275–299.

Lord, J.A.H., Waterfield, A.A., Hughes, J., Kosterlitz, H.W., 1977. Endogenous opioid peptides: multiple agonists and receptors. Nature 267, 495–499.

Lovenberg, W., Miller, R.C., 1990. Endothelin: A review of its effects and possible mechanisms of action. Neurochem. Res. 15, 407–417.

Malmberg, A.B., Yaksh, T.L., 1992. Isobolographic and dose–response analyses of the interaction between intrathecal *mu* and *delta* agonists: Effects of naltrindole and its benzofuran analog (NTB). J. Pharmacol. Exp. Ther. 263, 264–275.

Mansour, A., Fox, C.A., Burke, S., Meng, F., Thompson, R.C., Akil, H., Watson, S.J., 1994. Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. J. Comp. Neurol. 350, 412–438.

Misra, A.L., Pontani, R.B., Vadlamani, N.L., Mule, S.J., 1976. Physiological disposition and biotransformation of [allyl-1′,3′-^{14}C] naloxone in the rat and some comparative observations on nalorphine. J. Pharmacol. Exp. Ther. 196, 257–268.

Mjanger, E., Yaksh, T.L., 1991. Characteristics of dose-dependent antagonism by β-funaltrexamine of the antinociceptive effects of intrathecal *mu* agonists. J. Pharmacol. Exp. Ther. 258, 544–550.

Morgan, M.M., Heinricher, M.M., Fields, H., 1994. Inhibition and facilitation of different nocifensor reflexes by spatially remote noxious stimuli. J. Neurophysiol. 72, 1152–1160.

Ness, T.J., Gebhart, G.F., 1991. Interactions between visceral and cutaneous nociception in the rat. I. Noxious cutaneous stimuli inhibit visceral nociceptive neurons and reflexes. J. Neurophysiol. 66, 20–28.

Omote, K., Kitahata, L.M., Nakatani, K., Collins, J.G., 1991. δ receptor involvement in morphine suppression of noxiously evoked activity of spinal WDR neurons in cats. Brain Res. 554, 299–303.

Ossipov, M.H., Kovelowski, C.J., Wheeler-Aceto, H., Cowan, A., Hunter, J.C., Lai, J., Malan Jr., T.P., Porreca, F., 1996. Opioid antagonists and antisera to endogenous opioids increase the nociceptive response to formalin: demonstration of an opioid kappa and delta inhibitory tone. J. Pharmacol. Exp. Ther. 277, 784–788.

Pitcher, G.M., Yashpal, K., Coderre, T.J., Henry, J.L., 1995a. μ-, δ- and k-opiate receptors mediate antinociception in the rat tail flick test following noxious thermal stimulation of one hindpaw. J. Pharmacol. Exp. Ther. 273, 1428–1433.

Pitcher, G.M., Yashpal, K., Coderre, T.J., Henry, J.L., 1995b. Mechanisms underlying antinociception provoked by heterosegmental noxious stimulation in the rat tail-flick test. Neuroscience 65, 273–281.

Pomeranz, B., 1987. Scientific basis of acupuncture. In: G. Stux and B. Pomeranz (Eds.), Acupuncture: Textbook and Atlas, Springer-Verlag, Heidelberg, p. 1.

Pomeranz, B., Cheng, R., 1979. Suppression of noxious responses in single neurons of cat spinal cord by electroacupuncture and its reversal by the opiate antagonist naloxone. Exp. Neurol. 64, 327–341.

Ribeiro-da-Silva, A., De Koninck, Y., Cuello, A.C., Henry, J.L., 1992. Enkephalin-immunoreactive nociceptive neurons in the cat spinal cord. NeuroReport 3, 25–28.

Romita, V.V., Henry, J.L., 1994. β-FNA, a μ-opiate receptor antagonist, attenuates antinociception evoked by high intensity, low frequency peripheral stimulation in rats. Soc. Neurosci. Abstr. 20 (Part 1), 762 (Abstract).

Romita, V.V., Henry, J.L., 1996. Intense peripheral electrical stimulation differentially inhibits tail vs. limb withdrawal reflexes in the rat. Brain Res. 720, 45–53.

Rusin, K.I., Randic, M., 1991. Modulation of NMDA-induced currents by μ-opioid receptor agonist DAGO in acutely isolated rat spinal dorsal horn neurons. Neurosci. Lett. 124, 208–212.

Schiller, P.W., Weltrowska, G., Nguyen, T.M.-D., Wilkes, B.C., Chung, N.N., Lemieux, C., 1993. TIPP[¥]: A highly potent and stable pseudopeptide δ opioid receptor antagonist with extraordinary δ selectivity. J. Med. Chem. 36, 3182–3187.

Shah, S., Davis, T., Yoburn, B.C., 1994. The effect of naltrindole on spinal and supraspinal delta opioid receptors and analgesia. Life Sci. 55, 1451–1458.

Sofuoglu, M., Portoghese, P.S., Takemori, A.E., 1991. Differential antagonism of *delta* opioid agonists by naltrindole and its benzofuran analog (NTB) in mice: evidence for *delta* opioid receptor subtypes. J. Pharmacol. Exp. Ther. 257, 676–680.

Stein, E.A., Hiller, J.M., Simon, E.J., 1992. Effects of stress on opioid receptor binding in the rat central nervous system. Neuroscience 51, 683–690.

Stevens, C.W., Lacey, C.B., Miller, K.E., Elde, R.P., Seybold, V.S., 1991. Biochemical characterization and regional quantification of μ, δ and k opioid binding sites in rat spinal cord. Brain Res. 550, 77–85.

Sutters, K.A., Miaskowski, C., Taiwa, Y.O., Levine, J.D., 1990. Analgesic synergy and improved motor function produced by combinations of μ-δ- and μ-k-opioids. Brain Res. 530, 290–294.

Takemori, A.E., Ho, B.Y., Naseth, J.S., Portoghese, P.S., 1988. Nor-binaltorphimine, a highly selective *kappa*-opioid antagonist in analgesic and receptor binding assays. J. Pharmacol. Exp. Ther. 246, 246–255.

Taylor, J.S., Pettit, J.S., Harris, J., Ford, T.W., Clarke, R.W., 1990. Noxious stimulation of the toes evokes long-lasting, naloxone-reversible suppression of the sural-gastrocnemius reflex in the rabbit. Brain Res. 531, 263–268.

Toda, K., Ichioka, M., 1978. Electroacupuncture: Relations between forelimb afferent impulses and suppression of jaw-opening reflex in the rat. Exp. Neurol. 61, 465–470.

Ulett, G.A., 1982. Principles and Practice of Physiological Acupuncture. Warren H. Green, St. Louis, MO, p. 3.

Vaccarino, A.L., Marek, P., Liebeskind, J.C., 1992. Stress-induced analgesia prevents the development of the tonic, late phase of pain produced by subcutaneous formalin. Brain Res. 572, 250–252.

Vaught, J.L., Rothman, R.B., Westfall, T.C., 1982. Mu and delta receptors: Their role in analgesia and in the differential effects of opioid peptides on analgesia. Life Sci. 30, 1443–1455.

Villanueva, L., Bing, Z., Bouhassira, D., Le Bars, D., 1991. Depressive effects of *mu* and *delta* opioid receptor agonists on activities of dorsal horn neurones are enhanced by dibencozide. J. Pharmacol. Exp. Ther. 257, 1198–1202.

Visconti, L.M., Standifer, K.M., Schiller, P.W., Pasternak, G.W., 1994. TIPP [¥], a highly selective δ-ligand. Neurosci. Lett. 181, 47–49.

Ward, S.J., Portoghese, P.S., Takemori, A.E., 1982. Pharmacological profiles of β-funaltrexamine (beta-FNA) and β-chlornaltrexamine (beta-CNA) on the mouse vas deferens preparation. Eur. J. Pharmacol. 80, 377–384.

Watkins, L.R., Wiertelak, E.P., Grisel, J.E., Silbert, L.H., Maier, S.F., 1992a. Parallel activation of multiple spinal opiate systems appears to mediate ''non-opiate'' stress-induced analgesias. Brain Res. 594, 99–108.

Watkins, L.R., Wiertelak, E.P., Maier, S.F., 1992b. Kappa opiate receptors mediate tail-shock induced antinociception at spinal levels. Brain Res. 582, 1–9.

Welch, S.P., Dunlow, L.D., 1993. Antinociceptive activity of intrathecally administered potassium channel openers and opioid agonists: a common mechanism of action?. J. Pharmacol. Exp. Ther. 267, 390–399.

Yaksh, T.L., Rudy, T.A., 1977. Studies on the direct spinal action of narcotics in the production of analgesia in the rat. J. Pharmacol. Exp. Ther. 202, 411–428.

Zerari, F., Zouaoui, D., Gastard, M., Apartis, E., Fischer, J., Herbrecht, F., Cupo, A., Cucumel, K., Conrath, M., 1994. Ultrastructural study of δ-opioid receptors in the dorsal horn of the rat spinal cord using monoclonal anti-idiotypic antibodies. J. Chem. Neuroanat. 7, 159–170.

Zhang, L.J., Wang, X.J., Han, J.S., 1990. Phorbol ester suppression of opioid analgesia in rats. Life Sci 47, 1775–1782.