JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 1355–1363

Received March 3, 2008
Accepted June 11, 2008
DOI 10.1211/jpp/60.10.0012
ISSN 0022-3573

Microinjection of morphine into thalamic nucleus submedius depresses bee venom-induced inflammatory pain in the rat

Jie Feng, Ning Jia, Ling-Na Han, Fen-Sheng Huang, Yu-Feng Xie, Jian Liu and Jing-Shi Tang

Abstract

Previous studies have provided evidence of the existence of a pain modulatory feedback pathway consisting of thalamic nucleus submedius (Sm)–ventrolateral orbital cortex–periaqueductal grey pathway, which is activated during acute pain and leads to depression of transmission of nociceptive information in the spinal dorsal horn. The aim of this study was to test the hypothesis that morphine microinjection into the Sm decreased spontaneous pain and bilateral thermal hyperalgesia, as well as ipsilateral mechanical allodynia, induced by subcutaneous injections of bee venom into the rat hind paw. Morphine (1.0, 2.5 or 5.0 μg in 0.5 μL) injected into the Sm, contralateral to the bee venom-injected paw, depressed spontaneous nociceptive behaviour in a dose-dependent manner. Furthermore, morphine significantly decreased bilateral thermal hyperalgesia and ipsilateral mechanical allodynia 2 h after bee venom injection. These morphine-induced effects were antagonized by 1.0 μg naloxone (an opioid antagonist) microinjected into the Sm 5 min before morphine administration. The results provided further support for the important role of the Sm and Sm-opioid receptors in inhibiting nociceptive behaviour and indicated for the first time that Sm opioid receptors were also effective in inhibiting the hypersensitivity provoked by bee venom-induced inflammation.

Department of Physiology and Pathophysiology, Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education, Xi'an Jiaotong University School of Medicine, Xi'an, Shaanxi 710061, P.R. China

Jie Feng, Ning Jia, Ling-Na Han, Fen-Sheng Huang, Yu-Feng Xie, Jian Liu, Jing-Shi Tang

Correspondence: J.-S. Tang, Department of Physiology and Pathophysiology, Xi'an Jiaotong University School of Medicine, Xi'an, Shaanxi 710061, P.R. China. E-mail: jstang@mail.xjtu.edu.cn

Acknowledgements and funding: The authors wish to thank Professor J. O. Dostrovsky and Liwen Bianji (Edanz Editing China) for their expert help in preparing the manuscript, and Professor J. Chen for providing the bee venom. The project was supported by the National Natural Science Foundation of China (No. 30270453; 30570592).

Introduction

Previous studies have shown that subcutaneous injection of bee venom (BV) or melittin, the major component of BV, into the rat hind paw induces inflammation, spontaneous nociceptive behaviour, bilateral thermal hyperalgesia, and ipsilateral mechanical allodynia (Chen et al 1999a, b; Chen & Chen 2000; Li & Chen 2004). It is well known that descending pathways are responsible for modulating transmission of nociceptive information, and that this descending activity can be altered in inflammatory and neuropathic pain states (Millan 2002; Porreca et al 2002; Ren & Dubner 2002; Vanegas & Schaible 2004). Although the peripheral and spinal mechanisms involved in BV-induced inflammatory pain have been studied (Chen & Chen 2000; Chen et al 2000; Chen & Chen 2001: Chen et al 2001, 2003; Li et al 2000; Li & Chen 2003; You et al 2003), the precise role of descending inhibitory modulation in these mechanisms is still unknown.

Studies in our laboratory have indicated that the nucleus submedius (Sm) of the medial thalamus is part of an endogenous analgesic system (a feedback loop), consisting of a spinal cord–Sm–ventrolateral orbital cortex (VLO)–periaqueductal grey (PAG)–spinal cord loop (Tang & Yuan 1999). For example, electrical or chemical-evoked activation of the Sm depresses spinal and trigeminal nocifensive reflexes, such as the tail flick and jaw-opening reflex. These anti-nociceptive effects can be eliminated by electrolytic lesion or microinjection of γ-aminobutyric acid (GABA) into the VLO or PAG (Zhang et al 1995, 1996, 1998, 1999). Furthermore, microinjection of opioid agents into the Sm produces antinociception in the tail flick (Dong et al 1999) and formalin (Yang et al 2002) tests. The BV model of inflammation differs in several important aspects from the formalin-induced pain model. For example, spontaneous nociceptive behaviour persists longer and exhibits only one phase following a BV injection. Importantly, there is a

prolonged period of hyperalgesia, allodynia, and mirror image thermal hyperalgesia, which outlasts the spontaneous nociceptive behaviour, phenomena that are not observed after formalin injections (Chen et al 1999a). The major aim of this study was to examine the effect of morphine injected into the Sm on hypersensitivity and nociception induced by subcutaneous injection of BV into the rat hind paw.

Materials and Methods

Animal preparation

Male Sprague–Dawley rats (220–250 g) were provided by the Experimental Animal Center of Shaanxi Province, China. The experimental protocol was approved by the Institutional Animal Care Committee of the Xi'an Jiaotong University. According to the ethical guidelines of the International Association for the Study of Pain (Zimmermann 1983), all efforts were made to minimize the number of animals used, as well as distress to the animals.

The animals were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.) and the head was fixed in a stereotaxic frame. A small craniotomy was performed just above the thalamus. A stainless steel guide cannula (0.8-mm diam.) was stereotaxically inserted, with its tip 2.5 mm dorsal to the Sm, at the following coordinates: 2.3–2.8 mm posterior to bregma, 0.5–0.9 mm lateral, 6.0–7.0 mm from cortical surface (Paxinos & Watson 1986), and attached to the skull with four microscrews and dental cement. Once the animals had recovered from anaesthesia, they received sodium penicillin (0.2 million U per day for four days, i.p.) to prevent wound and intracerebral infections. The animals were carefully nursed, and were housed and fed in clear cages.

Bee venom testing paradigms

One week following surgery, behavioural testing was performed during the day portion of the circadian cycle (08:00–18:00) at room temperature (22°C). All rats were habituated in the experimental arena for 30 min daily during the three days before testing. Behavioural testing was performed two times in each animal over a time interval of 10 days to ensure that pain hypersensitivity and local inflammation, which was induced by the first BV injection, disappeared (Chen et al 1999a). At each testing, spontaneous nociceptive behaviour, thermal paw withdrawal latency (PWL), or mechanical paw withdrawal threshold (PWT) were measured before BV injection, which served as control values. Saline, injected into rat hind paw, was used as vehicle control. All experiments were performed in a blind fashion.

Spontaneous nociceptive behaviour

On the experimental day, the animal was moved to the laboratory and placed for approximately 30 min on a glass plate covered with a transparent plastic frame (280 × 250 × 210 mm) to adapt to the experimental environment until cage exploration and major grooming activity ceased. The animal was then removed from the test chamber and a solution of BV (50 μL, 4.0 μg μL^{-1}, RBI/Sigma Co., St Louis, MO, USA) was subcutaneously administered to the hind paw pad. The rat was immediately returned to the chamber for observation. A mirror was placed under the glass plate at an angle of 45° to make observations of spontaneous movements of the injected paw easier. The number of times the rat lifted the injected paw from the glass floor, and the duration (s) of licking and lifting of the injected paw within successive 5-min time periods, were recorded for 60 min by two experimenters using a counter and a stopwatch, respectively.

Thermal paw withdrawal latency (PWL)

Noxious heat stimulation applied to the hind paw was used to measure the PWL 2 h after BV injection, the time point when heat-evoked hyperalgesia has been shown to occur (Chen et al 1999a). A focused radiant heat light source (100 W, 5.0 V projector lamp) was placed under the glass floor of the chamber and applied alternately to the centre of the ipsilateral and contralateral hind paw pads to observe baseline PWL. Changes in PWL after intracerebral injection of drug or saline were observed (see below) at successive 10-min intervals during a 60-min observation period.

Mechanical paw withdrawal threshold (PWT)

Similarly, PWT in response to mechanical stimulation (von Frey filaments) was measured 2 h after BV injection using the up–down method (Dixon 1980; Chaplan et al 1994). This time point has been shown to coincide with mechanical allodynia (Chen et al 1999a). The rats were placed in a transparent plastic box (280 × 250 × 210 mm) with a metal wire mesh floor that allowed full access to the paws from underneath. Ten von Frey filaments were chosen (Stoelting Company, Wood Dale, IL, USA), with approximately equal logarithmic incremental (0.17) bending force (von Frey numbers: 3.61 to 5.18, equivalent to 0.4 to 15.0 g, respectively). Starting with a middle series of filaments, 4.31 (2.0 g), von Frey filaments were repeatedly applied from below for approximately 6–8 s, perpendicularly to the mid-plantar surface of the hind paw, with sufficient force to cause slight bending. The number of positive and negative withdrawal responses was recorded and converted to a 50% threshold value using the formula previously described by Dixon (1980) and Chaplan et al (1994). The mechanical PWT measurements were performed alternately on the ipsilateral and contralateral hind paws to determine the baseline PWT. Changes in PWT were measured at 10-min intervals during a 60-min observation period after intracerebral injection of drug or saline.

Intracerebral drug injection

The rats were lightly anaesthetized with enflurane (Baxter Caribe Inc., Guayama, Puerto Rico, USA), a very short-acting anaesthetic, to perform a microinjection of morphine into the Sm. A 1-μL microsyringe (0.4-mm diam.), with the

tip extending 2.5 mm beyond the end of the guide cannula, was inserted into the Sm through the guide cannula. Morphine hydrochloride (1.0, 2.5 or 5.0 μg, Shenyang Medicament Co., Shenyang, China) was dissolved in saline (0.5 μL) and slowly infused over 60 s into the Sm, contralateral to the BV injected paw. In the spontaneous nociceptive behavioural test, morphine was injected 5 min before the BV injection. However, in the hypersensitivity tests, morphine was injected immediately after baseline PWL or PWT was measured. Saline was microinjected into the Sm and served as a control.

For another series of experiments, an opioid receptor antagonist, naloxone hydrochloride (1.0 μg in 0.5 μL, RBI/Sigma Co., St Louis, MO, USA), was microinjected into the Sm 5 min before morphine application to determine whether the morphine effect could be blocked. Saline plus saline and saline plus morphine were injected into the Sm in the control experiments to ensure similar methods and times of injections. In other experiments the effects of naloxone (1.0 μg) itself were also observed.

Histological examination verified that the injection sites were located within the Sm, and the effective diffusion distance of the drugs (0.5 μL) was $\leq$0.5 mm from the injection site, similar to results reported by Zhang et al (1998).

Data analysis

All values were expressed as mean ± s.e.m. To normalize results, the PWT and PWL measurements were converted to score according to the following formula (Ren et al 2006): score = test value – baseline value 2 h after BV injection. Time course curves of the drug effects were plotted against a 60-min observation period. A linear regression analysis of the areas under the time course curves for each morphine dose was performed. The maximal percentage inhibition (MPI) of morphine on nociceptive behaviour (paw flinching and paw lifting/licking) was calculated using the following formula: MPI (%) = (test value – saline control)/(0 – saline control) × 100, where '0' represents maximal inhibition (Xin et al 1997). Differences in entire observation time, as well as at each time point, among the various groups were statistically tested using one- or two-way repeated measures analysis of variance, followed by a post-hoc Fisher LSD test (Milligan et al 2003). P values < 0.05 were considered to be statistically significant. Sigmastat software 2.03 was used for analysing the data.

Results

Inhibitory effects of Sm morphine on spontaneous nociceptive behaviour

Immediately following subcutaneous injections of BV (50 μL, 4.0 μg μL^{-1}) into the hind paw, the rats displayed nociceptive behaviour. As shown in Figure 1, the degree of paw flinching and duration of paw lifting/licking in the first or second 5-min time period reached peak values, respectively, and gradually subsided over 60 min. Saline (50 μL) was injected unilaterally into the hind paw and induced only a small number of paw flinches (1.5 ± 0.7, n = 6), and no paw lifting/licking responses were observed during the 60-min observation period. Microinjection of saline (0.5 μL) into the Sm, which is associated with BV injection into the contralateral hind paw, did not influence BV-induced nociceptive behaviour.

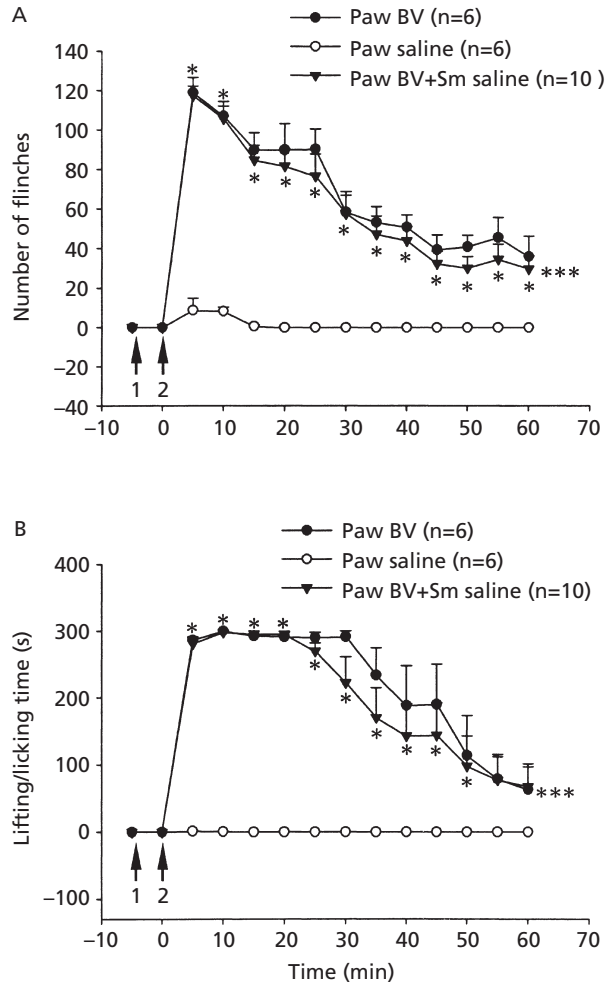

Figure 1 Time course curves showing that subcutaneous injections of bee venom (BV), not saline, into the hind paw pad induced spontaneous nociceptive behaviour, including flinching (A) and lifting/licking (B) in the ipsilateral hind paw. This effect was not influenced with microinjection of saline into the submedius. ***P < 0.001, compared with paw saline-injected group during the 60-min observation period, *P < 0.05, compared with paw saline-injected groups at those time points. Time point '–5' represents the value measured before the BV injection. Arrow 1 indicates the Sm saline injection, arrow 2 indicates the paw-BV or paw-saline injection.

However, when morphine (1.0, 2.5 or 5.0 μg) was microinjected into the Sm, contralateral to the BV-injected paw, the animals displayed reduced paw flinching in a dose-dependent manner (r = 0.998, P = 0.001, y = 3871.88 – 535.62x, by analysis of areas under the curves in Figure 2A). Morphine also decreased paw lifting/licking behaviour; however, these changes were not in a dose-dependent manner (r = 0.802, P = 0.104, y = 11341.61-945.2x, Figure 2B). Two-way

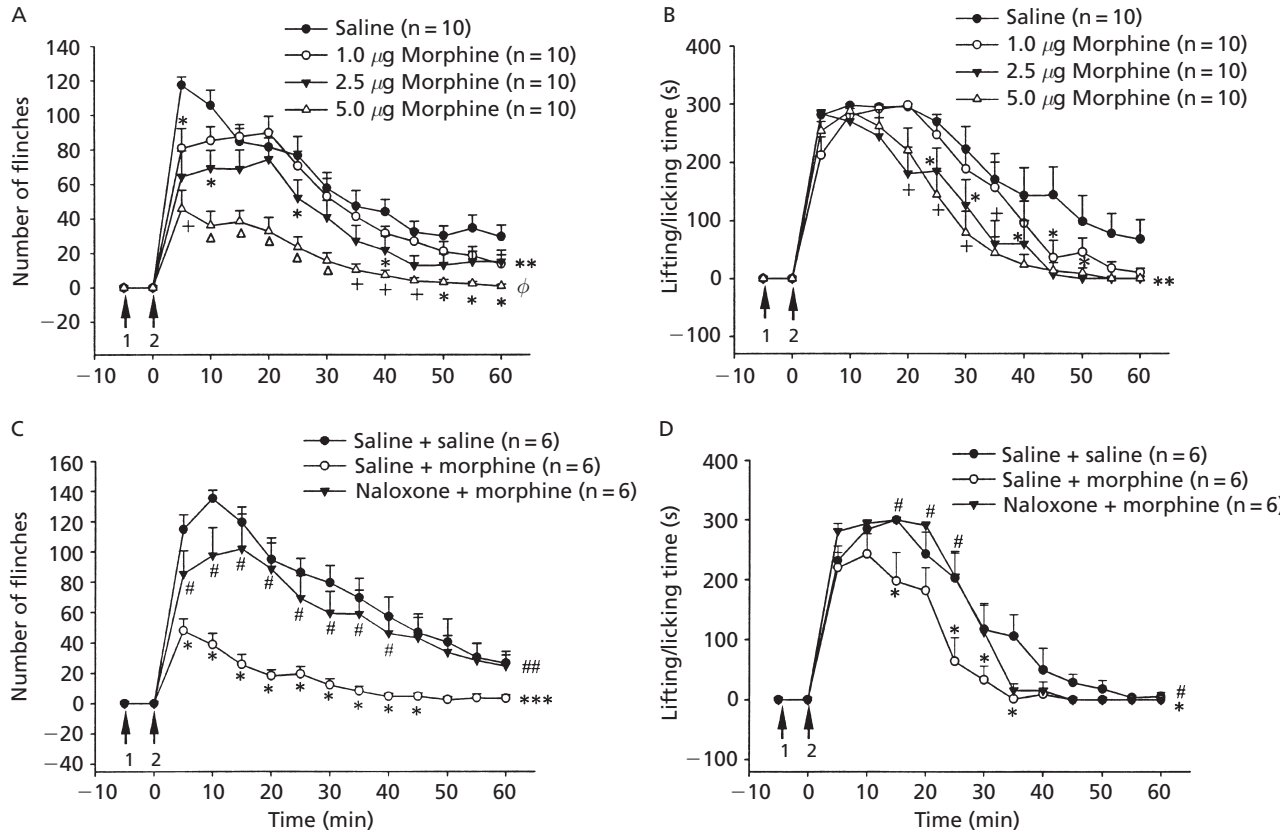

Figure 2 Time course curves showing the inhibitory effects of submedius (Sm) morphine microinjections (1.0, 2.5, 5.0 μg) on bee venom (BV)-induced spontaneous nociceptive behaviour (s.c. injection into the contralateral hind paw pad), and the antagonizing effects of naloxone on morphine-induced (5.0 μg) inhibition of nociceptive behaviour. A. Effects of morphine on the number of flinches. B. Effects of morphine on licking/lifting time (s). C. Effects of naloxone on morphine-induced inhibition of the number of flinches. D. Effects of naloxone on morphine-induced inhibition of the licking/lifting time. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with saline groups; $^{\varphi}P < 0.05$ compared with 2.5 μg morphine group; $\#P < 0.05$ (right side) and $\#\#P < 0.01$ compared with 5 μg morphine group during the 60-min observation period; $^{+}P < 0.05$ (inside) compared with 1.0 μg morphine group; and $^{\triangle}P < 0.05$ compared with 2.5 μg morphine group; $\#P < 0.05$ (inside) compared with 5 μg morphine group at those time points. Time point '–5' represents the value measured before BV injection. Arrow 1 indicates the Sm injection, arrow 2 indicates the BV injection.

repeated measures analysis of variance indicated that, as shown in Figure 2A and B, the time course curves (i.e. saline and different doses of morphine) varied greatly between the various treatments ($F_{(3,396)} = 14.725$, $P < 0.001$ for flinching test, $F_{(3,396)} = 5.024$, $P < 0.01$ for lifting/licking test). Further analyses indicated that during the 60-min observation period, 2.5 and 5.0 μg of morphine injected into the Sm significantly decreased the number of paw flinches and the duration of paw lifting/licking when compared with the saline-treated group. However, when 1.0 μg morphine was injected into the Sm it did not influence nociceptive behaviour. Detailed comparisons of the individual time points among the various groups are illustrated in Figure 2A and B. In addition, 5.0 μg morphine resulted in significantly different ($P < 0.001$, n = 10) paw flinching (MPI = 70.8 ± 2.9%) and paw lifting/licking (MPI = 40.8 ± 2.8%) behaviour.

Microinjection of the opioid receptor antagonist naloxone (1.0 μg) into the Sm 5 min before morphine (5.0 μg) significantly antagonized the morphine-induced inhibition of paw flinching and paw licking/lifting responses ($F_{(2,165)} = 13.564$, $P < 0.001$ for flinching test; $F_{(2,165)} = 6.127$, $P = 0.01$ for lifting/licking test). The specific time points when naloxone significantly decreased the morphine depression of the nociceptive behaviour are shown in Figure 2C and D.

Inhibitory effects of morphine on primary hypersensitivity when applied to Sm

Two hours after BV injection to the hind paw, ipsilateral thermal PWL and mechanical PWT were significantly reduced (Figure 3). This indicated that primary thermal hyperalgesia and mechanical allodynia were successfully established. BV-induced thermal hyperalgesia and mechanical allodynia were significantly reduced in a dose-dependent manner by morphine injections (1.0, 2.5 or 5.0 μg) into the contralateral Sm (r = 0.962, $P = 0.02$, y = –31.08 + 63.21x for PWL test, Figure 4A; r = 0.957, $P = 0.02$, y = 29.30 + 96.01x for PWT test, Figure 4B). Two-way repeated measures analysis of variance indicated that, as shown in Figure 4A and B, time course curves (i.e. saline and various morphine doses) were significantly different between the treatments

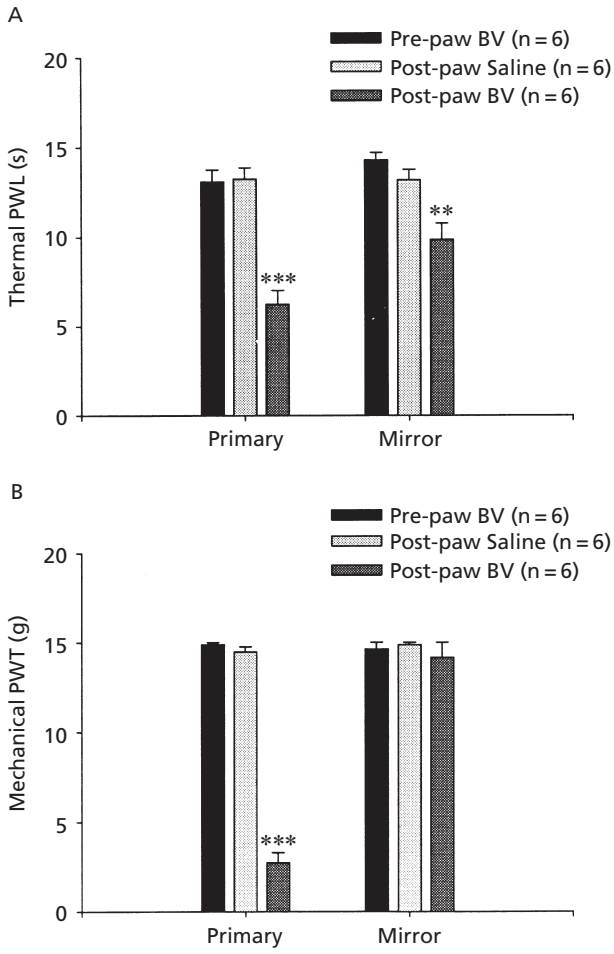

Figure 3 Bar graphs showing that subcutaneous injections of bee venom (BV solution 50 μL, 4 μg μL^{-1}), not saline, into the hind paw pad induced primary (ipsilateral) and mirror-image (contralateral) hypersensitivity. Two hours after BV injection, (a) the ipsilateral and contralateral paw withdrawal latency (PWL) and (b) ipsilateral paw withdrawal threshold (PWT) significantly decreased. ***$P < 0.001$ and **$P < 0.01$, compared with pre-BV and post-saline injection values.

($F_{(3,90)} = 14.133$, $P < 0.001$ for PWL test; $F_{(3,115)} = 36.297$, $P < 0.001$ for PWT test). Further analyses indicated that, compared with the 2.5-μg morphine-treated group, a 5.0-μg morphine injection into the Sm resulted in significantly increased mean PWL and PWT scores. In addition, 2.5 μg morphine was capable of inducing a greater increase in PWL scores than 1.0 μg morphine; the increased PWT score was also larger than that of the saline group (Figure 4A and B).

The pretreatment of naloxone (1.0 μg) 5 min before morphine (5 μg) administration significantly reduced morphine-evoked inhibition. As shown in Figure 4C and D, the difference between the treatment groups (saline plus saline, saline plus morphine, and naloxone plus morphine) was significant ($F_{(2,75)} = 29.167$, $P < 0.001$ for PWL test; $F_{(2,70)} = 22.969$, $P < 0.001$ for PWT test). Naloxone pretreatment resulted in significantly smaller mean PWL and PWT scores compared with treatment with morphine, but resulted in no difference compared with the saline group (Figure 4C and D).

Morphine-induced inhibitory effects on mirror-image hypersensitivity when applied to the Sm

Two hours after BV injection, thermal PWL, contralateral to the injected paw, was decreased when compared with pre-BV injection time points (see Figure 3A). Morphine microinjection (1.0, 2.5 or 5.0 μg) into the Sm significantly decreased this mirror-image thermal hyperalgesia ($F_{(3,95)} = 9.323$, $P < 0.001$), although not in a dose-dependent manner ($r = 0.847$, $P = 0.08$, $y = 5.85 + 27.18x$, Figure 5A). Furthermore, inhibition induced by 5.0 μg morphine was significantly larger than with saline, 1.0 or 2.5 μg morphine; however, no significant difference was found between 1.0 or 2.5 μg morphine and the saline groups.

Pretreatment with naloxone significantly reduced morphine-evoked (5.0 μg) inhibition of mirror-image hypersensitivity ($F_{(2,75)} = 14.491$, $P < 0.001$, Figure 5B), and the mean PWL score during the 60-min observation period was significantly smaller ($P < 0.001$) than the morphine group. No differences, however, were detected between the naloxone and saline groups. A detailed comparison of the individual time points among treatments is shown in Figure 5A and B.

BV injection did not induce any changes in mechanical PWT scores of the contralateral paw (mirror-image mechanical allodynia) (see Figure 3B). Injection of morphine (1.0, 2.5 or 5.0 μg) into the Sm had no significant effect on mechanical PWT (not shown).

Effects of naloxone injection to the Sm on BV-induced nociceptive behaviour

Naloxone (1.0 μg) microinjected into the Sm did not produce any significant effect on spontaneous nociceptive behaviour, primary thermal hyperalgesia, mechanical allodynia, or mirror-image thermal hyperalgesia; however, it slightly reduced PWT scores in the contralateral unaffected paw, as shown in Table 1.

Discussion

The behavioural effects after BV injection into the rat hind paw observed in this study were consistent with previous reports (Lariviere & Melzack 1996; Chen et al 1999a; Chen & Chen 2000; Chen et al 2000, 2001; Li & Chen 2004; Sumikura et al 2006), confirming that the BV test was a reliable tool for investigating inflammatory pain. Compared with the formalin-test model, the BV test produced minimal tissue damage, yet evoked a striking inflammation associated with prolonged spontaneous nociceptive behaviour. In addition, BV injection resulted in pronounced primary hypersensitivity to mechanical and heat stimuli in the treated hind paw, as well as thermal (but not mechanical) hypersensitivity in the contralateral hind paw. In contrast, formalin has been shown to produce significant tissue damage, followed by a biphasic spontaneous nociceptive response; the injected region becomes hyposensitive or even analgesic (Chen et al 1999a). Therefore, the use of BV injection in this study has provided novel information compared with the formalin test.

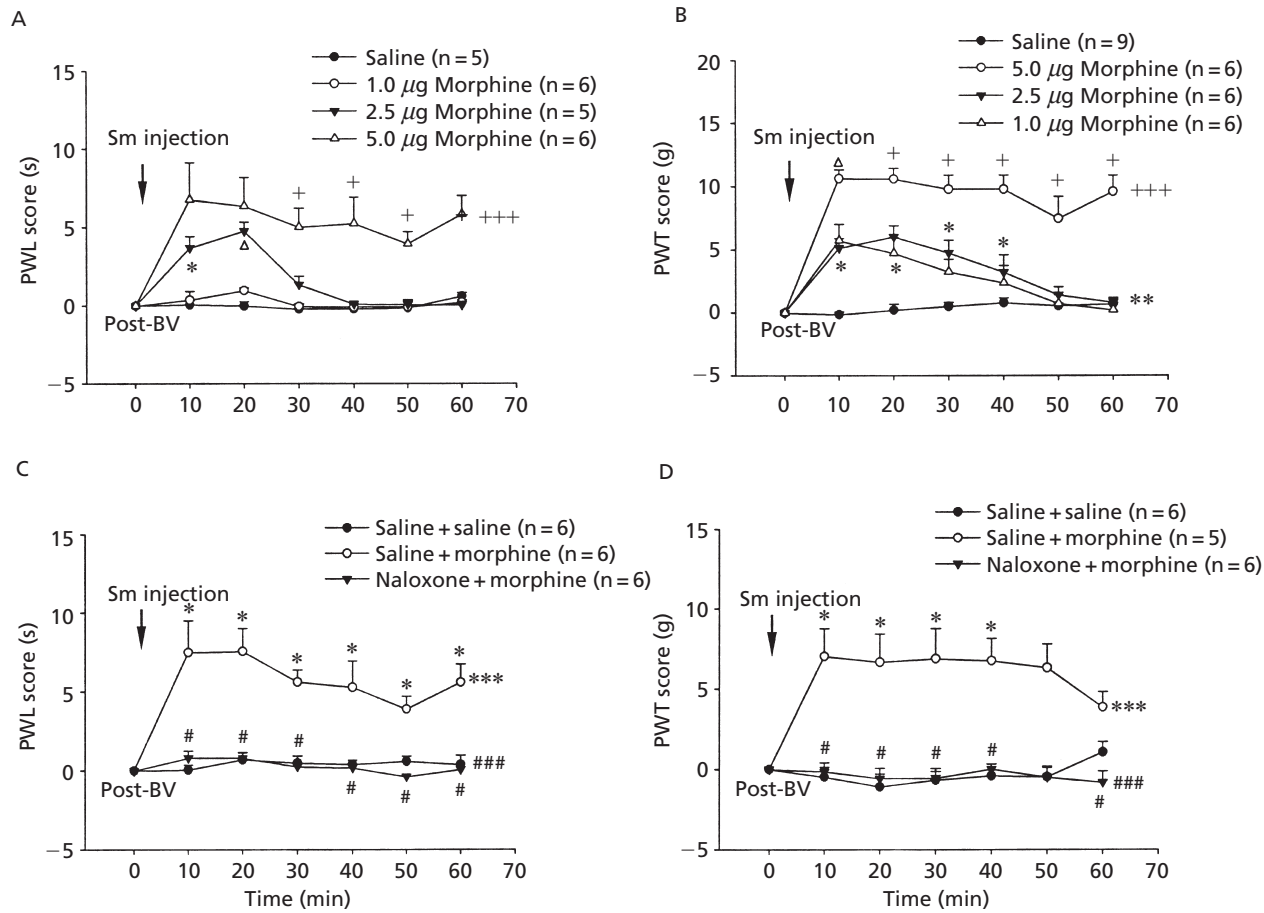

Figure 4 Time course curves showing the inhibitory effects of submedius (Sm) morphine microinjections (1.0, 2.5, 5.0 μg) on bee venom (BV)-induced primary hypersensitivity (s.c. injection into the hind paw pad) and the antagonizing effects of naloxone (1.0 μg) on 5.0 μg morphine-induced inhibition of primary thermal and mechanical hypersensitivity. A. Effects of morphine on the ipsilateral thermal paw withdrawal latency score (PWL score). B. Effects of morphine on the ipsilateral mechanical paw withdrawal threshold score (PWT score). C. Effects of naloxone on morphine-induced inhibition of the PWL score. D. Effects of naloxone on morphine-induced inhibition of the PWT score. $^{+++}P < 0.001$ compared with 2.5 μg morphine groups, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with saline group, $^{\#\#\#}P < 0.001$ compared with 5.0 μg morphine group during the 60-min observation period. $^{*}P < 0.05$ compared with saline group, $^{\Delta}P < 0.05$ compared with 1.0 μg morphine group, $^{+}P < 0.05$ compared with 2.5 μg morphine, and $^{\#}P < 0.05$ compared with 5 μg morphine group at those time points. Time point '0' shows the baseline score value measured 2 h after BV injection.

This study has demonstrated that morphine microinjection into the Sm reduced BV-induced spontaneous nociceptive behaviour, primary thermal hyperalgesia, mechanical allodynia, and mirror-image thermal hyperalgesia. These effects could be antagonized by pretreating with an injection of the opioid receptor antagonist, naloxone, into the same site. Although naloxone functions as a lipophilic opioid receptor antagonist, previous studies have established that it remains effective for longer than 60 min in similar conditions (Xin et al 1997; Halliday et al 1999; Wang et al 1999; Succu et al 2003). Results from this study complement our previous results demonstrating the effects of Sm morphine microinjection on nociceptive behaviour induced by the tail flick (Dong et al 1999), as well as previous formalin tests (Yang et al 2002). In addition, these results support evidence that the Sm was involved not only in mediating opioid receptor-mediated anti-nociception of acute and persistent pain (Dong et al 1999; Yang et al 2002), but also in mediating inflammation-induced hypersensitivity, including thermal hyperalgesia contralateral to the inflammation site.

Previous studies have shown that systemic morphine suppresses increased spinal neuronal firing induced by BV injected into the hind paw; this effect can be antagonized by naloxone (Chen et al 1998). When rats were treated with Freund's complete adjuvant, inflammatory hypersensitivity was induced; however, morphine or opioid agonist administration into the rostral ventromedial medulla reversed this effect; in contrast, naloxone or selective μ-opioid antagonists antagonized this effect (Hurley & Hammond 2000, 2001). The results of this study show that morphine administration into the Sm inhibited inflammatory hypersensitivity induced by BV. These results expand upon previous studies by indicating that opioids acting at the thalamic level (Sm) could induce anti-hypersensitivity, presumably by activating the descending pathway originating in the VLO and were effective in modulating ongoing inflammatory pain. These

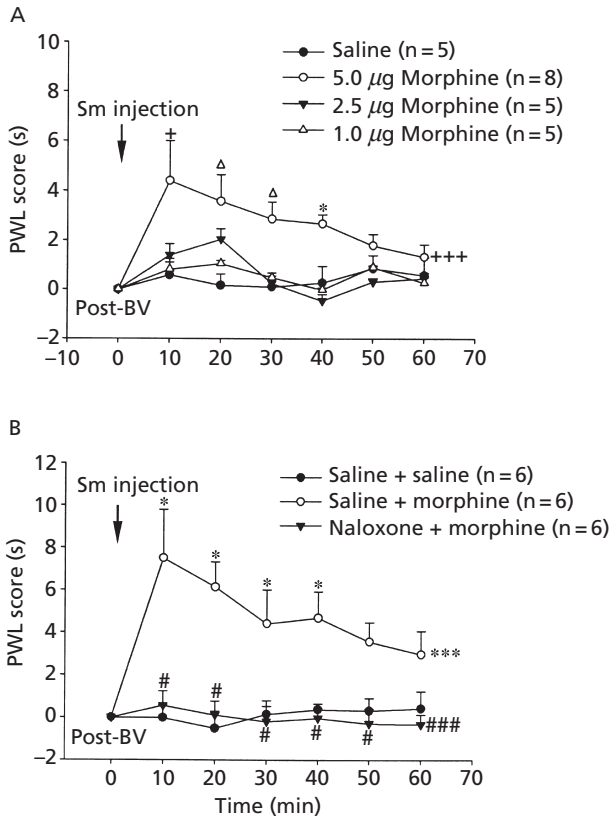

Figure 5 Time course curves showing the inhibitory effects of submedius (Sm) microinjected morphine (1.0, 2.5, 5.0 μg) on bee venom (BV)-induced mirror-image thermal hyperalgesia (s.c. injection into the contralateral hind paw pad) and the antagonizing effects of naloxone (1.0 μg) on morphine-induced (5.0 μg) inhibition of mirror-image thermal hyperalgesia. A. Effects of morphine on the contralateral paw withdrawal latency score (PWL score). B. Effects of naloxone on morphine-induced (5.0 μg) inhibition of the PWL score. $^{+++}P < 0.001$ compared with 2.5 μg morphine; $^{***}P < 0.001$ compared with saline group; $^{\#\#\#}P < 0.001$ compared with 5.0 μg morphine group during the 60-min observation period; $^{+}P < 0.05$ compared with 2.5 μg morphine group; $^{\triangle}P < 0.05$ compared with 1.0 μg morphine group; $^{*}P < 0.05$ compared with saline group; $\#P < 0.05$ compared 5 μg morphine group at those time points. Time point '0' shows the baseline score value measured 2 h after BV injection.

findings suggested that the analgesic effects of systemically administered opioids may have been mediated in part by acting on the Sm to increase descending modulation in the VLO–PAG–spinal cord pathway.

It is worth noting that inhibitory effects of morphine injection into the Sm were more pronounced on spontaneous paw flinching, compared with the paw licking/lifting behaviour. This may explain why the inhibitory effects on paw licking/lifting behaviour were not found to be effective during the 0–20 min observation period (see Figure 2B) and dose-dependent (lack of sufficient statistical power). These results were similar to Watanabe et al (2003), who found that the inhibitory effect of intrathecal morphine on the paw flinching response was stronger compared with the paw licking/lifting responses. One possible interpretation was that the paw flinching response was due to a flexion reflex mediated at the spinal cord level, while the paw licking/lifting response was a more complex reflex mediated by activity at supraspinal levels and was thus less sensitive to descending inhibition (Le Bars et al 2001; Okuda et al 2001; Watanabe et al 2003).

When the opioid receptor antagonist, naloxone, was applied to the Sm, spontaneous nociceptive behaviour, primary thermal hypersensitivity, mechanical hypersensitivity, and mirror-image thermal hypersensitivity were not affected; however, it reduced PWT measurements in the contralateral unaffected paw. This may provide support for the notion that nerve injury (or BV-induced inflammatory injury) triggered descending inhibition of secondary neuronal pools, but not primary ones as previously reported by Monhemius et al (2001). This suggested that opioid receptors in the Sm lacked significant tonic inhibitory action on inflammatory hypersensitivity arising from the injured side, but may have tonic inhibitory action of central sensitization of inputs from the contralateral unaffected paw. The descending inhibitory system may involve endogenous opioid release or activity in the Sm, thus preventing mirror-image mechanical allodynia that could be unmasked by naloxone.

The underlying mechanisms of peripheral and central increased sensitivity have been investigated by Urban et al (1999) and Chen et al (2003). In addition to continued input from primary afferents of the injured side, central sensitization may contribute to mirror-image hypersensitivity (Chen et al 2001), and may be closely related to central changes mediated by spinal commissural interneurons (Koltzenburg et al 1999). Furthermore, the brainstem's descending facilitatory system from the rostral medial medulla (RMM) has been found to contribute to secondary or mirror-image hypersensitivity, but not primary hypersensitivity (Urban et al 1999; Chen et al 2003). These studies have suggested that mechanisms of mirror-image hypersensitivity were different from primary hypersensitivity. We could, therefore, assume that Sm activity from the endogenous opioid system may have been different in primary and mirror-image hypersensitivity.

As mentioned above, the Sm has been shown to be involved in an endogenous analgesic system, consisting of the

Table 1 Effects of naloxone injected into submedius on the bee venom-induced nociceptive behaviour

Group	Number of flinches	Ipsilateral paw		Contralateral paw	
		PWL score (s)	PWT score (g)	PWL score (s)	PWT score (g)
Saline (n)	56.16 ± 5.56 (12)	0.02 ± 0.20 (5)	0.44 ± 0.28 (10)	0.43 ± 0.50 (5)	0.39 ± 0.31 (10)
Naloxone (n)	72.43 ± 5.42 (10)	0.09 ± 0.43 (7)	–0.07 ± 0.15 (6)	0.39 ± 0.34 (7)	–1.33 ± 0.56* (6)

Note: all data were expressed as mean ± s.e.m. during the 60-min observation period. $^{*}P < 0.05$ compared with saline group.

spinal cord–Sm–VLO–PAG–spinal cord loop. Opioids from the Sm primarily decrease spinal nociceptive responses induced by BV (as mentioned above). Therefore, it is reasonable to suggest that the nociceptive modulation pathway mediated the inhibitory effects of BV-induced inflammatory pain, when morphine was applied to the Sm. Jia et al (2004) reported that opioids injected into the Sm may have reduced the inhibitory actions of GABAergic neurons on the output neurons projecting to the VLO (disinhibition), leading to activation of the VLO–PAG brainstem descending inhibitory system and depression of nociceptive transmission at the spinal cord level. However, further investigation is required to understand the mechanisms of descending modulation in an inflammatory pain model.

Conclusion

This study has provided strong support that the thalamic Sm nucleus was involved in opioid-receptor mediated depression of nociceptive behaviour induced by peripheral BV administration. In combination with findings from previous studies, we speculate that morphine acted by reducing GABAergic inhibition in the Sm, thereby increasing activity in the Sm–VLO–PAG brainstem descending inhibitory system, which led to decreased nociceptive activity in the lumbar dorsal horn.

References

Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M., Yaksh, T.L. (1994) Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods* **53**: 55–63

Chen, H. S., Chen, J. (2000) Secondary heat, but not mechanical, hyperalgesia induced by subcutaneous injection of bee venom in the conscious rat: effect of systemic MK-801, a non-competitive NMDA receptor antagonist. *Eur. J. Pain* **4**: 389–401

Chen, H. S., Chen, J., Sun, Y. Y. (2000) Contralateral heat hyperalgesia induced by unilaterally intraplantar bee venom injection is produced by central changes: a behavioral study in the conscious rat. *Neurosci. Lett*. **284**: 45–48

Chen, H. S., Chen, J., Guo, W. G., Zheng, M. H. (2001) Establishment of bee venom-induced contralateral heat hyperalgesia in the rat is dependent upon central temporal summation of afferent input from the site of injury. *Neurosci. Lett*. **298**: 57–60

Chen, H. S., Li, M. M., Shi, J., Chen, J. (2003) Supraspinal contribution to development of both tonic nociception and referred mirror hyperalgesia: a comparative study between formalin test and bee venom test in the rat. *Anesthesiology* **98**: 1231–1236

Chen, J., Chen, H. S. (2001) Pivotal role of capsaicin-sensitive primary afferents in development of both heat and mechanical hyperalgesia induced by intraplantar bee venom injection. *Pain* **91**: 367–376

Chen, J., Luo, C. M., Li, H. L. (1998) The contribution of spinal neuronal changes to development of prolonged, tonic nociceptive responses of the cat induced by subcutaneous bee venom injection. *Eur. J. Pain* **2**: 359–376

Chen, J., Luo, C., Li, H., Chen, H. (1999a) Primary hyperalgesia to mechanical and heat stimuli following subcutaneous bee venom injection into the plantar surface of hindpaw in the conscious rat: a comparative study with the formalin test. *Pain* **83**: 67–76

Chen, J., Li, H., Luo, C., Li, Z., Zheng, J. (1999b) Involvement of peripheral NMDA and non-NMDA receptors in development of persistent firing of spinal wide-dynamic-range neurons induced by subcutaneous bee venom injection in the cat. *Brain Res*. **844**: 98–105

Dixon, W. J. (1980) Efficient analysis of experimental observation. *Ann Rev. Pharmacol. Toxicol.* **20**: 441–462

Dong, Y. F., Tang, J. S., Yuan, B., Jia, H. (1999) Morphine applied to the thalamic nucleus submedius produces a naloxone reversible antinociceptive effect in the rat. *Neurosci. Lett*. **271**: 17–20

Halliday, A. J., Bartlett, S. E., Colditz, P., Smith, M. T. (1999) Brain region-specific studies of the excitatory behavioral effects of morphine-3-glucuronide. *Life Sci*. **65**: 225–236

Hurley, R. W., Hammond, D. L. (2000) The analgesic effects of supraspinal mu and delta opioid receptor agonists are potentiated during persistent inflammation. *J. Neurosci.* **20**: 1249–1259

Hurley, R. W., Hammond, D. L. (2001) Contribution of endogenous enkephalins to the enhanced analgesic effects of supraspinal μ opioid receptor agonists after inflammatory injury. *J. Neurosci*. **21**: 2536–2545

Jia, H., Xie, Y. F., Tang, J. S. (2004) Involvement of GABAergic modulation of the nucleus submedius (Sm) morphine-induced antinociception. *Pain* **108**: 28–35

Koltzenburg, M., Wall, P. D., McMahon, S. B. (1999) Does the right side know what the left is doing? *Trends Neurosci*. **22**: 122–127

Lariviere, W. R., Melzack, R. (1996) The bee venom test: a new tonic-pain test. *Pain* **66**: 271–277

Le Bars, D., Gozariu, M., Cadden, S. W. (2001) Animal models of nociception. *Pharmacol. Rev*. **53**: 597–652

Li, K. C., Chen, J. (2003) Differential roles of spinal protein kinase C in development of primary heat and mechanical hypersensitivity induced by subcutaneous bee venom chemical injury in the rat. *Neurosignals* **12**: 292–301

Li, K. C., Chen, J. (2004) Altered pain-related behaviors and spinal neuronal responses produced by s.c. injection of melittin in rats. *Neuroscience* **126**: 753–762

Li, K. C., Zheng, J. H., Chen, J. (2000) Involvement of spinal protein kinase C in induction and maintenance of both persistent spontaneous flinching reflex and contralateral heat hyperalgesia induced by subcutaneous bee venom in the conscious rat. *Neurosci. Lett*. **285**: 103–106

Millan, M. J. (2002) Descending control of pain. *Prog. Neurobiol*. **66**: 355–474

Milligan, E. D., Twining, C., Chacur, M., Biedenkapp, J., O'Connor, K., Poole, S., Tracey, K., Martin, D., Maier, S. F., Watkins, L. R. (2003) Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. *J. Neurosci.* **23**: 1026–1040

Monhemius, R., Green, D. L., Roberts, M. H. T., Azami, J. (2001) Periaqueductal gray mediated inhibition of responses to noxious stimulation is dynamically activated in a rat model of neuropathic pain. *Neurosci. Lett*. **298**: 70–74

Okuda, K., Sakurada, C., Takahashi, M., Yamada, T., Sakurada, T. (2001) Characterization of nociceptive responses and spinal releases of nitric oxide metabolites and glutamate evoked by different concentrations of formalin in rats. *Pain* **92**: 107–115

Paxinos, G., Watson, C. (1986) *The Rat brain in stereotaxic coordinates*. 2nd edn, Academic Press, Sydney

Porreca, F., Ossipov, M. H., Gebhart, G. F. (2002) Chronic pain and medullary descending facilitation. *Trends Neurosci*. **25**: 319–326

Ren, K., Dubner, R. (2002) Descending modulation in persistent pain: an update. *Pain* **100**: 1–6

Ren, W. H., Guo, J. D., Cao, H., Wang, H., Wang, P. F., Sha, H., Ji, R. R., Zhao, Z. Q., Zhang, Y. Q. (2006) Is endogenous D-serine in the rostral anterior cingulate cortex necessary for pain-related negative affect? *J. Neurochem*. **96**: 1636–1647

Succu, S., Mascia, M. S., Melis, T., Melis, M. R., Deghenghi, R., Argiolas, A. (2003) Activation of GABAA and opioid receptors reduce penile erection induced by hexarelin peptides. *Pharmacol. Biochem. Behav*. **76**: 563–570

Sumikura, H., Andersen, O. K., Drewes, A. M., Arendt-Nielsen, L. (2006) Secondary heat hyperalgesia induced by melittin in humans. *Eur. J. Pain* **10**: 121–125

Tang, J. S., Yuan, B. (1999) The role of thalamic nucleus submedius – ventrolateral orbital cortex – periaqueductal gray pathway in pain modulation and acupuncture analgesia. *Chin. J. Neurosci*. **15**: 247–251

Urban, M. O., Zahn, P. K., Gebhart, G. F. (1999) Descending facilitatory influences from the rostral medial medulla mediate secondary, but not primary hyperalgesia in the rat. *Neuroscience* **90**: 349–352

Vanegas, H., Schaible, H. G. (2004) Descending control of persistent pain: inhibitory or facilitatory? *Brain Res. Rev.* **46**: 295–309

Wang, D., Ye, H. H., Yu, L. C., Lundeberg, T. (1999) Intra-periaqueductal grey injection of galanin increases the nociceptive response latency in rats, an effect reversed by naloxone. *Brain Res*. **834**: 152–154

Watanabe, C., Okuda, K., Sakurada, C., Ando, R., Sakurada, T., Sakurada, S. (2003) Evidence that nitric oxide-glutamate cascade modulates spinal antinociceptive effect of morphine: a behavioral and microdialysis study in rats. *Brain Res*. **990**: 77–86

Xin, L., Geller, E. B., Adler, M. (1997) Body temperature and analgesic effects of selective mu and kappa opioid receptor agonists microdialyzed into rat brain. *J. Pharmacol. Exp. Therapy* **281**: 499–507

Yang, Z. J., Tang, J. S., Jia, H. (2002) Morphine microinjections into the rat nucleus submedius depress nociceptive behavior in the formalin test. *Neurosci. Lett*. **328**: 141–144

You, H. J., Morch, C. D., Chen, J., Arendt-Nielsen, L. (2003) Role of central NMDA versus non-NMDA receptor in spinal withdrawal reflex in spinal anesthetized rats under normal and hyperexcitable conditions. *Brain Res*. **981**: 12–22

Zhang, Y. Q., Tang, J. S., Yuan, B., Jia, H. (1995) Inhibitory effects of electrical stimulation of thalamic nucleus submedius area on the rat tail flick reflex. *Brain Res*. **696**: 205–212

Zhang, Y. Q., Tang, J. S., Yuan, B. (1996) Inhibitory effects of electrical stimulation of thalamic nucleus submedius on the nociceptive response of neurons in the spinal dorsal horn in the rat. *Brain Res*. **737**: 16–24

Zhang, S., Tang, J. S., Yuan, B., Jia, H. (1998) Inhibitory effects of glutamate-induced activation of thalamic nucleus submedius are mediated by ventrolateral orbital cortex and periaqueductal gray in rats. *Eur. J. Pain* **2**: 153–163

Zhang, S., Tang, J. S., Yuan, B., Jia, H. (1999) Electrically-evoked inhibitory effects of the nucleus submedius on the jaw-opening reflex are mediated by ventrolateral orbital cortex and periaqueductal gray matter in the rat. *Neuroscience* **93**: 867–875

Zimmermann, M. (1983) Ethical guideline for investigations of experimental pain in conscious animals. *Pain* **16**: 109–110