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ORIGINAL PAPER

Mu-Opioid Receptor in the Nucleus Submedius: Involvement in Opioid-Induced Inhibition of Mirror-Image Allodynia in a Rat Model of Neuropathic Pain

Jun-Yang Wang · Mei Zhao · Fen-Sheng Huang · Jing-Shi Tang · Yu-Kang Yuan

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Abstract The current study investigated the roles of various subtypes of opioid receptors expressed in the thalamic nucleus submedius (Sm) in inhibition of mirror-image allodynia induced by L5/L6 spinal nerve ligation in rats. Morphine was microinjected into the Sm, which produced a dose-dependent inhibition of mirror-image allodynia; this effect was antagonized by pretreatment with non-selective opioid receptor antagonist naloxone. Microinjections of endomorphin-1 (μ-receptor agonist), or [D-Ala^2, D-Leu^5]-enkephalin (DADLE, δ-/μ-receptor agonist), also inhibited mirror-image allodynia, and these effects were blocked by the selective μ-receptor antagonist, β-funaltrexamine hydrochloride. The DADLE-induced inhibition, however, was not influenced by the δ-receptor antagonist naltrindole. The κ-receptor agonist, spiradoline mesylate salt, failed to alter the mirror-image allodynia. These results suggest that Sm opioid receptor signaling is involved in inhibition of mirror-image allodynia; this effect is mediated by μ- (but not δ- and κ-) opioid receptors in the rat model of neuropathic pain.

Keywords Mirror-image allodynia · Neuropathic pain · Thalamic nucleus submedius · Opioid · Opioid receptor · Rat

Introduction

Mirror-image hypersensitivity that arises from the site contralateral to pathology is an enigmatic phenomenon observed in patients with multiple clinical pain syndromes, particularly those with peripheral nerve trauma or inflammation [1–3]. Evidence has shown that primary, secondary and mirror-image hypersensitivity could also be induced in animal models of neuropathic and inflammatory pain. In these models, the underlying mechanisms of peripheral and central increased sensitivity have been well investigated [4, 5]. In addition to ongoing inputs from primary afferents of the injured side, central sensitization may contribute to mirror-image hypersensitivity [6], and may be closely related to a central change mediated by spinal commissural interneurons [7]. Furthermore, the brainstem's descending facilitatory system from the rostral medial medulla (RMM) has been found to contribute to secondary hypersensitivity, as well as mirror-image pain, but not primary hypersensitivity [4, 5]. However, little is known about the supraspinal modulatory mechanism of mirror-image pain, especially the descending inhibitory modulation at the forebrain level.

Previous studies have shown that the thalamic nucleus submedius (Sm) of the medial thalamus is involved in the ascending component of an endogenous analgesic system (feedback loop), which consists of the spinal cord-Sm-ventrolateral orbital cortex (VLO)-periaqueductal gray (PAG)-spinal cord loop [8–11]. A microinjection of morphine into the Sm can depress the tail flick reflex [12], formalin-induced nociceptive behavior, and c-fos protein

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J.-Y. Wang · Y.-K. Yuan
Department of Immunology and Pathogenic Biology, Xi'an Jiaotong University School of Medicine, Xi'an, Shaanxi 710061, People's Republic of China

M. Zhao · F.-S. Huang · J.-S. Tang (✉)
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, People's Republic of China
e-mail: jstang@mail.xjtu.edu.cn

expression of neurons in the spinal cord dorsal horn [13, 14]. These effects can be reversed by application of an opioid receptor antagonist, such as naloxone, to the Sm. Further study has shown that microinjection of morphine into the Sm depressed the primary allodynia that was induced by L5/L6 spinal nerve ligation [15], and this effect was mediated by the μ-opioid receptor. In this study, we present data obtained from the same rats with neuropathic pain, showing that a microinjection of morphine into the Sm can also inhibit mirror-image allodynia; this inhibitory effect is mediated by the μ-opioid receptor, and not the δ- or κ-opioid receptor.

Methods

Animals

The experiments were performed on male Sprague-Dawley rats (180–200 g) provided by the Experimental Animal Center of Shaanxi Province, China, and approved by the Institutional Animal Care Committee of the University. According to the guidelines of the International Association for the Study of Pain [16], all efforts were made to minimize the number of animals used and their suffering.

Spinal Nerve Ligation

The neuropathic pain model was induced by ligating the right L5 and L6 spinal nerves, as previously described [17]. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). A dorsal midline incision was made from L3 to S2 and the right L5 and L6 spinal nerves were isolated and tightly ligated with 6–0 silk sutures. The incision was then closed in two layers. Sham animals were treated the same way; however, the nerves were not ligated. The animal model of mirror hypersensitivity was considered to be successfully established if (1) the paw withdrawal threshold of the contralateral paw was less than 10 g in response to von Frey filament stimulation and (2) the number of contralateral paw lifts was greater than 4 times during a 5 min observation period on a 4°C plate. Rats that met these criteria were included in the experimental design (about 80%).

Catheterization and Drug Administration

Two weeks after spinal nerve ligation, rats were anesthetized with sodium pentobarbital and a stainless steel guide cannula (0.8 mm in diameter) was stereotactically implanted at a position 2.5 mm dorsal to the Sm and contralateral to the nerve injury at the following coordinates: 2.3–3.0 mm from bregma, 0.6–0.9 mm lateral, and 6.0–7.0 mm below cortical surface [18]. The guide cannula was fixed to the skull. A stainless-steel plug was inserted into the guide cannula and kept in place until the intracerebral injection. After surgery, the animals were injected intraperitoneally with penicillin (0.2 million units per day) for four consecutive days. One week later (i.e., three weeks after spinal nerve ligation), rats were lightly anesthetized with Enflurane (Baxter Caribe Inc., Guayama, Puerto Rico, USA), and a 1.0 µl microsyringe (0.4 mm in diameter), with the tip extending 2.5 mm beyond the end of the guide cannula, was inserted into the Sm through the guide cannula. Drugs were dissolved in normal saline (0.5 µl) and then slowly infused into the Sm over a 60 s period. The microsyringe was left in place for an additional 30 s to minimize backflow of the drug solution into the injection tract. At the end of the experiment, the microinjection sites were histologically verified to be within the Sm.

Drugs used in this study included the following: morphine hydrochloride (1.0, 2.5, and 5.0 µg, Shenyang Medicament Co., Shenyang, China), naloxone hydrochloride (1.0 µg, RBI/Sigma Co., St. Louis, MO, USA), endomorphin-1 (5.0 µg, RBI/Sigma), [D-Ala^2, D-Leu^5]-enkephalin (DADLE; 10 µg, RBI/Sigma), Spiradoline mesylate salt (U-62066; 100 µg, RBI/Sigma), β-funaltrexamine hydrochloride (β-FNA; 3.75 µg, RBI/Sigma), naltrindole hydrochloride (5.0 µg, RBI/Sigma). Antagonists were administered 5 min prior to the agonist, with the exception of β-FNA, which was administered 24 h before the agonist. Endomorphin-1, DADLE, β-FNA and naltrindole were all freshly prepared. In this study, injections were performed twice in each animal with the same drug and dose to perform the mechanical and cold stimulation tests, respectively. A time interval of 2 days was allowed before the same animal was tested again to assure that the residual drug effects were eliminated and that allodynic responses had returned to baseline levels. Drug doses were chosen according to previous studies, where they were reported to be effective [19–24]. The same volume of 0.9% saline was injected in the control animals.

Behavioral Measurements

One week after catheterization (i.e., three weeks after spinal nerve ligation), behavioral testing was performed during the day portion of the circadian cycle (08:00–18:00 h) at room temperature (22°C). All rats were habituated in the experimental arena (20 min for mechanical test and 5 min for cold test) daily during the 3 days before testing. Animal behavior was measured before and 5, 15, 25, 35, 45, 55, and 65 min after drug or saline administration. The investigator was blind to the treatment condition of the rats.

Tactile Test

The hind paw withdrawal threshold, in response to tactile stimulation (von Frey filaments), was measured using the up-down method [25, 26]. The rat was 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 (Stoelting Company, Wood Dale, IL), with approximately equal logarithmic incremental (0.17) bending force, were chosen. Starting with filament 4.31, which is one of the middle series of filaments, von Frey filaments were applied repeatedly from underneath. Pressure was perpendicular to the ventral surface of the third or fourth toe of the hind paw and had sufficient force to cause slight bending; this was held for 6–8 s. The pattern of positive and negative responses was converted into a 50% threshold using the formula given by Dixon [25].

Cold Plate Test

Cold-induced hind paw withdrawal response was determined as described by Jasmin [27]. The rat was placed on a metal plate kept at a temperature of 4 ± 1°C, and covered with a transparent plastic box (280 × 250 × 210 mm). Throughout a 60 min observation period, the number of times that the rat lifted its hind paw (number of paw lifts) from the floor during a 5 min period was recorded during a time interval of 10 min. The steps accompanied by walking were not counted.

Data Analysis

All data were presented as mean ± S.E.M. The non-parametric Kruskal–Wallis test was used for analysis of the difference in more than two groups, and the Mann–Whitney test was used to analyze the difference between two groups at each time point. The Spearman correlation analysis (two tailed) was performed for analysis of the correlation between the effects and doses of morphine. A P value less than 0.05 was considered to be statistically significant. The data were analyzed by using the SPSS 13.0 software.

Results

General

Two weeks after spinal nerve ligation, the paw withdrawal threshold and number of paw lifts (8.1 ± 0.4 g; 6.6 ± 0.2, $n = 25$), which were measured contralateral to the nerve injury site, were significantly different ($P < 0.001$) from the pre-injury (12.9 ± 0.5 g and 1.5 ± 0.3, $n = 25$) and sham-test animals (12.8 ± 0.7 g and 1.6 ± 0.3, $n = 6$). These results are similar to a previous report [3] and suggest that mirror-image allodynia was successfully induced, although the degree of mirror-image allodynia was significantly smaller than the primary (ipsilateral) allodynia, as reported previously in the same rats [15]. Normal saline (0.9%, 0.5 µl) microinjected into the Sm had no significant ($P > 0.05$) effect on mirror-image allodynia. The mean paw withdrawal threshold and number of paw lifts were 7.7 ± 0.6 g and 6.0 ± 0.2 ($n = 6$), respectively (Fig. 1a, b).

Inhibitory Effects of Morphine on Mirror-Image Allodynia

Microinjections of morphine (1.0, 2.5, and 5.0 µg) into the Sm, contralateral to the nerve injury, depressed mechanical

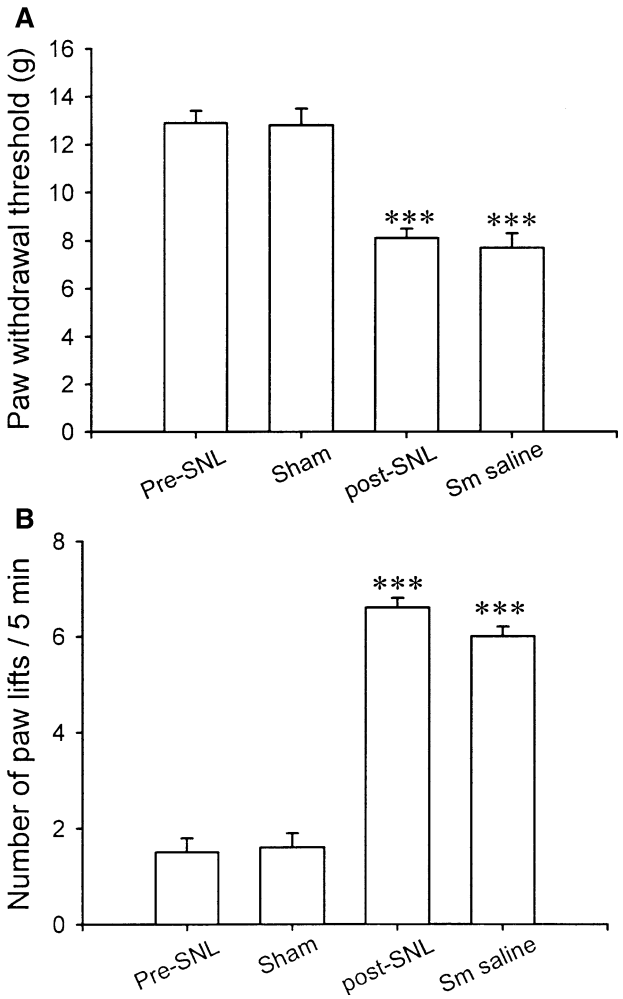

Fig. 1 Bar graphs showing mirror-image allodynia that developed in the rat ligated L5/L6 spinal nerve (SNL), and the effect of saline applied to the Sm on mirror-image allodynia. (**a**) Mechanical paw withdrawal threshold; (**b**) number of paw lifts. ***$P < 0.001$, compared with pre-SNL and sham treated group. $n = 25$ for pre-SNL and post-SNL, $n = 6$ for sham test and Sm saline injection

and cold-induced mirror-image allodynia in a dose-dependent manner ($r = 0.619$, $P < 0.001$, $n = 24$; $r = -0.682$, $P < 0.001$, $n = 24$, respectively). During the 60 min observation period, 5.0 μg morphine applied to the Sm significantly increased the paw withdrawal threshold (12.1 ± 0.9 g, $P = 0.05$), when compared to the 2.5 and 1.0 μg morphine, and well as the saline treated groups. However, no significant differences were observed between the 2.5, 1.0 μg morphine, and saline-treated groups (Fig. 2a). The anti-allodynic effect of morphine was also shown with the cold plate test: 5.0 and 2.5 μg morphine significantly decreased the number of paw lifts (3.3 ± 0.8, 4.6 ± 0.2, $P < 0.05$), when compared to the 1.0 μg morphine and saline-treated groups. However, no significant differences were observed between the 1.0 μg morphine and saline-treated groups (Fig. 2b).

A microinjection of naloxone (1.0 μg), a non-selective opioid receptor antagonist, into the Sm, 5 min prior to morphine (5.0 μg) administration, prevented morphine-induced inhibition of mirror-image allodynia, both in tactile stimulation and in cold plate tests. During the 60 min observation period, the mean paw withdrawal threshold of the naloxone plus morphine-treated group was significantly less, and the mean number of paw lifts was significantly larger than the morphine only group. However, these results were not significantly different from the saline group (Fig. 2a, b).

Naloxone (1.0 μg) injected alone into the Sm enhanced mirror-image allodynia; paw withdrawal threshold and number of paw lifts during the 60 min observation period were significantly different ($P < 0.01$; $P < 0.05$) from the saline group. However, the changes of the paw withdrawal threshold and the number of paw lifts were only 34.4% and 48.0% of the blocking effect of naloxone on morphine-induced inhibition (Fig. 2a, b).

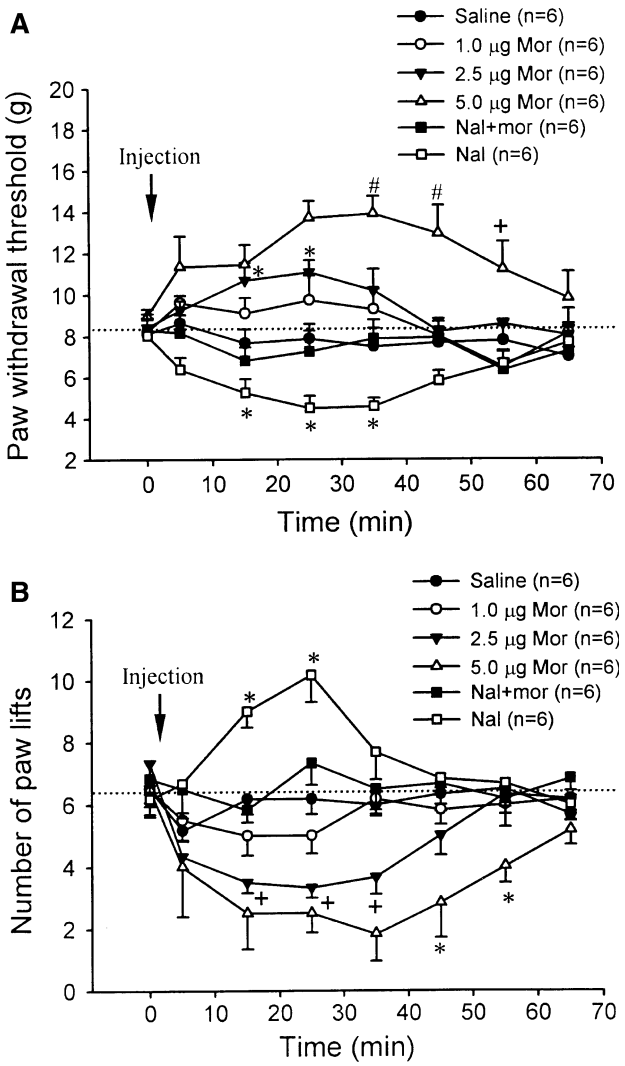

Fig. 2 Time course curves showing morphine (Mor) effects of different doses (1.0, 2.5, and 5.0 μg) applied to the Sm on mirror-image mechanical (**a**) and cold (**b**) allodynia in the rat ligated spinal nerve, as well as the influence of naloxone (Nal., 1.0 μg) on mirror-image allodynia and on morphine (5.0 μg)-induced inhibition of mirror-image allodynia. *$P < 0.05$, compared with saline group; #$P < 0.05$, compared with 2.5 μg morphine; +$P < 0.05$, compared with 1.0 μg morphine at those time points. The 0 time point represents the baseline values measured before drug injections

Blocking Effects of β-FNA on Endomorphin-1 Evoked Inhibition of Mirror-Image Allodynia

When endomorphin-1 (5.0 μg), a highly selective μ-opioid receptor agonist, was microinjected into the Sm, it was able to reduce spinal nerve ligation-induced mirror-image allodynia. This effect was blocked by β-FNA (3.75 μg), a selective μ-opioid receptor antagonist, which was administered into the same Sm site 24 h prior to endomorphin-1 application. As shown in Fig. 3a and b, following endomorphin-1 microinjection, the mean paw withdrawal threshold and number of paw lifts during the 60 min observation period were significantly different from the saline group ($P < 0.05$). Following β-FNA treatment, the paw withdrawal threshold and number of paw lifts were the same as the baseline values (at 0 time point in Fig. 3a, b); however, under this condition, endomorphin-1 induced inhibition was completely blocked, and the mean paw withdrawal threshold and number of paw lifts values were maintained at the saline control levels (Fig. 3a, b).

Effects of Naltrindole on DADLE-Evoked Inhibition of Mirror-Image Allodynia

DADLE (10 μg), a δ- and partial μ-opioid receptor agonist, was microinjected into the Sm and significantly reduced spinal nerve ligation-induced mirror-image allodynia.

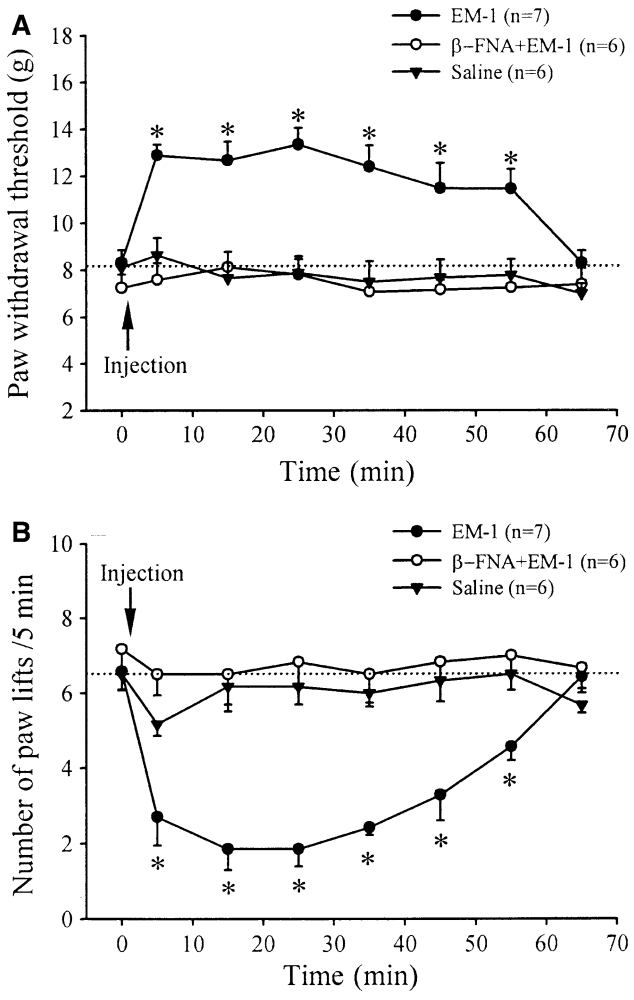

Fig. 3 Time course curves showing the effects of endomorphin-1 (EM-1, 5.0 μg) microinjected into Sm on mirror-image mechanical (**a**) and cold (**b**) allodynia induced by ligation to the rat spinal nerve, and the influence of *β*-FNA (3.75 μg) on these effects. $^*P < 0.05$, compared with saline and *β*-FNA plus EM-1 groups at those time points. The 0 time point represents the values measured 24 h after *β*-FNA injection and the baseline values before other drug injections

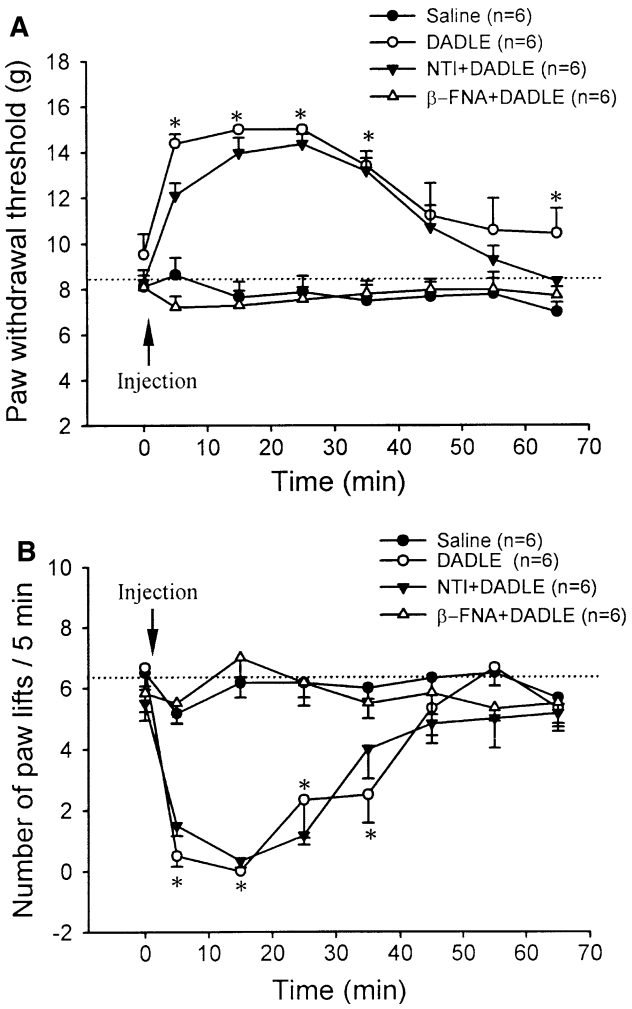

Fig. 4 Time course curves showing the effects of DADLE (10 μg) microinjected into the Sm on mirror-image mechanical (**a**) and cold (**b**) allodynia in a rat model of spinal nerve ligation, and the influence of naltrindole (NTI, 5.0 μg) and *β*-FNA (3.75 μg) on these effects. $^*P < 0.05$, compared with saline and *β*-FNA plus DADLE groups at those time points. The 0 time point represents the values measured 24 h after *β*-FNA injection or the baseline values before other drug injections

However, this anti-allodynic effect was not influenced by pretreatment with naltrindole (5.0 μg), a selective *δ*-opioid receptor antagonist. As shown in Fig. 4a and b, following the DADLE injection, the paw withdrawal threshold and number of paw lifts during the 60 min observation period were significantly greater and less than the saline group, respectively. Naltrindole pretreatment, however, did not influence DADLE-induced inhibition of mirror-image allodynia; the mean paw withdrawal threshold and number of paw lifts were not significantly different from those of the DADLE only group (Fig. 4a, b). Microinjection of naltrindole alone into the Sm had no significant effect on mirror-image allodynia, as compared with the saline group (data not shown).

Blocking Effects of *β*-FNA on DADLE-Evoked Inhibition of Mirror-Image Allodynia

Microinjection with the *μ*-opioid receptor antagonist *β*-FNA (3.75 μg), 24 h prior to DADLE application, was effective in preventing DADLE-induced inhibition of mirror-image allodynia. As shown in Fig. 4a and b, when *β*-FNA was applied together with DADLE, the mean paw withdrawal threshold and number of paw lifts during the 60 min observation period were significantly different from the DADLE only group; however, there were no differences when compared to the saline control group.

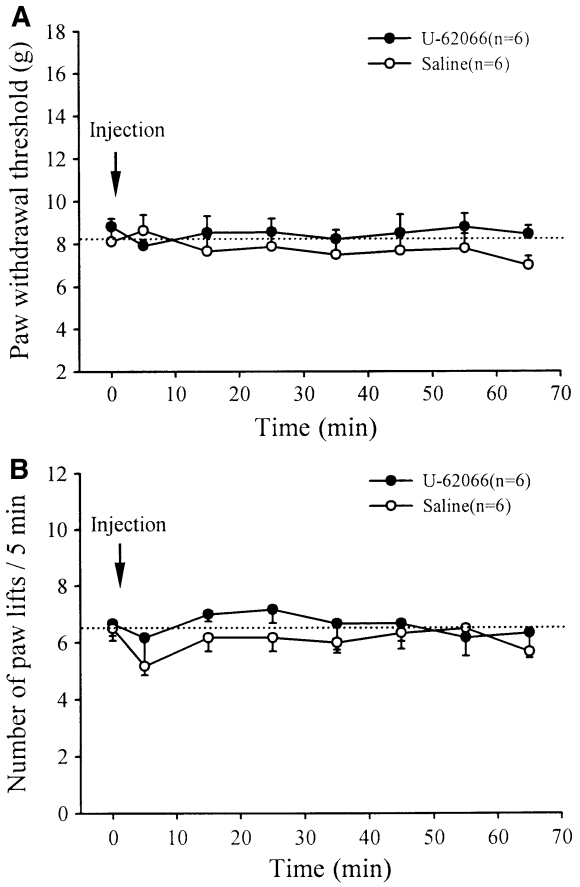

Fig. 5 Time course curves showing the effects of U-62066 (100 μg) microinjected into the Sm on mirror-image mechanical (**a**) and cold (**b**) allodynia induced by spinal nerve ligation in rats. There are no statistical differences between the U-62066 and saline groups

Effects of U-62066 on Mirror-Image Allodynia

U-62066 (100 μg), a selective non-peptide κ-opioid receptor agonist, microinjected into the Sm, did not influence spinal nerve ligation-induced mirror-image allodynia. Neither the paw withdrawal threshold nor the number of paw lifts were significantly different between the U-62066 microinjection group and saline control group, as shown in Fig. 5a and b.

Discussion

Role of Opioid in Sm-Mediated Inhibition of Mirror-Image Allodynia

Previous studies have shown that L5 spinal nerve injury-induced mechanical allodynia is enhanced bilaterally in μ-opioid receptor knockout mice, and systemic administration of morphine attenuated the mechanical paw withdrawal response bilaterally in wild type mice [28]. Intrathecal injection of endomorphin inhibited contralateral mechanical allodynia induced by peripheral inflammation [29]. These results suggest that endogenous opioid peptide is involved in the inhibition of mirror-image allodynia. This current study has shown that morphine microinjections into the Sm are capable of reducing tactile and cold-induced mirror-image allodynia in the spinal nerve ligation rat in a dose-dependent manner. These effects can be prevented by pretreatment at the same site with naloxone, a non-selective opioid receptor antagonist. These results are consistent with those reported previously in primary neuropathic pain induced in the same animal experiments [15], which suggests that the Sm is involved in an opioid receptor-mediated anti-allodynic effect and it plays a role in not only primary neuropathic pain, but also in mirror-image pain induced by spinal nerve ligation.

Interestingly, different from the primary allodynia [15, 30], injections of the opioid receptor antagonist naloxone alone into the Sm significantly enhanced the mirror-image allodynia, suggesting that the opioid receptors in the Sm in the nerve injured rats are tonically activated by activity of an endogenous opioid system which causes a reduction of the mirror-image allodynia. Since morphine microinjection into Sm alone completely blocks the mirror-image allodynia and naloxone alone enhances the allodynia one would expect that in the experiments where both naloxone and morphine are given, the morphine effect would be blocked but that the naloxone-induced enhancement of allodynia would still be present. Since this was not the case perhaps the dose of naloxone was not high enough to block both the endogenous opioid actions and the exogenous morphine or the morphine induced effect was partially mediated by a non-naloxone reversible mechanism.

Involvement of μ-Opioid Receptors in Sm Opioid-Evoked Inhibition of Mirror-Image Allodynia

The present study has demonstrated that the highly selective μ-opioid receptor agonist endomorphin-1 depressed spinal nerve ligation-induced mirror-image allodynia when microinjected into the Sm; the selective μ-opioid receptor antagonist, β-FNA, can block this inhibition. This suggests that the μ-opioid receptor mediates Sm opioid-induced inhibition of mirror-image allodynia in the spinal nerve ligation rat. Results from the present study support this hypothesis. Specifically, the application of β-FNA, but not naltrindole, into the Sm selectively blocks the DADLE-induced inhibition of mirror-image allodynia, although DADLE has a higher affinity to δ-receptor than μ-receptor [22]. Our results show that μ-opioid receptors mediate the Sm opioid-evoked anti-allodynic effect, and also provide functional support for the anatomic finding that μ-, not δ-, opioid receptors are present in the rat Sm [31, 32]. Moreover, previous studies have shown that systemic application

of the μ-opioid receptor agonist DAMGO ([D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]-Enkephalin), or intrathecal applications of endomorphin, reduced mechanical allodynia in the paw contralateral to the peripheral sciatic mononeuropathy or ipsilateral injection of complete Freund's adjuvant (CFA) in rats [29, 33]. The present study has further shown that the forebrain (thalamus) is involved in μ-opioid receptor-mediated inhibition of mirror-image pain and suggests that the Sm might, in part, mediate the anti-allodynic effect produced by the systemic μ-opioid receptor agonist. Since mechanical or cooling stimulation-induced paw withdrawal responses are considered to be a spinal reflex, we presumed that when opioids are applied to the Sm, activation of the brainstem descending inhibitory system might mediate inhibition of mirror-image allodynia via the Sm-VLO-PAG-RVM (rostral ventromedial medulla) pathway [8–11] by reducing nociceptive transmission at the spinal dorsal horn. As previously reported in an acute pain model [34], the GABAergic disinhibition mechanism might be involved in the μ-opioid receptor activation-induced anti-allodynic effect in the Sm. Opioid injected into the Sm might depress inhibitory actions of GABAergic neurons on output neurons projecting to the VLO, which leads to activation of the descending inhibitory pathway and an anti-allodynic effect. However, further investigation is needed to clarify the mechanisms of descending modulation in neuropathic pain models.

In addition, our results also suggest that δ-receptors do not play a role in Sm opioid-evoked inhibition of mirror-image pain in a rat model of L5/L6 spinal nerve ligation. These results are consistent with previous reports that the selective δ-receptor antagonist naltrindole, when applied to the Sm in the same neuropathic pain model, does not influence DADLE-induced inhibition of primary tactile and cold allodynia [15]. However, contrasting results were reported by Hurley and Hammond [35, 36]; levels of [Met^5]-enkephalin and [Leu^5]-enkephalin, which bind preferentially to the δ-opioid receptor, increased in the RVM and in other brainstem nuclei of CFA-treated rats. Microinjection of the δ-receptor agonist, [D-Ala^2, Glu^4] deltorphin, into the RVM of CFA-treated rats produced anti-hyperalgesia in the ipsilateral injured hind paw, and anti-nociception in the contralateral uninjured hind paw. However, application of the δ-receptor antagonist, naltriben, to the RVM enhanced hyperalgesia in the ipsilateral hind paw, and induced hyperalgesia in the contralateral uninjured hind paw. This suggests that the δ-opioid receptor in the RVM is involved in the modulation of primary and mirror-image hyperalgesia in rat models of inflammation injury. These differences strongly support the hypothesis provided by Fields and Basbaum that, at supraspinal sites, the role of δ-opioid receptors in mediating antinociception may be site-dependent [37].

Previous studies indicated that systemic administration of (−)-*trans*-(IS,25)-U-50488 hydrochloride (U-50488H), a κ-opioid agonist, bilaterally and dose-dependently depressed mechanical withdrawal responses of the contralateral hind paw in L5 nerve injured rats [7]. In addition, contralateral, but not systemic, administration of U-50488H depressed mirror-image and primary hypersensitivity induced by inflammation [38]. These results suggest that the κ-opioid receptor is involved in opioid-evoked inhibition of mirror-image allodynia. The present study demonstrated that the selective κ-opioid agonist, U-62066, when applied to the Sm, failed to alter mirror-image allodynia. Although the κ-opioid receptor is expressed in the Sm [32], the Sm is unlikely to be a specific site where κ-opioid receptors are involved in opioid-evoked inhibition of mirror-image pain in a rat model of spinal nerve ligation.

In conclusion, the present study suggests that the Sm is involved in opioid-induced inhibition of mirror-image allodynia, and the μ- (but not δ- and κ-) opioid receptor mediates the anti-allodynic effect in the rat ligated spinal nerve.

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