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Pharmacology, Biochemistry and Behavior 75 (2003) 447–457
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Possible antinociceptive mechanisms of opioid receptor antagonists in the mouse formalin test

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Received 25 September 2002; received in revised form 25 February 2003; accepted 5 May 2003

Abstract

It has been reported that opioid receptor antagonist can induce antinociception in several nociceptive tests. In the intraplantar formalin pain model, however, opioid antagonist-induced antinociception, as well as its underlying mechanism, has not been well characterized. Therefore, in the mouse formalin test, we attempted to characterize the site of action and the possible opioid receptor subtypes. We found that naltrexone (a nonselective opioid antagonist) injected intraperitoneally (ip, 1–20 mg/kg), intrathecally (it, 0.1–10 μg) and intracerebroventricularly (icv, 0.1–10 μg) inhibited nociceptive behaviors only during the second phase (20–40 min) but not during the first (0–5 min) phase. Administration of β-funaltrexamine (β-FNA, 10–40 mg/kg ip, 1.25–5 μg it or icv), naltrindole (1–10 mg/kg ip, 1.25–5 μg it or icv) and nor-binaltorphimine (nor-BNI, 1–10 mg/kg ip, 10–40 μg it or icv), which are selective μ-, δ- and κ-opioid antagonists, respectively, also produced antinociception during the second phase. Additionally, we examined the involvement of the descending monoaminergic systems in the naltrexone-induced antinociception in the formalin test. Pretreatment with 5,7-dihydroxytryptamine (5,7-DHT, a serotonergic neurotoxin, 20 μg it), but not *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4, a noradrenergic neurotoxin, 20 μg it), reversed the naltrexone-induced antinociception during the second phase. Our results suggest that blockade of supraspinally or spinally located opioid receptors may play roles in the regulation of antinociception during the tonic painful stage. In addition, opioid receptors localized at the neuroterminal of the descending serotonergic, but not noradrenergic, inhibitory system in the spinal cord appear to be involved in opioid antagonist-induced antinociception during the second tonic phase of the formalin test.

Keywords: Opioid receptor antagonist; Naltrexone; Formalin test; Antinociception; Descending serotonergic system; Mouse

1. Introduction

Generally, opioid receptor antagonists do not induce antinociception or analgesic effect in the nociceptive tests. However, in mid-1980s, Levine and Gordon (1986) have shown that administration of naloxone with a programmable infusion pump exclusively produces analgesia. Subsequently, several groups have reported that the nonselective opioid receptor antagonist (naloxone or naltrexone) itself produced paradoxical antinociception in various nociceptive tests. For example, single acute injection or repeated administration of naloxone or naltrexone can induce antinociceptive responses in the hot-plate test (Cappell et al., 1989; Poulos et al., 1990; Foo and Westbrook, 1991; Rochford and Stewart, 1992; Bianchi and Panerai, 1993; Walker et al., 1994). Furthermore, in this thermal nociceptive test, the possible involvement of κ-opiate receptors (Bianchi and Panerai, 1993) or 5-HT_2 receptors (Walker et al., 1994) has been suggested in naloxone-induced antinociception and/or in plasticity of the opiatergic system. In addition, naloxone has shown antinociception at very low doses (1 μg/kg) and hyperalgesia at higher doses (100 μg/kg) in the tail-flick tests in mice (Ueda et al., 1986). Kamei et al. (1992a) have found that subcutaneous (sc) injection of naloxone produces a dose-related increase in tail-flick latency in streptozotocin-induced diabetic mice. They also have demonstrated that naloxone-induced paradoxical analgesia in nondiabetic mice may be mediated by δ-opioid receptor (Kamei et al., 1992a,b).

Formalin injection into the mouse hindpaw induces nociceptive behaviors such as licking, shaking/flinching or biting the injured hindpaw, and this formalin-induced nociceptive test has been used as a model of tonic inflammatory

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doi:10.1016/S0091-3057(03)00144-8

pain (Hunskaar and Hole, 1987; Wheeler-Aceto et al., 1990). For the first time, Foo and Westbrook (1993) have described that treatment of naloxone enhanced the hypoalgesic responses to the formalin injection in rat. In another model of inflammatory pain (carrageenan-induced inflammation), low dose of naloxone induces an antinociceptive response to noxious thermal stimuli for both the inflamed and the noninflamed paws 4 h after carrageenan injection (Tsuruoka et al., 1998). They have suggested the involvement of noradrenergic mechanisms in naloxone-induced antinociception only in the early phase of carrageenan-induced inflammation (Tsuruoka et al., 1998; Tsuruoka and Willis, 1998). In the formalin test, however, opioid receptor antagonist-induced antinociceptive responses have not been characterized in detail. Moreover, the underlying mechanism of antinociception produced by opioid receptor antagonist in the formalin test is not known.

It has been well established that the monoaminergic descending inhibitory system has a modulatory role in formalin-induced nociception (Fasmer et al., 1985; Tjolsen et al., 1991; Omote et al., 1998; Martin et al., 1999). Pharmacological and biochemical studies have revealed that the painful state produced by formalin-induced inflammation is under the modulation of the monoaminergic (noradrenergic and serotonergic) descending inhibitory system (Omote et al., 1998; Martin et al., 1999). Moreover, it has been reported that the selective depletion of the serotonergic or noradrenergic neurons in brainstem reduced the nociceptive behavioral responses to formalin injection, which confirms that the descending monoaminergic inhibitory system participates in the regulation of tonic nociception in the spinal cord (Fasmer et al., 1985; Tjolsen et al., 1991; Martin et al., 1999). Accordingly, it is needed to evaluate relationship between the antinociceptive effect of opioid receptor antagonist and the descending pain inhibitory system in the formalin test.

Thus, it can be presumed that some possible links between the opioid receptor antagonist-induced antinoci-

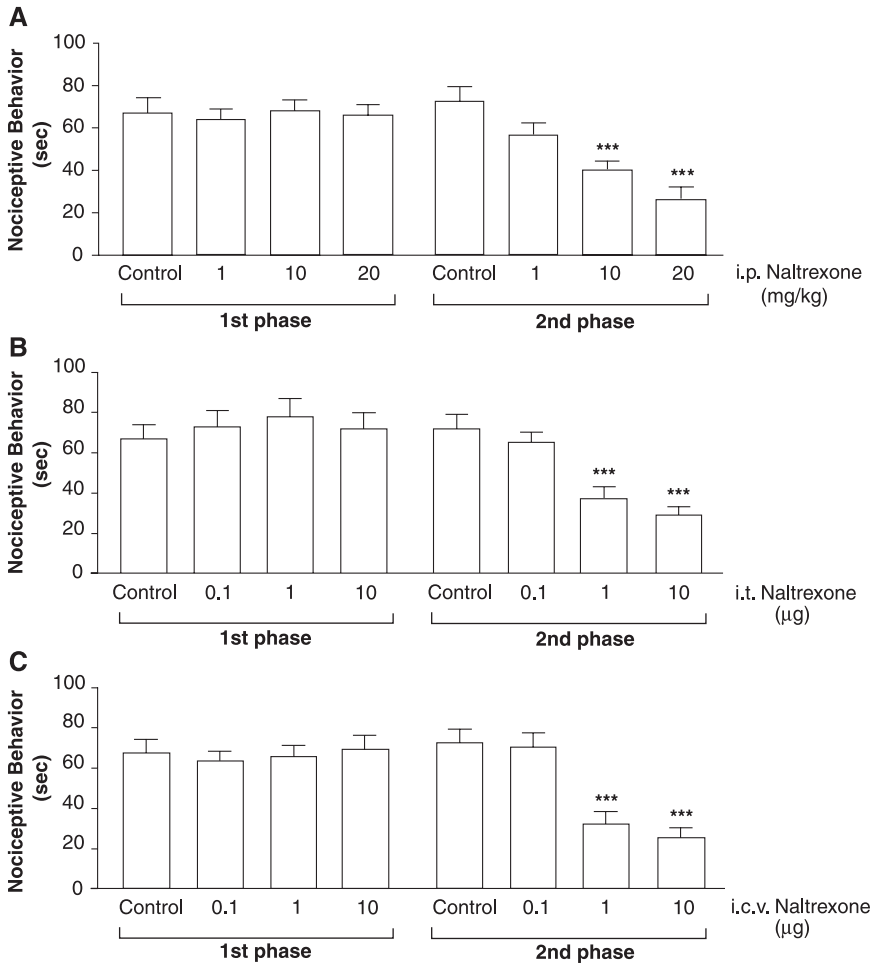

Fig. 1. Effects of naltrexone on the formalin-induced nociceptive behavioral response manifested during the first and second phases. Animals were treated intraperitoneally (A), intrathecally (B) and intracerebroventricularlly (C) with naltrexone at various doses for 15, 5 and 5 min, respectively, prior to the formalin (1%, 10 μl) injection into the plantar aspect of the left side hindpaw subcutaneously. The cumulative nociceptive response time of licking, shaking and biting the injected paw was measured during the period of 0–5 min (first phase) and 20–40 min (second phase). The vertical bars denote S.E.M. The number of animals used for each group was 10. $***P<.001$, compared with the control group of mice.

ception and the descending monoaminergic systems may exist. We have hypothesized that the opioid receptor antagonist-induced antinociception may be involved in the opioid receptor located at descending monoaminergic neuroterminal in the spinal cord. Therefore, in the present study, we investigated whether administration of naltrexone (a nonselective opioid receptor antagonist) through various routes of administration inhibits the nociceptive behaviors in the formalin pain model in mice as well as determine which subtype of opioid receptors is involved. Furthermore, to determine if the descending serotonergic and noradrenergic pain inhibitory systems were involved in the opioid antagonist-induced antinociception in the formalin test, effects of 5,7-dihydroxytryptamine (5,7-DHT) and *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) pretreated spinally on the naltrexone-induced antinociception were also examined.

2. Methods

These experiments were approved by the University of Hallym Animal Care and Use Committee. All procedures were conducted in accordance with the ''Guide for Care and Use of Laboratory Animals'' published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain.

2.1. Experimental animals

Male ICR mice (MJ, Seoul, Korea) weighing 20–25 g were used for all the experiments. Animals were housed five per cage in a room maintained at 22 ± 0.5 °C with an alternating 12 h light–dark cycle. Food and water were available ad libitum. The animals were allowed to adapt to the laboratory for at least 2 h before testing and were only

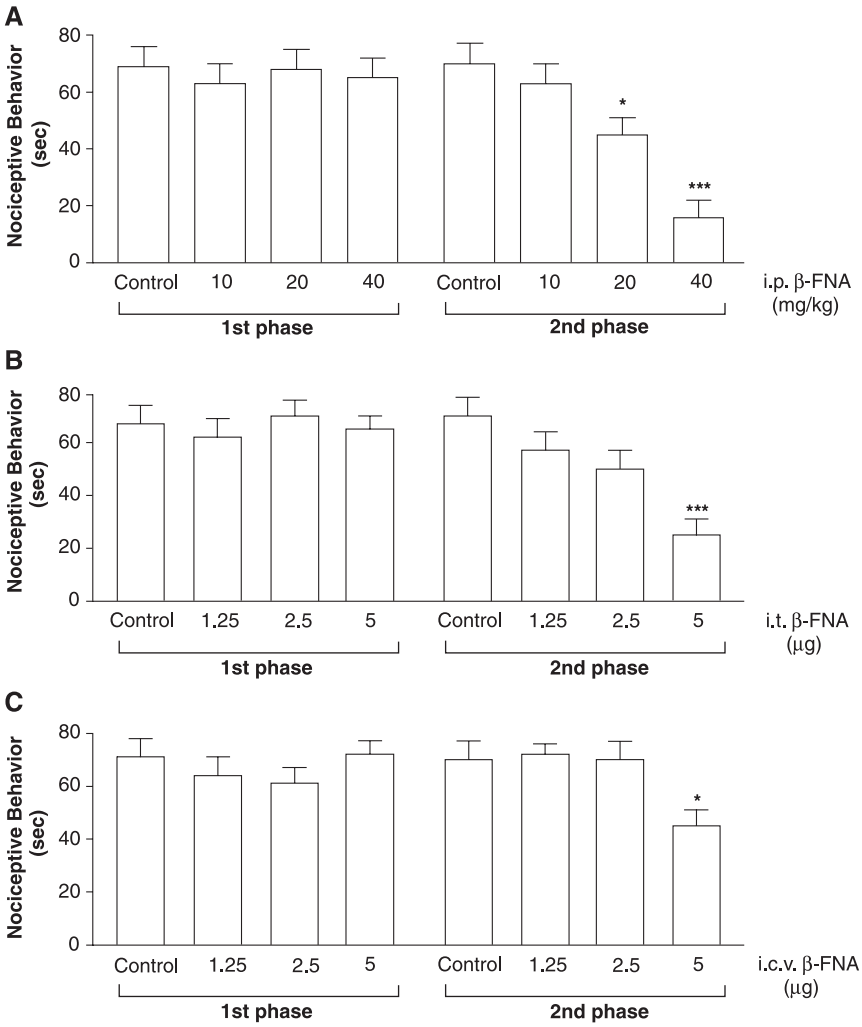

Fig. 2. Effects of β-FNA on the formalin-induced nociceptive behavioral response manifested during the first and second phases. Animals were treated intraperitoneally (A), intrathecally (B) and intracerebroventricularlly (C) with β-FNA at various doses for 24 h prior to the formalin (1%, 10 μl) injection into the plantar aspect of the left side hindpaw subcutaneously. The cumulative nociceptive response time of licking, shaking and biting the injected paw was measured during the period of 0–5 min (first phase) and 20–40 min (second phase). The vertical bars denote S.E.M. The number of animals used for each group was 10. * $P < .05$ and *** $P < .001$, compared with the control group of mice.

used once. To reduce variation, all experiments were performed during the light phase of the cycle (10:00–17:00 h).

2.2. Intraperitoneal (ip), intracerebroventricular (icv) and intrathecal (it) injections

Intraperitoneal injection was conducted to unanesthesized mice with volume of 10 ml/kg body weight. Intracerebroventricular injections were made according to the procedure of Haley and McCormick (1957). The intrathecal administration was performed following the method described by Hylden and Wilcox (1980, 1981) using a 30 G needle connected to a 25 μl Hamilton syringe with polyethylene tubing. Intracerebroventricular and intrathecal injection volume was 5 μl and the injection sites were verified by injecting a similar volume of 1% methylene blue solution and determining the distribution of the injected dye in the ventricular space or in the spinal cord. The dye injected intracerebroventricularly was found to be distributed through the ventricular spaces and reached the ventral surface of the brain and upper cervical portion of the spinal cord. The dye injected intrathecally was distributed both rostrally and caudally but with short distance (about 0.5 cm from the injection site) and no dye was found visually in the brain. The success rate for the injections was consistently found to be over 95%.

2.3. Formalin-induced nociceptive behavioral test

This test, which was previously published by Hunskaar et al. (1985), was carried out in mice. Ten microliters of 1.0% formalin solution, made up in physiologic saline (0.9% NaCl), was injected subcutaneously under the plantar surface of the left hindpaw. Following intraplantar injection of

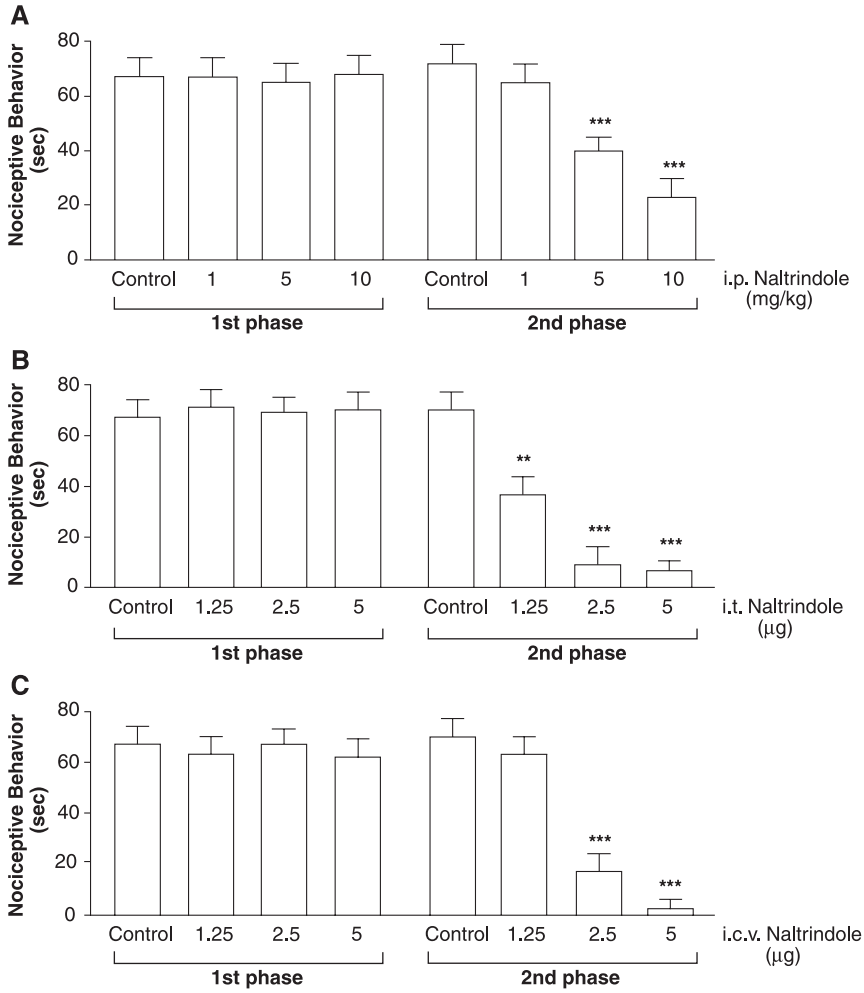

Fig. 3. Effects of naltrindole on the formalin-induced nociceptive behavioral response manifested during the first and second phases. Animals were treated intraperitoneally (A), intrathecally (B) and intracerebroventricularlly (C) with naltrindole at various doses for 10 min prior to the formalin (1%, 10 μl) injection into the plantar aspect of the left side hindpaw subcutaneously. The cumulative nociceptive response time of licking, shaking and biting the injected paw was measured during the period of 0–5 min (first phase) and 20–40 min (second phase). The vertical bars denote S.E.M. The number of animals used for each group was 10. $^{**}P<.01$ and $^{***}P<.001$, compared with the control group of mice.

formalin, the animals were immediately placed on an acrylic observation chamber (20 cm high, 20 cm diameter), and the time spent licking, shaking and biting the injected paw was measured with a stopwatch timer and considered as indicative of nociception. This was based on a previous finding that the formalin test for nociceptive response, especially second phase, is best characterized by the cumulative time spent biting/licking the injected paw using multiple regression analysis (Sufka et al., 1998). Control animals for formalin nociceptive test received a similar volume of physiologic saline into the left hindpaw. All assessments were carried out by two blind observers.

2.4. Drugs

Formalin and 5,7-DHT were purchased from Sigma (St. Louis, MO, USA). Naltrexone, β-funaltrexamine (β-FNA), naltrindole, nor-binaltorphimine (nor-BNI) and DSP-4 were purchased from Research Biochemicals (Natick, MA, USA). All drugs were dissolved in physiologic saline (0.9% w/v NaCl), except for 5,7-DHT and DSP-4, which were dissolved in 10% dimethyl sulfoxide (DMSO). All drugs were prepared just before use.

2.5. Experimental protocols

2.5.1. Treatment of opioid receptor antagonists

All treatment times and doses were determined from our previous studies, and the preliminary time course studies that showed effects reached a maximum after injection (Suh and Tseng, 1988, 1990; Suh et al., 2000).

2.5.1.1. Treatment of nonselective opioid receptor antagonist. Mice were injected once (1–20 mg/kg ip) with naltrexone for 15 min prior to the intraplantar formalin injection. Also, naltrexone was injected once intrathecally or intra-

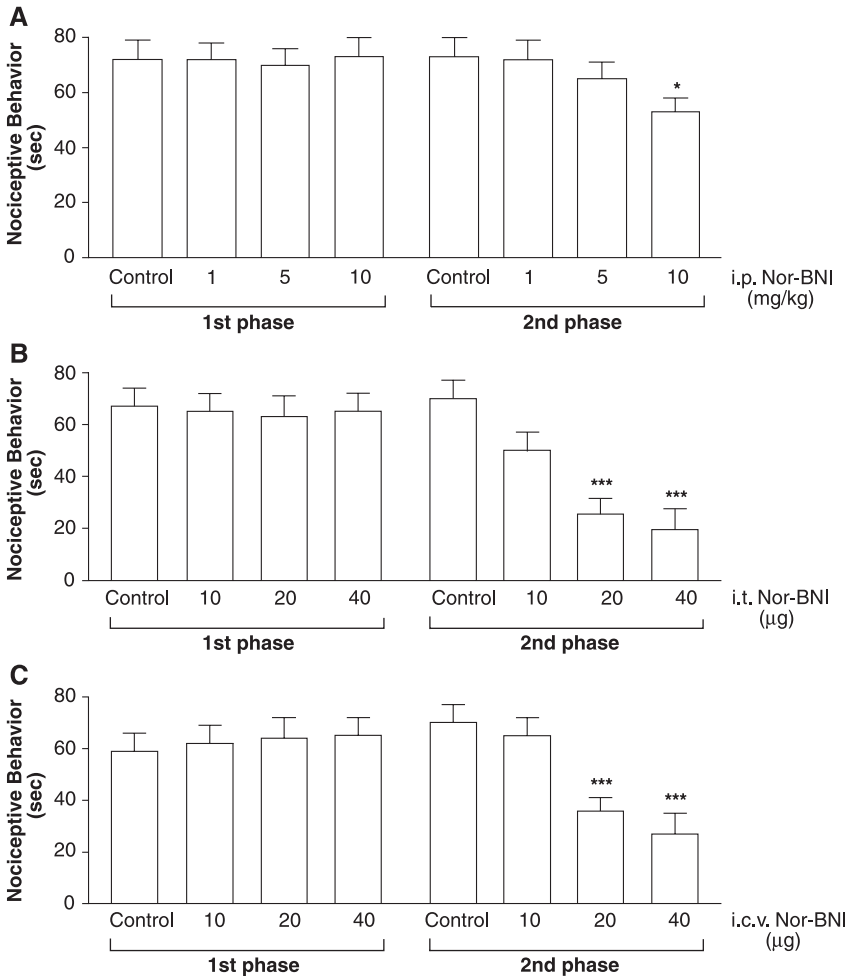

Fig. 4. Effects of nor-BNI on the formalin-induced nociceptive behavioral response manifested during the first and second phases. Animals were treated intraperitoneally (A), intrathecally (B) and intracerebroventricularlly (C) with nor-BNI at various doses for 6 h prior to the formalin (1%, 10 μl) injection into the plantar aspect of the left side hindpaw subcutaneously. The cumulative nociceptive response time of licking, shaking and biting the injected paw was measured during the period of 0–5 min (first phase) and 20–40 min (second phase). The vertical bars denote S.E.M. The number of animals used for each group was 10. $*P<.05$ and $***P<.001$, compared with the control group of mice.

cerebroventricularly at various doses (0.1–10 μg) for 5 min prior to the formalin injection. Control group received a similar volume of physiologic saline through the same routes of administration.

2.5.1.2. Treatment of selective μ-opioid receptor antagonist. Mice were injected once intraperitoneally (10–40 mg/kg), intrathecally (1.25–5 μg) or intracerebroventricularly (1.25–5 μg) with β-FNA for 24 h prior to the intraplantar formalin injection. Control animals received a similar volume of physiologic saline through the same routes of administration.

2.5.1.3. Treatment of selective δ-opioid receptor antagonist. Mice were injected once intraperitoneally (1–10 mg/kg), intrathecally (1.25–5 μg) or intracerebroventricularly (1.25–5 μg) with naltrindole for 10 min prior to the intraplantar formalin injection. Control group of animals received a similar volume of physiologic saline through the same routes of administration.

2.5.1.4. Treatment of selective κ-opioid receptor antagonist. Mice were injected once intraperitoneally (1–10 mg/kg), intrathecally (10–40 μg) or intracerebroventricularly (10–40 μg) with nor-BNI for 6 h prior to the intraplantar formalin injection. Control group of animals received a similar volume of physiologic saline through the same routes of administration.

2.5.2. Pretreatment of monoaminergic toxins

Mice were pretreated intrathecally with 5,7-DHT (20 μg), DSP-4 (20 μg) or vehicle (10% DMSO) for 72 h prior to intrathecal injection of naltrexone (20 μg) or saline as a control. After 5 min, the intraplantar formalin test was performed. The pretreatment time and dose used were based on the results of previous reports (Kuraishi et al., 1983; Berge et al., 1985; Fasmer et al., 1985; Suh et al., 1992). Intrathecal pretreatment of mice or rats with DSP-4 or 5,7-DHT at the doses used in the present study has been previously shown by others to selectively deplete norepinephrine and serotonin, respectively (Kuraishi et al.,

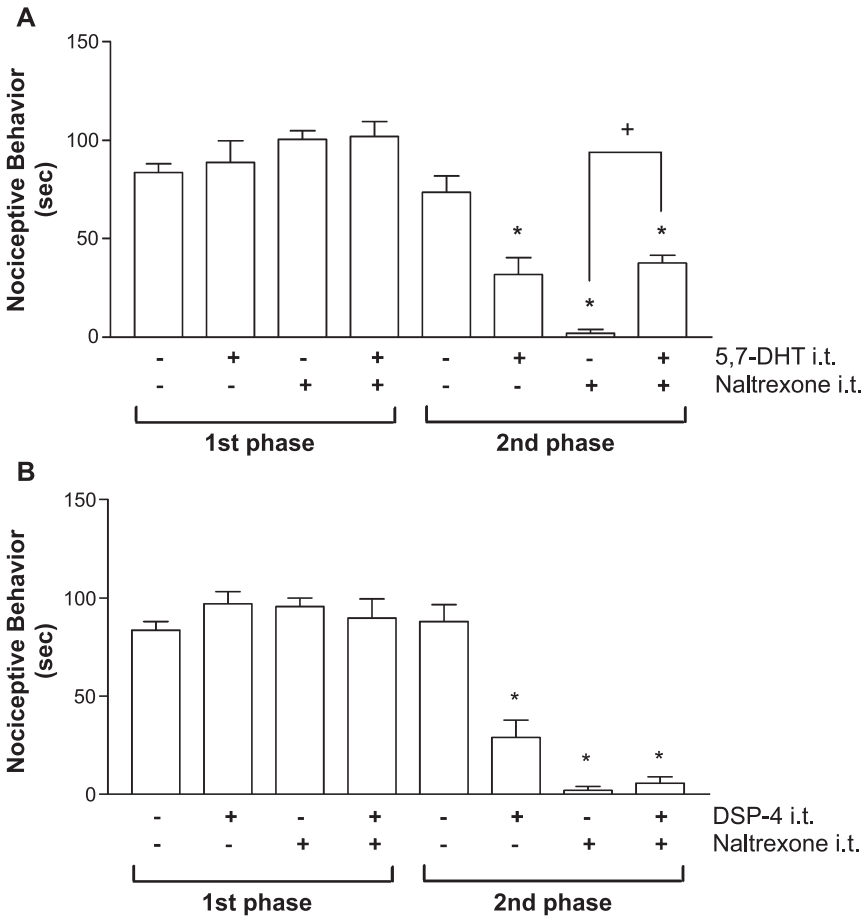

Fig. 5. Effects of 5,7-DHT and DSP-4 administered intrathecally on the antinociception induced by intrathecal naltrexone during the first and second phases of the formalin test. Animals were pretreated with intrathecally with 5,7-DHT (20 μg, A) or DSP-4 (20 μg, B) for 72 h prior to it injection of naltrexone (20 μg). Then, after 5 min, the formalin (1%, 10 μl) was injected into the plantar aspect of the left side hindpaw subcutaneously. The cumulative nociceptive response time of licking, shaking and biting the injected paw was measured during the period of 0–5 min (first phase) and 20–40 min (second phase). The vertical bars denote S.E.M. The number of animals used for each group was 20. * $P<.05$, compared with the control group of mice. $^{+}$ $P<.05$, compared with the naltrexone-treated group of mice.

1983; Berge et al., 1985; Nakazawa et al., 1991; Zhong et al., 1985; Pang and Vasko, 1986; Sawynok et al., 1991). This indicates that intrathecal pretreatment with DSP-4 or 5,7-DHT at the doses used in the present study selectively depletes norepinephrine and serotonin, respectively, in the spinal cord.

2.6. Statistical analysis

Statistical analyses for the antinociceptive effect of opioid receptor antagonists were carried out by two-way analysis of variance (ANOVA) with Bonferroni's post hoc test using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, USA), the factors being Treatment (opioid receptor antagonist vs. saline) and Nociceptive test (formalin vs. saline) (Figs. 1–4). In Fig. 5, three-way ANOVA tested the effect of 5,7-DHT or DSP-4 on the naltrexone-induced antinociception using SigmaStat version 2.03 for Windows (SPSS, Chicago, IL, USA), the factors being Pretreatment [5,7-DHT (or DSP-4) vs. DMSO], Treatment (naltrexone vs. saline) and Nociceptive test (formalin test vs. saline). When appropriate, Student's–Newman–Keuls' post hoc tests were carried out. P values less than .05 were considered to indicate statistical significance. All values were expressed as the means ± S.E.M.

3. Results

3.1. The effect of naltrexone on the nociceptive behavioral response elicited by intraplantar injection of formalin

After saline treatment (intraperitoneal, intrathecal or intracerebroventricular), subcutaneous injection of 1% formalin into the left hindpaw caused an acute, immediate nociceptive response, i.e., licking, shaking and biting the injected paw, which lasted for 5 min (first phase). The second phase formalin response began about 20 min after formalin administration and lasted for about 20 min. However, subcutaneous injection of saline into the left hindpaw did not induce any considerable nociceptive behavior (Table 1).

A two-way ANOVA indicated significant effects of intraperitoneal naltrexone (nonselective opioid receptor antagonist) treatment and formalin nociceptive test [$F(3,72)=12.89$, $P<.0001$ and $F(3,72)=277.73$, $P<.0001$, respectively], intrathecal naltrexone treatment and formalin nociceptive test [$F(3,72)=13.41$, $P<.0001$ and $F(3,72)=300.47$, $P<.0001$, respectively] and intracerebroventricular naltrexone treatment and formalin nociceptive test [$F(3,72)=14.90$, $P<.0001$ and $F(3,72)=230.12$, $P<.0001$, respectively] during the second phase. Moreover, Naltrexone treatment × Formalin test interaction during late phase reached a significant level [intraperitoneal, $F(3,72)=13.02$, $P<.0001$; intrathecal, $F(3,72)=13.56$, $P<.0001$; intracerebroventricular, $F(3,72)=15.05$, $P<.0001$]. In intraperitoneal naltrexone-treated group of mice, Bonferroni's post hoc comparison showed that cumulative time of intraplantar formalin-induced nociceptive behaviors was decreased as compared with control only during the second tonic phase, but not the first phase, at the doses of 10 mg/kg [$F(1.36)=16.61$, $P<.001$] and 20 mg/kg [$F(1.36)=27.59$, $P<.0001$] (Fig. 1A). In addition to systemic administration, naltrexone given spinal (intrathecal) or supraspinal (intracerebroventricular) route at the doses of 1 and 10 μg showed that the cumulative response time of the formalin-induced nociceptive behavior was significantly attenuated during the second tonic phase [1 μg it, $F(1.36)=14.20$, $P<.001$; 10 μg it, $F(1.36)=27.08$, $P<.0001$; 1 μg icv, $F(1.36)=18.55$, $P<.001$; 10 μg icv, $F(1.36)=28.58$, $P<.0001$] but not the first phase (Fig. 1B and C).

Table 1
Control experiments of intraplantar injection of saline versus formalin in each experiment

		Intraplantar saline injection		Intraplantar 1% formalin injection	
		First phase	Second phase	First phase	Second phase
Experiment 1-1	A	1.2 ± 1.0	1.3 ± 1.0	71.9 ± 5.2	72.6 ± 6.8
	B	1.2 ± 0.5	1.9 ± 0.9	67.0 ± 7.0	72.0 ± 7.5
	C	1.0 ± 1.0	1.1 ± 0.3	67.5 ± 6.0	71.5 ± 7.0
Experiment 1-2	A	1.5 ± 0.5	1.4 ± 0.6	69.8 ± 6.5	70.4 ± 6.9
	B	0.7 ± 0.3	1.7 ± 1.0	67.5 ± 9.0	71.0 ± 7.0
	C	2.0 ± 0.9	1.0 ± 0.2	71.0 ± 4.4	70.5 ± 5.0
Experiment 1-3	A	2.0 ± 2.0	1.9 ± 1.0	66.7 ± 6.4	71.2 ± 6.0
	B	1.5 ± 0.4	1.0 ± 1.0	70.0 ± 5.1	73.0 ± 7.0
	C	1.5 ± 1.0	2.0 ± 1.0	67.0 ± 7.0	69.6 ± 3.7
Experiment 1-4	A	1.4 ± 1.0	1.1 ± 0.5	71.7 ± 6.5	72.9 ± 7.0
	B	3.0 ± 2.0	1.4 ± 1.0	67.0 ± 7.0	70.3 ± 4.1
	C	1.0 ± 1.0	1.2 ± 1.0	69.0 ± 9.2	69.5 ± 5.0
Experiment 2	A	1.4 ± 1.0	1.3 ± 1.0	70.5 ± 4.5	73.5 ± 8.5
	B	1.2 ± 0.5	1.8 ± 1.0	78.0 ± 8.5	75.0 ± 8.5

The cumulative nociceptive response time (s) of licking, shaking and biting the injected paw was measured during the period of 0–5 min (first phase) and 20–40 min (second phase) after injection. The number of animals used for each group was 10–20.
Each value denotes the mean ± S.E.M. of three or more independent experiments.

3.2. The effect of β-FNA on the nociceptive behavioral response elicited by intraplantar injection of formalin

As shown in Table 1, after saline treatment (intraperitoneal, intrathecal or intracerebroventricular), formalin injection into the left hindpaw caused the nociceptive responses during the first and second phases. However, subcutaneous injection of saline into the left hindpaw did not induce any considerable nociceptive behavior.

A two-way ANOVA indicated significant effects of intraperitoneal β-FNA (selective μ-opioid receptor antago-

nist) treatment and formalin nociceptive test [$F(3,72) = 15.49$, $P < .0001$ and $F(3,72) = 234.92$, $P < .0001$, respectively], intrathecal β-FNA treatment and formalin nociceptive test [$F(3,72) = 7.60$, $P < .001$ and $F(3,72) = 206.88$, $P < .0001$, respectively] and intracerebroventricular β-FNA treatment and formalin nociceptive test [$F(3,72) = 4.25$, $P < .01$ and $F(3,72) = 411.15$, $P < .0001$, respectively] during the second phase. Moreover, β-FNA treatment × Formalin test interaction during late phase reached a significant level [intraperitoneal, $F(3,72) = 15.66$, $P < .0001$; intrathecal, $F(3,72) = 7.69$, $P < .001$; intracerebroventricular, $F(3,72) = 4.34$, $P < .01$]. In intraperitoneal β-FNA-treated group of mice, Bonferroni's post hoc comparison showed that cumulative time of intraplantar formalin-induced nociceptive behaviors was attenuated as compared with control during the second tonic phase, but not the first phase, at the doses of 20 mg/kg [$F(1.36) = 7.17$, $P < .05$] and 40 mg/kg [$F(1.36) = 38.32$, $P < .0001$] (Fig. 2A). β-FNA given spinal [$F(1.36) = 22.92$, $P < .0001$] or supraspinal [$F(1.36) = 7.05$, $P < .05$] route caused a significant reduction of cumulative response time of the formalin-induced nociceptive behaviors only during the second phase at the dose of 5 μg (Fig. 2B and C).

3.3. The effect of naltrindole on the nociceptive behavioral response elicited by intraplantar injection of formalin

As shown in Table 1, after saline treatment (intraperitoneal, intrathecal or intracerebroventricular), formalin injection into the left hindpaw caused the nociceptive responses during the first and second phases. However, subcutaneous injection of saline into the left hindpaw did not induce any considerable nociceptive behavior.

A two-way ANOVA indicated significant effects of intraperitoneal naltrindole (selective δ-opioid receptor antagonist) treatment and formalin nociceptive test [$F(3,72) = 13.99$, $P < .0001$ and $F(3,72) = 260.13$, $P < .0001$, respectively], intrathecal naltrindole treatment and formalin nociceptive test [$F(3,72) = 20.81$, $P < .0001$ and $F(3,72) = 82.71$, $P < .0001$, respectively] and intracerebroventricular naltrindole treatment and formalin nociceptive test [$F(3,72) = 26.79$, $P < .0001$ and $F(3,72) = 129.23$, $P < .0001$, respectively] during the second phase. Moreover, Naltrindole treatment × Formalin test interaction during late phase reached a significant level [intraperitoneal, $F(3,72) = 14.15$, $P < .0001$; intrathecal, $F(3,72) = 20.90$, $P < .0001$; intracerebroventricular, $F(3,72) = 26.98$, $P < .0001$]. In intraperitoneal naltrindole-treated group of mice, Bonferroni's post hoc comparison showed that cumulative time of intraplantar formalin-induced nociceptive behaviors was decreased as compared with control during the second tonic phase, but not the first phase, at the doses of 5 mg/kg [$F(1.36) = 15.71$, $P < .001$] and 10 mg/kg [$F(1.36) = 28.87$, $P < .0001$] (Fig. 3A). In addition to systemic administration, naltrindole given intrathecally showed that the cumulative response time of the formalin-induced nociceptive behaviors was significantly attenuated during the second tonic phase but not the first phase [1.25 μg, $F(1.36) = 11.09$, $P < .01$; 2.5 μg, $F(1.36) = 37.25$, $P < .0001$; 5 μg, $F(1.36) = 58.59$, $P < .0001$] (Fig. 3B). When naltrindole was administered intracerebroventricularly, the nociceptive behaviors induced by formalin were inhibited at the doses of 2.5 μg [$F(1.36) = 28.30$, $P < .0001$] and 5 μg [$F(1.36) = 67.44$, $P < .0001$] only during the second phase (Fig. 3C).

3.4. The effect of nor-BNI on the nociceptive behavioral response elicited by intraplantar injection of formalin

As shown in Table 1, after saline treatment (intraperitoneal, intrathecal or intracerebroventricular), formalin injection into the left hindpaw caused the nociceptive responses during the first and second phases. However, subcutaneous injection of saline into the left hindpaw did not induce any considerable nociceptive behavior.

A two-way ANOVA indicated significant effects of intraperitoneal nor-BNI (selective κ-opioid receptor antagonist) treatment and formalin nociceptive test [$F(3,72) = 2.86$, $P < .05$ and $F(3,72) = 332.79$, $P < .0001$, respectively], intrathecal nor-BNI treatment and formalin nociceptive test [$F(3,72) = 10.67$, $P < .0001$ and $F(3,72) = 126.28$, $P < .0001$, respectively] and intracerebroventricular nor-BNI treatment and formalin nociceptive test [$F(3,72) = 9.35$, $P < .0001$ and $F(3,72) = 194.40$, $P < .0001$, respectively] during the second phase. Moreover, nor-BNI treatment × Formalin test interaction during late phase reached a significant level [intraperitoneal, $F(3,72) = 2.93$, $P < .05$; intrathecal, $F(3,72) = 10.75$, $P < .0001$; intracerebroventricular, $F(3,72) = 9.46$, $P < .0001$]. In the nor-BNI-treated group of mice, Bonferroni's post hoc comparison showed that cumulative time of intraplantar formalin-induced nociceptive behavior was decreased as compared with control only during the second tonic phase, but not the first phase, at a higher dose used [10 mg/kg, $F(1.36) = 6.15$, $P < .05$] (Fig. 4A). In addition to systemic administration, nor-BNI given spinal (intrathecal) or supraspinal (intracerebroventricular) route at the doses of 20 and 40 μg attenuated significantly the cumulative response time of the formalin-induced nociceptive behaviors during the second phase [20 μg it, $F(1.36) = 22.96$, $P < .0001$; 40 μg it, $F(1.36) = 21.91$, $P < .0001$; 20 μg icv, 15.36, $P < .001$; 40 μg icv, 15.87, $P < .001$] but not the first phase (Fig. 4B and C).

3.5. The effect of 5,7-DHT and DSP-4 pretreatment on the intrathecal naltrexone-induced antinociception in the formalin test

To determine whether the descending inhibitory serotonergic and noradrenergic systems localized at the spinal level were involved in the naltrexone-induced antinociception in the formalin test, serotonergic (5,7-DHT, 20 μg) or noradrenergic (DSP-4, 20 μg) neurotoxin was pretreated intrathecally.

Formalin injection to control group (vehicle pretreatment before intrathecal saline injection) caused the nociceptive responses during the first and second phases. However, subcutaneous injection of saline into the left hindpaw did not induce any considerable nociceptive behavior (Table 1).

A three-way ANOVA during the second phase of formalin test indicated statistically significant effects of naltrexone treatment [$F(1,152)=25.23$, $P<.001$] and formalin nociceptive test [$F(1,152)=115.07$, $P<.001$] but not pretreatment of 5,7-DHT [$F(1,152)=0.21$, $P=0.648$] or DSP-4 [$F(1,152)=0.22$, $P=0.637$]. Moreover, Naltrexone treatment × Formalin nociceptive test [$F(1,152)=25.38$, $P<.001$], 5,7-DHT pretreatment × Naltrexone treatment × Formalin nociceptive test [$F(1,152)=35.00$, $P<.001$] and DSP-4 pretreatment × Naltrexone treatment × Formalin nociceptive test [$F(1,152)=23.28$, $P<.001$] interactions reached a significant level. Interestingly, Student's–Newman–Keuls' post hoc test revealed that 5,7-DHT ($P<.05$) or DSP-4 ($P<.05$) pretreated alone for 72 h decreased significantly the cumulative time of intraplantar formalin-induced nociceptive behavior only during the second tonic phase but not during the first phase. Pretreatment with 5,7-DHT, but not DSP-4, reversed the naltrexone-induced antinociception during the second phase up to the level of 5,7-DHT pretreatment alone (Student's–Newman–Keuls' post hoc test, $P<.05$) (Fig. 5A and B).

4. Discussion

The results of present study clearly demonstrated that the treatment of naltrexone decreased the formalin-induced nociceptive behaviors only during the second tonic phase but not during the first phase. When naltrexone was administered spinally or supraspinally, this antinociceptive response was also observed during the tonic phase of formalin test. In addition, selective μ-, δ- or κ-opioid receptor antagonist administration through intraperitoneal, intrathecal or intracerebroventricular routes induced antinociception during the second phase, suggesting that μ-, δ- or κ-opioid receptors, which are located at the supraspinal brain sites and the spinal cord, are involved in naltrexone-induced paradoxical antinociception in the tonic inflammatory pain.

A previous report has shown that morphine depresses the high K^+-evoked release of Met-enkephalin from slices of the rat brainstem, while naloxone significantly enhances the release by 80.6% (Ueda et al., 1986). They have suggested that naloxone can produce analgesia by blocking autoinhibition of enkephalin release in the mouse brain (Ueda et al., 1986). It also has been reported that the naloxone administration to rats showed supersensitivity to the analgesic effects of morphine as well as analgesia in hot-plate test (Poulos et al., 1990). Paronis and Holtzman (1991) have demonstrated that 7-day naloxone infusion increases the analgesic potency of opioids with μ-agonist activity. Kamei et al. (1992b) have demonstrated that chronic pretreatment with 5 mg/kg of naloxone for 5 days significantly enhances the analgesic effect of DPDPE, selective δ-opioid receptor agonist, in nondiabetic mice. They have suggested that naloxone-induced paradoxical analgesia in mice may be attributable to the supersensitivity of δ-opioid receptors in mice. In the hot-plate test, naloxone or naltrexone-induced analgesic effect is blocked by the MR 1452 (a κ-opiate receptor antagonist), which is modulated by the κ-opiate receptor agonist U50-488 (Bianchi and Panerai, 1993). Taken together with the result of the present study, it is speculated that μ-, δ- or κ-opioid receptors may play an important role in the naltrexone-induced antinociception during the tonic painful stage. Thus, it is hypothesized that the nonspecific antagonism of opioid receptors may induce supersensitivity of opioid receptors and that blockade of autoinhibition may enhance the release of certain neurotransmitters or neuropeptides responsible for the opioid receptor antagonist-induced antinociception. However, it cannot be ruled out that the involvement of interneuron remains to be elucidated.

Because the descending serotonergic and noradrenergic pain inhibitory systems are activated during the tonic phase in the formalin test (Fasmer et al., 1985; Tjolsen et al., 1991; Omote et al., 1998; Martin et al., 1999), we investigated the possible involvement of the descending serotonergic or noradrenergic pain inhibitory system in naltrexone-induced antinociception in the formalin test. The intrathecal pretreatment of 5,7-DHT, but not DSP-4, caused a reversal of naltrexone-induced antinociception during the second phase of formalin test. Our results using chemical ablation of the descending monoaminergic system strongly suggest that the descending serotonergic, but not noradrenergic, pain inhibitory system may play an important role in naltrexone-induced antinociception during the tonic inflammatory pain at the spinal level. We propose that presynaptic opioid receptors localized at the neuron terminal of the descending serotonergic, but not noradrenergic, inhibitory system in the spinal cord may be involved in the opioid receptor antagonist-induced antinociception during the tonic phase of formalin test. In several pain models, it has been reported that some evidence is related with the serotonergic system in the opioid receptor antagonist-induced antinociception. In hot-plate test, Foo and Westbrook (1991) have revealed that rats pretreated with adrenergic receptor antagonists against several subtypes of adrenoceptors did not affect the naloxone-induced hypoalgesia. In addition, involvement of 5-HT_2 receptors in naloxone-induced analgesia has been reported, although systemic administration of yohimbine blocked the reduction of paw lick latency induced by naloxone in the hot-plate test (Walker et al., 1994). These findings are in line with our results, which serotonergic, but not noradrenergic system, may participate in the naltrexone-induced antinociception especially at the spinal cord level. In carrageen-

an-induced inflammatory pain, it was demonstrated that significantly prolonged paw withdrawal latency in response to noxious thermal stimuli for both the inflamed and the noninflamed paws was produced by a low dose of naloxone (Tsuruoka et al., 1998). However, they have found that the noradrenergic mechanisms in the locus coeruleus may be involved in naloxone-induced antinociception only in the early phase of carrageenan-induced inflammation (Tsuruoka et al., 1998; Tsuruoka and Willis, 1998). This discrepancy can be explained partially with our previous reports that nociceptive behaviors produced by formalin, substance P, excitatory amino acids or capsaicin may be mediated by different nociceptive processing involved in various pain models and the regulation of nociceptive response also may have differential modulatory effects (Chung et al., 2000, 2001; Choi et al., 2001).

Interestingly, we found in the present study that intrathecal pretreatment with 5,7-DHT or DSP-4 alone significantly decreased the formalin-induced nociceptive behavior only during the second tonic phase but not during the first phase. Oyama et al. (1996) have demonstrated that intrathecal administration of selective 5-HT_3 receptor antagonists, granisetron and ondansetron, reduced the second phase of the formalin-induced aversive responses without affecting the first phase. Moreover, when 5-HT are depleted from the lumbar spinal cord by pretreatment with 5,7-DHT, it facilitated the aversive responses in the second phase. Eide and Hole (1988) have observed that intracerebroventricular 5,7-DHT treatment produces supersensitivity to 5-HT. Further experiment of this group has reported that supersensitivity to the antinociceptive effect of a 5-HT_{1B} receptor agonist after lesion of raphe spinal serotonergic neurons on the thermal nociception (tail-flick test) (Eide et al., 1992). Similarly, spinal norepinephrine depletion can potentiate the analgesic effects of intrathecal administration norepinephrine, which can be explained by the receptor supersensitivity (Archer et al., 1986). Another report has shown that depletion of descending noradrenergic system induces the functional supersensitivity both to norepinephrine and to selective α_2-adrenoceptor agonists at the spinal level (Post et al., 1987). Therefore, it can be speculated that supersensitivity to 5-HT or adrenergic receptor located at the spinal level may be involved in intrathecal pretreated 5,7-DHT- or DSP-4-induced antinociceptive responses respectively during the second tonic phase of formalin test.

In conclusion, we found that opioid receptor antagonists administered through the intraperitoneal, intrathecal or intracerebroventricular routes could induce antinociceptive effects during the second phase of the formalin test. It is suggested that μ-, δ- or κ-opioid receptors, which are located at supraspinal brain sites and the spinal cord, are involved in naltrexone-induced paradoxical antinociception in the tonic inflammatory pain. In addition, we establish the hypothesis that presynaptic opioid receptors localized at the neuron terminal of descending serotonergic, but not noradrenergic, pain inhibitory system may be involved in the production of opioid receptor antagonist-induced antinociception during the second tonic phase of formalin test, although the exact mechanisms remain to be elucidated in the future study.

Acknowledgements

This research was supported by research grants from the Ministry of Public Health and Welfare (01-PJ8-PG1-01CN01-0034), South Korea.

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