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# Roles of serotonin receptor subtypes for the antinociception of 5-HT in the spinal cord of rats

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**Abstract**

The contribution of 5-HT (5-hydroxytryptamine) receptor subtypes to the antinociception produced by intrathecal 5-HT in the formalin test was investigated in rats. Intrathecal 5-HT suppressed both phases of behaviors produced by 5% formalin, and this was blocked by antagonists for 5-$HT_{1B}$ (3-[3-(Dimethylamino)propyl]-4-hy-droxy-*N*-[4-(4-pyridinyl)phenyl]benzamide dihydrochloride, GR 55562), 5-$HT_{2C}$ (*N*-ormethylclozapine/8-Chloro-11-(1-piperazinyl)-5*H*-dibenzo[*b*,*e*][1,4]diazepine, D-MC), 5-$HT_3$ (1-Methyl-*N*-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-1*H*-indazole-3-carboxamide maleate, LY-278,584) and 5-$HT_4$ receptors (4-Amino-5-chloro-2-metho-xy-benzoic acid 2-(diethylamino)ethyl ester hydrochloride, SDZ-205,557), but not the 5-$HT_{1D}$ receptor antagonist 3-[4-(4-Chlorophenyl)piperazin-1-yl]-1,1-diphenyl-2-propanol hydrochloride (BRL 15572). The 5-$HT_{1A}$ receptor antagonist *N*-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]-*N*-2-pyridinyl-cyclohexanecarboxamide maleate (WAY-100635) decreased only the second phase antinociception of 5-HT. Intrathecal administration of agonists for 5-$HT_{1A}$ (3-(*N*,*N*-Dipropylaminoethyl)-1*H*-indole-5-carboxamide maleate, Dipropyl-5CT), 5-$HT_{1B}$ (7-Trifluoromethyl-4(4-met-hyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline maleate, CGS-12066A), 5-$HT_{2C}$ (6-Ch-loro-2-(1-piperazinyl)pyrazine hydrochloride, MK 212), 5-$HT_3$ (*N*-(3-Chlorophenyl)imidodicarbonimidic diamide hydrochloride, *m*-CPBG) and 5-$HT_4$ receptors (2-[1-(4-Piperonyl)piperazinyl]benzothiazole, BZTZ) suppressed both phases of the formalin response. The results of the present study indicate that spinal 5-$HT_{1B,}$ 5-$HT_{2C,}$ 5-$HT_3$ and 5-$HT_4$ receptors, but not the 5-$HT_{1D}$ receptor, mediate antinociception produced by 5-HT in the formalin test. The relevance of the 5-$HT_{1A}$ receptor is less clear because of the different effects of antagonist and agonist.


*Keywords:* Antinociception; 5-HT; 5-HT receptor subtype; Intrathecal; (Rat)

## 1. Introduction

Several lines of evidence suggest that the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) plays an important role in the modulation of nociceptive transmission and that the major site of action of 5-HT is the spinal cord (Yaksh and Wilson, 1979; Fields et al., 1991; Furst, 1999). Although multiple 5-HT receptor subtypes have been defined in the central nervous system (Barnes and Sharp, 1999), at least four types of 5-HT receptors (5-$HT_1$, 5-$HT_2$, 5-$HT_3$ and 5-$HT_4$) with several subtypes have been identified in the spinal cord (Marlier et al., 1991; Kidd et al., 1993; Hoyer et al., 1994; Fonseca et al., 2001). Spinal administration of certain agonists and antagonists for 5-HT receptor subtypes has resulted in different effects on nociceptive transmission according to the nociceptive circumstances (Xu et al., 1994; Bardin et al., 2000; Obata et al., 2001; Sasaki et al., 2001; Nadeson and Goodchild, 2002; Sasaki et al., 2003). This indicates that the individual role of these 5-HT receptor subtypes for nociception may be complicated and dependent on the context in which they are given. The effect of 5-HT on the formalin stimulation has been examined less frequently than other nociceptive stimulations at the spinal level. Moreover, intrathecal 5-HT produced the antinociception in the formalin test (Bardin et al., 1997), which subtypes of 5-HT receptor are involved in the antinociceptive effect has not been not synthetically determined.

The aim of the current study was to gain insight into the functional significance of 5-HT receptors in the control of

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nociceptive information at a spinal level using the formalin test. Thus, several selective 5-HT receptor antagonists were intrathecally administered to investigate the ability of 5-HT receptor subtypes to reverse the antinociception induced by 5-HT in the formalin test. Furthermore, selective agonists were intrathecally given to verify the properties of the receptor subtypes involved.

## 2. Materials and methods

### 2.1. Animal preparation

All procedures undertaken were approved by The Institutional Animal Care Committee of the Research Institute of Medical Science at Chonnam National University. Male Sprague–Dawley rats, weighting 250–300 g, were housed four per cage on a 12-h night/day cycle and provided with food and water at all times. Catheter installation into the subarachnoid space was performed as described elsewhere (Yaksh and Rudy, 1976). The rats were anesthetized with enflurane and a 3 cm midline incision was made over the atlantooccipital junction. The atlantooccipital membrane was cut with a needle until clear cerebrospinal fluid flowed freely. A polyethylene-10 catheter was carefully inserted 8.5 cm caudally to the lumbar enlargement and externalized through the anterior part of the scalp. The tip of catheter was plugged with a steel wire and the skin was closed with 3-0 silk sutures. The rats were closely observed and, if motor abnormalities appeared, they were killed through a volatile anesthetics overdose. All the animals were kept in individual cages, and the formalin test started approximately 4–5 days after intrathecal catheterization.

### 2.2. Drugs

The drugs used in this study were as follows: serotonin hydrochloride (Biomol Research Laboratories, USA), *N*-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]-*N*-2-pyri-dinyl-cyclohexanecarboxamide maleate (WAY-100635, Research Biochemical Internationals [RBI], USA), 3-[3-(Dimethylamino)propyl]-4-hydroxy-*N*-[4-(4-pyrid-dinyl)phenyl]benzamide dihydrochloride (GR 55562, Tocris Cookson, UK), 3-[4-(4-Chlorophenyl)piperazin-1-yl]-1,1-diphenyl-2-propanol hydrochloride (BRL 15572, Tocris), Normethylclozapine/8-Chloro-11-(1-piperazinyl)-5*H*-dibenzo[*b*,*e*][1,4]diazepine (DMC, Tocris), 1-Methyl-*N*-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-1*H*-indazole-3-carboxamide maleate (LY-278,584, RBI), 4-Amino-5-chloro-2-meth-oxy-benzoic acid 2-(diethylamino)ethyl ester hydrochloride (SDZ-205,557, RBI), 3-(*N*,*N*-Dipropylaminoethyl)-1*H*-indole-5-carboxamide maleate (Dipropyl-5CT, RBI), 7-Trifluoromethyl-4(4-methyl-1-piperazinyl)-pyrrolo[1,2-*a*]quinoxaline male-ate (CGS-12066A, RBI), 3-[3-(2-Dimethylaminoethyl)-1-*H*-indol-5-yl]-*N*-(4-metho-xybenzyl)acrylamide (GR 46611, Tocris), 6-Chloro-2-(1-piperazinyl)pyrazine hydrochloride (MK 212, Tocris), *N*-(3-Chlorophenyl)imidodicarbonimidic diamide hydrochloride (*m*-CPBG, RBI) and 2-[1-(4-Piperonyl)piperazinyl]benzothiazole (BZTZ, Tocris). BRL 15572, DMC, GR 46611 and BZDZ were dissolved in dimethylsulfoxide (DMSO). The other drugs were dissolved in normal saline. Intrathecal administration of drugs was performed using a hand-driven, gear-operated syringe pump. All the drugs were delivered in a volume of 10 μl solution.

### 2.3. Nociceptive test

In order to evoke a nociceptive state, 50 μl of 5% formalin solution was injected subcutaneously into the plantar surface of the hindpaw with a 30-gauge needle. The injection caused the affected paw to flinch or shake. This formalin-induced behavior was regarded as a pain response and monitored for 60 min. The numbers of flinching/shaking responses were counted over 1-min period at 1–2 and 5–6 min, and at 5-min intervals at 10–60 min. As a biphasic flinching response was observed after the formalin injection, a 0- to 10-min interval was defined as phase 1 of the formalin test, whilst the interval from 10 to 60 min was defined as phase 2. The rats were killed using a volatile anesthetics overdose directly after the formalin test was conducted.

### 2.4. Experimental protocol

Four to five days after intrathecal catheterization, the rats were placed in a restraining cylinder for the experiments. After a 20-min habituation period, the rats were then randomly assigned to one of the drug treatment groups. Experiments were carried out in a blind fashion. The drug vehicles (saline or DMSO) were used as a control. The rats were used only once for the formalin test.

### 2.5. Effects of 5-HT, 5-HT antagonists and agonists

Self-righting and place-stepping reflexes were used in order to examine motor tone after intrathecal administration of the experimental drugs. The former was assessed by placing the rat horizontally with its back on the table, which normally gives rise to an immediate coordinated twisting of the body to an upright position. The latter was evaluated by drawing the dorsum of either hindpaw of the rat across the edge of the table, resulting in the rat trying to put the paw ahead in a walking position.

To evaluate the time course and dose–response to the antinociceptive effects of 5-HT (30, 100 and 300 μg), 5-HT was intrathecally administered 10 min prior to the formalin injection. Next, in order to determine which subtypes of 5-HT receptor mediated the effect of 5-HT, several 5-HT receptor antagonists were intrathecally administered 10 min before the delivery of 5-HT (300 μg), and formalin was injected 10 min later. The 5-HT receptor antagonists were selected on the basis of relevant receptor affinity and selectivity, and the doses were

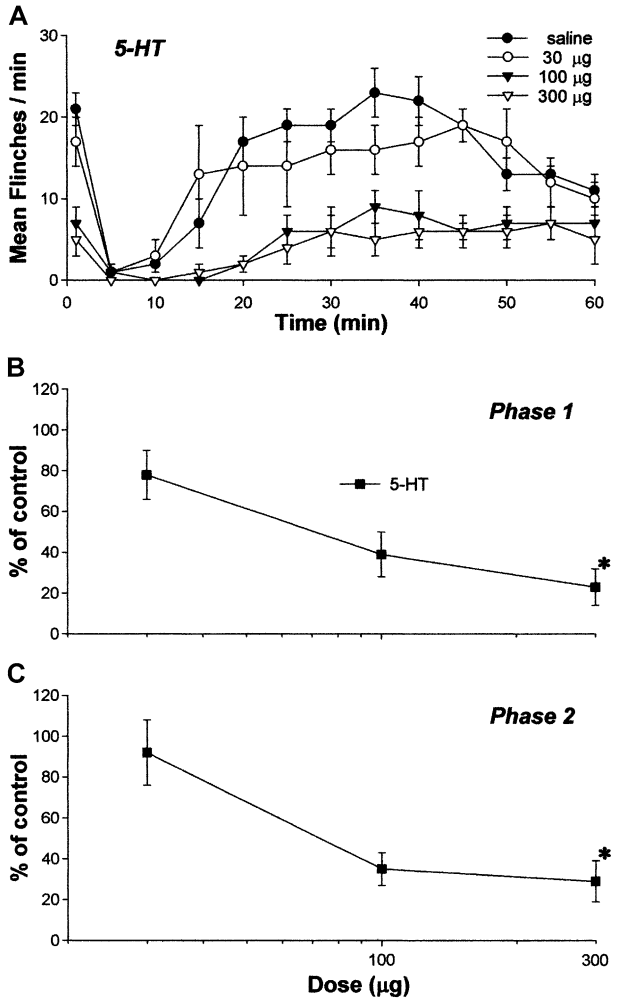


Fig. 1. Time effect (A) and dose–response curves (B and C) of intrathecal 5-HT for recorded flinching in the formalin test. 5-HT was administered 10 min prior to the formalin injection. Formalin was injected at time 0. The data represents the number of flinches (A) and the sum of flinches (B and C). Each line represents mean±S.E.M. for six to nine rats. *$P$<0.05.

chosen based on pilot experiments, in which the maximum dosage that did not affect the control formalin response or cause side effects such as motor impairment was determined. The 5-HT receptor antagonists used were as follows: 5-$HT_{1A}$ receptor antagonist, WAY-100635 (3 μg); 5-$HT_{1B}$ receptor antagonist, GR 55562 (30 μg); 5-$HT_{1D}$ receptor antagonist, BRL 15572 (30 μg); 5-$HT_{2C}$ receptor antagonist, DMC (30 μg); 5-$HT_3$ receptor antagonist, LY-278,584 100 (μg); and 5-$HT_4$ receptor antagonist, SDZ-205,557 (30 μg). Finally, to further characterize the subtypes of the receptor involved, several 5-HT receptor agonists were intrathecally delivered. The 5-HT receptor agonists used were as follows: 5-$HT_{1A}$ receptor agonist, Dipropyl-5CT (1, 3 and 10 μg); 5-$HT_{1B}$ receptor agonist CGS-12066A (3, 10 and 30 μg); 5-$HT_{1D}$ receptor agonist, GR 46611 (1 μg); 5-$HT_{2C}$ receptor agonist, MK 212 (10, 30 and 100 μg); 5-$HT_3$ receptor agonist, *m*-CPBG (10, 30 and 100 μg); and 5-$HT_4$ receptor agonist, BZTZ (3, 10 and 30 μg). In the present study, unfortunately, the rats showed signed of motor impairment at more than 30 μg of BRL 15572 (5-$HT_{1D}$ receptor antagonist) or 1 μg of GR 46611 (5-$HT_{1D}$ receptor agonist), thus, further experiments were not performed regarding the role of the 5-$HT_{1D}$ receptor for the antinociception of 5-HT.

### 2.6. Data and statistics

The data collected is expressed as mean±S.E.M. The time response data are presented as the number of flinches. The dose–response data are presented as percentage of control in each phase. The number of flinches was converted to a percentage of control as shown below:

$$\%\ \text{of control} = \frac{\text{Sum of phase 1(2) flinching count with drug}}{\text{Sum of control phase 1(2) flinching count}} \times 100$$

The dose–response data was analyzed using one-way analysis of variance (ANOVA) with Scheffe post hoc

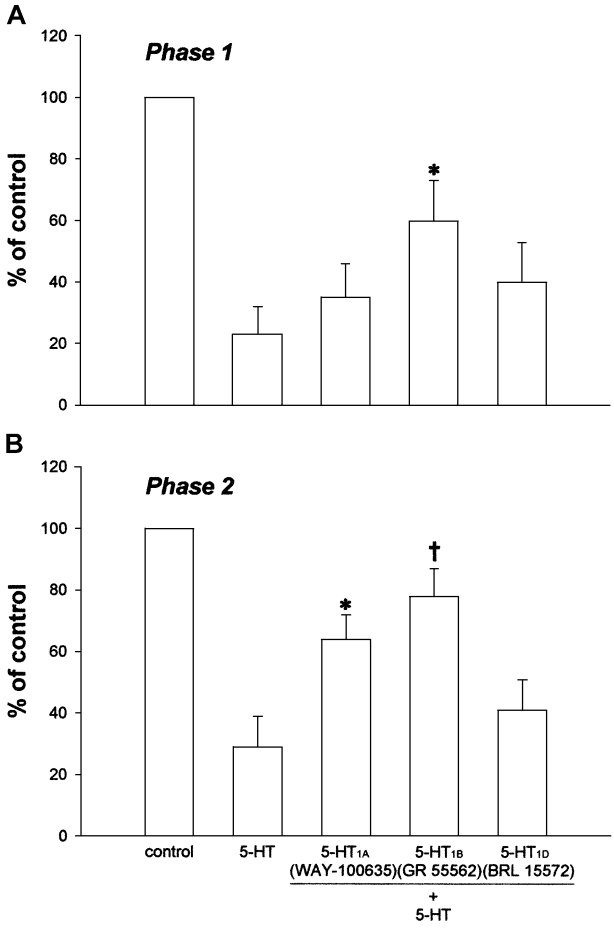


Fig. 2. The antagonistic effects of intrathecal 5-$HT_{1A}$ antagonist (WAY-100635, 3 μg), 5-$HT_{1B}$ antagonist (GR 55562, 30 μg) and 5-$HT_{1D}$ antagonist (BRL 15572, 30 μg) for the antinociception of intrathecal 5-HT (300 μg) during phase 1 (A) and phase 2 (B) of the formalin test. All three antagonists and 5-HT were administered 20 or 10 min prior to the injection of formalin, respectively. The data is presented as a percentage of control. Each bar represents mean±S.E.M. for five to eight rats. Compared with 5-HT. *$P$<0.05. †$P$<0.01.

analysis. Comparison of antagonism for the effects of 5-HT was analyzed by unpaired *t*-test. $P<0.05$ was considered to be statistically significant.

## 3. Results

### *3.1. Behavioral effects of formalin, 5-HT and 5-HT receptor antagonists and agonists*

A subcutaneous injection of formalin into the hindpaw resulted in a biphasic flinching response of the injected paw. The sum of the number of flinches in the control group (saline vs. DMSO) was not statistically different from those in either of the phases (21±1 vs. 19±1 in phase 1, 166±15 vs. 158±16 in phase 2).

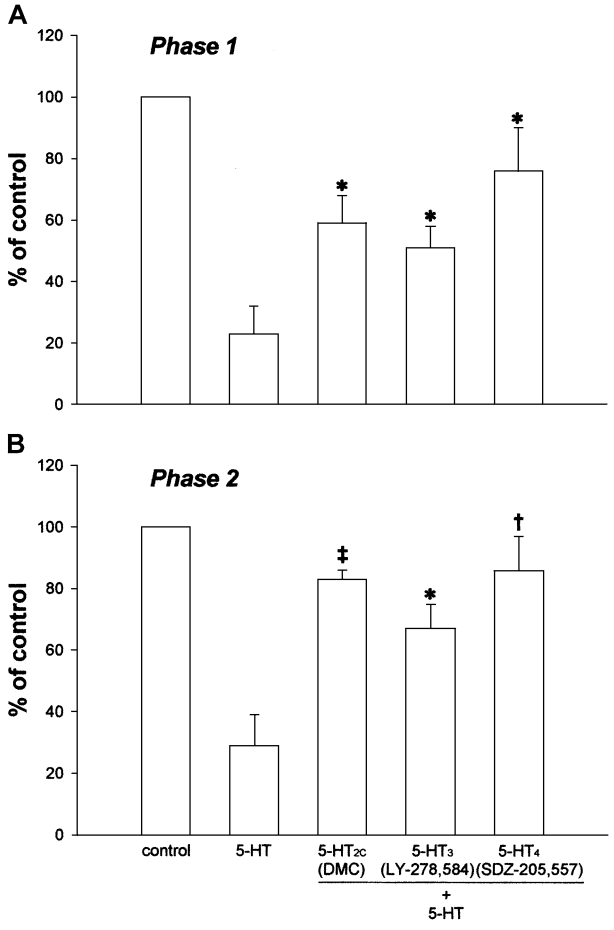


Fig. 3. The antagonistic effects of intrathecal 5-$HT_{2C}$ antagonist (DMC, 30 μg), 5-$HT_3$ antagonist (LY-278,584, 100 μg) and 5-$HT_4$ antagonist (SDZ-205,557, 30 μg) for the antinociception of intrathecal 5-HT (300 μg) during phase 1 (A) and phase 2 (B) of the formalin test. All three antagonists and 5-HT were administered 20 or 10 min prior to the injection of formalin, respectively. The data is presented as a percentage of control. Each bar represents mean±S.E.M. for five to eight rats. Compared with 5-HT. *$P<0.05$. †$P<0.01$. ‡$P<0.001$.

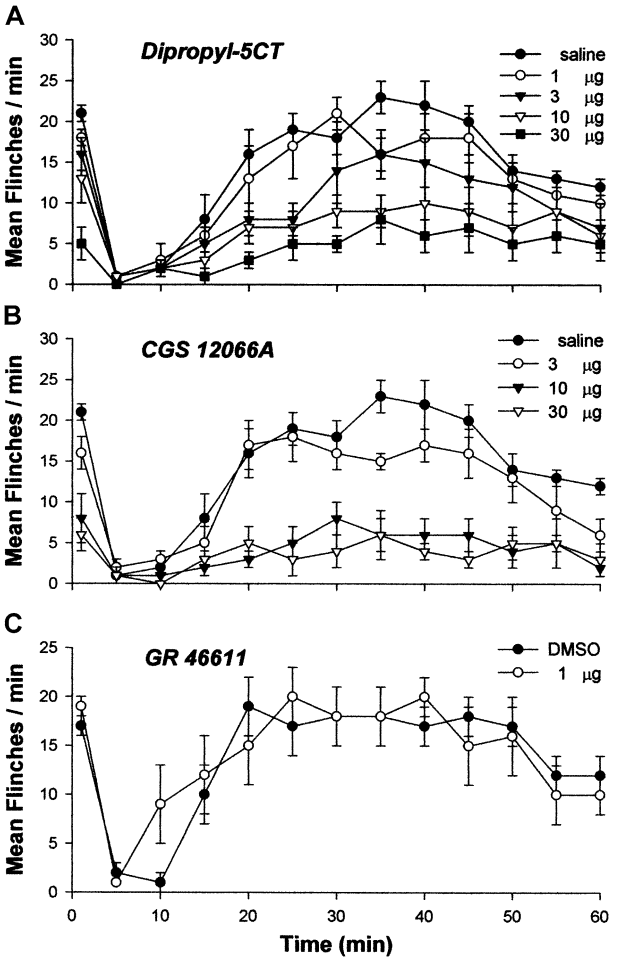


Fig. 4. Time effect curves of intrathecal Dipropyl-5CT (A), CGS 12066A (B) and GR 46611 (C) for recorded flinching in the formalin test. Each drug was administered 10 min prior to the formalin injection. Formalin was injected at time 0. The data represents the number of flinches. Each line represents the mean±S.E.M. for six to eight rats.

Motor function of the rats was found to be normal after intrathecal administration of 5-HT, 5-HT receptor antagonists and agonists at the doses used for this study.

### *3.2. Effects of intrathecal selective 5-HT receptor antagonists on antinociception by 5-HT*

Intrathecal 5-HT produced a dose-dependent suppression of the flinching responses during phase 1 and phase 2 in the formalin test (Fig. 1).

Intrathecal 5-$HT_{1A}$ receptor antagonist WAY-100635 reversed the antinociceptive effect of 5-HT during phase 2, but not during phase 1, of the formalin test (Fig. 2). The 5-$HT_{1D}$ receptor antagonist BRL 15572 did not alter the antinociception of 5-HT in either phase (Fig. 2). The 5-$HT_{1B}$ receptor antagonist GR 55562, 5-$HT_{2C}$ receptor antagonist DMC, 5-$HT_3$ receptor antagonist LY-278,584 and 5-$HT_4$ receptor antagonist SDZ-205,557 reversed the effects of 5-HT in both phases (Figs. 2 and 3).

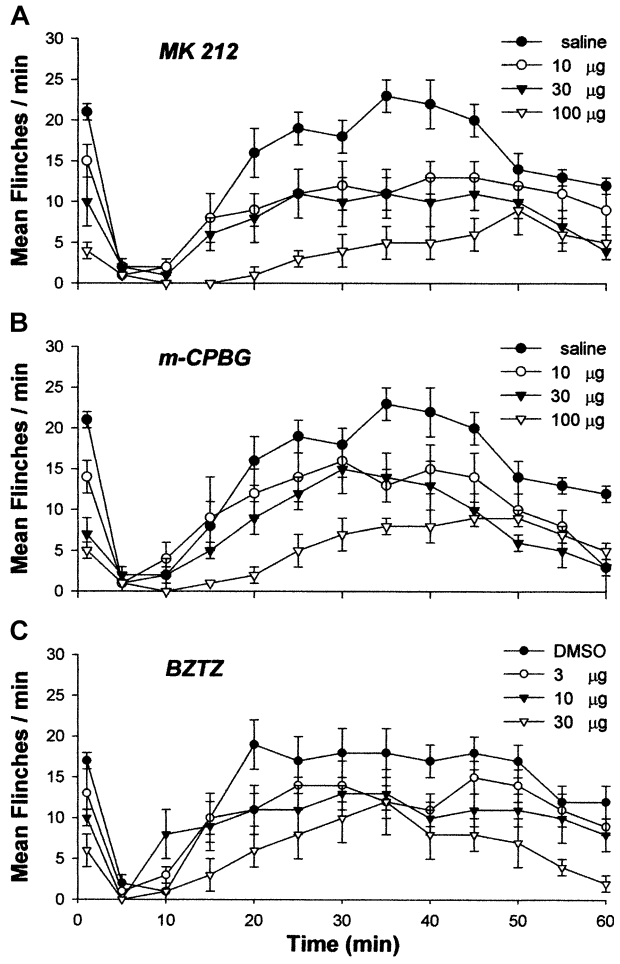


Fig. 5. Time effect curves of intrathecal MK 212 (A), *m*-CPBG (B) and BZTZ (C) for recorded flinching in the formalin test. Each drug was administered 10 min prior to the formalin injection. Formalin was injected at time 0. The data represents the number of flinches. Each line represents the mean±S.E.M. for six to eight rats.

### *3.3. Effects of intrathecal selective 5-HT receptor agonists on formalin behaviors*

Intrathecal 5-$HT_{1A}$ receptor agonist Dipropyl-5CT, 5-$HT_{1B}$ receptor agonist CGS-12066A, 5-$HT_{2C}$ receptor agonist MK 212, 5-$HT_3$ receptor agonist *m*-CPBG and 5-$HT_4$ receptor agonist BZTZ dose-dependently suppressed the flinching responses during phase 1 and phase 2 of the formalin test (Fig. 4–6). Intrathecal 5-$HT_{1D}$ receptor agonist GR 46611 did not reduce the flinching response in either of the phases (Figs. 4 and 6).

## 4. Discussion

In the present study, 5-HT attenuated the flinching responses in phase 1 and phase 2 of the formalin test. The antinociceptive effect of 5-HT was blocked by 5-$HT_{1B}$, 5-$HT_{2C}$, 5-$HT_3$ and 5-$HT_4$ receptors antagonists in both phases, and selective 5-$HT_{1B}$, 5-$HT_{2C}$, 5-$HT_3$ and 5-$HT_4$ receptors agonists decreased the formalin-induced pain behavior in both phases. However, the 5-$HT_{1D}$ receptor antagonist failed to reverse the antinociceptive effects of 5-HT in either phase. Furthermore, the 5-$HT_{1D}$ receptor agonist did not suppress the formalin-induced flinching responses in either phase. On the other hand, the antagonism by the 5-$HT_{1A}$ receptor antagonist was noted during phase 2, but not during phase 1. However, the 5-$HT_{1A}$ receptor agonist alleviated the flinching response in both phases.

The aforementioned findings suggest that the 5-$HT_{1B}$, 5-$HT_{2C}$, 5-$HT_3$ and 5-$HT_4$ receptors, but not the 5-$HT_{1D}$ receptor, may be involved in the antinociception induced by 5-HT for the facilitated state as well as acute nociception at a spinal level. The antinociception of 5-HT may also be mediated by the 5-$HT_{1A}$ receptor in a facilitated state, but this mediation in acute nociception is obscure due to the observed discrepancies between the effects of the antagonist and agonist used.

The phase 1 response of the formalin test is considered to be a result of the direct activation of the primary afferent fibers, and correspond to the high level of activity in the

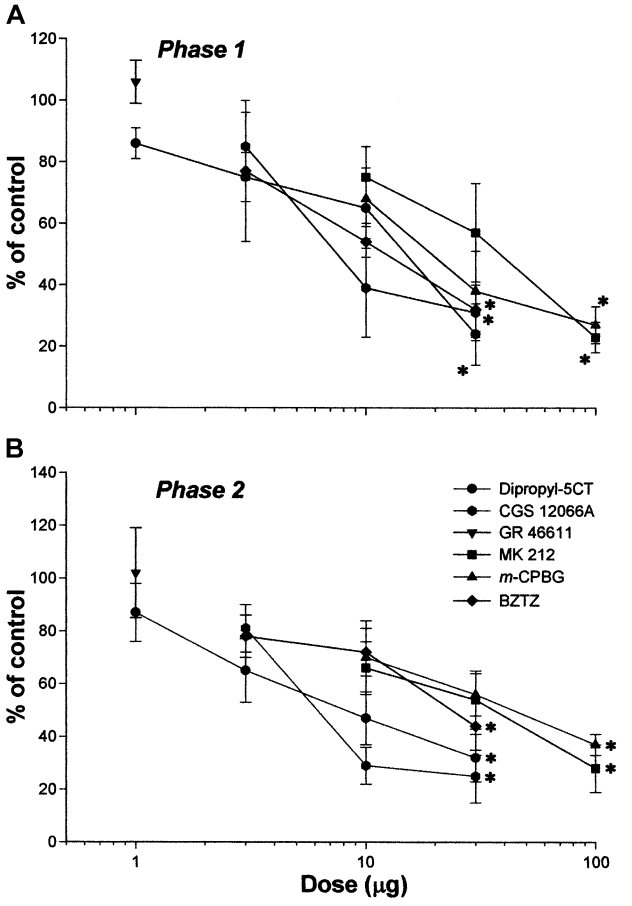


Fig. 6. Dose–response curves of intrathecal Dipropyl-5CT, CGS 12066A, GR 46611, MK 212, *m*-CPBG and BZTZ for flinching during phase 1 (A) and phase 2 (B) of the formalin test. The data is presented as a percentage of control. Each line represents the mean±S.E.M. for six to eight rats. *$P<0.05$.

primary afferent. On the other hand, the phase 2 response seems to result from the activation of wide dynamic range neurons with a continuously low level of activity in the primary afferent. Therefore, phase 1 and phase 2 reflect acute pain and the facilitated state, respectively. The advantage of this formalin pain model is that it may provide a tool for observing the effects of analgesic agents for the facilitated state as well as acute pain at once. In the current study, we used 5% concentration of formalin because peak of the flinching response was reached at 5% than 2.5% or 10% (Lee and Jeong, 2002).

It has been acknowledged that intrathecal 5-HT produces an antinociceptive effect, which is mediated by spinal 5-HT receptors. Autoradiographic and binding studies have demonstrated the existence of 5-HT receptors in the spinal cord. Correspondingly, the 5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1D}$ and 5-$HT_2$ receptors were present in the spinal cord (Marlier et al., 1991; Thor et al., 1993; Castro et al., 1997). Furthermore, the 5-$HT_3$ receptor was founded to exist on the primary afferent fibers terminating within the superficial layers of the dorsal horn (Kidd et al., 1993). In terms of biology, the 5-$HT2_A$ receptor mRNA was only expressed at higher levels in the lamina IX of the spinal cord, whereas the 5-$HT2_C$ receptor mRNA was strongly expressed in most spinal cord laminae except for the lamina II. The 5-$HT_3$ receptor mRNA was expressed in low levels with a broad expression in the spinal cord (Fonseca et al., 2001). The 5-$HT_{1A}$ receptor mRNA was widely distributed throughout the dorsal horn of the spinal cord (Zhang et al., 2002).

Pharmacological studies with selective 5-HT agents have shown a different involvement by the 5-HT receptors in the spinal cord. The 5-$HT_{1B}$, 5-$HT_{2A}$, 5-$HT_{2C}$ and 5-$HT_3$ receptors, excluding the 5-$HT_{1A}$ nor 5-$HT_4$ receptors, seemed to be involved in mediating 5-HT antinociception in the pressure test (Bardin et al., 2000). The 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors, except for the 5-$HT_3$ receptor, played a role in the antinociceptive effect of 5-HT against acute thermal stimulation (Xu et al., 1994). The 5-$HT_2$ receptor contributed to the antinociceptive action of 5-HT, although none of the 5-$HT_{1A}$, 5-$HT_{1B}$ and 5-$HT_3$ receptors were involved (Obata et al., 2001). The 5-$HT_2$ and 5-$HT_3$ receptors, except for the 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors, mediated the antinociceptive effect of 5-HT to formalin nociception (Sasaki et al., 2001; Sasaki et al., 2003). However, the role of these 5-$HT_2$ and 5-$HT_3$ receptors in the present study resembled those, while that of 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors differed from those. Such differences may be caused by the experimental methodology. They examined the effects of 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors agonists themselves, whereas we evaluated the effects of 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors antagonists on the antinociception of 5-HT as well as the effects of 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors agonists. The 5-$HT_{1A}$ receptor was identified in the nociception mechanisms assessed through noxious electrical stimulation, although not through thermal stimulation. (Nadeson and Goodchild, 2002). These combined observations suggest that spinal 5-HT receptors are involved in the modulation of nociception, but that the role of each receptor is distinct according to the receptor subtypes. Moreover, the complex picture of the roles of 5-HT receptor subtypes could arise from the different types of noxious stimulation (Bardin et al., 1997), different kinds of animal, difference in drugs, route of administration of drugs, and dosages.

Furthermore, the serotonergic system may interact with other neurotransmitters in the modulation of nociception. 5-HT via an action at the 5-$HT_3$ receptor may evoke the release of γ-aminobutyric acid (GABA), which may in turn inhibit thermal nociceptive transmission (Alhaider et al., 1991). 5-HT operating through the 5-$HT_2$ receptor acutely activated GABAergic interneurons in the prefrontal cortex (Abi-Saab et al., 1999). The 5-$HT_2$ or 5-$HT_4$ receptor agonists exerted an antinociceptive effect mediated by cholinergic activity (Ghelardini et al., 1996; Obata et al., 2002; Sasaki et al., 2003). 5-HT has been shown to release adenosine and noradrenaline from the spinal cord, creating the antinociceptive effect (Sawynok and Reid, 1996).

Another possibility that explains the antinociception of 5-HT would be the role of the descending inhibitory pathway. Noxious stimulation is able to activate the intrinsic descending inhibitory system, which promote the release of 5-HT and increase its turnover in the dorsal horn of the spinal cord (Hammond et al., 1985; Rivot et al., 1982). Although the effect of formalin stimulation on the descending inhibitory pathway has not been evaluated, considering the above observations, it could be assumed that a formalin stimulus may activate the descending inhibitory pathway, which in turn increase the level of spinal 5-HT, thereby resulting in the antinociception of 5-HT.

In addition to the 5-$HT_{1–4}$ receptors, other 5-$HT_{5–7}$ receptors were identified in the central nervous system (Barnes and Sharp, 1999). 5-$HT_{5A}$ and 5-$HT_7$ receptors were present in the rat lumbar dorsal root ganglion (Wu et al., 2001). Therefore, further research into the roles of 5-$HT_{5–7}$ receptor subtypes for nociception is needed in order to better understand the antinociceptive mechanisms of 5-HT.

Taken together, spinal 5-$HT_{1B}$, 5-$HT_{2C}$, 5-$HT_3$ and 5-$HT_4$ receptors, but not the 5-$HT_{1D}$ receptor, were involved in the antinociceptive action of 5-HT for a formalin-induced facilitated state and acute nociception. Additionally, 5-$HT_{1A}$ receptor-mediated antinociception of 5-HT develops in the facilitated state with the unknown involvement in acute nociception.

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