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Effects of 5-HT_2 and 5-HT_3 receptors on the modulation of nociceptive transmission in rat spinal cord according to the formalin test

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Abstract

We used the formalin test to clarify the 5-hydroxytryptamine (5-HT) receptor subtypes involved in the modulation of spinal nociceptive transmission in rats. Intrathecal administration of a 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)-tetraline (8-OH-DPAT; 1, 10, and 30 μg), or a 5-HT_{1B} receptor agonist, 1, 4-dihydro-3-(1, 2, 3, 6-tetrahydro-4-pyridinyl)-5H-pyrrol (3, 2-*b*) pyridin-5-one (CP 93129; 1 and 10 μg), produced no significant change in the number of flinches. A 5-HT_2 receptor agonist, (±)-2, 5-dimethoxy-4-iodoamphetamine (DOI ; 10, 30, and 100 μg), and a 5-HT_3 receptor agonist, 2-methyl-5-HT (100 and 300 μg), produced dose-dependent decreases in the number of flinches in phases 1 (1 to 6 min) and 2 (10 to 61 min) of the test. The antinociceptive effects of DOI and 2-methyl-5-HT were antagonized by intrathecal pretreatment with a 5-HT_2 receptor antagonist, ketanserin, and a 5-HT_3 receptor antagonist, 3-tropanyl-3, 5-dichlorobenzoate (MDL-72222), respectively. These results suggest that 5-HT_2 and 5-HT_3 receptors in the spinal cord mediate antinociception to chemical stimuli. © 2001 Published by Elsevier Science B.V.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); 5-HT receptor subtype; Antinociception; Spinal cord; Formalin test; (Rat)

1. Introduction

The neurotransmitter 5-hydroxytryptamine (5-HT) is important in the spinal inhibition of nociceptive transmission. Activation of the descending serotonergic bulbospinal system inhibits behavioral and dorsal horn neuronal responses to noxious stimuli (Mayer et al., 1971; Zemlan et al., 1980). Direct microiontophoretic application of 5-HT to dorsal horn neurons mimics the inhibitory effects of activation of the descending serotonergic pathway (Belcher et al., 1978; Davies and Roberts, 1981; Yassir et al., 1988). Intrathecal administration of 5-HT in rats produces antinociceptive effects that are antagonized by a nonselective 5-HT receptor antagonist, methysergide (Yaksh and Wilson, 1979). The models in which an antinociceptive effect of intrathecally administered 5-HT has been demonstrated in rats include the tail-flick test (Schmauss et al., 1983; Xu et al., 1994), the paw-pressure test, and the formalin test (Bardin et al., 1997a).

Multiple 5-HT receptor subtypes within the central nervous system have been defined pharmacologically and demonstrated by radioligand binding studies, indicating the presence of at least three 5-HT receptor families in the spinal cord (5-HT_1, 5-HT_2, and 5-HT_3) (Hamon et al., 1990). The 5-HT_1 receptor has been classified further into five subtypes designated by subscripts, A, B, D, E, and F (Hoyer et al., 1994). While the three 5-HT receptor types are thought to be involved in the modulation of nociceptive transmission, controversy exists about the 5-HT receptor subtypes involved in antinociception. For example, in the tail-flick test, intrathecal administration of a 5-HT_{1A} receptor agonist has variously been reported to facilitate (Solomon and Gebhart, 1988; Crisp et al., 1991; Alhaider and Wilcox, 1993), inhibit (Eide and Hole, 1991; Xu et al., 1994), or not affect nociception (Mjellem et al., 1992; Millan, 1994), while a 5-HT_{1B} receptor agonist has been reported either to facilitate (Solomon and Gebhart, 1988) or inhibit nociceptive transmission (Alhaider and Wilcox, 1993; Xu et al., 1994). Also, intrathecally administered 5-HT_2 and 5-HT_3 receptor agonists have been found to produce antinociceptive effects (Solomon and Gebhart, 1988; Glaum et al., 1990; Crisp et al., 1991; Eide and Hole, 1991), while Xu et al. (1994) did not find evidence

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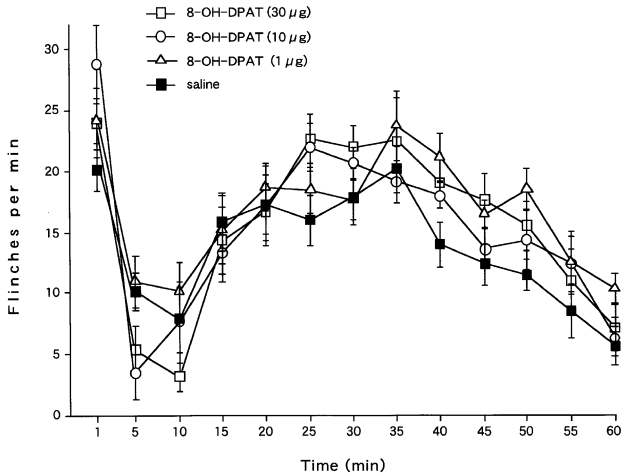

Fig. 1. Effects of intrathecal administration of 8-OH-DPAT (1 and 10 μg) on number of flinches in the formalin test. Time course of effect of 8-OH-DPAT administered 5 min before formalin injection. Data are expressed as means ± S.E.M. ($n = 6$ for each group).

for spinal 5-HT_2 or 5-HT_3 receptor involvement in antinociception.

Most behavioral tests used in studies of the 5-HT or 5-HT receptor subtypes involved in antinociceptive effects are thermal tests, especially the tail-flick test. Tests using a chemical irritant such as the formalin are used less frequently (Giordano, 1991; Bardin et al., 1997a). In the formalin test, intrathecally administered 5-HT produced dose-related antinociception in both the early and late phases (Bardin et al., 1997a). These results suggest that 5-HT modulates nociceptive transmission in the spinal cord induced by chemical stimuli. Nevertheless, which 5-HT receptor subtypes are involved in the antinociceptive effects is not clear. In the present study, we determined the subtypes of spinal 5-HT receptors involved in modulating nociceptive transmission in the formalin test, to better understand the pharmacological mechanisms of 5-HT-induced antinociception.

2. Materials and methods

This investigation was conducted according to a protocol approved by the Animal Care Committee of Gunma University. All responses were recorded without knowledge of the drug or dose administered. Drugs were given intrathecally in a randomized manner.

2.1. Animal preparation

Experiments were conducted on male Wistar rats (300 to 350 g). After rats were anesthetized with isoflurane in oxygen, an intrathecal catheter was inserted using a modification of the method described by Yaksh and Rudy (1976). Briefly, a polyethylene catheter (PE 10) was advanced 8.5 cm caudally through an incision in the atlanto-occipital membrane, extending to the rostral segments of the lumbar enlargement. A subcutaneous tunnel was created so that the catheter emerged at the vertex, and the wound was closed with 3-0 silk sutures. After implantation of the catheters, rats were housed in individual stainless steel cages. Rats showing neurologic deficits postoperatively were killed promptly by barbiturate overdose. All testing was carried out within 8 days after intrathecal catheter implantation.

2.2. Formalin test

Formalin (50 μl of a 5% solution) was injected subcutaneously into the plantar surface of the right hindpaw with a 30-gauge needle. The rat was placed in an open clear plastic enclosure to allow unhindered observation of the formalin-injected paw. Flinching was readily identified and was characterized as rapid, brief flexion withdrawal of the injected paw. Pain-related behavior was quantified by periodically counting spontaneous flinching movements of the injected paw. In phase 1, flinches were counted for two 1-min periods from 1 to 2 min and from 5 to 6 min; then, from 10 to 61 min after formalin injection (phase 2), flinches were counted for 1 min at 5-min intervals. After observations, rats were killed promptly by barbiturate overdose.

2.3. Drugs and intrathecal injection

Agonists given included a selective 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)-tetraline hydrobromide (8-OH-DPAT, molecular weight 328.29; Research Biochemicals, Natick, MA, USA); a selective 5-HT_{1B} receptor agonist, 1, 4-dihydro-3-(1, 2, 3, 6-tetrahydro-4-pyridinyl)-5H-pyrrol (3, 2-*b*) pyridin-5-one (CP 93129 dihydrochloride, molecular weight 301.68; Tocris Cookson,

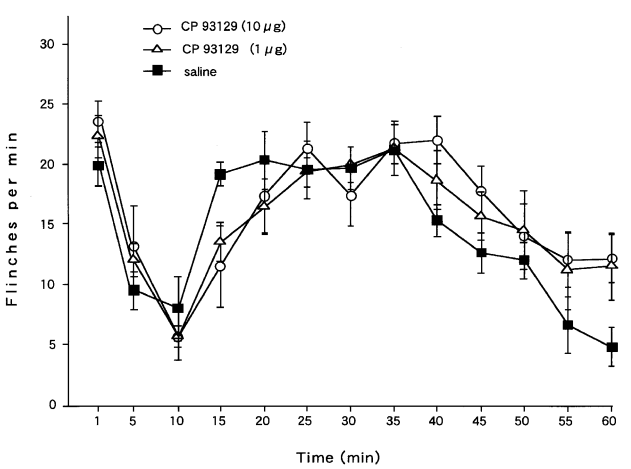

Fig. 2. Effects of intrathecal administration of CP-93129 (1 and 10 μg) on number of flinches in the formalin test. Time course of effect of CP-93129 administered 5 min before formalin injection. Data are expressed as means ± S.E.M. ($n = 6$ for each group).

Langford, Bristol, UK); a selective 5-HT_2 receptor agonist, (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI, molecular weight 357.6; Research Biochemicals); and a 5-HT_3 receptor agonist, 2-methyl-5-HT (molecular weight 306.32; Research Biochemicals). Antagonists used included a selective 5-HT_2 antagonist, ketanserin (molecular weight 545.5; Research Biochemicals), and a 5-HT_3 receptor antagonist, 3-tropanyl-3, 5-dichlorobenzoate (MDL-72222, molecular weight 314.2; Research Biochemicals). Because of solubility limitations, MDL-72222 was dissolved in a 50% solution of dimethylsulfoxide (vehicle). Other drugs were dissolved in sterile water, at concentrations that allowed administration of the intended intrathecal doses in a 10-μl volume. All injections were administered manually over 10 s, and were followed by 10 μl of saline to flush the catheter. Control animals were injected similarly with sterile physiological saline. Because 8-OH-DPAT (Eide et al., 1990) and 2-methyl-5-HT (Glaum et al., 1990) have been reported to produce short-term antinociception, all agonists were administered intrathecally 5 min before formalin injection. All antagonists were administered intrathecally 5 min prior to agonist injection.

2.4. Expression of results and statistical analysis

Statistical comparisons were made by analysis of variance (ANOVA), and Dunnet's test was applied for multiple comparisons. The level of statistical significance was defined as $P < 0.05$. Values are expressed as the means ± S.E.M.

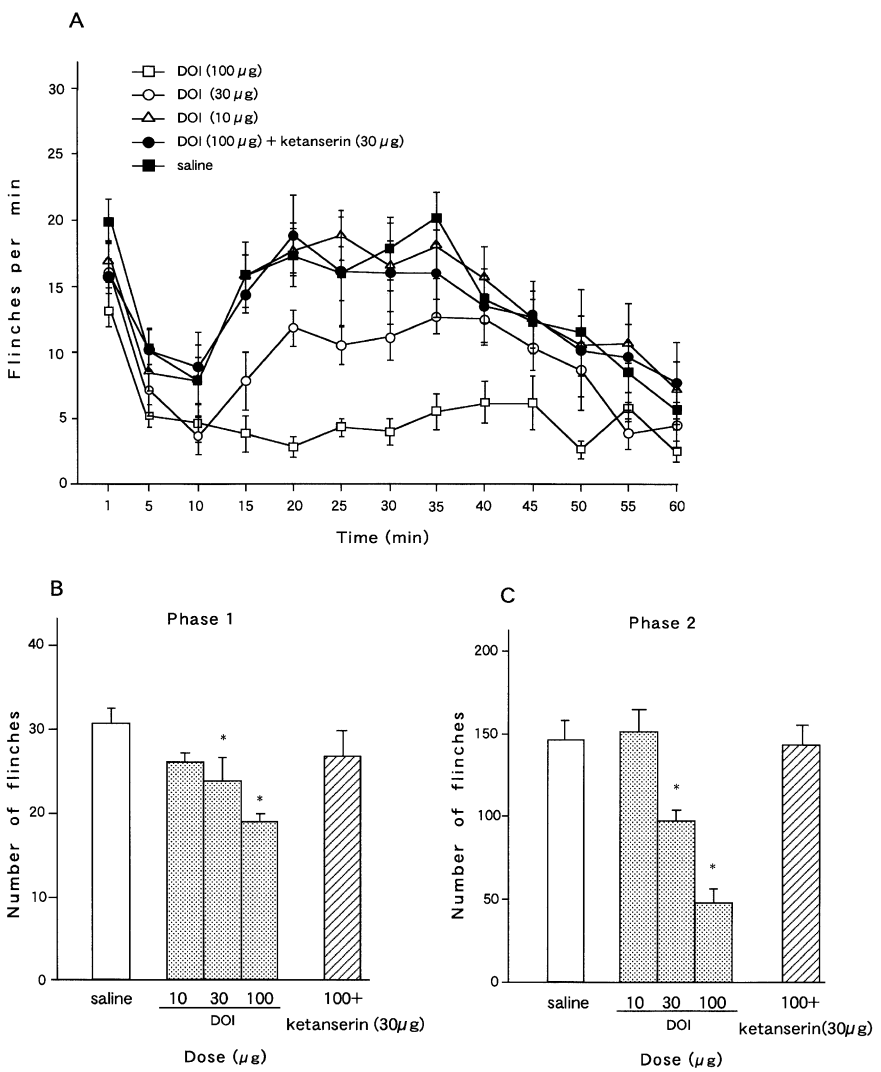

Fig. 3. Effects of intrathecal administration of DOI (10, 30, and 100 μg) on number of flinches in the formalin test. (A) Time course of effect of DOI administered 5 min before formalin injection. (B and C) Cumulative scores indicating the effect produced by intrathecal administration of DOI as changes in flinching in phase 1 (B) and phase 2 (C). Ketanserin (30 μg) was administered intrathecally 5 min prior to DOI injection in one group. Data are expressed as means ± S.E.M. ($n = 6$ for each group). * $P < 0.05$ compared to saline-treated rats.

3. Results

3.1. Effects of 5-HT1 receptor agonists

Administration of the 5-HT_{1A} receptor agonist 8-OH-DPAT (1, 10, and 30 μg) or the 5-HT_{1B} receptor agonist CP 93129 (1 and 10 μg) did not change the number of flinches in either phase 1 or 2 compared with those in the saline-treated group (Figs. 1 and 2). Intrathecal administration of 8-OH-DPAT at 30 μg produced mild motor weakness in the paws so that the limb was able to move but could not support a normal posture, while righting and stepping reflexes were preserved. At 50 μg, this drug produced agitation, restlessness, and mild motor weakness. CP 93129 in doses higher than 30 μg produced biting and scratching behavior, and vocalization in response to gentle handling, with increased motor activity immediately after injection.

3.2. Effects of 5-HT2 receptor agonist

The 5-HT_2 receptor agonist DOI (10, 30, and 100 μg) caused a dose-dependent reduction in the number of flinches of the formalin injected paw in both phases 1 and 2 (Fig. 3). Intrathecal pretreatment with the 5-HT_2 receptor antagonist ketanserin (30 μg) completely antagonized the antinociceptive effects produced by DOI. Intrathecal administration of ketanserin alone did not modify the number of flinches compared with those of the saline-treated group (data not shown).

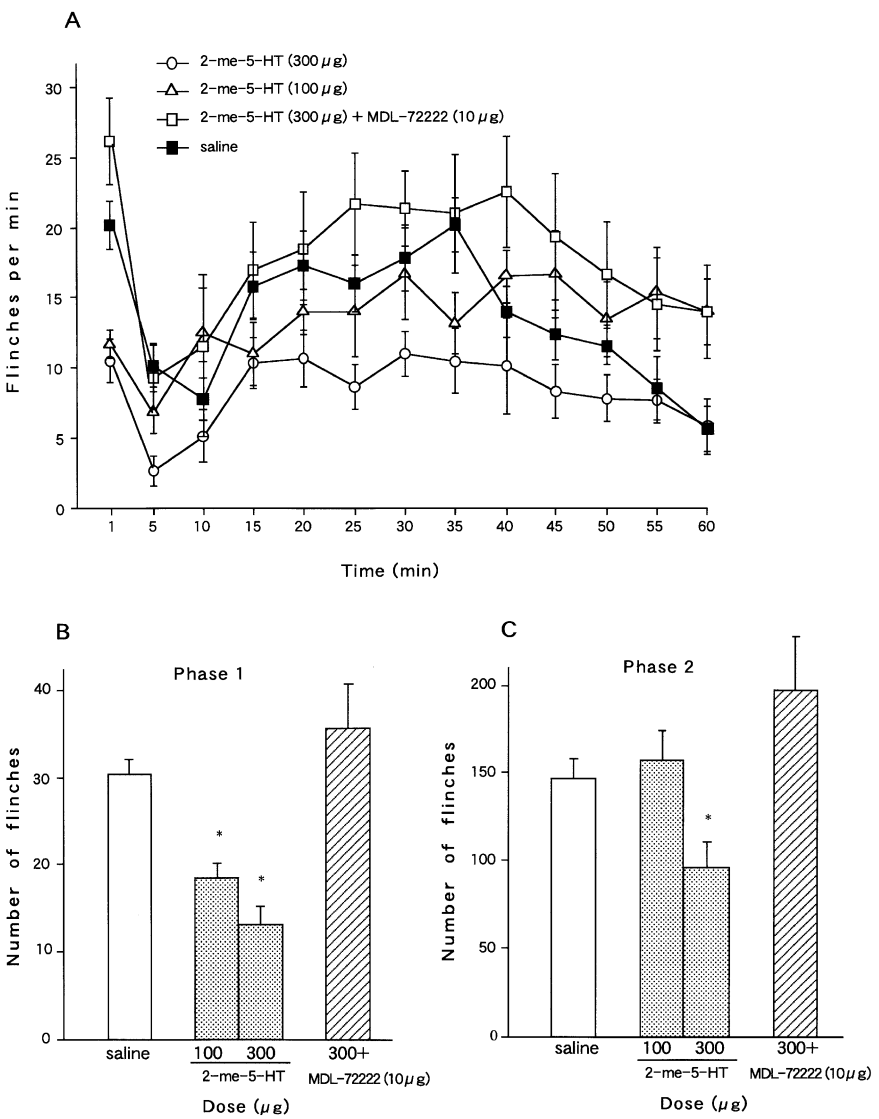

Fig. 4. Effects of intrathecal administration of 2-methyl-5-HT (100 and 300 μg) on number of flinches in the formalin test. (A) Time course of effect of 2-methyl-5-HT administered 5 min before formalin injection. (B and C) Cumulative scores indicating the effect produced by intrathecal administration of 2-methyl-5-HT as changes in flinching in phase 1 (B) and phase 2 (C). MDL-72222 (10 μg) was administered intrathecally 5 min prior to 2-methyl-5-HT injection in one group. Data are expressed as means ± S.E.M. ($n = 6$ for each group). $^{*}P < 0.05$ compared to saline-treated rats.

3.3. Effects of 5-HT3 receptor agonist

The 5-HT_3 receptor agonist 2-methyl-5-HT (100 and 300 μg) mediated dose-dependent antinociception in phases 1 and 2 (Fig. 4). Intrathecal pretreatment with the 5-HT_3 receptor antagonist MDL-72222 (10 μg) completely antagonized the antinociceptive effects produced by 2-methyl-5-HT. Vehicle treatment had no effect on the number of flinches (phase 1; 22.3 ± 3.8, phase 2; 143.2 ± 10.3, $n = 6$) compared with a saline-treated group (phase 1; 29.0 ± 1.5, phase 2; 155.8 ± 10.1, $n = 6$). Intrathecal administration of MDL-72222 alone did not modify the number of flinches compared with those of the saline- or vehicle-treated group (data not shown).

4. Discussion

We demonstrated that 5-HT_2 and 5-HT_3 receptor agonists produced antinociceptive effects that were antagonized by intrathecal pretreatment with the corresponding antagonists. However, 5-HT_{1A} and 5-HT_{1B} receptor agonists did not produce antinociception. Our results suggest that 5-HT_2 and 5-HT_3 receptors in the spinal cord mediate antinociception to chemically induced inflammatory stimuli.

At peripheral sites, 5-HT facilitates pain responses (Fozard, 1984; Hong and Abbott, 1994). In contrast, intrathecally administered 5-HT mediates antinociception (Yaksh and Wilson, 1979; Schmauss et al., 1983; Xu et al., 1994; Bardin et al., 1997a). The existence of multiple subtypes of 5-HT receptors within the central nervous system was suggested by the results of radioligand binding studies. Initially, two subtypes of 5-HT binding sites, designated 5-HT_1 and 5-HT_2, were identified on the basis of the binding characteristics of [^{3}H] 5-HT and [^{3}H] spiperone, respectively (Peroutka and Snyder, 1979). Investigations of 5-HT binding in the spinal cord have indicated that this tissue contains both 5-HT_1 and 5-HT_2 binding sites (Pazos and Palacios, 1985; Pazos et al., 1985). Additionally, a 5-HT_3 binding site has been identified in spinal cord synaptosomal membrane preparations from rats (Glaum and Anderson, 1988).

In the rat spinal cord, 5-HT_2 receptors are present in both superficial and deep laminae of the dorsal horn, although their density is low (Marlier et al., 1991; Thor et al., 1993). Stimulation of 5-HT_2 receptors may facilitate nociceptive transmission, including the release of substance P from presynaptic terminals (Iverfeldt et al., 1986). Eide and Hole (1991) reported that, in mice, intrathecal administration of DOI (5 to 20 μg) produced a dose-dependent behavioral syndrome consisting of biting or licking, directed towards the caudal part of the body, and reciprocal hindlimb scratching, and that this effect was reversed by a substance P antagonist. Similarly, a 5-HT_2 receptor agonist, α-methyl-5-HT, elicited dose-related biting and scratching after intrathecal injection in mice (Wilcox and Alhaider, 1990). However, several studies indicated that the 5-HT_2 receptor was involved in antinociception, using the tail-flick test (Solomon and Gebhart, 1988; Eide and Hole, 1991), the hot-plate test (Crisp et al., 1991), or the colorectal distension test (Danzebrik and Gebhart, 1991). In the present study, intrathecal administration of DOI had an antinociceptive effect in the formalin test and this effect was reversed by intrathecal pretreatment with ketanserin. Discrepancies concerning the action of the 5-HT_2 receptor in spinal pain modulation may be due to differences in the pain test or animal species used. The mechanism underlying the antinociceptive effects of an intrathecally administered 5-HT_2 agonist is not clear. Sugiyama and Huang (1995) suggested that 5-HT activated 5-HT_2 receptors on interneurons and promoted the release of γ-aminobutyric acid (GABA) or glycine in trigeminal neurons. Recently, Abi-Saab et al. (1999) reported that DOI activated GABAergic interneurons in the rat prefrontal cortex. By analogy, one possible explanation for the mechanisms of the antinociceptive effects of 5-HT_2 receptor agonists is that they induce the release of an inhibitory neurotransmitter, such as GABA, by interneurons in the rat spinal cord.

The 5-HT_3 receptors located in the dorsal horn of the rat spinal cord have been shown to mediate an antinociceptive effect. The 5-HT_3 receptor agonist 2-methyl-5-HT has been found to inhibit nociceptive behavior induced by intrathecally administered substance P or *N*-methyl-D-aspartic acid (NMDA) (Wilcox and Alhaider, 1990; Alhaider et al., 1991). The antinociceptive effect of 5-HT was blocked by a 5-HT_3 receptor antagonist in the tail-flick test, the hot-plate test (Glaum and Anderson, 1988) and the paw-pressure test (Bardin et al., 2000). In addition, intrathecal administration of 2-methyl-5-HT produced antinociception in the tail-flick test, the hot-plate test (Glaum et al., 1990), and the colorectal distension test (Danzebrik and Gebhart, 1991). Other 5-HT_3 receptor agonists, namely, 1-(*m*-chlorophenyl)-bi-guanide (mCPBG) and phenylbiguanide (PBG) produced antinociceptive effects in the paw-pressure test (Bardin et al., 1997b, 2000) and the hot-plate test (Crisp et al., 1991), respectively. In contrast, another report showed that intrathecal injection of 2-methyl-5-HT produced no significant change in a mechanical pain test (Giordano, 1991) or in the tail-flick test (Xu et al., 1994). As for inflammatory pain, Giordano (1991) reported that intrathecal administration of 2-methyl-5-HT produced an antinociceptive effect in the formalin test, and generally our results were similar. The antinociceptive effect produced by 2-methyl-5-HT has a rapid onset and a short duration, with peak effect within 5 min and a return to baseline values 25 min after injection (Glaum et al., 1990). At a dose of 100 μg, 2-methyl-5-HT did not significantly reduce the number of flinches in phase 2 of the test in the present study. Like opioid receptors, 5-HT_3 receptors are reported to be localized in

presynaptic sites on unmyelinated primary afferent fiber terminals in the superficial layers of the dorsal horn (Hamon et al., 1989; Kidd et al., 1993). Interactions with opioid and GABAergic systems may occur as part of the effect of spinal 5-HT_3 receptor agonist on pain from chemical inflammation (Giordano, 1991).

Neither 5-HT_{1A} nor 5-HT_{1B} receptor agonists produced a significant change in the formalin test. Controversy prevails about possible roles of these receptor subtypes in the modulation of pain in the spinal cord. Intrathecal administration of 8-OH-DPAT (300 nmol) has produced antinociception in the hot-plate test (Crisp et al., 1991), but hyperalgesia in the tail-flick test (Solomon and Gebhart, 1988; Crisp et al., 1991). Xu et al. (1994) reported that lower doses of 8-OH-DPAT (0.25 to 1 μg) mediated a dose-dependent increase in tail-flick latency, but induced a hyperalgesic effect at higher doses (2 to 100 μg). Moreover, Danzebrik and Gebhart (1991) demonstrated that intrathecal administration of 8-OH-DPAT (25 μg) produced agitation and restlessness. In the present study, intrathecally administered 8-OH-DPAT at doses higher than 30 μg produced mild motor weakness in the hindpaws, and at 50 μg this agent produced agitation and restlessness. This motor weakness continued for about 30 min after injection, although righting/stepping reflexes were essentially normal. Therefore, we used 1, 10, and 30 μg of 8-OH-DPAT so that motor effects would not affect the number of flinches induced by formalin injection. With regard to the 5-HT_{1B} receptor, intrathecal administration of 5-HT_{1B} receptor agonists produced antinociception in some experiments (Crisp et al., 1991; Alhaider and Wilcox, 1993; Xu et al., 1994), while in others these agents facilitated pain responses (Solomon and Gebhart, 1988) or had no apparent effect (Mjellem et al., 1992). 5-Methoxy-3 (1, 2, 3, 6-tetrahydropyridine-4-yl)-1*H*-indole (RU-24969) and 1-(3-(trifluoromethyl)phenyl)-piperazine hydrochloride (TFMPP), the agonists used in those studies, were not sufficiently selective for 5-HT_{1B} receptors (Hamon et al., 1986; Feniuk and Humphrey, 1989; Schoeffter and Hoyer, 1989). CP-93129 is the most selective 5-HT_{1B} agonist available (Macor et al., 1990). In our preliminary investigation, intrathecal administration of the agonist (30 and 100 μg) produced biting and scratching behavior as well as hyperalgesia, resembling the behavior observed with intrathecally administered substance P (Cridland and Henry, 1986) or NMDA (Aanonsen and Wilcox, 1986; Mjellem et al., 1992). We took care to use lower doses than those inducing such abnormal behavior.

Formalin injection into the paw of a rat produces a biphasic behavioral syndrome including flinching and licking (Dubuisson and Dennis, 1977). Hunskaar et al. (1985) demonstrated that licking was the most relevant behavior in mice. Wheeler-Aceto et al. (1990), however, reported that biphasic flinching responses were more typical than licking in rats. We counted the number of flinches periodically to assess the pain intensity. This method has been established for pharmacological studies in rats (Yamamoto and Yaksh, 1992; Malmberg et al., 1995). It is unlikely that motor effects induced by intrathecal administration of 5-HT agonists modified flinching behaviour in our study, because we used doses lower than those inducing abnormal behavior such as biting and scratching. The first-phase behavior in the formalin test is considered to result from direct activation of Aβ-, Aδ- and C-fibers, whereas the second-phase may involve ongoing input from C-fibers located at the injection site, together with Aδ- and C-fibers from adjacent sites activated by the spread of inflammation (Puig and Sorkin, 1995). Repetitive C-fiber stimulation is known to induce facilitated discharge of "wide dynamic range neurons" (wind-up) (Mendell, 1966). This mechanism is also thought to be involved in the second-phase response (Dickenson and Sullivan, 1990; Haley et al., 1990; Coderee et al., 1990; Yamamoto and Yaksh, 1992). In the present study, DOI and 2-methyl-5-HT decreased the number of flinches not only in phase 1 but also in phase 2.

Recently, our group demonstrated that intrathecal administration of a 5-HT_2 agonist, but not a 5-HT_3 agonist, had an antiallodynic action in a rat model with L5 and L6 spinal nerve ligation (Obata et al., 2001). We do not yet understand the reason for the discrepancy between our two studies concerning the effects of the 5-HT_3 agonist. Neuropathic pain induced by nerve injury is known to be mediated not only by the wind up of "wide dynamic range neurons", but also by anatomical changes, such as sprouting of Aβ fibers into lamina II of the dorsal horn (Woolf et al., 1992), trans-synaptic change and degeneration of inhibitory interneurons (Sugimoto et al., 1990; Ibuki et al., 1997), and sympathetic sprouting within dorsal root ganglia (McLachlan et al., 1993). In contrast, chemical inflammation does not induce such anatomical changes in the nervous system. The difference in morphology may explain the discrepancy between the two models. Further investigation is necessary to determine the specific involvement of 5-HT receptor subtypes by comparing various animal models, including those of inflammatory pain and neuropathic pain.

In summary, intrathecally administered 5-HT_2 receptor agonist and 5-HT_3 receptor agonist decreased the number of flinches in both phases of the formalin test. This effect was reversed by intrathecal pretreatment with the respective antagonists. Dorsal horn 5-HT_2 and 5-HT_3 receptors, then, inhibit nociceptive transmission in response to chemical inflammatory stimuli.

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