

Spinal 5-HT₇ Receptors Play an Important Role in the Antinociceptive and Antihyperalgesic Effects of Tramadol and Its Metabolite, O-Desmethyltramadol, via Activation of Descending Serotonergic Pathways

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ABSTRACT

Background: Tramadol is an analgesic drug, and its mechanism of action is believed to be mediated by the μ -opioid receptor. A further action of tramadol has been identified as blocking the reuptake of serotonin (5-HT). One of the most recently identified subtypes of 5-HT receptor is the 5-HT₇ receptor. Thus, the authors aimed to examine the potential role of serotonergic descending bulbospinal pathways and spinal 5-HT₇ receptors compared with that of the 5-HT_{2A} and 5-HT₃ receptors in the antinociceptive and antihyperalgesic effects of tramadol and its major active metabolite O-desmethyltramadol (M1) on phasic and postoperative pain models.

Methods: Nociception was assessed by the radiant heat tail-flick and plantar incision test in male Balb-C mice (25–30 g). The serotonergic pathways were lesioned with an intrathecal injection of 5,7-dihydroxytryptamine. The selective 5-HT₇, 5-HT₂, and 5-HT₃ antagonists; SB-269970 and SB-258719; ketanserin and ondansetron were given intrathecally.

Results: Systemically administered tramadol and M1 produced antinociceptive and antihyperalgesic effects. The antinociceptive effects of both tramadol and M1 were significantly diminished in 5-HT-lesioned mice. Intrathecal injection of SB-269970 (10 μ g) and SB-258719 (20 μ g) blocked both tramadol- and M1-induced antinociceptive and antihyperalgesic effects. Ketanserin (20 μ g) and ondansetron (20 μ g) were unable to reverse the antinociceptive and antihyperalgesic effects of tramadol and M1.

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Conclusions: These findings suggest that the descending serotonergic pathways and spinal 5-HT₇ receptors play a crucial role in the antinociceptive and antihyperalgesic effects of tramadol and M1.

What We Already Know about This Topic

❖ Tramadol, a widely used analgesic, works in part by stimulation of the opioid receptor and in part by increasing catecholamine signaling in the nervous system

What This Article Tells Us That Is New

❖ In mice, the antinociceptive and antihypersensitivity effects of tramadol and one of its metabolites is blocked by the destruction of descending spinal serotonergic pathways and by intrathecal injection of a serotonin receptor type 7 antagonist

TRAMADOL is a widely used central analgesic drug and a synthetic 4-phenylpiperidine analog of codeine.^{1–3} The clinical benefits of tramadol in the treatment of mild to moderate pain in human surgical patients are well known.² The analgesic effects of tramadol is thought to be mediated by μ -opioid receptors.³ However, tramadol affinity for the μ -opioid receptor is weak and approximately 6,000-fold less than that of morphine.^{4–6} Tramadol is rapidly and extensively metabolized by O-demethylation, and one of its major active metabolite O-desmethyltramadol (M1) binds to μ -opioid receptors with high affinity.^{6–10} Thus, it has been suggested that tramadol is a prodrug, and M1 is important for the analgesic effects of tramadol.^{8,9,11} Interestingly, the analgesic action of tramadol is not completely blocked by naloxone, an opioid receptor antagonist. Therefore, it has been suggested that a nonopioid mechanism contributes to tramadol-induced analgesia.

One of the nonopioid mechanisms of tramadol consists in the enhancement of the extraneuronal concentration of 5-HT by inhibiting the 5-HT transporter.^{9,12–14} Because tramadol increases 5-HT in the central nervous system, the serotonergic system has been suggested to be involved in tramadol analgesia.^{15,16} Oliva *et al.*¹⁶ reported that the antinociceptive effect of tramadol in the mouse formalin test

was mediated by the serotonergic component. However, there are conflicting results in the literature about the role of serotonergic receptor subtypes in systemic tramadol-induced antinociception. Some studies have shown that the blockade of 5-HT₂ or 5-HT₃ receptors reduce the antinociceptive effect of systemic tramadol.^{3,12,17} In contrast, others have found that the blockade of 5-HT_{1A} increases¹⁵ and the blockade of 5-HT₃ receptors did not systemically change tramadol-induced analgesia.¹⁸

It is well known that most of the serotonergic innervations of the spinal cord are derived from supraspinal sources, and serotonergic systems comprise one of the major components of descending pain inhibitory pathways.^{19,20} In this regard, it is possible that there is an existence of specific neuronal circuits whereby systemic tramadol or its main metabolite M1 can modulate nociception through the activation of a descending serotonergic system to the spinal cord and act on serotonergic receptors in the dorsal horn. Currently, there are seven families of 5-HT receptors (5-HT₁–7), and one of the most recently identified subtypes is the 5-HT₇ receptor.²¹ A recent study has reported that spinal 5-HT₇ receptors play an important role in the antinociceptive effects of systemic morphine.²² Immunocytochemical studies found that 5-HT₇ receptors are localized in the superficial layers of the spinal cord dorsal horn²³ consistent with a predominant role of the 5-HT₇ receptors in the control of nociception. Interestingly, Doly *et al.*²⁴ reported that the 5-HT₇ receptor was also located in the dorsolateral funiculus, and the dorsolateral funiculus has been generally accepted as the main route for the bulbospinal descending inhibition on the spinal transmission of nociceptive inputs.²⁵

This study was undertaken to evaluate the potential role of the descending serotonergic system and spinal 5-HT₇ receptors in the antinociceptive and antihyperalgesic effects of tramadol and its active metabolite M1 on phasic and postoperative pain models.

Materials and Methods

Animals

Adult male Balb-C mice (25–30 g) were used. They were housed in a room maintained at 22° ± 3°C and 50–55% humidity on a 12-h light-dark cycle (the lights on at 8:00 AM). All the experiments were conducted during the light phase of the light-dark cycle (between 8:00 AM and 2:00 PM), in accordance with the guidelines set forth by the International Association for the Study of Pain²⁶ and approved by the GATA Institute Animal Care and Use Committee (Ankara, Turkey).

Drugs

Tramadol hydrochloride and M1 were provided by Grünenthal GmbH (Aachen, Germany) and diluted with 0.9% saline in a volume of body weight of 5 ml/kg for subcutaneous injections. The selective serotonergic toxin, 5,7-dihydroxytryptamine (5,7-DHT) creatinine sulfate salt, was

obtained from Sigma Chemical Co. (St. Louis, MO). Potent and selective 5-HT₇ receptor antagonists, SB-269970 and SB-258719, having 100-fold selectivity over a range of other serotonergic receptors^{27–29} were obtained from Sigma Chemical Co. and Tocris (Ellisville, MO), respectively. The selective 5-HT₂ receptor antagonist,³⁰ ketanserin, was obtained from Sigma Co., and the selective 5-HT₃ receptor antagonist ondansetron³¹ was obtained from Glaxo-Smith-Kline (Istanbul, Turkey). All the drugs were dissolved in 0.9% saline and were freshly given.

Intrathecal Injections

Intrathecal injections were performed with the methods of Hylden and Wilcox³² in which a 30-gauge needle is inserted into the lumbar space between the L5 and L6 vertebrae of unanesthetized mice, and a volume of 10 µl is injected. Control animals received 0.9% sterile saline.

Surgery

Plantar incision was performed as previously described³³ with minor modifications. Briefly, mice were anesthetized with ketamine and xylazine (80 and 4 mg/kg intraperitoneal, respectively). After antiseptic preparation of the right hind paw with 10% povidone-iodine solution, a 5-mm longitudinal incision was made with a no. 11 blade, through the skin and fascia of the plantar surface of paw, starting 2 mm proximal to the edge of the heel and extending toward the toes. The plantaris muscle was elevated and longitudinally incised. After hemostasis, the skin was opposed with two single interrupted sutures using 6-0 silk, and animals were allowed to recover in a home cage. The behavioral tests were performed 24 h after paw incision.

Lesion of the Descending Serotonergic Pathways

The selective denervation of spinal serotonergic neurons was performed according to the method of Hung *et al.*³⁴ According to this method, to block the uptake of 5,7-DHT into noradrenergic terminals, desipramine (25 mg/kg) was intraperitoneally administered 30 min after an intrathecal injection of 5,7-DHT (50 µg) 3 days before testing. On the fourth day, to confirm 5,7-DHT efficacy by determining 5-HT lumbar levels using high-performance liquid chromatography, a group of mice were killed by high-dose ether anesthesia followed by a cut made at the lumbar spine region. The spinal cord containing lumbar regions were weighed and homogenized with 0.2 M perchloric acid solution including 100 M EDTA (1 ml/30 mg of spinal tissue). The sample was centrifuged at 20,000g for 15 min at 0°C. The clear supernatant was filtered using 0.45-µm membrane filters for the assay of 5-HT. The contents of 5-HT in the spinal extract were determined by a high-performance liquid chromatography–fluorescence detector (Agilent 1100, Santa Clara, CA) at 344-nm wavelength with an excitation wavelength at 285 nm using a commercially available kit (Eureka, Chiaravalle, Italy). According to kit procedure, 200 µl of deproteinization solution with internal standard was added to 400 µl of

homogenized sample, vortexed at least 10 s, centrifuged at 5,000 *g* for 5 min, and 200 μ l was pipetted in a tube of clear supernatant. After the addition of a 200- μ l stabilization solution, we injected 50 μ l of this solution onto a reverse-phase column (VertiSep GES C18; 4.6 \times 150 mm; particle size, 4 μ m) with a guard precolumn (Vertical Chromatography, Bangkok, Thailand). At a flow rate of 1.2 ml/min, retention times of internal standard and serotonin were approximately 10.4 and 15.8 min, respectively.

Behavioral Assessment of Nociception

Tail-flick Test. The radiant heat tail-flick test (type 812; Columbus Instruments, Columbus, OH) was used to assess antinociception. The intensity of the beam was adjusted to produce mean control reaction times of 2–3 s. A cutoff time of 6 s was used to prevent tissue damage. Baseline tail-flick latencies for each mouse was determined before treatment. After drug injections, test latencies were measured. To generate a dose–response curve, data were converted to % antinociception by the formula % antinociception = [(test latency – baseline latency)/(cutoff time – baseline latency)] \times 100.

Paw Withdrawal Latency to Radiant Heat Stimulus. The method of Hargreaves *et al.*³⁵ was used to assess thermal hyperalgesia. Each mouse was placed on a glass platform within a clear plastic chamber. After acclimation, a radiant heat source was focused from a 50-W light bulb placed in a custom-built case, which allowed focusing of the light source (COMMAT Company Ltd., Ankara, Turkey). The intensity of the beam was adjusted to produce mean baseline paw flick latencies of 8–10 s. The time required to cause withdrawal of the hind paw from thermal stimulus was measured after a sham procedure and after plantar incision. To generate a dose–response curve, data were converted to % reversal of hyperalgesia = [(postdrug latency – baseline latency)/(pre-surgery latency – baseline latency)] \times 100.

Experimental Protocol

We first performed dose–response effects of tramadol and M1, a main metabolite of tramadol, in the tail-flick test in naive animals. Tramadol hydrochloride and M1 were subcutaneously administered into different groups of mice in a volume of 5 mg/kg of body weight and nociceptive latencies were monitored for 2 h. We then examined the antinociceptive effects of tramadol hydrochloride and M1 after lesioning the serotonergic bulbospinal pathways using 5,7-DHT. Furthermore, we assessed the involvement of spinal 5-HT7 receptors in the systemic tramadol- and M1-induced antinociception by intrathecal injection of SB-269970 and SB-258719, 30 min after subcutaneous tramadol and M1 treatments. In our previous study, we found that 10 μ g of an intrathecal dose of SB-269970 totally blocked the antinociceptive effects of systemically administered morphine. Thus, we chose a 10- μ g dose for the intrathecal injection of SB-269970. Because of the lower inhibitor antagonist potency of SB-258719 over 5-HT7 receptors,³⁶ we choose a 20- μ g dose for the intrathecal injection of SB-258719. We also com-

pared the involvement of spinal 5-HT7 receptors with that of the spinal 5-HT2 and 5-HT3 receptors in systemic tramadol- and M1-induced analgesia by an intrathecal injection of ketanserin (20 μ g) and ondansetron (20 μ g) 30 min after subcutaneous tramadol and M1 treatments.

In the second set of experiments, we assessed the antihyperalgesic effects of tramadol and M1 in the plantar heat test after paw incision. In a protocol similar to the tail-flick test, tramadol hydrochloride and M1 were subcutaneously administered 24 h after plantar incision and paw flick latencies were monitored in 2 h. We then examined the antihyperalgesic action of tramadol hydrochloride and M1 after lesioning the serotonergic bulbospinal pathways using 5,7-DHT. After these, we assessed the involvement of spinal 5-HT7 receptors compared with that of the spinal 5-HT2 and 5-HT3 receptors in systemic tramadol- and M1-induced antihyperalgesia by an intrathecal injection of SB-269970 (10 μ g), SB-258719 (20 μ g), ketanserin (20 μ g), and ondansetron (20 μ g) 30 min after subcutaneous tramadol and M1 treatments.

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism 4 software (GraphPad, San Diego, CA). After demonstrating that the data were normally distributed, the significance of any differences in thermal thresholds was assessed using two-way repeated-measures ANOVA. A significant effect on the main factor(s) was taken as the criterion for progressing to *post hoc* testing. The Bonferroni *post hoc* test was used to compare more than two groups at corresponding time points among different groups. A comparison of the two groups was done by using the unpaired Student *t* test. Data were expressed as mean \pm SEM. Groups of 8–12 mice were used. Statistical tests were two tailed, and differences were considered significant at $P < 0.05$. Three doses of each drug were used to determine the ED₅₀ value. The ED₅₀ values and the 95% confidence limits of tramadol, M1 alone, and M1 in combination with 5-HT antagonists were calculated from dose–percent inhibition relations by computerized log-linear regression analysis.³⁷ If the 95% confidence limits of tramadol and M1 alone did not overlap with the 95% confidence limit of the combination treatment with 5-HT antagonists, statistical significance was considered to be present between groups.

Results

The Effects of Tramadol and M1 in the Tail-flick Test

The mean baseline tail-flick latency of naive animals was 2.63 \pm 0.03 s. Two-way repeated-measures ANOVA analyses indicated that systemically administered tramadol (20, 40, and 80 mg \cdot kg⁻¹) and M1 (10, 20, and 30 mg/kg) had significant effects on tail-flick latencies ($F(3,176) = 118.2$, $P < 0.001$ and $F(3,176) = 192.4$, $P < 0.001$, respectively, $n = 12$). Tramadol and M1 elicit a dose-dependent increase in the tail-flick latencies (figs. 1A and B). Tramadol- and M1-induced antinociception were indicated by the prolon-

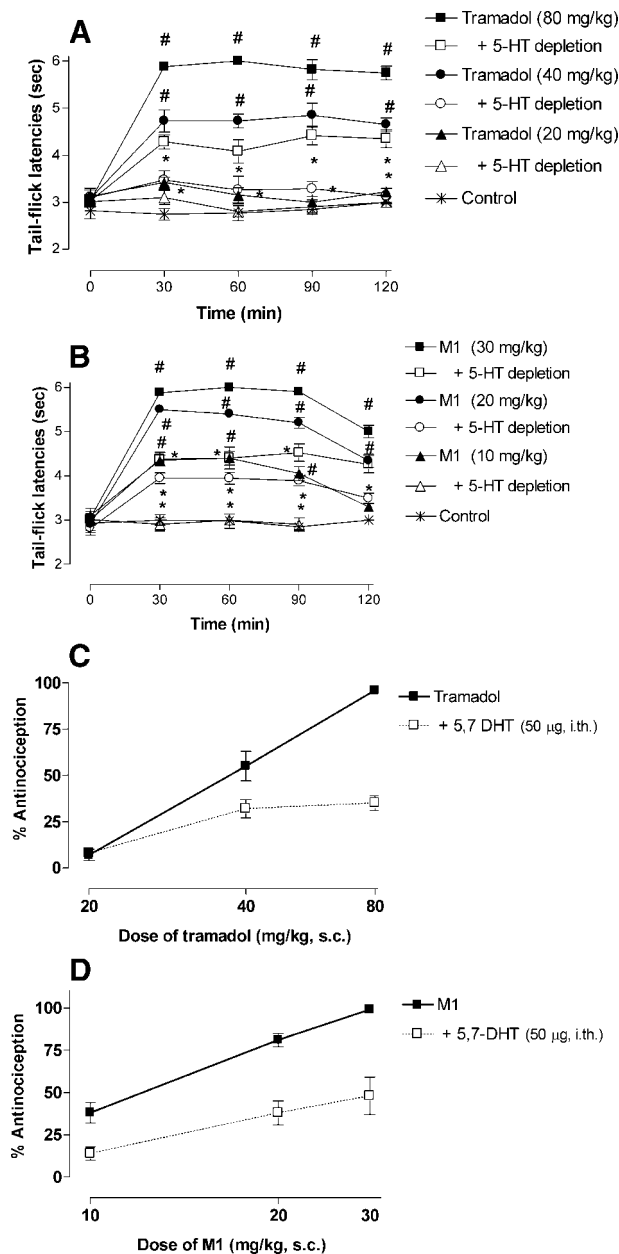


Fig. 1. The involvement of spinal serotonergic neurons in the antinociceptive effects of tramadol (A) and O-desmethyltramadol (M1) (B). 5,7-Dihydroxytryptamine (5,7-DHT) was intrathecally injected to deplete spinal serotonin. Three days after 5,7-DHT administration, tramadol or M1 was subcutaneously injected. A total of 0.9% saline was intrathecally injected as a control. The results are presented as mean \pm SEM. Tail-flick latencies at 60 min after tramadol (C) or M1 (D) administration were converted to % antinociception to generate the dose-response curve. N = 12 per group. # Differences from 0.9% saline control group, $P < 0.05$ (Bonferroni *post hoc* test). * Differences corresponding to the dose of tramadol and M1 alone, $P < 0.05$ (Bonferroni *post hoc* test).

gation of tail-flick latencies and reached peak effect 30 min (approaching the cutoff latency of 6 s in the 80 and 30 mg/kg tramadol and M1 group, respectively) after injections. Bonferroni *post hoc* test showed that tramadol at the dose of 40 and 80 mg/kg (fig. 1A) and M1 at the dose of 10, 20, and 30 mg/kg (fig. 1B) displayed increased tail-flick latencies at 30,

60, 90, and 120 min after drug injection compared with saline injection ($P < 0.001$ for each time points for tramadol and M1). We next evaluated the antinociceptive effects of systemically administered tramadol (20, 40, and 80 mg/kg) and M1 (10, 20, and 30 mg/kg) in spinal 5,7-DHT-lesioned animals. We found that intrathecal treatment with 5,7-DHT (50 μ g) significantly reduced 5-HT levels in the spinal cord by $88 \pm 6.4\%$ ($1,348 \pm 244$ ng/g vs. 134.35 ± 48 ng/g of spinal cord tissue for control and 5,7-DHT-treated mice, respectively, $P < 0.001$, $n = 8$). The mean baseline tail-flick latency of spinal 5,7-DHT (50 μ g)-administered animals was found to be 2.84 ± 0.03 s, which was not significantly different from the saline groups ($P = 0.138$, $n = 12$). Tramadol-induced (20, 40, and 80 mg/kg) ($F(5,168) = 27.24$, $P < 0.001$, $n = 8$) and M1-induced (10, 20, and 30 mg/kg) antinociception ($F(5,168) = 41.78$, $P < 0.001$, $n = 8$) were significantly diminished in spinal 5,7-DHT (50 μ g)-pretreated animals when compared with naive animals (figs. 1A and B). Figures 1C and D illustrate the dose-response curves of tramadol and M1 generated from the data 60 min after tramadol and M1 administration in naive mice or in mice with lesioned serotonergic bulbospinal pathways by 5,7-DHT.

Then, to explore the contribution of spinal 5-HT₇ receptors in the systemic tramadol- and M1-induced antinociception, we intrathecally injected SB-269970 (10 μ g/10 μ l) or SB-258719 (20 μ g/10 μ l) 30 min after tramadol (20, 40, and 80 mg/kg) and M1 (10, 20, and 30 mg/kg) administration. Intrathecally administered SB-269970 (10 μ g) and SB-258719 (20 μ g) alone did not produce any significant effect in tail-flick latencies (data not shown). However, two-way repeated-measures ANOVA analyses indicated that intrathecally administered SB-269970 (10 μ g) had significant effects on tramadol- and M1-induced antinociception ($F(5,252) = 42.34$, $P < 0.001$ and $F(5,252) = 71.08$, $P < 0.001$, respectively, $n = 8$). The intrathecal injection of SB-269970 produced a significant, time-dependent reduction in the effect of tramadol (40 and 80 mg/kg) and M1 (10, 20, and 30 mg/kg) on tail-flick latencies (figs. 2A and B). The inhibitory effects of SB-269970 peaked within 10 min and weakened time dependently. Inhibition of the antinociceptive effect of tramadol and M1 lasted 90 min, and tail-flick latencies returned to the corresponding response latencies for 80 mg/kg of tramadol and 30 mg/kg of M1 120 min after intrathecal administration of SB-269970 (figs. 2A and B). Figures 2C and D illustrate the dose-response curves of tramadol and M1 generated from the data 10 min after an intrathecal administration of SB-269970 (10 μ g), respectively. Similar to SB-269970, intrathecally administered SB-258719 (20 μ g) had significant effects on tramadol- and M1-induced antinociception ($F(5,252) = 19.18$, $P < 0.001$ and $F(5,252) = 103.4$, $P < 0.001$, respectively, $n = 8$). Intrathecal administration of SB-258719 (20 μ g) 30 min after tramadol and M1 administration produced a significant reduction in the effect of tramadol and M1 on tail-flick latencies (figs. 3A and B). The inhibitory effects of SB-258719 peaked within 30 min and did not weaken time dependently during the obser-

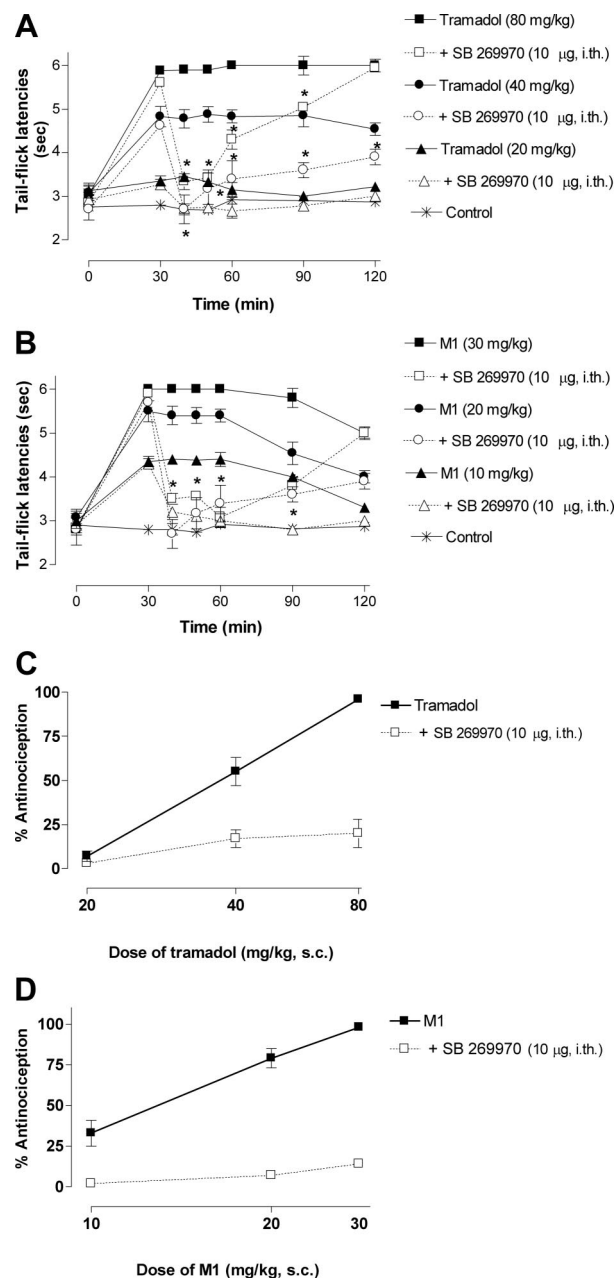


Fig. 2. The effects of an intrathecal injection of SB-269970 (10 μg) on systemic tramadol-induced (A) and O-desmethyltramadol (M1)-induced (B) antinociception. SB-269970 was intrathecally given 30 min after subcutaneous administration of tramadol and M1. The results are presented as mean ± SEM. Tail-flick latencies at the time of peak blocking effects (10 min) were converted to % antinociception to generate the dose-response curve of tramadol (C) and M1 (D). N = 8 per group. * Differences corresponding to the dose of tramadol and M1 alone, $P < 0.05$ (Bonferroni *post hoc* test).

vation period of 90 min after its administration. Figures 3C and D demonstrate the dose-response curves of tramadol and M1 generated from the data 30 min after the intrathecal administration of SB-258719 (20 μg), respectively.

In contrast to 5-HT₇ antagonists, *post hoc* analysis showed that intrathecal administration of ketanserin (20 μg) did not alter the antinociceptive effects of tramadol (40 and

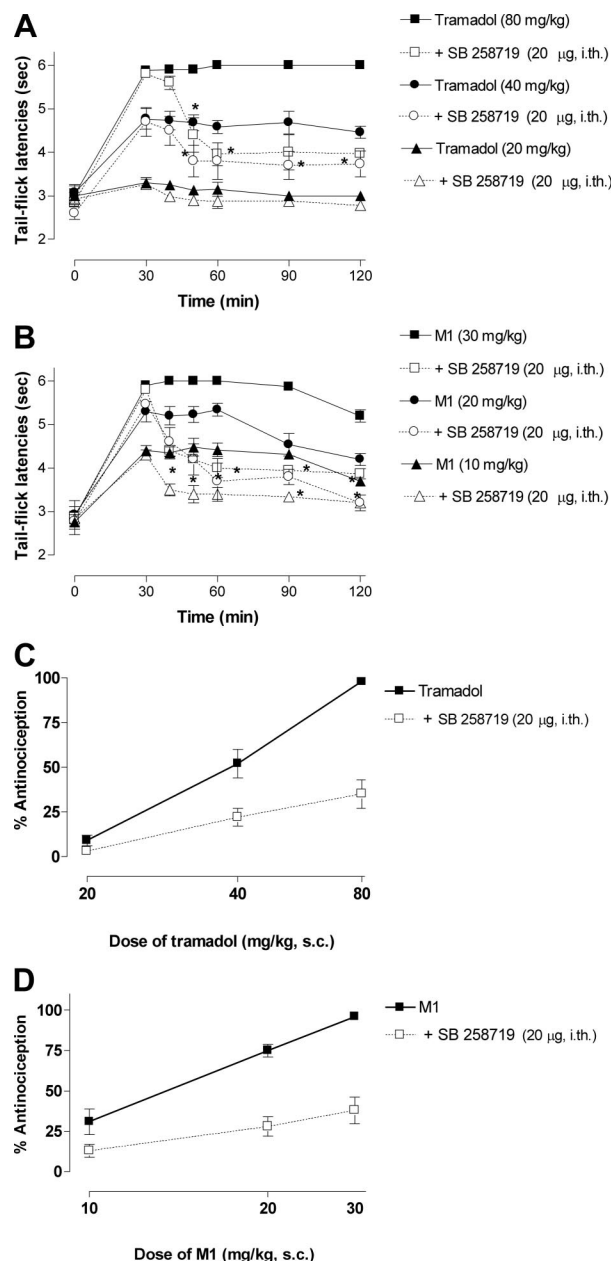


Fig. 3. The effects of an intrathecal injection of SB-258719 (20 μg) on systemic tramadol-induced (A) and O-desmethyltramadol (M1)-induced (B) antinociception. SB-258719 was intrathecally given 30 min after subcutaneous administration of tramadol and M1. The results are presented as mean ± SEM. Tail-flick latencies at the time of peak blocking effects (30 min) were converted to % antinociception to generate the dose-response curve of tramadol (C) and M1 (D). N = 8 per group. * Differences corresponding to the dose of tramadol and M1 alone, $P < 0.05$ (Bonferroni *post hoc* test).

80 mg/kg) and M1 (10, 20, and 30 mg/kg) at any time point during observation (figs. 4A and B). Figures 4C and D demonstrate the dose-response curves of tramadol and M1 generated from data the 30 min after the administration of ketanserin (20 μg), respectively. In addition, intrathecally administered ondansetron (20 μg) also did not significantly change tramadol-induced (40 and 80 mg/kg) and M1-induced (10, 20 and 30 mg/kg) antinociception (figs. 5A and

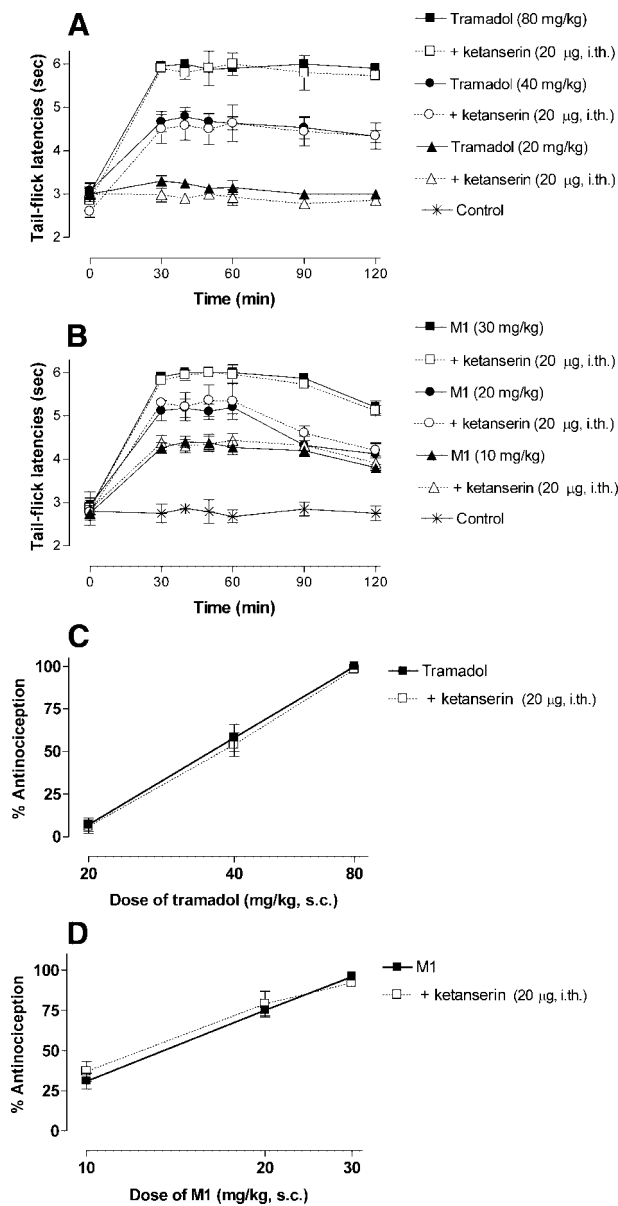


Fig. 4. The effects of an intrathecal injection of ketanserin (20 µg) on systemic tramadol-induced (A) and O-desmethyltramadol (M1)-induced (B) antinociception. Ketanserin was intrathecally given 30 min after subcutaneous administration of tramadol and M1. The results are presented as mean ± SEM. Tail-flick latencies at 60 min after tramadol (C) and M1 (D) administration were converted to % antinociception to generate the dose–response curve. N = 8 per group.

B). Figures 5C and d demonstrate the dose–response curves of tramadol and M1 generated from the data 30 min after the administration of ondansetron (20 µg), respectively.

The ED₅₀ values and 95% confidence limits for systemic tramadol and M1 alone or with intrathecal SB-269970 (10 µg), SB-258719 (20 µg), ketanserin (20 µg), and ondansetron (20 µg) are shown in table 1. The ED₅₀ values for tramadol and M1 with intrathecal SB-269970 (10 µg) or intrathecal SB-258719 (20 µg) were significantly greater than those for tramadol and M1 alone (table 1).

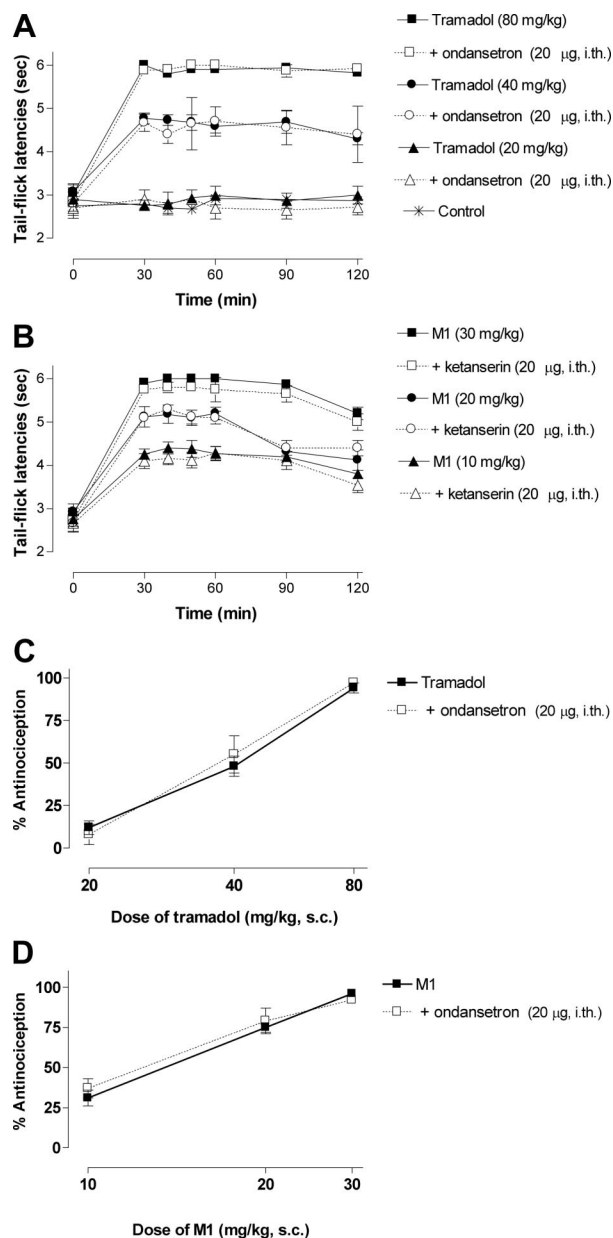


Fig. 5. The effects of an intrathecal injection of ondansetron (20 µg) on systemic tramadol-induced (A) and O-desmethyltramadol (M1)-induced (B) antinociception. Ondansetron was intrathecally given 30 min after subcutaneous administration of tramadol and M1. The results are presented as mean ± SEM. Tail-flick latencies at 60 min after tramadol (C) and M1 (D) administration were converted to % antinociception to generate the dose–response curve. N = 8 per group.

Next, to examine the potency of intrathecally administered SB-269970 and SB-258719 on systemic tramadol- and M1-induced antinociception, the different doses of SB-269970 (1, 3, and 10 µg) and SB-258719 (5, 10, and 20 µg) were tested against the maximal antinociceptive dose of tramadol (80 mg/kg) (figs. 6A and B) and M1 (30 mg/kg) (figs. 6C and D). Intrathecal administration of both SB-269970 and SB-258719 produced dose-dependent inhibition of tramadol-induced ($F(3,168) = 48.9, P < 0.001$

Table 1. The Effects of Intrathecally Administered 5-HT7, 5-HT2, and 5-HT3 Antagonists on Tramadol-induced and Its Major Active Metabolite (O-desmethyltramadol [M1])-induced Antinociception

Drug (mg/kg, Subcutaneous)	Antagonist (μ g/Mouse, Intrathecal)	ED ₅₀ and 95% Confidence Limit (mg/kg, Subcutaneous)
Tramadol (20, 40, and 80 mg/kg)	0.9% saline, 10 μ l	36.90 (32.94–41.33)
Tramadol (20, 40, and 80 mg/kg)	SB-269970, 10 μ g/mouse	2222.88 (1305.43–3785.08)
Tramadol (20, 40, and 80 mg/kg)	SB-258719, 20 μ g/mouse	117.18 (76.45–179.59)
Tramadol (20, 40, and 80 mg/kg)	Ketanserin, 20 μ g/mouse	39.56 (33.34–46.94)
Tramadol (20, 40, and 80 mg/kg)	Ondansetron, 20 μ g/mouse	31.21 (28.47–38.73)
M1 (10, 20, and 30 mg/kg)	0.9% saline, 10 μ l	11.14 (10.3–12.39)
M1 (10, 20, and 30 mg/kg)	SB-269970, 10 μ g/mouse	622.44 (357.78–1082.85)
M1 (10, 20, and 30 mg/kg)	SB-258719, 20 μ g/mouse	62.51 (55.14–70.86)
M1 (10, 20, and 30 mg/kg)	Ketanserin, 20 μ g/mouse	11.43 (10.21–12.79)
M1 (10, 20, and 30 mg/kg)	Ondansetron, 20 μ g/mouse	11.19 (10.02–12.49)

The ED₅₀ (the dose that produces 50% of its maximum antinociceptive effects) and confidence limits were calculated from the dose-percent inhibition relations by log-linear regression analysis on systemically administered tramadol- and M1-induced antinociception followed by 10 min and 30 min after intrathecal administration of SB-269970 and SB-258719, ketanserin, or ondansetron.

and $F(3,168) = 97.23$, $P < 0.001$, respectively, $n = 8$) and M1-induced ($F(3,168) = 154.5$, $P < 0.001$ and $F(3,168) = 86.77$, $P < 0.001$, respectively, $n = 8$) antinociception (figs. 6A–D).

The Effects of Tramadol and M1 in Incisional Pain

Consistent with a previous study,³³ plantar incision led to induction of thermal hyperalgesia in mice. Cumulative mean paw withdrawal latency to radiant heat was 9.6 ± 0.64 s and significantly decreased to 2.6 ± 0.18 s ($P < 0.0001$, $n = 12$) 24 h after incision. Incisional surgery did not alter paw withdrawal latency to radiant heat stimuli of the contralateral hind paws, which was 9.4 ± 0.39 s ($P = 0.8931$, $n = 12$). Thermal hyperalgesia remained for more than 3 days and recovered to baseline values by 8 days in the ipsilateral paw after surgery (data not shown).

Two-way repeated-measures ANOVA analyses indicated that systemically administered tramadol (10, 20 and 30 mg/kg) and M1 (1, 3 and 10 mg/kg) had significant effects on thermal paw withdrawal latencies after surgery ($F(3,112) = 29.11$, $P < 0.001$ and $F(3,112) = 35.67$, $P < 0.001$, respectively, $n = 8$). Tramadol and M1 elicited dose-dependent thermal antihyperalgesia (figs. 7A and B). A higher dose of tramadol (30 mg/kg) and M1 (10 mg/kg) increased paw withdrawal latencies to 10.2 ± 1.2 s and 9.025 ± 1.63 s at 30 min after injection ($P < 0.001$) compared with 2.58 ± 0.18 s and 2.86 ± 0.33 s for baseline values, respectively.

We next evaluated the antihyperalgesic effects of systemically administered tramadol (10, 20, and 30 mg/kg) and M1 (1, 3, and 10 mg/kg) in spinally 5,7-DHT-lesioned animals. The mean paw withdrawal latency of spinal 5,7-DHT (50 μ g) administered animals was 9.76 ± 0.96 s, which was not significantly different from saline-treated naive animals ($P = 0.64$, $n = 12$). The plantar incision significantly decreased paw withdrawal latency to 2.9 ± 0.76 s ($P < 0.001$, $n = 12$) 24 h after incision in spinally 5,7-DHT-lesioned animals. In contrast to antinociception experiments, *post hoc* analysis showed that tramadol-induced (20 and 30 mg/kg) and M1-

induced (1, 3, and 10 mg/kg) thermal antihyperalgesia remained unchanged in spinally 5,7-DHT-lesioned animals at the observation period (figs. 7A and B). Figures 7C and D illustrate the dose–response curves of tramadol and M1 generated from the data 60 min after tramadol and M1 administration in naive mice or in mice with lesioned serotonergic bulbospinal pathways by 5,7-DHT in paw incision model.

Then, we explored the contribution of spinal 5-HT7 receptors in the antihyperalgesic effects of systemically administered tramadol (10, 20, and 30 mg/kg) and M1 (1, 3, and 10 mg/kg). Although intrathecal administration of SB-269970 (10 μ g) was inactive alone (data not shown), it blocked the antihyperalgesic effects of tramadol ($F(5,168) = 20.7$, $P < 0.001$, $n = 8$) and M1 ($F(5,168) = 16.89$, $P < 0.001$, $n = 8$) (figs. 8A and B). Figures 8C and D demonstrate the dose–response curves of tramadol and M1 generated from the data 30 min after the intrathecal administration of SB-269970 (10 μ g), respectively. Intrathecally administered SB-258719 (20 μ g), inactive alone (data not shown), inhibited the antihyperalgesic effects of tramadol ($F(5,168) = 26.35$, $P < 0.001$, $n = 8$) and M1 ($F(5,168) = 13.23$, $P < 0.001$, $n = 8$) (figs. 9A and B). Figures 9C and D show the dose–response curves of tramadol and M1 generated from the data 30 min after the intrathecal administration of SB-258719 (20 μ g), respectively. In contrast to 5-HT7 antagonists, *post hoc* analysis showed that the intrathecal administration of ketanserin (20 μ g) was unable to reverse the antihyperalgesic effects of tramadol and M1 at any time point during observation (figs. 10A and B). Figures 10C and D demonstrate the dose–response curves of tramadol and M1 generated from the data 30 min after ketanserin (20 μ g) administration. Similar to ketanserin, intrathecally administered ondansetron (20 μ g) was unable to reverse the antihyperalgesic effects of tramadol and M1 at any time point during observation (figs. 11A and B). Figures 11C and D demonstrate the dose–response curves of tramadol and M1 generated from the data 30 min after the administration of ondansetron (20 μ g).

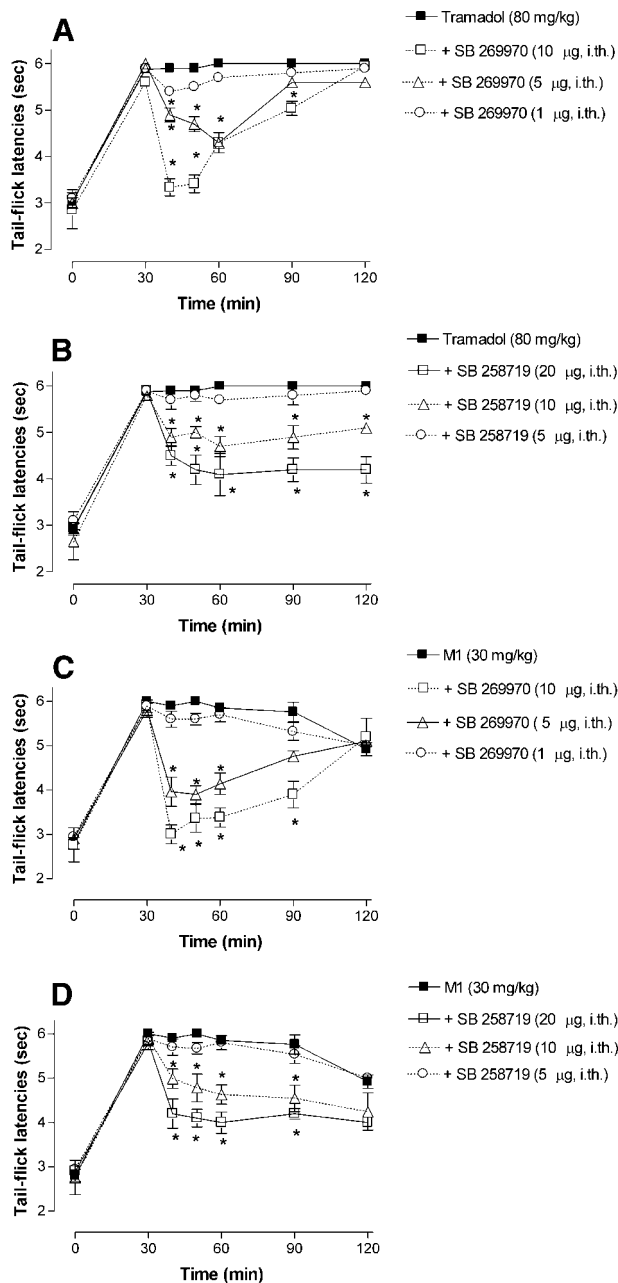


Fig. 6. The effects of an intrathecal injection of different doses of SB-269970 (1, 5, and 10 µg) and SB-258719 (5, 10, and 20 µg) on the fixed maximal dose of systemic tramadol-induced (80 mg/kg) (A and B) and O-desmethylntramadol (M1)-induced (10 mg/kg) (C and D) antinociception. SB-269970 and SB-258719 were intrathecally given 30 min after the subcutaneous administration of tramadol (80 mg/kg) and M1 (10 mg/kg). N = 8 per group. * Differences corresponding to the dose of tramadol and M1 alone, $P < 0.05$ (Bonferroni *post hoc* test).

The ED₅₀ values and 95% confidence limits for systemic tramadol- and M1-induced antihyperalgesia with intrathecal SB-269970 (10 µg), SB-258719 (20 µg), ketanserin (20 µg), and ondansetron (20 µg) are shown in table 2. The ED₅₀ values for tramadol and M1 and intrathecal SB-269970 (10 µg) or intrathecal SB-258719 (20 µg) were significantly greater than those for tramadol and M1 alone (table 2).

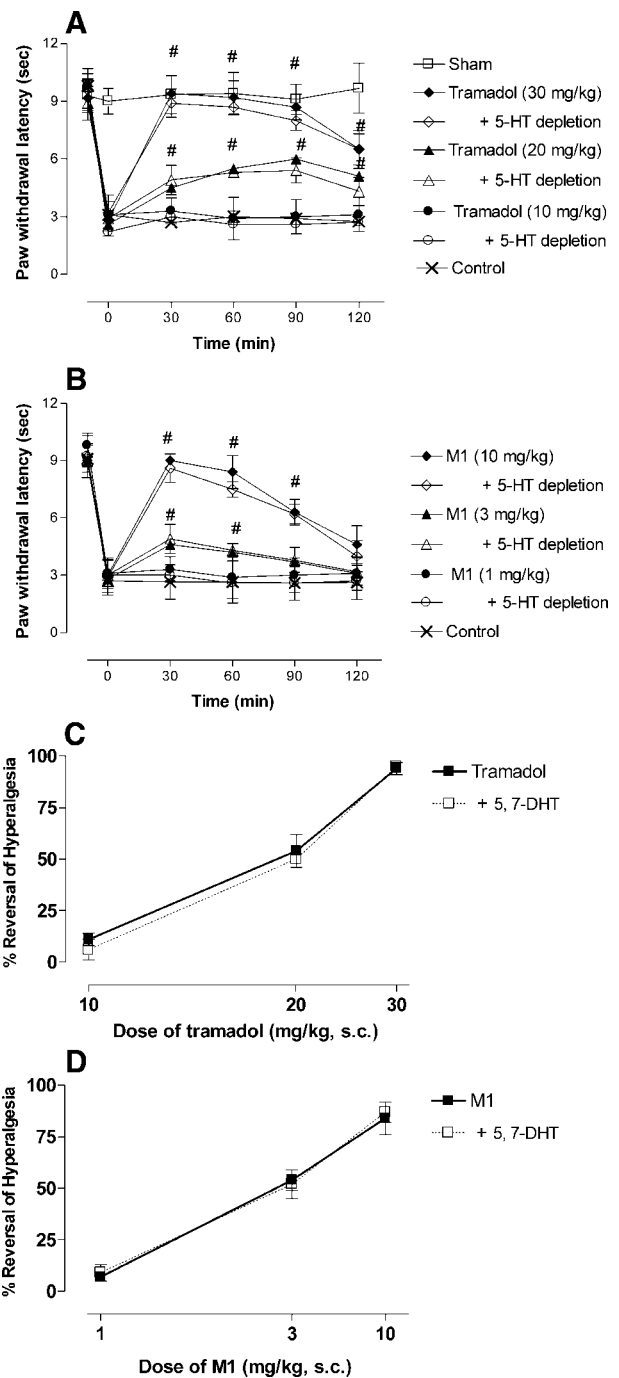


Fig. 7. The effects of subcutaneous administration of tramadol and O-desmethylntramadol (M1) on thermal hyperalgesia induced by paw incision. 5,7-dihydroxytryptamine (5,7-DHT) was intrathecally injected to deplete spinal serotonin. Three days after 5,7-DHT administration, mice were tested. Paw withdrawal latencies (PWLs) were determined before surgery and after surgery. PWLs to radiant heat in mice after plantar incision treated with 0.9% saline, tramadol (A), or O-desmethylntramadol (M1) (B) are shown in 0.9% saline-treated and 5-HT-depleted animals. The results are presented as mean ± SEM. PWLs at 60 min after tramadol (C) or M1 (D) administration were converted to % reversal of hyperalgesia to generate the dose-response curve. N = 8 per group. # $P < 0.05$ versus 0.9% saline control (Bonferroni *post hoc* test).

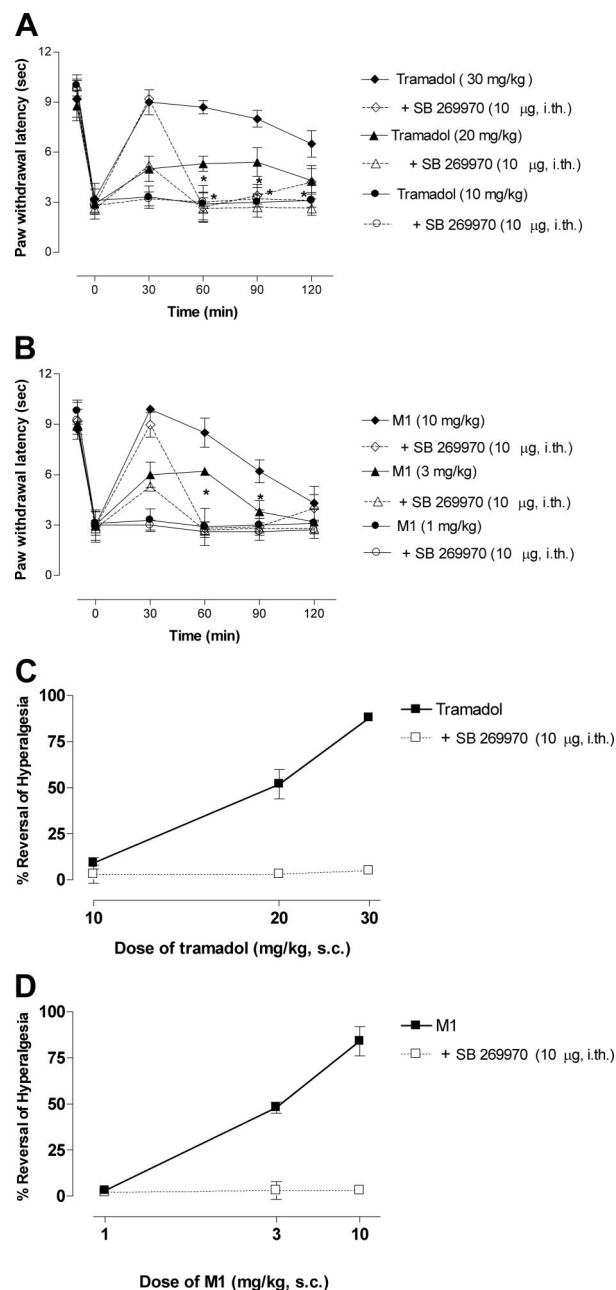


Fig. 8. The effects of an intrathecal injection of SB-269970 (10 µg) on systemic tramadol-induced (A) and O-desmethyltramadol (M1)-induced (B) thermal antihyperalgesia. SB-269970 was intrathecally given 30 min after subcutaneous administration of tramadol and M1. The results are presented as mean ± SEM. Paw withdrawal latencies at 60 min after tramadol (C) or M1 (D) administration were converted to % reversal of hyperalgesia to generate the dose-response curve. N = 8 per group. * Differences corresponding to the dose of tramadol and M1 alone $P < 0.05$ (Bonferroni *post hoc* test).

Next, we evaluated the potency of intrathecally administered SB-269970 and SB-258719 on systemic tramadol- and M1-induced antihyperalgesia. The different doses of SB-269970 (1, 3, and 10 µg) and SB-258719 (5, 10, and 20 µg) were tested against the maximal antihyperalgesic dose of tramadol (80 mg/kg) (figs. 12A and B) and M1 (30 mg/kg) (figs. 12C and D), respectively. Intrathecal ad-

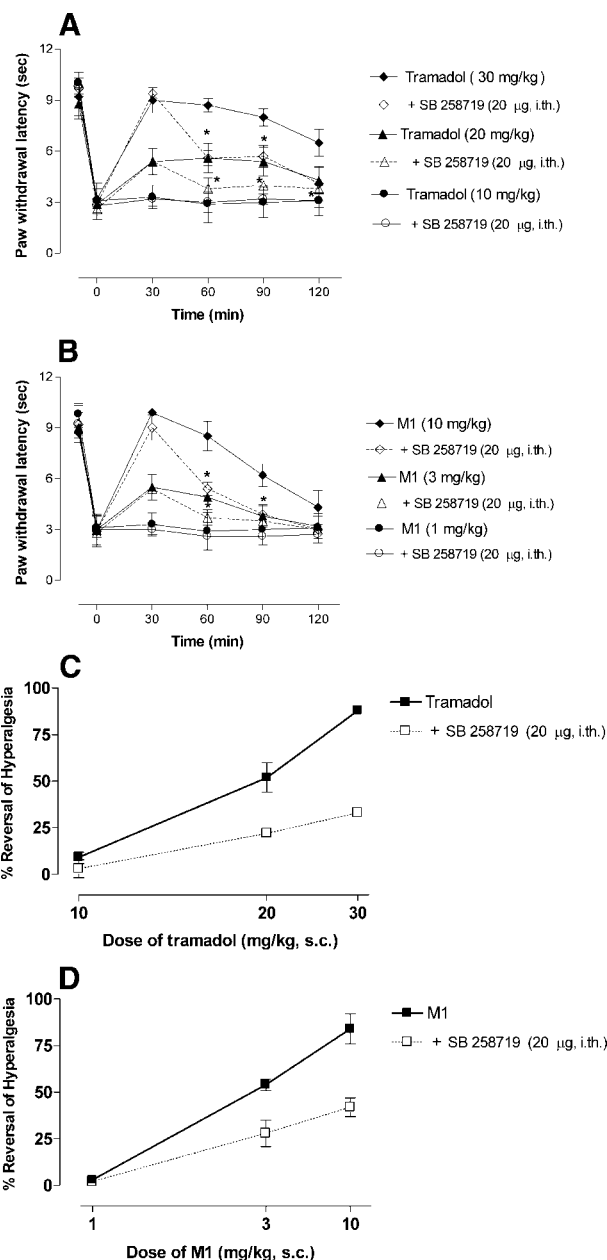


Fig. 9. The effects of an intrathecal injection of SB-258719 (20 µg) on systemic tramadol-induced (A) and O-desmethyltramadol (M1)-induced (B) thermal antihyperalgesia. SB-258719 was intrathecally given 30 min after subcutaneous administration of tramadol and M1. The results are presented as mean ± SEM. Paw withdrawal latencies at 60 min after tramadol (C) or M1 (D) administration were converted to % reversal of hyperalgesia to generate the dose-response curve. N = 8 per group. * Differences corresponding to the dose of tramadol and M1 alone $P < 0.05$ (Bonferroni *post hoc* test).

ministration of both SB-269970 and SB-258719 produced dose-dependent inhibition of tramadol-induced ($F(4,140) = 29.74$, $P < 0.001$ and $F(4,140) = 20.90$, $P < 0.001$, respectively, $n = 8$) and M1-induced ($F(4,140) = 27.75$, $P < 0.001$ and $F(4,140) = 29.19$, $P < 0.001$, respectively, $n = 8$) elevations in paw-flick latencies in incised animals.

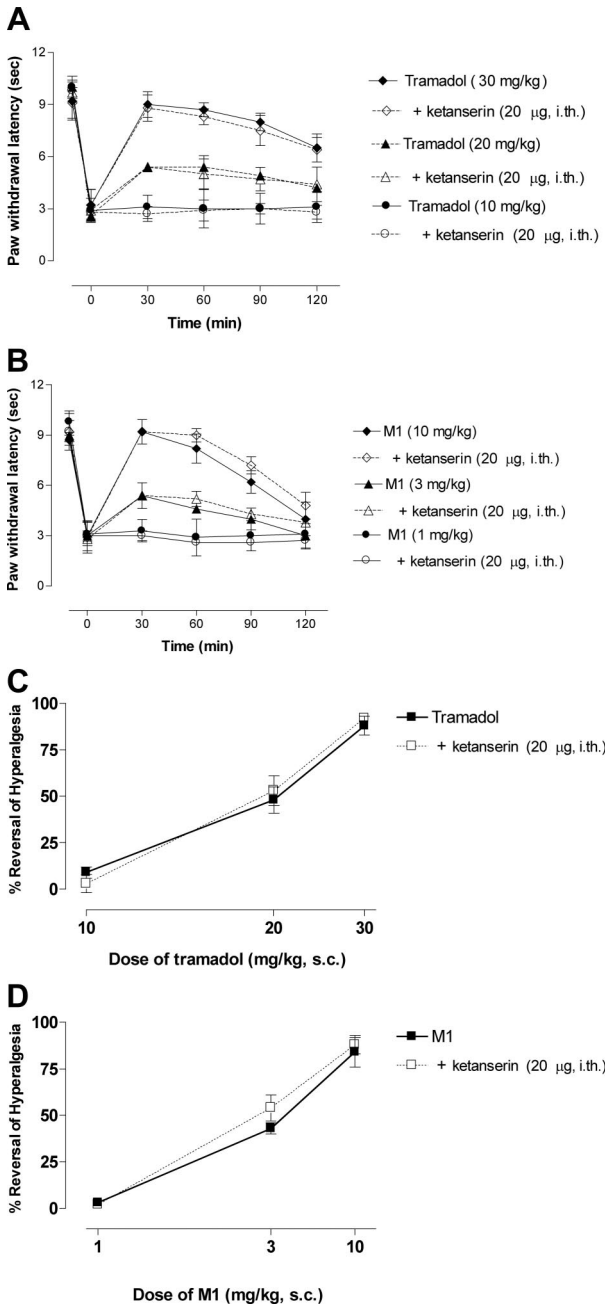


Fig. 10. The effects of an intrathecal injection of ketanserin (20 µg) on systemic tramadol-induced (A) and O-desmethyltramadol (M1)-induced (B) thermal antihyperalgesia. Ketanserin was intrathecally given 30 min after subcutaneous administration of tramadol and M1. The results are presented as mean ± SEM. Paw withdrawal latencies at 60 min after tramadol (C) or M1 (D) administration were converted to % reversal of hyperalgesia to generate the dose-response curve. N = 8 per group.

Discussion

In this study, we provide behavioral evidence that descending serotonergic pathways play an important role in the antinociceptive effects of tramadol and M1, and spinal 5-HT7 receptors play a key role in the antinociceptive effects and antihyperalgesic effects of tramadol and M1.

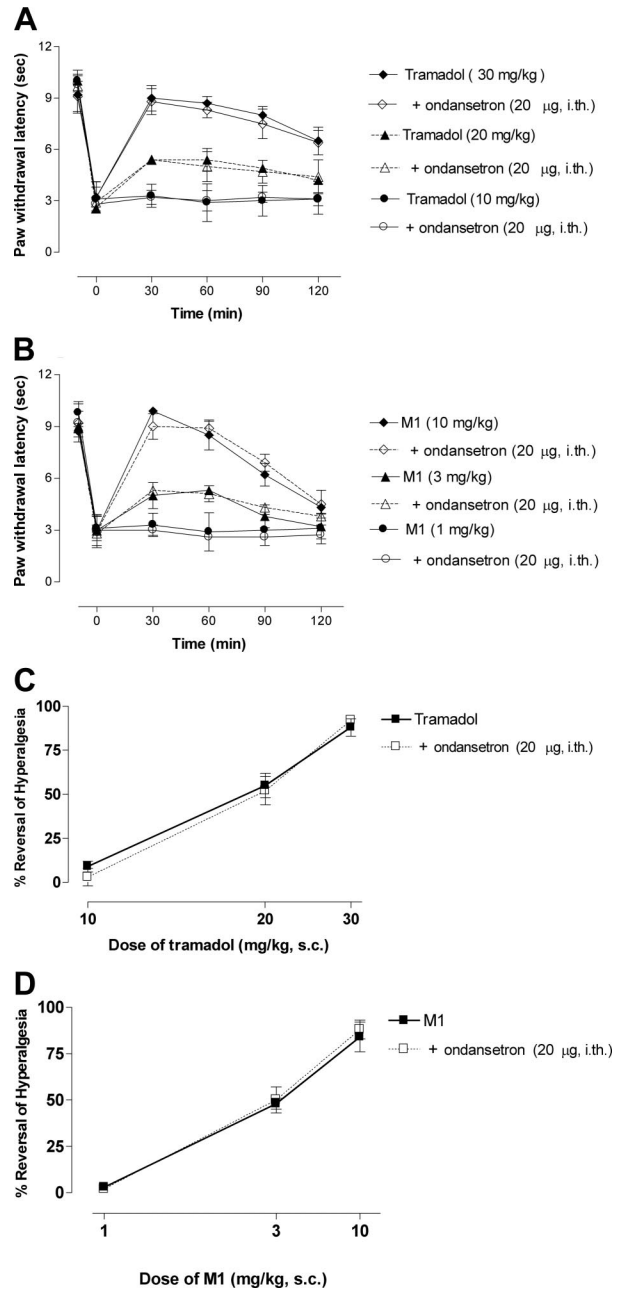


Fig. 11. The effects of an intrathecal injection of ondansetron (20 µg) on systemic tramadol-induced (A) and O-desmethyltramadol (M1)-induced (B) thermal antihyperalgesia. Ondansetron was intrathecally given 30 min after subcutaneous administration of tramadol and M1. The results are presented as mean ± SEM. Paw withdrawal latencies at 60 min after tramadol (C) or M1 (D) administration were converted to % reversal of hyperalgesia to generate the dose-response curve. N = 8 per group.

It is well known that the antinociceptive effect of some narcotic and nonnarcotic analgesics is dependent, in part, on the integrity of the serotonergic system.²⁰ In the brain, the highest concentration of serotonergic neurons can be detected in midbrain and brain stem areas called raphe nuclei.^{15,20,38} The bulbospinal descending serotonergic system that originates from the dorsal raphe nucleus is involved in the control of transmission of noxious inputs at the spinal

Table 2. The Effects of Intrathecally Administered 5-HT7, 5-HT2, and 5-HT3 Antagonists on Tramadol-induced and Its Major Active Metabolite (O-desmethyltramadol [M1])-induced Antihyperalgesia

Drug (mg/kg, Subcutaneous)	Antagonist (μ g/Mouse, Intrathecal)	ED ₅₀ and 95% Confidence Limit (mg/kg, Subcutaneous)
Tramadol (10, 20, and 30 mg/kg)	0.9% saline, 10 μ l	18.68 (16.40–21.28)
Tramadol (10, 20, and 30 mg/kg)	SB-269970, 10 μ g/mouse	1355.49 (1209.23–1519.38)
Tramadol (10, 20, and 30 mg/kg)	SB-258719, 20 μ g/mouse	75.74 (71.50–80.24)
Tramadol (10, 20, and 30 mg/kg)	Ketanserin, 20 μ g/mouse	19.04 (17.10–21.20)
Tramadol (10, 20, and 30 mg/kg)	Ondansetron, 20 μ g/mouse	19.73 (17.46–22.29)
M1 (1, 3, and 10 mg/kg)	0.9% saline, 10 μ l	3.06 (2.42–3.87)
M1 (1, 3, and 10 mg/kg)	SB-269970, 10 μ g/mouse	290.43 (165.76–509.45)
M1 (1, 3, and 10 mg/kg)	SB-258719, 20 μ g/mouse	66.23 (49.73–88.20)
M1 (1, 3, and 10 mg/kg)	Ketanserin, 20 μ g/mouse	2.63 (2.18–3.16)
M1 (1, 3, and 10 mg/kg)	Ondansetron, 20 μ g/mouse	3.46 (2.66–3.52)

The ED₅₀ (the dose that produces 50% of its maximum antinociceptive effects) and confidence limits were calculated from the dose-percent inhibition relations by log-linear regression analysis on systemically administered tramadol- and M1-induced thermal antihyperalgesia followed by 30 min after intrathecal administration of SB-269970 and SB-258719, ketanserin, or ondansetron.

cord, and a predominant source of serotonergic input to spinal cord arises within vicinity of raphe nucleus.^{15,19,20} We used two approaches such as selective lesions of serotonergic pathways and administration of selective 5-HT antagonist to identify the specific role of 5-HT in the antinociceptive and antihyperalgesic effects of systemically administered tramadol and its active metabolite M1.

It has been previously reported that tramadol and M1 produce antinociceptive effects on acute pain models.³⁹ In addition to its antinociceptive effects, tramadol also elicited thermal antihyperalgesic effects in a persistent pain model such as plantar incision.⁴⁰ This study supports and extends these findings by showing that M1 can also elicit thermal antihyperalgesic effects in paw incision model. Hyperalgesia is the physiologic expression of central nervous system sensitization induced by nociceptive inputs from the surgical wound.⁴⁰ The antihyperalgesic effect deserves attention because hyperalgesia that occurs after tissue incision may contribute to postoperative pain.⁴¹ Previous studies have shown tramadol to be an effective postoperative analgesic. We confirm that tramadol reduces thermal hypersensitivity in acute and postoperative pain models in mice.

The depletion of spinal 5-HT by intrathecal administration of the neurotoxin 5,7-DHT has been previously reported as a method for characterizing the role of 5-HT in nociceptive modulation.^{42,43} It has been reported that intrathecal administration of 5,7-DHT at a dose of 50 μ g selectively depleted 5-HT contents more than 92% in the spinal cord 3 days after toxin administration in mice.³⁴ Consistent with this study, we found an 88% decrease of 5-HT contents in spinal cord levels after intrathecal treatment of 5,7-DHT at a dose of 50 μ g. Early studies on the effects of neurotoxic destruction of spinal serotonergic neurons elicits no change in the thermal nociceptive threshold in naive animals.^{34,44,45} Consistent with these studies, we observed a similar thermal nociceptive threshold in naive and lesioned mice in tail-flick and paw incision tests.

We found that both tramadol and M1 produced antino-

ceptive effects in naive animals in the tail-flick test. In addition, lower doses of tramadol and M1 than those used in the tail-flick test blocked incision-induced thermal hyperalgesia. It is well known that opioids are very sensitive to thermal nociceptive tests and produce antinociceptive and thermal antihyperalgesic effects by a common mechanism, most likely activity at the μ -opioid receptor. The strong antinociceptive and antihyperalgesic action of M1 after systemic administration confirms the finding in previous studies that M1 metabolite was a major opioid component of tramadol. In this study, the antinociceptive effects of both tramadol and M1 were clearly attenuated after the neurotoxic destruction of descending serotonergic pathways. Thus, our results strongly indicate that the release of 5-HT in the spinal cord plays a crucial role in the antinociceptive effects of systemically administered tramadol and M1. The role of serotonin in tramadol analgesia has been suggested by Oliva *et al.*,¹⁶ and they speculate that tramadol reduces formalin-induced pain by increasing 5-HT concentration at the spinal cord level. Our results that the depletion of spinal 5-HT reduced the antinociceptive effects of systemically administered tramadol and its major metabolite M1 strongly support this hypothesis and provide additional support for the involvement of descending serotonergic pathways in the antinociceptive effects of tramadol. Nevertheless, the antihyperalgesic effects of tramadol and M1 were not changed after neurotoxic destruction of descending serotonergic pathways.

Multiple serotonin subtypes have been identified within the central nervous system, and there are conflicting findings about 5-HT receptor subtypes responsible for mediating the antinociceptive effect of systemically administered tramadol.^{3,12,15–18} In these previous studies, selective serotonin antagonists were given systemically to explore the involvement of serotonin receptor subtypes.^{3,12,15–18} Despite the importance of the findings obtained from these studies, complexity may arise when systemically administered serotonin antagonists access multiple sites within the pain transmission system (periphery, spinally, and supraspinally). To clarify the

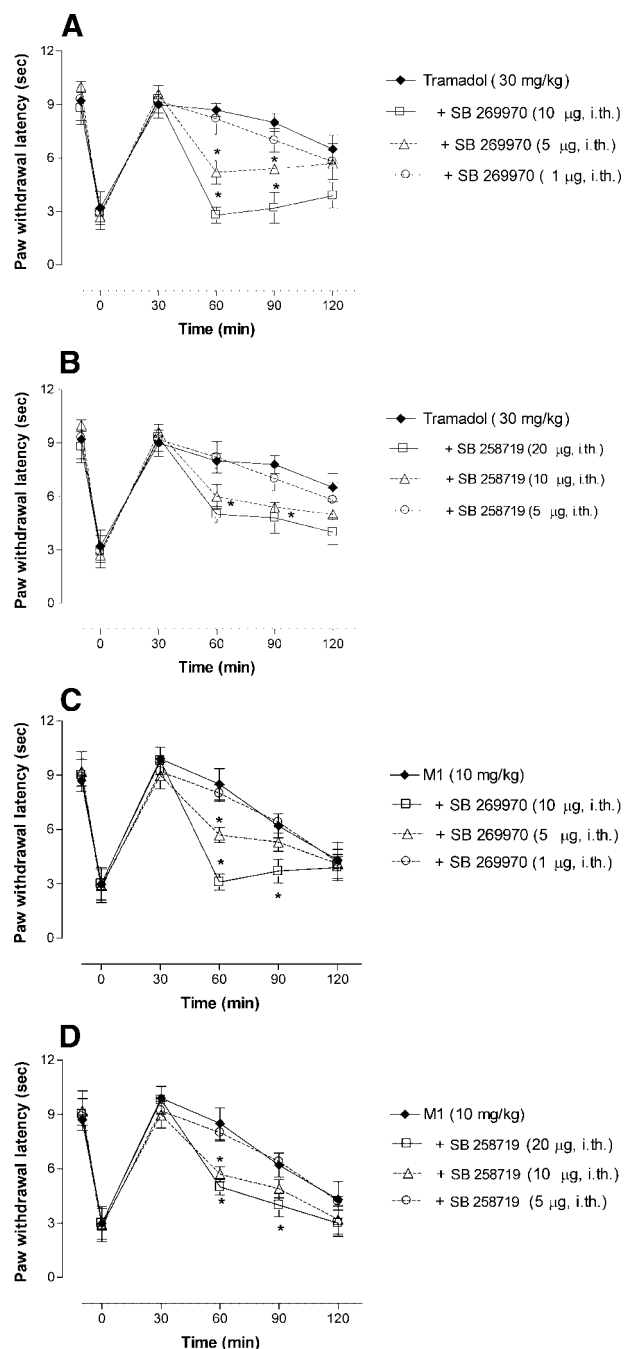


Fig. 12. The effects of an intrathecal injection of different doses of SB-269970 (1, 3, and 10 µg) and SB-258719 (5, 10, and 20 µg) on the fixed maximal dose of systemic tramadol-induced (30 mg/kg) (A and B) and O-desmethyltramadol (M1)-induced (10 mg/kg) (C and D) thermal antihyperalgesia. SB-269970 and SB-258719 were intrathecally given 30 min after subcutaneous administration of tramadol (80 mg/kg) and M1 (10 mg/kg). N = 8 per group. * Differences corresponding to the dose of tramadol and M1 alone $P < 0.05$ (Bonferroni *post hoc* test).

role of spinal-specific 5-HT receptors responsible for mediating analgesia and antihyperalgesia induced by tramadol and its active metabolite M1 we intrathecally (locally) administered serotonin antagonists, which has been rarely performed. It has been suggested that spinal 5-HT₇ receptors play an important role in the nociceptive process, and the

anatomic localization of 5-HT₇ receptors in the superficial layers of spinal cord dorsal horn support this hypothesis.²³ In a recent study, Dogrul and Seyrek²² reported the important role of spinal 5-HT₇ receptors in systemic morphine-induced analgesia. In our study, the finding that spinal selective blockade of 5-HT₇ receptors by SB-269970 and SB-258719 inhibits the antinociceptive and antihyperalgesic effect of systemic tramadol and M1 suggests that the spinal 5-HT₇ receptors play a crucial role in tramadol-induced antinociception and antihyperalgesia. These effects were not reproduced by spinal administration of the selective 5-HT₂ and 5-HT₃ receptor antagonists, supporting the notion that selective activation of spinal 5-HT₇ receptors plays a crucial role in the antinociceptive and antihyperalgesic effects of systemically administered tramadol and M1.

We found that the inhibitory potency of spinal SB-269970 against systemic tramadol-induced antinociception and antihyperalgesia was higher than that of spinal SB-258719. It has been reported that SB-258719 had a moderate affinity for 5-HT₇ receptors, indicated by $pIC_{50} = 6.7$ when compared with the pKi of SB-269970 that was 7.96.³⁶ In addition, 5-HT₇ receptor is a member of the family of G-protein-coupled 5-HT receptors and couples to the stimulation of adenylate cyclase. *In vitro* antagonist potency of SB-269970 and SB-258719 for 5-HT-mediated cyclic adenosine monophosphate formation in stably transfected HEK-293F/rat 5-HT₇ cells have been evaluated and indicated by $pIC_{50} = 8.75$ and 6.8 for SB-269970 and SB-258719, respectively.⁹ Thus, the lower inhibitory effect of SB-258719 on systemic tramadol- and M1-induced antinociception and antihyperalgesia when compared with SB-269970 may be due to the lower antagonist potency of SB-258719 against 5-HT₇ receptors.

An interesting finding in this study was that SB-269970, which is structurally different from SB-258719, showed a different time course of effect on systemic tramadol- and M1-induced antinociception and antihyperalgesia. The duration of the inhibitor effect of spinally administered SB-269970 against systemic tramadol- and M1-induced antinociception and antihyperalgesia were markedly shorter compared with that induced by SB-258719. Our results correlated with the study of Dogrul and Seyrek,²² who showed a similar time course effect of spinally administered SB-269970 on systemic morphine-induced antinociception and reinforced previous results that SB-269970 had an extremely high clearance rate and possessed a very short half-life of less than 0.5 h when compared with that of SB-258719.⁴⁶ In addition, it has been known that highly lipophilic drugs rapidly transfer from the cerebrospinal fluid after intrathecal injection,⁴⁷ and it has been reported that SB-269970 was a lipophilic drug.⁴⁸ Thus, the significantly shorter duration of action of SB-269970 may be explained by its higher lipophilicity. Furthermore, lipid solubility is also a major determinant of the rate of onset of spinally administered drugs.⁴⁷ Thus, in our study, a much more rapid onset of action of SB-269970 than SB-258719 to antagonize the

antinociceptive and antihyperalgesic effects of tramadol and M1 supports that SB-269970 may easily spread into spinal 5-HT7 receptors. In our study, the lack of effects of selective 5-HT2 and 5-HT3 antagonists on the antinociceptive and antihyperalgesic effects of tramadol and M1 supports the specific involvement of spinal 5-HT7 receptors but not of 5-HT2 and 5-HT-3 receptors in the modulation of nociception by tramadol and M1.

Surprisingly, although there is a total blockade of the antinociceptive and antihyperalgesic effects of both tramadol and M1 by spinally administered 5-HT7 antagonists at some time point, some antinociceptive effects of tramadol and M1 still remained, and the antihyperalgesic effects of tramadol and M1 were unable to reverse under neurotoxic destruction of descending serotonergic pathways. A possible resolution of this paradox is that the neurotoxic lesion has still left some 5-HT to produce antinociceptive and antihyperalgesic effects by tramadol and M1 on spinal 5-HT7 receptors. Besides, it is possible that much lower spinal release of 5-HT is necessary to produce an antihyperalgesic effect than the antinociceptive effect by tramadol and M1.

It has been reported that systemically administered morphine activates the descending serotonergic pathways, and 5-HT7 receptors in the spinal cord plays an important role in systemic morphine antinociception.²² In this regard, it is possible that there is an existence of specific neuronal circuits whereby systemic tramadol can modulate nociception through activation of a descending serotonergic system to the spinal cord and act on 5-HT7 receptors in the spinal system. Supporting our data, the involvement of the 5-HT7 receptor subtype in the control of pain in conditions involving central sensitization such as capsaicin-induced hypersensitivity has been reported and raises the notion that systemically administered selective 5-HT7 receptor agonists may represent a new potential therapeutic approach for pain alleviation.⁴⁹

In our study, it is unlikely that activation of spinal 5-HT7 receptors by tramadol could directly inhibit primary afferents or nociceptive dorsal horn neurons because these receptors are positively coupled to adenylate cyclase, and their stimulation is excitatory in neurons.²⁰ However, the serotonergic system in the spinal cord may interact with other neurotransmitters in the modulation of nociception. Thus, it is likely that activation of 5-HT7 receptors localized on spinal inhibitory γ -aminobutyrgic or enkephalinergic interneurons, to evoke the release of γ -aminobutyric acid or enkephalins, would produce an inhibition of nociceptive transmission.⁴⁹

Morphine exert its analgesic effects on binding to opioid receptors.⁵⁰ It has been reported that morphine can interact with descending serotonergic pathways, and spinal 5-HT7 receptors play a crucial role in morphine-induced analgesia. In our study, we observed that descending serotonergic pathways and spinal 5-HT7 receptors play a crucial role in both tramadol- and its active metabolite M1-induced analgesia and antihyperalgesia. It is well known that approximately 70% of tramadol is metabolized to M1 that binds μ -opioid

receptors with approximately 300-fold higher affinity than the parent compound.⁵ It has been suggested that the expression of the opioid component of tramadol is primarily due to its metabolic conversion to M1. Specifically, the ratio of tramadol to M1 in brain increases with increasing doses of tramadol after its systemic administration in mice.⁶ In the light of these findings, it is possible that tramadol expresses an opioid component in the mechanism of its antinociceptive and antihyperalgesic effects. In this regard, it would seem reasonable to assume that tramadol metabolizes into M1, which may access supraspinal sites and binds opioid receptors such as morphine and activate descending serotonergic pathways to the spinal cord and produce antinociceptive effects *via* spinal 5-HT7 receptors in the dorsal horn. However, because tramadol or its enantiomer is an effective 5-HT reuptake inhibitor,^{6,38,51} it is tempting that tramadol itself and its M1 metabolite might activate serotonergic and opiodergic systems, respectively, and this dual mechanism of tramadol may contribute to the activation of descending serotonergic pathways and elicit antinociceptive and antihyperalgesic effects *via* spinal 5-HT7 receptors.

Despite our effort to achieve a well-designed study, it might be prudent to warn about the problems associated with design and the statistical complexity in this study. It is well known that when multiple significant tests are performed, it is possible, if not probable, to have both false-positive and false-negative errors within the same study.⁵² Because of the sheer number of the statistical models conducted and thorough evaluation of the hypotheses required a substantial number of significance tests, and probability errors are of concern in our study.

In conclusion, our study suggests that descending serotonergic pathways play an important role in the antinociceptive effects of tramadol and M1, and spinal 5-HT7 receptors contribute to the antinociceptive and antihyperalgesic effects of tramadol and M1.

References

1. Grond S, Sablotzki A: Clinical pharmacology of tramadol. *Clin Pharmacokinet* 2004; 43:879-923
2. Moore RA, McQuay HJ: Single-patient data meta-analysis of 3453 postoperative patients: Oral tramadol versus placebo, codeine and combination analgesics. *Pain* 1997; 69:287-94
3. Xie H, Dong ZQ, Ma F, Bauer WR, Wang X, Wu GC: Involvement of serotonin 2A receptors in the analgesic effect of tramadol in mono-arthritic rats. *Brain Res* 2008; 1210:76-83
4. Bamigbade TA, Davidson C, Langford RM, Stamford JA: Actions of tramadol, its enantiomers and principal metabolite, O desmethyltramadol, on serotonin (5-HT) efflux and uptake in the rat dorsal raphe nucleus. *Br J Anaesth* 1997; 79:352-6
5. Minami K, Uezono Y, Ueta Y: Pharmacological aspects of the effects of tramadol on G-protein coupled receptors. *J Pharmacol Sci* 2007; 103:253-60
6. Tao Q, Stone DJ, Borenstein MR, Codd EE, Coogan TP, Desai-Krieger D, Liao S, Raffa RB: Differential tramadol and O-desmethyl metabolite levels in brain *vs.* plasma of mice and rats administered tramadol hydrochloride orally. *J Clin Pharm Ther* 2002; 27:99-106

7. Campanero MA, García-Quetglas E, Sádaba B, Azanza JR: Simultaneous stereoselective analysis of tramadol and its primary phase I metabolites in plasma by liquid chromatography. Application to a pharmacokinetic study in humans. *J Chromatogr A* 2004; 1031:219-28
8. Gillen C, Haurand M, Kobelt DJ, Wnendt S: Affinity, potency and efficacy of tramadol and its metabolites at the cloned human mu-opioid receptor. *Naunyn Schmiedeberg Arch Pharmacol* 2000; 362:116-21
9. Raffa RB: Basic pharmacology relevant to drug abuse assessment: Tramadol as example. *J Clin Pharm Ther* 2008; 33:101-8
10. Shipton EA: Tramadol—present and future. *Anaesth Intensive Care* 2000; 28:363-74
11. Dayer P, Desmeules J, Collart L: Pharmacology of tramadol. *Drugs* 1997; 53(suppl 2):18-24
12. Arcioni R, Della Rocca M, Romanò S, Romano R, Pietropoli P, Gasparetto A: Ondansetron inhibits the analgesic effects of tramadol: A possible 5-HT(3) spinal receptor involvement in acute pain in humans. *Anesth Analg* 2002; 94:1553-7
13. Barann M, Urban B, Stamer U, Dorner Z, Bönisch H, Brüß M: Effects of tramadol and O-demethyl-tramadol on human 5-HT reuptake carriers and human 5-HT_{3A} receptors: A possible mechanism for tramadol-induced early emesis. *Eur J Pharmacol* 2006; 531:54-8
14. Frink MC, Hennies HH, Englberger W, Hanrand M, Willfert B: Influence of tramadol on neurotransmitter systems of the rat brain. *Arzneimittelforschung* 1996; 46:1029-36
15. Berrocoso E, Rojas-Corrales MO, Mico JA: Differential role of 5-HT_{1A} and 5-HT_{1B} receptors on the antinociceptive and antidepressant effect of tramadol in mice. *Psychopharmacology (Berl)* 2006; 188:111-8
16. Oliva P, Aurilio C, Massimo F, Grella A, Maione S, Grella E, Scafuro M, Rossi F, Berrino L: The antinociceptive effect of tramadol in the formalin test is mediated by the serotonergic component. *Eur J Pharmacol* 2002; 445:179-85
17. De Witte JL, Schoenmaekers B, Sessler DI, Deloof T: The analgesic efficacy of tramadol is impaired by concurrent administration of ondansetron. *Anesth Analg* 2001; 92:1319-21
18. Erhan E, Onal A, Kocabas S, Parlar A, Yegul I, Kosay S: Ondansetron does not block tramadol-induced analgesia in mice. *Methods Find Exp Clin Pharmacol* 2005; 27:629-32
19. Braz JM, Basbaum AI: Genetically expressed transneuronal tracer reveals direct and indirect serotonergic descending control circuits. *J Comp Neurol* 2008; 507:1990-2003
20. Millan MJ: Descending control of pain. *Prog Neurobiol* 2002; 66:355-474
21. Thomas DR, Atkinson PJ, Hastie PG, Middlemiss DN, Price GW: [³H]-SB-269970 radiolabels 5-HT₇ receptors in rodent, pig and primate brain tissues. *Neuropharmacology* 2002; 42:78-81
22. Dogrul A, Seyrek M: Systemic morphine produce antinociception mediated by spinal 5-HT₇, but not 5-HT_{1A} and 5-HT₂ receptors in the spinal cord. *Br J Pharmacol* 2006; 149:498-505
23. Meuser T, Pietruck C, Gabriel A, Xie GX, Lim KJ, Pierce Palmer P: 5-HT₇ receptors are involved in mediating 5-HT-induced activation of rat primary afferent neurons. *Life Sci* 2002; 71:2279-89
24. Doly S, Fischer J, Brisorgueil MJ, Verge D, Conrath M: Pre- and postsynaptic localization of the 5-HT₇ receptor in rat dorsal spinal cord: Immunocytochemical evidence. *J Comp Neurol* 2005; 26:256-69
25. Liu RJ, Zhang RX, Qiao JT, Dafny N: Interrelations of opioids with monoamines in descending inhibition of nociceptive transmission at the spinal level: An immunocytochemical study. *Brain Res* 1999; 830:183-90
26. Zimmermann M: Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983; 16:109-10
27. Forbes IT, Dabbs S, Duckworth DM, Jennings AJ, King FD, Lovell PJ, Brown AM, Collin L, Hagan JJ, Middlemiss DN, Riley GJ, Thomas DR, Upton N: (R)-3, N-dimethyl-N-[1-methyl-3-(4-methyl-piperidin-1-yl) propyl] benzenesulfonamide: The first selective 5-HT₇ receptor antagonist. *J Med Chem* 1998; 41:655-7
28. Lovell PJ, Bromidge SM, Dabbs S, Duckworth DM, Forbes IT, Jennings AJ, King FD, Middlemiss DN, Rahman SK, Saunders DV, Collin LL, Hagan JJ, Riley GJ, Thomas DR: A novel, potent, and selective 5-HT₇ antagonist: (R)-3-(2-(2-(4-methylpiperidin-1-yl)ethyl)pyrrolidine-1-sulfonyl) phenol (SB-269970). *J Med Chem* 2000; 43:342-5
29. Pouzet B: SB-258741: A 5-HT₇ receptor antagonist of potential clinical interest. *CNS Drug Rev* 2002; 8:90-100
30. Herndon JL, Ismael A, Ingher SP, Teitler M, Glennon RA: Ketanserin analogues: Structure-affinity relationships for 5-HT₂ and 5-HT_{1C} serotonin receptor binding. *J Med Chem* 1992; 35:4903-10
31. Freeman AJ, Cunningham KT, Tyers MB: Selectivity of 5-HT₃ receptor antagonists and anti-emetic mechanisms of action. *Anticancer Drugs* 1992; 3:79-85
32. Hylden JL, Wilcox GL: Intrathecal morphine in mice: A new technique. *Eur J Pharmacol* 1980; 17:313-6
33. Pogatzki EM, Raja SN: A mouse model of incisional pain. *Anesthesiology* 2003; 99:1023-7
34. Hung KC, Wu HE, Mizoguchi H, Leitermann R, Tseng LF: Intrathecal treatment with 6-hydroxydopamine or 5,7-dihydroxytryptamine blocks the antinociception induced by endomorphin-1 and endomorphin-2 given intracerebroventricularly in the mouse. *J Pharmacol Sci* 2003; 93:299-306
35. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988; 32:77-88
36. Romero G, Pujol M, Pauwels PJ: Reanalysis of constitutively active rat and human 5-HT_{7(a)} receptors in HEK-293F cells demonstrates lack of silent properties for reported neutral antagonists. *Naunyn Schmiedeberg Arch Pharmacol* 2006; 374:31-9
37. Tallarida RJ, Murray RB: *Manual of Pharmacologic Calculations with Computer Programs*, 2nd edition. New York, Springer-Verlag, 1987, pp 26-31
38. Häring M, Marsicano G, Lutz B, Monory K: Identification of the cannabinoid receptor type 1 in serotonergic cells of the raphe nuclei in mice. *Neuroscience* 2007; 146:1212-9
39. Ide S, Minami M, Ishihara K, Uhl GR, Sora I, Ikeda K: Mu opioid receptor-dependent and independent components in effects of tramadol. *Neuropharmacology* 2006; 51:651-8
40. Girard P, Pansart Y, Coppe MC, Gillardin JM: Nefopam reduces thermal hypersensitivity in acute and postoperative pain models in the rat. *Pharmacol Res* 2001; 44:541-5
41. Wilder-Smith OHG, Arendt-Nielsen L: Postoperative hyperalgesia. Its clinical importance and relevance. *Anesthesiology* 2006; 104:601-7
42. Zhao ZQ, Gao YJ, Sun YG, Zhao CS, Gereau RW, Chen ZF: Central serotonergic neurons are differentially required for opioid analgesia but not for morphine tolerance or morphine reward. *Proc Natl Acad Sci USA* 2007; 104:14519-24
43. Zhao ZQ, Chiechio S, Sun YG, Zhang KH, Zhao CS, Scott M, Johnson RL, Deneris ES, Renner KJ, Gereau RW, Chen ZF: Mice lacking central serotonergic neurons show enhanced inflammatory pain and an impaired analgesic response to antidepressant drugs. *J Neurosci* 2007; 27:6045-53
44. Li JY, Wong CH, Huang EY, Lin YC, Chen YL, Tan PP, Chen JC: Modulations of spinal serotonin activity affect the development of morphine tolerance. *Anesth Analg* 2001; 92:1563-8
45. Geranton MG, Fratto V, Tochiki KK, Hunt SP: Descending serotonergic controls regulate inflammation-induced mechanical hypersensitivity and methyl-CpG-binding protein 2 phosphorylation in the rat superficial dorsal horn. *Mol Pain* 2008; 4:35
46. Forbes IT, Douglas S, Gribble AD, Ife RJ, Lightfoot AP,

- Garner AE, Riley GJ, Jeffrey P, Stevens AJ, Stean TO, Thomas DR: SB-656104-A: A novel 5-HT7 receptor antagonist with improved in vivo properties. *Bioorg Med Chem Lett* 2002; 12:3341-4
47. Kristensen JD, Hartvig P, Karlsten R, Gordh T, Halldin M: CSF and plasma pharmacokinetics of the NMDA receptor antagonist CPP after intrathecal, extradural and i.v. administration in anaesthetized pigs. *Br J Anaesth* 1995; 74:193-200
48. Mahé C, Loetscher E, Feuerbach D, Müller W, Seiler MP, Schoeffter P: Differential inverse agonist efficacies of SB-258719, SB-258741 and SB-269970 at human recombinant serotonin 5-HT7 receptors *Eur J Pharmacol* 2004; 495:97-102
49. Brenchat A, Romero L, García M, Pujol M, Burgueño J, Torrens A, Hamon M, Baeyens JM, Buschmann H, Zamanillo D, Vela JM: 5-HT7 receptor activation inhibits mechanical hypersensitivity secondary to capsaicin sensitization in mice. *Pain* 2009; 141:239-47
50. Taylor BK, Basbaum AI: Systemic morphine-induced release of serotonin in the rostroventral medulla is not mimicked by morphine microinjection into the periaqueductal gray. *J Neurochem* 2003; 86:1129-41
51. Driessen B, Reimann W: Interaction of the central analgesic, tramadol, with the uptake and release of 5-hydroxytryptamine in the rat brain in vitro. *Br J Pharmacol* 1992; 105:147-51
52. Smith RA, Levine TR, Lachlan KA, Fediuk TA: The high cost of complexity in experimental design and data analysis: Type I and type II error rates in multiway ANOVA. *Hum Commun Res* 2002; 28:515-30

ANESTHESIOLOGY REFLECTIONS

The Red Barn by Vandam



Sometime after William T.G. Morton (1819–1868) turned 8 yr of age, his family's farmhouse burned down all the way to its stone foundation. His parents responded by moving the family in 1827 to old Charlton Center in Worcester County, Massachusetts. Where Cemetery Road crossed Stafford Street, Morton's father began selling supplies to farmers. Eventually a red barn was constructed there at that intersection. Years later *The Red Barn* and its tenuous connection to celebrated etherizer W.T.G. Morton would be memorialized (see above), by a retired Editor of ANESTHESIOLOGY, artist-anesthesiologist Leroy D. Vandam (1914–2004). Dr. Vandam autographed 100 prints of this watercolor to benefit the Wood Library-Museum. (Copyright © the American Society of Anesthesiologists, Inc. This image appears in color in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

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