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Interaction between midazolam and serotonin in spinally mediated antinociception in rats

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Abstract
Purpose. Intrathecal administration of serotonin (5-HT) is antinociceptive through the involvement of spinal cord γ-aminobutyric acid (GABA) receptors. Therefore, 5-HT would interact with the GABA agonist, midazolam, which is well known to exert spinally mediated antinociception in the spinal cord. The present study investigated the antinociceptive interaction between spinally administered 5-HT and midazolam, using two different rat nociceptive models.
Methods. Sprague-Dawley rats with lumbar intrathecal catheters were tested for their thermal tail withdrawal response and paw flinches induced by formalin injection after the intrathecal administration of midazolam or 5-HT, or the midazolam/HT combination. The effects of the combination were tested by isobolographic analysis, using the combination of each 1, 1/2, 1/4, 1/8, and 1/16 of the 50% effective dose (ED50). The total fractional dose was calculated. Behavioral side effects were also examined.
Results. 5-HT alone and midazolam alone both showed dose-dependent antinociception in both the tail flick test and the formalin test. The ED50 of the combination was not different from the calculated additive value either in the tail flick test or in phase 2 of the formalin test, but it was significantly smaller than the calculated additive value in phase 1 of the formalin test. The total fractional dose value was 0.90 in the tail flick test, 0.093 in phase 1 of the formalin test, and 1.38 in phase 2 of the formalin test. The agitation, allodynia, or motor disturbance observed with either agent alone was not seen with the combination treatment.
Conclusion. The antinociceptive effects of intrathecal midazolam and 5-HT were additive on thermal acute and inflammatory facilitated stimuli, and synergistic on inflammatory acute stimulation.

Key words Midazolam · Serotonin · Spinal cord · Antinociception · Interaction

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Introduction

Midazolam is well known to have antinociceptive effects exerted through the benzodiazepine-γ-aminobutyric acid $(GABA)_A$ receptor complex in the spinal cord [1]. We have already shown that intrathecally administered midazolam exerts dose-dependent antinociception in both the tail flick test and the formalin test in rats [2].

Serotonin (5-HT) is also an important neurotransmitter that mediates nociceptive transmission. 5-HT can produce either excitation or inhibition of single motoneurons [3], as well as the enhancement or suppression of motor behavior. Depending on the type of stimulation, the dose, and the route of administration, 5-HT may either inhibit [4–6] or facilitate [4,7,8] nociceptive transmission.

Intrathecally administered 5-HT induced its antinociceptive effects on electrical stimuli by the involvement of spinal cord GABA and μ opioid receptors, but no such interactions occurred for the tail flick latency [9,10]. Therefore, 5-HT may have different interactions with GABA agonists depending on the type of stimuli; it seems that the interactions could be synergistic or additive for electrical stimuli, but there may be no interaction for thermal stimuli. There is no report of an interaction between 5-HT and GABA agonists in inflammatory-induced nociception. The present study was performed to investigate the interaction between intrathecally administered 5-HT and midazolam in thermal-induced and inflammatory-induced nociception.

Materials and methods

After obtaining the approval of the Research Committee of the University of Tokyo, we implanted male Sprague-Dawley rats (280–300 g; Nippon Bio-Supply, Tokyo, Japan) with lumbar intrathecal catheters under

halothane (2%) in 100% oxygen inhalation. An 8.5-cm polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ, USA) was inserted caudally to the thoracolumbar level in the intrathecal space through the atlanto-occipital membrane. The rostral part of the catheter was plugged with a 28-gauge (G) steel wire and put through to the top of the skull. Only rats with normal motor function and behavior 7 days later were used. After the experiment, the location of the catheter was confirmed anatomically and the data of the rats with mal-location of the catheter were excluded. In each dose group, eight rats were used after the exclusion. For the midazolam group, we had already studied eight rats in the tail-flick test and eight in the formalin test [2]; therefore, to save animals in the present study, we added four rats for each test and deleted the older data from each test in our previous study [2]. Thus, half of the data in the midazolam group were the same as the data in our previous study [2]. Each rat was used only once.

Midazolam (Sigma, St. Louis, MO, USA) and 5-HT (Sigma) were dissolved in normal saline to make a solution of 1, 3, 10, 30, or 100 μg (midazolam), or a solution of 10, 30, 100, or 300 μg (5-HT) in 10 μL. For the interaction study, the combination of each of 1, 1/2, 1/4, 1/8, or 1/16 of the 50% effective dose (ED50) was adjusted to make a 10-μL solution. Normal saline was used as a control. After injection of the drug, the catheter was flushed with 10 μL (8 ± 0.7 μL; mean ± SD) normal saline to clear the dead space of the catheter.

The tail-flick test was performed with the Tail-Flick Analgesia Meter (MK-330A; Muromachi Kikai, Tokyo, Japan). Rats were placed in a clear plastic cage with their tails extending through a slot located at the rear of the cage. Thermal stimulation was given by a beam of high-intensity light focused on the tail 2 to 3 cm proximal to the end. The time between the start of the stimulation and the tail withdrawal response was measured as the tail-flick latency. The intensity of the light beam was adjusted to induce a tail-flick latency of about 3 s in a normal rat. The cutoff time in the absence of a response was set at 14 s to prevent tissue injury of the tail. The test was done at 5, 10, 15, 30, 60, 90, 120, 180, and 240 min after drug injection. Data values are shown as the percentage of the maximum possible effect (% MPE):

$$\%\ \text{MPE} = (\text{postdrug latency} - \text{predrug latency}) \times 100/(\text{cutoff time} - \text{predrug latency}).$$

The formalin test was performed 10 min after intrathecal drug injection. Fifty microliters of 5% formalin was injected subcutaneously into the dorsal surface of the right hind paw with a 30-G needle. Immediately after the injection, the rat was placed in an open clear plastic chamber and its flinching or paw-shaking response was observed for 60 min. The number of flinches was counted every 5 min, for 1 min. Usually two phases were observed: phase 1, from 0 to 6 min after the formalin injection; and phase 2, beginning about 10 min after the injection, with an interval of no flinches between the two phases.

In the tail-flick test, side effects were also examined, and were judged as present (abnormal) or absent (normal). Agitation was judged as spontaneous irritable movement, vocalization, or both. Allodynia-like behavior was judged as escape, vocalization, or both, induced by lightly stroking the flank of the rat with a small probe. The placing or stepping reflex was evoked by drawing the dorsum of either hind paw across the edge of the table. Normal rats try to put the paw ahead into a position to walk. The righting reflex was assessed by placing the rat horizontally with its back on the table. Normally rats twist the body to an upright position immediately. Flaccidity was judged as muscle weakness by placing the forepaw 3 to 5 cm higher than the hind paw. Normal rats will walk up. Pinna or corneal reflexes were examined with a paper string. When a string is put into the ear canal or touches the cornea, rats normally shake their heads.

First, both the tail-flick test and the formalin test were performed to determine the dose-dependency of the antinociceptive effects and to obtain the ED50 values of intrathecal midazolam and 5-HT. As mentioned above, half of the midazolam data were derived from our previous data [2]. The ED50 was obtained using the maximum effects in the tail-flick test and the area under the curve of the number of flinches in the formalin test. Secondly, to investigate the interaction between midazolam and 5-HT, an isobolographic analysis was performed, with the method based on that of Tallarida et al. [11]. The combinations of each ED50, 1/2 ED50, 1/4 ED50, 1/8 ED50, or 1/16 ED50 adjusted to 10 μL were administered to perform the tail-flick test and the formalin test, and the ED50 of the combination in each test was determined. The total fractional dose value was calculated as follows:

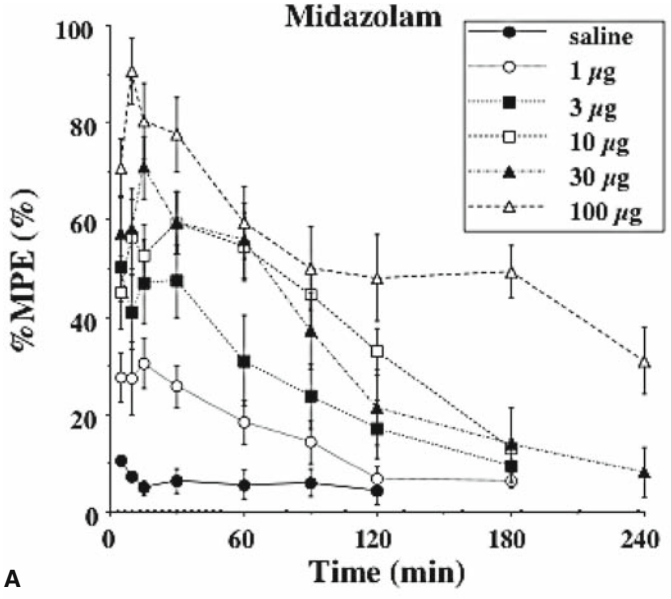
$$(\text{ED50 dose of midazolam in combination})/(\text{ED50 dose of midazolam alone}) + (\text{ED50 dose of 5-HT in combination})/(\text{ED50 dose of 5-HT alone}).$$

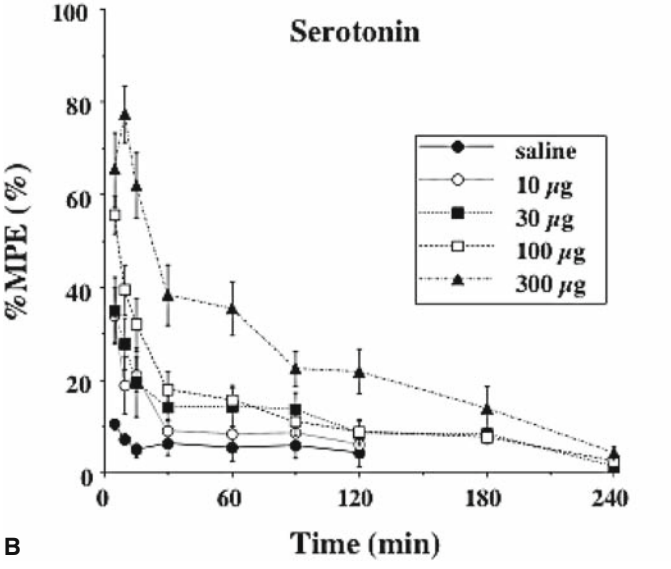
The value was normalized by assigning the ED50 value of each drug given alone as 1. Values near 1 suggest an additive interaction, values of more than 1 imply an antagonistic interaction, and values of less than 1 indicate a synergistic interaction. The data values are shown as means ± SD or 95% confidence intervals (CIs). Statistical analysis was performed with Student's *t*-test to compare the calculated ED50 values with the theoretical additive values. A P value of less than 0.05 was considered to be statistically significant.

Results

Intrathecally administered midazolam alone and 5-HT alone both produced dose-dependent increases in the tail-flick latency (Fig. 1). The control tail flick latency (before treatment) was 3.0 ± 0.4 s. The combination of midazolam and 5-HT also induced dose-dependent increases in the tail-flick latency (Fig. 1). The ED50 values of the combination were not different from the calculated additive values (P = 0.52; Fig. 2, Table 1). The total fractional dose value was 0.90.

Midazolam alone and 5-HT alone, and their combination, produced dose-dependent decreases in the numbers of flinches in both phase 1 and phase 2 of the formalin test (Fig. 3). The ED50 values of the combination in phase 1 of the formalin test were significantly lower than the calculated additive values (P < 0.01; Fig. 4A, Table 1). The ED50 values of the combination in phase 2 of the formalin test were not different from the calculated additive values (P = 0.61; Fig. 4B, Table 1). The total fractional dose value was 0.093 in phase 1 of the formalin test and 1.38 in phase 2.





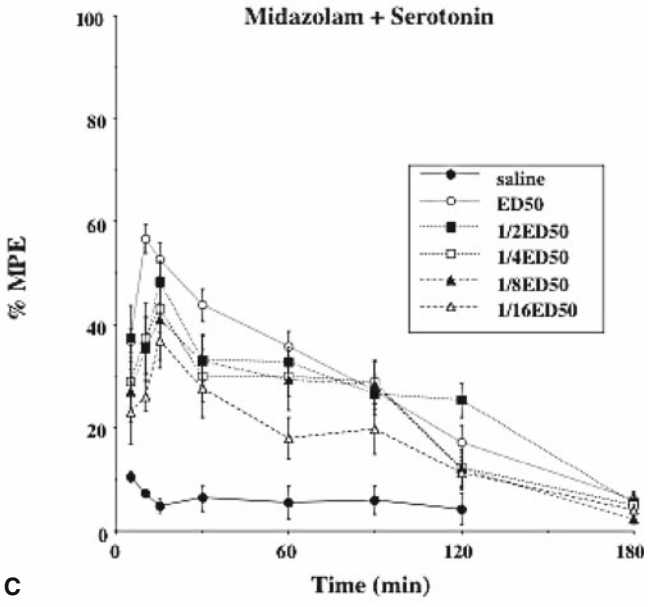


Fig. 1A–C. Time response curves for analgesia induced by midazolam (**A**), serotonin (5-HT; **B**) and their combination (**C**) in the tail-flick test. Values are means, and *bars* indicate SD (n = 8). *MPE (%)*, percentage of the maximum possible effect; 50% effective dose (*ED50*), midazolam 1.6 μg + serotonin 34.4 μg; *1/2 ED50*, midazolam 0.8 μg + serotonin 17.2 μg; *1/4 ED50*, midazolam 0.4 μg + serotonin 8.6 μg; *1/8 ED50*, midazolam 0.2 μg + serotonin 4.3 μg; *1/16 ED50*, midazolam 0.1 μg + serotonin 2.15 μg

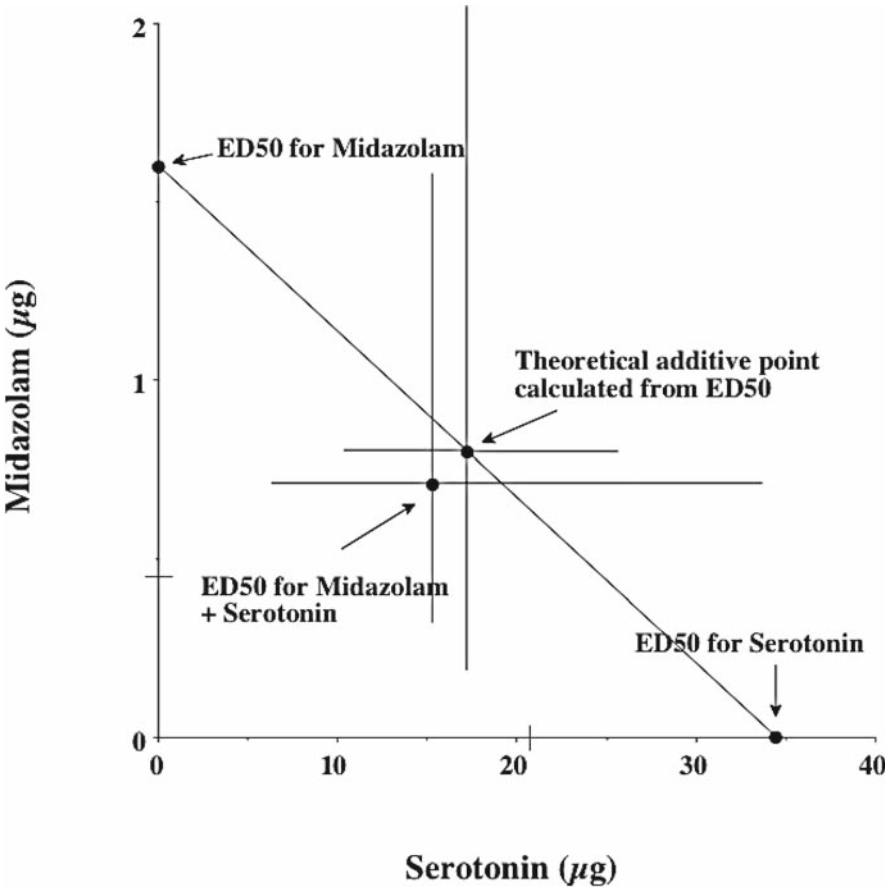


Fig. 2. Isobolograph for the interaction of midazolam and serotonin (5-HT) in the tail-flick test. *Bars* indicate 95% confidence intervals. The *x* and *y* axes show the doses (μg) of midazolam and serotonin, respectively

Table 1. Fifty percent effective dose values (μg)

	Tail-flick test	Formalin test phase 1	Formalin test phase 2
Midazolam	1.57 (0.42–5.11)	1.30 (0.32–3.24)	1.22 (0.33–4.20)
Serotonin (5-HT)	34.4 (21.3–55.6)	12.6 (5.5–31.0)	1.3 (0.04–8.2)
Midazolam in combination	0.71 (0.32–1.61)	0.11 (0.03–0.41)	0.84 (0.05–13.7)
Serotonin (5-HT) in combination	15.3 (6.8–34.7)	0.12 (0.03–0.44)	0.89 (0.05–14.6)

Figures in parentheses are 95% confidence intervals

The combination of midazolam and 5-HT did not show any observable side effects, whereas each agent alone induced agitation, allodynia, and motor disturbance (Table 2).

Discussion

The present study showed that midazolam and 5-HT had additive antinociceptive effects on thermal-induced acute nociception and formalin-induced stimuli. Both agents had synergistic effects on formalin-induced acute nociception. The side effects of the combination were decreased compared to findings for each single agent alone.

The tail-flick response represents acute thermal nociception that is mediated by primary afferent fibers and, in part, by a spinal reflex [12]. Formalin directly activates peripheral nociceptors on primary afferent fibers, producing an acute barrage of activity into the dorsal horn, which constitutes the first phase of the response [13]. Therefore, the present results suggest that midazolam and 5-HT exert their additive or synergistic interaction in primary afferent fibers. The difference between the additive antinociceptive effects on thermal-induced acute nociception and the synergistic effects on formalin-induced acute nociception may be due to the different stimuli; i.e., thermal and inflammatory.

The GABAergic system plays an important role in the presynaptic inhibition of primary afferents. $GABA_A$ receptor agonists inhibit the excitatory effects of glutamate [14]. The $5\text{-}HT_3$ receptor selective agonist, 2-methyl 5-HT, administered intrathecally, inhibited behaviors elicited by intrathecally administered *N*-

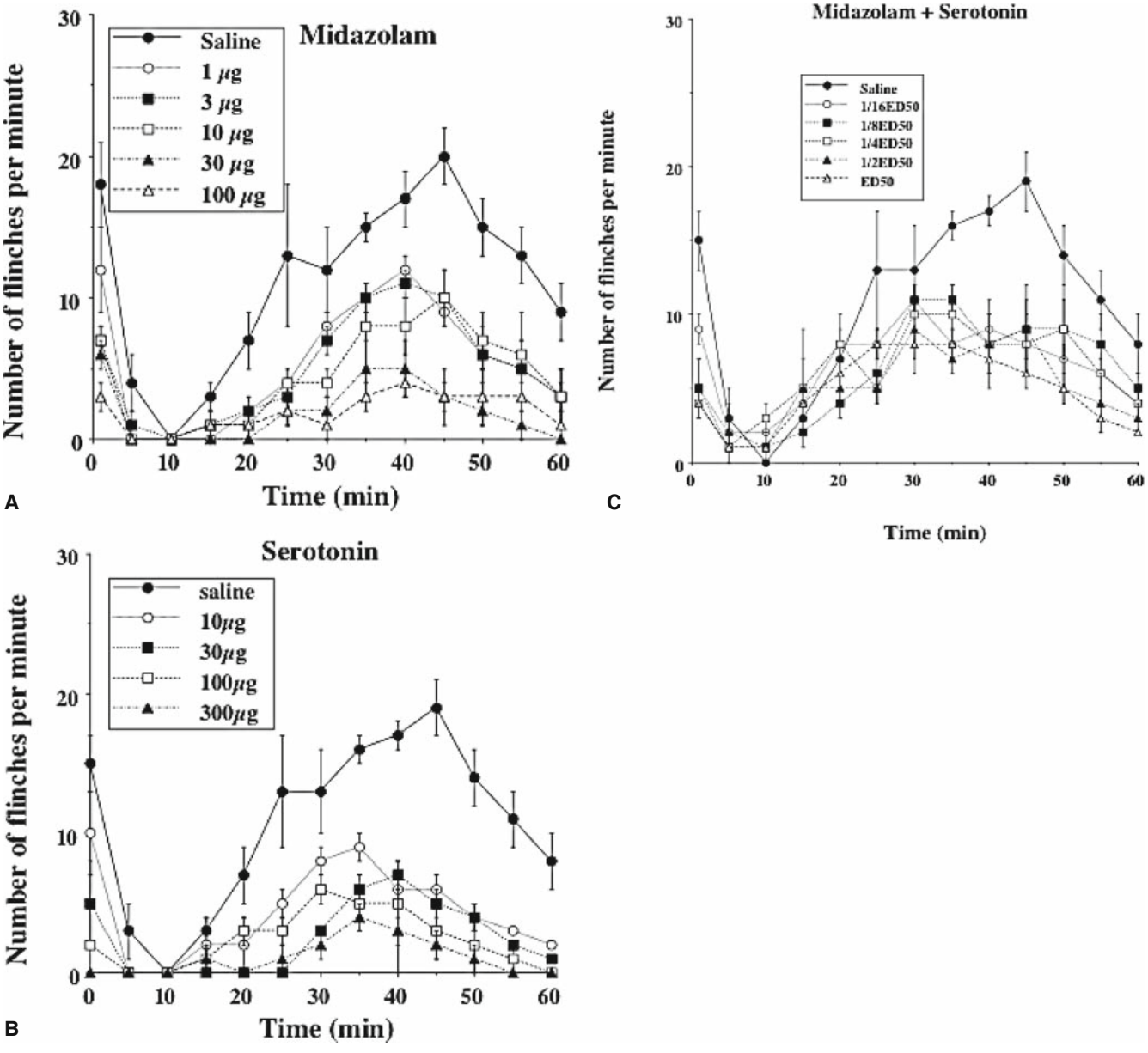
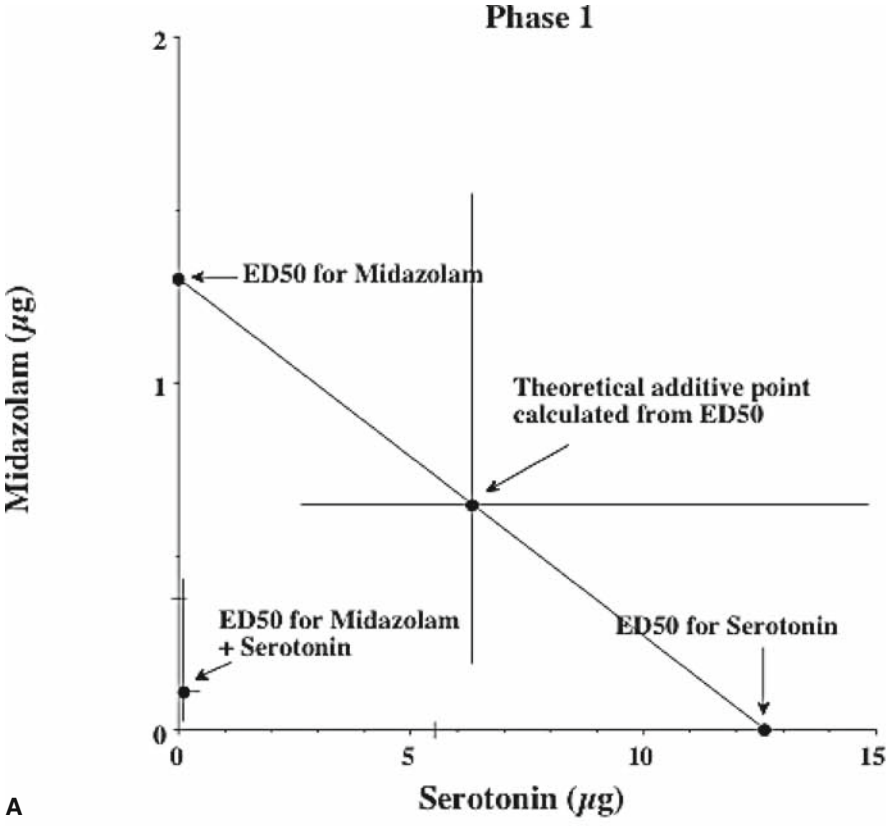


Fig. 3A–C. Time response curves for analgesia induced by midazolam (**A**), serotonin (5-HT; **B**), and their combination (**C**) in the formalin test. Values are means, and *bars* indicate SD (n = 8). *ED50*, midazolam 1.2 μg + serotonin 1.3 μg; *1/2 ED50*, midazolam 0.6 μg + serotonin 0.65 μg; *1/4 ED50*, midazolam 0.3 μg + serotonin 0.325 μg; *1/8 ED50*, midazolam 0.15 μg + serotonin 0.1625 μg; *1/16 ED50*, midazolam 0.075 μg + serotonin 0.08125 μg

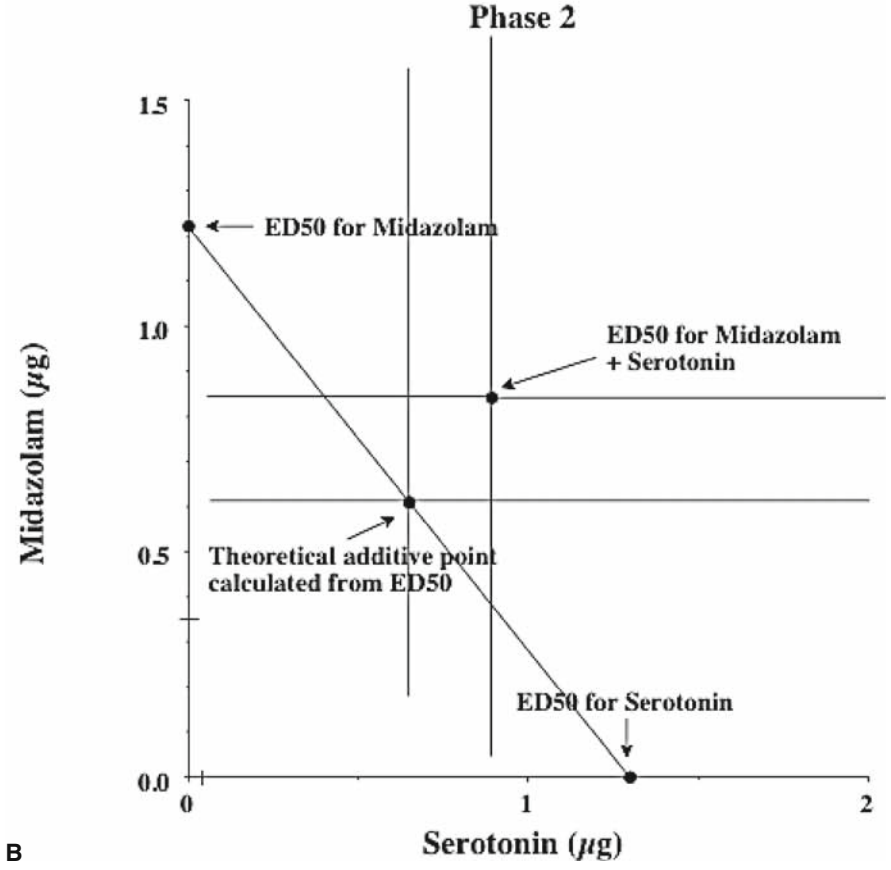
Table 2. Side effects for midazolam alone, serotonin (5-HT) alone, and their combination

		Midazolam (μg)					Serotonin (5-HT; μg)				Midazolam + Serotonin (5-HT; fraction of ED50)				
Dose	Saline	1	3	10	30	100	10	30	100	300	1	1/2	1/4	1/8	1/16
Agitation	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0
Allodynia	0	0	1	1	1	1	0	0	1	0	0	0	0	0	0
Loss of pinna reflex	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Flaccidity	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
Disturbance of righting reflex	0	0	0	0	2	6	0	0	0	0	0	0	0	0	0
Disturbance of placing and stepping	0	0	0	0	1	4	0	0	0	0	0	0	0	0	0

The number of rats showing each side effect is indicated. The total number of animals tested was eight for each dose
ED50, 50% effective dose



A



B

Fig. 4. Isobolograph for the interaction of midazolam and serotonin (5-HT) in phase 1 (**A**) and phase 2 (**B**) of the formalin test. *Bars* indicate 95% confidence intervals. The *x* and *y* axes show the doses (μg) of midazolam and serotonin, respectively

methyl-D-aspartate (NMDA) [15]. Therefore, both midazolam and 5-HT may inhibit neuronal excitation by glutamate, and this would contribute to their interaction.

There are many subtypes of 5-HT receptors. The major class of 5-HT receptors in the spinal cord is 5-HT_1 receptors, and the intrathecal injection of a 5-HT_{1A} receptor agonist strengthened hyperalgesia [16]. Other 5-HT_1 receptors are reported to be involved in spinally mediated antinociception [17]. Thus, the additive or synergistic interaction between midazolam and 5-HT may be due to their action on 5-HT_1 receptors other than 5-HT_{1A} receptors. 5-HT may exert its antinociceptive effects by causing the release of GABA, which inhibits nociceptive impulses by an effect on postsynaptic membranes in the dorsal horn [15]. The release of 5-HT is physiologically regulated by distinct subtypes of GABA receptors at presynaptic and postsynaptic sites [18]. 2-Methyl 5-HT caused antinociceptive effects that involved spinal opioid and GABA receptor systems [19]. Therefore, in the present study, it is possible that the administered 5-HT may have activated the intrinsic GABA system or opioid receptors to induce the additive or synergistic antinociception shown with midazolam.

5-HT receptors and GABA receptors in the brain interact in the modulation of emotional behavior [20] and midazolam has been shown to increase central serotonergic activity and provoke retrograde amnesia [21]. However, there are no reports of any antinociceptive interaction between 5-HT receptors and GABA receptors in the brain. We cannot deny that, in the present study, the interaction between midazolam and 5-HT may have happened in the brain; however, this seems unlikely, because the combination showed no centrally mediated side effects.

Clinically, neither midazolam nor 5-HT is available for intrathecal use. However, from the results of the present study, and when it has been confirmed that there are no side effects (such as neurotoxicity) of intrathecal midazolam and 5-HT, it is possible that the combination of these two agents may be useful for analgesia.

In conclusion, the antinociceptive effects of intrathecal midazolam and 5-HT were additive on thermal acute and inflammatory-facilitated stimuli, and synergistic on inflammatory acute stimulation.

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