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Analgesic Interaction between Intrathecal Midazolam and Glutamate Receptor Antagonists on Thermal-induced Pain in Rats

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Background: **Two major neurotransmitters, γ-aminobutyric acid (GABA) and the excitatory amino acid, glutamate, may be involved in nociception in the spinal cord. GABA and glutamate receptors may operate in concert to modify signals in the central nervous system. The purpose of this study was to investigate the spinal analgesic interaction between midazolam, a benzodiazepine–$GABA_A$ receptor agonist, and two glutamate receptor antagonists on acute thermal nociception.**

Methods: **Sprague-Dawley rats were implanted with chronic lumbar intrathecal catheters and were tested for their tail withdrawal response by the tail flick test after intrathecal administration of saline, midazolam (1–100 μg), AP-5 (1–30 μg), or YM872 (0.3–30 μg). AP-5 is an *N*-methyl-D-aspartate (NMDA) receptor antagonist and YM872 is an α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist. The combination of midazolam and the other two agents were also tested by isobolographic analyses. Motor disturbance and behavioral changes were observed.**

Results: **Dose-dependent increases in the tail flick latency were observed with midazolam, AP-5, and YM872 with 50% effective dose values of 1.57 ± 0.34 (SEM) μg, 5.54 ± 0.19 μg, and 1.0 ± 0.22 μg, respectively. A potent synergy in analgesia with decreased behavioral changes and motor disturbance was obtained when combining midazolam with AP-5 or YM872.**

Conclusions: **Spinally administered midazolam and an NMDA- or an AMPA-receptor antagonist exhibited potent synergistic analgesia on acute thermal nociception in rats. Side effects, shown by behavioral changes and motor disturbance, decreased with the combination of the agents. These results point out an important direction for the study of acute nociception. (Key words: α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid; analgesia; midazolam; *N*-methyl-D-aspartate; spinal cord.)**

TWO major neurotransmitters, γ-aminobutyric acid (GABA)[1] and the excitatory amino acid, glutamate, may be involved in nociception in the spinal cord. GABA is found in high concentration in the spinal cord.[2] Specific benzodiazepine receptors are associated with dorsal-horn systems in the spinal cord that encode pain related information.[3] Benzodiazepine receptor agonists appear to increase the intrinsic efficacy of GABA at the $GABA_A$ receptor coupling with benzodiazepine receptor by increasing the chloride conductance for a given GABA-ergic stimulus.[4] Midazolam, a benzodiazepine derivative, depresses spinal nociceptive neurotransmission, as measured by changes in the nociception related slow ventral root potential.[5] It is also reported that midazolam has spinally mediated analgesic effects in behavioral studies.[3,6]

On the other hand, glutamates, excitatory amino acids, exist in primary afferents and interneurons.[7] Ionotropic glutamate receptors in the spinal cord are well known to mediate nociception. They may be mainly classified into two classes: the *N*-methyl-D-aspartate (NMDA) receptors and the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. NMDA receptor antagonists block the facilitated states of pain processing but have little effect on acute nociception.[8] In contrast, the AMPA receptor antagonists have analgesic effects on acute nociception.[9,10]

GABA and glutamate receptors may operate in concert

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to regulate nociceptive signals in various regions of the brain.[11,12] Because there were few reports of the relation of these two receptor systems in the spinal cord, we investigated the spinally mediated analgesic interaction of these two receptor systems on acute nociception using an intrathecally catheterized rat model.

Materials and Methods

Animal Preparations

The protocol was approved by the Research and Education Institute of Harbor-UCLA Medical Center. Sprague-Dawley rats (280–300 g; B. K, Universal, Fremont, CA) were implanted with chronic lumbar intrathecal catheters under halothane (2%) anesthesia according to the method described by Yaksh and Rudy.[13] Briefly, an 8.5-cm polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ) was advanced caudally through an incision in the atlantooccipital membrane, to the thoracolumbar level of the spinal cord. The external part of the catheter was tunneled subcutaneously to exit on the top of the skull and plugged with a 28-gauge stainless steel wire. Only rats with normal motor function and behavior 5 days after surgery were used.

Drugs and Administration

Midazolam (a benzodiazepine-$GABA_A$ receptor agonist; Sigma, St. Louis, MO) 1, 3, 10, 30, and 100 μg, and AP-5 (2-amino-5-phosphonovaleic acid, an NMDA receptor antagonist; Sigma) 1, 3, 10, and 30 μg were dissolved in saline 10 μl. YM872 ([2,3-Dioxo-7-(1H-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinoxalinyl] acetic acid), an AMPA receptor antagonist (Yamanouchi Pharmaceutical, Tsukuba, Japan) 10 mg was dissolved in 0.97 ml distilled water with 30 μl 1 N NaOH to adjust *p*H to 7.3–7.5. Solutions of 0.3 (0.86), 1 (2.86), 3 (8.59), 10 (28.63), and 30 (85.89) μg (nM) per 10 μl were made using normal saline. After intrathecal drug injection, the catheter was flushed with a subsequent injection of 10 μl of normal saline to clear the dead space of the catheter (7 ± 0.4 μl, mean ± SE). Microinjector syringes were used for all injections. In each dose group, eight randomly selected rats were used. Normal saline 10 μl was injected in the control group.

Nociceptive Test: Tail Flick Test

Each rat was placed in a clear plastic cylindrical cage with its tail extended through a slot provided in the rear of the tube. Noxious stimulation was provided by a beam of high-intensity light (Tail-flick Analgesia Meter 0570-001L, Columbus Instruments International, Columbus, OH) focused on the tail 2–3 cm proximal to the end. The response time was measured and defined as the interval between the onset of the thermal stimulation and the abrupt flick of the tail. The cutoff time in the absence of a response was set to 14 s to prevent tissue injury.

Behavioral and Motor Function Test

The general behavior (including agitation and allodynia), motor function, pinna reflex, and corneal reflex were examined. Their presence or absence was recorded. Agitation was judged to be present when the rat spontaneously vocalized or became restless. The presence of allodynia was examined by looking for agitation (escape or vocalization) evoked by lightly stroking the flank with a pencil. The stimulus was sufficient to move hair but not dent the skin. Motor function was evaluated by the placing/stepping reflex and by the righting reflex. The former was evoked by drawing the dorsum of either hind paw across the edge of the table. The latter was assessed by placing the rat horizontally with its back on the table, which normally gives rise to an immediate, coordinated turning of the body back to an upright position. Flaccidity was judged as a muscle weakness. Pinna and corneal reflexes were examined with a paper string.

Experimental Paradigm

The first series of experiments was performed to determine the dose dependency and time course of the analgesic actions of intrathecally administered midazolam, AP-5, and YM872 on acute thermal nociception. The tail flick test, behavioral test, and motor function test were performed before and 5, 10, 15, 30, 60, 90, 120 min after drug injection and at 1-h intervals until the response time returned to baseline (maximum 360 min).

To investigate the interaction between midazolam and AP-5 or YM872, an isobolographic analysis was used.[14] The method is based on comparisons of dose ratios that are determined to be equieffective. First, the respective 50% effective dose (ED_{50}) values are determined from the dose-response curves of the agents alone. Subsequently, a dose-response curve is obtained by coadministration of the two drugs in a constant dose ratio based on the ED_{50} values of the single agents. From the dose-response curve of the combined drugs, the ED_{50} value of the mixture was calculated.

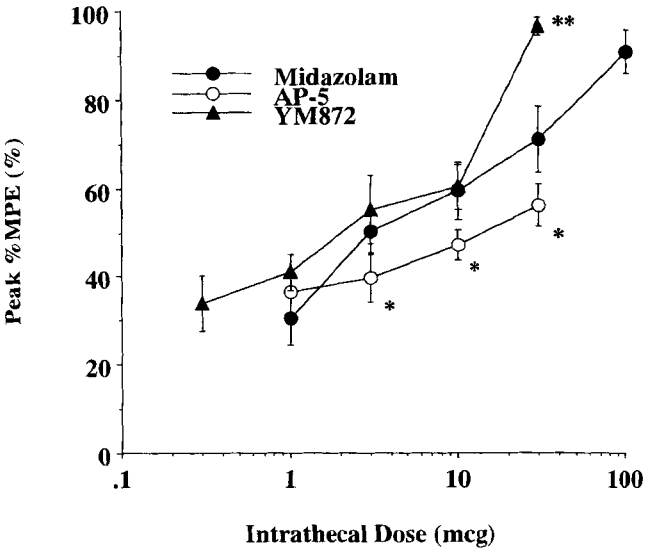

Fig. 1. Dose–response curves of intrathecal midazolam, AP-5 (NMDA antagonist), and YM872 (AMPA antagonist) on the tail flick latency expressed as the peak percentage maximum possible effect. Each point presents the mean ± SEM of eight animals. *$P < 0.05$ *versus* the other two agents, **$P < 0.01$ *versus* the other two agents.

Data Analysis and Statistics

Data were expressed as mean ± standard error (SEM). Tail flick response latency was converted to percentage maximum possible effect (%MPE) according to the formula: %MPE = [(postdrug latency − baseline latency)/(cutoff time − baseline latency)] × 100. ED_{50} was calculated by a computer program, which was created in the laboratory of University of California, San Diego, according to Tallarida and Murray,[15], as the dose that produces a value of 50% MPE.

To describe the magnitude of interaction between the agents, a total fractional dose value was calculated as follows: [(ED_{50} dose of drug 1 in combination)/(ED_{50} value for drug 1 alone)] + [(ED_{50} dose of drug 2 in combination)/(ED_{50} value for drug 2 alone)]. The values were normalized by assigning the ED_{50} values of the agents given alone a value of 1. Values near 1 indicate an additive interaction, values greater than 1 imply an antagonistic interaction; values less than 1 indicate a synergistic interaction. To compare the theoretic additive point with experimentally derived ED_{50}, isobolographic analysis[14] was used.

Differences between doses were analyzed with two-way analysis of variance followed by the Newman-Keuls test. Student *t* test was used to compare the calculated ED50 values with the theoretic additive values. A *P* value less than 0.05 was considered statistically significant.

Results

Analgesic Effects of Midazolam, AP-5, and YM872

The baseline latency (before drug injection) in the tail flick test was 3.0 ± 0.2 s (mean ± SE). Intrathecal administration of midazolam, AP-5, and YM872 resulted in dose-dependent increases in the tail flick latency (fig. 1). The ED_{50} values were 1.57 ± 0.34 μg, 5.54 ± 0.19 μg, and 1.0 ± 0.22 μg with midazolam, AP-5, and YM872, respectively.

Interaction between Midazolam and NMDA Antagonist

Coadministration of midazolam and AP-5 intrathecally shifted a dose response curve to the left (fig. 2) and showed a significant increase in the thermal escape latency compared with the agents alone by an isobolographic analysis (fig. 3). The experimentally obtained ED_{50} of the combination of midazolam and AP-5 was midazolam 0.21 ± 0.18 μg and AP-5 0.75 ± 0.18 μg. These doses were significantly lower than the theoretic additive doses (midazolam 0.79 ± 0.38 μg and AP-5 2.77 ± 0.24 μg). The total fractional dose value of the combination was calculated to be 0.27 ± 0.11, which indicates a synergistic interaction.

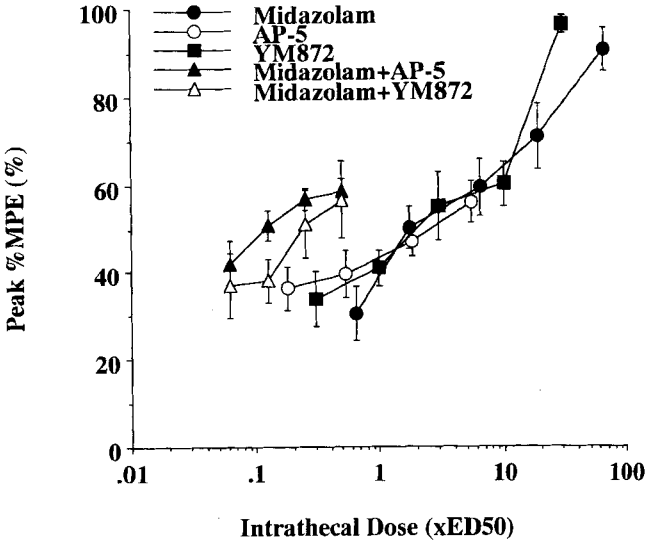

Fig. 2. Comparison of the dose–response curves of intrathecal midazolam, AP-5 (NMDA antagonist), YM872 (AMPA antagonist), midazolam plus AP-5, and midazolam plus YM872 on the tail flick latency expressed as the peak percentage maximum possible effect. Intrathecal dose is indicated as the percentage of ED_{50}. Each point presents the mean ± SEM of eight animals.

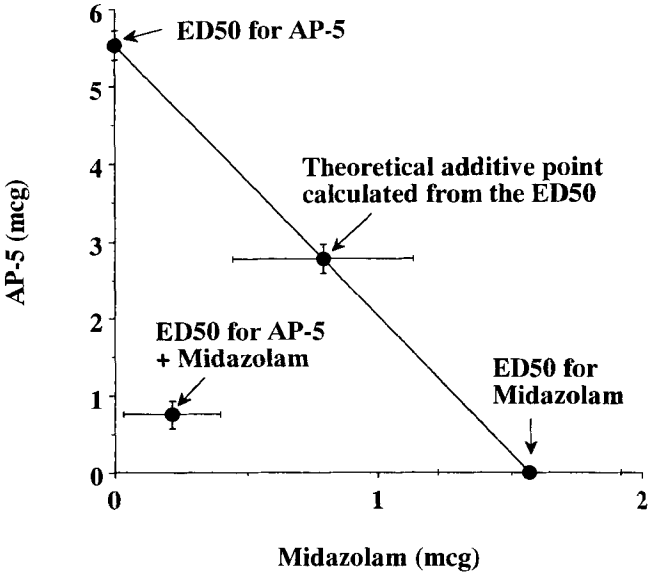

Fig. 3. Isobologram for the intrathecal interaction of midazolam and AP-5. Horizontal and vertical bars indicate SEM. The oblique line between the x-axis and y-axis is the theoretic additive line. The point in the middle of this line is the theoretic additive point calculated from the separate ED_{50} values. The experimental point lies far below the additive line, indicating a significant synergism.

Interaction between Midazolam and AMPA Antagonist

Coadministration of midazolam and YM872 intrathecally shifted a dose-response curve to the left (fig. 2) and showed a significant increase in the thermal escape latency compared to the agents alone (fig. 4). The experimentally obtained ED_{50} of the combination of midazolam and YM872 was midazolam 0.15 ± 0.09 μg and YM872 0.24 ± 0.09 μg. These doses were significantly lower than the theoretic additive doses (midazolam 0.79 ± 0.38 μg and YM872 0.5 ± 0.22 μg). The total fractional dose value of the combination was calculated to be 0.34 ± 0.12, which indicates a synergistic interaction.

Behavior and Motor Function

Midazolam ≥ 3 μg (each one rat in 3, 10, 30, and 100 μg) and AP-5 ≥ 10 μg (one in 10 μg and two in 30 μg) induced agitation and allodynia. Motor disturbances (tested by the placing/stepping reflex and by the righting reflex) occurred with midazolam ≥ 30 μg (two rats in 30 μg and six in 100 μg), AP-5 ≥ 10 μg (two in 10 μg and three in 30 μg) or YM872 ≥ 10 μg (three in 10 μg and four in 30 μg). Flaccidity was seen in the rats with midazolam ≥ 30 μg (one in 30 μg and two in 100 μg), or YM872 ≥ 10 μg (two in 10 μg and six in 30 μg). AP-5 30 μg induced loss of pinna reflex (two rats). In contrast, combination of midazolam and AP-5 induced no observable side effects. Allodynia was seen with midazolam 0.2 μg plus YM872 0.125 μg (one rat), and loss of righting reflex occurred with midazolam 0.8 μg plus YM872 0.5 μg (one rat). The combinations of midazolam and AP-5 or YM872 displayed fewer side effects than the equieffective doses of the individual agents (table 1). No rats showed paralysis in this study.

Discussion

We found that intrathecally administered midazolam (benzodiazepine-$GABA_A$ receptor agonist), AP-5 (NMDA receptor antagonist) and YM872 (AMPA receptor antagonist) produced dose- dependent increases in tail flick latency. Midazolam showed synergistic analgesic effects with both AP-5 and YM872.

In the dorsal horn of the spinal cord, GABA receptors mediate presynaptic inhibition on the primary afferent terminals.[16] At these endings, GABA produces a mild depolarization of the primary afferents and thereby reduces the release of the excitatory transmitter onto the second-order neurons in the spinal cord.[17] Binding sites

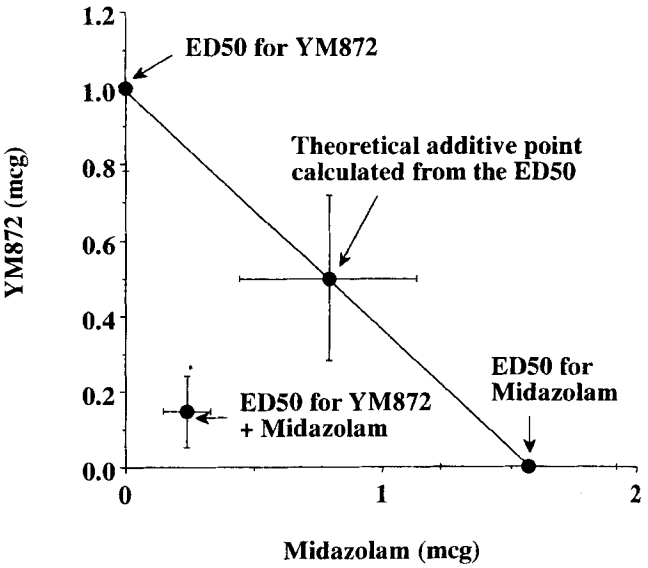

Fig. 4. Isobologram for the intrathecal interaction of midazolam and YM872. Horizontal and vertical bars indicate SEM. The oblique line between the x-axis and y-axis is the theoretic additive line. The point in the middle of this line is the theoretic additive point calculated from the separate ED_{50} values. The experimental point lies far below the additive line, indicating a significant synergism.

Table 1. Side Effects with the Comparable Doses

	Saline (control)	Midazolam 3 μg	AP-5 10 μg	YM872 1 μg	Midazolam 0.2 μg + AP-5 0.69 μg	Midazolam 0.2 μg + YM872 0.125 μg
Agitation	0	0	1	0	0	0
Allodynia	0	1	1	0	0	1
Loss of righting reflex	0	1	2	0	0	0
Loss of placing and stepping reflex	0	0	1	0	0	0
Flaccidity	0	0	0	0	0	0
Loss of corneal reflex	0	0	0	0	0	0
Loss of Pinna reflex	0	0	0	0	0	0

Values are the number of rats that showed each side effect. Total number of rats tested in each group is 8.

for benzodiazepine are in lamina II of the dorsal horn.[18] Radioligand binding assays and electrophysiologic studies showed the linkage of the benzodiazepine sites to the $GABA_A$ receptor complex in the spinal cord.[19,20] Enhancement of presynaptic inhibition might be a possible mechanism for the action of midazolam, because benzodiazepines are known to increase GABA transmission *via* their specific binding site colocated with the $GABA_A$ receptor.[21] Benzodiazepines were reported not to block the transmission of sensory impulses through nerve fibers.[22]

Yanez *et al.*[23] reported that intrathecally administered midazolam 20–60 μg produced dose-dependent antinociception on thermally induced pain and larger doses (60–100 μg) induced motor dysfunction. These published data are similar to those of our current study, in which midazolam 1–100 μg induced dose-dependent analgesia and doses higher than 30 μg induced motor dysfunction. In the study of Bahar *et al.*[6], 75 μg intrathecal midazolam induced sleep. However, 100 μg did not induce sleep in our study. We did not apply higher doses of midazolam because of the limitation of its solubility in saline and because the 30 and 100 μg doses already induced motor dysfunction.

NMDA receptors are involved in the wind-up phenomena of deep dorsal-horn cells evoked by C-fiber activation.[24] NMDA receptor antagonists are therefore the most efficacious against the continuously stimulated state of nociception, induced for example by formalin.[8] Generally, they are inefficacious on acute nociception,[11] although some studies[25,26] have shown analgesic effects on acute thermal stimuli. In the present study, AP-5 (NMDA receptor antagonist) produced dose-dependent analgesic effects on acute thermal stimulus, although the ED_{50} value was relatively high. In a previous study, AP-5 had only weak analgesic effects at the maximum usable dose in the hot-plate test.[27] Considering these results together, NMDA antagonists might have some analgesic effects on acute nociception depending on the experimental settings.

AMPA receptors are found throughout all superficial laminae of the dorsal horn pre- and postsynaptically.[28,29] These receptors are thought to mediate the acute excitation from primary afferent fibers to dorsal horn neurons evoked by high intensity stimuli. Intrathecal application of AMPA receptor antagonists produces dose-dependent antinociception on acute pain in animal models.[27,30] The results of the present study are consistent with these previous studies.[22,30]

No single agent of these classes (benzodiazepines, NMDA, or AMPA receptor antagonists) administered alone is effective enough to block nociception without any adverse effects. One reason is that pain is not mediated by a single receptor or a single neurotransmitter. The other is that the receptors and neurotransmitters mediating pain are also connected to other neuronal networks in the central nervous system that may induce adverse effects. Thus, combination of agents acting through different mechanisms may be one of the best ways to arrive at better analgesic methods.

The present study showed a significant synergistic antinociception between midazolam, a benzodiazepine-$GABA_A$ receptor agonist, and AP-5, an NMDA receptor antagonist, or YM872, a new AMPA receptor antagonist, on acute thermal stimulation. We used only the tail flick test. To confirm the results of the present study, further investigation using other methods is necessary. Aanonsen *et al.*[12] reported that $GABA_A$ receptor agonist inhibited behavioral effects of NMDA, quisqualic acid, and kainic acid. Only in the presence of NMDA, did $GABA_A$ receptor agonist have antinociceptive effect in the tail flick test. GABA produces a mild depolarization of the primary afferents and thereby reduces the release of the excitatory transmitter onto the second-order neurons in the spinal cord.[17] The $GABA_A$ receptor might have some functional coupling with glutamate receptor.

With regard to the side effects, no paralysis was seen in this study. Therefore, we considered tail flick latency not to be affected by motor dysfunction. Midazolam plus AP-5 and midazolam plus YM872 decreased behavioral changes and motor dysfunction and enhanced analgesic effects. These combinations could enhance the therapeutic efficacy of the acute pain treatment and safety. However, one of the important concerns in applying the results to clinical pain management is toxicity of the agents. There are still some controversies surrounding the neurotoxicity of intrathecal midazolam.[31,32] Current formulations of NMDA receptor antagonists are also neurotoxic.[33] AMPA receptor antagonists have poor water solubility and nephrotoxicity.[34] YM872 is a new AMPA receptor antagonist, which is much more water-soluble than the other formulations of AMPA receptor antagonists.[35] YM872 had no neurotoxicity in cat,[36] rat and monkey brains in toxicologic studies (unpublished data). However, there are no studies investigating the toxicity of YM872 on the spinal cord. Therefore, further studies of their toxicity and of new compounds should be performed before applying the results to humans.

In conclusion, intrathecal coadministration of midazolam (a benzodiazepine-$GABA_A$ receptor agonist) with AP-5 (an NMDA receptor antagonist) or midazolam with YM872 (an AMPA receptor antagonist) produced significant synergistic analgesia with decreased side effects on acute thermal nociception measured by tail flick test. These results suggest a functional coupling of benzodiazepine-$GABA_A$ receptors with NMDA and AMPA receptors in acute nociception in the spinal cord.

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