Synergistic analgesic effects of intrathecal midazolam and NMDA or AMPA receptor antagonists in rats

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Purpose: To investigate the interaction of midazolam and N-methyl-D-aspartate (NMDA) receptor or -amino-3-hydroxy-5-methyl isoxazole-4-propionic acid (AMPA) receptor antagonist on the effects of persistent inflammatory nociceptive activation.

Methods: Male Sprague-Dawley rats were implanted with lumbar intrathecal catheters and were tested for their responses to subcutaneous formalin injection into the hindpaw. Saline, midazolam (1 to 100 µg), AP-5 (1 to 30 µg), a NMDA receptor antagonist, or YM872 (0.3 to 30 µg), an AMPA receptor antagonist was injected intrathecally 10 min before formalin injection. The combinations of midazolam and AP-5 or YM872 in a constant dose ratio based on the 50% effective dose (ED₅₀) were also tested and were analysed with an isobologram.

Results: Dose-dependent effects were observed with midazolam (ED₅₀ was 1.34 µg and 1.21 µg in phase 1 and 2 of the formalin test, respectively), AP-5 (7.64 µg and 1.4 µg) and YM872 (0.24 µg and 0.21 µg). Synergistic effects in both phases were obtained when combining midazolam with AP-5 or YM872. The ED₅₀ of midazolam decreased to 0.012 µg (phase 1) and 0.27 µg (phase 2) with AP-5 and to 0.09 µg (phase 1) and 0.35 µg (phase 2) with YM872 ($P < 0.01$).

Conclusions: These results suggest a functional coupling of benzodiazepine—aminobutyric acid (GABA)_A receptor with NMDA and AMPA receptors in acute and persistent inflammatory nociceptive mechanisms in the spinal cord.

Objectif : Examiner l'interaction du midazolam et d'un antagoniste du N-méthyl-D-aspartate (NMDA) ou d'un antagoniste du récepteur de l'acide -amino-3-hydroxy-5-méthylisoxazole-4-propionique (AAMP) sur les effets de l'activation nociceptive de l'inflammation persistante.

Méthode : On a implanté à des rats mâles Sprague-Dawley des cathéters intrathécaux lombaires et on a testé leurs réactions à l'injection sous-cutanée de formoline dans la patte arrière. Une solution salée, du midazolam (1 à 100 µg), de l'AP-5 (1 à 30 µg), un antagoniste du récepteur NMDA ou le YM872 (0,3 à 30 µg), un antagoniste du récepteur AMPA a été administré en injection intrathécale 10 min avant l'injection de formoline. Les combinaisons de midazolam et d'AP-5 ou de YM872 en dose au rapport constant, fondée sur la dose efficace 50 % (ED₅₀) ont été aussi testées et analysées à l'aide d'un isobogramme.

Résultats : Des effets dépendants de la dose ont été observés avec le midazolam (ED₅₀ a été de 1,34 µg et 1,21 µg en phases 1 et 2 du test de formoline, respectivement), avec AP-5 (7,64 µg et 1,4 µg) et avec YM872 (0,24 µg et 0,21 µg). Les effets synergiques des deux phases ont été obtenus en combinant le midazolam et AP-5 ou YM872. La ED₅₀ du midazolam a baissé à 0,012 µg (phase 1) et à 0,27 µg (phase 2) avec AP-5 et à 0,09 µg (phase 1) et à 0,35 µg (phase 2) avec YM872 ($P < 0,01$).

Conclusion : Ces résultats suggèrent un couplage fonctionnel des récepteurs NMDA ou AMPA avec les récepteurs benzodiazépine-acide -aminobutyrique (GABA)_A comme partie des mécanismes nociceptifs de l'inflammation aiguë et persistante dans la moelle épinière.

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IN the spinal cord, both the excitatory neurotransmitter, glutamate¹ and the inhibitory neurotransmitter, γ -aminobutyric acid (GABA)² are involved in nociceptive mechanisms. The N-methyl-D-aspartate (NMDA) receptors are thought to mediate persistent nociceptive activation,¹ while the γ -amino-3-hydroxy-5-methyl isoxazole-4-propionic acid (AMPA) receptors are considered to have a role in acute nociception.³ The AMPA receptors also mediate facilitated state of pain processing.⁴ Both GABA_A and GABA_B receptors also have roles in spinally mediated nociception.⁵ The benzodiazepine binding site is present on the GABA_A receptor (Cl⁻ channel) in the spinal cord⁶ and benzodiazepines have spinally mediated antinociceptive effects.⁷

The relations between different receptors involved in spinal nociceptive mechanisms are studied primarily with opioid receptors. Morphine, a μ -opioid receptor agonist, possesses a synergistic antinociceptive interaction with α_2 adrenergic agonist,⁸ midazolam,⁹ or AMPA receptor antagonist.³ However, it did not show synergy with NMDA receptor antagonist or NMDA glycine site antagonist.³ The GABA and glutamate receptors may operate in concert to regulate nociceptive signals.¹⁰ There are few studies that address the relation between GABA receptors and glutamate receptors in spinal nociceptive mechanisms. We have reported that midazolam, a benzodiazepine-GABA_A receptor complex agonist, exhibited synergistic analgesia for thermally induced acute nociception both with NMDA and AMPA receptor antagonists.¹¹ However, there are no reports of the interaction between these receptors in other types of nociceptive mechanisms. The purpose of the present study was to investigate the relationships of the benzodiazepine-GABA_A receptor complex and the NMDA receptor or the AMPA receptor in the mechanisms of persistent inflammatory nociceptive activation in the formalin test of intrathecally catheterized rat model.

Materials and methods

Animal preparations

The protocol was approved by the Research and Education Institute of Harbor - UCLA Medical Center. Male Sprague-Dawley rats (280–300 g; BK Universal, Fremont, CA, USA) were implanted with chronic lumbar intrathecal catheters under halothane (2%) anesthesia according to the previously described method.¹² Briefly, an 8.5-cm polyethylene (PE-10; Clay Adams, Parsippany, NJ, USA) catheter was advanced caudally through an incision in the atlanto-occipital membrane, to the thoracolumbar level of the spinal cord. The external part of the catheter was tunnelled subcutaneously to exit on the top of the skull and plugged with a 28G stainless steel wire. The position of the catheter was checked by the aspiration of cerebrospinal fluid at the implantation and was directly verified after sacrificing the rat. Only rats with normal motor function and behaviour five days after surgery were used. In total, 176 rats were used.

Drugs and administration

Midazolam (a benzodiazepine-GABA_A receptor agonist; Sigma, St. Louis, MO, USA) 1, 3, 10, 30, and 100 μ g, and AP-5 (2-amino-5-phosphonovaleic acid, a NMDA receptor antagonist; Sigma, St. Louis, MO, USA) 1, 3, 10, and 30 μ g were dissolved in 10 μ L saline. YM872 {[2,3-Dioxo-7-(1H-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinolinyll] acetic acid}, an AMPA receptor antagonist (Yamanouchi Pharmaceutical Co. Ltd., Tsukuba, Japan) 10 mg was dissolved in 0.97 mL distilled water with 30 μ L, 1N NaOH added to adjust pH to 7.3–7.5. Solutions of 0.3 (0.86), 1 (2.86), 3 (8.59), 10 (28.63), or 30 (85.89) μ g (nMol) per 10 μ L were made using normal saline. Normal saline, 10 μ L, was used in the control group. After intrathecal drug or saline injection, the catheter was flushed with an injection of 10 μ L normal saline to clear the dead space of the catheter (7

TABLE ED₅₀ (50% effective dose) values (μ g)

	<i>Each single agent</i>		<i>AP-5 + Midazolam</i>		<i>YM872 + Midazolam</i>	
	<i>Phase 1</i>	<i>Phase 2</i>	<i>Phase 1</i>	<i>Phase 2</i>	<i>Phase 1</i>	<i>Phase 2</i>
ED ₅₀ of						
Midazolam	1.34 (0.29–6.19)	1.21 (0.19–7.63)	0.012* (0.001–0.13)	0.27* (0.15–0.49)	0.09* (0.027–0.30)	0.35* (0.21–0.58)
AP-5	7.64 (4.3–13.6)	1.4 (0.05–35.86)	0.014* (0.001–0.13)	0.32* (0.18–0.57)	N/A	N/A
YM872	0.24 (0.08–0.75)	0.21 (0.06–0.73)	N/A	N/A	0.015* (0.005–0.05)	0.058* (0.035–0.097)

In the parenthesis, 95% confidence intervals are shown

* $P < 0.01$ vs the values at each single agent

N/A=not available

FIGURE 1 Time course of the effects of intrathecal midazolam (A), AP-5 (NMDA receptor antagonist, B), YM872 (AMPA receptor antagonist, C), AP-5 + midazolam (D), and YM872 + midazolam (E) on the formalin test. Each point represents mean $\pm$ SEM of eight animals. AP: AP-5, Mid: midazolam, YM: YM872. The numbers after symbols, AP, Mid and YM are the doses expressed in μ g.

FIGURE 2 Dose response curves of the effects of intrathecal midazolam, AP-5 (NMDA receptor antagonist), YM872 (AMPA receptor antagonist) (A, B), AP-5 + midazolam, and YM872 + midazolam (C, D) on phase 1 and 2 of the formalin test. Each point represents mean $\pm$ SEM of eight animals. AP: AP-5, Mid: midazolam, YM: YM872.

$\pm 0.4 \mu$ L, mean $\pm$ standard error). Micro injector syringes were used for all injections. In each dose group, eight randomly selected rats were used.

Nociceptive test

Rats were kept in an open Plexiglas chamber with a diameter of 40 cm for 30 min before drug injection. Ten minutes after the intrathecal administration of the agent, 50 μ L formalin 5% were injected subcutaneously into the dorsal surface of the right hindpaw with a 30 G needle. Immediately after injection, the rat was

placed in an open Plexiglas chamber and observed for 60 min. Quantification of pain behaviour was made by counting the incidence of spontaneous flinches/shaking of the injected paw at 1–2 min, 5–6 min and at 5-min intervals during 10- to 60-min periods after formalin injection. The animals were then sacrificed with an overdose of halothane. As previously described,¹³ two distinct phases were observed after formalin injection: phase 1, during 0–6 min after injection, and phase 2, beginning about 10 min after injection.

FIGURE 3 Isobolograms for the intrathecal interaction of AP-5 or YM872 and midazolam in both phase 1 and 2 of the formalin test. Horizontal and vertical bars indicate 95% confidence interval. The oblique lines between the x axis and y axis are the theoretical additive lines. The points in the middle of this line are the theoretical additive points calculated from separate ED_{50} values. The experimental points lie far below the additive line, indicating very marked significant synergism ($P < 0.01$ in phase 1 (A) and $P = 0.038$ in phase 2 (B) of AP-5 + midazolam, $P < 0.01$ in phase 1 (C) and $P = 0.029$ in phase 2 (D) of YM872 + midazolam).

Experimental paradigm

The first series of experiments were performed to determine the dose-dependency of the antinociceptive effects of intrathecally administered midazolam, AP-5, and YM872 on the formalin test.

To investigate the interaction between midazolam and AP-5 or YM872, an isobolographic analysis¹⁴ was used. The method is based on comparisons of the dose ratios that are determined to be equieffective. First, the respective 50% effective dose (ED_{50}) values were determined from the dose-response data of each agent alone. Subsequently, dose-response data were obtained by coadministration of the two drugs in a constant dose ratio based on the ED_{50} values of the phase 2 of the single agents; i.e., combinations of each $\frac{1}{2} ED_{50}$, $\frac{1}{4} ED_{50}$, $\frac{1}{8} ED_{50}$, or $\frac{1}{16} ED_{50}$ doses. From the dose-response data of the combined drugs, the ED_{50} value of the mixture was calculated (calculated ED_{50} values). The theoretical additive values were the $\frac{1}{2} ED_{50}$ s of the single agents. Eight rats were used for each dose combination.

Data analysis and statistics

The ED_{50} values were calculated using the % maximum possible effect (% MPE) by a computer program made in the Anesthesiology Laboratory of University of California, San Diego.^{3,11} The % MPE was the % against the value in the control group.

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 normalised by assigning the ED_{50} values of the agents given alone as 1. Values near to 1 suggest an additive interaction, values >1 imply an antagonistic interaction, while values <1 indicate a synergistic interaction.

Student's *t* test was used to compare the calculated ED_{50} values with the theoretical additive values. A *P* value < 0.05 was considered statistically significant.

Results

Analgesic effects of each agent

Midazolam, AP-5 and YM872 dose-dependently decreased the number of flinches in both phase 1 and phase 2 (time course was shown in the Figures 1A, 1B, and 1C, and dose response was shown in the Figures 2A and 2B). The ED_{50} values are shown in the Table.

Analgesic interaction between midazolam and AP-5 or YM872

Intrathecal administration of the combination of midazolam and AP-5 produced dose-dependent analgesic effects on both phase 1 and phase 2 of the formalin test (time course was shown in the Figure 1D, and dose response was shown in the Figures 2C and 2D). The ED_{50} values for midazolam and AP-5 decreased in the combination compared with each single agent (Table). Total fractional dose values were 0.01 in phase 1 and 0.45 in phase 2. Isobolograms are shown in the Figure (Figures 3A, 3B).

The combination of midazolam and YM872 also induced dose-dependent decreases of flinches in both phase 1 and phase 2 of the formalin test (time course was shown in the Figure 1E, and dose response was shown in the Figures 2C and 2D). The ED_{50} values for midazolam and YM872 decreased in the combination compared with each single agent (Table). Total fractional dose values were 0.07 in phase 1 and 0.57 in phase 2. Isobolograms are shown in the Figure (Figures 3C, 3D).

Discussion

Intrathecal administered midazolam (benzodiazepine-GABA_A receptor agonist), AP-5 (NMDA

receptor antagonist) and YM872 (AMPA receptor antagonist) produce dose-dependent decreases in the number of flinches in both phase 1 and phase 2 of the formalin test in rats. Midazolam showed synergistic suppressive effects with AP-5 and YM872 on the number of flinches of both phase 1 and phase 2. The synergism of midazolam was more pronounced with AP-5 than with YM872 in phase 1 of the formalin test.

The GABA_A receptors are proposed to exist at the primary afferent terminal in the spinal cord; and the GABAergic system plays an important role in the presynaptic inhibition of primary afferents.¹³ Benzodiazepine receptors are concerned with pain transmission in the dorsal horn of the spinal cord.⁷ Benzodiazepine receptor agonists increase the intrinsic efficacy of GABA at the GABA_A receptor coupling with benzodiazepine receptor in the spinal cord⁶ by increasing Cl⁻ conductance.⁵ Midazolam, a benzodiazepine derivative, has spinally mediated analgesic^{7,11} and anesthetic¹⁶ effects in animal experiments. In the present study, we could show analgesic effects of intrathecally administered midazolam on both formalin induced acute nociception and persistent nociceptive activation. Goodchild *et al.* reported that intrathecal midazolam caused spinally mediated antinociception in rats by a mechanism involving opioid receptor activation.¹⁷ We did not use any antagonists for GABA or opioid receptors in the present study. Therefore, further studies are necessary to verify the detailed mechanism of the analgesic action of midazolam in the spinal cord.

Glutamates also exist in primary afferents and in interneurons.¹⁸ The NMDA receptors are involved in persistent nociceptive input of deep dorsal horn cells.¹⁹ Accordingly, NMDA receptor antagonists are effective in phase 2 of the formalin test¹ rather than on acute nociception.³ This is consistent with our present results in which the ED₅₀ value of AP-5 was smaller in phase 2 than in phase 1 of the formalin test.

The AMPA receptors are localized in superficial laminae of the dorsal horn pre- and postsynaptically.²⁰ These receptors alter acute afferent evoked excitation. Intrathecal application of AMPA receptor antagonists are commonly thought to decrease the responses to acute noxious stimulus.^{11,21} However, on the formalin test of the animals, Hunter *et al.* reported that the AMPA receptor antagonist inhibited only phase 1, but not phase 2 responses.¹³ In contrast, Simmons *et al.* reported that the AMPA receptor antagonist reduced phase 2 response but not phase 1.⁴ In our present study, YM872 inhibited both phase 1 and phase 2 responses. The discrepancies in these results might be due to the different compounds used: NBQX used by

Hunter and Singh,¹³ YM872 used in our present study, and other compounds in the study of Simmons *et al.*⁴ have different affinities to the AMPA and kainate subtypes of ionotropic glutamate receptors.^{4,22} Further experiment to compare the affinity of these compounds to different subtypes in the same study is necessary to discuss the discrepancies of the analgesic effect furthermore.

As we already reported,¹¹ midazolam and NMDA receptor antagonist or AMPA receptor antagonist had synergistic antinociception for acute thermal stimulation. Similarly in the present study, midazolam was very markedly synergistic with NMDA receptor antagonist and AMPA receptor antagonist in suppressing phase 1 response of the formalin test. Curiously, AP-5, which is usually less effective for acute pain, enhanced the effect of midazolam in phase 1 of the formalin test more than YM872. The phase 1 response is due to the direct stimulation of nociceptors by formalin or tissue damage and is thought to be an acute pain reaction.²³ In addition, in the phase 2 response of the formalin test, midazolam had synergistic antinociception with NMDA and AMPA receptor antagonists in the present study. The phase 2 response is due to subsequent inflammation after formalin injection and central sensitization related to C fibre activity.²⁴ Therefore, in the spinal cord, benzodiazepine-GABA_A receptor and NMDA or AMPA receptor might have some functional coupling in both acute and persistent inflammatory nociception.

Benzodiazepines increase the Cl⁻ conductance of GABA_A on the primary afferents hyperpolarizing the afferent,⁶ therefore, reducing the release of glutamate in the spinal cord.²⁵ The glutamate receptor antagonists inhibit the binding of glutamate post-synaptically reducing the depolarization of the postsynaptic cell. Reduction of glutamate release and binding would decrease transmission and, therefore, pain perception. It is also reported that a GABA_A receptor agonist inhibited the behavioural effects of NMDA, quisqualic acid and kainic acid.¹⁰ Thus, GABA_A receptors might have some functional coupling with glutamate receptors in the spinal cord with regard to nociceptive transmission.

Regarding side effects, we did not investigate any behavioural effects in this study. However, in our previous study,¹¹ the combinations of midazolam with AP-5 or YM872 decreased side effects compared to each single agent. Therefore, we also expected decreased side effects in the same combinations in the present study.

In conclusion, intrathecal coadministration of midazolam (a benzodiazepine-GABA_A receptor agonist) with AP-5 (a NMDA receptor antagonist) or

midazolam with YM872 (an AMPA receptor antagonist) produced synergistic analgesia on formalin induced acute nociception and persistent nociceptive activation. These results suggest a functional coupling of benzodiazepine-GABA_A receptor with NMDA and AMPA receptors in various nociceptive mechanisms in the spinal cord. These synergisms can reduce dose requirements (increase therapeutic index) in consequence can reduce toxicity when projected to possible clinical application.

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