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Actions of Midazolam on Excitatory Transmission in Dorsal Horn Neurons of Adult Rat Spinal Cord

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Background: **Although intrathecal administration of midazolam, a water-soluble imidazobenzodiazepine derivative, has been found to produce analgesia, how it exerts this effect at the neuronal level in the spinal cord is not fully understood.**

Methods: **The effects of midazolam on electrically evoked and spontaneous excitatory transmission were examined in lamina II neurons of adult rat spinal cord slices using the whole cell patch clamp technique.**

Results: **Bath-applied midazolam (1 μM) diminished Aδ- and C-fiber evoked polysynaptic excitatory postsynaptic currents in both amplitude and integrated area. However, it affected neither Aδ- and C-fiber evoked monosynaptic excitatory postsynaptic currents in amplitude nor miniature excitatory postsynaptic currents in amplitude, frequency, and decay time constant. In the presence of a benzodiazepine receptor antagonist, flumazenil (5 μM), midazolam (1 μM) did not diminish Aδ-fiber evoked polysynaptic excitatory postsynaptic currents, suggesting that midazolam modulate the γ-aminobutyric acid interneurons in the dorsal horn.**

Conclusions: **Midazolam reduced excitatory synaptic transmission by acting on the γ-aminobutyric acid type A/benzodiazepine receptor in interneurons, leading to a decrease in the excitability of spinal dorsal horn neurons. This may be a possible mechanism for the antinociception by midazolam in the spinal cord.**

THE water-soluble imidazobenzodiazepine derivative midazolam is widely used as a hypnotic, anxiolytic, and amnestic. Administration of midazolam directly to the spinal cord by intrathecal delivery produces analgesia in both laboratory animals[1–4] and humans.[5,6] Intrathecal administration of the γ-aminobutyric acid type A ($GABA_A$) receptor antagonist bicuculline or the benzodiazepine receptor antagonist flumazenil reduces the analgesic action of midazolam, showing that the spinal cord is one of the sites for midazolam-induced analgesic action.[1–3]

Agonists of the $GABA_A$/benzodiazepine receptors are known to modulate synaptic transmission, both presynaptically and postsynaptically, in neurons of the central nervous system,[7,8] including the spinal dorsal horn.[9] Benzodiazepines activate $GABA_A$ receptors and inhibit synaptic transmission in primary-afferent central terminals presynaptically, reducing excitatory transmitter release in spinal dorsal horn neurons.[10] Furthermore, benzodiazepines augment the activation of $GABA_A$ receptors in postsynaptic spinal cord neurons, which results in a decrease of excitatory activity.[11] Binding and immunohistochemical studies support these findings, that the highest density of $GABA_A$/benzodiazepine receptors has been found in the superficial layers of the spinal dorsal horn, especially lamina II (substantia gelatinosa [SG]).[12–15] In addition, the binding sites of the $GABA_A$ receptor in the superficial dorsal horn were reduced in number, but not abolished, after neonatal capsaicin treatment.[16] This indicates that the $GABA_A$ receptors are present in both primary-afferent central terminals and interneurons.[13] Alternatively, it has been demonstrated that SG neurons contain abundant endogenous GABA, which is the primary inhibitory neurotransmitter in the central nervous system and plays a role in controlling nociceptive information.[17–19]

We previously reported that midazolam enhanced inhibitory transmission in the SG.[11] Although midazolam augments the γ-aminobutyric acid–mediated (GABAergic) inhibitory transmission, it is not known whether excitatory function is similarly affected. It is possible that the midazolam-induced inhibition of pain transmission is due to a modulation of excitatory transmission in addition to the augmentation of the inhibitory system. SG neurons preferentially receive thin myelinated Aδ- and unmyelinated C-primary afferent fibers, both of which carry nociceptive information and thus are thought to play an important role in modulating nociceptive transmission.[20,21] To clarify whether midazolam modulates excitatory transmission in the dorsal horn, we examined the action of midazolam on Aδ- and C-fibers electrically evoked or spontaneous glutamatergic excitatory transmission in SG neurons of adult rat spinal cord slices by using the whole cell patch clamp technique.

Materials and Methods

Spinal Cord Slice Preparations and Electrophysiologic Recording

This study was approved by the Animal Research Committee of Niigata University Graduate School of Medical and Dental Sciences in Niigata, Japan. The lumbar spinal cord was removed under urethane anesthesia (1.5 g/kg, intraperitoneal) from adult rats (6-8 weeks). Transverse slices of 600 μm in thickness, which included the L4 dorsal root (10–20 mm), were cut as described previously.[18,22] After preparation, the slices were perfused

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with Krebs solution (10 ml/min, 36° ± 1°C), which was saturated with 95% O_2 and 5% CO_2 for at least 30 min before recording. The Krebs solution contained 117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose. Electrodes were positioned in lamina II, which is identifiable as a distinct translucent band across the superficial dorsal horn under a dissecting microscope with transmitted illumination. Placing the electrode in such a fashion targets a heterogeneous group of intrinsic stalk and islet neurons in inner and outer lamina II.[23] Blind whole cell patch clamp recordings were made from the SG neurons with patch pipette electrodes with a resistance of 5–10 MΩ. The patch pipette solution contained 110 mM Cs-sulfate, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, 5 mM tetraethylammonium, and 5 mM ATP-Mg salt. Signals were amplified with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and were filtered at 2 kHz and digitized at 5 kHz. Data were collected and analyzed using pClamp8.0 (Axon Instruments). Spontaneous and synaptically evoked fast excitatory postsynaptic currents (EPSCs) were recorded from SG neurons voltage clamped to −70 mV.[22,24]

Synaptically evoked currents were elicited at a frequency of 0.1 Hz by relative low-intensity dorsal root stimulation sufficient to recruit Aδ fibers (approximately 20–100 μA, 0.05 ms), and a relatively higher intensity and longer duration for C fibers (approximately 200–1,000 μA, 0.5 ms), respectively. The stimulus intensity necessary to activate Aδ and C fibers and the afferent fiber conduction velocity was determined by extracellular recording of compound action potentials from the dorsal root.[22,23] A-fiber EPSCs were classified as monosynaptic if response latency remained constant and there was an absence of failure upon high-frequency (20-Hz) stimulation.[23] Identification of C-fiber monosynaptic EPSCs was based on an absence of failure with low-frequency (1-Hz) repetitive stimulation.[25,26]

Application of Drugs

Drugs were applied by superfusion, through changing solutions in the recording chamber without alterations to the perfusion rate and temperature. Drugs used were 6-cyano-7-nitroquinoxaline-2,3-dion (CNQX), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA; Sigma, St. Louis, MO), midazolam, flumazenil (given from Hoffmann-LaRoche, Basel, Switzerland), and tetrodotoxin (Wako, Osaka, Japan).

Data Analysis

Numerical data were expressed as mean ± SD. Statistical significance was determined as $P < 0.01$ using either the paired Student *t* test or the Kolmogorov-Smirnov test. In all cases, n refers to the number of neurons studied.

Results

Midazolam Diminishes Dorsal Root Evoked Polysynaptic EPSCs in SG Neurons

Stimulation of dorsal root with a low stimulus strength evoked Aδ-fiber polysynaptic EPSCs in SG neurons as shown in figure 1A. Superfusion of midazolam (1 μM) suppressed the peak amplitude of the glutamatergic Aδ-fiber evoked EPSCs (78 ± 8% of control, n = 10; $P < 0.01$; fig. 1A). Next, the effects of midazolam on C-fiber evoked polysynaptic EPSCs were evaluated. Superfusion of midazolam (1 μM) similarly affected the peak amplitude of C-fiber evoked EPSCs (71 ± 10% of control, n = 5; $P < 0.01$; fig. 1B). The integrated area of these evoked polysynaptic EPSCs was also used to evaluate the effect of midazolam. We integrated from rising of the first wave to restore to baseline. Midazolam significantly reduced the integrated area of Aδ- and C-fiber intensity stimulation evoked polysynaptic EPSCs (74 ± 12 and 69 ± 11% of control, respectively, n = 10 and 5; $P < 0.01$; fig. 1C). Midazolam did not change the latency or the number of failures of the primary afferent evoked polysynaptic EPSCs. The inhibition by midazolam was dose dependent (fig. 1D). A previous report demonstrated the biphasic effects of midazolam: GABAergic enhancement at 1 nM but antagonism at higher concentration of 100 nM.[27] However, we did not observe the biphasic effects of midazolam in the current study. These inhibitory effects of midazolam (1 μM) were blocked by a benzodiazepine receptor antagonist, flumazenil (5 μM, 95 ± 10% of control, n = 6; $P = 0.44$; fig. 1E), indicating an activation of $GABA_A$/benzodiazepine receptors. Superfusing flumazenil (5 μM) alone did not have any effects on the polysynaptic EPSCs (102 ± 8% of control, n = 4; $P = 0.51$).

Midazolam Does Not Affect Dorsal Root Evoked Monosynaptic EPSCs

Stimulation of a dorsal root with Aδ- or C-fiber intensity evoked a monosynaptic EPSC as described above (figs. 2A and B). Previous work has demonstrated that these Aδ- and C-fiber evoked EPSCs were completely blocked by CNQX (20 μM), indicating an activation of non–*N*-methyl-D-aspartate receptors.[28] Midazolam (1 μM) had no effect on Aδ- or C-fiber evoked monosynaptic EPSCs in all recorded neurons (figs. 2A and B). The peak amplitude did not change significantly (103 ± 5 and 98 ± 9% of control, respectively, n = 11 and n = 7; $P = 0.53$ and 0.38; fig. 2C). At a higher concentration of 10 μM, midazolam still had no effect on the amplitude of Aδ-fiber evoked monosynaptic EPSCs (97 ± 8%, n = 5; data not shown). In addition, we investigated the effect of midazolam on the AMPA–induced current in SG neurons. Superfusing AMPA (10 μM) elicited an inward current (fig. 2D). The amplitude of the AMPA-induced current was not affected by midazolam as well (105 ± 6% of

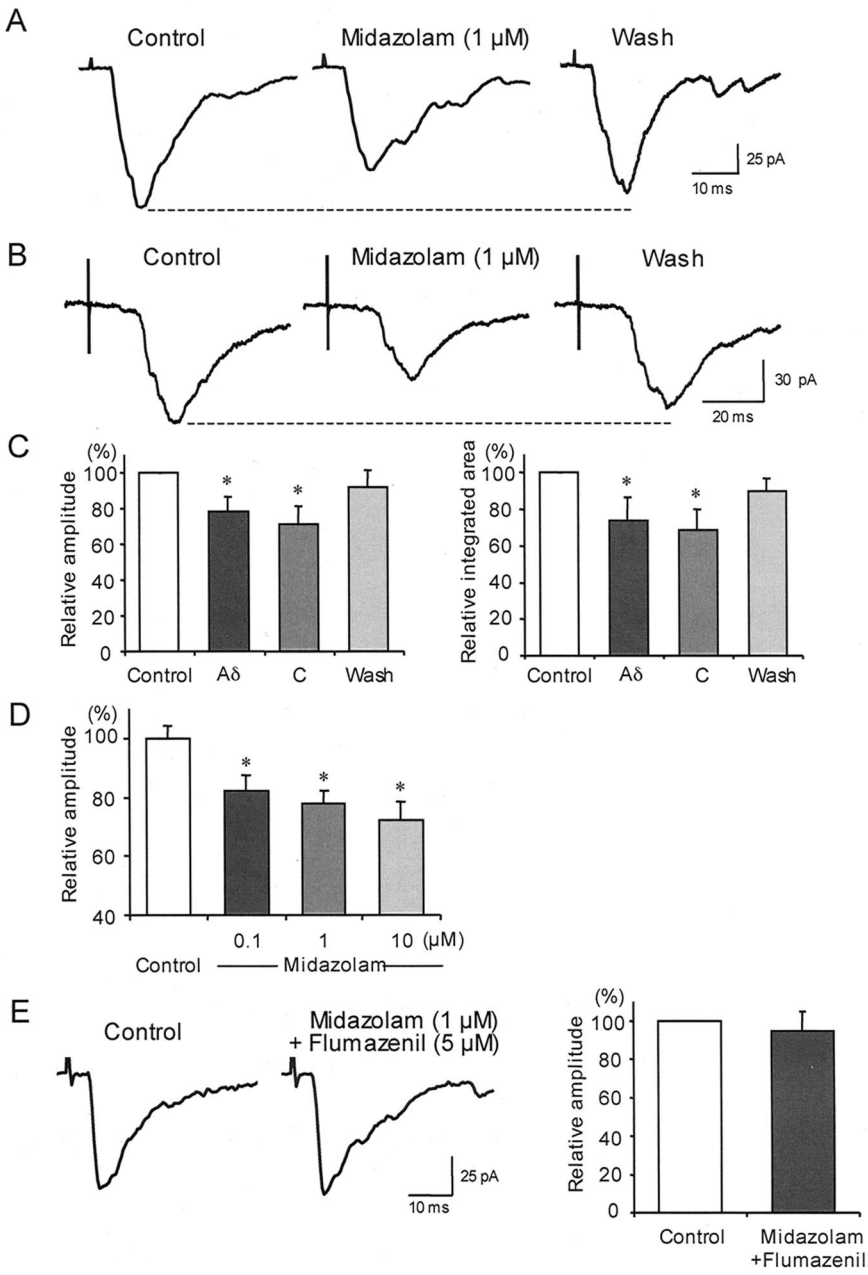

Fig. 1. Midazolam inhibits dorsal root evoked polysynaptic excitatory postsynaptic currents (EPSCs). (*A* and *B*) Averaged traces of six consecutive Aδ-fiber (*A*) and C-fiber (*B*) intensity stimulation evoked polysynaptic EPSCs before and during the application of midazolam (1 μM) and after washout. These were recorded in two different neurons. (*C*) Amplitude and integrated area of Aδ- and C-fiber intensity stimulation evoked polysynaptic EPSCs in the presence of midazolam, relative to those in the control (n = 10 and n = 5, respectively). (*D*) Dose-dependent inhibition of Aδ-fiber intensity stimulation evoked polysynaptic EPSCs by midazolam. For each dose, n > 6. (*E*) Inhibitory action of Aδ-fiber evoked polysynaptic EPSCs by midazolam (1 μM) was not observed in the presence of flumazenil (5 μM). *Vertical bars* show SDs. * $P < 0.01$.

control, $P = 0.56$, n = 6; fig. 2D). These results indicate that midazolam has no effect on activation of non–*N*-methyl-D-aspartate receptors on the postsynaptic membrane or on glutamate release from primary afferent fibers.

No Effect of Midazolam on Miniature EPSCs

We further tested the effect of midazolam on glutamatergic miniature EPSCs (mEPSCs). The mEPSCs were isolated by adding tetrodotoxin (1 μM), an indicator of action at presynaptic terminals and postsynaptic responsiveness to glutamate (fig. 3A). mEPSCs were completely blocked by CNQX (20 μM, data not shown) as well evoked EPSCs, indicating an activation of non–*N*-methyl-D-aspartate receptors.[28] In all cells tested (n = 7), amplitude and interevent interval distributions were not changed by midazolam (figs. 3B and C). Superfusion of midazolam (1 μM) had no effect on the amplitude, frequency, or decay time constant of mEPSCs; they were 101 ± 6, 98 ± 5, and 104 ± 6% of control, respectively (n = 7; $P = 0.43$, $P = 0.68$, and $P = 0.24$, respectively; fig. 3D). These data indicate that midazolam does not affect glutamate release from presynaptic terminals of primary afferents or excitatory interneurons.

Midazolam Does Not Affect Polysynaptic EPSCs in the Presence of Bicuculline

We tested whether the inhibitory effect of midazolam on Aδ-fiber polysynaptic EPSCs is eliminated under the blockade of GABAergic transmission. In the presence of

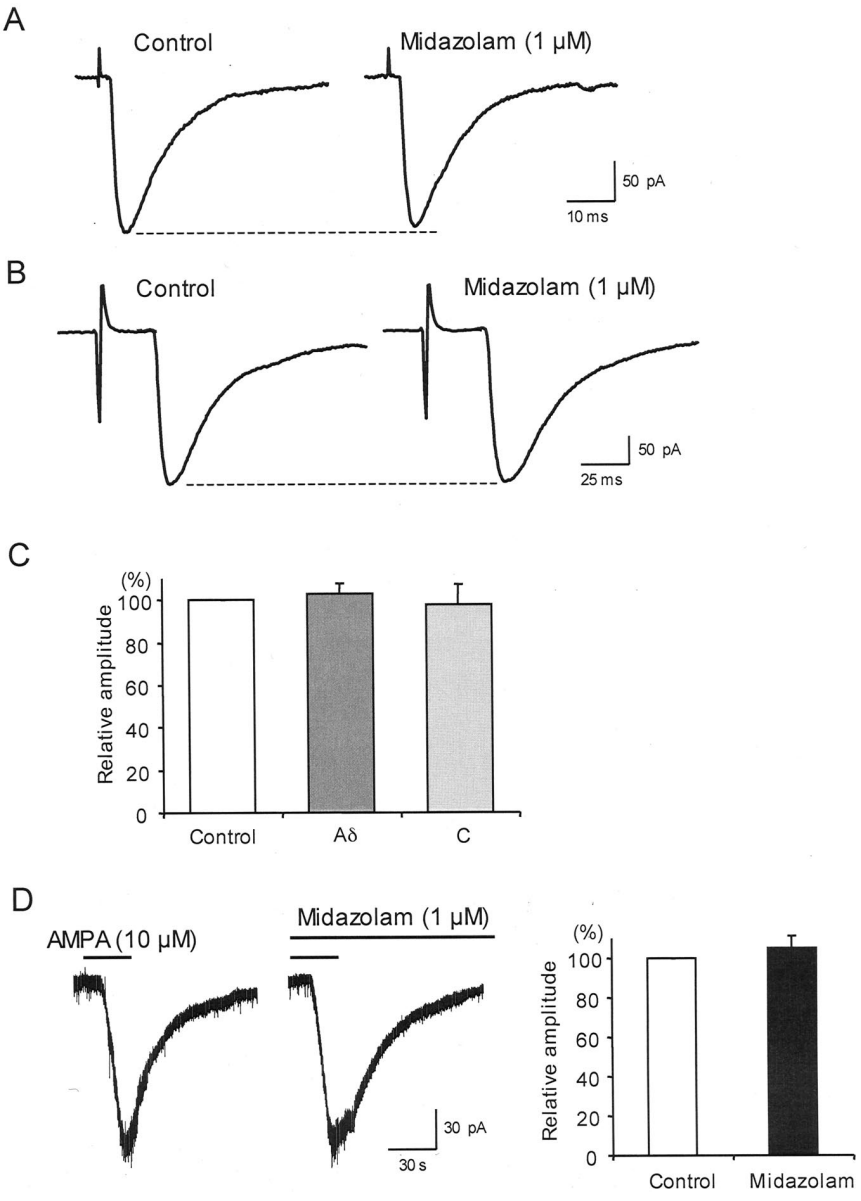

Fig. 2. Effect of midazolam on dorsal root evoked monosynaptic excitatory postsynaptic currents. (*A* and *B*) Averaged traces of six consecutive monosynaptic excitatory postsynaptic currents evoked by Aδ-fiber (*A*) and C-fiber (*B*) intensity stimulation before and during the application of midazolam (1 μM). These were recorded in two different neurons. (*C*) The amplitude in the presence of midazolam, relative to that in the control. Midazolam had no effect on Aδ- and C-fiber evoked monosynaptic excitatory postsynaptic currents (n = 11 and n = 7, respectively). (*D*) Amplitude of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA, 10 μM)–induced current (n = 6) in the presence of midazolam (1 μM) in substantia gelatinosa neurons, relative to that in the control. *Vertical bars* show SDs.

bicuculline (20 μM), Aδ-fiber intensity stimulation usually evokes repetitive, long-lasting polysynaptic EPSCs that follow the initial fast monosynaptic or polysynaptic EPSCs.[19] Midazolam affected neither the amplitude nor the integrated area of the Aδ-fiber evoked polysynaptic EPSCs in all recorded neurons (93 ± 8 and 94 ± 6% of control, respectively, n = 5; $P = 0.27$ and $P = 0.18$; fig. 4).

Discussion

γ-Aminobutyric acid type A receptors are classically thought to exist on primary afferent terminals in the spinal cord and are involved in presynaptic inhibition *via* primary afferent depolarization.[7] However, we failed to observe presynaptic inhibition of Aδ- and C-fiber evoked monosynaptic EPSCs by midazolam in the current study (fig. 2). Our group previously reported that muscimol, a $GABA_A$ receptor agonist, had no effect on the amplitude of dorsal root evoked monosynaptic EPSCs.[19] This result suggested that facilitation of presynaptic GABAergic inhibition (primary afferent depolarization) by midazolam is not prominent, at least in the fine afferent fibers in the superficial dorsal horn. Most likely, primary afferent depolarization by $GABA_A$ receptor activation may exert its action on primary afferent terminals of large myelinated Aα/β fibers in the deep dorsal horn.[29] Moreover, the current study shows that midazolam does not affect spontaneous glutamate release from presynaptic terminals of primary afferents and excitatory interneurons. Taken together, glutamatergic transmission in the SG is not a primary target for midazolam.

Dorsal root evoked monosynaptic EPSCs were not affected by midazolam, whereas midazolam inhibited the amplitude of polysynaptic EPSCs. Primary afferent stim-

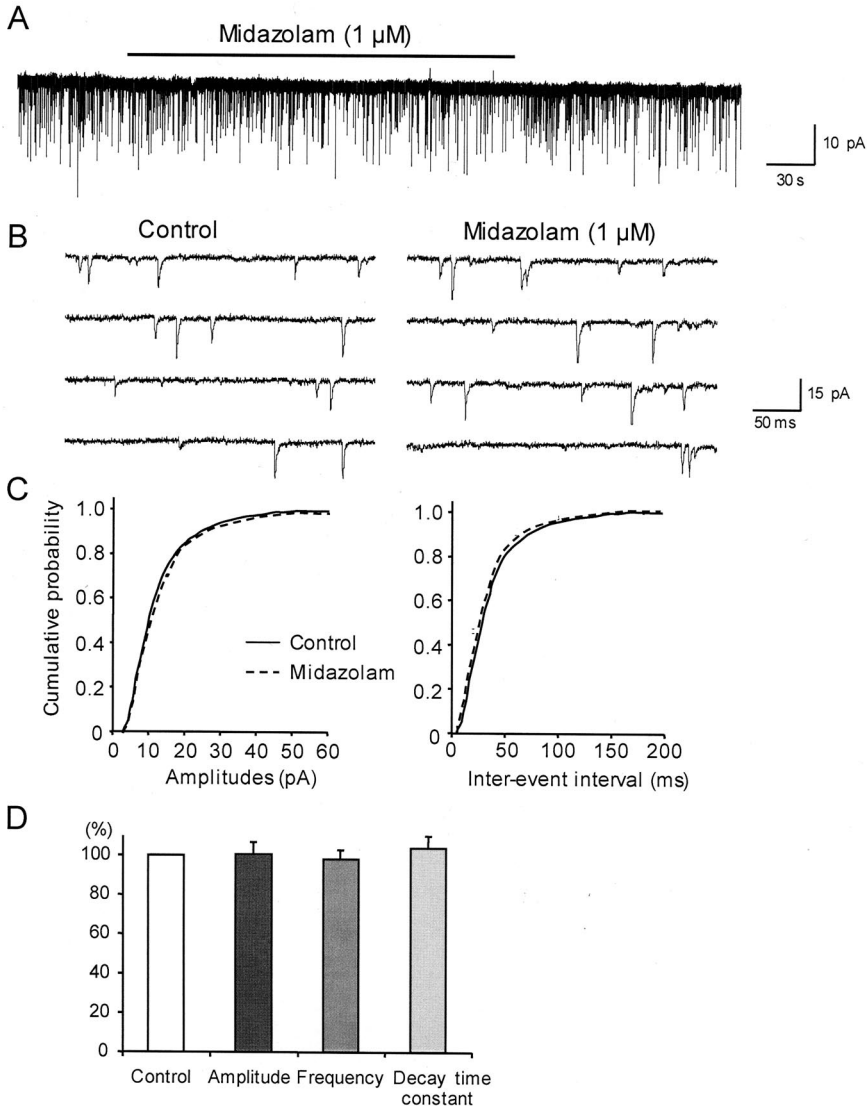

Fig. 3. Effect of midazolam on miniature excitatory postsynaptic currents (mEPSCs). mEPSCs were recorded at −70 mV, in the presence of tetrodotoxin (1 μM). (*A*) Superfusion of midazolam (1 μM) had no effect on mEPSCs. (*B*) The consecutive traces of mEPSCs before (*left*) and during (*right*) application of midazolam (1 μM). (*C*) Cumulative distributions of amplitude (*left*) and interevent interval (*right*) of mEPSC, before (*straight line*) and under (*dotted line*) the action of midazolam. Each datum was constructed from continuous recording for 60 s. Midazolam had no effect on the distribution of the amplitude or frequency ($P = 0.82$ and $P = 0.76$, respectively; Kolmogorov-Smirnov test). (*D*) The mean amplitude, frequency, and decay time constant in the presence of midazolam, relative to those in the control. Midazolam did not affect the amplitude, frequency, and decay time constant of mEPSCs (n = 7). *Vertical bars* show SDs.

ulation evoked excitatory synaptic responses in SG neurons, which consist of monosynaptic or polysynaptic EPSCs or both.[22,23] The monosynaptic EPSCs are recorded from SG neurons with a direct connection from primary afferents, whereas those with only polysynaptic EPSCs do not have direct primary afferent input. SG neurons with exclusively monosynaptic EPSCs are relatively rare.[22,23] To what extent polysynaptic EPSCs contributed to the excitability of SG neurons could not be determined. However, we can reasonably speculate that the inhibition of polysynaptic EPSCs by midazolam has considerable effects on nociceptive transmission in the superficial dorsal horn, given that more than 70% of SG neurons exhibit polysynaptic EPSCs,[22,23] and the amplitude and duration of polysynaptic EPSCs are almost identical to those of monosynaptic EPSCs.

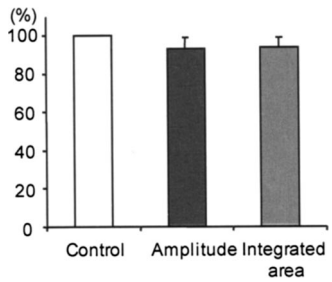

Fig. 4. Midazolam has no inhibitory action in the presence of bicuculline. Aδ-fiber intensity dorsal root stimulation evoked polysynaptic excitatory postsynaptic currents in the presence of bicuculline (20 μM). The amplitude and integrated area of the Aδ-fiber evoked polysynaptic excitatory postsynaptic currents were not affected by midazolam (n = 5). *Vertical bars* show SDs.

What underlies the inhibition of midazolam on the primary afferent fiber evoked polysynaptic EPSCs but not monosynaptic EPSCs? Taking into consideration the absence of an effect on miniature EPSCs, the augmentation of GABAergic inhibition located on somatodendritic sites of excitatory interneurons in the SG is the most likely mechanism of action for midazolam. To reinforce this hypothesis, we tested the effect of midazolam under the blockade of GABAergic inhibitions. In this situation,

the inhibitory action of midazolam was eliminated. Moreover, we have suggested that midazolam prolonged the decay time course of GABAergic evoked and miniature inhibitory postsynaptic currents in SG neurons.[11] These results support the notion that the effect on the $GABA_A$ receptor is a major action of midazolam in the inhibition polysynaptic EPSCs. The actions of benzodiazepines, such as midazolam, are due to interactions with a specific binding site on the $GABA_A$ receptor complex, and these interactions subsequently increase the probability of GABA to open the chloride channel associated with the receptor inducing inhibition. Therefore, the effects of midazolam depend on the level of GABA activity. The spinal dorsal horn, especially SG, is a primary receiving area for somatosensory, presumably nociceptive inputs, which contain a high density of $GABA_A$ receptors and also of endogenous GABA.[13] Therefore, midazolam easily activate $GABA_A$ receptors in SG neurons.

The spinal dorsal horn neurons receive direct fine primary afferent input, and the excitatory postsynaptic potentials are generally followed by GABAergic or glycinergic inhibitory postsynaptic potentials or both. Therefore, under circumstances where $GABA_A$ receptor antagonists are applied, excitatory postsynaptic potentials often lead to a bursting activity of action potentials in SG neurons in response to a single stimulus, which previously had evoked only a single excitatory postsynaptic potential. This indicates that a normally inhibitory circuitry may prevent a recurrent excitation in the SG.[30] Therefore, when the duration of GABAergic inhibitory postsynaptic currents is prolonged by midazolam, the number of spikes should be decreased, and consequently, the peak amplitude and integrated area of polysynaptic EPSCs can be reduced in the recorded SG neuron.

In summary, the current study provides a further possible physiologic underpinning for behavioral studies, which have demonstrated the antinociceptive action of midazolam at the spinal cord level.

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