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Enhancement of GABAergic Tonic Currents by Midazolam and Noradrenaline in Rat Substantia Gelatinosa Neurons *In Vitro*

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ABSTRACT

Background: Substantia gelatinosa of the spinal dorsal horn is crucial for transmission and modification of noxious stimuli. Previous studies have demonstrated that intrathecal midazolam, a benzodiazepine agonist, enhanced perioperative analgesia. Not only synaptic but also extrasynaptic inhibitory currents contribute to modification of noxious stimuli. Thus, the effects of midazolam on extrasynaptic γ-aminobutyric acid (GABA) type A receptors in substantia gelatinosa neurons and interaction with noradrenaline, a transmitter of the descending inhibitory systems, were investigated.

Methods: Using whole cell patch-clamp technique in the adult rat spinal cord slices, extrasynaptic GABAergic currents were recorded in substantia gelatinosa neurons in the presence of gabazine (1 μM), which blocked synaptic GABAergic currents, and then midazolam (5 μM) and noradrenaline (20 μM) were applied.

Results: Bath application of midazolam induced tonic outward currents in the presence of gabazine. Although the decay time of synaptic current was prolonged, neither frequency nor amplitude was affected by midazolam. In contrast, the application of noradrenaline markedly increased both frequency and amplitude of synaptic currents with a slight enhancement of tonic currents. Coapplication of noradrenaline and midazolam markedly increased tonic currents, and the increase was much greater than the sum of currents induced by noradrenaline and midazolam.

Conclusions: Midazolam had much larger effects on extrasynaptic GABA type A receptors than the synaptic receptors, suggesting a role of the enhancement of GABAergic extrasynaptic currents in the midazolam-induced analgesia. Because noradrenaline is shown to increase extrasynaptic GABA concentration, simultaneous administration of noradrenaline and midazolam may enhance the increased GABA action by midazolam, thereby resulting in an increase in tonic extrasynaptic currents.

What We Already Know about This Topic

- Intrathecal midazolam and spinally released norepinephrine may produce analgesia by stimulating γ-aminobutyric acid (GABA) receptors

What This Article Tells Us That Is New

- In single-cell recordings in the substantia gelatinosa in the spinal cord slices from rats, midazolam enhanced tonic, extrasynaptic $GABA_A$ receptor currents, and coapplication of norepinephrine, which increases GABA release, further enhanced these inhibitory currents

SUBSTANTIA gelatinosa (SG), the lamina II of the spinal dorsal horn, is one of the crucial sites for the transmission and modification of noxious stimuli. Noxious stimuli are delivered particularly to the superficial dorsal horn through fine myelinated Aδ- and unmyelinated C-primary afferent fibers from the periphery and then transmitted to the upper central nervous system.[1–3] In the SG of the spinal cord, the noxious stimuli are modified by at least two inhibitory systems. One is γ-aminobutyric acid (GABA)-containing interneurons whose terminals are abundant in the superficial dorsal horn.[4–7] It has been shown that the primary afferent fibers activate GABAergic and/or glycinergic interneurons through the glutamatergic receptors, resulting in the suppression of nearby SG neurons.[8] Another is the descending inhibitory

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system projecting from the brainstem, in which noradrenaline is one of the representative neurotransmitters.[9–12]

GABA, an important inhibitory neurotransmitter in the SG, has been known to affect neuronal excitability at the synaptic clefts. However, recent electrophysiologic studies have shown that GABA acts on extrasynaptic GABA type A ($GABA_A$) receptors, which causes extrasynaptic or tonic currents (inhibition),[13,14] whereas synaptic $GABA_A$ receptors produce synaptic or phasic currents. Thus, a clinical importance of $GABA_A$ receptor-mediated tonic inhibition has been suggested as a target of anesthetic or sedative drugs. It has been reported that an anticonvulsant that increases GABA concentration exerts its action by potentiating tonic rather than phasic inhibition in the hippocampus.[15] Despite the important role of extrasynaptic inhibitory currents, the effects of anesthetics on extrasynaptic $GABA_A$ receptors in the SG neurons remain to be elucidated.

It is well known that benzodiazepines enhance the affinity of GABA–$GABA_A$ receptors through allosteric modulation at benzodiazepine-binding sites.[16,17] Midazolam, an agonist of benzodiazepine receptors, has been shown to prolong the decay time of the inhibitory postsynaptic currents (IPSCs) in the rat SG neurons.[18] However, little is known about a direct effect of midazolam on extrasynaptic $GABA_A$ receptors in the SG neurons. Because midazolam does not activate $GABA_A$ receptors directly in the absence of GABA,[19] it is possible that ambient GABA concentration is important for the action of midazolam. Conversely, noradrenaline has been shown to increase GABA release through α_1-adrenergic receptors located at the cell body or presynaptic terminals of GABAergic neurons in the SG.[20,21] Therefore, noradrenaline may induce spillover of GABA from the synapses to act on extrasynaptic $GABA_A$ receptors, thereby influencing the effects of midazolam. In the current study, we first divided GABAergic inhibitory currents into synaptic and extrasynaptic ones and then examined the effects of midazolam on each current using whole cell patch-clamp technique in the adult rat spinal cord slices. Furthermore, the effects of coapplication of midazolam and noradrenaline were investigated to determine the interaction of the drugs.

Materials and Methods

All the experimental procedures involving the use of animals were approved of by the Ethics Committee on Animal experiments, Kyushu University (Fukuoka, Japan) and were in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and associated guidelines.

Spinal Cord Slice Preparation

Methods for obtaining adult rat spinal cord slice preparations have been described previously.[20] In brief, male adult Sprague–Dawley rats (6–8 weeks old) were deeply anesthetized with intraperitoneal urethane (1.5 g/kg), and then lumbosacral laminectomy was performed. The lumbosacral spinal cord (L1–S3) was removed and immersed in preoxygenated ice-cold Krebs. Immediately after the removal of spinal cord, the rats were given an overdose of urethane and were killed by exsanguination. After the dura mater, the ventral and dorsal roots, and the pia-arachnoid membrane were removed, a 500- to 600-μm-thick transverse slice was made using vibrating microslicer (DTK 1500; Dosaka Co. Ltd., Kyoto, Japan). The slice was placed on the nylon mesh in the recording chamber, which had a volume of 0.5 ml, and perfused with Krebs solution saturated with 95% O_2 and 5% CO_2 at a rate of 15 ml/min and maintained at 36° ± 1°C. 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.

Patch-clamp Recordings from SG Neurons

Blind whole cell patch-clamp recordings were made from SG neurons with patch-pipette electrodes having the resistance of 5–10 MΩ. The SG was clearly discernible as a distinct translucent band across the superficial dorsal horn under a dissecting microscope with transmitted illumination as reported previously.[1,22] The patch-pipette solution was composed of 110 mM Cs_2SO_4, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, 5 mM tetraethylammonium, and 5 mM ATP-Mg (pH 7.2) for inhibiting postsynaptic effects on K^+ channels. In addition, 2 mM guanosine-5′-O-(2-thiodiphosphate)-β-S, which inhibits the activation of guanosine triphosphate-binding proteins through GABA type B receptors in postsynaptic neurons, was added to the patch-pipette solution. Recording of signals was performed at least 20 min after the establishment of whole cell patch clamp. Signals were acquired with patch-clamp amplifier (Axopatch 200B; Axon Instruments, Union City, CA) at an acquisition rate of 10 kHz. Currents obtained in the voltage clamp mode were low-pass-filtered at 5 kHz and digitized with an A/D converter (Digidate 1440; Axon Instruments). Data were stored and analyzed with a personal computer using the pClamp acquisition program (version 10.1, Axon Instruments).

Application of Drugs

Drugs were dissolved in Krebs solution and applied by perfusion *via* a three-way stopcock without any change in either the perfusion rate or the temperature. The time necessary for the solution to flow from the stopcock to the surface of the spinal cord slice was approximately 20 s. Drugs perfused in this study were midazolam (Wako, Osaka, Japan), flumazenil (Wako), noradrenaline (Wako), strychnine (Sigma-Aldrich, St. Louis, MO), bicuculline (Sigma-Aldrich), guanosine-5′-O-(2-thiodiphosphate)-β-S (Sigma-Aldrich), gabazine (Tocris Cookson, Bristol, United Kingdom), and GABA (Sigma-Aldrich).

Data Analysis

To measure the baseline current of each trace, 30 s of the digitized current trace was plotted as all-points in 0.1-pA bins, and the peak of the histogram was determined by pClamp 10.1 software (Axon Instruments). Frequency, amplitude, decay-time constant, and averaged charge transfer of

IPSCs (Q_{IPSCs}, area between the averaged IPSC and the baseline) were analyzed from recordings for 60 s using Mini analysis program (Synaptsoft, Decatur, GA). The change in charge transfer for 1 s produced by drugs (δQ_{PC}) was calculated by the equation: $\delta Q_{PC} = F \times (Q'_{IPSCs} - Q_{IPSCs}) \times t$, where F is the frequency (Hz) of IPSCs, Q'_{IPSCs} and Q_{IPSCs} are the averages of charge transfer per IPSC in the presence and absence of drugs, and t is 1 s. An increased charge transfer (δQ_{TC}) produced by drugs was calculated according to the equation: $\delta Q_{TC} = I_{TC} \times t$, where I_{TC} is the average amplitude of the baseline current calculated from 60-s recordings after drug application, and t is 1 s. The average of the total area under IPSCs was calculated by recordings for 60 s before drug application using pClamp 10.1 software. Then the area was again measured for 60 s during peak of the response after drug administration. All numerical data were expressed as the mean ± SE (SEM). Statistical significance was determined as $P < 0.05$ using Student paired *t* test to compare the frequency, amplitude, decay-time constant, and baseline shift. Student unpaired *t* test was used to compare the charge transfers of δQ_{PC} and δQ_{TC}. Significant difference in the total area was analyzed by one-way analysis of variance followed by Student–Newman–Keuls test. In all cases, n refers to the number of neurons studied.

Results

Stable whole cell recordings were obtained from 128 SG neurons, of which membrane potentials were more negative than −55 mV (average, 68.7 ± 1.6 mV). All the SG neurons exhibited IPSCs with an average frequency of 3.8 ± 0.5 Hz and amplitude of 16.9 ± 1.3 pA, and the holding current was 38.2 ± 4.8 pA at the membrane holding potential of 0 mV. Spontaneous excitatory postsynaptic currents were invisible because of the reversal potential for excitatory postsynaptic currents to be close to 0 mV.

Effects of Gabazine on Phasic and Tonic GABAergic Currents

First effects of two $GABA_A$ receptor antagonists, bicuculline and gabazine, on IPSCs and baseline membrane currents were examined. To block the glycine-evoked IPSCs, a glycine receptor antagonist, strychnine (2 μM) was always present in the following experiments. As shown in figure 1A, bath application of gabazine (1 μM) suppressed IPSCs without significant changes in basal current (boxes a and b). Expanded time scale traces before (box a) and after (box b) application of gabazine are shown in figure 1B, which correspond to figures 1Aa and Ab, respectively. In six of eight (75%) neurons, examined coadministration of bicuculline (20 μM, for 3 min) induced a slow inward current as shown in figure 1Ac. The mean current shown in figures 1Ca–c corresponds to figures 1Aa–c, respectively. The peak of all-points histogram shifted to more negative values (fig. 1Cd). The baseline shift after additional application of bicuculline

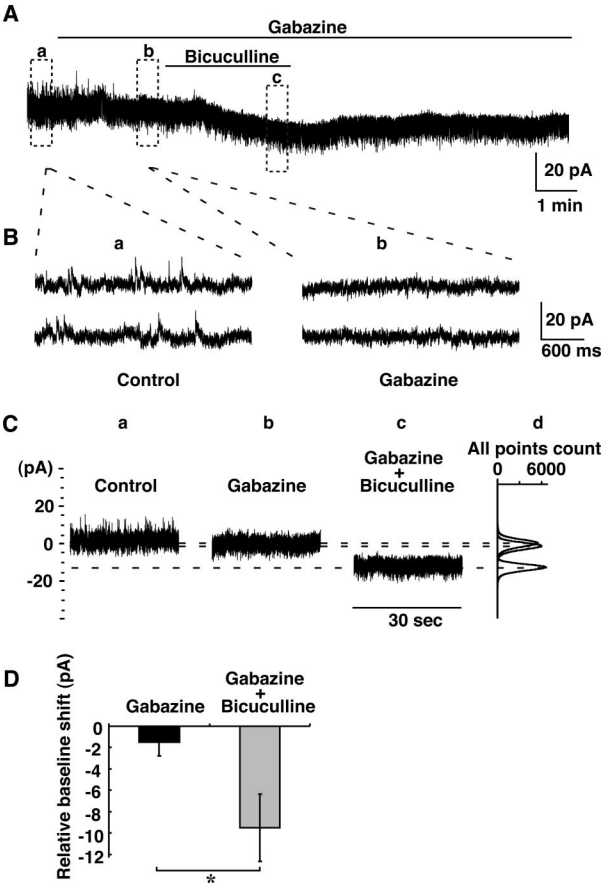

Fig. 1. Effects of gabazine on γ-aminobutyric acid (GABA)ergic inhibitory synaptic and extrasynaptic currents in the substantia gelatinosa neurons. (*A*) In the presence of strychnine (2 μM), bath application of gabazine (1 μM) completely abolished inhibitory postsynaptic currents (*boxes a* and *b*) without any changes in baseline current. In contrast, the application of bicuculline (20 μM) evoked a slow inward current (*box c*). The holding membrane potential was 0 mV. (*B*) Two consecutive traces in the expanded time scale in *boxes a* (control) and *b* (gabazine), indicating the suppression of inhibitory postsynaptic currents by gabazine (1 μM). (*C*) Baseline currents in 30 s of *box a* (control), *box b* (gabazine), and *box c* (gabazine with bicuculline) in fig. 1A. The *dashed lines* indicate the averages of the each baseline current. Corresponding all-points histograms are shown (*d*). (*D*) Relative baseline shifts produced by gabazine (−1.5 ± 1.3 pA) and bicuculline (−9.5 ± 3.2 pA, n = 6, *$P < 0.05$).

(9.5 ± 3.2 pA, $P < 0.05$, Student *t* test, n = 6) was significantly larger than that induced by gabazine only (1.5 ± 1.3 pA, $P > 0.05$; fig. 1D). When bicuculline (20 μM) was applied without gabazine, IPSCs were abolished as gabazine application, and a baseline current also shifted inwardly in 15 of 19 (79%) SG neurons (8.0 ± 1.1 pA, $P < 0.05$, n = 15). In addition, the GABA-induced (1 mM) outward current (65.92 ± 21.6 pA) was suppressed by bicuculline (20 μM) but not by gabazine (1 μM; 64.6 ± 20.0 pA, n = 5), suggesting different effects of bicuculline and gabazine at this concentration (<10 μM) on the baseline current, as reported previously.[23–25]

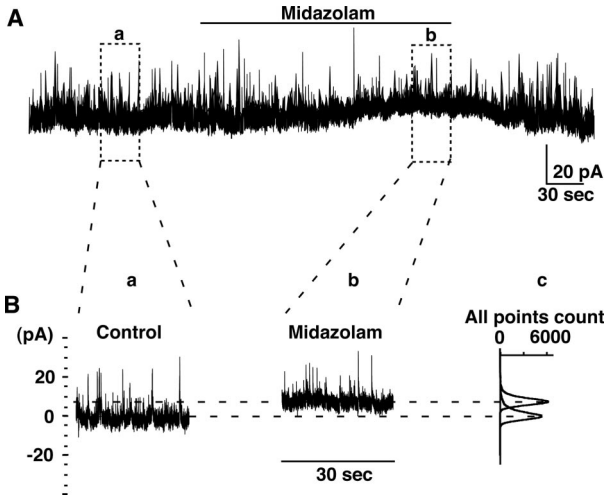

Fig. 2. Effects of midazolam on γ-aminobutyric acid (GABA)ergic inhibitory currents. (*A*) In the presence of strychnine (2 μM), application of midazolam (5 μM) induced slow outward current without any changes in frequency and amplitude of inhibitory postsynaptic currents (*dashed boxes a* and *b*). The holding membrane potential was 0 mV. (*B*) The baseline currents for 30 s of before (*a*, control) and during application of midazolam (*b*). The *horizontal dashed lines* indicated the mean amplitude of the baseline currents. Corresponding all-points histograms are shown (*c*).

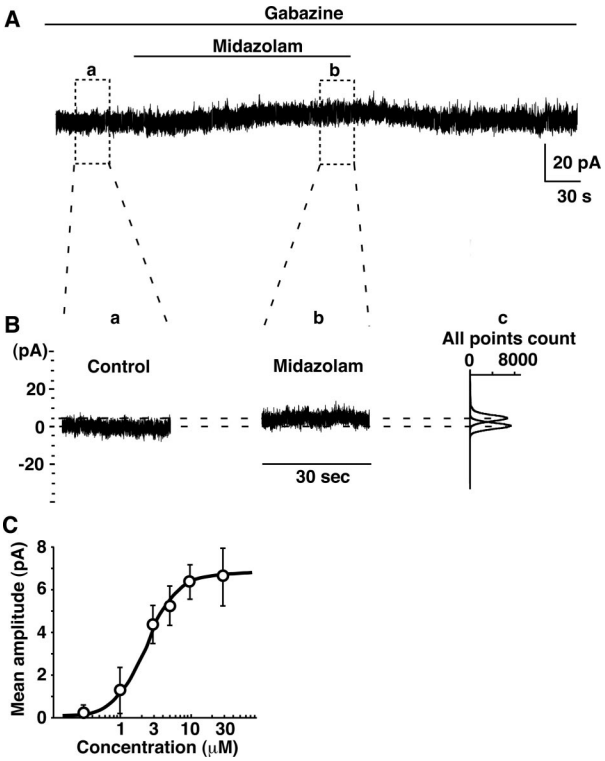

Fig. 3. Effects of midazolam on γ-aminobutyric acid (GABA)ergic inhibitory extrasynaptic currents in the presence of gabazine. (*A*) After inhibitory postsynaptic currents were abolished by gabazine (1 μM) and strychnine (2 μM), midazolam (5 μM) was applied for 3 min. Slow outward current was induced by midazolam (*dashed boxes a* and *b*). The holding membrane potential was 0 mV. (*B*) The baseline currents for 30 s before (*a*, control) and during application of midazolam (*b*). The *horizontal dashed lines* indicate the mean amplitude of the baseline currents. Corresponding all-points histograms are shown (*c*). (*C*) Dose dependence of the midazolam-induced outward current. EC_{50} (effective concentration producing half-maximal response) was 2.1 ± 0.03 μM (n = 7 at each point).

Effects of Midazolam on Baseline Membrane Currents

Application of midazolam (5 μM, for 3 min) induced slow outward current at the membrane holding potential of 0 mV in the presence of strychnine (fig. 2A). The baseline current and all-points count showed a significant outward shift from the control in 7 of 13 (54%) neurons (4.4 ± 0.8 pA, $P < 0.05$, n = 7; figs. 2Ba–c). It has been reported that midazolam induces prolongation of decay-time constant of IPSCs in the SG neurons at concentrations more than 1 μM.[18,26] Therefore, although IPSCs did not seem to be affected by midazolam in the time scale of figures 2A and B, upward shift of all-points count might include changes in the decay time of IPSCs. Thus, we again examined the effects of midazolam in the presence of gabazine (1 μM) to block IPSCs evoked by synaptic $GABA_A$ receptors. As shown in figure 3A, application of midazolam (5 μM, for 3 min) again evoked an outward current in the presence of gabazine (1 μM) in 7 of 12 (58%) neurons. The upward shift of baseline current shown in figures 3Ba and b corresponds to figures 3Aa and b, respectively. All-points count is demonstrated in figure 3Bc. The average of the shift was 4.1 ± 0.9 pA (n = 7), which was not different from that induced by midazolam in the absence of gabazine (4.4 ± 0.8 pA; fig. 2). Figure 3C shows the dose-dependent curve for the midazolam-induced outward current, in which the effective concentration producing half-maximal response (EC_{50}) was 2.1 ± 0.03 μM. In the following experiments, the slow outward current or baseline shift recorded in the presence of gabazine (1 μM) was designated as tonic inhibition or current and IPSCs as phasic inhibition. The midazolam-induced outward current was abolished in the presence of flumazenil (1 μM), an antagonist of benzodiazepine receptor, suggesting an involvement of $GABA_A$-benzodiazepine receptors (n = 10, data not shown).

Effects of Midazolam on GABAergic IPSCs

In the presence of strychnine (2 μM), the frequency and amplitude of GABAergic IPSCs were 2.1 ± 0.2 Hz and 17.1 ± 1.1 pA, respectively, at the membrane holding potential of 0 mV. As shown in figure 4A, neither frequency nor amplitude of IPSCs (fig. 4Aa, control) was affected by perfusion with midazolam (5 μM, for 3 min, fig. 4Ab). The decay-time constant was, however, increased from 28.7 ± 2.5 to 39.3 ± 2.9 ms ($P < 0.01$, n = 18, paired *t* test, 144.1 ± 9.3%) by the application of midazolam (fig. 4B), as reported previously.[18,26] Figure 4C shows the summary of the relative frequency, amplitude, and decay time after the administration of midazolam.

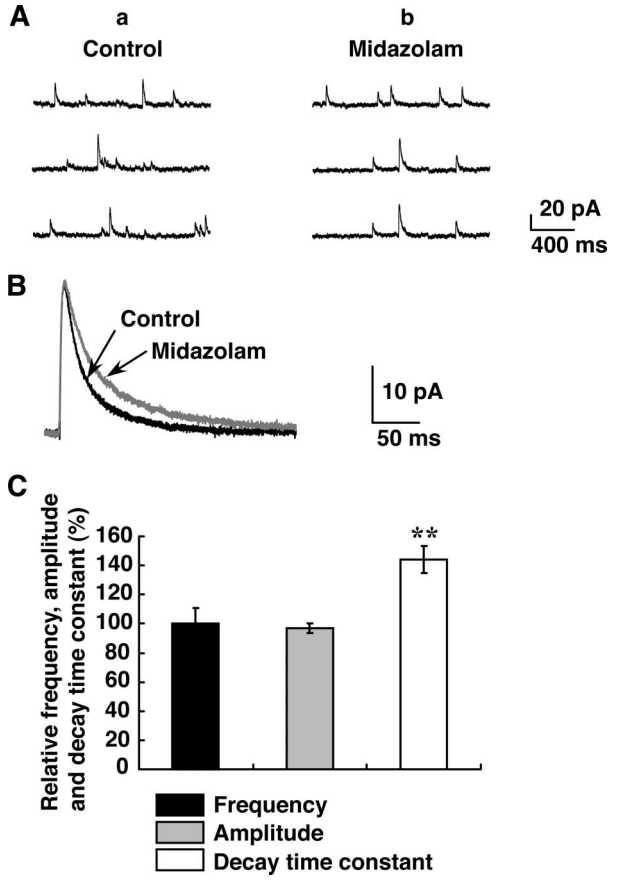

Fig. 4. Effects of midazolam on γ-aminobutyric acid (GABA)ergic inhibitory postsynaptic currents (IPSCs). (*A*) Three consecutive traces of GABAergic IPSCs before (*a*, control) and during application of midazolam (5 μM) for 3 min (*b*) in the presence of strychnine (2 μM). The holding membrane potential was 0 mV. (*B*) Averaged traces of 54 and 50 GABAergic IPSCs before (*black*) and during (*gray*) administration of midazolam. Superimposed traces indicate a prolongation of decay time of IPSCs by midazolam. (*C*) Summary of relative frequency (100.2 ± 10.8%, $P > 0.05$), amplitude (97.0 ± 3.1%, $P > 0.05$) and decay-time constant (144.1 ± 9.3%, $^{**}P < 0.01$) of GABAergic IPSCs after application of midazolam (n = 18).

Increase in Charge Transfers of Phasic and Tonic Currents by Midazolam

To differentiate the effects of midazolam on phasic and tonic inhibition, charge transfers through $GABA_A$ receptors for IPSCs and baseline currents were measured separately. The midazolam-induced increase in charge transfers for phasic currents (δQ_{PC}) was calculated by averaged charge transfers of control (Q_{IPSCs}) and during midazolam (Q'_{IPSCs}), frequency (F), and duration (1 s, t; fig. 5Aa), whereas that for tonic current (δQ_{TC}) was from baseline shift of the tonic currents (I_{TC}) and 1 s (t) (fig. 5Ab). As shown in figure 5B, the effect of midazolam on tonic currents was much larger than that on phasic currents (δQ_{PC}, 0.14 ± 0.02 pC, n = 18 and δQ_{TC}, 4.3 ± 0.7 pC, n = 7, $P < 0.01$, Student *t* test).

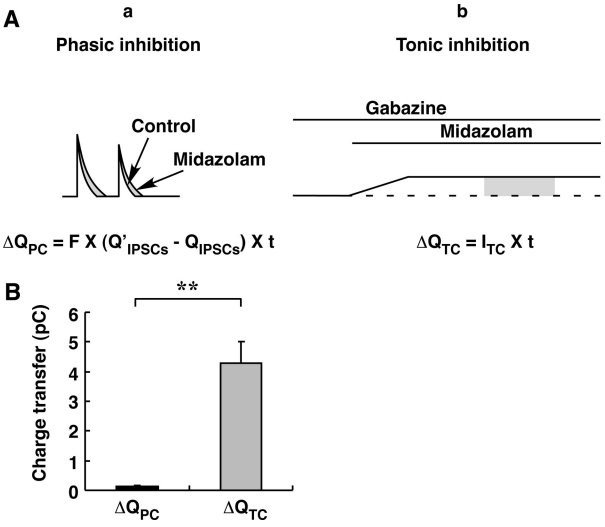

Fig. 5. An increase in charge transfer of γ-aminobutyric acid (GABA)ergic phasic (inhibitory postsynaptic currents [IPSCs]) and tonic inhibitory currents induced by administration of midazolam. (*A*) Schematic diagram and equations illustrating the methods for calculating charge transfer. The *gray areas* are increases in charge transfer of phasic current (*a*, ΔQ_{PC}) and tonic current (*b*, ΔQ_{TC}) induced by midazolam (5 μM). For details of equations, see Materials and Methods and Data Analysis. (*B*) Summary of ΔQ_{PC} (0.14 ± 0.02 pC, n = 18) and ΔQ_{TC} (4.3 ± 0.7 pC, n = 7). There was a significant difference between ΔQ_{PC} and ΔQ_{TC} ($^{**}P < 0.01$).

Effects of Noradrenaline and Midazolam on Inhibitory Currents

Effects of noradrenaline were examined in 10 of 14 (71%) SG neurons that showed the midazolam-induced tonic inhibitory currents. Administration of noradrenaline (20 μM, for 1 min) increased both frequency and amplitude of GABAergic IPSCs (fig. 6Aa, control frequency and amplitude, 3.8 ± 0.5 Hz and 16.9 ± 1.3 pA, respectively; noradrenaline, 14.6 ± 1.8 Hz and 20.6 ± 2.1 pA) in the presence of strychnine. Lower traces show IPSCs in the expanded time scale before (fig. 6Aa1, control) and during noradrenaline application (fig. 6Aa2). After the responses were completely abolished, midazolam (5 μM) was applied for 3 min, and then noradrenaline (20 μM) was again administered for 1 min together with midazolam (fig. 6Ab). The interval of noradrenaline applications was more than 10 min. As shown in figure 6Ab, noradrenaline again increased frequency and amplitude of IPSCs (14.8 ± 1.1 Hz and 20.7 ± 1.6 pA, n = 10), but there were no differences from those during noradrenaline only. Nevertheless, baseline current shifted upward by the simultaneous application of noradrenaline and midazolam (figs. 6Ab1 and b2). The total areas of the currents during control and after noradrenaline application were 0.11 ± 0.02 and 0.86 ± 0.15 × 10^6 pA· ms ($P < 0.01$), respectively. Conversely, the area after simultaneous application of noradrenaline and midazolam was 1.5 ± 0.22 × 10^6 pA·ms, which was significantly larger than noradrenaline only ($P < 0.01$) and much greater than the sum of areas after the administration of noradrenaline (0.86 × 10^6) and mida-

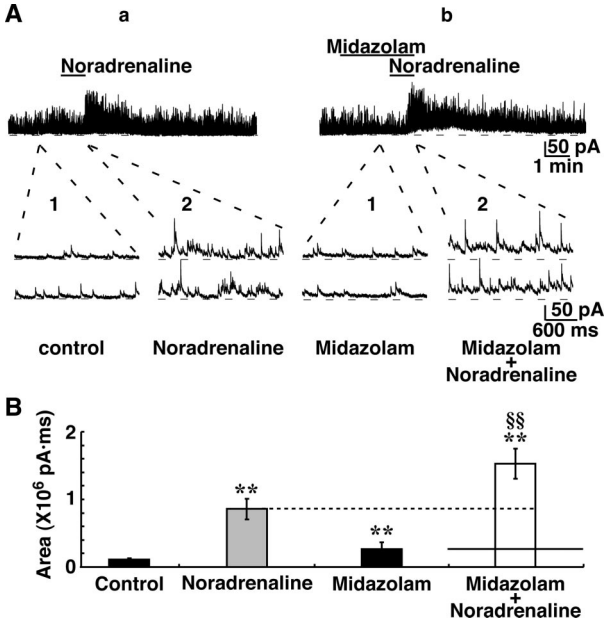

Fig. 6. Effects of coapplication of noradrenaline and midazolam on inhibitory currents. (*A*) Application of noradrenaline (20 μM) increased frequency and amplitude of inhibitory postsynaptic currents (*a*). Slight outward shift was observed after application of noradrenaline. Lower two consecutive traces in the expanded time scale were before (*a1*) and during application of noradrenaline (*a2*). *Horizontal dashed lines* indicate a level of baseline current before noradrenaline. Application of noradrenaline (20 μM) in the presence of midazolam (5 μM) again increased frequency and amplitude of inhibitory postsynaptic currents (*b*). Although the increases in frequency and amplitude were not different from noradrenaline only (see Results), an apparent outward shift of the baseline current was observed. Lower two consecutive traces were before (*b1*) and during application of noradrenaline (*b2*). The holding membrane potential was 0 mV. (*Aa*, *Ab*) Same neuron. (*B*) Summary of total areas under currents of control during application of noradrenaline, midazolam (5 μM), and noradrenaline in the presence of midazolam. Note that difference in areas between noradrenaline in the presence of midazolam and noradrenaline only is much larger than area during midazolam only. Each *bar*, n = 10; ** *versus* control, $P < 0.01$, and §§ *versus* noradrenaline only, $P < 0.01$.

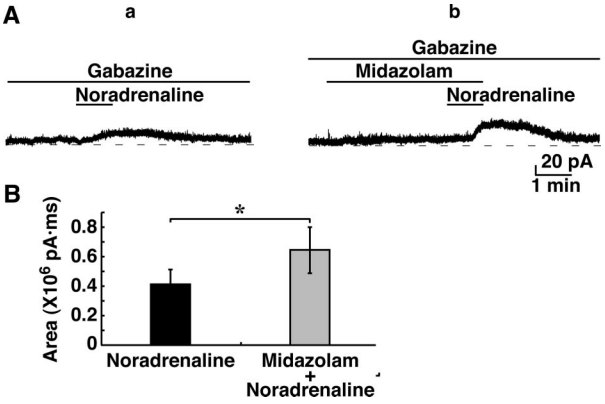

Fig. 7. Effects of coapplication of noradrenaline and midazolam on inhibitory tonic currents. (*A*) In the presence of strychnine (2 μM) and gabazine (1 μM), application of noradrenaline (20 μM) induced an outward current (*a*). The same dose of noradrenaline induced larger outward current when midazolam (5 μM) was coapplied with noradrenaline (*b*). (*Aa*, *Ab*) Same neuron. The holding membrane potential was 0 mV. (*B*) Summary of total areas under tonic currents. There was a significant difference between areas of noradrenaline only and coapplication of noradrenaline and midazolam. Each *bar*, n = 9, and * $P < 0.05$.

zolam ($0.27 \pm 0.05 \times 10^6$ pA·ms) separately (fig. 6B, n = 10). The significant difference between the total areas following the coapplication and noradrenaline only was abolished in the presence of flumazenil (1 μM, n = 4, data not shown), indicating that the effect of midazolam was mediated by $GABA_A$ receptors.

Increase in Noradrenaline-induced Tonic Inhibitory Currents by Midazolam

The total area under the currents includes both IPSCs (phasic currents) and tonic currents. Therefore, to confirm that the increase in the area after simultaneous application of noradrenaline and midazolam was due to an activation of GABAergic tonic current, noradrenaline and midazolam were again administered in the presence of gabazine and strychnine. As shown in figure 7Aa, an application of noradrenaline, which was performed for 1 min after IPSCs were completely blocked by gabazine, induced an outward shift of the baseline current (tonic current). When noradrenaline was administered together with midazolam, the outward current was larger than after the administration of noradrenaline only (fig. 7Ab). There was a significant difference in areas between noradrenaline only ($0.41 \pm 0.10 \times 10^6$) and coapplication of noradrenaline and midazolam ($0.64 \pm 0.16 \times 10^6$ pA · ms, n = 9, $P < 0.05$; fig. 7B).

Discussion

To investigate the direct effects of midazolam on extrasynaptic GABAergic currents, we first sought to distinguish pharmacologically the synaptic and extrasynaptic currents. $GABA_A$ receptors are known to be pentameric assemblies of subunits that form a central ion channel.[27] Nineteen $GABA_A$ receptor subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3) have been cloned from the mammalian central nervous system,[13,28] consequently numerous combinations of $GABA_A$ receptors have been synthesized. However, $GABA_A$ receptors of only a few subunit combinations have been shown to exist.[13,29,30] Particularly, the receptors containing a γ2 subunit with α1, α2, or α3 subunit are predominantly present in the synaptic clefts, whereas receptors that contain α4, α5, or α6 subunit predominantly or exclusively exist in extrasynaptic sites.[13] The composition of subunits, especially the difference of α subunits, determines their pharmacologic and electrophysiologic properties.[31–34] It has been reported that gabazine is a competitive drug for bicuculline, a nonselective $GABA_A$ receptor antagonist, but a submicromolar concen-

tration of gabazine selectively blocks synaptic currents in the hippocampal and cortical neurons.[23–25] Therefore, we tested whether a low concentration of gabazine blocked only synaptic currents or not in SG neurons. As shown in figure 1, gabazine (1 μM) completely abolished synaptic currents (IPSCs) without any changes in baseline current. However, the simultaneous application of bicuculline (20 μM) produced an inward current, indicating the presence of intrinsic GABA acting on extrasynaptic $GABA_A$ receptors. The bicuculline-induced inward current might result from a blockade of the outward GABA current. Furthermore, bath application of GABA (1 mM) evoked an outward current in the presence of gabazine (1 μM) but not in the presence of 20 μM bicuculline. These findings suggest that this concentration of gabazine blocks only synaptic IPSCs without affecting extrasynaptic GABAergic current. The difference in the effects of gabazine and bicuculline is considered to be due to the different subunit compositions between synaptic and extrasynaptic $GABA_A$ receptors.[13,35] Thus, the effects of midazolam and noradrenaline on extrasynaptic currents were investigated in the presence of 1 μM gabazine in the current study.

Effects of Midazolam on Extrasynaptic GABAergic Currents in SG Neurons

Several studies have demonstrated that intrathecal administration of midazolam produces analgesic effects in human[36–38] and rats.[39–41] In the current study, it was demonstrated that midazolam evoked an outward current in more than half of the SG neurons tested in the presence of strychnine, an antagonist of glycine receptors (fig. 2), suggesting an inhibitory influence of midazolam on noxious transmission. The similar amplitude of the midazolam-induced outward current was again observed in the presence of gabazine (fig. 3), indicating that the outward baseline shift was not due to the summation IPSCs but due to an activation of extrasynaptic $GABA_A$ receptors. Because midazolam does not directly activate $GABA_A$ receptors but potentiates GABA–$GABA_A$ receptor affinity,[19] it is suggested that the midazolam-induced outward current is caused by GABA in the extrasynaptic space. Several reports have shown that ambient GABA concentration was controlled by spillover from inhibitory synaptic cleft[42,43] or reverse operation of GABA cotransporters located on the neurons and astrocytes.[44,45] The presence of intrinsic GABA in the extrasynaptic space in the spinal cord slices has been demonstrated by the bicuculline-induced inward currents in mice[14,35] and rats in the current study (fig. 1).

Difference in Effects of Midazolam on Extrasynaptic and Synaptic GABAergic Currents in SG Neurons

Midazolam prolonged the decay-time constant but did not affect the frequency or amplitude of synaptic GABAergic currents (IPSCs; fig. 4). It is suggested that this effect is also due to a potentiation of GABA–$GABA_A$ receptor affinity at the benzodiazepine sites of synaptic $GABA_A$ receptors, as reported previously.[18,26] However, the midazolam-induced increase in charge transfers through the extrasynaptic GABAergic receptors (δQ_{TC}, average, 4.3 pC) was about 30 times greater than that through the synaptic receptors (δQ_{PC}, 0.14 pC; fig. 5). Therefore, it is possible that the effect of midazolam on extrasynaptic $GABA_A$ receptors may play an important role in analgesia induced by the intrathecal midazolam, which has been shown in clinical[36–38] and experimental[39–41] studies. It seems to be difficult to maintain a 5 μM or more concentration of midazolam in the spinal cord by intravenous injection because of its effects on the cardiovascular system. According to the previous studies, however, intrathecal injection of midazolam can achieve this concentration without hemodynamic change or other marked side effect.[36–38] As far as our results, the EC_{50} for the midazolam-induced outward current was approximately 2 μM (fig. 3). Further studies may be required to define the appropriate dose for the clinical intrathecal analgesia.

Effects of Coapplication of Midazolam and Noradrenaline

Noradrenaline is a representative neurotransmitter of the descending inhibitory system that acts on presynaptic and postsynaptic neurons in the dorsal horn of the spinal cord, thereby modulating nociceptive transmission. In the current study, the application of noradrenaline increased both frequency and amplitude of IPSCs (fig. 6Aa). These findings are consistent with previous studies showing that noradrenaline excites GABAergic neurons through somatodendritic α1-adrenergic receptors[21] and increases frequency of miniature IPSCs also through α_1 receptors located at presynaptic terminals.[20] In addition, the application of noradrenaline induced an outward current in the presence of gabazine (fig. 7Aa). Because the noradrenaline-induced outward current was observed in the presence of blockers of K^+ channels and G protein-coupled receptors in the current study, it is likely that the outward current was not induced by postsynaptic α_2-adrenergic receptors in the SG neurons as reported previously,[46] but because of an activation of extrasynaptic GABAergic receptors such as midazolam. However, the total area of the noradrenaline-induced outward current in the absence of gabazine, which included both synaptic and extrasynaptic currents, was 0.86×10^6 pA · ms (fig. 6B), whereas that of extrasynaptic current in the presence of gabazine was 0.41×10^6 pA·ms (fig. 7B). Thus, the activation ratio of extrasynaptic to synaptic currents during noradrenaline application was $0.41/(0.86 - 0.41) = 0.91$, suggesting that the effects of noradrenaline on synaptic and extrasynaptic currents were almost equal. Conversely, the effects of midazolam on extrasynaptic and synaptic currents calculated by changes in charge transfer were 4.3 pC (δQ_{TC}) and 0.14 pC (δQ_{PC}), respectively, and the ratio was more than 30 as mentioned in the previous section.

Finally, we applied noradrenaline in the presence of midazolam and found that the total area under the current (1.5×10^6 pA·ms) was much greater than the sum of areas during application of noradrenaline (0.86×10^6) and mida-

zolam (0.27×10^6 pA · ms), separately. Because the increases in frequency and amplitude were not affected by the simultaneous application of midazolam (fig. 6), it was suggested that an application of noradrenaline enhanced the midazolam-induced extrasynaptic inhibitory current. This was confirmed by the finding that the outward current induced by noradrenaline was increased significantly by the simultaneous application of midazolam in the presence of gabazine, which blocked synaptic GABAergic IPSCs (fig. 7). Previous studies have shown that the concentration of extrasynaptic GABA can be changed by synaptic[42,43,47] and nonsynaptic (activity of neuron–astrocyte unit)[44,45,48,49] origin of GABA. It is likely that noradrenaline excites GABAergic neurons and increases the possibility of GABA release from the presynaptic terminals through α_1-adrenergic receptors,[20,21] thereby increasing the ambient GABA concentration because of more frequent spillover of GABA from inhibitory synapses. In addition, it is suggested that monoamines such as noradrenaline can induce GABA release from the astrocytes into the extracellular space.[49] Either way, noradrenaline increases ambient GABA concentration, resulting in an enhancement of extrasynaptic inhibitory current in the presence of midazolam.

In conclusion, our results demonstrated that midazolam had greater effects on extrasynaptic $GABA_A$ receptors than the synaptic ones, suggesting a predominant role of the enhancement of GABAergic extrasynaptic currents in midazolam-induced analgesia. In addition, the noradrenaline-induced increase in ambient GABA concentration acts to enhance the extrasynaptic GABAergic currents in the SG of the spinal cord. Although the interaction of midazolam with noradrenaline should be further examined using *in vivo* preparations, the current findings suggest a clinical availability of intrathecal administration of midazolam and a drug that increases GABA release such as noradrenaline.

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