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

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Abbreviated Title: GABAERGIC CURRENTS BY MIDAZOLAM AND NORADRENALINE

Summary Statement: Midazolam produces extrasynaptic γ-aminobutyric acid currents in substantia gelatinosa neurons, which may explain the midazolam-induced analgesia. Simultaneous application of noradrenaline further enhances midazolam-induced extrasynaptic currents by increasing extracellular γ-aminobutyric acid concentration.

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 spinal cord slices from rats, midazolam enhanced tonic, extra-synaptic $GABA_A$ receptor currents and co-application of norepinephrine, which increases GABA release, further enhanced these inhibitory currents

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 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/or 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, either frequency or amplitude was not affected by midazolam. In contrast, application of noradrenaline markedly increased both frequency and amplitude of synaptic currents

with a slight enhancement of tonic currents. Co-application 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. Since 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.

Introduction

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, 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 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 system projecting from the brain stem, 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 electrophysiological studies have shown that GABA acts on extrasynaptic GABA type

A ($GABA_A$) receptors, which causes extrasynaptic/tonic currents (inhibition)[13,14], while synaptic $GABA_A$ receptors produce synaptic/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 elevates 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 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 SG neurons. Since 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. On the other hand, 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 effects of midazolam. In the present study, we first divided GABAergic inhibitory currents into synaptic and extrasynaptic ones, 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 co-application 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 exsanguinations. After the dura mater, the ventral and dorsal roots and the pia-arachnoid membrane were removed, a 500-600 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 0.5ml, 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 (mM): NaCl 117, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11.

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 (mM): Cs_2SO_4 110, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, tetraethylammonium 5 and ATP-Mg 5 (pH 7.2), for inhibiting post synaptic effects on K^+ channels. In addition, 2 mM guanosine-5'-O-(2-thiodiphosphate) (GDP-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 pCLMP 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 sec. Drugs perfused in this study were midazolam (Wako, Osaka, Japan), flumazenil (Wako), noradrenaline (Wako), strychnine (Sigma-Aldrich, St Louis, MO), bicuculline (Sigma-Aldrich), GDP-β-S (Sigma-Aldrich), and gabazine (Tocris Cookson, Bristol, United Kingdom), and GABA (Sigma-Aldrich).

Data Analysis

To measure the baseline current of each trace, 30 sec 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 sec using Mini analysis program (Synaptsoft, Decatur, GA). The change in charge transfer for 1 sec 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 sec. 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 sec-recordings after drug application and t is 1 sec. The average of the total area under IPSCs was calculated by recordings for 60 sec before drug application using pClamp 10.1 software (Axon Instruments). Then the area was again measured for 60 sec during peak of the

response after drug administration. All numerical data were expressed as the mean ± standard error (S.E.M.). Statistical significance was determined as $P<0.05$ using Student's paired *t* test to compare the frequency, amplitude, decay time constant and baseline shift. Student's un-paired *t* test was used to compare 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 of 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 owing to 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 Fig. 1A, bath application of gabazine (1 μM) suppressed IPSCs without significant changes in basal current (box a and b). Expanded time scale traces before (a) and after (b) application of gabazine were shown

in Fig. 1B, which correspond to Fig. 1Aa and Ab, respectively. In 6 of 8 (75%) neurons examined co-administration of bicuculline (20 μM, for 3 min) induced a slow inward current as shown in Fig. 1Ac. The mean current shown in Fig. 1Ca, b, and c correspond to a-c in Fig. 1A, respectively. The peak of all point histogram shifted to more negative values (Fig. 1Cd). The baseline shift following additional application of bicuculline (9.5 ± 3.2 pA, $P<0.05$, Student's 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 (less than 10 μM) on the baseline current, as reported previously[23-25].

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, Fig. 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 Fig. 2A and B, upward shift of all points count might include changes in the decay time of IPSCs. Thus we again examined effects of midazolam in the presence of gabazine (1 μM) to block IPSCs evoked by synaptic $GABA_A$ receptors. As shown in Fig. 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 Fig. 3Ba and b correspond to a and b in Fig. 3A, respectively. All points count were demonstrated in Fig. 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). Fig. 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 Fig. 4A, either frequency or amplitude of IPSCs (a, control) was not affected by perfusion with midazolam (5 μM, for 3 min, b). 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 application of midazolam (Fig. 4B), as reported previously[18,26]. Fig. 4C shows the summary of the relative frequency, amplitude and decay time following midazolam.

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 sec, t) (Fig. 5Aa), while that for tonic current (ΔQ_{TC}) from baseline shift of the tonic currents (I_{TC}) and 1 sec (t) (Fig. 5Ab). As shown in Fig. 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's t-test).

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 amplitude and frequency of GABAergic IPSCs (Fig. 6Aa,

control amplitude and frequency, 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 (a1, control) and during noradrenaline application (a2). After the responses were completely abolished, midazolam (5 μM) was applied for 3 min, then noradrenaline (20 μM) was again administered for 1min together with midazolam (Fig. 6Ab). The interval of noradrenaline applications was more than 10 min. As shown in Fig. 6Ab, noradrenaline again increased amplitude and frequency 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 (b1 and b2). The total areas of the currents during control and after noradrenaline application were 0.11 ± 0.02 and 0.86 ± 0.15 $\times 10^6$ pA·ms ($P<0.01$), respectively. On the other hand, the area after simultaneous application of noradrenaline and midazolam was 1.5 ± 0.22 $\times 10^6$ pA·ms, which was significantly larger than noradrenaline only ($P<0.01$) and much greater than the sum of areas following noradrenaline (0.86 $\times 10^6$) and midazolam (0.27 ± 0.05 $\times 10^6$ pA·ms)

administered separately (Fig. 6B, n = 10). The significant difference between the total areas following the co-application 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 following 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 Fig. 7Aa, an application of noradrenaline, which was performed for 1min 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 that following noradrenaline only (Fig. 7Ab). There was a significant difference in areas between noradrenaline only ($0.41 \pm 0.10 \times 10^6$) and co-application of noradrenaline and

midazolam ($0.64 \pm 0.16 \times 10^6$ pA·ms, n = 9, $P<0.05$, Fig. 7B).

Discussion

To investigate 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 being synthesized numerous combinations of $GABA_A$ receptors. However, $GABA_A$ receptors of only a few subunits combinations have been shown to exist[13,29,30]. Particularly, receptors containing a γ2 subunit with α1, α2 or α3 subunit are predominantly present in synaptic clefts, while receptors that contain α4, α5 or α6 subunit predominantly or exclusively exist in extrasynaptic sites[13]. The composition of subunits, especially difference of α subunits, makes their pharmacological and electrophysiological properties[31-34]. It has been reported that gabazine is a competitive drug for bicuculline, a non-selective $GABA_A$ receptor antagonist, but a sub-micromolar concentration of gabazine selectively blocks synaptic currents in hippocampal and cortical neurons[23-25]. Therefore, we tested whether low concentration of gabazine

blocked only synaptic currents or not in SG neurons. As shown in Fig. 1, gabazine (1 μM) completely abolished synaptic currents (IPSCs) without any changes in baseline current. However, 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/or noradrenaline on extrasynaptic currents were investigated in the presence of 1 μM gabazine in the present 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 present study it was demonstrated that midazolam evoked an outward current in more than half of 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. Since midazolam does not directly activate $GABA_A$ receptors but potentiates GABA-$GABA_A$ receptors 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 spill-over from inhibitory synaptic cleft[42,43] or reverse operation of GABA co-transporters located on neurons and/or 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], as well as in rats in the present study (Fig. 1).

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

Midazolam prolonged the decay time constant but didn't affect 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 at 5 μM or more concentration of midazolam in the spinal cord by the intravenous injection, because of its effects on the cardio-vascular system. According to 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 about 2 μM (Fig. 3). Further studies may be required to define the appropriate dose for the clinical intrathecal analgesia.

Effects of Co-application of Midazolam and Noradrenaline

Noradrenaline is a representative neurotransmitter of the descending inhibitory system that acts on pre- and postsynaptic neurons in the dorsal horn of the spinal cord, thereby modulating nociceptive transmission. In the present study, 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, application of noradrenaline induced an outward current in the presence of gabazine (Fig. 7Aa). Since the noradrenaline-induced outward current was observed in the presence of blockers of K^+ channels and G-protein coupled receptors in the present study, it is likely that the outward current was not induced by postsynaptic α_2 adrenergic receptors in SG neurons reported previously[46], but due to an

activation of extrasynaptic GABAergic receptors like 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), while 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. On the other hand, 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 midazolam (0.27×10^6 pA·ms), separately. Since 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 significantly increased by the simultaneous application of midazolam in the presence of gabazine, which blocked synaptic GABAergic IPSCs (Fig. 7). Previous studies have shown that concentration of extrasynaptic GABA can be changed by synaptic[42,43,47] and/or non-synaptic (activity of neuron-astrocyte unit)[44,45,48,49] origin of GABA. It is likely that noradrenaline excites GABAergic neurons and increases possibility of GABA release from the presynaptic terminals through α_1 adrenergic receptors[20,21], thereby increasing ambient GABA concentration due to more frequent spillover of GABA from inhibitory synapses. In addition, it is suggested that monoamines such as noradrenaline can induce GABA release from astrocytes into the extracellular space[49]. In 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 predominant role of the enhancement of GABAergic extrasynaptic currents in the 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 present findings suggest a clinical availability of intrathecal administration of midazolam and a drug that increases GABA release like noradrenaline.

References

1. Yoshimura M, Jessell TM: Primary afferent-evoked synaptic responses and slow potential generation in rat substantia gelatinosa neurons in vitro. J Neurophysiol 1989; 62: 96-108

2. Kumazawa T, Perl ER: Excitation of marginal and substantia gelatinosa neurons in the primate spinal cord: Indications of their place in dorsal horn functional organization. J Comp Neurol 1978; 177: 417-34

3. Sugiura Y, Lee CL, Perl ER: Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin. Science 1986; 234: 358-61

4. Todd AJ, McKenzie J: GABA-immunoreactive neurons in the dorsal horn of the rat spinal cord. Neuroscience 1989; 31: 799-806

5. Todd AJ, Sullivan AC: Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. J Comp Neurol 1990; 296: 496-505

6. Hantman AW, Perl ER: Molecular and genetic features of a labeled

class of spinal substantia gelatinosa neurons in a transgenic mouse. J Comp Neurol 2005; 492: 90-100

7. Hantman AW, van den Pol AN, Perl ER: Morphological and physiological features of a set of spinal substantia gelatinosa neurons defined by green fluorescent protein expression. J Neurosci 2004; 24: 836-42

8. Yoshimura M, Nishi S: Primary afferent-evoked glycine- and GABA-mediated IPSPs in substantia gelatinosa neurones in the rat spinal cord in vitro. J Physiol 1995; 482 (Pt 1): 29-38

9. Hagihira S, Senba E, Yoshida S, Tohyama M, Yoshiya I: Fine structure of noradrenergic terminals and their synapses in the rat spinal dorsal horn: An immunohistochemical study. Brain Res 1990; 526: 73-80

10. Rajaofetra N, Ridet JL, Poulat P, Marlier L, Sandillon F, Geffard M, Privat A: Immunocytochemical mapping of noradrenergic projections to the rat spinal cord with an antiserum against noradrenaline. J Neurocytol 1992; 21: 481-94

11. Satoh K, Kashiba A, Kimura H, Maeda T: Noradrenergic axon terminals in the substantia gelatinosa of the rat spinal cord: An electron-microscopic

study using glyoxylic acid-potassium permanganate fixation. Cell Tissue Res 1982; 222: 359-78

12. Westlund KN, Bowker RM, Ziegler MG, Coulter JD: Descending noradrenergic projections and their spinal terminations. Prog Brain Res 1982; 57: 219-38

13. Farrant M, Nusser Z: Variations on an inhibitory theme: Phasic and tonic activation of $GABA_A$ receptors. Nat Rev Neurosci 2005; 6: 215-29

14. Ataka T, Gu JG: Relationship between tonic inhibitory currents and phasic inhibitory activity in the spinal cord lamina II region of adult mice. Mol Pain 2006; 2: 36

15. Overstreet LS, Westbrook GL: Paradoxical reduction of synaptic inhibition by vigabatrin. J Neurophysiol 2001; 86: 596-603

16. Hanson SM, Czajkowski C: Structural mechanisms underlying benzodiazepine modulation of the $GABA_A$ receptor. J Neurosci 2008; 28: 3490-9

17. Olsen RW, Chang CS, Li G, Hanchar HJ, Wallner M: Fishing for allosteric sites on $GABA_A$ receptors. Biochem Pharmacol 2004; 68: 1675-84

18. Kohno T, Kumamoto E, Baba H, Ataka T, Okamoto M, Shimoji K, Yoshimura M: Actions of midazolam on GABAergic transmission in substantia gelatinosa neurons of adult rat spinal cord slices. Anesthesiology 2000; 92: 507-15

19. Lavoie AM, Twyman RE: Direct evidence for diazepam modulation of $GABA_A$ receptor microscopic affinity. Neuropharmacology 1996; 35: 1383-92

20. Baba H, Shimoji K, Yoshimura M: Norepinephrine facilitates inhibitory transmission in substantia gelatinosa of adult rat spinal cord (part 1): Effects on axon terminals of GABAergic and glycinergic neurons. Anesthesiology 2000; 92: 473-84

21. Baba H, Goldstein PA, Okamoto M, Kohno T, Ataka T, Yoshimura M, Shimoji K: Norepinephrine facilitates inhibitory transmission in substantia gelatinosa of adult rat spinal cord (part 2): Effects on somatodendritic sites of GABAergic neurons. Anesthesiology 2000; 92: 485-92

22. Yajiri Y, Yoshimura M, Okamoto M, Takahashi H, Higashi H: A novel slow excitatory postsynaptic current in substantia gelatinosa neurons of the rat spinal cord in vitro. Neuroscience 1997; 76: 673-88

23. Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF, Orser BA: Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by γ-aminobutyric $acid_A$ receptors in hippocampal neurons. Mol Pharmacol 2001; 59: 814-24

24. Semyanov A, Walker MC, Kullmann DM: GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. Nat Neurosci 2003; 6: 484-90

25. Yeung JY, Canning KJ, Zhu G, Pennefather P, MacDonald JF, Orser BA: Tonically activated $GABA_A$ receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. Mol Pharmacol 2003; 63: 2-8

26. Takahashi A, Tokunaga A, Yamanaka H, Mashimo T, Noguchi K, Uchida I: Two types of GABAergic miniature inhibitory postsynaptic currents in mouse substantia gelatinosa neurons. Eur J Pharmacol 2006; 553: 120-8

27. Lester HA, Dibas MI, Dahan DS, Leite JF, Dougherty DA: Cys-loop receptors: New twists and turns. Trends Neurosci 2004; 27: 329-36

28. Simon J, Wakimoto H, Fujita N, Lalande M, Barnard EA: Analysis

of the set of $GABA_A$ receptor genes in the human genome. J Biol Chem 2004; 279: 41422-35

29. Wisden W, Laurie DJ, Monyer H, Seeburg PH: The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci 1992; 12: 1040-62

30. Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G: $GABA_A$ receptors: Immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 2000; 101: 815-50

31. Sieghart W: Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. Pharmacol Rev 1995; 47: 181-234

32. Bohme I, Rabe H, Luddens H: Four amino acids in the alpha subunits determine the γ-aminobutyric acid sensitivities of $GABA_A$ receptor subtypes. J Biol Chem 2004; 279: 35193-200

33. Colquhoun D: Binding, gating, affinity and efficacy: The interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. Br J Pharmacol 1998; 125: 924-47

34. Minier F, Sigel E: Positioning of the α-subunit isoforms confers a functional signature to γ-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 2004; 101: 7769-74

35. Takahashi A, Mashimo T, Uchida I: GABAergic tonic inhibition of substantia gelatinosa neurons in mouse spinal cord. Neuroreport 2006; 17: 1331-5

36. Bharti N, Madan R, Mohanty PR, Kaul HL: Intrathecal midazolam added to bupivacaine improves the duration and quality of spinal anaesthesia. Acta Anaesthesiol Scand 2003; 47: 1101-5

37. Ho KM, Ismail H: Use of intrathecal midazolam to improve perioperative analgesia: A meta-analysis. Anaesth Intensive Care 2008; 36: 365-73

38. Serrao JM, Marks RL, Morley SJ, Goodchild CS: Intrathecal midazolam for the treatment of chronic mechanical low back pain: A controlled comparison with epidural steroid in a pilot study. Pain 1992; 48: 5-12

39. Bahar M, Cohen ML, Grinshpon Y, Chanimov M: Spinal anaesthesia with midazolam in the rat. Can J Anaesth 1997; 44: 208-15

40. Goodchild CS, Serrao JM: Intrathecal midazolam in the rat:

Evidence for spinally-mediated analgesia. Br J Anaesth 1987; 59: 1563-70

41. Shih A, Miletic V, Miletic G, Smith LJ: Midazolam administration reverses thermal hyperalgesia and prevents gamma-aminobutyric acid transporter loss in a rodent model of neuropathic pain. Anesth Analg 2008; 106: 1296-302, table of contents

42. Isaacson JS, Solis JM, Nicoll RA: Local and diffuse synaptic actions of GABA in the hippocampus. Neuron 1993; 10: 165-75

43. Scanziani M: GABA spillover activates postsynaptic $GABA_B$ receptors to control rhythmic hippocampal activity. Neuron 2000; 25: 673-81

44. Liu QY, Schaffner AE, Chang YH, Maric D, Barker JL: Persistent activation of $GABA_A$ receptor/Cl^- channels by astrocyte-derived GABA in cultured embryonic rat hippocampal neurons. J Neurophysiol 2000; 84: 1392-403

45. Wu Y, Wang W, Richerson GB: GABA transaminase inhibition induces spontaneous and enhances depolarization-evoked GABA efflux via reversal of the GABA transporter. J Neurosci 2001; 21: 2630-9

46. North RA, Yoshimura M: The actions of noradrenaline on neurones

of the rat substantia gelatinosa in vitro. J Physiol 1984; 349: 43-55

47. Timmerman W, Westerink BH: Electrical stimulation of the substantia nigra reticulata: Detection of neuronal extracellular GABA in the ventromedial thalamus and its regulatory mechanism using microdialysis in awake rats. Synapse 1997; 26: 62-71

48. Attwell D, Barbour B, Szatkowski M: Nonvesicular release of neurotransmitter. Neuron 1993; 11: 401-7

49. Del Arco A, Segovia G, Fuxe K, Mora F: Changes in dialysate concentrations of glutamate and GABA in the brain: An index of volume transmission mediated actions? J Neurochem 2003; 85: 23-33

Figure legend

Figure 1. Effects of gabazine on γ-aminobutyric acid (GABA)-ergic inhibitory synaptic and extrasynaptic currents in substantia gelatinosa neurons. (A) In the presence of strychnine (2 μM), bath application of gabazine (1 μM) completely abolished inhibitory postsynaptic currents (box a and b) without any changes in baseline current. In contrast, 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 during box a (control) and box b (gabazine), indicating suppression of inhibitory postsynaptic currents by gabazine (1 μM). (C) Baseline currents in 30 sec 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 in d. (D) Relative baseline shifts produced by gabazine (-1.5 ± 1.3 pA) and bicuculline (-9.5 ± 3.2 pA, n = 6, *, $P<0.05$).

Figure 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 box a and b). The holding membrane potential was 0 mV. (B) The baseline currents for 30 sec 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 in c.

Figure 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 minutes. Slow outward current was induced by midazolam (dashed box a and b). The holding membrane potential was 0 mV. (B) The baseline currents for 30 sec 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 in c. (C) Dose-dependency of the midazolam-induced outward current. EC_{50} was 2.1 ± 0.03 μM (n = 7 at each point).

Figure 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 minutes (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 following application of midazolam (n = 18).

Figure 5. Increase in charge transfer of γ-aminobutyric acid (GABA)-ergic phasic (inhibitory postsynaptic currents) and tonic inhibitory currents induced by administration of midazolam. (A) Schematic diagram and equations illustrating methods for calculating charge transfer. The gray areas were increases in charge transfer of phasic current (a, ΔQ_{PC}) and tonic current (b, ΔQ_{TC}) induced by midazolam (5 μM). 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} (**, $P<0.01$).

Figure .6. Effects of co-application 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 following 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 and 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. **, vs. control, $P<0.01$, and §§, vs. noradrenaline only, $P<0.01$.

Figure. 7. Effects of co-application 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 co-applied with noradrenaline (b). Aa and 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 co-application of noradrenaline and midazolam. Each bar, n = 9, and *, $P<0.05$.

Figure .1

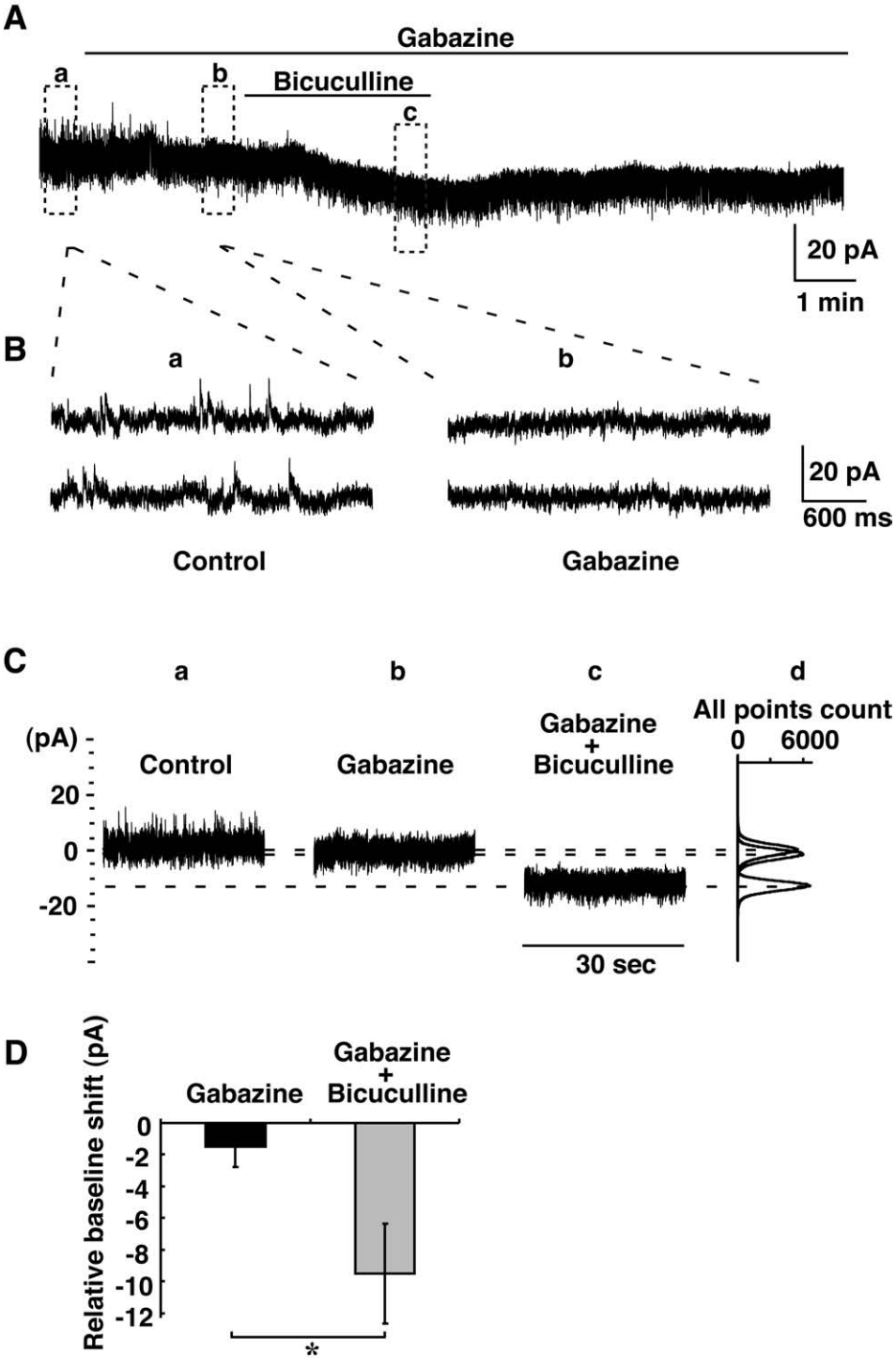

A
Gabazine
a
b
Bicuculline
c
20 pA
1 min
B
a
b
20 pA
600 ms
Control
Gabazine
C
a
b
c
d
All points count
0 6000
(pA)
Control
Gabazine
Gabazine
+
Bicuculline
20
0
-20
30 sec
D
Relative baseline shift (pA)
Gabazine
Gabazine
+
Bicuculline
0
-2
-4
-6
-8
-10
-12
*

Figure .2

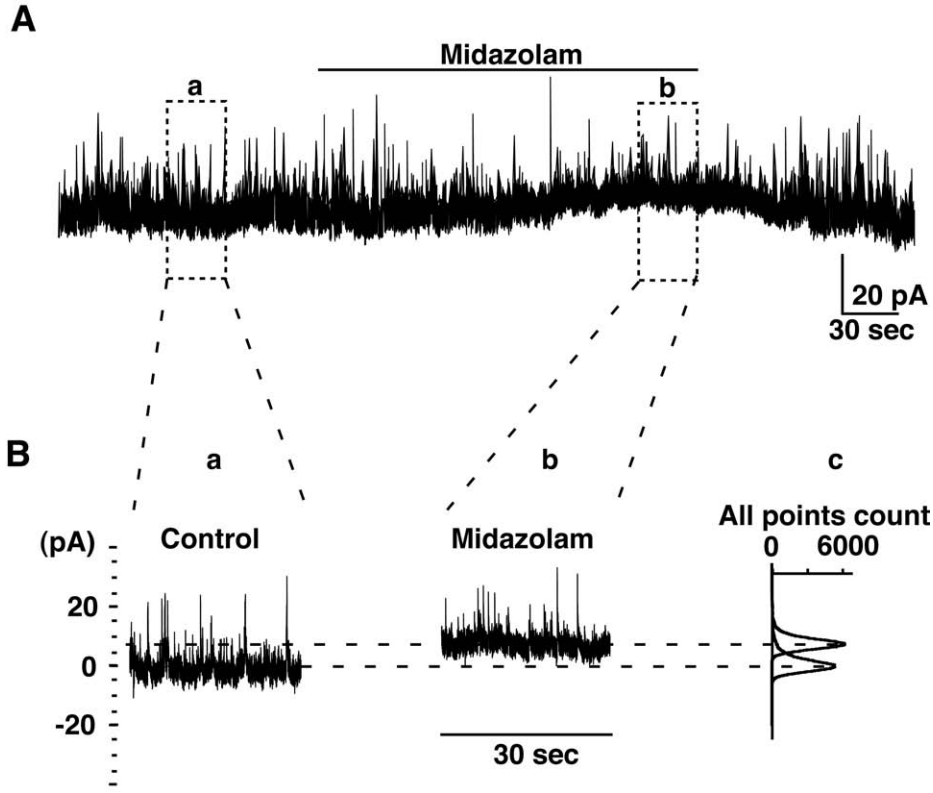

A
Midazolam
a
b
20 pA
30 sec
B
a
b
c
(pA)
Control
Midazolam
All points count
0
6000
20
0
-20
30 sec

Figure .3

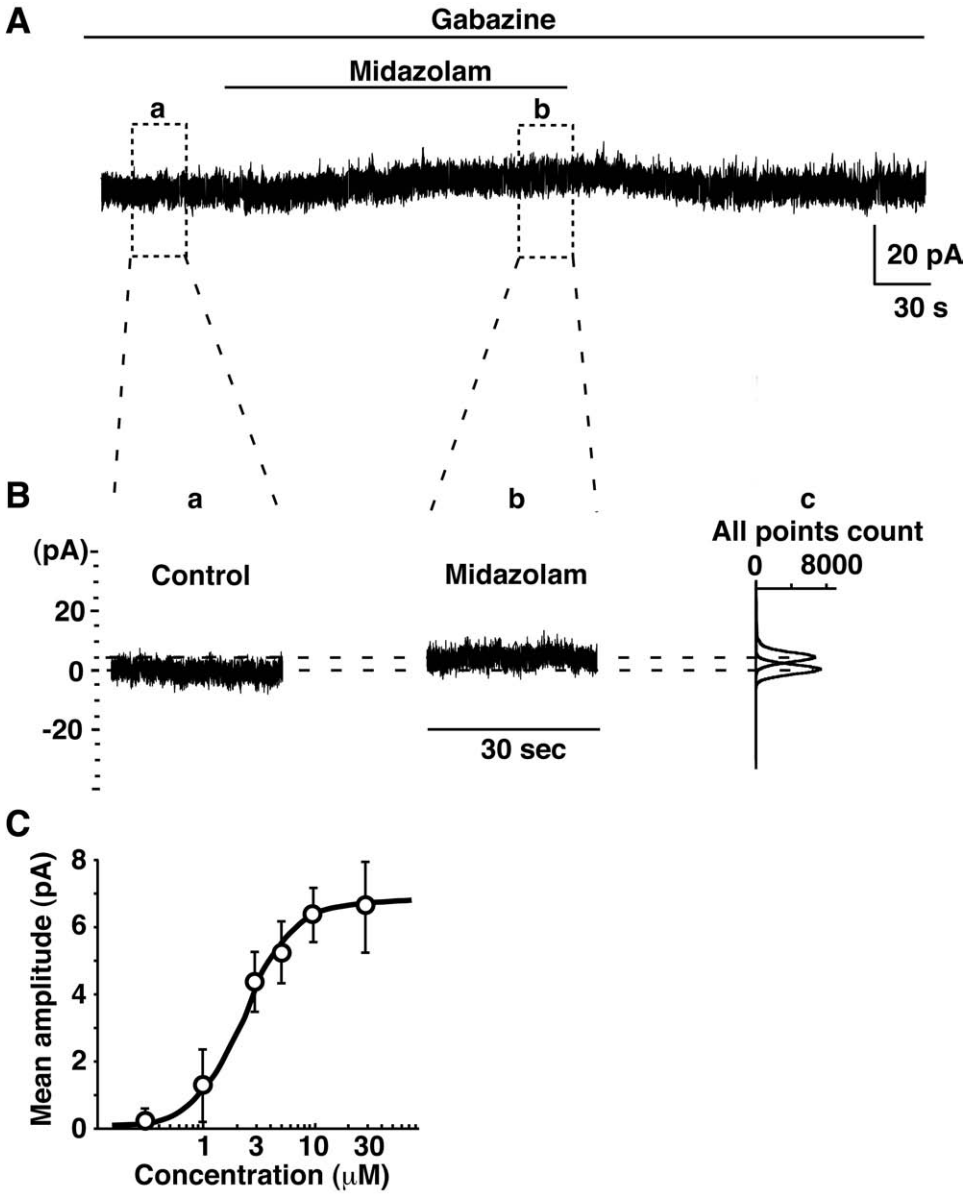

A
Gabazine
Midazolam
a
b
20 pA
30 s
B
a
b
c
All points count
0 8000
(pA)
20
0
-20
Control
Midazolam
30 sec
C
Mean amplitude (pA)
8
6
4
2
0
1 3 10 30
Concentration (μM)

Figure .4

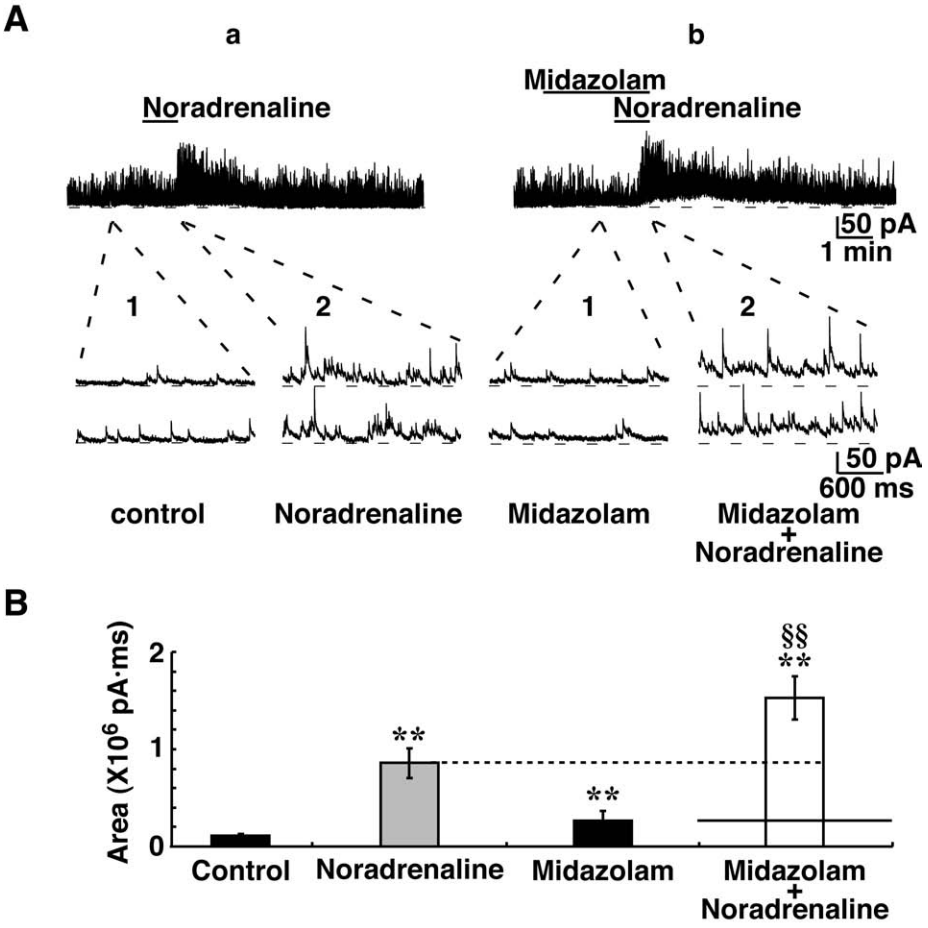

A
a
b
Noradrenaline
Midazolam
Noradrenaline
50 pA
1 min
1
2
1
2
50 pA
600 ms
control
Noradrenaline
Midazolam
Midazolam
+
Noradrenaline
B
Area (X10^6 pA·ms)
2
1
0
**
**
§§
**
Control
Noradrenaline
Midazolam
Midazolam
+
Noradrenaline

Figure .5

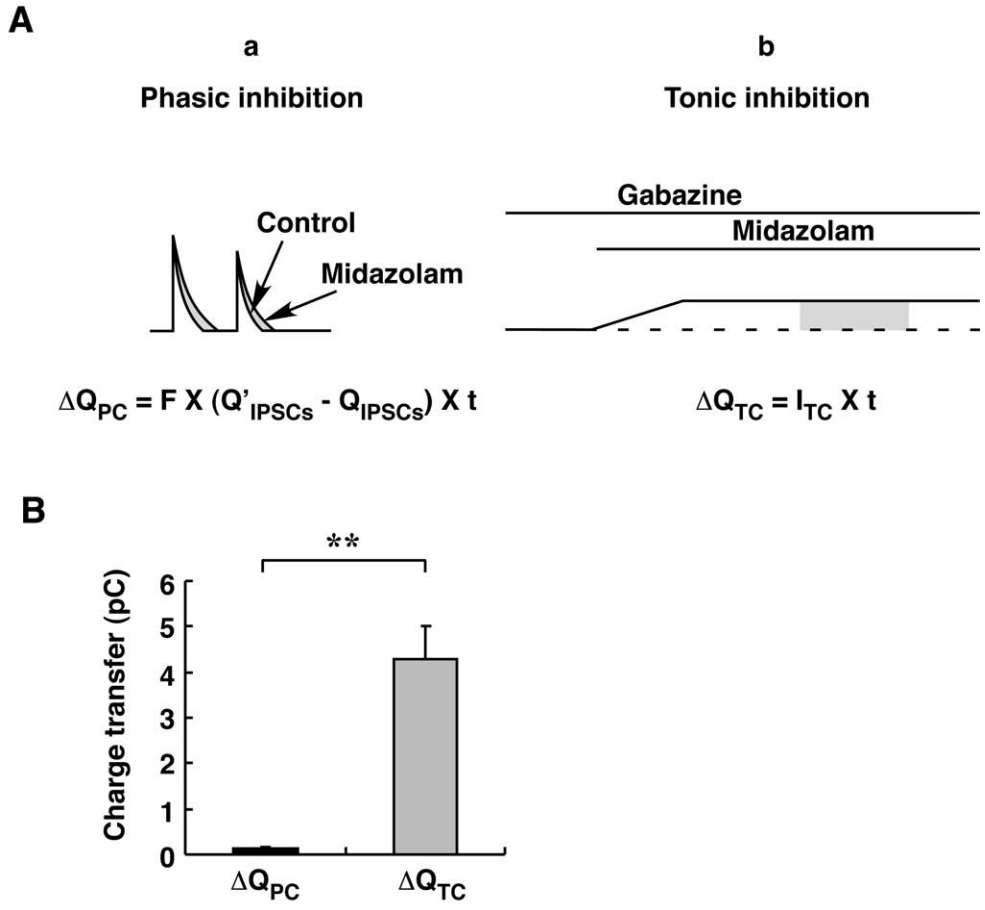

A
a
Phasic inhibition
b
Tonic inhibition
Control
Midazolam
Gabazine
Midazolam
$\Delta Q_{PC} = F \times (Q'_{IPSCs} - Q_{IPSCs}) \times t$
$\Delta Q_{TC} = I_{TC} \times t$
B
**
Charge transfer (pC)
0
1
2
3
4
5
6
ΔQ_{PC}
ΔQ_{TC}

Figure .6

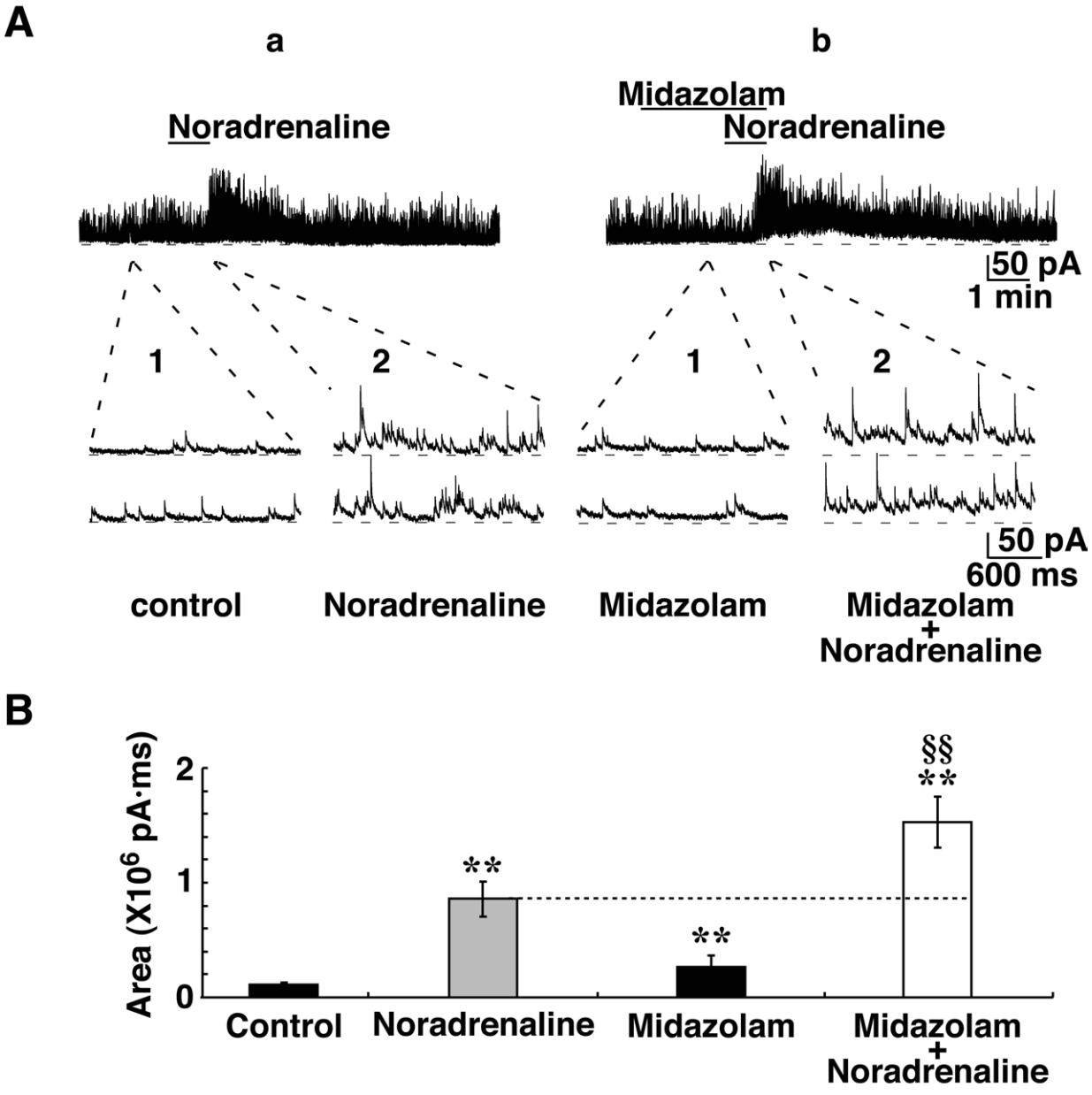
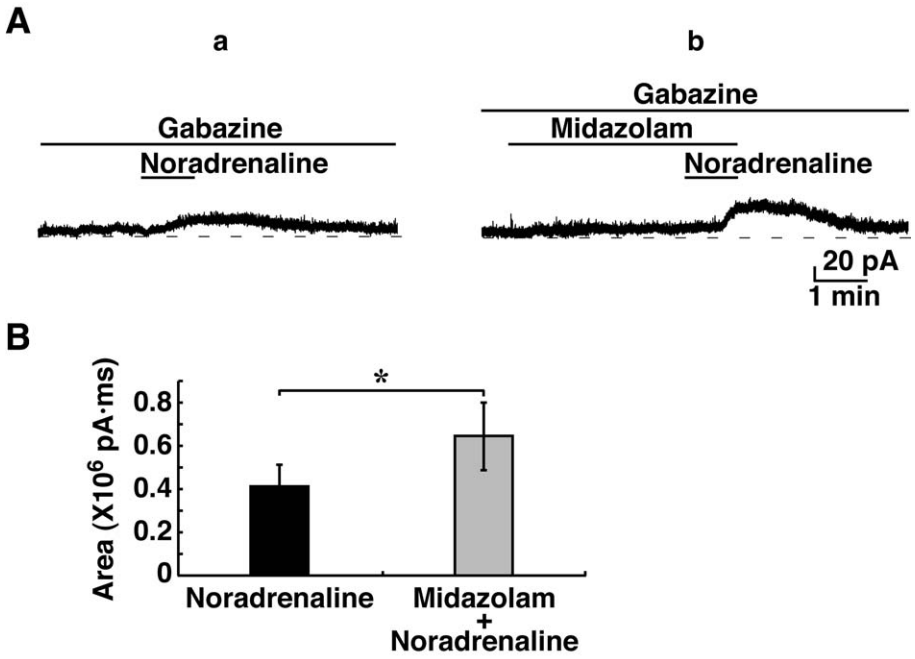

A
a
b
Noradrenaline
Midazolam
Noradrenaline
50 pA
1 min
1
2
1
2
50 pA
600 ms
control
Noradrenaline
Midazolam
Midazolam
+
Noradrenaline
B
Area (X10^6 pA·ms)
2
1
0
**
**
§§
**
Control
Noradrenaline
Midazolam
Midazolam
+
Noradrenaline

Figure .7


A
a
b
Gabazine
Noradrenaline
Gabazine
Midazolam
Noradrenaline
20 pA
1 min
B
Area (X10^6 pA·ms)
0.8
0.6
0.4
0.2
0
*
Noradrenaline
Midazolam
+
Noradrenaline