

# Bupivacaine Inhibits Glutamatergic Transmission in Spinal Dorsal Horn Neurons

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## ABSTRACT

**Background:** The local anesthetic bupivacaine is thought not only to block sodium channels but also to interact with various receptors. Here, the authors focus on excitatory glutamatergic transmission in the superficial dorsal horn of the spinal cord with respect to its importance for nociceptive processing.

**Methods:** The effects of bupivacaine on the response to exogenous administration of *N*-methyl-D-aspartate (NMDA) receptor agonists were examined in lamina II neurons of adult rat spinal cord slices using the whole-cell patch-clamp technique.

**Results:** Bupivacaine (0.5, 2 mM) dose-dependently reduced the peak amplitudes of exogenous NMDA-induced currents. However, this inhibitory effect of bupivacaine (2 mM) was not blocked by the presence of tetrodotoxin, a sodium channel blocker, or  $\text{La}^{3+}$ , a voltage-gated  $\text{Ca}^{2+}$  channel blocker, and was unaffected by changes in pH conditions. Moreover, intrapipette guanosine-5'-*O*-(2-thiodiphosphate) (1 mM), a G-protein inhibitor, did not block the reduction of NMDA current amplitudes by bupivacaine. Similarly, lidocaine, ropivacaine, and mepivacaine also reduced the amplitudes of NMDA-induced currents.

**Conclusions:** These findings raise the possibility that the antinociceptive effect of bupivacaine may be due to direct modulation of NMDA receptors in the superficial dorsal horn. In addition to voltage-gated sodium channels, glutamate NMDA receptors are also important for analgesia induced by local anesthetics.

It is well established that local anesthetics block impulses in peripheral nerves through the inhibition of voltage-gated sodium channels. However, the underlying mecha-

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## What We Already Know about This Topic:

- ❖ Local anesthetics block sodium channels, but also other membrane-bound proteins.
- ❖ In simple cell systems, local anesthetics block glutamate receptors of the *N*-methyl-D-aspartate type.

## What This Article Tells Us That Is New:

- ❖ Bupivacaine and other local anesthetics, in concentrations found after spinal anesthesia, block *N*-methyl-D-aspartate receptors.
- ❖ Spinal anesthesia may be accompanied by blockade of *N*-methyl-D-aspartate receptors, which are important to acute pain and to generation of sensitized states.

nisms of spinal and epidural anesthesia may be more complex than simply the blockade of impulses in nerve roots. Local anesthetics may not only block the impulses in nerve roots, but could also interact with many membrane phospholipids and proteins, including various receptors, and thereby affect a variety of cellular activities.

*N*-methyl-D-aspartate (NMDA)-type glutamate receptors are one of the major receptor channel types mediating rapid excitatory neurotransmission in the central nervous system; they also play an important role in central sensitization regarding long-term pain.<sup>1,2</sup> Some previous studies have examined the interactions between local anesthetics and NMDA receptors. These have shown that local anesthetics inhibit NMDA receptors in *Xenopus* oocytes<sup>3,4</sup> and in mouse CA1 pyramidal neurons.<sup>5</sup> Furthermore, bupivacaine, a long-acting local anesthetic, inhibits phosphorylated extracellular signal-regulated kinase activation induced by NMDA in the rat spinal cord, as revealed by immunohistochemistry.<sup>6</sup> Therefore, local anesthetics may have a direct antinociceptive effect in the spinal dorsal horn by modulating NMDA receptors, and if so, they could play an important role in preventing the development of pain by spinal and epidural anesthesia. Lamina II (substantia gelatinosa [SG]) neurons of the spinal cord preferentially receive thin myelinated A $\delta$ - and unmyelinated C-primary afferent fibers. Both of these fiber types carry nociceptive information and, therefore, are thought to play an important role in modulating nociceptive transmission.<sup>7,8</sup>

To test the hypothesis that local anesthetics directly inhibit NMDA receptors in the spinal dorsal horn, we evalu-

ated the action of bupivacaine, a local anesthetic commonly used for spinal, epidural anesthesia and neuraxial blockade, on excitatory NMDA-mediated synaptic responses in the SG of rat spinal cord slices using the whole-cell patch-clamp method.

## Materials and Methods

This study was approved by the Animal Care and Use Committee at Niigata University Graduate School of Medical and Dental Sciences (Niigata, Japan). Adult Wistar rats (aged 5–8 weeks) were anesthetized with urethane (1.5 g/kg, intraperitoneal). A dorsal laminectomy was performed, and the lumbosacral segment of the spinal cord with ventral and dorsal roots attached was removed.<sup>9,10</sup> The rats were then immediately killed by exsanguination, and the spinal cords were placed in preoxygenated ice-cold Krebs solution. After the arachnoid membrane was removed, the spinal cord was placed in an agar block and mounted on a metal stage. A transverse slice (500  $\mu\text{m}$  thick) was cut on a DTK-1500 microslicer (Dosaka, Japan) and placed on a nylon mesh in the recording chamber. The slice was perfused continuously with Krebs solution (10–20 ml/min) equilibrated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  gas mixture at 36°C. The Krebs solution contained 117 mM NaCl, 3.6 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 1.2 mM  $\text{NaH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , and 11.5 mM D-glucose. Whole-cell patch-clamp recordings were made from SG neurons in voltage-clamp mode with patch pipette electrodes having a resistance of 10 M $\Omega$ . The patch pipette solution contained 110 mM Cs-sulfate, 0.5 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , 5 mM EGTA, 5 mM HEPES, 5 mM tetraethylammonium, and 5 mM ATP-Mg salt. Guanosine-5'-*O*-(2-thiodiphosphate) (GDP- $\beta$ -S; 1 mM) was used as a G-protein blocker when necessary. Signals were amplified using an Axopatch 200B amplifier (Molecular Devices, Union City, CA) and were filtered at 2 kHz and digitized at 5 kHz. Data were collected and analyzed using pClamp 10.0 software (Molecular Devices). All experiments were performed in voltage-clamp mode at a holding potential of  $-40$  mV. Drugs were applied by superfusion without alteration of the perfusion rate and temperature. NMDA (100  $\mu\text{M}$ ) was applied to slices for 30 s. Tetrodotoxin (0.5  $\mu\text{M}$ ) was used in a subset of experiments. Drugs were purchased as follows: bupivacaine hydrochloride, lidocaine hydrochloride, NMDA, and GDP- $\beta$ -S from Sigma-Aldrich (St. Louis, MO); tetrodotoxin and  $\text{LaCl}_3$  from Wako (Osaka, Japan); and mepivacaine hydrochloride and ropivacaine hydrochloride from AstraZeneca (Osaka, Japan).

## Statistical Analysis

Data are expressed as mean  $\pm$  SD. Statistical significance was determined as  $P < 0.05$  using either the Student paired *t* test or one-way analysis of variance.

## Results

To study the effects of bupivacaine on excitatory synaptic transmission, whole-cell patch-clamp recordings were made

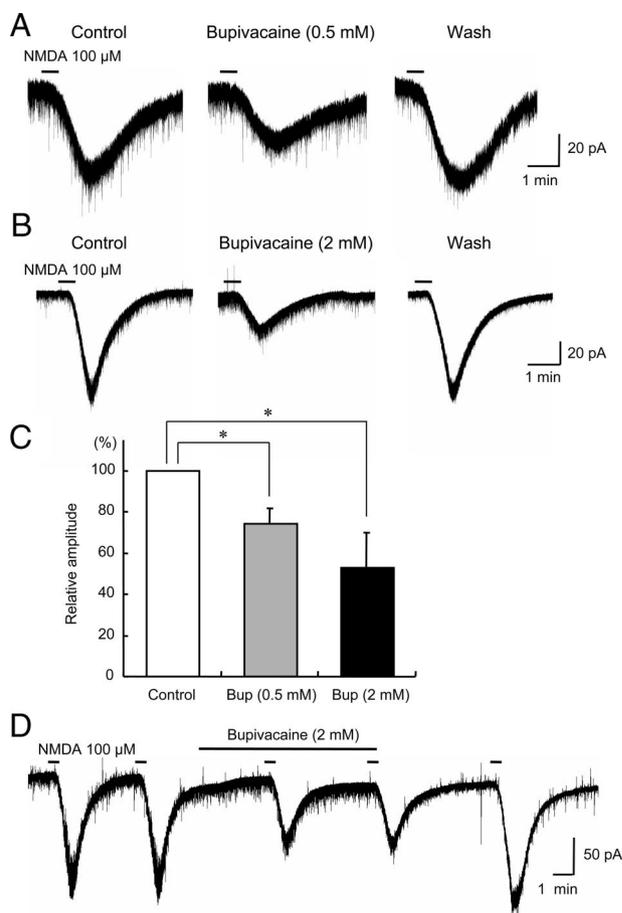


Fig. 1. Bupivacaine (Bup) inhibits the response to exogenous *N*-methyl-D-aspartate (NMDA). (A, B, and D) Representative traces of NMDA-induced current, recorded at  $-40$  mV before, during, and after bupivacaine application. Bupivacaine (0.5 mM, 2 mM) reversibly inhibited NMDA-induced currents. (C) The relative amplitudes were 75  $\pm$  8% of the control level in the presence of 0.5 mM bupivacaine and 53  $\pm$  18% in the presence of 2 mM bupivacaine. \*  $P < 0.01$ .

from 80 rat SG neurons. Bupivacaine did not alter the level of holding current required to maintain neurons at  $-40$  mV.

## Bupivacaine Inhibits NMDA Receptor-mediated Responses

Exogenous application of NMDA (100  $\mu\text{M}$ , 30 s) elicited an inward current at  $-40$  mV (fig. 1), reflecting the activation of NMDA receptors. Preapplication of bupivacaine for 3 min reduced the amplitudes of NMDA-induced currents to 75  $\pm$  8% (0.5 mM,  $n = 6$ ;  $P < 0.01$ ; figs. 1A and C) and 53  $\pm$  18% (2 mM,  $n = 10$ ;  $P < 0.01$ ; figs. 1B and C) of the control values, respectively. These effects of bupivacaine were reversible and the amplitudes of currents recovered to the control values within 5–10 min (fig. 1D), although the effect of bupivacaine on voltage-gated sodium channels was longer lasting.<sup>11</sup>

## Tetrodotoxin, a Sodium Channel Blocker, or $\text{La}^{3+}$ , a Voltage-gated $\text{Ca}^{2+}$ Channel Blocker, Does Not Affect NMDA Receptor-mediated Responses

To test the possibility that the observed effects were not specific for bupivacaine, we used tetrodotoxin, a sodium

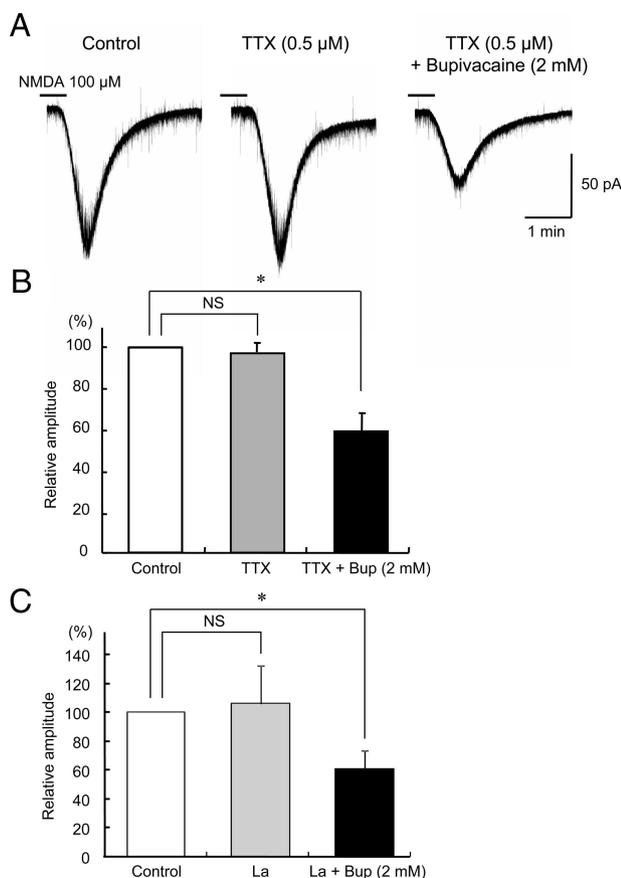


Fig. 2. Effect of tetrodotoxin (TTX) and  $\text{La}^{3+}$  (La) on *N*-methyl-D-aspartate (NMDA)-induced currents. (A) Tetrodotoxin ( $0.5 \mu\text{M}$ ) did not affect the amplitudes of NMDA-induced currents. However, bupivacaine (Bup;  $2 \text{ mM}$ ) inhibited NMDA-induced currents in the presence of tetrodotoxin. (B) The relative amplitudes were  $101 \pm 18\%$  of the control level in the presence of tetrodotoxin and  $59 \pm 10\%$  in the presence of tetrodotoxin +  $2 \text{ mM}$  bupivacaine. (C)  $\text{La}^{3+}$  ( $30 \mu\text{M}$ ) could not affect NMDA-induced currents, but bupivacaine ( $2 \text{ mM}$ ) reduced the amplitudes of NMDA-induced currents in the presence of  $\text{La}^{3+}$ . The relative amplitudes were  $106 \pm 26\%$  of the control level in the presence of  $\text{La}^{3+}$  and  $61 \pm 13\%$  of control level in the presence of  $\text{La}^{3+}$  +  $2 \text{ mM}$  bupivacaine. \*  $P < 0.01$ . NS = not significant.

channel blocker. Tetrodotoxin ( $0.5 \mu\text{M}$ ) did not affect the amplitudes of NMDA-induced currents ( $101 \pm 18\%$  of control,  $n = 8$ ;  $P = 0.82$ ; figs. 2A and B). In addition, we further investigated the effects of bupivacaine on NMDA-induced currents in the presence of tetrodotoxin. As expected, bupivacaine ( $2 \text{ mM}$ ) reduced the amplitudes of NMDA-induced currents ( $59 \pm 10\%$  of control,  $n = 5$ ;  $P < 0.01$ ; figs. 2A and B), as it did in the absence of tetrodotoxin. Moreover, we used  $\text{La}^{3+}$  ( $30 \mu\text{M}$ ) to block the  $\text{Ca}^{2+}$  entry through voltage-gated  $\text{Ca}^{2+}$  channels.<sup>12</sup>  $\text{La}^{3+}$  could not affect NMDA-induced currents ( $106 \pm 26\%$  of control,  $n = 11$ ;  $P = 0.49$ ; fig. 2C), however, bupivacaine ( $2 \text{ mM}$ ) reduced the amplitudes of NMDA-induced currents ( $61 \pm 13\%$  of control,  $n = 7$ ;  $P < 0.01$ ; fig. 2C) even in the presence of  $\text{La}^{3+}$ . Therefore, the observed current inhibition by bupivacaine is not likely to occur *via* blockage of sodium channels or voltage-gated  $\text{Ca}^{2+}$  channels.

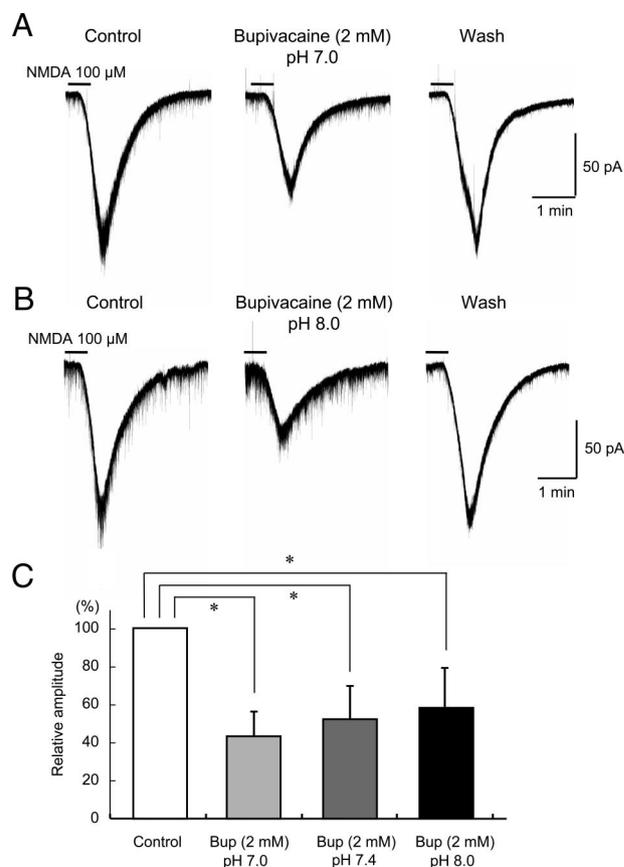


Fig. 3. Bupivacaine inhibits the response to exogenous *N*-methyl-D-aspartate (NMDA) in substantia gelatinosa neurons in different pH conditions. (A and B) Bupivacaine (Bup;  $2 \text{ mM}$ ) reversibly inhibited NMDA-induced currents under pH 7.0 or pH 8.0 conditions. (C) The relative amplitudes were  $43 \pm 13\%$  of the control level at pH 7.0 and  $58 \pm 21\%$  at pH 8.0. Comparison of the inhibitory effects of bupivacaine among the three groups (pH 7.0, 7.4, and 8.0) showed that the levels of inhibition were the same ( $P = 0.30$ , one-way analysis of variance). \*  $P < 0.01$ .

### Bupivacaine Inhibits NMDA Receptor-mediated Responses in Different pH Conditions

To test whether the above actions of bupivacaine are dependent on the level of  $\text{H}^+$ , we further examined the effects of bupivacaine on NMDA-induced currents at several different pH values (pH 7.0 or 8.0). Bupivacaine ( $2 \text{ mM}$ ) was dissolved in Krebs solution and titrated by HCl or NaOH up to pH 7.0 or 8.0. Before titration, the pH of the bupivacaine solution was approximately 7.4. The amplitudes of NMDA-induced currents were reduced by bupivacaine to  $43 \pm 13\%$  of the control level ( $n = 7$ ;  $P < 0.01$ ) at pH 7.0 and  $58 \pm 21\%$  of the control level ( $n = 6$ ;  $P < 0.01$ ; fig. 3) at pH 8.0. However, no significant inhibitory effects of bupivacaine on NMDA-induced currents were seen by one-way analysis of variance among the three groups (pH 7.0, 7.4, and 8.0;  $P = 0.30$ ).

### Bupivacaine Inhibits NMDA Receptor-mediated Responses without Activation of G Proteins

There are a few reports investigating the interactions between local anesthetics and G protein-coupled receptors.<sup>6,13,14</sup> We

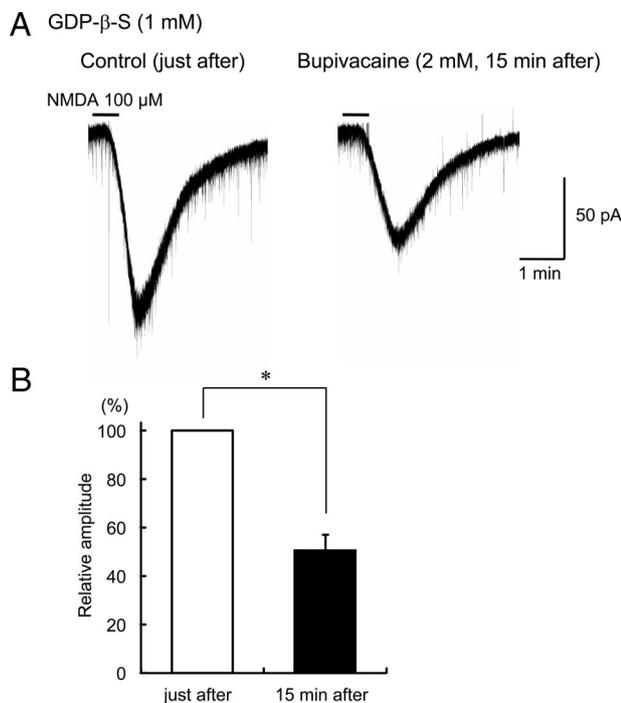


Fig. 4. Effect of a G-protein blocker on the inhibition of *N*-methyl-D-aspartate (NMDA)-induced currents by bupivacaine. (A) Intrapipette administration of the G-protein blocker guanosine-5'-*O*-(2-thiodiphosphate) (GDP- $\beta$ -S; 1 mM) did not block the reduction of NMDA-induced currents by bupivacaine (2 mM). (B) The amplitudes of NMDA-induced currents decreased to  $50 \pm 7\%$  of the control level (just after establishing the whole-cell configuration). \*  $P < 0.01$ .

therefore examined to determine whether G proteins are responsible for the observed effect of bupivacaine. GDP- $\beta$ -S (1 mM), a G-protein inhibitor that competitively inhibits G-proteins, was added to the pipette solution to prevent the postsynaptic activation of G proteins.<sup>15-17</sup> NMDA-induced currents were recorded just after establishing the whole-cell configuration (fig. 4A). These currents did not change when NMDA was again applied 15 min later ( $92 \pm 4\%$  of control,  $n = 4$ ;  $P = 0.26$ ). However, these currents were reduced in the presence of bupivacaine (2 mM,  $50 \pm 7\%$  of control,  $n = 4$ ;  $P < 0.01$ ; figs. 4A and B). These findings suggested that the observed current inhibition by bupivacaine was not mediated by the activation of G proteins.

#### Lidocaine, Ropivacaine, and Mepivacaine Also Inhibit NMDA Receptor-mediated Responses

To test whether other local anesthetics inhibit NMDA-induced currents like bupivacaine, we used lidocaine, ropivacaine, and mepivacaine. NMDA-induced currents were also inhibited by lidocaine (2 mM,  $72 \pm 19\%$  of control,  $n = 5$ ;  $P < 0.05$ ), ropivacaine (2 mM,  $61 \pm 19\%$  of control,  $n = 5$ ;  $P < 0.01$ ), and mepivacaine (2 mM,  $74 \pm 15\%$  of control,  $n = 6$ ;  $P < 0.01$ ), shown in figures 5A and B. These local anesthetics other than bupivacaine also inhibited NMDA-induced currents, suggesting that the inhibition was not specific for bupivacaine.

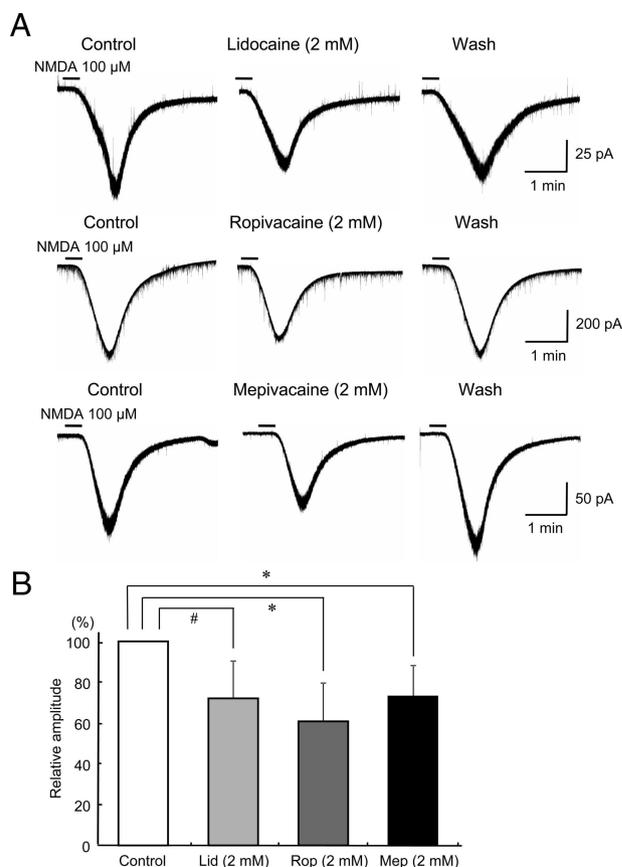


Fig. 5. Lidocaine (Lid), ropivacaine (Rop), and mepivacaine (Mep) inhibit *N*-methyl-D-aspartate (NMDA)-induced currents. (A) NMDA-induced currents were also inhibited by lidocaine (2 mM), ropivacaine (2 mM), and mepivacaine (2 mM). (B) The relative amplitudes were  $72 \pm 19\%$  (2 mM lidocaine),  $61 \pm 19\%$  (2 mM ropivacaine), and  $74 \pm 15\%$  (2 mM mepivacaine) of control level, respectively. \*  $P < 0.01$ . #  $P < 0.05$ .

#### Discussion

This is the first study to show the inhibition of NMDA-induced currents by bupivacaine electrophysiologically in the SG. The SG is thought to play an important role in modulating nociceptive transmission. The inhibitory effect lasted for several minutes only, despite being known as a "long-lasting" local anesthetic. Similarly, lidocaine, ropivacaine, and mepivacaine also inhibited NMDA-induced currents. However, tetrodotoxin and  $La^{3+}$  did not affect these currents. In addition, mixing a G-protein blocker with the pipette solution did not block the inhibition of NMDA-induced currents by bupivacaine. These results suggest that inhibition of voltage-gated sodium channels, voltage-gated  $Ca^{2+}$  channels, or G proteins is not the mechanism by which bupivacaine modulates these currents.

Some investigators have reported the interactions between NMDA receptors and local anesthetics.<sup>3-6</sup> In these reports, bupivacaine exerted inhibitory effects toward NMDA-induced responses. The obtained  $IC_{50}$  values of bupivacaine were 1.0 in *Xenopus* oocytes<sup>3</sup> and 2.8 mM in mouse CA1 pyramidal neurons,<sup>5</sup> respectively. Hahnenkamp *et al.*<sup>4</sup> reported that they could not calculate  $IC_{50}$  because the inhi-

bition of currents did not exceed 50% in the presence of 0.1 mM bupivacaine. Furthermore, bupivacaine (2 mM) reduced NMDA-induced phosphorylated extracellular signal-regulated kinase activation to 59% of the control levels.<sup>6</sup> These concentrations and the inhibitory potency of bupivacaine correspond to our data and suggest that bupivacaine exerts an inhibitory effect on NMDA-induced responses in the millimolar concentration range, even though bupivacaine blocks voltage-gated sodium channels at micromolar levels for as long as 30 min in rat dorsal horn neurons.<sup>11</sup> We used a racemic mixture of bupivacaine in this study and did not examine the difference between levobupivacaine and dexbupivacaine. Ueta *et al.*<sup>18</sup> reported that the inhibitory effects of racemic bupivacaine and its enantiomers were similar at NMDA receptors in *Xenopus* oocytes. However, Hahnenkamp *et al.*<sup>4</sup> reported that *S*(-)-ropivacaine inhibited NMDA signaling, whereas *R*(+)-ropivacaine was without effect. Therefore, there remains the possibility that the stereoselectivity may contribute to the inhibition of NMDA-induced current in the dorsal horn.

The concentration of 0.5% bupivacaine, widely used in spinal anesthesia, is approximately 15 mM. There are several reports about the concentrations of local anesthetics in cerebrospinal fluid (CSF) after spinal or epidural injection. In humans, the CSF concentration of bupivacaine ranged from 0.1 to 3 mM after injection of plain bupivacaine (17.5 or 20 mg),<sup>19</sup> whereas that of bupivacaine after epidural administration (150 mg) was up to approximately 0.1 mM.<sup>20</sup> Moreover, the maximal CSF concentration of tetracaine after intrathecal administration was 0.7 mM.<sup>21</sup> In addition, Kamiya *et al.*<sup>22</sup> reported that the CSF concentration of lidocaine 10 min after epidural administration of 100 mg was around 0.25 mM. Similarly, the CSF concentration was in the millimolar range after intrathecal or epidural administration of bupivacaine and other local anesthetics in animals.<sup>23-25</sup> The intraspinal concentration of local anesthetics after intrathecal injection should be lower than that in CSF, but superficial dorsal horn neurons would receive a relatively higher concentration, close to the CSF concentration. These investigations indicate that the concentrations of bupivacaine used in this study are clinically relevant during spinal anesthesia. Studies using an *in vivo* model will be valuable to confirm whether its concentration is relevant during spinal anesthesia.

Local anesthetics have also been reported to have an inhibitory effect on other ion channel-coupled receptors, including 5-hydroxytryptamine type 3,<sup>26</sup>  $\gamma$ -aminobutyric acid, glycine,<sup>27</sup> and nicotinic acetylcholine<sup>18</sup> receptors. These broad actions of bupivacaine suggest that it may act on membrane phospholipids but not at any defined sites on receptors, because its potency is low and it is not specific among ionotropic receptors.<sup>6</sup> However, we observed that bupivacaine inhibited NMDA-induced inward currents, *i.e.*, cation influx, in this study. This suggests that bupivacaine directly inhibits NMDA receptor-coupled channels and prevents depolarization of dorsal horn neurons. On the other hand, there are a few reports investigating the interactions between

local anesthetics and GPCRs. For example, local anesthetics can affect guanosine triphosphate-binding proteins.<sup>13</sup> Local anesthetics inhibit certain GPCRs (lysophosphatidic acid, thromboxane A<sub>2</sub>, platelet-activating factor, and m1 muscarinic receptors) expressed in *Xenopus* oocytes.<sup>14</sup> However, Yanagidate *et al.*<sup>6</sup> reported that bupivacaine did not attenuate phosphorylated extracellular signal-regulated kinase activation by GPCR agonists like substance P, bradykinin, and (*R*, *S*)-3,5-dihydroxyphenylglycine (an agonist of metabotropic glutamate receptors). Similarly, in our study, G proteins were not involved in the inhibition of NMDA-induced responses by bupivacaine. Therefore, local anesthetics might inhibit ion channel-coupled receptors more potently than they inhibit GPCRs.

It has been known that an increase in the pH of local anesthetics accelerates the onset and decreases the required concentration to block nerve conduction due to increasing the amount of drug in the uncharged base form,<sup>28</sup> which diffuses easily across the nerve membrane. However, Hahnenkamp *et al.*<sup>4</sup> reported that intracellular QX314 and extracellular benzocaine inhibited NMDA-induced currents, but that extracellular QX314 produced no such inhibition. QX314 is a permanently charged and non-membrane-permeable lidocaine analog, and benzocaine is a completely uncharged local anesthetic in physiologic conditions. They concluded that the site of action of local anesthetics toward NMDA-induced currents is intracellular and charge independent. Although we did not examine the charge independency by bupivacaine in this study, this charge independency for inhibiting the current is consistent with our data, because the inhibition of NMDA-induced currents by bupivacaine did not change in different pH conditions.

There was a paradox between the dependence of local anesthetic function on charge and pH, but not its interaction with NMDA receptors. It is suggested that bupivacaine may have the different route to reach its effect site to inhibit NMDA-induced currents. However, there remains the possibility that we could not detect the charge dependency because there is little difference in the amount of uncharged base form between pH 7.0 and 8.0. However, we did not examine the lower or higher pH conditions because we were afraid that neurons may not survive in those conditions.

*N*-methyl-D-aspartate receptors play an important role in central sensitization regarding long-term pain.<sup>1,2</sup> Therefore, NMDA receptor antagonism results in an antinociceptive effect, especially in patients with chronic pain, although there is little evidence that NMDA receptor antagonism is involved in antinociceptive effect of local anesthetics. Local anesthetics have been widely used in these patients *via* neuraxial blockade or intravenous administration and have provided them with pain relief. These effects have been discussed from one aspect, sodium channel blockade, but our study suggests that not only sodium channel blockade but also the NMDA receptor antagonism of bupivacaine may have the possibility to prevent these patients from developing chronic pain. However, we think that studies using intrathe-

cal application of bupivacaine *in vivo* will be required to fully understand the physiologic implication of NMDA receptor antagonism by bupivacaine.

In summary, the current study suggests that bupivacaine inhibits NMDA-induced glutamatergic transmission in rat dorsal horn neurons and that this inhibition is not affected by sodium channel blockers, Ca<sup>2+</sup> channel blockers, G-protein blockers, or different pH conditions. This suggests that not only voltage-gated sodium channels but also glutamate NMDA receptors are important for the analgesia induced by bupivacaine and other local anesthetics, especially during spinal and epidural anesthesia.

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