

# Action of Isoflurane on the Substantia Gelatinosa Neurons of the Adult Rat Spinal Cord

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**Background:** Although isoflurane, a volatile anesthetic, can block the motor response to noxious stimulation (immobility and analgesia) and suppress autonomic responsiveness, how it exerts these effects at the neuronal level in the spinal cord is not fully understood.

**Methods:** The effects of a clinically relevant concentration (1 rat minimum alveolar concentration [MAC]) of isoflurane on electrically evoked and spontaneous excitatory/inhibitory transmission and on the response to exogenous administration of the  $\gamma$ -aminobutyric acid type A receptor agonist muscimol were examined in lamina II neurons of adult rat spinal cord slices using the whole cell patch clamp technique. The effect of isoflurane on the action potential-generating membrane property was also examined.

**Results:** Bath-applied isoflurane (1.5%, 1 rat MAC) diminished dorsal root-evoked polysynaptic but not monosynaptic excitatory postsynaptic currents. Glutamatergic miniature excitatory postsynaptic currents were also unaffected by isoflurane. In contrast, isoflurane prolonged the decay phase of evoked and miniature  $\gamma$ -aminobutyric acid type A receptor-mediated inhibitory postsynaptic currents and increased the amplitude of the muscimol-induced current. Isoflurane had little effect on action potential discharge activity.

**Conclusions:** Isoflurane augments  $\gamma$ -aminobutyric acid-mediated inhibitory transmission, leading to a decrease in the excitability of spinal dorsal horn neurons. This may be a possible mechanism for the antinociceptive effect of isoflurane in the spinal cord.

MOST general anesthetics can induce unconsciousness, block the motor response to noxious stimulation, and suppress autonomic responsiveness. Recent studies have revealed that the minimum alveolar concentration (MAC) is spinally mediated.<sup>1–4</sup> The depression of the motor response to noxious stimulation may be caused by immobilization and antinociceptive effects at the spinal cord level.<sup>5,6</sup> Cheng and Kendig<sup>7</sup> showed that enflurane directly depresses glutamatergic  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) currents in mouse spinal cord motor neurons, suggestive of the immobilization effect of inhaled anesthetics and its possible mechanism in the spinal cord. However, the antinociceptive effect of inhaled anesthetics may not be fully accepted because

they cannot prevent the increase of blood pressure or heart rate caused by noxious stimuli at clinically relevant concentrations. Recently, Yamauchi *et al.*<sup>8</sup> reported that halothane depressed the extracellular activity of single spinal dorsal horn wide-dynamic-range neurons to noxious stimuli, suggesting that halothane has an antinociceptive effect at the spinal cord level. Furthermore, they reported that the inhibitory effect of halothane was blocked by bicuculline ( $\gamma$ -aminobutyric acid type A [GABA<sub>A</sub>] receptor antagonist), which suggests that the antinociceptive effect of volatile anesthetics may be related to  $\gamma$ -aminobutyric acid-mediated (GABAergic) transmission in the spinal dorsal horn. In fact, inhaled anesthetics enhance GABA<sub>A</sub> currents in cultured neurons,<sup>9</sup> human embryonic kidney cells expressing GABA<sub>A</sub> receptors,<sup>10,11</sup> and brain slices<sup>12,13</sup> at clinically relevant concentrations. Inhaled anesthetics have many effect sites other than GABA<sub>A</sub> receptors, such as K<sup>+</sup> channels,<sup>14–17</sup> Ca<sup>2+</sup> channels,<sup>18–21</sup> nicotinic acetylcholine receptors,<sup>22–24</sup> glycine receptors,<sup>25</sup> AMPA receptors,<sup>7,26</sup> and NMDA receptors.<sup>7,27–30</sup> Collectively, it is considered that multiple potential anesthetic targets exist.<sup>31–33</sup> Therefore, also in the spinal dorsal horn, the antinociceptive action of isoflurane may be mediated by the GABAergic inhibitory system and some or all of the many other mechanisms listed above.

To clarify whether isoflurane has an antinociceptive effect in the dorsal horn and whether the GABAergic system is its major anesthetic (analgesic) target, we investigated the action of isoflurane on the excitatory and inhibitory synaptic responses in lamina II (substantia gelatinosa [SG]), where the GABAergic system plays a major role in controlling nociceptive information.<sup>34,35</sup>

## Materials and Methods

### Spinal Cord Slice Preparation and Electrophysiologic Recording

This study was approved by the Animal Research Committee of Niigata University Graduate School of Medical and Dental Sciences in Niigata, Japan. Thick (600- to 650- $\mu$ m) spinal cord slices containing the L4 dorsal root (10–20 mm) were prepared from adult rats (aged 7–10 weeks) as described previously.<sup>36,37</sup> After preparation, slices were perfused with oxygenated Krebs solution (10 ml/min; 36° ± 1°C; composition: 117 mM NaCl, 3.6 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, and 11 mM D-glucose) in the recording chamber for at least 30 min before recording. Blind whole cell patch clamp recordings were made from

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Received from the Division of Anesthesiology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan. Submitted for publication June 15, 2004. Accepted for publication November 5, 2004. Supported by Grant-in-Aid for Scientific Research Nos. 13470318 and 16591529 from the Ministry of Education, Science, Sports and Culture of Japan, Tokyo, Japan.

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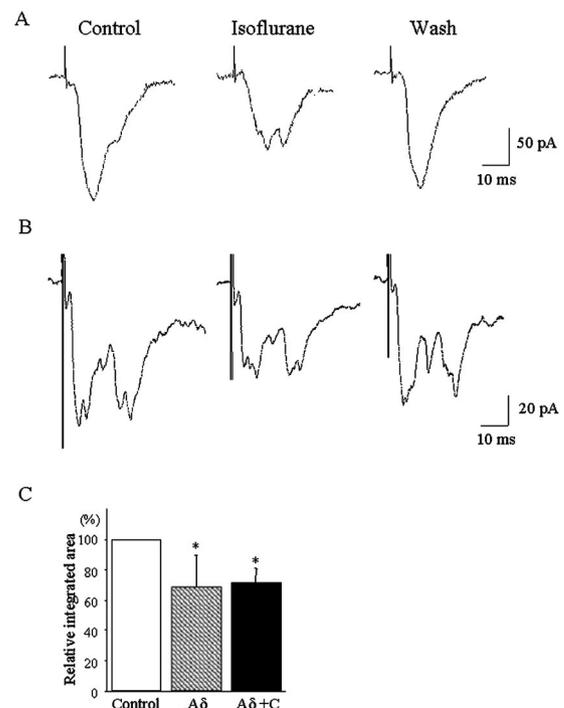
neurons located in the SG. After the whole cell configuration was established, voltage clamped neurons were held at  $-70$  mV for recording non-NMDA receptor-mediated excitatory postsynaptic currents (EPSCs), at  $+40$  mV for NMDA receptor-mediated EPSCs and at  $0$  mV for inhibitory postsynaptic currents (IPSCs).<sup>38,39</sup> The dorsal root was stimulated using a suction electrode at  $100$   $\mu$ A ( $0.05$  ms) for A $\delta$  fibers and  $1,000$   $\mu$ A ( $0.5$  ms) for C fibers.<sup>34-39</sup> A $\delta$  fiber-evoked EPSCs were judged to be monosynaptic on the basis of both their short and constant latencies and the absence of failures with repetitive stimulation at  $20$  Hz.<sup>40,41</sup> Identification of C fiber-evoked monosynaptic EPSCs was based on an absence of failures with low-frequency ( $1$  Hz) repetitive stimulation.<sup>42,43</sup> In contrast, polysynaptic EPSCs had variable latencies and showed failures with such stimulation protocols. The monopolar silver-wire electrode (diameter,  $50$   $\mu$ m) was used for focal stimulation, insulated except for the tip, and located within  $150$   $\mu$ m of the recorded neurons. Whole cell patch pipettes were constructed from borosilicate glass capillaries ( $1.5$  mm OD; World Precision Instruments, Sarasota, FL). The resistance of a typical patch pipette was  $5$ – $10$  M $\Omega$  when filled with internal solution. Two pipette solutions were used: (1) Cs sulfate-based solution for voltage clamp recording, containing  $110$  mM Cs<sub>2</sub>SO<sub>4</sub>,  $0.5$  mM CaCl<sub>2</sub>,  $2$  mM MgCl<sub>2</sub>,  $5$  mM TEA-Cl,  $5$  mM ATP-Mg salt,  $5$  mM EGTA, and  $5$  mM HEPES, which used Cs and TEA as K<sup>+</sup> channel blockers; and (2) potassium gluconate-based solution for current clamp recording, containing  $135$  mM K-gluconate,  $5$  mM KCl,  $0.5$  mM CaCl<sub>2</sub>,  $2$  mM MgCl<sub>2</sub>,  $5$  mM EGTA, and  $5$  mM HEPES. Membrane currents were amplified with an Axopatch 200A (Axon Instruments, Foster City, CA). Signals were filtered at  $2$  kHz and digitized at  $5$  kHz. Data were collected and analyzed using pClamp6.3 (Axon Instruments).

#### Application of Drugs

Drugs were dissolved in Krebs solution, and the solution was applied by perfusion without an alteration in the perfusion rate and temperature. Isoflurane was applied using a carrier gas ( $95\%$  O<sub>2</sub>,  $5\%$  CO<sub>2</sub>) and calibrated *via* a specific vaporizer, and the concentration of isoflurane in the Krebs solution was measured by gas chromatography. We ascertained that the concentration saturated with  $1.5\%$  isoflurane was approximately  $0.37$  mM, nearly equal to  $1$  rat MAC. The drugs used in this work were strychnine,  $6$ -cyano- $7$ -nitroquinoxaline- $2,3$ -dione (CNQX), DL- $2$ -amino- $5$  phosphonovaleric acid (Sigma, St. Louis, MO), tetrodotoxin (Wako, Osaka, Japan), muscimol (Sigma), NMDA (Sigma), and isoflurane (Abbott Japan, Tokyo, Japan).

#### Data Analysis

Numerical data are expressed as mean  $\pm$  SD. Statistical differences were assessed using the paired *t* test and the



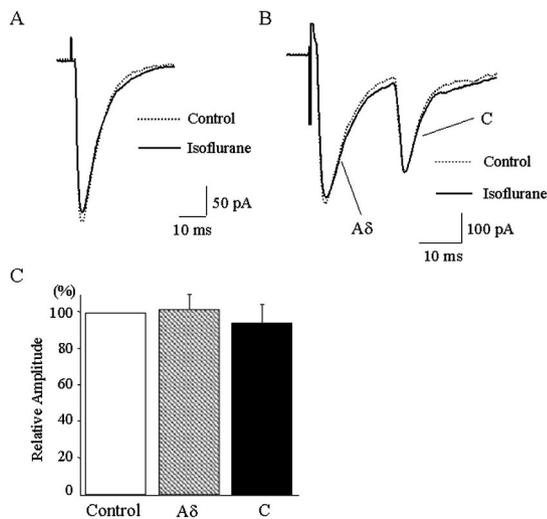
**Fig. 1.** Isoflurane inhibits dorsal root-evoked polysynaptic excitatory postsynaptic currents. (A and B) Averaged traces of several consecutive A $\delta$  fiber (A) and C fiber (B) intensity stimulation-evoked polysynaptic excitatory postsynaptic currents before and during the application of isoflurane ( $3$  min) and after washout ( $5$  min). (C) Integrated area of A $\delta$ - and C-fiber intensity stimulation-evoked polysynaptic excitatory postsynaptic currents under the action of isoflurane, relative to those in the control ( $n = 6$  and  $7$ , respectively). Vertical bars represent SDs. \*  $P < 0.05$ . The excitatory postsynaptic currents were recorded in the presence of strychnine ( $2$   $\mu$ M) at  $-70$  mV.

Kolmogorov-Smirnov test.  $P < 0.05$  was considered significant.

## Results

### Isoflurane Diminishes Dorsal Root-evoked Polysynaptic EPSCs

The dorsal root stimulation at A $\delta$ - or C-fiber intensity evoked monosynaptic EPSCs, polysynaptic EPSCs, or both in all SG neurons tested. In some SG neurons, solely polysynaptic EPSCs were recorded at a holding potential of  $-70$  mV, and the effects of isoflurane on these polysynaptic EPSCs were evaluated in the presence of strychnine (glycine receptor antagonist;  $2$   $\mu$ M). Bath application of  $1$  MAC isoflurane reversibly suppressed A $\delta$ - and C-fiber intensity stimulation-evoked polysynaptic EPSCs in all recorded neurons (figs. 1A and B). The integrated area of these evoked polysynaptic EPSCs was used to evaluate the isoflurane effect. Isoflurane significantly reduced the integrated area of A $\delta$ -fiber intensity stimulation-evoked polysynaptic EPSCs from  $1,328 \pm 1,132$  pA  $\cdot$  ms to  $756 \pm 328$  pA  $\cdot$  ms ( $68 \pm 22\%$  of control,  $n = 6$ ,  $P < 0.05$ ; fig. 1C) and C-fiber intensity

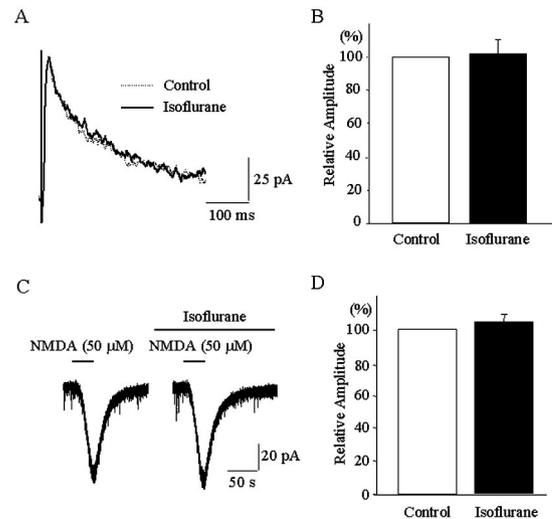


**Fig. 2.** Isoflurane does not affect dorsal root-evoked monosynaptic excitatory postsynaptic currents. (A and B) Averaged traces of monosynaptic excitatory postsynaptic currents evoked by A $\delta$ - (A) and C-fiber (B) intensity stimulation before and during the application of isoflurane (3 min). These were recorded in two different cells. (C) The amplitude under the action of isoflurane, relative to that in the control. Isoflurane had no effect on A $\delta$ - and C-fiber-evoked monosynaptic excitatory postsynaptic currents (n = 9 and 6, respectively).

stimulation-evoked polysynaptic EPSCs from  $3,833 \pm 2,053$  pA  $\cdot$  ms to  $2,772 \pm 1,701$  pA  $\cdot$  ms ( $70 \pm 9\%$  of control, n = 7,  $P < 0.05$ ; fig. 1C).

Stimulation of a dorsal root with A $\delta$ -fiber intensity, C-fiber intensity, or both evoked a monosynaptic EPSC in a population of SG neurons (figs. 2A and B). These dorsal root-evoked monosynaptic EPSCs were completely blocked by  $20 \mu\text{M}$  CNQX, indicating an activation of non-NMDA receptors.<sup>38</sup> In clear contrast to polysynaptic EPSCs, isoflurane had no effect on A $\delta$ - or C-fiber-evoked monosynaptic EPSCs in all SG neurons tested (figs. 2A and B). The peak amplitude was not significantly affected by isoflurane (A $\delta$  fiber:  $102 \pm 8$  of control, n = 9,  $P = 0.531$ ; C fiber:  $95 \pm 10\%$  of control, n = 6,  $P = 0.493$ ; fig. 2C), suggesting that isoflurane does not affect glutamate release from primary afferent terminals and the postsynaptic non-NMDA glutamatergic receptors.

Next, we tested whether isoflurane affects the activity of the NMDA receptors. In the presence of CNQX ( $20 \mu\text{M}$ ), bicuculline ( $20 \mu\text{M}$ ), and strychnine ( $2 \mu\text{M}$ ), we recorded dorsal root-evoked monosynaptic NMDA receptor-mediated EPSCs at a holding potential of +40 mV (fig. 3A). The peak amplitude of these evoked NMDA currents was not affected by isoflurane ( $102 \pm 9\%$  of control, n = 6,  $P = 0.66$ ; fig. 3B). In addition, we investigated the effect of isoflurane on the NMDA-induced current in SG neurons. Superfusing NMDA ( $50 \mu\text{M}$ ) elicited an inward current at -50 mV. The amplitude of the NMDA-induced current was not affected, nor was that of evoked EPSCs ( $104 \pm 5\%$  of control, n = 9,  $P = 0.09$ ; figs. 3C and D), suggesting that



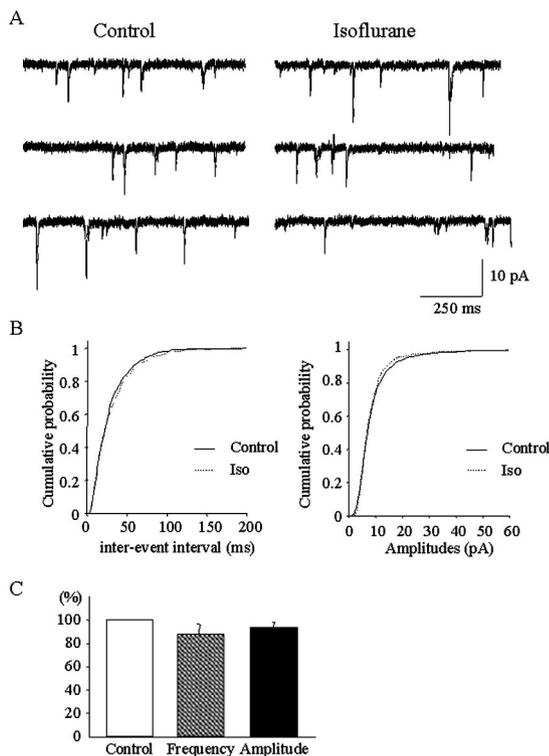
**Fig. 3.** Isoflurane does not affect the N-methyl-D-aspartate (NMDA) current. (A) Monosynaptic NMDA currents evoked by dorsal root stimulation were recorded at +40 mV, in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX;  $20 \mu\text{M}$ ), bicuculline ( $20 \mu\text{M}$ ), and strychnine ( $2 \mu\text{M}$ ). Averaged traces of several consecutive monosynaptic NMDA currents before and during the application of isoflurane (3 min). (B) The amplitude under the action of isoflurane, relative to that in the control. Isoflurane had no effect on NMDA currents (n = 6). (C) The effect of isoflurane on the current evoked by bath application of NMDA ( $50 \mu\text{M}$ ). NMDA-induced currents before (left) and during (right) application of isoflurane at -50 mV. (D) The amplitude under the action of isoflurane, relative to that in the control. Isoflurane had no effect on the NMDA-induced current (n = 9).

isoflurane has no effect on activation of NMDA receptors in SG neurons.

We further tested the effect of isoflurane on the frequency and amplitude of miniature EPSCs (mEPSCs), indicators of action at presynaptic terminals and postsynaptic responsiveness to glutamate, respectively (fig. 4).<sup>36</sup> In the presence of tetrodotoxin ( $0.5 \mu\text{M}$ ), SG neurons exhibited mEPSCs with a frequency of 2.3–50.3 Hz and a mean amplitude of 8.1–24.0 pA. Neither the frequency (n = 8,  $P = 0.225$ ) nor the mean amplitude (n = 8,  $P = 0.373$ ) of mEPSCs was affected by 1 MAC isoflurane (fig. 4C). In all cells tested (n = 8), interevent interval and amplitude distributions were not changed by isoflurane (fig. 4B). These data indicate that isoflurane affects glutamate release from presynaptic terminals of neither primary afferents nor excitatory interneurons. Therefore, a clinically relevant concentration of isoflurane does not affect monosynaptic glutamatergic excitatory transmission, but instead it may suppress nociceptive transmission by acting somewhere in the polysynaptic pathways of the dorsal horn.

#### *Isoflurane Enhances GABAergic IPSCs*

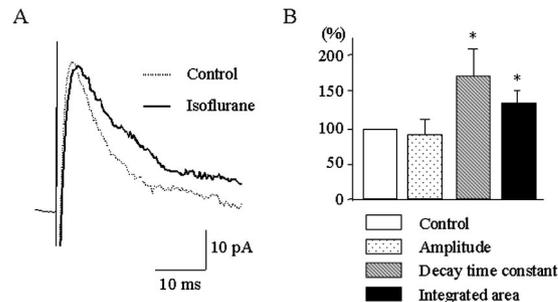
As mentioned above, the most likely target of isoflurane action in the polysynaptic pathway is the GABA<sub>A</sub> receptor. Therefore, we next examined the effect of isoflurane on inhibitory GABAergic transmission. Mono-



**Fig. 4.** Effect of isoflurane on miniature excitatory postsynaptic currents (mEPSCs). mEPSCs were recorded at  $-70$  mV, in the presence of tetrodotoxin ( $0.5 \mu\text{M}$ ). (A) The consecutive traces of mEPSCs before (left) and during (right) application of isoflurane (Iso; 3 min). (B) Cumulative distributions of interevent intervals and amplitudes of mEPSC, before (straight line) and under (dotted line) the action of isoflurane. Data were constructed from continuous recording for 30 s each. Isoflurane did not affect the distributions of interevent intervals and amplitudes. (C) The frequency and mean amplitude under the action of isoflurane, relative to those in the control. Isoflurane affected neither the frequency nor mean amplitude of mEPSCs ( $n = 8$ ).

synaptic GABAergic IPSCs were evoked by focal stimulation after a blockade of glutamatergic and glycinergic transmission by CNQX ( $20 \mu\text{M}$ ), DL-2-amino-5 phosphonovaleric acid ( $50 \mu\text{M}$ ), and strychnine ( $2 \mu\text{M}$ ), respectively (fig. 5A). Isoflurane had little effect on the amplitude of monosynaptic GABAergic IPSCs ( $91 \pm 23\%$ ,  $n = 6$ ,  $P = 0.886$ ). However, the decay time constant was significantly prolonged from  $13.3 \pm 6.3$  to  $24.4 \pm 14.6$  ms ( $177 \pm 52\%$  of control,  $P < 0.05$ ). Moreover, isoflurane significantly augmented the integrated area from  $1,409 \pm 588$  to  $1,847 \pm 578$  pA  $\cdot$  ms ( $138 \pm 23\%$  of control,  $P < 0.05$ ; fig. 5B). These results provide evidence that isoflurane potentiates GABAergic transmission in the spinal dorsal horn neurons. In addition to these effects on the IPSC duration, isoflurane significantly delayed the time to peak of IPSC in all SG neurons tested ( $\Delta t = 1.1 \pm 0.5$  ms,  $n = 6$ ,  $P < 0.05$ ).

We next investigated how isoflurane enhanced GABAergic transmission, *i.e.*, via presynaptic or postsynaptic mechanisms. GABAergic miniature IPSCs (mIPSCs) were isolated by adding tetrodotoxin ( $0.5 \mu\text{M}$ ) and



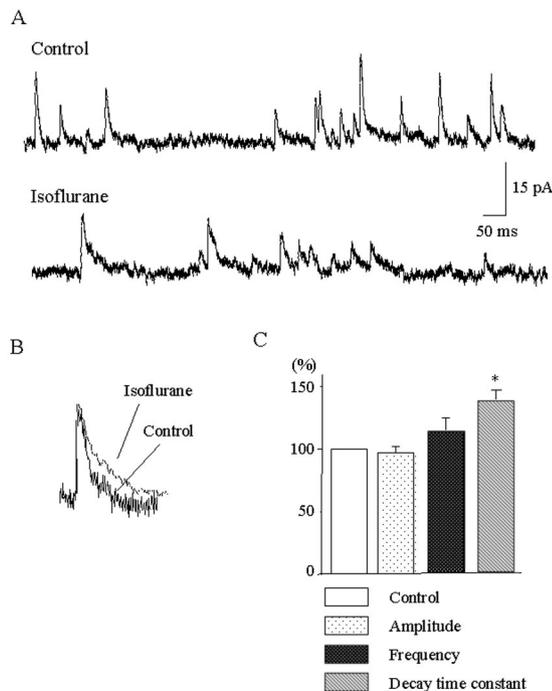
**Fig. 5.** Isoflurane augments  $\gamma$ -aminobutyric acid receptor-mediated monosynaptic inhibitory postsynaptic currents. (A) Averaged traces of focal stimulation evoked  $\gamma$ -aminobutyric acid receptor-mediated monosynaptic inhibitory postsynaptic currents in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX;  $20 \mu\text{M}$ ), DL-2-amino-5 phosphonovaleric acid ( $50 \mu\text{M}$ ), and strychnine ( $2 \mu\text{M}$ ) before and during the application of isoflurane (3 min). Isoflurane slightly delayed the time to peak of inhibitory postsynaptic currents. (B) Amplitude, decay time constant, and integrated area of under the action of isoflurane, relative to those in the control. The amplitude was not affected by isoflurane ( $n = 6$ ); however, decay time constants and integrated area were significantly increased by isoflurane. \*  $P < 0.05$ .

strychnine ( $2 \mu\text{M}$ ) (fig. 6A). Neither the amplitude (from  $20.2 \pm 6.2$  pA to  $19.3 \pm 5.2$  pA,  $97 \pm 12\%$  of control,  $n = 8$ ,  $P = 0.470$ ) nor the frequency (from  $6.4 \pm 3.2$  to  $6.8 \pm 2.7$  Hz,  $113 \pm 29\%$  of control,  $P = 0.245$ ) of mIPSCs was affected by isoflurane. However, the decay time constant of mIPSCs was significantly augmented by isoflurane, as was that of evoked IPSCs (from  $19.4 \pm 3.8$  to  $26.1 \pm 3.6$  ms,  $137 \pm 22\%$  of control,  $P < 0.05$ ; figs. 6B and C). We did not measure the time to peak of mIPSC because this amplitude was very small and hence it was difficult to measure the time to peak exactly. These results suggest that isoflurane does not alter GABA release from presynaptic terminals, but instead enhances the response of GABA<sub>A</sub> receptor on the postsynaptic membrane.

Superfusing muscimol ( $5 \mu\text{M}$ ) elicited an outward current at 0 mV in SG neurons. When we applied isoflurane before muscimol, the muscimol-induced current was potentiated markedly (fig. 7A), and the peak amplitude was augmented significantly ( $161 \pm 57\%$  of control,  $n = 8$ ,  $P < 0.05$ ; fig. 7B). This result reinforces the notion that isoflurane changes the responsiveness of the postsynaptic GABA<sub>A</sub> receptor and augments GABAergic transmission in the dorsal horn neurons.

#### *Isoflurane Does Not Affect Polysynaptic EPSCs in the Presence of Bicuculline*

We tested whether the inhibitory effect of isoflurane on polysynaptic EPSCs is due to augmentation of GABAergic transmission. In the presence of bicuculline ( $20 \mu\text{M}$ ) and strychnine ( $2 \mu\text{M}$ ), dorsal root stimulation-evoked polysynaptic EPSCs were recorded. Under these conditions, A $\delta$ - or C-fiber intensity stimulation usually evokes repetitive, long-lasting polysynaptic EPSCs that

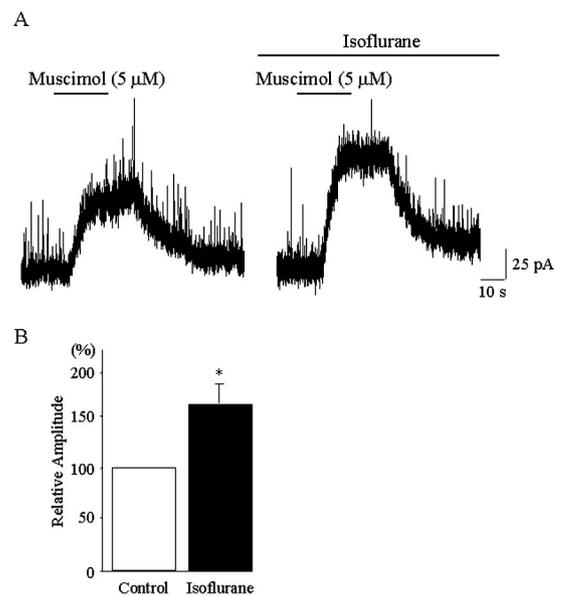


**Fig. 6.** The effect of isoflurane on  $\gamma$ -aminobutyric acid–mediated miniature inhibitory postsynaptic currents.  $\gamma$ -Aminobutyric acid receptor–mediated miniature inhibitory postsynaptic currents were recorded at 0 mV in the presence of strychnine (2  $\mu$ M) and tetrodotoxin (0.5  $\mu$ M). (A) The consecutive traces of  $\gamma$ -aminobutyric acid–mediated inhibitory postsynaptic currents before (*upper*) and during (*lower*) the application of isoflurane (3 min). (B) Averaged traces of several  $\gamma$ -aminobutyric acid–mediated miniature inhibitory postsynaptic currents before and after the addition of isoflurane, respectively; these are superimposed for comparison. (C) The amplitude, frequency, and decay time constants under the action of isoflurane, relative to those in the control. Isoflurane affected neither the amplitude nor the frequency ( $n = 8$ ). However, the decay time constants were significantly prolonged. \*  $P < 0.05$ .

follow the initial fast monosynaptic or polysynaptic EPSCs.<sup>34</sup> Isoflurane affected neither the initial fast polysynaptic nor the long-lasting polysynaptic EPSCs under the blockade of GABAergic transmission in all recorded neurons (A $\delta$  fiber:  $n = 9$ ,  $P = 0.053$ ; C fiber:  $n = 9$ ,  $P = 0.105$ ; figs. 8A and B).

#### Isoflurane Had No Effect on Action Potential Discharge Activity in SG Neurons

Finally, another possibility is that isoflurane would affect ionotropic channels, such as the K<sup>+</sup> and Ca<sup>2+</sup> channels, and, as a result, would alter the intrinsic membrane properties of SG neurons. We studied the action potential discharge activity in SG neurons in current clamp mode using patch pipettes filled with potassium gluconate instead of Cs sulfate. The resting membrane potential was not changed by isoflurane. In response to a depolarizing current injection (100 pA, 400 ms), SG neurons exhibited a train of action potentials (fig. 9A). Isoflurane had little effect on the number (from  $7.2 \pm 2.9$  to  $6.4 \pm 3.2$ ,  $n = 5$ ,  $P = 0.10$ ; fig. 9B) and the

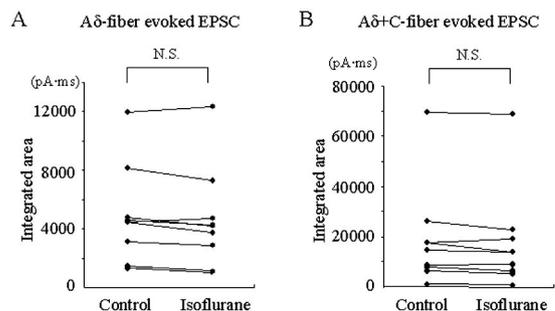


**Fig. 7.** Isoflurane augments the muscimol-induced  $\gamma$ -aminobutyric acid current. (A) Muscimol (5  $\mu$ M)-induced outward currents before (*left*) and during (*right*) application of isoflurane. The membrane potential was clamped at 0 mV. (B) Amplitude of the current under the action of isoflurane relative to that in the control ( $n = 8$ ). \*  $P < 0.05$ .

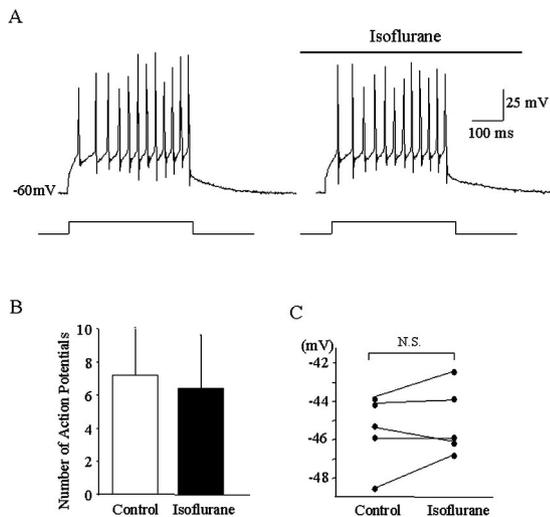
threshold of action potential discharges (from  $-45.5 \pm 1.8$  mV to  $-45.0 \pm 1.8$  mV), suggesting that the effect of isoflurane on action potential-generating activity is minimal.

## Discussion

We have shown that 1 MAC isoflurane inhibits glutamatergic polysynaptic EPSCs in the SG neurons, leaving monosynaptic EPSCs unchanged. Conversely, isoflurane augments GABA<sub>A</sub> receptor–mediated inhibitory transmission. Under conditions in which GABAergic and glycinergic inhibitory transmission were eliminated, the



**Fig. 8.** Isoflurane has no inhibitory action in the presence of bicuculline. (A and B) The dorsal root stimulation–evoked polysynaptic excitatory postsynaptic currents (EPSCs) were recorded in the presence of bicuculline (20  $\mu$ M) and strychnine (2  $\mu$ M). The integrated area of the A $\delta$ - (A) and C-fiber (B) intensity stimulation–evoked polysynaptic EPSCs were not affected by isoflurane ( $n = 9$  and 9, respectively).



**Fig. 9.** Effect of isoflurane on action potential discharge activity. Recordings were made in whole cell current clamp. (A) Representative examples of isoflurane effects on action potential discharge activity. (B) The effect of isoflurane on the number of action potentials induced by current injection (100 pA, 400 ms). The number was not significantly changed by isoflurane ( $n = 5$ ). (C) The effect of isoflurane on the threshold of action potential discharges. Isoflurane had little effect on the threshold.

inhibitory action of isoflurane on polysynaptic EPSCs almost disappeared.

#### Implication of Inhibition of Polysynaptic EPSCs

Dorsal root-evoked excitatory synaptic responses in the SG consist of monosynaptic and polysynaptic EPSCs or solely polysynaptic EPSCs. SG neurons with solely monosynaptic EPSCs are relatively rare.<sup>41</sup> The exact contribution of polysynaptic EPSCs to the excitability of SG neurons cannot be determined. However, one can reasonably speculate that the inhibition of polysynaptic EPSCs by isoflurane has considerable effects on nociceptive transmission in the superficial dorsal horn, given that more than 80% of SG neurons exhibit polysynaptic EPSCs,<sup>41</sup> and the amplitude and duration of polysynaptic EPSCs are almost identical to those of monosynaptic EPSCs.<sup>44</sup>

#### Minimal Action of Isoflurane on Glutamatergic Transmission in the SG

We have shown that isoflurane does not affect glutamate release from presynaptic terminals of primary afferents and excitatory interneurons. In addition, the responses of postsynaptic non-NMDA and NMDA receptors are also unaffected by isoflurane. These data indicate that glutamatergic transmission in the SG is not a primary target for isoflurane.

Volatile anesthetics have been reported to depress glutamate transmission *via* presynaptic<sup>45</sup> and postsynaptic actions.<sup>7</sup> MacIver *et al.*<sup>45</sup> have reported that clinically relevant concentrations of isoflurane and halothane reduced glutamate release in rat hippocampal brain slices.

Haseneder *et al.*<sup>46</sup> reported that isoflurane reduced glutamatergic transmission in SG neurons of immature rat spinal cord in presynaptic manner. An article provided evidence that enflurane directly depresses AMPA currents in mouse spinal cord motor neurons.<sup>7</sup> Several reports have shown that isoflurane diminishes the NMDA current in cultured cortical neurons<sup>9</sup> and *Xenopus* oocytes.<sup>29</sup> Cheng and Kendig<sup>7</sup> showed that enflurane reduced the NMDA current in mouse spinal cord motor neurons. However, our results do not support the data listed above, at least in the superficial dorsal horn of adult rat. The exact reason for the discrepancies is unknown, but they might be caused by differences in organs and maturity of animals used. It was reported that changes in subtypes of NMDA receptors can occur during growth.<sup>47,48</sup> Alternatively, differences of the type or concentration of volatile anesthetics used may also contribute.

#### Augmentation of GABAergic Transmission by Isoflurane

Classically, GABA<sub>A</sub> receptors were thought to be located on primary afferent terminals and involved in presynaptic inhibition *via* primary afferent depolarization.<sup>49</sup> In this study, however, we did not observe inhibition of dorsal root-evoked monosynaptic EPSCs by isoflurane (fig. 2). Previously, we demonstrated that muscimol (a GABA<sub>A</sub> receptor agonist) affected neither the amplitude of dorsal root-evoked monosynaptic EPSCs nor the frequency of mEPSCs.<sup>34</sup> Taken together, these results indicate that facilitation of presynaptic GABAergic inhibition by isoflurane (*via* primary afferent depolarization) is not prominent, at least in the fine afferent fibers in the superficial dorsal horn. Instead, postsynaptic GABA<sub>A</sub> receptors located on somatodendritic sites of excitatory interneurons are the most likely sites of action for isoflurane.

Isoflurane is known to have two distinct effects on synaptic GABA<sub>A</sub> responses: prolongation of the decay phase and reduction of the peak amplitude (blocking effect) of GABAergic IPSCs in rat hippocampal slices<sup>12</sup> and human embryonic kidney 293 cells.<sup>11</sup> Furthermore, Banks and Pearce<sup>12</sup> have reported that the concentration of isoflurane revealed a dissociation between the effects on the time course and the amplitude of IPSCs (prolonging and blocking effects), suggesting that distinct mechanisms underlie the two actions. These results indicate that the blocking effect of isoflurane at 1 MAC was not remarkable, but this concentration was enough to observe the prolongation of the decay phase. Our current results showed the amplitude of GABAergic evoked and miniature IPSCs were not significantly reduced by isoflurane at 1 MAC. However, we observed a prolongation of the decay time course of both of GABAergic evoked and miniature IPSCs. We speculate that similar blocking ef-

fects of IPSCs may be observable also in the dorsal horn at higher concentrations of isoflurane.

On the other hand, the amplitude of the bath-applied muscimol-induced current was apparently augmented by isoflurane at 1 MAC (fig. 7), although isoflurane had no effect on the peak amplitude of GABAergic IPSCs (figs. 5B and 6C). There are several possibilities for the difference in the action of isoflurane between exogenous and synaptic GABA responses. First, isoflurane may positively modulate extrasynaptic but not synaptic GABA<sub>A</sub> receptors in SG neurons. Naturally expressed GABA<sub>A</sub> receptors are thought to be heteromeric and comprised of forms of some subunits, such as  $\alpha$ ,  $\beta$ , and  $\gamma$ . This variety of GABA<sub>A</sub> receptor subunits and a different combination of subunits results in the formation of GABA<sub>A</sub> receptors exhibiting a distinct pharmacology, including isoflurane actions. Second, an augmentation of IPSC amplitude by isoflurane may have been occluded, owing to the release of GABA into the synaptic cleft at a concentration high enough to saturate the GABA<sub>A</sub> receptors on the postsynaptic neurons. A single quantum of GABA is known to saturate postsynaptic GABA<sub>A</sub> receptors.<sup>50</sup> Hapfelmeier *et al.*<sup>11</sup> showed that isoflurane significantly increased the amplitude and rise times of currents elicited by subsaturating GABA concentrations ( $10^{-7}$  M~ $10^{-5}$  M) but decreased the amplitude of currents elicited by a saturating GABA concentration ( $10^{-4}$  M). They suggested that isoflurane would decrease the dissociation rate of GABA from GABA<sub>A</sub> receptors. In addition to the prolonging effect, we also demonstrated that isoflurane significantly delayed the time to peak of the evoked IPSC. These results may support dissociation rate theory.

#### *Is the Facilitation of GABAergic Transmission a Major Mechanism of Isoflurane on Polysynaptic EPSCs?*

As discussed above, isoflurane inhibits polysynaptic excitatory transmission, and the augmentation of GABAergic inhibition may be a most likely mechanism for this. To reinforce this hypothesis, we tested the effect of isoflurane in the presence of bicuculline and strychnine, a condition in which both GABAergic and glycinergic inhibitions are eliminated. In this situation, the inhibitory action of isoflurane almost disappeared (figs. 8A and B), suggesting that the effect on the GABA<sub>A</sub> receptor is a major action of isoflurane in the inhibition of polysynaptic EPSCs. In the dorsal horn neurons that receive direct primary afferent input, the monosynaptic EPSC is generally followed by GABAergic IPSCs, glycinergic IPSCs, or both. Therefore, when the duration of these IPSCs is prolonged, the number of spikes should be decreased, and consequently the peak amplitude and integrated area of polysynaptic EPSCs can be reduced in the recorded neuron.

The remaining possibility was that isoflurane might

directly suppress the excitability of excitatory interneurons by affecting membrane properties for generating action potentials. Many studies have reported that inhaled anesthetics affect several types of K<sup>+</sup> and Ca<sup>2+</sup> channels,<sup>14-21</sup> which may alter action potential-generating membrane properties. However, our data have shown that isoflurane had no effect on the resting membrane potential and the threshold of action potential discharges (fig. 9). Therefore, a direct inhibitory action of isoflurane on membrane excitability is unlikely as a mechanism for the inhibition of polysynaptic EPSCs.

It is well known that inhaled anesthetics also enhance the glycine-induced Cl<sup>-</sup> current.<sup>31</sup> However, in this study, we did not examine the isoflurane action on the glycine receptor, because the inhibitory effect of glycinergic transmission in nociceptive transmission is much less prominent than that of GABAergic transmission in the SG.<sup>34</sup> The glycine receptor may also play a role, at least in part, in the antinociceptive action of isoflurane in the dorsal horn.

In conclusion, we have demonstrated that 1 MAC isoflurane markedly inhibits dorsal root-evoked polysynaptic EPSCs. The augmentation of GABAergic transmission by isoflurane is the most likely mechanism for this phenomenon. Our results presented here may provide a cellular basis for the antinociceptive action of isoflurane at the spinal cord level.

The authors thank Hidemasa Furue, Ph.D. (Lecturer, Department of Integrative Physiology, Graduate School Sciences, Kyusyu University, Fukuoka, Japan), and Naoshi Fujiwara, Ph.D. (Professor and Chairman, Department of General Education, Niigata University School of Health Sciences, Niigata, Japan), for technical advice.

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