

# Isoflurane Inhibits Transmitter Release and the Presynaptic Action Potential

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**Background:** Isoflurane inhibits the excitatory postsynaptic current (EPSC) at many synapses. Accumulated evidence suggests the involvement of a presynaptic mechanism. However, the extent of the presynaptic contribution has not been quantitatively studied. Furthermore, the mechanism underlying the presynaptic contribution remains unclear.

**Methods:** To estimate the presynaptic contribution, the authors compared the effects of isoflurane on the presynaptic capacitance jump, which is proportional to vesicle release, and the postsynaptic glutamate receptor-mediated EPSC at a calyx-type synapse in rat brainstem. The authors determined whether isoflurane affects the waveform of the action potential recorded from nerve terminals. By studying the relation between the EPSC and the presynaptic action potential at the same synapse, the authors determined whether isoflurane inhibits the EPSC by decreasing the presynaptic action potential.

**Results:** Isoflurane at 0.35–1.05 mM reduced the EPSC and the presynaptic capacitance jump to a similar degree without affecting the miniature EPSC (an indicator of quantal size), suggesting that isoflurane inhibits the EPSC predominantly by reducing glutamate release. Isoflurane reduced the presynaptic action potential by approximately 3–8%. The EPSC was proportional to the presynaptic action potential amplitude raised to a power of 10.2. Based on this relation, inhibition of the presynaptic action potential contributed to 62–78% of isoflurane-induced inhibition of the EPSC.

**Conclusions:** Isoflurane inhibits the EPSC predominantly by inhibition of transmitter release. Isoflurane reduces the presynaptic action potential amplitude, which may contribute significantly to its inhibitory effect on the EPSC.

VOLATILE anesthetics inhibit the excitatory postsynaptic current (EPSC) or potential at many synapses.<sup>1,2</sup> This inhibitory effect, together with an enhancement of in-

hibitory synaptic transmission, may contribute to the generation of general anesthesia.<sup>1,2</sup> The mechanism underlying volatile anesthetic-induced inhibition of the EPSC is not completely understood. Multiple lines of evidence indirectly suggest a presynaptic contribution.<sup>3–12</sup> However, direct evidence indicating the extent of a presynaptic contribution is missing.

The mechanism mediating inhibition of transmitter release by volatile anesthetics remains not well understood. Transmitter release is triggered by  $\text{Ca}^{2+}$  influx through  $\text{Ca}^{2+}$  channels, which are activated by action potentials. Each of these steps could be the target for volatile anesthetics, as proposed by studies on somatic or expressed channels.<sup>9,13–18</sup> The relative contribution of each of these presynaptic mechanisms remains unclear.

Here, we studied how isoflurane, a clinically used volatile anesthetic, inhibits the EPSC at a large, glutamatergic calyx-type synapse in the medial nucleus of the trapezoid body (MNTB) in rat brainstem. We chose this synapse because both the nerve terminal and the postsynaptic neuron can be patch clamped,<sup>19</sup> which allows for a direct and quantitative study of presynaptic mechanisms underlying isoflurane-induced inhibition of the EPSC. By monitoring vesicle release with presynaptic capacitance measurements, combined with measurements of EPSCs, we found that isoflurane inhibited the EPSC by depression of vesicle release. By quantitatively examining the relation between the EPSC and the presynaptic action potential, we found that isoflurane reduced the presynaptic action potential amplitude, which may contribute significantly to its inhibition of the EPSC.

## Materials and Methods

### *Slice Preparation and Electrophysiology*

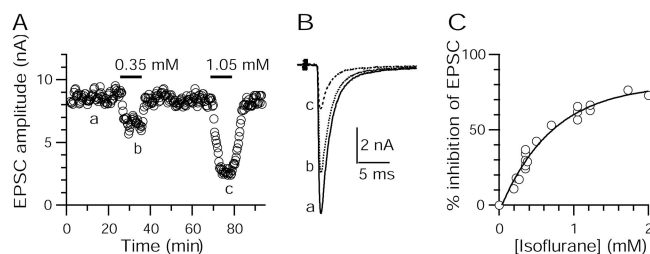
The experiments were performed with permission from the Animal Studies Committee of Washington University (St. Louis, Missouri). All methods were described previously.<sup>20,21</sup> Briefly, Wistar rats (7–10 days old) were decapitated. Transverse or parasagittal slices, 200  $\mu\text{m}$  thick, were cut from the auditory brainstem with a vibratome. Recordings were made at room temperature (23–25°C). The holding potential was –80 mV, and the potential was corrected for a liquid junction potential of –11 mV between the extracellular and the pipette solution. Currents were low-pass filtered at 5 KHz and digitized at 20 KHz with a 16-bit analog-to-digital converter (Instrutech, Greatneck, NY). Data were expressed as

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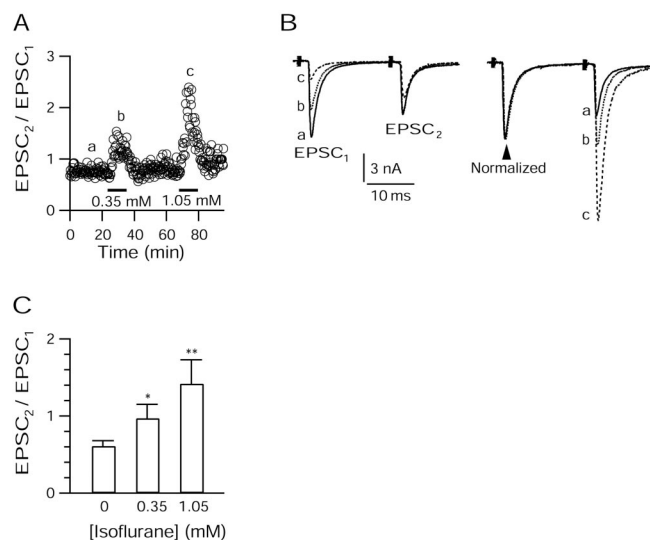
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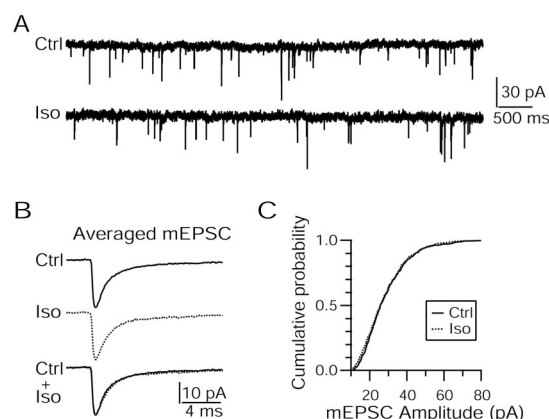
**Fig. 1.** Isoflurane inhibits the excitatory postsynaptic current (EPSC) dose dependently. (A) Isoflurane at 0.35 and 1.05 mM (bars) inhibited the EPSC to different levels. The EPSC was measured every 20 s. (B) Sampled EPSCs (a, b, and c) taken at times indicated in A. Stimulation artifacts are truncated. (C) The percent inhibition of the EPSC amplitude plotted versus isoflurane concentration in the slice chamber from eight synapses. With the use of the Igor program (WaveMetrics, Inc., Lake Oswego, OR) using the principle of the least squares optimization, the data were fit with a Hill function (curve) with an  $EC_{50}$  of 0.49 mM, a Hill coefficient of 1.7, and a maximal inhibition of 80%.

mean  $\pm$  SEM. The statistical test for calculation of  $P$  values was a  $t$  test.

For postsynaptic whole cell voltage clamp recordings of EPSCs and miniature EPSCs (mEPSCs; figs. 1–3) in transverse slices, an Axopatch 200B amplifier (Axon Instruments Inc., Foster City, CA) was used. The postsyn-



**Fig. 2.** Isoflurane relieves paired-pulse depression. (A) Isoflurane at 0.35 and 1.05 mM increased the ratio ( $EPSC_2/EPSC_1$ ) between the second ( $EPSC_2$ ) and the first ( $EPSC_1$ ) excitatory postsynaptic current (EPSC) amplitude during paired-pulse stimulation with an interval of 20 ms. The pair of EPSCs was evoked every 20 s, and the ratio was plotted. The bars indicate the isoflurane application time. The labels a, b, and c indicate the times at which the sampled EPSCs shown in B were taken. Data were taken from the same synapse as shown in figures 1A and B. (B) (Left) Sampled EPSCs recorded in control (a, solid) and in the presence of 0.35 mM (b, dotted) and 1.05 mM (c, dash) isoflurane. (Right) The traces in the left were scaled so that the first EPSCs were the same. (C) The ratio (mean  $\pm$  SEM) between the second and the first EPSC amplitudes (20 ms interval) in control ( $n = 16$ ) and in the presence of 0.35 mM ( $n = 6$ ) and 1.05 mM ( $n = 3$ ) isoflurane. The ratios in the presence of isoflurane were significantly larger than the ratio in control ( $t$  test). \*  $P < 0.05$ . \*\*  $P < 0.01$ .

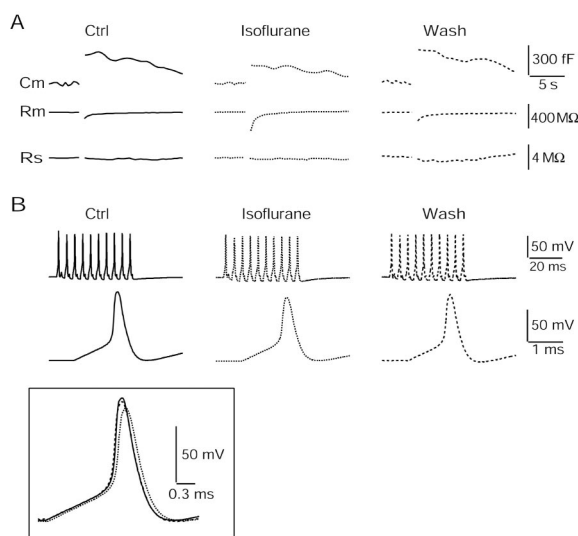


**Fig. 3.** Isoflurane does not affect the miniature excitatory postsynaptic current (mEPSC) amplitude. (A) Sampled mEPSCs in control (Ctrl, top) and isoflurane (Iso, 0.7 mM, bottom). (B) Averaged mEPSCs in control (top solid, 505 mEPSCs in 4 min) and in 0.7 mM isoflurane (middle dotted, 454 mEPSCs in 4 min). These two traces are superimposed (bottom). (C) The mEPSC amplitude distribution in control and in 0.7 mM isoflurane. Data in A, B, and C were from the same synapse.

aptic pipette (2–3 M $\Omega$ ) solution contained 125 mM K-gluconate, 20 mM KCl, 4 mM MgATP, 10 mM Na<sub>2</sub>-phosphocreatine, 0.3 mM GTP, 10 mM HEPES, and 0.5 mM EGTA, at a pH of 7.2, adjusted with KOH. The series resistance ( $< 15$  M $\Omega$ ) was compensated by 95% (lag, 10  $\mu$ s). The mEPSCs were analyzed with use of a software program (Jaevin Software, Leonia, NJ). The bath solution contained 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM dextrose, 0.4 mM ascorbic acid, 3 mM myo-inositol, 2 mM sodium pyruvate, 0.05 mM D-APV, 0.01 mM bicuculline, and 0.01 strychnine, at a pH of 7.4 when bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

For capacitance recordings at calyces (fig. 4), an EPC-9 amplifier, together with the software lock-in amplifier (HEKA, Lambrecht, Germany), was used at parasagittal slices (see Sun and Wu<sup>21</sup> for selection of calyces). The bath solution was the same as described in the previous paragraph (starting with “The bath solution contained . . .”). The series resistance ( $< 20$  M $\Omega$ ) was compensated by 60%. A sinusoidal stimulus (1,000 Hz) was applied in addition to the DC holding potential ( $-80$  mV). The peak-to-peak voltage of the sine wave was less than 60 mV. The presynaptic pipette (3.5–5 M $\Omega$ ) solution contained 125 mM K-gluconate, 20 mM KCl, 4 mM MgATP, 10 mM Na<sub>2</sub>-phosphocreatine, 0.3 mM GTP, 10 mM HEPES, and 0.05 mM BAPTA, at a pH of 7.2, adjusted with KOH.

For voltage clamp recordings of presynaptic Ca<sup>2+</sup> currents (figs. 5 and 6), the presynaptic pipette (3.5–5 M $\Omega$ ) solution contained 125 mM Cs-gluconate, 20 mM CsCl, 4 mM MgATP, 10 mM Na<sub>2</sub>-phosphocreatine, 0.3 mM GTP, 10 mM HEPES, and 0.05 mM BAPTA, at a pH of 7.2, adjusted with CsOH. The bath solution contained 105 mM NaCl, 20 mM TEA-Cl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>,

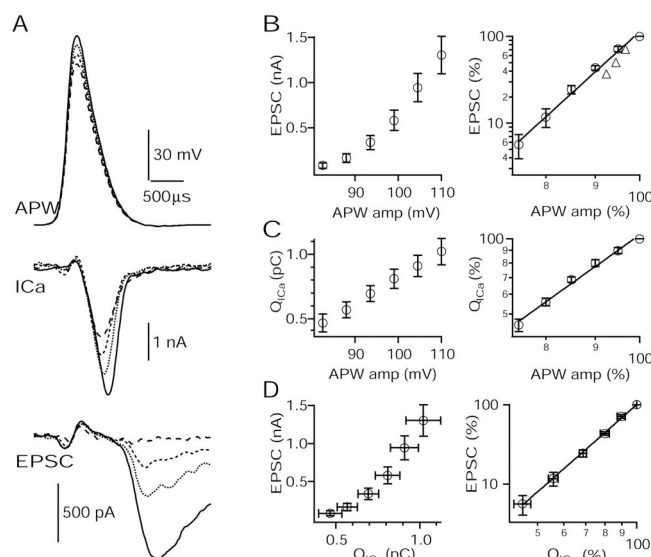


**Fig. 4.** Isoflurane reduces the capacitance jump and the action potential amplitude. (A) Sampled capacitance jump (Cm, *top*) induced by 10 action potentials at 200 Hz before application of (Ctrl, *left*), during application of (*middle*), and after washout of (*right*) 0.7 mM isoflurane. The gaps indicate the time of stimulation. Capacitance jumps were not accompanied by parallel changes in the membrane resistance (Rm, *middle*) and series resistance (Rs, *bottom*). The traces were low-pass filtered at 20 Hz. (B) Sampled action potentials induced by current injection (10 pulses at 200 Hz; each pulse was a 3-ms step current of 400 pA) before application of (*left*, *solid*), during application of (*middle*, *dotted*), and after washout of (*right*, *dashed*) 0.7 mM isoflurane. The first action potential in each train is shown below the train. They are superimposed in the *inset* in larger scale.

2 mM CaCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM dextrose, 0.4 mM ascorbic acid, 3 mM *myo*-inositol, 2 mM sodium pyruvate, 0.001 mM tetrodotoxin, 0.1 mM 3,4-diaminopyridine, and 0.05 mM D-APV, at a pH of 7.4 when bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

#### Delivery of Isoflurane and Measurements of Isoflurane Concentration

Delivery of isoflurane and measurements of isoflurane concentration at the room temperature were similar to those described previously.<sup>22,23</sup> Briefly, the 95% O<sub>2</sub>-5% CO<sub>2</sub> mixture was directed *via* a flowmeter through a calibrated commercial vaporizer (Fortec; Fraser Harlake, Orchard Park, NY) containing isoflurane. The gas mixture of O<sub>2</sub>-CO<sub>2</sub>-isoflurane was then used to bubble the experimental saline in a reservoir bottle for at least 20 min before it was applied to the slice chamber with a pump (approximately 4 ml/min, Ismatec pump; Cole-Parmer, Vernon Hills, IL). The aqueous isoflurane concentration, measured with the standard gas chromatography methods,<sup>24</sup> dropped from the saline reservoir to the slice chamber by  $28 \pm 2\%$  ( $n = 3$ ). The aqueous isoflurane concentration was linearly proportional to the gaseous partial pressure of isoflurane, detected by a Raman light-scattering gas analyzer (Rascal II; Ohmeda, Salt Lake City, UT; data not shown). Based on these data,



**Fig. 5.** Relation between the excitatory postsynaptic current (EPSC) and the action potential waveform (APW) amplitude (amp). (A) Sampled presynaptic Ca<sup>2+</sup> currents (ICa, *middle*) and EPSCs (*bottom*) evoked by various amplitudes (*solid line* = 110 mV; *dotted line* = 104.5 mV; *short-dash line* = 99 mV; *long-dash line* = 93.5 mV) of APW commands (*top*) at the same synapse. (B) (*Left*) Relation between the EPSC amplitude and the presynaptic APW amplitude obtained from six synapses. (*Right*) Same data as in the *left* but normalized to that evoked by 110 mV APW and plotted in double log scale (*circles*). The *line* was obtained from fitting with equation 1 (see Results for fit parameters). For comparison, the mean EPSCs and the mean action potential amplitudes obtained with 0.35, 0.7, and 1.05 mM isoflurane were plotted (*triangles*). (C) (*Left*) Relation between the normalized ICa charge (Q<sub>ICa</sub>) and the APW amplitude obtained from six synapses. (*Right*) Same data as in the *left* but normalized to that evoked by 110 mV of APW. The *line* was obtained from fitting with equation 2 (see Results). (D) (*Left*) Relation between the EPSC amplitude and Q<sub>ICa</sub> obtained from six synapses. (*Right*) Same data as in the *left* but normalized to that evoked by 110 mV of APW. The *line* was obtained from fitting with equation 3 (see Results).

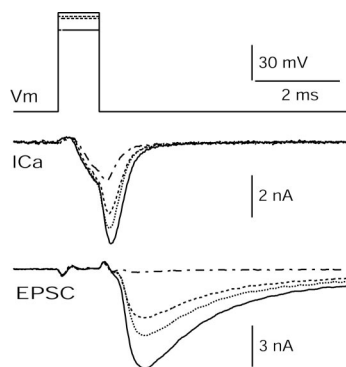
the continuously measured gaseous partial pressure was used to estimate the aqueous isoflurane concentration in the slice chamber.

## Results

### Isoflurane Inhibits the EPSC Dose Dependently at Calyx-type Synapses

The postsynaptic neurons of the MNTB were whole cell voltage clamped at a holding potential of  $-80$  mV, a potential at which the EPSC is mediated by AMPA receptors.<sup>25</sup> A bipolar stimulating electrode was positioned at the midline of the trapezoid body, through which the presynaptic axons of MNTB synapses pass. A stimulus (5–20 V, 0.1 ms) was delivered every 20 s to induce a presynaptic action potential,<sup>20</sup> and the resulting EPSC was recorded at the postsynaptic cell. The EPSC amplitude in control was  $5.6 \pm 0.7$  nA ( $n = 8$ ). Application of 0.35 mM isoflurane (see Materials and Methods) reduced the EPSC amplitude by  $34 \pm 3\%$  ( $n = 5$  synapses)





**Fig. 6.** Relation between the excitatory postsynaptic current (EPSC) and the amplitude of the step depolarization. Sampled recordings of presynaptic  $\text{Ca}^{2+}$  currents (ICa, middle) and EPSCs (bottom) evoked by steps of 1-ms depolarization ( $V_m$  = membrane voltage; top) from  $-80$  to various amplitudes (solid line =  $7$  mV; dotted line =  $4$  mV; dashed line =  $2$  mV; dotted and dashed line =  $-8$  mV).

without significantly affecting the 20–80% rise and decay time (fig. 1). The blocking effect reached steady state in less than 10 min after applying isoflurane and reversed in 5 min after washout (fig. 1A). This effect was dose dependent (e.g., fig. 1, A and B). The percent inhibition as a function of the isoflurane concentration (eight synapses) was fit with a Hill function in which the  $\text{EC}_{50}$ , the Hill coefficient, and the maximal inhibition were free parameters. The fitting results were an  $\text{EC}_{50}$  of  $0.49$  mM, a Hill coefficient of  $1.7$ , and a maximal inhibition of  $80\%$  (fig. 1C). Here, the  $\text{EC}_{50}$  referred to the concentration at which isoflurane achieved 50% of its maximal inhibition, i.e.,  $40\%$  ( $= 50\% \times 80\%$ ) inhibition of the EPSC. These results are quantitatively similar to those observed at hippocampal synapses.<sup>5,23</sup> The minimum alveolar concentration (MAC) of isoflurane, at which general anesthesia occurs in 50% of animals, is  $0.35$  mM in rats.<sup>26</sup> Therefore, clinically relevant concentrations ( $\leq 2$  MAC) of isoflurane may significantly inhibit the EPSC at MNTB synapses.

#### *Isoflurane Reduces Paired-pulse Depression*

When a pair of electrical stimuli with a short interval (e.g.,  $< 50$  ms) is applied to axons, it generates a pair of action potentials, which in turn causes a pair of EPSCs. The ratio between the amplitudes of the second and the first EPSC is less than 1 at synapses, including MNTB synapses,<sup>27</sup> which release a large number of vesicles during the first stimulus. Such a paired-pulse depression is conventionally interpreted as a result of depletion of the releasable pool by the first pulse.<sup>28</sup> Consequently, if isoflurane reduces transmitter release, depletion is relieved, and thus, paired-pulse depression is reduced. We tested this prediction as follows. A pair of electrical stimuli ( $5$ – $20$  V,  $0.1$  ms) with an interval of  $20$  ms was applied to generate a pair of EPSCs (e.g., fig. 2, A and B). The ratio between the second and the first EPSC amplitudes was  $0.61 \pm 0.07$  ( $n = 16$ ; fig. 2). This ratio was

significantly increased to  $0.97 \pm 0.18$  ( $n = 5$ ;  $P = 0.02$ ) and  $1.42 \pm 0.31$  ( $n = 3$ ;  $P = 0.0002$ ) in the presence of  $0.35$  and  $1.05$  mM isoflurane, respectively (fig. 2). These results, similar to those observed at hippocampal synapses,<sup>5–7</sup> are consistent with the hypothesis that isoflurane inhibits the EPSC by a presynaptic mechanism.

#### *Isoflurane Does Not Affect the Miniature EPSC*

If isoflurane inhibits the EPSC by decreasing the sensitivity of postsynaptic glutamate receptors, it should decrease the mEPSC, the postsynaptic response to release of a single vesicle. We tested this prediction by collecting mEPSCs for 3–5 min before and after a 10-min application of  $0.7$  mM isoflurane (fig. 3A). Before application of isoflurane, the peak amplitude of mEPSCs was  $24.1 \pm 3.1$  pA ( $n = 5$  synapses). Application of isoflurane did not significantly reduce the amplitude of the mean mEPSC, 20–80% rise time and half decay time (e.g., fig. 3, B and C;  $n = 5$  synapses;  $P > 0.4$ ). The frequency of mEPSCs ranged from approximately 1 to 5 Hz in control. Isoflurane reduced the frequency to  $91 \pm 4\%$  of control, which was not statistically significant ( $P = 0.18$ ;  $n = 5$ ). Because AMPA receptors were not saturated by release of a single quantum at MNTB synapses,<sup>29</sup> these results suggest that isoflurane does not inhibit the EPSC by decreasing the sensitivity of postsynaptic AMPA receptors.

#### *Isoflurane Reduces the Presynaptic Capacitance Jump*

To determine whether isoflurane inhibits the EPSC by reducing the number of released vesicles, we measured the membrane capacitance increase at the nerve terminal (the calyx of Held), which is proportional to the number of released vesicles.<sup>21,30</sup> Capacitance measurements were made in the voltage clamp mode (fig. 4A). The voltage clamp mode was switched to a current clamp mode for 500 ms, during which 10 pulses of 3-ms current of  $300$ – $600$  pA at  $200$  Hz were injected into the calyx to induce 10 action potentials (fig. 4B). We induced 10 action potentials instead of 1 to increase the signal-to-noise ratio. The capacitance jump, calculated as the difference between the capacitance values before and 500 ms after stimulation, was  $293 \pm 31$  fF ( $n = 6$ ; fig. 4A). The jump returned to the baseline in 10s of seconds (not shown, but see Sun and Wu,<sup>21</sup> which reflects the time course of endocytosis). Therefore, slow endocytosis does not significantly affect measurements of capacitance jumps after stimulation. Consistent with our previous study,<sup>21</sup> the capacitance change did not parallel the change of the membrane resistance or the series resistance (fig. 4A). Application of isoflurane ( $0.7$  mM) significantly reduced the capacitance jump by  $43 \pm 6\%$  ( $n = 6$ ) of control. This decrease was similar to the decrease in the EPSC (approximately 50% at  $0.7$  mM as calculated from the fit curve in fig. 1C), suggesting that

isoflurane inhibits the EPSC largely by reducing vesicle release.

#### *Isoflurane Decreases the Amplitude of the Presynaptic Action Potential*

The effects of isoflurane on the capacitance jump were accompanied by a decrease in the amplitude of the presynaptic action potential. For example, isoflurane at 0.7 mM reduced the presynaptic action potential amplitude from  $106.3 \pm 1.3$  mV ( $n = 6$ ) to  $100.5 \pm 1.7$  mV ( $n = 6$ ;  $P < 0.01$ ; fig. 4B). That is, isoflurane reduced the action potential amplitude by  $5.8 \pm 1.3$  mV or  $5.5 \pm 1.3\%$  ( $n = 6$ ). Isoflurane (0.7 mM) also slightly hyperpolarized the resting membrane potential (ranged from  $-56$  to  $-68$  mV) by  $1.2 \pm 0.3$  mV ( $n = 6$ ;  $P < 0.01$ ). These effects were reversible on drug washout (fig. 4B). Isoflurane (0.7 mM) did not significantly affect the half-width of the action potential ( $0.36 \pm 0.02$  ms in control;  $0.39 \pm 0.03$  ms in isoflurane;  $n = 6$ ;  $P > 0.2$ ). The effect of isoflurane on the action potential amplitude was dose dependent. At 0.35 mM, isoflurane reduced the amplitude by  $3.3 \pm 1.5\%$  ( $n = 4$ ). At 1.05 mM, isoflurane reduced the amplitude by  $7.6 \pm 0.8\%$  ( $n = 4$ ).

The above analysis of the action potential waveform (APW) was made at the first action potential during a train of 10 action potentials (fig. 4B). Similar results were obtained when the last action potential during the 10-action-potential train was analyzed. For example, isoflurane at 0.7 mM reduced the amplitude of the last action potential by  $4.1 \pm 1.1\%$  ( $n = 6$ ), without significantly affecting its half-width ( $P > 0.4$ ). Therefore, the inhibition of the action potential during the 10-action-potential train was not activity dependent.

#### *Relation between the EPSC and the Presynaptic Action Potential Amplitude*

To determine the extent to which a decrease in the action potential accounts for inhibition of release, we examined the relation among the presynaptic action potential, the action potential-induced presynaptic  $\text{Ca}^{2+}$  current (ICa), and the EPSC. All of these measurements were made in the absence of isoflurane. Both the nerve terminal and the postsynaptic neuron were whole cell voltage clamped at the same synapse. ICa was recorded by pharmacologically blocking  $\text{Na}^+$  and  $\text{K}^+$  channels (see Materials and Methods). An experimentally recorded action potential of 110 mV evoked by nerve stimulation (stimulation electrode positioned at the mid-line of the trapezoid body)<sup>20</sup> was used as the voltage command to mimic an action potential. Application of this command induced a presynaptic ICa and a postsynaptic EPSC at the same synapse (fig. 5A). By scaling the typical APW to 95, 90, 85, 80, or 75%, the APW amplitude was reduced by 5.5, 11, 16.5, 22, or 27.5 mV, respectively. Consequently, the presynaptic ICa and the postsynaptic EPSC were reduced (e.g., fig. 5A). The am-

plitude of the resulting EPSC was highly nonlinearly related to the presynaptic APW amplitude (fig. 5B). For example, the EPSC amplitude was decreased to  $72 \pm 5\%$  ( $n = 7$ ) or  $43 \pm 3\%$  ( $n = 7$ ) of control when the APW amplitude was decreased from 110 mV to 104.5 or 99 mV, respectively (fig. 5B). The normalized EPSC amplitude and the normalized APW amplitude obtained from seven synapses was fit with the following equation:

$$\text{EPSC} = c_1 \cdot \text{APW}^n \quad (1)$$

in which  $c_1 = 1.15$  and  $n = 10.2$  (fig. 5B, right).

To study why there was such an extremely nonlinear relation, we plotted the ICa charge *versus* the APW amplitude (fig. 5C) and the EPSC amplitude *versus* the ICa charge (fig. 5D). The relation between the normalized ICa charge ( $Q_{\text{ICa}}$ ) and the normalized APW amplitude was fit with the following equation:

$$Q_{\text{ICa}} = c_2 \cdot \text{APW}^k \quad (2)$$

in which  $c_2 = 1.04$  and  $k = 2.8$  (fig. 5C, right). Therefore, a small decrease in the APW amplitude may cause a larger decrease in the  $Q_{\text{ICa}}$ . The relation between the normalized EPSC amplitude and the normalized  $Q_{\text{ICa}}$  was fit with the following equation:

$$\text{EPSC} = c_3 \cdot (Q_{\text{ICa}})^m \quad (3)$$

in which  $c_3 = 1.01$  and  $m = 3.7$  (fig. 5D, right). This nonlinear relation was consistent with previous studies at MNTB synapses and other synapses.<sup>19</sup> Therefore, equations 2 and 3 explain why the EPSC amplitude was extremely nonlinearly related to the APW amplitude.

The EPSC induced by a typical APW command was  $1.3 \pm 0.3$  nA ( $n = 7$ ), which was smaller than the EPSC induced by a real action potential.<sup>20,31</sup> This is likely caused by imperfect voltage clamp. To determine whether the results shown in figure 5 still hold when the EPSC is similar to that induced by a real action potential, we induced EPSC by a 1-ms step voltage command from  $-80$  to  $+7$  mV (fig. 6, solid traces). We have shown such a step depolarization to induce release similar to that induced by a real action potential.<sup>32</sup> Here, this depolarization induced an EPSC of  $5.2 \pm 1.4$  nA ( $n = 4$ ; e.g., fig. 6). By decreasing the 1-ms step voltage command by 5 mV (the step was from  $-80$  to  $+2$  mV; fig. 6, dash trace), *i.e.*, by approximately 6% ( $5/87 \approx 6\%$ ), the charge of the presynaptic calcium current and the postsynaptic EPSC decreased to  $88 \pm 2$  and  $67 \pm 8\%$  ( $n = 4$ , e.g., fig. 6) of control, respectively. These results were similar to those shown in figure 5, confirming that a small decrease in a brief presynaptic depolarization results in a large decrease in the EPSC.

Based on the relation between the EPSC and the isoflurane concentration (fit curve in fig. 1C), 0.35, 0.7, and 1.05 mM isoflurane inhibited the EPSC by 29, 52, and 63%, respectively. Isoflurane at these concentrations reduced the action potential amplitude by means of 3.3,

5.5, and 7.6%, respectively. These results (fig. 5B, right, triangles) were plotted together with those obtained with voltage clamp experiments in control (fig. 5B, right, circles). It was clear from the plot that the decrease in the action potential amplitude may largely, but not completely account for the decrease in the EPSC induced by isoflurane. According to equation 1, 3.3, 5.5, and 7.6% decreases in the action potential amplitude would decrease the EPSC by 18, 35, and 49%, respectively. Therefore, on average, the decrease in the EPSC induced by the decrease in the action potential amplitude accounted for approximately 62% (= 18%/29%), 70% (= 35%/50%), or 78% (= 49%/63%) of inhibition of the EPSC by isoflurane at 0.35, 0.7, and 1.05 mM, respectively.

The amplitude of the APW we used was 110 mV rather than 106 mV as measured here. This was to be consistent with several previous studies that used a 110-mV APW as the voltage command.<sup>31,33,34</sup> However, such a small difference should not significantly affect our conclusion because a similar extent of decrease in the EPSC was observed when the APW amplitude was decreased by 5.5 mV from 110 or 104.5 mV (fig. 5B).

## Discussion

### *Isoflurane Depresses the EPSC by Inhibition of Vesicle Release*

We found that isoflurane dose-dependently inhibited the EPSC at MNTB synapses. By the use of capacitance measurements to monitor vesicle release at nerve terminals, we showed that isoflurane inhibits vesicle release induced by a train of 10 action potentials at 200 Hz. The stimulation train lasted for only 50 ms, during which mobilization of synaptic vesicles from the reserve pool to the releasable pool is negligible.<sup>34,35</sup> Therefore, the capacitance jump induced by the stimulation train was proportional to the averaged release evoked by an action potential during the train. Because isoflurane inhibited the capacitance jump and the EPSC to a similar degree and reduced paired-pulse depression but did not affect the mEPSC amplitude, we concluded that isoflurane inhibited the EPSC predominantly by a presynaptic mechanism. These results provide strong evidence confirming many previous studies suggesting a presynaptic contribution to volatile anesthetic-induced inhibition of synaptic transmission.<sup>3-12</sup>

Isoflurane inhibited the presynaptic capacitance jump ( $43 \pm 6\%$ ) and the EPSC (50%) to slightly different extents. Therefore, the possibility that isoflurane reduced the EPSC to a minor extent by a mechanism other than presynaptic mechanisms cannot be ruled out. These minor mechanisms might include effects on the number of glutamate receptors,<sup>12</sup> glutamate receptor desensitization, and clearance of glutamate from the synaptic cleft. However, the slight difference in the inhibition of the capacitance jump and the EPSC might also be

caused by the difference in the stimuli used to evoke capacitance jump and the EPSC. The EPSC was evoked by a single action potential. Such an EPSC was linearly proportional to the capacitance jump, suggesting that postsynaptic receptor saturation does not occur at such a minimal stimulus.<sup>21</sup> In contrast, the capacitance jump was evoked by 10 action potentials at 200 Hz, which may cause a larger extent of depletion of a limited pool of vesicles immediately available for release<sup>21,34,35</sup> and thus might reduce the percent inhibition of the capacitance jump by isoflurane.

### *Isoflurane Inhibits the EPSC Largely by Depressing the Presynaptic Action Potential*

We found that 0.35–1.05 mM isoflurane reduced the presynaptic action potential amplitude by approximately 3.3–7.6%. This is consistent with many previous studies showing little effect or minor effects of general anesthetics on the action potential.<sup>1,2</sup> However, the EPSC was proportional to the presynaptic APW amplitude raised to a power of 10.2 (equation 1). Such an extremely nonlinear relation was caused by two highly nonlinear relations, one between the charge of the ICa induced by the APW and the APW amplitude (equation 2), and the other between the EPSC amplitude and the ICa charge (equation 3). Based on these quantitative relations, we concluded that inhibition of the presynaptic action potential accounts for approximately 62–78% of the inhibitory effects of isoflurane on the EPSC, whereas the remaining fraction might be mediated by mechanisms downstream of the action potential.

The decrease in the action potential amplitude could be due to inhibition of Na<sup>+</sup> channels<sup>13,36</sup> or activation of K<sup>+</sup> channels.<sup>14,37-39</sup> The mechanism downstream of the action potential may be mediated by inhibition of Ca<sup>2+</sup> channels<sup>15,17,18</sup> or the release machinery downstream of the calcium influx.<sup>16</sup> It would be of great interest to dissect each of these potential mechanisms. We attempted to perform voltage clamp recordings of Na<sup>+</sup> currents at the calyx and found that the current was too large (> 5–10 nA) and too fast to reliably voltage clamp. We also attempted to study whether isoflurane (0.7 mM) inhibits the presynaptic Ca<sup>2+</sup> currents. We did not observe consistent inhibition of Ca<sup>2+</sup> current by more than 15% (unpublished results, Xin-Sheng Wu, M.D., Ph.D., and Ling-Gang Wu, M.D., Ph.D., National Institute of Neurologic Disorders and Stroke, Bethesda, Maryland, May–September 2002). However, the Ca<sup>2+</sup> current evoked by an APW was extremely sensitive to a very small change (e.g., 1- to 2-M $\Omega$  change) in the series resistance. As a result, stable recordings of the Ca<sup>2+</sup> current for as long as 10 min, which is needed for the effects of isoflurane to reach a steady state, was extremely difficult (note that experiments shown in figures 5A and 6 may take < 2 min). We could not consistently resolve an inhibition of less than 10–15% in a 10 min,



which may well be in the range of inhibition induced by isoflurane. In summary, resolving small changes (perhaps < 5–10%) in presynaptic  $\text{Na}^+$  and  $\text{Ca}^{2+}$  currents is a difficult and substantial undertaking that is beyond the scope of the current study.

A single calyx in the MNTB contains approximately 600 conventional active zones<sup>40</sup> and thus is viewed as equivalent to approximately 600 boutons in parallel.<sup>19,41</sup> Except for this difference, the calyx-type MNTB synapse shares many similar properties with central synapses, such as paired-pulse depression or facilitation, a nonlinear relation between release and  $\text{Ca}^{2+}$  currents,  $\text{Ca}^{2+}$  channel types and their modulation by transmitters, AMPA and *N*-methyl-D-aspartate receptors, and the ultrastructure of active zones.<sup>19,41</sup> More importantly, isoflurane dose-dependently inhibited the EPSC and relieved paired-pulse depression while producing a minor reduction of the presynaptic action potential at MNTB synapses. These effects are qualitatively and quantitatively similar to those observed with volatile anesthetics at other synapses.<sup>5–8,10,11,23,42</sup> Isoflurane inhibited the capacitance jump evoked by action potentials at MNTB synapses, which is consistent with the result that volatile anesthetics inhibit glutamate release induced by prolonged depolarization at synaptosomes.<sup>3,4</sup> Therefore, the principles learned in the current study are likely to apply to other glutamatergic synapses.

We performed experiments in brain slices of 7- to 10-day-old rats at room temperature (23–25°C). Whether our findings can be applied to adult rats at body temperature remains unclear. We think this possibility is likely for the following reasons. The electrical properties of MNTB synapses of 9-day-old rats, such as the action potential width, the EPSC size, the quantal size, the calcium channel types, and paired-pulse depression and facilitation, are qualitatively similar but quantitatively slightly different from those of adult rats.<sup>19</sup> Therefore, the results observed here might be quantitatively slightly different in adult rats. However, the major conclusions are likely to remain the same. Volatile anesthetics at similar aqueous concentrations inhibit excitatory synaptic transmission to a similar degree at other synapses at both room temperature and a temperature close to body temperature.<sup>9,22,23,43–45</sup> Therefore, it is likely that the principles learned from brain slices at room temperature apply to those at body temperature. In conclusion, isoflurane-induced inhibition of the presynaptic action potential amplitude may contribute to inhibition of glutamate release, which may be relevant to certain end points of anesthesia.<sup>1,2</sup>

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