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Halothane modulates NMDA and non-NMDA excitatory synaptic transmission in rat cortical neurons

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Abstract
Purpose. Although general anesthetics may decrease neuronal excitation, their detailed effects on spontaneous excitatory postsynaptic currents (EPSCs) remain controversial. We investigated and compared the effects of halothane on *N*-methyl-D-asparate (NMDA) and non-NMDA receptor-mediated postsynaptic currents.
Methods. Spontaneous synaptic currents were recorded by the patch clamp technique in cultured rat cortical neurons. They were isolated by specific pharmacological blocking agents and their electrophysiologic properties were examined.
Results. The frequency of NMDA EPSCs was preferentially decreased as compared with that of non-NMDA EPSCs at halothane 1.2 mM. The total net charge of EPSCs mediated by NMDA and non-NMDA receptors was depressed to 56% ± 6% (mean ± SD) and 71% ± 7% of control by halothane 0.6 mM, and to 11% ± 9% and 59% ± 11% of control by halothane 1.2 mM, respectively.
Conclusion. These results show that halothane causes decrease of excitatory synaptic activity, with NMDA EPSCs being more sensitive than non-NMDA EPSCs.

Key words Halothane · NMDA · Non-NMDA · Patch clamp

Introduction

Although the mechanisms of general anesthesia have not been well elucidated, it is possible that anesthesia is induced by enhancing neuronal inhibition, by decreasing neuronal excitation, or by a combination of both effects. There is ample evidence that most general anesthetics potentiate GABAergic inhibitory synaptic responses [1–3]. The excitatory synaptic system may also be a potential target of general anesthetics [4,5]. Based on their pharamacologic sensitivity, ion permeability, and channel kinetics, the glutamate receptors are broadly divided into two subgroups determined by specific agonists: which are *N*-methyl-D-asparate (NMDA) receptors and non-NMDA receptors [i.e., α-amino-3-hydroxy-5-methyl-4-isoxazole propionate acid (AMPA) and kinate receptors].

Previous studies have shown that volatile anesthetic agents can depress NMDA and non-NMDA receptor-mediated excitatory postsynaptic potentials [6–8]. Perouansky et al. [9] reported that halothane depressed glutamatergic excitatory synaptic transmission irrespective of the receptor subtype, most likely by inhibiting the presynaptic release of glutamate. They also reported that halothane similarly depressed the amplitude of NMDA and non-NMDA receptor-mediated excitatory postsynaptic currents in hippocampal pyramidal cells [10] and inhibitory interneurons. In contrast, it has also been reported that halothane may selectively block NMDA receptor channels. Population spikes mediated by NMDA receptors in the CA1 region of the rat hippocampus appear to be more sensitive to halothane than non-NMDA receptor-mediated responses [11]. Thus, the relative extent of the depression of NMDA and non-NMDA receptor-mediated responses by volatile anesthetic agents remains controversial. One of the reasons for the variable data may be the different preparations and techniques used in different studies. In order to assess the relative importance of various factors underlying the effects of general anesthetics on synaptic transmission, it is important to systematically investigate the parameters likely to be involved using the same preparation established synaptic network.

In this study, we investigated the effects of halothane on NMDA and non-NMDA receptor-mediated postsynaptic currents in rat cultured cortical neurons, where spontaneous excitatory postsynaptic currents

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(EPSCs) and inhibitory postsynaptic currents (IPSCs) could be recorded [12,13], and compared their electrophysiologic properties.

Materials and methods

Cell preparation

Cortical neurons were cultured by the method previously reported by Marszalec et al. [14]. In brief, 17-day fetuses were removed from pregnant Sprague-Dawley rats under methoxyflurane anesthesia. Small wedges of the frontal cortex were excised and incubated in phosphate-buffered saline containing 0.25% (w/v) trypsin (Sigma Chemical, St. Louis, MO, USA) for 25 min at 37°C. After mechanical trituration by repeated passages through a Pasteur pipette, the dissociated cells were suspended in Dulbecco's modified Eagle's medium with 10% (v/v) Ham's F-12 supplement, 2 mM glutamine, 20 $U \cdot ml^{-1}$ of penicillin, and 20 $ng \cdot ml^{-1}$ of streptomycin. The cells were placed into 35-mm culture dishes at a concentration of 200,000 cells·3 ml^{-1}. Each dish contained five 12-mm glass coverslips with a confluent layer of glia that had been plated 2 to 4 weeks earlier. The cortical/glial cocultures were maintained in a humidified atmosphere of 90% air and 10% CO_2 at 37°C. The spontaneous activity of these cells increased over time and was probably related to the development of synapses [15]. The cells used in these experiments were cultured for 2 to 4 weeks. All current recordings were made from pyramidal-shaped neurons that were 30 to 50 μm in diameter. Smaller oval bipolar cells were also present in the cultures, but they were not used for recording.

Electrophysiological methods

Spontaneous synaptic currents were recorded by the standard patch clamp technique [16] using an Axopatch-1B amplifier (Axon Instruments, Foster City, CA, USA) at room temperature (22°–25°C). Recording electrodes were pulled from borosilicate glass (Kimble, Vineland, NJ, USA) on a vertical puller to a final resistance of 1.5 to 2.5 MΩ when filled with the internal solution.

The external solution used for recording spontaneous synaptic currents consisted of (mM): NaCl 150, KCl 5, $CaCl_2$ 2.5, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES-acid) 5.5, HEPES-Na^+ 4.5, and glucose 10. This solution was adjusted to a pH of 7.3 with NaOH. During observation of the NMDA-mediated component of the EPSCs, Mg^{2+} was omitted from the external solution to avoid voltage-dependent Mg^{2+} blockade. The internal pipette solution contained (mM): K^+ gluconate 140, $MgCl_2$ 2.0, $CaCl_2$ 1.0, ethylene glycoltetraacetic acid (EGTA) 11, HEPES-acid 10, Mg^{2+}-ATP 2, and Na^+-GTP 0.2. It was adjusted to a pH of 7.3 with KOH.

Drug application

A neuron-coated glass coverslip was placed into a microscope-mounted recording chamber (0.5 ml volume), and then control and drug-containing solutions were perfused at a rate of 1 to 2 $ml \cdot min^{-1}$. A saturated halothane (Ayerst Laboratories, New York, NY, USA) solution was made by stirring halothane into the external solution over 8 h in a sealed glass container with a minimal air space. Halothane test solutions were then prepared by diluting the saturated halothane solution with the external solution immediately prior to the experiment using sealed glass containers and glass pipettes. By 19F-NMR spectroscopy (GE NMR instruments, Tallahassee, FL, USA), the saturated solution was found to contain 18.0 ± 0.5 mM halothane at 25.0°C, a value identical to that determined previously [17]. The 80-fold diluted saturated solution was found to contain 0.23 mM halothane.

The EPSCs were isolated by application of 20 μM bicuculline (Sigma) to block GABAergic currents, and 1 μM 6-cyano-7-nitroquinoxaline (CNQX) (Tocris Cookson, St. Louis, MO, USA) or 30 μM 2-amino-5-phosphonovaleric acid (APV) (Research Biochemicals, Natick, MA, USA) was used to block the AMPA or NMDA-mediated currents, respectively. In the previous studies using the same cultured rat hippocampal and cortical neurons, spontaneous EPSCs and IPSCs that were isolated by applying each specific receptor blocker or by manipulating the external and internal ionic balance were recorded [12,13]. All other chemicals were purchased from Sigma.

Data acquisition and analysis

Postsynaptic currents were continuously recorded for 10 min at a holding potential of −40 mV after 10 min of application of each concentration of halothane. Signals were filtered at 2 kHz and digitized at a sample interval of 200 μs. The algorithm for synaptic event detection started with estimation of the baseline signal variance from a user-specified segment of digitized data that was free of events. Synaptic currents were analyzed with a software package (Mini Analysis Program, Jaejin Software, Leonia, NJ, USA). The amplitude threshold was set as 3× noise, with noise being measured during periods of no visually detectable events. The peak current was determined and the event was followed until the current declined to 5% of the baseline mean. The decay phase of synaptic current $I(t)$, in which t is the time

measured from the peak of the current, was fitted by a biexponential equation of the form

$$I(t) = I_{\text{fast}} e^{-t/\tau \text{fast}} + I_{\text{slow}} e^{-t/\tau \text{slow}}$$

where I_{fast} and I_{slow} are the amplitudes and τ_{fast} and τ_{slow} are the time constants of the fast and slow components, respectively. The frequency, and the amplitude and decay phase of the EPSCs were taken as a measure of presynaptic and postsynaptic effects, respectively [18,19]. According to the quantum theory of vesicular release, miniature postsynaptic currents are assumed to represent the spontaneous release of individual vesicles or neurotransmitter quanta from the presynaptic membrane. Thus, drug-induced changes in the frequency of EPSCs are indicative of a presynaptic action.

The current amplitude (pA), the time constant of current decay (ms), the frequency, and the total charge integrated between the peak and the 5% baseline value were tabulated for statistical comparisons between control and halothane-treated cells.

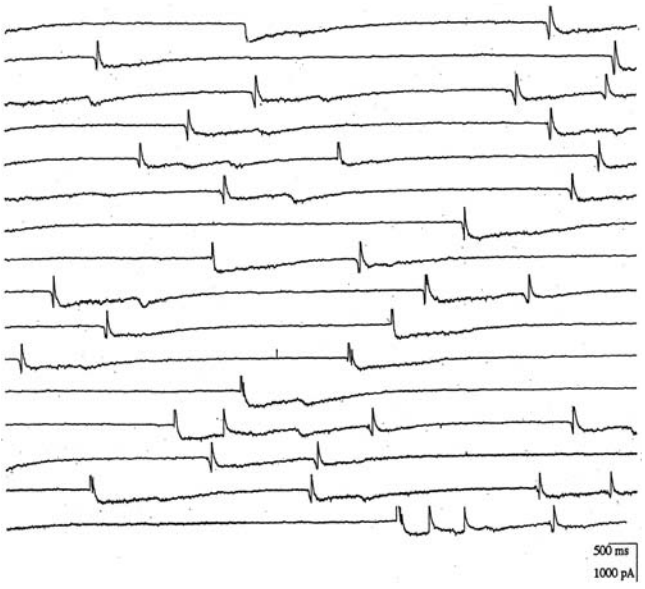

Fig. 1. Records of spontaneous postsynaptic currents. At a holding potential of −40 mV, inhibitory postsynaptic currents (IPSCs) and excitatory postsynaptic currents (EPSCs) were recorded as upward and downward deflections, respectively

Statistical analyses

All analyses, including curve fitting, were performed using pCLAMP software and the Jaejin software (see above). The amplitude and the interevent interval of the spontaneous synaptic current under control and test conditions were compared by the Kolmogorov-Smirnoff test, with $P < 0.05$ indicating significance. The results were analyzed for significant differences by the two-tailed paired Student's *t*-test or one-way analysis of variance (ANOVA). The amplitude, frequency, and area during 10 min of the currents are expressed relative to the control values. Unless otherwise stated, data are presented as means ± SD.

Results

The internal and external recording solutions produced glutamate- and GABA-induced currents that had reversal potentials of approximately 0 and −75 mV, respectively (data not shown). Therefore, spontaneous IPSCs and EPSCs were respectively recorded as the inward and outward responses of membrane potentials clamped between −40 and −30 mV. Spontaneous IPSCs were observed as upward currents and EPSCs as downward currents (Fig. 1). To observe spontaneous EPSCs, 20 μM bicuculline was added to the external solution in order to block $GABA_A$ receptors [13]. The NMDA EPSCs or non-NMDA EPSCs could be recorded separately by using 1 μM CNQX or 30 μM APV, respectively. Figures 2A and 3A (control) clearly show the relatively slow rise time and prolonged decay time of the NMDA components compared with the non-NMDA EPSCs. Each component of EPSCs under 0.6 mM halothane is shown in Figs. 2B and 3B.

Halothane at concentrations ranging from 0.15 to 0.6 mM had little effect on the amplitude of NMDA EPSCs and non-NMDA EPSCs. However, 1.2 mM halothane caused the amplitude of NMDA to decrease significantly (90.5% ± 6.5% of control, $n = 6$, $P < 0.05$) and preferentially depressed the amplitude of NMDA EPSCs when compared with non-NMDA EPSCs ($P < 0.05$) (Fig. 4).

The macroscopic onset and recovery kinetics of the anesthetic effect were determined as the time constant derived from fitting an exponential function to the onset and recovery phases of EPSC amplitude depression. Halothane had little effect on the decay time of non-NMDA and NMDA EPSCs (Fig. 5). This indicates that the kinetics of the synaptic currents underlying the EPSCS were not appreciably changed by halothane.

In contrast, the frequency of NMDA and non-NMDA EPSCs was significantly decreased by halothane at 0.6 and 1.2 mM (NMDA: 60.7% ± 7.8% and 15.5% ± 10.9% of control in response to 0.6 mM and 1.2 mM halothane, respectively, $n = 6$, $P < 0.01$ vs. control; non-NMDA: 57.2% ± 6.6% and 50.5% ± 9.5% of control in response to 0.6 mM and 1.2 mM halothane, respectively, $n = 6$, $P < 0.01$ vs. control) (Figs. 2, 3, and 6). At 1.2 mM, the effect on NMDA EPSCs was significantly greater than that on non-NMDA responses ($P < 0.01$, ANOVA).

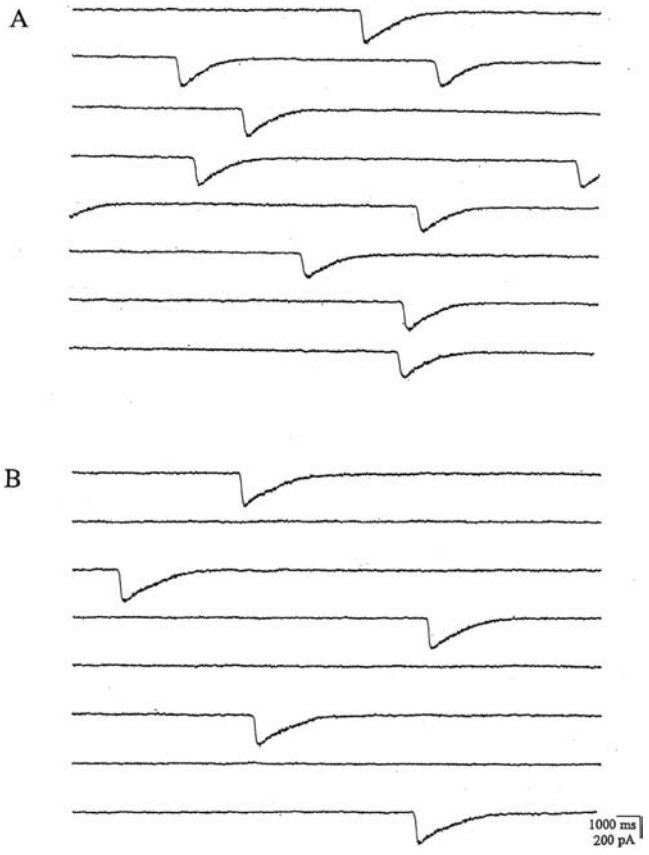

Fig. 2. Records of spontaneous *N*-methyl-D-aspartate (NMDA) EPSCs (**A**) before (control) and (**B**) during application of 0.6 mM halothane. The frequency of NMDA EPSCs was decreased

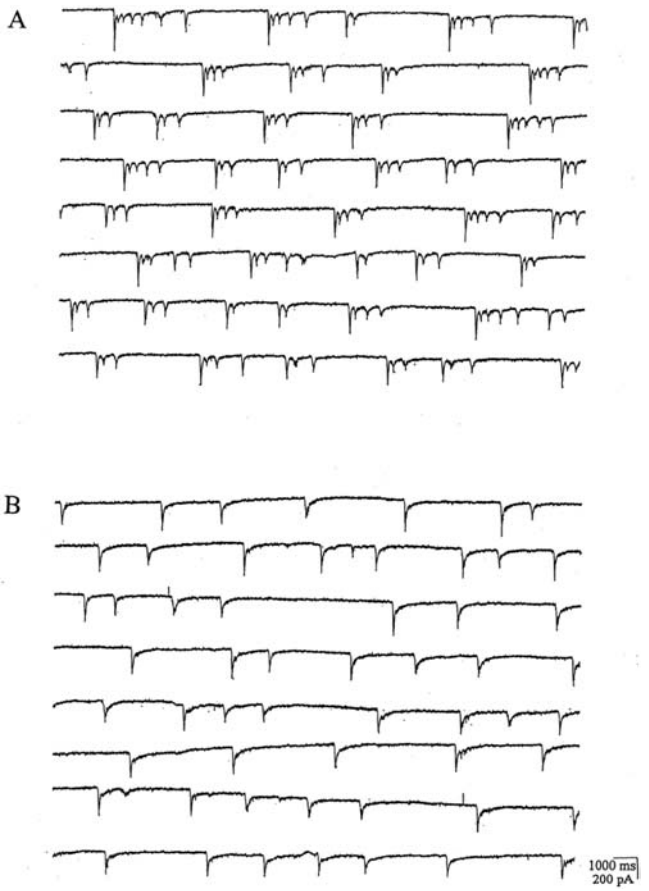

Fig. 3. Records of spontaneous non-NMDA EPSCs (**A**) before (control) and (**B**) during application of 0.6 mM halothane. The frequency of non-NMDA EPSCs was decreased

To evaluate the net effect of halothane on the excitatory current in cortical neurons, we measured the total charge transfer by calculating the integral of the current trace during 10 min. Figure 7 shows the normalized data on the total charge transfer, cumulated during 10 min, plotted as a function of the absolute anesthetic concentration. Halothane caused a net decrease in charge transfer of EPSCs mediated by NMDA and non-NMDA receptors at concentrations of 0.6 and 1.2 mM (mean ± SD: 55.5% ± 6.2% and 11.2% ± 8.9% of control, $n \pm 6$; 71.2% ± 6.6% and 58.5% ± 10.5% of control, $n = 6$, respectively; $P < 0.01$ vs. control). The effects on NMDA EPSCs were significantly greater than those on non-NMDA responses ($P < 0.01$, ANOVA).

Discussion

We studied the effects of halothane on spontaneous EPSCs during an interval of 10 min by the patch clamp technique in an established synaptic network of cultured rat cortical neurons. The major finding was that halothane decreased the frequency and consequently depressed the total net charges of NMDA and non-NMDA EPSCs during an interval of 10 min, with NMDA EPSCs being more sensitive to halothane concentrations of 0.6 mM or more than non-NMDA EPSCs.

The amplitude of the EPSCs was hardly affected by halothane, except at a high concentration of 1.2 mM. Whereas non-NMDA EPSCs were not inhibited by this concentration of halothane, NMDA EPSCs showed slight, but significant, inhibition (Fig. 4). In contrast, the frequency of both non-NMDA EPSCs and NMDA EPSCs was decreased by 0.6 and 1.2 mM halothane (Figs. 2, 3, and 6). These results are in keeping with the data on glutamate-, kainate-, quisqualate-, and NMDA-induced currents in rat tractus solitarius neurons, which were slightly inhibited by halothane at a high concentration (1 mM) [20]. Evoked EPSCs mediated by NMDA and AMPA/kinate in hippocampal neurons were also decreased by isoflurane (0.31–1.2 mM), with a resultant

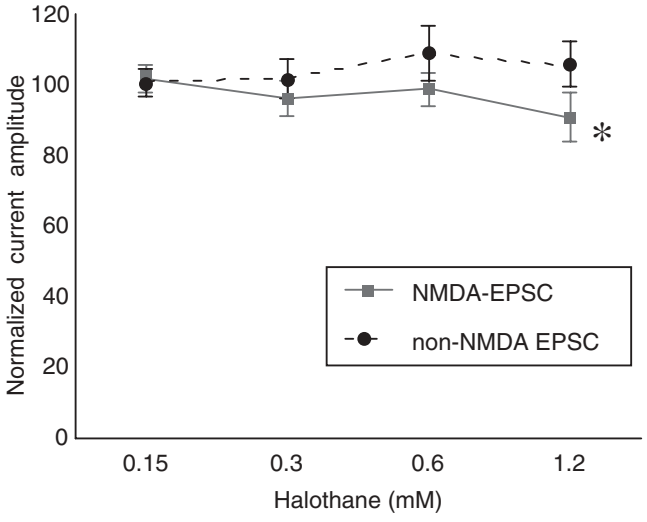

Fig. 4. *Halothane* concentration-dependence of the amplitude of EPSCs. Data are normalized to control values and plotted versus the aqueous anesthetic concentration. Halothane at 1.2 mM decreased the amplitude of *NMDA EPSC*s more than that of *non–NMDA EPSC*s ($P < 0.05$, ANOVA), but there was no significant difference in the decay time constant between the two types of EPSCs (*vs control value; #vs NMDA-EPSCs)

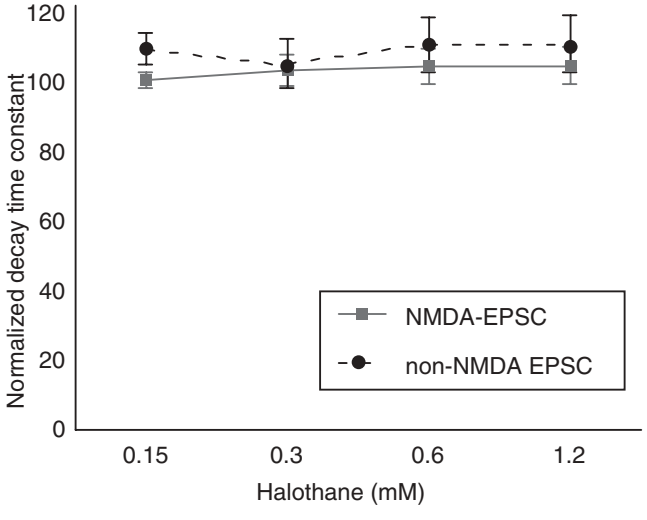

Fig. 5. *Halothane* concentration-dependence of the decay time constant of EPSCs. Data are normalized to control values and plotted versus the aqueous anesthetic concentration. There was no significant difference in the decay time constant between the two types of EPSCs

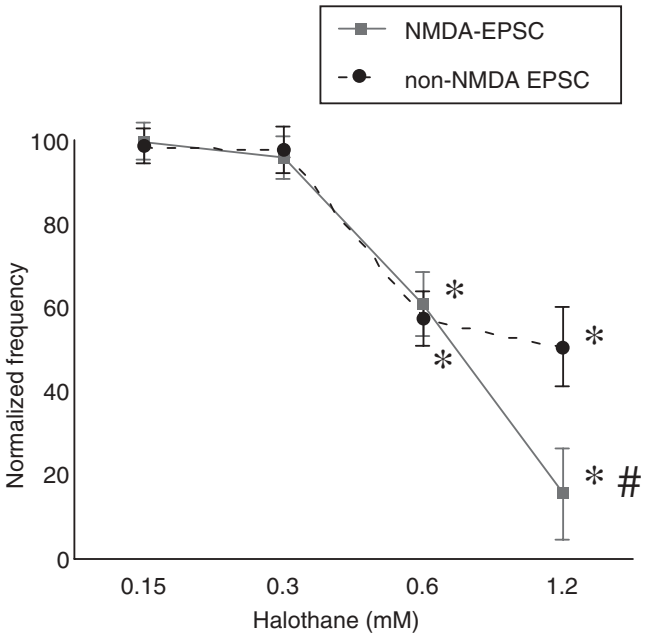

Fig. 6. Effects of *halothane* on the frequency of *NMDA* and *non-NMDA EPSC*s. Data are normalized to control values and plotted against the anesthetic concentration. Halothane at 0.6 and 1.2 mM decreased the frequency of NMDA and non-NMDA EPSCs (*$P < 0.01$, ANOVA). The effects on NMDA EPSCs were significantly greater than those on non-NMDA responses at 1.2 mM halothane (#$P < 0.01$, ANOVA)

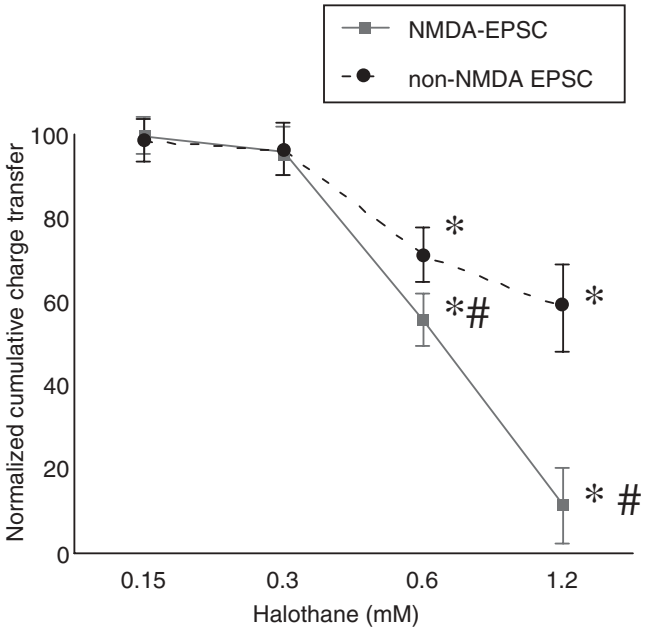

Fig. 7. Effects of *halothane* on the cumulative net charge transfer during 10 min of *NMDA* and *non-NMDA EPSC*s. Data are normalized to control values and plotted against the anesthetic concentration. The effects of halothane at 0.6 and 1.2 mM on NMDA EPSCs were significantly greater than those on non-NMDA responses ($P < 0.01$, ANOVA, *vs control value; #vs NMDA-EPSCs)

decrease in charge transfer [21], which could have resulted from presynaptic inhibition. In the present study, the effect of halothane on the frequency of EPSCs, together with its minimal effect on amplitude, suggest that halothane inhibited presynaptic neurotransmitter release [8,9,22,23]. Recently, volatile anesthetics have been found to inhibit calcium currents [8,24,25]. These studies did not, however, establish that such inhibition was related to the halothane-mediated change in neurotransmitter release, and the actions by each glutamate agonist application might be useful to assess the postsynaptic effects of halothane on each receptor. Thus, further research is needed to assess this correlation.

Several authors have reported that anesthetics can suppress sodium channels [26,27]. In this study, 1.2 mM halothane decreased the NMDA EPSC frequency more than the non-NMDA EPSC frequency (50.5% vs. 15.5%). It is possible that the difference in the process of neurotransmitter release between NMDA and non-NMDA, the direct effect on postsynaptic receptor, and the effect of the sodium channel block might be the reason for the higher sensitivity of the NMDA component compared with the non-NMDA component [11,23]. Although we observed the action potential-dependent postsynaptic current on the cortical neurons established the neuronal network in this study, this tended to complicate analysis of the effects of anesthetics. There is a mixture of action potential-dependent and -independent events in the data. Perhaps experiments conducted in the presence of sodium channel blockers would be useful by permitting direct observation of the effects of anesthetics on the frequency and amplitude of spontaneous vesicular transmitter release in the absence of the action potential [13]. However, we could not determine the NMDA component of EPSCs in the presence of tetrodotoxin, perhaps because the peak amplitude was too small and the rise time too slow to detect in this experiment [11,28]. Moreover, dose dependency of the effect of halothane on the frequency of non-NMDA EPSCs was not observed. Studies on a larger number of samples are necessary, and more work is needed to establish the role of synaptic effects in the overall clinical manifestations of anesthetics.

Some of the observations made in the present study differ from those reported previously. Whereas depression of the amplitude of NMDA receptor-mediated field excitatory postsynaptic potentials in the CA1 region of the hippocampus was more sensitive to isoflurane than non-NMDA receptor-mediated responses, this selective effect was not observed for 0.5 mM halothane [23]. This discrepancy might be due to the different brain regions used and the different anesthetics. In this study, the synaptic effects of halothane were exerted at almost clinically relevant concentrations, as the data on cumulative total net charge transfer show. Although minimum alveolar concentration (MAC) for humans is 0.25 mM halothane, 1 MAC for rats is approximately 0.35 mM. Whereas the presynaptic block occurred at 0.6 mM halothane (~1.7 MAC), the synaptic effects of halothane were exerted at almost clinically relevant concentrations in this study. Each glutamate receptor has important modulation of neuronal damage, disease, pain. NMDA antagonists have neuroprotective effects after injury [29] and function for pain modulation [30]. General anesthetics, including halothane, might have the possibility of clinical effectiveness in some fields.

In summary, clinically relevant concentrations of halothane caused a decrease in excitatory synaptic activity. The NMDA component of EPSCs was more sensitive to halothane than the non-NMDA component. This effect appeared to be related to a decrease in the release of glutamate from nerve terminals mediated via a presynaptic action.

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