Halothane Blocks Synaptic Excitation of Inhibitory Interneurons

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Background: Activation of principal hippocampal neurons is controlled by feedforward and feedback inhibition mediated by γ-aminobutyric acidergic interneurons. The effects of halothane on glutamate receptor-mediated synaptic excitation of inhibitory interneurons have not been reported yet.

Methods: The effects of halothane on glutamatergic excitatory post-synaptic currents and on spike threshold in visually identified interneurons were studied with tight-seal, whole-cell voltage- and current-clamp recordings in thin slices from adult mouse hippocampus. The excitatory postsynaptic currents were pharmacologically isolated into their N-methyl-D-aspartate and non-N-methyl-D-aspartate receptor-mediated components using selective antagonists.

Results: Halothane (0.37–2.78 μm) reversibly blocked non-N-methyl-D-aspartate and N-methyl-D-aspartate excitatory postsynaptic currents in hippocampal oriens-aleveus interneurons. Half-maximal inhibition was observed at similar concentrations (0.59 μm and 0.50 μm, respectively). Halothane inhibited synaptically generated action potentials at concentrations that did not elevate the spike threshold.


HALOTHANE affects synaptic transmission mediated by glutamate and γ-aminobutyric acid (GABA), the major excitatory and inhibitory transmitters in the mammalian central nervous system.† Halothane was shown consistently to inhibit glutamatergic excitation,6–9 whereas its effects on GABAergic inhibition have been more contradictory. In isolated preparations, halothane generally enhanced GABA currents,10 but in intact preparations, depressant effects of halothane on GABAergic inhibition were found.9,10 This suggests that halothane also may modulate the synaptic recruitment and the excitability of GABAergic interneurons.

Inhibitory interneurons are instrumental in the synchronization of neuronal activity in the hippocampus11,12 and may contribute to the generation of hippocampal high-frequency13 and cortical 40-Hz oscillations implicated in cognitive function.11 Thus understanding the effects of volatile anesthetics on synaptic responses and membrane properties of interneurons is important for the development of a comprehensive view of the central nervous system effects of this class of general anesthetics. No studies investigating the effects of anesthetics on these cells have been published yet. Therefore, we have obtained tight-seal, whole-cell recordings from visually identified interneurons in adult mouse hippocampal slices. The effects of halothane on excitatory postsynaptic currents (EPSCs) and potentials (EPSPs) were investigated and compared with its effect on intrinsic excitability. Some of these results have been reported previously in abstracts.15

Materials and Methods

Preparation of Slices

Institutional approval for the experiments was obtained. Experiments were performed on thin hippocam-
pal slices from adult mice (7 to 13 weeks old), as described previously. Briefly, each mouse was anesthetized with ether and decapitated. The brain was quickly removed and a block of tissue containing one hippocampus was glued to the stage of a vibratome. Transverse slices (150 μm) were cut from the hemispherical region containing the hippocampus and transferred to an incubation chamber perfused with oxygenated saline (95% oxygen/5% carbon dioxide) at 34°C. They were used, one at a time, at least 1 h of incubation. In the recording chamber (200 μl), the slice was perfused continuously (2.5 ml/min) with oxygenated saline at room temperature (21–23°C).

Solutions and Drugs

The standard saline used to dissect and maintain slices consisted of 125 mM NaCl, 2.5 mM KCl, 13 mM HEPES, 1.25 mM NaH₂PO₄, 12.5 mM glucose, 1.5 mM CaCl₂, and 4 mM MgSO₄, pH 7.3. The concentration of NaHCO₃ was 26.7 mM in the dissection and experimental solutions and 13 mM in the incubation solution. Experimental salines contained 5 μM glycine to saturate glycine-binding sites on N-methyl-D-aspartate (NMDA) receptors and 10 μM bicuculline methiodide to block GABAₐ receptor-mediated chloride currents. Bicuculline was omitted in the current-clamp experiments. In the experimental saline studies, the concentration of CaCl₂ was increased to 2.5 mM, MgCl₂ was either omitted completely, as in experiments isolated NMDA EPSCs, or added at the specified concentrations. DL-2-amino-3-phosphonovaleric acid (APV) or 6-nitro-7-cyano-quinoxaline-2,3-dion (CNQX) were added to block NMDA or non-NMDA receptor-mediated currents, respectively.

The intracellular (pipette) solution was composed of 130 mM cesium fluoride (CsF), 10 mM NaCl, 10 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, 1 mM CaCl₂, and 10 mM tetra-ethyl ammonium. For current-clamp recordings, CsF was replaced by potassium fluoride (KF), tetra-ethyl ammonium was omitted, and EGTA was reduced to 0.1 mM. The pH was adjusted to 7.2–7.3. Drugs were purchased from Sigma Chemical Company (St. Louis, MO), with the exception of CNQX (Tocris, Neuramin, UK) and halothane (Trophicel Surficals, Switzerland).

Application and Measurement of Halothane

The fresh oxygen-carbon dioxide mixture was directed through a flowmeter through an Enfluretec vaporizer (Cyprane, Keighley, England) containing halothane. This gas mixture was used to bubble through the experimental saline for at least 15 min before it was applied to the slice with a peristaltic pump.

Halothane concentrations in the recording chamber were determined as described previously, using a Tocrac 540 gas chromatograph (Tocrac Instruments, Austin, TX). A modified aquarium pump was used to scavange for waste gases.

Stimulation and Recording

Interneurons in the CA1 field of the hippocampal slice were visualized at ×400 magnification with a water immersion objective using an upright microscope equipped with Nomarski optics (Zeiss Standard 18, Oberkochen, Germany). Tight-seal whole-cell recordings were made from multipolar neurons located close to the oriens-alcveus border. Previous work identified them as GABAergic interneurons. Recording pipettes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany) on a vertical puller (List-medical, Darmstadt, Germany). The series resistance (5–10 MΩ) was compensated to 50–90% by the patch-clamp amplifier (List L-EPSC 7 or Axopatch 200 integrated amplifier, Axon Instruments, Foster City, CA). Stimulating pipettes were pulled from "Boralex" disposable micropipettes (Rochester Scientific, Rochester, NY) and filled with saline. They were positioned 20–70 μm from the patched cell. Stimuli were applied at 0.1 Hz by a Master 8 stimulator (AMPI, Jerusalem, Israel) via an isolation unit (World Precision Instruments, Sarasota, FL). Stimulus strength was adjusted to evoke sub maximal stable EPSCs.

Data Analysis

All current records were filtered at 1 kHz, digitized on-line at sampling rates between 1 and 2 kHz, and analyzed off-line using a personal computer and commercial software (pClamp, Axon Instruments). Kinetic analysis was performed on averaged signals (usually five to ten consecutive EPSCs). The EPSC rise times were measured at the 10–90% peak. Their decays were fitted with an exponential function: y = Ae⁻τx, where A is the peak current amplitude and τ is the time constant of decay. Measurements are given as mean ± SD or SE, as indicated. Differences among multiple groups were tested using one- or two-way analysis of variance, as appropriate. When significant differences were indicated in the F ratio test (P < 0.001), the significance of differences between any two of these groups was evaluated by the method for multiple comparisons with a confidence level of 0.05. Differences were considered significant when P < 0.05.

The dose-dependent method of GABAergic transmission was tested by fitting the following equation:

\[ y = a \times (1 - e^{-bx}) \]

in which \( a \) is the maximum current amplitude and \( b \) is the threshold of GABAergic transmission.

Results

GABAergic transmission was dose dependent. Slices were loaded with 95% oxygen/5% carbon dioxide for 1 h, followed by 3 h of washout. Halothane decayed after 3 h of washout. The halothane concentration was recorded as described above. A modified aquarium pump was used to scavange for waste gases.

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Fig. 1. Experimental preparation. (A) Diagram of the circuitry within a transverse hippocampal slice, showing the trisynaptic hippocampal excitation pathway. Axons of dentate gyrus (DG) granule cells innervate CA3 pyramidal cells, which send recurrent (Schaffer) collaterals to innervate CA1 pyramidal cells. The local CA1 circuitry mediates feedforward and feedback inhibition of CA1 pyramidal cells is illustrated inside the box. The Schaffer collaterals (1) in stratum radiatum (SR) and axon collaterals of CA1 pyramidal cells (2) in the alveus (Al) form excitatory (glutamatergic) synapses on an interneuron situated in stratum oriens (SO). Axon collaterals of the interneuron (3), in turn, form inhibitory (GABAergic) synapses on the CA1 pyramidal cell situated in stratum pyramidale (SP). (B) Expanded description of the CA1 circuitry, shown in the box in A. Closed and open circles denote excitatory (glutamatergic) and inhibitory (GABAergic) synapses, respectively. The arrows indicate the direction of impulse propagation (PC = pyramidal cell). (C) Typical nonaccommodating response of an oriens-alveus interneuron to a long (400 ms) depolarizing current pulse injected into the soma.

Glutamatergic Excitatory Postsynaptic Currents in Adult Oriens-alveus Interneurons

Previous analyses of glutamatergic EPSCs in hippocampal interneurons were performed in slices from immature (2–3 weeks old) rats. The EPSCs were composed of two components: a fast, voltage-insensitive component that was blocked by the DL-α-amino-3-hydroxy-5-methylisoxazolepropionic acid/kainate receptor blocker CNQX (non-NMDA EPSC), and a slow, voltage-sensitive component that was blocked by the NMDA receptor blocker APV (NMDA EPSC). Because the densities and properties of glutamate receptors may change during development, it was important first to characterize the pharmacologic and kinetic properties of glutamatergic EPSCs in adult interneurons.

Native EPSCs reversed polarity close to 0 mV. At a holding potential of −60 mV, they had a fast rise time and a dual component decay. With depolarization, the slow component of decay became more prominent. This is consistent with the voltage-dependent relief of the Mg²⁺-block of NMDA receptor channels. Accordingly, this component increased in Mg²⁺-free saline at negative potentials and was blocked by 100 μM APV (fig. 2A). The fast component of the EPSC was blocked by 5 μM CNQX, indicating its mediation by non-NMDA receptors (fig. 2B).

Results

Glutamatergic excitation of oriens-alveus interneurons originates predominantly from recurrent axon collaterals of either CA3 (Schaffer collaterals) or CA1 pyramidal cells. Accordingly, oriens-alveus interneurons mediate either feedforward or feedback inhibition of CA1 pyramidal cells. Figures 1A and 1B summarize the relevant neuronal circuitry.

This article is based on recordings from 26 CA1 oriens-alveus interneurons. Under current-clamp, these neurons fired repetitively in a nonaccommodating manner in response to a prolonged depolarizing current pulse (fig. 1C).

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The rise times and the decay time constants of the pharmacologically isolated non-NMDA EPSCs evoked at a membrane potential of \(-60\) mV were (mean ± SD) \(1.12 ± 0.26\) and \(3.4 ± 1.7\) ms (n = 8), respectively. The respective rise times and decay time constants of NMDA EPSCs in Mg\(^{2+}\)-free saline were \(10.36 ± 4\) ms (n = 10) and \(176 ± 33\) ms (n = 6).

**Effects of Halothane on Excitatory Postsynaptic Currents**

As illustrated in figure 3, native EPSCs at both negative and positive potentials were reversibly depressed by 0.64 mM halothane (n = 4). At both potentials, halothane similarly depressed the fast and slow EPSC components, suggesting that it blocks indiscriminately both non-NMDA and NMDA EPSCs.

This notion was further tested by investigating the effects of halothane on pharmacologically isolated non-NMDA and NMDA EPSCs. In the experiment illustrated in figure 4A, 0.64 mM halothane reduced the peak amplitude of non-NMDA (a) and NMDA (b) EPSCs by 64% and 58%, respectively. While depressing EPSC amplitude, halothane affected neither the rise time (fig. 4B) nor the decay time course (fig. 4C) of non-NMDA (a) and NMDA EPSCs (b).

![Graph A and B with legend](image)

Fig. 2. Dual-component excitatory postsynaptic currents (EPSCs) in adult oriens-aleveus interneurons and their pharmacologic separation. (a,b) Two representative experiments conducted in saline containing 1 mM Mg\(^{2+}\). The EPSCs were evoked by stimulating afferent fibers while the neurons were voltage-clamped at either \(-60\) mV (lower) or \(30\) mV (upper). At \(-60\) mV, only a small, slow component of the native EPSC was blocked by 100 \(\mu\)M APV. The dominating DL-2-amino-phosphonovaleric acid (APV)-insensitive fast current was blocked by 5 \(\mu\)M 6-nitro-7-cyano-quinoxaline-2,3-dione. At \(+30\) mV, the relative size of the slow, APV-sensitive EPSC component markedly increased.

![Graph A and B with legend](image)

Fig. 4. Halothane depresses dual-component excitatory postsynaptic currents (EPSCs) in an adult oriens-aleveus interneuron. The experiment was conducted in saline containing 1 mM Mg\(^{2+}\). The EPSCs were evoked while the cell was voltage-clamped at either \(-60\) mV (a) or \(30\) mV (b). At these two potentials, the native EPSCs consisted predominantly of either non-V-methyl-\(\alpha\)-aspartate or N-methyl-\(\alpha\)-aspartate receptor-mediated components, respectively. (a) Control EPSCs. (b) EPSCs after 10 min of exposure to 0.64 mM halothane. (c) EPSCs after 20 min of wash. Both fast and slow components of the EPSCs were similarly depressed by halothane in a reversible manner.

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Fig. 4. Halothane depresses pharmacologically isolated non-N-methyl-D-aspartate (NMDA) and NMDA excitatory postsynaptic currents (EPSCs) without affecting their kinetic properties. (A) Halothane (0.64 mM) reduced the peak amplitudes of non-NMDA (a) and NMDA (b) receptor-mediated EPSCs by 64% and 58%, respectively. The EPSCs were evoked in the presence of the selective blockers aminophosphonovaleric acid and 6-nitro-2-cyano-quinolinic acid-2,3-dion, as indicated. The NMDA EPSCs were evoked in Mg²⁺-free saline. The illustrated non-NMDA EPSC was recorded in saline containing 1 mM Mg²⁺. (B) The rising phases of the EPSCs recorded in 0.64 mM halothane were normalized and superimposed on the rising phase of the control EPSC. Neither the rise time of the non-NMDA (a) nor that of the NMDA (b) EPSC were affected by halothane. (C) The same procedure as in B was applied to the decaying phases of the non-NMDA (a) and the NMDA EPSCs (b), which also remained unaffected by halothane. Note the different time calibrations. Traces in B and C were taken from the EPSCs shown in A.

Concentration-Response Relation

To quantify the inhibitory effect of halothane on glutamatergic EPSCs in oriens-alveus interneurons, we tested halothane at concentrations ranging from 0.37 to 2.79 mM. Sample traces for non-NMDA (fig. 5A, upper) and NMDA EPSCs (fig. 5A, lower) in 0.64 mM and 2.79 mM halothane are shown together with the control cur-

Fig. 5. Dose-dependent suppression of non-N-methyl-D-aspartate (NMDA) and NMDA excitatory postsynaptic currents (EPSCs) by halothane. (A) Exemplary records of non-NMDA (upper) and NMDA (lower) EPSCs evoked at −60 mV and recorded at the indicated halothane concentrations. Note that the time calibration is different. (B) Dose-response relation of halothane-induced depression of the EPSCs. The curve summarizes the results from 13 oriens-alveus interneurons clamped at −60 mV. The NMDA receptor-mediated currents were recorded in Mg²⁺-free saline. Each cell was exposed to several concentrations of halothane. Non-NMDA and NMDA receptor-mediated currents were isolated pharmacologically with 100 μM aminophosphonovaleric acid and 5 μM 6-nitro-2-cyano-quinolinic acid-2,3-dion, respectively. Solid lines were fitted through the data points, as described in Materials and Methods. The dotted lines indicate the halothane concentrations producing 50% inhibition: 0.59 mM for non-NMDA and 0.50 mM for NMDA EPSCs.
halothane blocks synaptically generated spikes in an oriens-alueus interneuron without elevating spike threshold. (A) Stimulation of afferent fibers induced solitary action potentials (left), which were suppressed by 1.5 mM halothane (middle) in a reversible manner (right). (B) Hyperpolarization of the cell by steady negative current injection unmasked the underlying excitatory postsynaptic potentials (left), which were markedly depressed by halothane (middle). (C) At the same time, action potentials evoked by injection of just-supra-threshold, brief depolarizing current pulses (left), were not inhibited by halothane (middle). Samples represent unaveraged traces recorded in saline containing 1 mm Mg²⁺. Spikes were truncated. Note the differences in calibrations.

The results obtained from 13 oriens-alueus interneurons are summarized in figure 5B. Halothane inhibited both EPSC components in a dose-dependent manner. At the lowest concentration tested (0.37 mM), non-NMDA and NMDA EPSCs were inhibited by 16.5 ± 4.9% (n = 3) and 27.7 ± 4.9% (n = 3), respectively (difference not significant). Nearly complete blockade (96.3 ± 3.7% and 97.2 ± 2.8%, n = 5) was achieved at 2.79 mM. The calculated concentrations producing 50% inhibition were 0.59 mM and 0.50 mM for non-NMDA and NMDA EPSCs, respectively.

Effects of Halothane on Synaptically and Electrically Evoked Action Potentials
In current-clamp recordings, afferent fiber stimulation evoked glutamatergic EPSPs in oriens-alueus interneurons. As shown in figure 6, large EPSPs triggered action potentials (fig. 6A, left). These could be blocked by hyperpolarizing the IN with negative current, thereby unmasking the underlying EPSP (fig. 6B, left). Single action potentials also could be evoked by injecting the interneuron with brief positive current pulses (fig. 6C, left).

The effects of halothane on synaptically and electrically evoked spikes were compared in four oriens-alueus interneurons. The intensities of the orthodromic and the depolarizing stimuli were adjusted to be just more than the threshold value for eliciting a single spike. As illustrated in figure 6A, 1.5 mM halothane reversibly inhibited synaptically evoked action potentials. This effect was due to the suppression of the EPSP (fig. 6B), because halothane did not increase the threshold for evoking spikes by direct electrical stimulation (fig. 6C).

Discussion
The main finding in this study is that halothane suppresses synaptic excitation of oriens-alueus interneurons. This effect is due to a dose-dependent suppression of glutamatergic EPSCs in these neurons, rather than to changes in their intrinsic excitability.

Glutamatergic Excitatory Postsynaptic Currents in Adult Oriens-alueus Interneurons
As in the immature preparation, native glutamatergic EPSCs in adult oriens-alueus interneurons comprised both non-NMDA and NMDA receptor-mediated components, which could be studied in isolation in APV- or CNQX-containing salines, respectively. Non-NMDA EPSCs had faster kinetics than did NMDA EPSCs, although we did not detect any NMDA EPSCs with very slow rise times, similar to those previously seen in the juvenile preparation.

Depression of Glutamatergic Excitatory Postsynaptic Currents
Halothane indiscriminately inhibited the non-NMDA and the NMDA receptor-mediated EPSCs in adult oriens-alueus interneurons in a dose-dependent manner. This block was not associated with changes in the rise time or decay time course of these currents, which suggests that halothane may inhibit glutamatergic EPSCs by a predominantly presynaptic mechanism of action. Supporting this hypothesis, we showed previously that halothane indiscriminately reduces non-NMDA and NMDA EPSCs in normal saline. Since the EPSC time course and GABAergic synapse interaction blockers do not suppress EPSCs, these findings suggest that the ORS block is due to a reduction of the GABAAergic synapses.

Implications
We performed experiments by the in vivo technique (2°C), but these findings also indicate that the effects of halothane on EPSCs in ORS may be influenced by other factors. Such factors could include the type of current clamp recording, the use of volatile anesthetic, and the presence of NMDA receptor agonists.
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EPSCs in adult CA1 pyramidal cells without affecting EPSC time course or currents induced by bath-applied glutamate agonists. In contrast, halothane prolonged GABA, receptor-mediated responses, presumably by interacting with the postsynaptic GABAergic receptors.

Suppression of Synaptically Evoked Action Potentials
As expected from its effect on native EPSCs, halothane suppressed glutamatergic EPSPs in oriens-alevus interneurons. Suppression of glutamatergic EPSPs was previously noted in spinal motoneurons. Halothane blocked EPSP-evoked action potentials in oriens-alevus interneurons. Under the same conditions, halothane did not suppress spike generation by direct current injection. Thus halothane inhibits the synaptic recruitment of oriens-alevus interneurons by depressing glutamatergic EPSCs. This notion is supported by previous findings that 1.5 mM halothane did not affect the threshold current necessary to elicit a single spike in hippocampal CA1 neurons.

Implications for the Mechanisms of Anesthesia
We performed the experiments at temperatures that, by themselves, appear anesthetic in mammals (21-23°C), but other researchers have shown that halothane also affects excitatory neurotransmission at close to normal body temperatures . However, the concentrations producing 50% inhibition expressed as partial pressures are very temperature dependent because of the increase of halothane solubility in aqueous solutions with decreasing temperature. This problem can be addressed by comparing the free aqueous concentrations of volatile anesthetics. The depression of glutamatergic EPSCs in oriens-alevus interneurons occurred at concentrations (0.5 to 0.6 mM) about twice the reported "aqueous minimum alveolar concentration" for mice (0.27 mM ≅ 0.95% at normal body temperature). Thus glutamatergic EPSCs probably become increasingly inhibited with deepening levels of halothane anesthesia.

Under experimental conditions that test only its post-synaptic effect, halothane consistently enhances GABA, receptor-mediated responses, an action that may help induce anesthesia. However, in preparations incorporating the presynaptic element, halothane depresses GABAergic inhibitory synaptic potentials in spinal motoneurons and in CA1 pyramidal cells, possibly by pre-synaptically inhibiting evoked GABA release. Here we show that, at similar concentrations, halothane also blocked the synaptic recruitment of GABAergic interneurons. Therefore we expect that the overall effect of halothane on feedforward and feedback GABAergic inhibition would be depressant in nature. The potentiation of tonic GABAergic inhibition mediated by asynchronous GABA release may contribute to the anesthetic action of halothane. Halothane's inhibitory action on oriens-alevus interneurons may affect other neurotransmitter systems as well, because oriens-alevus interneurons have been found to colocalize various neuropeptides in addition to GABA. Together the data presented here and in our previous study in pyramidal cells suggest that halothane indiscriminately reduces glutamatergic excitation of interneurons and principal neurons in the hippocampus. Because glutamate is the most ubiquitous excitatory neurotransmitter in the central nervous system, this action may be important for achieving or maintaining the anesthetic state.

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References

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sion of hippocampal inhibitory post synaptic potentials and spontaneous firing by volatile anesthetics. Brain Res 1985; 340:363-8


20. Perouansky M, Yarai Y: Kinetic properties of NMDA receptor mediated synaptic currents in rat hippocampal pyramidal cells versus interneurons. J Physiol (London) 1995; 465:223-44


