ANESTHESIOLOGY

Isoflurane Suppresses Hippocampal High-frequency Ripples by Differentially Modulating Pyramidal Neurons and Interneurons in Mice

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Volatile anesthetics can induce anterograde amnesia
- Hippocampal ripples are high-frequency oscillatory events involved in memory processes
- The actions of volatile anesthetics on hippocampal ripples are incompletely understood

What This Article Tells Us That Is New

- In mice, isoflurane at 0.5% impaired hippocampus-dependent memory processing and ripple oscillations without inducing loss of righting reflex
- At the cellular level, these effects were associated with decreased fast-spiking interneuron activity and a concomitantly enhanced activity of excitatory neurons
- These observations suggest that the suppression of hippocampal ripples by isoflurane *via* the differential modulation of principal neurons and interneurons contributes to the amnestic action of this drug

ABSTRACT

Background: Isoflurane can induce anterograde amnesia. Hippocampal ripples are high-frequency oscillatory events occurring in the local field potentials of cornu ammonis 1 involved in memory processes. The authors hypothesized that isoflurane suppresses hippocampal ripples at a subanesthetic concentration by modulating the excitability of cornu ammonis 1 neurons.

Methods: The potencies of isoflurane for memory impairment and anesthesia were measured in mice. Hippocampal ripples were measured by placing recording electrodes in the cornu ammonis 1. Effects of isoflurane on the excitability of hippocampal pyramidal neurons and interneurons were measured. A simulation model of ripples based on the firing frequency of hippocampal cornu ammonis 1 neurons was used to validate the effects of isoflurane on neuronal excitability *in vitro* and on ripples *in vivo*.

Results: Isoflurane at 0.5%, which did not induce loss of righting reflex, impaired hippocampus-dependent fear memory by 97.4 ± 3.1% (mean ± SD; n = 14; $P < 0.001$). Isoflurane at 0.5% reduced ripple amplitude (38 ± 13 *vs.* 42 ± 13 μV; n = 9; $P = 0.003$), rate (462 ± 66 *vs.* 538 ± 81 spikes/min; n = 9; $P = 0.002$) and duration (36 ± 5 *vs.* 48 ± 9 ms; n = 9; $P < 0.001$) and increased the interarrival time (78 ± 7 *vs.* 69 ± 6 ms; n = 9; $P < 0.001$) and frequency (148.2 ± 3.9 *vs.* 145.0 ± 2.9 Hz; n = 9; $P = 0.001$). Isoflurane at the same concentration depressed action potential frequency in fast-spiking interneurons while slightly enhancing action potential frequency in cornu ammonis 1 pyramidal neurons. The simulated effects of isoflurane on hippocampal ripples were comparable to recordings *in vivo*.

Conclusions: The authors' results suggest that a subanesthetic concentration of isoflurane can suppress hippocampal ripples by differentially modulating the excitability of pyramidal neurons and interneurons, which may contribute to its amnestic action.

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Although general anesthetics have been used in the clinic for more than 170 yr, the mechanisms by which general anesthetics produce their various pharmacologic effects are still poorly understood.[1,2] Compared to the intravenous general anesthetic propofol, which has relatively clear molecular targets (such as γ-aminobutyric acid type A [$GABA_A$] receptors),[3] volatile anesthetics like isoflurane and sevoflurane can modulate the functions of multiple molecular targets at clinical concentrations, including ion channels[1] and neurotransmitter receptors.[2] Understanding the complex mechanisms of volatile anesthetics at the molecular, cellular, and systems levels as they pertain to amnesia and anesthesia is a major challenge to the fields of anesthesiology and neuroscience.

Volatile anesthetics including isoflurane produce anterograde amnesia, which is one of their most important and useful pharmacologic actions.[4] Anterograde amnesia during

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general anesthesia prevents distressing memories and unintended intraoperative awareness.[5] How volatile anesthetics impede memory consolidation and retrieval, which occur at lower concentrations than loss of consciousness and immobility, is unknown.[4]

Various ion channels and/or neurotransmitter receptors that modulate neuronal excitability may contribute to the amnesic effects of volatile anesthetics.[1] Mice lacking HCN1 channels in the forebrain exhibit resistance to the amnestic effects of volatile anesthetics.[6] Genetic inactivation of the α4 subunit-containing $GABA_A$ receptors reduced the amnestic effects of isoflurane in mice, but minimally affected loss of righting reflex, and produced no effect on immobility.[7] However, it remains unclear how volatile anesthetics interfere with memory formation and retrieval at the network level.

The hippocampus region of the cortex is critical for memory retrieval and consolidation.[8] High-frequency ripples are characteristic local field potentials in hippocampal cornu ammonis 1, caused by excitatory input from hippocampal cornu ammonis 3, and are associated with synchronous neural firing in the hippocampal cornu ammonis 1 subfield.[9,10] Hippocampal ripples are believed to participate in information transmission between brain regions and contribute to memory consolidation.[9] Given that hippocampal ripples support both memory consolidation and memory retrieval, ripple-related interventions can alter subsequent memory performance *in vivo*.[11,12] It is unclear whether isoflurane can affect hippocampal high-frequency ripples at low concentrations that induce anterograde amnesia.

The current study was designed to test the hypothesis that the volatile anesthetic isoflurane suppresses hippocampal high-frequency ripples at a concentration lower than minimum alveolar concentration (MAC) by modulating both excitability and firing patterns of hippocampal cornu ammonis 1 neurons. This may contribute to the amnestic effect of isoflurane.

Materials and Methods

Animals

All experimental protocols were approved by the Institutional Animal Experimental Ethics Committee of West China Hospital of Sichuan University (Chengdu, China), and relevant aspects of the Animal Research Reporting *In Vivo* Experiments (ARRIVE) guidelines were followed throughout. C57BL/6J mice were housed in humidity- and temperature-controlled rooms, with a 12-h light–dark cycle and free access to food and water. Adult mice (approximately 7 to 8 weeks old) were used for behavioral tests, including fear-conditioning testing (n = 14/group; 7/7 male/female), measurement of MAC of isoflurane (n = 20; 10/10 male/female), recording of local field potentials (n = 9/group; 5/4 male/female), and measurement of locomotor activity of mice under 0.5% isoflurane (n = 14/group; 7/7 male/female). Neonatal mice (postnatal day 7 to 10; 30 mice in total with both sexes) and adult mice (6 to 8 weeks old; 22 mice in total of both sexes) were used for acute brain slice recording. Mice were numbered, randomly assigned to experimental groups using a random number table, and tested in sequential order. All efforts were made to minimize the number of animals used and the suffering of the animals. Animals were euthanized using carbon dioxide and isoflurane after experiments. Although this study was not designed to investigate potential sex differences, comparisons between male and female mice for all behavioral results are provided in Supplemental Digital Content 1 (http://links.lww.com/ALN/C619).

Fear-conditioning Test

A fear-conditioning test was used to measure the effects of isoflurane on memory. Typically, contextual fear conditioning is hippocampus-dependent.[13] The paradigm followed has been described.[14] Experiments were performed over 2 consecutive days using a fear-conditioning system (Med Associates, Inc., USA). Mice were randomly assigned to five groups (n = 14/group; 7/7 male/female) receiving 0.00%, 0.17%, 0.33%, 0.50%, and 0.63% isoflurane in 40% O_2/60% N_2, respectively. A gas monitor (Datex-Ohmeda, USA) was used to measure isoflurane concentration. The shape, color, odor, and floor texture of the training chamber comprised the training context. Before day 1 training, a similar-sized equilibration chamber was used for 15-min pre-exposure with isoflurane before entry into the training chamber. During training, mice were continuously exposed to isoflurane at the concentration assigned to the group. After a 3-min exploratory period, mice received a 30-s tone (2,000 Hz; 90 dB) four times followed by a 2-s foot shock (0.6 mA). On day 2, mice were tested for fear-conditioned memory to the context and cue tone (no isoflurane during test), respectively. For the context memory test, each mouse was placed back in the fear-conditioning chamber containing the exact the same context for 3 min in the absence of a tone or foot shock. For the cue tone memory test, each mouse was placed in a new chamber with a different context during training. Freezing behavior (no movement other than breathing and heartbeats) was analyzed by a video tracking system and software (Med Associates, Inc.). The percentage of freezing time during the test period was calculated as memory performance.

Measurement of Isoflurane MAC

The MAC values of isoflurane for loss of righting reflex and loss of response to tail clamping were measured in adult mice (n = 20; 10/10 male/female), as measures of hypnosis[15] and immobility,[6] respectively. Mice were placed in a transparent gas-tight plastic chamber (25 × 15 × 12 cm) and allowed to breathe spontaneously. Carrier gas flow was 2 l/min (40% O_2/60% N_2). The concentration of isoflurane

and partial pressures of oxygen and carbon dioxide were continuously monitored using an infrared gas monitor (Datex-Ohmeda). Mice were warmed with a heating blanket to maintain rectal temperature of 36 to 38°C. The MAC values of isoflurane for loss of righting reflex and immobility were estimated as described.[6,15] Concentrations of isoflurane were continuously increased to 0.44%, 0.52%, 0.61%, 0.72%, 0.85%, 1.00%, and 1.18% for determining MAC for loss of righting reflex; and 0.72%, 0.85%, 1.00%, 1.18%, 1.40%, 1.65%, and 1.94% for determining MAC for immobility. Each concentration was maintained for at least 15 min, and then the righting reflex and/or tail-clamping responses were tested. For loss of righting reflex, the mouse did not roll over during the 10-s observation period. No purposeful reactions to tail clamping by an alligator clip (type 85, Newark Electronics, USA) during the 10-s observation period was defined as immobility.

Recording of Local Field Potentials in Hippocampal Cornu Ammonis 1 in Mice

Adult mice were anesthetized with isoflurane (2% in 98% O_2) and placed in a stereotactic apparatus (RWD, China). Electrode implantation was performed as described.[16,17] Three electrodes (Brotschi Decolletage AG, Switzerland) were implanted into the hippocampal cornu ammonis 1 region: left parietal site (−2.06 mm to bregma; −2.0 mm from midline; 1.5 mm below the surface of the cortex), right parietal site (−2.06 mm to bregma; 2.0 mm from midline; 1.5 mm below the surface of the cortex), and interparietal site over the cerebellum (−2.0 mm from lambda, 0.0 mm from midline) as a reference electrode. All electrodes were secured with dental cement, and animals were allowed 1 week to recover. Accuracy of electrode placement was confirmed after recordings by histologic examination.

For recording of local field potentials, the mouse was placed in a transparent gas-tight plastic chamber (20 × 15 × 10 cm) with carrier gas (40% O_2/60% N_2) at a flow of 2 l/min for a duration of approximately 30 min. The chamber was put on a heating blanket to maintain rectal temperature of 36 to 38°C. Local field potentials were recorded for 4 min in carrier gas as baseline, and then with 0.5% isoflurane in carrier gas for 4 to 14 min, and back to carrier gas until full recovery. The concentration of isoflurane was continuously monitored using an infrared gas monitor (Datex-Ohmeda).

For local field potentials analysis, raw signals were preamplified, digitized, and recorded using a PowerLab system (16/35, AD Instruments, Australia). Local field potentials were digitized at 2,000 Hz and filtered at 0.1 to 500 Hz (raw data), 0.1 to 4 Hz (delta), 4 to 12 Hz (theta), and 100 to 200 Hz (ripples). Notch filtering at 50 Hz was applied to remove noise. As previously described,[11,16,18] a ripple event was defined as threshold crossings 3 times SD above the mean, with beginning and ending of duration at 1 SD. The amplitude of a ripple was defined as the maximum peak of the ripple envelope. The frequency of a ripple was calculated as the inverse of the interpeak interval within the ripple duration. The interarrival time of ripples was the time elapsed between the ending of the former ripple and the onset of the next ripple. The rate of ripples was defined as the number of ripple events per minute. LabChart software (Version 8, AD Instruments) and MATLAB (MathWorks, USA) were used for the analysis.

Measurement of Locomotor Activity

To explore the pharmacologic effects of 0.5% isoflurane in mice, locomotor activity without head-fixed cable was recorded with the same protocol used for local field potential recordings. An infrared gas monitor (Datex-Ohmeda) was used to measure isoflurane concentration continuously. The behaviors and movement trajectories of mice were video recorded and analyzed by behavioral tracking software (Smart 2.5, Panlab, DL Naturgene Life Science, Inc., China). Total travel distance, mean speed, and percentage of resting time were measured. The resting state was defined as an average change in trajectory of less than 1 cm for at least 1 s during a continuous period.

Preparation of Acute Brain Slices

Neonatal (postnatal day 7 to 10) and adult (6 to 8 weeks old) mice were anesthetized with ketamine/xylazine (60/10 mg/kg, intraperitoneal) and decapitated. The brain was removed and quickly put into ice-cold dissecting solution containing (in mM) 260 sucrose, 3 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, and 1 kynurenic acid. Transverse hippocampal slices (270 μm) were cut using a vibratome (Leica VT1000 A, USA), and incubated for 30 min at 37°C and then at room temperature (23 to 25°C) in incubation solution composed of (in mM) 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose. Both dissecting and incubation solutions were oxygenated with 95% O_2 and 5% CO_2. After incubation, brain slices were mounted in a recording chamber for electrophysiologic recordings at room temperature (23 to 25°C).

Whole Cell Patch Clamp Recording

Pyramidal neurons and fast-spiking interneurons were visualized in the hippocampal cornu ammonis 1 region using infrared-differential interference contrast imaging microscopy (Zeiss Axioimager FS, Germany). Pyramidal neurons were identified by their typical shape and size.[19] Fast-spiking interneurons were identified on the basis of their nonpyramidal shape, multipolar dendrites, fast-spiking behavior (action potential duration less than 1 ms),[20] large postspike after-hyperpolarization, and sustained high-frequency discharges after current injection.[21] Whole cell patch clamp recordings were conducted using an Axopatch 700B amplifier, 1440 Digidata digitizer, and pClamp 10.2 software (Molecular Devices, USA). Whole cell current clamp recordings were

made of action potentials in response to current injections from −120 to +150 pA with a duration of 1 s. Resting membrane potential was measured when current injection = 0. Currents were sampled at 20 kHz and filtered at 10 kHz. Glass recording microelectrodes were made using a microelectrode puller (P-1000, Sutter Instruments, USA) with resistances of 3 to 5 MΩ. Series resistance was compensated by approximately 75 to 80%, and data were rejected when series resistance exceeded 15 MΩ. Pipette solution contained (in mM) 120 KCH_3SO_3, 4 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 10 EGTA, 3 Mg–adenosine triphosphate, and 0.3 guanosine 5′-triphosphate tris salt (pH 7.3, 290 mOsm).

A saturated solution of isoflurane (12 to 15 mM, measured by gas chromatography) was prepared in extracellular solution (in mM: 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose). Isoflurane-saturated solution was prepared by rotating up and down in gas-tight vial bottles for at least 24 h. Working solutions were prepared by diluting the saturated solution of isoflurane to experimental concentrations, and final concentrations of isoflurane were confirmed by gas chromatography. Isoflurane 0.27 mM at 25°C was used as predicted MAC.[19]

Simulation of Hippocampal Ripples

A computational model using MATLAB was used to simulate the effects of isoflurane on hippocampal ripples. The ripple model was based on firing frequency of hippocampal cornu ammonis 1 pyramidal neurons and basket interneurons (code is available on Model DB, https://senselab.med.yale.edu/ModelDB/ShowModel.cshtml?model=188977).[18] Briefly, 800 pyramidal neurons and 160 interneurons were contained in this ripple model, which is a ratio consistent with hippocampal cornu ammonis 1 anatomy to represent a small patch of hippocampal cornu ammonis 1 subfield. To model each neuron, this system was based on the Adaptive Exponential Integrate-and-Fire model of neuronal activity; its simple and controllable variables have been shown to reproduce many reliable neuronal spiking behaviors.[22] Detailed information of model implementation and justifications for parameter adjustments are provided in Supplemental Digital Content 1 (http://links.lww.com/ALN/C619).

Control ripples were simulated using the default parameter values, including leak reversal potential, spike-triggered adaptation, and adaptation time constant. Simulated effects of isoflurane on ripples were conducted by adjusting parameters according to the electrophysiologic effects of isoflurane on pyramidal neurons and interneurons from cornu ammonis 1. The simulations consisted of 76 ripples with a total duration of 20 s.

Statistical Analysis

No specific power calculation was conducted. Sample sizes for electrophysiologic, local field potential recordings and the fear-conditioning test were based on our experience with similar experimental designs.[19,23,24] There were no missing data. For measurement of isoflurane effects on fear-conditioned memory and MAC of isoflurane, concentration-response curves were fitted by a sigmoidal dose-response model with a four-parameter logistic by nonlinear regression using GraphPad Prism 8.0 (GraphPad, USA). For analyzing the effects of isoflurane on local field potentials *in vivo* and simulated hippocampal ripples *in silico*, a two-tailed paired *t* test was performed to determine significance. For analyzing the effects of isoflurane on locomotor activity, two-way repeated-measures ANOVA followed by the Bonferroni *post hoc* multiple comparison test was performed. For patch clamp recordings, two-way repeated-measures ANOVA or two-tailed paired *t* test was performed to determine the difference between control and isoflurane. Normal distribution was tested by the Shapiro–Wilk test. Data are presented as mean ± SD or EC50 (95% CI). The exact statistical method is indicated in the figure legends and statistical significance was set as $P < 0.05$. No outliers were observed, and no data were excluded from statistical analyses. All statistical analyses were performed using GraphPad Prism 8.0 software, SPSS 22.0 (IBM Corp., USA), LabChart software (AD Instruments), or MATLAB software.

Results

Isoflurane Impairs Fear-conditioning Memory at Nonimmobilizing Concentrations

Isoflurane incrementally suppressed freezing behavior related to both context and cue tone memory in mice (n = 14/group; 7/7 male/female). According to the concentration-response curves, the EC50 value of isoflurane for suppressing context memory was 0.23% (95% CI, 0.21 to 0.25%; fig. 1A), and the EC50 value of isoflurane for suppressing cue tone memory was 0.39% (95% CI, 0.36 to 0.42%; fig. 1B). No difference was observed between male and female mice (Supplemental Digital Content 1, fig. 1, A and B, http://links.lww.com/ALN/C619). The MAC values of isoflurane for loss of righting reflex and immobility were measured in adult mice (n = 20; 10/10 male/female). The MAC value of isoflurane for loss of righting reflex was 0.82% (95% CI, 0.80 to 0.83%; fig. 1C), and the MAC value of isoflurane for immobility was 1.32% (95% CI, 1.25 to 1.39%; fig. 1D). No difference was observed between male and female mice (Supplemental Digital Content 1, fig. 1, C and D, http://links.lww.com/ALN/C619).

Thus, context memory (hippocampus-dependent) was almost completely suppressed by 0.5% isoflurane (approximately 0.6 MAC for loss of righting reflex and approximately 0.4 MAC for immobility). This concentration did not induce loss of righting reflex or immobility. Isoflurane at 0.5% was chosen for the subsequent experiments.

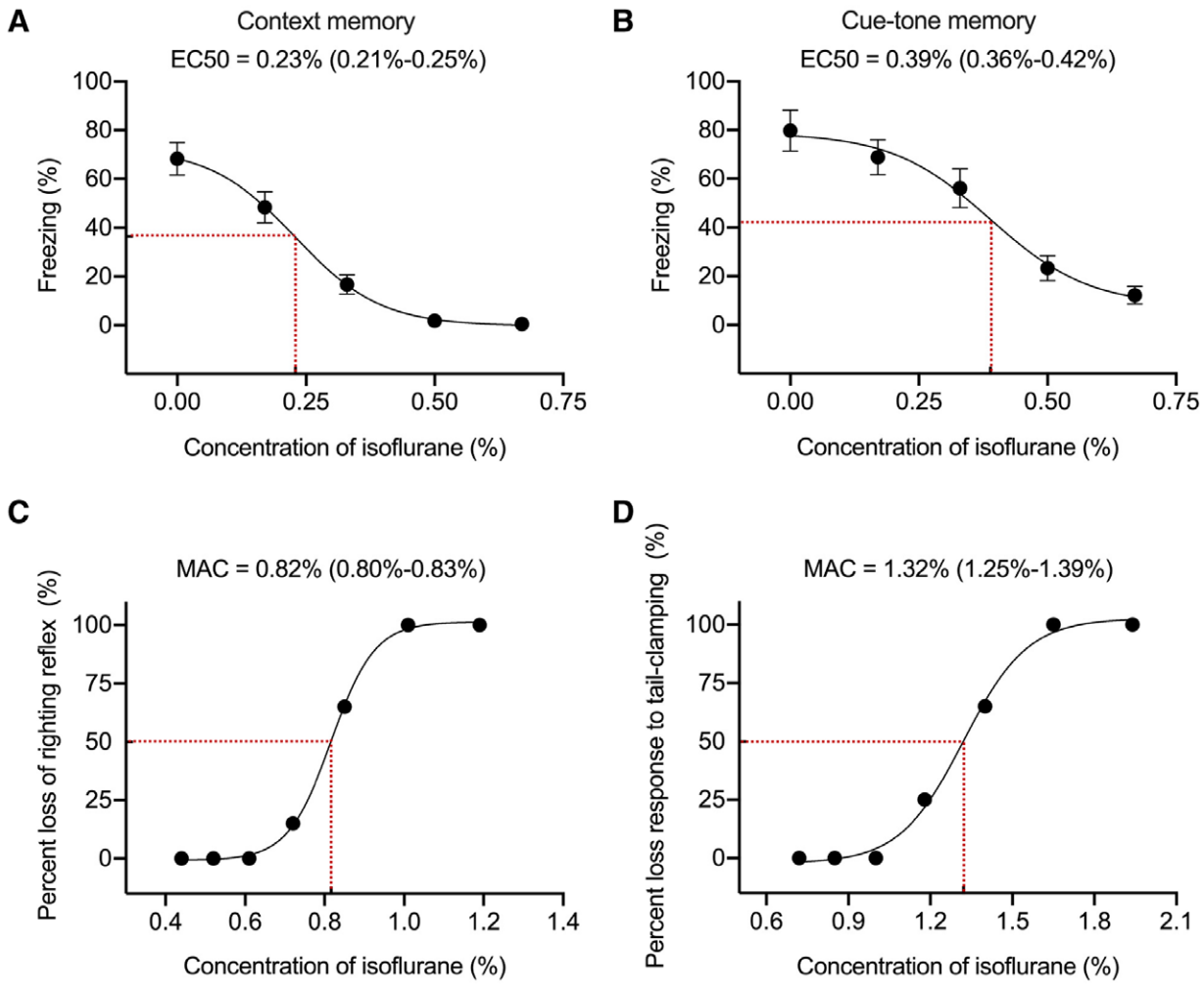

Fig. 1. Isoflurane impairs fear conditioning memory at a subanesthetic concentration. Concentration-response curves of isoflurane for suppressing fear conditioning memory to context (*A*) and cue tone (*B*), respectively (n = 14/group; 7/7 males/females). EC50 for inhibition of context memory was 0.23% (95% CI, 0.21 to 0.25%); EC50 for inhibition of cue-tone memory was 0.39% (95% CI, 0.36 to 0.42%). Concentration-response curves of isoflurane for inducing loss of righting reflex (*C*) and loss of response to tail clamping (*D*) in mice (n = 20, 10/10 males/females). Minimum alveolar concentration (MAC) of isoflurane for inducing loss of righting reflex was 0.82% (95% CI, 0.80 to 0.83%); MAC of isoflurane for inducing loss of response to tail clamping was 1.32% (95% CI, 1.25 to 1.39%). The concentration-response curves were fitted by a sigmoidal dose-response model with a four-parameter logistic by nonlinear regression. Data are presented as EC50 (95% CI).

Isoflurane Suppresses Local Field Potentials Recorded in the Hippocampal Cornu Ammonis 1 Subfield *In Vivo*

Local field potentials were recorded by deep electrodes inserted into the cornu ammonis 1 subfield (n = 9/group; 5/4 male/female) and filtered to the frequency components between 0.1 and 500 Hz (fig. 2, A and B). The strength of local field potentials activity was determined by power spectral density for each frequency component, including delta (0.1 to 4 Hz; fig. 2, D and E), theta (4 to 12 Hz; fig. 2, G and H), and ripples (100 to 200 Hz; fig. 2, J and K). A two-tailed paired *t* test was performed to determine the significance between control and isoflurane within each frequency component. For delta (0.1 to 4 Hz) frequency components (fig. 2F), isoflurane at 0.5% did not affect peak frequency (2.1 ± 0.9 *vs.* 2.1 ± 0.9 Hz; n = 9; $P = 0.904$; $t_{(8)} = 0.13$) or power spectral density ($2.8 \times 10^{-11} \pm 2.3 \times 10^{-11}$ *vs.* $1.6 \times 10^{-11} \pm 6.2 \times 10^{-12}$ mV^2/Hz; n = 9; $P = 0.127$; $t_{(8)} = 1.70$). For theta (4 to 12 Hz) frequency components (fig. 2I), there was no difference in peak frequency (6.4 ± 1.6 *vs.* 5.9 ± 1.1 Hz; n = 9; $P = 0.554$; $t_{(8)} = 0.62$) and power spectral density ($1.6 \times 10^{-11} \pm 1.7 \times 10^{-11}$ *vs.* $5.8 \times 10^{-12} \pm 3.5 \times 10^{-12}$ mV^2/Hz; n = 9; $P = 0.091$; $t_{(8)} = 1.92$) between isoflurane and control. For ripple (100

to 200 Hz) frequency components (fig. 2L), isoflurane at 0.5% increased ripple peak frequency (146.0 ± 10.6 *vs.* 123.7 ± 15.2 Hz; n = 9; $P = 0.002$; $t_{(8)} = 4.51$) and reduced ripples power spectral density ($1.5 \times 10^{-13} \pm 2.3 \times 10^{-14}$ *vs.* $2.3 \times 10^{-13} \pm 5.9 \times 10^{-14}$ mV²/Hz; n = 9; $P = 0.008$; $t_{(8)} = 3.48$) compared to control. All of the actions of isoflurane on local field potentials were quickly reversed after washout of isoflurane.

Isoflurane Suppresses High-frequency Ripples *In Vivo*

High-frequency ripples were filtered as the frequency component of 100 to 200 Hz (fig. 3, A and B) for analysis of amplitude, frequency, duration, interarrival time, and rate. A two-tailed paired *t* test was performed to determine the difference between control and isoflurane. Isoflurane at 0.5% decreased ripple amplitude from 42 ± 13 to 38 ± 13 μV (n = 9; $P = 0.003$; $t_{(8)} = 4.26$; fig. 3C) and duration from 48 ± 9 to 36 ± 5 ms (n = 9; $P < 0.001$; $t_{(8)} = 6.16$; fig. 3D). Accordingly, interarrival time was increased from 69 ± 6 to 78 ± 7 ms (n = 9; $P < 0.001$; $t_{(8)} = 9.80$; fig. 3E) and rate of ripple events was decreased from 538 ± 81 to 462 ± 66 spikes/min (n = 9; $P = 0.002$; $t_{(8)} = 4.71$; fig. 3F). Unexpectedly, isoflurane enhanced ripple frequency from 145.0 ± 2.9 to 148.2 ± 3.9 Hz (n = 9; $P < 0.001$; $t_{(8)} = 5.31$; fig. 3G).

Isoflurane Produces Time-dependent Hyperactivity and Sedation *In Vivo*

Representative trajectories of a mouse before, during, and after inhalation of 0.5% isoflurane are shown in figure 4A. Isoflurane induced hyperactivity during the induction (4 to 8 min) compared to the control group, followed by slight sedation (8 to 14 min; fig. 4, B and C). Based on the decreased activity in the open field test, it can be inferred that isoflurane induced sedation in mice, since this effect simply indicated decreased mobility. Ripples were suppressed despite hyperactivity or sedation (fig. 4B). During induction (4 to 8 min), the total travel distance (by two-way ANOVA: $P < 0.001$ for interaction between groups × time, $F[27,702] = 12.46$; $P = 0.064$ for

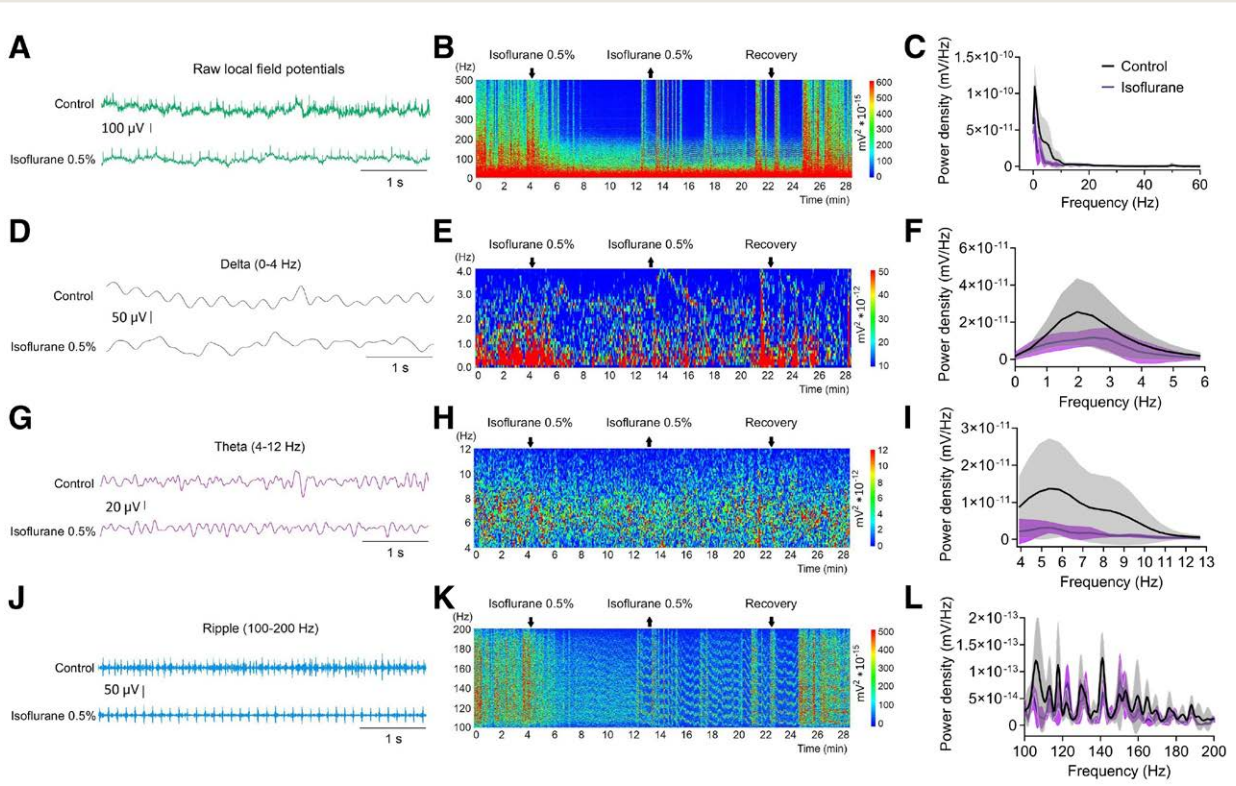

Fig. 2. Isoflurane suppresses local field potentials recorded in the hippocampal cornu ammonis 1 subfield *in vivo*. (*A*) Representative traces of raw local field potentials (0.1 to 500 Hz) recorded in hippocampal cornu ammonis 1 region for control or isoflurane (0.5%). (*B*) Power spectrogram of raw cornu ammonis 1 showing isoflurane effect. (*C*) Power spectral density of raw local field potentials for control or isoflurane. Representative traces (*D*), power spectrogram (*E*), and power spectral density (*F*) of delta frequency band (0.1 to 4 Hz). Representative traces (*G*), power spectrogram (*H*), and power spectral density (*I*) of theta frequency band (4 to 12 Hz). Representative traces (*J*), power spectrogram (*K*), and power spectral density (*L*) of ripple frequency band (100 to 200 Hz). *Solid lines* represent mean values, and *shaded regions* represent 95% CI. Data were analyzed by a two-tailed paired *t* test (*C*, *F*, *I*, and *L*; n = 9/group; 5/4 males/females).

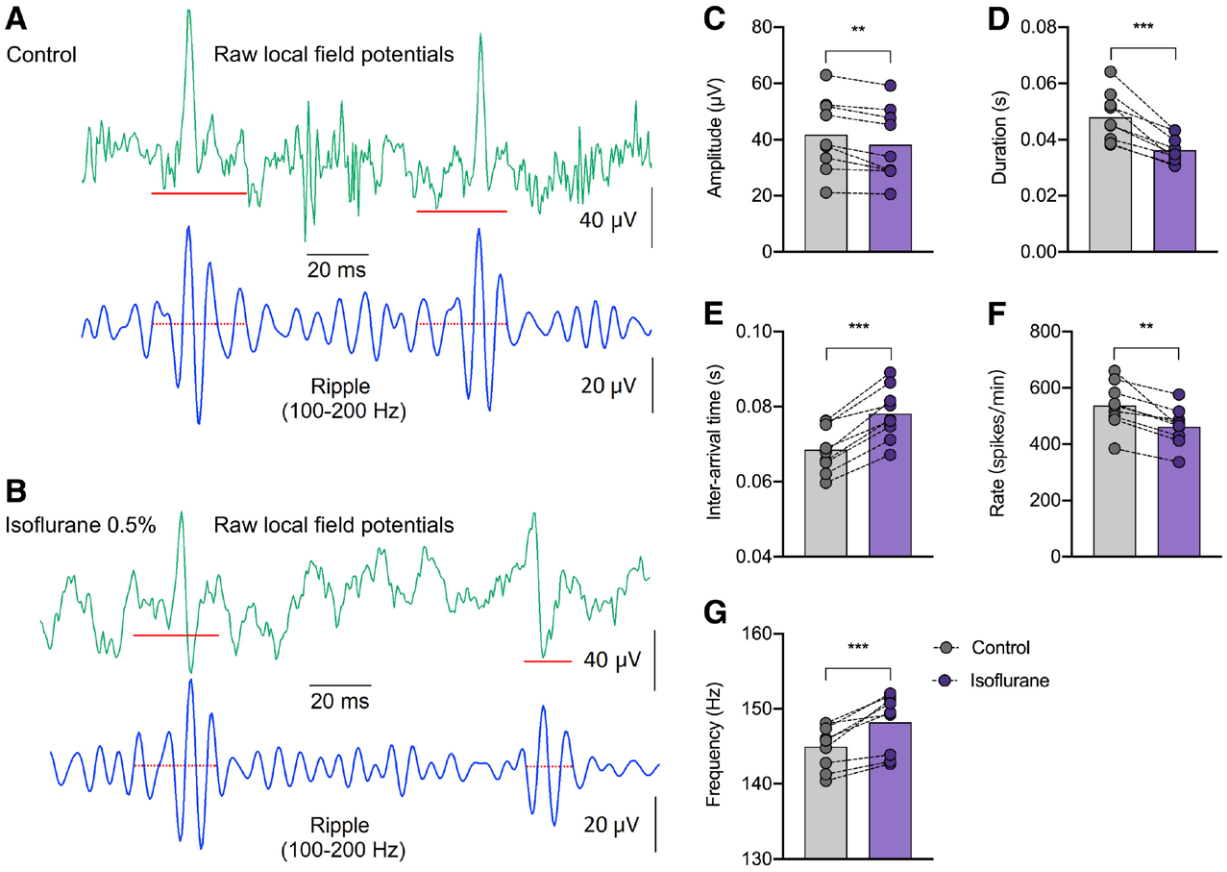

Fig. 3. Isoflurane suppresses hippocampal cornu ammonis 1 ripples *in vivo*. Representative traces of raw local field potentials (*green*) and ripples (100 to 200 Hz; *blue*) traces for control (*A*) and isoflurane (0.5%; *B*). The *red lines* indicate the duration of each ripple based on the criteria described in "Materials and Methods." Isoflurane decreased the amplitude (*C*) and shortened the duration (*D*) of ripples and increased the interarrival time (*E*) and intrinsic frequency within ripples (*G*). The rate of ripple events was decreased by isoflurane compared to control (*F*). Data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ by a two-tailed paired *t* test (n = 9/group; 5/4 males/females).

control *vs.* isoflurane, F[1,26] = 3.73; $P < 0.001$ for time, F[8,219] = 28.20; n = 14; fig. 4D) and mean speed (by two-way ANOVA: $P < 0.001$ for interaction between groups × time, F[27,702] = 12.40; $P = 0.060$ for control *vs.* isoflurane, F[1,26] = 3.86; $P < 0.001$ for time, F[8,219] = 28.07; n = 14; fig. 4E) were markedly increased compared to control. The percentage of resting time (by two-way ANOVA: $P < 0.001$ for interaction between groups × time, F[27,702] = 27.47; $P < 0.001$ for control *vs.* isoflurane, F[1,26] = 38.45; $P < 0.001$ for time, F[11,286] = 31.56; by Bonferroni *post hoc* test, isoflurane decreased the resting time at 6 to 7 min, $P < 0.001$; n = 14; fig. 4F) during induction (4 to 8 min) was accordingly decreased by 0.5% isoflurane compared to control. The sample size was 14/group mice (7/7 male/female), and no difference was observed between male and female mice (Supplemental Digital Content 1, fig. 1, E and F, http://links.lww.com/ALN/C619).

Isoflurane Differentially Modulates Neuronal Excitability of Pyramidal Neurons and Fast-spiking Interneurons in Adult Mice

Frequency of action potentials in fast-spiking interneurons was much higher than in pyramidal neurons with the same current injection. The effect of 0.12 to 0.15 mM (approximately 0.43 to 0.53 MAC, 25°C[25]) isoflurane on the excitability of pyramidal neurons and fast-spiking interneurons was evaluated in the hippocampal cornu ammonis 1 region of acute brain slices of adult mice. For pyramidal neurons, isoflurane increased action potential frequency when injecting currents from 60 to 150 pA for 1 s (by two-way ANOVA: $P = 0.031$ for interaction between groups × current injection, F[1,120] = 4.74; $P = 0.017$ for control *vs.* isoflurane, F[1,20] = 6.82; $P < 0.001$ for current injection, F[2,36] = 38.86; by Bonferroni *post hoc* test, isoflurane increased action potential frequency at current

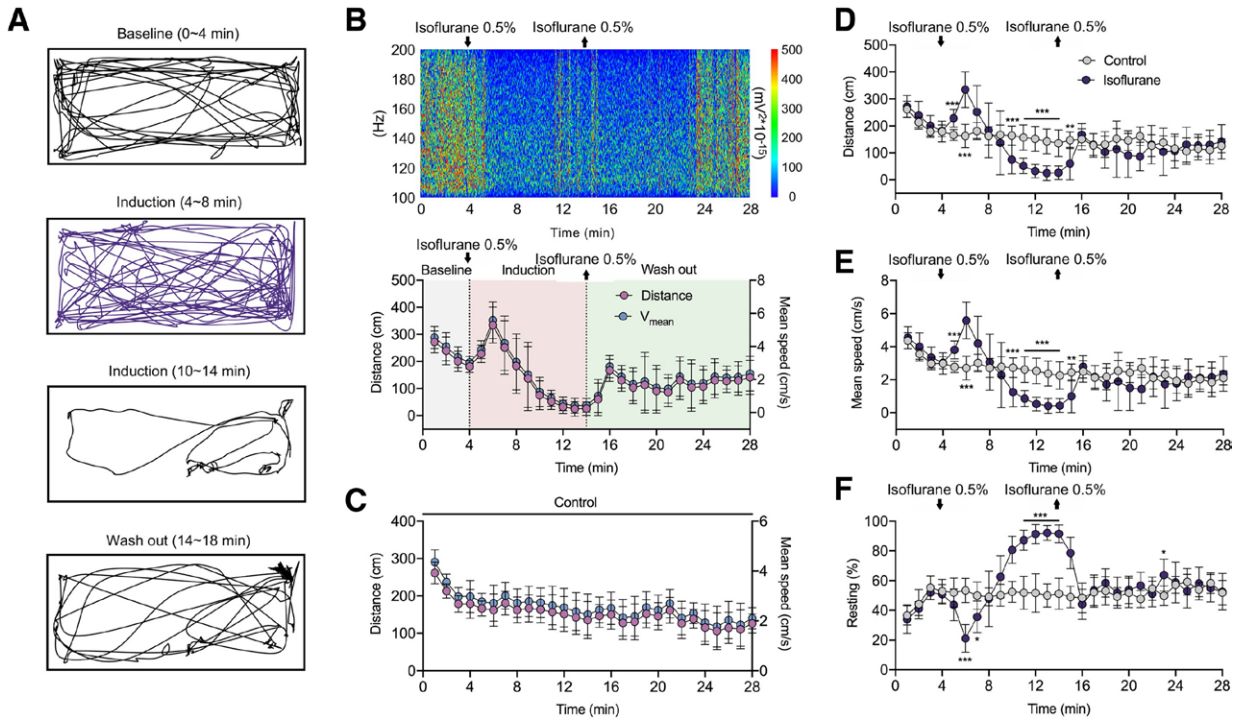

Fig. 4. Isoflurane at 0.5% produces time-dependent hyperactivity and sedation *in vivo*. (*A*) Representative traces of a mouse in control (*upper*) and 0.5% isoflurane (*middle two*), as well as during recovery (*lower*). (*B*) *Upper*: Representative power spectrogram of ripple recording. *Lower*: The time-course of travel distance and mean speed under 0.5% isoflurane. *Purple dots* are travel distance, and *blue dots* are mean speed. (*C*) Time-course of travel distance and mean speed of control mice. *Purple dots* are travel distance, and *blue dots* are mean speed. The time-course of travel distance (*D*), mean speed (*E*), and percentage of resting time (*F*) of mice between control and 0.5% isoflurane. Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by a two-way repeated-measures ANOVA followed by *post hoc* Bonferroni multiple comparison test (*B* to *F*; n = 14/group; 7/7 males/females).

injections of 60 pA [$P = 0.034$], 90 pA [$P = 0.024$], 120 pA [$P = 0.022$], and 150 pA [$P = 0.013$]; n = 11; fig. 5, A and B). For fast-spiking interneurons, isoflurane decreased action potential frequency when injecting 120 to 150 pA currents for 1 s (by two-way ANOVA: $P = 0.003$ for interaction between groups × current injection, $F[1,108] = 9.03$; $P = 0.006$ for control *vs.* isoflurane, $F[1,18] = 9.86$; $P < 0.001$ for current injection, $F[2,31] = 24.54$; by Bonferroni *post hoc* test, isoflurane decreased action potential frequency at current injections of 120 pA [$P = 0.012$] and 150 pA [$P = 0.004$]; n = 10; fig. 5, C and D). Isoflurane (0.12 to 0.15 mM) depolarized the resting membrane potential from −63.2 ± 4.6 mV to −60.9 ± 4.4 mV in pyramidal neurons (n = 11; $P = 0.007$; $t_{(10)} = 3.40$; fig. 5, E and F), but had no effect on the resting membrane potential of fast-spiking interneurons from −62.3 ± 2.9 mV to −63.2 ± 3.9 mV with no significance (n = 9; $P = 0.260$; $t_{(8)} = 1.21$; fig. 5, I and J). For analyzing the effect of isoflurane on the resting membrane potentials, a two-tailed paired *t* test was performed. This concentration of isoflurane produced no effect on neuronal input resistance in pyramidal neurons (fig. 5, G and H) or in fast-spiking interneurons (fig. 5, K and L). Of note, similar results were recorded in acute brain slices from neonatal mice (Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/C619).

Computational Model of Hippocampal Cornu Ammonis 1 Ripples Reproduces Isoflurane Effect

A model of hippocampal cornu ammonis 1 ripples based on neuronal excitability of the cornu ammonis 1 subfield was used to simulate the effects of isoflurane on ripples *in silico*. Modulation by isoflurane of hippocampal cornu ammonis 1 pyramidal neurons and fast-spiking interneurons at approximately 0.5% concentration was based on our recordings from acute brain slices. The Adaptive Exponential Integrate-and-Fire neuronal model was used.[22] Adaptation parameters including leak reversal potential, spike-triggered adaptation, and adaptation time constant were critical for neuronal firing rate. Control neuronal parameters were set as default in this model: leak reversal potential = −58 mV; spike-triggered adaptation = 100 pA; adaptation time constant = 120 ms for pyramidal neurons, and leak reversal potential = −70 mV; spike-triggered adaptation = 10

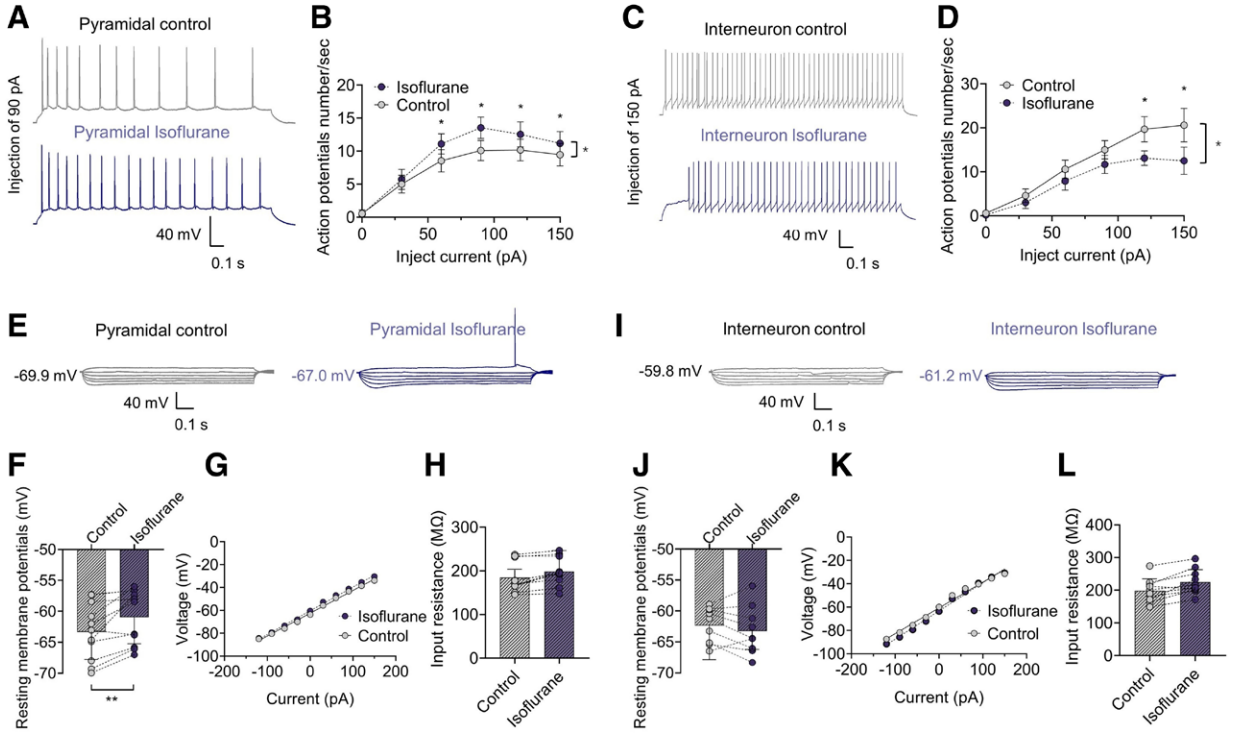

Fig. 5. Isoflurane differentially regulates action potential firing of pyramidal neurons and fast-spiking interneurons in adult mice. (*A*) Representative traces of action potentials recorded in a pyramidal neuron without (*upper*) and with (*lower*) isoflurane (0.12 to 0.15 mM; approximately 0.43 to 0.53 minimum alveolar concentration; injecting current = 90 pA). (*B*) Isoflurane enhanced action potential firing in pyramidal neurons (n = 11). (*C*) Representative traces of action potentials recorded in a fast-spiking interneuron without (*upper*) and with (*lower*) isoflurane (0.12 to 0.15 mM; injecting current = 150 pA). Interneurons were identified by their fast-spiking properties. (*D*) Isoflurane suppressed firing of action potentials in fast-spiking interneurons (n = 9). (*E* and *I*) Representative current-voltage traces recorded in a pyramidal neuron and fast-spiking interneuron. Of note, sag currents produced by activation of hyperpolarization-activated cation channels were observed with hyperpolarized current injection. (*E* and *F*) Isoflurane depolarized the resting membrane potentials of pyramidal neurons, with no effect on the current-voltage curve (*G*) of input resistance (*H*) of pyramidal neurons. For fast-spiking interneurons, isoflurane did not influence the resting membrane potential (*I* and *J*), current-voltage curve (*K*), or input resistance (*L*). Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ by a two-way repeated-measures ANOVA (*B*, *D*, *G*, and *K*) or a two-tailed paired *t* test (*F*, *H*, *J*, and *L*).

pA; adaptation time constant = 30 ms for fast-spiking interneurons. Based on the electrophysiologic recording results from adult mice, isoflurane at approximately 0.5% increased action potential frequency by 35.7 ± 35.7% in pyramidal neurons and reduced action potential frequency by 28.5 ± 26.6% in fast-spiking interneurons (calculated as area under the curve in figure 5, B and D). The neuronal parameters of pyramidal neurons and/or fast-spiking interneurons under the 0.5% isoflurane condition were set to change action potential frequency according to our recordings in acute adult mice brain slices as leak reversal potential = −55.6 mV; spike-triggered adaptation = 135.7 pA; adaptation time constant = 77.2 ms for pyramidal neurons and leak reversal potential = −70 mV; spike-triggered adaptation = 7.1 pA; adaptation time constant = 38.6 ms for fast-spiking interneurons. The simulated results indicated that isoflurane increased firing of pyramidal neurons by approximately 36% and suppressed firing of interneurons by approximately 29% (fig. 6, A and B), consistent with the extent of modulation by 0.5% isoflurane on action potential frequency in pyramidal neurons and/or fast-spiking interneurons, respectively. A representative simulated ripple is shown in figure 6, C and D. Ripple amplitude decreased from 47 ± 6 to 42 ± 8 μV (n = 76 ripples; $P < 0.001$; $t_{(75)} = 4.21$; fig. 6E), and ripple duration decreased from 42 ± 7 to 25 ± 12 ms (n = 76 ripples; $P < 0.001$; $t_{(75)} = 11.76$; fig. 6F), while ripple frequency increased from 176.0 ± 15.7 to 185.1 ± 16.7 Hz (n = 76 ripples; $P = 0.002$; $t_{(75)} = 3.16$; fig. 6G) and interarrival time between ripples increased from 178 ± 7 to 195 ± 11 ms (n = 76 ripples; $P < 0.001$; $t_{(75)} = 8.17$; fig. 6H; a total of 76 ripple events were included in the analysis of a 20-s simulation). A two-tailed paired *t* test was performed to determine the difference between control and isoflurane. The simulated effects of isoflurane on hippocampal cornu ammonis 1 ripples were comparable to the recordings of local field potentials *in vivo* (fig. 6, I to L). The simulated results based

on the electrophysiologic recordings in neonatal mice are presented in Supplemental Digital Content 1 (fig. 3, http://links.lww.com/ALN/C619).

Discussion

Isoflurane at a nonimmobilizing concentration of 0.5% impaired fear memory *in vivo* and suppressed hippocampal high-frequency ripples. At such low concentrations, which did not produce loss of righting reflex or immobility, isoflurane differentially modulated neuronal action potentials by increasing action potential frequency of cornu ammonis 1 pyramidal neurons and depressing action potential frequency of fast-spiking interneurons. Such differential modulation by isoflurane of neuronal excitability resulted in a similar reduction in ripple rate *in vivo* and *in silico*. Therefore, our results suggest that isoflurane can downregulate hippocampal high-frequency ripples, at least in part through differential modulation of hippocampal neuronal excitability.

Anterograde amnesia is an important action of volatile anesthetics.[4,5] Both isoflurane and sevoflurane produce anterograde amnesia at concentrations lower than they induce unconsciousness or immobility.[14,26] Volatile anesthetics can modulate many hippocampal functions including neuronal excitability,[19,27] synaptic transmission,[28,29] and neuroplasticity.[30,31] However, the mechanisms of the amnestic effects of volatile anesthetics are not well-characterized at the network level. Our results suggest that isoflurane modulates neuronal firing of hippocampal cornu ammonis 1 neurons to interfere with hippocampal high-frequency ripples, which may potentially disrupt memory retrieval and/or consolidation.

Actions of general anesthetics at the network level are critical for understanding the mechanisms of general anesthetics,[2,32] but explaining their network actions based on their effects on specific molecular targets is challenging. We combined behavioral, neurophysiological, electrophysiologic, and simulation experiments in an attempt to understand how isoflurane modulates critical local field potential changes *in vivo* and neuronal function at the network level. More sophisticated models may eventually simulate the effects of general anesthetics at the whole brain level.

Mice showed impaired fear memory performance associated with context and tone when exposed to isoflurane. Many brain regions are involved in fear conditioning learning and memory, including the amygdala and hippocampus.[33] Typically, contextual fear conditioning is hippocampus-dependent.[13,33] Another reason for using fear conditioning rather than a water maze or other hippocampus-dependent behavioral tests is technical compatibility with inhalation of isoflurane. Of note, the underlying analgesic action of isoflurane might weaken the response to electrical stimulus and thus also suppress the freezing behaviors. However, the electrical shock of 0.6 mA is a large nociceptive stimulus to mice,[34] and we confirmed that the analgesic action of approximately 0.4 to 0.5% isoflurane was very small (data not shown). Therefore, the suppression by isoflurane of freezing behaviors should result mostly from a direct impairment of learning and memory, which was also shown in a previous study.[35]

Isoflurane at 0.5% induces opposite effects on neural excitability between pyramidal neurons and fast-spiking interneurons. For pyramidal neurons, isoflurane increased firing rates and depolarized resting membrane potentials but depressed the firing rates of interneurons. Pyramidal neurons are the principal excitatory neurons in the hippocampus, and isoflurane increased their action potential frequency. Excitatory behaviors are common during induction and emergence from volatile anesthesia.[36] While the mechanisms of the differential modulation by isoflurane of pyramidal neurons and interneurons are unknown, they may result from varied expression of anesthetic-sensitive ion channels or neurotransmitter receptors between cell types.[1,37] For example, isoflurane at 0.5% can activate a sodium leak conductance, which may contribute to the excitatory effects of isoflurane at low concentrations.[38] Other ion channels such as potassium channels and hyperpolarization-activated cation channels maybe also be involved in modulation by isoflurane of intrinsic neuronal excitability.[39] Interestingly, isoflurane at 0.12 to 0.15 mM produced similar effects in both adult and neonatal mice. Excitation-inhibition transformation along with development of γ-aminobutyric acid–mediated networks contributes little to the effects of isoflurane between different ages, perhaps due to the negligible effect of isoflurane on $GABA_A$ receptors at low concentrations.[40] Of note, the development of γ-aminobutyric acid–mediated networks changes some neuronal properties affecting excitability (*e.g.*, resting membrane potential) with age.

Isoflurane at 0.5% decreased ripple amplitude by approximately 9%, duration by approximately 24%, and rate by approximately 14%, compared to control. Accordingly, 0.5% isoflurane increased ripple frequency by approximately 2% and interarrival time by approximately 14% compared to control. These effects are comparable to previously reported effects of anesthetic agents on hippocampal ripples, which may impair memory. The general anesthetic thiopental at a clinical concentration that induced explicit memory impairment significantly reduced the number of ripples and the duration of ripple episodes by approximately 20% *in vitro*.[41] Diazepam at a dose (1 mg/kg) that induced immobility with a gradual transition to slow-wave sleep reduced ripple amplitude by approximately 11% and duration by approximately 17% and intraripple frequency by approximately 3% in rats.[42]

Hippocampal ripple oscillations may be coordinated by recurrent interactions between pyramidal and inhibitory neurons.[43] Isoflurane at 0.5% inhibited fast-spiking interneurons and may impair recurrent interactions between

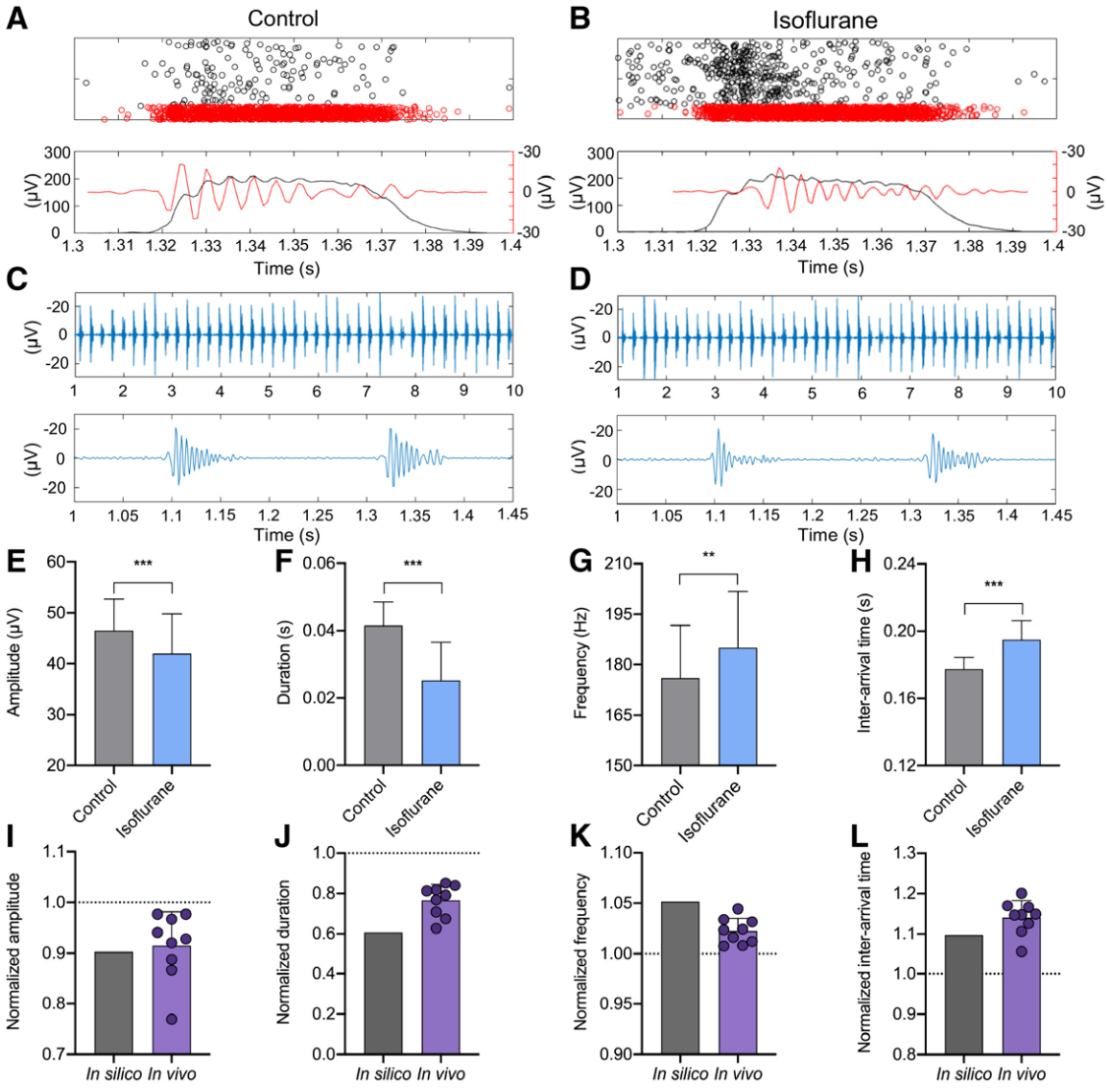

Fig. 6. Computational model of hippocampal cornu ammonis 1 ripples based on spike firing of pyramidal neurons and fast-spiking interneurons in adult mice. Rastergram of pyramidal neuron (*black*) and interneuron (*red*) spikes during a ripple and cornu ammonis 3 input for control (*A*, *upper*) and isoflurane at 0.5% (*B*, *upper*). Raw (*black*) and ripple (*red*) local field potentials (filtered 100 to 300 Hz) in the network for control (*A*, *lower*) and isoflurane at 0.5% (*B*, *lower*). Representative simulated traces of ripples with a duration of 10 s (*upper*) and amplification of two ripple events (*lower*) for control (*C*) and isoflurane at 0.5% (*D*). Isoflurane decreases ripple amplitude (*E*) and duration (*F*) and increases frequency (*G*) and interarrival time (*H*). A total of 76 ripple events were included in the analysis of a 20-s simulation. Normalized effects of isoflurane on ripples *in silico* are similar to the effects seen on local field potentials recordings *in vivo*, including amplitude (*I*), duration (*J*), frequency (*K*), and interarrival time (*L*). The effects of isoflurane used in the simulation model are based on the results of patch clamp recordings in acute brain slices. Data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ by a two-tailed paired *t* test.

pyramidal and inhibitory neurons to decrease ripple amplitude, duration, and rate. Interestingly, isoflurane increased intrinsic ripple frequency, which might result from its excitatory effects on pyramidal neurons. How isoflurane at 0.5% suppresses the action potential frequency of fast-spiking interneurons is unclear, but it is known that inhibition by isoflurane is activity-dependent, and high-frequency activity is more sensitive.[19,44]

It is likely that isoflurane at 0.5% affects all frequency components of local field potentials (delta, theta, and ripples). Isoflurane significantly suppressed ripples by decreasing power spectral density, while the effect on the power spectral density of theta oscillations was not statistically significant. A previous study reported that isoflurane at 0.32% slowed theta 1 type peak frequency by 1 Hz recorded in the cornu ammonis 1 region of the dorsal hippocampus in rats,

but no systematic change was observed in theta 1 power.[14] Two reasons may contribute to this discrepancy. First, theta 1 was defined as 4 to 12 Hz previously, but the theta band in this study was analyzed as 4 to 8 Hz. Second, the concentration of isoflurane used in this study was higher (0.5% in mice *vs.* 0.32% in rats). However, both studies supported possible modulation by isoflurane of theta oscillations at low concentrations. Given that hippocampal ripples and theta oscillations are both involved in memory processes,[9,45] high-frequency ripples may be more sensitive to isoflurane at low concentrations.

Although the detailed mechanisms of memory encoding and storage are not fully understood, the hippocampus is widely recognized as a critical brain region for memory retrieval and consolidation.[45] Hippocampal high-frequency ripples represent the firing activity in cornu ammonis 1 caused by excitatory input from cornu ammonis 3 and are critical for conversion from short-term to long-term memory storage in the neocortex.[9,11,18] Interventions to alter ripple-related electrical activity can alter subsequent memory performance *in vivo*.[11,12] Although the mechanisms underlying hippocampal cornu ammonis 1 function are not known, the firing patterns of inhibitory interneurons and excitatory pyramidal neurons are major determinants.[18] Many interneuron subtypes exist in the hippocampal cornu ammonis 1, but fast-spiking interneurons such as basket cells are most critical for ripple activity in mediating recurrent inhibition.[18] Our simulation included excitatory pyramidal and basket interneurons. Excitatory input from the cornu ammonis 3 activates cornu ammonis 1 inhibitory interneurons, which then inhibit cornu ammonis 1 pyramidal neurons, a putative mechanism for ripples.[9] We did not identify the interneuron subtypes recorded in brain slices by immunofluorescence or genetic tagging, but the interneurons recorded were selected based on their high-frequency discharges, which is the main identifying characteristic of basket cells.[46,47]

General anesthesia and sleep share certain neurobiological mechanisms.[48] However, modulation by isoflurane of hippocampal ripples is distinct from natural sleep. Hippocampal high-frequency ripples are more active during immobility and slow-wave sleep, which is consistent with a role for sleep in memory consolidation.[9] In this study, isoflurane at 0.5% induced time-dependent hyperactivity during induction, followed by sedation until washout of isoflurane. Ripples were suppressed throughout, indicating that modulation by isoflurane of ripple activity may be different than physiologic modulations such as sleep.

Although neuronal activity of the hippocampal cornu ammonis 1 region is a determinant of ripples, other regions such as cornu ammonis 3 and neocortex can also modulate ripples.[49] The effects of isoflurane on neuronal activity can result from modulation of intrinsic neuronal excitability, cornu ammonis 1 synaptic input, or input from other brain regions. The neuronal firing patterns we observed in brain slices represented the overall effects of isoflurane on hippocampal local circuits. The limitation is that acute brain slices do not contain brain remote structures providing synaptic connections required for memory processing.

There are limitations to our study. First, we cannot artificially modulate hippocampal ripples *in vivo* to rescue or recapitulate the amnestic effects of isoflurane; therefore, the causality between modulation of hippocampal ripples and amnestic effects of isoflurane is not established. A recent study prolonged spontaneously occurring ripples by optogenetic stimulation, which increased memory during maze learning.[11] A challenging but possible approach may be to inhibit pyramidal neurons while activating fast-spiking interneurons in the same hippocampal cornu ammonis 1 subfield. Second, the patch clamp recordings were performed at room temperature for electrophysiologic stability. Although we corrected the MAC of isoflurane to 37°C,[50] the effects of isoflurane maybe different between room temperature and physiologic temperature. Third, the parameters of the simulation model were not directly determined by our recordings. Therefore, the model cannot simulate absolute values of action potential frequencies of pyramidal neurons and/or interneurons. Instead, we can only simulate the relative change compared to default baseline.

In summary, our findings suggest that the volatile anesthetic isoflurane at a nonimmobilizing concentration can suppress hippocampal high-frequency ripples by differentially modulating firing of hippocampal cornu ammonis 1 neurons. High-frequency hippocampal activity may be a critical mediator of memory function during general anesthesia.

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Competing Interests

Dr. Hemmings is editor-in-chief of the *British Journal of Anesthesia*, has consulted for Elsevier (Philadelphia, Pennsylvania), and has received research support from Instrumentation Laboratory (Bedford, Massachusetts). The other authors declare no competing interests.

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