ANESTHESIOLOGY

Isoflurane Modulates Hippocampal Cornu Ammonis Pyramidal Neuron Excitability by Inhibition of Both Transient and Persistent Sodium Currents in Mice

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ABSTRACT

Background: Volatile anesthetics inhibit presynaptic voltage-gated sodium channels to reduce neurotransmitter release, but their effects on excitatory neuron excitability by sodium current inhibition are unclear. The authors hypothesized that inhibition of transient and persistent neuronal sodium currents by the volatile anesthetic isoflurane contributes to reduced hippocampal pyramidal neuron excitability.

Methods: Whole-cell patch-clamp recordings of sodium currents of hippocampal cornu ammonis pyramidal neurons were performed in acute mouse brain slices. The actions of isoflurane on both transient and persistent sodium currents were analyzed at clinically relevant concentrations of isoflurane.

Results: The median inhibitory concentration of isoflurane for inhibition of transient sodium currents was 1.0 ± 0.3 mM (~3.7 minimum alveolar concentration [MAC]) from a physiologic holding potential of −70 mV. Currents from a hyperpolarized holding potential of −120 mV were minimally inhibited (median inhibitory concentration = 3.6 ± 0.7 mM, ~13.3 MAC). Isoflurane (0.55 mM; ~2 MAC) shifted the voltage-dependence of steady-state inactivation by −6.5 ± 1.0 mV (n = 11, $P < 0.0001$), but did not affect the voltage-dependence of activation. Isoflurane increased the time constant for sodium channel recovery from 7.5 ± 0.6 to 12.7 ± 1.3 ms (n = 13, $P < 0.001$). Isoflurane also reduced persistent sodium current density (median inhibitory concentration = 0.4 ± 0.1 mM, ~1.5 MAC) and resurgent currents. Isoflurane (0.55 mM; ~2 MAC) reduced action potential amplitude, and hyperpolarized resting membrane potential from −54.6 ± 2.3 to −58.7 ± 2.1 mV (n = 16, $P = 0.001$).

Conclusions: Isoflurane at clinically relevant concentrations inhibits both transient and persistent sodium currents in hippocampal cornu ammonis pyramidal neurons. These mechanisms may contribute to reductions in both hippocampal neuron excitability and synaptic neurotransmission.

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Neurotransmitter release from presynaptic nerve terminals is hindered by volatile anesthetics through inhibition of voltage-gated sodium channels
- Depression of neuronal activity by volatile anesthetics through direct inhibition of sodium currents in excitatory neurons has not been previously reported

What This Article Tells Us That Is New

- Electrophysiologic studies show that isoflurane, at clinically relevant concentrations, inhibits both transient and persistent sodium currents on mouse cornu ammonis hippocampal neurons *ex vivo*
- The isoflurane-induced inhibition of sodium channels on excitatory neurons may contribute to the reduction of neuronal excitability and synaptic transmission

Although general anesthetics have been used clinically for more than 170 yr, their molecular and cellular mechanisms of action are still not clear. Compared with intravenous general anesthetics like propofol, the molecular mechanisms of volatile anesthetics such as isoflurane and sevoflurane are more complex because they interact with multiple molecular targets.[1,2] Volatile anesthetics produce the desirable pharmacologic endpoints of amnesia, unconsciousness, and immobility, but also unwanted side effects including respiratory and cardiovascular depression and developmental neurotoxicity.[3–5] Therefore, understanding the various molecular targets that contribute to specific desired and undesired endpoints is important for a complete pharmacologic understanding and for development of novel anesthetics with improved safety profiles.

Volatile anesthetics depress neurotransmitter release with greater potency at excitatory than inhibitory synapses, which contributes to volatile anesthetic depression of central nervous system function.[6] Volatile anesthetics at clinically relevant concentrations inhibit sodium currents in transfected cells,[7,8] nerve terminals,[8,9] and dorsal root ganglion neurons.[10] Voltage-gated sodium channels are important targets for the presynaptic effects of volatile anesthetics on

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excitatory neurotransmitter release.[11,12] However, whether volatile anesthetics can directly depress activity of excitatory neurons by inhibiting sodium currents is unclear.

Initiation and propagation of action potentials are important for synaptic transmission and neuronal plasticity.[13] Even a small change in action potential properties can lead to significant modulation of synaptic transmission.[14] Transient sodium currents are crucial for membrane excitability, including initiation and propagation of action potentials.[13] Voltage-gated sodium channels can also produce persistent and resurgent sodium currents to modulate excitability of neuronal networks.[15,16] Slowly inactivating or noninactivating sodium currents (persistent sodium currents) are activated at sub-threshold voltages and enhance repetitive firing.[17] Resurgent sodium currents occur with repolarization after a previous period of depolarization; neurons with significant resurgent sodium currents share the capacity for rapid spontaneous firing or burst firing.[16] Changes in persistent or resurgent sodium currents are implicated in several diseases including epilepsy, paramyotonia congenita, and extreme pain syndromes.[17,18] However, whether volatile anesthetics directly modulate persistent sodium currents and/or resurgent sodium currents is unknown.

The hippocampus is critical to many brain functions including learning and memory,[19] which are sensitive to the actions of general anesthetics.[20–22] Pyramidal neurons are the principal excitatory neurons in the hippocampus and express multiple subtypes of voltage-gated sodium channels.[23] We designed the present study to test the hypothesis that the volatile anesthetic isoflurane directly modulates excitability of hippocampal pyramidal neurons by inhibiting both transient and persistent sodium currents.

Materials and Methods

Materials

Working solutions of isoflurane were prepared from saturated solutions (12 to 15 mM isoflurane, as measured by gas chromatography[24]) in extracellular solution consisting of (in mM) 125 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, and 15 glucose, pH = 7.3 ± 0.5. The saturated solution was prepared by rotation in a gas-tight vial for at least 24 h. The saturated solution was diluted to experimental concentrations immediately before perfusion, and final concentrations of isoflurane were confirmed by gas chromatography.[24] Isoflurane 0.27 mM was used as the predicted minimum alveolar concentration (MAC, equivalent to the EC_{50} for immobilization) for mouse adjusted to room temperature (~25°C).[25–27] Isoflurane was purchased from Abbott Pharmaceutical Co. Ltd. (China), tetrodotoxin was purchased from Alomone Labs (Israel), and other compounds were obtained from Sigma-Aldrich (China).

Preparation of Mouse Hippocampal Slices

Procedures were approved by the Animal Ethics Committee of Sichuan University (Chengdu, China). Randomization and blinding methods were not used in these electrophysiologic recordings. C57BL/6 mice (28 male and 25 female, 53 in total) at 7 to 10 postnatal days were anesthetized with ketamine/xylazine (60/10 mg/kg) and decapitated. The brain was rapidly removed and put into ice-cold oxygenated (95% O_2/5% CO_2) sucrose-substituted dissecting solution containing (in mM): 87 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 7.5 $MgCl_2$, 75 sucrose, and 25 glucose. Horizontal hippocampal slices (thickness of 270 μm) were cut using a vibratome (Leica VT1000 A, USA), then incubated for 30 min at 37°C and then at room temperature (23° to 25°C) in extracellular solution aerated with 95% O_2/5% CO_2. After incubation, hippocampal slices were mounted in the recording chamber for electrophysiologic recordings at room temperature.[8]

Whole-cell Patch-clamp Recording

Hippocampal slices were placed in a recording chamber and continuously perfused with extracellular solution at 2 ml/min. Pyramidal neurons in the hippocampal cornu ammonis region were directly visualized and identified by their shape and size with infrared differential interference contrast imaging microscopy. Whole-cell voltage-clamp recordings were applied to record sodium currents. Electrophysiologic recordings were conducted using an Axopatch 200B amplifier, 1,440 Digidata and coupled with pClamp 10.2 software (Molecular Devices, USA). Currents were sampled at 20 kHz and filtered at 5 kHz. The external solution was the same as the incubation solution but supplemented with 25 mM TEA-Cl to block potassium currents. The resistance of glass pipettes was 3 to 4 MΩ and the internal pipette solution contained (in mM): 110 CsF, 9 NaCl, 1.8 $MgCl_2$, 4 Mg-ATP, 0.3 Na-GTP, 0.09 EGTA, 0.018 $CaCl_2$, 9 HEPES, 10 TEA-Cl. CsOH was used to adjust to pH = 7.38 (290 to 310 mOsm). Tetrodotoxin-sensitive currents were confirmed by subtraction after application of 500 nM tetrodotoxin. Series resistance was compensated by approximately 70 to 75%, and cells were rejected when series resistance exceeded 15 MΩ. Action potentials were recorded under current-clamp mode with an extracellular solution containing (in mM): 125 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 15 glucose, and 10 μM bicuculline or 100 μM picrotoxin to block possible effects from γ-aminobutyric acid–mediated (GABAergic) inputs. The internal pipette solution for current-clamp mode contained (in mM): 122 K-methanesulfonate, 9 NaCl, 1.8 $MgCl_2$, 4 Mg-ATP, 0.3 Na-GTP, 0.09 EGTA, 0.018 $CaCl_2$, and 9 HEPES (pH adjusted to 7.35 with KOH, 290 to 310 mOsm).

Statistical Analysis

No power calculations were conducted before the study. Sample sizes were based on our experience with similar experimental designs. No data were lost, but data were not collected when cell series resistance exceeded 15 MΩ.

Electrophysiologic data were analyzed using Clampfit 10.0 software (Molecular Devices), Graph-Pad Prism 6 (Graph-Pad Software Inc., USA), and Origin 10.0 (OriginLab, USA). Half-maximal inhibitory concentration (IC_{50}) values were obtained by least squares fitting to the Hill equation: $Y = 1/(1 + 10((\log IC_{50} - X) \cdot h)$, where Y is the effect, X is the measured concentration of isoflurane, and h is the Hill slope. For transient sodium currents, voltage-dependence of half-maximal activation and half-maximal inactivation were fitted to the Boltzmann equation: $G/G_{max} = 1/[1 + \exp(V_{½} - V/k)]$, where G = conductance, G/G_{max} = normalized conductance; $V_{½}$ = voltage of half-maximal activation or inactivation, and k = slope factor. Sodium current recovery traces and time constants (τ) were determined by fitting to the mono-exponential function: $Y = \exp(-\tau \cdot n) + A_p$, where τ is the time constant, A_p is the plateau, and n is stimulus number based on complete recovery time. Inhibition of peak transient sodium current was normalized as %inhibition *versus* control and compared by chi-square test. For persistent sodium currents, currents were measured as the preserved currents from 450 to 475 ms after stimulus. Resurgent sodium current refers to the peak current during the first 50 ms following step back. Both persistent and resurgent sodium currents were corrected for each individual trace by subtracting current recorded in the presence of tetrodotoxin. Normal distribution of data was tested by the Shapiro–Wilk test. All data are presented as mean ± SD. Repeated measures data were analyzed by two-way ANOVA with Bonferroni *post hoc* testing. Two-tailed independent-samples or paired Student's *t* test were used for comparison between control and isoflurane conditions. Statistical significance was set as $P < 0.05$.

Results

Isoflurane Inhibits Transient Sodium Channel Current

Transient sodium channel current was activated by depolarization to 0 mV from holding potentials of −70 or −120 mV. At a clinically relevant concentration (0.55 mM, ~2

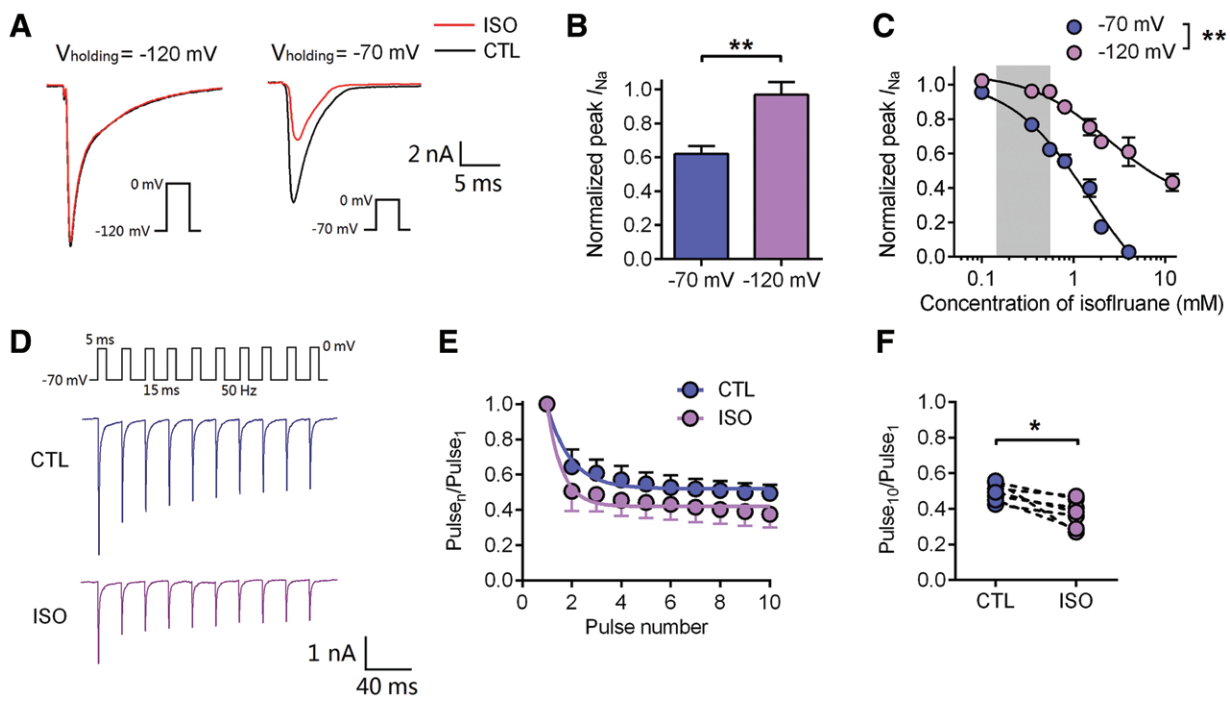

Fig. 1. Isoflurane inhibits sodium currents in a voltage- and activity-dependent manner. (*A* and *B*) Representative current traces recorded from the same pyramidal neuron before and after isoflurane at a clinically relevant concentration (0.55 mM, ~2 minimum alveolar concentration [MAC]) application from holding potentials of −70 mV and −120 mV (*A*), and the normalized inhibition (*B*) showing voltage-dependent inhibition of Na^+ currents with greater inhibition by isoflurane from a holding potential of −70 mV than of −120 mV (n = 9, $P = 0.012$). (*C*) Concentration-effect curves of isoflurane on sodium currents from holding potentials of −70 mV or −120 mV, respectively. The shadow indicates clinically relevant concentrations of isoflurane (0.5 to 2 MAC). The median inhibitory concentration (IC_{50}) from a holding potential of −70 mV was 1.0 ± 0.3 mM (~3.7 MAC) and 3.6 ± 0.7 mM (~13.3 MAC) at a holding potential of −120 mV (n = 9 to 12, $P < 0.0001$). (*D* through *F*) Activity-dependent inhibition by isoflurane. (*d*) Representative currents from the same neuron before (*top*) and after (*bottom*) isoflurane. (*E* and *F*) Normalized sodium currents (*E*) and the last pulse (*F*) from individual normalized currents ($Pulse_{10}/Pulse_1$) in the absence or presence of isoflurane (n = 7, $P = 0.010$). Data are mean ± SD. *$P < 0.05$, **$P < 0.01$. Analyzed by two-tailed unpaired *t* test (*B*), two-tailed paired *t* test (*F*). CTL, control; ISO, isoflurane.

MAC), isoflurane inhibited transient sodium channel current in a voltage-dependent manner (fig. 1A). Inhibition was significantly greater at the physiologic holding potential of −70 mV (37.8% ± 4.6% inhibition, n = 9) than at a hyperpolarized holding potential of −120 mV (3.1% ± 7.6% inhibition, n = 9, $P = 0.012$ *vs.* −70 mV; fig. 1B). The median inhibitory concentration of isoflurane for inhibition of transient sodium channel current was 1.0 ± 0.3 mM (~3.7 MAC) from a holding potential of −70 mV, whereas the currents from a holding potential of −120 mV were minimally inhibited (median inhibitory concentration = 3.6 ± 0.7 mM, ~13.3 MAC; fig. 1C). With repeated 5-ms pulses depolarizations at 50 Hz (fig. 1D), normalizing sodium channel current of each pulse to that of the first pulse ($Pulse_n/Pulse_1$) removed the effect of resting block by isoflurane.[24] Thus the reduced sodium channel current at the 10th pulse reflected activity-dependent inhibition as a result of repeated membrane depolarizations (fig. 1E). From a holding potential of −70 mV, isoflurane reduced $Pulse_{10}/Pulse_1$ ratio from 0.49 ± 0.02 to 0.38 ± 0.03 (fig. 1F, n = 7, $P = 0.010$). This suggests that isoflurane leads to progressive inhibition of sodium channel current during trains of action potentials as a result of delayed recovery from inactivation produced by isoflurane.

Isoflurane Modulates Transient Sodium Channel Gating

To test the effects of isoflurane on voltage-gated sodium channel activation, sodium current was activated by a series of voltage steps from −70 to +70 mV preceded by a

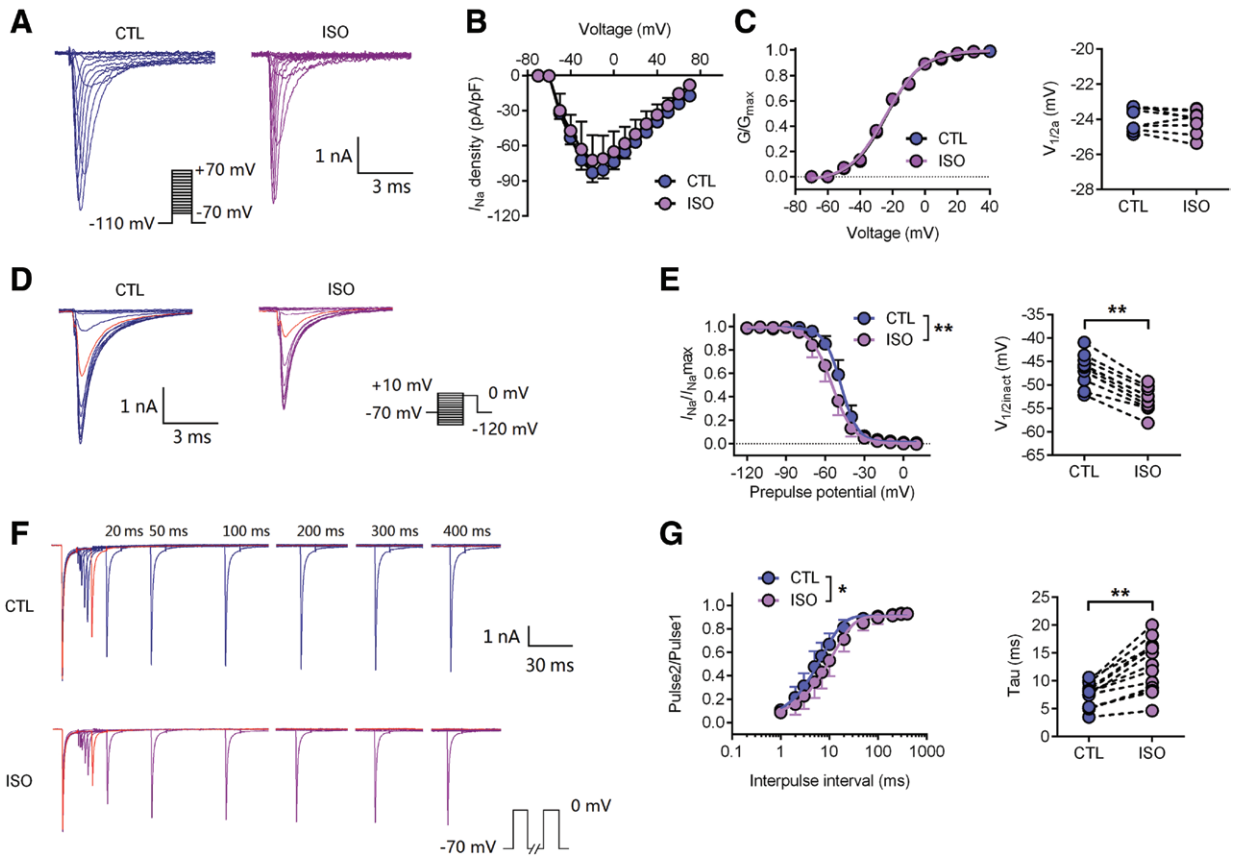

Fig. 2. Isoflurane modulates voltage-gated sodium channel gating. (*A* and *B*) Isoflurane at a clinically relevant concentration (0.42 mM, ~1.6 minimum alveolar concentration [MAC]) does not alter the voltage-dependence of voltage-gated sodium channel activation. (*A*) Representative current traces recorded from the same pyramidal neuron before (*left*) and after (*right*) isoflurane application. (*C*) Voltage of half-maximal activation ($V_{½act}$) was not altered by isoflurane (n = 8; $P = 0.144$). (*D* and *E*) Isoflurane shifts the voltage-dependence of voltage-gated sodium channel inactivation in the hyperpolarized direction. (*D*) Steady-state current curves recorded from the same neuron in the absence (left) or presence (right) of isoflurane. (*E*) Voltage of half-maximal inactivation ($V_{½inact}$) is shifted in the hyperpolarized direction by isoflurane (n = 8, $P < 0.0001$). (*F* and *G*) Isoflurane significantly delays recovery from inactivation of voltage-gated sodium channels. (*F*) Traces recorded in the absence (*top*) or presence (*bottom*) of isoflurane using a paired-pulse protocol. (*G*) Normalized peak current was plotted against duration of the inter-pulse interval, which varied from 1 to 400 ms (*inset*), for CTL and ISO, and time constants were determined from mono-exponential fits for individual neuron data (*left*; n = 13, $P = 0.0002$). Data are mean ± SD. *$P < 0.05$, **$P < 0.01$ by two-tailed paired *t* test. CTL, control; ISO, isoflurane.

−110 mV prepulse to relieve channel inactivation (fig. 2A). Isoflurane at 0.42 mM (~1.6 MAC) did not shift the current–voltage relationship, and maximum sodium channel current for both control and isoflurane conditions occurred at −20 mV (fig. 2B). Voltage-dependent activation was unchanged by isoflurane: voltage-dependence of half-maximal activation was −23.9 ± 0.2 mV for control compared to −24.2 ± 0.2 mV with isoflurane (fig. 2C, n = 8, $P = 0.144$). The effect of isoflurane on steady-state inactivation was determined by eliciting currents at 0 mV after a 20-ms prepulse to voltages from −120 to +10 mV (fig. 2D). Normalized sodium channel current/maximum sodium channel current values reflected the fraction of channels inactivated during the prepulse.[9] Isoflurane (0.42 mM, ~1.6 MAC) shifted the steady-state inactivation curve in the hyperpolarizing direction; with a voltage of half-maximal inactivation of −46.7 ± 0.9 mV for control compared with −53.2 ± 0.8 mV with isoflurane (fig. 2E, n = 11, $P < 0.0001$). These data suggest that isoflurane inhibits peak sodium channel current by increasing the fraction of inactivated channels.

As neuronal firing frequency depends in part on how fast voltage-gated sodium channels can cycle through its various activation states, we measured the time-course of recovery from inactivation. Peak sodium channel current was recorded in response to two 10-ms pulses to 0 mV from a holding potential of −70 mV, where the duration between the two pulses was varied from 1 to 400 ms (fig. 2F). Recovery time-courses were fit to a mono-exponential function for both control and isoflurane conditions, indicating that channels predominantly entered a single fast-inactivated state.[24] Isoflurane increased the time required for channel recovery, with the recovery time constant increasing from 7.5 ± 0.6 to 12.7 ± 1.3 ms (fig. 2G, n = 13, $P < 0.0001$).

Isoflurane Inhibits Persistent and Resurgent Sodium Currents

Persistent currents after fast-inactivated transient currents were recorded with 500-ms voltage steps from −70 to +20 mV (fig. 3A). The mean residual currents during 450 to 475 ms were calculated as persistent sodium currents.[18] The largest persistent currents were found at a voltage step to −40 mV (fig. 3B). Isoflurane inhibited persistent sodium currents at nearly all voltage steps from −60 to 0 mV (fig. 3C). The median inhibitory concentration of isoflurane for persistent sodium currents was 0.4 ± 0.1 mM (~1.5 MAC) from a holding potential of −70 mV with depolarization to −40 mV (the voltage for maximal persistent currents; fig. 3D). Persistent sodium currents were corrected for each individual trace by subtracting current recorded in the presence of tetrodotoxin (500 nM). When persistent sodium current was evoked by a ramp depolarization stimulus (from −80 to 0 mV at 30 mV/s),[28] isoflurane decreased persistent sodium current density (fig. 3E and 3F, n = 11). Persistent sodium current was tetrodotoxin-sensitive as it was inhibited completely by 500 nM tetrodotoxin (fig. 3E). Resurgent sodium current is the residual fraction of the slow sodium current that can be activated by the next depolarization.[16] Isoflurane inhibited the resurgent current evoked by voltage steps from −70 to 0 mV with a 20-ms prepulse to +30 mV (fig. 3G-H, n = 7, all $P < 0.05$ for voltages from −70 to −10 mV).

Isoflurane Inhibits Sub-threshold Currents

Sodium currents at sub-threshold voltages determine the threshold of neuronal activation and are critical to synaptic transmission.[29] To test the effects of isoflurane on sub-threshold sodium currents, we applied successive 5-mV step depolarizations at the same rate as the ramp depolarization[29] (fig. 4A). Both transient and steady-state sodium channel current were evoked at these potentials. The largest transient and steady-state sodium channel current densities (current/conductance) were found at a voltage step to −60 mV, with current densities of −24.0 ± 7.9 and −2.0 ± 0.3 sodium channel current densities (current/conductance), respectively (fig. 4B). Isoflurane (0.42 mM, ~1.6 MAC) inhibited the transient sodium current at voltage steps to −50 and −45 mV, and steady-state sodium currents at voltage steps to −60 mV (fig. 4C and 4D, n = 9).

Isoflurane Depresses Action Potentials

Single action potential were evoked by injection of 60 pA current for 100 ms in current-clamp mode (fig. 5A). Isoflurane reduced action potential amplitude from 104.5 ± 5.1 to 90.0 ± 3.0 mV (fig. 5B left, n = 13, $P = 0.001$) and increased action potential width from 2.3 ± 0.2 to 2.6 ± 0.2 ms (fig. 5B right, n = 13, $P = 0.001$). By phase-plane plot voltage-time derivative analysis, isoflurane inhibited the whole time course of somatic spikes recorded from hippocampal cornu ammonis pyramidal neurons (fig. 5C). Firing of action potentials was activated by 1-s series of current injection from 120 to 60 pA (fig. 5D). Isoflurane reduced firing frequency from 10.2 ± 1.6 to 3.3 ± 1.0 Hz for 30 pA injections (fig. 5E, n = 20, $P = 0.0002$), from 13.2 ± 1.5 to 7.2 ± 1.0 Hz for 60 pA injections (fig. 5E, n = 20, $P < 0.0001$), and from 12.5 ± 1.5 to 7.6 ± 1.0 Hz for 90 pA injections (fig. 5E, n = 20, $P < 0.0001$). Isoflurane hyperpolarized the resting membrane potential from −54.6 ± 2.3 to −58.7 ± 2.1 mV (fig. 5F, n = 16, $P = 0.001$). Rheobase was increased by isoflurane from 21.4 ± 5.7 to 42.9 ± 11.5 pA (fig. 5G, n = 14, $P = 0.019$), and input resistance was increased from 220 ± 17 to 241 ± 17 MΩ (fig. 5H, n = 12, $P < 0.0001$). The effects of isoflurane on action potentials firing frequency, rheobase, neuronal input resistance, and resting membrane potential represent the combined results with recordings under bicuculline or picrotoxin.

Discussion

Specific molecular targets mediate the neurophysiologic actions of general anesthetics on critical properties and

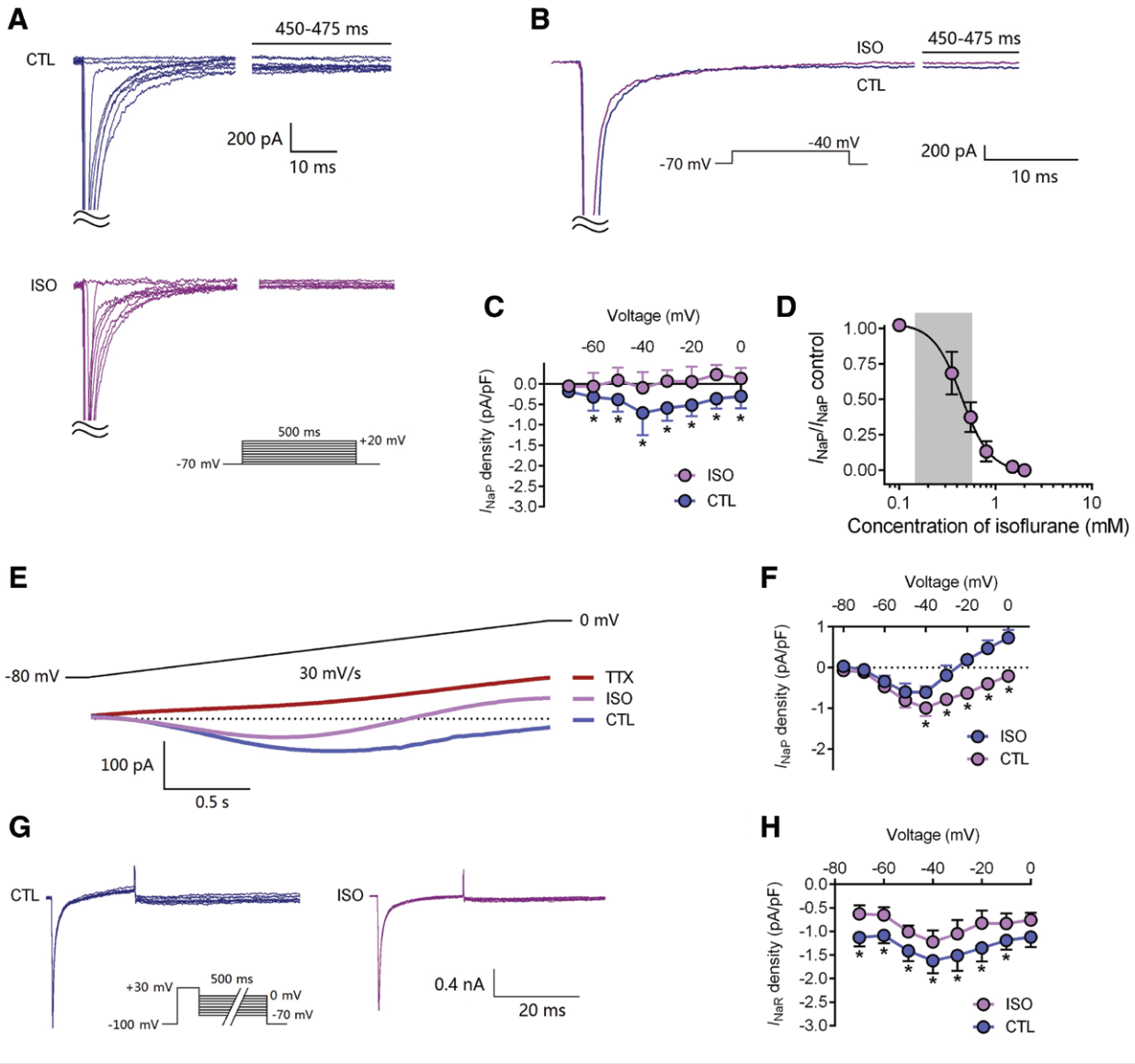

Fig. 3. Isoflurane inhibits persistent (I_{NaP}) and resurgent (I_{NaR}) sodium channel currents. (*A* through *F*) Isoflurane at a clinically relevant concentration (0.49 mM, ~1.8 minimum alveolar concentration [MAC]) inhibits I_{NaP} in pyramidal neurons. (*A*) Representative traces recorded from the same neuron using a prolonged step protocol shown in the *inset* (I_{NaP} was determined as the mean persistent currents from 450 to 475 ms). (*B*) The largest currents were elicited by depolarization to −40 mV; recordings before and after isoflurane perfusion are shown. (*C*) Density of I_{NaP} evoked by depolarization from −70 mV to 0 mV (n = 7). (*D*) Concentration-effect curve of isoflurane for I_{NaP} from a holding potential of −70 mV. The shading indicates clinically relevant concentrations of isoflurane (0.5 to 2 MAC). (*E*) Mean I_{NaP} curves obtained from seven neurons, evoked with a ramp protocol from −80 mV to 0 mV (30 mV/s). (*F*) Isoflurane reduced the density of I_{NaP}. (*G* and *H*) Representative resurgent sodium currents (*G*) and density (*H*) indicates that isoflurane depressed I_{NaR} in pyramidal neurons (n = 17). I_{NaR} refers to the peak current during the first 50 ms following step back. Data are mean ± SD. *$P < 0.05$ by two-way ANOVA. CTL, control; ISO, isoflurane.

processes such as neuronal excitability, axonal conduction, and synaptic transmission.[30,31] Voltage-gated sodium channels are essential ion channels in mediating the rising phase of action potentials[13,32] and are implicated as presynaptic targets for general anesthetics.[3] Although early studies on the squid giant axon found that action potentials were relatively insensitive to clinical concentrations of general anesthetics,[33] more recent studies report significant sensitivity of sodium channels to volatile anesthetics at clinically relevant concentrations.[9,34] Supporting *in vivo* animal studies show that intravenous administration of the voltage-gated sodium channels blocker lidocaine reduces MAC for several volatile anesthetics both in animals and humans.[35–37] Moreover, intrathecal injection of tetrodotoxin reduces MAC for isoflurane in rats,[35] whereas intrathecal veratridine, a

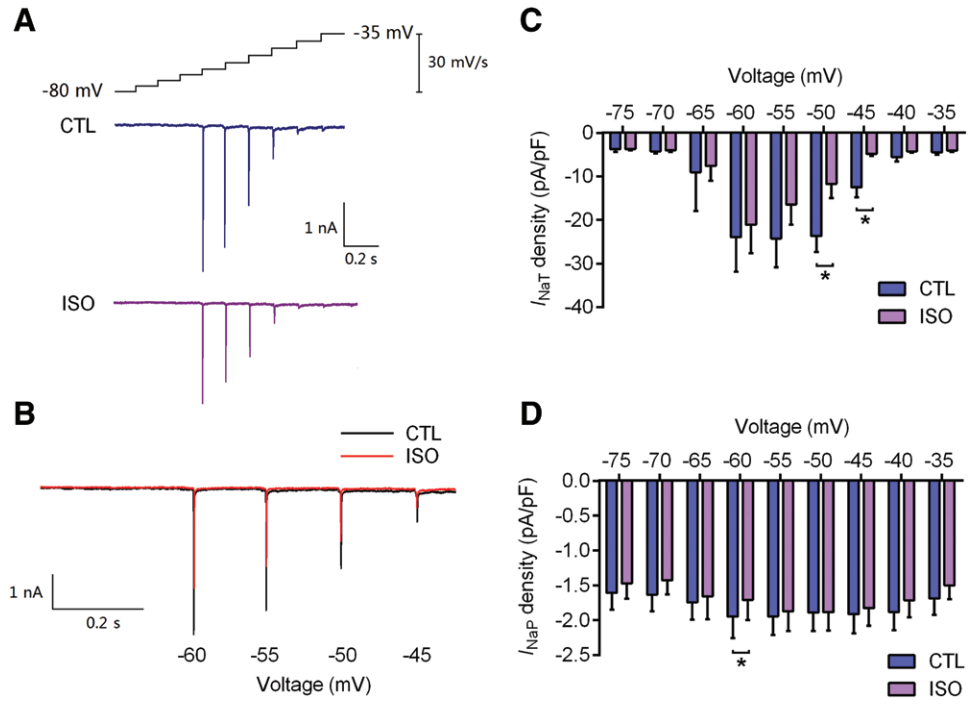

Fig. 4. Isoflurane modulates sub-threshold transient and persistent currents. (*A*) Representative currents recorded by a stair ramp protocol. Transient (I_{NaT}) and state-steady persistent (I_{NaP}) currents at each potential were calculated. (*B*) Sample of initial part of recording. I_{NaT} was inhibited by isoflurane at a clinically relevant concentration (0.42 mM, ~1.6 minimum alveolar concentration [MAC], n = 8, $P = 0.047$, and $P = 0.011$) at potentials of –50 and –45 mV (*C*), whereas I_{NaP} was inhibited at a potential of –60 mV (*D*, n = 9, $P = 0.044$). Data are mean ± SD. *$P < 0.05$ by two-way ANOVA. CTL, control; ISO, isoflurane.

toxin that promotes the voltage-gated sodium channels open state, increases MAC and antagonizes the effect of intrathecal tetrodotoxin.[38]

Volatile anesthetics including isoflurane and sevoflurane inhibit heterologously expressed recombinant brain voltage-gated sodium channels at clinical concentrations by selectively interacting with the inactivated state of the channel.[31] Our findings support and extend these findings by demonstrating effects of isoflurane on sodium channel current and action potentials in native hippocampal neurons *in situ*. Voltage- and activity-dependent inhibition of voltage-gated sodium channels by volatile anesthetics can be explained by preferential interaction with the inactivated state to impede transition from the inactivated state back to the resting state.[24,31] The median inhibitory concentration of isoflurane for inhibition of transient sodium channel currents from a holding potential of −70 mV is relevant to the clinically used concentrations, whereas isoflurane did not inhibit transient sodium channel currents at clinically relevant concentrations from a holding potential of −120 mV. Even perfusion with saturated isoflurane stock solution (12 to 14 mM, ~48 MAC) only depressed transient sodium channel current by ~60% from a holding potential of −120 mV. The partial inactivation state sodium channels existing at a holding potential of −70 mV can explain this result. Volatile anesthetics including isoflurane also display activity-dependent inhibition of neuronal sodium channels in isolated rat neurophysical nerve terminals,[9] neuroblastoma cells,[24] and dorsal root ganglion neurons,[10] and in the bacterial voltage-gated sodium channels homologue voltage-gated sodium channel of Bacillus halodurans,[39,40] but there was no pervious evidence that volatile anesthetics directly depress activity of excitatory neurons by inhibiting neuronal sodium channels.

The major anion in our pipette solution was fluorion, which has been reported to affect inactivation of voltage-gated sodium channels in squid giant axons.[41] We used fluorion instead of chlorine in the pipette solution for voltage-gated sodium channels recordings[7,42] to avoid changes in intracellular chlorine that might affect neuronal excitability, signaling, and plasticity of hippocampal pyramidal neurons.[43,44] Since isoflurane may also potentiate chlorine permeable γ-aminobutyric acid type A receptors, the level of intracellular chlorine has important consequences for its actions in a manner similar to that recently shown for propofol.[45,46]

We provide evidence that isoflurane at clinically relevant concentrations inhibits transient sodium channel currents, persistent sodium currents, and resurgent sodium currents at physiologic holding potentials in mouse hippocampal cornu ammonis pyramidal neurons. For transient sodium

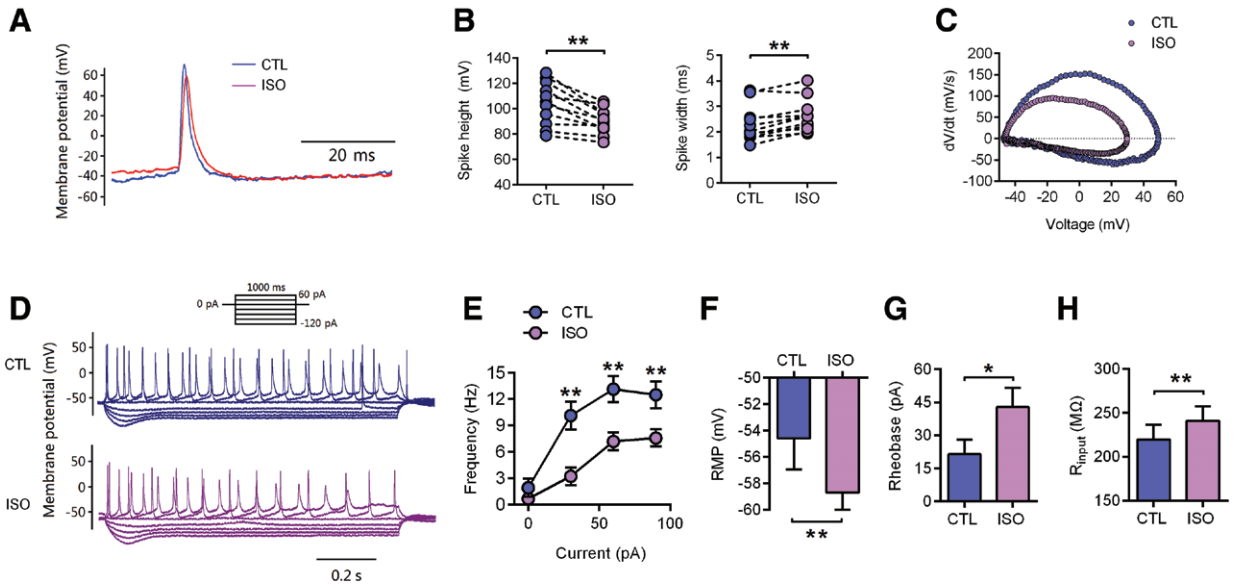

Fig. 5. Effects of isoflurane on hippocampal action potentials. (*A*) The effects of isoflurane at a clinically relevant concentration (0.48 mM, ~1.8 minimum alveolar concentration [MAC]) on single action potentials. (*B*) Action potential amplitude was reduced and width increased by isoflurane (n = 13, $P = 0.001$), as illustrated by the action potential dynamics of voltage-time derivative (*C*). (*D* and *E*) Isoflurane significantly reduced action potential frequency (n = 20). (*F*) Isoflurane significantly hyperpolarized pyramidal neuron resting membrane potential (n = 16, $P = 0.001$). (*G*) Isoflurane significantly increased the rheobase that evoked action potentials (n = 14, $P = 0.019$). (*H*) Isoflurane significantly increased the input resistant of neurons (n = 12, $P < 0.0001$). Data are mean ± SD. *$P < 0.05$, **$P < 0.01$ by two-tailed paired *t* test (*b*, *g*, *h*) or two-way ANOVA (*E*). CTL, control; ISO, isoflurane.

channel current, isoflurane inhibits peak sodium current and modulates channel gating by shifting the voltage-dependence of voltage-gated sodium channels inactivation, and by delaying recovery from inactivation. Isoflurane also inhibits persistent sodium currents and resurgent sodium currents at clinically relevant concentrations.[6,47] From a holding potential of −70 mV, the median inhibitory concentration of isoflurane for persistent sodium current inhibition was lower than for transient sodium channel current (0.4 ± 0.1 *vs.* 1.0 ± 0.3 mM), indicating that isoflurane selectively inhibits the persistent currents of sodium channels. This inhibition by isoflurane of persistent sodium current at clinically relevant concentrations might reduce neuronal excitability such as lower action potentials firing frequency and increased rheobase.

At least three activation states have been identified for neuronal sodium channels depending on membrane potential: resting (closed), activated (open), and inactivated.[16] transient sodium channel currents are evoked by step depolarization and rapidly activate, whereas the subsequent residual sodium currents that last throughout the step are referred to as persistent sodium currents.[17] Upon repolarization, closed voltage-gated sodium channels reopen to produce resurgent currents, known as resurgent sodium currents.[16] Although these three components of sodium currents all flow through the same channels, they have different kinetics and physiologic roles. Transient sodium channel currents are pivotal for initiation and propagation of action potentials,[32] whereas persistent sodium currents and resurgent sodium currents amplify responses of neurons to synaptic input and enhance repetitive firing.[18] The rapid kinetics and all-or-none properties of transient sodium channel currents mediate the upstroke of fast neuronal action potentials.[13] Persistent and resurgent sodium currents are activated by small synaptic depolarization and are slower than transient sodium channel currents. Despite their small amplitudes compared with transient sodium channel currents, persistent and resurgent sodium currents can profoundly alter neuronal firing behavior, especially at subthreshold voltages.[16,17] In dendrites, persistent sodium currents can boost distal synaptic potentials to propagate to the neuronal soma.[15] In proximal axons and peripheral axons, persistent sodium currents can affect initiation of action potentials.[48,49] Both persistent and resurgent sodium currents also play critical roles in the regulation of neuronal firing behavior because of their high densities near the axon initial segment.[50] Resurgent sodium current is enhanced by specific pathogenic mutations in sodium channels, thereby increasing action potential firing rate and duration, causing neuronal hyperexcitability.[51] Inhibition by isoflurane of these neuronal sodium channel currents provides neurophysiologic mechanisms to support previous reports that

isoflurane reduces excitability of cornu ammonis pyramidal neurons by depressing action potentials.

Neuronal information coding occurs by varying frequencies and patterns of action potentials between somata and axons, where they can rapidly propagate information over distance. action potentials are fundamentally important for synaptic transmission and neuroplasticity.[52,53] Neurotransmitter release is largely determined by the shape, frequency, and pattern of presynaptic action potentials during the process of information transfer.[14] Release of many neurotransmitters is inhibited by volatile anesthetics in a sodium channel–dependent manner.[54,55] Thus voltage-gated sodium channels is a plausible presynaptic molecular target for volatile anesthetics.

Isoflurane decreased excitability of mouse cornu ammonis pyramidal neurons by shaping the action potentials and hyperpolarizing the resting membrane potential. The generation of action potentials depends on multiple ion channels including voltage-gated sodium channels for rapid depolarization, voltage-gated calcium channels for slow depolarization, and potassium channels for repolarization.[56] Analysis of action potential kinetics (voltage-time derivative) showed that isoflurane slowed action potential kinetics throughout, consistent with net inhibition. Isoflurane decreased action potential amplitude mainly from inhibition of transient voltage-gated sodium channel currents, as seen in rat neurohypophysial terminals.[9] Prolonged action potential duration impairs neuronal responses to high frequency stimuli, enhancing the depression of synaptic transmission. The increased action potential half-width by isoflurane is likely attributable to inhibition of potassium channels.[57] Isoflurane at clinical concentrations can enhance and stabilize the inactivation state of voltage-gated sodium channels, resulting in activity-dependent reduction of sodium channel current with high-frequency stimuli, which could partly contribute to slow the rapid depolarization of action potential by isoflurane. Inhibition by isoflurane of persistent sodium channel current may also contribute to its effect on action potential amplitude and duration. Isoflurane slightly hyperpolarized resting membrane potential possibly as a result of enhancement of volatile anesthetic-sensitive background K^+ channels.[58] The effects of isoflurane on resting membrane potential have been reported to be varied[47,59] and voltage-dependent;[60] expression of different K^+ channel subtypes may contribute to these differential effects.[58]

In conclusion, isoflurane can inhibit three components of sodium channel current, which contribute to its depression of neuronal excitability and action potentials in hippocampal cornu ammonis pyramidal neurons. Voltage-dependent and activity-dependent inhibition by isoflurane, and possibly other volatile anesthetics, depresses neuronal excitability and may contribute to anesthetic effects on neurotransmitter release and synaptic plasticity.

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Competing Interests

The authors declare no competing interests.

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References

1. Hemmings HC Jr, Akabas MH, Goldstein PA, Trudell JR, Orser BA, Harrison NL: Emerging molecular mechanisms of general anesthetic action. Trends Pharmacol Sci 2005; 26:503–10
2. Franks NP: General anaesthesia: From molecular targets to neuronal pathways of sleep and arousal. Nat Rev Neurosci 2008; 9:370–86
3. Doi M, Ikeda K: Respiratory effects of sevoflurane. Anesth Analg 1987; 66:241–4
4. Karanovic N, Pecotic R, Valic M, Jeroncic A, Carev M, Karanovic S, Ujevic A, Dogas Z: The acute hypoxic ventilatory response under halothane, isoflurane, and sevoflurane anaesthesia in rats. Anaesthesia 2010; 65:227–34
5. Bi SS, Deng CH, Zhou TY, Guan Z, Li L, Li HQ, Zhang LP, Yang L, Lu W: Remifentanil-sevoflurane interaction models of circulatory response to laryngoscopy and circulatory depression. Br J Anaesth 2013; 110:729–40
6. Westphalen RI, Hemmings HC Jr: Volatile anesthetic effects on glutamate *versus* GABA release from isolated rat cortical nerve terminals: Basal release. J Pharmacol Exp Ther 2006; 316:208–15
7. Herold KF, Nau C, Ouyang W, Hemmings HC Jr: Isoflurane inhibits the tetrodotoxin-resistant voltage-gated sodium channel Nav1.8. ANESTHESIOLOGY 2009; 111:591–9
8. Yamada-Hanff J, Bean BP: Activation of Ih and TTX-sensitive sodium current at subthreshold voltages during CA1 pyramidal neuron firing. J Neurophysiol 2015; 114:2376–89

9. Ouyang W, Wang G, Hemmings HC Jr: Isoflurane and propofol inhibit voltage-gated sodium channels in isolated rat neurohypophysial nerve terminals. Mol Pharmacol 2003; 64:373–81
10. Scholz A, Appel N, Vogel W: Two types of TTX-resistant and one TTX-sensitive Na+ channel in rat dorsal root ganglion neurons and their blockade by halothane. Eur J Neurosci 1998; 10:2547–56
11. Pal D, Jones JM, Wisidagamage S, Meisler MH, Mashour GA: Reduced Nav1.6 sodium channel activity in mice increases *in vivo* sensitivity to volatile anesthetics. PLoS One 2015; 10:e0134960
12. Baumgart JP, Zhou ZY, Hara M, Cook DC, Hoppa MB, Ryan TA, Hemmings HC Jr: Isoflurane inhibits synaptic vesicle exocytosis through reduced Ca2+ influx, not Ca2+-exocytosis coupling. Proc Natl Acad Sci U S A 2015; 112:11959–64
13. Bean BP: The action potential in mammalian central neurons. Nat Rev Neurosci 2007; 8:451–65
14. Deng PY, Rotman Z, Blundon JA, Cho Y, Cui J, Cavalli V, Zakharenko SS, Klyachko VA: FMRP regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels. Neuron 2013; 77:696–711
15. Crill WE: Persistent sodium current in mammalian central neurons. Annu Rev Physiol 1996; 58:349–62
16. Lewis AH, Raman IM: Resurgent current of voltage-gated Na(+) channels. J Physiol 2014; 592:4825–38
17. Stafstrom CE: Persistent sodium current and its role in epilepsy. Epilepsy Curr 2007; 7:15–22
18. Sittl R, Lampert A, Huth T, Schuy ET, Link AS, Fleckenstein J, Alzheimer C, Grafe P, Carr RW: Anticancer drug oxaliplatin induces acute cooling-aggravated neuropathy via sodium channel subtype Na(V)1.6-resurgent and persistent current. Proc Natl Acad Sci U S A 2012; 109:6704–9
19. Moser MB, Moser EI: Functional differentiation in the hippocampus. Hippocampus 1998; 8:608–19
20. Zhong T, Qing QJ, Yang Y, Zou WY, Ye Z, Yan JQ, Guo QL: Repression of contexual fear memory induced by isoflurane is accompanied by reduction in histone acetylation and rescued by sodium butyrate. Br J Anaesth 2014; 113:634–43
21. Lee BH, Hazarika OD, Quitoriano GR, Lin N, Leong J, Brosnan H, Chan JT, May LD, Yu D, Alkhamisi A, Stratmann G, Sall JW: Effect of combining anesthetics in neonates on long-term cognitive function. Int J Dev Neurosci 2014; 37:87–93
22. Lee BH, Chan JT, Hazarika O, Vutskits L, Sall JW: Early exposure to volatile anesthetics impairs long-term associative learning and recognition memory. PLoS One 2014; 9:e105340
23. Hotson JR, Prince DA, Schwartzkroin PA: Anomalous inward rectification in hippocampal neurons. J Neurophysiol 1979; 42:889–95
24. Purtell K, Gingrich KJ, Ouyang W, Herold KF, Hemmings HC Jr: Activity-dependent depression of neuronal sodium channels by the general anaesthetic isoflurane. Br J Anaesth 2015; 115:112–21
25. Ouyang W, Herold KF, Hemmings HC Jr: Comparative effects of halogenated inhaled anesthetics on voltage-gated Na+ channel function. Anesthesiology 2009; 110:582–90
26. Taheri S, Halsey MJ, Liu J, Eger EI 2nd, Koblin DD, Laster MJ: What solvent best represents the site of action of inhaled anesthetics in humans, rats, and dogs? Anesth Analg 1991; 72:627–34
27. Franks NP, Lieb WR: Selective actions of volatile general anaesthetics at molecular and cellular levels. Br J Anaesth 1993; 71:65–76
28. Lunko O, Isaev D, Maximyuk O, Ivanchick G, Sydorenko V, Krishtal O, Isaeva E: Persistent sodium current properties in hippocampal CA1 pyramidal neurons of young and adult rats. Neurosci Lett 2014; 559:30–3
29. Carter BC, Giessel AJ, Sabatini BL, Bean BP: Transient sodium current at subthreshold voltages: activation by EPSP waveforms. Neuron 2012; 75:1081–93
30. Ratnakumari L, Hemmings HC Jr: Inhibition of presynaptic sodium channels by halothane. Anesthesiology 1998; 88:1043–54
31. Rehberg B, Xiao YH, Duch DS: Central nervous system sodium channels are significantly suppressed at clinical concentrations of volatile anesthetics. Anesthesiology 1996; 84:1223–33; discussion 27A
32. Hodgkin AL, Huxley AF: A quantitative description of membrane current and its application to conduction and excitation in nerve. 1952. Bull Math Biol 1990; 52:25–71; discussion 5–23
33. Franks NP, Lieb WR: Molecular and cellular mechanisms of general anaesthesia. Nature 1994; 367:607–14
34. Herold KF, Hemmings HC Jr: Sodium channels as targets for volatile anesthetics. Front Pharmacol 2012; 3:50
35. Acevedo-Arcique CM, Ibancovichi JA, Chavez JR, Gutierrez-Blanco E, Moran-Muñoz R, Victoria-Mora JM, Tendillo-Cortijo F, Santos-González M, Sanchez-Aparicio P: Lidocaine, dexmedetomidine and their combination reduce isoflurane minimum alveolar concentration in dogs. PLoS One 2014; 9:e106620
36. Moran-Muñoz R, Ibancovichi JA, Gutierrez-Blanco E, Acevedo-Arcique CM, Victoria Mora JM, Tendillo FJ, Santos-Gonzalez M, Yamashita K: Effects of lidocaine, dexmedetomidine or their combination on the minimum alveolar concentration of sevoflurane in dogs. J Vet Med Sci 2014; 76:847–53
37. Hamp T, Krammel M, Weber U, Schmid R, Graf A, Plöchl W: The effect of a bolus dose of intravenous lidocaine on the minimum alveolar concentration of sevoflurane: A prospective, randomized, double-blinded, placebo-controlled trial. Anesth Analg 2013; 117:323–8

38. Zhang Y, Sharma M, Eger EI 2nd, Laster MJ, Hemmings HC Jr, Harris RA: Intrathecal veratridine administration increases minimum alveolar concentration in rats. Anesth Analg 2008; 107:875–8
39. Ouyang W, Jih TY, Zhang TT, Correa AM, Hemmings HC Jr: Isoflurane inhibits NaChBac, a prokaryotic voltage-gated sodium channel. J Pharmacol Exp Ther 2007; 322:1076–83
40. Sand RM, Gingrich KJ, Macharadze T, Herold KF, Hemmings HC Jr: Isoflurane modulates activation and inactivation gating of the prokaryotic Na+ channel NaChBac. J Gen Physiol 2017; 149:623–38
41. Chandler WK, Hodgkin AL, Meves H: The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. J Physiol 1965; 180:821–36
42. Castelli L, Biella G, Toselli M, Magistretti J: Resurgent Na+ current in pyramidal neurones of rat perirhinal cortex: Axonal location of channels and contribution to depolarizing drive during repetitive firing. J Physiol 2007; 582(Pt 3):1179–93
43. Raimondo JV, Richards BA, Woodin MA: Neuronal chloride and excitability: The big impact of small changes. Curr Opin Neurobiol 2017; 43:35–42
44. Kaila K, Price TJ, Payne JA, Puskarjov M, Voipio J: Cation-chloride cotransporters in neuronal development, plasticity and disease. Nat Rev Neurosci 2014; 15:637–54
45. Garcia PS, Kolesky SE, Jenkins A: General anesthetic actions on GABA(A) receptors. Curr Neuropharmacol 2010; 8:2–9
46. Puskarjov M, Fiumelli H, Briner A, Bodogan T, Demeter K, Lacoh CM, Mavrovic M, Blaesse P, Kaila K, Vutskits L: K-Cl cotransporter 2-mediated Cl- extrusion determines developmental stage-dependent impact of propofol anesthesia on dendritic spines. Anesthesiology 2017; 126:855–67
47. Wu XS, Sun JY, Evers AS, Crowder M, Wu LG: Isoflurane inhibits transmitter release and the presynaptic action potential. Anesthesiology 2004; 100:663–70
48. Astman N, Gutnick MJ, Fleidervish IA: Persistent sodium current in layer 5 neocortical neurons is primarily generated in the proximal axon. J Neurosci 2006; 26:3465–73
49. Tokuno HA, Kocsis JD, Waxman SG: Noninactivating, tetrodotoxin-sensitive Na+ conductance in peripheral axons. Muscle Nerve 2003; 28:212–7
50. Yue C, Remy S, Su H, Beck H, Yaari Y: Proximal persistent Na+ channels drive spike afterdepolarizations and associated bursting in adult CA1 pyramidal cells. J Neurosci 2005; 25:9704–20
51. Jarecki BW, Piekarz AD, Jackson JO 2nd, Cummins TR: Human voltage-gated sodium channel mutations that cause inherited neuronal and muscle channelopathies increase resurgent sodium currents. J Clin Invest 2010; 120:369–78
52. Hoppa MB, Gouzer G, Armbruster M, Ryan TA: Control and plasticity of the presynaptic action potential waveform at small CNS nerve terminals. Neuron 2014; 84:778–89
53. Poage RE, Zengel JE: Repolarization of the presynaptic action potential and short-term synaptic plasticity in the chick ciliary ganglion. Synapse 2002; 46:189–98
54. Westphalen RI, Desai KM, Hemmings HC Jr: Presynaptic inhibition of the release of multiple major central nervous system neurotransmitter types by the inhaled anaesthetic isoflurane. Br J Anaesth 2013; 110:592–9
55. Westphalen RI, Hemmings HC Jr: Selective depression by general anesthetics of glutamate *versus* GABA release from isolated cortical nerve terminals. J Pharmacol Exp Ther 2003; 304:1188–96
56. Storm JF: Potassium currents in hippocampal pyramidal cells. Prog Brain Res 1990; 83:161–87
57. Suzuki A, Aizawa K, Gassmayr S, Bosnjak ZJ, Kwok WM: Biphasic effects of isoflurane on the cardiac action potential: An ionic basis for anesthetic-induced changes in cardiac electrophysiology. Anesthesiology 2002; 97:1209–17
58. Patel AJ, Honoré E, Lesage F, Fink M, Romey G, Lazdunski M: Inhalational anesthetics activate two-pore-domain background K+ channels. Nat Neurosci 1999; 2:422–6
59. Wilde DW: Isoflurane reduces K+ current in single smooth muscle cells of guinea pig portal vein. Anesth Analg 1996; 83:1307–13
60. MacIver MB, Kendig JJ: Anesthetic effects on resting membrane potential are voltage-dependent and agent-specific. Anesthesiology 1991; 74:83–8