BJA

British Journal of Anaesthesia 115 (1): 112–21 (2015)

doi: 10.1093/bja/aev203
Translational Research

TRANSLATIONAL RESEARCH

Activity-dependent depression of neuronal sodium channels by the general anaesthetic isoflurane

K. Purtell[1], K. J. Gingrich[3], W. Ouyang[2,4], K. F. Herold[2,*] and H. C. Hemmings Jr[1,2,*]

[1]Department of Pharmacology, [2]Department of Anesthesiology, Weill Cornell Medical College, New York, NY 10065, USA, [3]Department of Anesthesiology, University of Texas Southwestern Medical Center, Dallas, TX 75235, USA, and [4]Present address: College of Physical Education and Health Sciences, Zhejiang Normal University, Jinhua, Zhejiang, China

*Corresponding authors. E-mail: kah2016@med.cornell.edu; hchemmi@med.cornell.edu

Abstract

Background: The mechanisms by which volatile anaesthetics such as isoflurane alter neuronal function are poorly understood, in particular their presynaptic mechanisms. Presynaptic voltage-gated sodium channels (Na_v) have been implicated as a target for anaesthetic inhibition of neurotransmitter release. We hypothesize that state-dependent interactions of isoflurane with Na_v lead to increased inhibition of Na^+ current (I_{Na}) during periods of high-frequency neuronal activity.
Methods: The electrophysiological effects of isoflurane, at concentrations equivalent to those used clinically, were measured on recombinant brain-type $Na_v1.2$ expressed in ND7/23 neuroblastoma cells and on endogenous Na_v in isolated rat neurohypophysial nerve terminals. Rate constants determined from experiments on the recombinant channel were used in a simple model of Na_v gating.
Results: At resting membrane potentials, isoflurane depressed peak I_{Na} and shifted steady-state inactivation in a hyperpolarizing direction. After membrane depolarization, isoflurane accelerated entry ($\tau_{control}$=0.36 [0.03] ms compared with $\tau_{isoflurane}$=0.33 [0.05] ms, $P<0.05$) and slowed recovery ($\tau_{control}$=6.9 [1.1] ms compared with $\tau_{isoflurane}$=9.0 [1.9] ms, $P<0.005$) from apparent fast inactivation, resulting in enhanced depression of I_{Na}, during high-frequency stimulation of both recombinant and endogenous nerve terminal Na_v. A simple model of Na_v gating involving stabilisation of fast inactivation, accounts for this novel form of activity-dependent block.
Conclusions: Isoflurane stabilises the fast-inactivated state of neuronal Na_v leading to greater depression of I_{Na} during high-frequency stimulation, consistent with enhanced inhibition of fast firing neurones.

Key words: anaesthetics, general; isoflurane; presynaptic terminals; voltage-gated sodium channels

General anaesthesia is a composite pharmacological state of amnesia, unconsciousness, and immobility. The molecular pharmacology of this state involves multiple target proteins, prominently including ligand-gated and voltage-gated ion channels.[1–3] Optimization of anaesthetic drug design and clinical use requires detailed understanding of the roles of specific targets involved in the therapeutic (amnesia, unconsciousness, immobility) and undesirable (cardiovascular and respiratory depression, neurotoxic) effects of various anaesthetics.

A role for voltage-gated Na^+ channels (Na_v) in the modulation of neurotransmission by general anaesthetics, is supported by evidence that presynaptic blockade of Na^+ current (I_{Na})

Accepted: April 13, 2015

Editor's key points

- The mechanisms of the effects of isoflurane on neuronal function are unclear.
- Isoflurane may inhibit neurotransmitter release through effects on presynaptic voltage gated sodium channels (Na_v).
- The effects of isoflurane were measured in Na_v in neuroblastoma cells and in isolated rat nerve terminals.
- Isoflurane stabilized the fast inactivated state of neuronal Na_v.
- This was consistent with increased inhibition of fast firing neurones.

contributes to suppression of the release of multiple neurotransmitters, by volatile anaesthetics (VAs).[4–6] The widely used anaesthetic isoflurane blocks multiple subtypes of Na_v,[7–11] and reduces action potential amplitude in rat hippocampal neurones[12] and isolated rat neurohypophysial terminals.[13] Moreover, intrathecal delivery of the highly specific Na_v inhibitor tetrodotoxin (TTX) in adult rats enhances isoflurane potency in producing immobilization, a primarily spinal cord-mediated effect, whereas the Na_v activator veratridine reduces isoflurane potency and antagonizes the effect of TTX.[14]

Inhibition of Na_v by diverse compounds, including local anaesthetics, anti-arrhythmic drugs, antiepileptic drugs, and neurotoxins, is state-dependent[15–17]: their ability to interact with or bind to an ion channel is determined by the conformational state of the channel (resting - open - inactivated). The state of the channel is in part governed by membrane potential. State-dependent inhibition of Na_v by isoflurane is supported by the observations that isoflurane inhibition is voltage-dependent, and is characterised by enhanced inactivation and delayed recovery from inactivation, which is consistent with stabilisation of fast inactivation.[8 10 18] If isoflurane stabilises the fast-inactivated state, block of Na_v should increase with repeated stimulation at frequencies high enough for fast-inactivated channels to accumulate, contributing to overall block of Na_v. Block of Na_v at clinical concentrations of general anaesthetics was previously considered too modest to be physiologically relevant.[3] However, this conclusion was based on studies of tonic block which would be insensitive to possible 'activity-dependent block' and would therefore underestimate the magnitude of isoflurane effects on Na_v at more physiologically relevant fast firing frequencies.

We examined activity-dependent block of isoflurane on $Na_v1.2$, the principal neuronal Na_v subtype, and endogenous rat neuronal Na_v, using high-frequency stimulation protocols to elucidate the underlying kinetic mechanism. We show that isoflurane stabilises the fast-inactivated state of Na_v, resulting in a novel form of activity-dependent anaesthetic block. This effect contributes significantly to overall block of I_{Na} and supports a role for Na_v inhibition in presynaptic anaesthetic action, through reduction of presynaptic action potential amplitude and consequent neurotransmitter release.[19]

Methods

Anaesthetic solutions

External bath solutions were saturated with isoflurane (12–12.5 mM; Abbott Laboratories, North Chicago, IL USA) and diluted to final concentrations of 0.45–0.5 mM in gas-tight glass syringes. Isoflurane solutions were perfused using a pressure driven microperfusion system (ALA BPS-8; ALA Scientific, Westbury, NY USA), positioned 100–150 μm away from the cell. Concentrations of isoflurane sampled at the perfusion pipette tip were measured using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu, Tokyo, Japan), after extraction into octane (1:1 v/v), and reflected ~10% loss from the syringe to the bath.[11]

Cell culture and Na_v transfection of ND7/23 cells

Neuroblastoma ND7/23 cells (Sigma-Aldrich, St. Louis, MO USA) were plated on 12-mm glass coverslips and incubated in a humidified atmosphere at 37°C in 5% CO_2, in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (all reagents from Sigma-Aldrich unless specified).

Wild-type rat $Na_v1.2a$ (accession number NM_012647) was kindly provided by William Catterall (University of Washington, Seattle, USA). TTX-resistance was engineered into $Na_v1.2a$ by site-directed mutagenesis (F385S)[20] (referred to as $Na_v1.2_R$) to allow isolation from endogenous channels and expression in a neuronal background, which is crucial to measuring the effect of isoflurane on Na_v in heterologous expression systems.[11] Cells were transiently transfected with $Na_v1.2_R$ and pEGFP-N1 (Clontech, Mountain View, CA USA) cDNA using Lipofectamine LTX (Invitrogen, Carlsbad, CA USA) to allow identification of transfected cells by eGFP fluoresence imaging. Experiments were performed in the presence of 250 nM TTX (Sankyo Kasei, Tokyo, Japan) to block endogenous I_{Na}.

Electrophysiological recording of ND7/23 cells

Whole-cell patch-clamp experiments were performed at room temperature (23–24°C) using an Axopatch 200B amplifier (Axon Instruments, Burlingame, CA USA), digitized via a Digidata 1321A interface, and analysed using pClamp 10.2 software (Axon Instruments). Whole-cell currents were sampled at 50 kHz and low-pass filtered at 5 kHz. Whole-cell seal resistance was 2–8 GΩ before patch rupture. Pipette resistance was 1.5–2.5 MΩ when filled with internal solution containing (in mM): 120 CsF, 10 NaCl, 10 HEPES, 10 EGTA, 10 TEA-Cl, 1 $CaCl_2$, and 1 $MgCl_2$ and adjusted to pH 7.3 (with CsOH) and 310 mOsm kg^{-1} H_2O. External solution contained (in mM): 130 NaCl, 10 HEPES, 3.25 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 20 TEA-Cl, 5 D-glucose, 0.00025 TTX and was adjusted to pH 7.4 with NaOH and 310 mOsm kg^{-1} H_2O with sucrose. The liquid–junction potential (~7.8 mV) was not corrected.

Only cells expressing 2–8 nA of peak current were analysed in order to minimize space clamp and series resistance errors. Capacitive transients were electronically cancelled and voltage error was minimized using 70–80% series resistance compensation. Series resistance was typically 2–4 MΩ and data were discarded if >10 MΩ. Experiments began 5 min after attaining whole-cell patch to allow equilibration of the pipette solution with the cytosol. Voltage protocols were applied from a holding potential (V_h) of −70 or −90 mV with 5-s intervals between sweeps. Protocols were applied in control solution and again after 5 min perfusion with isoflurane. Perfused cells showed stable responses (rundown <10%) for up to 5 min in control experiments (data not shown). Linear leak currents were subtracted using the P/4 method.[21]

Electrophysiological recording of isolated nerve terminals

Animal protocols were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College and relevant aspects of the ARRIVE guidelines and in the AVMA Guidelines for Euthanasia of Animals (https://www.avma.org/KB/Policies/

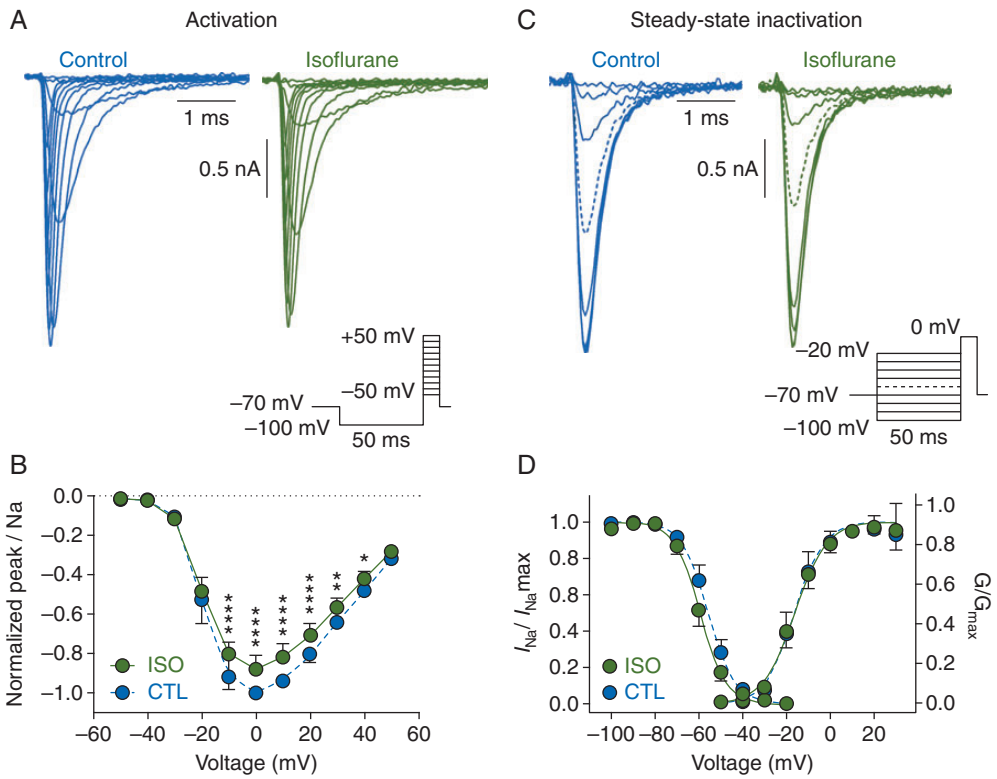

Fig 1 Effects of isoflurane on activation and inactivation properties of brain-type sodium channel $Na_v1.2_R$ in a neuronal cell line. (A) Representative whole-cell $Na_v1.2_R$ current traces in the absence (*left*) or presence (*right*) of 1.5 MAC isoflurane using the protocol in *inset*. (B) Normalized peak current (I_{Na}) plotted against test potential in the absence (CTL) or presence (ISO) of isoflurane [mean (SD), n=7, *P<0.05, **P<0.01, ****P<0.0001 compared with respective control value by two-way ANOVA]. (C) Representative current traces in the absence (*left*) or presence (*right*) of isoflurane using the protocol in *inset* to measure steady-state fast inactivation (prepulse to –60 mV indicated with a dashed line). (D) Steady-state fast inactivation (n=6), where I_{Na} at 0 mV was normalized to the maximum I_{Na} for each condition (I_{Na}/I_{Na}max) and normalized conductance (G/G_{max}) values [mean (SD), n=7] were plotted against the voltage command and fit with a Boltzmann function.

Documents/euthanasia.pdf). Neurohypophysial nerve terminals were prepared as described[9] with minor modifications. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA USA) were anaesthetized by slowly replacing the air in their cage with regulated inflow from a tank of 80 CO_2/20% O_2 to avoid hypoxaemia and exposure to potent anaesthetics. Upon loss of righting reflex, animals were swiftly killed by decapitation. Animals showed no signs of distress with this technique. The neurohypophysis was removed and gently homogenized in 270 mM sucrose, 10 mM HEPES, and 0.01 mM K-EGTA, pH 7.25, using a 0.5-ml Teflon/glass homogenizer. The homogenate was pipetted into a plastic 35-mm Petri dish and allowed to settle for 5–8 min.

Dissociated nerve terminals were superfused with modified Locke's solution consisting of (in mM) 145 NaCl, 5 KCl, 2.2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 2 D-glucose, pH 7.3 with NaOH. Large terminals (11–16 μm diameter), identified by their bright refraction, were selected for study. An amphotericin B-perforated patch-clamp technique was used to reduce rundown of I_{Na}, that occurs with whole-terminal patch-clamp. Pipette tips were fire-polished and coated with SYLGARD (Dow Corning Corporation, Midland, MI USA) to reduce background noise and pipette capacitance. Pipette resistance was 3–7 MΩ, and seal resistance was 1–5 GΩ. Pipettes were filled with a solution containing (in mM) 10 NaCl, 135 Cs-glutamate, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5 D-glucose, 10 TEACl and 300–350 μg ml^{-1} amphotericin B, pH 7.25 with CsOH.

Gating model simulation and parameter estimation

A three-state Markov gating model was designed in MATLAB v7.5 (The MathWorks, Natick, MA USA) by solving the matrix equation $X(t)=e^{Q(t)} \cdot X(0)$, where $X(t)$ is a 3×1—state variable vector indicating the probability of resting (R), open (O), and the fast-inactivated (I_F) states at time t, $X(0)$ is the initial state vector at time 0, and $Q(t)$ =the 3×3—state transition matrix of rate constants governing the transition rates between all connected states, given the R-O-I_F gating scheme. Simulation paradigms involved two periods, or a repetitive sequence of these periods when addressing pulse trains, where membrane voltage was initially V_h followed by a depolarized pulse triggering channel activation. Parameter estimation used a least-squares method (MATLAB Optimization Toolbox 4.1; The MathWorks).

Statistical analysis

Data were analysed using Prism v6.05 (Graph-Pad Software Inc., San Diego, CA USA) and SigmaPlot 6.0 (SPSS Science Software Inc., Chicago, IL USA). Conductance (G) values were derived

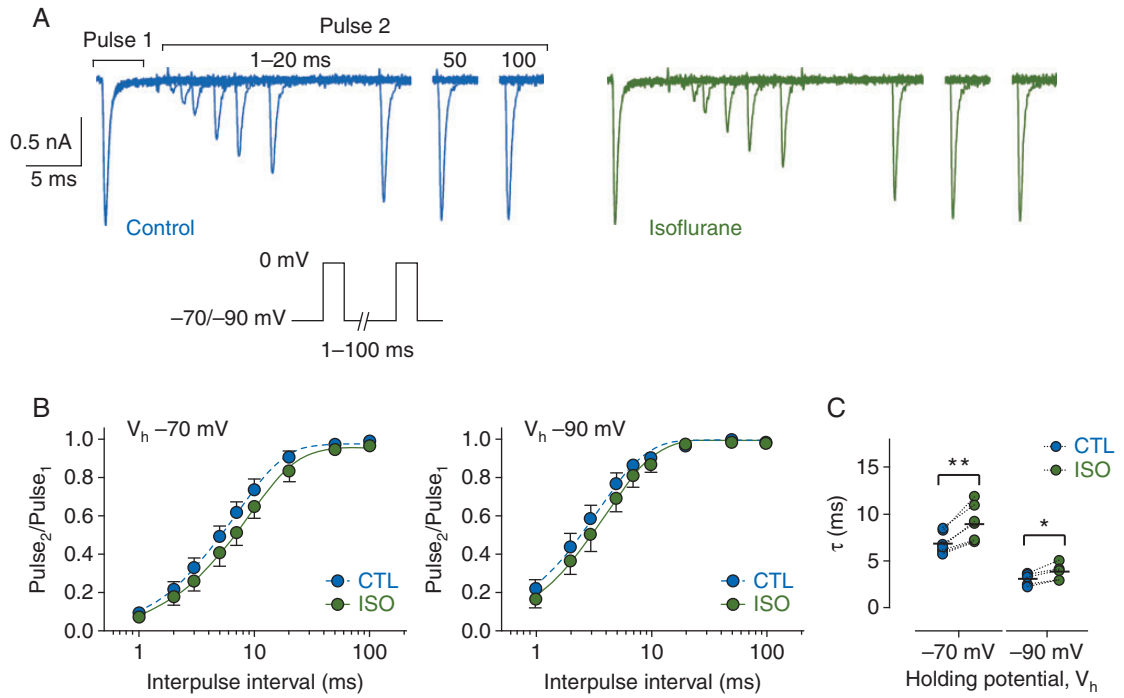

Fig 2 Effects of isoflurane on recovery of brain-type sodium channel $Na_v1.2_R$ from fast inactivation. (A) Families of whole-cell $Na_v1.2_R$ current traces recorded in the absence (*left*) or presence (*right*) of 1.5 MAC isoflurane and evoked from a V_h of –70 mV using a paired-pulse protocol where the duration between two 5-ms pulses to 0 mV was varied from 1–100 ms (protocol in *inset*). (B) Normalized peak current ($Pulse_2/Pulse_1$) was plotted against duration of the interpulse interval for control (CTL) and isoflurane (ISO) from a V_h of –70 mV (n=7) or –90 mV (n=6). Data are presented as mean (SD) and fit with a mono-exponential function. (C) Time constants (τ) determined from mono-exponential fits of data from individual cells in B in the absence (CTL) or presence (ISO) of isoflurane from a V_h of –70 (*left*) or –90 mV (*right*) (*P<0.05, **P<0.005; by two-tailed, paired Student's t-test).

from the I–V relationship using the equation $G=I/(V-V_{rev})$, where I is the peak I_{Na} at a given voltage (V) and V_{rev} is the measured Na^+ reversal potential. Voltages at half-maximal activation ($V_{½act}$) were obtained from fitting the data for each cell to a Boltzmann equation of the form $G/G_{max}=1/[1+\exp(V_{½act}-V/k)]$, where G/G_{max} is the normalized fractional conductance and k is the slope factor. The voltage at which fast inactivation is half-maximal ($V_{½}$) was measured by fitting normalized steady-state I_{Na} values to a Boltzman function, of the form $I_{Na}/I_{Na}max=1/[1+\exp(V_{½}-V/k)]$. Time course data were fitted to the mono-exponential function $Y=\exp(-\tau^*n)+A_P$, where τ is the time constant, A_P is the plateau and n is stimulus number. To determine the kinetics of macroscopic inactivation the decay phase of the current trace was fit with a bi-exponential equation, of the form $A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2)+B$, where A_n is the nth component amplitude, B is the plateau, t is time and τ_n are time constants.

Data are expressed as mean and standard deviation (SD), and were analysed using two-tailed paired Student's t-test or ANOVA with post hoc testing as indicated, with statistical significance set as P=0.05.

Results

We first measured inhibition of peak Na^+ current (I_{Na}) by a clinically relevant concentration of isoflurane. Fig. 1A shows whole-cell $Na_v1.2_R$ currents recorded from ND7/23 cells in the absence or presence of isoflurane at a concentration that produces anaesthesia in rats [0.42 mM; equivalent to ~1.5 times MAC (minimum alveolar concentration of anaesthetic required to abolish movement upon a painful stimulus in 50% of subjects)].[22] Current was activated by a series of voltage steps from –50 to +50 mV preceded by a 50-ms prepulse to –100 mV to relieve channel inactivation. Isoflurane inhibited peak I_{Na} at test potentials of –10–+40 mV [n=7, *P<0.05, two-way (voltage x drug) ANOVA with Sidak's multiple comparisons test], without altering the current-voltage (I–V) relationship; maximum I_{Na} for both control and isoflurane conditions occurred at 0 mV (Fig. 1B).

The effect of isoflurane on steady-state inactivation was determined by eliciting currents at 0 mV after a 50-ms prepulse to voltages from –100 to –20 mV (Fig. 1C). Normalized $I_{Na}/I_{Na}max$ values reflected the fraction of channels inactivated during the prepulse. As isoflurane alters the voltage-dependence of $Na_v1.2$ gating,[8–10] we plotted conductance (G) and steady-state inactivation against voltage (Fig. 1D). Isoflurane shifted the voltage-dependence of steady-state inactivation in a hyperpolarizing direction [$V_{½}$ control= – 55.5 (2.1) mV; $V_{½}$ isoflurane= – 59.3 (2.0) mV, n=6, P<0.001, by paired Student's t-test]. The voltage-dependence of activation was not affected [$V_{½act}$ control= – 15.3 (2.7) mV; $V_{½act}$ isoflurane= – 15.4 (2.0) mV, n=6, n.s.]. These data suggest that isoflurane inhibits peak I_{Na} by increasing the fraction of inactivated channels at normal resting membrane potentials.

As neuronal firing frequency depends in part on how fast Na_v can cycle through their various conformational states, we measured the time-course of recovery from inactivation (Fig. 2). Peak I_{Na} was recorded in response to two 5-ms pulses to 0 mV,

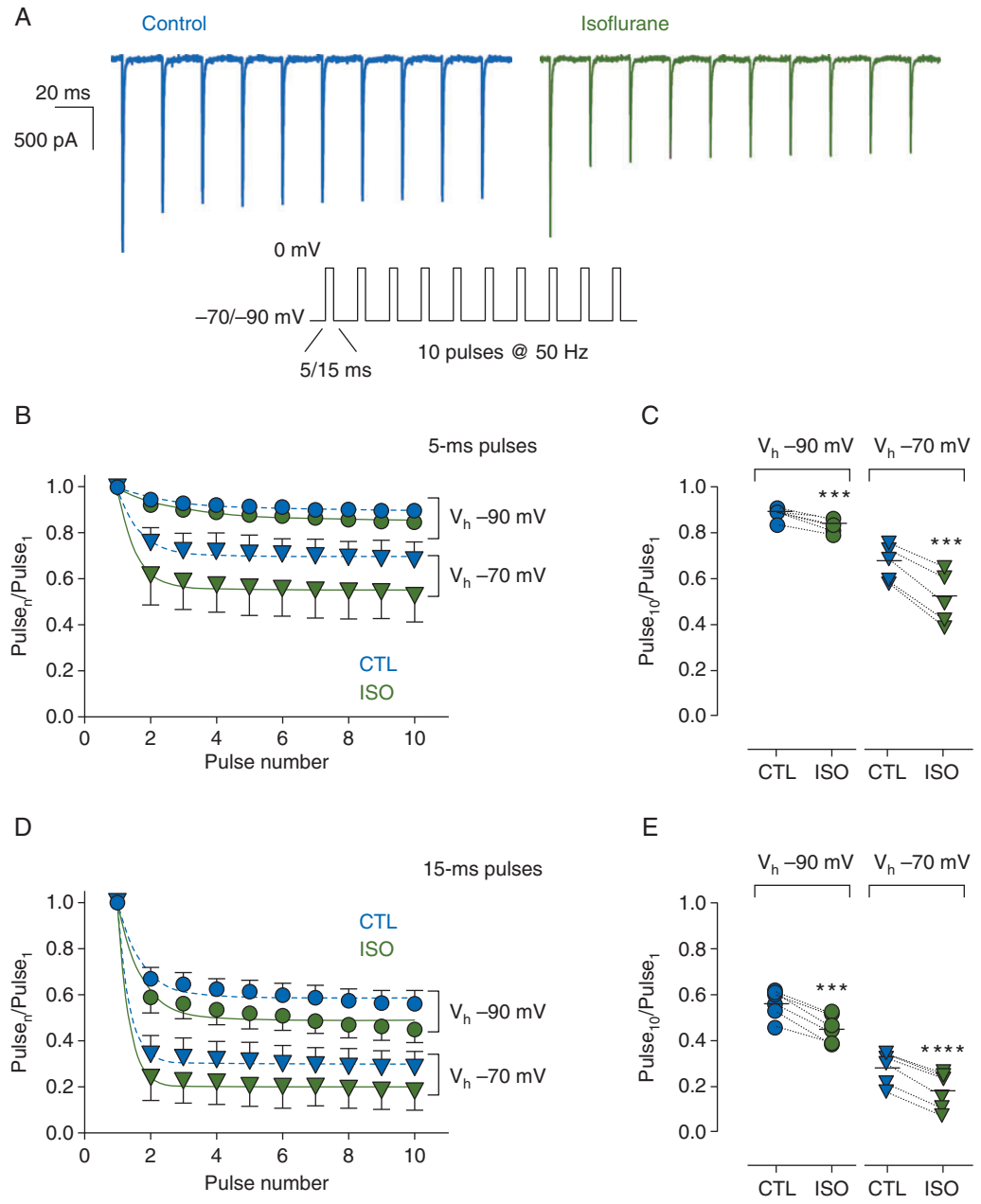

Fig 3 Effects of isoflurane on brain-type sodium channel $Na_v1.2_R$ expressed in a neuronal cell line stimulated at high-frequency. (A) Representative current traces in response to a 50 Hz train of 5-ms depolarizing pulses to 0 mV from a V_h of –70 mV in the absence (*left*) or presence (*right*) of 1.5 MAC isoflurane (protocol in *inset*). (B) Peak I_{Na} elicited by a 50 Hz train of 5-ms pulses in the absence (CTL) or presence (ISO) of isoflurane from a V_h of –70 mV (*triangles*, n=5) or –90 mV (*circles*, n=6) normalized to the first pulse of the train ($Pulse_n/Pulse_1$). Mean (SD) data were fit to a mono-exponential function. (C) Normalized I_{Na} at $Pulse_{10}$ for each cell in B. (D) Similar experiments as *B* but with 15-ms depolarizing pulses from a V_h of –70 mV (n=6) or –90 mV (n=6). (E) Normalized I_{Na} at $Pulse_{10}$ for each cell in D. Dotted lines denote paired data. ***P<0.001, ****P<0.0001 by two-tailed, paired Student's *t*-test.

where the duration between the two pulses was varied. Recovery time-courses were fit with a mono-exponential function in both control and isoflurane conditions, indicating that channels predominantly entered a single 'fast'-inactivated state and not any 'slow'-inactivated states. Isoflurane increased the time required for full channel recovery at a hyperpolarized V_h of –90 mV [$\tau_{control}$=3.1 (0.6) ms; $\tau_{isoflurane}$=3.9 (0.8) ms, n=6, P<0.05 by paired Student's *t*-test]; this effect was enhanced at more physiological V_h [recovery at –70 mV, $\tau_{control}$=6.9 (1.1) ms; $\tau_{isoflurane}$=9.0 (1.9) ms, n=7, P<0.005 by paired Student's *t*-test] (Fig. 2B and C).

We posited that this delay in recovery from inactivation after membrane depolarization would lead to progressive inhibition of I_{Na} during trains of action potentials. We tested this by administering depolarizing stimuli at 50 Hz (Fig. 3A), normalizing I_{Na} of each pulse to that of the first pulse ($Pulse_n/Pulse_1$) to remove

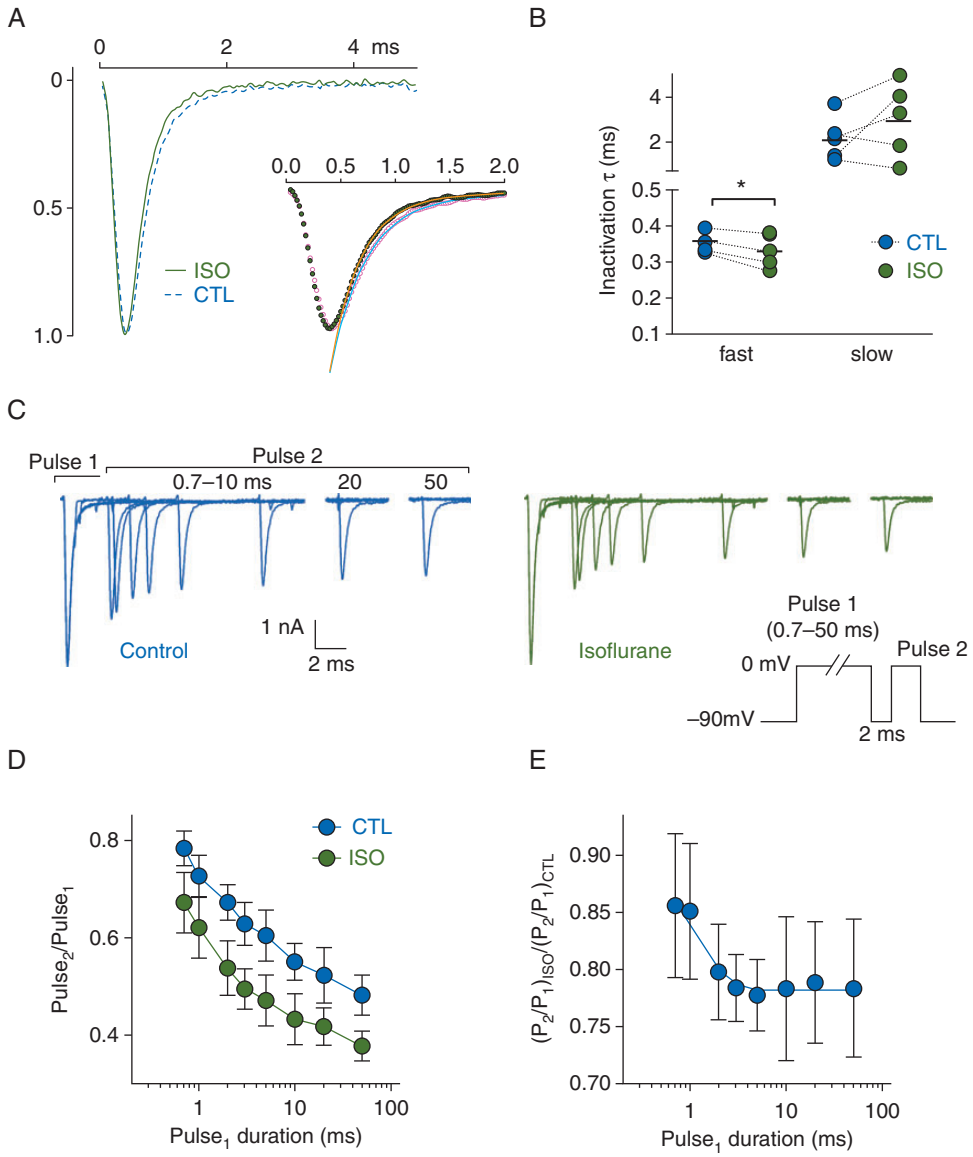

Fig 4 Acceleration by isoflurane of inactivation in brain-type sodium channel $Na_v1.2_R$ expressed in a neuronal cell line. (A) Representative responses to a 5-ms stimulus pulse to 0 mV from a V_h of −70 mV for control (CTL) or 1.5 MAC isoflurane (ISO) treated cells normalized to the peak of CTL. *Inset* shows a bi-exponential fit of the decay phase for CTL (*blue line*) and ISO (*orange line*). (B) Slow and fast τ derived from bi-exponential fitting of individual cells in the absence (CTL) or presence (ISO) of 1.5 MAC isoflurane. Dotted lines denote paired data. (*$P<0.05$ by two-tailed, paired Student's *t*-test). (C) Representative current traces recorded in the absence (*left*) or presence (*right*) of 1.5 MAC isoflurane using a two-pulse protocol (*inset*) to investigate onset of isoflurane inhibition. From a V_h of −90 mV, cells were stimulated with two pulses to 0 mV with the duration of the first pulse varied from 0.7–50 ms. (D) Peak I_{Na} of the test pulse ($Pulse_2$) was normalized to that of the conditioning pulse ($Pulse_1$) to yield fractional I_{Na} ($Pulse_2/Pulse_1$). Mean [SD] fractional I_{Na} (n=8) plotted vs $Pulse_1$ duration. (E) Fractional I_{Na} [mean (SD), n=8] for ISO normalized to that of CTL to determine the onset of isoflurane inhibition fitted with a mono-exponential function.

the effect of resting block by isoflurane (Fig. 3B and D). The reduced I_{Na} at the 10th pulse was thus 'activity-dependent' as a result of repeated membrane depolarization. For 5-ms pulses delivered at 50 Hz, from a V_h of −90 mV, isoflurane reduced the fraction of current at $Pulse_{10}$ ($Pulse_{10}/Pulse_1$) from 0.90 [0.03] to 0.85 [0.03] (n=6, $P<0.001$). From a V_h of −70 mV, isoflurane reduced $Pulse_{10}/Pulse_1$ from 0.68 [0.08] to 0.53 [0.11] (n=5, $P<0.0005$) (Fig. 3C). For 15-ms pulses delivered at 50 Hz from a V_h of −90 mV, isoflurane reduced $Pulse_{10}/Pulse_1$ from 0.56 [0.06] to 0.45 [0.06] (n=6, $P<0.0005$) and from a V_h of −70 mV, isoflurane reduced $Pulse_{10}/Pulse_1$ from 0.28 [0.07] to 0.18 [0.08] (n=6, $P<0.0001$) (Fig. 3E). Activity-dependent block was not seen in time control experiments, which used a mock perfusion to ensure the reduction in I_{Na} was as a result of isoflurane block and not experimental time (data not shown).

Block was increased by more depolarized V_h and shorter recovery intervals, conditions that promote inactivation, implicating stabilisation of fast-inactivation as the mechanism of

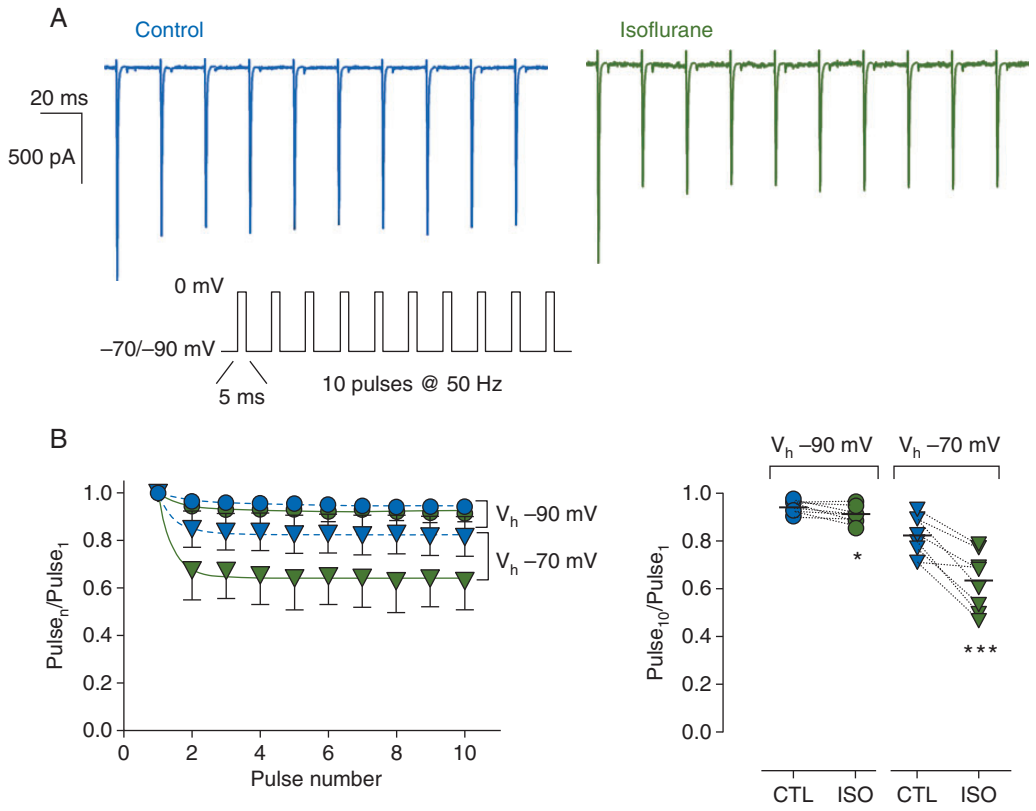

Fig 5 Effects of isoflurane on peak Na^+ current (I_{Na}) in isolated rat neurohypophysial nerve terminals during high-frequency stimulation. (A) Representative current traces for a 50 Hz train of 5-ms depolarizing pulses to 0 mV from V_h of –70 mV in the absence (*left*) or presence (*right*) of 1.5 MAC isoflurane (protocol in *inset*). (B) *Left panel*, shows normalized peak I_{Na} [mean (SD), n=8] in the absence (*blue symbols*) or presence (*green symbols*) of isoflurane from V_h of –70 mV (*triangles*) or –90 mV (*circles*). Peak amplitude of each pulse was normalized to the first pulse ($Pulse_n/Pulse_1$) and fitted with a mono-exponential function. *Right panel*, individual normalized responses for the last pulse ($Pulse_{10}/Pulse_1$) in the absence (*blue symbols*) or presence (*green symbols*) of isoflurane. Dotted lines denote paired data. *$P< 0.05$, ***$P<0.001$ by two-tailed, paired Student's t-test.

activity-dependent block. This mechanism predicts that higher frequency stimulation will enhance channel block. To test this prediction we examined the pulse train responses with 100 Hz frequency (V_h= – 90 mV, 5-ms pulses, data not shown), in control and isoflurane which we compared with those at the 50 Hz frequency (Fig. 3B). To focus on the fractional block of channels available for opening in control at near steady-state we applied the following calculation: [fractional block=(1– (ISO P_{10})/(CTL P_{10}))]. Increasing the stimulation frequency from 50 to 100 Hz more than doubled the fractional block by isoflurane [50 Hz, 0.058 (0.018); 100 Hz, 0.137 (0.034), n=6, P<0.001, data not shown]. The results provide additional support for the activity-dependent block mechanism.

We measured the effect of isoflurane on the kinetics of inactivation by comparing the rates of macroscopic I_{Na} decay. We normalized current traces to peak I_{Na} of paired control and isoflurane experiments and fit the decay phases with a double exponential function (Fig. 4A, *inset*). The majority of the decay phase (~95%) could be described by the fast time constant (τ). Isoflurane reduced the fast τ from 0.36 [0.03] ms in control to 0.33 [0.05] ms with isoflurane (n=5, P<0.05, paired Student's t-test). Isoflurane had little effect on the slow time constant (2.11 [0.99] for control; 2.93 [1.67] for isoflurane, n=5, n.s.) (Fig. 4B). These results are summarized in Supplementary Table S1 and are consistent with isoflurane enhancing fast inactivation or promoting a unique inhibited state with entry rates similar to that of fast inactivation.

We investigated the onset of isoflurane inhibition using a double-pulse protocol (Fig. 4C). Conditioning pulse durations (Pulse 1) greater than 3 ms were used, which typically produce complete channel inactivation. Consequently, the protocol briefly (2 ms) returns to V_h to allow partial recovery of fast-inactivated and inhibited channels, which inversely reflect the degree of inactivation and inhibition induced by Pulse 1. In control conditions, longer Pulse 1 durations reduced fractional I_{Na} (Pulse 2 normalized to Pulse 1), reflecting a growing population of inactivated channels that fail to fully recover between pulses. Isoflurane depressed fractional I_{Na} for all Pulse 1 durations relative to control (Fig. 4D). To investigate the time course of inhibition onset, we normalized the isoflurane response to that of control (Fig. 4E). The response reached an apparent plateau at around 10 ms and was well fit by a mono-exponential function with kinetics [τ=2.43 (1.62) ms, A_P=0.76 (0.04), n=8], comparable with fast inactivation. These findings further support the involvement of fast inactivation in isoflurane action.

We examined whether activity-dependent block by isoflurane occurs in isolated rat neurohypophysial nerve terminals, an intact preparation that contains endogenous nerve terminal Na_v channel complexes. Isoflurane produced activity-dependent block of normalized peak I_{Na} at a stimulation frequency (50 Hz)

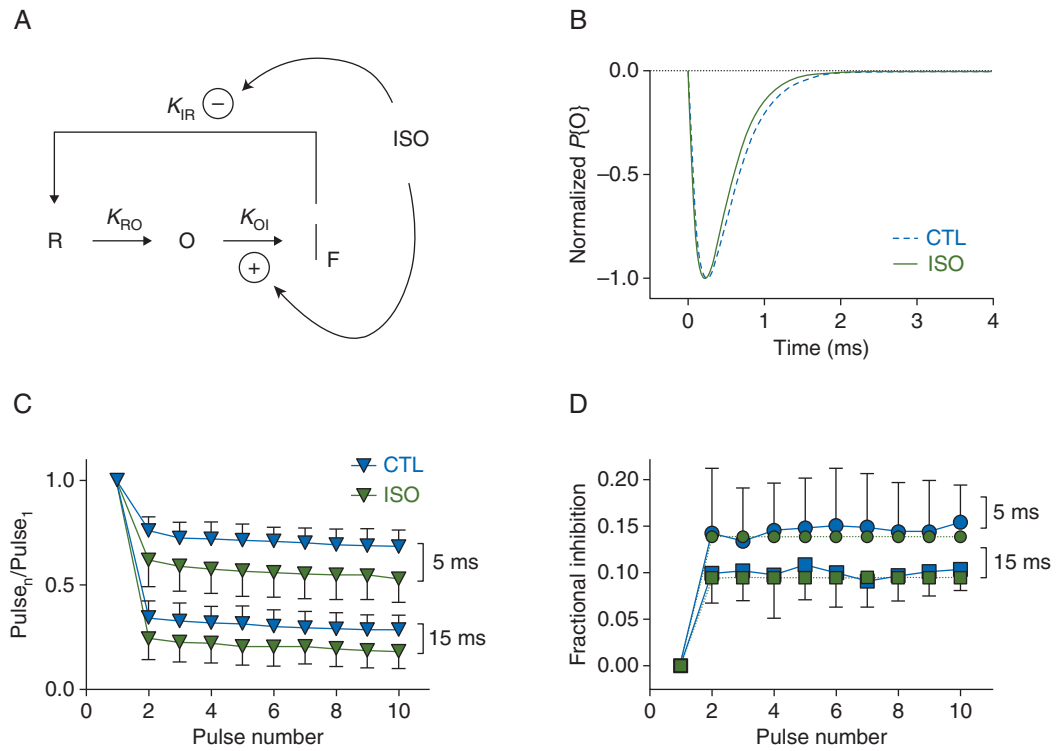

Fig 6 Gating model for isoflurane inhibition of $Na_v1.2_R$ involving stabilisation of fast inactivation. (A) Simple Markov gating model of isoflurane inhibition involving primary resting (*R*), open (*O*) and fast inactivated (I_F) states (*R*-*O*-I_F model). Transitions between states are indicated by arrows shown with associated rate constants. During activation, channels transition from *R* to *O* to I_F, and upon repolarization channels recover to availability by transitioning from I_F to *R*. It is assumed that transitions from *O* to *R* are negligible and I_F is an absorbing state during activation. Curved arrows identify transitions modulated by isoflurane (ISO). Isoflurane enhances (+) transitions from *O* to I_F and slows (−) transitions from I_F to *R*, which together represents I_F stabilisation. (B) Simulated responses to single depolarizations (5 ms, 0 mV, V_h= − 70 mV). Normalized probability of state *O* (*P*{*O*}), proportional to current, plotted against time for control (CTL) and isoflurane (ISO). (C) Empirical pulse trains replotted from Fig. 3B and D (V_h= − 70 mV), with pulse durations as indicated. (D) Time course of isoflurane inhibition during a pulse train determined by plotting the fraction of channels inhibited (Fractional Inhibition; 1 − $Pulse_n/Pulse_1$) against pulse number. Empirical responses were derived from the pulse trains of panel C shown as *blue symbols*. Simulated responses are shown as *green symbols*, Data shown as mean [SD], with pulse durations indicated.

and pulse duration (5 ms) that produced minimal decrements in control (Fig. 5A). Isoflurane produced rapidly developing activity-dependent block, evident in enhanced reduction in plateau amplitude of normalized peak I_{Na} (Fig. 5B). At a V_h of −90 mV, the fractional peak of the last pulse of the train was reduced from 0.94 [0.02] in control to 0.91 [0.03] with isoflurane (n=8, P<0.05), and at a V_h of −70 mV, from 0.82 [0.08] for control to 0.63 [0.12] for isoflurane (n=8, P<0.001). Combined with resting block, activity-dependent block significantly enhanced overall inhibition of I_{Na} by isoflurane with repetitive stimulation.

We constructed a simple gating model to examine whether stabilisation of fast inactivation is sufficient to quantitatively account for our experimental observations. Figure 6A shows a 3-state (*R*-*O*-I_F) Markov model, in which isoflurane accelerates transitions from open to fast-inactivated states and slows recovery from fast-inactivated states upon repolarization. Rate constants k_{RO} (3.6 ms^{-1}) and k_{OI} (control, 2.77 ms^{-1}; isoflurane, 3.03 ms^{-1}) were derived from mono-exponential fits to activation (not shown) and fast-inactivation (Fig. 4A) time courses. Model parameters derived from empirical responses, demonstrate that the model accounts for changes in macroscopic current time course during single depolarizations (Fig. 6B). The derived empirical time course of isoflurane inhibition during pulse trains (Fig. 6C and D) was well accounted for by the *R*-*O*-I_F model, using estimated values of k_{IR} (control, 89.4 s^{-1}; isoflurane, 59.7 s^{-1}) comparable with those observed experimentally (control, 137 s^{-1}; isoflurane, 100 s^{-1}, see Fig. 2B). We conclude that stabilisation of the fast-inactivated state is sufficient to quantitatively account for our experimental observations of activity-dependent block of I_{Na} by isoflurane.

Discussion

We show here that isoflurane enhances activity-dependent depression of I_{Na} in both brain-type $Na_v1.2$ and endogenous nerve terminal sodium channels. This novel anaesthetic effect on Na_v contributes significantly to overall block during high-frequency stimulation. This should lead to greater sensitivity to isoflurane of fast firing neuronal networks, including depression of presynaptic excitability and reduced neurotransmitter release. Use of both a neuronal expression system and neuronal tissue, allowed us to demonstrate activity-dependent block of the major neuronal Na_v subtype and of endogenous nerve terminal Na_v subtypes *in situ*, in a physiological context as heterologous expression could influence anaesthetic effects.[10–11 23] We further demonstrate that this novel form of Na_v block involves stabilisation of the fast-inactivated state.

Neuronal signals are transmitted via trains of action potentials, so activity-dependent block of I_{Na} is a potentially important mechanism of volatile anaesthetic action on neuronal networks. Previous assessments of the role of Na_v inhibition by general anaesthetics *in vitro* have not considered the impact of activity-dependent block, which significantly enhances the efficacy of isoflurane inhibition of neuronal Na_v, under physiological conditions. For example, the magnitude of tonic I_{Na} inhibition by clinical concentrations of isoflurane is relatively modest: we observed ~10% reduction of peak *INa*, comparable with previous reports.[8 10] In comparison, we observed an additional ~20% block at 50 Hz stimulation, from a physiological V_h of −70 mV. Even modest block of Na_v can strongly affect neuronal transmission, as small reductions in peak I_{Na} alter both frequency of action potential firing[24] and neurotransmitter release.[5] Small reductions in I_{Na} produce significant effects on oscillatory activity in neuronal networks,[25] which is relevant to systems level mechanisms of general anaesthesia. By analogy with use-dependent block by local anaesthetics, volatile anaesthetics would preferentially inhibit more active neurones to selectively suppress fast firing networks.[26 27] Neurohypophysial nerve terminals express Na_v in high density,[9] comparable with hippocampal mossy fibre boutons, in which Na_v amplify presynaptic action potential amplitude and enhance Ca^{2+} influx coupled to transmitter release.[28] This high concentration of Na_v at the bouton could explain how small changes in I_{Na} lead to substantial inhibition of synaptic vesicle exocytosis.

To our knowledge this is the first report of activity-dependent decay of I_{Na} in intact nerve terminals. This preparation has the advantage over recombinant Na_v of reflecting the gating properties of native nerve terminal Na_v *in situ*. Electrophysiology is limited in its ability to measure ionic currents at the synapse, where volatile anaesthetics exert their most potent effects.[1–3] We show that isoflurane inhibits endogenous neurohypophysial nerve terminal Na_v,[29] which are coupled to neurotransmitter release by depolarizing the membrane and thus lead to activation of voltage-gated Ca^{2+} channels, Ca^{2+} influx and exocytosis. Magnocellular neurones of the supraoptic nucleus that innervate the neurohypophysis, express both $Na_v1.2$ and $Na_v1.6$,[30] which are likely to mediate I_{Na} in these central nervous system nerve terminals. Heterologously expressed $Na_v1.2$ and $Na_v1.6$ are inhibited by isoflurane and other volatile anaesthetics at clinically relevant concentrations.[8 18] Interestingly, heterologously expressed $Na_v1.6$ currents exhibit use-dependent potentiation compared with $Na_v1.2$ currents, which show use-dependent inhibition.[31] As neurohypophysial nerve terminals exhibited rapid activity-dependent reductions in peak I_{Na}, amplitude with repetitive stimulation comparable with recombinant $Na_v1.2_R$, the dominant Na_v isoform in neurohypophysial terminals is most likely $Na_v1.2$.

Our mechanistic analysis of $Na_v1.2_R$ revealing stabilisation of fast inactivation, is relevant to the intrinsic neurophysiological behaviour of presynaptic Na_v, and has important implications for the anaesthetic sensitivity of nerve terminals at high firing frequencies. Although the molecular details underlying our proposal that isoflurane stabilises the fast-inactivated state are beyond the scope of the current investigation, the mechanism could involve isoflurane binding to a freely accessible receptor, that then allosterically modulates free energy profiles to stabilise the fast-inactivated state. Isoflurane can participate in hydrogen bonding by forming dipoles, and has been shown to bind hydrophobic macromolecules.[32] Consistent with this possibility, anaesthetic binding has been demonstrated in prokaryotic voltage-gated ion channels,[33 34] and molecular dynamics simulations of a homology model of NaChBac[35] revealed a possible pathway for isoflurane to enter the pore via hydrophobic side fenestrations.[36] Further structural and molecular dynamics studies are necessary to determine where isoflurane binds and what residues are involved in stabilizing the bound state of the channel.

In summary, we show that isoflurane stabilises the fast-inactivated state of neuronal Na_v such that recovery from fast inactivation is delayed and entry into fast inactivation is accelerated, resulting in activity-dependent inhibition. This enhanced inactivation leads to progressive inhibition of I_{Na}, with high-frequency stimulation and contributes significantly to overall inhibition of I_{Na} by isoflurane, through activity-dependent inhibition compared with tonic inhibition. At high stimulus frequencies, Na_v inhibition by isoflurane and probably other anaesthetics that exhibit state-dependent inhibition, will be greater than that suggested by previous studies of resting block alone.

Authors' contributions

H.C.H. contributed to the study design and writing the paper. K.P. contributed to the data collection, data analysis and writing the paper. K.J.G., and W.O. contributed to the data collection and data analysis. K.F.H. contributed to the study design and data analysis. All of the authors read, revised and approved the final manuscript.

Supplementary material

Supplementary material is available at *British Journal of Anaesthesia* online.

Declaration of interests

H.C.H. is an Editor of the *BJA* and of *Anesthesiology*. K.P., K.J.G., W. O. and K.F.H. declare no interests.

Funding

Supported by NIH grant GM58055 and GM58055S1.

References

1. Hemmings HC Jr, Akabas MH, Goldstein PA, Trudell JR, Orser BA, Harrison NL. Emerging mechanisms of general anesthetic action. *Trends Pharmacol Sci* 2005; **26**: 503–10
2. Eger EI 2nd, Raines DE, Shafer SL, Hemmings HC Jr, Sonner JM. Is a new paradigm needed to explain how inhaled anesthetics produce immobility? *Anesth Analg* 2008; **107**: 832–48
3. Franks NP, Lieb WR. Molecular and cellular mechanisms of general anaesthesia. *Nature* 1994; **367**: 607–14
4. Westphalen RI, Hemmings HC Jr. Selective depression by general anesthetics of glutamate versus GABA release from isolated cortical nerve terminals. *J Pharmacol Exp Therap* 2003; **304**: 1188–96
5. Wu XS, Sun JY, Evers AS, Crowder M, Wu LG. Isoflurane inhibits transmitter release and the presynaptic action potential. *Anesthesiology* 2004; **100**: 663–70
6. Hemmings HC Jr, Yan W, Westphalen RI, Ryan TA. The general anesthetic isoflurane depresses synaptic vesicle exocytosis. *Mol Pharmacol* 2005; **67**: 1591–9
7. Urban BW. Differential effects of gaseous and volatile anaesthetics on sodium and potassium channels. *Br J Anaesth* 1993; **71**: 25–38

8. 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
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. Shiraishi M, Harris RA. Effects of alcohols and anesthetics on recombinant voltage-gated Na channels. *J Pharmacol Exp Ther* 2004; **309**: 987–94
11. 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
12. Fujiwara N, Higashi H, Nishi S, Shimoji K, Sugita S, Yoshimura M. Changes in the spontaneous firing patterns of rat hippocampal neurones induced by volatile anaesthetics. *J Physiol* 1988; **402**: 155–75
13. Ouyang W, Hemmings HC Jr. Depression by isoflurane of the action potential and underlying voltage-gated ion currents in isolated rat neurohypophysial nerve terminals. *J Pharmacol Exp Ther* 2005; **312**: 801–8
14. Zhang Y, Guzinski M, Eger EI 2nd, *et al*. Bidirectional modulation of isoflurane potency by intrathecal tetrodotoxin and veratridine in rats. *Br J Pharmacol* 2010; **159**: 872–8
15. Schulze V, Stoetzer C, O'Reilly AO, *et al*. The opioid methadone induces a local anaesthetic-like inhibition of the cardiac Na^+ channel, Na(v)1.5. *Br J Pharmacol* 2014; **171**: 427–37
16. Ragsdale DS, Scheuer T, Catterall WA. Frequency and voltage-dependent inhibition of type IIA Na^+ channels, expressed in a mammalian cell line, by local anesthetic, antiarrhythmic, and anticonvulsant drugs. *Mol Pharmacol* 1991; **40**: 756–65
17. Sheets MF, Fozzard HA, Lipkind GM, Hanck DA. Sodium channel molecular conformations and antiarrhythmic drug affinity. *Trends Cardiovasc Med* 2010; **20**: 16–21
18. Ouyang W, Hemmings HC Jr. Isoform-selective effects of isoflurane on voltage-gated Na^+ channels. *Anesthesiology* 2007; **107**: 91–8
19. Hemmings HC Jr. Sodium channels and the synaptic mechanisms of inhaled anaesthetics. *Br J Anesth* 2009; **103**: 61–9
20. Leffler A, Herzog RI, Dib-Hajj SD, Waxman SG, Cummins TR. Pharmacological properties of neuronal TTX-resistant sodium channels and the role of a critical serine pore residue. *Pflugers Arch* 2005; **451**: 454–63
21. Benzanilla F, Armstrong CM. Inactivation of the sodium channel. I. Sodium current experiments. *J Gen Physiol* 1977; **70**: 549–66
22. 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
23. John V, Main M, Powell A, *et al*. Heterologous expression and functional analysis of rat $Na_V1.8$ (SNS) voltage-gated sodium channels in the dorsal root ganglion neuroblastoma cell line ND7–23. *Neuropharmacology* 2004; **46**: 425–38
24. Scholz A, Kuboyama N, Hempelmann G, Vogel W. Complex blockade of TTX-resistant Na^+ currents by lidocaine and bupivicaine reduce firing frequency in DRG neurons. *J Neurophysiol* 1998; **79**: 1746–54
25. Århem P, Klement G, Nilsson J. Mechanisms of Anesthesia: Towards Integrating Network, Cellular, and Molecular Level Modeling. *Neuropsychopharmacology* 2003; **28**: S40–7
26. Stewart A, Lambert DH, Concepcion MA, *et al*. Decreased incidence of tourniquet pain during spinal anesthesia with bupivicaine. *Anesth Analg* 1988; **67**: 833–7
27. Wildsmith JAW, Brown DT, Paul D, Johnson S. Structure-activity relationships in differential nerve block at high and low frequency stimulation. *Br J Anaesth* 1989; **63**: 444–52
28. Engel D, Jonas P. Presynaptic action potential amplification by voltage-gated Na^+ channels in hippocampal mossy fiber boutons. *Neuron* 2005; **45**: 405–17
29. Westphalen RI, Yu J, Krivitski M, Jih TY, Hemmings HC Jr. Regional differences in nerve terminal Na^+ channel subtype expression and Na^+ channel-dependent glutamate and GABA release in rat CNS. *J Neurochem* 2010; **113**: 1611–20
30. Tanaka M, Cummins TR, Ishikawa K, Black JA, Ibata Y, Waxman SG. Molecular and functional remodeling of electrogenic membrane of hypothalamic neurons in response to changes in their input. *Proc Natl Acad Sci* 1999; **96**: 1088–93
31. Zhou W, Goldin AL. Use-dependent potentiation of the $Na_v1.6$ sodium channel. *Biophys J* 2004; **87**: 3862–72
32. Zhang T, Johansson JS. An isothermal titration calorimetry study on the binding of four volatile general anesthetics to the hydrophobic core of a four-alpha-helix bundle protein. *Biophys J* 2003; **85**: 3279–85
33. Nury H, Van Renterghem C, Weng Y, *et al*. X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel. *Nature* 2011; **469**: 428–31
34. Spurny R, Billen B, Howard RJ, *et al*. Multisite binding of a general anesthetic to the prokaryotic pentameric Erwinia chrysanthemi ligand-gated ion channel (ELIC). *J Biol Chem* 2013; **288**: 8355–64
35. Raju SG, Barber AF, LeBard DN, Klein ML, Carnevale V. Exploring volatile general anesthetic binding to a closed membrane-bound bacterial voltage-gated sodium channel via computation. *PLoS Comput Biol* 2013; **9**: e1003090
36. Payandeh J, Scheuer T, Zheng N, Catterall WA. The crystal structure of a voltage-gated sodium channel. *Nature* 2011; **475**: 353–8

Handling editor: H. F. Galley