

Isoflurane Inhibits the Tetrodotoxin-resistant Voltage-gated Sodium Channel Na_v1.8

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Background: Voltage-gated sodium channels (Na_v) mediate neuronal action potentials. Tetrodotoxin inhibits all Na_v isoforms, but Na_v1.8 and Na_v1.9 are relatively tetrodotoxin-resistant (TTX-r) compared to other isoforms. Na_v1.8 is highly expressed in dorsal root ganglion neurons and is functionally linked to nociception, but the sensitivity of TTX-r isoforms to inhaled anesthetics is unclear.

Methods: The sensitivities of heterologously expressed rat TTX-r Na_v1.8 and endogenous tetrodotoxin-sensitive (TTX-s) Na_v to the prototypic inhaled anesthetic isoflurane were tested in mammalian ND7/23 cells using patch-clamp electrophysiology.

Results: From a holding potential of -70 mV, isoflurane (0.53 ± 0.06 mM, 1.8 minimum alveolar concentration at 24°C) reduced normalized peak Na⁺ current (*I*_{Na}) of Na_v1.8 to 0.55 ± 0.03 and of endogenous TTX-s Na_v to 0.56 ± 0.06. Isoflurane minimally inhibited *I*_{Na} from a holding potential of -140 mV. Isoflurane did not affect voltage-dependence of activation, but it significantly shifted voltage-dependence of steady-state inactivation by -6 mV for Na_v1.8 and by -7 mV for TTX-s Na_v. IC₅₀ values for inhibition of peak *I*_{Na} were 0.67 ± 0.06 mM for Na_v1.8 and 0.66 ± 0.09 mM for TTX-s Na_v; significant inhibition occurred at clinically relevant concentrations as low as 0.58 minimum alveolar concentration. Isoflurane produced use-dependent block of Na_v1.8; at a stimulation frequency of 10 Hz, 0.56 ± 0.08 mM isoflurane reduced *I*_{Na} to 0.64 ± 0.01 versus 0.78 ± 0.01 for control.

Conclusion: Isoflurane inhibited the tetrodotoxin-resistant isoform Na_v1.8 with potency comparable to that for endogenous tetrodotoxin-sensitive Na_v isoforms, indicating that sensitivity to inhaled anesthetics is conserved across diverse Na_v family members. Block of Na_v1.8 in dorsal root ganglion neurons could contribute to the effects of inhaled anesthetics on peripheral nociceptive mechanisms.

VOLTAGE-GATED Na⁺ channels (Na_v) are critical to neuronal excitability, neurotransmitter release, and action potential initiation and propagation.¹ These channels consist of a highly processed 260-kDa α-subunit that contains the ion channel pore formed by four homologous domains associated with auxiliary β-subunits (β1-β4) of 33-36 kDa.¹ At least nine α-subunits (Na_v1.1-Na_v1.9) have been

identified, but the functional significance of the multiple isoforms is largely unclear.² All Na_v isoforms can be blocked by the puffer fish toxin tetrodotoxin, but three isoforms (Na_v1.5, Na_v1.8, and Na_v1.9) are relatively resistant (200 to 10,000-fold less sensitive) compared to other isoforms.^{3,4} This suggests that pharmacological differences in anesthetic sensitivity might also apply to other drugs, such as inhaled anesthetics.

Peripheral sensory neurons express both tetrodotoxin-sensitive (TTX-s) (Na_v1.1, Na_v1.2, Na_v1.6, and Na_v1.7) and TTX-resistant (TTX-r) (Na_v1.8 and Na_v1.9) α-subunit isoforms.⁴⁻⁶ TTX-r Na⁺ currents found in dorsal root ganglion (DRG) neurons show distinctive biophysical properties, such as persistent and slowly inactivating currents.⁷ The persistent current has been attributed to Na_v1.9, and the slowly inactivating current to Na_v1.8.^{8,9} Na_v1.8 is exclusively expressed in small to medium-sized DRG neurons that give rise to C- and Aδ-fibers.^{4,10} These neurons play an important role in pain pathways as the majority of Na_v1.8-containing afferents transmit nociceptive signals to the spinal cord.¹⁰ After peripheral nerve damage, functional expression of Na_v1.8 decreases in injured neurons but is upregulated in adjacent uninjured axons.¹¹ Activation of uninjured neurons appears critical to the hyperalgesia seen in neuropathic pain states,¹² and upregulation of Na_v1.8 in these neurons is an important component of this sensitization.¹¹ Antisense oligonucleotides against Na_v1.8 markedly reduce hyperalgesia and allodynia in animals with nerve injury.¹³ These findings support an important role for Na_v1.8 in pain and identify it as an interesting target for the development of new analgesic drugs. Indeed, Na_v1.8 has been reported to be fourfold more sensitive to inhibition by lidocaine than the TTX-s channel Na_v1.7.¹⁴

Halogenated inhaled (volatile) anesthetics inhibit endogenous TTX-s neuronal Na⁺ channels,¹⁵⁻¹⁷ including TTX-s Na⁺ channels in DRGs,¹⁸ as well as various Na_v α-subunit isoforms heterologously expressed in mammalian cell lines^{19,20} or amphibian oocytes.²¹ Inhibition of presynaptic Na⁺ channels contributes to depression of neurotransmitter release by volatile anesthetics.²²⁻²⁵ In contrast to the TTX-s isoforms tested, Na_v1.8 expressed in *Xenopus* oocytes has been reported to be insensitive to inhaled anesthetics.²¹ Such reduced inhaled anesthetic sensitivity, opposite to that of local anesthetics,¹⁴ would have important implications for analgesic mechanisms and the structural basis of Na⁺ channel anesthetic sensitivity and would be remarkable given the close sequence homologies between Na⁺ channel iso-

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forms.² We have therefore reexamined this more closely by using a mammalian neuronal expression system.

To test the sensitivity of Na_v1.8 to inhaled anesthetics under more physiologic conditions, we investigated the effects of the halogenated ether isoflurane on heterologously expressed TTX-r Na_v1.8 and endogenously expressed TTX-s Na_v in ND7/23 cells. Previous attempts to express Na_v1.8 in nonneuronal mammalian cell lines for electrophysiological analysis have been unsuccessful.^{26,27} However the hybrid ND7/23 cell line derived from rat DRG neurons and a mouse neuroblastoma cell line (N18TG2) exhibits sensory neuron-like properties²⁸ and can express functional heterologously transfected Na_v1.8,^{27,29-31} which probably reflects a requirement for additional subunits and/or a neuronal background for functional expression. In contrast to a previous report,²¹ transfected TTX-r Na_v1.8, like endogenous TTX-s Na_v isoforms, was inhibited by clinical concentrations of isoflurane.

Materials and Methods

Transient Transfection and Cell Culture

Rat Na_v1.8-cDNA was subcloned into the mammalian expression vector pCMV-Script (Stratagene, La Jolla, CA) and the sequence of the entire Na_v1.8 channel protein (NCBI nucleotide access number U53833) was verified by dideoxynucleotide sequencing. The ND7/23 rat DRG/mouse neuroblastoma fusion cell line was purchased from Sigma (Sigma-Aldrich, St. Louis, MO) and cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C under 95% air/5% CO₂. Cells were grown on 12-mm glass coverslips in 35-mm polystyrene culture dishes and transiently transfected with rat Na_v1.8-pCMV-Script (1–3 μg) and the pEGFP-N1 (Clontech, Mountain View, CA) reporter plasmid (0.5–1 μg) or reporter plasmid alone using Lipofectamine LTX (Invitrogen). At 48 h after transfection, the transfected cells were identified by expression of enhanced green fluorescent protein (EGFP) by using fluorescence microscopy. Average transfection efficiency was 60–70% for Na_v1.8/EGFP. Untransfected ND7/23 cells were plated and seeded as described above and incubated for 48 h to study endogenous TTX-s Na⁺ channels.

Electrophysiological Technique and Recordings

Coverslips containing ND7/23 cells were transferred into a small-volume open bath perfusion chamber (Warner Instruments, Hamden, CT) and continuously perfused with external solution containing (in mM): 129 NaCl, 10 HEPES, 3.25 KCl, 2 MgCl₂, 2 CaCl₂, 20 tetraethylammonium-Cl, 5 D-glucose, 0.0003 tetrodotoxin

(Sankyo Kasei Co., Tokyo, Japan), adjusted to pH 7.4 (with NaOH) and 310 mOsm/kg H₂O. Voltage-clamp recordings were performed at room temperature (23–24°C) in standard whole-cell configuration³² using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Experiments were performed at room temperature to minimize anesthetic losses, maximize recording stability, and facilitate comparisons to other studies of isoflurane on Na⁺ currents. Fire-polished patch pipettes were pulled from borosilicate glass capillaries (Warner Instruments) by using a Sutter P-97 puller (Sutter Instruments, Novato, CA) and had a resistance of 1.0–2.0 MΩ when filled with the following internal pipette solution (in mM): 120 CsF, 10 NaCl, 10 HEPES, 11 EGTA, 10 tetraethylammonium-Cl, 1 CaCl₂, 1 MgCl₂, adjusted to pH 7.3 (with CsOH) and 315 mOsm/kg H₂O.

Currents were low-pass filtered at 5 kHz and sampled at 20 kHz. Capacitive transients were electronically cancelled, and voltage errors were minimized by using 70–80% series resistance compensation. Series resistance was typically 1–5 MΩ, and recordings were discarded if resistance exceeded 8 MΩ. Initial seal after establishing the whole-cell patch was 2–4 GΩ, and recordings were discarded if the seal dropped below 1 GΩ. Liquid junction potential was not corrected. Linear leakage currents were digitally subtracted online by the P/4 protocol (except for inactivation and use-dependent experiments).

Isoflurane-saturated external solutions (containing 12–12.5 mM isoflurane) were prepared by shaking in gas-tight glass vials for 24 h as previously described.¹⁶ This stock solution was further diluted on the day of the experiment into gas-tight glass syringes, from which a sample was taken for determination of aqueous isoflurane concentration by gas chromatography. Solutions were perfused focally onto recorded cells *via* a 150-μm diameter perfusion pipette by using polytetrafluoroethylene tubing to minimize isoflurane loss. Perfusate samples were also taken to determine isoflurane concentrations at the tip of the perfusion manifold, and reflected approximately 10% loss that occurred from the syringe through the tubing to the pipette tip. Isoflurane concentrations were determined by extraction into *n*-heptane (1:1 v/v) followed by analysis using a Shimadzu GC-8A gas chromatograph (Shimadzu, Tokyo, Japan) with external standard calibration as described.^{16,20} Isoflurane solution flow of 0.05 ml/min was controlled by a pressurized perfusion system (ALA Scientific, Westbury, NY).

Stimulation Protocols and Data Analysis

The holding potential (V_h) used was either –70 mV or –140 mV. To analyze the voltage-dependence of activation, currents were evoked by 5-ms pulses ranging from –80 to +70 mV in steps of 10 mV. The conductance (G_{Na}) was calculated by using the equation: $G_{Na} = I_{Na} / (V_m - V_{rev})$, where I_{Na} is peak current, V_m is the test

potential, and V_{rev} is the calculated reversal potential (+65 mV). Normalized conductance (G/G_{max}) was plotted against test potentials and fitted to the Boltzmann function: $G/G_{max} = 1/[1 + \exp(V_{1/2} - V/k)]$, where $V_{1/2}$ is the voltage that elicits half-maximal activation and k is the slope factor. Steady-state and fast inactivation were measured by applying a double-pulse protocol that consisted of a 500 ms (b_{∞}) or 15 ms (fast inactivation) prepulse ranging from -120 to +20 mV in steps of 10 mV, followed by a test pulse to +10 mV (Na_v1.8) or -10 mV (TTX-s Na_v). Peak currents of the test pulse were measured, normalized ($I_{Na}/I_{Na,max}$), plotted against the prepulse potential, and fitted with a Boltzmann function. Decay time constants of peak I_{Na} were obtained from a monoexponential fit to the decay phase of the macroscopic Na⁺ current from 90% of peak current. Use-dependent block for Na_v1.8 was studied at 1, 3, and 10 Hz with 60 10-ms test pulses up to a final potential of +10 mV. Peak currents were measured, normalized to the first pulse and plotted against pulse number. IC₅₀ values were determined by least squares fitting of data to the Hill equation: $Y = 1/(1 + 10((\log IC_{50} - X)^h))$, where Y is the current amplitude, X is the isoflurane concentration, and h is Hill slope. Statistical significance was assessed by analysis of variance with Newman-Keuls *post hoc* test, or paired or unpaired Student *t* test. $P < 0.05$ was considered statistically significant. The programs used for data acquisition and analysis were pClamp 10 (Axon/Molecular Devices), Excel (Microsoft Inc., Redmond, WA), and Prism 5 (GraphPad Software Inc., San Diego, CA). Values are reported as mean \pm SEM unless otherwise stated.

Results

Properties of Na⁺ Currents in Wild-type and Na_v1.8-transfected Cells

ND7/23 cells express endogenous TTX-s Na⁺ currents (I_{Na}) with properties similar to TTX-s currents in isolated DRG neurons.²⁷ Though the specific Na⁺ channel isoforms responsible for TTX-s currents in ND7/23 cells are unknown, DRG neurons express a mixed population of Na⁺ channels that includes Na_v1.1, Na_v1.2, Na_v1.6, and Na_v1.7.^{5,6,33} We compared the effects of isoflurane on endogenously expressed TTX-s I_{Na} in untransfected ND7/23 cells and on TTX-r I_{Na} in ND7/23 cells transiently transfected with rat Na_v1.8 in the presence of 300 nM tetrodotoxin to block endogenous TTX-s I_{Na} , since Na_v1.8 is resistant to tetrodotoxin (IC₅₀ > 100 μ M).³ ND7/23 cells expressed voltage-gated TTX-s I_{Na} (-1,620 \pm 880 pA at $V_h = -70$ mV, $n = 6$; -2,220 \pm 680 pA at $V_h = -140$ mV, $n = 6$, mean \pm SD) that rapidly activated and inactivated upon depolarization (fig. 1A, left). These Na⁺ currents were completely inhibited by 300 nM tetrodotoxin, indicating that ND7/23 cells do not express detectable endogenous TTX-r Na⁺ channels (fig. 1A,

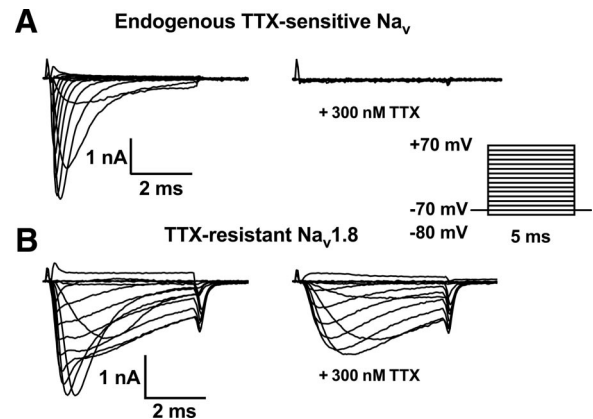


Fig. 1. Representative voltage-clamp recordings of Na⁺ currents. (A) Tetrodotoxin (TTX)-sensitive Na⁺ currents endogenously expressed in an ND7/23 cell in the absence (left) or presence (right) of 300 nM tetrodotoxin, which is sufficient to block all tetrodotoxin-sensitive Na⁺ channels without affecting Na_v1.8.³ (B) Recordings of an ND7/23 cell transfected with Na_v1.8 in the absence (left) or presence (right) of 300 nM tetrodotoxin, which blocks tetrodotoxin-sensitive Na⁺ channels and results in pharmacological isolation of the tetrodotoxin-resistant Na_v1.8 current. Inset shows stimulation protocol, $V_h = -70$ mV, voltage steps from -80 to +70 mV; pulse duration, 5 ms.

right). ND7/23 cells transfected with Na_v1.8 α -subunit showed prominent voltage-gated TTX-r I_{Na} (-1,830 \pm 840 pA at $V_h = -70$ mV, $n = 8$; -1,920 \pm 620 pA at $V_h = -140$ mV, $n = 8$, mean \pm SD) in the presence of 300 nM tetrodotoxin (fig. 1B).³⁰ Transfection of EGFP alone did not result in expression of TTX-r I_{Na} (data not shown).

Effect of Isoflurane on Peak Current and Activation

Current-voltage relationships were determined for TTX-r Na_v1.8 and TTX-s Na_v at the physiologic holding potential of -70 mV (fig. 2). Peak I_{Na} was activated at a command potential of +10 mV for TTX-r Na_v1.8 and -10 mV for TTX-s Na_v. At a concentration of isoflurane (0.53 \pm 0.06 mM) equivalent to 1.8 minimal alveolar concentration (MAC) in rat after temperature correction to 24°C,^{34,35} the normalized peak I_{Na} was 0.55 \pm 0.03 for TTX-r Na_v1.8 ($n = 8$) and 0.56 \pm 0.06 for TTX-s Na_v ($n = 6$) (fig. 2A). Inhibition was reversible upon washout of isoflurane (data not shown). Normalized conductance (G/G_{max}) plotted against command potential indicated no significant effects of isoflurane on voltage-dependence of activation for TTX-r Na_v1.8 or TTX-s Na⁺ currents (fig. 2B, table 1). From a more hyperpolarized holding potential of -140 mV, at which most channels are in the closed resting state, isoflurane was much less effective and reduced normalized peak I_{Na} to 0.91 \pm 0.03 for TTX-r Na_v1.8 ($n = 8$) and to 0.91 \pm 0.01 for TTX-s Na_v ($n = 6$) (fig. 3). There was no significant effect of isoflurane on the voltage-dependence of activation of TTX-r Na_v1.8 or TTX-s Na_v from either holding potential (for $V_{1/2}$ and k values, see table 1). Further analysis of time-to-peak values, the interval from the beginning of

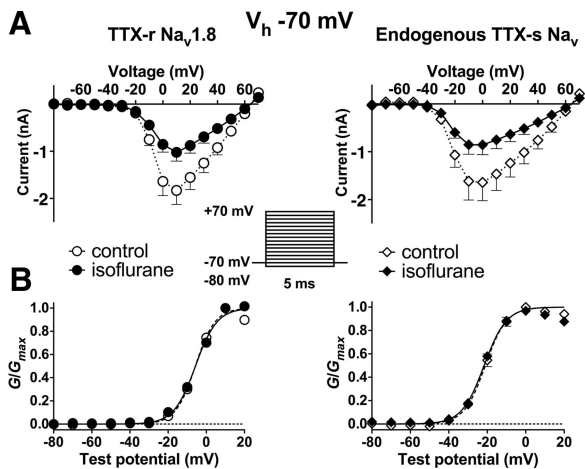


Fig. 2. Current-voltage relationships and voltage-dependence of activation for tetrodotoxin-resistant (TTX-r) $\text{Na}_v1.8$ (left) and tetrodotoxin-sensitive (TTX-s) Na_v (right) currents performed in the absence or presence of isoflurane. Whole-cell currents were evoked from a holding potential of -70 mV using a series of depolarizing steps from -80 mV to $+70$ mV in 10 -mV steps (inset). Data are shown as mean \pm SEM ($n = 6-8$) for control (open symbols) and isoflurane (0.53 ± 0.06 mM, equivalent to 1.8 minimum alveolar concentration [MAC] when corrected to 24°C , closed symbols). (A) Isoflurane reduced peak current amplitude but otherwise did not change the current-voltage relationship. (B) Activation curves (mean \pm SEM, $n = 6-10$) for TTX-r $\text{Na}_v1.8$ and TTX-s Na_v conductance in the absence (open symbols) or presence (closed symbols) of isoflurane. The data show the normalized Boltzmann function for conductance (G/G_{\max}) derived from the equation; $G/G_{\max} = 1/[1 + \exp(V_{1/2} - V/k)]$, where G is the measured conductance, G_{\max} is the maximal conductance, $V_{1/2}$ is the membrane potential for half-maximal activation, and k is the slope.

the test pulse to peak I_{Na} amplitude, also showed no significant difference between control and isoflurane for TTX-r $\text{Na}_v1.8$ and TTX-s Na_v (data not shown).

Effect of Isoflurane on Inactivation

The voltage-dependence of Na^+ channel inactivation was studied by using a 2-pulse protocol that consisted of a series of command potentials from -120 mV to $+20$ mV in 10 -mV steps, followed by a test pulse to elicit peak I_{Na} ($+10$ mV for TTX-r $\text{Na}_v1.8$ and -10 mV for TTX-s

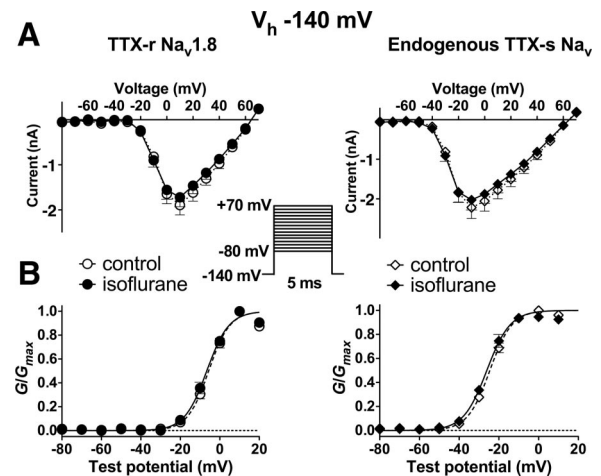


Fig. 3. Current-voltage relationships and voltage-dependence of activation for tetrodotoxin-resistant (TTX-r) $\text{Na}_v1.8$ (left) and tetrodotoxin-sensitive (TTX-s) Na_v (right) currents performed in the absence or presence of isoflurane. Whole cell currents were evoked from a hyperpolarized holding potential of -140 mV at which most Na^+ channels are in the closed resting state using a series of depolarizing steps from -80 mV to $+70$ mV in 10 -mV steps (inset). Data are shown as mean \pm SEM ($n = 6-8$) in the absence (open symbols) or presence of isoflurane (0.53 ± 0.06 mM, equivalent to 1.8 minimum alveolar concentration [MAC] when corrected to 24°C , closed symbols). (A) Isoflurane reduced peak current amplitude only minimally and did not change the shape of the current-voltage relationship. (B) Activation curves (mean \pm SEM) for TTX-r $\text{Na}_v1.8$ and TTX-s Na_v conductance in the absence (open symbols) or presence (closed symbols) of 0.53 ± 0.06 mM isoflurane. See Fig. 2 legend for details of curve fitting.

Na_v). This standard approach assesses the fraction of channels available for activation by the second pulse. As the membrane potential of the prepulse becomes more positive, more channels enter inactivated and nonconducting states such that fewer channels are available for activation by the second test pulse. Inactivation curves were determined by using two different prepulse durations; fast inactivation was measured by using a 15 -ms prepulse²⁷ and steady-state inactivation using a 500 -ms prepulse (fig. 4). The I_{Na} of the second test pulse was normalized to peak I_{Na} ($I_{\text{Na}}/I_{\text{Na,max}}$), plotted against prepulse potential, and fitted to a standard Boltzmann func-

Table 1. Parameters for Na^+ Current Activation and Inactivation

	Activation $V_h = -70$ mV			Activation $V_h = -140$ mV			Steady-state Inactivation (500 ms Prepulse)			Fast Inactivation (15 ms Prepulse)		
	$V_{1/2}$	k	n	$V_{1/2}$	k	n	$V_{1/2}$	k	n	$V_{1/2}$	k	n
$\text{Na}_v1.8$												
Control	-5.7 ± 0.4	5.1 ± 0.2	8	-5.2 ± 1.0	5.2 ± 0.2	8	-49.0 ± 1.2	9.6 ± 0.3	8	-29.5 ± 1.8	10.9 ± 0.8	8
Isoflurane	-5.7 ± 0.7	5.6 ± 0.1	8	-6.6 ± 1.3	5.4 ± 0.3	8	$-54.6 \pm 1.3^\dagger$	$10.8 \pm 0.4^\dagger$	8	$-41.7 \pm 2.4^\dagger$	13.1 ± 1.0	8
TTX-s												
Control	-20.9 ± 1.2	5.3 ± 0.3	6	-24.5 ± 0.9	5.5 ± 0.2	6	-67.0 ± 2.3	6.0 ± 0.3	4	-55.7 ± 0.6	13.6 ± 0.4	4
Isoflurane	-21.6 ± 0.9	5.8 ± 0.3	6	-25.9 ± 1.3	5.7 ± 0.4	6	$-74.1 \pm 2.1^\ddagger$	6.5 ± 0.4	4	$-70.7 \pm 2.6^\dagger$	16.6 ± 2.0	4

Mean values were derived from Boltzmann equation fits of individual data sets as described in the Methods. Isoflurane was tested at 0.53 ± 0.06 mM (equivalent to 1.8 minimum alveolar concentration [MAC] when corrected to 24°C).

$^\dagger P < 0.01$; $^\ddagger P < 0.001$ vs. control, compared by paired two-tailed Student t -test.

k = slope; n = number of experiments; TTX-s = tetrodotoxin-sensitive; V_h = holding potential; $V_{1/2}$ = voltage at which half maximal activation or inactivation occurs.

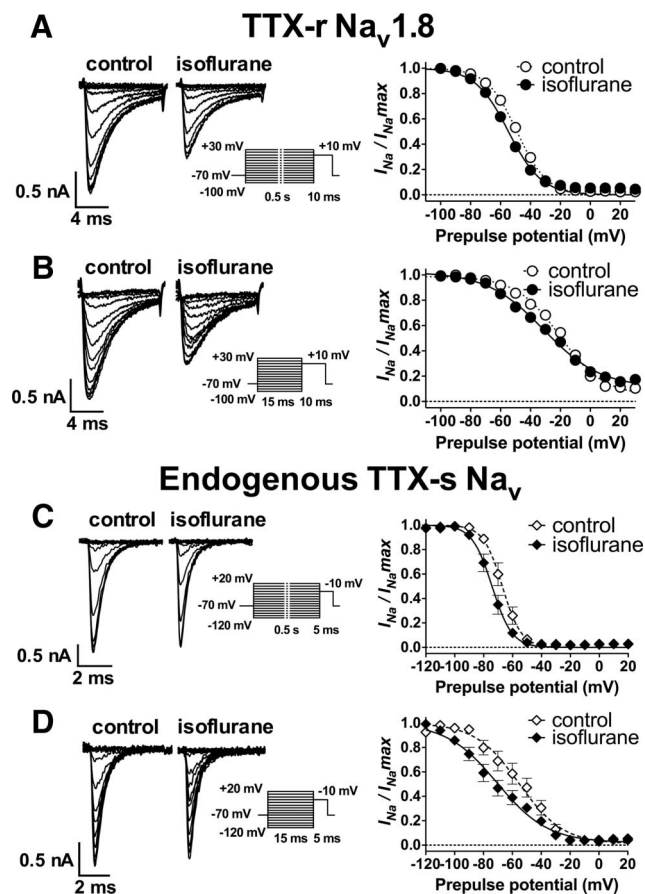


Fig. 4. Inactivation of tetrodotoxin-resistant (TTX-r) Na_v1.8 (A, B; n = 8) and tetrodotoxin-sensitive (TTX-s) Na_v (C, D; n = 4) Na⁺ currents. Peak Na⁺ current (I_{Na}) was normalized to the maximal value ($I_{Na,max}$) and plotted against the conditioning pulse potential. Data were fitted by a Boltzmann function according to the following equation: $I_{Na}/I_{Na,max} = 1/[1 + \exp(V_{1/2} - V/k)]$, where V is the prepulse potential, $V_{1/2}$ is the potential for half-maximal inactivation, and k is the slope. Data are shown for two prepulse durations of 500 ms (A, C) and 15 ms (B, D) (stimulation protocols shown in insets). Note the presence of a noninactivated fraction (10–20%) with the shorter prepulse seen in TTX-r Na_v1.8 (B). Isoflurane concentration used for the experiments was 0.53 ± 0.06 mM (equivalent to 1.8 minimum alveolar concentration when corrected to 24°C).

tion from which the voltage of half-inactivation ($V_{1/2}$) was determined (table 1). Isoflurane (0.53 ± 0.06 mM) produced a negative shift in the voltage-dependence of steady-state inactivation (500 ms prepulse) of -5.6 ± 0.8 mV for TTX-r Na_v1.8 and -7.1 ± 0.3 mV for TTX-s Na_v ($P < 0.001$), and a negative shift in the voltage-dependence of fast inactivation (15-ms prepulse) of -9.2 ± 2.1 mV for TTX-r Na_v1.8 and -15 ± 2.3 mV for TTX-s Na_v ($P < 0.01$). There were no significant effects on slope values. The time constant of Na⁺ current decay at peak I_{Na} (τ) was significantly increased by isoflurane for both TTX-r Na_v1.8 and TTX-s Na_v. The τ values for Na_v1.8 were 2.5 ± 0.1 ms in control and 3.0 ± 0.2 ms with isoflurane ($P < 0.05$, n = 8), and for TTX-s Na_v were 0.54 ± 0.04 ms in control and 0.58 ± 0.04 ms with isoflurane ($P < 0.05$, n = 6).

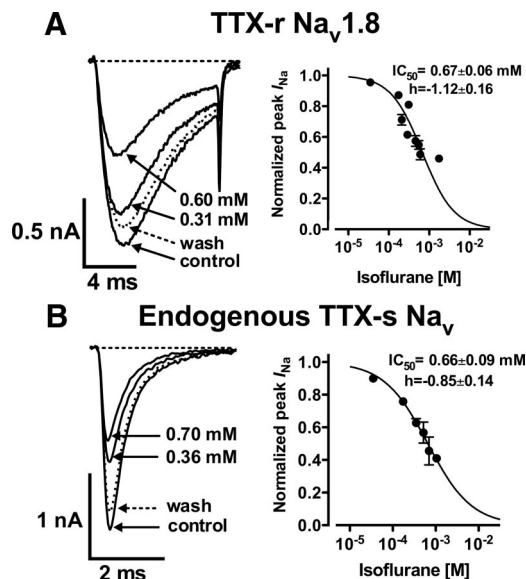


Fig. 5. Concentration-dependence for inhibition of tetrodotoxin-resistant (TTX-r) Na_v1.8 (A) and tetrodotoxin-sensitive (TTX-s) Na_v (B) by isoflurane. *Left panels* show representative traces of TTX-r Na_v1.8 (A) or TTX-s Na_v (B) Na⁺ currents in the absence (*control*) or presence of two concentrations of isoflurane, and the subsequent washout of isoflurane (*dotted line*). The *dashed line* represents the baseline. Normalized peak I_{Na} values for TTX-r Na_v1.8 (n = 33) and for TTX-s Na_v (n = 13) were fitted to the Hill equation to yield IC_{50} values and Hill slopes (b). The IC_{50} values and Hill slopes were not significantly different by sum-of-squares F test. Holding potential, $V_h = -70$ mV.

IC_{50} values for isoflurane inhibition of I_{Na} were obtained by eliciting peak I_{Na} from a holding potential of -70 mV. Normalized peak I_{Na} values were fitted to the Hill equation to yield IC_{50} and Hill slope values (fig. 5). The IC_{50} values of 0.67 ± 0.06 mM for TTX-r Na_v1.8 and 0.66 ± 0.09 mM for TTX-s Na_v, and Hill slopes of -1.12 ± 0.16 for TTX-r Na_v1.8 and -0.85 ± 0.14 for TTX-s Na_v were not significantly different. Significant inhibition occurred at isoflurane concentrations as low as 0.17 ± 0.01 mM (equivalent to 0.58 MAC after temperature correction to 24°C) for both TTX-r Na_v1.8 ($P < 0.01$; n = 6) and TTX-s Na_v ($P < 0.001$; n = 8).

Voltage-dependent Block of Na_v1.8

At the physiologic holding potential of -70 mV, isoflurane (0.53 ± 0.05 mM) significantly reduced the normalized peak I_{Na} to 0.55 ± 0.03 for TTX-r Na_v1.8 ($P < 0.001$, n = 8) and to 0.56 ± 0.06 for TTX-s Na_v ($P < 0.01$, n = 5) (fig. 6). Inhibition by isoflurane was significantly less from a holding potential of -140 mV, at which most channels are in the closed resting state (normalized peak I_{Na} was 0.91 ± 0.03 ($P < 0.05$, n = 8) for TTX-r Na_v1.8 and 0.91 ± 0.01 ($P < 0.001$, n = 6) for TTX-s Na_v).

Use-dependent Block of Na_v1.8

Preferential interaction of isoflurane with the inactivated state of Na⁺ channels results in accumulation of drug-bound channels during high-frequency stimula-

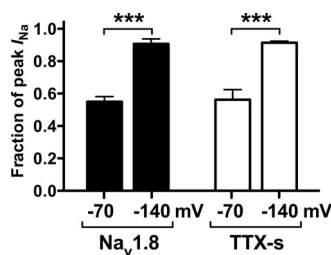


Fig. 6. Voltage-dependent effects of isoflurane on inhibition of peak I_{Na} . Tetrodotoxin-resistant $Na_v1.8$ (closed bars) and tetrodotoxin-sensitive (TTX-s) Na_v (open bars) Na^+ currents were inhibited strongly by isoflurane (0.53 ± 0.06 mM, equivalent to 1.8 minimum alveolar concentration [MAC] when corrected to 24°C) at the physiologic holding potential of -70 mV, but minimally when held at -140 mV. *** $P < 0.0001$, paired two-tailed Student t test, $n = 5-10$.

tion.³⁶ Native TTX-r Na^+ channels found in DRG neurons recover quickly from inactivation after membrane repolarization,³⁷ and similar behavior has been reported for $Na_v1.8$ in ND7/23 cells.³⁰ We used 60 pulses to $+10$ mV to determine the decay in peak amplitude of $Na_v1.8$ at various frequencies (1, 3, and 10 Hz). In control experiments, use-dependent block was more pronounced at higher stimulation frequencies (fig. 7). In the presence of isoflurane (0.56 ± 0.08 mM), $Na_v1.8$ currents showed a greater use-dependent decrement compared to control at all three stimulation frequencies tested. At a stimulation frequency of 1 Hz, the plateau of normalized peak I_{Na} , which was determined by fitting the data to a single exponential function, was 0.94 ± 0.01 in control and 0.85 ± 0.02 in the presence of isoflurane ($P < 0.001$, $n = 5$). At a stimulation frequency of 3 Hz, the plateau of normalized peak I_{Na} was 0.88 ± 0.01 in control and 0.81 ± 0.01 in the presence of isoflurane ($P < 0.001$, $n = 5$). At the highest frequency of 10 Hz, the plateau of normalized peak I_{Na} was 0.78 ± 0.01 in control and 0.64 ± 0.01 in the presence of isoflurane ($P < 0.001$, $n = 5$). Control data for use-dependent reductions in $Na_v1.8$ current are comparable to those published previously for this channel.^{27,30}

Discussion

Voltage-gated Na^+ channel isoforms are pharmacologically distinguishable, and are in fact classified, by their differential sensitivities to the specific inhibitor tetrodotoxin. Considerable evidence indicates that TTX-s isoforms are reversibly inhibited by clinical concentrations of volatile anesthetics, but anesthetic effects on the TTX-r isoforms are poorly characterized. A previously published study suggested that $Na_v1.8$ was unique among Na_v isoforms tested in its resistance to inhibition by isoflurane when tested using heterologous expression in amphibian oocytes,²¹ but this has not been confirmed in neuronal cells. We investigated the effects of the commonly used inhaled anesthetic isoflu-

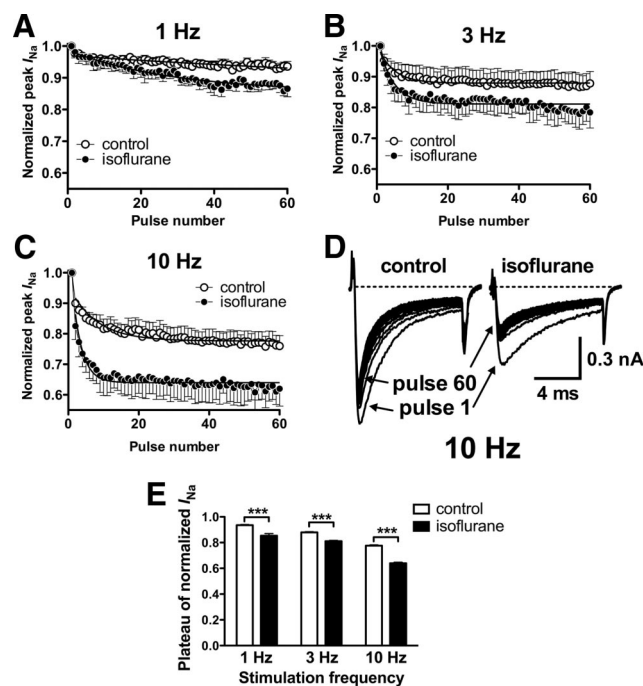


Fig. 7. Use-dependent block of tetrodotoxin-resistant $Na_v1.8$ currents in the absence (control, open symbols) or presence (closed symbols) of isoflurane (0.56 ± 0.08 mM; 1.6 minimum alveolar concentration [MAC]). Whole-cell currents were evoked by 60-step depolarization commands (holding potential -70 mV; test potential $+10$ mV; pulse duration 10 ms) delivered at 1 Hz (A), 3 Hz (B) or 10 Hz (C). Peak-current amplitude values (mean \pm SEM, $n = 5$) were normalized to that of the first response at each frequency and plotted against pulse number. The normalized first pulse amplitude was reduced to 0.54 ± 0.03 of control by isoflurane. (D) Representative recordings at 10 Hz in the absence (left) or presence (right) of isoflurane. Arrows mark traces for pulse 1 and pulse 60 (dashed line represents baseline). Data were fitted by a mono-exponential equation, and values for fractional block of the plateau of normalized I_{Na} are shown in E. *** $P < 0.001$, paired two-tailed Student t test, $n = 5$.

rane on endogenously expressed TTX-s and heterologously expressed TTX-r $Na_v1.8$ currents in a neuronal cell line.

Isoflurane inhibited both TTX-r $Na_v1.8$ and endogenous TTX-s Na_v with similar potencies ($IC_{50} = 0.67$ mM or 0.66 mM, respectively). These concentrations correspond to 2.3 MAC in rat after temperature correction to 24°C , and they are similar to those reported previously for inhibition of $Na_v1.2$, $Na_v1.4$, and $Na_v1.5$ heterologously expressed in Chinese hamster ovary cells by isoflurane ($IC_{50} = 0.70$, 0.61 , and 0.45 mM, respectively).²⁰ Although the IC_{50} values are somewhat higher than clinically relevant concentrations, significant inhibition occurs in the more clinically relevant concentration range of more than 0.5 times MAC.^{20,24,38} Moreover, small reductions in I_{Na} can have large physiologic effects due to nonlinear coupling.²⁴ The finding that isoflurane inhibits $Na_v1.8$ expressed in a mammalian neuronal cell line but not when expressed in *Xenopus* oocytes²¹ demonstrates the importance of an appropriate expression system for pharmacological studies of these channels.

It is now clear that both TTX-r and TTX-s Na_v isoforms are inhibited by inhaled anesthetics and do not exhibit major differences in anesthetic sensitivity.²⁰ Sensitivity to inhaled anesthetics is even present in the homologous prokaryotic Na⁺ channel NaChBac, indicating that anesthetic sensitivity is related to a fundamental evolutionarily conserved Na⁺ channel property.³⁹ In addition to their differential sensitivities to tetrodotoxin, Na_v isoforms have been reported to have different sensitivities to local anesthetics. In rat DRG neurons and with oocyte expression, TTX-r currents (primarily Na_v1.8) are more sensitive to inhibition by lidocaine than TTX-s channels, despite their highly conserved amino acid sequences.^{7,14} Sensitivity of Na_v isoforms to local anesthetics is determined primarily by conserved residues in the DIV-S6 segment,⁴⁰ whereas the greater sensitivity of Na_v1.7 and Na_v1.8 to lidocaine has been proposed to result from minor sequence differences in the DI and DII S6 segments,¹⁴ although this could be affected by differences in voltage dependence of inactivation. The comparable sensitivities of various Na_v isoforms to isoflurane suggest a conserved drug-binding domain, perhaps in DIV-S6.

Analysis of Na_v1.8 pharmacology has been hampered by difficulties in expressing functional channels. Initial expression in *Xenopus* oocytes showed relatively small currents,⁴ and attempts by other groups to express Na_v1.8 in mammalian cell lines, including COS-7,⁴¹ CHO,⁴² and HEK-293 cells,²⁷ resulted in very low levels of functional expression. However ND7/23 cells, derived from rat DRG and mouse neuroblastoma (N18TG2) cells, are suitable for transient and stable expression of recombinant Na_v1.8 in a mammalian neuronal environment.²⁸ These cells endogenously express Na_v β1- and β3-subunits,²⁷ which are sufficient for the functional expression and stability of Na_v1.8 α-subunits. Cotransfection of Na_v1.8 with the β1-subunit²⁹ or β3-subunit²⁷ does not alter current kinetics, activation, or inactivation characteristics of TTX-r currents in ND7/23 cells.

Isoflurane had negligible effects on the voltage dependence of Na_v1.8 activation, but it produced a hyperpolarizing shift in the voltage-dependence of fast and steady-state inactivation. This behavior is consistent with selective interaction of isoflurane with channels in the inactivated state as described previously for other Na⁺ channel isoforms including Na_v1.2 and Na_v1.4.¹⁹⁻²¹ The molecular basis of this block has yet to be determined for volatile anesthetics. Our results suggest that the shift in voltage-dependence of inactivation might result from slowing of inactivation evidenced by the increased time constants of current decay. The functional consequence is a reduction in the range of membrane potentials over which Na_v1.8 can operate, as confirmed by the voltage-dependence of isoflurane inhibition.

Other Na⁺ channel blockers such as local anesthetics (e.g., lidocaine) and certain anticonvulsants and antiarrhythmics also exhibit state-dependent drug interactions

with Na_v as described by the modulated receptor hypothesis.⁴³ Voltage-dependent block by local anesthetics results in a hyperpolarizing shift in steady-state inactivation, thus enhancing channel block at normal as opposed to hyperpolarized potentials. Isoflurane apparently inhibits Na⁺ channels by a similar mechanism involving enhanced inactivation. Selective interaction with inactivated states is consistent with the use-dependent block by isoflurane, which increases the fraction of channels in the inactivated state. Na⁺ channels undergo both fast and slow inactivation, and slow inactivation contributes to the use-dependent effects of some drugs.⁴⁴ The contribution of slow inactivation to the effects general anesthetics on Na_v block is an interesting question for future investigation.

The rate at which Na⁺ channels recover from inactivation (repriming) determines how well channels respond to high firing rates. Isoform-specific differences in repriming rates have been reported.⁴⁵ Interestingly, repriming rates of TTX-r Na⁺ currents, which are “slow” in terms of time to peak current and time constant of current decay, are about 10-fold faster than those of TTX-s Na⁺ currents in rat DRG neurons. Use-dependent block of Na_v1.8 by isoflurane could be due to its slow dissociation from blocked channels during repolarization, effectively slowing the repriming rate, but this is unlikely given the low affinity interaction. Lidocaine does not interfere with movement of the cytoplasmic inactivation loop, which is the underlying mechanism for fast inactivation, such that lidocaine-induced slowing of Na⁺ channel repriming does not result from slow recovery of the fast-inactivation gate.⁴⁶ This suggests that use-dependent block does not involve accumulation of fast-inactivated channels, but it could involve effects on slow inactivation mechanisms. By analogy with local anesthetics, stabilization of inactivated channel states and/or open channel block by isoflurane is currently a more plausible explanation.³⁸

The Na⁺ current underlying the depolarization phase of the action potential in nociceptive DRG neurons is carried primarily by Na_v1.8, which is expressed exclusively in this cell type.^{4,10} Slowly inactivating TTX-r Na⁺ currents are eliminated in DRG neurons of Na_v1.8 knockout mice, which confirms the role of Na_v1.8 in conducting these currents.⁸ Both antisense and knock-out studies support a role for Na_v1.8 activation in inflammatory pain.⁸ Previous studies using antisense nucleotides suggested a role for Na_v1.8 in neuropathic pain,¹³ but a recent study shows that Na_v1.8 is necessary for mechanical, cold, and inflammatory pain, but not for neuropathic and heat pain.⁴⁷ Visceral pain, a major consideration in the perioperative setting, has been attributed to Na_v1.8 since knockout mice show decreased visceral pain and referred hyperalgesia.⁴⁸ Subanesthetic concentrations of isoflurane, which would probably have relatively small effects on Na_v1.8, depress the nociceptive

reflex to single electrical stimuli in humans,^{49,50} whereas anesthetic concentrations of 1 MAC are required to depress the response to repetitive stimuli critical to central hyperexcitability in humans.^{49,50} In addition, volatile anesthetics, including isoflurane, significantly suppress development of spinal sensitization in the rat paw formalin test, which has implications for the development of postoperative pain.⁵¹ Moreover, isoflurane has peripheral antinociceptive effects in a number of animal models in which supraspinal modulatory and/or pronociceptive effects were surgically or pharmacologically eliminated.⁵²⁻⁵⁴ The anesthetic concentrations required for these effects on pain processing are consistent with the sensitivity of Na_v1.8 to inhibition by isoflurane and a possible role in nociceptive processing by DRG neurons. This inhibition would be enhanced at high firing frequencies and depolarized membrane potentials, conditions that occur with tissue injury and inflammation, based on the frequency- and voltage-dependence of isoflurane block. Recent studies also implicate volatile anesthetic activation and sensitization of TRPV1 ion channels in lowering the threshold for heat activation.⁵⁵ Anesthetic modulation of peripheral Na⁺ channels such as Na_v1.8, therefore, has the potential to modulate these poorly characterized pronociceptive mechanisms. The role of isoflurane inhibition of Na_v1.8 in acute perioperative pain and the development of hyperexcitability is an interesting topic for further investigation.

In conclusion, both TTX-r Na_v1.8 and TTX-s Na_v were inhibited by isoflurane at concentrations that occur during clinical anesthesia. This is consistent with a conserved drug-binding site among various Na_v isoforms. The critical role of Na_v1.8 in peripheral pain mechanisms suggests that its inhibition could contribute to the antinociceptive and possibly antiinflammatory effects of isoflurane and other inhaled anesthetics capable of blocking these channels.

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