

# Volatile anesthetics inhibit sodium channels without altering bulk lipid bilayer properties

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Although general anesthetics are clinically important and widely used, their molecular mechanisms of action remain poorly understood. Volatile anesthetics such as isoflurane (ISO) are thought to alter neuronal function by depressing excitatory and facilitating inhibitory neurotransmission through direct interactions with specific protein targets, including voltage-gated sodium channels ( $\text{Na}_v$ ). Many anesthetics alter lipid bilayer properties, suggesting that ion channel function might also be altered indirectly through effects on the lipid bilayer. We compared the effects of ISO and of a series of fluorobenzene (FB) model volatile anesthetics on  $\text{Na}_v$  function and lipid bilayer properties. We examined the effects of these agents on  $\text{Na}_v$  in neuronal cells using whole-cell electrophysiology, and on lipid bilayer properties using a gramicidin-based fluorescence assay, which is a functional assay for detecting changes in lipid bilayer properties sensed by a bilayer-spanning ion channel. At clinically relevant concentrations (defined by the minimum alveolar concentration), both the FBs and ISO produced prepulse-dependent inhibition of  $\text{Na}_v$  and shifted the voltage dependence of inactivation toward more hyperpolarized potentials without affecting lipid bilayer properties, as sensed by gramicidin channels. Only at supra-anesthetic (toxic) concentrations did ISO alter lipid bilayer properties. These results suggest that clinically relevant concentrations of volatile anesthetics alter  $\text{Na}_v$  function through direct interactions with the channel protein with little, if any, contribution from changes in bulk lipid bilayer properties. Our findings further suggest that changes in lipid bilayer properties are not involved in clinical anesthesia.

## INTRODUCTION

The public demonstration of the anesthetic properties of diethyl ether in 1846 initiated the era of modern anesthesia, enabling more complex and longer surgical procedures. Volatile anesthetics in current use are nonflammable fluorinated derivatives of ether that are capable of producing general anesthesia, a drug-induced coma-like state characterized by unconsciousness, amnesia, and immobility (Rudolph and Antkowiak, 2004; Hemmings et al., 2005). Despite their widespread clinical use, however, the molecular, cellular, and systems mechanisms underlying anesthesia remain incompletely understood.

For many years, general anesthetics were considered to produce their effects by altering membrane properties through unspecified interactions with the lipid bilayer, a concept rooted in the Meyer–Overton correlation between anesthetic potency and lipophilicity (Meyer, 1899; Overton, 1901). These nonspecific and usually undefined

interactions with the lipid bilayer formed the basis for a lipid-based theory of anesthetic action (Perouansky, 2012). The demonstration that anesthetics bind directly to globular proteins in the absence of lipids (Ueda and Kamaya, 1973; Franks and Lieb, 1984), and that anesthetics produce measurable changes in lipid structure only at supra-anesthetic concentrations (Franks and Lieb, 1979), led to the search for relevant protein targets. Volatile anesthetics have since been shown to affect the function of  $\gamma$ -aminobutyric acid ( $\text{GABA}_A$ ) receptors (Zimmerman et al., 1994), NMDA receptors (Dickinson et al., 2007; Hasenader et al., 2008), two-pore domain potassium background channels (Patel and Honoré, 2001; Sirois et al., 2002), and voltage-gated calcium (Study, 1994; Nikonorov et al., 1998) and sodium (Herold and Hemmings, 2012) channels ( $\text{Na}_v$ ).

It is now generally accepted that general anesthetics alter neuronal function by modulating excitatory and inhibitory synaptic transmission (Rudolph and Antkowiak, 2004; Hemmings et al., 2005), and that anesthetics interact directly with proteins rather than acting solely through the lipid bilayer (Franks and Lieb, 1994; Eckenhoof and

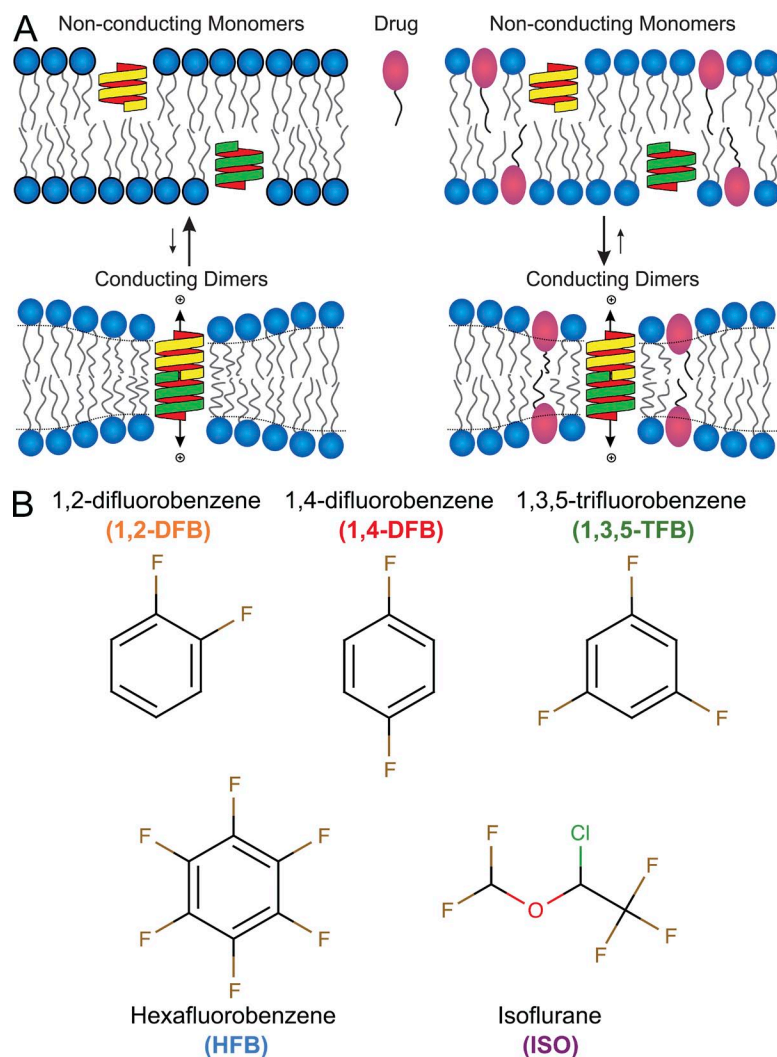
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Abbreviations used in this paper: ANTS, 8-aminonaphthalene-1,3,6-trisulfonate; DFB, difluorobenzene; GABA,  $\gamma$ -aminobutyric acid; GBFA, gramicidin-based fluorescence assay; HFB, hexafluorobenzene; ISO, isoflurane; LUV, large unilamellar vesicle; MAC, minimum alveolar concentration;  $\text{Na}_v$ , voltage-gated sodium channel(s); TFB, trifluorobenzene.

Johansson, 1997; Quillin et al., 2000). Indeed, prokaryotic ligand-gated channels of the Cys-loop family have been co-crystallized with anesthetics (Nury et al., 2011; Spurny et al., 2013), and anesthetic-binding sites in the bacterial sodium channel NaChBac have been inferred from molecular dynamics simulations (Raju et al., 2013). Although there is convincing evidence for the importance of direct anesthetic–protein interactions, it remains unclear if anesthesia can be ascribed solely to direct effects on membrane proteins or whether changes in lipid bilayer properties might also be involved. The diversity of targets that are sensitive to inhaled anesthetics (Eger et al., 2008), together with the inability to eliminate anesthesia in mice engineered to express volatile anesthetic-resistant mutant ion channels (Zeller et al., 2008; Werner et al., 2011), suggests that we need to look further—and modern theories of small-molecule interactions with lipid bilayers (Gruner and Shyamsunder, 1991; Cantor, 1997; Sonner and Cantor, 2013) provide a possible mechanism for lipid bilayer–based theories of anesthetic effects on ion channel function.

We therefore explored the effects of the family of fluorobenzene (FB) anesthetics (Solt et al., 2006), as well as the clinically used agent isoflurane (ISO), on the function of  $\text{Na}_v$  expressed in neuronal cells and on lipid bilayer mechanical properties as sensed by prokaryotic gramicidin channels (Lundbæk et al., 2010b; compare Fig. 1 A).

The principles underlying the use of gramicidin channels to detect changes in bilayer properties are illustrated in Fig. 1. Gramicidin channels are dimers (D) formed by the trans-bilayer association of monomeric subunits (M) from each bilayer leaflet. The channel's exterior is coupled to the lipid bilayer core through hydrophobic interactions and, as is the case for other membrane-spanning channels, gramicidin channels will locally reorganize the surrounding bilayer. Channel formation in a bilayer with a hydrophobic thickness ( $d_0$ ) that exceeds the channel hydrophobic length ( $l$ ) involves a local bilayer deformation (thinning) (Huang, 1986; Lundbæk and Andersen, 1994; Nielsen et al., 1998; Harroun et al., 1999). This local bilayer deformation has an associated energetic cost, the bilayer deformation energy ( $\Delta G_{\text{def}}^0$ ), which varies as



**Figure 1.** Use of the gramicidin (gA) channel as a molecular force probe to detect changes in lipid bilayer properties. (A; left) Schematic of gA channel formation in the bilayer. Two nonconducting channel monomers (yellow and green structures) form a conducting dimer, enabling monovalent cations to pass through the pore. In the GBFA, changes in the gramicidin monomer↔dimer equilibrium are evaluated from changes in the rate of thallium ( $\text{Tl}^+$ ) influx into fluorophore-loaded LUVs where we exploit that  $\text{Tl}^+$  is a fluorescence quencher (see Materials and methods for details). (Right) gA dimerization is altered in the presence of a bilayer-modifying drug (red ovals). The drug partitions between the aqueous solution and the bilayer, and redistributes (between the aqueous solution and the membrane and in the plane of the membrane) in response to local bilayer deformation, which may increase the degrees of freedom available for the membrane to adapt to the bilayer deformation. (B) Chemical structures of the anesthetics used in this study. Their properties are summarized in Table 1.

a function of the hydrophobic mismatch between the channel length ( $l$ ) and the bilayer thickness ( $d_0$ ), and the channel radius and bilayer material properties — thickness, intrinsic curvature, and the associated elastic moduli (Huang, 1986; Nielsen et al., 1998; Nielsen and Andersen, 2000; Rusinova et al., 2011). Changes in bilayer properties will alter the bilayer deformation energy associated with the monomers and dimers,  $\Delta G_{\text{def}}^{\text{M}}$  and  $\Delta G_{\text{def}}^{\text{D}}$ , respectively, and thereby the bilayer contribution to the free energy of dimerization ( $\Delta G_{\text{total}}^{\text{M} \rightarrow \text{D}} = \Delta G_{\text{protein}}^{\text{M} \rightarrow \text{D}} + \Delta G_{\text{bilayer}}^{\text{M} \rightarrow \text{D}}$ , where  $\Delta G_{\text{total}}^{\text{M} \rightarrow \text{D}}$  denotes intrinsic contributions, such as the formation of the hydrogen bond-stabilized interface between the two monomers and  $\Delta G_{\text{bilayer}}^{\text{M} \rightarrow \text{D}} = \Delta G_{\text{def}}^{\text{D}} - \Delta G_{\text{def}}^{\text{M}}$ ), and thus the gramicidin dimerization constant ( $K^{\text{M} \rightarrow \text{D}} = \exp\{\Delta G_{\text{total}}^{\text{M} \rightarrow \text{D}}/k_B T\}$ ) and the number of conducting channels per unit area.

Gramicidin channels have been calibrated as probes of changes in lipid bilayer mechanical properties (Lundbæk and Andersen, 1994; Lundbæk et al., 1997; Goulian et al., 1998). In the present context, it is imperative that gramicidin channels sense changes in intrinsic lipid curvature—or lateral pressure profile (Andersen and Koeppe, 2007; Marsh, 2007). Gramicidin channels have proven useful for exploring how small molecules alter lipid bilayer properties that are relevant for altering the function of channels formed by integral membrane proteins (Lundbæk et al., 1996, 2004, 2005; Suchyna et al., 2004; Artigas et al., 2006; Sogaard et al., 2006; Rusinova et al., 2011; Ingólfsson et al., 2014). For the present study, it is important that  $\text{Na}_v$  (Lundbæk et al., 2004, 2005; Rusinova et al., 2011) and  $\text{GABA}_A$  channels (Sogaard et al., 2006; Chisari et al., 2010), both of which are important anesthetic targets (Hemmings et al., 2005), are modulated by amphiphiles at concentrations that alter lipid bilayer properties, as sensed by gramicidin channels.

We chose the FBs as model volatile anesthetics because of their closely related structures (Fig. 1 B) and physicochemical properties. These compounds were once considered for clinical use as anesthetics but were rejected in favor of modern inhaled anesthetics (Burns et al.,

1961, 1964; Fang et al., 1996). They remain valuable model compounds for investigating molecular mechanisms of anesthetics (Solt et al., 2006). We find that at clinically relevant concentrations, the FBs and ISO inhibit  $\text{Na}_v$  function with no obvious effects on gramicidin channel function, demonstrating that their effects are unlikely to involve alterations in lipid bilayer properties but rather result from direct interactions with  $\text{Na}_v$ .

## MATERIALS AND METHODS

### Materials

1,2-difluorobenzene (DFB), 1,4-DFB, 1,3,5-trifluorobenzene (1,3,5-TFB), and hexafluorobenzene (HFB) were purchased from Sigma-Aldrich. ISO (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane) was from Abbott Laboratories. All other chemicals were from Sigma-Aldrich. Lipids were from Avanti Polar Lipids, Inc.

### Cell culture and electrophysiology

$\text{Na}^+$  currents were recorded from rodent neuronal ND7/23 cells, which express endogenous tetrodotoxin-sensitive  $\text{Na}^+$  currents, using conventional whole-cell patch-clamp electrophysiology (Hamill et al., 1981). Cells were cultured on 12-mm glass coverslips in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (Gibco) at 37°C under 95% air plus 5%  $\text{CO}_2$ . For recordings, coverslips were transferred into a small-volume laminar-flow perfusion chamber (Warner Instruments) and continuously perfused at 2 ml/min with extracellular solution containing (mM): 130 NaCl, 10 HEPES, 3.25 KCl, 2  $\text{MgCl}_2$ , 2  $\text{CaCl}_2$ , 0.1  $\text{CdCl}_2$ , 20 TEACl, and 5 D-glucose, adjusted to pH 7.4 (with NaOH) and 310 mOsm/kg  $\text{H}_2\text{O}$  (with sucrose).  $\text{Na}^+$  currents were recorded in voltage-clamp mode at room temperature (23–24°C) using a patch-clamp amplifier (Axopatch 200B; Molecular Devices) with a 10-kHz low-pass filter at a sampling rate of 50 kHz. Recording pipettes were pulled from borosilicate glass capillaries (Sutter Instrument) on a horizontal puller (P-97; Sutter Instrument) and fire-polished before use. Pipettes were filled with internal solution containing (mM): 120 CsF, 10 NaCl, 10 HEPES, 10 EGTA, 10 TEACl, 1  $\text{CaCl}_2$ , and 1  $\text{MgCl}_2$ , adjusted to pH 7.3 (with CsOH) and 310 mOsm/kg  $\text{H}_2\text{O}$  (with sucrose); pipette resistance was 1.5–2.5 M $\Omega$ . Access resistance (2–4 M $\Omega$ ) was reduced using 75–85% series resistance correction. Initial whole-cell seal resistance was 2–4 G $\Omega$ ; recordings were discarded if resistance dropped below 1 G $\Omega$ . Liquid-junction potentials were not corrected. Capacitive current transients were electronically cancelled with the internal amplifier circuitry, and leak currents were digitally subtracted online using the P/4 protocol (Bezannila and Armstrong, 1977).

Before each experiment, stock solutions of FBs in DMSO were diluted with external perfusion buffer; both test and control solutions contained 0.14% (vol/vol) DMSO. ISO solutions were prepared as described previously (Herold et al., 2009). Clinical concentrations of volatile anesthetics are expressed relative to the minimum alveolar concentration (MAC; see Table 1), which is defined as the concentration necessary to inhibit movement in 50% of subjects in response to a painful stimulus (Eger et al., 1965). We corrected these MAC values for our experimental conditions of 25°C (Franks and Lieb, 1993). To reduce loss of volatile compounds, the perfusion system consisted of gastight glass syringes with Teflon tubing connected to a 0.2-mm diameter perfusion pipette positioned in close proximity to the patch-clamped cell with a perfusion rate of  $\sim 0.2$  ml/min controlled by a pressurized

TABLE 1  
*Properties of anesthetics*

Anesthetic	MAC at 25°C	cLogP	Membrane concentration (at 1 MAC)	Mole fraction (at 1 MAC)
	mM		mM	
1,2-DFB	0.70	2.2	106	0.087
1,4-DFB	0.59	2.4	135	0.109
1,3,5-TFB	0.60	2.5	174	0.136
HFB	0.25	2.4	57	0.049
ISO	0.30	2.3	57	0.049

MAC values from Solt et al. (2006); cLogP values were estimated using the ACD/Labs LogP algorithm on ChemSpider. Concentrations and mole fractions were calculated as described in Materials and methods.



perfusion system (ALA Scientific Instruments). Anesthetic concentrations were determined using gas chromatography as described previously (Ratnakumari and Hemmings, 1998).

Mole fractions of the anesthetics in the lipid bilayer were estimated following Bruno et al. (2007). Membrane concentrations of anesthetic  $[\text{Drug}]_m$  were determined as:

$$[\text{Drug}]_m = K_p \cdot [\text{Drug}]_{\text{nom}} \cdot \frac{V_{\text{aq}}}{V_{\text{aq}} + K_p \cdot V_{\text{lip}}},$$

where  $K_p$  is the octanol/water partition coefficient,  $[\text{Drug}]_{\text{nom}}$  is the nominal concentration of (added) drug in the aqueous solution,  $V_{\text{aq}}$  is the aqueous volume in the vesicle system, and  $V_{\text{lip}}$  is the lipid volume in the vesicle system; the second term on the right accounts for depletion caused by drug redistribution between aqueous and membrane phases. The mole fraction of anesthetic,  $m_{\text{Drug}}$ , was determined as:

$$m_{\text{Drug}} = \frac{[\text{Drug}]_m}{[\text{Drug}]_m + [\text{Lipid}]_m},$$

where  $[\text{Lipid}]_m$  denotes the lipid concentration in the membrane phase ( $\sim 1$  M). Given the relatively modest anesthetic hydrophobicity, aqueous concentrations will be close to nominal concentrations, and corrections for depletion that occur because of anesthetic redistribution between the aqueous and membrane phases are minimal.  $K_p$  was estimated as  $10^{\text{cLogP}}$ , and cLogP was estimated using the ACD/Labs LogP algorithm (<http://www.chemspider.com>).

#### Stimulation protocols and data analysis

The holding potential ( $V_h$ ) was  $-80$  mV unless otherwise stated. Voltage-dependent inhibition of peak inward  $\text{Na}^+$  current ( $I_{\text{Na}}$ ) was analyzed using a 10-ms test pulse to 0 mV, preceded by a 300-ms prepulse alternating between either  $-130$  mV ( $V_0$ ) or the voltage of half-maximal inactivation ( $V_{1/2} = -69 \pm 4$  mV) applied every 5 s (Lundbæk et al., 2004; Rusinova et al., 2011).  $V_{1/2}$  was determined for each individual cell using the double-pulse protocol for steady-state inactivation described below.

Steady-state fast inactivation ( $h_\infty$ ) was measured using a double-pulse protocol with a 300-ms prepulse ranging from  $-120$  to  $-30$  mV in 10-mV steps, followed by a 10-ms test pulse to 0 mV. Peak currents during the test pulse were normalized to the maximal current ( $I/I_{\text{max}}$ , where  $I_{\text{max}}$  is the maximal current that is elicited at the test potential), plotted against the prepulse potential ( $V_m$ ), and fitted with a Boltzmann function (Rusinova et al., 2011):

$$I/I_{\text{max}} = 1 / \left( 1 + \exp \left\{ -e \cdot z_{1/2} \cdot (V_m - V_{1/2}) / k_B T \right\} \right),$$

where  $z_{1/2}$  and  $V_{1/2}$  denote the apparent gating valence and potential for half-maximal inactivation, respectively.

Recovery from fast inactivation was tested from a holding potential of  $-130$  mV using a two-pulse protocol with two identical 10-ms pulses to 0 mV separated by an interpulse interval with increasing durations from 1 to 100 ms. Current amplitudes were normalized ( $\text{Pulse}_2/\text{Pulse}_1$ ), plotted against interpulse interval, and fitted with the single-exponential equation to obtain the recovery time constants  $\tau$ :

$$Y_t = Y_0 + (\text{plateau} - Y_0) \cdot (1 - \exp\{-t/\tau\}),$$

where  $Y_t$  denotes the normalized current at time  $t$ ,  $Y_0$  denotes the normalized current at zero time, and  $\text{plateau}$  denotes the normalized current at infinity.

Programs used for data acquisition and analysis were pClamp 10 (Molecular Devices), Excel (Microsoft), Prism 6.0 (GraphPad

Software), and MATLAB (v7.4; The MathWorks, Inc.). Values are reported as mean  $\pm$  SD unless otherwise stated. Statistical significance ( $P < 0.05$ ) was assessed by two-tailed paired Student's  $t$  test.

#### Fluorescence quench measurements of changes in lipid bilayer properties

To probe for FB- and ISO-dependent changes in lipid bilayer properties, as sensed by a bilayer-spanning ion channel, we used a gramicidin-based fluorescence assay (GBFA) (Ingólfsson and Andersen, 2010; Ingólfsson et al., 2010, 2014; Rusinova et al., 2011). This method allows determination of how experimental interventions, such as the addition of anesthetics, alter the equilibrium distribution between nonconducting gramicidin monomers and conducting gramicidin dimers (channels) based on changes in influx rate of a gramicidin-channel permeant heavy ion quencher ( $\text{Ti}^+$ ) (Fig. 1 A). In brief, 1,2-dierucoyl-*sn*-glycero-3-phosphocholine ( $\text{DC}_{22:1}\text{PC}$ ) without or with gramicidin (molar ratio of 1,000:1) in chloroform was dried under a nitrogen stream, desiccated overnight, and rehydrated in an aqueous solution containing 25 mM of the fluorophore 8-aminonaphthalene-1,3,6-trisulfonate (ANTS), 100 mM  $\text{NaNO}_3$ , and 10 mM HEPES, pH 7. Experiments with cholesterol-containing vesicles were done using large unilamellar vesicles (LUVs) prepared from a 1:1 di-oleoyl-*sn*-3-phosphocholine ( $\text{DC}_{18:1}\text{PC}$ ) and cholesterol lipid mixture. LUVs were prepared by sonication, freeze-thawing, extrusion through a 100-nm polycarbonate membrane using a mini-extruder (Avanti Polar Lipids, Inc.), and elution on a desalting column to remove extravesicular ANTS. Changes in the gramicidin monomer $\leftrightarrow$ dimer equilibrium were estimated from the rate of ANTS fluorescence quenching by the gramicidin channel-permeable quencher  $\text{Ti}^+$  using a stopped-flow spectrofluorometer (SX.20; Applied Photophysics) with a dead time of  $\sim 1.5$  ms. Samples were excited at 352 nm, and fluorescence emission above 455 nm was recorded. Vesicles were incubated with anesthetics for 10 min at  $25^\circ\text{C}$  before measuring fluorescence quenching rate.

Data were analyzed using MATLAB (v7.9; The MathWorks, Inc.). Because of the unavoidable dispersion of LUV sizes, fluorescence quench curves cannot be described by single-exponential decays, so data were analyzed using a stretched exponential. This is a mathematically efficient way to express large sums of similar exponential decays (Peyron et al., 1996; Berberan-Santos et al., 2005). Fluorescence quench rates for the first 2–100 ms of the fluorescence time course for individual quench curves were fitted by the stretched exponential:

$$F(t) = F(\infty) + (F(0) - F(\infty)) \cdot \exp\left\{-(t/\tau_0)^\beta\right\},$$

where  $F(t)$  denotes the fluorescence intensity as a function of time  $t$ ,  $\tau_0$  is a parameter with units of time, and  $\beta$  ( $0 < \beta \leq 1$ ) is a measure of sample dispersity. The quenching rate at 2 ms was determined as (Berberan-Santos et al., 2005):

$$k(t) = (\beta/\tau_0) \cdot (t/\tau_0)^{(\beta-1)}.$$

Reported values are averages of quenching rates, normalized to the rate in the absence of anesthetic ( $n \geq 3$ ).

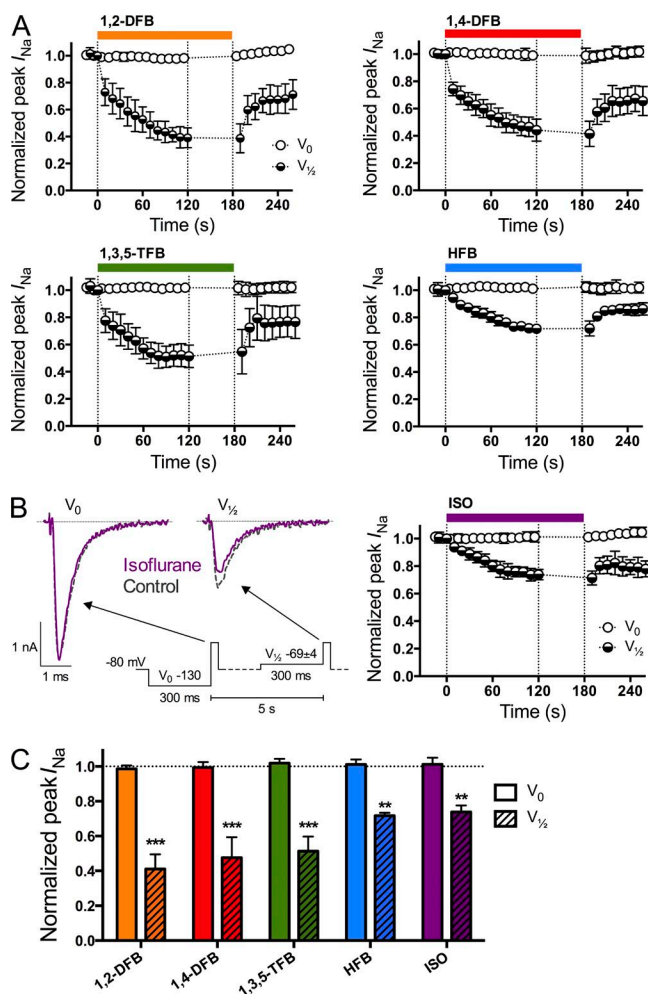
## RESULTS

#### Inhibition of peak $\text{Na}^+$ currents

The effects of FBs and ISO on neuronal  $\text{Na}^+$  currents were tested during drug wash-in and wash-out using a periodic stimulation protocol that allows determination of

voltage-dependent drug effects (Lundbæk et al., 2005; Rusinova et al., 2011; Fig. 2).

A depolarizing pulse to 0 mV was applied every 5 s to elicit peak Na<sup>+</sup> currents ( $I_{Na}$ ) after a 300-ms prepulse to either  $-130$  mV ( $V_0$ , at which most channels are in the resting state) or to the voltage of half-maximal steady-state inactivation ( $V_{1/2} = -69 \pm 4$  mV), which was determined for each cell under control conditions. Fig. 2



**Figure 2.** Inhibition of peak Na<sup>+</sup> current ( $I_{Na}$ ) by anesthetics. (A) Time course of the effects of wash-in and wash-out of the FBs and ISO at 1 MAC (see Table 1). (B; left) Macroscopic whole-cell current traces in the absence (gray discontinued traces) or presence (purple full traces) of ISO using a periodic stimulation protocol (inset) from a holding potential ( $V_h$ ) of  $-80$  mV. This alternating protocol was chosen to test voltage-dependent inhibition. A test pulse (10 ms at 0 mV) every 5 s to elicit peak  $I_{Na}$  was preceded by a 300-ms prepulse to either  $-130$  mV (denoted " $V_0$ "; B, left, left traces) or to a voltage at which approximately half of the channels were in the fast-inactivated state (denoted " $V_{1/2}$ ";  $-69 \pm 4$  mV; B, left, right traces). Peak  $I_{Na}$  was normalized to control. (C) The values after 120 s of drug treatment. All five anesthetics significantly inhibited peak  $I_{Na}$  with a prepulse to  $V_{1/2}$  but had no effect with a prepulse to  $V_0$  (Table 2). The gap between 120 and 180 s was used to record steady-state inactivation (see Fig. 3). Data are mean  $\pm$  SD;  $n = 6-9$ . \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$  versus control by paired two-tailed Student's  $t$  test.

(A and B) shows the time course of peak  $I_{Na}$  inhibition for each anesthetic; the rather slow time course of inhibition most likely represents drug partitioning among different intracellular compartments, as opposed to an intrinsic slow rate of binding to the anesthetic target, whether Na<sub>v</sub> or the host bilayer (Gingrich et al., 2009). Fig. 2 C summarizes results for peak  $I_{Na}$  inhibition (after a wash-in period of 120 s to reach steady state) after a prepulse to either  $V_0$  or  $V_{1/2}$  (see also Table 2). Results are expressed as the ratio of peak  $I_{Na}$  in the presence of drug to control  $I_{Na}$ .

With a prepulse to  $V_0$ , none of the anesthetics inhibited peak  $I_{Na}$ ; with a prepulse to  $V_{1/2}$ , all five anesthetics produced significant inhibition. 1,2-DFB had the greatest effect, and HFB and ISO had the least (Table 2). Inhibition was reversible after a 1-min washout period, although peak  $I_{Na}$  did not completely recover to control values. This presumably reflects that the steady-state inactivation curves did not reverse fully (see next section); reversal is complete when  $V_h$  is corrected by the difference between the control and washout  $V_{1/2}$  (not depicted). Although not tested here, ethanol, at concentrations similar to that used as a positive control in the stopped-flow bilayer experiments (see below and Fig. 5), also inhibits  $I_{Na}$  (Frenkel et al., 1997; Wu and Kendig, 1998; Klein et al., 2007; Horishita and Harris, 2008; Xiao et al., 2008).

#### Effect on steady-state inactivation

Inhibition of peak  $I_{Na}$  was observed only after a prepulse to  $V_{1/2}$ , consistent with a preferential anesthetic interaction with an inactivated state of the channel (Herold and Hemmings, 2012). This was tested further by determining steady-state inactivation curves in the absence or presence (after 120-s superfusion) of anesthetic using a double-pulse protocol. All five anesthetics shifted  $V_{1/2}$  (steady-state inactivation) significantly toward more hyperpolarized potentials (Fig. 3 and Table 3). Again, 1,2-DFB had the greatest effect, and HFB and ISO had the least.

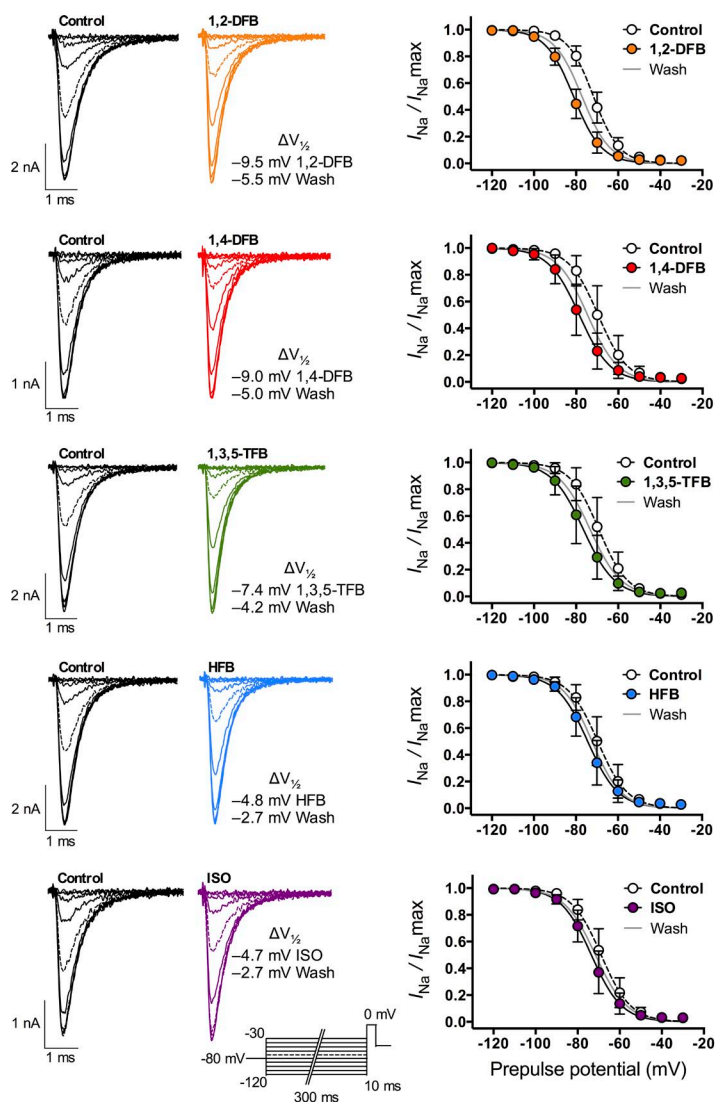
#### Recovery from inactivation

Recovery from inactivation was tested from a holding potential of  $-130$  mV at which the majority of channels

Anesthetic	Test pulse with prepulse to $V_0$	Test pulse with prepulse to $V_{1/2}$	<i>n</i>
1,2-DFB	0.99 $\pm$ 0.02	0.41 $\pm$ 0.08 <sup>a</sup>	7
1,4-DFB	0.99 $\pm$ 0.03	0.48 $\pm$ 0.12 <sup>a</sup>	7
1,3,5-TFB	1.02 $\pm$ 0.02	0.51 $\pm$ 0.08 <sup>a</sup>	7
HFB	1.01 $\pm$ 0.03	0.72 $\pm$ 0.02 <sup>b</sup>	7
ISO	1.01 $\pm$ 0.04	0.74 $\pm$ 0.04 <sup>b</sup>	9

<sup>a</sup> $P < 0.001$  versus control by two-tailed paired Student's  $t$  test.

<sup>b</sup> $P < 0.01$  versus control by two-tailed paired Student's  $t$  test.



**Figure 3.**  $\text{Na}^+$  channel steady-state inactivation.  $\text{Na}^+$  channel availability (or  $h_\infty$ ) was tested using a double-pulse protocol with a 300-ms prepulse ranging from  $-120$  to  $-30$  mV in 10-mV steps, followed by a 10-ms test pulse to 0 mV (inset at ISO traces shows stimulation protocol). Peak  $I_{\text{Na}}$  was normalized ( $I_{\text{Na}}/I_{\text{Na max}}$ ), plotted against prepulse potential, and fitted with a two-state Boltzmann distribution to calculate  $V_{1/2}$  (see Materials and methods for details). (Left) Families of  $\text{Na}^+$  current traces in the absence (black traces, control) or presence (colored traces, drug) of anesthetics at 1 MAC. (Right) Fitted data in the absence (open circles, dotted line) or presence (full colored circles, straight black line) of anesthetic, or after washout (straight gray line). All anesthetics caused a significant left-shift of  $V_{1/2}$  with values displayed next to each graph (see Table 3). Data are mean  $\pm$  SD;  $n = 5-7$ .

are in the resting, closed state. We compared 1,2-DFB, which showed the strongest effects in the previous experiments, to ISO (Fig. 4). Both anesthetics slowed recovery from inactivation;  $\tau$  increased from  $6.9 \pm 0.9$  ms to  $7.9 \pm 0.9$  ms for 1,2-DFB and from  $7.5 \pm 1.0$  ms to  $8.9 \pm 1.4$  ms for ISO (Fig. 4 C;  $n = 9$ ; mean  $\pm$  SD; \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$  vs. control).

### Effects on lipid bilayer properties

Lipid bilayer-perturbing properties were tested using a GBFA. The time course of fluorescence quenching resulting from the influx of the heavy ion quencher  $\text{Ti}^+$  into LUVs that have been loaded with the water-soluble fluorophore ANTS is monitored (Ingólfsson and Andersen, 2010; Ingólfsson et al., 2010). Gramicidin

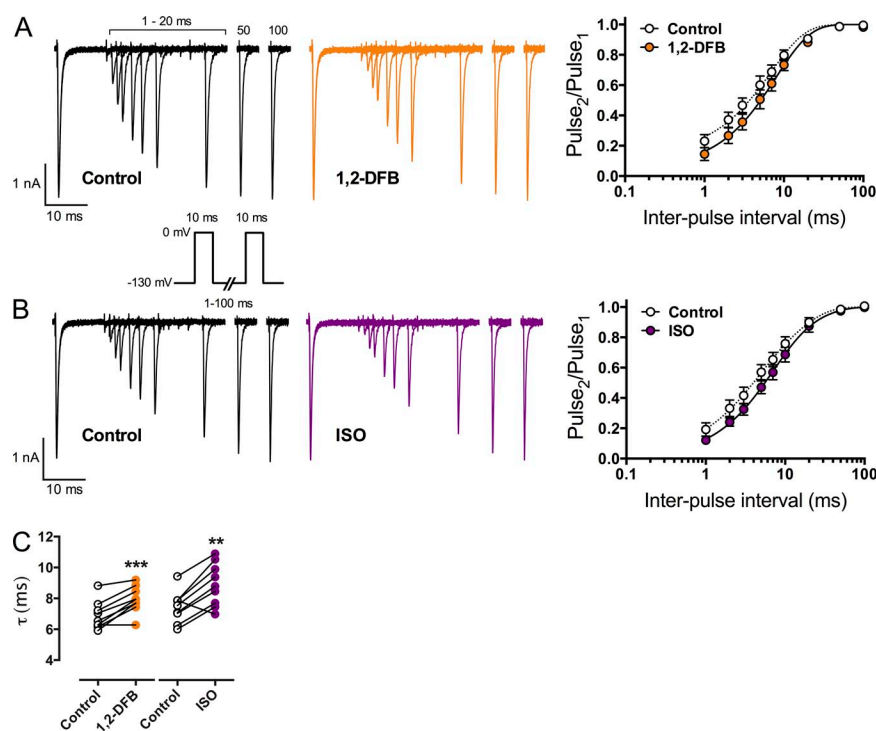
TABLE 3  
Anesthetic effects on the voltage dependence of  $\text{Na}_v$  inactivation

Anesthetic	$V_{1/2}$ control	$V_{1/2}$ drug	$V_{1/2}$ wash	$\Delta V_{1/2}$	$n$
1,2-DFB	$-71.7 \pm 2.8$	$-81.1 \pm 3.0^a$	$-77.1 \pm 3.6^b$	$-9.4 \pm 0.9$	6
1,4-DFB	$-69.5 \pm 5.6$	$-78.5 \pm 5.4^a$	$-74.6 \pm 5.6^a$	$-9.0 \pm 1.0$	6
1,3,5-TFB	$-69.2 \pm 5.4$	$-76.5 \pm 5.8^a$	$-73.3 \pm 5.9^b$	$-7.4 \pm 1.0$	5
HFB	$-69.6 \pm 4.9$	$-74.3 \pm 4.8^b$	$-72.2 \pm 5.0^c$	$-4.8 \pm 0.7$	5
ISO	$-68.8 \pm 4.2$	$-73.4 \pm 4.3^a$	$-71.6 \pm 3.3^b$	$-4.7 \pm 0.9$	6

<sup>a</sup> $P < 0.0001$  versus control by two-tailed paired Student's  $t$  test.

<sup>b</sup> $P < 0.001$  versus control by two-tailed paired Student's  $t$  test.

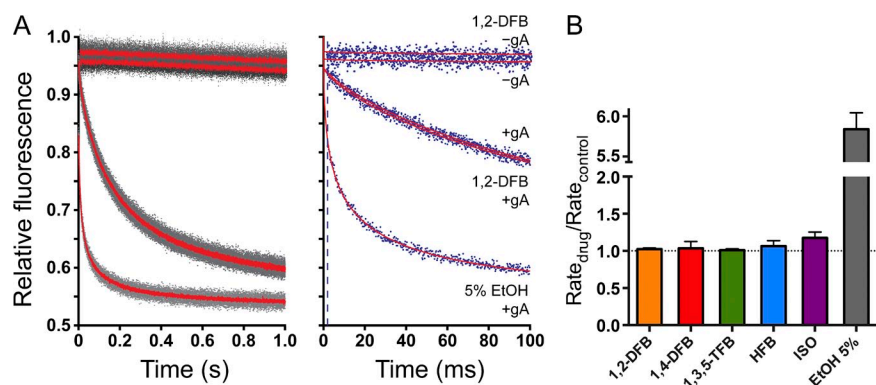
<sup>c</sup> $P < 0.01$  versus control by two-tailed paired Student's  $t$  test.



**Figure 4.**  $Na^+$  channel recovery from inactivation. A two-pulse protocol was used from a holding potential ( $V_h$ ) of  $-130$  mV, with two 10-ms test pulses to 0 mV separated by an interpulse interval from 1 to 100 ms (inset shows stimulation protocol). Peak  $I_{Na}$  of the second test pulse was normalized to the first ( $Pulse_2/Pulse_1$ ) and plotted against the interpulse interval. Data were fitted to a mono-exponential equation to calculate the time constant  $\tau$  (see Materials and methods for details). (A and B; left) Overlaid macroscopic  $Na^+$  current traces of the two pulses with increasing interpulse durations in the absence (black traces, control) or presence (colored traces, drug) of anesthetic at 1 MAC. Peak  $I_{Na}$  of the second test pulse slowly recovers to control values with increasing interpulse durations. (A and B; right) Fitted data in the absence (open circles, dotted line) or presence (closed colored circles, straight line) of anesthetic. (C) Recovery time constant  $\tau$  in the absence (open circles) or presence (colored circles) of anesthetic. Both anesthetics significantly increased  $\tau$ , thereby slowing recovery from inactivation. Data are mean  $\pm$  SD;  $n = 9$ . \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$  versus control by paired two-tailed Student's  $t$  test.

channels are very permeable to  $Tl^+$ , such that fluorescence quench rate varies with changes in the time-averaged number of conducting gramicidin channels in the LUV membrane, i.e., with changes in the dimerization constant (the free energy of dimerization). Gramicidin channels are sensitive reporters of changes in bilayer properties (Elliott et al., 1983; Lundbæk et al., 1997, 2010a) that are relevant for the function of voltage-gated calcium channels (Lundbæk et al., 1996) and  $Na_v$  (Lundbæk et al., 2004, 2005; Rusinova et al., 2011).

At 1 MAC, the FBs and ISO did not produce significant changes in quench rate (Fig. 5), meaning that they do not alter the gramicidin dimerization constant (or lipid bilayer properties that are sensed by gramicidin channels). This lack of effect should be compared with the changes produced by 5% ethanol ( $\sim 1$  M; Fig. 5). For ISO, only minimal changes in bilayer properties were observed at the supratherapeutic concentration of 4 MAC ( $\sim 1.1$  mM; Fig. 6 A). Only at extremely high concentrations of ISO (18 MAC or 5–6 mM, a lethal concentration) did ISO significantly alter fluorescence



**Figure 5.** Effects of anesthetics on lipid bilayer properties using a GBFA. (A) Examples of normalized fluorescence quench traces. (Left) Time course of normalized fluorescence decay over 1 s. Gray dots denote results from all repeats (more than five per condition), and red solid lines indicate the average of all repeats. (Right) The first 100 ms of the normalized fluorescence decay. Blue dots denote results from a single repeat for each condition; red lines correspond to the stretched exponential fits (2–100 ms) to those repeats; and the broken line marks the 2-ms time point, at which quenching rate is determined.

In both panels, the top two traces show the results without gramicidin (gA) in the presence (1,2-DFB – gA) or absence (–gA) of 1,2-DFB. The next two overlapping red traces show control gA in the absence (+gA) or presence (1,2-DFB + gA) of 1,2-DFB. The bottom trace corresponds to gA-containing vesicles treated with 5% ethanol (5% EtOH + gA) as a positive control. (B) The rate of quencher influx for anesthetics at 1 MAC and ethanol. None of the anesthetics significantly altered lipid bilayer properties at clinically relevant concentrations. Data are mean  $\pm$  SD;  $n = 3$ –4.



quench rate, and thus lipid bilayer properties as detected by the bilayer-spanning gramicidin channels (Fig. 6 A). We conclude that, at clinically relevant concentrations, the anesthetics tested cause minimal changes in the gramicidin monomer $\leftrightarrow$ dimer equilibrium, and therefore produce minimal changes in lipid bilayer properties.

To explore whether anesthetic effects might be affected by lipid bilayer composition, we also performed experiments using cholesterol-containing lipid bilayers (1:1 mixture of DC<sub>18:1</sub>PC and cholesterol). The results were similar to those obtained in experiments without cholesterol, and confirmed that neither ISO nor 1,2-DFB produces significant changes in lipid bilayer properties at concentrations as high as four times MAC (Fig. 6 B).

## DISCUSSION

Our key findings are that FB model anesthetics and the clinically used anesthetic ISO inhibit Na<sub>v</sub> function in neuronal cells at clinically relevant concentrations that do not perturb bulk lipid bilayer properties (as sensed by a bilayer-spanning reporter channel). These observations provide additional support for the notion that general anesthetics alter membrane protein function through direct (specific) interactions with ion channel proteins (in casu Na<sub>v</sub>) rather than indirect effects caused by altered lipid bilayer properties.

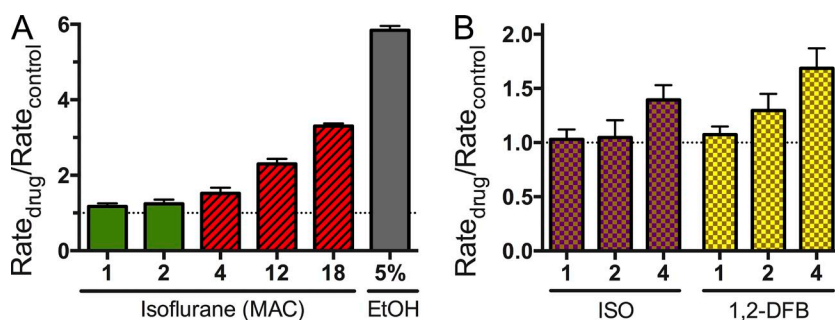
### Direct anesthetic effects on membrane proteins

Several putative molecular targets for volatile anesthetics have been identified based on their sensitivities to anesthetics at clinical concentrations; these include multiple ligand-gated and voltage-gated ion channels (Hemmings et al., 2005). Direct anesthetic–protein interactions likely involve binding to hydrophobic cavities, such as those identified in soluble proteins (Dubois et al., 1993; Franks et al., 1998; Quillin et al., 2000; Liu et al., 2005). Direct anesthetic binding has been demonstrated in prokaryotic ion channels (Barber et al., 2011;

Nury et al., 2011; Spurny et al., 2013) and in GABA<sub>A</sub> receptors by photolabeling experiments (Chen et al., 2012; Chiara et al., 2012; Yip et al., 2013). But a role for lipid bilayer-mediated effects on ion channel function has not been excluded. This is important because mice in which putative molecular targets have been genetically engineered to be resistant to volatile anesthetics remain sensitive to anesthetics (Liao et al., 2005; Sonner et al., 2007; Zeller et al., 2008; Werner et al., 2011).

One would expect anesthetic alteration of bilayer properties at higher concentrations (compare Franks and Lieb, 1979). Some anesthetics, including ISO (Haseneder et al., 2002) and pentobarbital (Gingrich et al., 2009), activate GABA<sub>A</sub> channels at supra-anesthetic concentrations (3–5 MAC for ISO) where they also alter lipid bilayer properties as sensed by gramicidin channels (Sun, Y.T., V. Jogini, and O.S. Andersen. 2002. Biophysical Society 46th Annual Meeting. Abstr. 550A; Fig. 6 A). It is in this context important that *n*-alkanols inhibit Na<sub>v</sub> function at concentrations that alter gramicidin channel function (compare Horishita and Harris, 2008; Ingólfsson and Andersen, 2011). Indeed, there is a remarkable correlation between alkanol inhibition of Na<sub>v</sub> function and alkanol-induced changes in bilayer properties as sensed by gramicidin channels (Ingólfsson and Andersen, 2011). These changes in gramicidin channel function occur at alcohol concentrations that are at the low end of the concentrations that alter lipid bilayer properties in micromechanical measurements (Ly and Longo, 2004), as would be expected for a functional assay of changes in bilayer properties.

In contrast, the anesthetics tested here do not alter lipid bilayer properties at clinical concentrations, as sensed by transmembrane gramicidin channels. It remains unclear whether anesthetic effects observed at supra-anesthetic concentrations, which would alter the function of numerous membrane proteins (and ion channels), involve changes in lipid bilayer properties. In this context, it is relevant that the bilayer-modifying potency (per molecule in the bilayer) varies considerably



**Figure 6.** Concentration dependence of the ISO effect on lipid bilayer properties. (A) At clinically relevant concentrations of 1 and 2 MAC (0.3 and 0.6 mM at 25°C; green columns), ISO did not significantly alter lipid bilayer properties. Even at the supratherapeutic concentration of 4 MAC (~1.1 mM; red hashed column), only minimal changes were seen. Only at very high toxic concentrations of 12 and 18 MAC (1.1–5.4 mM; red hashed columns) did ISO alter lipid bilayer properties. 5% ethanol (~1 M) was used as a positive control (gray column). (B) Effects of ISO and

1,2-DFB on lipid bilayer properties in the presence of cholesterol. Use of cholesterol-containing lipid bilayers (1:1 mixture of DC<sub>18:1</sub>PC and cholesterol) showed similar results to bilayers without cholesterol. Neither ISO nor 1,2-DFB altered lipid bilayer properties at clinically relevant concentrations of 1 and 2 MAC. Even at 4 MAC, only minimal changes were seen.



among different classes of amphiphiles. Bilayer-modifying effects occur at membrane mole fractions of  $\sim 0.001$  for troglitazone (Rusinova et al., 2011) to  $\sim 0.01$  for curcumin (Ingólfsson et al., 2007) and docosahexaenoic acid (Bruno et al., 2007), to  $\sim 0.1$  for *n*-alkanols (Ingólfsson and Andersen, 2011), whereas the intravenous anesthetic propofol is inert (Tibbs et al., 2013). The FBs, ISO, and propofol thus are notable for their remarkably low bilayer-perturbing effects at a mole fraction of about  $\sim 0.1$ , although they do alter lipid bilayer properties at higher concentrations (membrane mole fractions).

#### Anesthetic effects on $\text{Na}_v$

Inhibition of  $\text{Na}_v$  has been implicated in the neurodepressant effects of general anesthetics (Herold and Hemmings, 2012), as well as in the action(s) of some antiepileptic drugs (Yang et al., 2009; Jo and Bean, 2014) and intoxicants (Harris and Bruno, 1985; Shiraishi and Harris, 2004). Blockade of presynaptic  $\text{Na}_v$  results in reduced neurotransmitter release (Westphalen and Hemmings, 2006; Westphalen et al., 2010), thereby affecting synaptic transmission. Moreover,  $\text{Na}_v$  inhibition has been implicated in anesthetic-induced immobilization, a cardinal feature of general anesthesia, as demonstrated in rat for ISO using an in vivo pharmacological approach (Zhang et al., 2007, 2010).

The electrophysiological effects of volatile anesthetics on  $\text{Na}_v$  are characterized by voltage-dependent reduction in peak current largely caused by hyperpolarizing shifts in the voltage dependence of steady-state inactivation (Herold and Hemmings, 2012), as demonstrated here for a series of model FB anesthetics. The magnitude of the FBs' effects varied among drugs tested at equi-anesthetic concentrations equivalent to 1 MAC, as expected if they altered the function of more than one target protein.

At the clinical concentration of 1 MAC, the estimated anesthetic concentrations in the bilayer are high (Table 1), varying between  $\sim 60$  mM (for HFB and ISO) and  $\sim 170$  mM (for 1,3,5-TFB), which suggests that it is the membrane-dissolved anesthetics that are relevant for  $\text{Na}_v$ . In fact, molecular dynamics simulations of a homology model of the prokaryotic  $\text{Na}_v$  homologue NaChBac (Raju et al., 2013) revealed that ISO was able to access the pore through hydrophobic side fenestrations that were observed in the crystal structure of NavAb (Payandeh et al., 2011). Although all four fenestrations were occupied by ISO, it is unclear whether other volatile anesthetics and larger molecules might do the same, or whether ISO binding in the fenestrations alters  $\text{Na}_v$  function.

The agent-specific differences in FB effects on  $\text{Na}_v$  are comparable to those shown previously for inhibition of NMDA-type glutamate receptors by structurally diverse inhaled anesthetics (Solt et al., 2006). The relative potencies of aromatic anesthetics for inhibition of NMDA receptors correlate well with their abilities to form

cation- $\pi$  interactions rather than with their hydrophobic properties (Raines et al., 2004). This is consistent with our observations that the anesthetic with strong cation- $\pi$  interactions (1,2-DFB) had a strong effect on  $\text{Na}_v$ .

#### Modulation of ion channel function by anesthetics

The FB model anesthetics have qualitatively similar effects on  $\text{Na}_v$  as clinically used halogenated anesthetics such as ISO, sevoflurane, and desflurane, all of which produce voltage-dependent inhibition of  $\text{Na}_v$  function (Rehberg et al., 1996; Stadnicka et al., 1999; Horishita et al., 2008; Ouyang et al., 2009). These effects on  $\text{Na}_v$  function could result from both lipid bilayer-mediated and/or direct protein-mediated mechanisms, and the relative contributions of these mechanisms to anesthetic-induced changes in ion channel function remain uncertain (Yamakura et al., 2001; Hemmings et al., 2005; Eger et al., 2008; Perouansky, 2012; Sonner and Cantor, 2013). It thus becomes important to consider the different mechanisms by which anesthetics can modify protein (e.g., ion channel) function. Fig. 7 A shows a schematic representation of five major mechanisms by which volatile anesthetics could plausibly alter protein function.

Whatever the mechanism, or combination of mechanisms, anesthetics alter the distribution among various membrane protein conformations, i.e., shift the equilibrium between resting and inactivated  $\text{Na}_v$  toward the inactivated state(s). The free energy difference for conformational transitions in membrane proteins (in our case,  $\Delta G_{\text{total}}^{\text{rest} \rightarrow \text{inact}}$ ) is the sum of contributions from rearrangements within the protein ( $\Delta G_{\text{protein}}^{\text{rest} \rightarrow \text{inact}}$ ) and within the surrounding bilayer (the difference in the bilayer deformation energy associated with the two conformations,  $(\Delta G_{\text{bilayer}}^{\text{rest} \rightarrow \text{inact}} = \Delta G_{\text{def}}^{\text{inact}} - \Delta G_{\text{def}}^{\text{rest}})$ ). Anesthetic-induced changes in channel function could result from direct binding to the channel protein (sites 2 and 3 in Fig. 7 A), as a result of differences in binding energy to different channel states (Monod et al., 1965; Jackson et al., 1989) included in  $\Delta G_{\text{protein}}^{\text{rest} \rightarrow \text{inact}}$ , as well as from drug-induced changes in lipid bilayer properties included in  $\Delta G_{\text{bilayer}}^{\text{rest} \rightarrow \text{inact}}$ . Changes in  $\Delta G_{\text{protein}}^{\text{rest} \rightarrow \text{inact}}$  are expected to vary as a saturating function of anesthetic concentration, whereas changes in  $\Delta G_{\text{bilayer}}^{\text{rest} \rightarrow \text{inact}}$  are expected to vary as a linear function of anesthetic concentration (Alejo et al., 2013). Thus, as anesthetic concentration increases, not only will the function of an increasing number of membrane proteins be altered because of direct anesthetic-protein interactions (of decreasing specificity), but indiscriminate changes in the function of many membrane proteins will occur caused by changes in lipid bilayer properties that increase the magnitude of the contribution to the energetics of conformational changes in any membrane protein.

Bilayer-mediated effects arise because hydrophobic interactions between a bilayer-spanning protein and the

lipid bilayer in which it is embedded cause the relatively soft lipid bilayer to adapt itself to the exterior surface of the protein bilayer-spanning domain (Fig. 7 B, left). As is the case for gramicidin channels (Fig. 1), this bilayer adaptation (deformation) is associated with an energetic cost, the bilayer deformation energy ( $\Delta G_{\text{def}}^0$ ). When an amphiphilic drug partitions into the bilayer (Fig. 7 B, right), it may increase the number of degrees of freedom that are available to the bilayer as it adapts to the protein, which in general reduces the energetic cost of bilayer deformation.

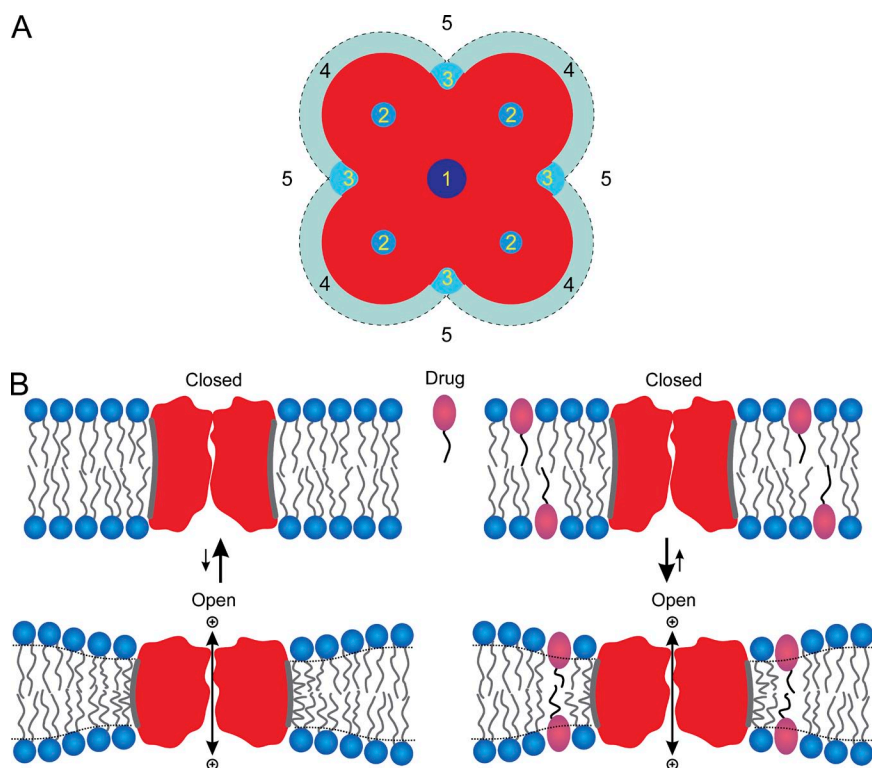
But the structure of the amphiphile is important. Experiments with *n*-alcohols of varying length show that short- and long-chain alcohols have opposite effects on chain ordering (Pope and Dubro, 1986) and on partial molar volume changes that occur upon partitioning into lipid bilayers (Aagaard et al., 2006). These compounds similarly have opposite effects on the energetics of the metarhodopsin I  $\leftrightarrow$  metarhodopsin II equilibrium (Mitchell et al., 1996) and the gramicidin monomer  $\leftrightarrow$  dimer equilibrium (Ingólfsson and Andersen, 2011). Although amphiphile-induced increases in lipid bilayer elasticity appear to be quite general (Lundbæk et al., 2010b), the sign of the change in bilayer deformation energy also reflects the ordering/disordering effects that amphiphiles impose on the acyl chains.

Bilayer deformation energy can be expressed using the theory of elastic bilayer deformations (Nielsen and Andersen, 2000; Rusinova et al., 2011):

$$\Delta G_{\text{def}}^0(l-d_0, c_0) = \Delta G_{\text{def}}^0(0,0) + H_B \cdot (l-d_0)^2 + H_x \cdot (l-d_0) \cdot c_0 + H_c \cdot c_0^2,$$

where  $l$  and  $d_0$  denote protein hydrophobic length and average thickness of the bilayer hydrophobic core, respectively;  $c_0$  is intrinsic curvature of the bilayer-forming lipids;  $\Delta G_{\text{def}}^0(0,0)$  is the energetic cost of embedding a protein into a bilayer with no hydrophobic mismatch ( $l-d_0=0$  and  $c_0=0$ ), such as the loss of conformational entropy by the acyl chains adjacent to the protein (Fattal and Ben-Shaul, 1993); and the  $H$  coefficients are functions of bilayer thickness, protein shape (for cylindrical inclusions, the inclusion radius), and the elastic area compression and bending moduli ( $K_a$  and  $K_c$ , respectively). Membrane proteins are not smooth cylinders, however, and there might not be perfect hydrophobic adaption between a protein and bilayer lipids, in which case there can be an additional residual exposure contribution,  $\Delta G_{\text{res}}^0$ , to the energetics of protein-imposed bilayer perturbations (Mondal et al., 2011).

$K_a$ ,  $K_c$ ,  $c_0$  (and  $d_0$ ) are functions of the lateral pressure profile across each bilayer leaflet (Helfrich, 1981;



**Figure 7.** Schematic representation of potential sites for drug-ion channel interactions. (A) 1, binding in the pore to block ion movement; 2, binding to sites formed by the channel to modify the free energy difference between different conformational states; 3, binding to specific sites composed of both the protein and bilayer lipids to alter the bilayer deformation energy contribution to the free energy difference between conformational states; 4, accumulation of drug at the protein-bilayer interface to alter local lipid packing; 5, partitioning into the lipid bilayer-solution interface to alter bulk lipid bilayer properties, which will alter the bilayer deformation energy associated with the channel conformational changes (adapted from Andersen, 2008). (B) Membrane protein conformational changes in the absence and presence of an amphiphile. (Left) Hydrophobic coupling between a channel protein and the host bilayer causes membrane protein conformational changes that involve the protein's bilayer-spanning domain to alter the packing of the adjacent membrane lipids. This bilayer deformation has an associated energetic cost ( $\Delta G_{\text{def}}^0$ ) that contributes to the total free energy of the protein con-

formational change. (Right) Amphiphiles partition between the aqueous solution and the bilayer, and will redistribute (between the aqueous solution and the membrane and in the plane of the membrane) in response to the local protein-induced bilayer deformation, which may increase the degrees of freedom available for the membrane to adapt to the protein and thus the value of  $\Delta G_{\text{def}}^0$  (adapted from Ingólfsson and Andersen, 2010).

Szleifer et al., 1990; Marsh, 2007), which in turn means that the  $H$  coefficients in the expression for  $\Delta G_{\text{def}}^0(l-d_0, c_0)$ , as well as  $c_0$  and  $d_0$ , vary with changes in lateral pressure profile. That is, the bilayer contribution to the free energy of gramicidin dimerization (and thus the average number of conducting channels per vesicle) will vary as a function of changes in the lateral pressure profile. The above analysis does not consider explicitly the  $\Delta A \cdot p$  contribution, where  $A$  is the channel's cross-sectional area and  $p$  is the lateral pressure, to the bilayer deformation energy (Cantor, 1997, 1999). This is likely to be minimal in the case of gramicidin channels but does not imply that gramicidin channels do not report changes in lateral pressure profile.

Early studies of anesthetic–lipid interactions tended to focus on poorly defined lipid bilayer-mediated effects (Perouansky, 2012). Overt changes in lipid bilayer properties occur only at very high concentrations (Franks and Lieb, 1979), and the magnitude of anesthetic-induced changes in bilayer properties appeared insufficient to explain the clinical effects of anesthetics (Yamakura et al., 2001). More recently, it was proposed that anesthetics could alter membrane protein function by altering more subtle bilayer properties, such as intrinsic lipid curvature (Gruner and Shyamsunder, 1991) or lateral pressure profile (Cantor, 1997; Sonner and Cantor, 2013), two equivalent descriptors of membrane protein–lipid bilayer interactions (Andersen and Koeppe, 2007; Marsh, 2007), which also could alter the free energy difference between different protein conformations. Whether or not the energetic consequences of changes in curvature or lateral pressure are sufficient to alter membrane protein function remains controversial (Lee, 2004; Marsh, 2007).

There is considerable experimental evidence that lateral pressure variations can be important for MscL (Martinac et al., 1990) and MscS (Kamaraju and Sukharev, 2008; Kamaraju et al., 2011) gating, and Teague et al. (2013) have demonstrated that rhodopsin is regulated by changes in lipid bilayer curvature under conditions where lipid bilayer thickness is maintained constant. Moreover, lipid bilayers respond to mechanical deformation and stress (stretch) by membrane area expansion (or thinning), which produces altered function of embedded proteins such as ion channels (Hamill and Martinac, 2001; Markin and Sachs, 2007; Morris and Juranka, 2007). However, experiments with  $\text{Na}_v$  (Lundbæk et al., 2005; Rusinova et al., 2011) and  $\text{GABA}_A$  (Søgaard et al., 2006; Chisari et al., 2010) channels show that amphiphiles that produce positive and negative changes in curvature—and thus rather different changes in lateral pressure profile, Marsh (2007)—produce similar changes in channel function. This suggests that the energetic consequences of changes in lateral pressure profile (or curvature) are dominated by other contributions. One such contribution could be changes in bilayer

elasticity (Evans et al., 1995; Zhelev, 1998; Bruno et al., 2013) that arise from reversible partitioning of amphiphiles between the aqueous solution and the bilayer–solution interface, as well as any lateral redistribution in the plane of the membrane (Bruno et al., 2007), as shown schematically in Fig. 7 B (right). It is, in fact, possible to predict changes in  $\text{Na}_v$  gating under situations where changes in bilayer elasticity were evaluated using gramicidin channels of different lengths (Lundbæk et al., 2010b).

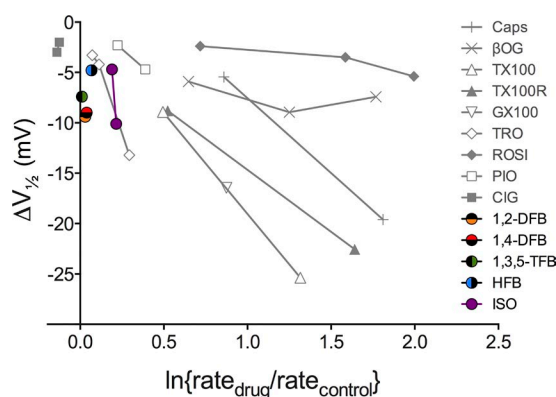
#### Lack of anesthetic-induced changes in lipid bilayer properties

Although anesthetic potency correlates with lipid solubility, the FBs and ISO had no discernable effects on lipid bilayer properties at clinical concentrations. At higher supratherapeutic and toxic (potentially lethal) concentrations, however, anesthetics can alter bilayer properties (Franks and Lieb, 1978, 1979; Goodman et al., 1996; Pickholz et al., 2005; Finol-Urdaneta et al., 2010; Cascales et al., 2011; Weinrich et al., 2012). Such changes, however, would be associated with promiscuous changes in membrane protein function because changes in lipid bilayer properties would alter the lipid bilayer contribution to the free energy of many membrane protein conformational transitions (Lundbæk et al., 2010b; Rusinova et al., 2011). These effects therefore are unlikely to be relevant for their desired clinical effects, but might account for their toxic effects.

Experiments with gramicidin channels in single-component bilayers have proven to be very sensitive to changes in lipid bilayer properties (Ingólfsson and Andersen, 2010; Ingólfsson et al., 2010; Rusinova et al., 2011). Amphiphile-induced changes in  $\text{Na}_v$  function (the hyperpolarizing shift in the inactivation curve) can be predicted from changes in gramicidin single-channel lifetimes (Lundbæk et al., 2004, 2005; Rusinova et al., 2011). Fig. 8 (gray symbols) shows a similar relationship when the changes in  $\text{Na}_v$  function ( $V_{1/2}$ ) are plotted against gramicidin channel function determined using the GBFA, meaning that gramicidin channels indeed sense changes in bilayer properties that are relevant for  $\text{Na}_v$  function. In contrast, anesthetic-induced shifts in  $V_{1/2}$  occur in the absence of changes in fluorescence quench rate, i.e., in the absence of changes in lipid bilayer properties detected as changes in gramicidin channel function (Fig. 8, colored symbols).

The question remains as to whether anesthetics might alter some bilayer property that is not sensed by gramicidin channels. This is unlikely because gramicidin channels are sensitive to changes in intrinsic curvature (Lundbæk et al., 1997), lateral stress (Goulian et al., 1998), and bilayer thickness (Lundbæk and Andersen, 1994). The lateral stress- and bilayer thickness-dependent changes in gramicidin channel function agree with predictions based on the theory of elastic bilayer deformations





**Figure 8.** Relation between bilayer-modifying effects and alteration of  $\text{Na}_v$  function for various drugs and other amphiphiles. Plot of amphiphile-induced changes in the midpoints of the  $\text{Na}_v$  inactivation curve ( $V_{1/2}$ ) against changes in the fluorescence quench rate measured using the GBFA. The gray points denote results for bilayer-modifying amphiphiles that have been examined in previous studies (Lundbæk et al., 2005; Rusinova et al., 2011). The colored points show results for the anesthetics from this study. The amphiphile-induced shifts in  $V_{1/2}$  are from Rusinova et al. (2011); the second ISO concentration is from Ouyang et al. (2009); except for the results for Genapol X-100 and reduced Triton X-100, which were done as part of this study, the amphiphile-induced changes in quench rates are from Ingólfsson and Andersen (2010) and Rusinova et al. (2011). Caps, capsaicin;  $\beta\text{OG}$ ,  $\beta$ -octyl-glucoside; TX100, Triton X-100; TX100R, reduced Triton X-100; GX100, Genapol X-100; TRO, troglitazone; ROSI, rosiglitazone; PIO, pioglitazone; CIG, ciglitazone.

(compare Huang, 1986; Helfrich and Jakobsson, 1990; Nielsen et al., 1998). Changes in the elastic moduli and intrinsic curvature arise from changes in the lateral pressure profile across the bilayer (Helfrich, 1981; Szleifer et al., 1990; Marsh, 2007), meaning that gramicidin channels sense changes in lateral pressure profile, as shown experimentally (Lundbæk et al., 1997; Goulian et al., 1998). Given that gramicidin channels, if anything, are more sensitive probes of changes in lipid bilayer properties than micromechanical measurements (compare Ly and Longo, 2004; Ingólfsson and Andersen, 2011), we conclude that it is most unlikely that anesthetics alter some bilayer property not reported by gramicidin channels.

We also examined the bilayer-modifying effects of anesthetics in LUVs formed from cholesterol-containing lipid mixtures (1:1 mixture of  $\text{DC}_{18:1}\text{PC}$  and cholesterol). In these mixed LUVs, their bilayer-modifying effects were, if anything, less, which strengthens our conclusion that anesthetics have minimal effects on lipid bilayer properties at anesthetic concentrations. Our results do not exclude the possibility that there might exist membranes with a lipid composition that makes them especially sensitive to anesthetics. We are not aware of evidence for such specialized membranes, and the generality of anesthetic effects across organisms ranging from microbes to man (Sonner and Cantor, 2013) makes the existence of such specialized domains unlikely.

## Conclusions

Model aromatic FB anesthetics and the clinical anesthetic ISO inhibit  $\text{Na}_v$  at clinically relevant concentrations at which they do not alter lipid bilayer properties, as sensed by gramicidin channel function. We therefore conclude that these anesthetics alter  $\text{Na}_v$  function through direct interactions with the ion channel protein rather than through alterations of lipid bilayer properties. These results provide strong support for the notion that the functionally relevant targets of volatile anesthetics are membrane proteins rather than the lipid bilayer itself.

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