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# Role of Voltage-Gated Sodium Channels in the Mechanism of Ether-Induced Unconsciousness

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**Abstract**—Despite continuous clinical use for more than 170 years, the mechanism of general anesthetics has not been completely characterized. In this review, we focus on the role of voltage-gated sodium channels in the sedative-hypnotic actions of halogenated ethers, describing the history of anesthetic mechanisms research, the basic neurobiology and pharmacology of voltage-gated sodium channels, and the evidence

for a mechanistic interaction between halogenated ethers and sodium channels in the induction of unconsciousness. We conclude with a more integrative perspective of how voltage-gated sodium channels might provide a critical link between molecular actions of the halogenated ethers and the more distributed network-level effects associated with the anesthetized state across species.

## I. Introduction

### A. Scope and Relevance

Despite more than 170 years of clinical use and scientific study, the molecular mechanism of action of ether anesthesia remains incompletely understood. Characterizing the molecular events by which anesthetic agents lead to the loss of consciousness is important for

both clinical and basic science. From a clinical perspective, there are more than 300 million major surgeries around the world each year, which typically occur under general anesthesia (Weiser et al., 2015). Understanding the structure–activity relationships of anesthetic action can potentially lead to the development of safer and more efficacious anesthetic drugs. In addition to the practical applications, elucidating the molecular mechanisms of

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anesthetic-induced unconsciousness has implications for pharmacology as well as the neurobiology of consciousness itself.

The clinical state of general anesthesia is characterized by the pharmacological induction of amnesia, unconsciousness, analgesia, and immobility. General anesthesia is reversible and can be induced by a structurally diverse range of molecules administered either intravenously or by inhalation. A large body of evidence now supports inhaled anesthetics acting on diverse anatomic sites and molecular targets within the nervous system to produce the array of clinical endpoints seen during general anesthesia. This review will focus on the class of inhaled anesthetics known as the halogenated ethers and the role that voltage-gated sodium channels (VGSCs) play in their mechanism of sedative-hypnotic action.

### *B. Historical Background and Clinical Use of the Halogenated Ethers*

In 1540, German botanist and physician Valerius Cordus heated a mixture of triply-distilled wine (ethanol) with vitriol (sulfuric acid) (Cordus, 1561). Cordus named the sweet-smelling remains “oleum dulci vitrioli,” which is Latin for the sweet oil of vitriol. Cordus’s sweet oil was diethyl ether, commonly referred to as ether.

For the following three centuries after Cordus’s initial discovery, ether was primarily used recreationally. Although not made public, Dr. Crawford Long performed surgeries under ether at his private practice in Jefferson, Georgia, from 1842 to 1846. He did not publish his findings until 1849 (Long, 1849). The widespread use of ether as a general anesthetic was ushered in on October 16, 1846 in the first successful public demonstration of surgery under diethyl ether anesthesia by dentist William T. G. Morton (Bigelow, 1846). Morton’s new “miracle” gas Letheon enabled renowned American surgeon John Collins Warren to remove a neck tumor from a patient without any pain or knowledge of the operation at a time when, without such interventions, surgical patients could be completely conscious of these major traumatic interventions (Fenster, 2001).

Subsequently, diethyl ether was used to achieve general anesthesia for over 75 years. Ether had many shortcomings, notably its flammable and explosive properties. The dangerous properties of diethyl ether arise from a combination of factors, including its low boiling point, high vapor pressure, and tendency to form explosive peroxides. The necessity for a safer and non-flammable alternative led to a wealth of research into general anesthetics in the first half of the 20th century.

As halogen chemistry developed, it was found that a halogenated derivative would greatly reduce the flammability of diethyl ether. In the 1930s, chemists Harold Booth and E. Max Bixby were the first to try synthesizing a fluorinated anesthetic. Although they failed with their compound monochlorodifluoromethane, Booth and Bixby (1932) recognized the importance of fluorine substitutions in developing a noncombustible volatile anesthetic. Thorough analysis of organic fluoride-based compounds led them to the observation that fluorination would improve molecular stability and toxicity.

Earl McBee directed a Manhattan Project initiative to refine  $^{235}\text{U}$  by first converting it to the uranium hexafluoride intermediate. His expertise in fluorination ultimately resulted in a grant from Mallinckrodt Chemical Works to produce new fluorinated hydrocarbons as potential anesthetics (Whalen et al., 2005). The McBee group synthesized dozens of compounds, none of which displayed anesthetic properties. Vanderbilt Pharmacologist Ben Robbins tested the fluorinated hydrocarbons. Although none succeeded, his analysis was optimistic, and he encouraged further exploration in the area (Robbins, 1946).

A University of Maryland pharmacology group led by John Krantz published dozens of articles in *The Journal of Pharmacology & Experimental Therapeutics*, and *Anesthesiology* from 1940 to 1961, investigating the properties of hundreds of ethers and hydrocarbons (Calverley, 1986). The 40th study in their series reported the effects of trifluoroethyl vinyl ether (Krantz et al., 1953). In 1954, the Ohio Chemical and Surgical Company introduced trifluoroethyl vinyl ether (fluroxene) into clinical use as the first halogenated ether for general anesthesia (Calverley, 1986). Although much safer than ether, fluroxene was potentially flammable at supraclinical concentrations. Additionally, it caused severe nausea and vomiting. In 1974, after 20 years on the market, fluroxene was withdrawn. This came shortly after it was discovered that fluroxene was metabolized into multiple toxic metabolites, including trifluoroethanol (Cascorbi and Singh-Amaranath, 1972). Methoxyflurane was introduced in 1960, sustaining popularity for a decade (Artusio et al., 1960). Its high lipid solubility caused long postoperative recovery times. Reports of methoxyflurane causing dose-related nephrotoxicity due to inorganic fluoride began to emerge (Crandell et al., 1966). It too was withdrawn from the market. Throughout the 1960s and 1970s, the chemistry group led by Ross Terrell synthesized hundreds of fluorinated compounds that were screened for anesthetic

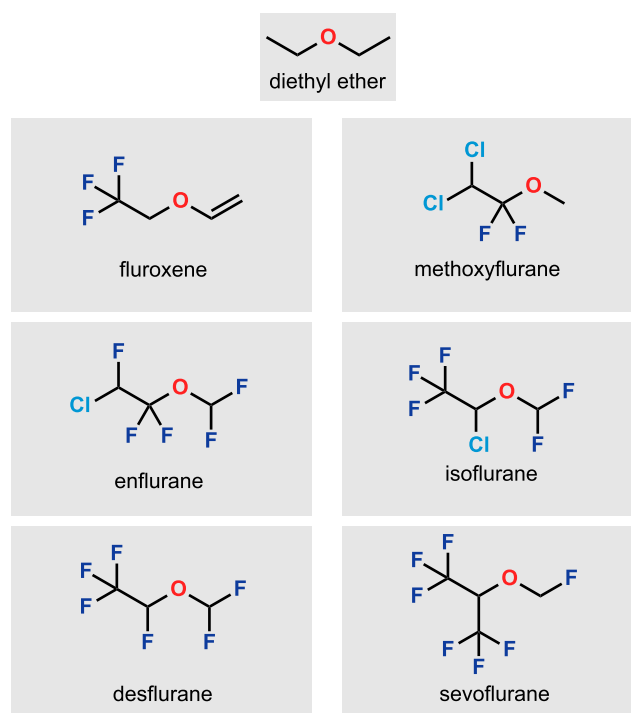
activity (Terrell, 2008). Four of those compounds (enflurane, isoflurane, desflurane, and sevoflurane) eventually were introduced into clinical care.

Due to its tendency to be cardiotoxic and proconvulsant, enflurane was never used widely in the clinical setting. Although isoflurane was synthesized in 1965, 2 years after its structural isomer enflurane, it was not introduced into general clinical practice in the United States until 1981. Technical difficulties in purifying the molecule delayed testing in animals, a problem ultimately resolved by Louise Speers. Delayed use came from concerns over the potential carcinogenic effects of isoflurane anesthesia. These fears motivated intense safety screening in animals during the 1970s. Due to the elevated scrutiny, isoflurane is widely considered the most thoroughly studied halogenated ether.

Desflurane and sevoflurane are the two most recently approved halogenated ethers (Young and Apfelbaum, 1995). With lower blood–gas solubility than their predecessors, desflurane and sevoflurane provide the anesthesiologist with nimbler titratability. The pharmacokinetic properties of desflurane and sevoflurane cause faster anesthetic induction and allow the patient to regain consciousness more rapidly upon emergence (Tempelhoff, 1997). In addition, desflurane and sevoflurane largely resist metabolism in vivo (Lavelle and Kurra, 2017). This lack of biotransformation contributes to the high safety profile of the two agents.

## II. Basic Pharmacology and Physicochemical Properties of the Halogenated Ethers

Despite the long clinical history and extensive characterization of the properties of halogenated ethers (see Fig. 1; Table 1), their precise molecular mechanisms of action remain unclear. Evidence points to pre- and postsynaptic ligand-gated ion channels and voltage-gated ion channels as highly relevant targets of halogenated ether-induced unconsciousness (Campagna *et al.*, 2003; Covarrubias *et al.*, 2015). Broadly speaking, general anesthetics act via depression of excitatory signaling and/or enhancement of inhibitory signaling. Under this generalization, many known protein targets exist. GABA<sub>A</sub>, glycine, glutamate, nicotinic acetylcholine, and 5-HT<sub>3</sub> receptors, among others, have been implicated (Mashour *et al.*, 2005; Forman and Chin, 2008). For further discussion of the promiscuous molecular pharmacology of general anesthetics, see the comprehensive reviews (Franks and Lieb, 1994; Campagna *et al.*, 2003; Rudolph and Antkowiak, 2004; Hemmings *et al.*, 2005b; Franks, 2006, 2008). In addition to neurotransmitter receptors, a growing body of evidence has emerged indicating VGSCs as mediators of some of the effects of halogenated ethers (Hemmings, 2009; Herold and Hemmings, 2012).



**Fig. 1.** Molecular structures of diethyl ether and the halogenated ethers.

## III. Basic Overview of Voltage-Gated Sodium Channel Biology

VGSCs are transmembrane protein complexes responsible for the initiation and propagation of action potentials in neurons as well as most excitable cells (Hille, 2001). VGSCs consist of a pore-forming  $\alpha$ -subunit (220–260 kDa) and smaller nonpore-forming  $\beta$ -subunits, 30–40 kDa in size (Beneski and Catterall, 1980). VGSC  $\alpha$ -subunits alone are sufficient for ion conduction and voltage sensing, whereas  $\beta$ -subunits play many roles, including the modification of channel expression, gating, localization, as well as functioning as a cell adhesion and signaling molecule (Goldin *et al.*, 1986; Hull and Isom, 2018). VGSCs transition between several conformations through the course of an action potential, including closed, open, and inactivated states (see Fig. 2). The predominant state of the channel at resting membrane potential is the closed state, where no Na<sup>+</sup> ions are conducted. Upon sufficient membrane depolarization, channels undergo a conformational change to the open state, where Na<sup>+</sup> ions enter the cell and drive further depolarization. After transition to the open state, fast inactivation is enhanced, in which intracellular residues (the inactivation gate) occlude the inside face of the channel, blocking further conduction. Such fast inactivation (recovery time constants on the scale of milliseconds) is distinct from slow inactivation (recovery time constants on the scale of hundreds of milliseconds to seconds), which is discussed in more detail below. The comparatively slower rate of inactivation to

TABLE 1  
Basic physicochemical properties of the halogenated ethers

Halogenated Ether	Clinical Introduction (US)	Boiling Point (°C)	Vapor Pressure (mmHg 20°C)	MAC % (Age 30–55, 1 atm)	Blood/Gas Partition Coefficient (37°C)	Oil/Gas Partition Coefficient (37°C)	EC <sub>50</sub> (mM)
Fluroxene	1954	43.2	286	3.4	1.37	47.7	—
Methoxyflurane	1960	105	22.5	0.2	12	950	0.27 <sup>a</sup>
Enflurane	1972	57	172	1.63	1.9	99	0.49 <sup>b</sup>
Isoflurane	1981	48	240	1.17	1.4	90.8	0.27 <sup>b</sup>
Desflurane	1992	23	669	6	0.45	19	0.53 <sup>b</sup>
Sevoflurane	1995	59	170	2	0.65	47–54	0.30 <sup>b</sup>

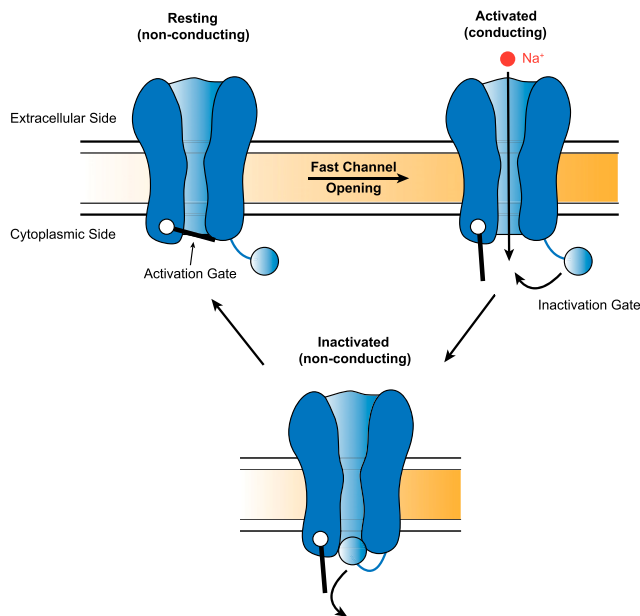
Values for fluroxene obtained from Morris (1972). All other physicochemical property values obtained from Miller et al. (2015) or Lavelle and Kurra (2017). EC<sub>50</sub> values in human obtained from <sup>a</sup>Franks and Lieb (1993), <sup>b</sup>Franks and Lieb (1996). atm, atmosphere.

activation and deactivation allows for timely termination of depolarization during an action potential and supports the refractory period between action potentials (Hille, 2001). Entry into the slow inactivated state requires repetitive or sustained depolarizations that accumulate channels in the slow inactivated state over the course of numerous action potentials (Schauf et al., 1976). Recovery from the slow inactivated state is much slower than entry, supporting longer-lasting effects on the channel than fast inactivation, which is limited to affecting fewer individual action potentials due to its fast recovery time constant. VGSC slow inactivation has the interesting property that at the depolarized potentials that support spiking (i.e.,  $-70$  mV), the recovery from slow inactivation is slow, whereas at hyperpolarized potentials (i.e.,  $-80$  mV), the recovery is much faster (Fleiderovich et al., 1996; Jung et al., 1997). This

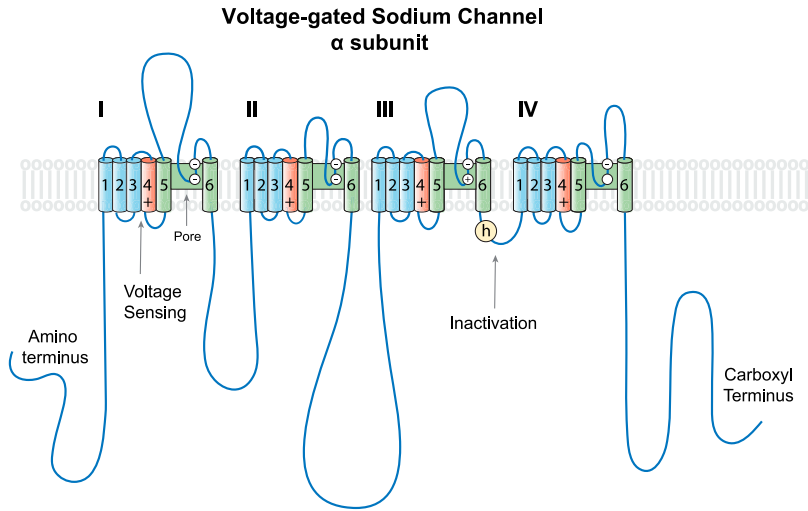
voltage-dependent effect is highly relevant to neuro-modulation, as discussed below.

The different conformational states rely on the activity of distinct regions of the channel, indicating that distinct channel states and their role in general anesthesia can be tested pharmacologically. VGSC  $\alpha$ -subunits consist of four homologous domains, each composed of six transmembrane segments (see Fig. 3). Segments 1–4 (S1–S4) are primarily involved in voltage sensing, whereas S5–S6 form the pore. Positively charged lysine and arginine residues in the S4 helices act as voltage sensors. A short loop on the intracellular side of the channel linking domains III and IV forms the inactivation gate. Slow inactivation utilizes molecular mechanisms that differ from fast inactivation. Mutations that impair fast inactivation do not prevent slow inactivation (Rudy, 1978; Featherstone et al., 1996; VEDANTHAM and Cannon, 1998; Todt et al., 1999; Mitrovic et al., 2000; Ong et al., 2000). Although numerous mutations in VGSCs have been found to affect slow inactivation, the S6 segments contain highly conserved asparagine residues that, when mutated to hydrophobic residues, completely block slow inactivation (Carr et al., 2003; Chen et al., 2006).

Ten different human VGSC  $\alpha$ -subunit isoforms (Na<sub>v</sub>1.1–1.9 + Na<sub>x</sub>) encoded by the *SCN(X)A* genes have been characterized (see Table 2). VGSC  $\alpha$ -subunits can be divided into two classes based on their sensitivity to tetrodotoxin (TTX). Na<sub>v</sub>1.1–1.4, Na<sub>v</sub>1.6, and Na<sub>v</sub>1.7 are TTX-sensitive, as half-maximal block of Na<sup>+</sup> conduction by TTX occurs at concentrations between 1 and 25 nM (Catterall et al., 2005). Na<sub>v</sub>1.5, Na<sub>v</sub>1.8, and Na<sub>v</sub>1.9 are referred to as TTX-resistant, requiring  $>1$   $\mu$ M TTX for block of half-maximal conduction. All of the human VGSC subtypes share greater than 75% amino acid sequence identity (Catterall et al., 2005). When VGSC  $\alpha$ -subunits were originally purified from rat brain, it was discovered that  $\beta$ -subunits were present in a heterotrimeric complex (Beneski and Catterall, 1980). Five  $\beta$ -subunit isoforms have been discovered (O'Malley and Isom, 2015). Heterologous expression of VGSC  $\alpha$ -subunits alone is sufficient for functional Na<sup>+</sup> conduction, but the presence of  $\beta$ 1 or  $\beta$ 3 and  $\beta$ 2 or  $\beta$ 4 confers further modularity to channel function and expression.  $\beta$ 1 or  $\beta$ 3 associates noncovalently, and  $\beta$ 2 or  $\beta$ 4 forms disulfide bonds with



**Fig. 2.** Transition states of voltage-gated sodium channels. At rest, the activation gate is closed and the channel is in a nonconducting state. After sufficient depolarization of the membrane, the channel undergoes a conformational change to enter the activated state where both gates are open, allowing the conduction of Na<sup>+</sup> ions. After transitioning to the activated state, the channel undergoes fast inactivation, where intracellular residues (the inactivation gate) occlude the inside face of the channel blocking further conduction of Na<sup>+</sup> ions. After recovery from inactivation, the channel returns to the resting state.



**Fig. 3.** Schematic of the eukaryotic VGSC  $\alpha$ -subunit. Roman numerals and numbers indicate domains and segments, respectively. The pore loop regions comprised of segments S5 and S6 are highlighted in green. Voltage-sensing S4 segments are highlighted in red. The inactivation gate and “h” particle containing the IFMT motif that is crucial for inactivation are highlighted in yellow. Small white circles of the re-entrant loops in each domain represent the amino acid residues that form the Na<sup>+</sup> ion selectivity filter.

$\alpha$ -subunits. In addition to modulation of channel expression and function,  $\beta$ -subunits act as cell adhesion molecules, with roles in cellular aggregation, neurite extension, and axonal fasciculation (Hull and Isom, 2018). Each VGSC subtype is uniquely distributed within and across cell and tissue types, indicating that specific regional- or cell type-selective mechanisms of general anesthesia may be investigated through manipulation of specific VGSCs. Each subtype has distinctive kinetic and voltage-sensing properties. VGSC subtypes are strategically expressed based on these properties to maximize physiologic function. Na<sub>v</sub>1.1–1.3 and Na<sub>v</sub>1.6 are the most prominent central nervous system VGSCs, whereas the Na<sub>v</sub>1.7–1.9 isoforms are mainly expressed in the peripheral nervous system. Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5 are mainly expressed in skeletal and cardiac muscle, respectively (Catterall et al., 2005). The different VGSCs expressed in brain exhibit isoform enrichment in a cell type- and subcellular region-directed manner. In this study, we focus on VGSC  $\alpha$ -subunits; for a review of  $\beta$ -subunit expression patterns, see Hull and Isom (2018). In the adult brain, Na<sub>v</sub>1.1, Na<sub>v</sub>1.2, and Na<sub>v</sub>1.6 are the predominant VGSCs, whereas Na<sub>v</sub>1.3 is highly expressed in the soma

of neurons during development, but shows low expression in the adult brain (Yu and Catterall, 2003). In the forebrain, Na<sub>v</sub>1.1 mRNA and protein are highly expressed in GABAergic inhibitory interneurons, although weaker expression exists in pyramidal neurons of the cortex and hippocampus (Westenbroek et al., 1989; Gong et al., 1999; Van Wart et al., 2007; Okaty et al., 2009). In interneurons, subcellular localization of Na<sub>v</sub>1.1 protein is enriched in the cell body and to a greater degree in the axon initial segment (Ogiwara et al., 2007). Na<sub>v</sub>1.2 expression is enriched in the axons of somatostatin- but not parvalbumin (PV)-positive interneurons of the cortex, showing further cellular subtype enrichment in VGSC expression (Li et al., 2014). Na<sub>v</sub>1.2 is also expressed highly in the proximal axon initial segment of pyramidal neurons and, during development, highly at nodes of Ranvier. Na<sub>v</sub>1.2 expression is present, but weaker in the cell body and dendrites of pyramidal neurons (Hu et al., 2009). Na<sub>v</sub>1.6 protein is highly expressed in the axons of PV-positive neurons and in the distal axon initial segment of pyramidal neurons and in nodes of Ranvier (Hu et al., 2009; Li et al., 2014). Na<sub>v</sub>1.6 is also expressed in the cell body and dendrites of pyramidal neurons (Caldwell

TABLE 2  
Human VGSC  $\alpha$ -subunits

Gene	$\alpha$ -Subunit	Human Chromosomal Location	Primary Tissue Expression	Tetrodotoxin Sensitivity
SCN1A	Na <sub>v</sub> 1.1	2q24	Neurons of the central nervous system	TTX-sensitive
SCN2A	Na <sub>v</sub> 1.2	2q23-24	Neurons of the central nervous system	TTX-sensitive
SCN3A	Na <sub>v</sub> 1.3	2q24	Neurons of the central nervous system	TTX-sensitive
SCN4A	Na <sub>v</sub> 1.4	17q23-25	Skeletal muscle	TTX-sensitive
SCN5A	Na <sub>v</sub> 1.5	3p21	Cardiac, denervated skeletal muscle	TTX-resistant
SCN8A	Na <sub>v</sub> 1.6	12q13	Neurons of the central nervous system	TTX-sensitive
SCN9A	Na <sub>v</sub> 1.7	2q24	Neurons of the peripheral nervous system	TTX-sensitive
SCN10A	Na <sub>v</sub> 1.8	3p22-24	Neurons of the dorsal root ganglia	TTX-resistant
SCN11A	Na <sub>v</sub> 1.9	3p21-24	Neurons of the dorsal root ganglia	TTX-resistant
SCN6/7A	Na <sub>x</sub>	2q21-23	Uterus, astrocytes, hypothalamus	N/A

et al., 2000; Krzemien et al., 2000; Lorincz and Nusser, 2010). The three major adult central nervous system VGSCs show both pre- and postsynaptic localization at synaptic terminals in the hippocampus (Johnson et al., 2017). Such localization appears to be regional/cell class specific, with the central hilus of the dentate gyrus showing predominantly presynaptic  $\text{Na}_v 1.1$  immunoreactivity, whereas in the stratum radiatum of CA1,  $\text{Na}_v 1.1$ , 1.2, and 1.6 exhibit stronger postsynaptic localization in spines and dendrites (Johnson et al., 2017). Although most of the work studying the cell type- and subregional-specific VGSC expression and function has focused on cortex and hippocampus, investigation has expanded to thalamic and subthalamic regions of the brain. Recently, it was found that targeted deletion of *Scn8a* (encoding  $\text{Na}_v 1.6$ ) in PV-positive neurons of the thalamic reticular nucleus of the thalamus was sufficient to generate absence epilepsy, a nonconvulsive seizure type that is characterized by sudden impairment of consciousness (Makinson et al., 2017).  $\text{Na}_v 1.1$  is also expressed in the thalamus, with deletion of *Scn1a* impacting function of thalamic reticular neurons (Kalume et al., 2015). The role of VGSCs in the mechanism(s) of the impairment of consciousness in absence epilepsy may further elucidate our understanding of the role of VGSCs in the molecular mechanism of halogenated ether-induced unconsciousness. Although the study of VGSCs in the consciousness promoting subcortical regions including the brainstem is nascent, cell type and regional effects of specific VGSCs in these regions are also likely to be highly relevant to mechanisms of general anesthesia.

To date, the successful crystallization of a human VGSC has yet to be accomplished. However, in 2018, the structure of human  $\text{Na}_v 1.4$  coexpressed with the  $\beta 1$ -subunit was resolved at a 3.2-Å resolution using cryogenic electron microscopy (Pan et al., 2018). This marks the highest-resolution structure of a eukaryotic VGSC currently available and may guide pharmacological investigation of VGSCs, including the action of the halogenated ethers. Selectivity of  $\text{Na}^+$  over other ions is an essential function for the action of VGSCs. This is achieved by the precise architecture of a VGSC region known as the selectivity filter. Approximately 4.6 Å in size, the selectivity filter is composed of an inner and outer ring of charged residues. The asymmetric charge distribution of each ring coordinates the solvated  $\text{Na}^+$  ions as they flow through the pore (Catterall, 2012). Through mutagenesis and subsequent electrophysiological analysis, four key residues (D: aspartate, E: glutamate, K: lysine, and A: alanine), each present in one of the domains, were identified as the inner ring of the mammalian VGSC selectivity filter (Lipkind and Fozzard, 2008). The DEKA motif found in human VGSCs is conserved across all isoforms. Mutating the KA (lysine and alanine) of the DEKA motif to glutamate (EE) abolishes  $\text{Na}^+$  selectivity of the channel and allows

$\text{Ca}^{2+}$  ions to pass through the pore (Heinemann et al., 1992).

#### A. Neuromodulation of Voltage-Gated Sodium Channels

VGSCs are affected by the action of various neuromodulators, including acetylcholine, monoamines, neuropeptides, and glutamate (Cantrell and Catterall, 2001). Such modulation by neurotransmitters that are involved in the induction or maintenance of consciousness may represent targets for the pharmacological disruption of consciousness. Administration of the cholinergic agonist carbachol reduces peak  $\text{Na}^+$  current and slows fast inactivation in dissociated hippocampal neurons (Cantrell et al., 1996). This modulation was shown to act via a protein kinase C (PKC)-dependent phosphorylation of VGSCs with similar effects in cortical pyramidal neurons. Cholinergic input to the hippocampus suppresses bursting activity and promotes tonic firing (Azouz et al., 1994; Alroy et al., 1999). The nucleus basalis, a major driver of cholinergic tone in the cortex, shifts cortical spiking from bursting to tonic firing, potentially utilizing modulation of VGSCs, as described above (Metherate et al., 1992). Dopaminergic modulation of VGSCs is also observed. Activation of D1-like dopamine receptors, which stimulate adenylyl cyclase activity, decreases  $\text{Na}^+$  current in acutely isolated hippocampal neurons (Cantrell et al., 1997, 1999). This modulation was shown to be protein kinase A (PKA) dependent. Alternatively, activation of D2 receptors, which are coupled to inhibition of adenylyl cyclase, increases  $\text{Na}^+$  current in medium spiny neurons, but has an overall inhibitory effect through activation of a PKC-mediated pathway. Both PKA and PKC phosphorylate VGSCs to mediate a reduction in  $\text{Na}^+$  current. Effects on  $\text{Na}^+$  current are limited to depolarized potentials, with similar features to slow inactivation, as described above, leading to the proposal that PKC and PKA modulation acted on the slow inactivation process (Carr et al., 2003). Indeed, it was found that mutation of an asparagine in S6 required for slow inactivation to alanine increased slow inactivation and potentiated the effects of PKC and PKA modulation, whereas mutation to aspartate blocked slow inactivation and prevented modulation by PKA and PKC. Modulation of VGSCs by PKA and PKC exhibits synergistic modulation (Li et al., 1993; Scheuer, 2011). Those residues that are phosphorylated by PKC, but not PKA, when mutated to block phosphorylation, also blocked the reduction in  $\text{Na}^+$  current by PKA activation. In the absence of PKC modulation, PKA requires membrane depolarization for effective modulation, whereas, in the presence of PKC modulation, PKA modulation occurs at all potentials (Cantrell et al., 1996). This interface between neuromodulation and activity dependence provides a rich environment for mechanisms of action for general anesthetics in the



disruption of consciousness, which is likely to depend on both neuronal activity patterns and neuromodulatory tone in the brain.

It is important to note the effects of phosphorylation on VGSC activity are specific to the cellular environment in which they are studied. Careful attention is required to understand the influence that an experimental expression system may have on the resulting VGSC modulation observed. Not surprisingly, given the high degree of differential expression of the VGSCs in cell type and subcellular domains, the different VGSC  $\alpha$ -subunits also exhibit differential sensitivity to neuromodulation by PKA and PKC.  $\text{Na}_v1.6$  is far less sensitive to PKA and PKC modulation than  $\text{Na}_v1.2$  (Maurice et al., 2001; Chen et al., 2008). Recent work has found that activation of 5-HT<sub>1A</sub> receptors in prefrontal cortical layer 5 pyramidal neurons differentially decreases  $\text{Na}^+$  current in the proximal axon attributable to  $\text{Na}_v1.2$  while not impacting those in the distal axon, attributable to  $\text{Na}_v1.6$  showing subcompartment-specific action of neuromodulators on VGSC function. As described above for PKA and PKC modulation, the effect of 5-HT<sub>1A</sub> receptor activation on reducing  $\text{Na}^+$  current could be attributed to enhancing slow inactivation and depolarizing the voltage dependence of channel activation (Yin et al., 2017). The importance of neuromodulation in regulating VGSC availability may provide insights into how the disruption or potentiation of such effects is involved in the mechanism of action of halogenated ethers in the disruption of consciousness.

#### IV. Molecular Interactions of Halogenated Ethers with Voltage-Gated Sodium Channels

In 1940, Heinbecker and Bartley reported a marked increase in the rate of accommodation to subthreshold depolarizations seen in the presence of 24 mM ether. It is now known that the accommodation rate is directly proportional to the rate of  $\text{K}^+$  channel activation and  $\text{Na}^+$  channel inactivation. Due to the low potassium conductance in frog nerve, this may have been the first electrophysiological indication of ether's action on sodium channel function (Heinbecker and Bartley, 1940). In 1956, Stephen Thesleff examined the effect of numerous nonvolatile anesthetics on the action potential of frog skeletal muscle using what was, at the time, the new voltage-clamp technique (Thesleff, 1956). All agents tested increased firing threshold and reduced action potential amplitude, mostly at concentrations higher than those relevant to clinical anesthesia. At the time, the idea that voltage-controlled channels in the membrane were carrying  $\text{Na}^+$  ions was hypothetical. Thesleff (1956) was aware of the ionic hypothesis underlying the action potential proposed during his time (Hodgkin and Katz, 1949; Hodgkin and Huxley, 1952). Decades before the molecular characterization of VGSCs, he hypothesized that the reduction in

excitability caused by anesthetic agents could be due to a reduction in the selective  $\text{Na}^+$  conductance across the membrane. He attributed the anesthetic reduction in action potential amplitude to what he called an "inhibition of the sodium carrying mechanism" (Thesleff, 1956).

The same sodium-dependent action potential blockade was also seen in isolated frog skeletal muscle fiber with diethyl ether and chloroform (Yamaguchi, 1961; Inoue and Frank, 1965). Combined with their finding that the local anesthetic (LA) procaine also blocked action potential conduction in isolated frog skeletal muscle fiber via a sodium-carrying mechanism, Inoue and Frank proposed that general and LAs acted via a unitary molecular mechanism in all excitable cells of the nervous system (Inoue and Frank, 1962; Frank and Sanders, 1963; Frank and Pinsky, 1966).

Motivated by the well-known positive correlation discovered between a compound's solubility in olive oil and anesthetic potency (Meyer, 1899; Overton, 1901; Harris and Bruno, 1985), research on the mechanism of anesthetic action during the 1970s focused on a non-specific drug perturbation of the lipid bilayer. It was proposed that volatile anesthetics acted on the lipid bilayer causing a lipid fluidization of the cell membrane, resulting in the depression of neurotransmission and eventually unconsciousness. Fluidization of the membrane was thought to cause distortions of membrane proteins (with an emphasis on ion channels such as  $\text{Na}^+$  channels), leading to a block of  $\text{Na}^+$  conductance and a breakdown of signaling in excitable cells of the central nervous system (Seeman, 1972). Phillip Seeman's hypothesis is regarded as one of the first proposing VGSCs as specific targets of volatile anesthetic action (Seeman, 1974).

Early voltage-clamp studies of the late 1970s and early 1980s, using the voltage-clamp technique in frog, crayfish, and squid giant axon, showed that diethyl ether reduced peak  $\text{Na}^+$  current by accelerating VGSC inactivation, but with little to no effect at concentrations of clinical relevance (Bean, 1979; Kendig *et al.*, 1979; Bean *et al.*, 1981; Haydon and Urban, 1983, 1986; Urban and Haydon, 1987; Elliott *et al.*, 1992). When investigating the effect of diethyl ether and the haloalkane halothane on  $\text{Na}^+$  current in the crayfish giant axon, Bean *et al.* (1981) observed a minor concentration-dependent enhancement of inactivation at all membrane potentials. Ether produced a hyperpolarizing shift in steady-state inactivation but did not alter the time-dependent recovery from inactivation, nor did it affect frequency-dependent stimulation (Bean *et al.*, 1981).

Haydon and Urban (1983) reached similar conclusions when investigating the squid giant axon. No significant difference was found in membrane capacitance or  $\text{Na}^+$  conductance. Steady-state inactivation was shifted by a few millivolts in the hyperpolarizing

direction (Haydon and Urban, 1983). The effect of ether, halothane, and enflurane inhibition on  $\text{Na}^+$  uptake using rodent brain synaptosomes was evaluated by Harris and Bruno (1985). All three of the anesthetics caused a 10%–20% reduction in  $\text{Na}^+$  uptake (Harris and Bruno, 1985).

According to the lipid-based hypotheses, anesthetics would be adsorbed by the bilayer, increasing membrane thickness, which would inhibit ion channels and subsequently axonal conduction (Franks, 2006). Controversy ensued when it was observed that the anesthetic-induced hyperpolarizing shift in sodium channel steady-state inactivation could not be due to the minor perturbations of bilayer thickness (Franks and Lieb, 1978, 1979, 1981; Elliott et al., 1984). Nicholas Franks and William Lieb demonstrated that anesthetics could act in a lipid-free environment, which brought lipid fluidization into question as the mechanism of anesthetic action (Franks and Lieb, 1981, 1982, 1984). They proposed that direct interactions with membrane proteins were responsible for anesthetic action at a molecular level. They did not, however, believe that VGSCs were among the main proteins targeted. Before dismissing the involvement of VGSCs in their landmark *Nature* review, Franks and Lieb (1994) conceded the plausibility of even minor ion channel modulation disrupting communication between neurons.

A unitary hypothesis of general anesthetic action based on membrane fluidization is now widely regarded as misleading. Changes in temperature cause similar effects as general anesthetics on the properties of the cell membrane, but do not alter consciousness. Herold et al. (2014) showed that volatile anesthetics inhibit VGSCs independent of any action on the lipid bilayer of the cell membrane. Recent studies by Herold et al. (2017a,b) examined the effects of a range of structurally diverse general anesthetics on lipid bilayer properties using a gramicidin-based fluorescence assay. The authors observed no detectable effects on lipid bilayer properties at clinically relevant concentrations of most anesthetics tested, with only minimal perturbations seen in some halogenated ethers or at supratherapeutic concentrations of the agents tested (Herold et al., 2017a,b). These findings provide strong support for the notion that general anesthetics act directly on membrane proteins to produce their clinical effects, excluding indirect ion channel modulation as a pharmacologically relevant mechanism.

Molecular advances and cloning techniques permitted the analysis of isolated VGSC isoforms expressed in mammalian cells or other heterologous systems such as *Xenopus* oocytes. Using rat brain IIA ( $\text{Na}_v1.2$ ) channels stably expressed in Chinese hamster ovary cells, Rehberg et al. (1996) were among the first to publish recordings showing halogenated ether activity with a specific VGSC isoform. All agents tested caused a reduction of peak  $\text{Na}^+$  current and a hyperpolarizing

shift in the half-maximal voltage ( $V_{1/2}$ ) of steady-state inactivation. The hyperpolarizing shift in the  $V_{1/2}$  of inactivation ranged from  $-20$  to  $-25$  mV, depending on the volatile anesthetic tested and holding potential of the protocol (Rehberg et al., 1996). Duch et al. (1998) confirmed this, reporting a similar reduction in peak  $\text{Na}^+$  current and a hyperpolarizing shift of inactivation with whole-cell recordings testing isoflurane in rat dorsal root ganglion neurons. Stadnicka et al. (1999) tested isoflurane and halothane on human embryonic kidney cells expressing human  $\text{Na}_v1.5$  (hH1a) and concluded both agents preferentially inhibit the inactivated state of  $\text{Na}_v1.5$ .

Why did the early electrophysiologists see no conduction block in response to halogenated ethers at similar concentrations to those used in the experiments above? The diminished effect seen with early voltage-clamp recordings of halogenated ethers may be specific to the experimental preparation (i.e., squid giant axon vs. mammalian cell line). Experimental conditions used with invertebrate preparations—including ion concentrations, recording temperature, ion channel expression patterns, and numerous other factors—can complicate the translation of results to mammalian physiology. A potential explanation is the large reserve of VGSCs present in axons, in which perturbations are likely to affect spike-timing precision and conduction velocity, but unlikely to result in overt action failure. Although axons contain the highest density of VGSCs in neurons, as discussed previously, VGSCs are expressed in a controlled manner in a variety of subcellular and cell type-specific contexts. Furthermore, as discussed above regarding neuromodulation, VGSCs exhibit differential sensitivity based on a variety of context-dependent factors, including neuromodulation, membrane potential, and isoform type.

To address this question, Ouyang et al. (2003) performed patch-clamp recordings in isolated rat neurohypophysial nerve terminals. Both isoflurane (and the intravenous anesthetic propofol) blocked  $\text{Na}^+$  current at clinically relevant concentrations. Isoflurane inhibited peak  $\text{Na}^+$  current with an  $\text{IC}_{50}$  of 0.45 mM when holding the voltage of the membrane at  $-70$  mV ( $V_h - 70$  mV) and 0.56 mM ( $V_h - 90$  mV). Isoflurane did not alter activation kinetics, but did cause a hyperpolarizing shift of the  $V_{1/2}$  of inactivation ( $-14$  mV with a 30-ms prepulse protocol) (Ouyang et al., 2003). The  $\text{IC}_{50}$  values seen in the terminals were lower than previously reported (Rehberg et al., 1996; Duch et al., 1998). This indicates several possibilities, including differential inhibition of VGSC isoforms, neuromodulatory state dependence, or modulation of channel sensitivity by accessory VGSC proteins. VGSC  $\alpha$ -subunit isoforms expressed in Chinese hamster ovary or human embryonic kidney cells do not possess the same VGSC accessory proteins present in rat neurohypophysial nerve terminals, although some



cell lines exhibit background  $\beta$ -subunit expression (Calhoun and Isom, 2014).

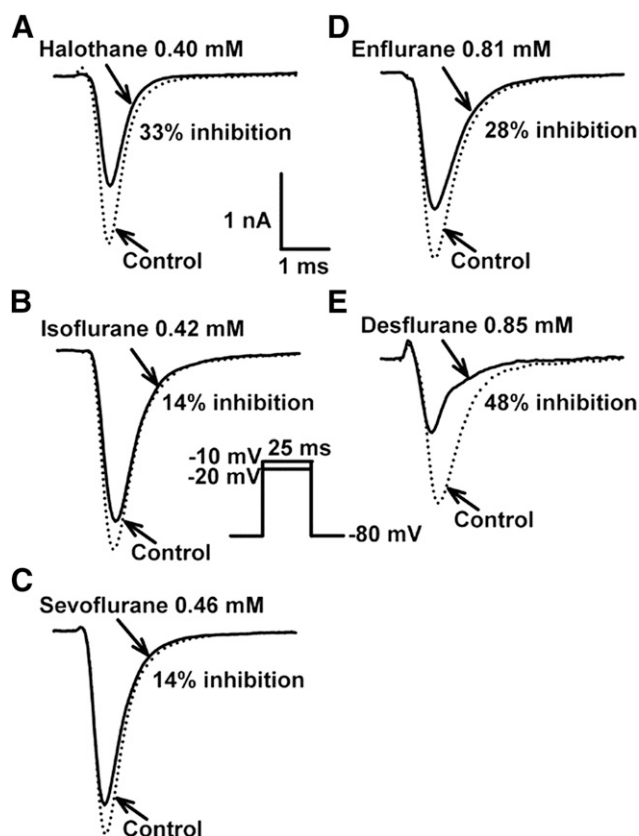
Using the two-electrode voltage-clamp technique, Shiraishi and Harris (2004) measured  $\text{Na}^+$  current in *Xenopus* oocytes expressing rat  $\text{Na}_v1.2$ , human  $\text{Na}_v1.4$ , rat  $\text{Na}_v1.6$ , or rat  $\text{Na}_v1.8$ . They also coexpressed rat  $\beta1$ -subunits, although their presence did not alter any channel's sensitivity to the anesthetics tested. Isoflurane inhibited peak  $\text{Na}^+$  current in oocytes expressing rat  $\text{Na}_v1.2$ , human  $\text{Na}_v1.4$ , or rat  $\text{Na}_v1.6$  by  $\sim 30\%$  (Shiraishi and Harris, 2004). The effect of isoflurane was more pronounced at depolarized holding potentials. The TTX-resistant isoform  $\text{Na}_v1.8$  was found to be insensitive to isoflurane at concentrations up to twice the minimum alveolar concentration (defined as the concentration at which 50% of subjects do not respond with movement to noxious stimuli) (Shiraishi and Harris, 2004). A later study using the rodent neuronal expression system, ND7/23 cells, expressing rat  $\text{Na}_v1.8$ , showed that  $\text{Na}_v1.8$  is sensitive to isoflurane in this expression system. At ( $V_h - 70$ ), 1.8 minimum alveolar concentration (MAC) blocked  $\sim 55\%$  of  $\text{Na}_v1.8$ -mediated current ( $\text{IC}_{50} 0.67 \pm 0.06$  mM) (Herold et al., 2009). The discrepancy seen in oocytes versus the ND7/23 line indicates the importance of a native neuronal environment, suggesting a potential role for nonpore-forming  $\beta$ -subunit proteins, cytoskeletal environment, or post-translational modification (e.g., phosphorylation) in VGSC sensitivity to halogenated ethers. Similar results of cell line- and cell type-specific modulation of VGSC function and expression are observed with VGSC  $\beta$ -subunits, suggesting a general need to investigate VGSC function in a physiologic and precisely defined context. Of note is that aromatic volatile anesthetics like fluorobenzene are also observed to cause significant inhibition of  $\text{Na}^+$  current in *Xenopus* oocytes (Horishita et al., 2008).

Ouyang and Hemmings (2007) tested the selectivity of isoflurane on Chinese hamster ovary cells stably expressing rat  $\text{Na}_v1.2$ , rat  $\text{Na}_v1.4$ , or rat  $\text{Na}_v1.5$ .  $\text{Na}^+$  current was significantly reduced, and a hyperpolarizing shift in steady-state inactivation was seen in all three isoforms. Importantly, isoflurane displayed selective blocking properties between isoforms. At  $V_h - 100$  mV, tonic block was observed for all three isoforms. When holding at  $V_h - 70$  mV, enhanced inactivation became the dominant mechanism of isoflurane inhibition of rat  $\text{Na}_v1.4$ - and rat  $\text{Na}_v1.5$ -mediated  $\text{Na}^+$  current (Ouyang and Hemmings, 2007).

Isoflurane, sevoflurane, enflurane, desflurane, and the haloalkane halothane were tested via whole-cell patch clamp from Chinese hamster ovary cells stably expressing rat  $\text{Na}_v1.4$  (Ouyang et al., 2009). Each anesthetic at  $\sim 1$  MAC significantly inhibited peak  $\text{Na}^+$  current. Peak current was elicited by a 25-ms step ( $V_h$  of  $-80$  mV) to  $-10$  or  $-20$  mV. Reduction in peak  $\text{Na}^+$  current by isoflurane and sevoflurane: 14%;

enflurane: 28%; and desflurane: 48% (see Fig. 4). Consistent with previous reports, each of the halogenated ethers and halothane caused a significant hyperpolarizing shift in the  $V_{1/2}$  of inactivation at  $\sim 1$  MAC (Ouyang et al., 2009). The differential agent-specific inhibition displayed by the halogenated ethers is important. The higher relative ability of desflurane in blocking  $\text{Na}^+$  current compared with the other halogenated ethers is notable. The underlying mechanism of desflurane's increased VGSC conduction inhibition is currently unknown.

Yokoyama et al. (2011) tested the effects of sevoflurane on rat  $\text{Na}_v1.4$ -, human  $\text{Na}_v1.7$ -, and rat  $\text{Na}_v1.8$ -mediated current expressed in oocytes. Sevoflurane (0.5 mM) did not cause a shift in activation or inactivation, nor did it influence peak current. Sevoflurane (1 mM) also did not change the current-voltage relationship, but did significantly inhibit the peak  $\text{Na}^+$  current of all three isoforms by 15%–19%. The authors hypothesized that sevoflurane inhibits VGSCs through a PKC-mediated pathway. Pretreatment with bisindolylmaleimide, a PKC inhibitor, reversed the effect of sevoflurane on all isoforms (Yokoyama et al., 2011).



**Fig. 4.** The halogenated ethers show differential inhibition of whole-cell  $\text{Na}^+$  current in Chinese hamster ovary cells expressing rat  $\text{Na}_v1.4$  at 1 MAC. (A) Halothane; (B) isoflurane; (C) sevoflurane; (D) enflurane; (E) desflurane. Currents were evoked via 25-ms depolarizing pulses from a holding potential of  $-80$  to  $-20$  or  $-10$  mV. Reproduced with permission from Ouyang et al. (2009).

## V. Structural and Computational Studies Using Prokaryotic Voltage-Gated Sodium Channels

The successful crystallization of a eukaryotic VGSC has yet to be accomplished. Therefore, the exact amino acid residues of the human VGSCs that the halogenated ethers interact with can only be inferred. However, prokaryotic VGSCs such as Na<sub>v</sub>AB from *Arcobacter butzleri*, Na<sub>v</sub>Ms from *Magnetococcus marinus*, and Na<sub>v</sub>Rh from *Rickettsiales* sp. *HIMB114* have been crystallized (Payandeh et al., 2011; McCusker et al., 2012; Zhang et al., 2012). These bacterial sodium channels serve as powerful models to study the structure and function of eukaryotic VGSCs.

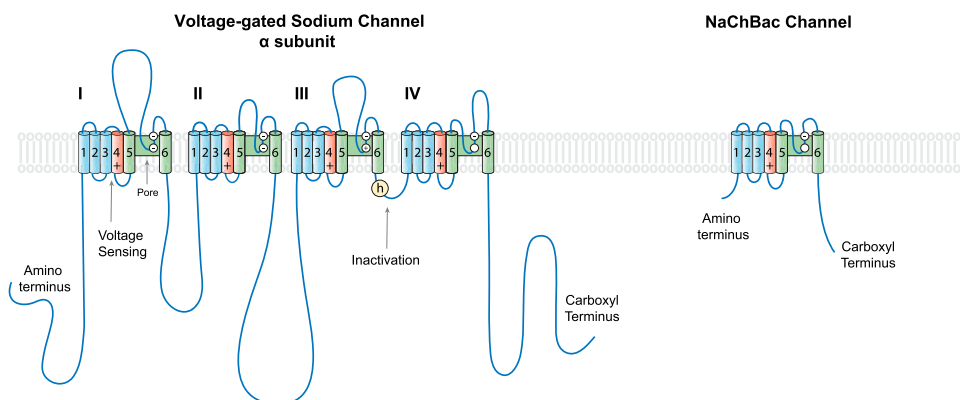
One bacterial sodium channel known as NaChBac, which has not yet been crystallized, from *Bacillus halodurans* is considered an exemplar that has been used as a model on which to base hypotheses of halogenated ether binding to a eukaryotic channel. Like all other bacterial VGSCs studied to date, NaChBac contains a single domain with six-transmembrane  $\alpha$ -helices (see Fig. 5) (Catterall, 2001). Despite being one-tenth the amino acid size of a mammalian VGSC, NaChBac still conserves many of its integral functions. The channel similarly activates in response to voltage changes. Both activation and inactivation kinetics are slower for NaChBac compared with mammalian channels. The Na<sup>+</sup> selectivity mechanism consists of a symmetric glutamate (EEEE) motif. This selectivity mechanism is used by voltage-gated calcium channels and diverges from the mammalian DEKA motif (Lipkind and Fozzard, 2008). Interestingly, NaChBac is not blocked by TTX, but is sensitive to the L-type calcium channel blocker class of dihydropyridines (Charalambous and Wallace, 2011). Its small size and homotetrameric organization make it a powerful tool to study VGSC structure, function, and pharmacology.

Ouyang et al. (2007) performed whole-cell patch-clamp recordings with isoflurane on human embryonic kidney cells expressing NaChBac. Isoflurane caused a concentration-dependent inhibition of peak Na<sup>+</sup>

current ( $IC_{50} = 0.35$  mM at  $V_h = -80$  mV). Both activation and inactivation kinetics were altered. At clinically relevant concentrations, isoflurane positively shifted the voltage dependence of activation. Consistent with earlier reports, isoflurane also shifted the  $V_{1/2}$  of NaChBac inactivation in the hyperpolarizing direction. The investigators found NaChBac 8-fold more susceptible to isoflurane-induced enhanced inactivation compared with tonic block of the activated state of the channel (Ouyang et al., 2007). This NaChBac study provides important evidence of phylogenetic conservation for halogenated ether binding to VGSCs.

Raju et al. (2013) performed molecular dynamics simulations of isoflurane binding to NaChBac and proposed three distinct binding sites that emerge at clinically relevant concentrations. The first is an extracellular site at “the intersubunit interface between the P-loops” (Raju et al., 2013). The second is a linker site between the N and C termini. The authors hypothesized that multiple isoflurane molecules occupy this site simultaneously, with mainly nonpolar drug-channel interactions. The third site was deemed the pore site. Even though the molecular dynamics simulations were carried out with the activation gate of NaChBac closed, isoflurane was still able to access the ionic pore to form hydrophobic interactions with the channel. Two of these residues (T220 and F227) align with residues from mammalian VGSCs that are known to mediate LA binding (F1764 and Y1771 rat Na<sub>v</sub>1.2 numbering) (Raju et al., 2013).

Using a combination of electrophysiology and molecular dynamics simulations, Barber et al. (2014) proposed that sevoflurane caused open channel blockade while also altering the activation and inactivation kinetics of NaChBac. Sevoflurane (0.2 mM) caused a  $-10$  mV shift in the  $V_{1/2}$  of activation and a  $-14$  mV shift in the  $V_{1/2}$  of inactivation. This resulted in a  $\sim 15\%$  increase in peak Na<sup>+</sup> current at 0.2 mM sevoflurane and a  $\sim 7\%$  decrease at 2 mM (Barber et al., 2014). Sevoflurane also accelerated the time-dependent recovery from inactivation, whereas



**Fig. 5.** Comparing the eukaryotic and prokaryotic VGSC structures. (Left) Two-dimensional schematic of the eukaryotic VGSC  $\alpha$ -subunit. (Right) Two-dimensional schematic of NaChBac, the prokaryotic VGSC  $\alpha$ -subunit found in *Bacillus halodurans*.

previous studies with halogenated ethers and mammalian VGSCs showed a delay in recovery from inactivation. Molecular dynamics simulations indicated sevoflurane binding to multiple sites. The authors noted a state-dependent interaction with the central cavity of the pore, leading them to emphasize an open channel block. Consistent with the findings of Raju et al. (2013), residues involved in LA binding (F1764 and Y1771 rat Na<sub>v</sub>1.2 numbering) were highlighted by Barber et al. (2014) as sevoflurane binding sites.

Kinde et al. (2016) sought to experimentally validate binding of isoflurane to NaChBac by nuclear magnetic resonance. <sup>19</sup>F probes were inserted into critical residues of the three putative binding sites (extracellular site, pore site, and linker site) suggested by the computational studies (Raju et al., 2013; Barber et al., 2014). <sup>19</sup>F NMR saturation transfer spectroscopy confirmed multiple major sites of isoflurane interaction with NaChBac. The study revealed two primary mechanisms by which isoflurane inhibits the channel. One mechanism is at the inner ring of the selectivity filter, occluding the pore. The other is at a hinge site, in which isoflurane binds to the S4–S5 linker that controls inactivation gating via interactions with S6 (Kinde et al., 2016).

The effects of isoflurane of NaChBac gating via site-directed mutagenesis were recently explored (Sand et al., 2017). After expressing NaChBac in human embryonic kidney cells, two point mutations were introduced to residues involved in activation: G229A, which inhibits activation, and G219A, which enhances activation. Their analysis suggested isoflurane primarily inhibits NaChBac via enhanced activation and inactivation, not through an open channel block of NaChBac (Sand et al., 2017).

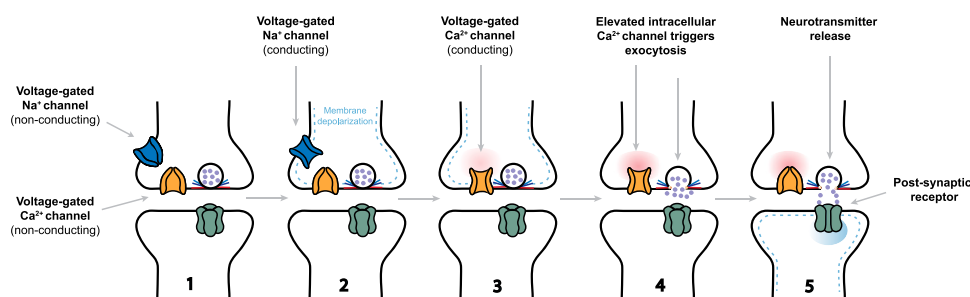
A recent study using whole-cell patch clamp examining the effects of propofol on NaChBac showed that this anesthetic also inhibits peak Na<sup>+</sup> current of the prokaryotic channel at clinically relevant concentrations (Yang et al., 2018). The authors propose that propofol acts via a hyperpolarizing shift in steady-state inactivation. Molecular dynamics simulations with propofol

suggest it directly interacts with NaChBac at multiple distinct sites on the channel (Wang et al., 2018). These results indicate that VGSC inhibition may be involved in the mechanism of action of anesthetic agents beyond the halogenated ethers.

## VI. Presynaptic Voltage-Gated Sodium Channel Inhibition Leads to Depressed Neurotransmission

What are the neurochemical consequences of activity-dependent VGSC inhibition by halogenated ethers? Hemmings (2009) has proposed a mechanism by which presynaptic VGSC inhibition leads to reduced depolarization of the presynaptic terminal. The VGSC inhibition reduces Ca<sup>2+</sup> influx into the terminal via voltage-gated calcium channels (Hemmings, 2009). Reduced Ca<sup>2+</sup> influx ultimately leads to reduced exocytosis and neurotransmission (see Fig. 6). Previous studies have revealed that isoflurane and other volatile anesthetics suppress synaptic vesicle exocytosis and neurotransmission via presynaptic actions (Schlame and Hemmings, 1995; MacIver et al., 1996; Hemmings et al., 2005a; Ouyang and Hemmings, 2005). Due to most neurons in the human brain utilizing glutamatergic neurotransmission, this mechanism would have important consequences for neural networks if occurring during volatile anesthesia *in vivo*.

Studies conducted by Berg-Johnsen and Langmoen (1986, 1990, 1992) revealed a depression of neuronal excitability and excitatory synaptic transmission by isoflurane in the rat hippocampus and human neocortex. Using isolated rat cerebrocortical nerve terminals, Westphalen and Hemmings (2006a,b) showed that isoflurane inhibition of presynaptic VGSCs was more highly coupled to glutamate release than GABA. By measuring the release of radiolabeled glutamate and GABA evoked by either 4-aminopyridine (4-AP; VGSC-dependent release) or elevated levels of extracellular K<sup>+</sup> (VGSC-independent release), the investigators found the VGSC-dependent release using 4-AP more sensitive to inhibition by isoflurane than VGSC-independent release



**Fig. 6.** Basic overview of voltage-gated Na<sup>+</sup> channels in synaptic transmission. (1) At rest, presynaptic voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> channels are closed. (2) Voltage-gated Na<sup>+</sup> channels open, driving membrane depolarization and action potential propagation. (3) Voltage-gated Ca<sup>2+</sup> channels open, raising intracellular levels of Ca<sup>2+</sup>. (4) Elevated intracellular Ca<sup>2+</sup> triggers exocytosis. (5) Neurotransmitters are released into the synaptic cleft, activating postsynaptic receptors leading to a depolarization of the postsynaptic membrane. Due to the instrumental role voltage-gated Na<sup>+</sup> channels play in this process, their inhibition by the halogenated ethers is likely to lead to dysfunction in neurotransmitter release.

using elevated extracellular  $K^+$ . Moreover, the  $IC_{50}$  values for 4-AP-evoked release support the preferential inhibition of isoflurane for glutamate over GABA release. Isoflurane  $IC_{50}$  values of 4-AP-evoked release were 0.44 and 0.58 mM for glutamate and GABA, respectively (Westphalen and Hemmings, 2003; Hemmings, 2009).

Additionally, even a small inhibition of the action amplitude in the presynaptic terminal induced by isoflurane inhibits excitatory postsynaptic current amplitude and exocytosis in the rat calyx of Held synapse with no significant effect on  $Ca^{2+}$  currents. Wu et al. (2004) reported that a majority (~70%) of the isoflurane-induced inhibition of the excitatory postsynaptic current resulted from inhibition of the presynaptic action potential. In cultured rat hippocampal neurons, isoflurane significantly inhibits synaptic vesicle exocytosis through a reduction in presynaptic  $Ca^{2+}$  influx without affecting the  $Ca^{2+}$  sensitivity of exocytosis. This effect was more pronounced when targeting glutamatergic over GABAergic neurons (Baumgart et al., 2015).

It has long been observed that ether and some halogenated derivatives display a depression of excitatory synaptic transmission that varies in different brain regions (Richards and White, 1975; Richards et al., 1975). The suppression of neurotransmitter release by isoflurane was shown to have regional specificity (Westphalen et al., 2011). This regional specificity could, in part, result from the differential localization of VGSC subtypes within nerve terminals of different cell types and their expression patterns within various regions of the brain (Westphalen et al., 2010). Isoflurane was recently shown to inhibit persistent and transient  $Na^+$  current in hippocampal excitatory pyramidal neurons from mice at clinically relevant concentrations (Zhao et al., 2019). Moreover, the differential localization of VGSC subtypes in pre- and postsynaptic compartments most likely contributes to localized variations in neurotransmitter suppression observed (Johnson et al., 2017).

## VII. In Vivo Modulation of Halogenated Ether Anesthesia

### A. Modulation of Anesthetic Sensitivity Mediated by Pharmacological Manipulation of Voltage-Gated Sodium Channels

Evidence of VGSC involvement in the action of volatile anesthetics came from a study of two structurally similar fluorinated cyclobutanes by Ratnakumari et al. (2000). Only one of these compounds showed anesthetic capability: the anesthetic compound F3 (1-chloro-1,2,2-trifluorocyclobutane) versus the nonanesthetic compound F6 (1,2-dichlorohexafluorocyclobutane).  $Na^+$  current was measured using whole-cell patch clamp in rat dorsal root ganglia neurons. Compound F3 induced a  $70\% \pm 9\%$  reduction in peak  $Na^+$  current at 0.6 MAC, whereas the nonanesthetic

compound F6 only caused a  $16\% \pm 2\%$  reduction in peak  $Na^+$  current at the predicted 1 MAC (Ratnakumari et al., 2000). The authors then used the voltage-gated  $K^+$  channel blocker 4-AP and the VGSC activator veratridine to assess the VGSC dependence of glutamate release by compound F3 or F6. The anesthetic compound F3 significantly inhibited VGSC-dependent glutamate release and significantly inhibited veratridine-evoked increases in intracellular  $Ca^{2+}$ . The nonanesthetic F6 had no significant effects at the concentrations tested (Ratnakumari et al., 2000). Ratnakumari et al. (2000) further investigated the role of VGSCs in the volatile anesthetic depression of glutamate release from synaptosomes prepared from three different mammalian species (Lingamaneni et al., 2001). Isoflurane (and propofol) preferentially inhibited veratridine-evoked, as opposed to high KCl-evoked, glutamate release. The authors used this result to suggest that interruption of presynaptic VGSC coupling to glutamate release is more relevant to anesthesia than  $Ca^{2+}$  channel-dependent glutamate release. Shiraishi and Harris (2004) measured the blocking capacity of compound F3 and compound F6 on rat  $Na_v1.2$ -mediated current. The anesthetic compound F3 inhibited peak  $Na^+$  current by  $7\% \pm 2\%$  ( $V_h - 90$  mV) and  $34\% \pm 5\%$  ( $V_h - 60$  mV), whereas the nonanesthetic F6 had no measurable effects (Shiraishi and Harris, 2004).

1. *Overlap with Local Anesthetic Mechanism of Action.* Infusion of the LA lidocaine significantly increases the anesthetic potency of isoflurane in rats (Zhang et al., 2007). Use-dependent LA drugs like lidocaine bind rapidly, preferentially blocking the open (activated) state of the VGSC. The term “use-dependent” means that the degree of channel block is proportional to the firing frequency of the neuron. Neurons firing repetitively quickly accumulate sodium channels in the open state, increasing the blocking effects of lidocaine, rendering them incapable of firing. The primary LA site has been determined to consist of residues located on the S6 segment of domains I, III, and IV (Catterall, 2012). Lingamaneni and Hemmings (2003) also showed that isoflurane and enflurane interact with the LA site of neuronal VGSCs by competition binding of [ $^3H$ ]-batrachotoxinin A 20- $\alpha$  benzoate to rat cerebrocortical synaptosomes.

Bertil Hille proposed another access pathway for LA drugs in his modulated receptor hypothesis in the 1970s. Hille posited that fenestrations in the lipid bilayer of the membrane provide LA drugs hydrophobic access to the receptor site when the channel is inactivated. This hypothesis is supported in the features of the bacterial VGSC  $Na_vAb$  via analysis of the crystal structure by Payandeh et al. (2011). It is possible that the halogenated ethers act on VGSCs via these fenestrations similarly to LAs. Of note is that the  $\beta 1$ - and  $\beta 3$ -subunits attenuate the binding of lidocaine to  $Na_v1.3$ , further supporting the hypothesis that the action of

halogenated ethers may similarly act in a context-dependent manner on VGSCs present in the central nervous system.

Xing et al. (2003) showed that intrathecal and intravenous infusion of the Food and Drug Administration–approved sodium channel blocker riluzole significantly decreased the MAC for isoflurane and halothane. Veratridine is a steroidal alkaloid that acts as an activator for VGSCs (Barnes and Hille, 1988). The toxin persistently activates the channel by shifting the activation threshold to more negative potentials (Barnes and Hille, 1988). Zhang et al. (2008) found that intrathecal administration of 1.6  $\mu$ M veratridine caused a  $\sim$ 21% increase in isoflurane MAC. In a follow-up study, Zhang et al. (2010) showed this effect to be reversible with intrathecal administration of TTX. TTX has a high affinity for neurotoxin receptor site on one of the TTX-sensitive VGSCs, occluding the pore. Intrathecal infusion of 0.63  $\mu$ M TTX decreased the MAC of isoflurane in rats by more than 50% (Zhang et al., 2010). The consistent increase in halogenated ether potency with coadministration of structurally diverse VGSC blockers and the ability to antagonize that effect with the channel activator veratridine provides pharmacological evidence of VGSC mediation of halogenated ether immobilization.

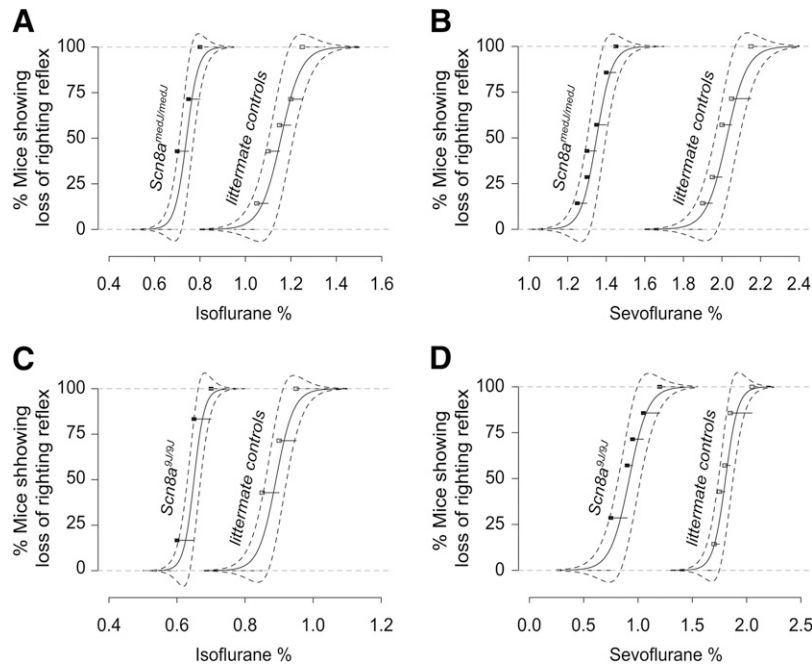
### *B. Modulation of Anesthetic Sensitivity Mediated by Genetic Manipulation of Voltage-Gated Sodium Channels*

Na<sub>v</sub>1.6 (encoded by *SCN8A*) is the most abundantly expressed VGSC subtype in the adult central nervous system (Goldin, 2001). The subtype is highly localized to the neuronal axon initial segment and nodes of Ranvier (Lorincz and Nusser, 2010). As a major contributor to neuronal excitability, Na<sub>v</sub>1.6 is a subtype of interest in halogenated ether anesthesia. Pal et al. (2015) showed that Na<sub>v</sub>1.6 was important for mediating in vivo sensitivity to isoflurane and sevoflurane anesthesia induction. Using mice with reduced Na<sub>v</sub>1.6 activity (*SCN8A*<sup>medJ/medJ</sup> and *SCN8A*<sup>9J/9J</sup> mice), the authors found the mutant mice to be significantly more sensitive to isoflurane and sevoflurane than their wild-type littermate controls (see Fig. 7). The *SCN8A*<sup>medJ/medJ</sup> mutation does not change the amino acid sequence, but reduces expression levels of Na<sub>v</sub>1.6 by 90%–95% of that of wild-type levels by disrupting splicing efficiency. The *SCN8A*<sup>9J/9J</sup> mutation is an in-frame deletion in segment 6 of domain IV, resulting in a dramatic decrease in channel activity (Pal et al., 2015). The concentration required for 50% of tested mice to lose their righting reflex (IC<sub>50</sub>) was significantly lower for the *SCN8A*<sup>medJ/medJ</sup> and *SCN8A*<sup>9J/9J</sup> mice compared with their littermate controls.

Notably, the time to emerge from anesthesia (return of righting reflex) was not different in the mutant mice versus control under isoflurane. There was a significant

difference in the return of righting reflex after sevoflurane for *SCN8A*<sup>9J/9J</sup> mice versus wild-type. Intracranial electroencephalogram was performed on the mice. Significant reductions in  $\theta$  power for both *SCN8A*<sup>medJ/medJ</sup> and *SCN8A*<sup>9J/9J</sup> mice in the waking state compared with controls excluded sleep deprivation as an explanation for the increased anesthetic sensitivity. Increased  $\theta$  power is a marker for sleep deprivation in the waking state of mice. The differential effect on induction sensitivity supports a potential role for VGSC involvement in the maintenance of consciousness. The authors noted that differential expression patterns of Na<sub>v</sub>1.6 could lead to nonspecific physiologic effects (Pal et al., 2015).

An accumulating body of evidence indicates halogenated ethers inhibit sodium channels via stabilization of the fast-inactivated state. Purtell et al. (2015) used the prototypical halogenated ether isoflurane to further investigate this effect on rat Na<sub>v</sub>1.2-mediated sodium current in ND7/23 cells. To eliminate the endogenous VGSC current in the neuronal rodent line, a phenylalanine to serine mutation (F385S) was introduced to rat Na<sub>v</sub>1.2 to render it TTX resistant, and whole-cell recordings were performed in the presence of 250 nM TTX. A modest reduction of peak Na<sup>+</sup> current ( $\sim$ 10%) was observed at 1.5 MAC isoflurane. A  $\sim$ –4 mV shift in the V<sub>1/2</sub> of steady-state inactivation was seen with no alterations in activation. The time-dependent recovery from inactivation was delayed following treatment with 1.5 MAC (Purtell et al., 2015). After observing that the delayed recovery from inactivation was more pronounced at depolarized holding potentials, the authors tested the activity-dependent effects of isoflurane. Following 10 depolarizing 5-ms pulses at 50 Hz (V<sub>h</sub> of –70 or –90 stepping to 0 mV), 1.5 MAC isoflurane significantly reduced the fraction of Na<sup>+</sup> current at pulse 10 to pulse 1. The reduction in activity-dependent Na<sup>+</sup> current was increased when starting from a V<sub>h</sub> of –70 mV instead of V<sub>h</sub> of –90 mV. Because V<sub>h</sub> of –70 mV is closer to the V<sub>1/2</sub> of inactivation, more VGSCs will be inactivated during these protocols than when holding at V<sub>h</sub> of –90 mV. Therefore, the larger reduction in activity-dependent Na<sup>+</sup> current further confirms isoflurane preferentially inhibits channels in the inactivated state. Increasing the frequency from 50 to 100 Hz further increased the block by isoflurane, although only a subset of neurons is capable of firing at this rate in vivo, including fast-spiking GABAergic interneurons. Using the same protocol, the activity-dependent inhibition by isoflurane was also observed in rat neurohypophysial nerve terminals. The authors then constructed a three-state Markov model with the data obtained from their electrophysiology experiments. The model provided quantitative support of the hypothesis that the activity-dependent block of isoflurane is achieved via stabilization of the inactivated state (Purtell, 2015; Purtell et al., 2015). The novel



**Fig. 7.** Increased anesthetic sensitivity in mice with loss-of-function mutations in *Scn8a* (the gene that encodes  $\text{Na}_v1.6$ ). Best-fit dose–response curves along with 95% confidence band (dashed lines) show the behavioral response (loss of righting reflex) of *Scn8a*<sup>medJ/medJ</sup> (A and B) and *Scn8a*<sup>9J/9J</sup> (C and D) mice and their respective littermate control groups to increasing concentrations of isoflurane and sevoflurane anesthesia.  $\text{EC}_{50}$  shows a statistically significant leftward shift in the mutant mice, indicating hypersensitivity to anesthesia mediated by  $\text{Na}_v1.6$ . Reproduced with permission from Pal et al. (2015).

activity-dependent action of isoflurane on  $\text{Na}_v1.2$  is nonetheless important as it is one of the major VGSC isoforms expressed in the central nervous system. The authors claim the additional  $\sim 20\%$  increase in  $\text{Na}^+$  current block at 50 Hz stimulation from  $V_h$  of  $-70$  mV has major implications for the in vivo systems level mechanism of isoflurane. If isoflurane increasingly blocks VGSCs as neurons fire more frequently, information-rich networks of fast-firing neurons will be differentially suppressed. Recent experiments have provided supporting evidence that isoflurane is a subtype-selective inhibitor of the neuronal VGSCs. In ND7/23 cells, clinically relevant concentrations of isoflurane showed a voltage-dependent reduction (between 15% and 20%) of peak  $\text{Na}^+$  current mediated by  $\text{Na}_v1.3$  and  $\text{Na}_v1.6$ , but no significant effect was observed in cells expressing  $\text{Na}_v1.1$  (Zhou et al., 2019). Analysis of the activity other halogenated ethers have on the neuronal subtypes  $\text{Na}_v1.1$ , 1.3, and 1.6 will be important to determine whether this activity-dependent and subtype-selective effect is agent-specific.

### VIII. Conclusion

The reduction in peak  $\text{Na}^+$  current by the halogenated ethers in the experiments reviewed was consistently minor, leading to a  $\sim 10\%$ – $20\%$  decrease in peak current, except for desflurane, which led to a 48% decrease in peak  $\text{Na}^+$  current at 0.85 mM (Ouyang et al., 2009). However, even small disturbances in  $\text{Na}^+$  channel function, such as disruption of normal inactivation

kinetics, can lead to significant changes in the formation, stability, and coherence of neural assemblies in the brain. Minor disruptions in voltage-gated ion channel function are predicted to cascade into more dramatic dysfunction in the oscillatory activity of neural networks (Arhem et al., 2003).

Subtype-specific VGSC inhibition by the halogenated ethers could lead to a heterogeneous reduction in action potential conduction velocity. This reduction may corrupt spike-timing precision in a way that is sufficient to disrupt higher-order network-level interactions, while leaving local network functions intact. Higher-order functions that rely on multiple integrated inputs are dependent on precise temporal coordination. Disruption of these higher-order functions likely contributes to the loss of consciousness under general anesthesia. Hentschke et al. (2017) investigated the effects of isoflurane on stimulated and spontaneous activity in mouse thalamocortical slices using a multi-electrode array. In the presence of clinically relevant concentrations of isoflurane ( $>1$  MAC), the authors found a more prominent suppression of polysynaptic signal propagation throughout the cortex than of the monosynaptic signal propagation traveling from the thalamus to the cortex in response to stimuli. The isoflurane-induced suppression had a more significant effect on burst propagation and long-latency signaling as opposed to burst initiation and shorter-latency signaling (Hentschke et al., 2017). Suppression of VGSCs by isoflurane, and the other halogenated ethers, may play a role in the



anesthetic-induced breakdown of long-range cortical communication and temporal coordination.

If halogenated ethers inhibit presynaptic VGSCs in excitatory neurons, for example, this can lead to depressed glutamate release. Altered neurotransmission via presynaptic sodium channel inhibition represents a potential link between the molecular actions of general anesthetics and the known alterations of brain network function during general anesthesia such as disrupted functional connectivity and increased modularity (Kim et al., 2018; Lee and Mashour, 2018). Importantly, these network-level mechanisms of anesthesia may be relevant across multiple species, including nonmammalian animals and even single-cell organisms (which are reversibly immobilized by general anesthetics). Because the evolution of sodium channels is thought to predate the origin of a nervous system in animals (Liebeskind et al., 2011), VGSC inhibition may provide insight into the known susceptibility to general anesthetics across phyla.

In this study, we have reviewed the mechanisms of action of halogenated ethers on VGSCs almost exclusively. Halogenated ethers, like many anesthetics, are promiscuous drugs known to target multiple proteins (Eckenhoff and Johansson, 1997). It is thus likely that the various therapeutic endpoints of the anesthetized state can be attributed to different protein targets, with some of these endpoints requiring the concerted action of multiple targets. Through understanding the role of VGSCs, we may gain further insight into both the molecular and network-level mechanisms of general anesthesia.

#### Authorship Contributions

Wrote or contributed to the writing of the manuscript: Denomme, Hull, Mashour.

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