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RECENT ADVANCES IN VOLTAGE-GATED SODIUM CHANNELS, THEIR PHARMACOLOGY AND RELATED DISEASES

Topic Editors
Mohamed Chahine and Jean-François Desaphy

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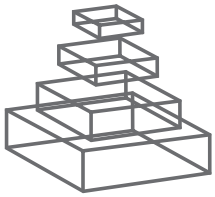

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RECENT ADVANCES IN VOLTAGE-GATED SODIUM CHANNELS, THEIR PHARMACOLOGY AND RELATED DISEASES

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It is our pleasure to co-edit a Research Topic on voltage-gated sodium channels pharmacology and related diseases. We are in a process to inviting submissions of novel research article, state-of-the-art review papers and viewpoints on this topic. All the papers will follow a peer-review process according to the guidelines of Frontiers in Pharmacology.

Voltage-gated sodium channel play a critical role in electrical signalling in many excitable cells such as neurons, skeletal muscle cells and cardiac myocytes. They are responsible for the initiation and the propagation of action potential, allowing integration of higher processes. They are formed by one alpha subunit that forms the pore of the channel and one or several regulatory subunits. Sodium channels are the target for local anaesthetics, antiarrhythmic drugs, and anticonvulsants. This class of ion channels is prime target to several toxins. Recently, a number of mutations in sodium channels that often results in alterations of rapid channel gating have been identified and linked to human diseases. Such channelopathies cause periodic paralysis, myotonia, long QT syndrome and other cardiac conductance disturbances, pain, hemiplegic migraine, and epilepsy. Sodium channels play also a negative role in malignant cellular proliferation, chronic pain, and other diseases. Considering these crucial physiological and pathological implications, it is not surprising that sodium channels have been and still are key targets for drug development.

This special issue of Frontiers of Pharmacology of Ion Channels and Channelopathies will focus on this important class of ion channels how they operate, their pharmacology and their implication in diseases.

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Recent advances in voltage-gated sodium channels, their pharmacology, and related diseases

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Because of their fundamental role in generating electrical impulses in many excitable tissues, sodium channels were among the first voltage-gated ion channels to be extensively investigated. Neurons bathed in a physiological solution containing 150 mM sodium ions respond to a threshold electrical stimulus by generating an action potential, whereas such a response is abolished in a Na^+-free medium. Since the classic 1952 studies of squid axon sodium conductance, the Hodgkin and Huxley model of sodium channel gating has served as a framework for understanding the time and voltage-dependent properties of these channels (Hodgkin and Huxley, 1952). The advent of sophisticated biochemical and molecular approaches eventually lead to sodium channel purification (Hartshorne and Catterall, 1981) and cloning (Noda et al., 1984). To date nine genes encoding voltage-gated sodium channels are found in the human genome. Dysfunction of these channels causes diseases known as sodium channelopathies. In the 1990's, the term "channelopathy" was first coined to describe skeletal muscle hereditary diseases, including periodic paralysis and myotonia, due to mutations in the SCN4A gene encoding the muscle isoform of voltage-gated sodium channels (Wang et al., 1993). Many of these aspects are reviewed in this special issue dedicated to voltage-gated sodium channels (Simkin and Bendahhou, 2011; Savio-Galimberti et al., 2012).

Despite this impressive track record, there are still a number of critical questions that need to be addressed regarding voltage-gated sodium channels. Up to day, many studies have focused on the main sodium channel α-subunit because it contains all the requisites for a functioning channel, but it has become clear that the α-subunit interacts with auxiliary β-subunits and other protein partners, that regulate the trafficking, the expression levels and the function of these channels (Brackenbury and Isom, 2011; Chahine and O'Leary, 2011; Savio-Galimberti et al., 2012).

Today voltage-gated sodium channels are the primary targets of drugs used as local anaesthetics, antiarrhythmics, anticonvulsants, and neuroprotectants (Conte Camerino et al., 2007). Several ongoing studies are aimed at understanding the intimate drug-channel molecular interactions to design more efficacious and safer drugs (Fozzard et al., 2011; Desaphy et al., 2012; Morris et al., 2012). Gating properties of these channels are also affected during trauma injury (Morris et al., 2012). As far as our knowledge increases regarding sodium channel biophysics and involvement in diseases, sodium channels represent a more, and more attractive druggable targets for other conditions such as neuropathic pain and general anesthesia (Theile and Cummins, 2011; Herold and Hemmings, 2012). Voltage-gated sodium channels are also the targets of numerous natural ligands, especially neurotoxins, which provide important tools for the definition of the channel structure–activity relationship, and ideally may serve as lead compounds in the development of novel drugs (Stevens et al., 2011). Thus, voltage-gated sodium channels will likely continue to exert much interest for basic scientists and the pharmaceutical industry. This topic is dedicated to voltage-gated sodium channels their pharmacology and related diseases.

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Voltage-gated sodium channels: biophysics, pharmacology, and related channelopathies

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Voltage-gated sodium channels (VGSC) are multi-molecular protein complexes expressed in both excitable and non-excitable cells. They are primarily formed by a pore-forming multi-spanning integral membrane glycoprotein (α-subunit) that can be associated with one or more regulatory β-subunits. The latter are single-span integral membrane proteins that modulate the sodium current (I_{Na}) and can also function as cell adhesion molecules. *In vitro* some of the cell-adhesive functions of the β-subunits may play important physiological roles independently of the α-subunits. Other endogenous regulatory proteins named "*channel partners*" or "*channel interacting proteins*" (ChiPs) like caveolin-3 and calmodulin/calmodulin kinase II (CaMKII) can also interact and modulate the expression and/or function of VGSC. In addition to their physiological roles in cell excitability and cell adhesion, VGSC are the site of action of toxins (like tetrodotoxin and saxitoxin), and pharmacologic agents (like antiarrhythmic drugs, local anesthetics, antiepileptic drugs, and newly developed analgesics). Mutations in genes that encode α- and/or β-subunits as well as the ChiPs can affect the structure and biophysical properties of VGSC, leading to the development of diseases termed sodium "*channelopathies*". This review will outline the structure, function, and biophysical properties of VGSC as well as their pharmacology and associated channelopathies and highlight some of the recent advances in this field.

Keywords: voltage-gated sodium channels, channelopathies, electrophysiology, sodium channels, pharmacology, biophysics

INTRODUCTION

In mammals, 11 genes (*SCN1A–SCN11A*) encode a family of nine functionally expressed voltage-gated sodium channels (VGSC; $Na_v1.1$–$Na_v1.9$) that share more than 50% amino acid sequence homology (Catterall et al., 2005). α-subunits encoded by these genes are organized into four homologous domains (DI–DIV), each one of which is composed of six transmembrane segments. Segments 1 through 4 of each domain form the voltage sensor, while segments 5 and 6 (and their connecting linker the P-loop) compose the pore region. In addition to the α-subunit, VGSC also include β-subunits (which are mainly regulatory molecules) as integral parts of the channel. VGSC can interact with other endogenous proteins called "*channel partners*" or "*channel interactive proteins*" (ChiPs) that modulate channel expression and/or function. The last group currently includes caveolin-3 (Lu et al., 1999; Yarbrough et al., 2002; Vatta et al., 2006; Cronk et al., 2007), calmodulin/calmodulin kinase II (CaMKII; Maier and Bers, 2002; Tan et al., 2002; Wagner et al., 2006; Pitt, 2007), connexin-43 (Sato et al., 2011; Chkourko et al., 2012), telethonin (Mazzone et al., 2008), plakophilin (Sato et al., 2009), ankyrins (Kordeli et al., 1995, 1998; Davis et al., 1996; Garrido et al., 2003; Mohler, 2006), neuronal precursor cell-expressed developmentally down regulated 4 (nedd4; Ingham et al., 2004; Rougier et al., 2005), fibroblast growth factor homologous factors (FHFs; Liu et al., 2001, 2003b; Laezza et al., 2007; Dover et al., 2010; Wang et al., 2011; Goldfarb, 2012), membrane-associated guanylate kinase synapse-associated proteins (SAPs; Petitprez et al., 2011; Milstein et al., 2012), and the syntrophin/dystrophin complex (Haenggi and Fritschy, 2006; Shao et al., 2009).

Mutations in the genes encoding the VGSC have been associated with a wide variety of diseases including Dravet syndrome and other types of epilepsy (Claes et al., 2001; Mantegazza et al., 2005; Mullen and Scheffer, 2009), pain-related syndromes [which includes congenital insensitivity to pain (CIP), primary erythromelalgia (PE), and paroxysmal extreme pain disorder (PEPD) (Dib-hajj et al., 2009; Lampert et al., 2010)], and cardiac arrhythmias [which includes congenital long QT syndrome (LQTS) type 3 (Wang et al., 1995), Brugada Syndrome (BS; Probst et al., 2003), progressive cardiac conduction defect (Scott et al., 1999), sick sinus syndrome (Benson et al., 2003), atrial fibrillation (AF; Olson et al., 2005; Darbar et al., 2008), slow ventricular conduction (Chambers et al., 2010; Sotoodehnia et al., 2010), and atrial stand still (Tan, 2006; Remme et al., 2008)]. Epigenetic up regulation of VGSC has recently been associated with aggressive metastatic carcinoma of prostate ($Na_v1.7$) and breast ($Na_v1.5$) (Onkal and Djamgoz, 2009). The up regulation of the VGSC seems to occur early in the dissemination of this type of cancer and ignite the metastatic status (the VGSC expression positively correlated *in vivo* with invasiveness and therefore metastatic spread) (Onkal and Djamgoz, 2009). Because of their central role in the pathophysiology of these diseases, VGSC are clear pharmacological targets as sites of action for antiepileptic drugs, newly developed analgesics and

antiarrhythmic drugs, and potential disease markers in metastatic carcinomas (prostate, breast).

This review will focus on the structure, function, and biophysics of the VGSC, as well as their pharmacology, the sodium channel "*partners*" (or "ChiPs") currently identified and the sodium "*channelopathies*".

STRUCTURE OF VGSC

Voltage-gated sodium channels are heteromeric integral membrane glycoproteins that can be differentiated by their primary structure, kinetics, and relative sensitivity to the neurotoxin tetrodotoxin (TTX). They are composed of an α-subunit of approximately 260 kDa (∼2000 amino acids), that is associated with one or more regulatory β-subunits (β1–β4) of approximately 35 kDa each (Catterall, 2000). We will describe in detail both subunits (α and β) that conform the VGSC.

α-SUBUNITS

Ten different mammalian α-subunit isoforms ($Na_V1.1$–$Na_V1.9$ and Na_X) have been characterized (**Table 1**) and at least seven of them are expressed in the nervous system. $Na_V1.1$, $Na_V1.2$, $Na_V1.3$, and $Na_V1.6$ isoforms are mainly expressed in the central nervous system (CNS). In contrast, $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$ isoforms are predominantly located in the peripheral nervous system (PNS; Ogata and Ohishi, 2002), are known to accumulate in the region of peripheral nerve injury and may be important in chronic, neuropathic pain (Devor, 2006; **Table 1**). In recent reports *SCN10A*/$Na_V1.8$ has also been identified in human hearts (Facer et al., 2011; Yang et al., 2012) and in intracardiac neurons (Verkerk et al., 2012), where genetic variations in the *SCN10A* gene have been associated with alterations in the PR interval, QRS duration, and ventricular conduction (Chambers et al., 2010; Sotoodehnia et al., 2010). Because these isoforms ($Na_V1.1$–1.3, $Na_V1.6$–1.9) are mainly localized in nervous tissue they are generally referred as "*brain type*" or "*neuronal-type*" sodium channels. $Na_V1.4$ isoform is mainly expressed in skeletal muscle, while $Na_V1.5$ is the cardiac-specific isoform. The isoform referred to as "Na_X channel" [also named NaG/SCL11 (rats), Nav2.3 (mice), and/or hNav2.1 (humans)] identifies a subfamily of sodium channel-like proteins (George et al., 1992). This channel has significant differences in the amino acid sequence in the voltage sensor, inactivation gate, and pore region when compared to the rest of VGSC (George et al., 1992; Goldin et al., 2000). Na_X is normally expressed in a variety of organs including the heart, skeletal muscle, uterus, dorsal root ganglia (DRG), and brain [mainly in the circumventricular organs (CVOs)]. The difficulties in the characterization of the biophysical properties of this channel are mainly due to lack of success in expressing the functional protein in heterologous expression systems. Hiyama et al. (2002) generated a mouse model in which the Na_X gene was knocked out. This group confirmed that Na_x channel was expressed in neurons in the CVOs that play a fundamental role controlling body fluid and ionic balance. This group reported that under thirst conditions, mice lacking Na_x showed hyperactivity of the neurons in these areas and ingested excessive salt, while wild-type mice did not. This led the investigators to propose that Na_X was involved in the mechanism that senses sodium levels in the brain, where this protein might sense extracellular sodium concentration (Hiyama et al., 2002; Noda, 2006).

Each α-subunit is arranged in four homologous domains (DI–DIV) that contain six transmembrane segments (S1–S6; **Figure 1**). Using cryo-electron microscopy Sato et al. (2001) showed that these four domains are arranged around the central pore of the channel. Segment 4 of each domain contains a high concentration of positive charges (mostly arginine) and functions as the core of the voltage sensor responsible for the voltage-dependent activation of the channels. Segment 6 from all four domains forms the inner surface of the pore. The hairpin-like loop between segments 5 and 6 [S5–S6 hairpin-like P(ore)-loop] is part of the pore of the channel and forms a narrow (ion-selective) filter that controls the ion selectivity and permeation at the extracellular side of the pore (Catterall, 2000; Yu and Catterall, 2003; George, 2005).

Payandeh et al. (2011) recently reported the crystal structure of Na_VAb, a VGSC found in the bacterium *Arcobacter butzleri*. Na_VAb is part of the NachBac channel family, which is a well-established model to study vertebrate Na_V and Ca_V channels (Ren et al., 2001; Koishi et al., 2004; Payandeh et al., 2011). Payandeh et al. (2011) were able to capture this channel in the close configuration when the pore was closed with four activated voltage sensors at a resolution limit of 2.7 Å. Payandeh's work provides the first insight into the structural basis for voltage-dependent gating ion selectivity and drug block in VGSC. The pore consists of an outer tubular vestibule, a selectivity filter, a central cavity (which can lodge partially hydrated sodium ions) and an intracellular activation gate. The helices that constitute the pore are positioned to stabilize cations in the central cavity through helical-dipole interactions (Doyle et al., 1998; Jogini and Roux, 2005). A second P2-helix forms an extracellular funnel and represents a highly conserved element in sodium channels (Payandeh et al., 2011).

Payandeh and coworkers proposed that in Na_VAb the ion conduction pathway is electronegative and the selectivity filter (mainly composed of negatively charged glutamate (Glu) side chains) forms the narrowest constriction near the extracellular side of the membrane. There are 4 Glu 177 side chains that form a 6.5-Å × 6.5-Å scaffold with an orifice of approximately 4.6 Å wide. A profuse mesh of amino acid residue interactions, including hydrogen bonds between glutamine from the P-helix and the carbonyl of Glu, stabilizes the selectivity filter. The radius of the pore suggests that hydrated Na^+ ions can conduct through the channel. Free diffusion then allows the hydrated Na^+ to enter the central cavity and move through the open activation gate toward the cytoplasm (Payandeh et al., 2011). This permeation pathway contrasts with the selectivity filter in K^+ channels, which is much narrower. In this case the smaller radius of the pore can only conduct dehydrated K^+ ions through direct interactions with backbone carbonyls through a long, narrow pore (Morais-Cabral et al., 2001; Ye et al., 2010).

Identification of the primary structure of VGSC led to the development of the "*sliding helix*" (Catterall, 1986b) and the "*helical screw*" (Guy and Seetharamulu, 1986) models (validated by structure-function studies) to better understand how the voltage sensor operates. Both models suggest that positively charged residues in segment 4 within each domain serve as the gating charges moving outward across the membrane as a consequence of membrane depolarization, initiating the activation process (Catterall, 1986a,b; Guy and Seetharamulu, 1986; Catterall et al., 2010). Catterall and coworkers have extensively described these

Table 1 | Summary of the different types of VGSC, and the channelopathies associated to mutations in the genes encoding the α subunits.

Gene	Chromosome	Channel	Expression	TTX	EC50	Human channelopathies
SCN1A	2q24.3	$Na_V1.1$	Cell bodies of central neurons ("Brain type I"), T-tubules in myocytes (Brette and Orchard, 2006)	S	6 nM (Clare et al., 2000)	Epilepsy and epileptic disorders, including febrile epilepsy and GEFS+ (generalized epilepsy with febrile seizure) (Escayg et al., 2000; Spampanato et al., 2001), Dravet syndrome [severe myoclonic epilepsy of infancy (SMEI)], Doose syndrome (myoclonic astatic epilepsy), intractable childhood epilepsy with generalized tonic-clonic seizures, infantile spasms (West syndrome), Rasmussen's encephalitis, and Lennox–Gastaut syndrome Non-epileptic disorders: familial hemiplegic migraine (FHM), familial autism, Panayiotopoulos syndrome (Lossin, 2009)
SCN2A	2q24.3	$Na_V1.2$	Central neurons ("Brain type II"), mainly localized to unmyelinated and premyelinated axons	S	12 nM (Noda et al., 1986)	Inherited febrile seizures and epilepsy (Sugawara et al., 2001)
SCN3A	2q24.3	$Na_V1.3$	Cell bodies of central neurons (primarily expressed in embryonic/early prenatal life), cardiac myocytes	S	4 nM (Meadows et al., 2002)	Potential contributor to peripheral neuropathic pain after spinal cord injury (Hains et al., 2003)
SCN4A	11 (human), 17q23.3 (mouse)	$Na_V1.4$	Skeletal muscle (high levels in adult muscle, low levels in neonatal muscle)	S (non-selective)	5 nM (rat; Trimmer et al., 1989), 25 nM (human; Chahine et al., 1994)	Muscle sodium channelopathies (hyperkalemic periodic paralysis, paramyotonia congenital, and potassium-aggravated myotonia, myasthenic syndrome, hypokalemic periodic paralysis type 2, malignant hyperthermia susceptibility; Cannon, 1997)
SCN5A	3p21–24	$Na_V1.5$	Cardiac myocytes, immature and denervated skeletal muscle, certain brain neurons	R	2–6 μM (Goldin, 2001)	Cardiac sodium channelopathies: Congenital long QT syndrome (Wang et al., 1995; Chen et al., 1998), Idiopathic ventricular fibrillation (Brugada syndrome; Chen et al., 1998; Akai et al., 2000), Isolated cardiac conduction system disease, atrial standstill, congenital sick sinus syndrome, sudden infant death syndrome, dilated cardiomyopathy, other conduction disorders and arrhythmias (George, 2005)
SCN8A	15 (human), 12q13 (mouse)	$Na_V1.6$	Somatodendritic distribution in output neurons of cerebellum, cerebral cortex, hippocampus; Purkinje cells in cerebellar granule cell layer; astrocytes, and Schwann cells; DRG; nodes of Ranvier in PNS and CNS; T-tubules in cardiac myocytes	S (non-selective)	1 nM (rat; Dietrich et al., 1998), 6 nM (mouse; Smith et al., 1998)	Cerebellar ataxia in jolting mice (Kohrman et al., 1996); motor end-plate disease in mice (Burgess et al., 1995)

(Continued)

Table 1 | Continued

Gene	Chromosome	Channel	Expression	TTX	EC50	Human channelopathies
SCN9A	2q24	$Na_V1.7$	All types of DRG neurons, sympathetic neurons, Schwann cells, neuroendocrine cells	S (non-selective)	4 nM (rat), 25 nM (human; Catterall et al., 2005)	Congenital insensitivity to pain (CIP), familial primary erythromelalgia, and paroxysmal extreme pain disorder (PEPD; Lampert et al., 2010)
SCN10A	3p22.2	$Na_V1.8$	DRG neurons, human heart (Facer et al., 2011; Yang et al., 2012), and intracardiac neurons (Verkerk et al., 2012)	R	60 mM (Catterall et al., 2005)	Peripheral pain syndromes; the channel is up regulated in some models of inflammatory pain; alterations in PR interval and ventricular conduction in the heart (Chambers et al., 2010; Sotoodehnia et al., 2010).
SCN11A	3p22.2	$Na_V1.9$	c-type neurons in DRG (nociception)	R	40 mM (Catterall et al., 2005)	Potential role in nociception and hyperalgesic syndromes
SCN7A	2q24.3	Na_X	DRG neurons; neurons of hippocampus, thalamus, and cerebellum, median preoptic nucleus, but mainly in the circunventricular organs (CVO); PNS; heart; skeletal muscle; uterus	Unknown	–	Potential role in temporal lobe epilepsy (Gorter et al., 2010); the lack of Na_X in neurons from CVO would affect the ability to control body fluids and ionic balance (Hiyama et al., 2002; Noda, 2006)

S, sensitive; R, resistant; CNS, central nervous system; PNS, peripheral nervous system; DRG, dorsal root ganglia.

two models. Basically, four to seven residues positively charged within segment 4 would pair negatively charged residues in segments 1, 2, and/or 3. In this configuration, positively charged residues in segment 4 are pulled inward by the electric field of the resting membrane potential which is negative. As depolarization progresses, the change in the polarity of the membrane potential relieve the electrostatic force and the segments 4 move outward allowing each positive charged amino acid in the segment 4 pairs a negatively charged one. As described by Catterall (2010), this outward movement of the gating charges in segments 4 pulls the linker between segments 4 and 5, curves the segment 6 and initiates the opening of the central pore of the channel. The movement of charged particles to activate the sodium conductance ("*gating charges*" or "*gating current*") was first predicted by Hodgkin and Huxley (Hodgkin and Huxley, 1952; Catterall, 2010), but Armstrong and Bezanilla (1973) were the first ones that measured it in 1973, combining the techniques of internal perfusion, voltage-clamp, and signal average. Using similar techniques, Keynes and Rojas (1973) confirmed the existence of the gating current the same year. Armstrong and Bezanilla (1974) reported additional properties of this current and strong evidence linking it to the gating of the sodium channels the following year.

β-SUBUNITS

These are integral proteins as well, composed of one extracellular domain (ECD, N-terminal domain), one transmembrane domain, and one intracellular domain (C-terminal domain). The β-subunits are expressed in excitable and non-excitable cells within the nervous system and the heart, and there is some evidence suggesting that these proteins can be expressed in the cells even in the absence of the α-subunit (Patino and Isom, 2010; **Table 2**). One or more regulatory β-subunits (β1–β4) can associate with one α-subunit. An individual α-subunit can be associated with one non-covalently (β1 or β3) and one covalently (β2 or β4) linked β-subunits (Yu and Catterall, 2003; Catterall et al., 2005; Patino and Isom, 2010). The role of β-subunits has been reviewed in detail by Patino and Isom (2010). The authors remark that β-subunits are regulatory proteins that can act both as cell adhesion molecules (CAMs) and modulate the cell surface expression of the VGSC, enhancing sodium channel density and cell excitability. The latter may be a very important mechanism that regulates nociceptor excitability *in vivo* (Lopez-Santiago et al., 2011). β1 association with contactin or neurofascin (NF)-186 also results in increased VGSC cell surface expression (Kazarinova-Noyes et al., 2001; McEwen and Isom, 2004). Furthermore, β1 and β2 are ankyrin-binding proteins. Mice lacking ankyrin exhibit reduced sodium current (I_{Na}) density and abnormal I_{Na} kinetics (Chauhan et al., 2000), suggesting that β-subunits play important roles in the VGSC–ankyrin complex (Patino and Isom, 2010). The interaction between α- and β-subunits may be particularly critical at the nodes of Ranvier of myelinated axons, since mice lacking β1-subunit have reduced numbers of nodes, alterations in the myelination process, and drastically altered contacts between neurons and glial cells (Chen et al., 2004). Even though proteins within nodal regions are localized normally in these mice, association between VGSC and contactin is disrupted. Loss of β1-subunit dependent protein–protein interactions can lead to changes in the structure of the

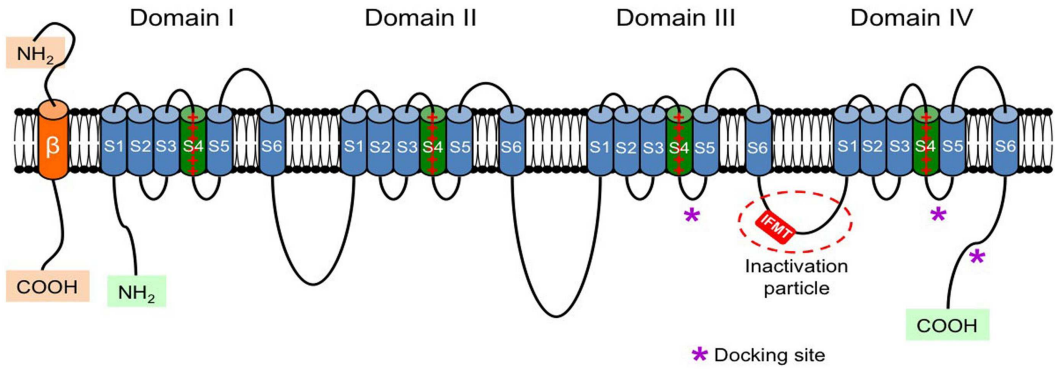

FIGURE 1 | Schematic representation of the α- and β-subunits of the VGSC. The four homologous domains (I–IV) of the α-subunit are represented; S5 and S6 are the pore-lining segments and S4 is the core of the voltage sensor. In the cytoplasmic linker between domains III and IV the IFMT (isoleucine, phenylalanine, methionine, and threonine) region is indicated. This is a critical part of the "inactivation particle" (inactivation gate), and substitution of aminoacids in this region can disrupt the inactivation process of the channel. The "docking site" consists of multiple regions that include the cytoplasmic linker between S4–S5 in domains III and IV, and the cytoplasmic end of the S6 segment in domain IV (*). Depending on the subtype of β-subunit considered they could interact (covalently or non-covalently) with the α-subunit.

Ranvier nodes and disrupted saltatory conduction (Chen et al., 2004; Davis et al., 2004). Similar to the β1- subunit, β2 can also modulate the expression of the channel at the cell surface and affect I_{Na} density (Isom et al., 1995). β2 (and β4) intracellular domains can translocate into the nucleus and enhance *SCN1A* expression, thus functioning as transcriptional regulators of the VGSC α-subunit.

β-subunits are also critical for cellular migration. β1 and β2 mediate migration of fibroblasts (Xiao et al., 1999) and cancer cells (Brackenbury and Isom, 2008), adhesion, and neurite outgrowth (β1 promotes and β2 inhibits this process, while β3 and 4 have no effect; Davis et al., 2004; McEwen et al., 2009). The effects of β-subunits on cell migration, adhesion, and neurite outgrowth also depends on intracellular transduction events like the activation of proto-oncogene tyrosine-protein kinase fyn by β1 to promote neurite (axon and/or dendrite) outgrowth (Brackenbury et al., 2008).

BIOPHYSICAL PROPERTIES OF VGSC

When the cell is depolarized, the outward movement of all segments 4 generates a conformational change that opens the pore and thus activates the channel. This allows sodium to flow into the cell down its electrochemical gradient. I_{Na} reaches a maximum within milliseconds and then becomes smaller as the sodium channel stops conducting ions and starts closing. The closure of the channel during maintained depolarization is called *inactivation*. The activation and inactivation processes and the coupling between them (in particular, the outward movement of the voltage sensor) are *voltage-dependent*. All the downstream rearrangements are *voltage-independent*. The refractoriness of the cell is related to the inactivated sodium channels, which cannot reopen until they are completely recovered. This mechanism protects the cell and prevents firing during prolonged depolarization (Goldin, 2003).

The time that the channel remains in a specific state (open or close) as well as the rate at which it transitions from one state to the other affects its ionic conductance and thus the shape of the action potential (AP). During the last decade most of the advances in the biophysics of the ion channels has been obtained by expressing the channels in isolated systems (cell lines or lipid bilayers). But the interpretation of this data has been somehow challenging due to the lack of the correct physiological context. The importance of computational biology was first emphasized by Rudy and Silva (2006) by trying to understand the context in which ion channels operate. Still, such theoretical approach in electrophysiology was initiated by Hodgkin and Huxley (1952). Hodgkin and Huxley (1952) were the first that formulated a model of the AP based on the findings of their pioneering work with voltage-clamp techniques showing voltage-dependent changes in ionic conductance in squid axons. The data reported by Hodgkin and Huxley (1952) suggested that the inward flow of Na^+ was responsible for the rapid initial positive upstroke of the membrane potential, whereas the outward flow of potassium determined the repolarization of the membrane back to resting levels. The mathematical model was designed to determine whether the sodium and potassium currents they identified could in fact generate an AP that was similar in morphology to their AP experimental recordings. The model proposed by Hodgkin and Huxley was reviewed in detail by Rudy and Silva (2006). Rudy and Silva explained that in this model the conductance of sodium and potassium currents was dependent upon the open probability of a series of activation gates. The activation gates in this model correspond to the α-subunits of the VGSC. Each gate can transition from a closed state (open probability = 0) to an open state (open probability = 1) that is independent of the state of the other gates. An ion can only pass through when all the gates (that is, the four α-subunits that constitute the tetramer VGSC) are open (open probability = 1). Rudy and Silva (2006) also pointed out that the original model has several limitations: the model assumes that intracellular concentrations of Na^+ and K^+ do not change during the AP, and it does not consider the inactivation of the channel. It also assumes that opening and closing transition rates are independent (Rudy and Silva, 2006). Rudy and Silva proposed that since the VGSC inactivation has a

Table 2 | Summary of the different types of β subunits associated with the different VGSC, and the related channelopathies associated with the mutations in the genes that encode them (modified from Patino and Isom, 2010).

Gene	Chromosome	β subunit	α subunit	Expression	Channelopathies	Model	Reference
SCN1B	19q13.1	β1	$Na_V1.1$–$Na_V1.7$	Central and peripheral neurons, glia, skeletal, and cardiac muscles.	Seizures and epileptic syndromes:		Coward et al. (2001), Audenaert et al. (2003), Chen et al. (2004), Pertin et al. (2005, 2007), Scheffer et al. (2007), O'Malley et al. (2009), Orrico et al. (2009), Patino et al. (2009)
					febrile seizures,	H	
					Dravet syndrome,	H,M	
					temporal lobe epilepsy	H	
					Traumatic nerve injury	H	
SCN2B	11q23	β2	$Na_V1.1$, $Na_V1.2$, $Na_V1.5$–$Na_V1.7$	Central and Peripheral neurons, glia, cardiac muscle.	Multiple sclerosis,	M	Coward et al. (2001), Pertin et al. (2005), Lopez-Santiago et al. (2006), O'Malley et al. (2009)
					Neuropathic pain (post-trauma)	M	
					Inflammatory pain,	M	
					traumatic nerve injury	H	
SCN3B	11q23.3	β3	$Na_V1.1$–$Na_V1.3$, $Na_V1.5$	Central and peripheral neurons, adrenal gland, kidney	Temporal epilepsy,	H	Casula et al. (2004), van Gassen et al. (2009)
					Traumatic nerve injury	H	
SCN4B	11q23.3	β4	$Na_V1.1$, Na 1.2, $Na_V1.5$	Central and peripheral neurons, glia, skeletal and cardiac muscles.	Huntington's disease	H,M	Oyama et al. (2006)

H, human; M, mouse.

greater probability of occurring when the channel is open, then inactivation is highly dependent on activation. Thus the independent gating of the Hodgkin and Huxley model would not be valid. To improve the accuracy of the mathematical model, Rudy and Silva (2006) suggest considering the dependence of a given transition on the occupancy of different states of the channel. In the case of the VGSC, the model should consider the dependence of the inactivation transition on the probability that the channel occupies the open state. Finally, the authors report that the different states and the dependence of transitions (both voltage-dependent and independent) between states can then be more accurately predicted using Markov-type models (the importance of computational biology to study the integrated electrophysiology of ion channels has been extensively reviewed by Rudy and Silva (2006)).

Inactivation is the process by which an open-channel enters a stable non-conducting conformation when the cell membrane depolarizes. The inactivation process includes *fast, slow* and *ultra-slow inactivation.* In addition, *long-term inactivation FHF-* and β4*-mediated* are processes distinct from slow inactivation (Goldfarb, 2012). In general, while isoforms $Na_v1.1$–$Na_v1.4$, $Na_v1.6$, and $Na_v1.7$ have faster inactivation kinetics, $Na_v1.5$, $Na_v1.8$, and $Na_v1.9$ have slower inactivation.

Fast inactivation mimics a "*ball-and-chain*" mechanism, where a cytoplasmic segment of the α-subunit of the VGSC (or inactivating particle) occludes the pore by binding to a docking site (Goldin, 2003). Rohl et al. (1999) were the group that first studied the inactivating particle. It consists of a portion of the cytoplasmic linker connecting domains III and IV, with the critical region centering on a 4-amino acid extent consisting of isoleucine (I), phenylalanine (F), methionine (M), and threonine (T) (IFMT; Goldin, 2003). Phenylalanine and threonine directly interact with the docking site and the distance between I- and T-residues correlates with the ability to inactivate the channel (Goldin, 2003). Amino acid substitution within this critical region can disrupt the inactivation of the channel (Kudora et al., 2000; Miyamoto et al., 2001a,b). The docking site includes the cytoplasmic linker connecting segments 4 and 5 in domains III and IV and the cytoplasmic end of segment 6 in domain IV. This "*ball-and-chain*" mechanism is equivalent to the N-type inactivation described for voltage-gated potassium channels (N-type fast inactivation), which involves occlusion of the intracellular mouth of the pore through binding of a short peptide segment from the N-terminal (Rasmusson et al., 1998). *Fast inactivation* is important during AP repolarization, and in some structures like mammalian nodes of Ranvier (which practically lack phasic potassium channels) it is the only repolarizing force besides the leakage current (Ulbricht, 2005).

Fast inactivation can be altered by the carboxyl (C)-terminus of the channel. This is due to electrostatic interactions involving the sixth helix in the C-terminus, which can modulate the interaction of the fast inactivating particle with its docking site. The different amino acid composition of the C-terminus explains the differences observed in fast inactivation between the VGSC isoforms (Mantegazza et al., 2001). Motoike et al. (2004) reported that the C-terminus is actually part of the inactivation gate, as it stabilizes the closed state minimizing the reopening of the channel. Mutations in the C-terminus disrupt fast inactivation and can lead to the LQTS type 3 (Goldin, 2003).

Fast inactivation can also be modulated by the interaction with β-subunits. The effect and mechanism is dependent on the specific α- and β-subunits involved and the heterologous expression system being used to express the channel. For example, the β1-subunit accelerates the recovery from inactivation of $Na_v1.5$

(Zimmer and Benndorf, 2002) and $Na_v1.2$ (Chen and Cannon, 1995; McCormick et al., 1998, 1999) and shifts the voltage-dependence of inactivation in the negative direction (Meadows et al., 2002). The β3-subunit has a similar effect on $Na_v1.5$, but it increases persistent current through $Na_v1.2$ in tsA-201 cells (cell line derived from human embryonic kidney cells; Goldin, 2003). β4-subunits disrupt VGSC inactivation in neurons, working as endogenous open-channel blockers. This subunit has a short cytoplasmic tail that basically blocks the channel in the open state but rapidly dissociates upon membrane repolarization to generate the resurgent current in some neurons like Purkinje cells (Grieco et al., 2005; Goldfarb, 2012).

Fast inactivation can be disrupted and transformed into *long-term inactivation* by the interaction of the α-subunit of the channel with a family of cytoplasmic proteins termed *fibroblast growth factor homologous factors* (FHFs) (Goldfarb, 2012). FHFs act as accessory channel subunits. Several FHFs delay fast inactivation by raising the voltage at which fast inactivation occurs. Dover et al. (2010) reported that all A-type FHFs (specially FHF2A and FHF4A) exert a rapid onset of a distinct mode of long-term inactivation of $Na_v1.6$ and other VGSC including cardiac $Na_v1.5$ (Dover et al., 2010; Goldfarb, 2012). A-type FHFs accomplish long-term inactivation by providing an independent cytoplasmic gating particle that competes with the channel's intrinsic inactivating particle for blockade of the channel upon membrane depolarization. The authors reproduced this mechanism by injecting a synthetic peptide corresponding to the A-type FHF particle that mimics the long-term inactivation and opposes sustained firing of excitable cells (Dover et al., 2010). β4-mediated channel block and A-FHF-mediated long-term inactivation have a similar physical mechanism. Both processes are mediated by small cytoplasmic particles that interact with the channel after the depolarization has driven the channels into the open state. In both cases, the blocking particles dock at similar sites deep within the cytoplasmic opening of the channel pore. The main difference between the two processes is the rate of particle dissociation, where β4 particle dissociates rapidly and FHF dissociates far more slowly (Goldfarb, 2012).

Slow inactivation is a different process that involves conformational changes of the channel leading to rearrangement of the pore. The process also involves segment 4 of domain IV and segments 5 and 6 of domain II (Goldin, 2003). This mechanism is equivalent to the C-type inactivation mechanism described for potassium channels (Rasmusson et al., 1998). *Slow inactivation* may play a role in regulating excitability by, for example, modulating burst discharges. However, this modulation is complicated since slow inactivation depends on both resting membrane potential and the previous history of AP firing (Ulbricht, 2005).

In addition to *fast* and *slow inactivation* there is a third type of inactivation named *ultra-slow inactivation*. This process was described in $Na_v1.4$ when the alanine in position 1529 (A1529) is replaced by aspartate (D) in the domain IV P-loop (Goldin, 2003). Binding of the fast inactivating particle inhibits this process. This result demonstrates that there are interactions (mostly, allosteric modulation) among the different inactivation events (Goldin, 2003).

"SODIUM CHANNEL PARTNERS" OR "CHANNEL INTERACTIVE PROTEINS"

The current density and gating properties of VGSC can also be modulated by the differential expression of *channel* "*partners*" or ChiPs (**Table 3**). These terms designate molecules that affect the aggregation, density, function, and regulation of VGSC. Up-to-day, the main identified VGSC partners include caveolin-3 (and the membrane compartment "caveolae"), CaMKII, connexin-43, telethonin, plakophilin, ankyrins, fibroblast growth factor homologous factors (FHFs), nedd4, SAPs, and syntrophin/dystrophin complex.

CAVEOLAE/CAVEOLIN-3

Caveolae are sarcolemmal membrane invaginations that have been implicated in cellular trafficking cascades involving the β-adrenergic receptors (β-AR; Schwencke et al., 1999; Rybin et al., 2000). These membrane invaginations also contain scaffolding proteins named "*caveolins*". Yarbrough et al. (2002) demonstrated both biochemically and functionally that caveolae are involved in VGSC regulation by a mechanism involving the α-subunit of the stimulatory G protein ($G\alpha_s$) through the activation of the β-ARs on the cell surface. Because direct $G\alpha_s$ activation induces an increase in the number of functional channels at the sarcolemma (Lu et al., 1999), they hypothesized that functional channels were recruited from an intracellular store, allowing a faster presentation of channels to the cell surface after β-stimulation. The authors purified the caveolin-3 rich fraction using immunoprecipitation. VGSC and $G\alpha_s$ are colocalized in the Cav3(+)-fraction, suggesting a physical association of both proteins with the caveolar (Cav3-rich) membrane (Rook et al., 2012). They also reported that the increase in I_{Na} induced by isoproterenol stimulation (10 μM) in the presence of a protein kinase A (PKA) inhibitor (PKA-independent increase in I_{Na}) was abolished when an anti-Cav3 antibody was injected into the cytoplasm of the cell through the pipette. This suggests a direct action of the $G\alpha_s$ on the caveolae, resulting in the presentation of caveolar VGSC to the sarcolemma. Palygin et al. (2008) also demonstrated that the histidine residue at position 41 of $G\alpha_s$ (H41) is a critical residue for the functional increase of I_{Na} observed.

CALMODULIN/CALMODULIN KINASE II

Tan et al. (2002) demonstrated that calmodulin regulates sodium channel gating through binding to a region of 25 amino acids located at the C-terminus of the intracellular domain. Wagner et al. further studied the downstream signaling through Ca^{2+}/CaM-dependent protein kinase II (CaMKIIδ) in heart cells from two heart failure animal models, where expression and activity of CaMKII are increased by twofold to threefold. They demonstrated that calmodulin regulates Na^+ channel gating in part via CaMKII. Using two cell models of CaMKII overexpression, they concluded that both acute and chronic overexpression of CaMKIIδc significantly shifted voltage-dependence of Na^+ channel availability by −6 mV, and the shift was Ca^{2+}-dependent. CaMKII also enhanced the inactivation of the channel and slowed its recovery from inactivation. These effects were prevented using CaMKII inhibitors (KN93 or AIP). CaMKII over-expression also increased persistent (late) inward I_{Na} and the intracellular Na^+ concentration (also

Table 3 | VGSC protein partners.

Gene	Chromosome	Protein	Expression site and function	Reference
Cav3	3p25.3	Caveolin-3	Scaffolding protein within caveolar membranes. Also involved in VGSC regulation by a mechanism involving the α subunit of the stimulatory G protein ($G\alpha_s$) through the activation of the βARs on the cell surface	Lu et al. (1999), Schwencke et al. (1999), Rybin et al. (2000), Yarbrough et al. (2002)
CALM 2	2p21	Calmodulin	"Calcium-Modulated Protein." Ca^{2+}-binding protein expressed in all eukaryotic cells	Tan et al. (2002)
CAMK 2A	5q32	CaMKII	Part of a family of serine/threonine kinases that mediate many of the second messenger effects of Ca^{2+}	Wagner et al. (2006)
GJA1	6q22.31	Connexin-43	Connexins are assembled in groups of six to form hemichannels, or connexons, and two hemichannels then combine to form a gap junction. The connexin gene family is diverse, with 21 identified members in the sequenced human genome	Sato et al. (2011)
TCAP	17q12	Telethonin	Small protein mainly expressed in skeletal muscle that binds to and is phosphorylated by titin kinase and protein kinase D. Both proteins serve as a scaffold to which myofibrils and other muscle related proteins are attached	Valle et al. (1997), Mayans et al. (1998), Mues et al. (1998), Furukawa et al. (2001), Knoll et al. (2002), Haworth et al. (2004), Kojic et al. (2004), Mazzone et al. (2008)
PKP2	12p11	Plakophilin-2	Fundamental component of the cardiac desmosome, structure present in the intercalated disc	Sato et al. (2009)
ANK2	4q25–27	Ankyrin-B (or ankyrin-2)	Cell membrane proteins that link the integral proteins of the membrane to the underlying spectrin-actin cytoskeleton. Mutations in these genes have been related to long QT syndrome type 4 (ANK2) and Brugada like-syndrome (ANK3)	Jenkins and Bennett (2001),Garrido et al. (2003), Lemaillet et al. (2003), Mohler et al. (2004)
ANK3	10q21	Ankyrin-G (or ankyrin-3)		
FGF3 FGF5 FGF6 FGF11 FGF12 FGF13 FGF14	11q13/13.3 4q21/21–21 12q13/13.32 17p13.1 3q28/29 Xq26.3/27.1 13q33.1/34	FHFs (FGFs)	Family of cytoplasmic proteins termed fibroblast growth factor homologous factors that can delay fast inactivation of VGSC	Dover et al. (2010)
Nedd4 Human	15q–15q21.3	Nedd4	Ubiquitin-protein ligases	Rougier et al. (2005)
SNTG 1	8q11.21	Syntrophin	The protein encoded by this gene is a member of the syntrophin family. Syntrophins are cytoplasmic peripheral membrane proteins that typically contain 2 pleckstrin homology (PH) domains, a PDZ domain that bisects the first PH domain, and a C-terminal domain that mediates dystrophin binding. This gene is specifically expressed in the brain	Gavillet et al. (2006), Haenggi and Fritschy (2006), Shao et al. (2009)
DMD	Xp21.2	Dystrophin	Rod-shaped cytoplasmic protein, and a vital part of a protein complex (*costamere* or dystrophin-assoc. prot.s) that connects the cytoskeleton of a muscle fiber to the surrounding extracellular matrix through the cell membrane	
SCN1B-SCN4B	19q13.1 (SCN1B) and 11q23 (SCN2B–4B)	β subunits of VGSC	Regulatory subunits of VGSC expressed in CNS, PNS, and heart (see also **Tables 1** and **2**)	Isom et al. (1994), Kazarinova-Noyes et al. (2001), Chen et al. (2004), McEwen and Isom (2004), Meadows and Isom (2005)

prevented using CaMKII inhibitors). They reported that CaMKII coimmunoprecipitates with and phosphorylates sodium channels. *In vivo* data suggested that CaMKII overexpression mice were more prone to suffer ventricular arrhythmias, particularly monomorphic ventricular tachycardia. The data as a whole supports the hypothesis that CaMKII regulates sodium channel function in myocytes most likely by association with and phosphorylation of the channels (Wagner et al., 2006).

TELETHONIN

Telethonin is a small protein (19 kDa) that is mainly expressed in striated muscle (Valle et al., 1997). This protein binds to and is phosphorylated by titin kinase (Mayans et al., 1998) and protein kinase D (Haworth et al., 2004). One of its many functions includes acting as a stretch sensor in the heart (Knoll et al., 2002), linking sarcomeres to K^+ channel subunits (Furukawa et al., 2001), and interacting with titin (Mues et al., 1998) and ankyrin-2 (also proposed to behave as stress sensors in muscle; Kojic et al., 2004). Mazzone et al. (2008) hypothesized that telethonin may be relevant in tissues different from striated muscle, where it might also behave as a ChiP. After screening 20 unrelated patients with primary intestinal pseudo-obstruction, the authors identified a patient with a heterozygous mutation, R76C, in the telethonin gene by direct DNA sequencing. The mutation is located in the region of telethonin where the protein has been shown to interact with sarcomeric proteins (muscle LIM protein and titin) in the heart. Using immunostaining and immunoprecipitation they demonstrated that telethonin and $Na_V1.5$ were colocalized in mouse hearts. They studied the effects of the R67C mutation on the *in vitro* electrophysiology of *SCN5A* expressed in a Human embryonic kidney cell line (HEK)-293. The coexpression of R67C telethonin with *SCN5A* resulted in a leftward shift in the steady-state activation of the sodium channel, leading to increased Na^+ entry at resting potential (depolarizing effect). The data supports the hypothesis that telethonin acts as a ChiP (Mazzone et al., 2008).

PLAKOPHILIN

Plakophilin-2 (PKP2) is a fundamental component of cardiac desmosomes. This structure is present in the intercalated disk, the site of end-to-end contact between cardiac myocytes, and provides mechanical integrity between adjacent cells. $Na_V1.5$ is also highly localized at the intercalated disks. Combining immunochemistry and electrophysiological studies Sato et al. (2009) demonstrated that PKP2 associates with $Na_V1.5$ in the same molecular complex, and that the knockout of PKP2 expression produced a decrease in peak current density, a shift in voltage-dependence inactivation, and a prolongation of time-dependence of recovery from inactivation.

CONNEXIN-43

Connexin-43 peptides are localized at intercalated disks, where they form gap junctions for electrical coupling of adjacent cells. Sato et al. (2011) showed that AnkG, plakophilin, and connexin-43 are associated at the intercalated disks and that this macromolecular complex may interact with clusters of $Na_V1.5$ also present in the disk. More recently, Chourko et al. characterized the remodeling of the gap junction (connexin-43) and VGSC in an ovine model of right ventricular pressure overload induced by pulmonary hypertension. The authors reported significant lateralization of connexin-43, which was colocalized with mechanical junction proteins and microtubule-associated proteins EB1 and Kifb5 (these proteins are responsible for the forward trafficking of connexin-43 to the intercalated disk). There was also a significant reduction in the peak I_{Na} and in $V_{1/2}$ activation, a slower recovery from inactivation, with no lateralization of the VGSC ($Na_V1.5$). The authors then speculate that the difference in the $Na_V1.5$ remodeling respect to the connexin-43 could be explained due to the fact that trafficking of $Na_V1.5$ might require molecules that cannot redirect the channel to the lateral membrane. In summary, the data reported support the idea of a partnership between these complexes, previously considered to be independent from each other (Chkourko et al., 2012).

ANKYRINS

Ankyrins are a widely expressed family of "adaptor" proteins responsible for the localization of proteins at specialized membrane domains. From all the members that are included in the ankyrin family, ankyrin-G ("G" from "general") was initially studied in the brain. In neurons, ankyrin-G colocalizes and copurifies with VGSC (Kordeli et al., 1995; Davis et al., 1996; Garrido et al., 2003; Mohler, 2006). Ankyrin-G is important for the clustering of $Na_V1.2$ and 1.6 isoforms at the nodes of Ranvier (Jenkins and Bennett, 2001; Garrido et al., 2003) and also colocalizes with VGSC at the neuromuscular junction (Flucher and Daniels, 1989; Kordeli et al., 1998). A role for ankyrin-G for VGSC targeting in cardiac muscle was hypothesized based on the role of this protein in clustering neuronal VGSC (Mohler, 2006). Ankyrin-G binds to a nine residue domain in the DII–DIII loop in the α-subunit of VGSC (Lemaillet et al., 2003). This binding is required for $Na_v1.5$ localization in heart cells (Garrido et al., 2003; Mohler et al., 2004). Since ankyrin-G is primarily expressed at the intercalated disk membrane and T-tubules, it colocalizes with $Na_v1.5$ at these specific sites (Lemaillet et al., 2003; Mohler et al., 2004; Bennett and Healy, 2008; Lowe et al., 2008). Mutations in either the sodium channel domain at which ankyrin binds, or in ankyrin itself, can affect the channel expression (Mohler et al., 2004).

FIBROBLAST GROWTH FACTOR HOMOLOGOUS FACTORS

Fibroblast growth factor homologous factors (FHFs) is a family of cytoplasmic proteins that can interact with VGSC and delay fast inactivation by raising the voltage at which fast inactivation occurs. The role of FHFs as VGSC modulators was already discussed under "Biophysical properties of VGSC."

NEURONAL PRECURSOR CELL-EXPRESSED DEVELOPMENTALLY DOWN REGULATED 4

Neuronal precursor cell-expressed developmentally down regulated 4 (Nedd4) is the prototypical protein in a family of E3 ubiquitin. They select specific proteins for conjugation to ubiquitin, which acts as a marker for protein degradation but also in the sorting of proteins at different steps in biosynthetic and endocytic pathways. They are found in the nucleus and at the plasma membrane. Need4-2 refers to a subgroup of ubiquitin-protein ligases that binds the PY motif of $Na_v1.5$ and reduces the sodium current (I_{Na}) in HEK293 cells by promoting its internalization (Rougier et al., 2005). For more details see the review written by Ingham et al. (2004).

SYNAPSE-ASSOCIATED PROTEINS

Synapse-associated proteins (also called MAGUK, membrane-associated guanylate kinase) are a family of proteins that include Dlg, SAP97/hDlg, SAP90/PSD-95, SAP102, and PSD-93/chapsyn110. They are composed of multiple sites of protein–protein interactions, like the PDZ domains. SAP are localized either to the pre- or postsynaptic sides of excitatory or inhibitory synapses and play a central role in the molecular organization of synapses, like PSD-95, SAP102, and distribution of the NMDA glutamate receptor at the postsynaptic level. One of the family members, SAP97, is also present in epithelial cells and localized at the lateral membrane between cells (Fujita and Kurachi, 2000). At the cardiomyocytes SAP97 colocalized with $Na_v1.5$ at the intercalated disks, determining the existence of a second pool of sodium channels in addition to the channels targeted at lateral membranes by the syntrophin-dystrophin complex (Petitprez et al., 2011).

SYNTROPHIN/DYSTROPHIN COMPLEX

Syntrophins (α, β, and γ) bind and localize signaling proteins to the plasma membrane (Shao et al., 2009). Syntrophins can also interact with multiple proteins via two pleckstrin homology domains, a PDZ domain and a conserved syntrophin unique region. The PDZ domain can bind to the last three residues of the C-termini intracellular domain of $Na_V1.4$ and 1.5 (Haenggi and Fritschy, 2006). The latter can also complex syntrophin and dystrophin (Gavillet et al., 2006). Syntrophin stabilizes the VGSC in the plasma membrane and reduce its internalization (Shao et al., 2009).

β-SUBUNITS

In addition to the modulation of VGSC function (Johnson and Bennett, 2006), β-subunits play critical roles in the intracellular trafficking of α-subunits, regulating the channel expression levels at the plasma membrane and their role in cell adhesion (Isom et al., 1994). *In vitro* data suggests that β-subunits constitute communication links between adjacent cells, extracellular space (via their interaction with tenascin-C and R), cytoskeleton and intracellular signaling mechanisms, and other ion channels. In particular, the β1-subunit seems to be critical for the interaction of the VGSC with other CAMs and cytoskeletal proteins (Kazarinova-Noyes et al., 2001; Chen et al., 2004; McEwen and Isom, 2004). Due to their roles in the interactions with cytoskeletal proteins, CAMs, and other ion channels, Meadows and Isom (2005) proposed that β-subunits should also be considered as molecular scaffolds of the ion conducting pore (α-subunits), therefore critically affecting channel function, subcellular localization and cell surface expression in a cell-specific and subcellular domain-specific manner (see **Table 2**).

PHARMACOLOGY OF VGSC

Voltage-gated sodium channels are the site of action of many toxins and drugs. At least six sites of action for neurotoxins (sites 1–6) and one receptor site for class I antiarrhythmic drugs, local anesthetics and related antiepileptic drugs are known to exist on the VGSC (Cestele and Catterall, 2000). All of them are located on the α-subunit of the channel. Receptor site 1 binds TTX and saxitoxin (Noda et al., 1989; Hille, 2001). This receptor site is formed by amino acid residues in the pore loops and on the extracellular side of them at the outer end of the pore. The sensitivity of the VGSC to TTX segregates them into two groups (**Table 1**):

1. TTX-sensitive channels (TTX-S; blocked with TTX in the nanomolar concentration range). This group includes $Na_v1.1$, $Na_V1.2$, $Na_v1.3$, $Na_v1.4$, $Na_v1.6$, and $Na_v1.7$ isoforms.
2. TTX-resistant channels (TTX-R; blocked with TTX in the micromolar-millimolar concentration range). This group includes $Na_v1.5$, $Na_v1.8$, and $Na_v1.9$ isoforms.

Biophysical and pharmacological properties of TTX-S and TTX-R Na^+ channels are different. TTX-R Na^+ channels can be blocked by inorganic (Co^{2+}, Mn^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, La^{3+}) and organic Ca^{2+} channel blockers. Typically, TTX-R Na^+ channels show smaller single-channel conductance, slower kinetics, and a more positive current-voltage relation than TTX-sensitive ones. Li and Zhu (2011) recently reported two chimeric peptides of drosotoxin that can block the activity of both TTX-R and TTX-S channels. The authors proposed that this approach of understanding the molecular determinants of toxins affecting VGSC would allow a more rational design of subtype-specific sodium channel blockers.

The overlapping sites of action of antiarrhythmic drugs, local anesthetics and related antiepileptic drugs are located in the inner cavity of the pore of the channel, and they are formed by amino acid residues located in S6 in domains I, III, and IV (Ragsdale et al., 1994, 1996; Hockerman et al., 1997; Catterall, 2000; Yarov-Yarovoy et al., 2001, 2002; Liu et al., 2003a). Drug affinity can be reduced by mutations in critical residues in the pore. Fundamentally, these drugs bind to their corresponding site of action to change the function of the channel (decrease the sodium current density). They can also change the affinity with which the channel binds the drug depending on the functional conformation or state in which the channel is found (rest, active, inactive; Bruton et al., 2011). Most sodium channel-blocking agents block the channel when it is open or inactivated, and have very little or no effect at all while the channel is in the resting state. Thus, with each AP, the drug binds to the VGSC and blocks them, and then dissociates during repolarization, with the consequent loss of blockage.

The dissociation rate is a key determinant of steady-state block of sodium channels. AP frequency and duration, membrane potential level, and the physicochemical properties of the drug will determine the rate of recovery from blockage. When depolarization frequency increases, the rest interval decreases, and so does the time available for drug dissociation as the drug remains attached to the channel for a longer time and consequently the steady-state channel block increases. The increase in depolarization frequency also represents repetitive openings of the pore that increase the access of drugs to the intracellular site of action (use-dependent block; Hille, 1977, 2001). The rate of recovery from blockage also slows as cells are depolarized, as occurs during ischemia. Increased AP duration results in a relative increase in the time the channel remains in the inactive state and this can also increase the block by drugs that mainly bind to sodium channels in the inactivated state such as lidocaine.

Current treatment of neuropathic pain includes tricyclic antidepressants (amitriptyline, nortriptyline), local anesthetics (lidocaine, mexiletine), and antiepileptic drugs (phenytoin, carbamazepine, lamotrigine). These drugs however have low efficacy in terms of pain control and are associated with adverse effects involving the heart and CNS.

DISEASES ASSOCIATED WITH VGSC MUTATIONS ("CHANNELOPATHIES")

Ten genes (*SCN1A–SCN11A*; **Table 1**) encoding the α-subunit isoforms of the VGSC and four genes encoding the β-subunits (*SCN1B–SCN4B*; **Table 2**) have been identified in the human genome. Mutations in any of these genes can affect the structure of the channel and, thus, its biophysical properties leading to the development of "*channelopathies*" (**Tables 1** and **2**). All these conditions are associated with autosomal dominant inheritance and *de novo* mutations have been identified. These channelopathies can be divided in four disease groups depending on the predominant organ involved (George, 2005):

1. *Brain sodium channelopathies*, which include mutations in *SCN1A, SCN2A, SCN3A,* and some mutations in *SCN8A* observed in cases of familial human ataxia and in mice models of ataxia and end-plate diseases (these genes encode the channels $Na_V1.1$, $Na_V1.2$, β1-subunit, and $Na_V1.6$ respectively). *SCN1A*, *SCN2A*, and *SCN3A* gene mutations may give rise to epilepsy and epileptic/convulsive disorders.
2. *Skeletal muscle sodium channelopathies*. This group involves mutations in the *SCN4A*, the gene that encodes the $Na_V1.4$ isoform (skeletal muscle specific isoform). *SCN4A* gene mutations are associated with myotonia, myasthenia syndromes, and paralysis.
3. *Cardiac sodium channelopathies*, which involve mutations in *SCN5A* (the gene that encodes $Na_V1.5$, which is predominantly found in cardiac muscle) and *SCN10A* (the gene that encodes $Na_V1.8$, which has been recently identified in the heart (Facer et al., 2011; Verkerk et al., 2012; Yang et al., 2012) and has been associated in genome-wide association studies (GWAS) with alterations in the ventricular conduction (Chambers et al., 2010; Sotoodehnia et al., 2010).
4. *Peripheral nerve sodium channelopathies*, which include mutations in *SCN9A* ($Na_V1.7$), *SCN10A* ($Na_V1.8$), and *SCN11A* ($Na_V1.9$). Mutations in these genes have been associated with peripheral pain syndromes (hyperalgesic syndrome) including neuropathic and inflammatory pain.

BRAIN SODIUM CHANNELOPATHIES

The most commonly affected gene is *SCN1A*[1,2]. Functional studies of *SCN1A* missense epileptogenic mutations *in vitro* have been controversial but several results are consistent with loss of function (hypoexcitability) mutations (Ragsdale, 2008; Mantegazza et al., 2010) and data obtained with animal models have confirmed this (Tang et al., 2009; Martin et al., 2010). Data obtained with a mouse model of Dravet syndrome expressing a truncated $Na_V1.1$ showed that loss of function of this VGSC causes reduced sodium current and excitability in GABAergic neurons, consistent with reduced GABAergic inhibition (Yu et al., 2006). $Na_V1.1$ missense mutations can induce loss of function because of folding defects and these mutants can be rescued by molecular interactions with co-expressed proteins and drugs; this may be one of the causes of the phenotypic variability in GEFS+ and may be exploited for therapeutic potential (Escayg et al., 2000; Meisler and Kearney, 2005; Rusconi et al., 2007, 2009; Catterall et al., 2008). Epilepsy has also been related with *SCN1A* mutations that alter channel inactivation, resulting in persistent inward sodium current [gain-of-function (hyperexcitability) mutations; Lossin et al., 2002]. The above paragraph describes functional studies with $Na_V1.1$ mutants that yield a wide range of biophysical phenotypes from loss of function to gain-of-function. At first sight this seems to be contradictory. Therefore, questions arise as to how mutations with such diverse functional effects can be associated with the same epileptic syndrome or disease. To better understand this, it is critical to remember that *SCN1A* is widely expressed in most neurons in the brain. It has also been previously reported that a single sodium channel mutation can produce hyper- or hypoexcitability in different types of neurons (Rush et al., 2006). Therefore, the net effect of the *SCN1A* mutations on the brain excitability will not only depend on the type of neuron where the mutant channel is expressed but also on the electrical balance between all the ionic currents that contribute to the neuronal AP and the mutant currents. There also might be several additional pathogenic mechanisms involved in the production of epilepsy that are not completely understood yet but still make a significant contribution to the production of the disease.

SMEI is a rare disorder characterized by generalized tonic, clonic, or tonic-clonic seizures that are initially induced by fever and begin during the first year of life. Typically, children with Dravet syndrome [or myoclonic epilepsy of infancy (SMEI); Claes et al., 2001] carry *de novo* mutations not inherited from their parents. Later, patients also manifest other seizure types, including absence, myoclonic, and simple and complex partial seizures. Psychomotor development delay is observed around the second year of life. SMEI is considered to be the most severe phenotype within the spectrum of generalized epilepsies with febrile seizures-plus. Because of this, genetic screening for *SCN1A* has become the diagnostic tool for children with early-onset seizures. More than half of the SMEI mutations cause loss of function as a result of stop codons or deletions, leading to decreased levels of functional sodium channels.

SCN1A mutations have also been associated with three other epileptic disorders: intractable childhood epilepsy with generalized tonic-clonic (ICEGTC) seizures, familial febrile convulsions type 3A (FEB3A), and familial hemiplegic migraine type 3 (FHM3). ICEGTC has been included in the Dravet syndrome (Mullen and Scheffer, 2009). A mutation causing simple familial febrile convulsions has been studied by Mantegazza et al. (2005). Familial hemiplegic migraine type 3 is a distinct disease caused by missense mutations of Na_V1. Here again the functional effects are controversial, but gain-of-function effects have been observed and this is consistent with the pathogenic mechanism of migraines with aura (Cestele et al., 2008; Kahlig et al., 2008).

[1] www.molgen.ua.ac.be/SCN1AMutations
[2] http://www.scn1a.info/

Missense mutations of *SCN2A* were also identified in a small percentage of GEFS+ patients and mainly in patients with benign familial neonatal-infantile seizures (BFNIS), a syndrome of mild seizures that remit during the first year of life without neurologic sequelae. BFNIS mutations produced abnormalities in the sodium channels that led to a reduced channel activity (loss of function; Misra et al., 2008). Other groups have reported mutations in *SCN2A* that result in a gain-of-function, consistently with the role of $Na_v1.2$ in excitatory cortical neurons (Scalmani et al., 2006; Liao et al., 2010).

The first *SCN3A* mutation (K353Q) was identified in a patient with partial epilepsy resistant to antiepileptic drugs (Holland et al., 2008). Even though the missense mutation described caused an increase in late current, the pathogenic role of mutated $Na_v1.3$ is still debated. In mouse models, mutations in *SCN8A* lead to ataxia and end-plate disease. These conditions can be reproduced by conditional knockout of *SCN8A* in cerebellar Purkinje and granule cell neurons (Levin et al., 2006). In rare cases of human familial ataxia, one frame-shift mutation has been identified in *SCN8A* that truncates the protein at the DIV and lead to loss of channel function (Vicart et al., 2005; Trudeau et al., 2006) (For more detailed information about mutations of brain VGSC see Catterall, 2010; Mantegazza et al., 2010).

SKELETAL MUSCLE SODIUM CHANNELOPATHIES

The second group of channelopathies includes mutations in the *SCN4A* gene, which is expressed in skeletal muscle. These skeletal muscle channelopathies (sodium channel myotonic disorders) are part of a group of diseases called non-dystrophic myotonias (Matheus et al., 2010). The clinical disorders can be split between two groups based on the presence or absence of episodic weakness: paramyotonia congenita (characterized by a marked worsening of myotonia by cold and by the presence of clear episodes of weakness), and sodium channel myotonia (notable for the absence of episodic weakness but still have cold sensitivity). The latter group includes all the pure myotonic phenotypes, including the potassium-aggravated myotonias (Fournier et al., 2004).

Causative mutations in the *SCN4A* gene result in a gain of sodium channel function that may show marked temperature dependence. Almost all mutations (over 40) that have been described are missense mutations with an exception of a three base pair deletion (Michel et al., 2007). Exons 22 and 24 are the main exons involved in paramyotonia congenita, including mutations T1313M, V1589M, and mutations at the R1448 and G1306 position (Vicart et al., 2005; Matheus et al., 2008). Lerche et al. (1993) reported a group of heterozygous mutations at the G1306 position of the *SCN4A* gene. Electrophysiological studies on patient muscle samples showed slower sodium fast channel inactivation and an increase in late channel opening resulting in a steady-state inward current, sustained muscle depolarization, and muscle fiber hyperexcitability. These findings suggest that *SCN4A* residue 1306 is important for sodium channel inactivation (Lerche et al., 1993).

Finally, numerous mutations in *SCN4A* gene have also been related to hypokalemic periodic paralysis. This is a muscle disease characterized by episodes of extreme muscle weakness, and it usually begins in infancy or early childhood. Most often, these episodes involve a temporary inability to move muscles in the arms and legs. Sokolov et al. (2007) reported three mutations in gating-charge-carrying arginine residues in an S4 segment that cause hypokalemic periodic paralysis. The mutations induce a hyperpolarization-activated cationic leak through the voltage sensor of the skeletal muscle $Na_V1.4$ channel, consistent with a gain-of-function. This "gating pore current" is active at the resting membrane potential and closed by depolarizations that activate the voltage sensor. The results reported by these authors showed a clear correlation between mutations that cause gating pore current and hypokalemic periodic paralysis.

CARDIAC SODIUM CHANNELOPATHIES

$Na_v1.5$, encoded by *SCN5A*, conducts the inward sodium current (I_{Na}) that initiates the cardiac AP. *SCN5A*-mediated late sodium current also influences repolarization and refractoriness. Mutations in the *SCN5A* gene result in alterations in the function of the α-subunit of the cardiac isoform $Na_v1.5$ channel that have been associated with several inherited arrhythmia syndromes. The main entities related to *SCN5A* mutations include an autosomal dominant form of the LQTS (LQT3; Wang et al., 1995), BS (Probst et al., 2003), progressive cardiac conduction disease (CCD; Scott et al., 1999), sinus node dysfunction (SND; Benson et al., 2003), AF (Olson et al., 2005; Darbar et al., 2008), atrial standstill (Tan, 2006; Remme et al., 2008), and dilated cardiomyopathy (DCM; McNair et al., 2004). Most of these diseases are associated with an increased risk of sudden cardiac death (SCD). The malfunction of the β-subunits (β1 through β4) as well as some of the protein partners that interact with $Na_v1.5$ α-subunit (like caveolin-3 and α-1 syntrophin) have been recently associated with diseases that resemble these arrhythmia phenotypes (Vatta et al., 2006; Cronk et al., 2007; Wu et al., 2008; Watanabe et al., 2009).

Long QT syndrome is characterized by a cardiac repolarization abnormality, with a prolonged QT interval duration observed on 12-lead ECG and vulnerability to a polymorphic ventricular tachycardia called Torsade de Pointes. About 5–10% of LQTS cases are related to mutations in *SCN5A* (LQT3) or the genes that encode the ChiPs. Mutations in *SCN5A* compromise the II–IV linker and disrupt fast inactivation, allowing repeated reopening of the channel during sustained depolarization. As a consequence, a small persistent sodium current is evoked during the AP plateau. This excessive inward current (gain-of-function) delays the repolarization of the cell, prolonging AP duration, and increasing the risk for ventricular arrhythmias.

Brugada Syndrome is a genetic disease that has been associated with ventricular fibrillation and SCD in young people. Approximately 20% of these patients have mutations in the *SCN5A* gene. More than 200 mutations have been associated with this disease (Kapplinger et al., 2010). In contrast to LQTS3 mutations, *SCN5A* mutations related to BS result in a loss of function of the channel. This can be produced by a confluence of different mechanisms, such as trafficking defects, generation of defective or truncated proteins, faster channel inactivation, shift of voltage-dependence inactivation toward a more depolarized membrane potential, or

even slow recovery from inactivation. The electrical consequence of this is the presence of a slower conduction substrate. BS has also been associated with mutations in the genes that encode β1- (*SCN1B*, BS type 5) and β3-subunits (*SCN3B*, BS type 7; Abriel, 2010) of the cardiac sodium channel.

Genetic mutations in *SCN5A* specific only to AF have recently been described. Recently, Li et al. (2009) identified a novel coding variant, K1493R, which altered a highly conserved residue in the DIII–IV linker and was located six amino acids downstream from the fast inactivation motif of sodium channels. Biophysical studies of K1493R in tsA-201 cells demonstrated a significant positive shift in voltage-dependence of inactivation and a large ramp current near resting membrane potential, indicating a gain-of-function. Enhanced cellular excitability was observed in transfected HL-1 atrial cardiomyocytes, including spontaneous AP depolarizations and a lower threshold for AP firing. These novel biophysical observations provide molecular evidence linking cellular "hyperexcitability" as a mechanism inducing vulnerability to AF.

Other pathologies related to mutations in *SCN5A* include progressive familial heart block type 1A (PFHB1A), sick sinus syndrome type 1 (SSS1), sudden infant death syndrome (SIDS), familial atrial standstill, and DCM. For a more detailed review on *SCN5A* channelopathies see Zimmer and Surber (2008), and Wilde and Brugada (2011).

In addition to *SCN5A* mutations, variants in *SCN10A* (the gene that encodes $Na_V1.8$) can also lead to alterations in the cardiac rhythm. $Na_V1.8$ has only recently been identified in the heart (Facer et al., 2011; Verkerk et al., 2012) and GWAS have identified common genetic variants in this gene that modulate ventricular conduction (Chambers et al., 2010; Sotoodehnia et al., 2010).

PERIPHERAL NERVE SODIUM CHANNELOPATHIES

Lampert et al. and Theile and Cummins, recently published extensive reviews on the role of sodium channels in chronic and neuropathic pain syndromes (Lampert et al., 2010; Theile and Cummins, 2011). Neuropathic pain is defined as "pain caused by a lesion or disease of the somatosensory nervous system," and can be divided into central and peripheral neuropathic pain. Typical examples of neuropathic pain include post-herpetic neuralgia, painful diabetic neuropathy, phantom limb pain, and spinal cord injury pain. The fundamental mechanism involved in the production of neuropathic pain is an increase in nerve excitability (and thus changes in VGSC properties), generally manifested in impulses generated ectopically or with minimal stimulation. Nerve injury (classically associated with neuropathic pain) can result in changes in sodium channel trafficking, gene expression, and/or channel kinetics, all of which contribute to neuronal membrane remodeling and hyperexcitability associated with neuropathic pain (Devor, 2006). VGSC $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$ have been particularly identified in the PNS (peripheral neurons and DRG neurons) and seem to have a central role in the genesis of neuropathic pain. Thus, these channels are the new targets for analgesia in peripheral neuropathy pain syndromes. In particular, $Na_v1.7$ is considered to be one of the main mediators of peripheral pain. It has been recently reported that $Na_v1.8$ sodium channel is part of the molecular machinery involved in mechanotransduction of joint pain and other pain syndromes (Schuelert and McDougall, 2012). On the other hand, the role of $Na_v1.3$ in diseased states is still controversial.

Recent human association studies have directly linked *SCN9A*, the gene that encodes $Na_v1.7$, to three human pain disorders: dominantly inherited gain-of-function mutations in inherited erythromelalgia (IEM; nine mutations), paroxysmal extreme pain disorder (PEPD; eight mutations), and recessively inherited loss-of-function mutations in $Na_v1.7$-related congenital insensitivity to pain (CIP; fourteen mutations) (Dib-hajj et al., 2009).

Inherited erythromelalgia (IEM) is a chronic neuropathic pain syndrome that is characterized by excruciating painful attacks in the extremities that begin in childhood and progress over life. A shift to voltage-dependent activation toward more negative potentials seems to be a common factor in all the mutations of *SCN9A* that lead to this disease. This leftward shift of activation can lead to a hyperexcitability state (gain-of-function mutations). Many mutations also delay inactivation, and therefore, larger currents result from slow depolarizing stimuli ("*ramp currents*").

Paroxysmal extreme pain disorder (PEPD), previously referred to as familial rectal pain (Fertleman et al., 2006), is characterized by severe pain accompanied by flushing which are induced by bowel movements or probing of the perianal areas, and are sometimes accompanied by tonic non-epileptic seizures, syncope, bradycardia, and occasionally asystole.

Congenital insensitivity to pain (CIP) is characterized by complete absence of pain perception in patients with non-functional $Na_v1.7$. These patients also exhibit partial anosmia. In this case, the mutations in *SCN9A* identified introduce a stop codon leading to the production of truncated proteins that are non-functional. For further details on the mutations related to each of these diseases see Lampert et al. (2010).

SUMMARY

Voltage-gated sodium channels are widely distributed in excitable and non-excitable cells, and play a critical role in electrical activation in the body. VGSC constitute macromolecular complexes, in which their function relies on both the specific structure of the channel protein (α- and β-subunits) as well as their protein partners (ChiPs). Since VGSC occur predominantly in the central and PNS, and striated (skeletal and cardiac) muscles, mutations in genes encoding VGSC and ChiPs will culminate in diseases named "*channelopathies*" that can be grouped into four main categories: epileptic syndromes, skeletal myopathies, cardiac arrhythmias, and neuropathies (with pain-related syndromes). Pathologic conditions can also arise from the up regulation of the VGSC, as for example in highly aggressive prostate ($Na_v1.7$) and breast ($Na_v1.5$) metastatic carcinomas, An improved understanding of the critical role of the molecular composition of ion channel complexes, the influence of protein partners, and the specific cellular domains underlying protein interactions, are essential for the development of new therapies to treat channelopathies associated with VGSC.

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Skeletal muscle Na^+ channel disorders

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Five inherited human disorders affecting skeletal muscle contraction have been traced to mutations in the gene encoding the voltage-gated sodium channel $Na_v1.4$. The main symptoms of these disorders are myotonia or periodic paralysis caused by changes in skeletal muscle fiber excitability. Symptoms of these disorders vary from mild or latent disease to incapacitating or even death in severe cases. As new human sodium channel mutations corresponding to disease states become discovered, the importance of understanding the role of the sodium channel in skeletal muscle function and disease state grows.

Keywords: sodium channel, skeletal muscle, $Na_v1.4$, treatment

INTRODUCTION

Voltage-gated sodium channels are essential in the generation and propagation of action potentials (APs) in excitable tissues such as muscle, heart, and nerve. Proper activity of these channels is crucial to the initiation of APs which ultimately lead to muscle contraction or neuronal firing. The necessity of Na^+ channels is better emphasized by the existence of human inherited skeletal muscle disorders caused by mutations in the Na^+ channel $Na_v1.4$ which is specifically expressed in skeletal muscle. Mutations in the *SCN4A* gene encoding the human skeletal muscle $Na_v1.4$ channel cause five different skeletal muscle disorders: potassium-aggravated myotonia (PAM), paramyotonia congenita (PMC), hyperkalemic periodic paralysis (HyperPP), hypokalemic periodic paralysis (HypoPP), and a form of congenital myasthenic syndrome (CMS). This review focuses on the role of $Na_v1.4$ channel in skeletal muscle and the complex clinical symptoms of these disorders. Also the most recent findings of new $Na_v1.4$ mutations causing a lethal form of myotonia will be discussed as well as treatment options for such disorders. An overview of skeletal muscle physiology is provided in order to illustrate the significance of ion channels within the skeletal muscle and their critical roles in muscle function.

SKELETAL MUSCLE PHYSIOLOGY

Skeletal muscles have complex structures working in concert to provide the appropriate response to nerve impulse and metabolic processes. Specialized compartments within skeletal muscle fibers such as neuromuscular junctions, sarcolemma membrane, traverse tubules, and the sarcoplasmic reticulum (SR) provide the mechanical architecture required for the excitation–contraction coupling mechanism to take place.

At the neuromuscular junction, motoneuron activity is transferred to skeletal muscle generating an acetylcholine (ACh) dependent endplate potential. ACh is released from the nerve terminal and binds to nicotinic acetylcholine receptors (AChR). A large enough endplate potential can induce a sarcolemmal AP that propagates from the endplate to the tendon and through the transverse tubular (T-tubules) system which is mediated by the opening of the voltage-gated $Na_v1.4$ Na^+ channels. Na^+ channels quickly inactivate and the depolarized potential enables the opening of delayed rectifier K^+ channels which mediate outward K^+ current during the repolarization stage of the muscle AP (Jurkat-Rott and Lehmann-Horn, 2005). High chloride channel (Cl^-) conductance then takes over to enforce the final repolarization or to reduce the afterdepolarization of the skeletal muscle fiber. This afterdepolarization is skeletal muscle AP specific and consists of an early and late phase mediated by different ionic currents (Jurkat-Rott et al., 2006). The early phase is caused by the spread of the depolarization spike in the T-tubules while the late phase is considered to be caused by accumulation of K^+ ions in the T-tubules which increases with frequency and duration of repetitive APs (Almers, 1980). Inward chloride conductance in the T-tubular system alleviates some of the depolarization caused by the extracellular K^+ accumulation by producing a more negative membrane potential than K^+ equilibrium, which stimulates inward potassium flux (Jurkat-Rott et al., 2006).

The contraction of the muscle occurs as a result of Ca^{2+} release from the SR which binds to troponin (a calcium binding protein which is part of the thin filaments necessary to produce muscle contraction) enabling filament sliding and contraction. The process, which allows Ca^{2+} release, is initiated by voltage changes of the AP. These changes will target in part the voltage sensor of the voltage-gated $Ca_v1.1$ Ca^{2+} channel (Dihydropyridine receptor or DHPR) leading to channel conformation rearrangements. The DHPR is believed to physically interact with a calcium release channel of the SR the ryanodine receptor (RYR) which releases calcium stores from the SR allowing calcium to bind to troponin (Rios et al., 1991). When the AP is over, the RYR close and Ca^{2+} is transported back to the SR *via* Ca^{2+}ATPases (SERCA).

SKELETAL MUSCLE Na^+ CHANNEL STRUCTURE AND GATING

Voltage-gated sodium channels are large integral membrane proteins expressed densely at the neuromuscular junctions where they

selectively conduct sodium ions into the muscle fibers in physiological conditions. The $Na_v1.4$ channel is composed of a 260-kDa α-subunit which consists of four homologous domains (I–IV), and each domain has six transmembrane segments (S1–S6; **Figure 1**; Noda et al., 1984; George et al., 1992a,b). The $Na_v1.4$ channels complex structure formed at the membrane incorporates several important gating domains facilitating the channel' three different gating states: resting (closed), activated (open), and inactivated (closed). When a voltage change occurs at cell surface, voltage sensing domains at the S4 segments sense this change and shift their conformation within the membrane relaying this change to the channels internal activation gate and opening it in a very fast manner. Within milliseconds of this fast activation, a ball and chain gate located at the intracellular loop between domains III and IV blocks the intracellular pore of the channel allowing the channel to quickly inactivate (Armstrong and Bezanilla, 1977; West et al., 1992). This fast inactivation process is voltage dependent and occurs at a greater extent and for a longer duration when membrane potentials are more depolarized. Before the channel can be opened again, the internal gate must close and deactivate and the ball and chain gate must be released, this process is called recovery from fast inactivation. Recovery from fast inactivation requires a hyperpolarized membrane potential to last several milliseconds. In addition to fast inactivation, slow inactivation occurs when the channel has been activated repeatedly during exercise, this prevents the channel from being available for further activation for hundreds of milliseconds to seconds. The slow (on the order of seconds to minutes) inactivation process enables the muscle to recover more quickly from exercise.

SKELETAL MUSCLE PATHOPHYSIOLOGY

Over 40 $Na_v1.4$ channel mutations leading to disease states have been found throughout each domain and segment of this channel (**Figure 1** and **Table 1**). These $Na_v1.4$ mutations produce changes that interfere with channel kinetics or function, producing changes in the micro or macroscopic electrophysiological properties of the skeletal muscle fibers thus underlying muscle hyperexcitability or inexcitability. Skeletal muscle Na^+ channel disorders are non-dystrophic and consist of myotonia which presents as an increased muscular activity causing muscle stiffness, and periodic paralysis which presents as a decrease in muscle activity causing transient weakness or paralysis of the muscle.

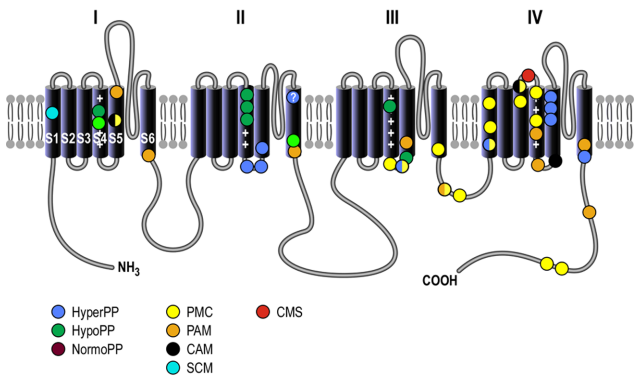

FIGURE 1 | Basic structure of the voltage-gated sodium channel. Structural arrangement of the $Na_v1.4$ channel α-subunit which is organized into four homologous domains (I to IV) and possesses six transmembrane segments (S1–S6), a pore-forming loop located between S5 and S6 segments, and cytosolic NH_3 and COOH *termini*. The schematic diagram of the $Na_v1.4$ channel shows the location of the mutations associated with hyperkalemic periodic paralysis (HyperPP), hypokalemic periodic paralysis (HypoPP), normokalemic periodic paralysis (NormoPP), paramyotonia congenita (PMC), potassium-aggravated myotonia (PAM), cold-aggravated myotonia (CAM), and congenital myasthenic syndrome (CMS).

MYOTONIA

A common feature of myotonia is delayed relaxation of the muscle after voluntary contraction or mechanical stimulation, electrophysiologically characterized by highly organized repetitive electrical activity of the muscle fibers. Non-dystrophic myotonias can be caused by mutations in the $Na_v1.4$ sodium channel which increases its function or the ClC-1 chloride channel decreasing channel function (Trip et al., 2008). Mutations causing up-regulation of the $Na_v1.4$ sodium channel can cause PMC or PAM depending on the type of mutation and its functional effect on this channel. Both PMC and PAM cause repetitive APs and increase activity of the muscle leading to myotonia or muscle rigidity.

POTASSIUM-AGGRAVATED MYOTONIA

Potassium-aggravated myotonia includes atypical myotonia congenita, moderate myotonia, myotonia fluctuans, myotonia permanens, acetazolamide-responsive myotonia, and painful myotonia which have overlapping clinical features. The prevalence of PAM is estimated at ∼1:400,000 (Lehmann-Horn et al., 2004). Myotonia fluctuans and moderate PAM patients exhibit stiffness during the first contractions after rest and thus complain that they are particularly stiff after rest. Upon further contractions, their muscles begin to warm up improving the initial stiffness, this occurrence is termed the warm-up phenomenon. However, other PAM patients become stiff within 10–30 min of strenuous work which may last for several hours (Lehmann-Horn et al., 2004).

Potassium-aggravated myotonia is exacerbated by potassium ingestion (but not cold temperatures) because increased K^+ ingestion causes cellular depolarization (George, 2005). Also unlike other myotonias PAM does not present with any major weakness.

Eight $Na_v1.4$ mutations causing PAM have been found in humans: V445M, S804F, I1160V, G1306A/V/E, V1458F, F1473S, and V1589M (McClatchey et al., 1992; Lehmann-Horn et al., 1993; Lerche et al., 1993; Mitrovic et al., 1994, 1995; Ptácek et al., 1994b; Richmond et al., 1997; Fleischhauer et al., 1998; Green et al., 1998; Hayward et al., 1999; Takahashi and Cannon, 1999; Wang et al., 1999; Groome et al., 2005). The clinical phenotypes of I1160V and V1589M mutations present as atypical myotonia congenita, G1306A presents myotonia fluctuans, and G1306E presents myotonia permanens. Most of these PAM mutations are situated in the interface between the cytosol and the membrane which is the location of the inactivation gate. An example of this is with the G1306 residue that is thought to act as the hinge for the inactivation gate. The more different the substituting mutation is from glycine the more severe are the clinical symptoms ranging from myotonia fluctuans (a benign form of myotonia) to myotonia

Table 1 | Disorders of the skeletal muscle and the associated Nav1.4 mutations.

Mutation	Location	Phenotype	References
I141V	IS1	SCM	Petitprez et al. (2008)
R222W	ISIV	HypoPP	Park and Kim (2010)
R225W	ISIV	SCM	Lee et al. (2009)
L266V	IS5	PMC/CAM	Wu et al. (2001)
Q270K	IS5	PAM	Carle et al. (2009)
V445M	IS6	PAM	Wang et al. (1999)
R669H	IIS4-1	HypoPP	Struyk et al. (2000), Kuzmenkin et al. (2002)
R672H	IIS4-2	HypoPP	Kuzmenkin et al. (2002)
R672G	IIS4-2	HypoPP	Kuzmenkin et al. (2002)
R672S	IIS4-2	HypoPP	Bendahhou et al. (2001)
R672C	IIS4-2	HypoPP	Kim et al. (2004)
R675G	IIS4-3	NormoPP	Vicart et al. (2004)
R675Q	IIS4-3	NormoPP	Vicart et al. (2004)
R675W	IIS4-3	NormoPP	Vicart et al. (2004)
L689I	IIS4S5	HyperPP	Bendahhou et al. (2002)
I693T	IIS4S5	HyperPP	Plassart-Schiess et al. (1998)
T704M	IIS4S5	HyperPP	Cannon and Strittmatter (1993), Cummins et al. (1993), Yang et al. (1994), Bendahhou et al. (1999b)
V781I	IIS6	HyperPP?	Baquero et al. (1995), Green et al. (1997)
A799S	IIS6	SCM/SNEL	Lion-Francois et al. (2010), Simkin et al. (2011)
S804F	IIS6	PAM	McClatchey et al. (1992), Green et al. (1998)
R1132Q	IIIS4	HypoPP	Carle et al. (2006)
R1135H	IIIS4	HypoPP	Matthews et al. (2009)
A1152D	IIIS4S5	PMC	Bouhours et al. (2005)
A1156T	IIIS4S5	PMC/HyperPP	McClatchey et al. (1992), Yang et al. (1994), Hayward et al. (1999)
P1158S	IIIS4S5	HypoPP/SCM	Sugiura et al. (2003)
I1160V	IIIS4S5	PAM	Richmond et al. (1997)
V1293I	IIIS6	PMC	Green et al. (1998)
N1297K	III–IV	CAM	Gay et al. (2008)
G1306A	III–IV	PAM	Mitrovic et al. (1995), Groome et al. (2005)
G1306V	III–IV	PAM	Mitrovic et al. (1995), Groome et al. (2005)
G1306E	III–IV	PAM/PMC	Mitrovic et al. (1995), Groome et al. (2005)
T1313M	III–IV	PMC	Yang et al. (1994), Hayward et al. (1997)
T1313A	III–IV	PMC	Bouhours et al. (2004)
M1360V	IVS1	PMC/HyperPP	Wagner et al. (1997)
I1363T	IVS1	PMC	Miller et al. (2004)
M1370V	IVS1	PMC	Okuda et al. (2001)
L1433R	IVS3	PMC	Yang et al. (1994)
L1436P	IVS3	PMC/CAM	Matthews et al. (2008), Bissay et al. (2011)
V1442E	IVS4	CMS	Tsujino et al. (2003)
R1448C	IVS4-1	PMC	Chahine et al. (1994), Yang et al. (1994)
R1448H	IVS4-1	PMC	Chahine et al. (1994), Yang et al. (1994)
R1448P	IVS4-1	PMC	Lerche et al. (1996), Featherstone et al. (1998)
R1448S	IVS4-1	PMC	Bendahhou et al. (1999a)
G1456E	IVS4	PMC	Sasaki et al. (1999)
V1458F	IVS4	PAM	Lehmann-Horn et al. (1993)
F1473S	IVS4S5	PAM	Fleischhauer et al. (1998)
M1476I	IVS4S5	SCM	Rossignol et al. (2007)
A1481D	IVS4S5	CAM	Schoser et al. (2007)
I1490L/M1493I	IVS5	HyperPP/SCM	Bendahhou et al. (2000)
I1495F	IVS5	HyperPP	Bendahhou et al. (1999b)
V1589M	IVS6	PAM	Mitrovic et al. (1994), Hayward et al. (1999)
M1592V	IVS6	HyperPP	Cannon and Strittmatter (1993), Rojas et al. (1999)
Q1633E	C-term	PAM	Kubota et al. (2009)
E1702K	C-term	PMC	Miller et al. (2004)
F1705I	C-term	PMC	Wu et al. (2005)

NormoPP, normokalemic periodic paralysis; HypoPP, hypokalemic periodic paralysis; HyperPP, hyperkalemic periodic paralysis; PMC, paramyotonia congenita; PAM, potassium-aggravated myotonia; CAM, cold-aggravated myotonia; SCM, sodium channel myotonia; SNEL, severe neonatal episodic laryngospasm; CMS, congenital myasthenic syndrome.

permanens a severe myotonia (Lerche et al., 1993; Mitrovic et al., 1994).

$Na_v1.4$ channel kinetics are altered by these mutations in such a way that the channel open probability is increased thus channel activity is up-regulated. With some of these PAM mutations, the rate of fast inactivation is slowed which allows the channels to stay open for a prolonged amount of time. However, the rate of recovery from inactivation is not found to be increased but deactivation is found to be slow for most of these PAM mutations. Most of these mutations have been reported to increase the size of the persistent Na^+ current two to fourfold. The increase in this inward Na^+ current generates after-depolarizations across the T-tubules and decreases the threshold required for AP generation consequently triggering repetitive AP and muscle contraction (Adrian and Marshall, 1976).

PARAMYOTONIA CONGENITA

Paramyotonia congenita is different from PAM in that muscle stiffness is usually followed by flaccid weakness or paralysis (Lehmann-Horn et al., 2004). This form of myotonia is exacerbated by cold temperatures and muscle stiffness is increased with continued activity which is considered a paradoxical effect

(Jackson et al., 1994; Vicart et al., 2005). Cold induced myotonia or weakness can last for several hours even if muscles are immediately re-warmed after short exposure to cold. The effect of temperature on cell excitability in PMC has been suggested to be a result of normal slowing of channel kinetics with cooling, and was not attributed to a consequence of altered $Na_v1.4$ temperature sensitivity (Lerche et al., 1996; Fleischhauer et al., 1998; Dice et al., 2004; Webb and Cannon, 2008). In some cases of PMC, patients are sensitive to serum potassium levels, however, unlike with PAM these patients exhibit weakness provoked by a hypokalemic challenge (Ptácek et al., 1993b; Lehmann-Horn et al., 2004). The main characteristic of PMC is both cold exacerbated myotonia and muscle weakness. This suggests that the PMC mutations under different physiological conditions lead to an increased Na^+ channel activity causing myotonia or inactivation of the channel with paralysis. The prevalence of PMC is ∼1:200,000 (Lehmann-Horn et al., 2004).

Sixteen mutations of the $Na_v1.4$ channel have been found to cause PMC in humans: L266V, A1152D, A1156T, V1293I, G1306E, T1313M/A, M1360V, M1370V, L1433R, R1448C/H/P/S, G1456E, and F1705I (McClatchey et al., 1992; Ptácek et al., 1992; Lerche et al., 1993, 1996; Chahine et al., 1994; Yang et al., 1994; Mitrovic et al., 1995; Wagner et al., 1997; Featherstone et al., 1998; Green et al., 1998; Bendahhou et al., 1999a; Hayward et al., 1999; Sasaki et al., 1999; Okuda et al., 2001; Wu et al., 2001, 2005; Bouhours et al., 2004, 2005; Groome et al., 2005; Gay et al., 2008). However, $Na_v1.4$ mutations A1156T and M1360V have been classified as both PMC and HyperPP. There are many similarities in electrophysiological properties and clinical observations between PMC and HyperPP. This led to a hypothesis that they are allelic disorders, supported by genetic linkage of this disease *locus* to the $Na_v1.4$ channel gene on chromosome 17 (Fontaine et al., 1990; Ptácek et al., 1991). Most of the PMC mutations are located either on the inactivation particle (IFM; isoleucine, phenylalanine, methionine) between domains III and IV, or on domain IV itself at the S4 voltage sensor (Yang et al., 1994; Fleischhauer et al., 1998; Bouhours et al., 2004).

Functional expression revealed that in most cases, PMC mutations cause a decreased rate of channel inactivation and increased rate of recovery from inactivation and other mutations cause channel deactivation to be slowed (Chahine et al., 1994; Goldman, 1999; Jurkat-Rott et al., 2010). The functional effects of PMC seem to be more severe than those of PAM in that the time constant of fast inactivation is greater due to increased channel activity. This may lead to the clinical state of weakness of paralysis more efficiently than with PAM by depolarizing the resting potential and inactivating Na^+ channels.

SEVERE NEONATAL EPISODIC LARYNGOSPASM

Recently a novel $Na_v1.4$ myotonia mutation was identified causing severe neonatal episodic laryngospasm (SNEL) found to be lethal in newborns (Lion-Francois et al., 2010). These newborns presented with episodic apneas and apparent life threatening events that included myotonia of the laryngeal muscles preventing proper ventilation. One infant who was not treated in time with either carbamazepine or mexiletine died due to respiratory arrest during an apneic episode. A *de novo* A799S missense mutation of the $Na_v1.4$ channel was found in this patient (Lion-Francois et al., 2010; Simkin et al., 2011). This mutation is located on the S6 transmembrane segment of domain II and results in such a severe phenotype by shifting the steady state of activation in a hyperpolarizing direction, slowing the kinetics of fast inactivation and deactivation, and dramatically increasing channel open probability (Simkin et al., 2011).

Na^+ channel mutations have already been described in neonates (Tsujino et al., 2003; Gay et al., 2008). These patients had hypotonia and were harboring mutations I693I and N1297K. In addition to the myotonic events, these patients experienced muscle weakness as well. Although muscle weakness can be triggered in patients with Na^+ channel myotonia, this clinical feature has not been observed in patients carrying the A799S mutation, making SNEL a distinct form of Na^+ channel myotonia in newborn babies.

PERIODIC PARALYSIS

Periodic paralysis can be caused by mutations of the $Na_v1.4$ sodium channel which decrease its function, the L-type calcium channel (DHPR), and the inwardly rectifying $K_{ir}2.1$ channel by inhibiting its function in Andersen's syndrome. In some cases of periodic paralysis, serum potassium levels are affected causing HyperPP, HypoPP, or normokalemic (where K^+ levels remain normal) thus periodic paralysis is defined in terms of serum K^+ levels.

HYPERKALEMIC PERIODIC PARALYSIS

Hyperkalemic periodic paralysis has been found in patients with sodium channel mutations that cause attacks of flaccid limb paralysis or weakness of the eye or throat muscles. HyperPP patients present with increased serum K^+ levels during the episodes of weakness. The triggers of HyperPP include K^+ ingestion, rest after exercise as well as cold temperatures, emotional stress, and fasting. HyperPP and PMC have overlapping symptoms because between episodes of periodic paralysis, HyperPP patients can experience a mild form of myotonia, which may be more pronounced before a paralytic attack. Also patients (which do not experience mild myotonia episodes between paralytic attacks) may be more prone to develop chronic progressive myopathy during midlife when paralytic attacks become more rare. Patients are usually diagnosed in the first decade of life and paralysis attacks increase in frequency and severity during puberty but begin to decrease after approximately 40 years of age. However, older individuals may develop permanent weakness related to the frequency and severity of prior attacks (Bradley et al., 1990; Ptácek et al., 1993a; Jurkat-Rott and Lehmann-Horn, 2007b). During a paralytic attack, patients may also experience respiratory insufficiency which could lead to death if not treated immediately. Attacks can last up to an hour and disappear as serum K^+ levels decrease due to elimination by the kidney and reuptake by skeletal muscle cells. However, after an attack, as serum K^+ levels drop, patients can become hypokalemic leading to misdiagnoses (Plassart et al., 1994; Jurkat-Rott and Lehmann-Horn, 2007b). Therefore, diagnoses are mostly based on the patient response to K^+ rather than the level of K^+ during an attack.

Several $Na_v1.4$ mutations lead to HyperPP including L689I, I693T, T704M, A1156T, M1360V, I1490L, M1493I, I1495F, and

M1592V (McClatchey et al., 1992; Cannon and Strittmatter, 1993; Cummins et al., 1993; Yang et al., 1994; Wagner et al., 1997; Plassart-Schiess et al., 1998; Bendahhou et al., 1999b, 2000, 2002; Hayward et al., 1999; Rojas et al., 1999). Mutations T704M and M1592V account for the majority of HyperPP cases (Venance et al., 2006). Most HyperPP mutations are located at the intracellular loop between transmembrane segments S4 and S5 of domains II and III or in the transmembrane segment S5 of domain IV. These areas (at least in domains III and IV) are thought to form a three-dimensional docking site for the fast inactivation particle. Structural malformations in those areas may reduce the affinity of the IFM particle to this docking site reducing fast inactivation of the $Na_v1.4$ channel. Sodium channels typically open for several milliseconds, but HyperPP mutations cause the $Na_v1.4$ channel to allow sodium current to flow even after tens of milliseconds (Cannon and Strittmatter, 1993). In the case of HyperPP, there is a persistent inward sodium current and sustained depolarization which leads to electrical inexcitability and muscle weakness (Lehmann-Horn et al., 1987). Also in excised fibers from patients there is an accumulation of intracellular Na^+ consistent with increased inward sodium current (Weber et al., 2006). It is suggested that persistent Na^+ conductance leads to membrane depolarization which drives K^+ out of the cell into the extracellular space causing severe hyperkalemia and further depolarization of the muscle fibers and Na^+ channel slow inactivation (Cannon et al., 1993; Cummins and Bendahhou, 2009). Indeed, it has been found that with HyperPP mutations L689I, I693T, T704M, and M1592V, the slow inactivation process (a process that modulates the availability of the channels) is impaired preventing the muscle from recovering after muscle contraction and subsequently causing paralysis, while in PMC the muscle is able to recover quicker (Cummins and Sigworth, 1996; Plassart-Schiess et al., 1998; Bendahhou et al., 1999b, 2002; Hayward et al., 1999; Cummins and Bendahhou, 2009; Platt and Griggs, 2009). Although, persistent current may underlay periodic paralysis and myotonia, other mechanisms have also been proposed. Some HyperPP mutations also shift the voltage dependence of activation in the negative direction which shifts the activation threshold and allows channels to open sooner (Cummins et al., 1993; Yang et al., 1994). This negative shift in activation leads to the rise of persistent currents that occur at voltage ranges between activation and fast inactivation or window currents (Cummins et al., 1993; Yang et al., 1994; Bendahhou et al., 1999b; Rojas et al., 1999). There are two mutations A1156T and M1360V that are associated with both HyperPP and PMC, and present with mixed phenotypes (Yang et al., 1994; Wagner et al., 1997). Neither slow inactivation nor activation is affected with these mutations, perhaps owing to the mixed clinical phenotype.

HYPOKALEMIC PERIODIC PARALYSIS

Hypokalemic periodic paralysis can be caused by mutations of the $Na_v1.4$ channel or the skeletal muscle calcium channel $Ca_v1.1$, or the inward rectifier potassium channel Kir2.1. HypoPP caused by Ca^{2+} channel mutations is referred to as HypoPP1 while HypoPP caused by Na^+ channel mutations is referred to as HypoPP2 (Jurkat-Rott et al., 1994, 2000; Ptácek et al., 1994a; Sternberg et al., 2001; Fontaine et al., 2007). The major differences between HypoPP1 and HypoPP2 are that HypoPP1 has an earlier onset, HypoPP2 presents with myalgias and can be aggravated by acetazolamide (a carbonic anhydrase inhibitor typically used to treat periodic paralysis), and in muscle biopsies there is a predominance of tubular aggregates in HypoPP2 and vacuoles in HypoPP1 (Fontaine et al., 2007; Platt and Griggs, 2009). HypoPP is characterized by reversible attacks of muscle weakness with decreased serum K^+ levels. During HypoPP attacks, patients can lose their muscle strength and reflexes and have subjective sensory symptoms. These attacks of weakness or paralysis are triggered by rest after strenuous exercise, ingestion of carbohydrates exposure to cold, or corticosteroid intake (Bendahhou et al., 2007). There is no evidence in electromyographic studies that HypoPP patients have any form of myotonia with the exception of eyelid myotonia which can sometimes even be present between attacks (Ptácek and Griggs, 1996). Attacks can last several hours or even days, unlike in HyperPP, and are more severe in nature. Patients are usually at their weakest during the later hours of the night and in the morning and progressively get stronger as the day goes by. Once an attack has resolved, patients appear completely normal. As patients get older, attack frequency decreases but some develop progressive persistent weakness in the form of proximal myopathy and can progress to debilitating weakness (to the point of wheelchair confinement) especially those who were untreated throughout previous attacks. Muscle biopsies from HypoPP patients reveal abnormal vacuoles resulting from SR dilatation (possibly as a result of osmotic fluxes) or atrophic changes even during attack free intervals. However, blood studies are unremarkable in these intervals.

A number of $Na_v1.4$ mutations resulting in HypoPP2 have been found including in humans: R222W, R669H, R672H/G/S, R1129Q, R1132Q, R1135H, and P1158S (Struyk et al., 2000; Bendahhou et al., 2001; Kuzmenkin et al., 2002; Sugiura et al., 2003; Carle et al., 2006; Matthews et al., 2009; Hong et al., 2010). All Na^+ or Ca^{2+} channel HypoPP mutations, are located on the voltage sensors of these two channels. Sodium channel HypoPP mutations are located primarily on the S4 voltage sensors of domains II and III where they neutralize positively charged residues hindering proper voltage sensor function (Kontis et al., 1997; Kuhn and Greeff, 1999; Matthews et al., 2009; Ruff, 2010). HypoPP $Na_v1.4$ mutations (unlike other $Na_v1.4$ mutations which cause a gain-of-function by enhancing activation or impairing inactivation) cause a channel loss of function by enhancing channel inactivation which, can be achieved by enhancing fast inactivation, slow inactivation or both reducing the availability of these sodium channels (Jurkat-Rott et al., 2000; Ruff and Cannon, 2000; Struyk et al., 2000; Bendahhou et al., 2001; Kuzmenkin et al., 2002). At normal muscle resting membrane potentials (RMP), about 70% of the $Na_v1.4$ channels are available for activation which is enough to generate an AP. However, in HypoPP there is a significantly depolarized RMP which causes more $Na_v1.4$ channels to enter the inactive and unavailable state for AP generation, while muscle is contracting, producing weakness. Other HypoPP $Na_v1.4$ mutations allow fewer channels to enter the slow inactivation state when they need to rest while muscle is active which prevents the turnover from inactive to active channels to happen as needed for the muscle and thus more Na^+ channels enter the inactive state subsequently causing paralysis. Nevertheless, this hypothesis of channel down regulation does not seem to explain the paradoxical sarcolemmal depolarization

with hypokalemia or paralysis induced by insulin and glucose during HypoPP attacks (Tricarico et al., 1998; Ruff, 1999).

Recent studies propose that the depolarization seen with some HypoPP mutations can be due to a cationic leak through the voltage sensor created by the mutations called the gating pore current (channel up-regulation) which may cause a cellular pH imbalance due to proton movement into the fibers (Kuzmenkin et al., 2002; Carle et al., 2006; Sokolov et al., 2007; Struyk and Cannon, 2007; Struyk et al., 2008). This gating pore is distinct from the main sodium ion pore between segments S5 and S6 and is suggested to be formed by segments S1–S4 in each domain (making up four possible gating pores) perhaps by the mutation of the S4 segment (Yang et al., 1997; Sato et al., 2001; Long et al., 2005; Sokolov et al., 2005; Tombola et al., 2005). This gating current is a hyperpolarization activated current of monovalent cations which counteracts the K^+ current and depolarizes the RMP thus it would explain why muscle fibers are depolarized during hypokalemia (Jurkat-Rott and Lehmann-Horn, 2007a; Jurkat-Rott et al., 2009). It has been suggested that the gating pore may lower the intracellular pH leading to (i) intracellular Na^+ ion accumulation through the activation of transporters such as the Na^+/H^+ exchanger, and/or (ii) the inhibition of the inwardly rectifying K^+ channels (see Matthews and Hanna, 2010). However, this has not been sufficiently elucidated because studies reporting on the gating currents corresponding to several HypoPP mutations were conducted in *Xenopus* oocytes only and have not been reproduced in mammalian cells (Sokolov et al., 2007; Struyk and Cannon, 2007).

CONGENITAL MYASTHENIC SYNDROME

Myasthenic syndrome (CMS) is a disorder with defective transmission of neuromuscular excitation resulting in muscle fatigue from defects in presynaptic, synaptic, or postsynaptic proteins (Engel et al., 2003). Patients experience fatigable generalized weakness and recurrent attacks of respiratory and bulbar paralysis beginning at birth. CMS can be caused by several types of defects. In one case, nerve stimulation at physiological rates rapidly decreased the compound muscle APs but no abnormalities in RMP evoked quantal release synaptic potentials, AChR channel kinetics, or endplate ultrastructure was found. However, endplate potentials depolarizing the resting potential to −40 mV failed to excite APs (Tsujino et al., 2003).

In this case, a $Na_v1.4$ channel mutation (V1442E) was found located at the extracellular linker between segments S3 and S4 of domain IV (Tsujino et al., 2003). Respiratory paralysis is not a common symptom of $Na_v1.4$ mutation defects despite the fact that $Na_v1.4$ is the predominant sodium channel in respiratory muscles. However, the CMS patient with the V1442E mutation did exhibit respiratory paralysis as well as paralysis of other muscles such as the bulbar muscles, limiting speech, and swallowing. This patient had also been on an apnea monitor since infancy and received ventilatory support during apneic attacks. In her early 1920s, the patient had limited ocular ductions, and weakness throughout her body worsened by activity. The V1442E mutation revealed an enhancement of fast inactivation even at hyperpolarized potentials and the availability of sodium channels was significantly reduced, even compared to HypoPP mutations, causing muscle AP failure even though the RMP was normal.

TREATMENTS FOR SKELETAL MUSCLE SODIUM CHANNEL MYOTONIAS AND PERIODIC PARALYZES

MYOTONIA

Treatment for myotonia is focused on reducing the involuntary AP bursts without blocking the voluntary high-frequency muscle stimulation. Although, it is important that PAM and PMC patients modify their lifestyle to avoid the triggers of their diseases such as potassium ingestion or cold temperatures, drug therapies are commonly used to relieve and prevent muscle stiffness (**Table 2**).

For PMC and PAM patients, anticonvulsants (phenytoin and carbamazepine), anti-arrhythmics of the class IB (mexiletine and tocainide), class IC (flecainide and propafenone), and local anesthetics have been shown to have some efficacy relieving stiffness in PAM and preventing stiffness and weakness from occurring in PMC (Trip et al., 2006; Alfonsi et al., 2007). Agents such as mexiletine and flecainide (orally absorbed methylated lidocaine derivatives) prevent repetitive AP firing and myotonia by reversibly blocking sodium channels (Wang et al., 2004; De Bellis et al., 2006). This therapeutic effect enhances sodium channel inactivation and shifts the voltage dependence of steady state inactivation in a hyperpolarizing direction and slows the recovery from inactivation. In most cases, $Na_v1.4$ mutations increased mexiletine sensitivity however, this was found to be dependent on the $Na_v1.4$ mutation as in some cases mexiletine sensitivity was reduced contributing to the heterogeneity of clinical symptoms observed with mexiletine treatment (Fan et al., 1996; Featherstone et al., 1998; Takahashi and Cannon, 2001; Desaphy et al., 2003).

Since PMC patients present with severe myotonia that overshadows their attacks of weakness most of these patients rarely require medications for weakness. However, sometimes diuretic carbonic anhydrase inhibitor medications such as acetazolamide and hydrochlorothiazide can be given to reduce serum K^+ levels or

Table 2 | Medication in the different forms of Na channel myotonia and paralysis.

Disorders	Therapies
PMC	Anticonvulsants (phenytoin and carbamazepine)
	Anti-arrhythmics of the class IB (mexiletine and tocainide)
	Anti-arrhythmics class IC (flecainide and propafenone)
	Local anesthetics (acetazolamide and hydrochlorothiazide)
PAM	Anticonvulsants (phenytoin and carbamazepine)
	Anti-arrhythmics of the class IB (mexiletine and tocainide)
	Anti-arrhythmics class IC (flecainide and propafenone)
	Local anesthetics
HyperPP	β-adrenergic agonists such as (salbutamol used as an inhalant)
	Glucose/insulin therapy
	Diuretic carbonic anhydrase inhibitors (acetazolamide and dichlorphenamide and thiazides)
HypoPP	Oral potassium
	Acetazolamide or dichlorphenamide, triamterene, aldosterone antagonists
	Potassium-sparing diuretics

lower the pH and decrease the frequency and severity of paralytic events (Rudel et al., 1980; Ricker et al., 1983; Ptácek et al., 1993b; Ptácek and Griggs, 1996).

PERIODIC PARALYSIS

Patients with HyperPP often find that reducing their carbohydrate intake and avoiding strenuous exercise and cold improves their condition. Other treatments include β-adrenergic agonists such as salbutamol used as an inhalant on patients without cardiac arrhythmia or glucose/insulin therapy (Hanna et al., 1998). Diuretic carbonic anhydrase inhibitors (acetazolamide and dichlorphenamide) and thiazides seem to be the most effective in HyperPP (Sansone et al., 2008). Treatments that enhance slow inactivation of sodium channels or shift the voltage dependence of activation may be more effective in treating some HyperPP patients.

Treatment of HypoPP is better achieved by administrating oral potassium and by avoidance of carbohydrates and sodium in the diet. Increasing K^+ levels usually helps to reduce the paradoxical membrane depolarization and shifts the resting potential to more normal hyperpolarized voltages. Administration of acetazolamide or dichlorphenamide has also proven to be useful, however in some cases these agents can exacerbate symptoms and triamterene (kidney sodium channel blocker used as a potassium-sparing diuretic) is used instead (Torres et al., 1981; Tawil et al., 2000; Sternberg et al., 2001; Venance et al., 2004). These carbonic anhydrase inhibitors also lower the intracellular sodium levels in addition to repolarizing the RMP (perhaps by opening calcium activated K^+ channels; Tricarico et al., 1999). Other aldosterone antagonists and potassium-sparing diuretics are also used.

CONCLUSION

Skeletal muscle sodium channel disorders show significant intra and interfamilial phenotypical variability as well as variability in the functional electrophysiological properties of the $Na_v1.4$ channel. It remains difficult to properly identify, classify, and treat myotonia and periodic paralysis patients due to an insufficient understanding of the mechanism by which these mutations bring about such variable phenotypes. $Na_v1.4$ channel gating defects to produce gain-of-function which slows the rate of inactivation and or deactivation typically cause myotonia while $Na_v1.4$ mutations that negatively shift the voltage dependence of activation typically cause HyperPP. Mutations that down-regulate $Na_v1.4$ channel function by enhancing slow inactivation or the newly suggested mechanism of increased gating pore currents which depolarize muscle fibers in the presence of low extracellular K^+ concentrations result in HypoPP. If gating pore currents do indeed underlie the abnormal depolarization associated with HypoPP, the "gating pore" may represent a novel therapeutic target for treating these patients. Nevertheless, functional studies may not lead to a full understanding of these diseases and careful analysis of clinical phenotypes, clinical trials, and genetic screening are clearly still lacking and needed. Understanding these different factors which underlie skeletal muscle sodium channel disorders may help to improve and develop new strategies of therapeutic treatment for these patients as well as treatments of related disorders of excitability.

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Na^+ channel β subunits: overachievers of the ion channel family

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Voltage-gated Na^+ channels (VGSCs) in mammals contain a pore-forming α subunit and one or more β subunits. There are five mammalian β subunits in total: β1, β1B, β2, β3, and β4, encoded by four genes: *SCN1B–SCN4B*. With the exception of the *SCN1B* splice variant, β1B, the β subunits are type I topology transmembrane proteins. In contrast, β1B lacks a transmembrane domain and is a secreted protein. A growing body of work shows that VGSC β subunits are multifunctional. While they do not form the ion channel pore, β subunits alter gating, voltage-dependence, and kinetics of VGSCα subunits and thus regulate cellular excitability *in vivo*. In addition to their roles in channel modulation, β subunits are members of the immunoglobulin superfamily of cell adhesion molecules and regulate cell adhesion and migration. β subunits are also substrates for sequential proteolytic cleavage by secretases. An example of the multifunctional nature of β subunits is β1, encoded by *SCN1B*, that plays a critical role in neuronal migration and pathfinding during brain development, and whose function is dependent on Na^+ current and γ-secretase activity. Functional deletion of *SCN1B* results in Dravet Syndrome, a severe and intractable pediatric epileptic encephalopathy. β subunits are emerging as key players in a wide variety of physiopathologies, including epilepsy, cardiac arrhythmia, multiple sclerosis, Huntington's disease, neuropsychiatric disorders, neuropathic and inflammatory pain, and cancer. β subunits mediate multiple signaling pathways on different timescales, regulating electrical excitability, adhesion, migration, pathfinding, and transcription. Importantly, some β subunit functions may operate independently of α subunits. Thus, β subunits perform critical roles during development and disease. As such, they may prove useful in disease diagnosis and therapy.

Keywords: adhesion, β subunit, development, excitability, voltage-gated Na^+ channel

INTRODUCTION

Mammalian voltage-gated Na^+ channels (VGSCs) exist as macromolecular complexes *in vivo*, comprising, at minimum, one pore-forming α subunit and one or more β subunits in a 1:1 stoichiometry for α:β (Catterall, 1992). Traditionally, VGSC β subunits have been termed "auxiliary." However, increasing evidence suggests that the β subunits are far from auxiliary, and, in fact, function as critical signaling molecules in their own right, perhaps even independently of α subunits. In this review, we will summarize the latest developments describing the growing, diverse, multifunctional roles of the β subunits, including their contribution to human disease.

MOLECULAR DIVERSITY AND FUNCTIONAL ARCHITECTURE

The topology of the canonical VGSC complex is shown in **Figure 1**. To date, five β subunits have been identified in mammals: β1, its alternative splice variant β1B (previously called β1A), β2, β3, and β4 (Isom et al., 1992, 1995; Kazen-Gillespie et al., 2000; Morgan et al., 2000; Qin et al., 2003; Yu et al., 2003). Each β subunit is encoded by one of four genes, *SCN1B–SCN4B*. With the exception of β1B, the β subunits share a similar type I membrane topology, including an extracellular N-terminal region immunoglobulin (Ig) loop, one transmembrane domain, and a small intracellular C-terminal domain (**Figure 2**). β2 and β4 are disulfide linked to VGSC α subunits, whereas β1 and β3 associate non-covalently (Isom et al., 1992, 1995; Morgan et al., 2000; Yu et al., 2003). The residues(s) responsible for the covalent interaction between β2/β4 and α have not yet been identified. Mutation studies have revealed that the A/A′ strand of the β1 Ig fold contains critical charged residues that interact with, and modulate the activity of, the α subunit whereas the intracellular domain is not involved (Mccormick et al., 1998). Less is known about the β subunit interaction sites on α subunits; however, an epilepsy-causing mutation, D1866Y, in the C-terminal cytoplasmic domain of $Na_v1.1$ disrupts modulation of Na^+ current by β1 (Spampanato et al., 2004). β1B shares the same N-terminal Ig domain as β1, but by virtue of retention of intron 3, has a different C-terminal region that lacks a transmembrane domain but contains a stop codon and polyadenylation site (Kazen-Gillespie et al., 2000; Qin et al., 2003). As a result, β1B is unique among the β subunits in that it is a soluble protein (Patino et al., 2011). For reasons that are not understood, the amino acid sequence of the β1B C-terminal domain is species-specific (Patino et al., 2011). Further species-specific alternative splicing events have been discovered within *SCN1B*, including splice variants of

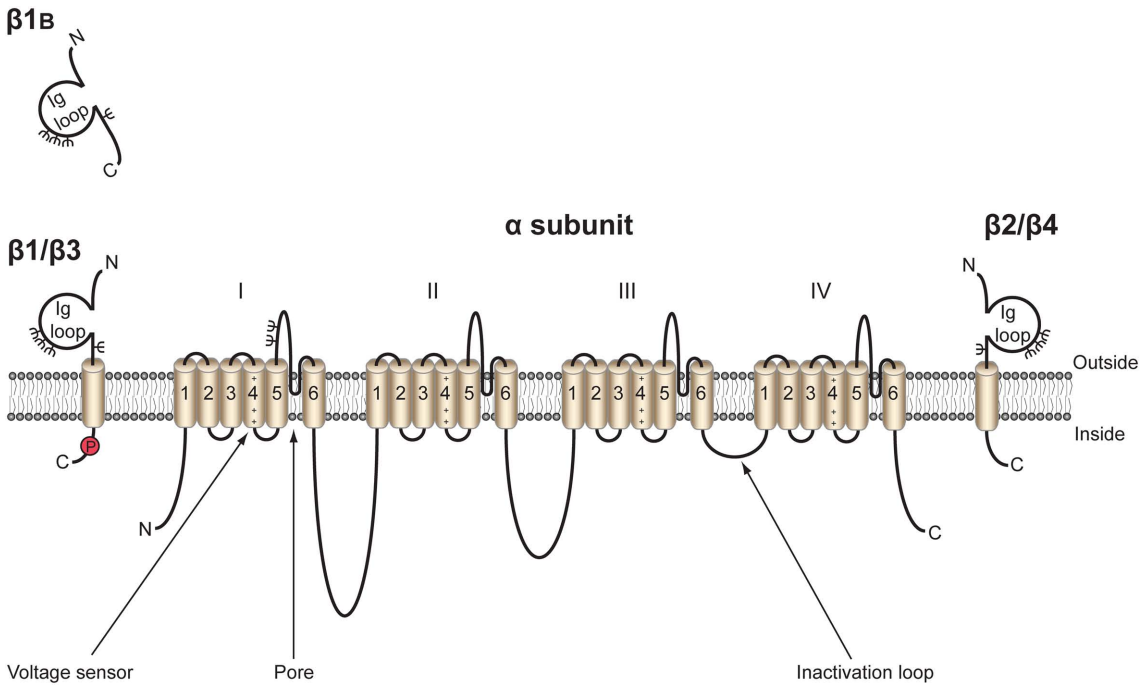

FIGURE 1 | Topology of the voltage-gated Na+ channel α and β subunits. VGSCs contain a pore-forming α subunit consisting of four homologous domains of six transmembrane segments (1–6). Segment 4 contains the voltage sensor (Catterall, 2000). VGSCs also contain one or more β subunits. β1, β2, β3, and β4 contain an extracellular immunoglobulin (Ig) loop, transmembrane domain, and an intracellular C-terminal domain (Isom et al., 1994). β1B also contains an Ig loop, but has a different C-terminus lacking a transmembrane domain, and is thus a soluble, secreted protein (Patino et al., 2011). β1 contains a tyrosine phosphorylation site in its C-terminus (Malhotra et al., 2004) ψ, glycosylation sites. β1 and β3 are non-covalently linked to α, whereas β2 and β4 are covalently linked through disulfide bonds. Figure was produced using Science Slides 2006 software.

the zebrafish *SCN1B* ortholog *scn1ba*, *scn1ba_tv1*, and *scn1ba_tv2* (Fein et al., 2007) with altered protein structure, and β1.2 in rat with an altered 3′ untranslated region (Dib-Hajj and Waxman, 1995). The tissue-specific expression profiles of each of the β subunits are subtly different, but clearly overlapping (**Table 1**). As with the α subunits, β subunits are highly expressed in excitable cells, including central and peripheral neurons, skeletal and cardiac muscle cells (Isom et al., 1992, 1995; Morgan et al., 2000; Yu et al., 2003; Maier et al., 2004; Lopez-Santiago et al., 2006, 2011; Brackenbury et al., 2010). Importantly, however, increasing evidence points to the expression of β subunits in a broad range of traditionally non-excitable cells, including stem cells, glia, vascular endothelial cells, and carcinoma cells (O'Malley and Isom, manuscript in preparation; Diss et al., 2008; Chioni et al., 2009; Andrikopoulos et al., 2011).

REGULATION OF EXCITABILITY BY INTERACTION WITH α SUBUNITS

Beginning with the initial report of β1 cloning in 1992 (Isom et al., 1992), numerous studies have demonstrated that all five β subunits alter gating and kinetics of α subunits expressed in heterologous cells (Catterall, 2000; Kazen-Gillespie et al., 2000; Qin et al., 2003; Yu et al., 2003). For example, in both *Xenopus* oocytes and mammalian cell lines, β1 and β2 increase the peak Na^+ current carried by $Na_v1.2$, accelerate inactivation, and shift the voltage-dependence of activation and inactivation to more negative potentials (Isom et al., 1992, 1994, 1995). However, inconsistencies between different reports documenting the magnitude and types of current modulation suggest that the cell background, including expression of endogenous β subunits and/or other interacting proteins, is a critical factor to consider when interpreting the data (Moran et al., 2000, 2003; Meadows and Isom, 2005). Furthermore, some of the more obvious effects of β1 and β2 on Na^+ current gating and kinetics in heterologous cells, especially *Xenopus* oocytes, do not appear to be reflected *in vivo*. In fact, results from null mouse models suggest that the effects of β1 and β2 on Na^+ currents *in vivo* are subtle and cell type-specific (Chen et al., 2002, 2004; Aman et al., 2009; Patino et al., 2009; Brackenbury et al., 2010).

Voltage-gated Na^+ channel β subunits have major effects on cellular excitability *in vivo*, suggesting that their subtle effects on Na^+ currents are functionally significant. For example, in *Scn1b* null mice, the fastest components of the compound action potential are slowed in the optic nerve (Chen et al., 2004). The heart rate is also slowed and action potentials in ventricular myocytes are slower to repolarize resulting in QT prolongation (Lopez-Santiago et al., 2007). *Scn1b* null mice are ataxic and, display frequent spontaneous bilateral myoclonic seizures from postnatal day (P)8–10 (Chen et al., 2004). Action potentials in *Scn1b* null CA3 neurons fire with a significantly higher peak voltage and significantly greater amplitude compared with wildtype neurons (Patino et al., 2009). In addition, the action potential firing

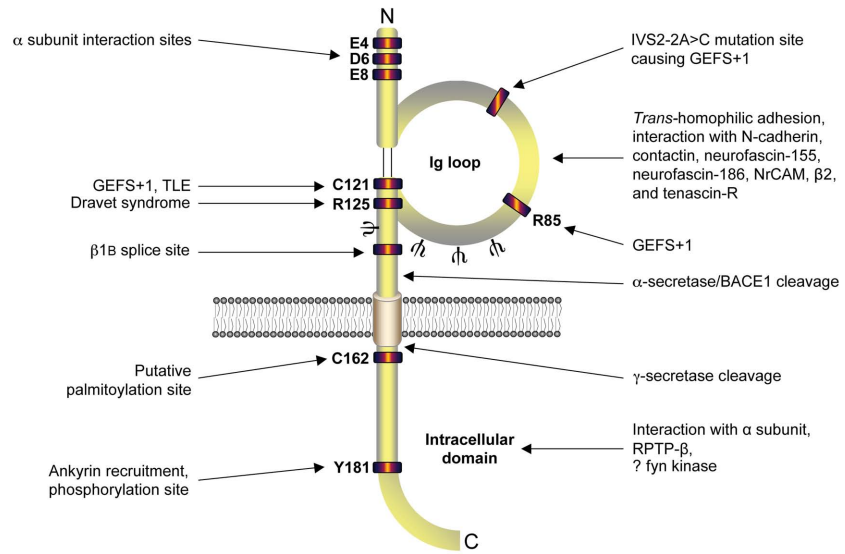

FIGURE 2 | Functional architecture of β1/β1B. β1 contains residues responsible for interaction with α subunit in its intracellular and extracellular domains (Mccormick et al., 1998; Spampanato et al., 2004). Mutation sites responsible for causing genetic epilepsy with febrile seizures plus (GEFS + 1), temporal lobe epilepsy (TLE), and Dravet syndrome are located in the extracellular immunoglobulin loop (Meadows et al., 2002; Wallace et al., 2002; Audenaert et al., 2003; Scheffer et al., 2007; Patino et al., 2009). Alternative splicing site for β1B (Kazen-Gillespie et al., 2000; Qin et al., 2003; Patino et al., 2011), putative palmitoylation site (Mcewen et al., 2004), ankyrin interaction site (Malhotra et al., 2002), tyrosine phosphorylation site (Malhotra et al., 2004), *N*-glycosylation sites (ψ; Mccormick et al., 1998), α/β/γ-secretase cleavage sites (Wong et al., 2005), receptor protein tyrosine phosphatase β (RPTPβ) interaction (Ratcliffe et al., 2000), and putative fyn kinase interaction (Malhotra et al., 2002, 2004; Brackenbury et al., 2008) are also marked. Figure was produced using Science Slides 2006 software.

rate is reduced in *Scn1b* null cerebellar granule neurons (Brackenbury et al., 2010). Reduced action potential firing in inhibitory interneurons (e.g., GABAergic granule neurons) may lead to overall hyperexcitability within the neuronal network, and result in hyperexcitability-related disorders, e.g., seizures (Oakley et al., 2011). In contrast, *Scn1b* null nociceptive dorsal root ganglion neurons are hyperexcitable (Lopez-Santiago et al., 2011). In the latter example, the hyperexcitability is proposed to be due to modulation of both Na^+ and K^+ currents by β1 or β1B (Lopez-Santiago et al., 2011). In support of this notion, β1 interacts with, and modulates, the gating of the inward rectifier $K_v4.3$ in heterologous cells (Deschenes et al., 2002, 2008).

β2 also regulates VGSC α subunits in neurons, and thereby electrical excitability. However, its role is proposed to be somewhat different to β1, and its effects on channel kinetics and voltage-dependence appear even subtler *in vivo*. Unlike *Scn1b* null mice, *Scn2b* null mice appear normal in neurological tests, although they display increased seizure susceptibility, and an elevated action potential threshold in the optic nerve (Chen et al., 2002, 2004). β2 associates with α subunits as the final step in neuronal VGSC biosynthesis, thereby permitting insertion of the complex into the plasma membrane, and increasing Na^+ current (Schmidt and Catterall, 1986; Isom et al., 1995). Thus, β2 plays an important role in stabilizing channel expression at the cell surface, and thereby maintaining normal action potential threshold. In agreement with this, there is ∼50% decreased expression of α subunits and Na^+ currents at the plasma membrane of *Scn2b* null hippocampal neurons (Chen et al., 2002). However, *Scn2b* deletion has no effect on Na^+ currents recorded from neurons isolated from the dentate gyrus, suggesting that, similar to β1, its effects are cell type-specific (Uebachs et al., 2010). In *Scn2b* null small-fast dorsal root ganglion neurons, tetrodotoxin-sensitive Na^+ current is reduced by ∼50% and kinetics of activation and inactivation are slowed. Consistent with this, the protein level of $Na_v1.7$ is reduced, whereas tetrodotoxin-resistant Na^+ current is unchanged (Lopez-Santiago et al., 2006). β2 may therefore specifically regulate tetrodotoxin-sensitive channels *in vivo*.

Similar to *Scn2b* null mice, *Scn3b* null mice behave normally and have full lifespans (Chen et al., 2002; Hakim et al., 2008). *Scn1b* may compensate for *Scn3b* deletion in brain, providing for an apparently normal neurological phenotype (Hakim et al., 2008, 2010a). *Scn3b* null hearts display ventricular arrhythmogenic properties, including shorter effective refractory periods, induced tachycardia, and shorter action potential durations, corresponding to reduced Na^+ current and hyperpolarized inactivation (Hakim et al., 2008). Atrial conduction abnormalities have also been reported in *Scn3b* null mice (Hakim et al., 2010a). These defects can be, in part, mitigated with the Class I antiarrhythmic agents flecainide and quinidine (Hakim et al., 2010b), supporting the conclusion that β3 modulates α subunit function in the heart.

The β4 intracellular domain may regulate α subunits in cerebellar Purkinje neurons by acting as an open-channel blocker of VGSCs that carry resurgent Na^+ current (Grieco et al., 2005). Silencing *Scn4b* in cerebellar granule neurons reduces resurgent and persistent Na^+ currents, hyperpolarizes voltage-dependence of inactivation of transient current, and reduces repetitive action

Table 1 | The β subunit family: tissue locations, interacting proteins, and disease association.

β subunit	Gene	Tissue locations	Interacting proteins	Disease	Reference
β1	*SCN1B*	CNS, heart, PNS, skeletal muscle	Ankyrin$_B$, ankyrin$_G$, β1, β2, contactin, K_v4.3, NF155, NF186, *N*-cadherin, NrCAM, tenascin-R, RPTPβ	Epilepsy, cardiac arrhythmia, cancer	Isom et al. (1992), Wallace et al. (1998), Xiao et al. (1999), Malhotra et al. (2000), Ratcliffe et al. (2000), Kaplan et al. (2001), Deschenes and Tomaselli (2002), Malhotra et al. (2002), Meadows et al. (2002), Wallace et al. (2002), Aronica et al. (2003), Audenaert et al. (2003), Chen et al. (2004), Davis et al. (2004), Malhotra et al. (2004), Mcewen and Isom (2004), Fein et al. (2007), Lopez-Santiago et al. (2007), Scheffer et al. (2007), Diss et al. (2008), Fein et al. (2008), Chioni et al. (2009), Watanabe et al. (2009), Brackenbury et al. (2010)
β1B	*SCN1B*	Adrenal gland, CNS, heart, PNS, skeletal muscle	β1	Epilepsy	Kazen-Gillespie et al. (2000), Qin et al. (2003), Patino et al. (2011)
β2	*SCN2B*	CNS, heart, PNS	Ankyrin$_G$ β1, β2, tenascin-C, tenascin-R	Altered pain response, cardiac arrhythmia, MS, seizure susceptibility	Isom et al. (1995), Srinivasan et al. (1998), Xiao et al. (1999), Malhotra et al. (2000), Chen et al. (2002), Yu et al. (2003), Mcewen et al. (2004), Pertin et al. (2005), Lopez-Santiago et al. (2006), O'Malley et al. (2009), Watanabe et al. (2009)
β3	*SCN3B*	Adrenal gland, CNS, heart, kidney, PNS	NF186	Epilepsy, cardiac arrhythmia, traumatic nerve injury	Morgan et al. (2000), Ratcliffe et al. (2001), Shah et al. (2001), Adachi et al. (2004), Casula et al. (2004), Chioni et al. (2009), Hu et al. (2009), Van Gassen et al. (2009), Valdivia et al. (2010), Wang et al. (2010), Olesen et al. (2011)
β4	*SCN4B*	CNS, heart, PNS, skeletal muscle	β1	Huntington's disease, long-QT syndrome	Yu et al. (2003), Davis et al. (2004), Oyama et al. (2006), Medeiros-Domingo et al. (2007), Aman et al. (2009)

CNS, central nervous system; MS, multiple sclerosis; NF155, neurofascin-155; NF186, neurofascin-186; PNS, peripheral nervous system; RPTPβ, receptor protein tyrosine phosphatase β.

potential firing (Bant and Raman, 2010). Resurgent Na^+ current is proposed to facilitate repetitive firing in cerebellar neurons (Khaliq et al., 2003; Bant and Raman, 2010). β4 thus appears to play a key role in regulating excitability. Finally, β4 plays an antagonistic role with β1 in regulating hippocampal neuron excitability: β4 slows inactivation and is proposed to promote excitability, whereas β1 promotes inactivation and is proposed to act as a brake on excitability (Aman et al., 2009). In summary, each of the β subunits regulates excitability through interaction with, and modulation of, α subunits in a cell type-specific and channel subtype-specific manner.

NON-CONDUCTING FUNCTIONS

β subunits are multifunctional (**Figure 2**). In addition to their "conducting" role in modulating Na^+ current kinetics and voltage-dependence, they are members of the Ig superfamily of cell adhesion molecules (CAMs) and participate in a number of "non-conducting" cell adhesion related activities (Isom et al., 1995; Yu et al., 2003). β1 and β2 both participate in *trans*-homophilic adhesion resulting in cellular aggregation and recruitment of ankyrin to points of cell–cell contact in *Drosophila* S2 cells (Malhotra et al., 2000). By contrast, β3, in spite of its high homology to β1, does not mediate homophilic adhesion (Mcewen et al., 2009) but participates in heterophilic adhesion (see below). Phosphorylation of tyrosine residue (Y)181 in the intracellular domain of β1 abrogates the recruitment of ankyrin$_B$ and ankyrin$_G$ in transfected Chinese hamster lung cells (Malhotra et al., 2002). In cardiac myocytes, phosphorylation of Y181 determines localization of β1 to intercalated disks with connexin-43, *N*-cadherin, and Na_v1.5, while non-phosphorylated β1 localizes in the t-tubules with ankyrin$_B$ (Malhotra et al., 2004). Thus, the phosphorylation state of Y181 may be important for regulating the subcellular distribution of β1. Interestingly, the intracellular domain of β1 interacts with receptor protein tyrosine phosphatase-β in rat brain neurons (Ratcliffe et al., 2000), potentially providing a mechanism for regulating Y181 phosphorylation. In addition, indirect evidence suggests that β1-mediated *trans*-homophilic adhesion results in fyn kinase activation in mouse cerebellar granule neurons (Brackenbury et al., 2008), which in turn could further fine tune phosphorylation of Y181.

The β subunits interact heterophilically with several other CAMs and extracellular matrix proteins. β1 interacts with VGSC β2, contactin, neurofascin-155, neurofascin-186, NrCAM, *N*-cadherin (Kazarinova-Noyes et al., 2001; Ratcliffe et al., 2001; Malhotra et al., 2004; Mcewen and Isom, 2004). Interaction between β1 and contactin, neurofascin-186, or VGSC β2 increases Na^+

current in heterologous systems (Kazarinova-Noyes et al., 2001; Mcewen et al., 2004), suggesting that β subunit-dependent adhesion may regulate α subunit function and excitability. Both β1 and β2 interact with the extracellular matrix protein tenascin-R (Xiao et al., 1999). β2 also interacts with tenascin-C (Srinivasan et al., 1998), but does not interact with contactin (Mcewen et al., 2004). Less is known about the heterophilic interactions of the other β subunits. β3, which does not interact with either β1 or contactin, does interact with neurofascin-186 (Ratcliffe et al., 2001; Mcewen et al., 2009). Although similar studies have not been performed for β1B, it has been proposed that its heterophilic binding partners are likely similar to those of β1, given that both molecules share an identical Ig domain (Patino and Isom, 2010).

β1, β2, β3, and β4 subunits are substrates for sequential proteolytic cleavage by enzymes from the secretase family. These β subunits contain cleavage sites for the β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) on the extracellular domain, adjacent to the transmembrane region (Wong et al., 2005; Gersbacher et al., 2010). β2 also contains a cleavage site for the α-secretase enzyme ADAM10 in the extracellular juxtamembrane region (Kim et al., 2005). Cleavage by BACE1 or α-secretase results in shedding of the extracellular Ig domain, leaving transmembrane C-terminal fragments (Kim et al., 2005; Wong et al., 2005). The C-terminal fragments are subsequently processed by γ-secretase at a site in the intracellular juxtamembrane region, thus releasing soluble intracellular domains into the cytoplasm (Kim et al., 2005; Wong et al., 2005). Although these four β subunits are cleaved by BACE1 *in vitro*, processing *in vivo* has so far been demonstrated only for β2 and β4 (Wong et al., 2005). Importantly, the signaling events responsible for initiating β subunit processing by the secretases, as well as potential developmental timing of these cleavage events *in vivo*, have not been investigated.

The functional effects of processing these β subunits appear critical to their *in vivo* function. For example, both the extracellular domain of β1, and its soluble splice variant, β1B, promote neurite outgrowth (Davis et al., 2004; Patino et al., 2011). Similarly, cleavage of the extracellular domain of β4 by BACE1 also increases neurite outgrowth (Miyazaki et al., 2007). Inhibition of γ-secretase activity reduces β2-dependent cell adhesion and migration, suggesting that the intracellular domain is important for promoting these functions (Kim et al., 2005). The β2 intracellular domain translocates to the nucleus of transfected SH-SY5Y cells and increases expression of *SCN1A*, suggesting that it may function, directly or indirectly, as a transcriptional regulator of VGSC α subunit expression (Kim et al., 2007). Further, the mRNA and protein levels of *Scn1a*/$Na_v1.1$ are reduced in the brains of *Bace1* null mice (Kim et al., 2011). Altered expression of α subunit mRNA and protein in the peripheral and central nervous systems of *Scn1b* and *Scn2b* null mice, as well as the hearts of *Scn1b* and *Scn3b* null mice (Chen et al., 2004; Lopez-Santiago et al., 2006, 2007, 2011; Hakim et al., 2008; Brackenbury et al., 2010), suggests that regulation of α subunit expression by β subunits may be widespread. Finally, cleavage of β4 by BACE1 in cerebellar Purkinje cells slows the decay of resurgent Na^+ current, thus promoting action potential firing (Huth et al., 2011), suggesting that secretase-mediated β subunit processing may modulate α subunit activity and thus neuronal excitability. However, another study from the same group indicated that BACE1 modulates α subunit gating in transfected human embryonic kidney cells and murine neuroblastoma cells, independent of its proteolytic effect on β2 and β4 (Huth et al., 2009). Thus the effects of BACE1 on α and β subunit function appear complex. Further work is required to understand the regulatory events involved in this putative signaling cascade and to establish whether BACE1 does indeed directly interact with α subunits in addition to β subunits.

ROLE OF β SUBUNITS IN DEVELOPMENT

β1 promotes neurite outgrowth in cerebellar granule neurons through *trans*-homophilic cell–cell adhesion (Davis et al., 2004). This mechanism operates through lipid rafts and requires fyn kinase, contactin, and Na^+ current (Brackenbury et al., 2008, 2010). β1-mediated neurite outgrowth also requires γ-secretase activity, suggesting that proteolytic processing of the intracellular domain may be important (**Figures 3A,B**). In contrast, neither β2 nor β4 promote neurite outgrowth in cerebellar granule neurons (Davis et al., 2004). However, β4 enhances neurite extension in neuroblastoma cells, and increases dendritic spine density in hippocampal neurons (Oyama et al., 2006; Miyazaki et al., 2007). In addition, β1 and β2 regulate the migration of fibroblasts away from the extracellular matrix protein tenascin-R (Xiao et al., 1999).

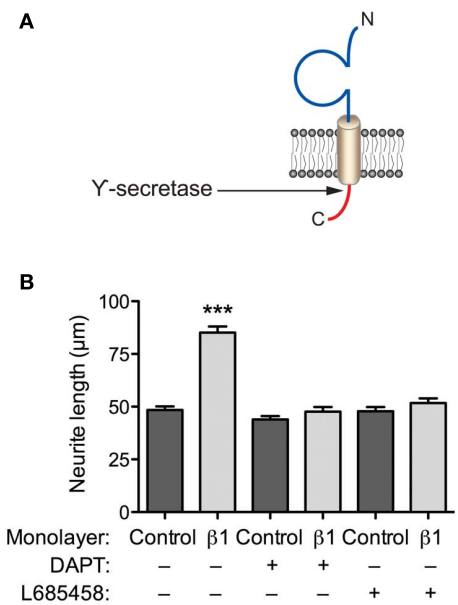

FIGURE 3 | β1-mediated neurite outgrowth requires γ-secretase activity. (A) Location of γ-secretase cleavage site on the intracellular domain of β1 (Wong et al., 2005). **(B)** Cerebellar granule neurons from postnatal day (P)14 wildtype mice were plated on top of monolayers of control or β1-expressing Chinese hamster lung cells, as described previously (Davis et al., 2004). Cultures were incubated with the either one of the γ-secretase inhibitors, L685458, or DAPT (both 1 μM); or control (DMSO) for 48 h (Kim et al., 2005). Cells were then fixed, processed for GAP43 immunocytochemistry, and neurite lengths measured, as described (Davis et al., 2004). Both L685458 and DAPT inhibited the increase in neurite length caused by β1 expressed in the monolayer. Data are mean + SEM ($n = 300$). Significance: $***P < 0.001$, ANOVA with Tukey's *post hoc* test.

Finally, β1 inhibits the migration of metastatic breast cancer cells (Chioni et al., 2009). Thus, β subunit-mediated neurite outgrowth and migration may be subtype and/or cell-specific.

The regulation of neurite outgrowth and migration by β subunits has consequences for development and organogenesis. In particular, β1 plays a critical role in neuronal pathfinding in postnatal-developing fiber tracts, coinciding with the onset of its expression from birth (Sutkowski and Catterall, 1990; Sashihara et al., 1995; Brackenbury et al., 2008). *Scn1b* null mice display a severe phenotype that includes growth retardation, ataxia, spontaneous seizures from P8–10, and death by P21 (Chen et al., 2004). In P14–16 *Scn1b* null mice, the pathfinding and migration of corticospinal axons is disrupted, leading to significant defasciculation of fibers at the pyramidal decussation (Brackenbury et al., 2008). The cerebellar parallel fibers are also defasciculated in P14 *Scn1b* null mice. The migration of granule neurons through the cerebellar molecular layer is disrupted, resulting in their accumulation in the external germinal layer, which is consequently thicker in *Scn1b* null mice than in wildtype littermates (Brackenbury et al., 2008). These cerebellar defects may contribute to the ataxic phenotype (Chen et al., 2004). Consistent with results in mice, abnormal pathfinding has also been reported in the olfactory nerve of zebrafish *scn1bb* morphants (Fein et al., 2008).

An important next step will be to determine whether defects in *Scn1b*-mediated cell–cell adhesion and migration in brain occur prior to the onset of convulsive seizures (Chen et al., 2004). It is possible that abnormal neuronal migration and pathfinding in the absence of β1/β1B-mediated cell adhesive interactions may lead to aberrant connections, resulting in neuronal hyperexcitability and epileptogenesis. Further work is required to establish the causational relationship between β1/β1B expression, cell adhesion, migration, and seizures during postnatal development of the nervous system.

ALTERATIONS IN VGSC PHARMACOLOGY BY β SUBUNITS

Studies have indicated that β subunits can alter the effect of pharmacological compounds on Na^+ currents carried by α subunits. For example, using heterologous systems, co-expression of β1 or β3 with $Na_v1.3$ in *Xenopus* oocytes attenuates the inhibitory effect of the antiarrhythmic agent and local anesthetic lidocaine on current amplitude and inactivation (Lenkowski et al., 2003). Further, the β1 C121W epilepsy mutation reduces tonic and use-dependent channel block in response to the antiepileptic drug phenytoin (Lucas et al., 2005). This altered sensitivity to phenytoin is proposed to be as a result of the altered gating caused by the mutation (Meadows et al., 2002), rather than a direct effect on the drug receptor. A recent *in vivo* study showed that the use-dependent reduction of transient Na^+ current caused by the anticonvulsant drug carbamazepine in wildtype hippocampal neurons was not observed in *Scn1b* or *Scn2b* null hippocampal neurons (Uebachs et al., 2010). However, carbamazepine caused a small hyperpolarizing shift in the voltage-dependence of activation of both transient and persistent Na^+ current. The hyperpolarizing shift in persistent Na^+ current was significantly increased in *Scn1b* null neurons at low carbamazepine concentrations, resulting in a complete loss in efficacy of the drug to reduce repetitive action potential firing (Uebachs et al., 2010). Thus, β1/β1B alters the pharmacological response of persistent Na^+ current to carbamazepine. Finally, in *Scn3b* null hearts, the VGSC-blocking antiarrhythmic agents flecainide and quinidine both modify ventricular effective refractory periods, resulting in anti-arrhythmogenic effects, in contrast to their effects in wildtype or *Scn5a* mutant hearts (Hakim et al., 2010b). Taken together, these findings have important clinical implications, suggesting that function-altering mutations and/or altered expression or localization of β subunits in patients may affect their sensitivity to VGSC-targeting drugs and thus therapeutic efficacy. Further work is required to establish whether or not β subunits alter pharmacological responses to additional VGSC-targeting therapeutics.

FUNCTIONAL RECIPROCITY BETWEEN α AND β SUBUNITS

Extensive evidence indicates that β subunits modulate channel gating of α subunits (see Regulation of Excitability by Interaction with α Subunits). Similarly, β1-mediated neurite outgrowth is inhibited by the VGSC-blocking toxin tetrodotoxin (Brackenbury et al., 2010). Thus, there is a potential for interplay between β1-mediated modulation of Na^+ current carried by α subunits and β1-mediated cell–cell adhesion/migration. β1 is required for normal high-frequency action potential firing in cerebellar granule neurons (**Figure 4A**). β1-mediated neurite outgrowth is abrogated in *Scn8a* null cerebellar granule neurons, suggesting that the mechanism requires Na^+ current carried by $Na_v1.6$ (**Figure 4B**). $Na_v1.6$ is vital for high-frequency repetitive firing in cerebellar neurons (Raman and Bean, 1997). Resurgent Na^+ current, carried by $Na_v1.6$, and which facilitates repetitive action potential firing (Raman and Bean, 1997; Khaliq et al., 2003; Bant and Raman, 2010), is reduced in *Scn1b* null cerebellar granule neurons (Brackenbury et al., 2010). The *Scn1b* null mutation disrupts the expression of $Na_v1.6$ at the axon initial segment (AIS) of cerebellar granule neurons (**Figure 4C**).

Taken together, these data suggest that there is a functional reciprocity between β1 and $Na_v1.6$ in cerebellar neurons, such that, on the one hand, β1 is required for normal localization of $Na_v1.6$ at the AIS, thus permitting resurgent Na^+ current, and repetitive action potential firing. On the other hand, $Na_v1.6$ is required for β1-mediated neurite outgrowth. Electrical activity generated at the AIS is proposed to provide a depolarizing signal to open $Na_v1.6$ channels at the growth cone, further promoting β1-mediated neurite outgrowth (**Figure 4D**; Brackenbury et al., 2008). This reciprocal relationship between β1 and $Na_v1.6$ is critical for postnatal cerebellar development (Chen et al., 2004; Van Wart and Matthews, 2006). Impaired localization of β1 at the AIS may thus lead to altered excitability. In agreement with this, in knock-in mice heterozygous for the β1C121W mutation, which disrupts β1-dependent adhesion and alters channel gating, mutant β1 protein is excluded from the AIS of pyramidal neurons, potentially contributing to febrile seizures (Meadows et al., 2002; Wimmer et al., 2010).

Future work will no doubt establish whether or not further complementary roles exist between VGSC α and β subunits, interacting in a coordinated fashion to regulate processes including excitability and neurite extension. It is already clear however, that β subunits function in macromolecular complexes with α subunits

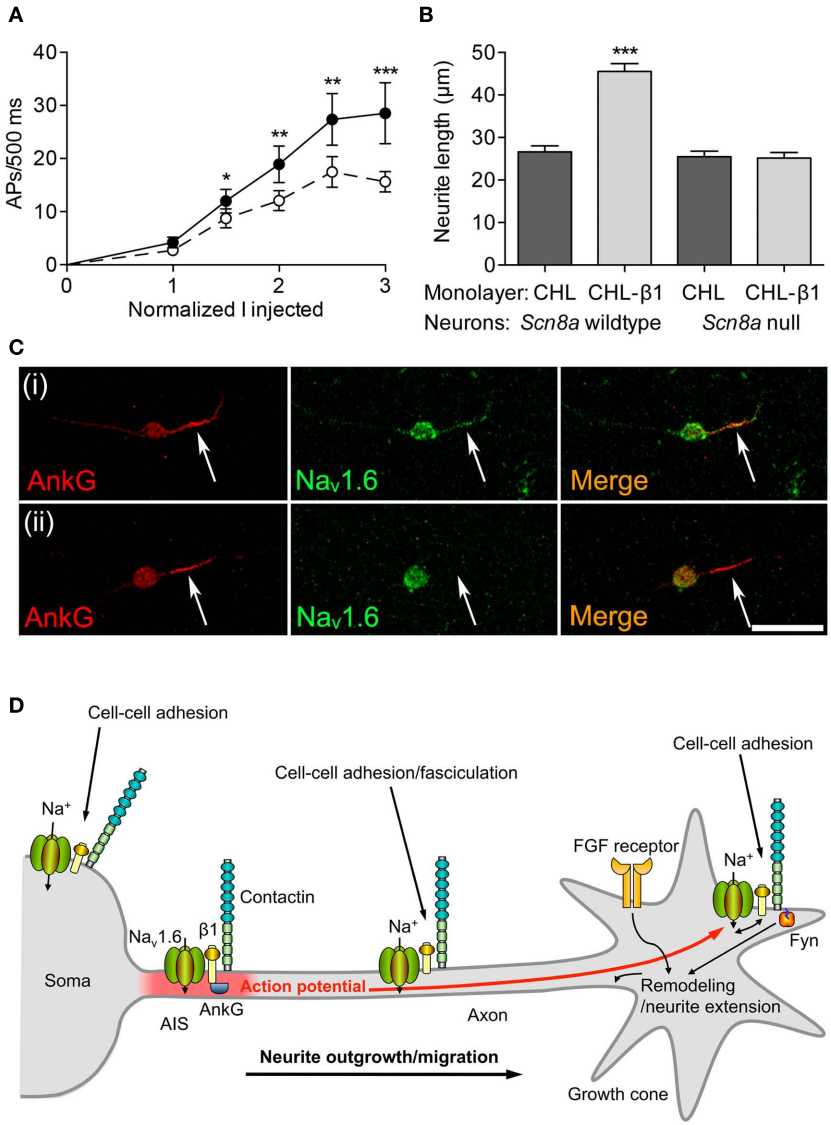

FIGURE 4 | Functional reciprocity between β1 and $Na_v1.6$. (A) Electrical excitability is impaired in *Scn1b* null cerebellar granule neurons. Action potential firing rate recorded from cerebellar granule neurons in brain slices from 12-day-old mice plotted as a function of injected current, normalized to action potential threshold for wildtype (filled circles) and *Scn1b* null (open circles). Data are mean ± SEM ($n \geq 15$). Significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; *t*-test. **(B)** β1-mediated neurite outgrowth is inhibited by the *Scn8a* null mutation. Neurite lengths of wildtype and *Scn8a* null cerebellar granule neurons grown on control Chinese hamster lung or β1-expressing monolayers ($n = 300$). Data are mean + SEM. Significance: $^{***}P < 0.001$, ANOVA with Tukey's *post hoc* test. **(C)** $Na_v1.6$ expression is reduced at the axon initial segment of *Scn1b* null cerebellar granule neurons. Wildtype and *Scn1b* null cerebellar granule neurons cultured *in vitro* for 14 days labeled with anti-ankyrin$_G$ (red) and $Na_v1.6$ antibodies (green). Scale bar, 20 μm. Arrows point to axon initial segment expressing ankyrin$_G$. **(D)** A model for Na^+ current involvement in β1-mediated neurite outgrowth. Complexes containing $Na_v1.6$, β1, and contactin are present throughout the neuronal membrane in the soma, neurite and growth cone. Localized Na^+ influx is necessary for β1-mediated neurite extension and migration. VGSC complexes along the neurite participate in cell–cell adhesion and fasciculation. β1 is also required for $Na_v1.6$ expression at the axon initial segment, and subsequent high-frequency action potential firing through modulation of resurgent Na^+current. Electrical activity may further promote β1-mediated neurite outgrowth at or near the growth cone. Thus, the developmental functions of β1 and $Na_v1.6$ are complementary, such that (1) Na^+ influx carried by $Na_v1.6$ is required for β1-mediated neurite outgrowth, and (2) β1 is required for normal expression/activity of $Na_v1.6$ at the axon initial segment. Fyn kinase and ankyrin$_G$ are likely also present in all complexes, but are only shown once in each panel for clarity. The FGF-mediated, β1-independent neurite outgrowth pathway is also shown. Figure reproduced with permission (Brackenbury et al., 2010).

to participate in signaling on multiple timescales to regulate excitability, adhesion, neurite outgrowth, and migration. A critical focus of future work will be to determine whether β subunits that are expressed independently of the ion-conducting pore also play roles in excitability *in vivo*, perhaps through regulation of axon guidance or fasciculation.

DYSREGULATION IN DISEASE

Voltage-gated Na^+ channel β subunits are implicated in a number of neurological diseases (**Table 1**) [reviewed extensively in Patino and Isom (2010)]. Of particular note is the growing list of mutations in *SCN1B* that are associated with genetic epilepsy with febrile seizures plus (GEFS) + 1 (OMIM 604233), a spectrum of disorders that includes mild to severe forms of epilepsy (Wallace et al., 1998, 2002; Audenaert et al., 2003; Burgess, 2005; Yamakawa, 2005; Scheffer et al., 2007; Patino et al., 2009, 2011). No GEFS + 1-causing mutations in the other β subunit genes have yet been identified. However, *Scn2b* null mice display increased seizure susceptibility (Chen et al., 2002). In addition, *SCN3B* is reduced in the hippocampus of patients with temporal lobe epilepsy, suggesting that altered β3 expression may contribute to or result from epilepsy (Van Gassen et al., 2009). The mutations in *SCN1B* may bias neurons toward hyperexcitability and epileptogenesis by one or both of two distinct mechanisms: (1) impaired regulation of α subunit-dependent excitability (Meadows et al., 2002; Chen et al., 2004; Spampanato et al., 2004; Patino et al., 2009; Wimmer et al., 2010); and/or (2) impaired cell–cell adhesive interactions (Meadows et al., 2002; Brackenbury et al., 2008; Fein et al., 2008; Patino et al., 2011). There may also be a causal relationship between VGSC α-β1 interactions, cell–cell adhesion, migration, and epilepsy. Further work is required to establish whether or not disrupted β1-dependent cell adhesion is indeed a prerequisite for seizure activity.

Changes in β2 expression have been implicated in altered pain sensation. *Scn2b* null mice are more sensitive to noxious thermal stimuli than wildtype mice (Lopez-Santiago et al., 2006). In addition, the spared nerve injury model of neuropathic pain results in increased β2 expression in rat sensory neurons, and mechanical allodynia-like behavior (Pertin et al., 2005). This behavior is absent in *Scn2b* null mice, suggesting that β2 expression may play an important role in neuropathic pain sensation (Pertin et al., 2005).

The β subunits play roles in neurodegenerative disease. The *Scn2b* null mutation is neuroprotective in the experimental allergic encephalomyelitis mouse model of multiple sclerosis (O'Malley et al., 2009). In addition, levels of *Scn4b* are reduced in mouse models of Huntington's disease prior to onset of motor symptoms, and a similar reduction has also been reported in patients (Oyama et al., 2006).

Indirect evidence suggests that β subunits may be involved in neuropsychiatric disorders. For example, $ankyrin_G$ and $Na_v1.6$, which both interact with β subunits, are linked genetically to bipolar disorder (Gargus, 2006; Wang et al., 2008). Migration defects observed in the cerebellum of *Scn1b* null mice may result in abnormal connections with the prefrontal cortex and posterior parietal cortex, thus providing a possible mechanism for β subunit involvement in mood disorders (Brackenbury et al., 2008; Strick et al., 2009). Similarly, migration defects in other brain areas in patients with *SCN1B* mutations may contribute to mental disorders. Pathological cellular migration regulated by β subunits extends beyond neurological diseases: β1 regulates cellular adhesion and migration in metastatic breast cancer cell lines (Chioni et al., 2009). β subunit transcripts are also expressed in prostate cancer cells and lung cancer cells (Roger et al., 2007; Diss et al., 2008), suggesting that their involvement in cancer may be widespread.

Finally, mutations in β subunits are associated with cardiac abnormalities (Wilde and Brugada, 2011). Mutations in *SCN1B* have been reported in patients with idiopathic ventricular fibrillation (Brugada syndrome; Watanabe et al., 2008; Hu et al., 2009; Valdivia et al., 2010). Mutations in *SCN1B*, *SCN2B*, and *SCN3B* are also associated with atrial fibrillation (Watanabe et al., 2009; Wang et al., 2010; Olesen et al., 2011). Mutations in *SCN3B* and *SCN4B* are associated with sudden infant death syndrome (Tan et al., 2010). These mutations are proposed to interfere with the ability of β subunits to modulate $Na_v1.5$ currents *in vivo* (Watanabe et al., 2008, 2009; Hu et al., 2009; Tan et al., 2010). A mutation in *SCN4B* results in long-QT syndrome (Medeiros-Domingo et al., 2007). Expression of a mutation linked to conduction disease and Brugada syndrome, $Scn5a^{1798insD/+}$, in a mouse strain (129P2) with markedly reduced *Scn4b* expression resulted in more severe cardiac conduction slowing than a strain with normal *Scn4b* levels (FVB/N), suggesting that *Scn4b* may be a genetic modifier of cardiac conduction (Remme et al., 2009). In summary, abnormal expression and/or function of β subunits appears to play an important role in a number of diseases, ranging from nervous system disorders, to cardiac abnormalities, and cancer.

CONCLUSION/OUTLOOK

Increasing new evidence supports the hypothesis that the VGSC β subunits are multifunctional. In addition, a growing list of mutations and *in vivo* studies indicate that the β subunits play important roles in a number of diseases due to abnormal function in both excitable and non-excitable cells. There is no doubt that the classical "conducting" role of β subunits as modulators of Na^+current is of paramount importance in regulating ion flux and excitability. However, there is a clear trend in the literature toward an increasingly important role for "non-conducting" functions, including cell adhesion, migration and pathfinding, and putative transcriptional regulation. As a result, the β subunits are integral components of VGSC macromolecular protein complexes, which can direct multiple signaling mechanisms on multiple timescales. Moreover, the cell adhesive, "non-conducting" properties of β subunits observed *in vitro* suggest that they may play critical functional roles independent of α subunits *in vivo*. The challenge now will be to clearly delineate the cell adhesive functions of β subunits from their roles in channel modulation during development and in pathophysiology. Clearer understanding of the interaction between the conducting and non-conducting functions of VGSC complexes will hopefully enable the full realization of their therapeutic potential.

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Regulatory role of voltage-gated Na^+ channel β subunits in sensory neurons

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Voltage-gated sodium Na^+ channels are membrane-bound proteins incorporating aqueous conduction pores that are highly selective for sodium Na^+ ions. The opening of these channels results in the rapid influx of Na^+ ions that depolarize the cell and drive the rapid upstroke of nerve and muscle action potentials. While the concept of a Na^+-selective ion channel had been formulated in the 1940s, it was not until the 1980s that the biochemical properties of the 260-kDa and 36-kDa auxiliary β subunits (β_1, β_2) were first described. Subsequent cloning and heterologous expression studies revealed that the α subunit forms the core of the channel and is responsible for both voltage-dependent gating and ionic selectivity. To date, 10 isoforms of the Na^+ channel α subunit have been identified that vary in their primary structures, tissue distribution, biophysical properties, and sensitivity to neurotoxins. Four β subunits (β_1–β_4) and two splice variants (β_{1A}, β_{1B}) have been identified that modulate the subcellular distribution, cell surface expression, and functional properties of the α subunits. The purpose of this review is to provide a broad overview of β subunit expression and function in peripheral sensory neurons and examine their contributions to neuropathic pain.

Keywords: voltage-gated sodium channel, pain, β subunit, peripheral nervous system

INTRODUCTION

Ten isoforms of voltage-gated Na^+ channels have been identified that vary in tissue distribution, structure, biophysical properties, and sensitivity to neurotoxins (**Table 1**; Chahine et al., 2005). In standardized nomenclature, the nine confirmed members with >50% common amino acid identity in the transmembrane and extracellular loop regions have been designated as $Na_v1.1$ through $Na_v1.9$. The prefix Na_v indicates the chemical symbol of the principal permeating ion (Na) with the principal physiological regulator (voltage; Catterall et al., 2003). The tenth isoform has not yet been fully identified because it has not been functionally expressed. However, this isoform plays an important role in the detection of body fluid Na^+ levels and the regulation of salt intake (Watanabe et al., 2000, 2003). At least eight of the mammalian α subunits are expressed in the nervous system: $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$ are widely expressed in the central nervous system (CNS) while $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$ are preferentially expressed in the peripheral nervous system (PNS; Black et al., 1996).

Primary sensory neurons in the dorsal root ganglia (DRG) give rise to afferent nerve fibers that convey information about thermal, mechanical, and chemical stimulations from peripheral tissues to the CNS. These neurons express a unique combination of tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) Na^+ currents that produce the rapid rising phase of action potentials. Much of what is currently known about the Na^+ channels expressed in sensory neurons is derived from electrophysiological studies of cultured DRG neurons (Cummins et al., 2007; Rush et al., 2007). Small-diameter DRG neurons (<25 μm) are the cell bodies of unmyelinated C-fiber nociceptors that preferentially express TTX-R Na^+ currents. This contrasts with the myelinated large-diameter (>30 μm) neurons typically associated with low-threshold A-fibers that predominately express TTX-S Na^+ currents. Primary sensory neurons express a variety of Na^+ channel isoforms that display properties similar to the endogenous TTX-S ($Na_v1.1$, $Na_v1.2$, $Na_v1.6$, $Na_v1.7$) and TTX-R ($Na_v1.8$, $Na_v1.9$) Na^+ currents observed in these neurons (Black et al., 1996; Dib-Hajj et al., 1998; Amaya et al., 2000; Ho and O'Leary, 2011).

In vivo, most Na^+ channel α subunits are associated with one or more auxiliary β subunits (Isom, 2002). Four distinct isoforms (β_1, β_4) and two splice variants (β_{1A}, β_{1B}) have been identified (**Table 2**). They share a common structure (Chahine et al., 2005) consisting of a single membrane spanning domain, a small intracellular C-terminal domain, and a large extracellular N-terminal domain incorporating an immunoglobulin-like fold similar to that of cell adhesion molecules (**Figure 1**; Isom, 2001; Yu et al., 2003). The β_{1A} and β_{1B} subunits are splice variants of β_1. They share an identical N-terminal domain but have a novel C-terminal domain resulting from intron retention (Kazen-Gillespie et al., 2000; Qin et al., 2003). Na^+ channel β subunits can be broadly classified based on sequence homology and molecular interactions with α subunits. The β_1, $\beta_{1A–B}$, and β_3 subunits have similar amino acid sequences and form non-covalent interactions with α subunits (Isom et al., 1992; Morgan et al., 2000). This contrasts with the β_2 and β_4 subunits, which are best characterized as closely related (sharing 35% amino acid sequence), and which are covalently linked to α subunits via a disulfide bridge (Yu et al., 2003).

Table 1 | Gene location and distribution of Na channels α subunits in subpopulations of DRG sensory neurons.

Channel	Gene	Human chromosome location	TTX sensitivity	Expression in DRG subpopulations*	Expression levels
$Na_v1.1$	*SCN1A*	2q2a	Sensitive	Large myelinated	++
$Na_v1.2$	*SCN2A*	2q23–24	Sensitive	Large myelinated/small unmyelinated	+
$Na_v1.3$	*SCN3A*	2q24	Sensitive	Not present	Increased after nerve injury
$Na_v1.4$	*SCN4A*	17q23–25	Sensitive	Not present	–
$Na_v1.5$	*SCN5A*	3p21	Resistant	Not present	–
$Na_v1.6$	*SCN8A*	12q13	Sensitive	Large myelinated	+++
$Na_v1.7$	*SCN9A*	2q24	Sensitive	Large myelinated/small unmyelinated	+++
$Na_v1.8$	*SCN10A*	3p22–24	Resistant	Small unmyelinated/some large	+++
$Na_v1.9$	*SCN11A*	3p21–24	Resistant	Small unmyelinated	+++

**Small- (<25 μm) and Large-diameter (>30 μm) DRG neurons. State of myelination determined by overlapping expression of peripherin, NF200 and Necl-1. +,++,+++: different levels of expression.*

Table 2 | Tissue distribution of auxiliary β subunits.

Subunit	Apparent M_r (kDa)	Tissue expression	Expression in DRG sensory neurons
β_1	36	Heart, skeletal muscle, CNS, glial cells, PNS	Large, intermediate diameter, and low levels in small-diameter
β_{1A}	45	Heart, skeletal muscle, adrenal gland, PNS	Large, intermediate, and small
β_{1B}	30.4	Human brain, spinal cord, DRG, cortical neurons, and skeletal muscle	Large, intermediate, and small
β_2	33	CNS, PNS, heart	Large, intermediate, and small
β_3	–	CNS, adrenal gland, kidney, PNS	Predominately in small-diameter
β_4	38	Heart, skeletal muscle, CNS, PNS	Large-diameter very low levels in intermediate and small

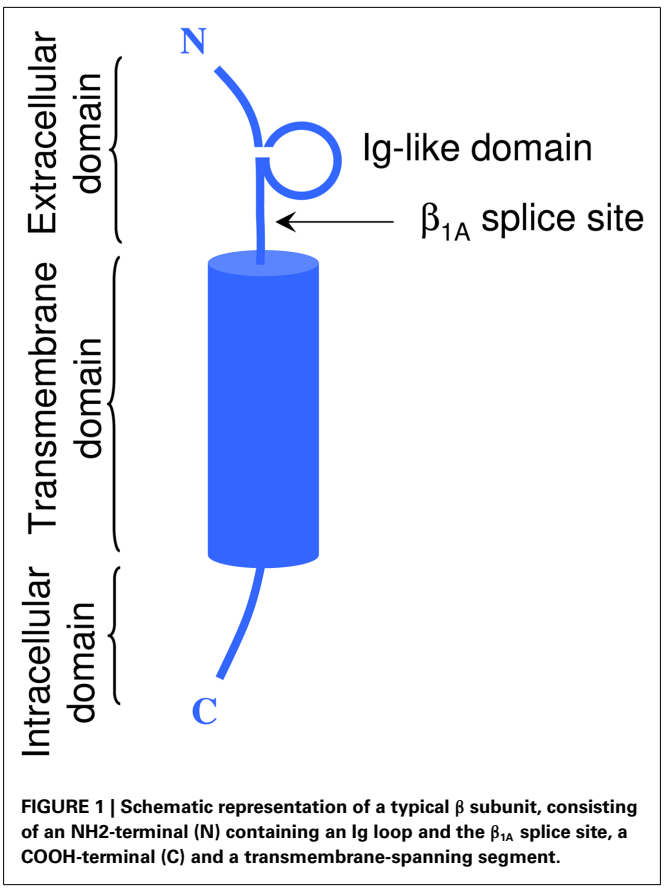

FIGURE 1 | Schematic representation of a typical β subunit, consisting of an NH2-terminal (N) containing an Ig loop and the β_{1A} splice site, a COOH-terminal (C) and a transmembrane-spanning segment.

In vivo Na^+ channel α subunits are believed to form heteromultimeric complexes consisting of one non-covalently associated (β_1, β_3) and one covalently (β_2, β_4) linked β subunit (Catterall et al., 2005). Depending on the composition of the α–β subunit, these interactions have been shown to modulate the gating kinetics, voltage-dependence, and cell surface expression of the associated α subunits (Catterall, 2000). β subunits also function as adhesion molecules that interact with cytoskeleton proteins, the extracellular matrix, and other molecules that regulate cell migration and aggregation (Yu and Catterall, 2003; Brackenbury et al., 2008).

Voltage-gated Na^+ channels are important determinants of sensory neuron excitability, and changes in the expression and gating properties of these channels have been implicated in the development of neuropathic pain (Cummins et al., 2007; Chahine et al., 2008). Immunohistochemistry and *in situ* hybridization studies have shown that all four isoforms of β subunits and both slice variants are present in DRGs (Kazen-Gillespie et al., 2000; Morgan et al., 2000; Coward et al., 2001; Qin et al., 2003). Given the close physical and functional interactions between α and β subunits, it is not surprising that these auxiliary subunits are also important contributors to pain sensation (Isom, 2001). However, the precise role of these subunits in nociception and neuropathic pain has not been fully elucidated.

THE β_1 SUBUNIT AND ITS SPLICE VARIANTS

It has been convincingly demonstrated that the β_1 subunit regulates the expression and gating properties of Na^+ channels and thereby modulates the electrical excitability of both nerves

and muscles (Chahine et al., 2008). Immunohistochemistry and transcript analyses have shown that the β_1 subunit is differentially expressed in subpopulations of primary sensory neurons (Oh et al., 1995; Black et al., 1996; Takahashi et al., 2003; Zhao et al., 2011). β_1 is abundantly expressed in intermediate- and large-diameter (>30 μm) DRG neurons but is present at comparatively low levels in small-diameter (<25 μm) neurons. The preferential expression of β_1 subunits in medium and large neurons suggests that these subunits may contribute significantly to the excitability of low-threshold A-fibers but play a reduced role in small-diameter nociceptors. This is consistent with rodent models of nerve injury where β_1 expression is not significantly altered, suggesting that these subunits do not contribute significantly to the development of neuropathic pain (Shah et al., 2001; Takahashi et al., 2003).

The co-expression of β_1 subunits with sensory neuron $Na_v1.7$ and $Na_v1.8$ Na^+ channels in *Xenopus* oocytes accelerates current kinetics and produces a hyperpolarizing shift in steady-state inactivation (Vijayaragavan et al., 2001). In addition, β_1 selectively increases $Na_v1.8$ current density but has no effect on $Na_v1.7$ expression. These findings indicate that β_1 subunits regulate both the gating and cell surface expression of sensory neuron Na^+ channels in an isoform-specific manner. More recent work using mammalian cell lines revealed a twofold increase in $Na_v1.8$–β_1 peak current density and hyperpolarizing shifts in both activation and inactivation (Zhao et al., 2011). Studies on β subunit chimeras showed that the intracellular C-terminus, but not the membrane spanning or extracellular domains of β_1, was critical for retaining the functional regulation of $Na_v1.8$ gating (Zhao et al., 2011).

The role of β_1 subunits in sensory neuron excitability has been addressed using *SCN1b* null mice (Lopez-Santiago et al., 2007). The β_1 knockouts exhibit numerous neuronal deficits, including symptoms of epilepsy and ataxia consistent with a broad distribution of this subunit in the CNS (Chen et al., 2004; Lopez-Santiago et al., 2007; Patino et al., 2009). In DRG neurons, the β_1 knockout produces a slight reduction in persistent Na^+ current associated with small changes in the amplitudes and gating properties of the predominant TTX-S and TTX-R Na^+ currents (Lopez-Santiago et al., 2011). Overall, the subtle β_1 regulation of DRG Na^+ channels coupled with the low level expression in small-diameter neurons and the absence of change in models of nerve injury are inconsistent with the idea that β_1 subunits contribute significantly to the development of neuropathic pain.

THE β_{1A} AND β_{1B} SUBUNITS

There are two splice variants of the β_1 subunit, the β_{1A} subunit in the rat and β_{1B} subunit in humans (Kazen-Gillespie et al., 2000; Qin et al., 2003). These variants have N-terminal domains that are identical to that of the β_1 subunit, but have novel C-terminals resulting from intron retention. The retained β_{1B} intron codes for a novel membrane spanning and intracellular domain that shares little sequence homology with β_1 (17%) or β_{1A} (33%). When co-expressed in oocytes, the β_{1B} subunit increases peak $Na_v1.2$ currents twofold but does not alter the current kinetics or gating properties of the channels (Qin et al., 2003).

The β_{1A} subunit is highly expressed during embryonic development but decreases after birth (Kazen-Gillespie et al., 2000). Western blotting analyses have revealed that β_{1A} is expressed in the heart, brain, spinal cord, and DRGs. When co-expressed in Chinese hamster ovary (CHO) cells, the β_{1B} subunit produces a 2.5-fold increase in $Na_v1.2$ current density and a slight depolarizing shift in activation (<3 mV), but no change in steady-state inactivation or current kinetics. β_{1A} appears to preferentially increase the cell surface expression of $Na_v1.2$ channels, a feature it shares with the parent β_1 subunit. These findings suggest that β_{1B} regulation may involve the homologous N-terminal domain that is common to the β_1 and β_{1A} variants.

THE β_2 SUBUNIT

The β_2 subunit is widely expressed in DRG neurons of all sizes (Coward et al., 2001; Takahashi et al., 2003) and throughout the CNS, including the spinal cord, cerebral cortex, and cerebellum (Gastaldi et al., 1998). $Na_v1.2$ channels expressed in *Xenopus* oocytes result in currents that display abnormally slow activation and inactivation kinetics (Auld et al., 1988; Krafte et al., 1988). Co-expressing the β_2 subunit induces more rapid activation and inactivation, which is consistent with a shift of $Na_v1.2$ channels from a slow to a fast mode of gating (Isom et al., 1995). The slow gating observed in oocytes contrasts sharply with the properties of $Na_v1.2$ channels expressed in CHO (West et al., 1992) and tsA201(O'Leary, 1998; Qu et al., 2001) cell lines, where rapid kinetics similar to those of native tissues are typically observed. In addition to changes in current kinetics, co-expressing the β_2 subunit in oocytes results in a hyperpolarizing shift in $Na_v1.2$ inactivation (2 mV) and a twofold increase in peak current (Isom et al., 1995). Again, this contrasts with results from tsA201 cells, where the β_2 subunit produces small depolarizing shifts (3–4 mV) in $Na_v1.2$ activation and inactivation but no changes in current kinetics or recovery from inactivation (Qu et al., 2001). This suggests that β_2 regulation of $Na_v1.2$ depends on the host cells used for expression, which may be related to differences in cellular genetic background, post-translational protein modification, or regulation by endogenous signal transduction pathways (West et al., 1992; Qu et al., 2001).

Recent work has focused on β_2 subunit regulation of Na^+ channel isoforms that are preferentially expressed in sensory neurons. The co-expression of $Na_v1.8$ and β_2 subunits in *Xenopus* oocytes results in a relatively modest depolarizing shift in inactivation (4 mV) but no change in activation, current kinetics, or peak Na^+ current (Vijayaragavan et al., 2004). Subsequent studies of $Na_v1.8$–β_2, $Na_v1.6$–β_2, and $Na_v1.3$–β_2 channels expressed in mammalian cells largely confirmed these findings, demonstrating little or no effect of β_2 on voltage-dependence, kinetics, or current density (Cummins et al., 2001; Zhao et al., 2011). Similar results have been observed in preliminary studies of heterologously expressed $Na_v1.7$–β_2 channels (Ho et al., 2011). Overall, the β_2 subunit appears to weakly regulate many of the voltage-gated Na^+ channels expressed in sensory neurons.

This contrasts with studies of null mice, where the knockout of the β_2 subunit is associated with reductions in TTX-S Na^+ current amplitude, mRNA, and protein (Lopez-Santiago et al., 2006). This suggests that β_2 expression in DRG neurons increases TTX-S Na^+ current amplitude and accelerates current kinetics, effects that are not widely observed in α–β co-expression studies. The underlying cause of this discrepancy is not known. One possibility is that β_2

subunits in native DRG neurons interact with endogenous proteins or are the target of signal transduction processes that are not reconstituted in heterologous expression systems. This possibility has gained credence from studies showing that the expression of $Na_v1.3$ in DRG cells results in a depolarizing shift in activation and faster recovery from inactivation compared to $Na_v1.3$ channels expressed in HEK293 cells (Cummins et al., 2001). Interactions with endogenous β subunits or other cell-specific proteins could account for the observed differences in gating properties. Alternatively, the apparent differences in Na^+ channel function observed in knockout and heterologous expression studies may stem from the compensatory upregulation of related β subunits and Na^+ channel isoforms in null mice (Chen et al., 2004; Yu et al., 2006). Additional studies of the changes in α and β subunit expression that occur in β_2 null mice, or the development of conditional β_2 knockouts that reduce the opportunity for subunit compensation, may shed light on the apparent discrepancy between the *in vivo* and *in vitro* effects of β_2 subunit regulation.

Several studies have examined the contribution of β_2 subunits to the development of pain behaviors in rodent models of nerve injury. A study investigating β subunit expression using RT-PCR and *in situ* hybridization found that β_2 mRNA levels in DRG neurons are not significantly altered following peripheral nerve injury (Takahashi et al., 2003). However, subsequent studies of β_2 protein expression using immunohistochemistry and Western blotting revealed that the β_2 protein is upregulated following nerve injuries (Pertin et al., 2005). β_2 upregulation has been observed in both injured and uninjured sensory neurons, suggesting that the β_2 subunit contributes to the excitability of both these populations. This possibility is supported by studies showing that the β_2 knockout decreases the expression of TTX-S Na^+ channels in DRG neurons (Lopez-Santiago et al., 2006). Importantly, the mechanical allodynia associated with peripheral nerve injury is attenuated in β_2 null mice, which is consistent with a role for this subunit in the development of neuropathic pain (Pertin et al., 2005).

THE β_3 SUBUNIT

In situ hybridization has shown that β_3 subunit mRNA is highly expressed in small- (<25 μm) and medium-diameter (25–45 μm) DRG neurons and to a lesser extent in large-diameter (>45 μm) neurons (Shah et al., 2000, 2001). The cellular distribution of β_3 expression extensively overlaps that of TTX-R $Na_v1.8$ and $Na_v1.9$ channels, which are primarily expressed in nociceptors (Akopian et al., 1996; Sangameswaran et al., 1996). In rodent models of neuropathic pain, β_3 mRNA increases in C-fiber nociceptors following chronic constriction (Shah et al., 2000), spared nerve ligation, and sciatic nerve transection (Takahashi et al., 2003), and in medium-diameter Aδ fibers in the streptozocin rodent model of diabetes (Shah et al., 2001). The upregulation of β_3 observed in animal models of nerve injury is consistent with the increase in β_3 protein in human DRG neurons following avulsion injuries (Casula et al., 2004). The preferential expression of β_3 subunits in small DRG neurons and their upregulation in models of nerve injury support the idea that β_3 is an important contributor to both acute and chronic pain.

β_3 Subunits also appear to play a major role in the development of neuropathic pain. Chronic constriction injury and sciatic nerve axotomy have been shown to induce an increase in TTX-S Na^+ currents (Cummins and Waxman, 1997) that has been linked to the enhanced expression of $Na_v1.3$ channels in small- and medium-sized DRG neurons (Waxman et al., 1994; Dib-Hajj et al., 1996, 1999; Black et al., 1999; Kim et al., 2002; Takahashi et al., 2003). The injury-induced increase in $Na_v1.3$ expression is paralleled by a similar increase in β_3 mRNA and protein levels (Shah et al., 2000; Takahashi et al., 2003). Heterologous expression studies have shown that co-expressing the β_3 subunit produces depolarizing shifts in $Na_v1.3$ activation and inactivation, faster recovery from inactivation, and slower current kinetics (Cummins et al., 2001). One possibility is that the upregulation of $Na_v1.3$ channels and β_3 subunits may be an attempt by the neurons to compensate for the injury-induced decrease in the expression of $Na_v1.8$ and $Na_v1.9$ channels (Dib-Hajj et al., 1996, 1999; Sleeper et al., 2000). Replacing the slowly gating TTX-R $Na_v1.8$ current with the more rapid TTX-S current of $Na_v1.3$–β_3 channels is predicted to reduce the action potential threshold and promote high-frequency firing, thereby contributing to the hyperexcitability of injured DRG neurons (Cummins et al., 2001). However, immunohistochemical analysis suggests that $Na_v1.3$ channels are preferentially upregulated in medium to large size DRG neurons after nerve injury (Kim et al., 2001; Fukuoka et al., 2008) and therefore may not extensively overlap with $Na_v1.8$ channels primarily expressed in small-diameter nociceptors.

Early studies of $Na_v1.8$ channels expressed in *Xenopus* oocytes found that co-expressing β_3 increases Na^+ current density and produces a hyperpolarizing shift in activation (Shah et al., 2000). This contrasts with later studies showing that co-expressing β_3 in oocytes produces a depolarizing shift in $Na_v1.8$ inactivation but no change in current density (Vijayaragavan et al., 2004). Studies on $Na_v1.8$ expressed in mammalian cells revealed that β_3 causes a 31% decrease in peak current density but no change in activation or steady-state inactivation (Zhao et al., 2011). Collectively, these findings suggest that co-expressing the β_3 subunit either has no effect or reduces $Na_v1.8$ current density, without altering voltage-dependence or gating kinetics. Similar findings have been reported for the β_3 regulation of $Na_v1.6$, a rapidly gating TTX-S Na^+ channel that is preferentially expressed at the nodes of Ranvier of peripheral nerve fibers (Krzemien et al., 2000; Tzoumaka et al., 2000; Ulzheimer et al., 2004) and in large-diameter sensory neurons (Black et al., 1996; Fukuoka et al., 2008; Ho and O'Leary, 2011). Heterologous expression studies have indicated that co-expression with the β_3 subunit does not alter the peak current density, current kinetics, or voltage-dependence of $Na_v1.6$ channels (Zhao et al., 2011).

THE β_4 SUBUNIT

The mature β_4 subunit protein has a large extracellular Ig-like fold, a single membrane spanning segment, and a short cytoplasmic C-terminal domain that is structurally similar to those of the β_1–β_3 subunits. β_4 shares high amino acid identity (35%) with β_2 and includes an extracellular unpaired cysteine that enables β_4 to covalently associate with Na^+ channel α subunits via disulfide bonds (Yu et al., 2003). The β_4 subunit is highly expressed

in DRGs and at lower levels in the brain and spinal cord. At the cellular level, β_4 is abundantly expressed in large-diameter sensory neurons and at lower levels in intermediate and small neurons (Yu et al., 2003).

The co-expression of β_4 with the $Na_v1.2$ channel in tsA201 cells produces a hyperpolarizing shift in activation (−7 mV) but no change in steady-state inactivation (Yu et al., 2003). The effects of β_4 on the gating properties of the TTX-S $Na_v1.6$ and TTX-R $Na_v1.8$ channels have also been studied (Chen et al., 2008; Zhao et al., 2011). Co-expressing β_4 produces pronounced hyperpolarizing shifts in activation (−17 mV) and steady-state inactivation (−9 mV) of $Na_v1.8$, and a smaller hyperpolarizing shift (−8 mV) in $Na_v1.6$ activation (Zhao et al., 2011). β_4 subunits produce similar negative shifts in the activation of the neuronal $Na_v1.1$ and skeletal muscle $Na_v1.4$ channels (Yu et al., 2003; Aman et al., 2009). The consistent hyperpolarizing shift in activation produced by the β_4 subunit suggests that this subunit may modulate neuronal excitability by causing Na^+ channels to activate at more hyperpolarized voltages.

Resurgent currents were initially described in Purkinje neurons where they were found to promote the discharge of multiple action potentials in response to brief depolarizations (Raman and Bean, 1997, 1999). Subsequent work found that the open-channel block at depolarized voltages coupled with rapid unblocking and slow Na^+ channel deactivation at voltages near threshold produce an inward Na^+ current (resurgent current) that transiently depolarizes the neurons (Grieco et al., 2005). These resurgent currents increase excitability and are believed to underlie the high-frequency firing of Purkinje neurons (Raman and Bean, 2001). The cytoplasmic C-terminus of the β_4 subunit has emerged as a likely candidate for the endogenous blocking particle responsible for resurgent currents (Grieco et al., 2005; Bant and Raman, 2010). This possibility is supported by studies showing that siRNA targeting *SCN4b* abolishes resurgent currents in cultured cerebellar granule cells and that the exogenous application of synthetic β_4 C-terminal peptide ($\beta4_{154-167}$) blocks Na^+ currents and induces resurgent currents in inactivation-impaired Purkinje neurons. Resurgent currents are substantially reduced in Purkinje neurons isolated from $Na_v1.6$ null mice, indicating that these channels play an important role in the production of resurgent currents (Raman et al., 1997). However, persistent resurgent currents have been reported in the subthalamic nucleus and Purkinje neurons isolated from $Na_v1.6$ null mice, suggesting that other Na^+ channel isoforms may also produce these currents (Do and Bean, 2004; Grieco and Raman, 2004).

The role of β subunits in the generation of resurgent currents has been further investigated *in vitro*. Co-expressing the β_4 subunit does not induce resurgent currents in heterologously expressed $Na_v1.1$ (Aman et al., 2009), $Na_v1.6$ (Zhao et al., 2011), or $Na_v1.8$ (Zhao et al., 2011) channels, indicating that the association with the intact β_4 subunit alone is insufficient to produce resurgent current. Additional proteins or post-translational modifications appear to be required to recapitulate the resurgent currents observed in native neurons (Grieco et al., 2002). These endogenous proteins and regulatory pathways may be highly specific to particular cell types and may thus be absent in the mammalian cells lines that are widely used for heterologous expression and cellular electrophysiology studies (Theile and Cummins, 2011). Alternatively, β_4-mediated resurgent currents may involve cell-specific enzymatic cleavage by proteases such as β-site amyloid precursor protein cleaving enzyme 1 (BACE1) or other proteases that are required to produce the functionally active blocking peptide (Huth et al., 2011).

Resurgent currents are observed in 40% of large-diameter (35–50 μm) DRG neurons and are substantially reduced in neurons from $Na_v1.6$ null mice (Cummins et al., 2005). Large DRG neurons express both the $Na_v1.6$ (Black et al., 1996; Fukuoka et al., 2008; Ho and O'Leary, 2011) and β_4 subunits (Yu et al., 2003), further supporting the idea that $Na_v1.6$–β_4 channels may play a role in these currents. This contrasts with small-diameter DRG neurons that do not routinely produce resurgent currents (Cummins et al., 2005) and that express low levels of $Na_v1.6$ (Black et al., 1996; Fukuoka et al., 2008; Ho and O'Leary, 2011) and β_4 subunits (Zhao et al., 2011).

Resurgent currents have recently been implicated in the neuronal hyperexcitability and pain associated with paroxysmal extreme pain disorder (PEPD; Jarecki et al., 2010; Theile and Cummins, 2011). In particular, the I1467T mutation in the interdomain III–IV linker of the $Na_v1.7$ channel reduces the rate of inactivation, increases the persistent Na^+ current, and induces a depolarizing shift in steady-state inactivation (Fertleman et al., 2006; Jarecki et al., 2008). These changes are consistent with impaired fast inactivation, which increases the probability of open-channel block, a suspected contributor to the generation of resurgent currents (Grieco and Raman, 2004). When heterologously expressed in cultured DRG neurons, the $Na_v1.7$–I1467T mutant channel increases both the percentage of neurons displaying resurgent currents and the peak current amplitude (Theile et al., 2011). Computer simulations further support the idea that PEPD mutations that alter $Na_v1.7$ inactivation induce resurgent currents in DRG neurons that contribute to aberrant action potential firing and increased cellular excitability. The evidence supporting a role for resurgent currents in the development of neuropathic pain is compelling and warrants further investigation.

SUMMARY

All four isoforms (β_1–β_4) and both splice variants (β_{1A}, β_{1B}) of β subunits are broadly expressed in the PNS. These subunits interact with many of the Na^+ channel isoforms in sensory neurons and alter the expression, voltage-dependence, and gating properties of these channels. β subunits are differentially expressed in large-diameter mechanoreceptors (β_1, β_4) and small-diameter nociceptors (β_3). This pattern of β subunit expression suggests that these auxiliary subunits may differentially regulate voltage-gated Na^+ currents and the excitability of these neuronal populations. Injury-induced changes in β subunit expression and the altered functional regulation of the Na^+ channels expressed in sensory neurons contribute to the hyperexcitability and ectopic firing of sensory neurons. Current evidence suggests that β subunits are important contributors to sensory physiology, nociception, and neuropathic pain.

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The sodium channel as a target for local anesthetic drugs

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Na channels are the source of excitatory currents for the nervous system and muscle. They are the target for a class of drugs called local anesthetics (LA), which have been used for local and regional anesthesia and for excitatory problems such as epilepsy and cardiac arrhythmia. These drugs are prototypes for new analgesic drugs. The drug-binding site has been localized to the inner pore of the channel, where drugs interact mainly with a phenylalanine in domain IV S6. Drug affinity is both voltage- and use-dependent. Voltage-dependency is the result of changes in the conformation of the inner pore during channel activation and opening, allowing high energy interaction of drugs with the phenylalanine. LA drugs also reduce the gating current of Na channels, which represents the movement of charged residues in the voltage sensors. Specifically, drug binding to phenylalanine locks the domain III S4 in its outward (activated) position, and slows recovery of the domain IV S4. Although strongly affecting gating, LA drugs almost certainly also block by steric occlusion of the pore. Molecular definition of the binding and blocking interactions may help in new drug development.

Keywords: lidocaine, molecular modeling, Na channel, local anesthetics, gating currents

INTRODUCTION

Local anesthetic (LA) drugs entered clinical use over a 100 years ago for surgical pain control. They continue to be important for local and regional anesthesia as well as for their cardiac antiarrhythmic actions. Their target is the voltage-gated Na channel, and a great deal of research over the recent years has located the binding site within the channel's inner pore. As the first class of channel-active drugs to be studied extensively, they serve as a model for other drug studies. In addition to cardiac arrhythmias, the voltage-gated Na channel is an important player in many pathophysiological processes, including pain and epilepsy. Many drugs with dissimilar structures resemble LA drugs in their channel action and perhaps also in their binding sites. Consequently, efforts to develop LA-like drugs with specific nerve blocking abilities are widespread. Recently there has been considerable progress in understanding the mechanism of voltage-dependent binding of LA drugs. This article focuses on new insights into LA molecular mechanism of action, which may assist in developing better and more specific drugs.

CLINICAL ROLE OF LOCAL ANESTHETIC DRUGS TODAY

The primary use of LA drugs at this time is for local or regional nerve block. Specific examples are dental procedures, eye surgery, epidural anesthesia for lower abdominal or leg surgery, large nerve block in the extremities for orthopedic surgery, and surgical biopsy. High concentrations are achieved by local injection, so that specificity is achieved at the site of injection while systemic effects of the drugs are minimized. Desirable features are rapid onset of nerve block, sufficient block duration, and lack of local reaction. LA drugs are also useful as antiarrhythmic agents for short-term use for suppression of cardiac excitability during rapid arrhythmias, and they can be lifesaving in acute ischemia. However, their action can also establish the conditions for development of arrhythmias, and their long-term use, especially in individuals with ischemic heart disease, must be considered cautiously.

Several nervous system conditions can be treated with LA-type drugs. Some of the commonly used anticonvulsant drugs block Na channels with LA-like characteristics. Because pain is transmitted to the central nervous system via nerve cells that depend on specific isoforms of the Na channel for excitation and conduction, agents that selectively target these Na channel isoforms that transmit pain signals are eagerly sought, in order to avoid the use of analgesic drugs that are addicting or have dangerous side effects.

STRUCTURE OF THE DRUG TARGET

The mammalian Na channel is an intrinsic membrane glycoprotein. It is a molecular complex of a ~2000 amino acid α-subunit, which contains the main channel functions and drug interaction sites, and a variable number of smaller β-subunits, which modulate membrane expression and channel functional properties (Catterall, 2000; Patino and Isom, 2010). The α-subunit contains 24 transmembrane segments organized into four homologous, but not identical, domains (DI–DIV). The six transmembrane, mostly α-helical segments in each domain (S1–S6) are further divided into a voltage-sensing unit (S1–S4) and a pore-forming unit (S5–S6), connected mainly through the S4–S5 cytoplasmic linker. Much of the protein is not within the membrane, with the glycosylated outside loops involved in toxin interactions, and the larger cytoplasmic loops involved in hormonal and metabolic regulation. There are multiple isoforms of the Na channel α-subunit. This discussion will focus on mammalian brain ($Na_V1.2$) skeletal ($Na_V1.4$), and cardiac ($Na_V1.5$) Na channels, which are very homologous in their intramembrane regions and which have been the major isoforms used for structure–function studies.

No direct detailed three-dimensional structural information for the mammalian Na channel is available to date because of the difficulty in developing crystals of these large amphipathic proteins. The primary and secondary structures of Na channels are similar to the better-studied K channel (e.g., Doyle et al., 1998; Jiang et al., 2002; Long et al., 2005), and it is expected that tertiary structure will be also. The crystal structure of a bacterial voltage-gated Na channel (Na_VAb) has been recently reported (Payandeh et al., 2011). In contrast to the mammalian Na channel, it is comprised of four identical subunits, rather than four connected non-identical domains. Nevertheless, its structure will be of great value in the interpretation of experimental studies. The structure of K channels and the bacterial Na channel, combined with studies of mammalian channel chimeras and point mutations, have allowed the development of molecular models of the mammalian Na channel α-subunit (Guy and Seetharamulu, 1986; Lipkind and Fozzard, 2005; Bruhova et al., 2008).

The part of the channel that is best characterized by modeling is the pore region, which has been useful in locating drug and toxin binding sites. The pore is formed mainly by a bundle of the four non-identical S6 α-helical segments, one from each domain. The pore outer vestibule is lined by four infolded hairpin loops of the S5–S6 extracellular regions (P loops) that form the outer vestibule and selectivity filter of the channel, which is located about 40% through the pore from the outside. The outer vestibule and selectivity filter contain several charged amino acid residues, but the inner pore is composed of neutral, mostly hydrophobic amino acid residues.

The voltage-sensing S4 segments are similar to those of voltage-gated K channels, as well as to those of the recently crystalized bacterial Na channel (Payandeh et al., 2011). The S4 segments contain several positively charged residues that are responsive to changes in the transmembrane potential, and their function has been explored by selective mutation (for review, see Bezanilla, 2000). Critical to the function of the Na channel is its ability to open and close its pore in response to changes in the transmembrane potential, a process called voltage-dependent gating. The channel is closed at the resting potential, and opens briefly upon depolarization. Based upon crystal structures of K channels (Doyle et al., 1998; Jiang et al., 2002) and the Na_VAb channel (Payandeh et al., 2011), the activation gate of Na channels is located at the cytoplasmic end of the pore, where all four S6 segments converge. The current understanding on opening of the activation gate is that a hinged motion of the S6 segments allows them to separate at their cytoplasmic ends resulting in an open pore (Jiang et al., 2002). Changes in the S6 conformation are linked to movement of the positive charges in the S4 segments, i.e., the voltage sensors, toward the extracellular surface of the membrane during depolarization. Each individual S4 pulls on its S4–S5 cytoplasmic segment, which is adjacent to the S5–S6 pore-wall at its cytoplasmic end, resulting in opening the channel pore (Long et al., 2005). The pore then is occluded (inactivated) by the movement of the intracellular segment formed by the linker between domains III–IV, i.e., the inactivation particle, into the open pore (Patton et al., 1992), and facilitated by the voltage-dependent movement of the domain IV S4 segment (Chahine et al., 1994; Sheets et al., 1999). Upon repolarization these processes appear to be reversed.

LOCAL ANESTHETIC DRUG-BINDING SITE(S)

The functional characteristics of LA action at concentrations of clinical relevance are complex, but they can be divided into three categories (Sheets et al., 2010). Firstly, when the channel is in the closed conformation, i.e., at very hyperpolarized potentials, block of Na current occurs only at high concentrations (mM range). Secondly, when the channel is opened with batrachotoxin, rapid flicker block can occur by block at the selectivity filter. This flicker block, when evident, is low affinity, i.e., in the millimolar range. The third type is high-affinity block, and it represents interaction of LA's with the pore conformation that occurs when the voltage sensors are deployed outward and the channel is in its open state and/or open-inactivated state. This occurs at positive potentials and is simply called voltage-dependent block.

The long-standing explanation for high-affinity LA block is called the "modulated receptor hypothesis" (Hille, 2001). It proposes that LA high-affinity depends on structural modification of the binding site (the LA receptor) as a consequence of channel opening and inactivation. Sorting out the types of block using electrophysiology can be less than straight-forward because; (1) channel conformation is voltage dependent, (2) there may be limitations in access and egress to various channel conformations, depending on chemical properties of the drug, and (3) drug on and off rates can be shorter than, in the same order of magnitude as, or longer than channel conformational lifetimes. In voltage clamp assays, block of closed channels is usually tested by holding at very negative potentials, i.e., usually at least −120 mV or more negative with infrequent depolarization for a short duration. This is sometimes called "resting block," but experimental measures differ greatly, because the holding potential can fail to be sufficiently negative to remove all voltage-dependent block. Resting block (also called closed-state or lipophilic-block) can be confirmed as such when the amount of block is not decreased by further hyperpolarization. Strict open-channel, flicker block can occur at potentials that also support high-affinity block, but it requires much higher drug concentrations; it has been assayed by single channel recordings in channels where inactivation is prevented with batrachotoxin (e.g., Zamponi et al., 1993). Flicker block is not likely to be of clinical significance.

Other electrophysiological assays, which are often of the most physiological relevance, represent a mixture of several types of block in combination with drug access/egress properties. For example, for many LA drugs (and especially those considered to be antiarrhythmic), repetitive test depolarizations show accumulation of block, a property called "use-dependence." Use-dependent increase in block can occur because drug off rates from the high-affinity site are sufficiently slow that dissociation is not complete between test depolarizing steps either because of high drug-binding affinity (such as lidocaine compared to benzocaine; Hanck et al., 2009) or because of limited egress (such as flecainide; Liu et al., 2003). In fact, protocols with rapid trains of depolarizations are often used as a surrogate for assaying the high-affinity state, since it is non-conducting and therefore amenable to direct measurement only by methods that do depend on measuring ionic current, e.g., binding assays or gating current.

Early information about the location of the high-affinity LA binding site, summarized by Hille (2001), indicated that it was on

the Na channel α-subunit and accessible from the cytoplasmic side. Because homology modeling and chemical reactivity studies indicated that the inner pore was most likely lined with residues of the S6 segments, Catterall and associates systematically mutated these residues and determined the effects on LA block at depolarized potentials (Ragsdale et al., 1994; Yarov-Yarovoy et al., 2002). They found only two residues that had a major effect on high-affinity block; Phe-1764 and Tyr-1771 in DIVS6. This is $Na_V1.2$ (brain isoform) numbering; for the skeletal muscle isoform ($Na_V1.4$), it is Phe-1579, and for the cardiac isoform ($Na_V1.5$), it is Phe-1759 or Phe-1760 (depending on which clone is used). So far, no differences in the binding interactions have been identified for these isoforms. Consequently, to simplify further discussion of these residues independent of the isoform studied, they will be identified as IVS6-Phe and IVS6-Tyr. Mutation of Ile-1760 also had effects although it is probably not part of the binding site (see below). Small effects of uncertain mechanism were noted for mutations of Leu-1465, Asn-1466, and Ile-1469 in DIIIS6, and Ile-409 and possible Asn-418 in DIS6. In contrast, the low affinity, closed-state block seen with test depolarizations from very hyperpolarized potentials was not reduced by these mutations, and may have been slightly increased. This demonstrated clearly that the low affinity and the high-affinity sites are clearly different. Indeed, the high-affinity block can be abolished by removal of only IVS6-Phe (Hanck et al., 2009).

Docking of LA drugs into homology models of the open, inner pore of the Na channel (**Figure 1**) illustrate the structure of a possible high-affinity binding site (Lipkind and Fozzard, 2005). The typical LA drug has a tertiary amine hydrophilic domain and an aromatic ring hydrophobic domain, separated by an intermediate linker containing an amide or an ester group. Consistent with the suggestion by Ragsdale et al. (1994), the Lipkind–Fozzard model had the alkylamine group interact with IVS6-Phe and the aromatic ring interact with IVS6-Tyr. The alkylamine group fitted into a space between IVS6-Phe and the leucine in IIIS6, which was created by opening of the pore, with a van der Waals energy of about −4 to −5 kcal/mol, and the aromatic ring interacted with IVS6-Tyr with an energy of about −2 to −3 kcal/mol. Replacement of IVS6-Phe with alanine in the model reduced the interaction energy about 3 kcal/mol, consistent with experimental evidence that this substitution abolished measurable voltage-dependent LA block. Ahern et al. (2008) systematically reduced the π-electron charge of IVS6-Phe by incorporation of unnatural amino acids at this position, and they demonstrated that the LA interaction with phenylalanine is due to an electrostatic force. Block by benzocaine, which contains the hydrophobic aromatic ring, but not the partially charged alkylamine end, was not influenced by their unnatural amino acid substitutions. The aromatic end of LA drugs interacted in the model with IVS6-Tyr with an energy of about −3 kcal/mol, and the interaction was not an aromatic–aromatic one. Interaction of the drug aromatic moiety with IVS6-Tyr was investigated by Li et al. (1999) using tetracaine. They found that substitution of IVS6-Tyr by hydrophobic residues produced minimal change in use-dependent LA block, consistent with an aliphatic interaction rather than an aromatic-aromatic one. In keeping with experimental data the model did not show interaction with residues in DIS6 or DIIS6.

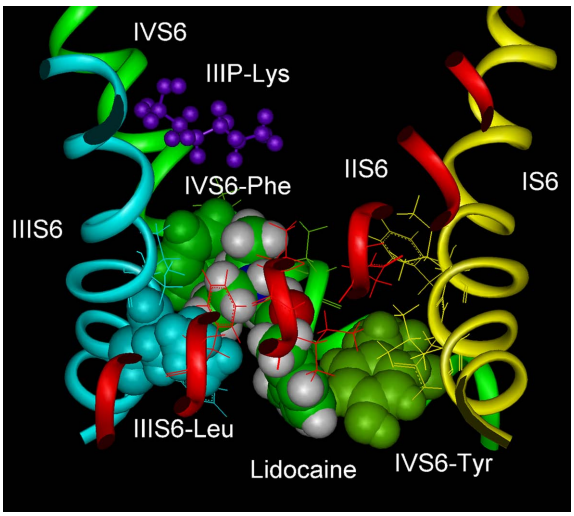

FIGURE 1 | Molecular model of lidocaine in its binding site in the Na channel inner pore. IVS6-Phe and IVS6-Tyr are shown as green space-filled images, and IIIS6-Leu is shown as blue spaced-filled images. IIIP-Lys is part of the DEKA selectivity ring and is shown as purple stick figures. Lidocaine in CPK space-filled images interacts with IVS6-Phe and IVS6-Tyr, almost filling the pore. The figure was prepared by Gregory Lipkind.

Because of their size, charged lidocaine-like molecules did not fit well into the inner pore of the Lipkind–Fozzard model of the closed Na channel. In the closed pore model both IVS6-Phe and IVS6-Tyr face the central pore lumen, but the cleft between IVS6-Phe and the leucine in IIIS6 is too small to allow the alkylamine to fit. IVS6-Tyr is part of the densely packed S6 crossing in the model. In the bacterial Na_VAb, the crossover is located lower (Payandeh et al., 2011), but experimental study of accessibility of MTS reagents supports the mammalian model (Sunami and Fozzard, unpublished data). According to the model, charged LA drugs may not be able to enter the inner pore, or if they do (possibly through pore-wall fenestrations, as noted in the Na_VAb structure; Payandeh et al., 2011), they do not form strong interactions with the key residues of IVS6-Phe and IVS6-Tyr. Therefore, LA drugs appear to bind with high-affinity to two residues in DIVS6 and possibly with low affinity to one or more in DIIIS6 in the inner pore when the binding site is optimized by the conformational changes that occur during channel voltage-dependent activation and opening. IVS6-Phe is the most important residue for LA binding, and its mutation abolishes voltage-dependent, i.e., use-dependent, block. The site of LA binding for low affinity closed-channel block is not yet clearly resolved, and may be simply a combination of hydrophobic interactions with the closed pore.

ROLE OF THE DRUG ACCESS PATH

The location of the LA binding site has depended upon the study of point mutational data that assume that impairment of drug block of Na current by point mutations involve parts of the drug-binding site. However, there are two other possible explanations for the effects of amino acid substitutions: (1) the mutation could interfere with a molecular sequence of events downstream of the binding step without being part of the binding site itself.

All evidence at this point supports the direct role in binding by IVS6-Phe and probably IVS6-Tyr, but roles of the others are less certain. (2) Another possible action of mutations is to alter the drug pathway to and from its binding site deep within the inner pore. Although LA drugs access their high-affinity binding site only from the inside for most Na channel isoforms (Narahashi and Frasier, 1971; Strichartz, 1973), two early experimental studies suggested that access to the inner pore site is possible from the outside. Alpert et al. (1989) used a cardiac cell preparation with independent perfusion of the outside and the inside and found that QX314, a permanently charged quaternary amine that is membrane impermeant, could block cardiac Na current from the outside. At that time, it was not clear whether QX314 could access the inner pore site directly or if there was another binding site. When Ragsdale et al. (1994) observed that replacement of Ile-1760 by alanine in $Na_V1.2$ reduced etidocaine block, they determined that this was not the result of a loss of affinity, but of a more rapid off-rate. This mutation also generated channels that were sensitive to extracellularly applied QX314. Subsequently, Qu et al. (1995) showed that an isoform difference in the upper part of DIVS6 between the nerve channel ($Na_V1.2$) and the cardiac channel ($Na_V1.5$) was responsible for an external LA access path in the cardiac isoform, and Sunami et al. (1997) found that an isoform difference in the selectivity region also allowed extracellular LA to block the cardiac channel. These external access paths can affect lidocaine block, but only in the cardiac isoform (Lee et al., 2001). Bruhova et al. (2008) have explored the possible molecular paths for external access in their model of the Na channel.

MECHANISM OF LA BLOCK OF THE Na CHANNEL

Location of the LA binding site in the open-channel inner pore suggests several possible mechanisms for high-affinity LA block. A key to approaching this question is recognition that high-affinity block is voltage dependent over the range that the Na channel activates to open the pore. These voltage-dependent conformational changes appear to create the high-affinity site composed mainly of IVS6-Phe and IVS6-Tyr. Block could occur because the LA molecule binds in the inner pore and physically blocks conductance, because drug-binding interferes with the gating machinery itself, or both. Experimental evidence can be found to support both possibilities.

Physical obstruction of the pore would be voltage dependent because the channel's conformational changes during activation create the high-affinity site. Molecular modeling is consistent with this idea, with the bound drug located horizontally across the pore (Hanck et al., 2009). LA drugs all produce complete block at the single channel level, i.e., reduction in probability of opening, associated with modest decrease in open times (Grant et al., 1989). The selectivity filter is the narrowest segment of the pore, and direct drug interaction with it can under some circumstances produce fast flicker block at very high concentrations (Zamponi et al., 1993). However, electrostatic interaction of the positively charged lidocaine with the negatively charged residues in the selectivity filter suggests that the high-affinity site is about 10 Å below the selectivity filter (Sunami et al., 1997). For charged LA drugs, the positive charge itself would be expected to interfere with permeation of Na, even if it does not occlude the pore completely (McNulty et al., 2007).

The alternative idea for mechanism of block is that it interferes with channel gating. The long-standing modulated receptor hypothesis for blocking action of LA drugs predicted a stabilization of the inactivated state of the Na channel (Hille, 1977). There is ample evidence of interaction between LA drug binding and gating. Voltage sensor movement, which is the mediator between membrane voltage and conformational changes of gating, can be measured directly by intramembranous charge movement, and LA binding reduces this gating charge as much as 40% (Hanck et al., 1994). The most dramatic effect of lidocaine on gating currents is loss of the entire contribution of the DIIIS4 gating charge, which normally contributes ∼30% to maximal charge (Sheets and Hanck, 2003), along with loss of a part of the DIVS4 charge. It appears that lidocaine stabilizes the DIIIS4 voltage sensor in its activated (depolarized) position, so that it is unable to reset upon repolarization. Muroi and Chanda (2008) studied the effects of lidocaine on fluorescence indicators of individual S6 segment movements in rat skeletal Na channels expressed in *Xenopus* oocytes. They found a somewhat smaller reduction in total gating charge (S1–S4) of ∼25%, with the greatest change in fluorescence-voltage curve in the DIIIS4. While the mechanism is not understood in detail, it appears that LA drugs interfere allosterically with return of DIIIS6 to its closed configuration and coupling to DIIIS4 via the S4–S5 linker prevents restoration of the DIIIS4.

However, it is the DIVS4 that controls fast inactivation (Chahine et al., 1994; Sheets et al., 1999). Measurement of the movement of the DIVS4 gating charge in drug-bound channels has shown more modest changes; a small (∼10%) reduction in total current (about 1/3 of the normal contribution of DIVS4) associated with a shift to the left of its voltage dependence (Sheets and Hanck, 2003). The shift to the left at more hyperpolarized potentials appears to result from the bound LA drug stabilizing the DIVS4 in a partially depolarized position, so its movement is facilitated upon depolarization (Sheets et al., 2010). The gating charge movements of DIS4 and DIIS4, which are presumably responsible for the fast activation of the Na current, were unchanged.

Does outward movement of DIIIIS4 and DIVS4 simply increase lidocaine affinity for steric block in the pore, or does lidocaine binding cripple the gating process resulting in altered kinetics and a non-conducting channel? Studies on Na channels that have had both their S4's in DIII and IV pre-stabilized in outward positions using MTSEA-biotin show dramatically increased affinity for lidocaine block, with perhaps the DIVS4 being the more important player, supporting the idea of an increase in steric block by lidocaine (Sheets and Hanck, 2007). Importantly, the pre-stabilization of both the DIIIS4 and DIVS4 did not, by themselves, prevent the channel from opening and closing normally and conducting current in the absence of LA drug. Consequently, the lidocaine-induced effects on gating currents do not directly cause block of channel conductance, but instead, they enhance lidocaine affinity. Arcisio-Miranda et al. (2010) mutated multiple residues in the S4–S5 and terminal S6 regions of DIII and found several mutations that dramatically reduced the stabilizing effect of lidocaine

on the DIIIS4 gating charge, but in most cases these mutations failed to prevent high-affinity (use-dependent) lidocaine block. This confirms the important role of the molecular link between DIIIS4–S5 and DIIIS6 in lidocaine effect on DIIIS4, and it also confirms that the DIIIS4 stabilization by lidocaine does not in itself produce the block. Arcisio-Miranda and colleagues have not reported comparable experiments on the DIVS4 movement.

High-affinity block is most likely the result of occlusion of the pore permeation path by the LA drugs (Hanck et al., 2009). This process is voltage dependent because it depends on conformational changes during activation to create the high-affinity site, as predicted by the modulated receptor hypothesis. These conformational changes also favor binding of the DIII–DIV inactivation loop, and its binding may help stabilize the LA binding site in its high-affinity conformation. A detailed understanding of how inactivation is related to the kinetics of LA block remains unclear, and there are conflicting reports on the effects of inactivation on lidocaine block. Vedantham and Cannon (1999) reported that the IFM blocking segment within the intracellular linker between domain III and IV, i.e., the inactivation particle, is not stabilized by lidocaine, but it returns quite normally to its unbound position upon repolarization in drug-bound Na channels. Chemical removal of inactivation has been reported to reduce LA affinity (Bennett et al., 1995). However, a mutation that removes inactivation apparently fails to reduce lidocaine affinity (Wang et al., 2004). It also remains possible that high-affinity LA drug-binding favors some ill-defined closed-state of the inner pore occurring naturally during slow inactivation (Ong et al., 2000) or a unique LA-induced closed-state. Recovery from LA block after repolarization would then depend on unbinding of the LA molecule accompanied by movements of the DIIIS4 and DIVS4 sensors and the inactivation particle, with resulting loss of the high-affinity site. Regardless of the details, which need careful experimental study, it is clear that the link between drug binding in the pore and voltage sensor movements provides a mechanism to control drug affinity dynamically and, thereby, provides for a range of therapeutic options. It would be helpful if future experimentation determined how drug binding to residues in the S6 segment of domain IV is coupled to the movements of the voltage sensor in domain III, and sorted out the relationship of drug affinity to conformational changes induced by movement of IVS4 and its associated inactivation processes. Although useful isoform differences in LA drug action have been difficult to find, many opportunities have not yet been explored.

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Molecular insights into the local anesthetic receptor within voltage-gated sodium channels using hydroxylated analogs of mexiletine

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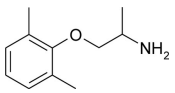
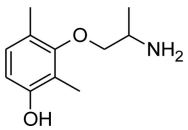
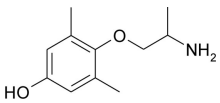
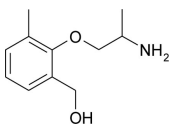
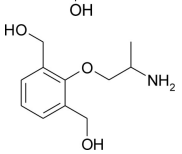
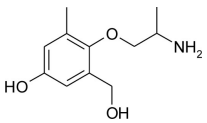
We previously showed that the β-adrenoceptor modulators, clenbuterol and propranolol, directly blocked voltage-gated sodium channels, whereas salbutamol and nadolol did not (Desaphy et al., 2003), suggesting the presence of two hydroxyl groups on the aromatic moiety of the drugs as a molecular requisite for impeding sodium channel block. To verify such an hypothesis, we synthesized five new mexiletine analogs by adding one or two hydroxyl groups to the aryloxy moiety of the sodium channel blocker and tested these compounds on hNav1.4 channels expressed in HEK293 cells. Concentration–response relationships were constructed using 25-ms-long depolarizing pulses at −30 mV applied from an holding potential of −120 mV at 0.1 Hz (tonic block) and 10 Hz (use-dependent block) stimulation frequencies. The half-maximum inhibitory concentrations (IC_{50}) were linearly correlated to drug lipophilicity: the less lipophilic the drug, minor was the block. The same compounds were also tested on F1586C and Y1593C hNav1.4 channel mutants, to gain further information on the molecular interactions of mexiletine with its receptor within the sodium channel pore. In particular, replacement of Phe1586 and Tyr1593 by non-aromatic cysteine residues may help in the understanding of the role of π–π or π–cation interactions in mexiletine binding. Alteration of tonic block suggests that the aryloxy moiety of mexiletine may interact either directly or indirectly with Phe1586 in the closed sodium channel to produce low-affinity binding block, and that this interaction depends on the electrostatic potential of the drug aromatic tail. Alteration of use-dependent block suggests that addition of hydroxyl groups to the aryloxy moiety may modify high-affinity binding of the drug amine terminal to Phe1586 through cooperativity between the two pharmacophores, this effect being mainly related to drug lipophilicity. Mutation of Tyr1593 further impaired such cooperativity. In conclusion, these results confirm our former hypothesis by showing that the presence of hydroxyl groups to the aryloxy moiety of mexiletine greatly reduced sodium channel block, and provide molecular insights into the intimate interaction of local anesthetics with their receptor.

Keywords: sodium channel, hNav1.4, mexiletine analogs, local anesthetic receptor

INTRODUCTION

Mexiletine is a class Ib antiarrhythmic drug, also considered as the first choice drug for treating myotonic syndromes (Conte Camerino et al., 2007). Moreover, recent clinical trials demonstrate its therapeutic value to relieve neuropathic pain (Challapalli et al., 2005). Mexiletine exerts its pharmacological action through blockade of voltage-gated sodium channels, thus reducing cell excitability. Preferential binding to inactivated channels and use-dependent block are the basis of the selective action of mexiletine on pathologic hyperactive tissues. The structure of mexiletine is related to that of local anesthetic (LA) drugs, presenting a protonable amine group connected to a hydrophobic aromatic ring through an intermediate ether link (**Table 1**). We have shown that little modification of these three components can substantially affect sodium channel blockade *in vitro* and antimyotonic effects *in vivo* (Desaphy et al., 1999, 2001; De Luca et al., 2000, 2003, 2004; De Bellis et al., 2006). The use of mexiletine analogs has allowed to get new important information on the molecular dynamic interaction between the drug and its receptor within the sodium channel pore (De Luca et al., 2000, 2003). Besides, sodium channel mutagenesis experiments have defined the amino acids most probably involved in the interaction with LAs (Ragsdale et al., 1994, 1996; Wright et al., 1998; Nau et al., 1999; Wang et al., 2000; Sunami et al., 2001; Yarov-Yarovoy et al., 2001, 2002; O'Leary and Chahine, 2002; McNulty et al., 2007; Ahern et al., 2008).

Table 1 | Chemical structures and physicochemical properties.

Compound	Structure	Log *P*	pK_a	Log *D* (pH 7.4)	Ionization (mol%, pH 7.4)	Electrostatic potential (kcal/mol)
Mexiletine		2.21 ± 0.01	9.28 ± 0.01	0.53	98.7	−50.3
mHM		1.67 ± 0.01	9.04 ± 0.01	0.02	97.7	−48.0
pHM		1.53 ± 0.01	8.97 ± 0.01	−0.05	97.3	−50.4
HMM		1.15 ± 0.01	9.13 ± 0.01	−0.59	98.1	−64.9
bHMM		0.25 ± 0.05	9.21 ± 0.03	−0.85	98.5	−62.6
pHHMM		0.23 ± 0.02	8.89 ± 0.02	−1.27	96.8	−65.0

The Log P, pK_a, Log D, and electrostatic potential values were determined experimentally, as described in the Section "Materials and Methods." Values of Log P and pK_a are given as mean ± SEM. Ionization of the amine group at pH 7.4 was calculated from Henderson–Hasselbalch equation, Ionization $= 1 - 10^{(pH-pK_a)}$.

In a previous study, we showed that drugs known as modulators of β-adrenergic receptors are able to block sodium channels in a manner reminiscent of LA drugs (Desaphy et al., 2003). The β-agonist clenbuterol and the β-blocker propranolol showed efficacies comparable to mexiletine in producing use-dependent inhibition of sodium currents in rat skeletal muscle fibers as well as in tsA201 cells transiently expressing the human muscle sodium channel isoform, hNav1.4. In contrast, the β-agonist salbutamol and the β-blocker nadolol had no effect on sodium currents at 1 mM concentration. Examination of chemical structures and physicochemical properties of these drugs revealed the presence of the two pharmacophores important for sodium channel blocking activity, the amine group and the aromatic ring, which respectively confer a high pK_a and high lipophilicity. However, the two inactive compounds were characterized by the presence of two hydroxyl groups on the aromatic moiety, which we proposed to be determinant for impeding sodium channel blockade.

To verify this hypothesis, we have synthesized new analogs of mexiletine by introducing one or two hydroxyl groups on the aryloxy moiety of the drug and tested them on sodium currents in mammalian cells permanently transfected with hNav1.4. Moreover site-directed mutagenesis was performed to introduce non-aromatic cysteine residues in place of the Phe1586 and Tyr1593 residues of hNav1.4, which are thought to be part of the binding site for LAs within the domain IV segment 6 of sodium channels (Ragsdale et al., 1994).

The results demonstrate that the presence of hydroxyl groups on the aromatic moiety drastically reduces the ability of compounds to block sodium channels and provide further insight in the molecular interaction between LA drugs and their binding site within the sodium channel pore.

MATERIALS AND METHODS

SITE-DIRECTED MUTAGENESIS AND EXPRESSION OF RECOMBINANT SODIUM CHANNELS

Full-length cDNA encoding the main α-subunit of wild-type (WT) skeletal muscle isoform of voltage-gated sodium channel (hNav1.4) was subcloned in the mammalian expression vector pRc–CMV. The two hNav1.4 mutants, F1586C and Y1593C, were engineered by standard two-step PCR-based site-directed mutagenesis. All PCR reactions were performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) for high-fidelity amplification (Desaphy et al., 2010). The complete coding region of channel mutant cDNAs was sequenced to exclude any polymerase errors. Permanent sodium channel expression was achieved by transfecting 1 mg ml^{-1} of plasmid in human embryonic kidney HEK293 cells using the calcium phosphate precipitation method and clone selection using geneticin (Gibco-Invitrogen, Milan, Italy).

SODIUM CURRENT MEASUREMENT IN HEK293 CELLS

Whole-cell sodium currents (I_{Na}) were recorded at room temperature (20–22°C) using an Axopatch 1D amplifier (Axon

Instruments, Union City, CA, USA). Voltage clamp protocols and data acquisition were performed with pCLAMP 9.2 software (Axon Instruments) through a 12-bit A–D/D–A interface (Digidata 1340, Axon Instruments). Pipettes made with Corning 7052 glass (Garner Glass, Claremont, CA, USA) had resistance that ranged from 1 to 3 MΩ. Currents were low-pass filtered at 2 kHz (–3 dB) by the four-pole Bessel filter of the amplifier and digitized at 10–20 kHz. After the patch membrane had been ruptured, a 25-ms-long test pulse to −30 mV from a holding potential (HP) of −120 mV was applied to the cell at a low frequency until stabilization of I_{Na} amplitude and kinetics was achieved (typically 5 min). Only those data obtained from cells exhibiting series resistance errors <5 mV were considered for analysis. Little (<5%) or no rundown was observed within the experiments. The concentration–response relationships were produced by obtaining the peak current amplitude measured in the presence of drug (I_{DRUG}), normalized to the peak current amplitude measured in the same cell before drug application (I_{CTRL}), as a function of drug concentration [DRUG]. Each data point is the mean ± SEM from at least three cells. The relationships were fitted with a first-order binding function:

$$I_{DRUG}/I_{CTRL} = I_{max}/[1 + \exp\ ([DRUG]/IC_{50})^{nH}] \quad (1)$$

where IC_{50} is the half-maximum inhibitory concentration, and n_H is the slope factor. The variable I_{max} was introduced only for dose–response relationships of compounds effects on Y1593C channel mutant at 10 Hz, otherwise I_{max} was fixed to 1.

MUTANT CYCLE ANALYSIS

Mutant cycle analysis was performed to evaluate the cross influence between amino acid mutations in the hNav1.4 channel and hydroxyl substitutions in mexiletine, in order to characterize the molecular interactions. The coupling energy ΔG is thus calculated as $RT \cdot \ln\Omega$, where R is the perfect gas constant (8.314 J K mol^{-1}), T is the temperature expressed in Kelvin (295.15 K = 22°C), and Ω is the coupling constant (Hidalgo and MacKinnon, 1995).

DRUGS AND SOLUTIONS

Patch clamp pipette solution contained in mM: 120 CsF, 10 CsCl, 10 NaCl, 5 EGTA, and 5 HEPES, and the pH was set to 7.2 with CsOH. Bath solution for patch clamp recordings contained (in mM): 150 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, and 5 glucose. The pH was set to 7.4 with NaOH. Mexiletine hydrochloride [Mex, 1-(2,6-dimethylphenoxy)propan-2-amine] was purchased from Sigma (Milan, Italy). The hydroxylated analogs of mexiletine {metahydroxymexiletine, mHM, 3-(2-aminopropoxy)-2,4-dimethylphenol; parahydroxymexiletine, pHM, 4-(2-aminopropoxy)-3,5-dimethylphenol; hydroxymethylmexiletine, HMM, [2-(2-aminopropoxy)-3-methylphenyl]methanol; *bis*(hydroxymethyl)mexiletine, bHMM, [2-(2-aminopropoxy)-1,3-phenylene]dimethanol; parahydroxyhydroxymethyl mexiletine, pHHMM, 4-(2-aminopropoxy)-3-(hydroxymethyl)-5-methylphenol} were synthesized in our laboratories (Catalano et al., 2004, 2010; Cavalluzzi et al., 2007). All drugs were dissolved directly in external patch solution at the desired final concentration. The patched cell was continuously exposed to a stream of control or drug-supplemented bath solution flowing out from a plastic capillary.

EXPERIMENTAL DETERMINATION OF DRUG PHYSICOCHEMICAL PROPERTIES

The pK_a and Log P values of mexiletine and its analogs were determined with a potentiometric method using Sirius GlpK_a (Sirius Analytical Instrument Ltd., Forest Row, East Sussex, UK) as described previously (Catalano et al., 2010). Because the compounds showed a low water solubility, methanol was used as co-solvent (methanol–water 10–30% w/w) for the determination of pK_a.

The electrostatic potential of each analog was determined as the molecular charge distribution of the corresponding methyl ether moiety [2,6-dimethylphenyl methyl ether, for Mex; 3-methoxy-2,4-dimethylphenol, for mHM; 4-methoxy-3,5-dimethylphenol, for pHM; (2-methoxy-3-methylphenyl)methanol, for HMM; (2-methoxybenzene-1,3-diyl)dimethanol, for bHMM; 3-(hydroxy methyl)-4-methoxy-5-methylphenol, for pHHMM] was investigated. Molecular models were constructed by fragments offered by SPARTAN PRO (Wavefunction, Inc., 18401 Von Karman Avenue, Suite 370, Irvine, CA 92715, USA) inner fragment library and assuming the suggested default starting geometries. The generated geometries were optimized by the molecular mechanics MMFF routine proposed by the software and then submitted to a systematic conformer distribution analysis. Conformers were classified according to the *ab initio* gas phase energy content calculated at the HF/3-21G* level. All conformers falling within a window of 5 kcal/mol above global minimum were retained. After removal of conformers differing for dihedral values lower than 10°, the retained conformers were submitted to HF/3-21G* geometry optimization. The most negative electrostatic potential (i.e., the highest energy of interaction with a positive charge used as a probe) value for the most stable conformer of each analog was used for structure–activity relationship considerations.

RESULTS

EFFECTS OF MEXILETINE AND ANALOGS ON WILD-TYPE hNav1.4 CHANNELS

To compare the effects of mexiletine and its hydroxylated analogs, block of sodium channels was evaluated by measuring the drug-induced reduction of I_{Na} elicited at −30 mV for 25 ms every 10 (0.1 Hz) or 0.1 (10 Hz) s from the HP of −120 mV. Applying this protocol in the absence of drug, there was no significant change in current amplitude for hNav1.4 channels (not shown). Typical time course of peak I_{Na} amplitude during application of 100 μM mexiletine is illustrated in **Figure 1A**. At the stimulation of 0.1 Hz, application of mexiletine reduced gradually I_{Na} down to a plateau. This block will be called tonic block hereafter. It is due mainly to the block of closed channels with low affinity, but depends also in part on the block of closed-state inactivated channels with high affinity (Desaphy et al., 2001, 2004). Then increasing the stimulation to 10 Hz further induced the reduction of I_{Na} to a lower level, determining the so-called use-dependent block, which depends

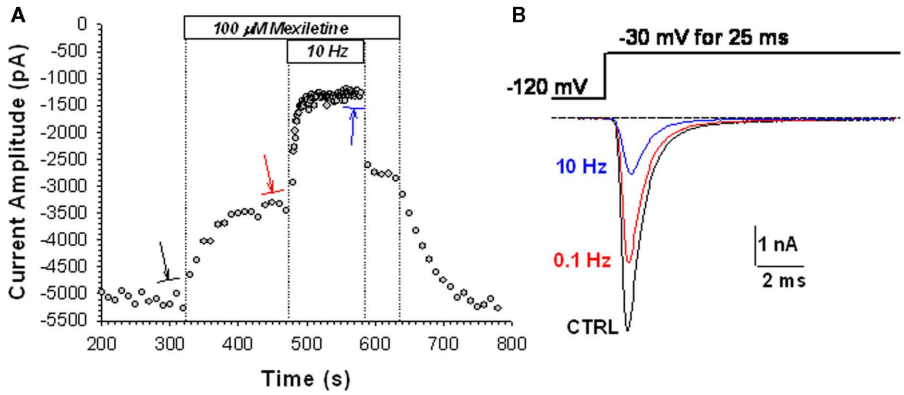


FIGURE 1 | Effects of 100 μM mexiletine on hNav1.4 currents. (A) Time course of sodium current amplitude measured in a representative HEK293 cell permanently transfected with hNav1.4 sodium channel subtype before, during, and after of application of 100 μM mexiletine. The sodium current was elicited by depolarizing the cell membrane to −30 mV for 25 ms every 10 (0.1 Hz) or 0.1 s (10 Hz), from a holding potential of −120 mV. **(B)** Typical sodium current traces are shown, which were obtained from the average of three records obtained at steady state in absence of drug using 0.1 Hz stimulation frequency (CTRL), and in presence of drug at 0.1 Hz (red) and 10 Hz (blue).

on the high-affinity binding to inactivated channels. These effects were fully reversible. Examples of current traces recorded at steady state before (control) and in the presence of 100 μM mexiletine at 0.1 Hz then 10 Hz are illustrated in **Figure 1B**. The reduction of I_{Na} induced by 100 μM mexiletine was 32 and 74% at 0.1 and 10 Hz, respectively.

The same protocol was used to test all the exploratory compounds at various concentrations. **Figure 2** shows representative current traces recorded at 0.1 and 10 Hz stimulation during application of 1 mM of each compound. Effects of mexiletine and mHM were quite similar, blocking I_{Na} by 77% at 0.1 Hz and 95% at 10 Hz. All the other compounds were less potent than mexiletine, following the rank order of potency: pHM > HMM > bHMM > pHHMM. The less potent pHHMM at 1 mM had no effect at 0.1 Hz and very little effect at 10 Hz. All the effects were fully reversible (not shown). The peak I_{Na} amplitude measured in presence of drug at 0.1 or 10 Hz was normalized with respect to control peak I_{Na} and reported as a function of drug concentration to draw concentration–response curves (**Figure 3**). All the curves were satisfactorily fitted with a first-order binding function, given the half-maximum inhibitory concentrations (IC_{50}) reported in **Table 2**. Addition of one hydroxyl group to mexiletine had different effects on efficacy depending on the position of substituent on the aryloxy moiety, ranging from merely no effect (mHM) to an eightfold increase of IC_{50} value (HMM) at 0.1 Hz. The addition of two hydroxyl groups induced a dramatic reduction of efficacy by more than 45 times for bHMM and 122 times for pHHMM. The effects of chemical maneuvers were more pronounced at 10 Hz stimulation, with an increase of IC_{50} values ranging from 1.7-fold for mHM to 567-fold for pHHMM, compared to mexiletine. Thus the 0.1- to 10-Hz IC_{50} ratio decreased from 9.8 for mexiletine to 6.3 for mHM and about 2.5 for the other compounds.

The most prominent effect of hydroxylation on the physicochemical properties of compounds is a reduction of the lipophilicity at the experimental pH, expressed as the Log *D* determined experimentally (**Table 1**). The relationships of IC_{50} values as a function of Log *P* or Log *D*, plotted on semilogarithmic axes, were linearly correlated: The higher the lipophilicity, the more efficient was the compound (**Figure 4**). Notwithstanding, if the lipophilicity was the unique factor influencing compound efficacy, mHM should have appeared less efficient than it was.

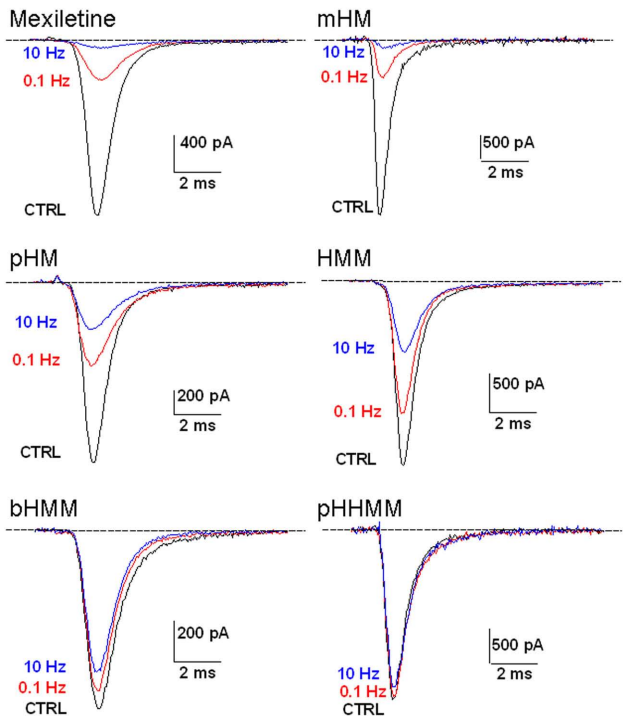


FIGURE 2 | Effects of 1 mM mexiletine or hydroxylated analogs on hNav1.4 currents. Sodium currents were recorded as described in **Figure 1**.

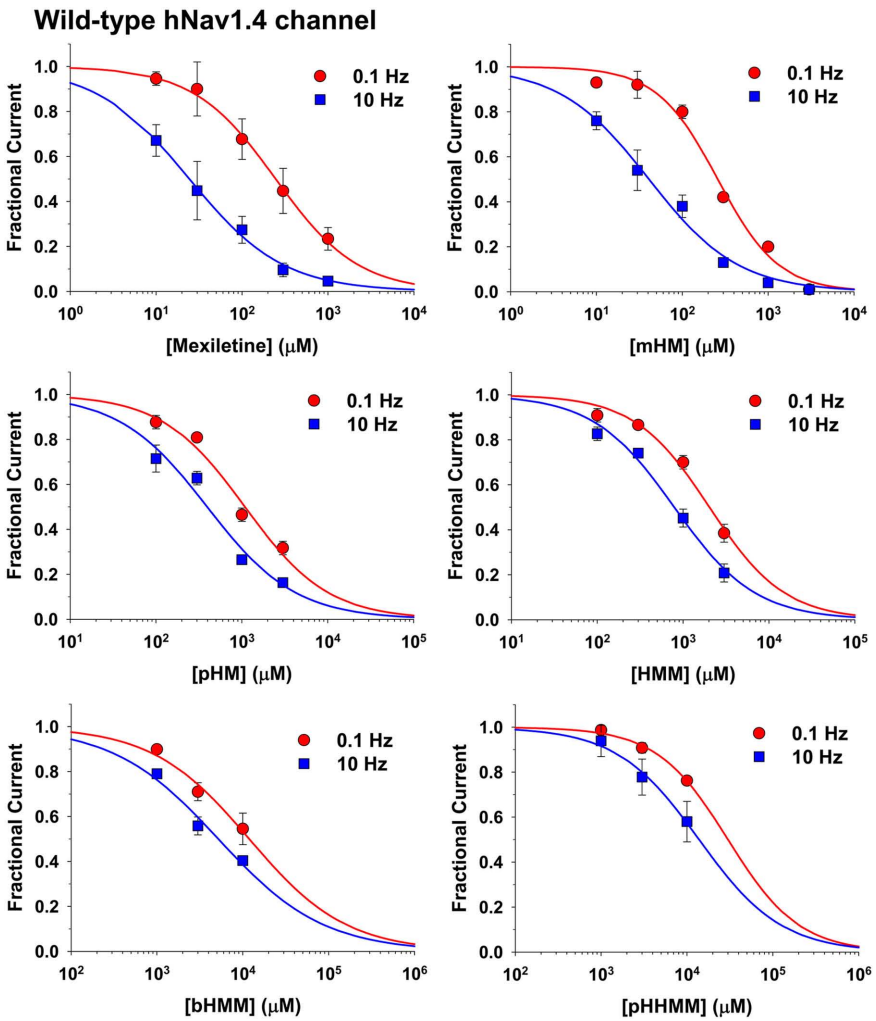


FIGURE 3 | Concentration–response relationships for mexiletine or hydroxylated analogs on hNav1.4 channels. The curves were constructed at 0.1 and 10 Hz frequency stimulation, and fitted with a first-order binding function (Eq. 1 in Materials and Methods). Each point is the mean ± SEM from at least three cells. The calculated IC_{50} values ± SE of the fit are reported in **Table 2**. The slope factors ranged between 0.71 and 1.22.

EFFECTS OF MEXILETINE AND DERIVATIVES ON F1586C hNav1.4 MUTANT

Mexiletine is thought to bind the putative high-affinity receptor for LAs within the pore of sodium channels. Indeed, the non-conservative mutation of phenylalanine to cysteine in position 1586 (hNav1.4) within the 6th segment of domain IV drastically reduced mexiletine block of inactivated channels (Desaphy et al., 2009). The hydroxylated compounds, except mHM, were tested on the same sodium channel mutant F1586C. Dose–response relationships of compound effects on F1586C I_{Na} are shown in **Figure 5**, and the IC_{50} values are reported in **Table 2**. On FC channels, the compound pHM appeared as much potent as mexiletine, whereas HMM was 2.2 times less efficient, bHMM 15–20 times less, and pHHMM 20–50 times less. Compared to WT channels, the mutation reduced sodium channel block for all the compounds, especially at 10 Hz stimulation. Indeed use-dependence was almost abolished for mexiletine, pHM, HMM, and bHMM, the 0.1- to 10-Hz IC_{50} ratio being reduced to 1.2–1.6. In contrast, use-dependent block of F1586C mutant by pHHMM was maintained.

EFFECTS OF MEXILETINE AND DERIVATIVES ON Y1593C hNav1.4 MUTANT

Another amino acid potentially involved in the binding of LA-like drugs is the tyrosine in position 1593 of hNav1.4 channel (Desaphy et al., 2010). This residue was proposed to interact with the hydrophobic moiety of sodium channel blockers through a π–π interaction (Ragsdale et al., 1994), although this hypothesis has been recently challenged (Ahern et al., 2008). We tested our compounds, except mHM, on the Y1593C mutant. Representative I_{Na} current traces recorded before and after application of 1 mM mexiletine are shown in **Figure 6**. In contrast to WT and F1586C channels, the Y1593C-mutant I_{Na} developed significant use-dependent reduction at 10 Hz in absence of drug, ranging from 15 to 30% of control current depending on the cell. The dose–response relationships are shown in **Figure 6**. To take into account

Table 2 | Half-maximum inhibitory concentration values (IC_{50}).

Drug	Stimulation frequency	WT hNav1.4	F1586C	Y1593C	
		IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	I_{max}
Mexiletine	0.1 Hz	246 ± 15	1340 ± 72	476 ± 36	1
	10 Hz	24.4 ± 1.8	1089 ± 181	169 ± 94	0.84 ± 0.17
	0.1-to-10 Hz ratio	10.1	1.2	2.8	
mHM	0.1 Hz	259 ± 28	n.d.	n.d.	n.d.
	10 Hz	40.9 ± 4.7	n.d.	n.d.	n.d.
	0.1-to-10 Hz ratio	6.3	n.d.	n.d.	
pHM	0.1 Hz	1086 ± 183	1132 ± 36	908 ± 30	1
	10 Hz	394 ± 82	847 ± 81	626 ± 45	0.74 ± 0.02
	0.1-to-10 Hz ratio	2.8	1.3	1.5	
HMM	0.1 Hz	2028 ± 255	2994 ± 74	1638 ± 14	1
	10 Hz	790 ± 91	2362 ± 187	1452 ± 111	0.76 ± 0.02
	0.1-to-10 Hz ratio	2.6	1.3	1.1	
bHMM	0.1 Hz	11883 ± 2992	28055 ± 10520	12740 ± 3141	1
	10 Hz	5190 ± 1021	17296 ± 2571	10353 ± 4102	0.78 ± 0.11
	0.1-to-10 Hz ratio	2.3	1.6	1.2	
pHHMM	0.1 Hz	30073 ± 7059	67730 ± 9125	24782 ± 2161	1
	10 Hz	13845 ± 2516	21056 ± 7650	13482 ± 1304	0.81 ± 0.03
	0.1-to-10 Hz ratio	2.2	3.2	1.8	

These values were determined at the holding potential of −120 mV from the fit of concentration–response curves with the first-order binding Eq. 1 described in Section "Materials and Methods." The IC_{50} values are given together with SE of the fit. Regarding Y1593C, the I_{max} value was fixed to 1 at 0.1 Hz, but was variable at 10 Hz. n.d., Not determined.

the use-dependent reduction of Y1593C-mutant I_{Na} in absence of drugs, an additional variable, namely I_{max}, was introduced in the fitting equation. The IC_{50} and I_{max} values are reported in **Table 2**. Regarding the IC_{50} values at 0.1 Hz, the Y1593C mutation was less disturbing than F1586C. Compared to WT, the IC_{50} of mexiletine on Y1593C I_{Na} at 0.1 Hz was twofold higher, whereas no significant change was observed for the other compounds. In contrast, the Y1593C mutation reduced dramatically use-dependent block at 10 Hz, increasing IC_{50} sevenfold for mexiletine, and 1.5- to 2-fold for the other compounds, except for pHHMM. Compared to WT, the 0.1- to 10-Hz IC_{50} ratio decreased to 2.8 for mexiletine and 1–1.5 for the other compounds. No significant effect of Y1593C mutation was observed for pHHMM.

MUTANT CYCLE ANALYSIS

Being modified solely on the aryloxy moiety, the derivatives of mexiletine can be considered as simple mexiletine mutants. Thus the combined evaluation of effects of mexiletine mutants on hNav1.4 channel mutants allows the calculation of mutant cycles (Hidalgo and MacKinnon, 1995; Lipkind and Fozzard, 2005). By this way, the cross influence of one mutation on the effect of the other can be quantified using a thermodynamic cycle in order to characterize the intimate molecular interactions. Such a mutant cycle analysis is illustrated for pHM in **Figure 7**, and coupling energies are reported in **Table 3**. Since the tonic block is determined mainly by interaction of drugs with the closed channel, mutant cycle calculation using the IC_{50} values for tonic block likely reveals information about interaction of drugs with the low-affinity binding site (**Figure 7A**). In these conditions, the coupling energy (ΔG) for interaction with F1586 is 1 kcal/mol, whereas ΔG is 0.5 kcal/mol for interaction with Y1593, thereby suggesting that mexiletine is closer to F1586 than to Y1593 in the closed channel. Since OH substitutions reduce ΔG for F1586 but leave unchanged ΔG for Y1593, we may hypothesize that the former amino acid may interact directly or indirectly with the aryloxy mexiletine moiety whereas the latter may not be involved in low-affinity binding. On the other hand, mutant cycle calculation using the IC_{50} values for use-dependent block (10 Hz stimulation) may allow to get information about the interaction of drugs with the high-affinity binding site (**Figure 7B**). In this case, the coupling energy between the drug and Y1593 is 0.9 kcal/mol, whereas ΔG is ∼1.8 kcal/mol for F1586, which suggests that mexiletine may interact with both amino acids, and that interaction with F1586 is more critical to high-affinity binding.

DISCUSSION

In this study, we used chemical maneuvers on mexiletine and site-directed mutagenesis on hNav1.4 voltage-gated sodium channel to get further insight in the intimate drug-channel interaction at the LA receptor. The binding affinity of LA drugs depends on the state of the channel, being higher for the open/inactivated with respect to the closed channel (Hille, 1977; Fozzard et al., 2011). Please note here that tonic block measured at the stimulation of 0.1 Hz results from the combination of low-affinity drug binding

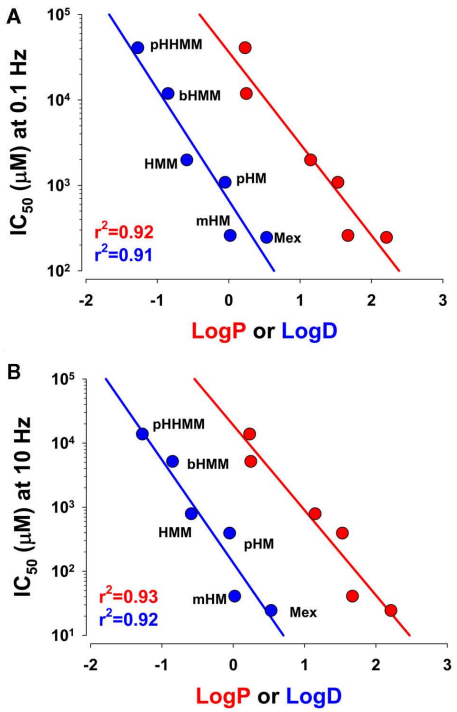


FIGURE 4 | Relationship between IC_{50} values for drug action on hNav1.4 channels and drug lipophilicity expressed either as Log *P* or Log *D*. The IC_{50} values were determined at the holding potential of −120 mV at 0.1 Hz **(A)** or 10 Hz **(B)** stimulation frequency, as described in **Figure 3**. The Log *P* and Log *D* values were measured as described in **Table 1**. The points were linearly correlated with $r^2 > 0.9$.

to closed channels and, to a lesser extent, high-affinity binding to closed-state inactivated channels, while use-dependent block measured at 10 Hz stimulation depends mainly on the high-affinity binding to inactivated channels (Desaphy et al., 2001, 2004).

LIPOPHILICITY OF DRUGS IS A KEY DETERMINANT OF SODIUM CHANNEL BLOCKADE

The results first confirm our former hypothesis (Desaphy et al., 2003), that the presence of two hydroxyl groups on the aryloxy moiety of LA-like drugs impedes high-affinity sodium channel blockade. We also show that the presence of a single hydroxyl group is less deleterious, depending however on the position of the OH group. The good correlation between the log *D* of compounds and the IC_{50} values, either at 0.1 or 10 Hz, suggests that compound lipophilicity is a major parameter. Because the putative LA receptor lies within the channel pore, lipophilicity might be determinant allowing the compound to reach/escape its receptor through the closed or inactivated channel. For instance, we previously showed that the drug pilsicainide, which has a negative log *D*, blocks channels inactivated from the closed state with very low efficiency compared to mexiletine, although affinities for inactivated channels of both drugs are comparable (Desaphy et al., 2010). Thus altering hydrophobicity of compounds can modify drug access to or escape from the binding site. A reduced hydrophobicity is expected to reduce drug access to closed channels, which will result in a reduced apparent affinity for the closed and closed-inactivated states. Such a mechanism likely contributes significantly to the effects observed in the present study. Nevertheless, such an explanation is not completely sufficient because inhibition of sodium current at 10 Hz stimulation is greatly reduced as well as use-dependence expressed as 0.1- to-10 Hz IC_{50} ratio, arguing against a trapping mechanism of the more hydrophilic compounds within the closed/inactivated channel (O'Leary et al., 2003). This result indicates that lipophilicity is also a critical determinant of the intimate interaction between the compound and its receptor. We thus tried to get more information by using contemporaneously mexiletine analogs and sodium channel mutants.

EFFECTS OF F1586C MUTATION ON SODIUM CHANNEL BLOCKADE BY MEXILETINE AND HYDROXYLATED DERIVATIVES

The phenylalanine in position 1586 is thought to be a key component of LA drug binding to the LA receptor (Ragsdale et al., 1994; Sheets et al., 2010). The aromaticity of this residue is required for use-dependent inhibition, while its hydrophobicity may be relevant for resting block (Li et al., 1999). By incorporating unnatural fluorinated phenylalanine analogs in place of the natural phenylalanine, it was recently demonstrated that disruption of the electrostatic potential at this position abolishes use-dependent inhibition without affecting resting block by lidocaine and mexiletine (Ahern et al., 2008; Pless et al., 2011). Since the fluorination did not affect blockade by the neutral benzocaine, the authors concluded that a π–cation interaction between phenylalanine side chain and the charged amine of drugs is a key player in high-affinity binding, at least for class Ib antiarrhythmics such as mexiletine (Pless et al., 2011). Accordingly, we observed that the mutation of F1586 by a non-aromatic cysteine nearly zeroed use-dependent inhibition by mexiletine and its derivatives, except pHHMM. In addition, we found that low-affinity block by mexiletine was also lightly affected by F1586C, because the IC_{50} value calculated at 0.1 Hz stimulation was 50% greater than mexiletine affinity for WT channels in the closed state (∼800 μM, Desaphy et al., 2001).

Interestingly, the inhibition of F1586C by pHM and mexiletine was comparable, indicating that the presence of one hydroxyl group in pHM mainly reduced high-affinity, use-dependent block of WT channels (as in mHM). Considering the π–cation interaction with F1586 as the main determinant of high-affinity binding, this suggests that alteration of the aryloxy moiety by introduction of the OH in mHM and pHM is able to weaken the critical interaction of the charged amine with F1586, through a mechanism resembling cooperativity between two pharmacophores. Our results suggest that this may be related to the reduction of drug lipophilicity (see Log *D* value in **Table 1**). Nevertheless, it has been recently shown that addition of a methyl group in para position of lidocaine is also able to weaken the π–cation interaction with F1586 (see supplemental data in Pless et al., 2011), suggesting that changes other than lipophilicity may be also involved. As hypothesized from previous experiments using aryloxy-substituted mexiletine analogs (Franchini et al., 2003), a possibility is that the presence of substituents on the aromatic ring may slightly modify the orientation of the molecule, thereby weakening the π–cation interaction. It is also interesting that while the overall hydrophobicity does not change between mHM and pHM, the IC_{50} for

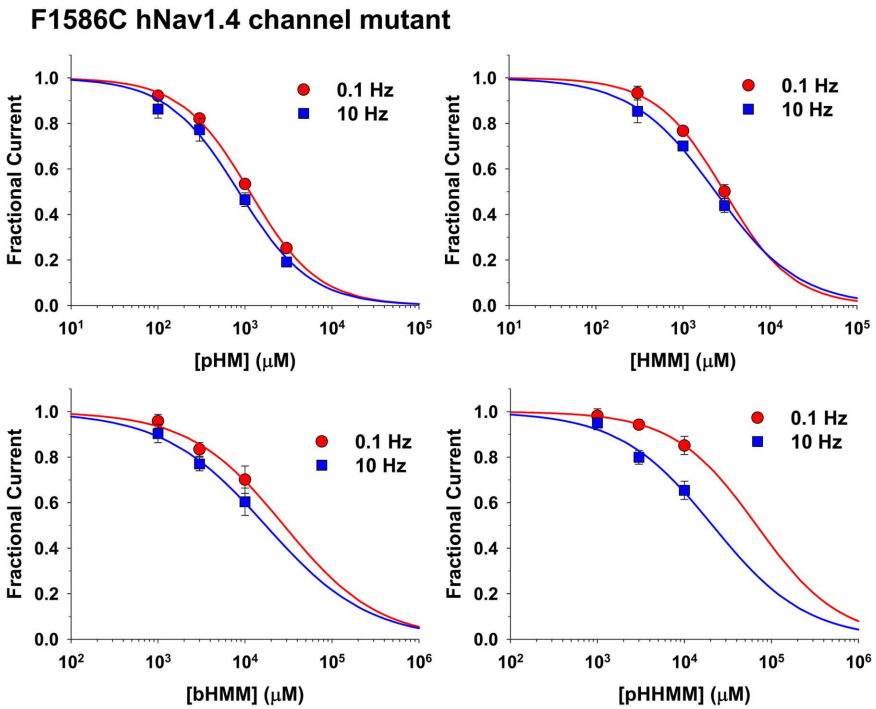


FIGURE 5 | Concentration–response relationships for hydroxylated mexiletine analogs on F1586C hNav1.4 channel mutants. The curves were constructed at 0.1 and 10 Hz frequency stimulation, and fitted with a first-order binding function (Eq. 1 in Materials and Methods). Each point is the mean ± SEM from at least three cells. The calculated IC_{50} values ± SE of the fit are reported in **Table 2**. The slope factors ranged between 0.74 and 1.11.

use-dependent block of WT channels decreases 10-fold with the OH in para position, thereby confirming that not only lipophilicity is involved. Thus, in function of its position, the substituent may modify more or less the orientation of the entire molecule within the binding site. It is possible to appreciate from **Figure 8** how OH addition in meta have limited effects on aryloxy hindrance and on electrostatic distribution within the aryloxy (compared to mexiletine), whereas the OH in para concentrates more electronegativity close to the molecule extremity and induces a major steric hindrance. We may speculate that, in case of π–π stacking between the aryloxy and Y1593 side chain (see below), the OH in para position as in pHM may interact very negatively with the OH in Y1593, whereas the OH in meta position may be less unfavorable. The former negative interaction may result from steric hindrance or local electrostatic repulsion between the two OH.

When the strong interaction between the charged amine group and F1586 is disrupted by cysteine substitution, pHM has nearly the same activity than mexiletine even if it displays a reduced Log *D*. This latter observation suggests that an additional OH, as in mHM and pHM, or substitution of F1586 have no additive effect on low-affinity binding. Such an observation would be in accord with the proposed horizontal orientation of LA drugs in the closed channel, where the aromatic moiety of drugs may be exposed toward F1586 (Bruhova et al., 2008; Hanck et al., 2009; Sheets et al., 2010). Thus the alteration of one or the other may determine similar effect on low-affinity binding.

Interestingly, we noted that mHM and pHM show an electrostatic potential similar to that of mexiletine (**Figure 8**). In addition we calculated the value of energy coupling for tonic block calculated from mexiletine/pHM and F1586C mutants cycle, which resulted close to 1 kcal/mol (**Table 3**), being in the range expected for a through-space electrostatic coupling (Hidalgo and MacKinnon, 1995). Altogether, these results suggest that the aromatic moiety of the drug may interact with F1586 in the closed channel through π–π stacking thereby accounting for low-affinity binding. It should be noted however that, in contrast with this hypothesis, the variation of F1586 electrostatic potential by fluorination was shown to have no effect on low-affinity binding of lidocaine (Ahern et al., 2008). Thus an alternative hypothesis to be considered may be an indirect effect of F1586C mutation, through an allosteric mechanism, on the electrostatic coupling between the aryloxy drug moiety and another aromatic residue in the closed pore.

Regarding HMM and bHMM, a similar impairment of positive cooperativity between the aromatic and protonable drug extremities may account for the reduction of high-affinity blockade of WT channels, and use-dependent block was also nearly zeroed by the F1586C mutation. Moreover, tonic block of WT channels by HMM and bHMM was greatly reduced compared to that exerted by mexiletine. Thus the IC_{50} values for tonic block by HMM and bHMM were more than 2- and 10-fold higher than the specific affinity of mexiletine for closed channels, respectively (∼800 μM, Desaphy et al., 2001), indicating that these compounds significantly affect the low-affinity binding. Interestingly, we observed that the electrostatic potentials of the aryloxy moiety of HMM and bHMM were 10-kcal/mol more negative than that of mexiletine, mHM, and pHM. We may thus hypothesized that alteration

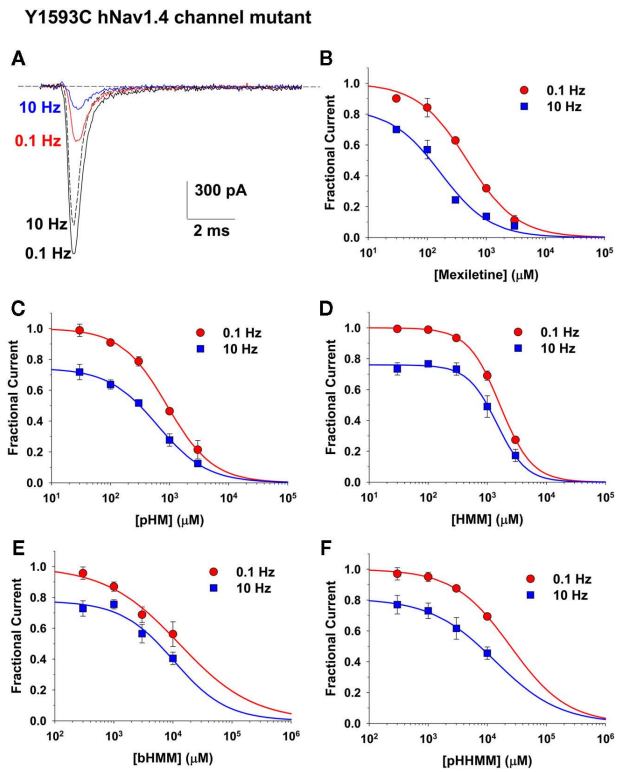


FIGURE 6 | Concentration–response relationships for mexiletine and hydroxylated analogs on Y1593C hNav1.4 channel mutants. (A) Representative sodium current traces recorded in a HEK293 cell permanently transfected with Y1593C hNav1.4 mutant, by depolarizing the cell to −30 mV from an holding potential of −120 mV at 0.1 and 10 Hz stimulation frequency, before and during application of 1 mM mexiletine. Note the use-dependent current reduction in absence of drug. **(B–F)** The concentration–response relationships were constructed at 0.1 and 10 Hz frequency stimulation, and fitted with a first-order binding function (Eq. 1 in Materials and Methods). Each point is the mean ± SEM from at least three cells. The calculated IC_{50} and I_{max} values ± SE of the fit are reported in **Table 2**. The slope factors ranged between 0.69 and 1.76.

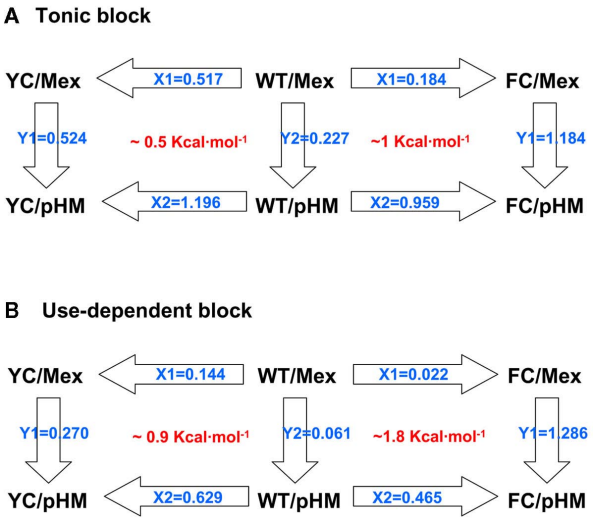


FIGURE 7 | Mutant cycle analysis of mexiletine and pHM binding to the wild-type hNav1.4 channel and its mutants Y1593C and F1586C. Mutant cycle analysis for tonic block considers IC_{50} values calculated at 0.1 Hz stimulation frequency **(A)**, while IC_{50} calculated at 10 Hz stimulation frequency allowed mutant cycle analysis for use-dependent block **(B)**. The *Xi* and *Yi* values represent the changes in affinity with each mutation, calculated as the ratio of IC_{50} values. The coupling constant Ω is calculated as the *X*1 to *X*2 ratio and the coupling energy ΔG is calculated as $RT \cdot \ln \Omega$, where *R* is the perfect gas constant (8.314 J K mol^{-1}) and *T* the temperature expressed in Kelvin (295.15 K = 22°C). The calculated ΔG shown in red was converted to kilocalorie per mole.

Table 3 | Coupling energy (ΔG, kcal/mol) calculated from mutant cycles, as shown in Figure 7.

Drug	Tonic block		Use-dependent block	
	F1586	Y1593	F1586	Y1593
pHM	0.97	0.49	1.79	0.87
HMM	0.75	0.50	1.60	0.78
bHMM	0.49	0.35	1.53	0.73
pHHMM	0.52	0.50	2.00	1.15

of electrostatic coupling may contribute to weaken the interaction of the aromatic tail of HMM/bHMM with the low-affinity receptor. Such a hypothesis is supported by the low energy coupling value (<1 kcal/mol) for tonic block by HMM and bHMM. Most probably, other parameters, including hydrophobicity, should be however involved to explain the large difference in tonic block exerted by HMM and bHMM. In addition, we observed that both HMM and bHMM exert a lower tonic block of F1586C channel with respect to mexiletine and pHM, indicating that substituents on HMM and bHMM have additional effects to those resulting from the suppression of aromatic side chain at position 1586.

The compound pHHMM shows an electrostatic potential similar to HMM/bHMM, and tonic block was similarly reduced twofold by F1586C. Quite surprisingly, the mutation had no significant effect on pHHMM use-dependence, indicating that the drug does not bind to F1586 at all. This latter observation suggests that F1586 is no more involved in the higher-affinity binding of pHHMM.

EFFECTS OF Y1593C MUTATION ON SODIUM CHANNEL BLOCKADE BY MEXILETINE AND HYDROXYLATED DERIVATIVES

The tyrosine in position 1593 was originally individuated as an important component of the intimate drug-receptor interaction, possibility through hydrophobic interaction with the aromatic moiety of LA drugs (Ragsdale et al., 1994). This hypothesis has been somewhat challenged because Y1593 mutations can modify sodium channel gating, which rends difficult the interpretation of pharmacological results. In addition to various effects on steady-state activation and inactivation, a slowing of recovery from inactivation has been reported (O'Reilly et al., 2000; Xiao et al., 2001), which may explain the use-dependent reduction of Y1593C currents we observed in the absence of drug. Recently, the contribution of Y1593 to π–cation or π–π interactions with lidocaine

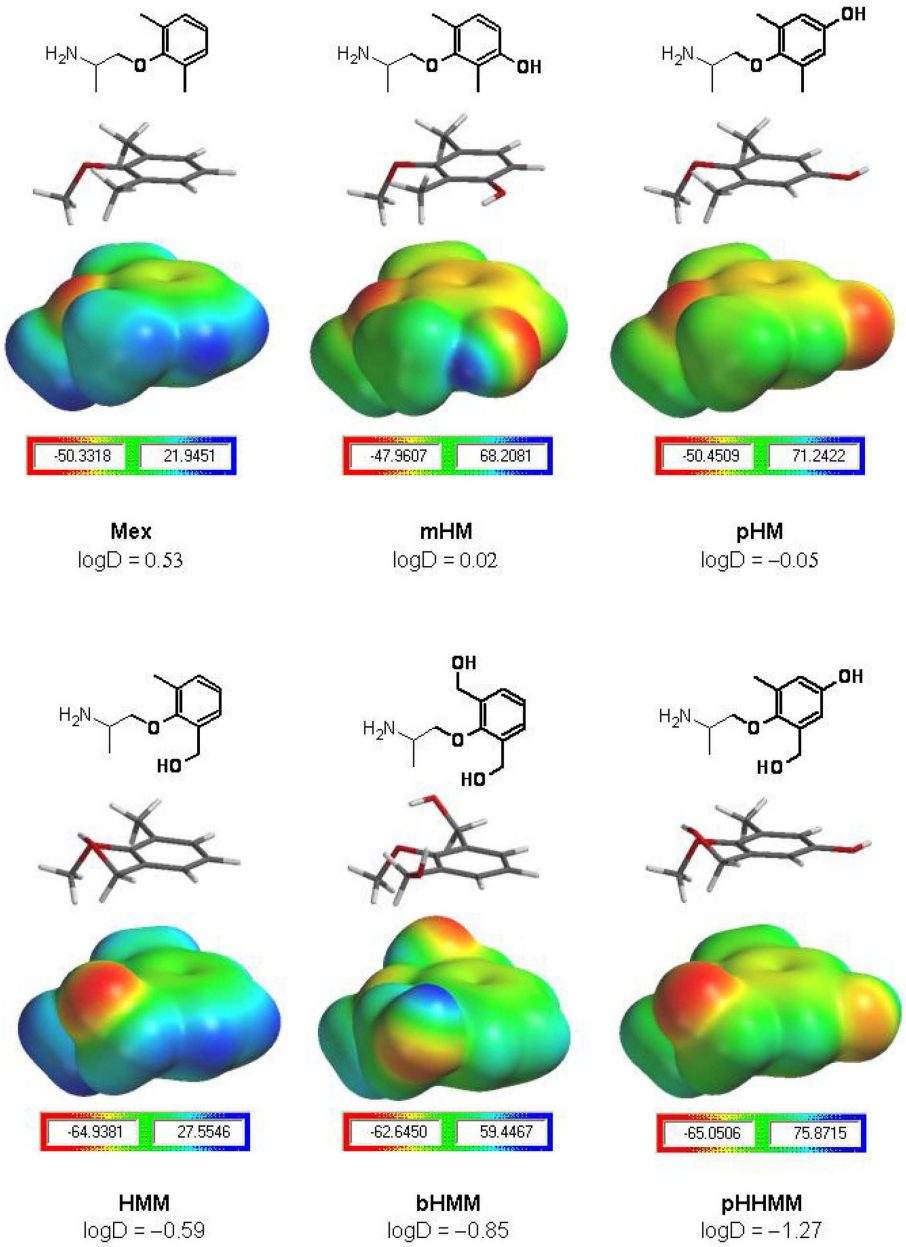


FIGURE 8 | Calculation of drug electrostatic potential. Mexiletine and its hydroxylated analogs are displayed as chemical structures presenting in bold the moiety studied by *ab initio* calculations (upper panels). For each compound, both the corresponding HF/3-21G∗ minimized three-dimensional structure and the electrostatic potential map is given in equatorial and lower panels, respectively. For each moiety, the scale bar indicates the electrostatic potential surface value range in kilocalorie per mole, the negative value being the one used in structure–activity evaluations. The HMM, bHMM, and pHHMM moieties (lower half of the figure) presented the most negative electrostatic potential values (around −65 kcal/mol), which most likely may allow less favorable interactions with electron-rich aromatic rings (e.g., F1586 or Y1593 rings), compared to the ones expected for Mex, mHM, and pHM moieties with a less negative electrostatic potential (around −50 kcal/mol). The Log *D* values experimentally measured at pH 7.4 are indicated.

has been ruled out by experiments using fluorinated amino acids (Ahern et al., 2008).

As for pilsicainide in a previous study (Desaphy et al., 2010), we observed that Y1593C reduced use-dependent sodium current blockade by mexiletine but to a lesser extent than F1586C.

The mutation may impede a direct interaction of Y1593 with the drug, or reduce the lipophilicity in proximity of the binding site. Thus considering Y1593 being involved either directly or indirectly on mexiletine binding, its mutation to C1593 may cooperatively reduce the strong interaction between the charged amine and F1586. The Y1593C mutation had a similar effect on mexiletine derivatives (except pHHMM), reducing use-dependence without altering tonic block. This observation indicates that Y1593 is not involved in the low-affinity binding, still in accord with

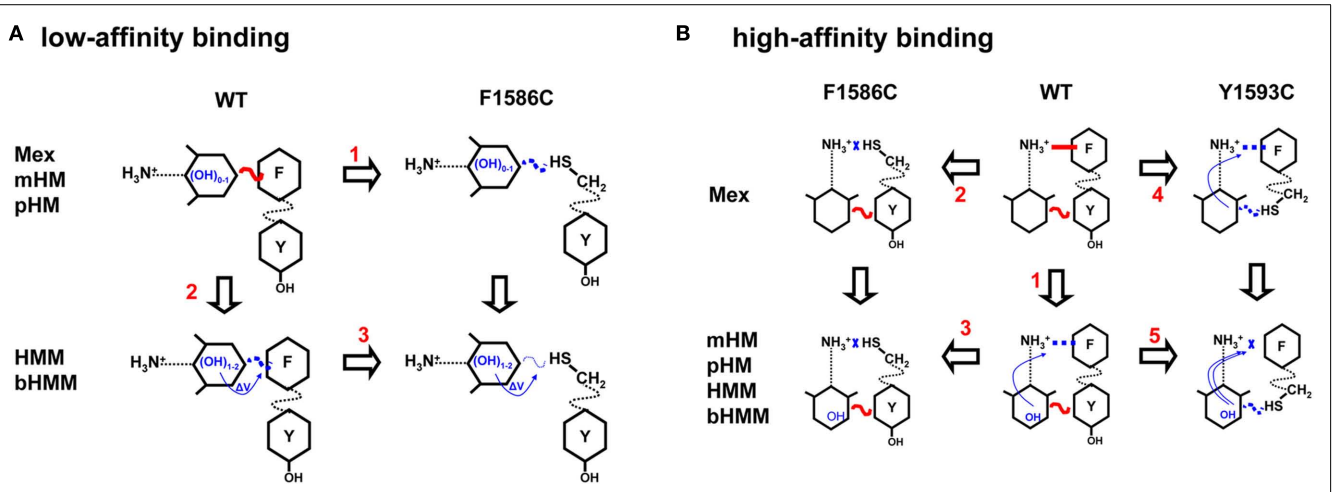


FIGURE 9 | Synopsis of possible interactions of mexiletine and hydroxylated analogs with the low (A) and high (B) binding site within the wild-type hNav1.4 channel and its mutants F1586C and Y1593C. (A) Regarding the low-affinity binding to closed channels, our results are in accord with an horizontal orientation of the drug, with the aryloxy moiety interacting either directly or indirectly with F1586 through electrostatic coupling. The addition of OH groups as in mHM and pHM does not modify aryloxy electrostatic potential thereby leaving this interaction unaltered. The F1586C mutation weakens interaction with Mex, mHM, and pHM to the same extent (arrow 1). Introduction of OH as in HMM and bHMM weakens the interaction due to modification of aryloxy electrostatic potential and possibly other parameters (arrow 2). The F1586C mutation produces additional weakening of the interaction with HMM and bHMM (arrow 3). Finally the Y1593C mutation has no effect on low-affinity binding (not illustrated). **(B)** Regarding the high-affinity binding to inactivated channels, addition of OH groups to the aryloxy moiety cooperatively reduced the interaction of charged amine group with F1586 (arrow 1). The amplitude of this effect depends on the OH position and lipophilicity. The F1586C mutation disrupts the π–cation interaction, so that OH substitutions have no more influence on binding (arrow 2 and 3). In contrast, the Y1593C mutation impairs only partially the π–cation interaction of mexiletine through cooperativity (arrow 4). The mutation also further impairs the binding of hydroxylated analogs (arrow 5).

the proposed position of LA drugs in the closed channel (Sheets et al., 2010). Tonic block and use-dependence of pHHMM was not altered by Y1593C, most probably because hydrophobicity is no more critical for a drug having a very negative Log *D*.

SYNOPSIS

We made an attempt to summarize the main findings in a graphical sketch (**Figure 9**). Regarding the low-affinity binding to closed channels (**Figure 9A**), our results suggest that the aryloxy moiety of drugs may interact either directly or indirectly with F1586 and suggest that electrostatic coupling is critical in drug binding. Regarding the high-affinity binding to inactivated channels (**Figure 9B**), addition of OH groups on the aryloxy moiety cooperatively reduced the interaction of drug charged amine group with F1586. The amplitude of this effect depends mainly on lipophilicity and also on the position of the OH substituent.

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Left-shifted Nav channels in injured bilayer: primary targets for neuroprotective Nav antagonists?

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Mechanical, ischemic, and inflammatory injuries to voltage-gated sodium channel (Nav)-rich membranes of axon initial segments and nodes of Ranvier render Nav channels dangerously leaky. By what means? The behavior of recombinant Nav1.6 (Wang et al., 2009) leads us to postulate that, in neuropathologic conditions, structural degradation of axolemmal bilayer fosters chronically left-shifted Nav channel operation, resulting in E_{Na} rundown. This "sick excitable cell Nav-leak" would encompass left-shifted fast- and slow-mode based persistent I_{Na} (i.e., I_{window} and slow-inactivating I_{Na}). Bilayer-damage-induced electrophysiological dysfunctions of native-Nav channels, and effects on inhibitors on those channels, should, we suggest, be studied in myelinated axons, exploiting $I_{Na}(V,t)$ hysteresis data from sawtooth ramp clamp. We hypothesize that (like dihydropyridines for Ca channels), protective lipophilic Nav antagonists would partition more avidly into disorderly bilayers than into the well-packed bilayers characteristic of undamaged, healthy plasma membrane. Whereas inhibitors using aqueous routes would access all Navs equally, differential partitioning into "sick bilayer" would co-localize lipophilic antagonists with "sick-Nav channels," allowing for more specific targeting of impaired cells. Molecular fine-tuning of Nav antagonists to favor more avid partitioning into damaged than into intact bilayers could reduce side effects. In potentially salvageable neurons of traumatic and/or ischemic penumbras, in inflammatory neuropathies, in muscular dystrophy, in myocytes of cardiac infarct borders, Nav-leak driven excitotoxicity overwhelms cellular repair mechanisms. Precision-tuning of a lipophilic Nav antagonist for greatest efficacy in mildly damaged membranes could render it suitable for the prolonged continuous administration needed to allow for the remodeling of the excitable membranes, and thus functional recovery.

Keywords: traumatic brain injury, spinal, riluzole, ranolazine, simulation, modeling

Nav CHANNEL GATING MODE TERMINOLOGY

As originally described by Hodgkin and Huxley (1952) voltage-gated sodium channels (Nav) have a predominant fast mode of gating. However, for any population of Nav channels, at any given time, a small fraction will be switching reversibly between fast-mode and slow mode (**Figure 1**). Terminology for Nav kinetics has evolved along with the structure/function studies propelling the voltage-gated channel field and it is sometimes unclear what is meant by "persistent sodium current." Thus, before embarking on a discussion of how membrane injury alters Nav channel behavior and what this might signify for drug therapies, we show (**Figure 1**) how we use kinetic terms. The simplified schematic is co-labeled with conventional (current-based) and newer (structure/function-based) terms: Nav channel conformations can be either Active mode (= fast) or Relaxed mode (= slow; Villalba-Galea et al., 2008). For transitions between modes, we prefer "mode-switch" to "mode-shift" (see Lin et al., 2007), since "switch" better evokes a protein conformation change than does "shift," which in any case is easily confounded with the graphical relocation of Boltzmann curves along a voltage axis, a routine occurrence for both Active mode and Relaxed mode channels (this will be seen, e.g., in **Figure 3C** and **Figures 4C,D**). The mode-switch conformation changes, though not well understood in Nav channels (Webb et al., 2009), are not dependent on voltage (Villalba-Galea et al., 2008).

THE PROBLEM: Nav CHANNELS GET LEAKY IN SICK EXCITABLE CELLS

As summarized in **Table 1**, Nav channels are dangerously leaky in a multitude of "sick-cell excitable cell" conditions. Mechanical trauma and/or ischemia and/or inflammation, and/or various genetic diseases result in damaged excitable cell membranes. More specifically, at Nav-rich membranes, bilayer structure degradation renders the Nav channels leaky. A CNS trauma example is depicted in **Figure 2A**: stretch trauma has caused axolemmal blebbing at the node of Ranvier. The fully blebbed bilayer, though detached from the specialized nodal spectrin skeleton (**Figure 2C**), would still be well-endowed with Nav1.6 channels. Even in such egregiously bleb-damaged plasma membrane, Nav channels are capable of voltage-dependent gating (Milton and Caldwell, 1990). In fact they gate "too well" (**Figure 2B**) in that they activate at inappropriately hyperpolarized (left-shifted) voltages (Shcherbatko et al., 1999; Tabarean et al., 1999; Wang et al., 2009; Beyder et al., 2010). A Nav channel whose operation becomes left-shifted in this manner is, in effect, leaky.

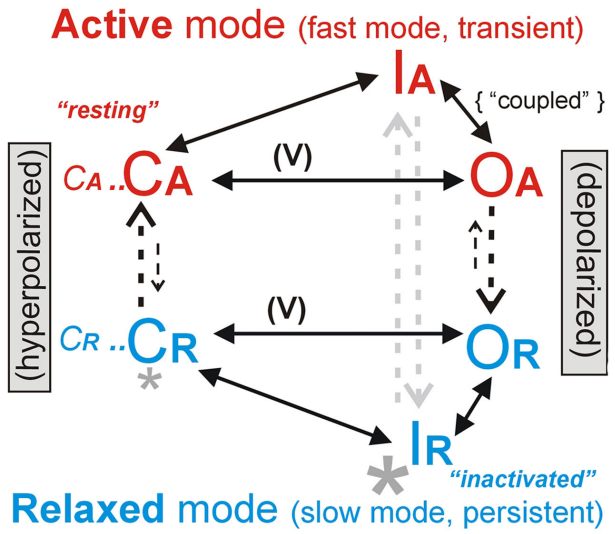

FIGURE 1 | Two major gating modes. A Nav channel has four voltage sensor domains and complex gating kinetics; this simplified scheme emphasizes voltage-dependent (*V*) activation [closed, open (C,O)], plus inactivation (I) to an open-like but occluded state in each of the two major modes (Active and Relaxed). The smaller letters (CA. . . and CR. . .) serve as a reminder that a full kinetic scheme (e.g., Taddese and Bean, 2002; Lenkey et al., 2011) would have many more states. Structural changes underlying mode switching, a feature of all voltage-gated proteins, are not yet understood (Villalba-Galea et al., 2008). During prolonged depolarizations and hyperpolarizations, inter-mode transitions (vertical arrows), which are voltage-independent (Villalba-Galea et al., 2008), would drive the Nav system toward I_R and C_A ("*inactivated*" and "*resting*" in the parlance of Lenkey et al., 2011), respectively. Macroscopic $I_{Na}(t)$ includes $I_{Na\text{-}fast}$ (fast, transient, or broadly speaking, Hodgkin–Huxley type) plus $I_{Na\text{-}slow}$ (also variously called "persistent," "slowly inactivating," "non-inactivating"), which flow through open Active and Relaxed mode Nav channels (O_A, O_R), respectively. Depolarization-induced repacking of voltage sensor domains (largely within the trans-bilayer electric field) yields net outward gating charge movement and facilitates C → O transitions. Activation curves, i.e., $g_{Na}(V)$ associated with C_A–O_A or with C_R–O_R, yield the "Boltzmann" sigmoid curves that summarize voltage-dependent fast or slow-gating energetics. Hyperpolarizing and depolarizing Boltzmann shifts are called "left shifts" or "right-shifts" respectively. The process by which the Nav "inactivation particle" binds ($O_A \rightarrow I_A$), though not inherently voltage dependent, is rate-limited by (hence *coupled* to) the voltage-dependent process of fast activation (Wang et al., 2009; Boucher et al., 2012). Thus, when activation [$g_{Na\text{-}fast}(V)$] left shifts due to bilayer damage, steady-state inactivation left shifts by the same number of millivolts, as depicted in **Figure 3**. This $g_{Na\text{-}fast}(V)$ phenomenon we term coupled-left shift (CLS). Importantly, the "subthreshold *persistent* current" of pacemaker neurons (Taddese and Bean, 2002) and of first-nodes (Kole, 2011) is a $g_{Na\text{-}fast}(V)$ based phenomenon, as is CLS. So-called "persistent current inhibitors" (e.g., riluzole, ranolazine) principally act by stabilizing non-conducting Relaxed mode states (large asterisk), but they also cause use-dependent ↓ $I_{Na\text{-}fast}$, perhaps through binding to Active mode channels (Lenkey et al., 2011; though inhibitor-bound C_R states (small asterisk) might contribute to ↓ [C_A] and thence to ↓ [O_A]).

By virtue of molecular coupling between the fast-mode activation and inactivation (availability) processes in a Nav channel (Banderali et al., 2010), these two processes left shift in synchrony: if the activation Boltzmann ["$m^3{}_\infty(V)$" in Hodgkin–Huxley parlance] left shifts by, say, 7 (or 11 or 20. . .) mV, then availability ["$h_\infty(V)$"] left shifts by 7 (or 11 or 20. . .) mV as well. This behavior of fast-mode Nav channels, shown for recombinant Nav1.6 channels in **Figures 3A,B** we term "coupled-left shift" (CLS). Crucially, as shown in **Figure 3C**, Nav-CLS yields a left-shifted steady-state window conductance ["$m^3h(V)$"]. Here, $m^3h(V)$ is shown before (black) and after a 20-mV CLS (gray). Note that the Nav-CLS version of $m^3h(V)$ now peaks at what would normally be a subthreshold voltage range (i.e., below the normal V_{rest}). Even if only a fraction (say 10%) of the nodal membrane suffered damage, that fraction, by virtue of its left-shifted $m^3h(V)$, should generate a "subthreshold persistent current" that would depolarize the adjacent (intact) Nav-rich membrane, causing the axon to fire ectopically. Because it facilitates rhythmic firing, a subthreshold persistent I_{Na} is used by pacemaker neurons (Taddese and Bean, 2002) and by the first node (beyond the axon initial segment) to elicit rhythmic firing (Kole, 2011), but injury-induced Nav-CLS based subthreshold persistent I_{Na} triggers pathological ectopic activity. We have modeled the consequences of Nav-CLS for axonal ion homeostasis and excitability (Boucher et al., 2012). In a nutshell, Nav-CLS causes [Na^+] gradients to run down, it overtaxes the Na/K-ATPase and, depending on CLS pervasiveness and severity, produces subthreshold voltage oscillations, bursting, and diverse other manifestations of hyper -and hypo-excitability.

After finding that reversible bilayer stretch elicits reversible Nav-CLS in recombinant Nav1.5 (Morris and Juranka, 2007), we turned to recombinant Nav1.6 (Wang et al., 2009). There, irreversible bilayer damage due to "membrane stretch" (cell-attached patch clamp, with stretch- and bleb-inducing pipette aspiration) yields irreversible CLS (**Figures 2B,C**) whether or not Nav1.6 channels are co-expressed with β-subunits. Once the irreversible damage has "saturated" (typically producing >20 mV of irreversible CLS), further stretch of the bilayer elicits reversible Nav-CLS because now, pipette aspiration is indeed causing reversible thinning/disordering (i.e., stretching) of the bilayer. For Nav1.6, as for Nav1.5 (Morris and Juranka, 2007; Banderali et al., 2010) for comfortably non-lytic stretch stimuli, reversible Nav-CLS (Figure 5 in Wang et al., 2009) is small (typically <5 mV). When studied in fast-mode, Nav1.4 and Nav1.2 channels (co-expressed with β-subunits) behave the same way as fast-mode Nav1.6 (Catherine E. Morris and P. F. Juranka, unpublished observations). It is now known, however, that expressed in HEK cells Nav1.5 channels exhibit large substantially irreversible Nav-CLS (Beyder et al., 2010). An interesting possible explanation for the isoform differences in oocytes is an idea that would be tested by immuno-biochemistry, namely that in oocytes, Nav1.5 traffics to bilayer subdomains with a high degree of disorderliness whereas Nav1.2, Nav1.4, and Nav1.6 traffic to highly ordered cholesterol-rich domains. It would, in fact, be worth exploiting this stark difference between Nav1.5 and the other Nav isoforms as a way to correlate channel-lipid interactions and kinetic behavior.

What appears to be irreversible Nav-CLS arising from metabolic (not mechanical) injury has been reported for the Nav channels of hippocampal neuronal somata subjected to prolonged epileptic discharge: the somatic Nav channels (presumably Nav1.2) show a measurably left-shifted I_{window} associated with left-shifted (fast-mode) activation and availability (Sun et al., 2006). Presumably these overworked neurons are ATP-depleted and their plasma

Table 1 | Sick excitable cells and their leaky, hyperactive, lethal Nav channels.

Excitable cell pathology	***A–E	Nav isoforms involved, notes on the bleb-like bilayer damage	• Additional notes. . . (I_{Na} changes, protective inhibitors, clinical issues, etc.)	Reference
Traumatic brain and spinal cord injury	A	Mechanically blebbed Nav1.6-rich nR and AIS Recombinant Nav1.6 in HEK cells and oocytes	• Ultrastructure of stretch-traumatized, rapidly fixed optic nerve shows blebs at nR, blebbed axolemma lacks dense-staining spectrin-cortex • TTX, mexiletine are protective in model systems. Without protection, positive feedback via Ca^{2+}-excitotoxic cascade elicits further Nav-leak	Maxwell (1996), Iwata et al. (2004), Ates et al. (2007), Underhill and Goldberg (2007), Wang et al. (2009), Wolf et al. (2001)
Stroke/cerebral ischemia	B,C,D	OGD-induced degradation is faster in AIS than nR AIS has Nav1.6 and Nav1.2 Dendrites bleb during OGD	• OGD causes classic excitotoxic cascades leading to spectrin breakdown; ATP depletion promotes bilayer destructuring. TTX-protects against OGD-induced disintegration of AIS. Low [dibucaine] selectively protects against OGD-induced spreading depression	Schafer et al. (2009), Douglas et al. (2011), Chen et al. (2011)
Muscular dystrophy	D,E	Chronic Nav1.4 leak; left-shifted Nav1.4 availability	• TTX fully protective against cell death in mdx mouse fibers. Sarcolemma lipidomics: abnormal lipid composition in mdx	Hirn et al. (2008), Benabdellah et al. (2009), Armstrong et al. (2001)
Multiple sclerosis	D	Disorganized Nav1.6-rich nR; focal axon swelling	• Nav antagonists promising, but severe side effects during clinical trials	Waxman (2008), Nikic et al. (2011)
Prolonged epileptic seizures	B,C,D	Metabolic overload induced changes CA1 hippocampal neurons	• Left-shifted Nav activation and availability and hence left-shifted, larger window current (↑ driving force) measured	Sun et al. (2006), Mantegazza et al. (2010)
Amyotrophic lateral sclerosis	B,C,D	ATP depletion characterizes ALS cortical motor neurons	• Riluzole effect taken as indicator that a persistent g_{Na} in familial ALS increases with extent of axonal degeneration	Vucic and Kiernan (2010), Browne et al. (2006), Pieri et al. (2009)
Dental pulp inflammation	D	Nav1.6 and Nav1.7 in disrupted nodal membrane	• Nav based painful signal discharges – locally acting Nav inhibitors are effective. Inflammation: associated with ROS-induced bilayer damage.	Henry et al. (2009)
Cardiac ischemia and reperfusion	B,C,D	Nav1.5, possibly Nav1.6 ventricular and atrial	• "Persistent I_{Na} inhibitors" riluzole, ranolazine, and F15845 counter reperfusion arrhythmias (assumption: an $I_{Na\text{-}slow}$ based Nav-leak)	Weiss et al. (2010), Antzelevitch et al. (2011), Létienne et al. (2009)
Critical illness myopathy	D,E	Nav1.4, HEK cells, DRG neuron sodium channels	• Endotoxin causes strong left shift of Nav availability, shuts down excitability in DRGs – muscle sepsis would engender bilayer damage	Novak et al. (2009), Haeseler et al. (2008)
Carpal tunnel	A,B,C,D	Presumably, Nav1.6 at nR	• "Persistent I_{Na}" becomes altered across the tunnel region; injury and inflammation. . . ectopic impulse generation. Mexiletine, etc., useful	Kuwabara et al. (2006)
Peripheral neuropathic pain	A,B,C,D	Ectopic excitation of axonal Nav channels	• In acutely damaged peripheral neurons, trauma may be exacerbated by OGD and inflammation, plus impaired membrane turnover	Zuliani et al. (2010), Boucher et al. (2012), Kuwabara and Misawa (2008)
Diabetic neuropathy	B,C,D	Nav1.6	• Neuropathic pain, periphery, an inflammatory neuropathy – indirect evidence of increased node of Ranvier "persistent I_{Na}"	Misawa et al. (2009)
Bladder, colon inflammation	D	Nav1.9	• Nav1.9 implicated in inflammation-related acute visceral hyperalgesia. Neuropathy presumably produces axolemma damage	Ritter et al. (2009), Martinez and Melgar (2008)

Abbreviations: AIS, axon initial segment; ALS, amyotrophic lateral sclerosis; Nav1.X refers to the implicated sodium channel isoform(s); nR, node of Ranvier; OGD, oxygen glucose deprivation; ROS, reactive oxygen species; TTX, tetrodotoxin.

****Known or likely cause(s) for degradation of healthy bilayer structure.*

A, direct mechanical blebbing (shear, stretch, or osmotic forces cause bilayer to detach from membrane skeleton; Milton and Caldwell, 1990; Maxwell, 1996; Sheetz et al., 2006).

B, degeneration secondary to ATP depletion (inactive Na/K and other ATPases yield Ca^{2+}-excitotoxicity, with hyperactivated. Ca^{2+}-proteases causing the spectrin–actin membrane skeleton to disintegrate; Armstrong et al., 2001; McGinn et al., 2009; Khanal et al., 2011).

C, bilayer leaflets become more symmetric secondary to ATP depletion (asymmetry requires P4-ATPase flippase activity; López-Marqués et al., 2011).

D, reactive oxygen species (ROS) damage lipases, bilayer lipids, and their arrangements (and possibly membrane skeleton; Nikic et al., 2011).

E, genetic or toxin-induced membrane skeleton impairments allow bilayer structure to relax toward a high entropy equilibrium. Structure similar to an abiotic bilayer. Duchenne's muscular dystrophy and the fungal toxin, cytochalasin, are examples (Allen and Whitehead, 2011).

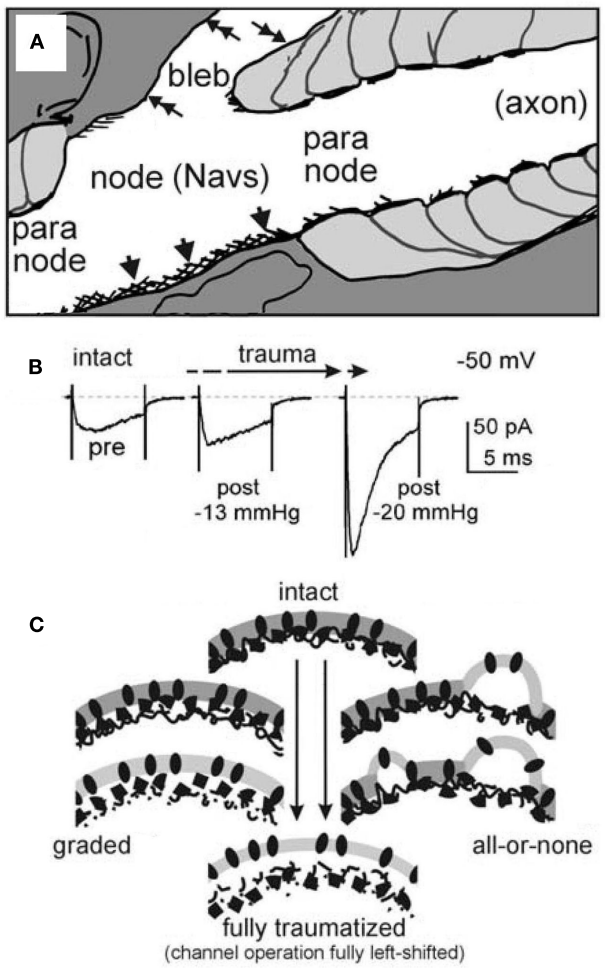

FIGURE 2 | (A) From a stretch-traumatized optic nerve, the nodal and paranodal regions of a node of Ranvier (cartooned by tracing an electron micrograph, Figure 2A of Maxwell, 1996) showing intact (arrowheads) and severely blebbed yet unruptured (double-headed arrows) nodal (hence Nav-rich) axolemma. **(B)** $I_{Na}(t)$ from recombinant αNav1.6/β1 in a cell-attached oocyte patch, before (intact) then again after bouts of pipette aspiration (experimental conditions as in Wang et al., 2009). The changing $I_{Na}(t)$ (at −50 mV) is consistent with increasingly strong coupled-left shift, the mechanism outlined in **Figure 3**. **(C)** In sick excitable cells, Nav channels that become "leaky," we assume, experience nano-structural environmental changes; here we depict a Nav-rich membrane going from intact (top) to blebbed (bottom). If I_{Na} changes such as seen in **B** are due to increasing bilayer entropy, then graded structural changes of each channel's nano-environments (left) would be expected. If, instead, as suggested by some (Shcherbatko et al., 1999; but see Morris et al., 2006), pipette aspiration induced-blebbing alters Nav kinetics by disrupting discrete channel/cytoskeleton interactions, then each Nav channel will see an all-or-none structural change, such as depicted at right.

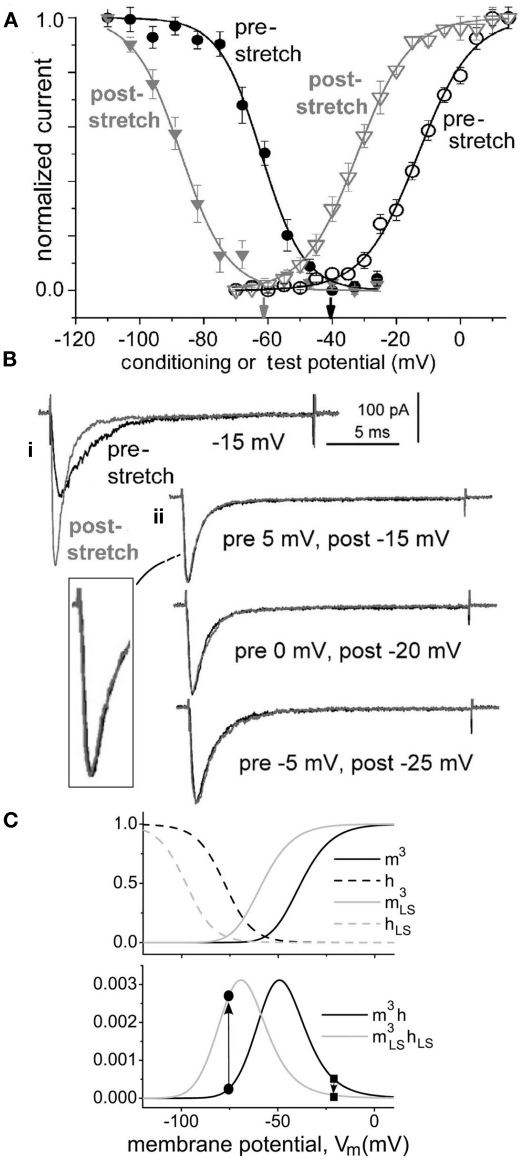

FIGURE 3 | Coupled-left shift (Nav-CLS). (A) Traumatic membrane stretch causes irreversible hyperpolarizing shifts of activation and steady-state fast inactivation (availability) for recombinant Nav1.6 (aspirated cell-attached oocyte patches; likewise in **B**). **(B)** CLS as seen from I_{Na} traces: **i,** $I_{Na}(t)$ during a step to −15 mV before and after stretch-induced membrane damage – the change is irreversible. **ii** Nav channels in this patch (same as **i**) underwent a membrane-injury-induced CLS found to be of magnitude 20 mV, as evidenced by the fact that amplitude-normalized pre/post $I_{Na}(t)$ traces measured at V_m steps 20 mV apart, as listed, have indistinguishable time courses (expanded section: pre 5 mV, post −15 mV traces). CLS like this is observed whether left shifts are small or large (see Figure 3 of Wang et al., 2009). This justifies co-left-shifting of HH Boltzmanns, $m^3_\infty(V)$ and $h_\infty(V)$ (= availability), along the voltage axis (**Figure 1** legend) to model axonal Nav channels in trauma-injured membrane (Boucher et al., 2012). **(C)** Activation $[m^3(V)]$ and inactivation $[h(V)]$ variables at $t = \infty$ for regular HH-Nav channels and after each function is left-shifted 20 mV (mimicking intact versus "strongly traumatized"). The product (lower panel) of overlapping activation and availability Boltzmann's, $m^3h(V)$ = steady-state open probability or non-dimensional g_{window}. Arrows: a 20-mV left-shift ($m^3_{LS}h_{LS}$) increases g_{window} near typical V_{rest} while decreasing it nearer 0 mV.

membranes are beset by the usual chemical insults of ischemia. Skeletal muscles subject to inflammatory conditions appear to have a comparable type of Nav-leak (Haeseler et al., 2008; Nikic et al., 2011). A recurring theme here is that it will be necessary to revisit native-Nav channels linked to disease states in **Table 1** to establish whether CLS correlates broadly with increasing Nav-leak.

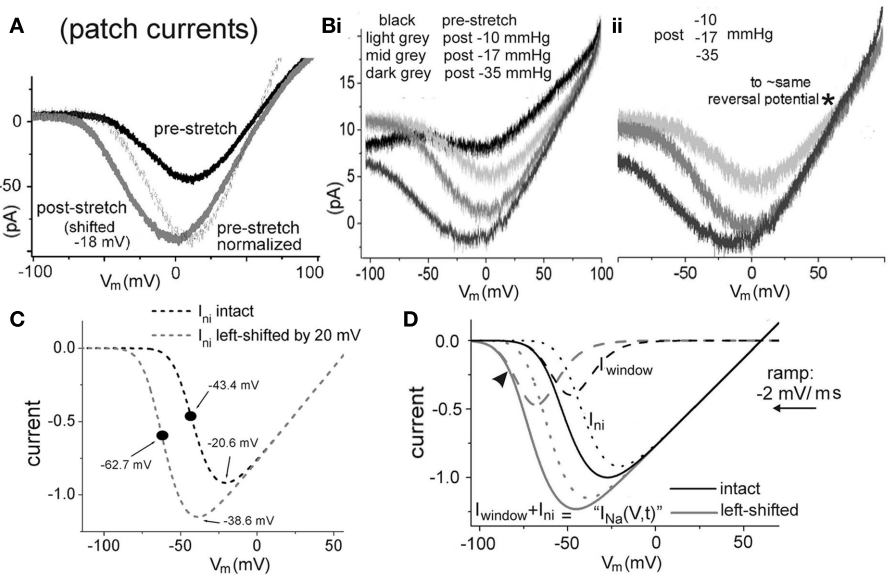

FIGURE 4 | Hyperpolarizing ramps. (A) Recombinant Nav1.6, oocyte patch, I_{Na} elicited by hyperpolarizing ramps from +100 to −100 mV, −2 mV/ms, before and after membrane damage. **(Bi)**. Similar data set before stretch then after increasingly strong ∼20 s bouts of pipette aspiration (conditions as in **(A)**; further explanation in text). **(Bii)** Damaged-membrane traces, *y*-axis adjusted, as described in text. **(C)** Simulated $I_{Na\text{-}ni}(V,t)$ for "intact" and 20 mV left-shifted non-inactivating HH-type Nav channels (ramps: −2 mV/ms). The I_{Na} midpoint shifts ∼−18 mV, as expected for the 20-mV shift imposed on $m^3(V)$ [equal-sized $I_{Na\text{-}ni}(V)$ and m^3 shifts would correspond to infinitely steep voltage-dependence in $m_{ni}(V)$; see Boucher, 2011]. **(D)** Simulated $I_{Na}(V,t) = (I_{Na\text{-}fast} + I_{Na\text{-}ni})$ for hyperpolarizing ramps from +150 to −150 mV at −2 mV/ms, $g_{ni}/g_{Na\text{-}fast} = 0.0075$; for control (black) and −20 mV shifted (gray). Total $I_{Na}(V,t)$ (solid lines) mimic experimental traces, given such a $g_{ni}/g_{Na\text{-}fast}$ mix; they would be comprised of the two components, as labeled.

BOTH FAST AND SLOW MODE Nav CURRENTS CONTRIBUTE TO Nav-LEAK

In injured membrane, as will be discussed further in **Figure 4** (see also Figure 2Ciii in Wang et al., 2009), Nav channels gating in slow mode exhibit left-shifted activation. However, whether slow inactivation is kinetically coupled to slow activation is unknown (in parallel to the coupling between fast activation and fast inactivation marked in between O_A and I_A in **Figure 1**). Typically, in a population of Nav channels, ∼1% would be in slow mode. Though slow mode current ($I_{Na\text{-}slow}$) is a kinetically distinct entity (see **Figure 1**) from any fast-mode based persistent currents (the coupled-"m^3h" or subthreshold g_{window} of Taddese and Bean, 2002), it too is called persistent current. In computational models, $I_{Na\text{-}slow}$ is typically simplified to a small stand-alone subpopulation of Nav channels with no inactivation process (e.g., Coggan et al., 2010). In reality, slow and fast modes interconnect (**Figure 1**) and the simplest assumption is that any injured membrane Nav channel (**Figure 3**) would show CLS when in fast mode and left-shifted activation when in slow mode. Thus, when we refer to "Nav-CLS"-injury, an implicit assumption is that the affected Nav channels "leak" in both fast and slow modes.

INCREASED ENTROPY AND Nav-CLS

Formally, plasma membrane blebs induced by pipette aspiration (Sheetz et al., 2006) or other shear forces in the mechanically traumatized CNS (Maxwell, 1996) are defined as regions of high fluidity bilayer dissociated from the cortical cytoskeleton. Clearly, the provenance of such injury-induced blebs differs radically from that of transient cell-mediated blebs used for ameboid-crawling (Charras et al., 2009); there, the blebbing-membrane loses its F-actin (due to precision cortical acto-myosin contractions) while retaining those elements of the spectrin skeleton needed for re-adhesion to the cortex. In these motility-blebs, the bilayer, we expect, would retain much of its healthy, far-from-equilibrium structure. By contrast, in excitable cell injury (Maxwell, 1996), as in pipette aspiration induced-blebbing (Morris et al., 2006; Sheetz et al., 2006), bleb formation, being an externally initiated unregulated process, is governed largely by raw physics. Bilayer structure in a blebbing-membrane region of a sick excitable cell will, thus, approach equilibrium, exhibiting increased disorder both vertically (between leaflets) and horizontally (within plane). By virtue of non-specific electrostatic binding of polar lipid headgroups to filamentous cortical proteins (Sheetz et al., 2006) plus the actions of lipid-ATPases (Shevchenko and Simons, 2010), certain phospholipids like phosphatidylserine are normally disproportionately high in the inner leaflet. As blebs develop, however, this vertical asymmetry dissipates: artificial bilayers and dead bilayers are symmetrical (Musters et al., 1993; Schlegel and Williamson, 2001; van Genderen et al., 2008). Simultaneously, lateral phase separation patterns found among the bilayer lipids will diminish in complexity (Maxwell, 1996; Kaiser et al., 2009; Lingwood and Simons, 2010; Shevchenko and Simons, 2010; Levental et al., 2011): within-plane organization will become more random, more disordered. In summary, in the plasma membrane of sick excitable cells, bilayer structure will slowly relax toward the minimum energy, maximum entropy situation of a self-organized bilayer. It is still a bilayer, but

one with a radically lower information content than the healthy far-from-equilibrium bilayer that various particular Nav channel isoforms have come (via evolution) to expect. Additionally, once it has areas denuded of cortical cytoskeleton, plasma membrane bilayer should be more prone to inappropriate insertion of intracellular membrane vesicles and to excessive uptake of free fatty acids (see Figure 1 of Milton and Caldwell, 1990; Morris and Homann, 2001). The expectation is that damaged Nav-rich bilayer will be disorderly and that its lipid-species composition too will render it abnormally fluidized.

Thus, while certain mutant variants of Nav channels are dangerously "leaky" due to structurally defective proteins (Catterall et al., 2008), Nav-CLS posits that genetically normal Nav channels in sick excitable cells become dangerously leaky when they find themselves embedded in structurally defective bilayers.

POSITIVE FEEDBACK: FROM SMALL Nav-RICH BLEB TO LARGE Nav-RICH BLEB

Consider, for a Nav-rich region of an excitable cell membrane (an axon initial segment, say, or the post-synaptic membrane of a skeletal muscle end-plate), an initially minor Nav-CLS leak due to a small bleb. Or, an entire Nav-rich membrane could be mildly damaged and have a similarly small Nav-CLS leak due, say, to inflammation-induced reactive oxygen species (ROS). A mild mechanically or chemically induced Nav-CLS leak should grow via a vicious cycle: the external event (trauma, infection, ischemia, etc.) degrades plasma membrane structural integrity. As detailed elsewhere (Morris, 2011a,b,c), abnormally fluidized, more easily thinned bilayers, like depolarization, lower the energy barrier between a voltage sensor's "down" and "up" conformations, and so will engender left-shifted Nav gating, i.e., Nav-CLS. The resulting Na^+-leak puts excess demands on Na^+/Ca^{2+} exchangers and on a cell's various ATP-driven pumps. When the demands can no longer be met an excitotoxic cascade occurs (Wolf et al., 2001; Yuen et al., 2009). Elevated $[Ca^{2+}]_{intracellular}$ hyperactivates Ca^{2+}-protease-mediated cleavage of cortical cytoskeleton elements, enlarging the extent of bleb-like membrane. Via Na-CLS, the initially minor mechanically induced bleb has subverted cellular processes so that those processes proceed to damage the Nav-rich membrane's bilayer structure. The process is irreversible once Ca^{2+}-protease dependent spectrin fragments become detectable (McGinn et al., 2009). In cerebral ischemia (Schafer et al., 2009) and in cardiomyocytes (Weiss et al., 2010) during post-ischemia/reperfusion, the initial insults that bring on Nav-leak are chemical. Dystrophic muscle cells are genetically bleb-prone and have left-shifted Nav availability linked to cell-lethal Nav-leaks (Hirn et al., 2008; Allen and Whitehead, 2011).

In the hours and days following traumatic or ischemic injury, mildly damaged excitable cells in "penumbra" zones abutting the primary insult zones succumb to deepening, self-induced (and, evidently, tetrodotoxin-sensitive) secondary injury. Thus, for various model systems there is a tetrodotoxin-sensitive axon loss in white matter, cell death in gray matter, loss of dystrophic skeletal muscle fibers, and, at cardiac infarct border-zones, loss of cardiomyocytes. **Table 1** signifies, we think, that sick excitable cells that suffer an initially minor Nav-CLS can expect excitotoxic demise if Nav-CLS based Na^+-leak is not stopped either by membrane remodeling that rids the cells of the damaged Nav-rich bilayer, or by therapeutic drugs that staunch the leak.

PROTEIN PARTNERS

For Nav1.6 channels expressed in oocytes, injury-induced CLS phenomena described here occur with or without co-expressed β subunits (Wang et al., 2009). For that reason, and since virtually nothing is known about *in situ* modulatory interactions of β subunits (or ankyrin-G, or other of Nav channels' many putative protein partners, Dib-Hajj and Waxman, 2010), we do not explicitly deal with them here. On the other hand, our insistence on how crucial it is to study sick-cell Nav-leak and its attendant Nav-pharmacology in native and not just recombinant systems, acknowledges that native lipid structures in conjunction with diverse protein partners in the immediate vicinity of native-Nav channels, are likely to determine the specifics of sick-cell Nav-leak in different types of excitable cells.

Nav INHIBITORS

Tetrodotoxin, being a pore blocker, inhibits both fast (Active) and slow (Relaxed) mode Nav channels. Its exclusive selectivity for Nav channels has made it a powerful tool in cell/tissue models of disease, as just described. Like many Nav inhibitors, tetrodotoxin is powerfully protective in cellular models of injury to Nav-rich excitable membranes (**Table 1**) but it is a universal Nav-pore blocker and as such, lethal upon systemic administration. Nav inhibitors with more appropriate clinical traits include heterocyclic molecules like ranolazine and riluzole (Antzelevitch et al., 2011; Cadotte and Fehlings, 2011). These lipophilic compounds preferentially bind and stabilize Nav channels in non-conducting slow-gating states (Song et al., 1997; Antzelevitch et al., 2011). Often called "persistent current" blockers, these drug molecules are especially effective at stabilizing slow mode Nav in non-conducting states and at higher concentrations they inhibit fast-mode channels (Jo and Bean, 2011; Lenkey et al., 2011). Because of severe side effects (Waxman, 2008), however, none of the available Nav antagonists is routinely used to counter the devastating, slow-developing consequences of traumatic brain injury described at the neurological level as diffuse axonal injury (Wolf et al., 2001; Iwata et al., 2004) though for spinal injury, riluzole trial are underway (Cadotte and Fehlings, 2011).

LIPOPHILICITY AND Nav INHIBITOR EFFICACY

Although it is recognized that clinically effective Nav inhibitors are lipophiles (or strongly lipophilic amphiphiles), what explains the importance of lipophilicity is unclear (Jo and Bean, 2011; Lenkey et al., 2011; Nesterenko et al., 2011). We formulate, below, a two-part hypothesis in which, for sick excitable cells, the known requirement for lipophilicity in effective Nav antagonists (Lenkey et al., 2011) correlates with the elevated bilayer-fluidity origin of Nav-CLS. Before doing so, we direct the reader to **Box 1** which itemizes some physiological, pharmacological, physico-chemical, and computational findings that bear on the idea.

To probe riluzole inhibition, Narahashi and colleagues clamped I_{Na} in DRG neurons (Song et al., 1997). Noting the drug's enhanced effectiveness in damaged neurons, they invoked high-affinity binding to the slow-inactivated Nav channels of

Box 1 | A brief history: bilayer partitioning and intra-bilayer orientation of lipophilic/amphiphilic molecules that bind voltage-gated channels and other membrane proteins•.**

**Herbette et al. (1989) partitioning of dihydropyridines (DHPs) into lipid bilayer could precede binding to voltage-gated Ca^{2+} channels. Sarcolemma/buffer partition coefficients: 5,000–150,000 range.
**Mason et al. (1992) voltage-gated Ca^{2+} channel antagonists and cholesterol. X-ray diffraction and equilibrium binding techniques: ↑membrane cholesterol →marked decrease in DHP partition coefficients (likewise verapamil, diltiazem).
**Mason (1993) Ca^{2+} channel DHP type antagonists – interactions with bilayers. Lipid composition (e.g., cholesterol content, acyl chain saturation) effects on membrane partitioning of antagonists should affect bioavailability under normal versus pathological conditions with altered membrane lipids. Specifics of bilayer composition may help concentrate and orient drug molecules relative to a hydrophobic binding site at channel/bilayer interface. For desirable pharmacokinetics, ↑'d efficacy, ↓'d side effects, drug design should anticipate contributions from membrane lipid compartment.
**Lee and MacKinnon (2004) amphiphilic voltage sensor toxins of arachnid venoms reach their target by partitioning into the lipid bilayer. Accumulation of toxin where voltage sensors reside and exploiting the free energy of partitioning of appropriately oriented amphiphilic toxins → high-affinity inhibition.
•Zhang et al. (2007) tetracaine/vesicle interactions: partitioning into solid-gel membrane depends mostly on steric accommodation between lipids, whereas in liquid-crystalline membrane (larger inter-lipid distances, lower steric hindrance), hydrophobic and ionic interactions between tetracaine and lipid molecules predominate. Bilayer partition coefficients ↓'d by cholesterol.
•Baenziger et al. (2008) bilayer lipid composition alters tetracaine action at nicotinic AChRs.
•Eckford and Sharom (2008) cholesterol-modulation of P-glycoprotein-mediated drug transport appears to operate via effects on drug partitioning into the bilayer and by changes in the protein's local lipid environment.
•Chisari et al. (2009) GABA-R both specific (e.g., enantiomer-dependent) and non-specific (e.g., bilayer partitioning) properties contribute to potency and longevity of steroid action.
•Lombardi et al. (2009) β2 agonist, indacaterol fluidizes membranes less than salmeterol and yields faster-onset, longer-duration therapeutic effects, perhaps because of synergy between indacaterol's better partitioning into raft micro domains and its faster membrane permeation.
**Schmidt and MacKinnon (2008) the mechanical state of bilayer lipids in the plasma membrane is crucial to the efficacy of amphiphilic peptide toxins that have evolved to bind to and right-shift voltage sensors.
**Milescu et al. (2009) conversion of sphingomyelin to ceramide shows that binding efficacy of an amphiphilic voltage sensor toxin to the membrane-embedded voltage sensors of Kv channels depends on the bilayer's lipid composition.
•Ali et al. (2010) loading of drugs (diazepam, ibuprofen, midazolam, propofol) into liposome bilayers is sensitive to cholesterol content: ↑'d cholesterol = ↓'d drug incorporation.
•Tejwani et al. (2011) all-atoms molecular dynamics simulations: a bilayer's hydrocarbon chain interior is the region most selective to the chemical structures of drug-type solutes. Multiple discrete polar groups (same solute) contribute additively to bilayer partitioning. Significant orientational preferences for the drugs are evident in specific bilayer regions.
•Jastrzebska et al. (2011) bilayer composition/organization regulates classic G protein-coupled receptor, rhodopsin.
**Lenkey et al. (2011) lipophilicity is a key factor in Nav antagonist potency, allowing inhibitor molecules to bind at two distinct sites, one most accessible at hyperpolarized potentials ("resting site"), the other at depolarized potentials ("inactivated site"). In addition, steric/orientation features involving aromatic rings, hydrogen acceptors, and sheer molecular bulk are important determinants of potency at the inactivated site.
Relevant to this Box, Muddana et al. (2012) recently showed differential partitioning of non-lipid amphiphiles (vitamin-E, benzyl alcohol, Triton-X 100) into bilayers depending on mechanical properties of different lipid subdomains.

damage-depolarized cells. In such situations, the Nav channels of interest are not only in damaged cells, we suggest, but damaged bilayer. This should affect the working concentration of lipophilic Nav antagonists (like riluzole) if they partition differently into damaged versus intact membrane. Further, riluzole efficacy might be higher in damaged than intact bilayer if its ability to orient appropriately with respect to intra-bilayer binding sites on the DRG Nav channels (see Lenkey et al., 2011) is better in less structured bilayers.

What little we could find in the literature relative to these issues of the partitioning and orientation of lipophilic drugs in different classes of bilayer structure is summarized in **Box 1**. Of particular note, highly ordered, cholesterol-rich membrane subdomains accumulate lipophilic drugs more poorly than cholesterol-poor fluidized membranes. Strikingly, the drugs in question are clinically important voltage-gated calcium channel antagonists, the dihydropyridines. The collection of findings sketched in **Box 1** suggest that at ones peril would one assume bilayer order/disorder to be irrelevant for the interactions of lipophilic drugs with dynamic membrane proteins (see also Benabdellah et al., 2009; Rajendran et al., 2010; Morris, 2011b). To our knowledge, however, these issues have not been investigated experimentally for Nav inhibitors.

Lenkey et al. (2011) showed that simple lipophilicity (measured from oil/water partition coefficients) correlates strongly with Nav inhibitor efficacy, and, beyond that, that molecular orientation and steric constraints are particularly important for inhibition of $I_{Na\text{-}slow}$ (Lenkey et al., 2011). The possibility that, for sick excitable cells, clinically useful Nav inhibitors could selectively target the malfunctioning Nav channels by (1) accumulating preferentially in disordered bilayers, and (2) exhibiting orientation/steric properties appropriate for within-bilayer binding to their targets (see Schmidt and MacKinnon, 2008; Morris, 2011a,b) directly echoes Mason's (1993) ideas regarding the dihydropyridines (see **Box 1**). It contrasts strikingly, however, with a recent model for the differential atrium/ventricle efficacy of ranolazine for (healthy) cardiac myocyte Nav channels. There, it is explicitly assumed that drug access to binding sites (Active mode and Relaxed mode conformations) is via hydrophilic pathways (Nesterenko et al., 2011).

HYPOTHESIS: IN SICK EXCITABLE CELLS, BILAYER ENTROPY ENGENDERS Nav-CLS BUT SIMULTANEOUSLY ENHANCES THE EFFICACY OF LIPOPHILIC Nav ANTAGONISTS

Against the above background material, we now restate some of the ideas formally in a two-part hypothesis. The hypothesis is grounded in our work on recombinant axon-isoform Nav channels in traumatized membranes (Wang et al., 2009), on Schmidt and MacKinnon's (2008) observations on Kv channel activation and drug interactions in intact versus mechanically disturbed plasma membranes, and on the Krepkiy et al. (2009) finding that isolated voltage sensors in "S4-up-states" locally thin/disorder the bilayer (explained in Morris, 2011a,b,c). In conjunction with the meta-analysis of Lenkey et al. (2011) for Nav-antagonist physical chemistry and efficacy, which postulates bilayer-embedded binding sites for fast and slow mode Nav channel conformations, these findings lead us to hypothesize:

H1: Mechanical and/or chemical insult causes the far-from-equilibrium order of a healthy bilayer to decay toward equilibrium, i.e., toward the more disordered dynamically self-organized arrangement of abiotic bilayers. In injured Nav-rich excitable cells including neurons, the Nav channels that leak are irreversibly "left-shifted" due to irreversibly increased bilayer disorder, with the shift proportionate to the extent of disorder in the Nav-embedding bilayer;

H2: Because complex lipophilic Nav inhibitors in aqueous solution partition into and may also orient more appropriately in damaged (disordered) bilayers than in well-packed bilayers of healthy Nav-rich membranes, lipophilic Nav inhibitors achieve their best global efficacy precisely at the loci of the leaky Nav channels.

Our title combines items H1, H2 as a question–hypothesis, and the question is not rhetorical. The hypothesis moreover, needs to be tested on native-Nav channels in their native-bilayer settings. The kinetics of native-Nav channels expressed and maintained at high-density (Kole et al., 2008) remain poorly understood (Fu et al., 2011); add trauma (or other sick excitable cell conditions) and this is even more true. We re-iterate: there is an unmet need to study the electrophysiological and pharmacological behavior of native-Nav/bilayer/membrane-skeleton assemblages before, during, and after imposing assorted types of membrane damage (see "A–E" column, **Table 1** and **Box 1**).

Nav-RICH NATIVE MEMBRANES NEED TO BE INVESTIGATED

Myelinated axon I_{Na} is carried by Nav1.6 channels (see Chatelier et al., 2010). Nodal I_{Na} has fast and slow components (Stys et al., 1993), but the discrete responses of these components to trauma or ischemia or other bilayer-damaging pathological states have not, to our knowledge, been studied electrophysiologically. Assuming fast and slow axonal I_{Na} arises from one Nav population (**Figure 1**), damaged axolemma should have co-existing left-shifted $I_{Na\text{-}fast}$ (hence left-shifted I_{window}) and left-shifted $I_{Na\text{-}slow}$. Carefully devised (though currently little-used) techniques exist, fortunately, to study drug actions on I_{Na} at voltage clamped nodes of Ranvier. An understanding of the pharmacological idiosyncrasies of nodal Nav1.6 channels in both modes is needed. Voltage clamp ramps (see **Box 2**) would be the most feasible way to simultaneously monitor fast and slow I_{Na} in intact versus damaged nodal membrane. Based on recombinant Nav1.6 ramp data and on simulations, the next three sections suggest what to watch for from nodal Nav1.6 current.

RAMP CLAMP, Nav1.6 CURRENT, AND MEMBRANE DAMAGE TO DATE

In our recordings of recombinant Nav1.6 current in oocyte patches, $I_{Na\text{-}slow}$ was hard to study since usually it was unmeasurable against background noise (see also Chatelier et al., 2010). Nevertheless, **Figures 4A,B** shows ramp-elicited I_{Na} traces from cell-attached oocyte patches. To minimize $I_{Na\text{-}fast}$ in favor of slow-gating current, hyperpolarizing ramps were used. The Nav-bearing membrane patches were injured via pipette aspiration induced-blebbing. Figure 3A is from Wang et al. (2009) while the **Figure 4B** data are from the same group of experiments, included here to help illustrate Nav1.6 ramp $I_{Na}(V,t)$ during progressively deepening membrane damage. The ramp-elicited $I_{Na}(V,t)$ progressively left shifts with stronger damage, its maximal amplitude increases and, in the g_{max} region, its slope conductance steepens. Offsets

Box 2 | Vaseline-gap voltage clamp of a node of Ranvier.

This technique (Conti et al., 1976; Benoit and Escande, 1991; Schwarz et al., 1995, 2006), though challenging, was the major electrophysiological approach used for Nav channel pharmacology several decades ago, before $I_{Na\text{-}slow}$ was recognized as a pharmacological target. It would be worth resurrecting the method to study native-Nav1.6 channels in damaged nodes of Ranvier. The specific aim would be to perform sawtooth ramp clamp of the nodal axolemma to concurrently detect $I_{Na\text{-}fast}$ and $I_{Na\text{-}slow}$ and their responses to membrane damage and to antagonists. Standard step voltage clamp would be used as well for purposes of technical, biophysical, and pharmacological benchmarking. In amphibians, nodes yield ~50 nA of $I_{Na\text{-}fast}$ and 1 nA traces show good signal-to-noise (Conti et al., 1976). Given a $g_{Na\text{-}slow}/g_{Na\text{-}fast}$ of ~1%, peak ramp $I_{Na}(V)$ for $I_{Na\text{-}slow}$ should be ~0.5 nA. Mechanically traumatizing a node after recording control data is not feasible. Nodal bilayer damage could, alternatively, be imposed by ATP depletion and/or Ca^{2+}-protease hyperactivation and/or generation of reactive oxygen species (Dong and Hare, 2005). Cyclodextrin extraction of membrane cholesterol could used to disrupt healthy bilayer structure. It is possible that accidental stretch, a recognized hazard while mounting axons for vaseline-gap clamp (see Methods section in Schwarz et al., 1995), could be exploited, or abrupt nerve stretch (Maxwell, 1996) prior to axon dissection. High resolution video-recording at all stages would allow for blinded *post hoc* classification of trauma (unintentional and intentional) intensity. Whatever the methods used to damage the nodal bilayer, ancillary imaging, and/or chemical evidence (Rajendran et al., 2010; Shevchenko and Simons, 2010) that initially intact nodal membranes had incurred structural/compositional damage would needed. Changes to nodal $I_{Na\text{-}fast}$ and $I_{Na\text{-}slow}$ would be most usefully tracked via the hysteresis information from sawtooth clamp. To illustrate, in **Figures 4** and **5**, we summarize what little information is available for ramp-elicited total $I_{Na}(V,t)$ from recombinant Nav1.6 channels before and after mechanical membrane damage, then we simulate some ramp clamp outcomes for a 0.0075 mixture of HH-type Nav channels without and with inactivation.

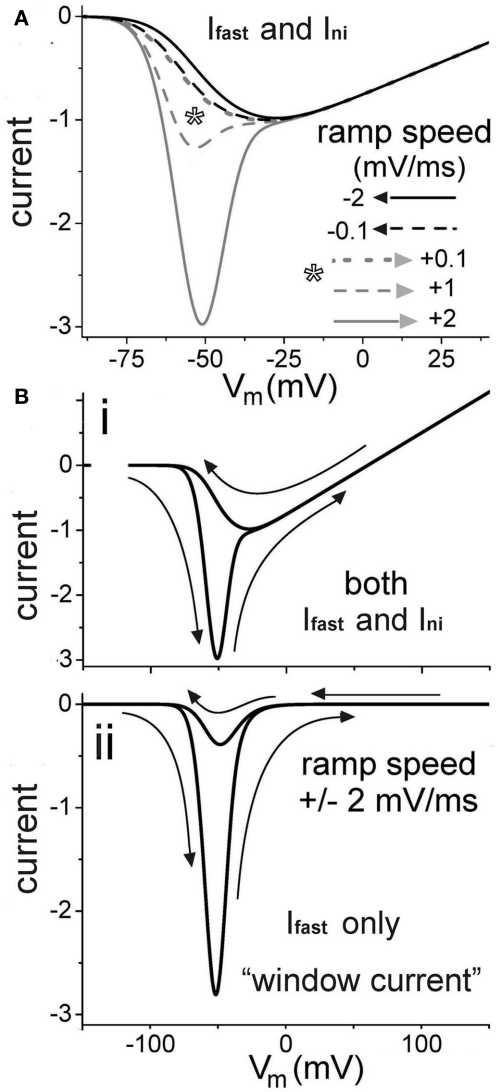

FIGURE 5 | Modeling how to dissect fast and slow I_{Na} using sawtooth hysteresis. (A) For $g_{ni}/g_{Na\text{-}fast} = 0.0075$ (also in **B**), total $I_{Na}(V,t)$ during negative (black) and positive-going (gray) voltage ramps of various speeds, normalized to peak I_{Na} for the negative 0.1 mV/ms ramp. Positive to ∼−25 mV, all plots overlap. Asterisk: speed range of commonly used *depolarizing* ramp protocols. **(Bi,ii)** Current hysteresis (arrows show direction of ramps) during sawtooth ramp clamp, as labeled, for both $I_{Na\text{-}fast}$ and $I_{Na\text{-}ni}$ [i.e., the total $I_{Na}(V,t)$ hysteresis trace as expected in a control experiment] and for $I_{Na\text{-}fast}$ only (expected outcome if a drug inhibited $I_{Na\text{-}slow}$ and not $I_{Na\text{-}fast}$).

and drift (though largely absent from **Figure 4A**) are the norm for I_{Na} from ramp clamp, as in **Figure 4Bi** [in **Figure 4Bii**, current offsets in the three post-trauma traces were handled by repositioning traces on the y-axis to a common reversal potential (asterisk, ∼−55 mV, E_{Na}), making more evident the post-trauma left shifts and the steepening slope conductance]. Steeper "g_{max}" slopes signify increased numbers of maximally activated Nav channels and/or greater maximal P_{open} values. Either way, these $I_{Na}(V,t)$ traces would be consistent with post-trauma "Nav-leak" through left-shifted Relaxed mode channels. In these experiments, strong patch-trauma left-shifted fast $I_{Na} \geq 20$ mV (Wang et al., 2009), but whether slow and fast I_{Na} in a given patch shifted the same amount was not established.

RUDIMENTARY SIMULATIONS FOR RAMP CLAMP – THE SIMPLEST MODEL

To concurrently monitor co-existing fast and slow I_{Na} in Nav-rich excitable cells is not straightforward. Simulations can help with experimental design. For slow I_{Na} we simply used g_{ni}, a non-inactivating version of fast g_{Na} [i.e., the usual Hodgkin–Huxley g_{Na} but with $h(V) = 1$], with $g_{ni}/g_{Na\text{-}fast} = 0.0075$. Eventually, more detailed modeling of I_{Na} (with slow mode inactivation and mode-switch transitions, **Figure 1**) will be needed, but even our rudimentary simulation illustrates some important points.

In **Figure 4C**, with fast-mode g_{Na} zeroed, $I_{Na\text{-}ni}(V,t)$ is depicted alone to illustrate the consequence of a left shift. As there is no inactivation or mode switching, the slope conductance in the g_{max} region is fixed. Maximal $I_{Na\text{-}ni}(V,t)$ increases because shifted channels activate where $V_m - E_{Na}$ is greater. In **Figure 4D** the $g_{Na\text{-}fast}$ and g_{ni} components are combined (black, control; gray, 20 mV left-shifted). Even though a hyperpolarizing ramp was used and even though the two-component $I_{Na}(V,t)$ elicited has the look of "just non-inactivating current," it is not. Encroaching on the V_m range near typical V_{rest} (arrowhead), the major component is, in fact, "window current" (I_{window}). Though g_{ni} is modeled as having precisely the same activation characteristics as $g_{Na\text{-}fast}$, it yields insignificant I_{ni} in the "subthreshold" V_m range where I_{window} flows, simply because of $g_{Na\text{-}fast}$:g_{ni} ratio (>100:1). The dominance of an I_{window}-based subthreshold Nav-leak could be consequential, especially if putative therapeutic inhibitors for mild injury were chosen to target $g_{Na\text{-}slow}$.

It is instructive to consider this scenario in light of Nav inhibitors and trauma. If a mild CLS-type injury depolarized an axon by a few millivolts, the key Nav-leak for the axon would be the shifted I_{window}. Accelerated Na/K pumping would be required to sustain a "resting" V_m below the zone ∼10 mV more depolarized (**Figure 4D**) where I_{ni}-leak starts to take over. The pharmacological goal, therefore: target the damaged-membrane's Nav-CLS channels with lipophilic inhibitors that, at voltages near V_{rest}, stabilize C_R or C_A conformations (see **Figure 1**). Strongly lipophilic antagonists that readily cross the blood–brain-barrier (e.g., Weston et al., 2009) would be ideal. While these may be relatively poorer inhibitors of slow I_{Na} (see Lenkey et al., 2011) staunching the Nav-CLS leak in mildly damaged cells *before* they depolarize is the "first responder" goal.

With this as the target, it becomes important (in pharmacologically relevant settings) to better understand what modulates the ratios of Active to Relaxed channels. This is a question of mode switching and it is unexplored territory. Inhibiting $O_A \rightarrow O_R$ (**Figure 1A**) or promoting $C_R \rightarrow C_A$ would minimize slow mode and maximize fast-mode channels. Based on Nav1.4 behavior in oocyte membranes (Tabarean et al., 1999) we know that in some cell types, membrane damage can dramatically modulate Nav channel mode switch transitions: conversion to fast-mode gating occurs. When examining native-Nav channels' responses to

injury, the possibility of mode switch modulation should be borne in mind.

EXPLOITING THE HYSTERESIS OF SAWTOOTH RAMPS

Figure 5A emphasizes how ramp rate and direction strongly influence the "shape" of $I_{Na}(V,t) = [I_{Na\text{-}fast} + I_{ni}](V,t)$. For either direction and for all speeds, $I_{Na}(V,t)$ is identical above $\sim$−25 mV since there, only g_{ni} is active [in its $P_{open}(V) \sim 1$ range]. For real Nav channels (which undergo slow inactivation), both ramp speed and direction will affect $I_{Na}(V,t)$ in this range, yielding information on Relaxed mode (slow) and mode-switch transitions. Relaxed and Active mode channels are from a single population dominated by Active mode, and sawtooth protocols allow for concurrent semi-quantitative monitoring of both. In membrane-damaged excitable cells, on-going left shift will be expected, making the system response a "moving target." Moreover, when following electrophysiologically the consequences of irreversible membrane damage, damage intensity, and its rate of change will not be tightly controlled. Within-preparation comparisons and time course data are therefore imperative (Tabarean et al., 1999; Morris et al., 2006; Wang et al., 2009).

With relatively fast ramps, obtaining larger within-preparation data sets is easier, but as our simulations illustrate, faster ramps increase the "contamination" of $I_{Na}(V,t)$ by $I_{Na\text{-}fast}$ (i.e., I_{window}) even though traces appear to be exclusively "$I_{Na\text{-}slow}$" (or, in the simulated form, I_{ni}). Standard practice in most labs is to use depolarizing ramps at $\sim$+0.1–1.0 mV/ms, but only at the slowest end of this range would I_{window} contamination be minimal. $I_{Na}(V,t)$ is essentially identical in either direction at +0.1 mV/ms, but fortunately, −2 mV/ms is almost as good (**Figure 5A**). As in **Figure 5Bi**, a sawtooth ramp with a fairly speedy depolarizing limb (i.e., $\sim$−2 mV/ms) can locate the $I_{Na\text{-}fast}(V)$ window current. The hyperpolarizing counterpart will then reveal the only slightly "contaminated" slow current. Ramps need not, of course, be mirror images; the empirical choice is for whatever yields informative and resolvable hysteresis traces. As membrane damage deepened, quick sawtooths like this could monitor both components' changes over time. Time course data over minutes could show whether, at minimal concentrations, lipophilic antagonists show evidence of accumulating in the bilayer, and if, say, they were acting more powerfully on slow or fast components. Depending on how rapidly injury-related membrane characteristics were changing, quick sawtooths could make it possible to capture multiple runs for better signal-to-noise. Intermittently obtained step protocol I/V data would, of course, be a useful supplement to the hysteresis data.

In **Figure 5Bii**, below the two-component hysteresis trace, is the $I_{Na\text{-}fast}$ only component. To a first approximation, these hysteresis sets would mimic start and end points of a notional experiment in which a "persistent current blocker" was added to the system, fully inhibiting slow mode current while having no effect on fast mode current. During Nav-antagonist time course experiments, changes of this ilk, in combination with damage-induced left-shifting (**Figure 3**) would be expected. It seems unlikely that all this could be captured in a timely fashion via the usual Nav voltage clamp approach of steps-for-$I_{Na\text{-}fast}$, slow-ramps-for-$I_{Na\text{-}slow}$.

To re-iterate, continual monitoring of nodal $I_{Na}(V,t)$ hysteresis via empirically worked-out sawtooth protocols could give a picture of Active and Relaxed mode Nav protein behavior in injured axons. For each mode, it could show if/how injury affects susceptibility to Nav antagonists. If Nav agonists showed lower efficacy in intact versus damaged membrane, this would raise a multitude of ancillary questions regarding the avidity of lipophilic drug partitioning into intact versus damaged membranes. Comparing inhibitor efficacy time courses in intact (cholesterol-packed) against cholesterol-extracted membranes (a recognized form of membrane damage) could, for example, be informative.

OLD IDEA, NEW WRINKLE

Differential partitioning of lipophilic drugs into different membrane subdomains (i.e., intact versus damaged) is a new idea for Nav channels, but a related concept is now general currency for G-proteins (Lombardi et al., 2009; Rajendran et al., 2010). Our proposal for Nav channels has a different twist, since we envisage disease states, not normal cell-mediated membrane modeling, as the factor underlying the therapeutically relevant structural heterogeneity in excitable Nav-rich membranes. Data have, interestingly, been available on membrane partitioning of the lipophilic antagonists of voltage-gated Ca channels for $\sim$20 years. Mason and colleagues (**Box 1**, first three entries) suggested, moreover, that these drugs would tend to partition differently into healthy membranes versus ones whose composition was altered due to pathological conditions. At about the same time, however, molecular-level descriptions of protein structure and of hydrophobic drug binding sites became increasingly dominant in pharmacology. The idea that bilayer structure must influence the activities of membrane proteins (Phillips et al., 2009) was largely put aside (see discussion in Finol-Urdaneta et al., 2010).

A recent finding from Andersen's group (Rusinova et al., 2011), that initially may seem tangential, is, on closer inspection, entirely germane to this issue: membrane-fluidizing insulin receptor sensitizers (insulin receptors are G-proteins) at clinically used levels cause "off-target" left shift of Nav channel gating. In effect, Nav left shift due to increased bilayer fluidity is such a robust phenomenon that it can be used as a bioassay for side effects! This, these authors point out, "underscores the importance of exploring bilayer effects of candidate drugs early in drug development." Framed in similar terms, we stress here the need to establish experimentally whether bilayer damage in sick excitable cells constitutes a "membrane-fluidizing Nav-antagonist sensitizer" for Nav channels. If so, it may be possible to modify antagonists to operate optimally (thus more selectively) on Nav channels in damaged membranes: this would be a "silver bullet" scenario – a "bullet" targeting only the "the bad guys." MacKinnon and colleagues (Schmidt and MacKinnon, 2008) among others (Lee and MacKinnon, 2004; Milescu et al., 2009, see **Box 1**) reported such an case for an amphiphilic voltage sensor toxin that targets Kv channels in damaged but not intact membrane (described in Morris, 2011a,b,c).

If lipophilic Nav antagonists can be fine-tuned for maximal efficacy in precisely the pathologically fluidized bilayers where they are most needed, the hoped-for result would be: hyper-accumulation of a drug that shows ultra-Nav-affinity in "sick" high entropy bilayers (see **Table 1**). Given the diversity of Nav channel

isoforms and the multitude of bilayer-structural features that differentiate intact from damaged Nav-rich membranes, silver bullet antagonists might be possible.

High throughput recombinant channel techniques make superb preliminary drug screens and could be adapted for first round studies of damaged Nav-bearing membrane. But to design optimally neuroprotective Nav agents for sick axons, the "retro" approach of the node of Ranvier vaseline-gap voltage clamp (**Box 2**) would be better.

CONCLUDING OVERVIEW

In diverse sick excitable cells, Nav channel leak causes hyperexcitability and excitotoxicity-induced cell demise (**Table 1**). Pharmacological mitigation is poor and side effects too severe (Waxman, 2008). There is uncertainty about the nature and subcellular location of the offending Nav channels and how they are accessed by clinically promising lipophilic antagonists. We argue that for pathologies featuring membrane damage, Nav channel operation, and antagonist binding in damaged versus intact membranes will be pivotal.

Native-Nav channel kinetics can differ appreciably from kinetics for the same isoform expressed in HEK cells or oocytes (Tabarean et al., 1999; Fu et al., 2011). The sick-Nav/sick-bilayer hypothesis presented here will need testing in native settings. The axolemma of a *Xenopus* sciatic nerve axon would, for instance, more closely resemble a human myelinated axon than does the plasma membrane of an undifferentiated human embryonic kidney cell. Too little is known about how, in cell-specific bilayer environments, fast and slow-gating native-Nav channels (plus whatever protein partners may accompany them) respond to sick-cell type membrane damage and then to lipophilic inhibitors. To illustrate how I_{Na}-hysteresis data (from sawtooth ramp clamp of nodes of Ranvier) could facilitate the concurrent study of fast and slow mode I_{Na} during damage and antagonist application, we simulated I_{Na} for various ramped voltage stimuli.

Our global hypothesis about sick excitable cells posits that the primary drug target for lipophilic antagonists is not "axonal Nav channels" or "cardiac channels" or "skeletal muscle Nav channels" but sick-bilayer Nav channels. Ignoring the physico-chemical consequences of bilayer damage could result in sub-optimal protective antagonists for sick excitable cells. Information on native-Nav/native-bilayer/Nav-antagonist interactions could underpin the design of safer neuroprotective drugs, i.e., ones that could be administered at low enough systemic concentrations to generate few side effects, while selectively accumulating in the high entropy bilayer locale of leaky Nav channels.

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Recent developments regarding voltage-gated sodium channel blockers for the treatment of inherited and acquired neuropathic pain syndromes

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Chronic and neuropathic pain constitute significant health problems affecting millions of individuals each year. Pain sensations typically originate in sensory neurons of the peripheral nervous system which relay information to the central nervous system (CNS). Pathological pain sensations can arise as result of changes in excitability of these peripheral sensory neurons. Voltage-gated sodium channels are key determinants regulating action potential generation and propagation; thus, changes in sodium channel function can have profound effects on neuronal excitability and pain signaling. At present, most of the clinically available sodium channel blockers used to treat pain are non-selective across sodium channel isoforms and can contribute to cardio-toxicity, motor impairments, and CNS side effects. Numerous strides have been made over the last decade in an effort to develop more selective and efficacious sodium channel blockers to treat pain. The purpose of this review is to highlight some of the more recent developments put forth by research universities and pharmaceutical companies alike in the pursuit of developing more targeted sodium channel therapies for the treatment of a variety of neuropathic pain conditions.

Keywords: voltage-gated sodium channel, neuropathic pain, TRPV1, Nav1.7, Nav1.8, resurgent currents

INTRODUCTION

The International Association for the Study of Pain (ISAP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage." The ability to experience painful stimuli is essential for an organism's survival by alerting the individual to engage in protective behaviors to prevent further tissue damage or to seek appropriate actions to ameliorate the painful condition. Pain sensations typically originate in nociceptors, peripheral nerve fibers which transduce noxious thermal, mechanical, or chemical stimuli into electrical impulses that are then encoded via central pathways. The cell bodies of these sensory nociceptors are located in the dorsal root ganglia (DRG) with afferent projections to the dorsal horn of the spinal cord. Persistent or chronic pain may linger beyond the initial acute pain-producing stimulus and can become debilitating, severely affecting an individual's quality of life. It is estimated that in the general population one out of five suffer from moderate or severe chronic pain. Whereas certain types of persistent *inflammatory* pain can be seen as an extension of the normal healing process, *neuropathic* pain serves no known protective or healing purpose. The ISAP defines neuropathic pain as "pain caused by a lesion or disease of the somatosensory nervous system," which can be subdivided into central and peripheral neuropathic pain. Common types of neuropathic pain for which many people seek treatment include post-herpetic neuralgia, painful diabetic neuropathy, phantom limb pain, and spinal cord injury pain.

Neuropathic pain is typically characterized by *allodynia* (pain produced by otherwise non-painful stimuli) and/or *hyperalgesia* (exacerbated responses to painful stimuli). Neuropathic pain can arise following an increase in intrinsic nerve excitability, generally manifested in impulses generated ectopically or with minimal stimulation. DRG neurons express a wide variety of voltage-gated sodium channels which regulate the excitability of these neurons (Rush et al., 2007). Nerve injury can result in changes in sodium channel trafficking, gene expression, and/or channel kinetics, all of which contribute to neuronal membrane remodeling and hyperexcitability associated with neuropathic pain (Devor, 2006). As such, voltage-gated sodium channels are attractive targets for the development of novel pain therapeutics. Currently used medications for the treatment of neuropathic pain which have demonstrable actions against sodium channels include tricyclic antidepressants (TCAs: amitriptyline and nortriptyline), local anesthetics (lidocaine, mexiletine), and anticonvulsants (carbamazepine, lamotrigine, phenytoin). However, most of the sodium channel blockers that are currently available are often associated with cardio-toxicity and central nervous system (CNS) side effects (Mulroy, 2002; Walia et al., 2004).

Several new strategies are emerging in the pursuit of providing effective pain relief in patients exhibiting neuropathic pain while minimizing adverse side effects typical of many currently available medications. In this review, we will highlight (1) the development of sodium channel blockers targeted at isoforms preferentially expressed in peripheral sensory neurons involved in the initiation and transduction of pain sensations, (2) techniques for limiting the action of sodium channel blockers to the periphery, and (3) the development of sodium channel modulators that target specific patterns of sodium channel activity associated with problematic pain.

OVERVIEW OF VOLTAGE-GATED SODIUM CHANNELS

Voltage-gated sodium channels mediate the inward sodium current of excitable cells and are thus key determinants regulating action potential generation and propagation (Hodgkin and Huxley, 1952). Voltage-gated sodium channels can also influence the resting potential of neurons and play critical roles in setting the threshold for generation of action potentials (Rush et al., 2007). Therefore, alterations in sodium channel function or expression can have profound effects on normal cell excitability. Sodium channels are dynamic transmembrane proteins consisting of a pore-forming α-subunit (220–260 kDa) and auxiliary β-subunits (32–36 kDa; Catterall, 2000). The α-subunit consists of four homologous domains (I–IV), each consisting of six transmembrane α-helices (S1–S6). Additional loops join S5–S6 segments of each domain to form the outer mouth of the channel pore, with residues of the α-helical S6 segment forming the inner mouth of the pore. The S1–S4 segments of each domain serve as the voltage-sensor, with translocation of the positively charged residues in the S4 segment initiating channel activation in response to changes in the membrane potential. At resting membrane potentials, the majority of the sodium channels are in the closed configuration, prohibiting sodium influx. Upon depolarization, the channels proceed through multiple activation states with translocation of all four S4 segments into the outward configuration allowing sodium influx. Within milliseconds, the channel inactivates via a hinged-lid mechanism whereby a short cytoplasmic loop (which includes an isoleucine, phenylalanine and methionine, IFM, motif) connecting domains III and IV folds into the channel structure, occluding the pore (Vassilev et al., 1988; West et al., 1992). Channel inactivation persists throughout the depolarizing pulse, thus underlying the action potential refractory period. Following hyperpolarization of the membrane potential, the channel recovers from inactivation by returning to the closed, resting state and is re-primed and available again for activation. Perturbations in channel activation and inactivation properties can modulate the onset, duration and frequency of action potentials, thus affecting physiological neuronal signaling.

To date, nine α-subunits (Nav1.1–1.9) and four β-subunits (β1–4) have been identified in mammals (Goldin et al., 2000). Voltage-gated sodium channel isoforms exhibit differential distribution (Felts et al., 1997) as well as distinguishing electrophysiological (Catterall et al., 2005; Rush et al., 2007) and pharmacological properties (England and De Groot, 2009). Seven of the nine isoforms are neuronal, with Nav1.4 and Nav1.5 expressed in skeletal and cardiac muscle, respectively. Nav1.7, Nav1.8, and Nav1.9 are expressed almost exclusively in the peripheral nervous system (PNS). Most of the sodium channel isoforms are sensitive to nanomolar concentrations of the puffer fish toxin, tetrodotoxin (TTX), while the Nav1.5, Nav1.8, and Nav1.9 are resistant to TTX up to millimolar concentrations. **Table 1** summarizes some of the properties of these channels.

Nav1.3, Nav1.7, Nav1.8, and Nav1.9 have been identified as possible targets for analgesics. Nav1.3 and Nav1.7 are very similar in structure to other neuronal sodium channels (Nav1.1, Nav1.2, and Nav1.6) and the skeletal muscle sodium channel (Nav1.4), but there are some interesting functional differences. By contrast, Nav1.8 and Nav1.9 exhibit pronounced differences in their sequences from other sodium channels (Goldin et al., 2000) and striking functional differences. The next section provides additional information on these four isoforms.

SODIUM CHANNEL ISOFORMS IMPLICATED IN ACQUIRED AND INHERITED DISORDERS OF NEUROPATHIC PAIN

Nav1.7

The Nav1.7 isoform (originally PN1, hNE) encoded by the *SCN9A* gene is expressed in the PNS, in both sensory and sympathetic neurons as well as in Schwann cells and neuroendocrine cells (Klugbauer et al., 1995; Felts et al., 1997; Sangameswaran et al., 1997; Toledo-Aral et al., 1997). Nav1.7 is TTX-sensitive (TTX-S) and like other TTX-S isoforms, it displays rapid activation and inactivation kinetics. However, Nav1.7 does exhibit several distinguishing characteristics with important functional effects including a slow recovery from inactivation and a slow onset of closed-state inactivation, resulting in the generation of prominent ramp currents – inward currents generated during a slow, depolarizing voltage ramp (Cummins et al., 1998; Herzog et al., 2003). As such, neurons expressing Nav1.7 are capable of amplifying slowly developing sub-threshold depolarizing inputs – such as generator potentials arising in peripheral nociceptor terminals – thereby directly modulating action potential threshold and ultimately, neuronal excitability.

Recent clinical and experimental studies have implicated Nav1.7 in playing a crucial role in inherited neuropathic pain mechanisms. Specifically, mutations in *SCN9A* have been linked to inherited pain syndromes (**Figure 1**). Mutations associated with congenital insensitivity to pain (CIP) result in truncated, non-functional Nav1.7 channels, and individuals unable to experience pain (Cox et al., 2006). In contrast, inherited erythromelalgia (IEM) and paroxysmal extreme pain disorder (PEPD) are distinct severe pain syndromes associated with gain-of-function mutations in *SCN9A*. IEM is characterized by episodes of burning pain, erythema, and mild swelling in the hands and feet (Waxman and Dib-Hajj, 2005). PEPD is characterized by severe rectal, ocular, and mandibular pain (Fertleman et al., 2007). Sensory neurons expressing either IEM or PEPD mutant channels are hyperexcitable, however, via distinct mechanisms (Rush et al., 2006; Dib-Hajj et al., 2008). Whereas IEM mutations hyperpolarize the voltage-dependence of activation and slow the rate of deactivation (Cummins et al., 2004; Dib-Hajj et al., 2005; Choi et al., 2006; Theile et al., 2011), PEPD mutations destabilize fast inactivation via a depolarizing shift in steady-state fast inactivation, slowed rate of open-channel fast inactivation and produce persistent currents (Fertleman et al., 2006; Dib-Hajj et al., 2008; Jarecki et al., 2008; Theile et al., 2011). PEPD mutant channels, likely due to destabilized inactivation, also enhance resurgent sodium currents (Jarecki et al., 2010; Theile et al., 2011), which have been demonstrated to facilitate high-frequency firing (Raman and Bean, 1997; Khaliq et al., 2003; Castelli et al., 2007) and as such, may contribute to increased neuronal excitability and extreme pain sensations associated with PEPD (Jarecki et al., 2010). Interestingly, a mutation in Nav1.7 was described in a patient with clinical characteristics of both IEM and PEPD. This mutation has biophysical characteristics common to both IEM and PEPD mutations, suggesting that these disorders may be part of

Table 1 | Summary of mammalian voltage-gated sodium channels.

Nav isoform	Tissue expression	Unique biophysical characteristics in DRG neurons	TTX sensitivity, kinetics	Role in pain
Nav1.1	CNS, PNS		TTX-S, fast	
Nav1.2	CNS, embryonic PNS	Depolarized voltage-dependence	TTX-S, fast	
Nav1.3	CNS, embryonic PNS	Rapid repriming; ramp currents; persistent currents	TTX-S, fast	Neuropathic; inflammatory
Nav1.4	Skeletal muscle		TTX-S, fast	
Nav1.5	Cardiac muscle		TTX-R, fast	
Nav1.6	CNS, PNS	Rapid repriming; resurgent currents	TTX-S, fast	
Nav1.7	PNS	Slow-repriming; slow closed-state inactivation; ramp currents	TTX-S, fast	Neuropathic; inflammatory; hereditary
Nav1.8	PNS	Depolarized voltage-dependence; rapid repriming; majority of AP upstroke	TTX-R, slow	Neuropathic; inflammatory
Nav1.9	PNS	Persistent currents; hyperpolarized voltage-dependence; window currents	TTX-R, very slow	Inflammatory

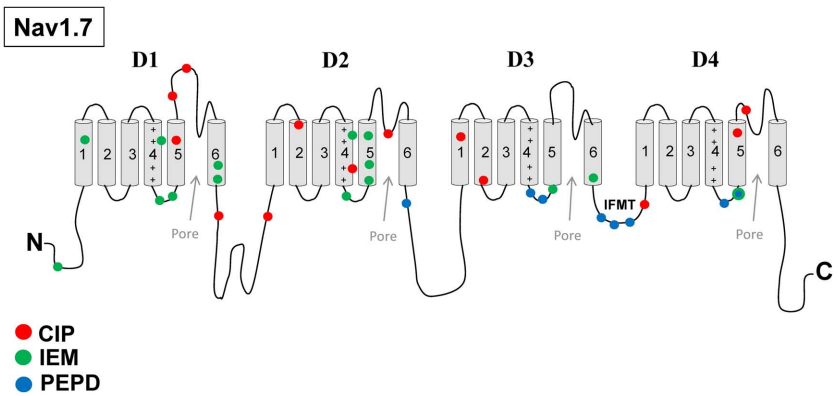

FIGURE 1 | Nav1.7 mutations associated with inherited pain syndromes. A linear representation of the Nav1.7 α-subunit showing the approximate mutation sites for CIP, congenital insensitivity to pain; IEM, inherited erythromelalgia; PEPD, paroxysmal extreme pain disorder.

a physiological continuum (Estacion et al., 2008). Furthermore, a single nucleotide polymorphism in the *SCN9A* (rs6746030 in the Single Nucleotide Polymorphisms database), is associated with increased pain perception in patients with osteoarthritis, sciatica, and phantom limb pain (Reimann et al., 2010), although the mechanism by which this polymorphism might increase pain sensations is not clear. Overall these studies indicate that alterations in Nav1.7 properties can profoundly impact pain sensitivity. Based on these findings, it has been proposed that Nav1.7 is also likely to play important roles in the more common acquired neuropathic pain syndromes.

Nav1.8

The Nav1.8 isoform (originally PN3, SNS), encoded by the *SCN10A* gene, is expressed in nociceptive trigeminal and DRG neurons (Akopian et al., 1996; Djouhri et al., 2003; Ho and O'leary, 2011). Nav1.8 is TTX-resistant (TTX-R), with an $IC_{50} > 50\,\mu M$ when expressed in *Xenopus* oocytes (Akopian et al., 1996; Sangameswaran et al., 1996). Early pharmacological characterization of Nav1.8 was limited due to its poor expression in several mammalian heterologous expression systems, although functional expression has been demonstrated in an immortalized rat DRG/mouse neuroblastoma hybridoma cell line, ND7–23 (John et al., 2004). DRG neurons express a second TTX-R isoform, Nav1.9 (Cummins et al., 1999). Due to differences in voltage-dependent properties and kinetics of Nav1.8 and Nav1.9, implementation of specific voltage-clamp protocols can allow for near complete isolation of either current in DRG neurons (Dib-Hajj et al., 1999; Priest et al., 2005; Sheets et al., 2008). In contrast to the fast and rapidly inactivating TTX-S channels, Nav1.8 channels exhibit ∼10-fold slower kinetics with a depolarized voltage-dependence of activation and inactivation (Akopian et al., 1996; Cummins and Waxman, 1997). Reported values for kinetics and voltage-dependent properties of Nav1.8 vary depending on a number of factors including: (1) cell background (John et al., 2004), (2) channel ortholog (Browne et al., 2009), splice variants (Kerr et al., 2004), and (3) possible contaminating expression of Nav1.9 currents. All of these factors can have an important impact on drug discovery, and must be taken into careful consideration.

Nav1.8 channel-mediated currents are important determinants in sensory neuron excitability and pain signaling. Studies using Nav1.8-null mice have demonstrated that Nav1.8 carries the majority of current underlying the upstroke of the action potential in nociceptive neurons (Renganathan et al., 2001). The use

of Nav1.8 knock-out mice and antisense oligodeoxynucleotide Nav1.8 knockdown have demonstrated a role for Nav1.8 in visceral and inflammatory pain (Akopian et al., 1999; Joshi et al., 2006). However, whether Nav1.8 plays any role in neuropathic pain is still a matter for debate (Kerr et al., 2001; Decosterd et al., 2002; Nassar et al., 2005; Siqueira et al., 2009; Leo et al., 2010). There are several lines of evidence that suggest some role of Nav1.8 in certain models of neuropathic pain. Increased levels of TNF-α in the cerebrospinal fluid and in DRG tissue following L5 ventral root transection results in the upregulation of Nav1.8 channel mRNA and protein levels (He et al., 2010). TNF-α mediated increase in Nav1.8 current density increases neuronal excitability, likely contributing to increased mechanical allodynia following motor nerve injury (Chen et al., 2011). Following sciatic nerve entrapment (SNE), a peripheral nerve injury model similar to that of the chronic constriction injury (CCI) model, upregulation of Nav1.8 mRNA is observed in sciatic nerve axons which may underlie the observed increase excitability as recorded from both A- and C-fibers (Thakor et al., 2009). However, in the SNE injury model, a concomitant decrease is observed in Nav1.8 in L4–L5 DRG neuron cell bodies, suggesting that the increased levels of mRNA may have occurred as a result of translocation from the cell bodies. Similar changes in Nav1.8 protein have been observed following CCI (Novakovic et al., 1998). As will be discussed below, several Nav1.8-selective inhibitors have been shown to be analgesic in several neuropathic pain animal models.

Nav1.3

The TTX-S isoform Nav1.3 is the predominant sodium channel isoform expressed in the CNS and PNS during embryogenesis, with very low levels in the PNS in adults (Waxman et al., 1994). However, Nav1.3 levels increase in the periphery following nerve injury and inflammation, suggesting that this channel could play a role in pain (Black et al., 2004). Nav1.3 levels are also increased in the dorsal horn following SCI (Lampert et al., 2006) and in DRG neurons following motor fiber injury (He et al., 2010). In addition, patients with trigeminal neuralgia, a disorder characterized by ectopic action potentials in trigeminal neurons, exhibit increased expression of Nav1.3 in gingival tissue (Siqueira et al., 2009). Nav1.3 channels exhibit fast kinetics with rapid recovery from inactivation, poising these channels to contribute to pathological high-frequency firing following nerve injury (Cummins and Waxman, 1997), although Nav1.3 knock-out animals still exhibit normal neuropathic pain behavior and ectopic discharges from damaged nerves (Nassar et al., 2006).

Nav1.9

Nav1.9-mediated TTX-R sodium currents are extraordinarily slow persistent currents with a relatively hyperpolarized voltage-dependency compared to the other isoforms (Cummins et al., 1999). Due to the slow, persistent nature of Nav1.9 currents these channels likely do not contribute to the upstroke of the action potential, though they may amplify sub-threshold depolarizations and lower the threshold for action potential induction (Cummins et al., 1999; Herzog et al., 2001; Baker et al., 2003). Studies using Nav1.9 KO mice suggest that this peripheral isoform plays a predominant role in inflammatory pain but not neuropathic pain (Priest et al., 2005; Amaya et al., 2006). However, even the role in inflammatory pain is somewhat unclear as antisense oligodeoxynucleotide-mediated knockdown of Nav1.9 does not reduce thermal hypersensitivity associated with complete Freund's adjuvant (CFA)-induced inflammatory pain (Yu et al., 2011). Although Nav1.9 currents are downregulated following nerve injury (Cummins and Waxman, 1997; Decosterd et al., 2002), in an L5 spinal nerve ligation model of neuropathy reduction of Nav1.9 mRNA expression is minimal in animals that exhibited the most pain and maximal in animals with minimal pain (Persson et al., 2009), suggesting that Nav1.9 may play a role in determining pain threshold. As Nav1.9 expression is restricted to the PNS, Nav1.9 is an attractive target for modulating pain sensitivity.

DEVELOPMENT OF DRUGS FOR NOCICEPTOR-SELECTIVE BLOCKADE

Nav1.7-SELECTIVE INHIBITORS

Given the role of Nav1.7 in pain and its expression limited to the periphery, it has been proposed that drugs selectively targeting Nav1.7 may be ideal analgesics. Despite the uncertain role of Nav1.7 in *acquired* neuropathic pain disorders (see above), recently developed Nav1.7-selective blockers have been shown to be efficacious in rat models of neuropathic pain. Researchers at Merck, using benzodiazepine as a molecular building block, developed a series of structurally novel benzazepinone-based state-dependent selective hNav1.7 blockers that displayed nearly complete inhibition of spontaneous neuronal firing *in vivo* in a rat peripheral axotomy model and reversed tactile allodynia in a rat model of spinal nerve ligation (SNL; Hoyt et al., 2007a). Following up on that study, two additional related compounds were developed with improved oral bioavailability and were highly efficacious in a rat model of SNL, with comparable or increased efficacy compared to the clinical standard, mexiletine (Hoyt et al., 2007b). However, these benzazepinone-based Nav1.7 blockers exhibited less than ideal pharmacokinetics (PK) with high clearance rates, thus Merck Research Laboratories developed a series of imidazopyridine-based blockers with improved PK and excellent efficacy in a rat SNL model with an associated reversal of inflammatory pain (London et al., 2008). Additional Nav1.7-selective compounds developed by Merck include the biphenyl thiazole carboxamides (Tyagarajan et al., 2010a) and biphenyl pyrazoles (Tyagarajan et al., 2010b,c). ProTx-II from the tarantula *Thrixopelma prurient* has also been reported to inhibit Nav1.7 by inducing a depolarizing shift in the voltage-dependence of activation and displays about 100-fold selectivity over the other Nav isoforms (Schmalhofer et al., 2008). Interestingly, ProTx-II seems to target the voltage-sensors of Nav isoforms (Schmalhofer et al., 2008; Xiao et al., 2010), suggesting that drugs targeting specific voltage-sensors of Nav1.7 may exhibit enhanced isoform selectivity. However, the therapeutic utility of ProTx-II *in vivo* may be limited by its nanomolar affinity to the other Nav isoforms and the ineffectiveness in reducing short-term or inflammatory pain following intravenous or intrathecal delivery, possibly related to its inability to cross the blood–nerve barrier (Schmalhofer et al., 2008). Amgen,

Inc., has recently reported on a state-dependent Nav1.7 inhibitor which displays efficacy in a formalin model of pain in rodents, although this compound displays only modest selectivity for Nav1.7 compared to Nav1.5 and Nav1.8 (Bregman et al., 2011). Interestingly, the binding site of this compound does not appear to overlap the local anesthetic binding site, as displacement of bound tritiated batrachotoxin (^{3}H-BTX) is not observed. Additionally, Xenon Pharmaceuticals currently has a Nav1.7-selective compound (XEN402) in the form of a topical ointment for the treatment of post-herpetic neuralgia in Phase II clinical trials.

Nav1.8-SELECTIVE INHIBITORS

Although early studies using Nav1.8 knock-out mice suggested a lack of a role of Nav1.8 in the development of neuropathic pain (Akopian et al., 1999; Nassar et al., 2005), the discovery of the μO-conotoxin MrVIB and the small molecule A-803476, both potent and selective Nav1.8 inhibitors demonstrating efficacy in attenuating neuropathic pain in rats, fueled interest in the search for selective Nav1.8 inhibitors for the treatment of neuropathic pain. MrVIB inhibition of sodium channels is not voltage-dependent and has only 10-fold higher selectivity for Nav1.8 over TTX-S currents in DRG neurons (Ekberg et al., 2006). While exhibiting 100-fold selectivity of Nav1.8 over Nav1.9, MrVIB inhibits Nav1.4 channels almost equally well as Nav1.8, potentially limiting the therapeutic effectiveness of MrVIB. In contrast, A-803467 reportedly exhibits >100-fold selectivity for both resting and inactivated hNav1.8 channels over hNav1.2, hNav1.3, hNav1.5, and hNav1.7 channels expressed in HEK293 cells (Jarvis et al., 2007). A-803467 has demonstrated 10-fold greater selectivity for inactivated Nav1.8 compared to the anticonvulsant compound V102862 and 100- to 1000-fold greater selectivity compared to the local anesthetic tetracaine, the α-amino amide derivative ralfinamide, and the lamotrigine derivative 227c89 (Browne et al., 2009). Systemic administration of A-803467 demonstrated acute antinociceptive activity as measured as a reduction in mechanical allodynia in several models of inflammatory and neuropathic pain in rats (Jarvis et al., 2007). Additionally, systemic and intraspinal delivery of A-803467 attenuates both evoked and spontaneous firing of wide dynamic range neurons in rats with spinal nerve ligations (Mcgaraughty et al., 2008). Following the discovery of A-803467, a series of structurally related compounds have been developed also exhibiting low nanomolar IC_{50} for Nav1.8 (Kort et al., 2008, 2010; Scanio et al., 2010).

More recently, scientists at Abbott Laboratories and Icagen, Inc., collaborated on the development of an additional Nav1.8-selective blocker, A-887826 (Zhang et al., 2010). A-887826 is structurally distinct from A-803467, and displays enhanced potency toward Nav1.8. At a holding potential of −40 mV, A-887826 blocks TTX-R currents in small diameter DRG neurons with an IC_{50} of 8 nM, compared to A-803467 with an IC_{50} of 140 nM (Jarvis et al., 2007). Although A-887826 displays greater potency for Nav1.8 compared to A-803467, its selectivity to other channels is reduced (3-fold, 30-fold, and 28-fold more potent for Nav1.8 compared to Nav1.2, Nav1.5, and Nav1.7 respectively). Oral administration of A-887826 dose-dependently attenuates tactile allodynia in a spinal nerve ligation model of neuropathic pain (Zhang et al., 2010). Both compounds attenuated evoked action potential firing in neurons clamped at −40 mV and suppressed spontaneous firing induced 2 days following CFA-induced inflammation. Interestingly, unlike most sodium channel blockers, both compounds demonstrate high nanomolar affinity for block of resting channels and neither compound displays frequency-dependent inhibition of Nav1.8, thus suggesting that the mechanism of alleviating mechanical allodynia likely does not involve selective inhibition of high-frequency firing neurons due to progressive block of Nav1.8, as is the case of typical anticonvulsant sodium channel blockers.

For both A-803467 and A-887826, there were considerable differences in IC_{50} values for different TTX-R currents, with hNav1.8 < rNav1.8 < native TTX-R currents in DRG neurons, suggesting that subtle differences in either channel sequence or in cell background can affect the drug/channel interaction. Pursuant to this, hNav1.8 and rNav1.8 channels display significant differences in the voltage-dependence of steady-state fast inactivation when both channels are expressed in ND7–23 cells (Browne et al., 2009). Furthermore, in that report, the half-inactivation for human (∼−80 mV) and rat (∼−64 mV) Nav1.8 channels expressed in ND7–23 cells are significantly hyperpolarized compared to other reports for Nav1.8 in *Xenopus* oocytes (∼−30 mV; Akopian et al., 1999), DRG neurons (∼−30 mV; Cummins and Waxman, 1997; Sheets et al., 2008), and HEK293 cells (∼−50 mV; Jarvis et al., 2007). Collectively, these reports underscore the importance of considering both the cell background and channel ortholog used in the drug development process. Additionally, due to the state-dependent binding characteristics of many of the small molecule Nav1.8 sodium channel blockers, careful consideration must be used in the design of voltage-protocols, as the membrane holding potential can influence both the half-inactivation potential as well as the relative drug binding affinity (John et al., 2004; Browne et al., 2009). Despite initial excitement regarding the ability of A-803467 to modulate pain sensations (Rush and Cummins, 2007), its usefulness as a research tool has been limited by these and other factors.

Ambroxol, a secretolytic used predominantly in the treatment of respiratory disorders and as a cough suppressant, inhibits both TTX-S and TTX-R sodium currents, although with a slightly higher potency for TTX-R channels (Weiser and Wilson, 2002). Ambroxol shares an overlapping binding site with local anesthetics and exhibits preferential use-dependent block, but not tonic block, for Nav1.8 (Leffler et al., 2010). Although weakly effective in acute pain models, ambroxol reduced mechanical allodynia in a partial nerve ligation (PNL) model and reduced thermal hyperalgesia, cold allodynia, and mechanical hyperalgesia in a CCI model (Gaida et al., 2005). Oral administration of ambroxol reversed below-injury level tactile and noxious heat hypersensitivity in SCI rats (Hama et al., 2010).

Nav1.3 AND Nav1.9-SELECTIVE INHIBITORS

Although Nav1.3 channels have been implicated in pain mechanisms (Hains et al., 2003; Siqueira et al., 2009), Nav1.3 specific blockers have not been identified. Nav1.3 is highly homologous to other sodium channels. For example, Nav1.2 and Nav1.3 are more than 85% identical. This likely limits the probability that Nav1.3 selective blockers can be developed (Catterall et al., 2005). In contrast, Nav1.9 is only about 45% identical to the other voltage-gated

sodium channel isoforms. However, it has been difficult to identify chemicals that modify Nav1.9 currents because Nav1.9 has proven very difficult to express in heterologous systems and typically exhibits rapid run-down in DRG neurons (Leffler et al., 2005). Recently a clever approach has been employed to help identify toxins that interact with Nav1.9 channels (Bosmans et al., 2011). In this study, individual voltage-sensor paddles of Nav1.9 were transplanted into potassium channel constructs and these chimeric constructs were used to screen for toxins that might target full-length Nav1.9 channels. Although this study indicated that Nav1.9 channels have distinctive pharmacological sensitivities and that the voltage-sensor paddles of Nav1.9 might be excellent targets, it is unclear to what extent the chimeric channels faithfully reproduce the pharmacological properties of the full-length Nav1.9 channels.

TECHNIQUES FOR LIMITING THE ACTION OF SODIUM CHANNEL BLOCKERS TO THE PERIPHERY

COMBINATIONAL THERAPY: TRPV1 AGONISTS AND LOCAL ANESTHETICS

Local anesthetics such as lidocaine block nociceptive nerve transmission via inhibition of sodium channels and are thus clinically useful for the treatment of pain. Local anesthetics are lipophilic in their uncharged form and gain access to the intracellular sodium channel pore binding site by diffusion across cell membranes. However, local anesthetics are non-selective across sodium channel isoforms causing block of cardiac sodium channels as well as non-nociceptive sensory, sympathetic and motor fibers leading to adverse and potentially toxic side effects. Thus, development of localized anesthesia via selective inhibition of nociceptive fibers would greatly enhance the clinical utility of local anesthetics in treating pain.

A novel strategy for such an approach was recently developed that exploited the relatively selective expression of the transient receptor potential vanilloid 1 (TRPV1) receptor to nociceptive sensory neurons (Binshtok et al., 2007). TRP channels are key regulators of sensory transduction (Patapoutian et al., 2009). Specifically, TRPV1 channels are polymodal nocitransducers activated by noxious stimuli including high heat (>43°C), low pH (<6.0) and the pungent ingredient of chili peppers, capsaicin (Caterina et al., 1997; Tominaga et al., 1998). TRPV1 channels have large pores capable of passing small charged molecules, such as QX-314, a permanently charged quaternary derivative of lidocaine. Normally, QX-314 has no effect on neuronal sodium channels when applied extracellularly but does induce block when applied intracellularly (**Figure 2**). Following activation of TRPV1 via capsaicin, Woolf and colleagues utilized the large pore of TRPV1 as a conduit by which to deliver QX-314 into the cell interior in order to block Na+ currents in DRG neurons (Binshtok et al., 2007). Furthermore, because TRPV1 channels are restricted primarily to nociceptive neurons, co-injection of capsaicin and QX-314 into the rat hindpaw or near the sciatic nerve produced a long-lasting decrease in pain sensitivity without any motor or tactile deficits. However, the use of capsaicin as a TRPV1 agonist presents an obvious downside for pain relief in that capsaicin itself produces acute pain upon application prior to the slowly developing QX-314 mediated block of nociceptive transmission. Interestingly, pre-treating with QX-314 prior to capsaicin injection imparted little or no response to capsaicin, possibly due to the immediate entry of QX-314 already in the extracellular space following activation of TRPV1, or inhibition of TRPV1 channels by low concentrations of QX-314 (Rivera-Acevedo et al., 2011). Although the use of a non-pungent TRPV1 agonist, such as eugenol (Yang et al., 2003) would circumvent the irritating aspect of capsaicin, formulation issues have so far prevented successful co-application of eugenol and QX-314 *in vivo* (Roberson et al., 2011). It should be noted that intrathecal administration of QX-314 in mice can be lethal, suggesting that QX-314 should be avoided for spinal anesthesia in humans (Schwarz et al., 2010).

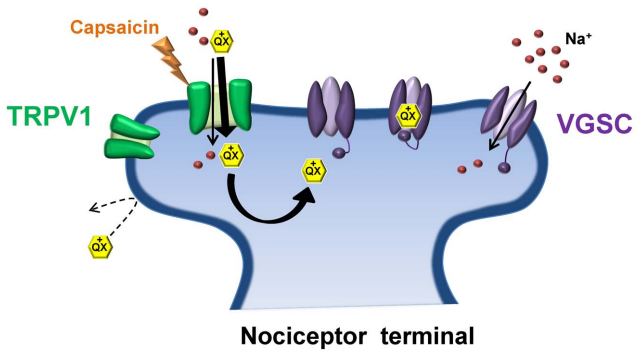

FIGURE 2 | Transient receptor potential vanilloid 1 and QX-314: No pain, no gain. Due to its positive charge, the lidocaine derivative QX-314 is unable to pass through the plasma membrane to gain access to its binding site on the intracellular face of the sodium channel pore. Activation of TRPV1 by capsaicin (the pungent ingredient in chili peppers) allows QX-314 to pass through the relatively large pore of TRPV1, shuttling QX-314 into the cytosol where it can bind and inhibit the sodium channel. TRPV1 receptors are preferentially expressed on nociceptive terminals thus allowing selective inhibition of pain-transmitting nerve fibers.

Interestingly, lidocaine itself is an activator of TRPV1 channels, although only at a concentration sufficient to produce appreciable block of sodium channels (Leffler et al., 2008). Nonetheless, co-application of lidocaine and QX-314 produces a long-lasting differential nerve block (with nociceptive block far outlasting motor block) better than lidocaine alone (Binshtok et al., 2009), without the initial irritation induced by capsaicin administration. Following pre-treatment with an amphipathic quaternary ammonium sodium channel blocker (*N*-methyl amitriptyline) and tertiary amine sodium channel blockers (amitriptyline, bupivacaine, and lidocaine), injection of capsaicin near the rat sciatic nerve results in long-lasting differential nerve block with the more hydrophobic drugs producing the largest differential block (Gerner et al., 2008). In an effort to determine the optimal concentration and ratio of lidocaine and QX-314 resulting in long-lasting duration of nociceptive-selective differential nerve block, Woolf and colleagues recently underwent a systematic study exploring multiple combinations of drug doses in a rat sciatic nerve model (Roberson et al., 2011). The authors determined that the combination of 0.5% QX-314 and 2% lidocaine administered near the sciatic nerve produced 1 h of non-selective sensory and motor block followed by >9 h of pain selective block. Though initially counter-intuitive (causing pain in order to alleviate pain), the

use of TRPV1 as a drug delivery portal represents an ingenious new platform for future researchers to build upon in the possible development of membrane-impermeable, subtype-selective sodium channel blockers that can be delivered to pain sensing terminals.

DEVELOPMENT OF SODIUM CHANNEL BLOCKERS THAT DO NOT CROSS THE BLOOD BRAIN BARRIER

Due to the restricted expression of Nav1.7, Nav1.8, and Nav1.9 to the periphery, development of poorly brain-penetrant Nav selective compounds may increase tolerability due to reduced actions on CNS Nav isoforms. Indeed, a small molecule Nav1.7 inhibitor developed by Merck, N-[(R)-1-((R)-7-chloro-1-isopropyl-2-oxo-2,3,4,5-tetrahydro-1H-benzo[b]aze pin-3-ylcarbamoyl)-2-(2-fluorophenyl)-ethyl]-4-fluoro-2-trifluoromethyl-benzamide (BZP), produces substantial reversal of inflammatory hyperalgesia and mechanical allodynia comparable to that of standard clinical drugs, but due to its poor brain-penetrance, BZP conferred fewer sedative, and motor-coordination impairments (Mcgowan et al., 2009). Although sodium channel blockers that do not penetrate the BBB may help reduce CNS associated side effects, actions against cardiac sodium channels could still be problematic, as could the use of such compounds in conditions which compromise the BBB (e.g., certain spinal cord injuries) or the blood-spinal cord barrier (Beggs et al., 2010).

TARGETING SODIUM CHANNEL ACTIVITY RATHER THAN THE ISOFORM

As detailed above, isoform-specific sodium channel blockers that are targeted at isoforms preferentially expressed in the peripheral sensory neurons (Nav1.7, Nav1.8, and Nav1.9) may be very effective at treating pain while minimizing significant side effects. An alternative approach is to identify sodium channel modulators that target specific patterns of sodium channel activity that are associated with problematic pain. Below we highlight several properties of sodium channel function which have shown to be promising pain targets.

SLOW-INACTIVATION

Within milliseconds following activation sodium channels enter a non-conducting fast-inactivated state; accounting for the action potential refractory period until the membrane potential has been sufficiently repolarized allowing the channels to become available again for activation. However, under conditions of prolonged (within seconds to minutes) depolarizations, sodium channels can also enter a slow-inactivated state (**Figure 3**). Whereas the mechanism of fast inactivation entails occlusion of the pore by translocation of the IFM motif of the DIII–DIV intracellular linker, the mechanism of slow-inactivation is believed to be due to structural rearrangement of the pore (Goldin, 2003). In neuropathic pain, sensory neurons become hyperexcitable, eliciting repetitive action potentials resulting in a progressively depolarized membrane potential. These sustained depolarizations reduce sodium channel availability as a result of accumulation into the slow-inactivated state, dampening neuronal excitability. As typical anticonvulsants curtail pathological high-frequency firing by preferentially binding open/inactivated channels, small molecules capable of stabilizing channels into the slow-inactivated state may represent an additional therapeutic approach for selectively targeting hyperexcitable neurons while sparing normal, low-frequency spiking activity.

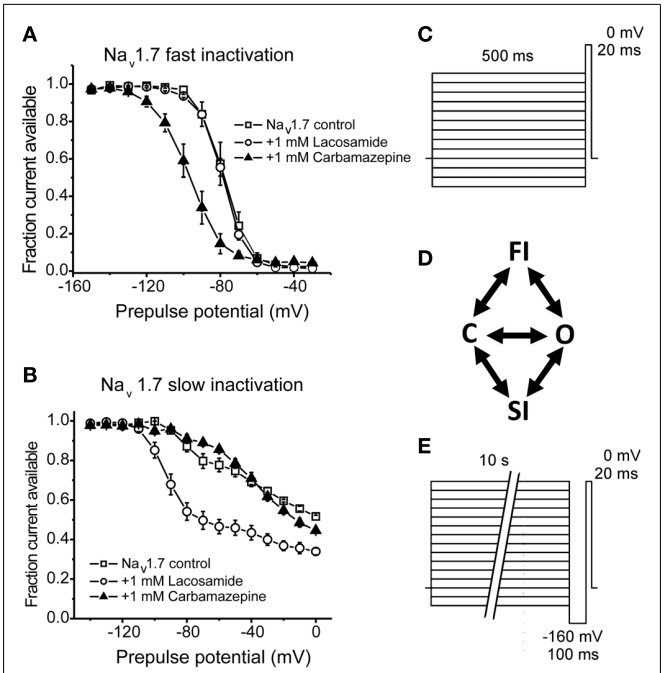

FIGURE 3 | Lacosamide enhances sodium channel slow-inactivation without altering fast inactivation. (A) Carbamazepine, but not lacosamide, enhances Nav1.7 fast inactivation as evident by a hyperpolarizing shift in the voltage-dependence of steady-state fast inactivation. **(B)** Lacosamide, but not carbamazepine, causes a significant shift in the voltage-dependence of Nav1.7 slow-inactivation. **(C,E)** Pulse protocols for determining the voltage-dependence of fast and slow-inactivation, respectively. **(D)** A simplified diagram showing the different channel occupancy states, where C indicates the closed-state, O the open state, FI the fast-inactivated state and SI the slow-inactivated state. **(A,B)** adapted from Sheets et al. (2008) with permission from *The Journal of Pharmacology and Experimental Therapeutics*.

Lacosamide is a functionalized amino acid that was synthesized during the development of anticonvulsant drug candidates and has displayed antinociceptive properties in inflammatory and neuropathic pain (Beyreuther et al., 2006; Hao et al., 2006; Stohr et al., 2006; Bee and Dickenson, 2009; Wymer et al., 2009; Ziegler et al., 2010). Lacosamide displays a unique mechanism of action in that it seemingly selectively stabilizes channels into the slow-inactivated state (Errington et al., 2008). In that study, lacosamide had no apparent effect on fast inactivation of sodium channels from neocortical neurons, nor did it exhibit rapid use-dependent block which is typical of the prototypical anticonvulsants. In a similar study, lacosamide inhibited currents from Nav1.3, Nav1.7, and Nav1.8, but only after prolonged depolarizations, consistent with an enhancement in slow-inactivation with no effect on fast inactivation (Sheets et al., 2008). Furthermore, Sheets et al. (2008) observed that lacosamide was better able to discriminate between resting and inactivated channels compared to lidocaine or carbamazepine (**Figure 3**), thus likely allowing for improved selectivity

over neurons with a depolarized membrane potential, with little tonic block.

Recently, several other compounds have been identified that also seem to selectively stabilize the slow-inactivated state (**Table 2**). Structurally novel derivatives of lacosamide have been developed that exhibit enhanced binding to the slow-inactivated state compared to lacosamide (Wang et al., 2011). Brilliant Blue G, also known as Coomassie blue, was recently demonstrated to be a potent inhibitor of sodium channels with a much higher affinity for the slow-inactivated state compared to the fast-inactivated state (Jo and Bean, 2011). Zalicus Pharmaceuticals recently developed a small, organic compound, Z123212, which selectively stabilizes slow-inactivation of recombinant Nav1.7, Nav1.8, and Cav3.2 T-type currents, as well as TTX-S and TTX-R currents from lamina I/II spinal cord neurons, with no effect on the voltage-dependence of fast inactivation for sodium or Cav3.2 currents (Hildebrand et al., 2011). In that study, Z123212 is considerably more potent ($IC_{50} = 480\,nM$) than lacosamide ($IC_{50} = 150\,\mu M$) in inhibiting AP firing elicited in response to a steady current injection. Furthermore, oral administration of Z123212 at doses within the range to enhance slow-inactivation resulted in a significant reversal of tactile allodynia and thermal hyperalgesia in SNL rats with no observable effects on motor-coordination or cardiovascular properties. These studies suggest that targeting slow-inactivation represents a potentially advantageous approach for the targeting of pathologically depolarized neurons associated with neuropathic pain. However, it should be noted that it can be very difficult to distinguish between a compound that targets slow-inactivation and one that simply exhibits very slow binding and unbinding to fast-inactivated channels. This caveat must be kept in mind when trying to definitively determine a mechanism of action with inactivation modifiers.

PERSISTENT SODIUM CURRENTS

Persistent sodium currents can arise when sodium channels fail to fully inactivate after opening, resulting in a very small residual sodium influx, representing only a fraction of the transient current (∼1%). Additionally, persistent currents can arise at voltages in which channels are activated but inactivation is sub-maximal ("window currents"). Persistent currents can play a critical role in modulating both resting membrane potential and action potential threshold, thus modulating neuronal excitability. Increased persistent currents have been observed under pathological conditions of hyperexcitability and may be associated with neuropathic pain. Nav1.7-PEPD mutations exhibited increased persistent currents (Jarecki et al., 2008; Theile et al., 2011), and inhibition of these currents is a proposed mechanism by which carbamazepine is effective in treating pain in PEPD patients (Fertleman et al., 2006). Upregulation of Nav1.3-mediated persistent currents in dorsal horn neurons is observed following contusive SCI in rats, and likely contributes to neuronal hyperexcitability seen in these neurons (Lampert et al., 2006). In rats, chronic constriction of L5 dorsal root ganglion (CCD) results in mechanical allodynia and increased persistent currents in injured A-type fibers, both of which are reduced by the persistent current-selective inhibitor, riluzole (Xie et al., 2011).

Riluzole is a neuroprotective agent mainly used in the treatment of amyotrophic lateral sclerosis (Bensimon et al., 1994), however it also displays anticonvulsant and antiepileptic properties in animals models (Romettino et al., 1991; Zgrajka et al., 2010). The neuroprotective properties of riluzole partially stem from its actions to reduce pre-synaptic glutamate release and/or via post-synaptic inhibition of NMDA receptors (Cheramy et al., 1992; Debono et al., 1993). Riluzole also displays activity against sodium channels, inhibiting TTX-S and TTX-R currents in DRG neurons equally well in a state-dependent manner (Song et al., 1997). In CNS and cardiac sodium channel isoforms, riluzole at low concentrations selectively inhibits persistent sodium currents compared to transient currents (Urbani and Belluzzi, 2000; Weiss et al., 2010). Recently, riluzole has demonstrated antinociceptive properties on below-level cutaneous hypersensitivity in rats with spinal cord injury, although it is not clear if this effect is mediated via sodium channel inhibition or a reduction

Table 2 | Summary of voltage-gated sodium channel blockers.

Compound	Selectivity	Likely mechanism of action	References
Benzazepinone series	Nav1.7 > Nav1.5 ≫ Nav1.8	State-dependent inhibition	Hoyt et al. (2007a,b)
Pyrazole 20	Nav1.7 > Nav1.8	State-dependent inhibition	Tyagarajan et al. (2010)
ProTx-II	Nav1.7 ≫ Nav1.2-Nav1.6, Nav1.8	Voltage-sensor trapper	Schmalhofer et al. (2008)
2,4-diaminotriazine 52	Nav1.7 ≈ Nav1.3, Nav1.4 > Nav1.5, Nav1.8	State-dependent inhibition	Bregman et al. (2011)
MrVIB	Nav1.8 ≈ Nav1.4 > Nav1.2, Nav1.3, Nav1.5, Nav1.7 ≫ Nav1.9	Blocks conduction pathway	Ekberg et al. (2006), Zorn et al. (2006)
A-803467	Nav1.8 ≫ Nav1.2, Nav1.3, Nav1.5, Nav1.7	State-dependent inhibition	Jarvis et al. (2007)
Ambroxol	Nav1.8 ≥ TTX-S channels	State-dependent inhibition	Leffler et al. (2010)
Capsaicin + QX-314	TRPV1 expressing neurons	TRPV1 activation paired with state-dependent inhibition	Binshtok et al. (2007)
Lacosamide	Chronically depolarized channels	Enhanced slow-inactivation	Errington et al. (2008)
Z123212	Chronically depolarized channels	Enhanced slow-inactivation	Hildebrand et al. (2011)
Riluzole	Persistent currents	Enhanced fast inactivation	Urbani and Belluzzi (2000)
Ranolazine	Persistent currents	Open-channel block	Wang et al. (2008)

in glutamatergic transmission (Hama and Sagen, 2011). The authors also observed general antinociceptive effects of riluzole on uninjured rats when administered peripherally but not centrally. Because persistent sodium currents, especially those that occur at or near the threshold for action potential generation, can substantially increase excitability, drugs that selectively target persistent currents in nociceptive neurons could be very useful in treating pain.

The analgesic potential of ranolazine, a drug that is FDA approved for treatment of chronic angina pectoris, has been investigated because ranolazine preferentially inhibits persistent cardiac sodium currents as well as sensory neuronal sodium channels (Rajamani et al., 2008, 2009; Wang et al., 2008). Ranolazine inhibits sensory neuronal excitability and the behavioral signs of inflammatory and neuropathic pain (Gould et al., 2009; Casey et al., 2010; Estacion et al., 2010). Although targeting persistent sodium currents in sensory neurons may be efficacious in treating pain, both riluzole and ranolazine interact with a broad spectrum of sodium channels, including cardiac and CNS channels, which may not be ideal.

RESURGENT SODIUM CURRENTS

Recently it has been proposed that resurgent sodium currents might also contribute to enhanced pain sensitivity (Jarecki et al., 2010). After opening, Nav channels normally undergo inactivation within milliseconds via occlusion of the pore with the IMF inactivation particle. Channels are refractory following inactivation until the membrane has been hyperpolarized to resting potentials, repriming the channel. Under certain conditions channels can re-open during repolarization to moderately negative potentials allowing a surge of inward current (resurgent current; **Figure 4**). Resurgent sodium currents were first identified in cerebellar Purkinje neurons (Raman and Bean, 1997) and more recently in DRG neurons (Cummins et al., 2005). These currents are proposed to flow following relief of ultra-fast open-channel block by an endogenous cytosolic blocking particle, proposed to be the C-terminal tail of the auxiliary Navβ4 subunit (Grieco et al., 2005; Bant and Raman, 2010). Resurgent currents facilitate recovery from inactivation and are maximal at potentials near the action potential threshold, and as such, may contribute to high-frequency firing (Raman and Bean, 1997; Khaliq et al., 2003; Castelli et al., 2007). Although Nav1.6 is the predominant carrier of resurgent current in DRG neurons (Cummins et al., 2005), other isoforms can carry this current under pathological conditions of impaired inactivation (Grieco and Raman, 2004; Jarecki et al., 2010). Furthermore, enhanced resurgent currents are associated with the Nav1.7-PEPD mutations (Jarecki et al., 2010; Theile et al., 2011). Thus, resurgent currents potentially represent a novel drug target for the treatment of pain. As resurgent currents arise following transition to a unique channel state (open-channel block), it may be possible to develop small molecules capable of selectively targeting resurgent currents. Indeed, a recent study showed that anandamide is able to inhibit resurgent sodium currents at concentrations that have little impact on peak transient currents (Theile and Cummins, 2011).

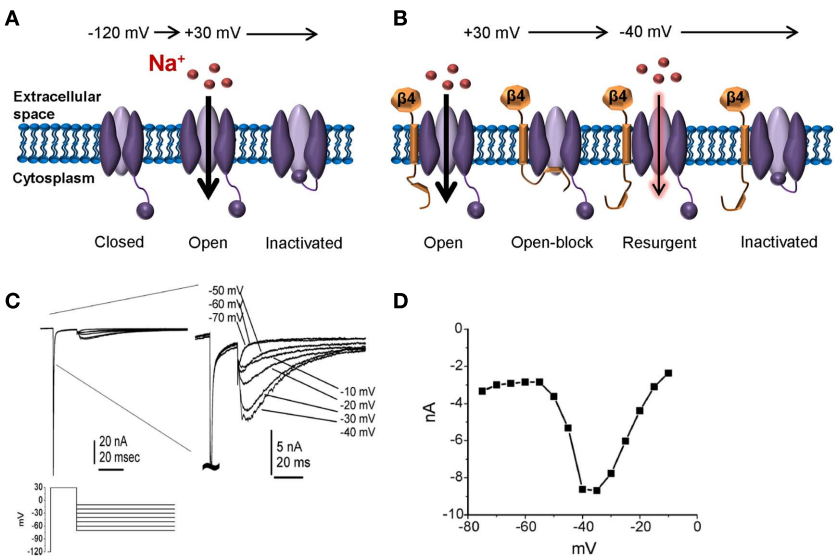

FIGURE 4 | Resurgent sodium currents. (A) Following a strong depolarization, sodium channels transition from the resting closed-state to open, allowing influx of sodium. Within milliseconds, the channel inactivates via a hinged-lid mechanism and remains inactivated until the membrane potential has been sufficiently hyperpolarized. This cycle of events underlies the action potential refractory period. **(B)** Following a strong depolarization, a blocking particle (likely the C-terminal portion of the auxiliary Navβ4 subunit) can occlude the open-channel before the inactivation gate can bind, thus resulting in open-channel block. Following a hyperpolarization to an intermediate potential, the blocker is expelled resulting in an additional surge in current. **(C)** Representative resurgent sodium currents recorded from a large Nav1.8-null DRG neuron. The traces are magnified in the right panel to better see the resurgent currents. **(D)** The voltage-dependence of the resurgent currents is shown by plotting the peak resurgent current amplitude against the repolarization pulse potential. **(C,D)** adapted from Cummins et al. (2005) with permission from *FEBS Letters*.

CONCLUSION

Voltage-gated sodium channels are emerging as exciting targets for the treatment of neuropathic pain, albeit several challenges must be overcome. The currently available sodium channel blockers used for treatment are non-specific among sodium channel subtypes, resulting in undesirable side effects thus limiting their clinical utility. However, as highlighted in this review, researchers have made considerable advances in the pursuit of developing sodium channel blockers with improved efficacy while minimizing off-target effects. Several pharmaceutical companies are engaged in the development of subtype-selective blockers targeted to the peripheral neuronal isoforms Nav1.7 and Nav1.8, which have a demonstrable role in pain mechanisms. In fact, several such blockers have entered into human clinical trials. Other exciting new approaches involve techniques limiting drug delivery to the periphery via the development of brain-impenetrable blockers and a unique approach that exploits the TRPV1 receptor. Additionally, as the widely used anticonvulsants antagonize aberrant sodium channel activity due to their state-dependence, identifying, and developing other state-dependent drugs which target pathological sodium channel activity may circumvent the challenges associated with developing drugs targeting the subtle differences between the highly homologous channel isoforms. Furthermore, resurgent current generation relies on the presence of the accessory Navβ4 protein. As resurgent currents may play a role in pathological pain, drugs targeting Navβ4 or other accessory proteins associated with sodium channels may represent yet another avenue for pain therapy. Overall, recent advances in our knowledge of sodium channel properties and increased understanding of the roles that specific isoforms play in abnormal pain syndromes makes this an exciting time for novel pain therapeutics that target voltage-gated sodium channels.

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Sodium channels as targets for volatile anesthetics

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The molecular mechanisms of modern inhaled anesthetics are still poorly understood although they are widely used in clinical settings. Considerable evidence supports effects on membrane proteins including ligand- and voltage-gated ion channels of excitable cells. Na^+ channels are crucial to action potential initiation and propagation, and represent potential targets for volatile anesthetic effects on central nervous system depression. Inhibition of presynaptic Na^+ channels leads to reduced neurotransmitter release at the synapse and could therefore contribute to the mechanisms by which volatile anesthetics produce their characteristic end points: amnesia, unconsciousness, and immobility. Early studies on crayfish and squid giant axon showed inhibition of Na^+ currents by volatile anesthetics at high concentrations. Subsequent studies using native neuronal preparations and heterologous expression systems with various mammalian Na^+ channel isoforms implicated inhibition of presynaptic Na^+ channels in anesthetic actions at clinical concentrations. Volatile anesthetics reduce peak Na^+ current (I_{Na}) and shift the voltage of half-maximal steady-state inactivation (h_∞) toward more negative potentials, thus stabilizing the fast-inactivated state. Furthermore recovery from fast-inactivation is slowed, together with enhanced use-dependent block during pulse train protocols. These effects can depress presynaptic excitability, depolarization and Ca^{2+} entry, and ultimately reduce transmitter release. This reduction in transmitter release is more potent for glutamatergic compared to GABAergic terminals. Involvement of Na^+ channel inhibition in mediating the immobility caused by volatile anesthetics has been demonstrated in animal studies, in which intrathecal infusion of the Na^+ channel blocker tetrodotoxin increases volatile anesthetic potency, whereas infusion of the Na^+ channels agonist veratridine reduces anesthetic potency. These studies indicate that inhibition of presynaptic Na^+ channels by volatile anesthetics is involved in mediating some of their effects.

Keywords: sodium channels, volatile anesthetics, presynaptic, anesthetic mechanism

BACKGROUND

It has been over 160 years since the use of diethyl ether as a general anesthetic was publicly demonstrated, yet our mechanistic understanding of these vitally important drugs lags far behind that of most other major drug classes. Most modern inhaled anesthetics are derivatives of ether, and over the years have been developed to have improved pharmacokinetics, but they are still plagued by a lack of specificity with significant cardiovascular and respiratory side effects. It remains unclear how these drugs produce general anesthesia, a pharmacologically induced coma characterized by amnesia, unconsciousness, and immobility in response to painful stimuli (Hemmings et al., 2005b). Studies into their molecular mechanisms in the 1960s, which have their origins in the Meyer–Overton correlation of anesthetic potency with lipophilicity from 1900, led to a lipid-based theory involving a unitary mechanism of non-specific actions on the lipid bilayer (Meyer, 1899; Overton, 1901).

With technical advances in biochemistry and biophysics, specific targets were studied and identified. Pioneering studies showed that anesthetic interactions with proteins themselves, not necessarily involving lipid interactions, could explain anesthetic effects at a biochemical level (Franks and Lieb, 1994). Animal studies showed that volatile anesthetics produce their immobilizing effects primarily by actions on the spinal cord (Antognini and Schwartz, 1993; Rampil et al., 1993), whereas unconsciousness and amnesia involve actions at supra-spinal centers (Eger et al., 2008). Membrane proteins including ion channels have been implicated as key mediators of the depressive effects of anesthetics on neuronal function. Many potential targets have been identified, and it has become clear that anesthetics act at multiple distinct targets in the central nervous system to produce the various component effects of the anesthetic state (multi-site hypothesis).

MECHANISMS OF GENERAL ANESTHETIC EFFECTS ON THE CENTRAL NERVOUS SYSTEM

The idea of general anesthetics acting both on excitatory and inhibitory synaptic transmission has lead to many studies pointing out the complexity of anesthetic mechanisms (Rudolph and Antkowiak, 2004; Hemmings et al., 2005b; Franks, 2006). General anesthetics, including both volatile and intravenous anesthetics, enhance synaptic inhibition via postsynaptic γ-aminobutyric acid type A ($GABA_A$) receptor modulation (Nicoll et al., 1975;

Zimmerman et al., 1994). More recent studies also point out the importance of extrasynaptic $GABA_A$ receptors as a target of anesthetics by potentiating tonic inhibitory currents (Orser, 2006; Rau et al., 2009) and by enhancing the release of GABA by a presynaptic increase in miniature inhibitory postsynaptic current (mIPSC) frequency (Nishikawa and MacIver, 2001). Depression of excitatory transmission by presynaptic effects is another target of anesthetic action (Perouansky et al., 1995; MacIver et al., 1996; Ouanonou et al., 1999; Wakasugi et al., 1999). Both volatile and intravenous anesthetics reduce excitatory postsynaptic potentials (EPSPs) in neurons, an effect most likely due to presynaptic mechanisms (Weakly, 1969; Richards and White, 1975; Kullmann et al., 1989; Berg-Johnsen and Langmoen, 1992). Recent evidence suggests that inhibition of glutamatergic synaptic transmission through *N*-methyl-D-aspartate (NMDA)-type glutamate receptor blockade by inhaled anesthetics might also contribute to depression of excitatory transmission (Dickinson et al., 2007; Haseneder et al., 2008).

It is now evident that ligand-gated ion channels are major targets for general anesthetics (Franks and Lieb, 1994). Both inhibition of excitatory NMDA receptors and potentiation of inhibitory $GABA_A$ and glycine receptors have come under scrutiny as important targets for both intravenous and inhaled anesthetic effects on synaptic transmission (Franks, 2006). These receptors are found throughout the central nervous system and are major transducers of excitatory and inhibitory neurotransmitter signaling.

Second-messenger regulated protein phosphorylation of Na^+ channels has been implicated as another possible target of volatile anesthetics. Halothane increases both purified (Hemmings and Adamo, 1994) and endogenous (Hemmings and Adamo, 1996) brain protein kinase C (PKC) activity. Phosphorylation of Na^+ channels by PKC and PKA reduces Na^+ channel activity by altering channel kinetics, for example by slowing inactivation, and is therefore an important component of neuromodulation (Cantrell and Catterall, 2001). It is possible that some of the inhibitory effects of volatile anesthetics on Na^+ channel activity are mediated through PKC phosphorylation.

More recent studies have extended the range of likely anesthetic targets to include neuronal nicotinic acetylcholine receptors (Flood et al., 1997), two pore domain K_{2P} channels and K^+ leak channels (Patel and Honore, 2001; Sirois et al., 2002), and presynaptic voltage-gated Na^+ channels. This review considers Na^+ channels as targets for the effects of volatile anesthetics (inhaled alkane and ether derivatives).

PRESYNAPTIC Na^+ CHANNELS AS ANESTHETIC TARGETS

Na^+ channels play a crucial role in cell-to-cell communication, as they are involved in initiating and propagating action potentials in excitable cells throughout the nervous system (Hodgkin and Huxley, 1952). Early reports in the 1970s associated the effects of volatile anesthetics on lipid bilayer properties to alterations of certain membrane bound ion channels, in particular voltage-gated Na^+ channels (**Figure 1**).

These reports were among the first to hypothesize a specific ion channel (Na^+ channels) as a potential target of volatile anesthetics, though at that time no specific binding site or specific mechanism could be identified. Early studies on the effects of general

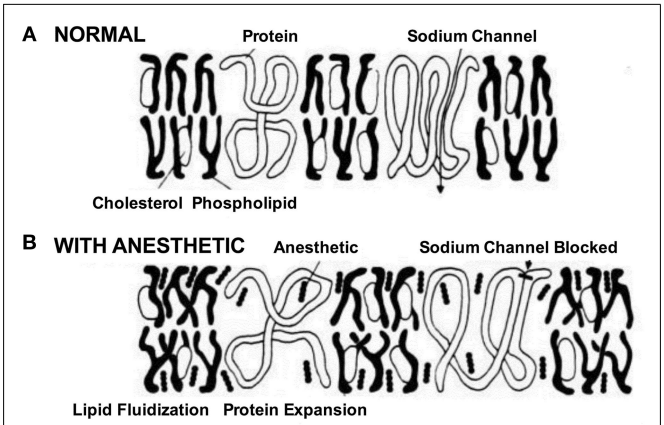

FIGURE 1 | Schematic of the effects of anesthetics on cell membrane and Na^+ channels. In the absence of the drug. **(A)** Na^+ channels initiate and propagate electrical signals, i.e., action potentials. **(B)** The anesthetic was believed to affect Na^+ channels by partitioning and interacting with the membrane. This process called lipid fluidification altered the cell membrane and subsequently distorted the channel protein leading to block of channel function (Seeman, 1974).

anesthetics on Na^+ and K^+ currents in the crayfish or squid giant axon showed inhibition of peak Na^+ (I_{Na}) current and effects on channel recovery, but in these preparations inhibition occurred at relatively high concentrations (Bean et al., 1981; Haydon and Simon, 1988). Subsequent studies examined the effects of various volatile anesthetics on mammalian brain derived Na^+ channels heterologously expressed in mammalian cell lines (Rehberg et al., 1996). Inhibition of peak I_{Na} due to stabilization of the inactivated state of Na^+ channels was evident as a hyperpolarizing "left-shift" in steady-state (or h_∞) inactivation. These experiments were among the first to demonstrate inhibition of neuronal Na^+ channels by volatile anesthetics. The sensitivity of Na^+ channels to clinically relevant concentrations of volatile anesthetics was confirmed in various *in vitro* expression systems and was subsequently extended to more physiologically relevant neuronal preparations.

Electrophysiological recordings performed in isolated rat neurohypophysial nerve terminals, an experimentally accessible nerve terminal preparation, showed that clinically relevant concentrations of isoflurane inhibited peak I_{Na} in nerve terminals in a concentration- and voltage-dependent manner (Ouyang et al., 2003; **Figure 2A**, upper panel). Similar to heterologous expression systems, a left-shift in the voltage-dependence of steady-state inactivation demonstrated stabilization of the fast-inactivated state. These results support the hypothesis that volatile anesthetics depress excitatory synaptic transmission by inhibiting presynaptic voltage-gated Na^+ channels. In addition, in the rat neurohypophysial nerve terminal preparation, isoflurane inhibited action potential amplitude and increased action potential half-width (Ouyang and Hemmings, 2005; **Figure 2A**, lower panel). The underlying current mediating the fast and rising depolarizing phase of the action potential is carried by tetrodotoxin (TTX)-sensitive Na^+ channels, which were inhibited by isoflurane using a voltage-stimulus based on an averaged action potential. The effects of non-immobilizers (structurally similar compounds without

anesthetic properties) in rat dorsal root ganglion neurons showed that compound F3, an anesthetic fluorinated cyclobutane, inhibited Na^+ channels similar to the effects of conventional volatile anesthetics, but the non-anesthetic (non-immobilizer) fluorinated cyclobutane F6 had only minimal effects (Ratnakumari et al., 2000; **Figure 4A**). These findings support the role of Na^+ channels as molecular targets for volatile anesthetic action.

Studies investigating subtype-specific effects of volatile anesthetics revealed small, but potentially significant, differences in isoflurane potency with IC_{50} values ranging from 0.45 to 0.7 mM (at V_h −70 mV) on $Na_v1.2$, $Na_v1.4$, $Na_v1.5$ expressed in Chinese hamster ovary cells (Ouyang and Hemmings, 2007). Despite the small potency differences, there were differences between isoforms in recovery from fast-inactivation tested by a double-pulse protocol. The effect of isoflurane on channel recovery was greatest in $Na_v1.2$, a major brain isoform (**Figure 2B**). Another study in which subtypes $Na_v1.2$, $Na_v1.4$, $Na_v1.6$, and TTX-resistant $Na_v1.8$ were expressed (with and without β1 subunit co-expression) in *Xenopus* oocytes also revealed that $Na_v1.2$, $Na_v1.4$, $Na_v1.6$ were sensitive to isoflurane, whereas the TTX-resistant subtype $Na_v1.8$, which is highly expressed in dorsal root ganglion nociceptive neurons, was insensitive (Shiraishi and Harris, 2004). Nerve terminals of nociceptive sensory neurons are the (main) origin of neuropathic and inflammatory pain signals (Dib-Hajj et al., 2010), but the pro- or anti-nociceptive effects of volatile anesthetics are not clearly defined. It is evident that these nociceptive neurons carry a distinct selection of Na^+ channel subtypes related to pain signaling (e.g., $Na_v1.7$, $Na_v1.8$, $Na_v1.9$; see review Dib-Hajj et al., 2010). Subsequently, $Na_v1.8$ expressed in mammalian neuronal cells revealed concentration- and voltage-dependent inhibition of $Na_v1.8$ by clinically relevant concentrations of isoflurane similar to other subtypes (Herold et al., 2009; **Figure 3A**, upper panel). This demonstrates the importance of choosing a suitable expression system for pharmacological studies of ion channels. In this case the neuronal cell line ND7/23, a hybrid cell line between rat dorsal root ganglion neurons and mouse neuroblastoma cells, may have provided auxiliary β-subunits or other neuron-specific signaling pathways that are important for inhibition by anesthetics. A comparative study showing the effects of several different volatile anesthetics on heterologously expressed Na^+ channels in mammalian cells revealed that desflurane, a highly fluorinated inhaled anesthetic, had the strongest effect on peak I_{Na} inhibition, but all agents in this class were effective at clinically relevant concentrations (Ouyang et al., 2009; **Figure 3B**). In contrast, the

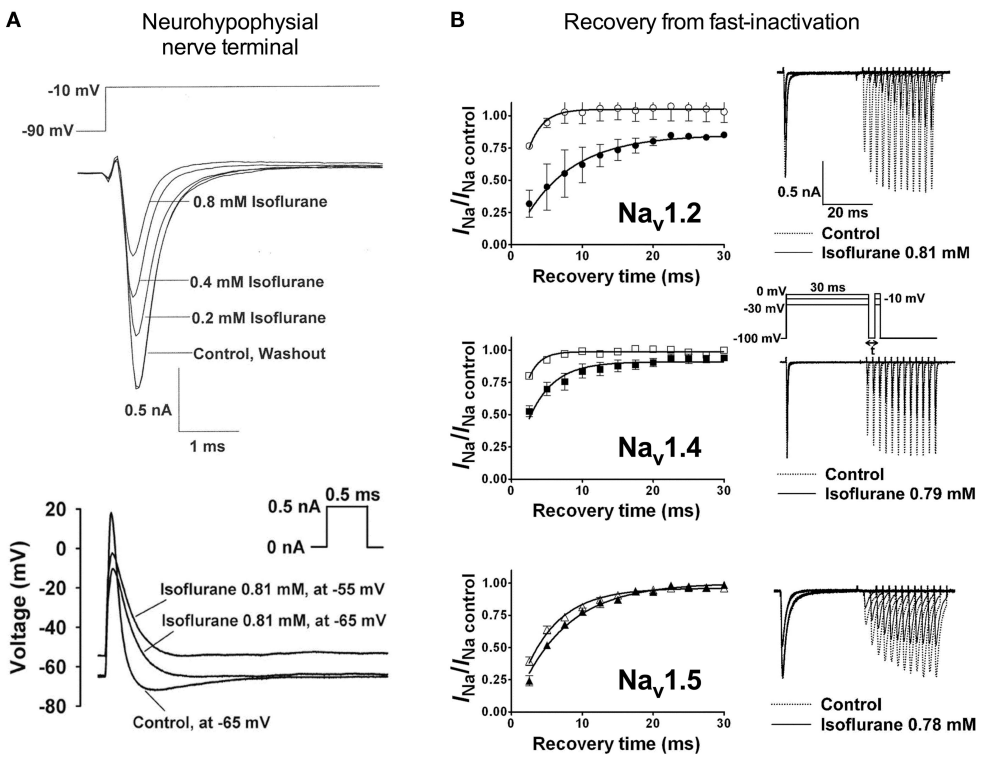

FIGURE 2 | Volatile anesthetics inhibit Na^+ channels in various expression systems. [**(A)**, upper panel] Electrophysiological recordings of isolated rat neurohypophysial nerve terminals show a reversible block of Na^+ currents and [**(A)**, lower panel] action potentials evoked by small current injections at clinically relevant concentrations of isoflurane (Ouyang et al., 2003; Ouyang and Hemmings, 2005). **(B)** Effects of isoflurane on channel recovery from fast-inactivation of three different Na^+ channel isoforms heterologously expressed in mammalian cells. Recovery was assessed using a two-pulse protocol with a 30-ms conditioning pulse followed by a variable recovery interval of up to 30 ms, and then a 5-ms test pulse to peak activation voltages. The time course of channel recovery from fast-inactivation was well fitted by a monoexponential function [**(B)**, left panels]. Representative current traces for a holding potential (V_h) of −100 mV are shown for all three subtypes [**(B)**, right panels; Ouyang and Hemmings, 2007].

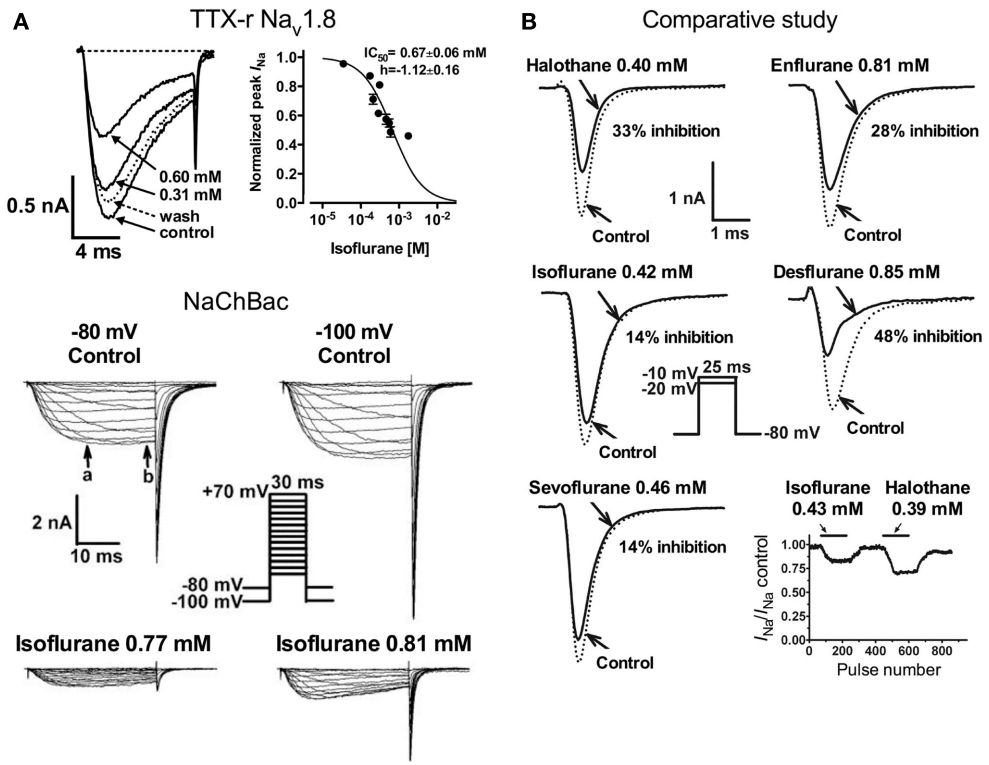

FIGURE 3 | [**(A)**, upper panel] Concentration-dependent inhibition of tetrodotoxin-resistant (TTX-r) $Na_v1.8$ by isoflurane. Current traces of TTX-r $Na_v1.8$ are shown in the absence or presence of two isoflurane concentrations. Normalized peak I_{Na} values for TTX-r $Na_v1.8$ were fitted to the Hill equation to yield IC_{50} values and Hill slopes (Herold et al., 2009). [**(A)**, lower panel] Effects of isoflurane on NaChBac expressed in HEK293 cells. Families of current traces are shown at two different holding potentials (V_h) in the absence or presence of isoflurane. Isoflurane significantly inhibited I_{Na} from a V_h of either −80 or −100 mV (Ouyang et al., 2007). **(B)** Inhibition of $Na_v1.4$ by equipotent concentrations of various inhaled anesthetics. Peak I_{Na} were recorded from a holding potential of –80 mV by 25-ms test steps as shown in the *inset*. The effects of clinically equipotent concentrations of halothane, isoflurane, sevoflurane, enflurane, and desflurane are shown in these representative traces. Desflurane had the greatest effect of peak I_{Na} reduction.

intravenous anesthetic propofol inhibits Na^+ channels only at supratherapeutic concentrations (Rehberg and Duch, 1999).

The prototypical halogenated ether isoflurane also inhibits the prokaryotic voltage-gated Na^+ channel of *Bacillus halodurans* (NaChBac; Ouyang et al., 2007; **Figure 3A**, lower panel). This was the first prokaryotic channel shown to be inhibited by an anesthetic, and demonstrates impressive evolutionary conservation of the mechanism responsible for this pharmacological effect. As with mammalian channels, inhibition of peak I_{Na} was concentration- and voltage-dependent, and was associated with a positive shift in the voltage-dependence of activation and a negative shift in the voltage-dependence of steady-state fast-inactivation. Furthermore use-dependent block occurred due to slowed recovery from inactivation. Despite the evolutionary difference between prokaryotic and eukaryotic voltage-gated Na^+ channels, the mechanisms by which volatile anesthetics act on the channel seem remarkably similar.

Aromatic compounds such as fluorobenzene, hexafluorobenzene, and 1,2-difluorobenzene have been shown to inhibit $Na_v1.2a$ expressed in *Xenopus* oocytes. Inhibition of peak I_{Na} as well as a shift in the $V_{1/2}$ of fast-inactivation occurs in an agent-dependent manner (Horishita et al., 2008). The exact mechanism of the differential effects of these structurally different compounds has yet to be elucidated. Differences also exist in the potency of volatile anesthetic inhibition of specific Na^+ channel subtypes (Ouyang et al., 2009), but again the mechanisms for these differences have to be studied in more detail. Such differences might underlie region-specific presynaptic effects of volatile anesthetics on neurotransmitter release (Westphalen et al., 2010, 2011).

Na^+ CHANNEL INHIBITION LEADS TO INHIBITION OF NEUROTRANSMITTER RELEASE BY ANESTHETICS

A physiological consequence of presynaptic Na^+ channel inhibition is depression of presynaptic action potential generation and conduction. Considerable evidence indicates that volatile anesthetics inhibit neurotransmitter release, and that this is due in part to inhibition of presynaptic Na^+ channels. Volatile anesthetics preferentially inhibit 4-aminopyridine (4AP)-evoked release of glutamate compared to GABA from isolated rat cortical nerve terminals (Westphalen and Hemmings, 2006). Action potential-evoked depolarization and release can be pharmacologically mimicked by 4AP, a K^+ channel blocker, while Na^+ channel independent release can be elicited by depolarization with elevated extracellular K^+ (Tibbs et al., 1989). Using this approach, 4AP-evoked

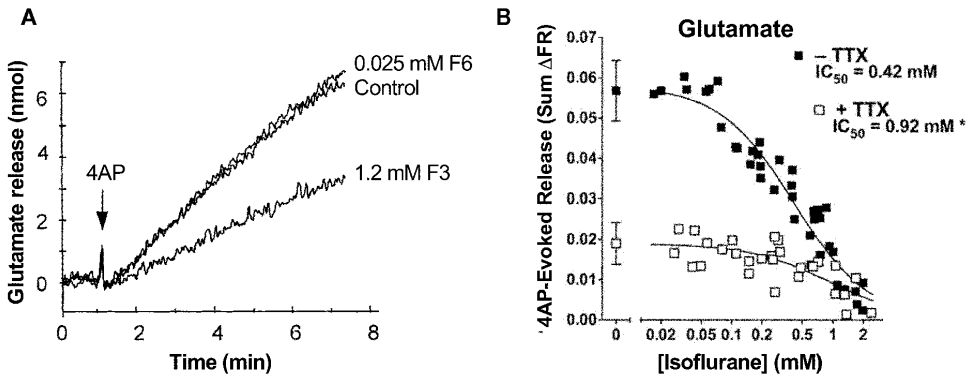

FIGURE 4 | Volatile anesthetics inhibit neurotransmitter release in nerve terminals. (A) Effects of the anesthetic compound F3 and the non-immobilizer F6 on 4-aminopyridine- (4AP) evoked glutamate release from cortical synaptosomes. The anesthetic cyclobutane F3 significantly inhibits glutamate release, whereas the non-anesthetic (non-immobilizer) cyclobutane F6 shown no inhibitory effect (Ratnakumari et al., 2000). **(B)** Isoflurane inhibition of 4AP-evoked glutamate release from rat cortical nerve terminals in the absence or presence of tetrodotoxin (TTX, 1 μM). The potency of isoflurane inhibition is much greater in the absence of the Na^+ channel blocker TTX indicating a strong involvement of Na^+ channels in inhibition of neurotransmitter release by volatile anesthetics (Westphalen et al., 2011).

release is significantly more sensitive to inhibition by volatile anesthetics as compared to KCl-evoked release, supporting a role for blockade of presynaptic Na^+ channels in the inhibitory effects of the anesthetics (Schlame and Hemmings, 1995; Westphalen and Hemmings, 2003). Interestingly, inhibition of glutamate release occurs with about 50% greater potency than inhibition of GABA release, consistent with pharmacologically relevant transmitter-specific specializations in neurotransmitter release regulation, perhaps involving differential coupling to Na^+ channels (Westphalen et al., 2010; **Figure 4B**). There is also evidence that volatile anesthetics inhibit neurotransmitter release in a brain region-specific manner (Westphalen et al., 2011), which suggests diversity in presynaptic Na^+ channel subtype expression and/or coupling to release (Westphalen et al., 2010).

Further experiments have examined the effects of volatile anesthetics on synaptic vesicle exocytosis, detected using fluorescence imaging, in cultured rat hippocampal neurons. This preparation allows electrical stimulation of release, and showed concentration-dependent and reversible inhibition of action potential-evoked exocytosis by isoflurane. Involvement of presynaptic Na^+ channels is supported by the observation that exocytosis, evoked by depolarization with elevated extracellular K^+ (which is insensitive to TTX), was relatively insensitive to isoflurane (Hemmings et al., 2005a). Isoflurane has also shown to inhibit excitatory postsynaptic currents (EPSCs) in the rat calyx of Held due to inhibition of neurotransmitter release caused by a reduction of presynaptic action potential amplitude (Wu et al., 2004). These effects of volatile anesthetics on synaptic transmission result primarily from inhibition of action potential-evoked synaptic vesicle exocytosis, most likely as a result of Na^+ channel blockade upstream of Ca^{2+} entry and exocytosis.

In vivo studies on rodents have implicated spinal Na^+ channels in immobilization, a major component of general anesthesia. Intravenous infusion of lidocaine, a classical local anesthetic, or intrathecal administration of riluzole, another potent Na^+ channel inhibitor, significantly increases the potency of volatile anesthetics as immobilizers (Xing et al., 2003; Zhang et al., 2007). The role of Na^+ channels in volatile anesthetic-mediated immobility is further supported by the observation that intrathecal infusion of the Na^+ channel activator veratridine, a plant neurotoxin that binds to site 2 and stabilizes the open state (Ulbricht, 1998), reduces the potency of isoflurane (Zhang et al., 2008), while intrathecal infusion of TTX increases the potency of isoflurane, and reverses the effect of veratridine (Zhang et al., 2010). Taken together, these results indicate that inhibition of spinal voltage-gated Na^+ channels by inhaled anesthetics is likely an important mechanism in anesthetic immobility.

NON-ANESTHETIC EFFECTS OF VOLATILE ANESTHETICS

A major side effect of volatile anesthetics is cardiovascular depression. Multiple ion channel types expressed in cardiomyocytes contribute to action potential conduction and myocardial contractility. Inhibition of L-type Ca^{2+} currents or voltage-gated transient and sustained outward K^+ currents by volatile anesthetics can lead to reduced contractility and delayed repolarization with mismatch of action potential duration (Huneke et al., 2004). In cardiac Na^+ channels ($Na_v1.5$), volatile anesthetics at clinically relevant concentrations inhibit peak I_{Na} and affect steady-state fast- as well as slow-inactivation (Stadnicka et al., 1999; Ouyang and Hemmings, 2007). This can, in combination with other cardiodepressant drugs, slow conduction and lead to tachyarrhythmias. Na^+ channels have also been implicated as potential targets for neuroprotection by volatile anesthetics (Hemmings, 2004). The possible role of voltage-gated Na^+ channels and other beneficial and detrimental side effects of volatile anesthetics in brain and other organs cannot be excluded.

CONCLUSION

Both electrophysiological and functional studies indicate that presynaptic voltage-gated Na^+ channels are inhibited by clinically used concentrations of volatile anesthetics. This leads to reductions in evoked neurotransmitter release that is both brain

region and neurotransmitter selective. The selective inhibition of glutamate release underlies a reduction in excitatory synaptic transmission with resultant nervous system depression. Detailed information regarding the presynaptic localization, function, and regulation of specific Na^+ channel subtypes is currently lacking. Further studies are necessary to identify the roles of specific presynaptic Na^+ channel subtypes in mediating neurotransmitter release and its inhibition by volatile anesthetics and other Na^+ channel inhibitors.

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Neurotoxins and their binding areas on voltage-gated sodium channels

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Voltage-gated sodium channels (VGSCs) are large transmembrane proteins that conduct sodium ions across the membrane and by doing so they generate signals of communication between many kinds of tissues. They are responsible for the generation and propagation of action potentials in excitable cells, in close collaboration with other channels like potassium channels. Therefore, genetic defects in sodium channel genes can cause a wide variety of diseases, generally called "channelopathies." The first insights into the mechanism of action potentials and the involvement of sodium channels originated from Hodgkin and Huxley for which they were awarded the Nobel Prize in 1963. These concepts still form the basis for understanding the function of VGSCs. When VGSCs sense a sufficient change in membrane potential, they are activated and consequently generate a massive influx of sodium ions. Immediately after, channels will start to inactivate and currents decrease. In the inactivated state, channels stay refractory for new stimuli and they must return to the closed state before being susceptible to a new depolarization. On the other hand, studies with neurotoxins like tetrodotoxin (TTX) and saxitoxin (STX) also contributed largely to our today's understanding of the structure and function of ion channels and of VGSCs specifically. Moreover, neurotoxins acting on ion channels turned out to be valuable lead compounds in the development of new drugs for the enormous range of diseases in which ion channels are involved. A recent example of a synthetic neurotoxin that made it to the market is ziconotide (Prialt®, Elan). The original peptide, ω-MVIIA, is derived from the cone snail *Conus magus* and now FDA/EMA-approved for the management of severe chronic pain by blocking the N-type voltage-gated calcium channels in pain fibers. This review focuses on the current status of research on neurotoxins acting on VGSC, their contribution to further unravel the structure and function of VGSC and their potential as novel lead compounds in drug development.

Keywords: voltage-gated sodium channel, neurotoxin, binding site

VOLTAGE-GATED SODIUM CHANNELS

Like many other voltage-gated ion channels, VGSCs are transmembrane complexes consisting of a large core protein, the α-subunit (220–260 kDa, ≈2000 amino acids), associated with one or more smaller regulatory β-subunits (22–36 kDa). Alpha-subunits contain the functional ion conduction pore as an aqueous cavity that is selectively permeable for sodium ions. In mammalian cells, nine α-subunit isoforms (classified as Na_v 1.1–Na_v 1.9) have been characterized so far. Additionally, sodium channel-like proteins, classified as Na_x, have been identified but are not yet functionally expressed (Catterall et al., 2005). The VGSC isoforms are distributed differentially throughout electrical excitable cells of the body, which correlates with different functional properties in the corresponding tissues. Na_v1.1, 1.2, 1.3, and 1.6 are mainly expressed in the central nervous system (CNS); Na_v1.7, 1.8, and 1.9 on the contrary are highly expressed in the peripheral nervous system (PNS) and finally, the Na_v1.4 and 1.5 isoforms are present in adult skeletal muscle and heart muscle, respectively (Goldin, 2001).

In contrast to potassium channels, no crystallographic image of a sodium channel could be assessed for a long time and information about the structural composition of the VGSC had to be deduced in an indirect way. Recently however, the group of Catterall enlightened the horizon with the determination of the crystal structure of a bacterial VGSC (Payandeh et al., 2011). In early molecular cloning studies, later confirmed by cryo-electron images (Sato et al., 2001) and the recent crystallographic image, the α-subunit turned out to be composed of four homologous domains, DI–DIV, which all contain six putative transmembrane segments, S1–S6 (Yu and Catterall, 2003). The four domains are connected by three cytoplasmic linker loops of different size and together they form a bell-shaped protein (Sato et al., 2001).

All of the four domains consist of two modules, the first being the voltage-sensing module formed by S1–4, the second being the pore-forming module, formed by S5 and S6 and the connecting loop. How the voltage-sensing module can "sense" voltage and thereby open the channel is a question that can be answered by looking at one particular segment of this module, the voltage sensor S4. These cylindrical α-helical structures display highly conserved positive residues at every third position. In the "sliding helix" (Catterall, 1986) or "helical screw" (Guy and Seetharamulu,

1986) models these positive charges are proposed to be drawn into the membrane by the negative internal resting membrane potential. The positive charges are stabilized by the formation of neutralizing ion pairs with neighboring negative charges in S1, S2, and/or S3. When the membrane depolarizes, the negative membrane potential is relieved and the S4 segments move outward, thereby causing a conformational change that opens the pore and initiates channel activation (Catterall, 2000, 2010).

Another question that has to be answered when discussing VGSC's function is how ion selectivity can be achieved. The answer lies in the pore region of the channel, formed by S5 and S6 and the connecting pore-loop (P-loop). By dipping into the membrane, the P-loops of DI–DIV form the outer pore of the channels. Herein, two rings determine ion selectivity. The inner selectivity ring is formed by the four amino acids Asp, Glu, Lys, and Ala in DI, DII, DIII, and DIV, respectively (DEKA-ring). The outer selectivity ring is formed in the same way and is represented by the residues Glu, Glu, Asp, and Asp of DI, DII, DIII, and DIV, respectively (EEDD-ring). The first studies that underlie the discovery of the DEKA-ring made use of the historical toxins tetrodotoxin (TTX) and saxitoxin (STX). Their mechanism of action and binding site is discussed in the following chapter. The intracellular part of the pore is formed by all four S6 segments (Yu and Catterall, 2003).

Within a few milliseconds after the opening of the channel, fast inactivation begins. This key feature was the third characteristic set by Hodgkin and Huxley when they first recorded sodium currents (Hodgkin and Huxley, 1952), besides the voltage-dependent activation and selective ion conductance. The part of the channel responsible for fast inactivation is the short, highly conserved intracellular linker that connects domains III and IV. In mutagenesis studies, the three hydrophobic amino acids Ile, Phe, and Met (IFM motif) turned out to be the key sequence necessary for fast inactivation. The inactivation gate receptor for this IFM motif is located within and near the inner mouth of the pore (Smith and Goldin, 1997). Mutations in the S4 segments, and more specifically S4 of DIV revealed that outward movement of S4 DIV initiates fast inactivation, which makes the inactivation process also voltage-dependent. This model was largely confirmed by studies with α-scorpion toxins and sea anemone toxins, of which some will be discussed later. Another mode of VGSC inactivation was described as slow inactivation and occurs in a time scale of seconds after activation, but still remains the topic of further investigations (Ulbricht, 2005).

The cytoplasmic linker loops between the four domains continue to be interesting sites at which the channel's modulation and regulation of gating processes happen. Several phosphorylation sites were found in the first and third intracellular linkers between DI–DII and DIII–DIV, respectively. Phosphorylation can be carried out by isoforms of protein kinase A (PKA) or C (PKC) and has different results on the channels' function depending on the kinase or affected VGSC isoform. An ankyrin binding site has also been identified in the linker between DII and DIII, which also may influence the channels' gating kinetics (Chahine et al., 2005).

The large α-subunit is associated with one or more smaller regulatory β-subunits, which modulate sodium currents and also regulate the cell surface expression of channels (Patino and Isom, 2010). They turned out to be essential pieces of the VGSC complexes as they are needed in the heterologous expression of α-subunits to display full native sodium channel characteristics, such as correct kinetics and voltage-dependence of the gating (Yu and Catterall, 2003). At least five regulatory β-subunits (β1–β4 and β1A) have been identified so far from four different genes (*SNC1B–4B*), with β1A as a splice variant of the β1-subunit. The β-subunits can be bound either non-covalently (β1- and β4-subunits) or covalently (β2- and β4-subunits) to form a heteromer with the α-subunit (Chahine et al., 2005).

NEUROTOXINS AND VGSC PHARMACOLOGY

NEUROTOXINS

From the early beginning of research on the basic principles of ion channel functioning, neurotoxins fulfilled an important job, especially concerning VGSCs. One particular neurotoxin that deserves special attention is tetrodotoxin (TTX). It was first isolated from the pufferfish (family of Tetraodontidae) and has a close relative: saxitoxin (STX). TTX played a great part in the discovery of the VGSC protein (Agnew et al., 1980) and in the determination of the selectivity filter (Heinemann et al., 1992) and binding site 1 for pore blockers of VGSCs (Noda et al., 1989). Nowadays TTX is still used to classify sodium channels according to their sensitivity to TTX: $Na_v1.5$, $Na_v1.8$, and $Na_v1.9$ are TTX-resistant (TTX-R), the others are TTX-sensitive (TTX-S).

The name "neurotoxin" already reveals at what those natural chemicals and peptides are best: interfering with the normal function of the nervous system. That is exactly what plants, animals, and microorganisms develop them for: to target a possible offender or prey, either by immobilizing or immediately killing it. Neurotoxins produced by those organisms mostly form part of a mixture of peptides and enzymes that act in concert. This mixture can be a venom (peptides and proteins, actively injected and used in defense or prey acquisition) or a poison (secondary products or metabolites, passively used to protect or defend the organism; Mebs, 2002). But their main target is always the CNS, PNS as herein lie the vital functions of the offender/prey. During evolution, venomous and poisonous organisms turned out to be excellent pharmacologists since their toxins evolved to be highly specific for targets in the CNS or PNS. They can act on membrane receptors and ion channels like voltage-gated sodium, potassium, and calcium channels. By doing so they give us outstanding keys to investigate the structure and function of their targets, to define underlying physiological processes, and most importantly, to design new, potent, and selective drugs useful in a wide variety of diseases like pain, diabetes, and multiple sclerosis (Lewis and Garcia, 2003). As an example of a rich and nearly unexplored library of valuable bioactive compounds, one can look at cone snails, which are venomous marine invertebrates. More than 700 species are known to date and it is estimated that a single *Conus* species may contain up to 200 different venom peptides, implying that a library of more than 140,000 compounds of possible pharmaceutical interest is available in their venom. At least one compound has led to a new therapy for severe chronic pain (ziconotide/Prialt®) and many other lead compounds can be suspected from these and other venomous and poisonous organisms (Terlau and Olivera, 2004).

NEUROTOXIN BINDING

Neurotoxins acting on VGSCs can aim at six different sites in the channels, distinguished not only by matters of localization of the toxin binding place but also by the results of the toxin's action. Apart from the neurotoxin receptor sites, two other sites are determined. The pyrethroid binding site is the binding site for some insecticidal agents like DDT and pyrethroids. Anticonvulsants and antiarrhythmics bind in a use-dependent manner to the local anesthetics (LA) site (**Table 1**). The latter two will not be discussed in this review because they are not affected by neurotoxins and therefore, are not in the scope of this review.

The interaction between neurotoxins and VGSCs can occur in two different ways. It either results in a pore block when the toxin physically occludes the pore and thereby inhibits the sodium conductance, or in a modification of the gating, which leads to altered gating kinetics and voltage-dependence of the channels. Toxins binding on site 1 use the first mechanism. The previously mentioned guanidinium toxins TTX and STX are such site 1 pore blockers; they will form a plug in the outer vestibule of the pore. Site 2 toxins like batrachotoxin and grayanotoxin will prevent inactivation and therefore persistently activate the channel. Scorpion α-toxins and sea anemone toxins are typical examples of site 3 toxins; they will slow or inhibit inactivation. Scorpion β-toxins and β-spider toxins bind to site 4 and shift the voltage-dependence of activation toward more hyperpolarized potentials. Site 5 neurotoxins like brevetoxins and ciguatoxins exhibit a real arsenal of effects upon VGSC binding, e.g., inhibition of activation and a hyperpolarizing shift of the voltage-dependence of activation. Lastly, δ-conotoxins acting on site 6 will cause similar effects as site 3 toxins by slowing or inhibiting inactivation (**Figure 1** and **Table 1**).

As mentioned previously, toxins can be differentiated based on their chemical composition. Organic hydrophilic or lipophilic neurotoxin compounds are mostly secondary metabolites, used by the producing organism as defense molecules. Examples of lipophilic compounds include the site 2 grayanotoxins and the brevetoxins acting on site 5. Hydrophilic secondary metabolites include site 1 toxins TTX and STX. On the other hand, organisms can use larger chemical entities like peptides to paralyze or kill their prey, like the peptides found in scorpions, spiders, wasps, cone snails, and others. But although their origin might be different, both kinds of neurotoxins can have the same effect (binding of μ-conotoxins and TTX for both results in block of the Na^+ conductance). For other reviews on neurotoxins acting on VGSCs see for example (Catterall et al., 2007; de la Vega and Possani, 2007; Billen et al., 2008; Andavan and Lemmens-Gruber, 2011).

NEUROTOXIN BINDING SITE 1

Among all putative binding sites on VGSCs, site 1 is probably the best defined and most straightforward of all sites. It is composed by residues at the reentrant P-loops connecting S5 and S6 of all four domains. The historical guanidinium molecules TTX and STX were the first neurotoxins shown to bind at this site. Upon their binding, Na^+ conductance is blocked. They are produced by some bacteria and dinoflagellate species, respectively (Narahashi, 2008; Chau et al., 2011). Later on, the μ-conotoxins found in cone snails, also turned out to bind to this specific site and to cause the same effects. At first it was thought that both types of toxins would interact with exactly the same residues but it seems to be a more complex interplay, as some mutations in $Na_v1.4$ channels affecting TTX binding did only affect binding of a μ-conotoxin to a minor extent. Therefore, it was suggested that TTX and μ-conotoxins share an overlapping but non-identical binding site (Stephan et al., 1994). In this model, the core of the binding site is situated more in the inner side of the pore mouth and can be occupied by TTX

Table 1 | Overview of neurotoxin binding sites according to the revised model.

Site	Neurotoxins	Examples	Peptide	Main binding area known up to date	Result
1	Guanidinium toxins	TTX, STX	–	DI–IV P-loop	Block of Na^+ conduction
	μ-Conotoxins	KIIIA, SIIIA, PIIIA	x		
2	Small lipid-soluble toxins	Batrachotoxin	–	DI DIV S6	Negative shift in voltage-dependency of activation
		Veratridine	–		Slowing down of inactivation
		Grayanotoxins	–		Block of Na^+ conductance
		Aconitine	–		Altering ion selectivity
3	Scorpion α-toxins	AaH II, LqhaIT, BMK M1	x	DIV S3–S4	Slowing down of inactivation
	Sea anemone toxins	ATX-II, AFTII	x		
4	Scorpion β-toxins	Css4, Tsγ, AahIT	x	DII S3–S4	Negative shift in voltage-dependency of activation
	Spider β-toxins	Magi 5, HWTX-IV	x		Block of Na^+ conductance
	μO-conotoxins	MrVIA	x		
5	Cyclic polyether compounds	Brevetoxins	–	DI S6	Negative shift in voltage-dependency of activation
		Ciguatoxins	–		Slowing down of inactivation
6	δ-Conotoxins		x	DIV S4	Slowing down of inactivation
7	Pyrethroids	DDT, Deltamethrin	–	DII–DIII	Slowing down of inactivation
LA	Local anesthetics	Lidocaine	–	DIV S6	Block of Na^+ conduction
	Anticonvulsants		–		
	Antiarrhythmics		–		
	Antidepressants		–		

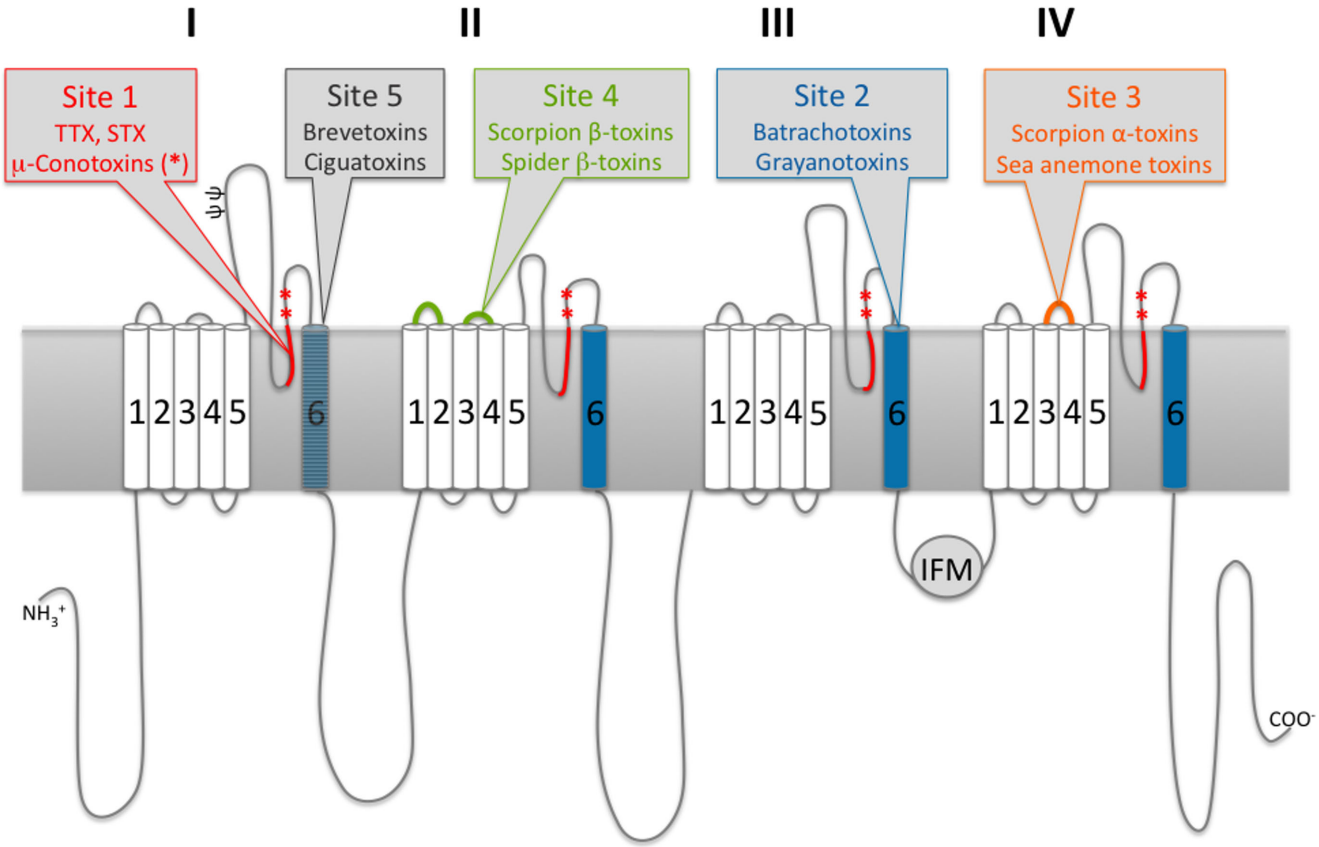

FIGURE 1 | Schematic two-dimensional representation of the functional α-subunit of voltage-gated ion channels and identification of known neurotoxin binding areas.

or STX as well as μ-conotoxins, the latter interacting with some extra residues laying at the outer vestibule of the pore. Recently, another TTX/μ-conotoxin binding model was proposed in which TTX, STX, and the μ-conotoxins were suggested to be "syntoxins," acting on adjacent sites in the VGSCs and influencing each other's binding kinetics. Zhang et al. (2009) first proposed the simultaneous binding of TTX and KIIIA, a μ-conotoxin from *Conus kinoshitai*. Addition of both toxins to oocytes expressing $Na_v1.2$ leads to the formation of a bi-liganded, ternary complex of Na_v·TTX/alkaloid·μ-conotoxin. If KIIIA binds first to the channel, this will slow the subsequent binding of TTX at its specific binding place, further down into the channel; and the other way around. But in another series of experiments done by the same group, this appeared not to be completely true for STX and its sulfated congener GTX2/3. Another, extended model was suggested, wherein the binary complex Na_v·μ-conotoxin could flicker between a permissive state, in which the alkaloid can "sneak" by the μ-conotoxin, and an unpermissive state (French et al., 2010; Zhang et al., 2010). Because STX has an extra positive charge compared with TTX, this could lead to electrostatic repulsion. The authors also checked this model with a docking model based on the current homology model for VGSCs. This was developed by Lipkind and Fozzard (2000), based on the crystal structure of the bacterial potassium channel KcsA. Docking of TTX and KIIIA was consistent with their experimental results; both molecules do fit simultaneously in the vestibule.

The VGSC homology model previously turned out to be valuable to define the structure of the outer channel vestibule (Cervenka et al., 2010). Although the outer vestibule was long time believed to be a rigid structure, this does not correspond to the large amount of studies that predict a highly flexible P-loop that even might undergo conformational changes that are linked to gating transitions upon binding of TTX or μ-conotoxins. This can be deduced, amongst other things, from mutagenesis studies examining the effects of mutations in this region. Those mutations mostly correlated with alterations in gating kinetics and more specifically decreased (mutations in the P-loop of DI) or enhanced inactivation (mutations in the P-loop of DIV; for a review see Cervenka et al., 2010). The latter can be explained by the proximity of DIV S6 to the P-loop of DIV, as DIV S6 plays an important role in the inactivation process. Moreover, this DIV S6 segment is supposed to enclose the binding site of LA, which on its turn connects site 1 and 6. Site 1 toxins such as μ-conotoxins can also influence the activation process by interacting with the voltage sensors. μ-Conotoxins such as KIIIA and GIIIA are strongly cationic peptides. Upon binding at site 1, these peptides electrostatically impede the outward movement of the positively charged residues in the S4 voltage sensor segments, which is a necessity for channel

activation. The electrostatic repulsion, induced by binding of the toxin at site 1, most probably stabilizes the closed state, resulting in channels which will open at more depolarized membrane potentials (French et al., 1996; Van Der Haegen et al., 2011).

Another neurotoxin believed to affect site 1 is Tx1 from *Phoneutria nigriventer* (Martin-Moutot et al., 2006). Though binding of labeled Tx1 was not inhibited by TTX, it was inhibited by the μ-conotoxin GIIIB (*Conus geographus*). Therefore it was suggested that Tx1, like TTX, binds to a micro site and that the binding site of μ-conotoxins overlaps the micro sites of TTX and Tx1. So far, only one other spider toxin is known to interact with binding site 1, that is Hainantoxin-I (HNTX-I) from *Ornithoctonus hainana* (Li et al., 2003). Other spider toxins like Huwentoxin-IV (HWTX-IV) from *Ornithoctonus huwena* and some Hainantoxins (HNTX III-V) were at first considered to be potential site 1 neurotoxins as they inhibit Na^+ conductance, an effect concerned to be typical for site 1 (Li et al., 2003; Nicholson, 2007). The same is valid for μO-conotoxins; but this hypothesis had to be adjusted as evidence occurred that these toxins do not bind to residues at site 1 but at residues in S3–S4 of DII (Leipold et al., 2007). Intriguingly, these residues form part of what is generally determined as "site 4." Together with other recent similar findings concerning the other "traditional" neurotoxin binding sites, this prompted us to consider a revision of the binding sites. Therefore the latter toxins will be discussed into more detail in the section about the revision of the binding sites.

NEUROTOXIN BINDING SITE 2

This site is targeted by a wide array of lipid-soluble toxins with greatly diverting chemical structures. Their structural non-relatedness is mirrored in their diverse source of origin as they can be found in plants, animals, and bacteria. Well-known examples of site 2 toxins from plants are alkaloids like veratridine (VTD; from *Liliaceae*) and aconitine (*Aconitum napellus*) and grayanotoxins (GTX) from *Ericaceae*. Batrachotoxin (BTX) and homologs are site 2 toxins produced by animals such as frogs (*Phyllobates* spp.) and birds (*Pitohui* and *Ifrita* spp.; Dumbacher et al., 2000). Recently, antillatoxin and hoiamide were isolated from some cyanobacteria (*Lyngbya majuscula*) and were found to bind to neurotoxin receptor site 2 (Pereira et al., 2009; Cao et al., 2010).

Site 2 toxins are known as activators as they modulate sodium channels in such a way that the channels open more easily and stay open longer. Activators preferentially bind to channels in the open state and their binding leads to Na_v channels with a unique and complex behavior (Hille, 2001). Several channel properties are altered upon site 2 binding: (i) the voltage-dependence of activation is shifted toward more negative potentials causing channels to open at resting potentials; (ii) the inactivation is slowed down or inhibited resulting in sustained, non-inactivating currents; (iii) the sodium conductance through toxin-bound channels is reduced; (iv) the ion selectivity of modified channels is altered due to a decreased discrimination for permeating ions (Tikhonov and Zhorov, 2005; Du et al., 2011). Numerous studies have been conducted to map the neurotoxin site 2 and to provide a better understanding of the molecular determinants responsible for these intriguing channel gating alterations upon toxin binding. Studies using photo-labeled BTX identified the first residues within the inner helixes of DI S6 (I433, N434, and L437) and DIV S6 (F1579 and N1584) that are crucial for BTX binding in $Na_v1.4$ (Wang and Wang, 1998, 1999). The key role of these residues in BTX binding was confirmed as point mutations of the equivalent residues in $Na_v1.5$ rendered these channels insensitive to BTX (Wang et al., 2007). Although site 2 is believed to be localized mainly at the S6 of DI and DIV, site-directed mutagenesis studies have shown that specific amino acid residues among all four S6 segments are contributing to the neurotoxin receptor site 2 (Wang et al., 2000, 2001). The exact location of all known molecular determinants contributing to the BTX binding site has recently been well summarized (Du et al., 2011).

The observed channel gating alterations upon binding to site 2 used to be explained by an allosteric model in which the lipid-soluble toxins bind at lipid-exposed sites distinct from the pore or the voltage sensors (Catterall et al., 2007). Even though this model of allosteric interactions provided a reasonable interpretation of modifications in ion selectivity, channel gating, and conductance at the time, an increasing number of studies have emerged the necessity to revise this model. Although the exact molecular mechanism of action is still poorly understood, there is increasing evidence suggesting that these toxins bind within the pore rather than at the lipid-exposed channel interface (Tikhonov and Zhorov, 2005; Du et al., 2011). Recently, a new model, which is consistent with most studies on BTX binding, has been proposed (Du et al., 2011). In this model, activators such as BTX bind within the inner pore with residues in the S6 segments of all four domains, exposing the activator directly to the permeation pathway. This model confirms the previous hypothesis that the observed inhibition of inactivation might be due to BTX interaction with the above-described residues in S6 of DIV. The DIV S6 segment is not only involved in fast inactivation, as it can also be seen that altered movements of toxin-bound S6 segments influence the movement of adjacent segments. Therefore, it is possible that BTX binding alters the voltage-dependent movement of the DIV S4 voltage sensor and thereby modifies channel activation and its coupling to inactivation (Linford et al., 1998; Catterall et al., 2007). The reduced sodium conductance can be seen as the outcome of a narrower Na^+ binding site due to the presence of a BTX molecule in the inner pore. In contrast, the altered ion selectivity could be a direct consequence of a wider selectivity filter in modified channels. It has been well established that the DEKA locus within the selectivity filter determines the sodium channel selectivity. Moreover, K1422 can be seen as a key residue since a point mutation of this residue into a glutamic acid conferred calcium conducting characteristics onto sodium channels (Heinemann et al., 1992). The new BTX binding model suggests that BTX does not interact directly with the DEKA locus but rather causes a deficiency of water molecules in the proximity of the selectivity filter. The displacement of water molecules may lead to a shift in the PKA of the ion selectivity-determining residue K1422 and in this way, lower the discrimination in permeating ions (Du et al., 2011). Although this model is consistent with most available experimental data, it should be noted that there are limitations to homology models and that more experiments are required to confirm the theoretical analysis of this BTX binding model.

Several studies have contributed in locating the binding site for the lipid-soluble grayanotoxins. Similar to the BTX binding residues, it was found that S251, I433, N434, L437, I1575, F1579 on $Na_v1.4$ and their equivalents on the cardiac sodium channel $Na_v1.5$ are involved in the binding of GTXs (Ishii et al., 1999; Kimura et al., 2001). Mutation of one specific residue at position 1586 in DIV S6 completely abolished grayanotoxin-induced effects on $Na_v1.4$ channels. Remarkably, the same mutation did not alter BTX binding (Kimura et al., 2000). Altogether it can be concluded that the GTX binding site is not completely identical to the BTX binding site but is overlapping as they share numerous molecular determinants.

In contrast with BTX, the channel modulation by veratridine is less investigated. In general it is assumed that VTD binds at the same site as BTX since VTD induces channel alterations that are similar to these of BTX (Wang and Wang, 2003). However, contrary to BTX, which does not dissociate from its receptor, VTD binding is reversible and it does dissociate from its receptor upon membrane hyperpolarization (Ulbricht, 1998). More recently, a bell-shaped relationship was described between the concentration of veratridine and the sodium current peak amplitude in murine vas deferens myocytes. It was observed that increasing concentrations of VTD enhance the peak amplitude, reaching a maximum around 10 μM while higher concentrations of VTD reduced the sodium conductance (Zhu et al., 2009).

The effects of aconitine still remain poorly studied. However, it is known that aconitine binding causes an incompletion of inactivation and an alteration of the ion selectivity in muscle VGSCs but not in nerve fibers (Campbell, 1982; Wang and Wang, 2003).

NEUROTOXIN BINDING SITE 3

Neurotoxins binding to site 3 include members of different phyla of the kingdom of Animalia, among which major players are toxins from scorpions, sea anemones, and spiders. In fact, binding site 3 was first determined by radiolabeling and mutagenesis studies performed with α-scorpion and sea anemone toxins (Tejedor and Catterall, 1988; Rogers et al., 1996). Scorpion toxins affecting the gating of VGSCs are historically classified into α- and β-toxins according to the effects that they cause. β-Scorpion toxins cause a strong hyperpolarizing shift in the voltage-dependence of activation, which is linked to neurotoxin binding site 4 and will be discussed in the following chapter. α-Scorpion toxins are long polypeptides of 60–70 amino acids and can be further subdivided into three groups according to their phylogenetic specificity (Gordon et al., 1996; Hamon et al., 2002). Classical α-scorpion toxins or anti-mammalian toxins will inhibit inactivation of mammalian VGSCs and have low affinity for insect neuronal membranes. Well-known examples are AaH II (*Androctonus australis* Hector; Jover et al., 1978) and Lqh II (*Leiurus quinquestriatus hebraeus*; Sautiere et al., 1998). Anti-insect α-scorpion toxins like LqhaIT (Eitan et al., 1990) only show minor activity against mammalian brain preparations but do show significantly higher inhibition of insect VGSCs. Finally, α-like scorpion toxins bind to both rat brain and insect VGSCs (e.g., BMK M1 (Hamon et al., 2002; Sun et al., 2003).

The second class of toxins that are famous for inhibiting VGSC inactivation is found in the venom of sea anemones. Sea anemone toxins targeting VGSCs are subdivided into several classes, but unlike α-scorpion toxins, this classification is not based on their phylogenetic preferences but on their amino acid sequences. Norton (1991) first proposed three classes to which a sea anemone toxin can belong. Type 1 and 2 toxins include larger peptides, composed of 46–49 amino acids and 3 disulfide bridges. Type 3 toxins are shorter peptides, composed of 27–30 amino acids only (Honma and Shiomi, 2006; Bosmans and Tytgat, 2007; Shiomi, 2009). Besides toxins from scorpions and sea anemones, site 3 is also targeted by toxins from other animals like spiders (e.g., δ-atracotoxins, Nicholson et al., 2004; Tx4(6-1), de Lima et al., 2002; and Magi 4, Corzo et al., 2003) and wasps (e.g., β-PMTX, Schiavon et al., 2010).

Parts of the VGSCs that were first identified to be involved in the binding of site 3 neurotoxins were located in the extracellular loops between S5 and S6 in DI and DIV (Thomsen and Catterall, 1989). Later on, residues in the extracellular loop between S3 and S4 in DIV also turned out to be involved in the binding of site 3 neurotoxins (Rogers et al., 1996). Fluorescence labeling studies indicated that site 3 neurotoxins stabilize the voltage sensor S4 of DIV in its deactivated position, thereby inhibiting its movement (Campos et al., 2008). As S4 of DIV is known to be involved in the voltage-dependent coupling between activation and fast inactivation (Chahine et al., 1994; Sheets and Hanck, 1995), it is logic that neurotoxin binding to this site causes an impairment of the fast inactivation. The inactivation can be slowed or even completely abolished and these effects can be associated with a minor hyperpolarizing shift in the activation. It was also shown that site 3 neurotoxins enhance the recovery from inactivation (e.g., work with the α-scorpion toxin Ts3 from *Tityus serrulatus*, Campos et al., 2004; and Anthopleurin B from the sea anemone *Anemonia xanthogrammica*, Sheets and Hanck, 1995). Another important characteristic is the voltage-dependency of the binding of these neurotoxins, such that they bind to a lesser extent at more depolarizing potentials (Catterall, 1977).

Site 3 neurotoxins turned out to be interesting tools for the investigation of gating currents, which are small transient currents that occur by movement of gating charges, mostly located on the S4 segments of the channel (Bezanilla, 2000). Via measurements of gating currents one can obtain much information about the S4 voltage sensors in ion channels and their contribution in sensing membrane potential. Sea anemone toxins ATX-II from *Anemonia sulcata* (Neumcke et al., 1985) and Anthopleurin A from *Anemonia xanthogrammica* (Sheets and Hanck, 1995) both reduced the maximum gating charge of VGSC and via mutagenesis studies it could nicely been demonstrated that this reduction resulted specifically from the inhibition of S4 of DIV, indicating clearly the main binding site of these toxins (Sheets et al., 1999).

The prior mentioned α-scorpion toxins, sea anemone toxins and spider toxins have all been shown to bind to the conventional site 3 and exert their conventional site 3 action: slowing or inhibiting the VGSC's fast inactivation. Recently however, this traditional view on site 3 was samewise challenged as with site 1, as the δ-palutoxins (from the spider *Paracoelotes luctuosus*) were suggested to bind to site 4, although they exhibit actions that are typical for site 3 neurotoxins (Corzo et al., 2005; Ferrat et al., 2005). Another study with some other spider toxins gaining similar unexpected findings related with binding sites is the

electrophysiological characterization of β/δ-agatoxins, originating from *Agelena orientalis* (Billen et al., 2010). It has been shown by Billen et al. that these toxins affect, in a voltage-dependent manner, both the inactivation process (site 3 effect) and the activation process (site 4 effect). These results even more questioned the generally accepted definition of "site 3" and the concepts of binding sites in general. The latter toxins will be discussed in the section about the revision of the binding sites, together with the unconventional site 1 neurotoxins.

NEUROTOXIN BINDING SITE 4

Receptor site 4 is recognized by the class of β-scorpion toxins targeting voltage-gated sodium channels (βNaScTxs) and by several spider toxins. These toxins exert their toxicity by acting as gating modifiers. Toxin binding at site 4 causes a shift in the voltage-dependence of activation toward more hyperpolarized membrane potentials and reduces the peak sodium current amplitude (Vijverberg and Lazdunski, 1984; Cestele et al., 2006). These alterations in channel gating are believed to be a direct result of toxin binding at site 4, hereby trapping the voltage sensor in its outward, activated position (Cestele et al., 2001, 2006). Receptor site 4 has been primarily defined to specific residues in the extracellular loops connecting the S1–S2 and S3–S4 segments of DII (Catterall et al., 2007). However, using the scorpion β-toxin Tz1 (*Tityus zulianus*) it was shown that three residues in the pore-loop of DIII are determining for the specificity of β-toxin for different sodium channel isoforms (Leipold et al., 2006). A recent report showed that specific mutations in the voltage sensor of DIII enhance the binding of β-toxins to S4 of DII, providing evidence for the involvement of the DIII voltage sensor in the action mechanism of βNaScTxs (Song et al., 2011).

Toxins belonging to the class of βNaScTxs are long chain peptides composed of 58–76 amino acids, cross-linked by four disulfide bridges. They belong to the structural superfamily of cysteine stabilized α/β motif containing proteins. This spatially conserved scaffold provides βNaScTxs with a high stability and a strong resistance against mutations in their sequence (Possani et al., 1999; de la Vega and Possani, 2007; Gurevitz et al., 2007). βNaScTxs are, similar to their αNaScTxs counterparts, classified into three groups according to their pharmacological properties exemplified by their preference for mammalian or insect sodium channels: mammalian-selective, β-like or insect-selective. (i) Mammalian-selective β-toxins such as Css4 (*Centruroides suffusus suffusus*) are highly toxic to mammals (Martin et al., 1987). (ii) β-like toxins are capable of competing for binding sites on both insect and mammalian Na_v channels. Tsγ, also known as Ts1 or Ts VII (*Tityus serrulatus*) and Lqhβ1 (*Leiurus quinquestriatus hebraeus*) are well-studied examples of such βNaScTxs acting on both insects and mammals (Possani et al., 1999; Gordon and Gurevitz, 2003). (iii) Insect-selective β-toxins fail to exert any affinity whatsoever for mammalian sodium channels, even in very high concentrations (de Dianous et al., 1987). Exactly this complete lack of mammal activity combined with their strong insect specificity and potency makes these insect-selective βNaScTxs interesting lead compounds in the design of new insecticides (Gurevitz et al., 2007).

The insect-selective β-toxins can be further subdivided into excitatory and depressant toxins according to the symptoms they evoke *in vivo*. Injection of excitatory toxins induces a fast repetitive activity of motor nerves that results in a reversible contraction paralysis. AahIT (*Androctonus australis* Hector), LqqIT1 (*Leiurus quinquestriatus quinquestriatus*) and Bj-xtrIT (*Hottentotta judaicus*, formerly known as *Buthotus judaicus*) belong to this group (Froy et al., 1999; Billen et al., 2008). These excitatory toxins differ from the other β-toxins as one disulfide bridge is located differently and furthermore they display extra secondary structural elements. The depressant toxins cause a transient contraction followed by a slow depressant and flaccid paralysis (Zlotkin et al., 1991; Karbat et al., 2007). Current-clamp experiments have shown that peptides belonging to this group suppress the evoked action potentials as a result of strong depolarization of the membrane (Strugatsky et al., 2005). Representatives of this group are LqqIT2 (*L. q. quinquestriatus*), BjIT2 (*H. judaicus*), and the highly potent toxin Lqh-dprIT3 (*L. q. hebraeus*; Zlotkin et al., 1993). It is interesting to note that LqqIT2 did not only cause a hyperpolarizing shift in the activation of channels but also affected the inactivation and the ion selectivity (Bosmans et al., 2005). Remarkably, when mammalian channels are excited by a long, preconditioning and depolarizing prepulse, insect-selective depressant β-toxins are given the opportunity to affect those channels. The same phenomenon is observed in the case of simultaneous binding of an α-toxin to site 3 (Cohen et al., 2007). As such, it can be seen that the presence of depressant β-toxins in the scorpion venom may still contribute significantly to the toxicity toward mammals.

Spiders are, similar to scorpions, capable of producing toxins that recognize the neurotoxin receptor site 4. Magi 5 (*Macrothele gigas*) was the first spider toxin shown to compete with the radioiodinated scorpion β-toxin ^{125}I-CssIV for site 4 (Corzo et al., 2003). Another group of site 4 spider toxins is constituted by the δ-palutoxins (*P. luctuosus*), curtatoxins (*Hololena curta*), μ-agatoxins (*Agelenopsis aperta*), and the recently characterized β/δ-agatoxins (*A. orientalis*; Stapleton et al., 1990; Corzo et al., 2000; Billen et al., 2010). All peptides belonging to this group are structurally related as they are composed of 36–37 residues and cross-linked by four disulfide bridges forming an inhibitor cystine knot (ICK) motif (Nicholson, 2007). Little is known about the mechanism of action of the curtatoxin but the highly homologous μ- and β/δ-agatoxins and δ-palutoxins have been well-studied. It was reported that the μ-agatoxins shift the voltage-activation curve toward more hyperpolarized potentials, like scorpion β-toxins do. However, these toxins also slowed down the inactivation process of the sodium channels, resulting in a non-inactivating persistent current (Adams, 2004). The same observations were made for the β/δ-agatoxins and a thorough electrophysiological characterization of the action of these agatoxins was performed. This indicated that β/δ-agatoxins induce a bell-shaped voltage-dependent modulation of both the activation and the inactivation, suggesting no strict correlation between the toxin binding site and its effect on channel gating (Billen et al., 2010). The δ-palutoxins compete with the depressant scorpion β-toxin Bj-xtrIT for site 4 but they fail to displace the binding of α-toxin LqhαIT from site 3 (Corzo et al., 2005). However, these toxins act as insect-selective modulators of sodium channels by slowing down the inactivation, a modulation typically seen upon toxin binding at site 3 (Corzo et al., 2000). All together these results indicate that βNaScTxs and

their spider homologs should be considered as macromolecular ligands that may have additional contact points with the extracellular domains of VGSCs, in addition to the neurotoxin receptor site 4. More details can be found in the section about the revision of the binding sites.

NEUROTOXIN BINDING SITE 5

Marine dinoflagellates produce highly lipophilic, cyclic polyether compounds which target the neurotoxin receptor site 5. Brevetoxins (*Karenia brevis*) and ciguatoxins *Gambierdiscus toxicus*) are such multi-ring polyether ladder toxins acting at site 5. Brevetoxins (PbTxs) consist of 11 transfused rings, 23 stereocenters and an overall linear low-energy conformation (Jeglitsch et al., 1998). PbTx-1 and PbTx-2 are two most potent brevetoxins and they are considered to be the parent toxins. Up to date at least 14 brevetoxins have been described. The two most potent brevetoxins are PbTx-1 and PbTx-2 that slightly differ from each other in their backbone structure. PbTx-1 and PbTx-2 are considered parent toxins since all other brevetoxins can be seen as derivates from one of these two structural backbones. All PbTxs possess a lactone in the A-ring and a strictly rigid region that forms a ladder structure and which is separated from the A-ring by a spacer region with limited flexibility (Gawley et al., 1995). Furthermore, they all possess a side chain that allows modification at the molecules' termini (Baden et al., 2005). PbTxs interact with VGSCs by intercalating in the membrane in a head-down orientation. Several studies have indicated that these toxins position themselves across the plasma membrane, parallel with the transmembrane segments, with the A-ring toward the intracellular side and the tail end of the molecule facing the extracellular side (Trainer et al., 1994; Jeglitsch et al., 1998). Experiments, in which a photoreactive PbTx-3 derivate was used as probe, could identify S6 of DI and S5 of DIV to participate in the formation of neurotoxin receptor 5 (Trainer et al., 1994). However, the key residues involved in brevetoxin activity still remain unknown. PbTx binding at site 5 leads to distinct alteration in channel gating: (i) the activation potential is shifted toward hyperpolarized potentials; (ii) channels remain longer in the open configuration which results in a longer mean open time; (iii) the inactivation is slowed down or inhibited; and (iv) brevetoxins have, among all known voltage-gated sodium channel modifying toxins, the unique capability to stabilize more than one conductance level. As such brevetoxin binding induces distinct sodium ion subconductance states in addition to the normal 21 pS rate (Schreibmayer and Jeglitsch, 1992; Jeglitsch et al., 1998; Baden et al., 2005). It is believed that the terminal, rigid four ring system is involved in channel binding while the functional lactone A-ring is responsible for the alterations in channel inactivation and prolongation of the mean open time (Jeglitsch et al., 1998; Purkerson-Parker et al., 2000).

Ciguatoxins (CTXs) are, like brevetoxins, lipid-soluble compounds with a structural backbone composed of 13 ether rings (Yasumoto, 2001). Although 29 ciguatoxin derivates have been identified, the information on their biological activity remains scarce, mainly due to difficulties in obtaining pure toxin (Perez et al., 2011). They compete with brevetoxins for site 5 and upon binding CTXs do cause similar modifications of channel gating. CTXs shift the activation of channels toward more negative potentials and they suppress the fast inactivation. Even though they bind at the same neurotoxin receptor, PbTxs and CTXs possibly differ from each other in their mechanism of action. This was first suggested by the observation that CTXs were, unlike PbTxs, capable of producing Na_v dependent oscillations in neuronal membrane potential (Hogg et al., 2002). More recently it was shown that CTX causes a concentration-dependent decrease of the sodium current amplitude in mammalian sensory neurons. This observation is in contrast to the observed amplitude increase after PbTx application (Cohen et al., 2008; Yamaoka et al., 2009; Perez et al., 2011).

NEUROTOXIN BINDING SITE 6

Among all sites, site 6 still is the most speculative and yet undefined site. A first proposal for this site arose when TxVIA and GmVIA, δ-conotoxins from the cone snails *Conus textile* and *Conus gloriamaris* respectively, were characterized (Fainzilber et al., 1994; Shon et al., 1994). As they turned out to slow down VGSC inactivation without the typical voltage-dependency that is seen with classical site 3 toxins, it was suggested that they bind to a novel, unidentified site (Fainzilber et al., 1994). To date, 19 δ-conotoxins sequences can be found in the ConoServer database (Kaas et al., 2008), but for none of them the exact binding site has been reported. Only for δ-SVIE an interaction with conserved residues in the linker between S3–S4 of DIV was shown (Leipold et al., 2005). This indicates that at least the S3–S4 of DIV is involved in the interaction of the toxin and VGSC. It remains to be determined if this is a general phenomenon and if δ-SVIE is a good representative of all δ-conotoxins, or if a difference exists between the two distinct groups that exist in δ-conotoxins. Those two groups or "clades" are based on the prey targeted by the according cone snail: namely the fish-hunting clade and the mollusk-hunting clade, as structural differences can be recognized between both groups (Bulaj et al., 2001). However, data on δ-conotoxins are still very limited and further studies are needed to elucidate the exact binding site(s) and mechanism of action of δ-conotoxins and to determine if other toxins apart from δ-conotoxins bind to this site.

REVISION OF NEUROTOXIN BINDING SITES

In the past, the classical nomenclature of neurotoxin binding sites has been a valuable tool for the investigation and classification of neurotoxins. However, more and more evidence emerges that these sites can not be as strictly delineated as once was believed (Catterall, 1980) and therefore the need for a revision of the binding sites comes forth. We would like to suggest a preliminary revision of the nomenclature of the neurotoxin binding sites, but are aware of the new insights that soon will be extracted from the recently discovered bacterial sodium channel crystal structure (Payandeh et al., 2011) which will definitely help in further revisions of the binding sites.

The revised binding site model can specifically signify a huge simplification of the conventional site 3 and site 4 since their current pharmacological profiles become more and more complex (Billen et al., 2010; Bosmans and Swartz, 2010; Liu et al., 2011). In general, the location of the site 4 binding area in the revised model can remain identical to the conventional site 4, located at DII, comprising the extracellular linkers between S1–S3 and

S3–S4. The same is valid for the revised site 3, remaining located at the conventional S3–S4 linker of DIV. Because of the increasing number of toxins displaying a structure–function relationship incompatible with the conventional binding site model, we propose an adjusted model that can provide a more straightforward and definite description of the binding sites, which will help us to further clarify the missing parts in the puzzle of VGSC's structure and function.

As mentioned in the section about site 1, μO-conotoxins (e.g., MrVIA and MrVIB) and some spider toxins (e.g., HWTX-IV) were at first considered to be site 1 toxins, as they exhibit the same effects as the conventional site 1 toxins. Nevertheless, these toxins are nowadays believed to bind at site 4 or close by. For μO-conotoxins for example, it was proven that they do not compete with STX for site 1 (Terlau et al., 1996). On the contrast, it was shown that they compete functionally with some classical β-scorpion toxins for modification of VGSCs and therefore are suggested to share, at least partially, a binding site similar to the classical defined site 4. However, in contrast to β-scorpion toxins that trap the S4 of DII in its outward position, μO-conotoxins will rather bind to the S4 voltage sensor in its inward position. As such they prevent the outward movement of the voltage sensor upon membrane depolarization and hence prevent the channels from opening, causing an inhibition of the Na^+ conductance (Leipold et al., 2007). Another example of a toxin interacting in the same way with site 4, is represented by the spider toxin Huwentoxin-IV (Xiao et al., 2008).

In the conventional definition, a site 4 neurotoxin is a toxin that (1) binds to an extracellular region on the channel, formed by the S3–S4 loop of DII, and (2) causes a hyperpolarizing shift in the voltage-dependence of the activation. However, the two examples mentioned in the previous paragraph, serve as prove that it makes sense to uncouple these two conditions. Instead, a site 4 neurotoxin could be defined by using only the first condition, indicating the toxin's main "binding area." The effect that a site 4 neurotoxin can cause could be the classical site 4 effect, a hyperpolarizing shift in the activation, or the μO-conotoxin/HWTX-IV effect of inhibiting Na^+ conductance. This effect will depend on specific residues or core regions that the toxin contains.

To define the main binding area one should be aware of the many contact points that a toxin can have on the VGSC, while only a few of them will be responsible for the exposed effects. To which *binding area* or "site" a toxin is assigned, should therefore only depend on which contact point causes the essential functional effect. Other contact points on the VGSC can rather be seen as *anchor points*, leading to non-functional binding of the toxin or serving as subtype-selective recognition sites for the toxin. Nevertheless, these non-functional contact points can also be a requirement for the full activity of the toxin. He et al. (2011) recently described this for binding of BmK IT2, a site 4 insect β-toxin from *Buthus martensii* Karsch, to insect $DmNa_v$ VGSCs. Mutations in the site 4 binding area (DII S3–S4) largely abolished the functional action of BmK IT2 on $DmNa_v$. However, some anchor points in DIII turned out to be responsible for the selective recognition of the insect channel over the rat $Na_v1.2$ channel. In addition, the N-terminal part of the S5–S6 linker (SS2–S6) in this DIII greatly influenced the potency of the BmK IT2 binding to $DmNa_v$ (He et al., 2011).

In competitive binding experiments, the occupancy of the functional binding area of a radiolabeled toxin can be diminished by another toxin that also recognizes this contact point, as it is one of its anchor points but not its functional binding area. On a macroscopic level this may raise the impression that the non-labeled toxin has an identical functional effect as the labeled toxin. In the proposed model however, the competitive binding of the displacing non-labeled toxin, does not necessarily correlates this contact point on the VGSC with the functional binding area of the non-labeled toxin. This can explain the apparent paradox as is the case for site 3 toxins like BmK I and BmK αIV (α-like scorpion toxins from *B. martensii* Karsch) that can displace radiolabeled site 4 toxins like BmK AS (β-like scorpion toxin) and BmK IT2 (depressant insect β-scorpion toxin) respectively (Li et al., 2000; Chai et al., 2006). For a comprehensive review on BmK toxins and another binding model for the complex sites 3 and 4 (see Billen and Tytgat, 2009; Liu et al., 2011).

Another group of toxins pleading for the revision of VGSC binding sites is that of the spider toxins β/δ-agatoxins, mentioned in the section about site 4. Thorough electrophysiological characterization of these toxins showed that both activation and inactivation of an insect VGSC is affected when the toxin is applied. The voltage-dependence of activation is shifted towards more hyperpolarized potentials and a non-inactivating persistent current is induced. In the classical view these effects suggest toxin binding to site 4 and site 3, respectively. Therefore it is difficult to "match" the toxin to one of those binding sites as both effects can never correlate with one of these classical defined sites. This problem is solved in the revised binding site model, as this does not couple the effects of a toxin to its binding site. Therefore, as long as other experimental evidence is lacking, β/δ-agatoxins could eventually be stated to be site 4 toxins based on their high sequence homology with site 4 δ-palutoxins (Billen et al., 2010). Moreover, it is more likely that the effect on the inactivation is a result of toxin biding at site 4 rather than that the effect on activation is an effect of toxin binding at site 3. As experimental data are still lacking at this point, these suggestions still have be confirmed in future experiments.

The revised binding model also provides additional insights in the recently developed innovative concept of transferring voltage paddles of the VGSC $Na_v1.2$ into fourfold symmetric potassium (K_v) channels (Bosmans et al., 2008). With these chimeric channels, the individual contribution of all four voltage paddles to toxin binding can be elucidated. Binding of the α-scorpion AaHII to the chimeric channels clearly involves only the paddle motif of DIV, which fits with the (revised) definition of site 3. Binding of the β-scorpion toxin revealed a less conventional pattern in which DII, III, and IV are all involved in toxin binding. Nevertheless, with the revised binding site model this can easily be explained and a distinction can be made between the toxin's functional binding area, located at the voltage paddle of DII, and its additional anchor points, located at DIII and DIV. If only one of the toxin's contact points is transplanted into a K_v channel to prove the original VGSC's sensitivity to the toxin, it makes sense to suggest that sensitivity of the chimeric channel to the applied toxin will be

induced anyway, no matter if the contact point is the functional binding area, or an additional anchor point.

In the same article, the interesting spider toxins ProTx-I and ProTx-II (*Thrixopelma pruriens*) are found to interact with DII and DIV of $Na_v1.2$ and ProTx-I also displayed an interaction with DI of $Na_v1.2$. Which domain comprises the main binding area for these toxins and which can serve as anchor point still remains the topic of discussion. Recently, Smith et al. (2007) conducted a thorough mutagenesis study of several predicted key residues in site 3 and 4 of the $Na_v1.5$ VGSC isoform. Contrary to the results in $Na_v1.2$, no main binding area could be detected. At the same time, they investigated which residues in ProTx-II were essential for its activity. The most important residues were hydrophobic and basic amino acids, making the bioactive surface amphiphatic in nature. This raises the exciting possibility that a yet undefined binding area or contact point exists, not located on the VGSC itself but somewhere in the surrounding membrane. This possibility was previously explored for K_v channels in several studies, examining for example partitioning of several toxins into model membranes (Milescu et al., 2007). All together, it was shown that a number of spider toxins do partition into membranes and that the composition of membrane lipids influences toxin effects. On the other hand, Cohen et al. (2006) demonstrated for several β-scorpion toxins that they do not interact with any binding area at the phospholipid bilayer. It should be noted however, that the tested spider toxins are members of a growing class of promiscuous toxins that not only target a particular class of ion channels but instead seem to bind to the voltage sensors of several classes of ion channels (e.g., Hanatoxin binds to several members of the K_v, Ca_v, and Na_v ion channel families, a detailed list of other promiscuous toxins can be found in Bosmans and Swartz, 2010). This could indicate that the mechanism of action and the binding area of promiscuous spider toxins differ from β-scorpion toxins and that an unidentified contact point in the lipid membrane may be responsible for at least part of the full activity of the ProTx-I and ProTx-II spider toxins. Interaction between the spider toxin and the VGSC may then result in a tri-molecular complex, comprising the toxin, and contact points at the voltage sensor and at the lipid membrane (Bosmans and Swartz, 2010).

Additional evidence for the influence of other contact points in the near environment of VGSC α-subunits resulted from recent experiments, investigating the influence of the expressed β-subunit on the action of μO-conotoxin MrVIB. Wilson et al. (2011) discovered that expression of $Na_v1.8$ channels with four different β-subunits resulted in a higher-affinity block of MrVIB compared to the α-subunit on its own. Contrary to this finding, the affinity of STX turned out to be independent of coexpression with β-subunits. One of their suggested mechanistic explanations for the influence of β-subunit expression on toxin function is that MrVIB could have a certain contact point on these β-subunits that contributes to its action. Further structure–function studies are necessary to confirm if the interaction between toxin and β-subunit is specific for μO-conotoxins or rather is the case for other toxins – apart from the guanidinium toxins, as well.

CONCLUSION AND FURTHER PERSPECTIVES

In the past, neurotoxins already have proven to be indispensible tools for the exploration of the structure and function of VGSCs. The long awaited elucidation of the crystal structure of the bacterial VGSC NavAb (Payandeh et al., 2011) certainly inaugurates a new era in VGSC research and will help to provide new insights into neurotoxin binding to VGSCs. Further examination of toxin–lipid interactions and toxin–β-subunit interactions, determination of the bioactive core of neurotoxins and detailed mutagenesis studies with possible key residues for neurotoxin binding are just a grasp out of the possible experiments of which we may gain interesting new concepts for VGSCs.

With our revised model for neurotoxin binding sites on VGSC we try to address the growing problems that arise with the traditional classification of neurotoxin binding sites. The uncoupling of the toxin's functional outcome from its binding area should help to get a more clear-cut classification of neurotoxins. However, to distinguish the toxin's main binding area from additional anchor points, one should not make preliminary conclusions by linking the effects of the toxin immediately to its binding area, nor should one deduct site classification or function of new toxins solely based on homology with other toxins. Therefore all available techniques (e.g., mutagenesis studies, chimeric channels, electrophysiological characterization) should be optimally used to characterize a toxin and its putative binding area on the channel.

Naturally occurring neurotoxins can be considered as valuable and promising tools, not only to further unravel structure–function of VGSCs but they also can serve as lead compounds in the development of novel drugs. Considering the fact that malfunctioning of VGSCs underlies a large range of diseases like epilepsy, neuropathic pain, and long QT syndrome, there is an urgent need for molecules that can selectively and potently target these membrane proteins. The quest for such molecules can start at the venom and poisons of organisms, providing us an almost endless library of possible lead compounds.

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