

CHAPTER 3

Voltage-Gated Na⁺ Channels: Not Just for Conduction

Larisa C. Kruger and Lori L. Isom

Department of Pharmacology, University of Michigan, Ann Arbor, Michigan 48109-5632

Voltage-gated sodium channels (VGSCs), composed of a pore-forming α subunit and up to two associated β subunits, are critical for the initiation of the action potential (AP) in excitable tissues. Building on the monumental discovery and description of sodium current in 1952, intrepid researchers described the voltage-dependent gating mechanism, selectivity of the channel, and general structure of the VGSC channel. Recently, crystal structures of bacterial VGSC α subunits have confirmed many of these studies and provided new insights into VGSC function. VGSC β subunits, first cloned in 1992, modulate sodium current but also have nonconducting roles as cell-adhesion molecules and function in neurite outgrowth and neuronal pathfinding. Mutations in VGSC α and β genes are associated with diseases caused by dysfunction of excitable tissues such as epilepsy. Because of the multigenic and drug-resistant nature of some of these diseases, induced pluripotent stem cells and other novel approaches are being used to screen for new drugs and further understand how mutations in VGSC genes contribute to pathophysiology.

Voltage-gated sodium channels (VGSCs) conduct inward current that depolarizes the plasma membrane and initiates the action potential (AP) in excitable cells, including neurons, cardiomyocytes, and skeletal muscle cells. Because of the intrinsic link between VGSCs and cellular excitability, it is not surprising that mutations in VGSC genes are linked with epilepsy, cardiac arrhythmia, neuropathic pain, migraine, and neuromuscular disorders. The goal of this review is to provide an overview of critical discoveries in VGSC physiology and discuss the challenges of studying VGSCs in disease, including some of the exciting techniques to address these challenges.

VGSC DISCOVERY AND STRUCTURE

In 1952, the Nobel Laureates Alan Lloyd Hodgkin and Andrew Fielding Huxley first recorded sodium current (I_{Na}) using their voltage-clamp technique on the squid giant axon. Their experiments showed three key features of I_{Na} —selective Na⁺ conductance, voltage-dependent activation, and rapid inactivation (Hodgkin and Huxley 1952). The Hodgkin–Huxley model mathematically described voltage-dependent initiation of the AP by inward I_{Na} , followed by fast inactivation of I_{Na} , and simultaneous activation of outward I_K . Outward I_K was postulated to reestablish the charge balance across the plasma membrane (Hodgkin and Huxley 1952). These results were supported by studies of peripheral motor neurons by Sir John Carew Eccles, who shared the Nobel Prize with Hodgkin and Huxley. Among other contributions, he identified deviations in membrane potential and APs because of injections of sodium into motor neurons (Coombs et al. 1955). In addition to this monumental discovery, refinement of the voltage-clamp technique opened the door for a plethora of research on membrane potential physiology.

Correspondence: lisom@umich.edu

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In the 1960s, Bertil Hille and Clay Armstrong proposed the idea that I_{Na} and I_K are conducted through specific ion channels. At that time, many studies centered on performing voltage-clamp recordings of squid axon with sodium-free solutions containing organic cations (e.g., ammonium) to determine the size of the ion-conducting pore. The axon membrane became transiently permeable to these ions following depolarization, and this permeability was abolished by tetrodotoxin (TTX) (Larramendi et al. 1956; Lorente De No et al. 1957; Tasaki et al. 1965, 1966; Tasaki and Singer 1966; Binstock and Lecar 1969). Hille added to this body of work by using additional metal and many organic cations to develop a model of the narrowest region of the ion-conducting pore of the channel (Hille 1971, 1972). Hille's model proposed a partial dehydration of Na^+ through interaction with a high-field-strength site at the extracellular end of the pore followed by rehydration in the lumen of the pore. This model is astonishingly close to what we now know to be true about VGSCs from crystal structure information (Payandeh et al. 2011; McCusker et al. 2012; Zhang et al. 2012). Armstrong and Bezanilla developed signal-averaging techniques to detect the movement of "gating charges" (Armstrong and Bezanilla 1973, 1974) corresponding to what we now understand to be the movement of voltage sensors, which respond to changes in membrane potential. Remarkably, without having any of the knowledge that we possess today about nucleotide/amino acid sequence and crystal structures, these early studies made many accurate predictions about VGSC structure and the functions of various channel domains.

Although early physiological studies provided evidence for the existence of a VGSC, the biochemical proof that tetrodotoxin/saxitoxin (STX) receptors were also ion-conducting did not yet exist. Membrane protein purification techniques were being established, but they required an extraordinarily large amount of time and biological starting material compared to today's techniques. Photoaffinity labeling of STX receptors with a scorpion toxin derivative by William Catterall's group identified a family of VGSC proteins that were designated α and β subunits (Beneski and Catterall 1980). Purification of VGSC protein to theoretical homogeneity was challenging because of the difficulty of solubilizing a high molecular weight, highly lipophilic membrane protein and the large amount of biological starting material required. Again, the Catterall laboratory was the first to overcome these challenges and purify VGSCs from rat brain (Hartshorne and Catterall 1981, 1984; Hartshorne et al. 1982). This work was closely followed by purification of VGSCs from rat and rabbit skeletal muscle (Barchi 1983; Kraner et al. 1985), and from chicken heart (Lombet and Lazdunski 1984). Reconstitution of purified VGSCs in a lipid bilayer membrane allowed observation of Na^+ flux and confirmed that a functional VGSC protein had been purified (Hartshorne et al. 1985). The evolution of VGSC purification methods is discussed in detail in Catterall (1992). Purification from rat brain revealed that VGSCs are heterotrimers, composed of a single α subunit and two non-pore-forming β subunits, β_1 and β_2 (Hartshorne and Catterall 1981). We now know that there are more than two VGSC β subunits. Subsequent homology cloning and heterologous expression studies showed that each VGSC α is associated at the plasma membrane with a noncovalently linked β_1 or β_3 subunit and a covalently linked β_2 or β_4 subunit (Morgan et al. 2000; Yu et al. 2003).

To clone the first VGSC α subunit complementary DNA (cDNA), researchers from Shosaku Numa's group took on the arduous task of purifying brain VGSCs and obtaining partial amino acid sequence by amino-terminal Edman degradation as well as through cleaved peptides from the α subunit. They then generated and screened multiple cDNA libraries based first on predicted degenerate cDNA sequences and then on cloned sequences. Assembly of the cloned VGSC cDNA fragments revealed what we now know to be *Scn1a* (Noda et al. 1984). Heterologous expression of the cloned cDNAs gave a functional channel, which we now recognize as the VGSC α subunit $Na_v1.1$.

VGSC β SUBUNITS ARE REQUIRED TO RECAPITULATE PHYSIOLOGICAL EXPRESSION OF I_{Na}

Early cloning of $Na_v1.2$ cDNA and its expression in oocytes resulted in I_{Na} that inactivated more slowly than I_{Na} recorded from neurons. Co-injection of low-molecular-weight rat brain messenger RNA (mRNA) was required for recapitulation of physiological I_{Na} in terms of rates of activation and

inactivation, and voltage dependence (Auld et al. 1988; Krafte 1990). Cloning and expression of the VGSC β 1 and β 2 subunits showed that they mimicked the effects of low-molecular-weight mRNA on α subunit expression in oocytes (Isom et al. 1992, 1995b). Coexpression of Na_v1.2 + β 1 in oocytes resulted in a larger I_{Na} that activated and inactivated more rapidly and had a negative shift in the voltage-dependence of inactivation compared to α alone. Coexpression of Na_v1.2 with both β 1 and β 2 further increased I_{Na} density (Isom et al. 1995b). As discussed by Calhoun and Isom, when expressed in heterologous systems, β subunits in general increase I_{Na} density, shift the voltage-dependence of current activation and inactivation, and accelerate the rates of activation and inactivation. However, the magnitude of current increase and direction of the shifts in activation and inactivation are dependent on the particular heterologous cell line and the identities of the β and α cDNAs expressed (Calhoun and Isom 2014). Importantly, heterologous expression systems cannot replicate the native cellular milieu, especially the multiprotein VGSC complexes that are known to form in specific subcellular domains of neurons and cardiac myocytes (Calhoun and Isom 2014). For example, in *Scn1b* null ventricular myocytes, peak and persistent sodium current is increased, mediated by increased Na_v1.5 expression, with no effect on channel kinetics and voltage dependence (Lopez-Santiago et al. 2007). Furthermore, loss of *Scn1b* also results in increased TTX-S current and *Scn3a* expression in ventricular myocytes (Lin et al. 2015). However, in the dorsal root ganglia (DRG), critical neurons for peripheral pain sensation, *Scn1b* deletion causes a depolarizing shift in the voltage dependence of VGSC inactivation and decreased persistent I_{Na} (Lopez-Santiago et al. 2011). As heterologous expression systems cannot accurately replicate these cell types, in vivo and transgenic mouse models offer more appropriate methods for studying the functional roles of VGSC physiology.

MECHANISTIC INSIGHTS ON VGSC STRUCTURE AND FUNCTION

VGSC α subunits are highly evolutionarily conserved. Each α subunit is composed of four homologous domains, each containing six transmembrane helices (S1–S6), which come together in pseudotetrafold symmetry to form the ion-conducting pore (Fig. 1). As discussed earlier, long before the emergence of crystal structures, much of VGSC structure and function was inferred through studies modeling gating and using site-specific antibodies and toxins during electrophysiological recordings.

The sliding-helix model of VGSC voltage-dependent activation was proposed in the 1980s by Catterall (1986a,b). The S4 segments of each domain that serve as voltage sensors contain arginine residues at every third position, which pair with negatively charged residues in nearby transmembrane segments. On membrane depolarization, these residues change ion-pair partners, causing each S4 helix to rotate or slide outward, pushing a single arginine residue in each of the four segments out, and generating gating current that activates, or opens, the channel pore (Payandeh et al. 2011). Data from potassium channel structures prompted the paddle model as an alternate mechanism of voltage-dependent activation (Jiang et al. 2003); however, the solving of multiple prokaryotic α subunit crystal structures showed the validity of the sliding-helix model for VGSCs (Payandeh et al. 2011; Vargas et al. 2012).

Another critical property of VGSCs is voltage-dependent inactivation, which occurs despite an ongoing depolarizing pulse. Early studies identified the intracellular loop between domains III and IV as the inactivation gate (Fig. 1). This was proposed to “swing” into the pore in a voltage-dependent manner, physically blocking ion conduction, and effectively inactivating the pore (Vassilev et al. 1988, 1989). A short amino acid sequence in the inactivation gate, isoleucine, phenylalanine, and methionine (IFM), was identified as critical to the process of inactivation. Mutation of these three residues to glutamine resulted in a noninactivating channel (West et al. 1992; Kellenberger 1997). These studies, combined with a three-dimensional nuclear magnetic resonance (NMR) structure of the inactivation gate (Rohl et al. 1999), helped form the current understanding of the molecular basis for fast inactivation of VGSCs.

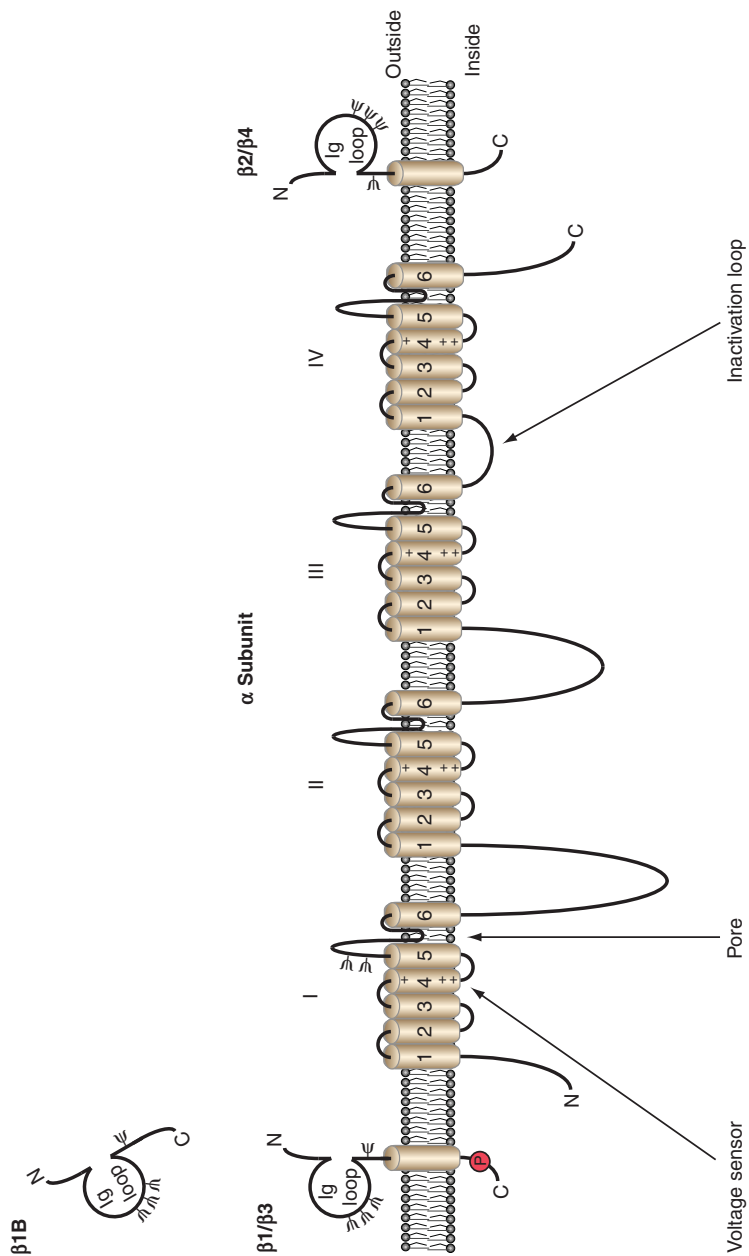


FIGURE 1. Voltage-gated sodium channel (VGSC) α and β subunit topology. The pore-forming α subunit is composed of four homologous domains (I–IV), each with six transmembrane segments. The fourth segment in each domain contains the voltage-sensing domain of the channel. The inactivation loop between domains III and IV contains the isoleucine, phenylalanine, and methionine (IFM) domain required for channel inactivation. Up to two β subunits may be associated with VGSCs. $\beta 1$ and $\beta 3$ subunits associate noncovalently, whereas $\beta 2$ and $\beta 4$ subunits associate via a disulfide link. (From Brackenbury and Isom 2011; reprinted, with permission, © Frontiers Media SD.)

Recent crystal structures of bacterial VGSCs have led to a more detailed understanding of the pore and selectivity filter. The membrane reentrant P-loop, located between transmembrane helices S5 and S6 of each domain that line the pore, was identified as the target for the pore-blocking TTX, suggesting its close location to the pore (Noda et al. 1989; Terlau et al. 1991). The crystal structure of a bacterial VGSC showed that the four P-loops together form a ring of glutamates, located near the extracellular end of the pore (Payandeh et al. 2011). As predicted in 1992, these highly conserved glutamates form a high-field-strength site critical in determining ion selectivity of the channel (Heinemann et al. 1992). These residues stabilize multiple ionic occupancy states, preferentially conducting hydrated Na⁺ through the pore (Payandeh et al. 2011). In contrast, potassium channels conduct dehydrated K⁺ (Doyle et al. 1998).

Capturing crystal structures of bacterial VGSCs in different activation states has provided new insights and biochemical modeling tools. A closed-pore conformation of the *Arcobacter* channel (Na_vAb), crystallized within a lipid-based bicelle, offered the first VGSC 3D structure (Payandeh et al. 2011). A putatively inactive Na_vRh channel from the marine bacterium *Rickettsiales* showed significant differences from Na_vAb and supplied new information about conformational rearrangements required for inactivation (Zhang et al. 2012). Finally, the apparently open conformation of the Na_vM structure from the marine bacterium *Magnetococcus* presented further insights into channel gating and selectivity (McCusker et al. 2012).

Despite the wealth of information provided by these prokaryotic VGSC structures, crystallization of a eukaryotic VGSC is the essential next step. Although prokaryotic VGSCs are homotetramers, mammalian VGSC α subunits are single polypeptides containing four nonidentical domains. Further, the extensive extracellular and intracellular loops of mammalian channels are not present in their prokaryotic orthologs. Thus, major gaps in the field include the crystal structure of a mammalian α subunit and the co-crystal structure of an α subunit associated with β subunits. This information will be critical in fully understanding mammalian VGSC structure.

VGSC Diversity

Originally, VGSC α subunit proteins were named according to the tissue from which they were purified. For example, brain type II, rat II, and R-II are all outdated terms for Na_v1.2 (Catterall et al. 2005). To date, nine VGSC α proteins (Na_v1.1–1.9) and five β proteins (β 1–4 and β 1B), encoded by *SCN(X)A* and *SCN1B-SCN4B* genes, respectively, have been identified in mammalian genomes.

A BLAST search of the cDNAs encoding β 1 and β 2 in 1995 revealed important, and unexpected, new information. Both proteins contained areas of homology with known CAMs of the immunoglobulin (Ig) superfamily, for example, contactin and myelin P0. This led to the hypothesis that β 1 and β 2 function as CAMs in addition to channel modulation (Isom et al. 1995b). We now know that all five β subunit proteins contain an extracellular Ig domain and have CAM function, as discussed later in this review. β 1–4 (encoded by *SCN1B-SCN4B*) are type 1 transmembrane proteins with an extracellular amino terminus (containing the Ig domain) and an intracellular carboxyl terminus (Isom et al. 1992, 1995b; Morgan et al. 2000). In contrast, β 1B, a splice variant of *SCN1B*, is soluble and, thus, a portion is secreted. Retention of, intron 3 results in generation of an alternate carboxy-terminal domain that does not contain a transmembrane region (Patino et al. 2011). Recently, the three-dimensional crystal structures of the human Ig domains of β 3 and β 4 were solved. β 3 formed a trimer when crystalized (Namadurai et al. 2014). In contrast, the β 4 Ig domain was monomeric in crystal form (Gilchrist et al. 2013). Although this could represent a physiological difference in homophilic interactions, it could also be because of the removal of 31 amino acids at the β 4 amino terminus to facilitate crystallization (Gilchrist et al. 2013). Comparisons of these structures will give new insights into the function of β subunits, but the co-crystal structure of α and β subunits remains the next frontier.

VGSC α and β subunits are highly expressed in central and peripheral neurons, cardiac myocytes, skeletal muscle, and some nonexcitable cells (Maier et al. 2004; Patino and Isom 2010; Brunklaus et al.

TABLE 1. VGSC genes, expression patterns, and disease associations

Gene symbol	Type	Tissue distribution	Consequence of mutations	TTX sensitivity
<i>SCN1A</i>	Na _v 1.1	CNS, PNS, heart, DRG	DS, familial autism, FHM3, FS ⁺ , GEFS ⁺ , SUDEP	+
<i>SCN2A</i>	Na _v 1.2	CNS, PNS, DRG	BFNIS, DS, EOEE, familial autism, GEFS, OS	+
<i>SCN3A</i>	Na _v 1.3	CNS, PNS, heart, DRG	Unclear	+
<i>SCN4A</i>	Na _v 1.4	Skeletal muscle, heart, DRG	PAM, PMC, HyperPP, HypoPP, SNEL	+
<i>SCN5A</i>	Na _v 1.5	Skeletal muscle, heart, CNS, DRG	AF, AS, BS, DCM, LQTS, PCCD, SIDS, SSS, SUDEP	-
<i>SCN8A</i>	Na _v 1.6	CNS, PNS, heart, DRG	EOEE; cognitive impairment, paralysis, ataxia, dystonia	+
<i>SCN9A</i>	Na _v 1.7	DRG	CIP, IEM, PEPD, PPN	+
<i>SCN10A</i>	Na _v 1.8	DRG	PPN	-
<i>SCN11A</i>	Na _v 1.9	DRG	PPN	-
<i>SCN1B</i>	β1	CNS, PNS, heart, DRG, glia (oligodendrocytes, Schwann cells, astrocytes, radial glia)	AF, BS, DS, GEFS ⁺ , LQTS, PCCD, TLE	N/A
<i>SCN2B</i>	β2	CNS, PNS, heart, DRG	AF, BS	N/A
<i>SCN3B</i>	β3	CNS, PNS, heart, DRG	AF, BS, PCCD, SIDS, ventricular fibrillation	N/A
<i>SCN4B</i>	β4	CNS, PNS, heart, DRG	LQTS, SIDS	N/A
<i>SCN1B</i>	β1B	Fetal CNS, PNS, heart, DRG	BS, PCCD, epilepsy	N/A

Modified, with permission, from Patino and Isom 2010, © Elsevier Ireland; Catterall 2012, © Wiley; Brunklaus et al. 2014, © BMJ. AF, Atrial fibrillation; AS, atrial standstill; BFNIS, benign familial neonatal-infantile seizures; BS, Brugada syndrome; CIP, channelopathy-associated insensitivity to pain; CNS, central nervous system; DCM, dilated cardiomyopathy; DRG, dorsal root ganglia; DS, Dravet syndrome; EOEE, early-onset epileptic encephalopathy; FHM3, familial hemiplegic migraine type 3; FS⁺, febrile seizures plus; GEFS⁺, genetic epilepsy with febrile seizures plus; HyperPP, hyperkalemic periodic paralysis, HypoPP, hypokalemic periodic paralysis; IEM, inherited erythromelalgia; LQTS, long QT syndrome; OS, Ohtahara syndrome; PAM, potassium-aggravated myotonia; PCCD, progressive cardiac conduction disease; PEPD, paroxysmal extreme pain disorder formally known as familial rectal pain syndrome; PMC, paramyotonia congenital; PNS, peripheral nervous system; PPN, painful peripheral neuropathies; SIDS, sudden infant death syndrome; SNEL, severe neonatal episodic laryngospasm; SSS, sick sinus syndrome; SUDEP, sudden unexplained death in epilepsys; TLE, temporal lobe epilepsy.

2014), including breast cancer cells (Fraser et al. 2005), astrocytes, and oligodendrocytes. Table 1 shows the gene name, known tissue expression, and diseases associated with mutations for each VGSC subunit (adapted from Patino and Isom 2010; Catterall 2012; Brunklaus et al. 2014). VGSC expression is developmentally regulated. For example, Na_v1.3, β3, and β1B are most prevalent in embryonic and neonatal rodent brain. This expression profile then changes such that Na_v1.1, Na_v1.2, Na_v1.6, β1, β2, and β4 are predominant in the adult brain, albeit distribution is not equivalent across brain regions (Kazen-Gillespie et al. 2000; Catterall et al. 2005; Patino et al. 2011).

VGSC subcellular localization is critical for normal physiological functions. In neurons, VGSCs are concentrated at the axon initial segment (AIS), where the AP is initiated, and nodes of Ranvier in myelinated axons, which are critical for saltatory conduction. In cardiomyocytes, VGSCs are differentially localized at intercalated discs and transverse tubules (see Bao and Isom 2014; Calhoun and Isom 2014 for reviews that discuss this in more detail).

Despite their high sequence similarity, each VGSC α subunit has subtle differences in biophysical properties and pharmacological sensitivities (as summarized in Catterall et al. 2005; Kwong and Carr 2015). Many of the agents that target α subunits are toxins, including TTX, STX, μ-conotoxins, and their derivatives, such as 4,9-anhydro-TTX, which preferentially blocks Na_v1.6 (Rosker et al. 2007; Kwong and Carr 2015). VGSCs are canonically separated into TTX-sensitive (TTX-S) and TTX-resistant categories (TTX-R), which are blocked by nanomolar or micromolar concentrations of TTX, respectively (Catterall et al. 2005). Interestingly, mutation of a single residue changes TTX sensitivity (Noda et al. 1989). Modulation of *I*_{Na} by these toxins (reviewed in Gilchrist et al. 2014) can be affected by β subunit expression. For example, expression of β1 and β3, but not β2 or β4, tend to increase the rate of association and binding affinity of μ-conotoxins for VGSCs. However, modulation of *I*_{Na} by STX or TTX was unaffected by β subunit expression (Isom et al. 1995a; Zhang et al. 2013). In vivo, a combination of electrophysiology and toxicology are used to infer which VGSCs contribute to

aspects of endogenous I_{Na} . Although challenging, *in vivo* studies are crucial, as heterologous results can be difficult to translate to *in vivo* physiological and pathophysiological mechanisms.

Posttranslational Modifications of α and β Subunits

VGSC α subunits are heavily glycosylated, although the extent of glycosylation varies among subunits (Bennett 2002). 40%–50% of these added carbohydrate residues are sialic acid moieties such that each channel contains ~100 sialic acid residues (Miller et al. 1983; Roberts and Barchi 1987). Differential glycosylation of VGSC subunits can affect channel gating, putatively because of the local negative charge of sialic acid residues. In general, when α subunits are less glycosylated, channel gating occurs at more depolarized voltages (Recio-Pinto et al. 1990; Bennett et al. 1997; Zhang et al. 1999; Tyrrell et al. 2001). For example, Na_v1.9 expressed in neonatal DRG neurons is more glycosylated than in adult DRG, suggesting developmental regulation of glycosylation. This may account for developmental differences in gating of persistent I_{Na} attributed to Na_v1.9 (Tyrrell et al. 2001). Voltage-dependent activation and inactivation of Na_v1.4, but not Na_v1.5, shifts when expressed in cells incapable of sialylation, indicating that not all VGSCs are affected equally (Bennett 2002). Glycosylation accounts for about one-third of the molecular weights of β subunits. Mature β subunits contain three to four *N*-linked glycosylation sites in the Ig domain (Isom et al. 1992; McCormick et al. 1998) all of which are exposed in the β 3 crystal structure (Namadurai et al. 2014). Sialic acid residues in these sites affect β 1- and β 2-mediated modulation of I_{Na} and channel surface expression (Johnson et al. 2004; Johnson and Bennett 2006). Critically, VGSC glycosylation varies developmentally and among cell types; for example, neonatal and adult myocytes have distinct differences in glycosylation, as do atrial and ventricular myocytes (Stocker and Bennett 2006). Therefore, tissue-specific variations in β subunit processing likely include altered sialylation/glycosylation and may contribute to differential modulation of I_{Na} by β subunits among cell types (Zhang et al. 1999; Bennett 2002).

β 1 can be phosphorylated on intracellular tyrosine residue Y-181 (Malhotra et al. 2002, 2004). Tyrosine phosphorylation regulates β 1-mediated recruitment of ankyrin to points of cell–cell contact (Malhotra et al. 2002) and likely occurs via *fyn* kinase, an *src* family kinase (Brackenbury et al. 2008; Nelson et al. 2014). Receptor tyrosine phosphatase β interacts with the intracellular domain of β 1 and may modulate β 1 phosphorylation (Ratcliffe et al. 2000). In cardiac myocytes, nonphosphorylated β 1 localizes at the T-tubules with ankyrinB, whereas phosphorylated β 1 localizes at intercalated discs with connexin-43, *N*-cadherin, and Na_v1.5 (Malhotra et al. 2004). Therefore, the phosphorylation state Y-181 regulates β 1 association with cytoskeletal proteins, as well as its subcellular localization.

β subunits are substrates for sequential proteolytic cleavage by BACE1 (β -secretase) and γ -secretase, similar to amyloid precursor protein. BACE1 cleavage releases the extracellular domain (ECD), followed by a γ -secretase-mediated cleavage event to release the carboxy-terminal fragment (CTF) (Wong et al. 2005). These cleaved peptides may have important physiological functions. For example, the β 2-CTF translocates to the nucleus and promotes *Scn1a* mRNA and protein expression when heterologously expressed in cultured neurons (Kim et al. 2005, 2007). Consistent with this proposed mechanism, total and surface expression of Na_v1.1 in acutely dissociated hippocampal slices is significantly reduced in BACE1 null mice compared to wild-type (Kim et al. 2011). The physiological importance of BACE1 and γ -secretase cleavage of the other β subunits is not understood. As β 1B can act as a CAM, it is possible that the β 1-ECD, which contains the identical Ig loop domain, functions as a ligand for cell adhesion (Patino et al. 2011). γ -Secretase activity and, thus, formation of the β 1-CTF is critical for β 1-mediated neurite outgrowth *in vitro* (Brackenbury and Isom 2011). Determining whether these functions occur *in vivo* represents a critical next step in our understanding of post-translational processing of VGSCs.

β Subunits Are Multifunctional

Effects on Surface Expression

Coexpression of β subunits with α results in increased I_{Na} density, in part, as a result of promotion of α cell-surface expression. Following posttranslational processing, α subunits are stored in an intracel-

lular pool associated with the plasma membrane (Schmidt et al. 1985; Schmidt and Catterall 1986). Concomitant with plasma membrane insertion, α and $\beta 2$ covalently associate via a disulfide bond (Chen et al. 2012). *Scn2b* null mice on the C57BL/6 \times 129SV background have a 50% reduction in hippocampal I_{Na} density and level of surface α subunits (Chen et al. 2002). However, a second study of *Scn2b* null mice congenic on the C57BL/6 background saw no change in hippocampal I_{Na} (Uebachs et al. 2010), suggesting genetic background influences on $\beta 2$ function. The effect of $\beta 2$ on channel surface expression may also depend on the particular α subunit. *Scn2b* null small-fast DRG neurons have reduced TTX-S I_{Na} , but left unchanged TTX-R I_{Na} (Lopez-Santiago et al. 2006). An *Scn2b*-linked Brugada syndrome mutation results in decreased surface Na_v1.5 protein compared to wild-type (Riuró et al. 2013). Thus, modulation of α subunit surface expression by $\beta 2$ may contribute to disease mechanisms. This is not unique to $\beta 2$; $\beta 1$ and $\beta 1B$ also promote VGSC surface expression (reviewed in Calhoun and Isom 2014).

Effects of $\beta 4$ on Resurgent I_{Na}

Resurgent I_{Na} is caused by the transient opening of VGSCs during recovery from inactivation following the AP. This specialized current is an important adaptation for high-frequency firing neurons, such as cerebellar Purkinje neurons (Raman and Bean 1997). For this to occur, a “blocking protein,” putatively the ICD of $\beta 4$, must bind to open channels during depolarization and unbind on repolarization, shortening the typical refractory period and producing a resurgent I_{Na} . Without $\beta 4$ expression, resurgent current is lost from cerebellar Purkinje neurons, but is rescued by a $\beta 4$ ICD peptide (Grieco et al. 2005). This same $\beta 4$ peptide is sufficient to invoke resurgent I_{Na} in Na_v1.7-expressing HEK cells (Theile and Cummins 2011). With $\beta 4$ knockdown in cerebellar granule neurons, resurgent I_{Na} is decreased, but is rescued by the $\beta 4$ peptide (Bant and Raman 2010). Other β subunits may also contribute to modulation of resurgent I_{Na} , as *Scn1b* null cerebellar granule neurons have reduced resurgent I_{Na} (Brackenbury et al. 2010).

Cell Adhesion and Neurite Outgrowth

β Subunits are multifunctional. They play critical roles in neurite outgrowth, migration, and maintenance of nodes of Ranvier. β Subunits are CAMs and, as such, recruit other signaling proteins to the VGSC complex. Most is known about the CAM interactions of $\beta 1$ and $\beta 2$, which participate in *trans*-homophilic adhesion, via the Ig loop, with $\beta 1$ or $\beta 2$ molecules on adjacent cells (Malhotra et al. 2000). $\beta 1$ or $\beta 2$ *trans*-homophilic adhesion recruits the cytoskeletal protein ankyrin to points of cell–cell contact (Malhotra et al. 2000). Ankyrin recruitment is abolished by phosphorylation of $\beta 1$ -Y181 (Malhotra et al. 2002). $\beta 1$ – $\beta 1$ *trans*-homophilic adhesion promotes neurite outgrowth via a pathway requiring fyn kinase, the CAM contactin, γ -secretase activity, and localized I_{Na} (Brackenbury et al. 2008, 2010; Brackenbury and Isom 2011). Because $\beta 1B$ contains the identical Ig loop, it similarly promotes neurite outgrowth of neurons expressing $\beta 1$ (Patino et al. 2011). Together, these properties may contribute to neuronal development *in vivo*; *Scn1b* null mice have neuronal pathfinding dysfunction and differences in subcellular localization of α subunits at AIS (Chen et al. 2004; Brackenbury et al. 2008).

$\beta 1$, and likely $\beta 1B$, interacts heterophilically with other CAMs and extracellular matrix proteins, including contactin, neurofascin-186, NrCAM, N-cadherin, and tenascin-R (Kazarinova-Noyes et al. 2001; Ratcliffe et al. 2001; Malhotra et al. 2004; McEwen and Isom 2004; Patino et al. 2011). Some of these interactions affect $\beta 1$ -modulation of α surface expression. For instance, coexpression of NF186 or contactin with Na_v1.2 and $\beta 1$ increases Na_v1.2 surface expression compared with Na_v1.2 and $\beta 1$ alone, although NF186 and contactin have no impact on Na_v1.2 expression in the absence of $\beta 1$ (Kazarinova-Noyes et al. 2001; McEwen and Isom 2004). Heterophilic interactions with tenascin-R, an extracellular matrix protein secreted by oligodendrocytes during myelination, repels cells expressing $\beta 1$ or $\beta 2$ and, therefore, may play a role in restricting VGSCs to nodes of Ranvier in myelinated axons (Xiao et al. 1999). The cell-adhesive properties of $\beta 1$ and $\beta 1B$ have likely clinical

relevance, as most known *SCN1B* epilepsy mutations are located in or near the Ig loop domain (reviewed in O'Malley and Isom 2015).

Much less is known about the CAM functions of $\beta 3$ and $\beta 4$. Studies of $\beta 3$ homophilic adhesion give conflicting results: the $\beta 3$ Ig domain appears to interact with full-length $\beta 3$ when expressed heterologously (Yerredy et al. 2013), yet another group reported that $\beta 3$ expression could not induce aggregation of *Drosophila* S2 cells, unlike $\beta 1$ and $\beta 2$ (McEwen et al. 2009). $\beta 3$ and $\beta 1$ likely do not associate, as $\beta 3$ does not interact with a peptide containing the $\beta 1$ -ECD (McEwen and Isom 2004). $\beta 4$ did not promote neurite outgrowth of cerebellar granule neurons, but this observation does not preclude other CAM interactions (Davis et al. 2004).

PATHOPHYSIOLOGICAL ROLES OF VGSCs

Mutations in VGSC genes are associated with many types of genetic epilepsy, peripheral neuropathy, long QT syndrome, neuromuscular disorders, and other diseases associated with dysfunction of excitable tissues. Recent reviews discuss the complex pathophysiological mechanisms of sodium channelopathies associated with pain and cardiac disease (Dib-Hajj et al. 2010; Adsit et al. 2013; Bao and Isom 2014; O'Malley and Isom 2015). Here, we will focus on the role of VGSCs in the genetic epilepsies and cancer as examples of disease resulting from aberrant or loss of VGSC function in mammals.

Epilepsy

Epilepsy results from an imbalance between excitation and inhibition in the brain. More than two million Americans have epilepsy, which can be caused by genetic mutations or brain injury (Hirtz et al. 2007). Epilepsy significantly impacts the quality of life for both patients and caregivers because of the unpredictability of seizures and the risk of comorbidities including cognitive decline, intellectual disability, developmental delay, and sudden unexpected death in epilepsy (SUDEP).

Mutations in genes encoding VGSC α and β subunits are associated with epilepsies with a wide range of phenotypic severities, including genetic epilepsy with febrile seizures plus (GEFS⁺), an inherited epilepsy with a wide range of phenotypes, and Dravet syndrome (DS), one of the most devastating pediatric epileptic encephalopathies. An intriguing discussion of the complex mechanisms underlying DS was published recently (Chopra and Isom 2014). In 1998, the first VGSC mutation associated with epilepsy was identified in *SCN1B* in a patient with GEFS⁺. However, GEFS⁺ is incompletely penetrant and family members with the same *SCN1B* mutation present with a wide range of epilepsy severities (Wallace et al. 1998), a now familiar characteristic of the genetic epilepsies. Since this initial discovery, a large number of mutations in *SCN1A*, *SCN2A*, *SCN8A*, and *SCN1B* (Escayg et al. 2000; O'Malley and Isom 2015) have been implicated in multiple types of epilepsy (see Table 1) (reviewed in part by Shi et al. 2012; Steinlein 2014; Wagnon and Meisler 2015). More than 1200 mutations have been identified in *SCN1A* alone (Meng et al. 2015). Identifying the pathophysiological mechanisms underlying DS is of particular importance, as traditional antiepileptic drugs often aggravate seizures in DS patients and development of new therapeutics to help these patients is especially critical. Although 70%–80% of DS patients have an identified heterozygous de novo *SCN1A* mutation (Marini et al. 2011), homozygous mutations in *SCN1B* have also been reported (Patino et al. 2009; Ogiwara et al. 2012).

Studies of DS *SCN1A* mutations have shown that a majority result in loss-of-function (Meng et al. 2015). Studies of *Scn1a*^{+/-} null mice and knockin mice expressing human mutations have led to the “interneuron hypothesis,” suggesting that selective I_{Na} loss in GABAergic interneurons causes epilepsy via loss of inhibitory tone (Yu et al. 2006). However, this has become controversial (Chopra and Isom 2014; Isom 2014). More recent studies of DS patient-derived induced pluripotent stem cell (iPSC) neurons showed either that both inhibitory and excitatory (Liu et al. 2013) or excitatory neurons (Jiao et al. 2013) are hyperexcitable compared to nonepileptic controls (Mistry et al. 2014). These studies

suggest that *SCN1A* haploinsufficiency may result in compensatory up-regulation of other VGSC α subunits. Hyperexcitability of both inhibitory and excitatory neurons may lead to seizures in DS via increased network excitability or synchronization of firing.

Scn1b null mice also model DS; they experience spontaneous seizures and early lethality. At least one DS *SCN1B* mutation causes $\beta 1$ to be retained intracellularly, preventing surface expression and, thus, function (Patino et al. 2009). *Scn1b* null mice have differences in neuronal pathfinding that are observed before seizure onset (Brackenbury et al. 2013). Thus, *SCN1B*-linked DS mutations may alter neuronal excitability by affecting α subunit surface expression/function as well as by altering neuronal development via changes in cell adhesion.

A major concern for epilepsy patients and their families is the risk of SUDEP. Research suggests that a “perfect storm” of seizures, cardiac arrhythmias, respiratory dysfunction, and autonomic and/or parasympathetic dysfunction contributes to SUDEP (Auerbach et al. 2013; Kalume 2013; Massey et al. 2014). Heterozygous *Scn1a-R1407X* knockin GEFs⁺/DS mice, as well as *Scn1b* null DS mice, have increased transient and persistent I_{Na} in cardiac myocytes, which may provide substrates for cardiac arrhythmias and contribute to SUDEP (Lopez-Santiago et al. 2007; Auerbach et al. 2013). Fully understanding the mechanisms underlying SUDEP will be essential to epilepsy treatment.

One of the greatest challenges in epilepsy research is the need for novel antiepileptic drugs. Even with optimal treatment, 20%–30% of all epilepsy patients are pharmacoresistant, defined as being unresponsive to at least two different, tolerated antiepileptic drugs (Kwan et al. 2010, 2011). Even in nonrefractory patients, antiepileptic drugs treat disease symptoms rather than preventing disease progression and often have intolerable side effects. A major goal of studying epilepsy-associated VGSC mutations is to further understand epileptogenesis (how a brain becomes epileptic) to inform new antiepileptic drug discovery.

Emerging Roles for VGSCs in Cancer

An exciting area of research is the role of VGSCs in nonexcitable cells, including multiple cancer cell types. Almost two decades ago, researchers observed that cancer cell lines with higher VGSC expression have increased cell motility and metastatic potential. Furthermore, the invasive capacity of these cells could be reduced by incubation with TTX (Grimes et al. 1995; Laniado et al. 1997). Since then, both α and β subunits have been detected in many cancer cell lines and in some patient biopsies. VGSC α subunits are expressed in some cervical, ovarian, breast, and colon tumors in vivo (Fraser et al. 2005; Gao et al. 2010; House et al. 2010; Hernandez-Plata et al. 2012), although some prostate, breast, cervical, and lung cancers express β subunits (reviewed in Patel and Brackenbury 2015). Studies have identified both positive and negative associations between VGSC expression and metastatic potential. $Na_v1.5$ expression in breast cancer cells correlates positively with increased risk of recurrence and metastasis (Fraser et al. 2005; Yang et al. 2012), and a similar trend has been described for colon, prostate, and ovarian cancers. However, there is an inverse correlation between α expression and clinical grade in glioma and no correlation in lung cancer cell lines (Schrey et al. 2002; Onganer and Djamgoz 2005; Roger et al. 2007). Expression of α subunits can potentiate lateral motility, adhesion, process extension, and other cellular behaviors associated with metastasis (Brackenbury 2012). It is not clear how increased expression of the pore-forming α subunits potentiates invasive changes in the tumor cells. Three potential models have been proposed: increased Na^+ influx enhances H^+ efflux, thus activating pH-dependent extracellular matrix degradation and invasion; regulation of an “invasion gene network,” particularly by *SCN5A*, and increased intracellular Ca^{2+} , which enhances formation of invasive projections (reviewed in Brackenbury 2012).

SCN1B and *SCN2B* expression are associated with metastatic potential in prostate cancer cells (Diss et al. 2008; Jansson et al. 2012). However, studies of the invasive potential of *SCN1B*-expressing breast cancer in vitro have been contradicted by in vivo studies. $\beta 1$ expression is associated with less invasive breast cancer cell lines (Chioni et al. 2009); however, in a mouse model of breast cancer, overexpression of $\beta 1$ increased tumor growth, metastasis, and angiogenesis (Nelson et al. 2014). $\beta 1$ *trans*-homophilic adhesion mediates process outgrowth from breast cancer cells (Nelson et al. 2014),

thus it is proposed that $\beta 1$ promotes metastasis by a mechanism similar to $\beta 1$ -mediated neurite outgrowth.

NEW FRONTIERS AND NOVEL TECHNIQUES

The list of pathogenic VGSC gene mutations is growing rapidly. The explosion of whole-genome sequencing has led to multi-institution efforts, such as the NINDS-funded Epi4K Project (www.ninds.nih.gov/news_and_events/news_articles/pressrelease_childhood_epilepsy_genes_08112013.htm), with the goal of sequencing the genomes of 4000 patients with epilepsy, and the England Department of Health funded 100,000 Genomes Project (genomicsengland.co.uk) (Kearney 2014; Siva 2015). However, even with familial genetic information in hand, determining whether mutations are causative and understanding their pathophysiology are not simple tasks. A given patient can have mutations in several genes, which may be causative, benign, or modify another mutation. A single causative mutation may result in a wide range of phenotypes among individuals, for example, DS and GEFS⁺ patients may express the same *SCN1A* or *SCN1B* mutation. Genetic background is critical. *Scn1a*^{+/-} mouse models of DS have phenotypes of varying severity depending on genetic background (Yu et al. 2006; Miller et al. 2014). This issue can now be addressed directly with the induced-pluripotent stem cell (iPSC) technique. Patient iPSCs, usually derived from a skin cell biopsy, can theoretically be differentiated into any cell type, including cardiomyocytes, and specific neuronal subtypes (Parent and Anderson 2015). With this remarkable technique, we now have the opportunity to study disease-causing mutations in human cells within the context of the patient's unique genomic background (Takahashi et al. 2007). Furthermore, endogenous genes that may contribute to pathology remain present. Brain organoids made from human iPSC-derived cells with a mutation in a gene involved in pluripotent cell maintenance were used to model microcephaly, providing proof-of-concept for use of organoids to model other neurological diseases (Lancaster et al. 2013). Another advantage of iPSCs is the opportunity to perform drug screening on differentiated human cells, alongside animal models, thus providing insight into toxicity and species-specific effects before clinical trials (reviewed in Ko and Gelb 2014). Patient-derived iPSC cells are critical for precision medicine approaches. For instance, antiepileptic drug effectiveness or toxicity may be optimized on patient-derived iPSC-neurons before patient treatment. As the iPSC technique is further refined and developed, it will provide additional, critical tools in therapeutic development.

Another challenge to the field is the non-specificity of clinically available VGSC-targeted drugs. There are six different potential sites for small molecule targeting on VGSCs, as shown by the wide variety of toxin binding sites. However, most of the current antiarrhythmic, antiepileptic, and analgesic VGSC drugs on the market, for example, lamotrigine, phenytoin, and lidocaine, target the "local anesthetic" site located in domain IVS6 (Kwong and Carr 2015). This site is highly conserved among α subunits; thus, these drugs have little selectivity for specific VGSCs and often cause adverse effects. Development of VGSC agents that target other binding sites remains an important task. Although, in most cases, toxins cannot be used directly for therapeutics, they can greatly inform drug design; small molecules can be developed that preserve beneficial effects while reducing harm. Drugs targeting specific toxin binding sites may allow more selective blockade of specific α subunits to treat disease, such as neuropathic pain, cardiac arrhythmias, epilepsies, and perhaps even cancer (Stevens et al. 2011; Xiao et al. 2014).

Transgenic zebrafish are an economical and rapid vertebrate system that can be used to screen small molecule libraries and FDA-approved drugs for novel applications. Zebrafish with mutations in *scn1Lab*, the gene orthologous to *SCN1A*, have spontaneous seizures that are resistant to many antiepileptic drugs and appear to model DS. Peter de Witte's group showed that DS zebrafish responded to fenfluramine (Zhang et al. 2015). Scott Baraban used locomotion tracking in mutant zebrafish to monitor behavioral seizures in a drug screen, which was validated using diazepam, valproate, and stiripentol, antiepileptic drugs effective in some DS patients. Out of 320 compounds

tested, clemizole, an FDA-approved antihistamine, suppressed spontaneous seizures in vivo (Baraban et al. 2013). This approach can be adapted for screening potential therapeutics for any monogenic epilepsy, especially if combined with the CRISPR/Cas9 technique. Discovered in 2010 as a bacterial defense mechanism against phages (Deveau et al. 2010), the CRISPR/Cas9 system has since been adapted to easily and quickly introduce heritable genetic mutations in mice, rats, zebrafish, and rabbits (Chang et al. 2013; Li et al. 2013; Yang et al. 2014a). Compared to zinc-finger nucleases and transcription activator-like effector nucleases (TALENs), CRISPR/Cas creates double-stranded DNA breaks in a sequence-specific manner at sites complementary to a “single-guide RNA.” Because these are easy to design, the CRISPR/Cas9 system vastly improves the precision, speed, and accuracy of creating transgenic cell lines (including iPSCs) and animal models for single gene mutations (Yang et al. 2014b). These and other cutting edge techniques will allow us to discover novel disease mechanisms and therapeutics for diseases linked to VGSC mutations for another six decades (Catterall 2012).

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Chapter 3

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