The Voltage-Dependent Sodium Channel Family

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Abstract and Keywords

In neurones and other electrically excitable tissues, voltage-dependent sodium (Nav) channels play an essential role in initiating and propagating the action potential. High-resolution structures of sodium channels have revealed new details concerning these macromolecules that provide insights into their ion-specificity and the conformational changes they undergo during the action potential. Nav channels typically exist in vivo as multicomponent macromolecular assemblies, containing auxiliary proteins that modulate channel gating and trafficking. The properties of some of these auxiliary proteins raise the possibility that Nav channels may exist as functionally coupled complexes. The close similarity between different Nav channel subtypes has frustrated attempts to develop isoform-specific inhibitors. However, the combination of new structural insights, together with antibody-based reagents and site-directed mutagenesis of protein-based toxin inhibitors, raises the possibility of higher target specificities than previously possible. Such reagents may form the basis for a new generation of Nav channel drugs.

Keywords: Sodium channels, Nav1.7, Nav1.5, Nav channel-specific toxins, Nav channel-specific antibodies

Activation of voltage-dependent sodium (Na_v) channels is responsible for the initial membrane depolarization phase of the action potential. Within a few milliseconds of opening, the Na_v channels typically enter an inactive state, during which they are functionally refractory to any further membrane depolarization signals. Restoration of the membrane potential by opening voltage-dependent potassium (K_v) channels permits Na_v channel recovery from inactivation back to their resting state, resetting the channel and permitting further activation (Vandenberg & Waxman, 2012). Na_v channels are of major research interest in neurobiology, pharmacology, biophysics, and structural biology. Over a thousand mutations have been identified in different Na_v channel isoforms that are related to a variety of inherited diseases, including epilepsy, cardiopathologies, myotonias, and chronic pain syndromes (Huang, Liu, Yan, & Yan, 2017; Kruger & Isom, 2016). Consequently, the development of drugs that target Na_v channels is of major pharmacological interest.

The minimum functional component of the eukaryotic Na_v channel is a single 250 kDa α-subunit that contains the ion-selective pore. Nine mammalian α-subunits, designated $Na_V1.1$–1.9, have been identified, as well as an atypical channel Na_{vx}—the product of distinct genes *SCN1A–10A* (Catterall, 2017; de Lera Ruiz & Kraus, 2015). Different isoforms vary in their gating behavior that reflect their physiological roles, and many are expressed in complex tissue-specific and developmentally regulated patterns. Further structural diversity is generated by alternative mRNA splicing and post-translational modifications, including N-linked glycosylation, phosphorylation, ubiquitination, arginine methylation, palmitylation, sulphation, and S-nitrosylation (Onwuli & Beltran-Alvarez, 2016).

The eukaryotic α-subunit is formed by a single polypeptide chain (approximately 2,000 amino acid residues long) containing four homologous-but non-identical-domains, designated DI–DIV (Figure 1A). Each domain contains six transmembrane α-helical segments, designated S1–S6, which are connected through short or moderate-length extracellular and intracellular loops. Helices S1–S4 from each domain form a voltage-sensing module (VSM). The pore module (PM) contains helices S5 and S6, connected to each other through extracellular loop regions and the re-entrant P-loop (pore loop) helices (Figure 1B). The domains create a pseudotetrameric unit in which the PMs from each domain line the central ion-conducting pore. Within each domain, the VSMs lie on the perimeter, and the VSM of one domain makes close contact with the clockwise PM from the adjacent domain, as viewed from above (Figure 2). This interleaved arrangement is characteristic of all known eukaryotic voltage-dependent ion channels and probably underlies and facilitates the coupling of VSM movement with pore opening (see further, this article). Both VSMs and PMs of Na_v channels can be expressed as functionally isolated modules (McCusker, D'Avanzo, Nichols, & Wallace, 2011; Paramonov et al., 2017). This suggests an independent evolutionary origin of the PMs and VSMs. Indeed, the subunits of some tetrameric prokaryotic ion channels consist of PMs only (Anderson & Greenberg, 2001). Furthermore, some VSM homologues occur in otherwise

functionally unrelated molecules. A particularly striking example is the voltage-sensitive phosphatase whose membrane-embedded VSM controls its phosphoinositide phosphatase activity in response to changes in membrane potential (Murata, Iwasaki, Sasaki, Inaba, & Okamura, 2005; Piao, Rajakumar, Kang, Kim, & Baker, 2015).

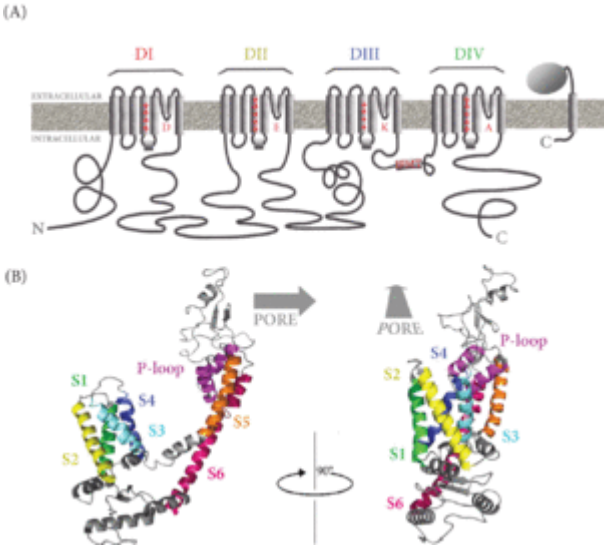

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Figure 1 A cartoon of the voltage-gated sodium channel (Na_v) and associated β-subunit. *A*. The primary structure of Na_v consists of four domains (DI–DIV), each of which contains six transmembrane α-helices (S1–S6) and two smaller P-loop α-helices. Ion-selectivity is governed by a ring of amino acids (DEKA, red text) that converge from each of the P-loop regions of all four domains. An α-helical inactivation gate between DIII and DIV contains a cluster of hydrophobic residues (IFMT) that can occlude the pore. Charged residues that act as a voltage sensor are found in S4 of each domain (+, red text; also see Figure 3). The β-subunit consists of a single transmembrane α-helix joined to an extracellular immunoglobulin domain. *B*. A single domain from the crystal structure of the cockroach Na_v channel (PDBID: 5X0M) showing the arrangement of segments S1–S6 and the P-loop. The right-hand side of the panel is rotated by 90^o, and viewed from outside of the channel as if looking towards the center of the pore. The α-helices are represented as cylinders and the adjoining polypeptide chains as black lines.

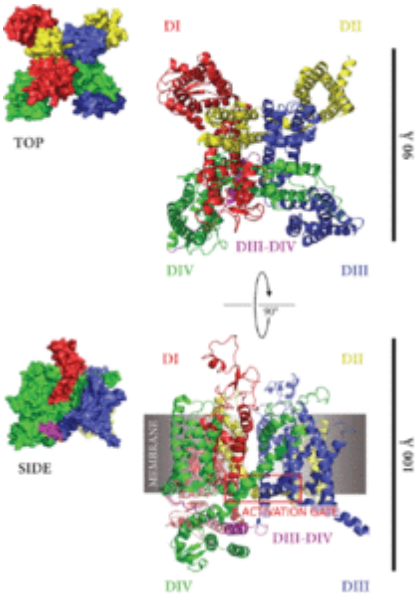

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Figure 2 The crystal structure of the cockroach voltage-gated sodium channel (PDBID: 5XOM) from above and from the side, showing the central ion-selective pore and the four domains that surround it. Each domain is colored differently, and the position of the cell membrane is shown as a grey box. The activation gate, mentioned in the text, is shown in the red-outlined box.

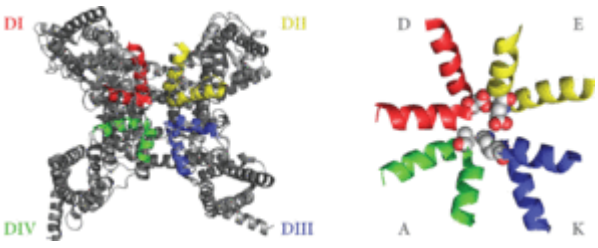
The Na_v channels belong to a large superfamily of voltage-gated ion channels, including voltage-gated potassium (K_v) and calcium (Ca_v) channels. Phylogenetic analysis suggests that the genes encoding Na_v and Ca_v channels evolved by two separate gene duplication events from an ancestral K_v-like channel containing a single domain. Subsequent duplication and divergence led to the separate evolution of Na_v channel and Ca_v channel gene families (Anderson & Greenberg, 2001; Moran, Barzilai, Liebeskind, & Zakon, 2015). Sodium-selective channels are also widespread in prokaryotes. Unlike their eukaryotic equivalents, the prokaryotic Na_v channels are tetramers of four identical subunits, with each subunit corresponding to an individual eukaryotic Na_v channel domain (Koishi et al., 2004). Their relative simplicity, and the availability of several high-resolution atomic structures, has made prokaryotic channels popular models to investigate the molecular mechanisms of gating behavior (Catterall & Zheng, 2015). However, it should be noted that detailed phylogenetic analysis strongly suggests that sodium-selectivity arose independently in prokaryotic and eukaryotic Na_v channel families (Liebeskind, Hillis, & Zakon, 2013). This is important to bear in mind when interpreting structural experiments, especially when applied to the mechanism of ion-selectivity and inactivation.

Although structures for related molecules such as a mammalian voltage-dependent calcium channels have been solved (Wu et al., 2016), at the time of writing, the structure of only one eukaryotic Na_v channel (from the American cockroach, *Periplaneta americana*) has been solved to near-atomic resolution (Shen et al., 2017) (Figures 1B, 2). Rapid technical developments in structural biology, especially in cryo-electron microscopy, should see structures for several mammalian Na_v channels becoming available in the near future. This will undoubtedly have a major impact on the field of Na_v channel research, and will greatly extend our understanding of these important molecules.

This review does not aim to provide a comprehensive analysis of current experimental approaches to Na_v channel biology. For reviews with such emphasis, we recommend, for example, Ahern, Payandeh, Bosmans, and Chanda (2016). Rather, we provide a general overview of Na_v channels for the non-specialist reader, place them in their broader physiological context, and note the pathological effects of some Na_v channel mutants. The potential for protein and peptide-based pharmacological tools to modulate Na_v channel behavior is then discussed in the light of this background.

The Na_v Channel Structure and Gating Mechanism

The S5 and S6 helices of each PM form the central pore cavity wall. At the intracellular face, the S6 helices from each domain draw together to form an intracellular cavity containing hydrophobic amino acids. This forms the activation gate and is constricted when the channel is closed (Figure 2) (Clairfeuille, Xu, Koth, & Payandeh, 2016). An extracellular linker connects helix S5 to a membrane-descending P-helix (P1), followed by an ascending P-helix (P2) and a further short extracellular loop connecting to helix S6 (Figures 1, 2). The extracellular loops from each domain create a turret-like structure for the outer mouth, which extends above the pore. They are glycosylated and form a pre-selection vestibule filter. In the cockroach structure, the extracellular loops contain disulphide bonds. Sequence comparison of the cockroach Na_v channel with mammalian Na_v channels shows that the cysteines (and thus, most likely, the disulphide bonds) are fully conserved, implying an important role in stabilizing the ion channel preselection filter (Shen et al., 2017). The narrowest point in the vestibule occurs where the P1 and P2 helices reverse direction within the membrane (Figures 1A, 3). Here charged residues provide a high field strength (HFS) site at the constriction point (Stephens, Guan, Zhorov, & Spafford, 2015). In eukaryotic Na_v channels, the residues constituting this site are aspartate (DI), glutamate (DII), lysine (DIII), and alanine (DIV). This creates an asymmetrical selectivity filter that is largely responsible for favoring sodium ions over other positively charged ions such as potassium or calcium (Heinemann, Terlau, Stuhmer, Imoto, & Numa, 1992).



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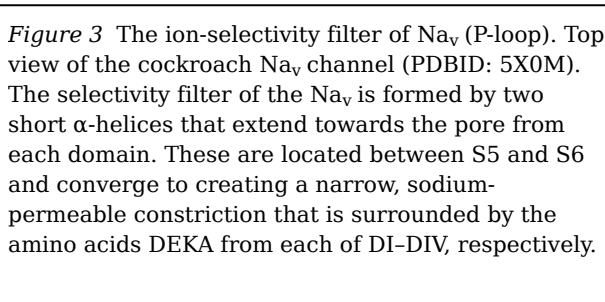

Figure 3 The ion-selectivity filter of Na_v (P-loop). Top view of the cockroach Na_v channel (PDBID: 5X0M). The selectivity filter of the Na_v is formed by two short α-helices that extend towards the pore from each domain. These are located between S5 and S6 and converge to creating a narrow, sodium-permeable constriction that is surrounded by the amino acids DEKA from each of DI–DIV, respectively.

To open the pore, the VSM must detect changes in membrane potential and transmit this information electromechanically to the PM by inducing an allosteric rearrangement of the S5 and S6 helices. Transmembrane helix S4 of the VSM contains four to six positively charged arginine and lysine residues that serve as the gating charges. They occur every three residues, so that the positive charges approximately lie along one face of the S4 helix (Noda et al., 1984) (Figure 4). For this arrangement to be thermodynamically feasible, the S4 positive charges within the membrane must be neutralized by forming ion-pairs with corresponding acidic groups from residues within the surrounding S1–S3 helices. According to the “sliding helix” (Catterall, 1986) and “helical screw” (Guy & Seetharamulu, 1986) models, the negative internal membrane potential provides a “pulling” force to keep these charges facing inward in the resting state. Following depolarization, this force is transiently weakened, enabling the S4 helix to move outward, probably in a spiral path, so that ion-pairs exchange partners. There is a wealth of evidence that supports the general outline of the model. This includes chemical modification, fluorescent labelling experiments, and mutagenesis studies guided by atomic-resolution structures of prokaryotic Na_v channels (Catterall, 2010; Clairfeuille et al., 2016; DeCaen, Yarov-Yarovoy, Zhao, Scheuer, & Catterall, 2008; Zhang et al., 2012). Nevertheless, some questions remain. For example, there is some debate as to whether the inner part of the S4 helix may transiently adopt a 3_{10} helix during this movement (Ahern et al., 2016; Villalba-Galea, Sandtner, Starace, & Bezanilla, 2008). If so, this would enable the inner region of the S4 helix to stretch during the activation process. A subtle point is that the transition from α-helix to high-energy 3_{10} helix, driven by the electrical field, would provide a means of capturing electrostatic potential energy, which can subsequently be used to drive the rearrangements needed to open the pore (Yarov-Yarovoy et al., 2012). Structures of prokaryotic Na_v channels trapped in distinct conformational states suggest that following activation, the VSM rotates in the membrane plane around the PM, thus exerting a torque on the S4–S5 linker (Catterall, Wisedchaisri, & Zheng, 2017; Clairfeuille et al., 2016). This pulls the lower end of the S5 helix outward and shifts the positions of the PM helices, with the S6 helix twisting in a counterclockwise manner (as seen from the intracellular face), and thereby opening the pore. The characteristic feature of Na_v channels, whereby each VSM is most closely associated with the PM of its neighbor (Figure 2), can now be rationalized, as this arrangement will facilitate gating by enforcing a concerted opening.

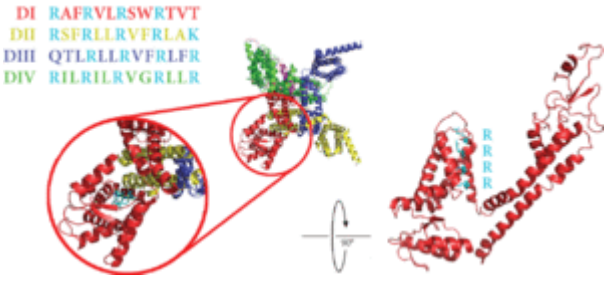

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Figure 4 The voltage-sensor: In each domain, charged residues in S4 detect changes in the cell membrane potential. Here the Na_v channel is viewed from below and DI is highlighted in more detail. Charged residues in S4 are shown in the enlarged image (cyan sidechains) with an accompanying alignment of S4 residues from each of the four domains. The image is of the cockroach Na_v channel crystal structure (PDBID: 5X0M).

In mammalian and probably other eukaryotic Na_v channels, the four VSMs activate with differing kinetics. Movement of the DI–DIII VSMs is the most rapid and is sufficient to begin ion flow (Chanda & Bezanilla, 2002). Interestingly, in the cockroach Na_v channel structure, two of the four S4 helices adopt a 3_{10} conformation and are in different relative positions (Shen et al., 2017), suggesting that this structure may have captured some of the presumed heterogeneity in eukaryotic VSM activation. The DIV VSM activates with the slowest kinetics (Bosmans, Martin-Eauclaire, & Swartz, 2008). Its movement frees an intracellular linker called the *inactivation gate* that connects DIII helix S6 to DIV helix S1 (Figure 1A) (Capes, Goldschen-Ohm, Arcisio-Miranda, Bezanilla, & Chanda, 2013) The inactivation gate contains a cluster of hydrophobic residues containing the amino acid sequence IFMT (the IFMT motif) that can now bind to a corresponding inactivation particle receptor lying within the S4–S5 linkers of DII, DIII, and DIV (Popa, Alekov, Bail, Lehmann-Horn, & Lerche, 2004). As a result, the inactivation gate occludes the pore and inactivates the channel within a few milliseconds of opening. This is the molecular basis of the fast inactivation pathway and ensures that the action potential can only be propagated in the forward direction.

The Na_v Channel Auxiliary Subunits

The Na_v channel α-subunit *in vivo,* typically exists in association with auxiliary subunits and other proteins within larger macromolecular assemblies localized to discrete regions of the plasma membrane (Abriel, 2010; Abriel, Rougier, & Jalife, 2015; Heine, Ciuraszkiewicz, Voigt, Heck, & Bikbaev, 2016; Lee, Fakler, Kaczmarek, & Isom, 2014; Meadows & Isom, 2005). The best-characterized auxiliary proteins are the Na_v β-subunits for which four genes (*Scn1b, Scn2b, Scn3b*, and *Scn4b*) encode the proteins β1, β2, β3, and β4. In addition, alternative splicing of the *Scn1b* gene can generate a secreted β1 Ig domain, lacking a transmembrane domain (Qin et al., 2003). All β-subunits have a type I membrane topology containing one extracellular amino-terminal single V-type immunoglobulin (Ig) domain connected through a short neck to a transmembrane alpha-helical domain and a small intracellular carboxy-terminal region. The β-subunits modulate intracellular Na_v channel traffic, surface expression, and protein stability; they generally

enhance rates of channel activation, inactivation, and recovery from inactivation, and they modulate the voltage-dependencies of these parameters. Some β-subunits are also involved in transmediated cell adhesion (Brackenbury & Isom, 2011; Cusdin, Clare, & Jackson, 2008; Namadurai et al., 2015).

A characteristic feature of the β-subunits is their ability to shift the half-maximal voltages ($V_{1/2}$) for activation and inactivation, usually in a hyperpolarizing direction (i.e., the voltage where half the channels activate or inactivate is shifted to more negative values compared to the values shown by the α-subunit alone). As a result, the action potential threshold is lowered, leading to an increased probability of firing (Namadurai et al., 2015). For the case of β1, these shifts can be abolished under conditions that inhibit the addition of sialic acid to N-linked sugar residues (Johnson, Montpetit, Stocker, & Bennett, 2004). All four of the β-subunit Ig domains are heterogeneously glycosylated in vivo. Hence, we have suggested that the β-subunit Ig domains might be positioned on the α-subunit in such a way that they can present negative charges from N-linked sugars close enough to one or more of the VSMs to influence its local field (Namadurai et al., 2015). Topological considerations indicate that the simplest way this can be achieved is for the Ig domain to bind a site or sites on the large extracellular S5–S6 pore loops and the β2-binding site has indeed been mapped to this loop on DII (Das, Gilchrist, Bosmans, & Van Petegem, 2016). Because of the close sequence and structural similarity between the β2 and β4 Ig domains (Das et al., 2016; Gilchrist, Das, Van Petegem, & Bosmans, 2013), it is likely that these two β-subunits bind to the same region on the α-subunit. In both cases, the β2 and β4 Ig domains bind covalently to the α-subunit through a disulphide bond. In contrast, the β1 and β3 Ig domains bind non-covalently to a different site. There is some evidence that the β1 Ig domain contacts sites on the S5–S6 extracellular loop from DI and DIV (Makita, Bennett, & George, 1996), but the binding site for the β3-subunit Ig domain has not yet been identified.

A striking feature of the β3-subunit is its ability to form homo-dimers and trimers in vivo,that can cross-link Na_v α-subunits (Namadurai et al., 2014). There are literature reports suggesting that even in the absence of β-subunits, the heart-specific channel Nav1.5 α-subunits can associate together on the plasma membrane (Clatot et al., 2012). So, the β3-subunit may promote and further stabilize this natural tendency for oligomerization (Namadurai et al., 2014). Furthermore, several β-subunits, including β3, can bind in a *cis* configuration to cell-adhesion molecules NrCAM, neurofascin, and contactins (Kazarinova-Noyes et al., 2001; Ratcliffe, Westenbroek, Curtis, & Catterall, 2001). Since these molecules contain multiple Ig-like binding sites, they could in principle bind more than one Na_v channel and thus further enhance and extend local Na_v channel clustering. There is some evidence that the β1-subunit can also cross-link Na_v channel α-subunits in vivo. For example, a mutant Na_v 1.5 channel is retained in the endoplasmic reticulum (ER) and thus leads to a form of Brugada syndrome. Remarkably, the wild-type Na_v1.5 channel is also trapped in the ER when co-expressed with both the mutant and in the presence of the β1-subunit (Mercier et al., 2012; Namadurai et al., 2015). This suggests that β1, like β3, may promote the formation of Na_v channel oligomers. The β1-subunit can also bind to the K_v4.2 potassium channel, a major regulator of neuronal

excitability (Marionneau et al., 2012), and proteomic analysis suggest that the heart-specific $Na_v1.5$ channel can also stably interact with potassium channels;although whether this is a direct association or mediated by additional proteins is not clear (Willis, Ponce-Balbuena, & Jalife, 2015). The existence of such cross-linked Na_v channel α-subunits raises the question of whether they become functionally coupled under these conditions. This is a controversial idea (McCormick, Shu, & Yu, 2007), but functional coupling between different α-subunits could contribute to rapid action potential initiation and gating. It may also help explain why many inherited sodium channel pathologies exhibit dominant-negative phenotypes (Hoshi et al., 2014; Keller et al., 2005; Poelzing et al., 2006; Sottas & Abriel, 2016). Dominant negative behavior is a common feature when a mutant subunit is incorporated into a multicomponent assembly and blocks the activity of all subunits in the complex (Veitia, 2007).

The β1 and β3 subunits also enhance the rates of inactivation and recovery from inactivation of the channel. The DIII–DIV linker region containing the inactivation gate also contains a separate binding site for the carboxy-terminus of the α-subunit, and binding between these two regions stabilizes the inactivated state (Kass, 2006). The likely importance of this interaction is illustrated by the existence of an epilepsy-inducing mutation in the carboxy-terminus of $Na_v1.1$ that disrupts the interaction and slows inactivation (Spampanato et al., 2004). The intracellular carboxy-termini of β1 and β3 also bind to the α-subunit carboxy-terminus. Thus, the β1 and β3-subunits may facilitate fast inactivation by enhancing the binding of the α-subunit carboxy-terminus and the inactivation gate. Furthermore, the α-subunit carboxy-terminal domain contains two EF hands and a calmodulin-binding IQ motif, both structural features involved in calcium sensing (Miloushev et al., 2009). In $Na_v1.4$, $Na_v1.5$, and $Na_v1.6$, calmodulin binds to both the α-subunit carboxy-terminal domain and the inactivation gate, and confers calcium-sensitivity on the inactivation properties of the channel (Gabelli et al., 2014; Sarhan, Tung, Van Petegem, & Ahern, 2012). The importance of this property is shown by several distinct arrhythmogenic mutations in the IQ motif of the $Na_v1.5$ carboxy-terminus (Rook et al., 1999).

As with almost all intrinsic membrane proteins, Na_v channels first fold and assemble in the lumen of the ER. It is not uncommon for large intrinsic membrane proteins to fold with relatively low efficiency, leading to accumulation in the ER. Under these circumstances, chaperones typically enhance folding efficiency (Araki & Nagata, 2011). One role of the β-subunits may be to act as a chaperone, since they increase Na_v channel trafficking out of the ER and to the plasma membrane (Cusdin et al., 2008). Na_v channel trafficking can also be disrupted by mutations that prevent the normal anchoring of the channel into the plasma membrane. For example, the membrane-bound cytoskeletal protein ankyrin-G enhances the clustering of $Na_v1.2$ and $Na_v1.6$ into the nodes of Ranvier and axon initial segment (Cusdin et al., 2008). In $Na_v1.6$, mutations in a cytoplasmic linker sequence between DII helix S6 and DIII helix S1 prevent the binding to ankyrin-G and inhibit channel association with the axon initial segment (Gasser et al., 2012).

“Non-classical” Roles for Na_v Channels

Na_v channels are also expressed in cells not usually thought to be electrically excitable (Black & Waxman, 2013). A notable example is the expression of the cardiac channel $Na_v1.5$ in phagosomes and late endosomes of activated macrophages. Both selective siRNA knockdown and tetrodotoxin treatment inhibit phagocytosis in these cells, suggesting that the Na_v channel has an important function in these processes. A likely role for $Na_v1.5$ is to allow sodium ion efflux from the endolysosomes, to provide charge counterbalance for the protons pumped into the organelles. Since the $Na_v1.5$ channel is inhibited by low pH, this could act as a feedback inhibitor of excessive acidification (Carrithers et al., 2007).

The $Na_v1.5$ channel is also expressed in astrocytes under pathological conditions leading to astrogliosis (Black, Newcombe, & Waxman, 2010). In murine models of multiple sclerosis, the relative abundance of $Na_v1.5$ in astrocytes is correlated with disease severity (Pappalardo, Liu, Black, & Waxman, 2014). An alternatively spliced neonatal form of $Na_v1.5$ was expressed in astrocytomas and its level correlated with increasing astrocytoma grade (Xing et al., 2014). Additionally, in the human U251 astrocytoma model cell-line, siRNA knockdown of $Na_v1.5$ expression conferred a loss or reduction in the proliferative, invasive, and migratory properties of these cells, and enhanced their apoptosis (Xing et al., 2014). The enhanced invasive properties that $Na_v1.5$ confers in this context has also been observed in human breast cancer cells (Nelson, Yang, Millican-Slater, & Brackenbury, 2015). The causal mechanisms connecting $Na_v1.5$ expression to enhanced invasiveness is not clear, but one attractive hypothesis suggests that the $Na_v1.5$ channels co-localize in lipid rafts with the Na^+/H^+ exchanger (NHE-1). Sodium influx activates the exchanger leading to enhanced proton extrusion and the subsequent activation of acid-dependent cell-surface proteases (Brisson et al., 2011). Similarly, an enhanced expression of $Na_v1.7$ has been observed in several prostate cancer cell-lines, where the expression correlates with invasive potential (Brackenbury & Djamgoz, 2007). Collectively, these findings suggest a key role for Na_v channels in many pathologies, including those that regulate neuroinflammatory processes and enhance the aggressive nature of cancer cells, with roles that are distinct from that of its classic function in cell excitability.

How Different Na_v Channel Isoforms Combine in Physiological Context: Examples from Cardiac Cells and Peripheral Pain Neurones

Most electrically excitable cells express multiple Na_v channel isoforms, each with different gating behaviors and distinct opening and inactivation kinetics. In such cases, the combined functional interactions between these distinct Na_v channel isoforms, acting together, can generate complex and emergent behavior. To illustrate this concept, two examples are discussed: cardiac cells and dorsal root ganglia (DRG) sensory neurones.

Nav Channel Behavior in Cardiac Cells

The heart expresses not only cardiac $Na_v1.5$, but also the neuronal, $Na_v1.1$, $Na_v1.3$, and $Na_v1.6$ channels (Maier et al., 2003). The occurrence and abundance of these different Na_v channel isoforms vary between different cardiac tissue types. Regenerative voltage-dependent Na_v channel opening triggers the large, rapid, inward depolarizing current that initiates the action potential upstroke. This is fundamental to *propagation* of the cardiac electrical activity through successive cardiomyocytes, triggering the heartbeat. In addition, Na_v channel activation thresholds determine the onset of the upstroke, relative to the background pacing ion channel activity. This determines the *frequency* of pacemaker excitation (Huang, Lei, Matthews, Zhang, & Lei, 2012). Both processes underlie function in the cardiac sino-atrial node (SAN), the source of the heart's natural pacemaker activity, in which co-expression of and interaction between Na_v channel α-subunit isoforms play central roles.

The SAN pacemaker comprises central and peripheral regions. The structure is in turn surrounded by the atrial tissue to which it conducts the resulting rhythmic excitation. Pacemaker activity begins from a repolarization of the preceding action potential. The extent of this depends upon the magnitude of the outward current mediated by the rapid K^+ channel (Verheijck, van Ginneken, Bourier, & Bouman, 1995). The resulting hyperpolarization activates an inward depolarizing, so-called *funny current*, carried primarily by the HCN4 hyperpolarization-activated cyclic nucleotide-gated (HCN) channel. Later phases of diastolic depolarization involve contributions from the depolarizing late, L-type, calcium current and transient, T-type, Ca^{2+} channel current (Sanders, Rakovic, Lowe, Mattick, & Terrar, 2006). The resulting membrane potential depolarization activates the Na_v channels that in turn trigger the action potential. However, SAN function not only involves a pacing process, but also requires conduction of the resulting excitation between its successive component cells from the center to the periphery of the SAN and between the outermost cells of the SAN and its coupled atrial cells. Here, different SAN regions and distinct Na_v isoforms play distinct roles. Whereas $Na_v1.1$ occurs throughout the SAN, occurrence of the $Na_v1.5$ channel is restricted to peripheral as opposed to central SAN cells (Lei et al., 2004). Cells in the central SAN pacemaker region are smaller in size and therefore also in total membrane capacitance. Their consequently larger input impedance, together with the rapid kinetics of activation shown by their $Na_v1.1$ channels, enhances excitability and therefore their primarily pacemaker role within the SAN. This contrasts with the larger size, larger total membrane capacitance and consequent lower input impedance of the peripheral region cells that express $Na_v1.5$. Consequently, their function consequently appears primarily to involve conduction of the resulting action potential from the central pacemaker region to the atrial cells that surround the SAN. Both conduction and activation processes vary with the relationship between active and passive electrophysiological properties between coupled cells within the SAN. They would also be affected by current-load matching

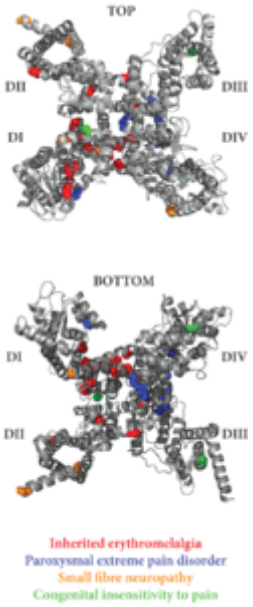
properties between peripheral SAN cells and the atrial myocytes to which they are directly coupled.

Such distinct pacing and conducting roles shown by $Na_v1.1$ and $Na_v1.5$ were demonstrated through experiments separating these contributions through the greater tetrodotoxin (TTX)-sensitivity of $Na_v1.1$ compared to $Na_v1.5$. Firstly, challenge by TTX at nM-concentrations that would selectively inhibit $Na_v1.1$ reduced pacemaker rates by 65%, 22%, and 15% in intact mouse hearts (Maier et al., 2002), isolated SA nodes, and isolated SAN pacemaker cells, respectively (Lei et al., 2004). Secondly, action potential clamp studies demonstrated that such TTX-sensitive Na_v currents are activated within voltage ranges traversed by the pacemaker potential, consistent with its additional participation in action potential conduction. Thirdly, block of both the TTX-sensitive and TTX-resistant Na^+ current by TTX at μM concentrations, but not selective block of TTX-sensitive Na^+ current by 10 or 100 nM TTX, increased SAN conduction times from the leading pacemaker site in the center of the SAN through the periphery to surrounding atrial muscle. Finally, modifications in either $Na_v1.1$ or $Na_v1.5$ influenced the emergent heart rates. Thus, $Na_v1.5$ haploinsufficient *Scn5a*$^{+/-}$ mice replicated the depressed heart rates and sino-atrial block clinically observed in sinus node disorder patients (Asseman et al., 1983). Their isolated hearts showed sinus bradycardia, with both slowed, and episodes of blocked, sino-atrial conduction. Isolated SAN and atrial tissue preparations from these *Scn5a*$^{+/-}$ mice similarly exhibited both slowed and blocked sino-atrial conduction. SAN cells from the *Scn5a*$^{+/-}$ mice demonstrated about a 30% reduction in maximum Na^+ currents compared to wild-type cells (Lei et al., 2005). These findings may also form the basis for human SAN syndromes associated with genetic defects in $Na_v1.5$, whose features are of significant clinical importance (Lei et al., 2004; Maier et al., 2003). Thus, sinus node dysfunction affects ~1 in 600 cardiac patients aged over 65 years and constitutes the clinical indication for ~50% of the million permanent pacemaker implants per year worldwide (Dobrzynski, Boyett, & Anderson, 2007).

Peripheral Pain-Sensing Neurones

Sensory neurones in the DRG detect painful stimuli that are transmitted to the spinal cord (see the chapter by Xiao et al., this volume). These neurones express a number of distinct Na_v channel isoforms, in particular, $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$, with smaller amounts of $Na_v1.3$ (Rogers, Tang, Madge, & Stevens, 2006). Associated β-subunits, particularly β3, whose expression correlates closely with that of $Na_v1.7$, may fine-tune the gating behavior of the channels (Shah et al., 2000). Acting together with voltage-dependent calcium and potassium channels, these molecules set the resting potential, action potential threshold, and neuronal firing rate (Waxman, 2012). To add further complexity, the relative expression of these channel isoforms is dynamic, and it can change dramatically following peripheral nerve damage (Chahine & O'Leary, 2014).

The existence of rare individuals congenitally insensitive to pain (CIP) led to the identification of $Na_v1.7$ as an Na_v channel with a critical role in pain perception (Cox et al., 2006). Most patients with congenital pain insensitivity possess $Na_v1.7$-deletion mutations that prevent functional expression of the protein. However, some missense $Na_v1.7$ mutations precipitate syndromes with the opposite pathology;a variety of extreme and often chronic pain conditions such as inherited erythromelalgia (IEM) and paroxysmal extreme pain disorder (PEPD) (Habib, Wood, & Cox, 2015). The IEM and PEPD mutant channels are hyperexcitable, but act in different ways. In IEM, the $Na_v1.7$ mutation leads to a pronounced hyperpolarizing shift in the voltage-dependenceof activation, causing a lowered action potential threshold (Cummins, Dib-Hajj, & Waxman, 2004). In contrast, PEPD mutations are more associated with depolarizing shifts in steady-state fast inactivation, which leads to a higher persistent current (Fertleman et al., 2006; Lampert, O'Reilly, Reeh, & Leffler, 2010). In both cases, the mutant channels become more easily activated by the initiating impulses. Figure 5 maps the locations of these mutations onto the cockroach Na_v channel (Shen et al., 2017). A striking feature of this analysis is how many of the mutations map to known functionally critical regions of the channels. Some 80% are found in the VSMs and PMs, with no clear distinction between different domains. In some cases, the position of a mutation immediately suggests an explanation for the functional impairment shown by the channel. For example, several of the IEM and PEPD mutants map to the S4 helix of one or more VSMs, and to the S4–S5 cytosolic linker connecting the VSM to the PMs. Other PEPD mutants map to the IFMT inactivation gate (Figures 1, 2, 5), most likely compromising fast inactivation and leading to persistent currents. On the other hand, it is not clear how some of the mutations affect activity. The rare cases of missense CIP mutants are good examples. All three of these mutations occur within the S5–S6 extracellular pore-loops where they are not obviously close to any of the recognized and important functional regions of the channel (Figure 5). Hence, the full atomic-resolution structure of $Na_v1.7$ may be required to further understand their pathology.



Click to view larger

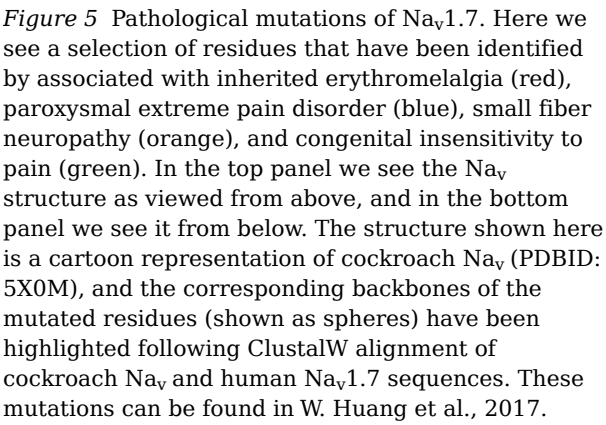


Figure 5 Pathological mutations of $Na_v1.7$. Here we see a selection of residues that have been identified by associated with inherited erythromelalgia (red), paroxysmal extreme pain disorder (blue), small fiber neuropathy (orange), and congenital insensitivity to pain (green). In the top panel we see the Na_v structure as viewed from above, and in the bottom panel we see it from below. The structure shown here is a cartoon representation of cockroach Na_v (PDBID: 5X0M), and the corresponding backbones of the mutated residues (shown as spheres) have been highlighted following ClustalW alignment of cockroach Na_v and human $Na_v1.7$ sequences. These mutations can be found in W. Huang et al., 2017.

The $Na_v1.7$ channels are mainly expressed in the terminals of sensory neurones. They activate and inactivate rapidly, but recover from inactivation relatively slowly. Their normal role is to activate in response to small depolarizations close to the resting potential. In doing so, they respond to and amplify ramp stimuli, and so bring the neurone closer to its action potential threshold (Herzog, Cummins, Ghassemi, Dib-Hajj, & Waxman, 2003). However, the $Na_v1.8$ isoform, also present in DRG neurones, activates at more depolarized potentials and has a fast recovery from inactivation profile. It will therefore respond to the initial $Na_v1.7$-driven depolarizations by repetitive firing (Renganathan, Cummins, & Waxman, 2001). The $Na_v1.3$ isoform, normally associated with the central nervous system, is up-regulated in DRGs in a variety of chronic pain conditions (Shah et al., 2000). This isoform shows a fast recovery from inactivation and a significant persistent current (Lampert, Hains, & Waxman, 2006). Both properties will probably contribute to hyperexcitability following nerve injury.

Towards the Specific Targeting of Voltage-Gated Sodium Channels: Toxins and Antibodies

Na_v channels are major drug targets, and inhibitors have been clinically exploited to provide therapeutics acting as antiarrthythmics, anticonvulsants, and local anesthetics. Agents such as flecainide and lidocaine are key examples (Salvage et al., 2017; Sheets, Fozzard, Lipkind, & Hanck, 2010). Unfortunately, the lack of Na_v channel isoform selectivity of these inhibitors can limit their therapeutic use. Given the prominent role of Na_v channels in such a wide range of diseases, there is significant interest in developing *isoform-selective* inhibitors. This is particularly important in the field of pain disorders (Kwong & Carr, 2015). The compelling genetic evidence for the role of $Na_v1.7$ in pain perception has made this Na_v channel isoform a major pharmacological target (Vetter et al., 2017). It should also be noted that CIP patients who lack functional $Na_v1.7$ show no other serious developmental defects—although they may be anosmic (Weiss et al., 2011). This indicates that, unlike some Na_v channel isoforms, $Na_v1.7$ is functionally specialized, making it a highly attractive pharmacological target. Recent reports have described a number of promising and selective small-molecule inhibitors that have advanced to the stage of clinical trials (Kwong & Carr, 2015), including aryl sulphonamides ICA-12143, PF-04856264, and GX-936, which target the S1–S4 voltage sensor domain of DIV (Ahuja et al., 2015; McCormack et al., 2013). The structural basis for binding GX-936 was elucidated by co-crystallizing the compound with a chimeric protein composed of the VSM from a bacterial Na_v channel engineered to contain portions of the DIV VSM of human Na_v 1.7 (Ahuja et al., 2015). This approach, combining traditional small-molecule screening methods, but guided by structural insights into the target protein, offers a powerful general method for new drug discovery.

Another area of research in the discovery of subtype selective inhibitors is the exploitation of natural toxins that target Na_v channels: in particular, peptide-based toxins isolated from the venoms of sea anemones, spiders, snails, scorpions, and centipedes (de Lera Ruiz & Kraus, 2015). This is a rich and still largely untapped resource, and it has been estimated that there may be millions of spider-venom peptides (Escoubas, Sollod, & King, 2006). Given their diversity, the study of these molecules has identified interesting therapeutic leads that are often more potent than small molecules. Despite their variety, these toxins act in a limited number of ways, usually by binding to the pore region and thus blocking sodium entry, or by binding to and inhibiting movement of the VSM (Gilchrist, Olivera, & Bosmans, 2014). Two promising examples are protoxin-II (ProTx-II) and huwentoxin-IV (HwTx-IV), which exhibit a degree of Na_v subtype selectivity. Both target the domain II voltage sensor of Na_v 1.7 (Klint et al., 2012). In the case of huwentoxin-IV, the toxin inhibits Na_v 1.7 with an IC_{50} of 26nM but has an IC_{50} of > 10μM for Na_v 1.5, demonstrating the pharmacological selectivity of the toxin (Revell et al., 2013). In other cases, the isoform specificity of the toxin may not be sufficient for immediate clinical use. Here, a variety of approaches might be adopted to engineer the

proteins to have improved potency and selectivity. These methods include generation of peptide libraries, directed evolution, saturation mutagenesis, and chemical modification (alone or in combination), and analysis of structure–activity relationships to identify improved variants (Flinspach et al., 2017). As an example, a structure–function study of HwTx-IV was performed in which chemically synthesized and oxidatively folded peptide analogues were made and studied by automated electrophysiology. Using this approach, several peptides with enhanced potency for $Na_v1.7$ (up to 45-fold compared to the wild-type peptide) were produced (Revell et al., 2013). As a second example, native tarantula ceratotoxin-1 (CcoTx1) binds to a number of different Na_v channel isoforms. Through a combination of directed evolution and structure-based mutagenesis, a variant was generated that was highly potent for $Na_v1.7$ compared to other Na_v channels (Shcherbatko et al., 2016).

Antibody therapeutics represent a further alternative approach (Reichert, 2012). A major part of their attraction is the ability to achieve exceptionally high specificity for the target. Protein engineering techniques can also be applied during lead optimization to enhance the affinity (in the nanomolar to subpicomolar range) and potency, as well as the effector function and pharmacokinetic properties to improve cellular cytotoxicity and plasma half-life (Wilkinson, Gardener, & Williams, 2015).

The potential for monoclonal antibody–based blocking of Na_v channels has been appreciated for some time (Meiri et al., 1986). However, for clinical use, monoclonal antibodies would have to be specific for a particular Na_v channel isoform. There are now several examples of functional antibodies that have been raised to peptides representing either the first or second loops of the DI or DII voltage sensors of Na_v channels or the pore loops (Finney et al., 2016; J. H. Lee et al., 2014; Macdonald, Murphy, Papadopoulos, Stahl, & Alessandri-Haber, 2014; Ulrichts et al., 2015; Xu et al., 2005). A number of antibodies bound to the pore loop with a K_D in the picomolar to nanomolar range, an affinity level that is requiredappropriate for therapeutic use. An example of a VSM antagonist is a monoclonal antibody (SVmAb1) raised against the second extracellular loop of the DII S3–S4 voltage sensor. This is the same site targeted by HwTx-IV and ProTx-II. Application of SVmAb1 stabilized the closed state, thus inhibiting activation, and was claimed to be efficacious in mouse models of neuropathic and inflammatory pain as well in suppressing acute and chronic itch. SVmAb1 was shown to inhibit the function of $Na_v1.7$ with high subtype specificity and potency in a state-dependent manner (J. H. Lee et al., 2014). Unfortunately, these claims were not confirmed by a subsequent study (Liu et al., 2016). Indeed, Liu et al. could not even demonstrate binding to $Na_v1.7$ of a recombinant antibody made to the same sequence as SVmAb1. The striking disparity between the results of these two groups is not resolved, but it highlights the challenging task of screening for anti-(Na_v channel) functional antibodies using electrophysiology techniques that can be extremely sensitive to many variables such as buffer components and pH.

Antibodies generally show a limited permeation through the blood–brain barrier. This this can be an advantage if the aim is to target only peripheral subtypes (such as the pain-associated $Na_v1.7$) and in doing so, to avoid antagonizing subtypes in the central nervous system, which may result in undesirable side-effects, but therelatively large size of antibodies can restrict penetration into tissues more generally. Recent developments have exploited camelid antibodies that lack the two immunoglobulin light-chains and the first immunoglobulin heavy-chain constant domain. These antibodies are thus smaller than conventional antibodies and allow penetration into more inaccessible epitopes (Nguyen, Desmyter, & Muyldermans, 2001).

Alternative biological approaches have been investigated that utilize micro RNAs/ short hairpin RNAs (shRNAs) to knockdown expression of the protein at the mRNA level (Muroi et al., 2011; Shao et al., 2016; Spencer, 2016). Providing suitable delivery methods are developed in the future, this type of drug could be more effective and more specific than a small molecule, antibody, or peptide antagonist.

Summary

In the 66 years since the Hodgkin Huxley model was first described (Hodgkin & Huxley, 1952), voltage-dependent Na_v channels have moved from a necessary mathematical abstraction to purified proteins, and now increasingly to atomic-resolution understanding. During this time, work on Na_v channels has inspired and sometimes driven experimental innovations in protein chemistry, electrophysiology, and pharmacology. The imminent arrival over the next few years of atomic-resolution structures for mammalian Na_v channels will undoubtedly inspire further hypotheses to provide better molecular insights into channel behavior, both normal and pathological. This in turn should encourage new approaches to rational drug development.

But there are still major unresolved questions. For example, the broader physiological and cell-biological context of Na_v channels, as they exist on the plasma membrane of neurones and muscle cells, is far from clear. This includes the roles of additional and auxiliary subunits and other Na_v channel interactors that modify channel behavior in vivo. To address such questions will probably require the application of high-resolution imaging techniques such as cryo-electron tomography, and of analytical techniques such as quantitative proteomics. This will represent a second revolution in understanding Na_v channel biology.

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