Selective Ligands and Drug Discovery Targeting the Voltage-Gated Sodium Channel Nav1.7

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Contents

Abstract

The voltage-gated sodium (Nav) channel Nav1.7 has been the focus of intense investigation in recent years. Human genetics studies of individuals with gain-of-function and loss-of-function mutations in the Nav1.7 channel have implicated

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Nav1.7 as playing a critical role in pain. Therefore, selective inhibition of Nav1.7 represents a potentially new analgesic strategy that is expected to be devoid of the significant liabilities associated with available treatment options. Although the identification and development of selective Nav channel modulators have historically been challenging, a number of recent publications has demonstrated progression of increasingly subtype-selective small molecules and peptides toward potential use in preclinical or clinical studies. In this respect, we focus on three binding sites that appear to offer the highest potential for the discovery and optimization of Nav1.7-selective inhibitors: the extracellular vestibule of the pore, the extracellular loops of voltage-sensor domain II (VSD2), and the extracellular loops of voltage-sensor domain IV (VSD4). Notably, these three receptor sites on Nav1.7 can all be defined as extracellular druggable sites, suggesting that non-small molecule formats are potential therapeutic options. In this chapter, we will review specific considerations and challenges underlying the identification and optimization of selective, potential therapeutics targeting Nav1.7 for chronic pain indications.

Keywords
Drug discovery · Nav1.7 · Pain · Subtype-selectivity · Voltage-gated sodium channel

1 Considerations for Selective, Therapeutic Targeting of Nav1.7

The voltage-gated sodium (Nav) channel Nav1.7 has been the focus of intense study in recent years. Numerous gain-of-function and loss-of-function mutations in the Nav1.7 channel have been implicated in inherited or spontaneous human pain syndromes (Cox et al. 2006; Goldberg et al. 2007; Vetter et al. 2017). Accordingly, our current understanding of the physiology of Nav1.7 has been the subject of many quality reviews (Vetter et al. 2017; Habib et al. 2015; Dib-Hajj et al. 2013; Fischer and Waxman 2010). Since Nav1.7 is primarily expressed in the peripheral nervous system, selective inhibition of Nav1.7 represents a potentially new analgesic strategy that is expected to be devoid of the significant liabilities associated with available treatment options, along with the potential to maintain high levels of efficacy and safety (Vetter et al. 2017; Skerratt and West 2015; Sun et al. 2014). Although the identification and development of selective Nav channel modulators have historically been challenging, a number of recent publications have summarized the extensive work that has progressed increasingly subtype-selective small molecule and peptide scaffolds forward for potential use in preclinical or clinical studies (Sun et al. 2014; Focken et al. 2016; Price et al. 2017; Marx et al. 2016; Flinspach et al. 2017; Deuis et al. 2017; Wu et al. 2017a; Kornecook et al. 2017; Swain et al. 2017; Storer et al. 2017; Murray et al. 2015a, b; Biswas et al. 2017; Schenkel et al. 2017; La et al. 2017; Graceffa et al. 2017; Shcherbatko et al. 2016; Alexandrou et al. 2016; Roecker

et al. 2017; Yang et al. 2014; Pero et al. 2017; Pineda et al. 2014; Bagal et al. 2015). It will not be the focus of this chapter, therefore, to exhaustively summarize work already presented by our colleagues in the field. Rather, we aim to focus on specific considerations and challenges underlying the identification of selective, potential therapeutics targeting Nav1.7 for chronic pain indications.

A diverse array of Nav channel modulators that target a vast number of receptor sites on Nav channels has been described and characterized in the literature (Cestele and Catterall 2000; de Lera Ruiz and Kraus 2015). Here, we focus primarily on the three binding sites that appear to offer the highest potential for the discovery and optimization of Nav1.7-selective inhibitors. The primary motivation in prioritizing these receptor sites for consideration includes available data that demonstrates that inhibition of Nav1.7 is possible at these sites, and that molecular selectivity can be achieved over closely related Nav channel subtypes. Accordingly, we focus on available chemical matter which is known to target: (1) the extracellular vestibule of the pore, the infamous tetrodotoxin (TTX) and saxitoxin (STX) binding site (Hille 1975); (2) the extracellular loops of voltage-sensor domain II (or VSD2); and (3) the extracellular loops of voltage-sensor domain IV (or VSD4). It is noteworthy that all three prioritized receptor sites on Nav1.7 can be defined as extracellular druggable sites, suggesting that non-small molecule formats are potential therapeutic options. However, we are immediately cautious about the challenges associated with subcutaneous or intravenous injection that would likely be necessary for the delivery of macrocycles, peptides, or antibody formats as required for long-term dosing and delivery in any potential chronic or neuropathic pain indications. Nevertheless, all molecular formats targeting these three prioritized Nav1.7 receptor sites are worthy of consideration and review, if for no other reason, as proof-of-concept modulators that may require further exploration, optimization, or development.

Focusing on these three receptor sites that appear to offer the most direct path to the discovery of a highly subtype-selective Nav1.7 inhibitor, it is important to point out the considerable physiological and functional distinctions that exist between these sites. First, inhibitors that target the extracellular vestibule of the pore, such as TTX and STX (Hille 1975), block in a largely voltage-independent manner (Cohen et al. 1981); therefore state-dependence of channel block would not be expected to contribute therapeutically or provide a potential increase in the safety window. Second, in the case of modulators targeting the extracellular loops of voltage-sensor domain II (or VSD2) (Bosmans et al. 2008), specific classes of peptide toxins are known that can inhibit channel activation, such as protoxin-II (ProTx2) (Middleton et al. 2002), which affords the distinct opportunity to inhibit a Nav1.7 channel that has not previously opened (Flinspach et al. 2017). Finally, while peptides such as α-scorpion toxins bind to the extracellular loops of voltage-sensor domain IV (or VSD4) to suppress fast-inactivation (Bosmans et al. 2008; Campos et al. 2008; Thomsen and Catterall 1989), a recently characterized class of highly subtype-selective small molecule inhibitor has been found to bind to this region of VSD4 (McCormack et al. 2013), but only after the channel has entered the inactivated state either following channel activation or via direct entry from a closed state (i.e., closed-state inactivation) (McCormack et al. 2013; Ahuja et al. 2015). How these

differentiating properties may be favorable or unfavorable for efficacy and safety in preclinical and clinical models for different pain indications remains unknown, but these are important considerations for the future as the field aims to develop and clinically validate useful and improved analgesics.

2 Introduction to Nav Channels

In a series of landmark studies (Hille 2001), Hodgkin and Huxley first posited that the membrane of an excitable cell must undergo rapid changes in the selective permeability of Na^+ and K^+ ions during an action potential, and that the movement of voltage-dependent gating particles controlled these changes (Hodgkin and Huxley 1952). The subsequent isolation, characterization, and molecular cloning of founding members of the voltage-gated ion channel (VGIC) superfamily confirmed the existence of membrane embedded pores that open and close ion selective passageways in response to small changes in the membrane potential (Papazian et al. 1987; Noda et al. 1986). Different classes of VGICs are now known to have distinct and diverse functional roles in neuronal and cellular excitability (Catterall et al. 2005a, b; Gutman et al. 2005). In humans, 10 voltage-gated sodium (Nav) channel, 40 voltage-gated potassium (Kv) channel, and 10 voltage-gated calcium (Cav) channel pore-forming α-subunit genes have been identified (Yu and Catterall 2004).

Nav channels initiate the upstroke of the action potential and are optimized for this specialized function by virtue of their rapid activation and inactivation properties (Hille 2001; Hodgkin and Huxley 1952). Nine Nav channel subtypes are functionally expressed in humans, Nav1.1–Nav1.9, whereas Nav2.1 or the Nax channel (encoded by the *SCN7A* gene) represents an atypical subtype whose biophysical and physiological properties remain poorly defined (Catterall et al. 2005a). The distinctive expression pattern of Nav channel subtypes along axons and within cell bodies helps to shape the firing properties of each excitable cell (Black et al. 1996; Fry et al. 2007; Ho and O'Leary 2011), but Nav channels are still often classified by their primary tissue and cellular expression profiles (Catterall et al. 2005a). The Nav1.1, Nav1.2, and Nav1.6 channel subtypes are best known for their predominant expression within the central nervous system (CNS), whereas Nav1.4 and Nav1.5 are most often referred to as the muscle and cardiac Nav channels, respectively (Catterall et al. 2005a). Not surprisingly, inherited or spontaneous mutations in Nav channel genes and their accessory subunits have been linked to various disorders in the brain, muscle, and heart (Escayg and Goldin 2010; O'Brien and Meisler 2013; Nicole and Fontaine 2015; Savio-Galimberti et al. 2017; O'Malley and Isom 2015).

3 Nav Channel Structure, Biophysics, and Receptor Sites

For decades, our structural-level understanding of Nav channels was rendered only through the analysis of rigorous biophysical studies that often utilized pharmacological and molecular biology tools as further probes of channel architecture (Hille

2001). At the turn of the century, the first high-resolution crystallographic structures of model K^+ channels began to serve as structural templates for homology modeling and further hypothesis-driven Nav channel research (Doyle et al. 1998; Long et al. 2005). Within the last decade, however, our molecular level view of Nav channels has been dramatically sharpened, first through the availability of ~3 Å resolution crystallographic models of simple homotetrameric bacterial Nav (BacNav) channel homologues (Payandeh et al. 2011, 2012; Zhang et al. 2012a; McCusker et al. 2012; Shaya et al. 2014), and more recently, further extended through advances in cryo-electron microscopy (cryoEM) techniques which have produced ~4 Å resolution structural models of intact eukaryotic Nav channels from insect and eel (Shen et al. 2017; Yan et al. 2017), respectively. These breakthrough structural data now set the stage for Nav channel pharmacology and drug discovery to finally enter the modern age of structure-guided drug design (Ahuja et al. 2015; Clairfeuille et al. 2017).

The pore-forming α-subunit of VGICs share a conserved architecture where four subunits or homologous domains create a central ion-conducting pore surrounded by four voltage-sensor domains (VSDs) (Yu and Catterall 2004; Long et al. 2005; Payandeh et al. 2011; Shen et al. 2017; Yan et al. 2017; Wu et al. 2015). The α-subunit of eukaryotic Nav channels contains 24-transmembrane (TM) segments linked in four homologous repeat domains (DI-DIV), where each domain contains six TM segments (S1–S6) (Fig. 1a, b). The S5 and S6 segments from each domain associate to form the central pore module (PM) which scaffolds the ion selectivity filter containing the signature sequence Asp-Glu-Lys-Ala (DEKA) locus that forms the Na^+ selectivity filter near the extracellular side (Shen et al. 2017; Yan et al. 2017). As anticipated from early receptor site mapping studies (Thomsen and Catterall 1989; Cohen et al. 2007; Leipold et al. 2007), the S1–S4 segments form the peripheral VSDs that exist in a domain-swapped arrangement around the pore in Nav channels (Fig. 1a, b). Mammalian Nav channels also associate with a family of immunoglobulin-like single-TM anchored auxiliary β-subunits that are known to modulate the trafficking, gating, and pharmacological profiles of Nav channels, often in a subtype or context-dependent manner (O'Malley and Isom 2015; Gilchrist et al. 2013; Chahine and O'Leary 2011; Ulbricht 2005; Das et al. 2016; Wilson et al. 2011a; Zhang et al. 2013). Recently, structural elucidation of the eel Nav1.4 channel-β1 subunit complex provided the first high-resolution insights into how β-subunits may exert these biochemical and biophysical properties onto the α-subunit (Yan et al. 2017) (Fig. 1b, c). Mammalian Nav channels are further known to interact with an array of intracellular proteins to form a signaling hub (Leterrier et al. 2010; Abriel and Kass 2005), and some of these interactions have also been characterized in structural detail (Pitt and Lee 2016). Although additional targetable sites may exist within these Nav channel-auxiliary subunit interfaces, we focus here on the potentially druggable receptor sites that have been established within the pore-forming α-subunit itself.

Central to the voltage-dependent gating of Nav channels, VSDs are capable of sensing changes in the membrane voltage by virtue of positively charged arginine (or lysine) residues known as gating charges that are found in a conserved RxxR motif along the S4 helix (Noda et al. 1986; Stuhmer et al. 1989). The architecture of Nav channels places the S4 gating charges within the membrane electric field, where

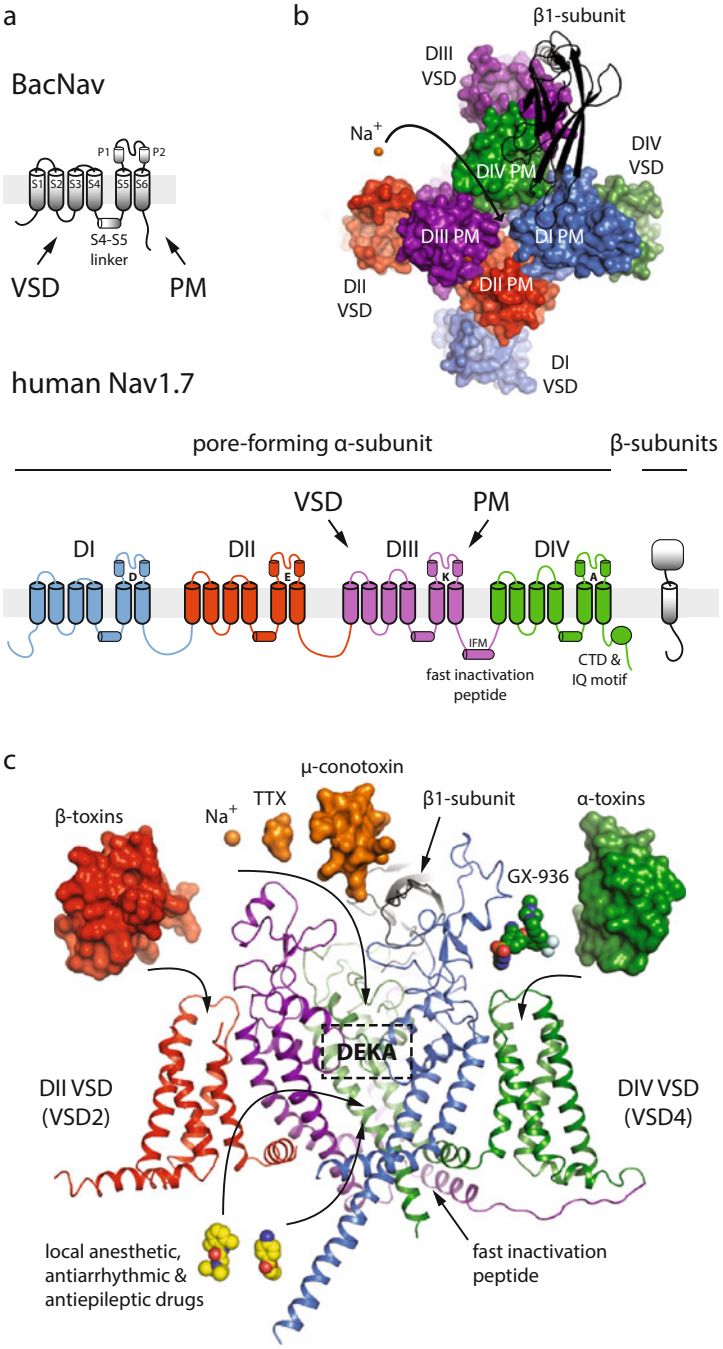

Fig. 1 Eukaryotic Nav channel architecture and major inhibitory receptor sites. (**a**) In gray, a schematic of the bacterial Nav (BacNav) channel subunit highlights the voltage-sensor domain (VSD, S1–S4) and the pore module (PM, S5–S6) connected through the S4–S5 linker. In color,

their outward displacement in response to membrane depolarization initiates opening of the central ion pore via an electromechanical coupling mechanism involving the intervening S4–S5 linker and associated VSD-PM contacts (Payandeh et al. 2011, 2012; Zhang et al. 2012a; Shen et al. 2017; Yan et al. 2017). High-resolution electrophysiological measurements of gating currents generated by the movement of gating charges have helped to confirm that the S4 segments are responsible for initiating processes of voltage-dependent activation and inactivation in Nav channels (Stuhmer et al. 1989; Armstrong and Bezanilla 1973; Bezanilla 2000). Using site-specific probes incorporated along the S4 and other regions within the VSD and channel, fluorometry measurements have further corroborated movement of the S4 during voltage-dependent activation and fast inactivation (Chanda and Bezanilla 2002; Chanda et al. 2004; Cha et al. 1999). In fact, a consensus in the field has begun to emerge suggesting displacements of the S4 on the order of 5–10 Å during gating (Vargas et al. 2012; Guo et al. 2016), although other extremes have been proposed (Chanda et al. 2005). However, the precise vector or mechanism of S4 displacement is still somewhat unsettled, since high-resolution structures of different VGICs so far hint that important differences likely exist between major channel subtypes (Guo et al. 2016; Whicher and MacKinnon 2016; Lee and MacKinnon 2017; Sun and MacKinnon 2017).

Physiological recordings and spectroscopic studies have confirmed that there is a functional specialization of the VSDs within Nav channels (Chanda and Bezanilla 2002; Chanda et al. 2004; Cha et al. 1999). Specifically, voltage-dependent activation of VSD1–3 is sufficient to open the central pore in mammalian Nav channels, whereas the activation of VSD4 is necessary to promote inactivation (Chanda and Bezanilla 2002; Capes et al. 2013). This is a key distinguishing functional feature between eukaryotic Nav channels and their homotetramic Kv channel counterparts (Ahern et al. 2016), where functional specialization of the VSDs is clearly permitted by the amino acid differences found within the eukaryotic Nav channel VSDs (Bosmans et al. 2008; Lacroix et al. 2013; Pless et al. 2014). Moreover, the sequence differences and resulting functional specialization of the four VSDs likely allowed for eukaryotic Nav channels to gain their contemporary role in initiating the action potential. Accordingly, pathogenic disease mutations and pharmacological modulators are known to target

Fig. 1 (continued) below, a schematic of the human Nav1.7 channel with each homologous domain (DI–DIV) shown in a different color and residues forming the "DEKA" motif of the selectivity filter indicated between the P1 and P2 helices. The fast inactivation peptide, which contains an IFM motif, is located between DIII and DIV; and the structured C-terminal domain (CTD) and presence of a calmodulin binding IQ motif are indicated. (**b**) Top view (extracellular) of the eel Nav1.4-β1 subunit complex structure (PDB 5XSY) with the pore forming α-subunit shown in surface representation colored according to the schematic of Nav1.7. The β1 subunit is shown in cartoon rendering (black). (**c**) Side view of the eel Nav1.4-β1 subunit complex structure colored as in part (**b**) with the VSD of DI and PM of DII removed for clarity. Representative peptide toxin and small molecule inhibitors are indicated with arrows pointing to the general location of their respective primary receptor sites. Note, the location of the selectivity filter is indicated by a dashed box (DEKA). PDB codes for peptide toxins are β-scorpion toxin (1BCG), μ-conotoxin (1TCG), and α-scorpion toxin (2ASC)

the VSDs of Nav channels with diverse effects on channel gating and biophysics (McCormack et al. 2013; Ahuja et al. 2015; Catterall 2010; Cannon 2010; Bosmans and Swartz 2010; Catterall et al. 2007). Here, sequence differences within the extracellular loops of the VSDs of Nav channels offer the distinct potential for subtype-selective channel targeting and pharmacological modulation.

Nav channels are targeted by a variety of clinically relevant drugs and naturally occurring toxins. These modulators represent important tools to probe the underlying structural and biophysical characteristics of Nav channels (Hille 2001); but in general terms, known Nav channel antagonists alter channel function through one of two distinct mechanisms: they either block ion conduction directly or modify channel gating (Bagal et al. 2015; de Lera Ruiz and Kraus 2015; Ahern et al. 2016; Israel et al. 2017). Historically, a number of distinct and non-overlapping binding sites for various drugs and toxins have been identified on Nav channels (Cestele and Catterall 2000); but these modulators are most easily considered in three general classes: (1) inner vestibule binders, (2) extracellular selectivity filter blockers, and (3) peripheral VSD binders (Fig. 1c). Here, in the following sections, we highlight recent literature describing modulators with the potential to selectively target Nav1.7 (Sun et al. 2014; Focken et al. 2016; Price et al. 2017; Marx et al. 2016; Flinspach et al. 2017; Deuis et al. 2017; Wu et al. 2017a; Kornecook et al. 2017; Swain et al. 2017; Storer et al. 2017; Murray et al. 2015a, b; Biswas et al. 2017; Schenkel et al. 2017; La et al. 2017; Graceffa et al. 2017; Shcherbatko et al. 2016; Alexandrou et al. 2016; Roecker et al. 2017; Yang et al. 2014; Pero et al. 2017; Pineda et al. 2014; Bagal et al. 2015) and the progress made towards ligand and drug discovery efforts on this emerging target for the potential treatment of chronic pain.

4 Introduction to Nav1.7 Physiology and Channelopathies

The critical role that Nav1.7 plays in the sensation of pain was initially discovered through genetics studies when it was found that individuals lacking functional Nav1.7 channels experienced congenital insensitivity to pain (CIP) (Cox et al. 2006; Goldberg et al. 2007). These individuals have a striking inability to feel both mechanical and thermal pain while apparently lacking defects in non-noxious somatosensation such as touch, proprioception, and the ability to distinguish (e.g.) hot, cold, vibration, and pinprick. Such individuals with Nav1.7-dependent CIP continuously injure themselves as a result of this lack of pain sensation. Among the most common types of injuries observed are biting of the tongue, self-mutilation of digits, frequent bone fractures, and frequent burn-related injuries (Cox et al. 2006, 2010; Goldberg et al. 2007; Kurban et al. 2010; Shorer et al. 2014; Sawal et al. 2016; Mansouri et al. 2014).

The expression pattern of Nav1.7 is consistent with its role in pain as this channel is highly expressed in the majority of sensory neurons present in the dorsal root ganglion (DRG) and the trigeminal ganglion (TG) (Black et al. 1996, 2012; Dib-Hajj et al. 2010). Anosmia or hyposmia is also a noted feature of the CIP phenotype, which is consistent with the observation that Nav1.7 is also highly expressed in olfactory epithelial neurons and olfactory neuron-specific knockout of Nav1.7

results in anosmia in mice (Weiss et al. 2011). Other locations where Nav1.7 is expressed are the sympathetic ganglia neurons as well as in restricted areas in the brain such as the hypothalamic/preoptic area, the subfornical organ, and several brainstem nuclei (Ahmad et al. 2007; Morinville et al. 2007), though autonomic and CNS-related defects have not been observed in CIP individuals, perhaps due to compensatory effects from other Nav channels.

It is interesting to consider the reason why Nav1.7 plays such an essential role in pain. This would be a simple question to answer if Nav1.7 were the only voltage-gated sodium channel expressed in sensory neurons. However, this is not the case as Nav1.1, Nav1.6, Nav1.8, and Nav1.9 are also expressed in sensory neurons in addition to Nav1.7 (Black et al. 1996; Dib-Hajj et al. 1998). This is particularly true for the TTX-resistant sodium channels Nav1.8 and Nav1.9, which are expressed in the majority of small-diameter DRG neurons involved in nociception. The leading hypothesis is that Nav1.7 is required for the initial generation of action potentials at the peripheral terminals of sensory neurons, where the detection of noxious stimuli takes place. The biophysical properties of Nav1.7 make this channel particularly suited for this role, as Nav1.7 is characterized by a comparatively slow closed state-inactivation (i.e., closed state-to-inactivated state transitions), which means that Nav1.7 is available to boost subthreshold stimuli arising from small, slow depolarizations of the cell membrane that occur during the detection of noxious stimuli (Cummins et al. 1998; Herzog et al. 2003). Nav1.7 also recovers slowly from inactivation (Klugbauer et al. 1995), which makes it ideally suited for the low-frequency firing characteristic of C-fiber DRG neurons. However, alternative hypotheses have been proposed, such as the idea that knockdown of Nav1.7 induces overexpression of endogenous enkephalin opioids by nociceptors that results in μ-opioid receptor dependent inhibition of pain (Minett et al. 2015).

Gain-of-function missense mutations in Nav1.7 have been found to cause clinically distinct painful neuropathies, depending on the location of the particular mutation within Nav1.7 (Vetter et al. 2017; Huang et al. 2017; Lampert et al. 2014). While many sub-variants have been described, three major Nav1.7 gain-of-function neuropathies have been extensively described: inherited erythromelalgia (IEM), paroxysmal extreme pain disorder (PEPD), and Nav1.7-dependent small fiber neuropathy (SFN) (Brouwer et al. 2014). IEM is in general caused by mutations that shift the activation $V_{1/2}$ toward more hyperpolarized voltages, allowing Nav1.7 to open with less robust membrane depolarization (Lampert et al. 2014). The clinical features of IEM generally include painful episodes that are triggered by a variety of stimuli including warm temperatures, exercise, humidity, and other various stresses. Commonly, the legs and/or arms of the affected individual will become reddened, concurrent with a powerful burning pain feeling that can be relieved to some extent by cooling (Fischer and Waxman 2010). PEPD, on the other hand, is generally caused by mutations in domains III or IV that shift steady-state inactivation to more depolarized voltages, making the channel more available to opening following membrane depolarization, and often with a small shift in the activation $V_{1/2}$ (also to more depolarized voltages) (Huang et al. 2017; Lampert et al. 2014). The clinical features of PEPD are extremely painful episodic sensations generally localized to the

recta, ocular, or submandibular regions that are triggered by various normally non-noxious stimuli such as defecation, yawning, chewing, or emotional stresses (Fischer and Waxman 2010; Brouwer et al. 2014).

5 Nav1.7 Receptor Sites: Potential for Selective Targeting

The strong genetics linking Nav1.7 to human pain syndromes has prompted an intense search for selective Nav1.7 inhibitors as potential next generation analgesics (Bagal et al. 2015; de Lera Ruiz and Kraus 2015). However, the high sequence and structural conservation shared between the Nav1.1–Nav1.9 pore-forming α-subunits presents a significant challenge in the identification of subtype-selective Nav channel modulators (Figs. 2, 3, and 4). Except where otherwise noted, we have used the eel Nav1.4 channel-β1 subunit cryoEM structure to model human Nav1.7 and the ConSurf software (Landau et al. 2005) to map and display sequence conservation among the closest related channel subtypes, Nav1.1–Nav1.7 (Figs. 2, 3, and 4). Although a range of Nav channel blockers have been historically clinically useful therapeutics (Fozzard et al. 2011; Glaaser and Clancy 2006; Abdelsayed and Sokolov 2013), a truly molecular subtype-selective Nav channel drug has yet to emerge from ongoing discovery efforts. Here, we review the main receptor sites that are known on mammalian Nav channels with an emphasis on their potential for exploring the development of novel Nav1.7 subtype-selective channel modulators.

6 Inner Vestibule Nav Channel Antagonists

The inner vestibule or central cavity within the pore module of Nav channels houses an important pharmacological modulatory site (Fig. 1c). Clinically relevant antiarrhythmic, antiepileptic, and local anesthetic drugs such as disopyramide, phenytoin, and lidocaine are thought to suppress cardiac or neuronal firing by binding within the inner vestibule of Nav channels to promote or stabilize channel inactivation (Fozzard et al. 2011; Glaaser and Clancy 2006; Abdelsayed and Sokolov 2013). However, the inner vestibule site is also known for the diversity of chemistries that are able to access it, consistent with the poor molecular selectivity of most inhibitors that bind and block within this site (Pless et al. 2011; Ahern et al. 2008). The poor molecular selectivity of drugs that target the inner vestibule arises because the surrounding pore-lining residues are highly conserved between Nav channel subtypes (Fig. 2a, b). In order to maintain safety, antagonists that modulate Nav channels at the inner vestibule receptor site must therefore block via a functionally selective mechanism, whereby they require Nav channels to cycle through open, closed, and inactivated states in highly active neurons to achieve their therapeutic effect and safety window (Fozzard et al. 2011; Glaaser and Clancy 2006; Abdelsayed and Sokolov 2013). Thus, while these drugs lack molecular selectivity among the Nav1.1–1.9 channel subtypes due to the high sequence conservation found within the inner vestibule (Fig. 2a, b), Nav channel block is highly use-dependent.

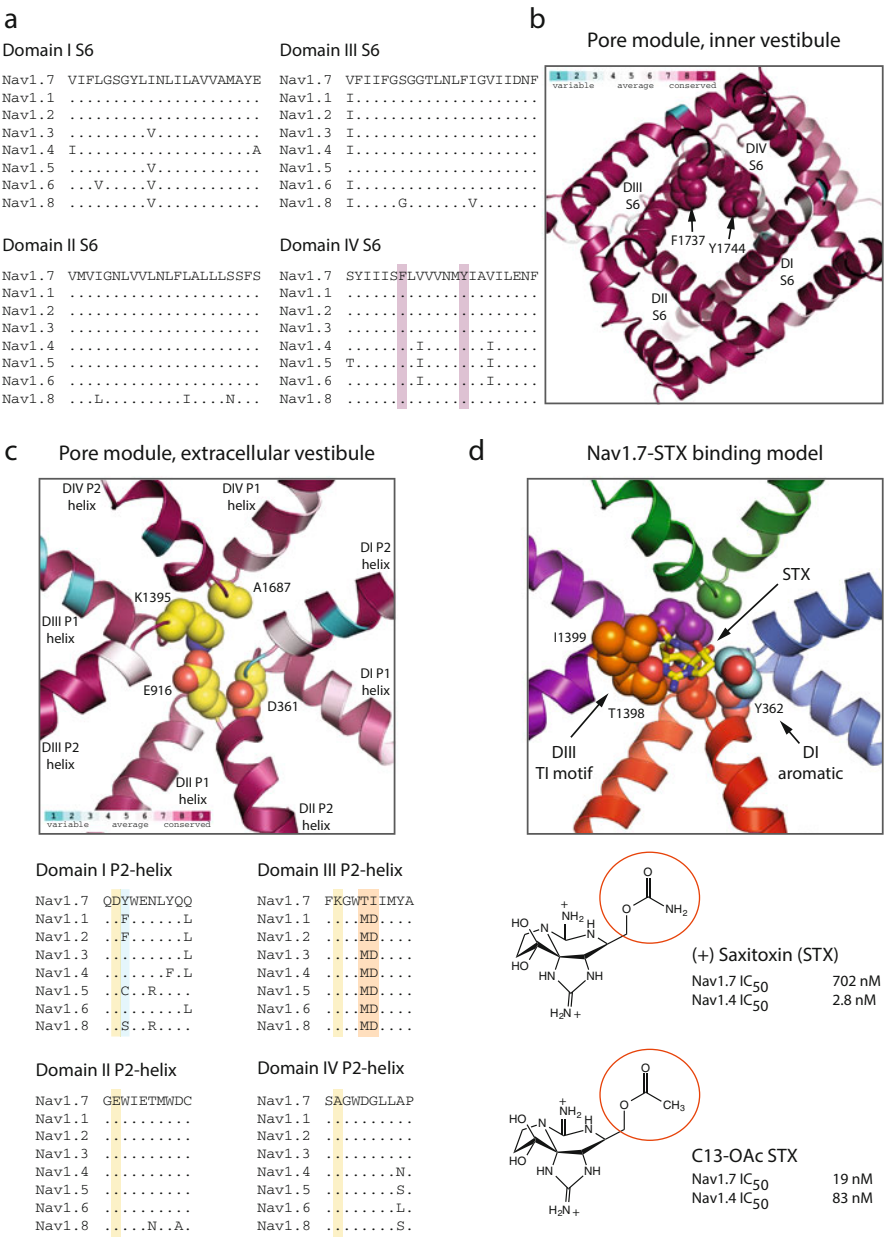

Fig. 2 The inner vestibule and extracellular vestibule receptor sites within the pore module of Nav channels. (**a**) Sequence alignment of the S6 regions comparing human Nav subtypes Nav1.1–Nav1.8. Highlighted in domain IV S6 are phenylalanine and tyrosine positions known to be important for local anesthetic binding; these are F1737 and Y1744 in human Nav1.7. (**b**) The inner vestibule receptor site. Homology model of human Nav1.7 was built based on the eel Nav1.4 structure (PDB 5XSY) and colored according to positional conservation comparing Nav1.1–Nav1.7

Despite the long-held view above, it is notable that molecularly selective inhibitors of Nav1.8 that appear to target the inner vestibule have been identified (Payne et al. 2015; Scanio et al. 2010). In this respect, however, Nav1.8 is more distinctive in its primary amino acid sequence within the inner vestibule compared to the Nav1.1–1.7 channel subtypes (Fig. 2a), and the same can be said for Nav1.9. Therefore, one can in theory envision the discovery of a Nav1.7-selective modulator based on the biophysical differences and minor sequence variations found within the inner vestibule of the Nav1.1–Nav1.7 channel subtypes (Fig. 2a, b); but we are presently unaware that any such inner vestibule antagonist with Nav1.7 molecular selectivity has been identified or advanced into preclinical studies.

7 Extracellular Vestibule Selectivity Filter Blockers

The small molecule guanidinium-containing toxins exemplified by tetrodotoxin (TTX) and saxitoxin (STX) are known to block Na^+ influx by binding directly into or near the selectivity filter from the extracellular side of the Nav channel α-subunit (Fig. 1c). In fact, since early studies on Nav channels, physiologists have long-postulated that the guanidinium moiety of TTX or STX directly engages and potentially competes for a Na^+ ion-binding site within the channel pore (Hille 1975; Lipkind and Fozzard 1994) (Fig. 2c, d). TTX, originally isolated from fugu pufferfish poison, has historically been used to divide subtypes within the Nav channel family into two groups: TTX-sensitive channel subtypes Nav1.1–Nav1.4 and Nav1.6–Nav1.7 are inhibited by nanomolar concentrations of TTX, whereas Nav1.8 and Nav1.9 require millimolar amounts to be blocked completely (Catterall et al. 2005a). Although Nav1.5 inhibition requires intermediate micromolar concentrations, TTX sensitivity can be substantially increased by replacing a cysteine in the domain I S5–S6 loop with a hydrophobic or aromatic residue (Lipkind and Fozzard 1994; Leffler et al. 2005), as this residue is a Phe or a Tyr in TTX-sensitive subtypes (Fig. 2c, d). These observations begin to highlight the molecular

Fig. 2 (continued) channel subtypes using the ConSurf software (Landau et al. 2005). The pore lining S6 residues are labeled and two key receptor site residues, F1737 and Y1744 (in human Nav1.7 numbering), are shown in sphere representation. (**c**) The extracellular vestibule receptor site. Sequence conservation mapping and homology modeling of human Nav1.7 were performed as in part (**b**). Residues of the DEKA motif in the selectivity filter are shown in yellow sphere representation (human Nav.17 numbering) for reference. Sequence alignment of the P2-helix regions comparing human Nav subtypes Nav1.1–Nav1.8 are shown, highlighting the aromatic side-chain in DI which is important for imparting TTX sensitivity and the MD/TI motif in DIII that influences STX potency. (**d**) Homology model of the human Nav1.7 extracellular vestibule receptor site highlighting the positions of the DIII TI motif (orange spheres) and the DI aromatic position (cyan spheres). Note, the structure of STX is shown in yellow stick representation and only placed approximately to indicate potential pose and region of contact within this receptor site. Shown below are chemical structures of STX and C13-OAc STX with potency data for Nav1.7 and Nav1.4 as reported by reference (Thomas-Tran and Du Bois 2016). The red circles highlight the differences between the two compounds

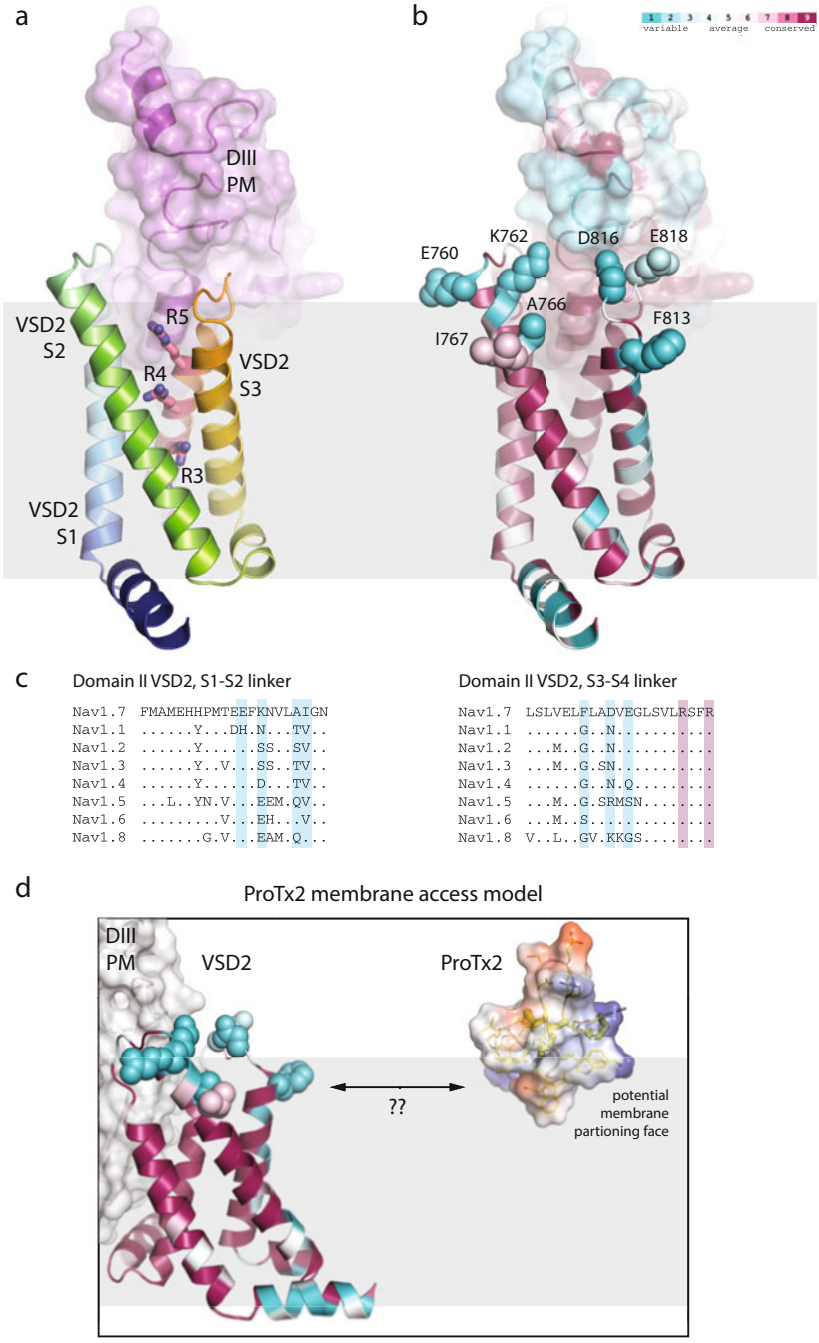

Fig. 3 The extracellular receptor site of voltage-sensor domain 2 (VSD2). (**a**) Homology model of human Nav1.7 was built based on the eel Nav1.4 structure (PDB 5XSY) and VSD2 is shown in

determinants of TTX sensitivity and suggest that it might be possible to identify or optimize subtype selective Nav channel blockers that bind to the extracellular selectivity filter pore site. Due to the complex molecular structure of TTX, however, the generation of extensive chemical derivatives remains challenging. Nevertheless, a natural TTX metabolite, 4,9-anhydro-TTX, has been shown to produce a shift in steady-state inactivation to more negative potentials in Nav1.6, but not in other TTX-sensitive subtypes (Rosker et al. 2007). Overall, these results begin to suggest that selectively targeting the extracellular vestibule in Nav channels is a possibility.

Recent progress has been made in using STX, a neurotoxin compound originally isolated from dinoflagellites, as a molecular scaffold to identify Nav1.7-selective inhibitors. A key observation that made STX of interest is the fact that it shows selectivity against human Nav1.7 (Walker et al. 2012). Despite this clearly undesirable inverse selectivity for Nav1.7, this observation indicated that selectivity motifs accessible to STX may be present in the extracellular vestibule binding site region. Accordingly, sequence analysis and mutagenesis within the outer vestibule have identified the residues responsible for this selectivity as a double amino acid variation located in the domain III pore-forming region, where Thr1398 and Ile1399 in human Nav1.7 (the TI motif) are found to be methionine and aspartic acid, respectively (the MD motif), in all other Nav channel subtypes in humans as well as non-primate Nav1.7 channels (Walker et al. 2012) (Fig. 2c, d). Detailed mutant-cycle analysis then led to the determination of the region of STX that likely directly interacts with these residues (Thomas-Tran and Du Bois 2016); this enabled a molecular model of the binding site region with sufficient resolution to allow structure-guided design efforts leading to the identification of a STX analog with true Nav1.7-subtype selectivity (Fig. 2d), although only ~5-fold selective relative to the human Nav1.4 channel. These results are exciting as further modifications of the STX scaffold may enable better interaction with the TI motif region present within human Nav1.7 and might allow for the identification of highly selective and therapeutically promising Nav1.7 blockers.

Given the intrinsic subtype selectivity of TTX and the progress made towards Nav1.7-directed targeting by STX analogs, it is important to note that subtype-selective peptide toxins that target the extracellular vestibule are also known, such

Fig. 3 (continued) carton rendering colored blue-to-red. Side-chains of S4 gating charge residues are shown in stick representation and colored pink. The abutting pore module (PM) of DIII is colored in purple and shown in transparent surface. (**b**) Homology model of human Nav1.7 from part (**a**) was colored according to positional conservation comparing Nav1.1–Nav1.7 channel subtypes using the ConSurf software (Landau et al. 2005). The side-chains near the extracellular side which are least conserved among subtypes (see part **c**) are indicated in sphere representation. (**c**) Sequence alignment of the S1–S2 and S3–S4 regions in VSD2 comparing human Nav subtypes Nav1.1–Nav1.8. Residues highlighted in cyan are those shown in sphere representation in part (**b**); two gating charges (R1 and R2) of S4 are also indicated in purple. (**d**) Transparent surface and electrostatic rendering of protoxin II (ProTx2; PDB 5O0U) are shown with an arrow to indicate its potential receptor site on VSD2 and highlight the proposed membrane access mechanism that is assumed for this class of peptides

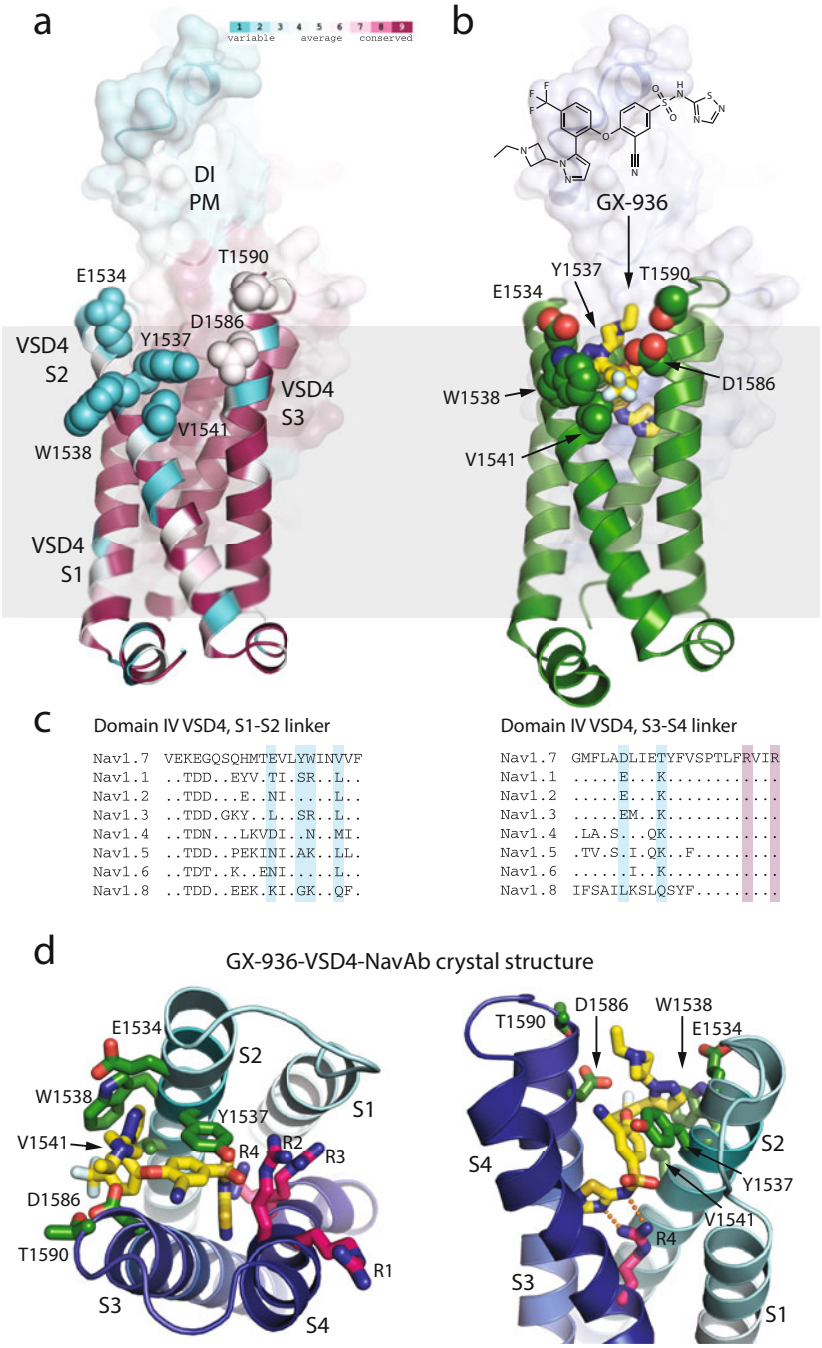

Fig. 4 The extracellular receptor site of voltage-sensor domain 4 (VSD4). (**a**) Homology model of human Nav1.7 was built based on the eel Nav1.4 structure (PDB 5XSY) and VSD4 is shown in

as μ-conotoxins (Wilson et al. 2011b; Knapp et al. 2012). μ-conotoxins (~20 amino acids) generally compete with TTX and STX for a binding site within the extracellular vestibule near the selectivity filter (French et al. 2010; Hui et al. 2002) (Fig. 1c). While some degree of molecular selectivity across Nav channel subtypes is known, no μ-conotoxin is particularly selective for Nav1.7. μ-PIIIA from *Conus purpurascens* is a notable example as it inhibits rat Nav1.4 with low nanomolar potency (IC_{50} 36 nM), rat Nav1.2 with high nanomolar potency (IC_{50} 620 nM), and rat Nav1.7 with low micromolar potency ($IC_{50} > 1$ μM) (Wilson et al. 2011b). In this light, as highlighted above for the inverse Nav1.7 selectivity noted for STX, μ-PIIIA-like peptides may serve as a viable scaffold to consider optimizing subtype-selectivity. Similarly, derivatives of the μ-KIIIA peptide from *Conus kinoshitai* have been demonstrated to have preference for the neuronal Nav1.2 channel (IC_{50} 5 nM) over the Nav1.4 muscle channel (IC_{50} 9–37 nM) subtype (Wilson et al. 2011b; McArthur et al. 2011). Here, structure–activity relationship (SAR) studies have indicated a possible direct interaction between Arg14 from μ-KIIIA with Asp1241 in the domain III P-loop of Nav1.2 or Nav1.4, where the equivalent P-loop residue in human Nav1.7 is Ile1410 and may offer a starting point to gain Nav1.7 subtype-selectivity (McArthur et al. 2011). Overall, the μ-conotoxins could be attractive starting point scaffolds since they likely exploit additional regions within the extracellular vestibule beyond the TTX or STX receptor sites to achieve selectivity (McArthur et al. 2011; Walewska et al. 2013; Brady et al. 2013). Nevertheless, μ-conotoxins are currently unsuitable to consider for oral dosing in their present form and generally lack state-dependence of block, which may or may not be ideal characteristics for achieving efficacy in therapeutically relevant pain settings.

8 Voltage-Sensor Targeting: Gating Modifying Peptides

Peptide toxins that target the VSDs of Nav channels have long been known because these neurotoxins played an essential role in early efforts to isolate, characterize, and ultimately clone the Nav channels (Beneski and Catterall 1980; Sharkey et al. 1984;

Fig. 4 (continued) carton rendering colored according to positional conservation comparing Nav1.1–Nav1.7 channel subtypes using the ConSurf software (Landau et al. 2005). The side-chains near the extracellular side which are least conserved among subtypes (see part **c**) are indicated in sphere representation. The abutting pore module (PM) of DI is colored similarly and shown in transparent surface. (**b**) Crystal structure of the human Nav1.7-VSD4-NavAb chimeric channel in complex with the aryl sulfonamide GX-936 is shown (PDB 5EK0), with GX-936 shown in yellow stick representation. Residues that are least conserved among subtypes (see part **c**) within ~5 Å of the bound GX-936 inhibitor are shown in green sphere representation. Note, the structure of GX-936 is shown above for reference, as is the homology model of the PM from DI. (**c**) Sequence alignment of the S1–S2 and S3–S4 regions in VSD4 comparing human Nav subtypes Nav1.1–Nav1.8. Residues highlighted in green are shown in sphere representation in part (**b**); two gating charges (R1 and R2) in S4 are indicated in purple. (**d**) Two close-in views of the GX-936 aryl sulfonamide (yellow sticks) in complex with the Nav1.7-VSD4-NavAb chimeric channel. Residues important for binding and potential selectivity are shown in stick representation and labeled

Rodriguez de la Vega and Possani 2005). These VSD-binding peptide toxins are generally thought to work principally as gating modifiers since they all target more peripheral binding sites on Nav channels (Fig. 1c), although effects on channel conductance or ion selectivity should not be ruled out *a priori*. Consistent with the functional specialization of the four VSDs within Nav eukaryotic channels (Chanda and Bezanilla 2002; Chanda et al. 2004; Capes et al. 2013; Lacroix et al. 2013; Pless et al. 2014), these toxins are mechanistically diverse; and there are many examples of VSD-binding peptide toxins that primarily inhibit channel activation, promote channel activation, or delay channel inactivation depending on their mechanism of action and primary interaction site with the α-subunit (Bosmans and Swartz 2010; Israel et al. 2017; Rodriguez de la Vega and Possani 2005).

Prototypical peptide toxins that target VSDs are often expected to be state-dependent modulators because they interact directly with the major gating apparatus of the channel (Pineda et al. 2014; Catterall et al. 2007; Rodriguez de la Vega and Possani 2005; Barhanin et al. 1983). However, the extent of state-dependent binding and modulation may depend on the peptide and conditions under study (Bosmans et al. 2008; Bosmans and Swartz 2010). Early studies with scorpion venoms capable of modulating Nav channels led to the identification of α-scorpion and β-scorpion toxins (Rodriguez de la Vega and Possani 2005; Barhanin et al. 1983; Catterall 1976, 1977; Couraud et al. 1978, 1982; Jaimovich et al. 1982; Wang and Strichartz 1982) (Fig. 1c). α-scorpion toxins target VSD4 where they limit S4 movement to inhibit fast inactivation and are often described as stabilizing VSD4 in a deactivated state (Campos et al. 2008; Rogers et al. 1996). Elegant chimeric studies using a Kv1.2 channel chassis has established the S3–S4 loop of VSD4 as a major binding determinant of the α-scorpion toxins (Bosmans et al. 2008), where more traditional approaches have additionally implicated a role for the S1–S2 loop (VSD4) and the S5–S6 loop (from domain I) (Thomsen and Catterall 1989; Rogers et al. 1996; Wang et al. 2011; Gur et al. 2011). The long-standing inference of a composite α-scorpion toxin receptor site on Nav channels is reasonable in light of the recent eukaryotic insect NavPas and eel Nav1.4 cryo-EM structures (Shen et al. 2017; Yan et al. 2017), as these channel structures suggest that the large α-scorpion toxins (~65 amino acids) could make multi-point contact with the pore-forming subunit to suppress S4 activation in VSD4 (Figs. 1c and 4a). Nevertheless, inhibiting channel fast inactivation of Nav1.7 is expected to phenocopy the genetics of GOF mutations, and this is in fact seen with the Nav1.7-selective α-like scorpion toxin OD1 that has proven to be a useful pharmacological tool to induce pain in animal models (Jalali et al. 2005; Maertens et al. 2006; Deuis et al. 2016). Notably, Hm1a, which is an unrelated toxin that can selectively inhibit fast inactivation in the Nav1.1 channel by targeting an apparently overlapping VSD4 receptor site, can also induce pain behavior in animal models (Osteen et al. 2016, 2017). Therefore, the utility of α-scorpion toxin-like molecules that suppress or delay fast inactivation is not regarded as a tractable approach to develop novel analgesics, but it is notable that high channel-subtype selectivity has been achieved from naturally isolated toxins.

In contrast to the modulation by α-scorpion toxins, classic β-scorpion toxins (65–70 amino acids) target VSD2 in Nav channels to shift the voltage dependence

of activation and promote channel opening at more hyperpolarized potentials (Rodriguez de la Vega and Possani 2005; Vijverberg and Lazdunski 1984; Cestele et al. 2006). Moreover, in some cases, β-scorpion toxins have been described as trapping the S4 of VSD2 in its activated state (Leipold et al. 2012; Cestele et al. 1998, 2001). Chimeric studies using a Kv1.2 channel chassis have established that β-scorpion toxins appear to interact primarily with S3–S4 loop of VSD2 in Nav channels (Bosmans et al. 2008), while other studies have implicated additional determinants within the S1–S2 loop or domain III pore-loop in binding and block as well (Zhang et al. 2011, 2012b; Leipold et al. 2006). Mechanistically, although overly simplistic, it seems that β-scorpion toxins may require three major points of contact on the channel (i.e., VSD2 S1–S2, VSD2 S3–S4, DIII pore loop) to maintain the S4 of VSD2 in its activated conformation (Figs. 1c and 3a, b). Similarly, α-scorpion toxins likewise appear to require three points of contact on the channel (i.e., VSD4 S1–S2, VSD4 S3–S4, DI pore loop) to suppress the activation of S4 in VSD4 (Figs. 1c and 4a). Nevertheless, it is important to note that toxin pharmacology may be influenced depending upon the presence or absence β-subunit co-expression (Gilchrist et al. 2013; Das et al. 2016; Wilson et al. 2011a; Zhang et al. 2013), further highlighting the complex nature of the interactions that Nav channels have with their surroundings and modulators.

Two features of the VSD-binding toxin gating modulators are notable: (1) their binding sites are accessible from the extracellular side of the membrane and therefore potentially subject to modulation by small and large molecules (Fig. 1c); and (2) sequence conservation within the extracellular loops of VSDs is lower across all channel subtypes (Figs. 3b, c and 4a, c), making the identification of a Nav subtype selective modulator a potential reality. In this respect, peptide toxins that target the VSDs of Nav channels have generated significant interest because subtype-selective toxins have been identified (Flinspach et al. 2017; Deuis et al. 2017; Murray et al. 2015a; Shcherbatko et al. 2016). Although clinical efficacy has yet to be demonstrated in humans, these toxin molecules are being seriously considered as starting points to further improve their selectivity and potential drug-like properties. As numerous reports continue to surface describing the isolation and characterization of novel Nav1.7 VSD-targeting peptide toxin modulators (Chow et al. 2015; Klint et al. 2015a), here we will only aim to capture a snapshot of progress and challenges towards their potential therapeutic validation and clinical application.

8.1 The Pn3a Peptide

Pn3a (or μ-TRTX-Pn3a) is a peptide toxin isolated from the tarantula *Pamphobeteus nigricolor* that potently inhibits human Nav1.7 (IC_{50} 0.9 nM) and produces a rightward shift (21.3 mV) in Nav1.7 voltage dependence of activation while having a remarkable 40–1,000-fold selectivity over other Nav channel subtypes (Deuis et al. 2017). Pn3a shows similar potency across mouse, rat, and human Nav1.7 channels, without significant impact on the Kv, Cav, and nicotinic acetylcholine receptors tested. Pn3a exhibits a conserved inhibitor cystine knot (ICK) fold (~30 amino

acids), which is common for peptides that modulate or inhibit Nav channels (Middleton et al. 2002; Cardoso et al. 2015), and a structural motif that may prove to be resistant to heat denaturation and proteolysis (Agwa et al. 2017a). Using a chimeric Kv2.1 channel chassis, Pn3a was shown to interact with the S3–S4 loops of VSD2 and VSD4 from Nav1.7. These receptor site-mapping studies are consistent with the observation that Pn3a inhibited Nav1.7 activation, presumably through interaction with VSD2, and also slowed fast inactivation and recovery from fast inactivation, presumably through interaction with VSD4 (Deuis et al. 2017).

Pn3a markedly inhibited the TTX-sensitive Nav currents in dissociated rat small diameter dorsal root ganglion neurons. Pn3a administered by intraperitoneal injection was able to dose-dependently reduce OD1-induced spontaneous pain behaviors in the absence of adverse effects, but Pn3a as a single agent displayed no analgesic activity in acute nociceptive or inflammatory pain in rodents when administered systemically despite its compelling apparent Nav1.7 selectivity, potency, and dual-VSD targeting mechanism (Deuis et al. 2017). Overall, these results identify Pn3a as a valuable scaffold to consider for the development of highly selective Nav1.7 targeting antagonists, but also highlight the challenges that may be associated with achieving broad analgesic efficacy in rodent models of pain using peripherally restricted selective Nav1.7 peptide toxin-based inhibitors.

8.2 ProTx2 and Derivatives

Protoxin II (or ProTx2) is a prototypical Nav1.7 targeting peptide toxin originally isolated from the tarantula *Thrixopelma pruriens* (Middleton et al. 2002). In contrast to α-scorpion or sea anemone toxins, spider toxins exemplified by ProTx2 typically do not affect the rate of inactivation but rather inhibit channel activity by shifting the voltage dependence of activation to more positive potentials and reducing conductance at all voltages. An early report on ProTx2 demonstrated marginal subtype-selectivity against Nav1.2, Nav1.5, Nav1.7, or Nav1.8, but noted recovery from inhibition varied widely, with Nav1.7 showing no recovery during a 20-min washout after ProTx2 exposure (Middleton et al. 2002). Subsequent reports have suggested that ProTx2 potently and selectively inhibits Nav1.7 with an IC_{50} of 0.3 nM compared to values of 26–146 nM for other Nav channel subtypes tested (Schmalhofer et al. 2008).

In contrast to the larger receptor sites assumed on Nav channels for the α-scorpion toxins (targeting VSD4) and β-scorpion toxins (targeting VSD2), which encompass respective S1–S2, S3–S4, and pore loops, mutagenesis and chimeric studies have demonstrated that the major determinants of ProTx2 binding appears to derive largely from a short stretch of residues in the S3–S4 loop of VSD2 (Bosmans et al. 2008; Bosmans and Swartz 2010; Schmalhofer et al. 2008; Sokolov et al. 2008). In this respect, it is notable that ProTx2 and related gating modifier peptide toxins (Milescu et al. 2009) have been suggested to partition into the membrane bilayer (Henriques et al. 2016; Smith et al. 2005), which may allow ProTx2 to gain considerable potency and selectivity for Nav1.7 (Lee and MacKinnon 2004; Agwa et al. 2017b; Klint et al. 2015b) (Fig. 3d). Here, the mechanism of ProTx2

antagonism is generally described as restricting S4 activation in VSD2 to produce a shift in channel activation to more positive potentials and a decrease in Na^+ current magnitude (Flinspach et al. 2017; Schmalhofer et al. 2008; Sokolov et al. 2008; Smith et al. 2005; Xiao et al. 2010). In addition to VSD2, it is important to note that potential multi-VSD targeting by ProTx2 on Nav1.7 has also been reported (Bosmans et al. 2008; Xiao et al. 2010).

The application of ProTx2 to desheathed cutaneous nerves has been shown to completely block the C-fiber compound action potential, but produces little effect on action potential propagation of an intact nerve (Schmalhofer et al. 2008). Accordingly, early reports using ProTx2 demonstrated no efficacy in rodent models of acute and inflammatory pain at tolerable doses, where lack of efficacy may result from an inability of ProTx2 to cross the blood-nerve barrier (Schmalhofer et al. 2008; Hackel et al. 2012). Intrathecal or perisciatic injections of ProTx2, however, did show efficacy in rodent pain models, furthering the notion that a blood-nerve barrier may be preventing access to the Nav1.7 target when the peptide is delivered systemically (Flinspach et al. 2017). Despite the apparent hurdles to achieving efficacy through intravenous injection or oral dosing, significant effort has been put forward to optimize the synthesis of ProTx2 (Park et al. 2012), and this has led to the understanding of important structure–activity relationships (Agwa et al. 2017a; Park et al. 2014). Structure–function studies have also extended beyond the ProTx2 parental sequence (Wright et al. 2017), including a rational approach to functionalize a non-Nav1.7 targeting toxin, whereby the Hhn2b toxin can be converted to a potent and selective Nav1.7 modulator through a few directed changes (Klint et al. 2015b).

Following the isolation of GpTx1 from the tarantula *Grammostola porteri*, a series of reports has described extensive characterization, synthesis, and structure – activity relationship studies that have produced a potent GpTx1 analog with improved Nav1.7 subtype-selectivity over ProTx2 (Murray et al. 2015a). Uniquely, this GpTx1 derivative shows low nanomolar potency on Nav1.7 (IC_{50} 1.6 nM) and >1,000-fold and >6,000-fold selectivity over the Nav1.4 and Nav1.5 muscle and cardiac channel subtypes, respectively (Murray et al. 2015a). In an effort to further explore the potential of GpTx1, homodimerization was tested and achieved through a bifunctional polyethylene glycol (PEG) linker, resulting in a compound with slightly increased potency for Nav1.7 (IC_{50} 0.6 nM) and a remarkably reduced off-rate where the recovery after washout had a $t_{1/2}$ of >45 min (Murray et al. 2015b). This observation warrants further consideration since multimerization of GpTx1 and related toxins may improve their intrinsic pharmacokinetic properties by increasing the molecular weight and/or hydrodynamic radius, although the in vivo stability and distribution of the bivalent-PEG-linked GpTx1 analog was not directly reported.

In a remarkable effort to improve circulating half-live and potentially alter biodistribution via an Fc receptor recycling mechanism, a GpTx1 derivative was conjugated site-specifically onto an engineered non-targeting monoclonal antibody (mAb) through PEG-based linkers (Biswas et al. 2017). This mAb-based conjugation of GpTx1 led to in vivo half-life extension from 0.6 to 80 h (~130-fold) relative to a nonconjugated GpTx1 peptide (Biswas et al. 2017). Moreover, differential

biodistribution of the GpTx1-mAb conjugate to nerve fibers was observed in wild-type but not Nav1.7 knockout mice. Unfortunately, mAb conjugation of GpTx1 significantly impacted the in vitro potency against Nav1.7 (IC_{50} 297 nM); however, the conjugate was still able to inhibit TTX-sensitive currents in DRG neurons with an IC_{50} of 68 nM. Disappointingly, the GpTx1-mAb conjugate was unable to impact scratching behavior in a histamine-induced pruritis model, and results of other pain assays were not reported, perhaps suggesting the GpTx1-mAb conjugate was unable to obtain sufficient concentrations in the nerve to adequately block the Nav1.7 channel (Biswas et al. 2017). Nevertheless, this impressive body of work highlights the opportunities and challenges associated with the development of novel Nav1.7-targeted peptide-based therapeutics for the treatment of pain, since it may well be possible to achieve in vivo efficacy with GpTx1 or ProTx-like molecules (Flinspach et al. 2017). In this light, it is important to note that engineered channel constructs containing viable ProTx2 receptor sites for potential ligand discovery and optimization purposes have been established (Rajamani et al. 2017), and that new approaches to discover and develop macrocyclic peptides may produce novel lead scaffolds with improved pharmacokinetic, pharmacodynamic, and efficacy profiles (Hosseinzadeh et al. 2017; Over et al. 2016).

9 Trapping VSD4: Identification of the Subtype Selective Aryl Sulfonamides

A breakthrough study recently described the characterization of a novel class of subtype-selective small molecule Nav channel antagonist that interacts with a unique site on the α-subunit, which is distinct from known receptor sites targeted by nonselective small molecule modulators such as TTX or local anesthetics (McCormack et al. 2013) (Fig. 1c). This class of aryl sulfonamide inhibitors was originally identified in high-throughput screening efforts for Nav1.3 inhibitors that showed selectivity over the cardiac Nav1.5 channel subtype. Moreover, a truly exciting development in the search for Nav1.7-selective modulators was the discovery of closely related aryl sulfonamide small molecule inhibitors exemplified by PF-04856264 and GX-674 (McCormack et al. 2013; Ahuja et al. 2015), which are highly potent on human Nav1.7 (IC_{50} 28 nM and 0.1 nM, respectively) and exhibit up to 1,000-fold selectivity against other Nav channel subtypes including Nav1.3 and Nav1.5. As with other drug-like small molecule Nav channel antagonists, block of Nav1.7 by PF-04856264 and GX-674 is highly state dependent, where potent inhibition by GX-674 is observed when binding is equilibrated at −40 mV (IC_{50} 0.1 nM), a membrane voltage that promotes steady-state inactivation of Nav1.7, whereas GX-674 inhibition is ~2,400-fold weaker at −120 mV, a voltage that promotes a resting closed state of the channel (Ahuja et al. 2015). Uniquely, and in line with the profound molecular selectivity observed for PF-04856264 and GX-674, electrophysiological characterization of chimeric and point mutant Nav channels has firmly established an extracellular VSD4-based receptor site for the aryl sulfonamide class of inhibitors (McCormack et al. 2013; Ahuja et al. 2015).

High-resolution experimental structures of human Nav channels still remain elusive (Shen et al. 2017; Yan et al. 2017). As such, bacterial Nav (BacNav) channels have been exploited as proximal model systems (Payandeh and Minor 2015) to enable the visualization of bound small molecule modulators to provide insight into mechanism of action and potentially guide rational structure-based drug design (Clairfeuille et al. 2017). In fact, this strategy has provided the first experimental views of approved drugs and drug-like modulators bound to the pore module of representative BacNav and bacterial Cav (BacCav) channels (Bagneris et al. 2014; Tang et al. 2016). In order to extend this approach to truly enable structure-based drug design methods for the optimization and development of subtype selective Nav channel antagonists, the human Nav1.7 VSD4 sequence encompassing the presumed binding site of the aryl sulfonamide inhibitors was engineered onto a bacterial NavAb channel chassis (Payandeh et al. 2011, 2012), and the X-ray crystallographic structure of this Nav1.7-VSD4-NavAb chimeric channel was determined to 3.53 Å resolution in complex with GX-936, a potent and Nav1.7-subtype selective inhibitor (Fig. 4b, d) (Ahuja et al. 2015). This approach revealed the molecular details of how the aryl sulfonamide class of compounds antagonizes Nav channels and, for the first time, uncovered the structural basis for how small molecule inhibitors like GX-936 and PF-04856264 can achieve high molecular selectivity for VSD4 of the Nav1.7 channel.

GX-936 and related aryl sulfonamide modulators inhibit Nav1.7 by trapping the S4 voltage-sensor of VSD4 in the "up" or activated state. When bound within the extracellular vestibule of VSD4, the negatively charged sulfonamide moiety of GX-936 forms a direct salt-bridge contact with the forth arginine (R4) of the VSD4 S4 voltage-sensor (Fig. 4d). The importance of the observed sulfonamide-R4 interaction was confirmed by a ~2,700-fold potency shift for the single alanine mutation (R4A) studied in the context of the full-length Nav1.7 channel (GX-674 on R4A human Nav1.7, IC_{50} 270 nM). This strong and direct ionic interaction ultimately prevents the S4 voltage-sensor of VSD4 from returning to the deactivated (or "down") resting position following voltage-dependent channel activation. Notably, VSD4 is known to play a key role in the Nav channel inactivation process, as S4 activation in VSD4 has been shown to be necessary and sufficient to produce fast inactivation (Chanda and Bezanilla 2002; Capes et al. 2013). Therefore, by effectively trapping the VSD4 S4 voltage-sensor in the activated conformation, this class of aryl sulfonamide inhibitors is able to prevent the Nav channel from recovering from fast [and slow (Osteen et al. 2017; Theile et al. 2016)] inactivation (Ahuja et al. 2015; Capes et al. 2013). As the crystal structure of the GX-936-VSD4-NavAb channel complex clearly reveals, the aryl sulfonamide modulators can only bind to R4 of the S4 voltage-sensor when VSD4 is in the activated conformation (Fig. 4d), rationalizing why the binding affinity is so strongly state-dependent (McCormack et al. 2013; Ahuja et al. 2015).

As discussed above for peptide toxins targeting the VSDs of Nav channels, the extracellular surface of VSD4 is not absolutely conserved between all Nav channel subtypes, explaining why subtype-selective modulators can be achieved by small molecule compounds that are able to make intimate contacts with the more poorly conserved residues in VSD4. In this regard, the most critical selectivity motif that the

aryl sulfonamide inhibitors like GX-936 and PF-04856264 target in human Nav1.7 VSD4 is Y1537/W1538 (the YW motif) (McCormack et al. 2013; Ahuja et al. 2015) (Fig. 4a–d). Specifically, the YW motif is present at this location of the S2 within VDS4 in only three of the nine Nav channel subtypes: Nav1.2, Nav1.6, and Nav1.7 (Fig. 4c). As aryl sulfonamides that can inhibit Nav1.7 make close contact with both side-chains of the YW motif (Fig. 4d), it is relatively straightforward to obtain inhibitors that are selective for these three channels over all other Nav channel subtypes. For example, GX-674 shows similar potencies on Nav1.2, Nav1.6, and Nav1.7 (IC_{50}'s all <1 nM), but it is at least 1,000-fold less potent on the other Nav channel subtypes that do not contain the YW motif (Ahuja et al. 2015). Importantly, additional residues within the aryl sulfonamide VSD4 binding site can in principle be used to make even more selective compounds (Ahuja et al. 2015), including: E1534 (S2), which is uniquely glutamate in Nav1.7 but asparagine in Nav1.2 and Nav1.6; V1541 (S2), which is uniquely valine in Nav1.7 but leucine in most other subtypes; D1586 (S3), which is aspartate in Nav1.4–Nav1.8 but glutamate in Nav1.1–Nav1.3; and T1590 (S3), which is uniquely threonine in Nav1.7 but lysine in most other subtypes (Fig. 4a–d). Likely by designing compounds that are able to interact with these specific residue side-chains, aryl sulfonamides with Nav1.7 selectivity ratios of >100 over Nav1.2 and Nav1.6 have been identified and reported, such as AMG8379 (Kornecook et al. 2017) and PF-06456384 (Storer et al. 2017).

With the recent discovery of the first truly Nav1.7-selective small molecule antagonists and elucidation of a high-resolution crystal structure of a representative aryl sulfonamide in complex within the VSD4 receptor site, significant efforts from multiple groups have since focused on optimizing the potency, selectivity, and drug-like properties of this class of molecules. Accordingly, a range of studies have begun to emerge that show promising results that aryl sulfonamide engagement of the VSD4 site of Nav1.7 in vivo leads to an analgesic effect in rodent models of acute and inflammatory pain (Focken et al. 2016; Roecker et al. 2017). In mice, engagement of the VSD4 receptor site in Nav1.7 by selective aryl sulfonamide inhibitors in vivo has been reported to reduce formalin-induced nociceptive pain behaviors (Pero et al. 2017; Wu et al. 2017b), histamine-induced scratching behavior (Marx et al. 2016; Weiss et al. 2017), capsaicin-induced licking in a nociception model of pain (Graceffa et al. 2017), and UVB radiation skin burn-induced thermal hyperalgesia (Kornecook et al. 2017). However, a notable lack of preclinical efficacy has also been reported for some aryl sulfonamide compounds (Storer et al. 2017; Wu et al. 2017c). Overall, results from these preclinical studies have warranted moving select aryl sulfonamides forward into human clinical trials (Jones et al. 2016).

10 Opportunities and Challenges in Nav1.7 Drug Discovery

Nav1.7 represents an extremely exciting target for the development of novel pain drugs. The challenge of identifying a selective Nav1.7 inhibitor with the proper drug-like properties to be a clinically useful pain drug, however, is immense. Each

potential class of selective Nav1.7 inhibitor has its advantages and disadvantages, which we will consider here.

10.1 Pharmacokinetic Properties

Effective Nav1.7 inhibitors require exquisite potency, selectivity, and metabolic stability to enable a safe, low dose, and orally bioavailable pain medication. Selective outer vestibule inhibitors such as those based on STX will likely possess poor oral bioavailability as a result of the positively charged guanidinium moiety. The need to be injected either intravenously or subcutaneously may limit the clinical use of such compounds, possibly restricting them to in-hospital settings. Similar concerns exist in the case of peptide-based inhibitors that bind to VSD2, which will also likely suffer from poor oral bioavailability and rapid clearance. On the other hand, the aryl sulfonamide class of inhibitors has demonstrated that good oral bioavailability and metabolic stability is achievable. However, the negatively charged aryl sulfonamide group, which is required for binding the positively charged R4 on the S4 voltage-sensor of VSD4, in general makes such molecules highly protein-bound in the blood, limiting the free drug available for blocking Nav1.7 channels. The clinical candidate PF-05089771 is an example of a highly protein-bound aryl sulfonamide, where >99% of the compound is bound to plasma proteins and thus unavailable to block channels (Swain et al. 2017). Recent work showing that aryl sulfonamides with a zwitterionic character can have much lower levels of plasma protein binding while retaining potency (Roecker et al. 2017) suggests a strategy for making aryl sulfonamide compounds that may achieve better target coverage in vivo without requiring very high levels of total plasma concentration. Ultimately, finding compounds with the right balance of potency, selectivity, and metabolic stability will be required to deliver best-in-class Nav1.7-targeted pain medications.

10.2 State-Dependence of Inhibition

While state-dependence is a key attribute for nonselective Nav blockers, especially those used systemically, it is currently unclear whether state-dependence is a desirable feature for a selective Nav1.7 blocker. State-dependence can in principle allow for selective block of more active sensory fibers versus less active sensory fibers. Among the three classes of potential Nav1.7-selective blockers profiled in this chapter, only aryl sulfonamides show state-dependence that is expected to be relevant in vivo since extracellular vestibule inhibitors like STX in general lack significant state-dependence, and ProTx2-like peptides that bind to VSD2 show a preference for antagonizing closed Nav channels (Flinspach et al. 2017). The potency of aryl sulfonamides is highly voltage-dependent since these compounds only bind to the activated state of VSD4, which occurs primarily (or only) when Nav1.7 is in the inactivated state. The amount of time a channel spends in inactivated

states depends on the membrane voltage, so aryl sulfonamides should inhibit Nav1.7 more effectively in more depolarized nerve fibers. This could in principle give rise to a safety factor between the desirable efficacy in chronic pain versus undesirable effects on acute nociception for the aryl sulfonamide class of inhibitors.

10.3 Efficiency of In Vivo Target Engagement

Even if a selective inhibitor can achieve high enough unbound concentrations in the plasma to cover the IC_{50} for Nav1.7 channel several-fold, it does not necessarily mean that it can access the Nav1.7 target within the micro-compartment in the nerve. A study of the in vivo efficacy of ProTx2, for example, showed no detectible effects of the peptide despite achieving high levels of coverage over the IC_{50} (>100-fold), whereas direct application of ProTx2 to the desheathed saphenous nerve was found to efficiently inhibit the C-fiber compound action potential (Schmalhofer et al. 2008). Accordingly, direct intrathecal or perisciatic injection of the ProTx2 peptide has shown in vivo efficacy (Flinspach et al. 2017). Additionally, a highly potent and selective aryl sulfonamide (PF-06456384) failed to show efficacy in the formalin-induced pain model despite achieving free plasma concentrations 62.5-fold over the mouse Nav1.7 IC_{50} (Storer et al. 2017). On the other hand, some aryl sulfonamide compounds are able to achieve much better target-engagement efficiencies. For example, compounds 5 and 9 from a recent medicinal chemistry effort were shown to inhibit formalin-induced flinching at free plasma concentrations only 11-fold and 3.5-fold over the mouse Nav1.7 IC_{50} (Roecker et al. 2017). Another example is compound AM8379, which showed efficacy in a histamine-induced itch model at free plasma concentrations of 5.3-fold over the mouse IC_{50} and showed efficacy in a UVB burn-induced heat hyperalgesia model at 23-fold over the mouse IC_{50} (Kornecook et al. 2017). Given that the efficiency of target engagement is clearly compound-dependent, careful examination of a potential inhibitor in Nav1.7-dependent target engagement models is required prior to considering human clinical trials.

10.4 Toxicity Considerations

Drug candidates based on any of the three aforementioned potentially Nav1.7-selective binding sites could in principle have off-target toxicity that will need to be evaluated preclinically and clinically. In addition, two types of on-target toxicity could occur with selective Nav1.7 inhibitors: those due to block of off-target Nav channels and those due to block of Nav1.7 itself. The effects on off-target Nav channels will depend in part on which receptor site a drug candidate targets. STX-like extracellular vestibule inhibitors may show equal potency on off-target Nav channels since only Nav1.7 contains the TI motif, whereas other Nav channels share an MD motif at this location. Peptide-based VSD2 binding inhibitors will likely have the weakest selectivity relative to Nav1.6 considering VSD2 sequence

similarities, although it is not clear what toxicity effects may occur by blocking peripheral Nav1.6 channels since such peptide-based drugs will likely not be able to cross the blood–brain barrier. As described above, Nav1.7-selective aryl sulfonamide inhibitors will tend to be the least selective against Nav1.2 and Nav1.6 channel subtypes due to the common YW motif found in the S2 of VSD4. Thus, since aryl sulfonamides will likely penetrate the blood–brain barrier at least to some extent, CNS-mediated side effects might occur unless the drug is very selective against these specific channel subtypes. By contrast, true on-target side effects of aryl sulfonamide inhibitors should follow the expression pattern of Nav1.7. Effects on olfaction might be expected given the high level of expression of Nav1.7 in the olfactory epithelium. Autonomic side effects might also be possible given the high level expression of Nav1.7 in the sympathetic ganglia as well as some parasympathetic neurons such as those with cell bodies in the dorsal nucleus of the vagus nerve within the medulla. While autonomic defects have not been observed in CIP individuals with germline Nav1.7 mutations, it is possible that acute block of Nav1.7 might reveal autonomic side effects due to a lack of developmental compensation. Finally, block of Nav1.7 in the hypothalamus might, in principle, produce some deleterious outcomes such as effects on body weight (Branco et al. 2016).

11 Perspective and Future Outlook

Among voltage-gated sodium channels, Nav1.7 has risen to prominence in recent years due to genetics studies that have implicated this channel as playing an important role in human pain perception. Since the known physiology and human genetics indicates that Nav1.7-selective blockers may lead to effective analgesia without the potential for significant adverse side effects or addiction liabilities, Nav1.7 has become a highly scrutinized target by academic and industrial biomedical researches. Accordingly, an intensive search for highly selective and highly potent Nav1.7 antagonists has ensued. However, as outlined in preceding sections, selectivity and potency alone do not guarantee an efficacious or safe therapeutic in vivo in any preclinical or clinical model. In fact, there are many considerations and significant hurdles to overcome on the path to the discovery, optimization, and development of a potentially transformative new pain medicine. As we discuss above in the sections on state-dependence and toxicity, for example, there are many key considerations around Nav1.7 that still remain largely unexplored or unreported. Moreover, challenges in reproducing results (Murray et al. 2015a; Liu et al. 2016) of some reports describing potential Nav1.7-targeting lead molecules (Lee et al. 2014; Yang et al. 2013), and other contributions that conclude lack of single agent in vivo efficacy (Deuis et al. 2017; Storer et al. 2017; Wu et al. 2017c; Hockley et al. 2017) tend to dampen or confuse enthusiasm around Nav1.7. Nevertheless, many researchers still firmly believe that rigorous investigation of the therapeutic hypothesis that Nav1.7 may be a transformative target for the treatment of chronic and potentially neuropathic pain is worth the steady and careful scientific and clinical evaluation that such a challenging drug discovery effort truly requires.

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