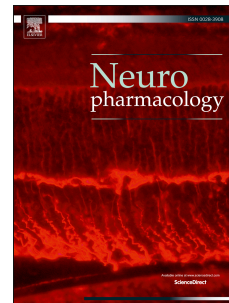# Accepted Manuscript

The pharmacology of voltage-gated sodium channel activators

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The Pharmacology of Voltage-gated Sodium channel Activators

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Abstract

Toxins and venom components that target voltage-gated sodium (Na_V) channels have evolved numerous times due to the importance of this class of ion channels in the normal physiological function of peripheral and central neurons as well as cardiac and skeletal muscle. Na_V channel activators in particular have been isolated from the venom of spiders, wasps, snakes, scorpions, cone snails and sea anemone and are also produced by plants, bacteria and algae. These compounds have provided key insight into the molecular structure, function and pathophysiological roles of Na_V channels and are important tools due to their at times exquisite subtype-selectivity. We review the pharmacology of Na_V channel activators with particular emphasis on mammalian isoforms and discuss putative applications for these compounds.

Na$_V$ activators

High-throughput screening assays

In vivo target engagement assays

Human physiology/ pathophysiology

Drug leads

Insecticides
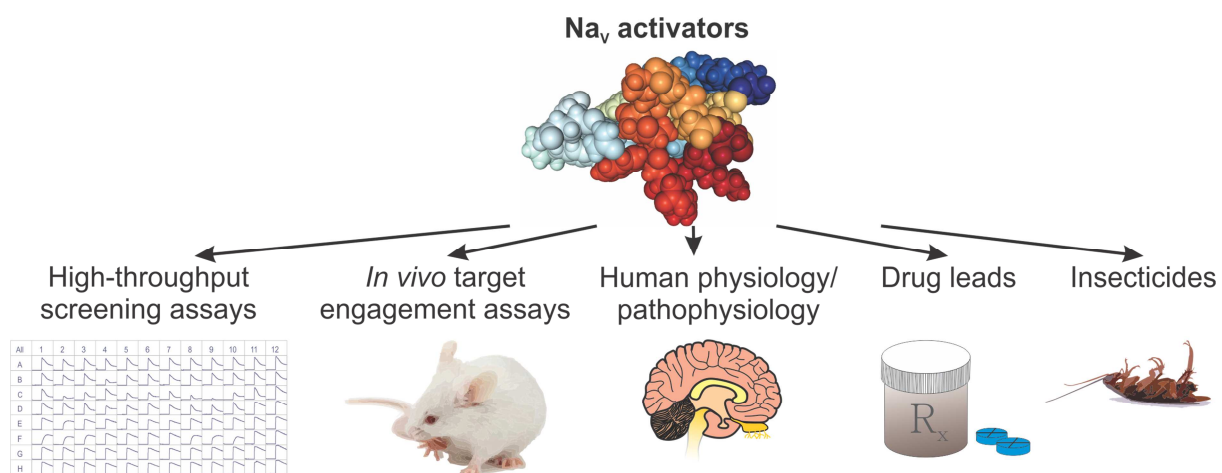
1 *Key Words*

2 Voltage-gated sodium channel; activator; toxin; voltage sensor; pharmacology.

3

4 *1. Introduction*

5 Voltage-gated sodium (Na$_V$) channels are key transmembrane proteins that permit

6 influx of Na$^+$ in excitable and non-excitable cells where they contribute to setting the

7 membrane potential, action potential initiation and propagation, as well as cell

8 motility and proliferation. While Na$_V$ channels can be found in many tissues and cell

9 types, they are particularly important for the function of central and peripheral

10 neurons as well as skeletal and cardiac muscle. Nine voltage-sensing pore forming α

11 subunit isoforms have been described in humans (Na$_V$1.1, Na$_V$1.2, Na$_V$1.3, Na$_V$1.4,

12 Na$_V$1.5, Na$_V$1.6, Na$_V$1.7, Na$_V$1.8, and Na$_V$1.9; encoded by the genes SCN1A,

13 SCN2A, SCN3A, SCN4A, SCN5A, SCN8A, SCN9A, SCN10A and SCN11A,

14 respectively), each with distinct expression profiles. The contribution of each of these

15 isoforms to tissue- and disease-specific physiology and pathology makes Na$_V$

16 channels important therapeutic targets for treatment of a range of diseases, including

17 epilepsy, pain, myotonias, cardiac arrhythmias and insufficiencies as well as cancer

18 (Luiz and Wood, 2016; Mantegazza et al., 2010; Novak et al., 2015; Zaklyazminskaya

19 and Dzemeshkevich, 2016). However, most drug discovery efforts to date have been

20 directed at the development of subtype-selective Na$_V$ channel inhibitors.

21 The endogenous "activator" of Na$_V$ channels is membrane voltage, more specifically

22 the distribution of charge across membrane bilayers which in turn affects the position

23 of charged transmembrane segments, leading to opening or closing of the central,

24 Na$^+$-permeable pore. Many toxins and venom components have evolved to hijack this

25 activation mechanism by stabilising the activated conformation of the channel *via*

1 interactions with extracellular or transmembrane domains. These compounds thus act

2 as net channel activators, although the specific effects on the electrophysiological

3 properties of the channel can vary significantly.

4 While they have been overlooked, in relative terms, for their clinical utility, Na_V

5 channel activators not only have applications in the treatment of disease, they are also

6 indispensable tool compounds and have provided key insights into the molecular

7 mechanisms governing function of this important class of ion channels. Moreover,

8 plant-derived or synthetic Na_V channel activators such as pyrethrins are one of the

9 most commonly used classes of insecticides, with uncontrolled Na^+ influx leading to

10 rapid spastic paralysis. In light of emerging resistance to these molecules (Silva et al.,

11 2014), novel classes of Na_V channel activators may play important roles in the control

12 of agricultural pests and vectors of insect-borne disease. Venom-derived peptides in

13 particular may confer benefits as more environmentally friendly compounds that are

14 less likely to accumulate and cause unwanted toxic effects on humans, wildlife, and

15 beneficial insects (Li et al., 2017).

16

17 Na_V channel activators have also provided key insights into the molecular

18 mechanisms underpinning channel activation and inactivation – key processes that are

19 essential for normal cellular function. Future insights into the molecular and structural

20 components involved in channel gating will undoubtedly facilitate the rational design

21 of highly subtype-selective Na_V channel modulators with therapeutic potential.

22

23 Lastly, Na_V channel activators also represent important tool compounds that permit

24 the dissection of the physiological and pathological roles of individual Na_V channel

25 isoforms. They have in addition been instrumental for the development of high-

1   throughput screening assays leading to the identification of subtype-selective Na_V

2   channel inhibitors, as control of membrane potential can be difficult to achieve in

3   traditional drug discovery assays (Vetter et al., 2012a).

4

5   This review will summarise effects of various classes of Na_V channel activators,

6   including peptide toxins from spider, wasp, snake, scorpion, cone snail and sea

7   anemone venoms as well as alkaloids and polyethers derived from bacteria and

8   dinoflagellates, with particular emphasis on pharmacology at mammalian Na_V

9   channels.

10

11   ## *2. Physiology of Na_V channels*

12   Na_V channels are a group of sodium (Na^+) selective ion channels that are normally

13   activated by voltage changes across cellular membranes, leading to channel opening

14   and a subsequent transmembrane influx of Na^+ ions along their electrochemical

15   gradient into the cell (Chandler and Meves, 1965; Hille, 1972; Hodgkin and Huxley,

16   1952a, b, c). Na_V channels are essential for the initiation and propagation of action

17   potentials in electrically excitable cells like nerves and muscle fibres. They are

18   responsible for the initial depolarization of the membrane and thus crucial for the fast

19   electrical signalling that is necessary to propagate vital information over long

20   distances in animals (Hodgkin and Huxley, 1952a, b, c, d).

21   The ion conducting α-subunits can form multi-protein signalling complexes by

22   coupling to one or two cell type specific auxiliary proteins, such as the β subunits β1 -

23   β4 (encoded by the genes SCN1B - SCN4B), which modulate Na_V channel biophysics

24   and trafficking (Meadows and Isom, 2005; O'Malley and Isom, 2015).

1   The individual Na_V channel subtypes are defined by the nine different α subunits that

2   also contain many binding sites for toxins and other modulating compounds. Seven

3   Na_V channels $Na_V1.1$, $Na_V1.2$, $Na_V1.3$, $Na_V1.6$, $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$ play

4   major roles in electrogenesis in neurons. The subtypes that are expressed at

5   considerable levels in the central nervous system (CNS) include $Na_V1.1$, $Na_V1.2$,

6   $Na_V1.3$ and $Na_V1.6$, where they can only be targeted by compounds that cross the

7   blood-brain barrier. Widely expressed in the peripheral nervous system (PNS) are

8   $Na_V1.1$, $Na_V1.6$, $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$. Impulse electrogenesis and conduction

9   in skeletal muscle cells and in cardiomyocytes is carried out by $Na_V1.4$ and $Na_V1.5$,

10 respectively (Catterall et al., 2005; Goldin, 1999). Additionally, multiple Na_V channel

11 subtypes are expressed in many non-excitable cell types like astrocytes, microglia,

12 macrophages, and cancer cells where they play a role in e.g. the release of bioactive

13 molecules or regulating motility, Na^+/K^+-ATPase activity, phagocytosis and

14 metastatic activity (Black and Waxman, 2013; de Lera Ruiz and Kraus, 2015).

15

16 Due to the important role of Na_V channels and their wide tissue distribution, it is not

17 surprising that Na_V channel dysfunction plays an important role in numerous severe

18 pathological conditions including epilepsy (Heron et al., 2002; Liao et al., 2010;

19 Sugawara et al., 2001), migraine (Dichgans et al., 2005), ataxia (Liao et al., 2010;

20 Trudeau et al., 2006), multiple sclerosis (Waxman, 2006), various painful conditions

21 (Dib-Hajj et al., 2013; Faber et al., 2012a; Faber et al., 2012b; Fertleman et al., 2006;

22 Vetter et al., 2017; Yang et al., 2004), the inability to experience pain (Ahmad et al.,

23 2007; Cox et al., 2006), heart disorders (Medeiros-Domingo et al., 2007; Song and

24 Shou, 2012; Tarradas et al., 2013) and neuromuscular diseases (Cannon, 2010; Jurkat-

25 Rott et al., 2010). These conditions are often caused by inherited or spontaneous

1 mutations, but can also be the result of injuries, adverse drug reactions or

2 intoxications that result in hypo- and hyper-excitable phenotypes (Dib-Hajj and

3 Waxman, 2010; Wood et al., 2004). The pharmacological modulation of any Na_V

4 channel in the CNS, in the heart or in muscle cells has the potential risk of life-

5 threatening side effects like seizures, cardiac arrhythmia or muscle paralysis resulting

6 in respiratory arrest. For this reason, high potency and subtype selectivity is a much

7 sought after characteristic in the development of pharmaceutical drugs. Both are

8 common features of many natural toxins and venom derived peptides as a result of

9 ongoing evolutionary specialisation and optimization.

10

11 The Na_x channel (also known as NaG or $Na_V2.1$, in humans encoded by the gene

12 SCN7A) also belongs to the family of the voltage-gated sodium channels, even

13 though it is not activated by changes in membrane potential. Instead it is activated by

14 an increased extracellular Na^+ concentration and is involved in sodium level sensing

15 in the central nervous system, in controlling salt intake behaviour as well as in

16 regulating epithelial sodium homeostasis (Hiyama et al., 2002; Watanabe et al., 2000;

17 Xu et al., 2015). We use the term "voltage-gated sodium (Na_V) channel" here to mean

18 the family of voltage activated $Na_V1.X$ channels.

19

20

21 *3. Structure of Na_V channels*

22 **3.1. Structure and function of α-subunits**

23 Structural information on Na_V channels has for a long time lagged behind functional

24 and pharmacological characterisation. The first insights into the principal Na_V channel

25 structure was obtained from studies with radiolabelled toxin-derivatives such as

5

1   modified [^{3}H]saxitoxin and a [^{125}I]β-scorpion toxin that covalently bind to their

2   receptor site. These toxins helped to identify distinct binding sites as well as the

3   existence of one larger alpha subunit (260 kDa) and several smaller beta subunits

4   (30–40 kDa) (Beneski and Catterall, 1980; Hartshorne et al., 1982). The same toxins

5   also enabled the purification of a Na$_V$ channel from the electric organ of an eel and

6   allowed for cloning and sequence analysis of the cDNA, which revealed the amino

7   acid sequence and thus the first primary structure of a Na$_V$ channel (Noda et al.,

8   1984). This started an era of educated guesswork on how the human Na$_V$ channel

9   structure might change during gating, and how toxins interact with the channel, that

10   continues to date. Although we are still lacking three-dimensional high-resolution

11   data from mammalian channels, many features can be deduced from insights from

12   site-directed mutagenesis studies, elegant functional and pharmacological studies as

13   well as crystal structures of the closely related bacterial channels and the cryo-EM

14   structure of a putative Na$_V$ channel from the American cockroach (Shen et al., 2017).

15

16   We now know that all human Na$_V$ channel α subunits likely can form functional

17   monomers that consist of one long polypeptide chain, which folds into four

18   homologous but markedly different repeat domains (DI - DIV), linked by three

19   intracellular loops, and congregate to a pseudo-tetrameric structure (Vetter et al.,

20   2017). Major structural differences between the nine human Na$_V$ channel α-subunits

21   Na$_V$1.1 – Na$_V$1.9 are unlikely, as indicated by the high sequence homology between

22   them. In contrast, the much simpler prokaryotic Na$_V$ channels, which have been

23   recently used to determine Na$_V$ channel crystal structures, are homo-tetramers, i.e.

24   formed by four identical and separate subunits, and thus more closely resemble the

25   structural arrangement of the mammalian K$_V$ channels.

1

2

3   All Na_V channels have similar functional characteristics and a common overall

4   structure, indicated by the high level of ==sequence identity== with about 50-90% identical

5   amino acids between any two isoforms (Vetter et al., 2017). However, the

6   pharmacological and kinetic properties as well as the voltage dependence of each

7   voltage-gated sodium channels isoform are different and contribute to their distinct

8   functional physiological and pharmacological roles (Catterall et al., 2005).

9

10   The characteristic features of voltage-gated sodium channels are fast gating (i.e.

11   channel opening and closing) with voltage-dependent activation, rapid inactivation

12   and selective sodium ion conductance (Hodgkin and Huxley, 1952d). The channels'

13   voltage sensors move in response to the cellular membrane potential changes and thus

14   determine the state in which the channels reside with the highest probability. Na_V

15   channels are closed at resting membrane potentials and require membrane

16   depolarization to be activated. Activation of the channel leads to a fast opening of the

17   pore domain due to a conformational change, resulting in selective Na^+ ion influx into

18   the cell and further membrane depolarization, thus contributing to the rapid upstroke

19   of the action potential in excitable cells. The subsequent fast inactivation leads to a

20   ==non-conducting channel state== within milliseconds of opening (Catterall, 2012).

21   ==Na_V1.9 is an exception, taking longer (>100 milliseconds) to inactivate than the other==

22   ==Na_V subtypes (Dib-Hajj et al., 2015).== A special form of inactivation is the slow

23   channel inactivation that recovers only very slowly and can occur during repetitive

24   neuronal firing, when membranes around Na_V channels are depolarized for long

25   periods (seconds) (Catterall, 2014).

1

2   Each of the four homologous domains DI - DIV of human Na$_V$ channels contains six

3   α-helical transmembrane segments (S1 - S6) and is divided into two functional

4   domains, a pore domain (PD) and a voltage-sensing domain (VSD) (**Figure 1**). The

5   ion-conducting pore of the channel is formed by the four assembled PDs of DI - DIV,

6   which are each shaped by segments S5 and S6 plus their extracellular linker. These

7   four linkers each contain a conserved pore loop (P-loop) that dips halfway back into

8   the membrane between S5 and S6, where they tightly associate with each other. This

9   creates a narrow Na$^+$ selective filter formed by the amino acids DEKA and EEDD at

10   the extracellular end of the pore. The pore region presents toxin binding sites on the

11   external opening, close to the selectivity filter (for pore blockers like tetrodotoxin and

12   the μ-conotoxins) as well as inside the pore (for gating modifiers like veratridine) (de

13   Lera Ruiz and Kraus, 2015).

14   The four functional and structural distinct VSDs are each shaped by the segments S1

15   – S4 (Catterall, 2000; Guy and Seetharamulu, 1986) and linked to the PD via the S4 –

16   S5 linker. The S4 segments function as voltage sensors of the channel as they carry

17   several highly conserved positively charged arginine and lysine residues (gating

18   charges) that are forced to move across the cell membrane upon changes in the

19   membrane potential and induce subsequent changes in the channel conformation (Guy

20   and Seetharamulu, 1986; Stuhmer et al., 1989). Especially the two small linkers

21   between S1 – S2 and S3 – S4, but also residues in the transmembrane segments of the

22   VSDs, present further extracellular binding sites for many gating modifier toxins like

23   spider and scorpion toxins (de Lera Ruiz and Kraus, 2015; Murray et al., 2016).

1 Another important structural feature of the Na$_V$ channel α subunits is the third

2 intracellular loop (L3) containing an IFM motif that connects S6 in DIII with S1 in

3 DIV and is crucial for inactivation of the channel (**Figure 1**).

4 While there is evidence for the importance of DIV for fast inactivation, the distinct

5 functional roles of the three other domains is still uncertain (Ahern et al., 2016), partly

6 due to a probable functional coupling between the different domains and a lack of

7 more detailed structural information.

8

9 Recent advances in single particle cryo-electron microscopy and membrane protein

10 crystallography have improved our general structural understanding of voltage-gated

11 ion channels, including the mammalian calcium and potassium channel (Hite et al.,

12 2015; Whicher and MacKinnon, 2016; Wu et al., 2015) and bacterial as well as

13 eukaryotic Na$_V$ channels (Catterall, 2014; Shen et al., 2017). However, the exact

14 three-dimensional structure of the pseudo-tetrameric human voltage-gated sodium

15 channel α-subunits is still unknown due to difficulties with its crystallization. Several

16 more or less accurate homology models exist, which are based on human Na$_V$ channel

17 sequences and the X-ray crystal structures of homo-tetrameric prokaryotic Na$_V$

18 channels (NavAb, NavRh, and NavMs) (Catterall, 2014; McCusker et al., 2012;

19 Payandeh et al., 2012; Payandeh et al., 2011; Zhang et al., 2012). One of the problems

20 with these models is that the much simpler bacterial channels lack several important

21 features of the eukaryotic version. Specifically, the four assembled identical subunits

22 do not have the intracellular loops that connect the four corresponding human

23 domains. Accordingly, the third loop (L3) with the conserved amino acid residues

24 IFM between DIII and DIV, which is essential for fast inactivation of the open

25 mammalian Na$_V$ channel (Stuhmer et al., 1989; Vassilev et al., 1989; West et al.,

1   1992), is not conserved. Furthermore, a symmetrical bacterial channel cannot explain

2   the distinct roles of the four individual domains, which each have different kinetics,

3   functional properties and contributions to channel gating as well as unique binding

4   sites for interacting compounds (Ahern et al., 2016). Another general problem of

5   crystallography studies is the rigidity of the crystallized channels, which makes it

6   difficult to analyse the *in vivo* flexible extracellular loops that determine the binding

7   site of many channel modulators. Nevertheless, information on the general design of

8   the Na_V channels obtained from these studies is still helpful for a better understanding

9   of these important proteins. With the continuing high interest in the field of Na_V

10 channels, it is likely that rapid advances in the field of high resolution single particle

11 cryo-electron microscopy will lead to additional structural details on mammalian Na_V

12 channels in the near future (Sato et al., 2001; Slowik and Henderson, 2015).

13

14

15 **3.2. Structure and function of β subunits**

16 When α subunits are heterologously expressed alone, they demonstrate full Na_V

17 channel function including sodium selectivity, rapid opening and fast inactivation

18 (Goldin et al., 1986; Noda et al., 1986). However, *in vivo* they typically couple to one

19 or two cell-type specific Na_V channel β subunits, which do not contribute to the ion-

20 conducting pore but modulate channel function. This makes the coexpression of the

21 appropriate β subunits in heterologous expression systems necessary to mimic the

22 kinetic properties of native sodium channels. β subunits modify Na_V channel gating

23 properties including kinetics and voltage dependence as well as channel expression

24 and trafficking (Catterall, 2000; Meadows and Isom, 2005; O'Malley and Isom, 2015).

25 However, β subunits do not only modify sodium currents but have additional

10

1   functions as cell adhesion molecules, playing roles in cell adhesion, migration,

2   invasion and neurite outgrowth (Brackenbury and Isom, 2011; Patino and Isom,

3   2010). Furthermore, the β4 subunit has been found to function as an endogenous

4   cytoplasmatic open channel blocker that directly competes with the fast inactivation

5   gate and thus enabling resurgent currents, which render certain Na_V channel isoforms

6   capable of high-frequency firing in excitable tissues (Lewis and Raman, 2013).

7   The identified genes SCN1B, SCN2B, SCN3B and SCN4B encode the four

8   membrane bound β subunits β1 – β4 as well as a fifth soluble splice variant β1b. All

9   are glycoproteins with a molecular weight of about 30 – 40 kDa, consisting of one

10 single transmembrane domain (which is lacking in β1b) and an immunoglobulin-like

11 extracellular domain. The subunits β1 and β3 interact non-covalently with α subunits,

12 whereas β2 and β4 bind via disulfide bonds covalently to Na_V channel α subunits.

13

14 β Subunits are involved in several Na_V channel-dependent pathologies, with changed

15 expression levels in conditions like pain and nerve injury and genetic mutations in all

16 four β subunit-encoding genes being linked to severe diseases (Eijkelkamp et al.,

17 2012).

18 Interestingly, changing expression levels of β subunits in heterologous expression

19 systems can significantly affect affinity and efficacy of activating and inhibitory

20 toxins and thus alter Na_V channel modulation (Gilchrist et al., 2013; Namadurai et al.,

21 2015). For instance, the presence of the β4 subunit dramatically reduces the inhibition

22 of Na_V1.2 by the spider toxin ProTx-II and decreases the maximal conductance of the

23 same channel induced by the activating β-scorpion toxin TsVII, while not affecting its

24 hyperpolarizing shift in voltage of activation. Co-expression of the Na_V channel β2

25 and β1 subunit significantly reduces the activation of Na_V1.2 channels by the sea

1 anemone toxin ATX-II and the α-scorpion toxin LqqIV, respectively (Gilchrist et al.,

2 2013). β subunit co-expression can also alter the kinetics of Na_V channel inhibition by

3 pore blocking modulators of the conotoxin family. Binding on-rates (k_{on}) of μ-

4 conotoxins at several Na_V channel isoforms can be increased in the presence of β1 and

5 β3 subunits, while they are decreased by β2 and β4 subunits (Zhang et al., 2013). In

6 contrast, all four β subunits increase the k_{on} of μO-conotoxin MrVIB at Na_V1.8

7 channels (Wilson et al., 2011). Furthermore, co-expression of β2 or β4 protects

8 Na_V1.1 -1.7 (excluding Na_V1.5) channels against block by an analogue of the μO§-

9 conotoxin GVIIJ (Gajewiak et al., 2014). Thus, coexpression of β-subunits can

10 strongly influence the affinity of conopeptides for Na_V channels.

11

12 Modification of toxin pharmacology by the presence of β subunits may thus explain

13 some tissue-specific differences in pharmacological activity, as the expression levels

14 of β subunits vary in different cell types (Gilchrist et al., 2013; Wilson et al., 2011). In

15 addition, the expression of β subunits has implications for the use of Na_V channel

16 modulators in pathological conditions where β subunit expression is changed.

17

18 **3.3. Gating mechanisms of Na_V channels: a toxin perspective**

19 Na_V channels exist in at least three, and likely many more, distinct states: resting

20 (closed), open, and inactivated (**Figure 2**). The process of channel gating refers to the

21 movement of the voltage sensors in response to changes in charge distribution across

22 the cellular membrane, resulting in structural re-arrangement of the channel. Toxins

23 have been indispensable tools to understand the complex transitions between these

24 states, and generally speaking interact with Na_V channels either by physically

25 occluding the pore, or by preventing movement of the voltage sensors either in the

1   outward or inward direction. Accordingly, to understand the pharmacological effects

2   and mechanism of action of toxins, it is imperative to understand the gating

3   mechanisms of Na_V channels.

4

5   In the resting or closed state of Na_V channels, all four VSD are presumably in the

6   "down" state as their positive gating charges on the S4 segments are forced toward the

7   intracellular side of the cell membrane. This is due to the strong electrical potential

8   generated by the negative resting membrane potential of unexcited neurons and

9   myocytes. Membrane depolarization leads to activation and very quick opening of the

10   channel as the more positive membrane potentials allow the S4 gating charges to

11   move toward the extracellular surface into the "up" position. This likely involves a

12   "helical screw" motion in which S4 helices slide and rotate around their axes, with the

13   charged residues exchanging ion-pair partners until they are stabilised again by highly

14   conserved negative charge clusters on the other segments (Chanda and Bezanilla,

15   2002; Guy and Seetharamulu, 1986; Payandeh et al., 2011). The movement in VSDs

16   is subsequently transferred to the PDs via the S4 - S5 linkers, changing channel

17   conformation and allowing the pore to become permeable to sodium ions (Li et al.,

18   2014; Payandeh et al., 2011).

19   Fast inactivation follows channel opening, resulting in a non-conducting inactivated

20   channel state before finally recovering from inactivation and returning to a resting,

21   closed state. Crucial for fast inactivation are the highly conserved residues IFM

22   (isoleucine – phenylalanine - methionine) on the third intracellular loop between DIII

23   and DIV (Vassilev et al., 1989; West et al., 1992), which bind to an unknown receptor

24   site involving several residues of the S4 - S5 linker of DIV, close to the intracellular

25   opening of the pore and thus closing it. The highly conserved DIV S4 - S5 linker is

1   made up of 15 amino acids of which 14 are identical in all nine human Na_V channel

2   isoforms. At least 7 of these residues have been found to be important for normal fast

3   inactivation in various mutagenesis studies and have thus been proposed to be part of

4   the putative receptor site of the docking region with the IFM motif (Filatov et al.,

5   1998; McPhee et al., 1998; Mitrovic et al., 1996; Tang et al., 1996).

6

7   It is possible that there is functional coupling between the four VSDs that affects

8   channel gating, but this is still unclear. Mutagenesis studies on S4 gating charges in

9   the four VSDs hinted at a predominant role of DIV in fast inactivation (Kuhn and

10   Greeff, 1999), while activation gating was affected by mutations of positive S4

11   charges in all four voltage-sensing domains, but predominantly DI-DIII (Kontis et al.,

12   1997). Structure-function studies with toxins that bind to the DIV S3 - S4 linker of the

13   VSD confirmed the role of DIV in fast inactivation (Benzinger et al., 1998; Rogers et

14   al., 1996). The results suggest that fast inactivation can only occur after a

15   conformational change in the VSD of domain IV makes it move toward the

16   extracellular side of the cell membrane, while fast inactivation is independent from

17   the movement of the VSDs of DI – DIII, which are likely to be mainly responsible for

18   initial channel opening. Consistent with these findings is the observation that upon

19   channel activation, the VSDs in DI - DIII move faster toward the extracellular surface

20   with time-constants correlating to the fast channel opening, while the movement of

21   the VSD in DIV is slower and correlates with the subsequent fast inactivation

22   (Chanda and Bezanilla, 2002). Moreover, Na_V channel modulating toxins that only

23   bind to the DIV VSD usually have no effects on channel activation but inhibit or

24   delay fast inactivation, resulting in slower decaying sodium currents. However, the

25   separate functions of the kinetically different voltage sensors in DI - DIII still remain

1   to be defined. The DII and DIV VSDs are targeted by many natural modulators, but

2   only very few toxins affect DI and DIII with binding sites rarely reported for these

3   two domains (Bosmans et al., 2008). This suggests a predominant role of DII in Na_V

4   channel activation as it was probably evolutionarily the most successful to target.

5

4. Na_V activator pharmacology

7   Due to its important role in action potential generation and propagation, many clinical

8   drugs have been developed and numerous toxins from venomous animals or plants

9   have evolved to target Na_V channels, often as part of prey capture or defence

10   strategies (Kaczorowski et al., 2008; Kalia et al., 2015). At least eight distinct binding

11   sites on Na_V channels (site 1-8) have been proposed, six of which are the receptor

12   sites for various toxins (Catterall et al., 2005; de Lera Ruiz and Kraus, 2015). The

13   definition of these binding sites is often complex as they include crucial amino acids

14   on multiple domains and segments of the channel that significantly overlap with other

15   binding sites. In a simplified model, binding sites can be differentiated structurally

16   into the pore region and the voltage-sensing domains, with additional elaboration on

17   functional effects distinguishing compounds that enhance or decrease channel

18   activation, as well as those that enhance or decrease channel inactivation. According

19   to the resultant effects on macroscopic Na_V channel currents, these compounds can

20   thus be classified as channel inhibitors or activators (**Figure 3**).

21   Specifically, the pore region binding modulators can be functionally divided into pore

22   occluding pore blocker (like tetrodotoxin and lidocaine) and compounds that facilitate

23   or prolong channel activation by modifying gating after binding into the pore (e.g.

24   veratridine). The VSD binding modulators can be broadly divided into another two

25   groups. The first group – functional Na_V channel activators – leads to an increase in

15

1 macroscopic Na^+ current which can be induced by facilitating activation

2 (hyperpolarizing shift in voltage of activation) and/or prolonging the open channel

3 state (inhibition of inactivation). Examples of this group are discussed in more detail

4 in the following sections. The second group – functional Na_V channel inhibitors –

5 leads to a decrease in Na^+ current, induced by reduced activation (depolarizing shift in

6 voltage of activation; e.g. ProTx-II) and/or shortened open channel states after

7 binding to a VSD (e.g. Pn3a) (Deuis et al., 2017).

8

9 Na_V channel activators have provided crucial tool compounds to delineate the

10 physiological and pathological roles of Na_V channels, and may in future find

11 applications as therapeutics for treatment of conditions such as epilepsy and cardiac

12 arrhythmias. However, in contrast to Na_V channel inhibitors, detailed pharmacological

13 characterisation of Na_V channel activators is relatively sparse, in part because more

14 complex electrophysiological protocols may be required to determine effects on

15 diverse gating parameters. Accordingly, it can be difficult to accurately define EC_{50}

16 values for Na_V channel activators, which depend on distinct, and finite, effects on a

17 single parameter.

18 Na_V channel activator toxins are typically gating modifiers toxins that bind either to

19 the inside of the pore or to the extracellular parts of DII VSD and/or DIV VSD,

20 sometimes involving extracellular pore domain residues in close proximity to the

21 VSDs (Murray et al., 2016; Osteen et al., 2016). Gating modifiers modulate

22 conformational changes of Na_V channels and the coupling between the different

23 channel states with varying effects on activating and inactivating gating properties. In

24 general terms, activator toxins binding to DII VSD generally affect activation by

25 shifting the voltage of activation to more hyperpolarized potentials, while activator

16

1 toxins that bind solely to the VSD of DIV generally affect inactivation by inhibiting

2 normal movement of S4 and the subsequent inactivation of the channel (Xiao et al.,

3 2014).

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10 **4.1. Classes of activators**

11 Due to their evolutionary success – based on the serious physiological effects caused

12 by uncontrolled Na_V channel activity – Na_V channel activators are found in many

13 different organisms, including plants, algae, frogs, cone snails, spiders, wasps, snakes,

14 sea anemone and scorpions. In addition to alkaloid and polyether Na_V channel

15 activators, many of the venom-derived Na_V channel activators are peptidic in nature,

16 although the size, sequence and structure of these compounds is diverse. The Na_V

17 channel selectivity and toxin-channel interactions for each class of activators are

18 discussed below, as well as being summarised in Table 1 and 2, respectively. It should

19 be noted that potency might differ between human and rodent orthologs of Na_V

20 channels, however this is generally not assessed systematically.

21

22 *4.1.1. Alkaloid Na_V channel activators*

23 Batrachotoxin, grayanotoxin, veratridine and aconitine are a chemically diverse group

24 of Na_V channel activators that share an overlapping binding site, designated as site 2

1   (Catterall, 1975). While often grouped together, these alkaloids have differential

2   effects on the biophysical properties of Na_V channels, all of which will be discussed

3   individually below.

4   Batrachotoxin, first isolated in 1965, is a lipophilic alkaloid secreted by the skin

5   glands of poison dart frogs (*Phyllobates* spp.), used by native South Americans to

6   poison the tips of blow-darts (Daly et al., 1965). Batrachotoxin is not produced by the

7   frog itself, but is bioaccumulated through dietary consumption of alkaloids from

8   insects, although the exact dietary source remains unknown (Daly et al., 1994).

9   Accordingly, frogs raised in captivity do not secrete batrachotoxin, and due to

10   difficulties in chemical synthesis, which were only recently overcome (Logan et al.,

11   2016), thousands of the now endangered *Phyllobates* poison dart frogs were captured

12   and killed to isolate batrachotoxin (Daly et al., 1994). Symptoms of batrachotoxin

13   intoxication include cardiac arrhythmias, motor impairment, respiratory depression

14   and convulsions, which can lead to death (Albuquerque et al., 1971).

15   Batrachotoxin preferentially binds to the open state of Na_V channels, requiring

16   repetitive depolarizing pulses (>1000) at a frequency of 1-2 Hz to observe a

17   functional effect under voltage-clamp conditions (Wang and Wang, 1998). Once

18   bound, batrachotoxin causes a hyperpolarizing shift in the voltage-dependence of

19   activation, inhibits fast and slow inactivation, and causes a change in ion selectivity

20   (Bosmans et al., 2004; Li et al., 2002; Wang and Wang, 1996; Wang and Wang,

21   1998). Batrachotoxin has an EC_{50} of 1 μM, and displays little Na_V channel selectivity,

22   inhibiting fast inactivation of $Na_V1.2$, $Na_V1.3$, $Na_V1.4$, $Na_V1.5$, $Na_V1.7$ and $Na_V1.8$

23   with a similar effect at a concentration of 10 μM (Bosmans et al., 2004; Li et al.,

24   2002; Logan et al., 2016).

1   The binding site of batrachotoxin is located within the inner pore region, with single

2   point mutations of amino acids located on S6 in DI (I433, N434, L437), DII (N784,

3   L788), DIII (F1236, S1276, L1280) and DIV (F1579, N1584) causing $rNa_V1.4$ to

4   become insensitive to the effects of batrachotoxin (**Figure 4-8**) (Toma et al., 2016;

5   Wang et al., 2001; Wang et al., 2000b; Wang and Wang, 1998, 1999). These amino

6   acids are conserved on $hNa_V1.1-1.8$, accounting for the limited selectivity of

7   batrachotoxin for Na_V channels. Interestingly, sequence alignment of frog $Na_V1.4$ has

8   identified similar single point mutations in *Phyllobates* poison dart frogs on S6 DI

9   (S429A, I433V, A446D) and S6 DIV (V1583I, N1584T), conferring resistance to

10   protect against self-intoxication (Tarvin et al., 2016).

11

12   Grayanotoxin is present in the leaves, twigs and flowers of *Rhododendron* plants. In

13   humans, intoxication can occur by consumption of honey (known as "mad honey")

14   produced by bees from the nectar of *Rhododendron* plants, with most reported cases

15   occurring from honey originating from Turkey (Gunduz et al., 2006). Mad honey

16   poisoning causes nausea, vomiting, dizziness, hypotension, bradycardia and other

17   cardiac disturbances, which can be fatal if left untreated.

18   Like batrachotoxin, grayanotoxin binds to the open state of Na_V channels and requires

19   repetitive depolarising pulses to see a functional effect under voltage-clamp

20   conditions (Yakehiro et al., 2000).   Once bound, grayanotoxin causes a

21   hyperpolarizing shift in the voltage-dependence of activation, but unlike

22   batrachotoxin, causes a reduction in peak current (Kimura et al., 2001). Grayanotoxin

23   also inhibits fast inactivation and causes a reduction in ion selectivity, similar to

24   batrachotoxin (Yuki et al., 2001). Compared with batrachotoxin, grayanotoxin is less

25   potent, with an EC_{50} of 31 μM, and has a greater effect on fast inactivation at $Na_V1.4$

1 compared to $Na_V1.5$, although activity at other Na_V channel subtypes in heterologous

2 expression systems remains to be determined (Kimura et al., 2001; Yakehiro et al.,

3 2000).

4 The binding site of grayanotoxin is less well defined, with studies focused on

5 assessing the activity of grayanotoxin on $rNa_V1.4$ channels with single point

6 mutations known to cause batrachotoxin insensitivity. While single point mutations of

7 amino acids located on S6 in DI (I433, N434, L437) cause grayanotoxin insensitivity,

8 activity is maintained with the single point mutation F1579A on S6 in DIV, indicating

9 that grayanotoxin and batrachotoxin share an overlapping but non-identical binding

10 site (**Figure 4**) (Ishii et al., 1999; Kimura et al., 2000). In addition, the poison dart

11 species *Phyllobates terribilis* is insensitive to batrachotoxin, but not to grayanotoxin,

12 confirming a non-identical binding site (Daly et al., 1980).

13

14 Veratridine is a steroid-derived alkaloid found in the roots of *Veratrum* plants from

15 the *Liliaceae* family. Unlike batrachotoxin and grayanotoxin, veratridine does not

16 require repetitive depolarising pulses to exert a functional effect on Na_V channels

17 under voltage-clamp conditions (Farrag et al., 2008; Ghatpande and Sikdar, 1999).

18 Veratridine causes a hyperpolarising shift in the voltage-dependence of activation and

19 decreases peak current, however compared to batrachotoxin and grayanotoxin, only

20 delays rather than inhibits fast inactivation, resulting in a comparatively small

21 persistent current (Ghatpande and Sikdar, 1999). Veratridine has an EC_{50} of 29 μM at

22 $Na_V1.7$, and appears to have minimal selectivity at $Na_V1.1$-1.7, based on its use as a

23 Na_V channel activator in fluorescence based assays (Deuis et al., 2015; Vetter et al.,

24 2012a; Vickery et al., 2004). In rodent dorsal root ganglion neurons, veratridine

Veratridine shares an overlapping but non-identical binding site with batrachotoxin, as the poison dart species *Phyllobates terribilis* is sensitive to veratridine, but not batrachotoxin (Daly et al., 1980). In addition, single point mutations of amino acids located on S6 in DI (I433, N434, L437) and S6 in DIV (F1579, N1584) cause $rNa_V1.4$ to become insensitive to the inactivation-modulating effects of veratridine (**Figure 4 and 8**), although all of the mutants remained sensitive to the inhibitory effects of veratridine on peak current (Wang et al., 2000a). The reason for this disparity remains unclear, but suggests that the persistent current and reduction in peak current are caused by two distinct channel interactions. The most likely explanation is that peak current is reduced after binding of veratridine due to partial occlusion of the ion permeation pathway, while gating/inactivation cannot be modulated anymore in these mutants due to removal of the essential gating-modifying interactions.

Aconitine is a steroid-derived alkaloid found in the *Aconitum napellus* (monkshood) plant. Like batrachotoxin and grayanotoxin, aconitine preferentially binds to the open channel state, requiring repetitive depolarising pulses to see a functional effect under voltage-clamp conditions (Rao and Sikdar, 2000). Aconitine causes a hyperpolarising shift in the voltage-dependence of activation, inhibits peak current, slows the rate of fast inactivation (although aconitine-modified channels can inactivate completely), and causes a reduction in ion selectivity (Rao and Sikdar, 2000; Wright, 2002). Aconitine has similar pharmacological effects at $Na_V1.2$, $Na_V1.4$ and $Na_V1.5$, although a small persistent current is seen at $Na_V1.5$ only (Rao and Sikdar, 2000; Wright, 2002). Activity at other Na_V channel subtypes in heterologous expression

1 systems remains to be determined. The binding site is assumed to overlap with other

2 site 2 toxins, based on similar chemical structures and mechanism of action, although

3 this remains to be experimentally confirmed.

4

5 *4.1.2. Spider-venom derived Na$_V$ channel activators*

6 Spider venom-derived peptides have diverse pharmacological effects at Na$_V$ channels

7 as they can interact with different voltage sensor(s). The resultant effects including

8 inhibition of channel opening, a shift the voltage-dependence of activation, and/or a

9 delay in fast inactivation. In general, spider venom-derived peptides that interact

10 *exclusively* with DIV delay fast inactivation, while peptides that interact with DI-III

11 result in inhibition of channel opening and/or shifts in the voltage-dependence of

12 activation (Bosmans et al., 2008). Accordingly, spider venom-derived peptides are

13 named using Greek symbols that denote their pharmacological activity, with the

14 prefix μ-, β-, or δ- used to described toxins that inhibit Na$_V$ channels, toxins that shift

15 the voltage dependence of activation or toxins that delay fast inactivation,

16 respectively, followed by taxonomic family, then a genus/species and numerical

17 descriptor (King et al., 2008). For consistency, the nomenclature utilised in

18 ArachnoServer will be used to identify spider venom-derived peptides in this review

19 and peptides will be grouped into Family's 1-12 based on sequence homology as

20 proposed by Klint et al. (Herzig et al., 2011; Klint et al., 2012).

21 Venom from two Australian funnel-web spider species, *Atrax robustus* and

22 *Hadronyche versuta*, and the Australian eastern mouse spider *Missulena bradleyi* are

23 the source of four homologous Na$_V$ channel activator toxins consisting of 42 amino

24 acid residues belonging to Family 4, named δ-hexatoxin-Ar1a (formally robustoxin),

1   δ-hexatoxin-Hv1a (formally versutoxin), δ-hexatoxin-Hv1b and δ-actinopoditoxin-

2   Mb1a. In dissociated rat DRG neurons, these toxins potently inhibit fast inactivation

3   of TTX-sensitive channels, with no effect on TTX-resistant channels (Alewood et al.,

4   2003; Gunning et al., 2003; Nicholson et al., 1996; Nicholson et al., 1998; Szeto et al.,

5   2000). Consistent with this activity, δ-hexatoxin-Ar1a and δ-hexatoxin-Hv1a

6   competitively inhibit binding of the α-scorpion toxin Lqh II in rat brain synaptosomes,

7   indicating they interact with DIV (Little et al., 1998). In addition, these toxins also

8   cause a hyperpolarizing shift in the voltage-dependence of activation and voltage-

9   dependence of steady-state inactivation (Alewood et al., 2003; Gunning et al., 2003;

10 Nicholson et al., 1996; Nicholson et al., 1998; Szeto et al., 2000), suggesting

11 additional interactions with DII, although the exact binding site(s) of this family

12 remains to be elucidated. The only member of Family 4 to be characterised at $Na_V1.1$-

13 1.8 is δ-hexatoxin-Mg1a (formally Magi 4) from the Japanese funnel-web spider

14 *Macrothele gigas*. At a concentration of 5 μM, δ-hexatoxin-Mg1a delays fast

15 inactivation, with a large effect at $Na_V1.1$ and $Na_V1.6$, a comparatively smaller effect

16 at $Na_V1.2$, $Na_V1.3$ and $Na_V1.7$, and no effect at $Na_V1.4$, $Na_V1.5$ and $Na_V1.8$ (Yamaji

17 et al., 2009). Like members of the Family 4 isolated from Australian spiders, δ-

18 hexatoxin-Mg1a also shifts the voltage-dependence of activation to more

19 hyperpolarized potentials.

20 Another Na_V channel activator isolated from the Japanese funnel-web spider

21 *Macrothele gigas* is β-hexatoxin-Mg1a (formally Magi 5; also identical primary

22 sequence to β-hexatoxin-Mr1a from *Macrothele raveni*), a 29-residue peptide that has

23 little sequence homology to δ-hexatoxin-Mg1a and belongs to Family 5 (Corzo et al.,

24 2007; Zeng et al., 2003). β-hexatoxin-Mg1a has only been characterised at $Na_V1.2$,

25 where it shifts the voltage-dependence of activation to more hyperpolarized

1 potentials, with no effect on the rate of fast inactivation (Corzo et al., 2007).

2 Consistent with this activity, in rat brain synaptosomes β-hexatoxin-Mg1a

3 competitively inhibits binding of the β-scorpion CssIV, but not the α-scorpion toxin

4 Lqh II, indicating it interacts with the DII voltage sensing domain (Corzo et al., 2003).

5 Over 90 peptides have been sequenced from the venom of the Chinese tarantula

6 species *Chilobrachys guangxiensis* (also known as *Chilobrachys jingzhao*). While the

7 pharmacological activity of most of these peptides remains to be confirmed, several

8 have been identified Na_V channel activators (Chen et al., 2008). δ-theraphotoxin-Cg1a

9 (formally Jingzhaotoxin-I) and δ-theraphotoxin-Cg3a (formally Jingzhaotoxin-II)

10 consist of 33 and 32 amino acid residues, respectively, with ~ 50% sequence

11 homology and belong to Family 7 (Wang et al., 2008b; Xiao et al., 2005). Both δ-

12 theraphotoxin-Cg1a and δ-theraphotoxin-Cg3a have similar pharmacological activity

13 and delay inactivation of Na_V channels, with little to no effect on the voltage-

14 dependence of activation or steady state inactivation (Huang et al., 2015; Tao et al.,

15 2016; Wang et al., 2008b; Xiao et al., 2005). Consistent with effects on inactivation,

16 site directed mutagenesis of D1609A on $hNa_V1.5$, which is located on the DIVS3-S4

17 linker (**Figure 9**), causes both δ-theraphotoxin-Cg1a and δ-theraphotoxin-Cg3a to

18 lose activity, confirming that these peptides interact with the voltage-sensing domain

19 of DIV (Huang et al., 2015; Tao et al., 2016). Interestingly, the selectivity profiles of

20 these peptides differs from each other, with δ-theraphotoxin-Cg3a most potently

21 delaying inactivation at $Na_V1.5$ (EC_{50} 125 nM), with minor effects on $Na_V1.3$, $Na_V1.4$

22 and $Na_V1.7$ at 5 μM, whilst δ-theraphotoxin-Cg1a is comparatively non-selective,

23 delaying inactivation at $Na_V1.2$, $Na_V1.3$, $Na_V1.4$, $Na_V1.5$ and $Na_V1.7$ with similar

24 potency (EC_{50} 335-870 nM) (Huang et al., 2015; Tao et al., 2016). At higher

25 concentrations, δ-theraphotoxin-Cg1a also inhibits $K_V2.1$ (Tao et al., 2013).

24

1 δ-theraphotoxin-Cg2a (formally Jingzhaotoxin-IV) is a 34-residue peptide also from

2 the venom of *Chilobrachys guangxiensis*, but that shares sequence homology to

3 Family 2. It delays inactivation of TTX-sensitive channels in rat DRG neurons, but

4 unlike the other spider-venom derived Na_V channel activators described so far, causes

5 a *depolarising* shift in the voltage-dependence of activation, as well as a

6 hyperpolarising shift in the voltage-dependence of inactivation (Wang et al., 2008a).

7 The binding site of δ-theraphotoxin-Cg2a remains to be elucidated but based on its

8 pharmacological activity, is likely to involve interactions with DII and DIV. While

9 interactions with other domains cannot be ruled out, DIII interactions are yet to be

10 described for spider toxins. Full Na_V channel selectivity is not available for δ-

11 theraphotoxin-Cg2a.

12 Many members of Family 2 inhibit K_V channels (denoted by the prefix κ). Despite

13 being originally identified as K_V inhibitors, an increasing number of peptides from

14 Family 2 are being found to act on Na_V channels. One notable example is δ-

15 theraphotoxin-Hm1a, which was originally described as a K_V2 and K_V4 inhibitor.

16 Interestingly, Hm1a selectively delays inactivation of $Na_V1.1$ (EC_{50} 38 nM), with no

17 effect on $Na_V1.2$-1.8 at a concentration of 100 nM (Osteen et al., 2016). Construction

18 of $K_V2.1$/$hNa_V1.1$ chimeric channels, where the S3-S4 linker from each domain of

19 $hNa_V1.1$ is inserted into the homotetrameric $K_V2.1$ channel, has identified that δ-

20 theraphotoxin-Hm1a binds exclusively to DIV, consistent with its pharmacological

21 activity (**Figure 9 and 10**) (Osteen et al., 2016).

22

23 *4.1.3. Wasp venom-derived Na_V channel activators*

25

1   Two 13 amino acid residue peptides, named α-pompilidotoxin and β-pompilidotoxin,

2   isolated from the venom of the solitary wasps *Anoplius samariensis* and *Batozonellus*

3   *maculifrons,* respectively, are known to activate Na$_V$ channels (Konno et al., 1998).

4   They are highly homologous, differing by only one amino acid, with most work

5   carried out using β-pompilidotoxin, as it is 5 times more potent than α-pompilidotoxin

6   (Konno et al., 2000).

7   β-pompilidotoxin has some selectivity between Na$_V$ channels subtypes: it delays fast

8   inactivation at Na$_V$1.1, Na$_V$1.2 (EC$_{50}$ 21 μM), Na$_V$1.3 (EC$_{50}$ 99 μM), and Na$_V$1.7,

9   inhibits fast inactivation at Na$_V$1.6 (EC$_{50}$ 30 μM), and has no effect on the fast

10   inactivation kinetics of Na$_V$1.4 and Na$_V$1.5 (up to 140 μM) (Schiavon et al., 2010).

11   Using a range of rNa$_V$1.2/rNa$_V$1.5 channel chimeras, the binding site of β-

12   pompilidotoxin was identified as the S3-S4 linker of DIV, consistent with its

13   pharmacological effects on delaying fast inactivation (Kinoshita et al., 2001). The

14   amino acid E1616 in the S3-S4 linker of DIV rNa$_V$1.2, which is conserved in Na$_V$1.1,

15   Na$_V$1.2, Na$_V$1.3, Na$_V$1.6, Na$_V$1.7 but not in Na$_V$1.4 or Na$_V$1.5, is essential for β-

16   pompilidotoxin binding, and accounts for this Na$_V$ channel selectivity profile (**Figure**

17   **9**) (Kinoshita et al., 2001).

18

19   *4.1.4. Snake venom-derived Na$_V$ channel activators*

20   Three-finger toxins (3FTxs) are a large family of snake venom-derived toxins with a

21   conserved structural fold but diverse pharmacology, which includes activity at

22   nicotinic and muscarinic acetylcholine receptors, acetylcholinesterase and L-type

23   voltage-gated calcium channels (Kini and Doley, 2010). δ-calliotoxin, a 57-amino

24   acid residue peptide isolated from the long-glanded blue coral snake (*Calliophis*

1 *bivirgatus*), is the first 3FTx to be identified as a Na$_V$ channel activator (Yang et al.,

2 2016). At Na$_V$1.4, δ-calliotoxin (200 nM) causes a small hyperpolarising shift in the

3 voltage-dependence of activation and delays fast inactivation, as well as causing a

4 persistent current (Yang et al., 2016). Activity and selectivity at other Na$_V$ channel

5 subtypes remains to be determined. Although the binding site of δ-calliotoxin has not

6 yet been defined, based on its pharmacological activity, it likely involves interactions

7 with residues of DIV.

8

9

10 *4.1.5. Scorpion venom Na$_V$ channel activators*

11 Scorpion neurotoxins that act on Na$_V$ channels are broadly categorised as α or β toxins

12 – both of which contain 60-80 amino acids linked by four disulphide bridges – based

13 on their effects on the electrophysiological properties of Na$_V$ channels. This

14 functional distinction is a reflection of distinct binding sites on Na$_V$ channels

15 (Bosmans and Tytgat, 2007; Pedraza Escalona and Possani, 2013), with α toxins

16 binding to site 3 to cause a delay in fast inactivation (Gordon et al., 1996). In contrast,

17 β-scorpion toxins bind to site 4 and alter the threshold for activation, allowing channel

18 opening at hyperpolarized potentials whilst also reducing peak current (Couraud et al.,

19 1982). Thus, both α- and β-scorpion toxins are appropriately described as Na$_V$

20 channels activators albeit with a distinct mechanism of action. Scorpion neurotoxins

21 have proved useful in characterising Na$_V$ channel binding sites and function, although

22 it is important to note that with increased understanding of Na$_V$ channel structure, a

23 more complex picture of toxin binding has emerged. For brevity, here we discuss only

24 those toxins that are only active at mammalian Na$_V$ channels and also display some

1   selectivity across the isoforms (for comprehensive review of scorpion toxins see

2   (Housley et al., 2016)).

3

4   Structural and functional assessment of α-scorpion toxins has led to a model of toxin

5   binding that impairs the movement of the VSD of domain IV (Cestele and Catterall,

6   2000), which as detailed above, is critical for inactivation but not activation gating.

7   Experimentally, classical α scorpion binding to site 3 manifests as a delay in

8   inactivation without major changes in channel activation (Bosmans and Tytgat, 2007).

9   Furthermore, α-scorpion toxins, which can in addition be classified into α or α-like

10 based on functional effects and target species, may be "pulsed off" with strong

11 depolarizations (Bosmans and Tytgat, 2007; Campos et al., 2004; Catterall, 1977).

12 The classical old world toxin, AaHII, isolated from *Androctonus australis*, is a

13 prototypical α-scorpion toxin. The selectivity of AaHII has not been reported,

14 however it readily binds to rat brain synaptosomes presumably expressing $Na_V1.2$

15 (Legros et al., 2005).

16

17 Venom from *Leiurus quinquestriatus hebraeus* contains α-toxins LqhII and LqhIII

18 that are highly toxic to mammals (Sautiere et al., 1998). LqhII and LqhIII modulate

19 $Na_V1.4$ and $Na_V1.5$ by removing fast inactivation as described above (Chen et al.,

20 2000; Chen and Heinemann, 2001). Additionally, LqhII and III enhance slow

21 inactivation of $Na_V1.5$ as some of the first α-scorpion toxins to cause this

22 phenomenon (Chen and Heinemann, 2001). LqhIII also alters the kinetics of $hNa_V1.2$

23 and $hNa_V1.7$ and is known as an α-like toxin, binding to both mammalian and insect

24 Na_V channels, but not rat synaptosomes (Chen et al., 2002; Gilles et al., 2000). LqTx

25 (*Leiurus quinquestriatus quinquestriatus)* highlighted the crucial role for E1613

1 (rNa$_V$1.2) in site 3 toxin binding (Rogers et al., 1996), which was further investigated

2 with LqhII and LqhIII. Interestingly, D1428 of rNa$_V$1.4 (analogous to E1613 rNa$_V$1.2)

3 is critical to LqhIII but not LqhII binding (Leipold et al., 2004). LqhII relies on

4 residues Y1433, F1434 and V1435 of DIV S3-S4 linker (rNa$_V$1.4) as site directed

5 mutagenesis causes loss of activity (**Figure 9**) (Leipold et al., 2005). Furthermore, a

6 secondary interaction site for LqhII is present in the DI S5-S6 linker (Wang et al.,

7 2011). In particular, an alanine scan of DI highlighted T393 (rNa$_V$ 1.2) as a crucial

8 binding partner of LqhII (**Figure 11**).

9

10 The α-scorpion toxin OD1, isolated from the Iranian scorpion *Odonthobuthus doriae*

11 was initially described to interact with the insect Na$_V$ channel isoform para/tipE.

12 However, OD1 is also an activator of hNa$_V$1.7, hNa$_V$1.4 and hNa$_V$1.6 as measured by

13 fluorescence based assays and electrophysiology (Durek et al., 2013a). At low

14 nanomolar concentrations, OD1 significantly impairs inactivation of Na$_V$1.7

15 (Maertens et al., 2006b; Motin et al., 2016), while at higher concentrations (300 nM),

16 OD1 also causes a hyperpolarising shift in the voltage dependence of activation,

17 considerably increases peak current values and induces a large persistent current

18 (Deuis et al., 2016b).

19 Ts2, from *T. serrulatus,* prevents inactivation of rat Na$_V$1.2, Na$_V$1.3, Na$_V$1.5, Na$_V$1.6

20 and Na$_V$1.7 (Cologna et al., 2012). However, Ts2 also selectively shifts the voltage

21 dependence of activation of rNa$_V$1.3 (Cologna et al., 2012). Accordingly, although

22 Ts2 is currently classified as an α scorpion toxin, this specific activation of rNa$_V$1.3 is

23 similar to β-scorpion Tf2 discussed below. Thus, the mixed pharmacological effects

24 of both OD1 and Ts2 blur the line between α- and β- scorpion toxins, inviting a

25 revision of the classification these Na$_V$ channel activators.

1

2   MeuNaTxαs are a large family of thirteen α-scorpion toxins isolated from the

3   scorpion *Mesobuthus eupeus*. Of these, the pharmacological activity of MeuNaTxα-1,

4   2 and 5 has been comprehensibly characterized. MeuNaTxα-1 modulates $Na_V1.2$,

5   $Na_V1.3$, $Na_V1.6$ and $Na_V1.7$, MeuNaTxα-2 affects $Na_V1.4$ only, whereas MeuNaTxα-

6   5 is the least selective modulating $Na_V1.3$-1.7 (Zhu et al., 2012). These peptides also

7   potently slow inactivation in the insect channel counterpart DmNaV1. MeuNaTxα-12

8   and MeuNaTxα-13 also preferentially affect $Na_V1.1$ (Zhu et al., 2013).

9   Bot IX, from *Buthus occitanus tunetanus* is a recently reported α-like toxin, inhibiting

10 fast inactivation at both insect Na_V and $rNa_V1.2$ (Martin-Eauclaire et al., 2016).

11 However, Bot IX also competed with AaHII binding, the first α-like toxin described

12 to do so (Martin-Eauclaire et al., 2016). Full selectivity of Bot IX at mammalian Na_V

13 channel isoforms is unknown.

14

15 Scorpions of the genus *Centruroides* produce several peptides that were critical for

16 early understanding of the mechanics of the VSD in Na_V channels (Cestele et al.,

17 1998). Specifically, the class of β-scorpion toxins was based on characterisation of

18 CssIV, from C. *suffusus suffusus*, which shifts the voltage dependence of activation of

19 $Na_V1.1$, 1.2 and 1.6 (Jover, 1980; Jover et al., 1980; Karbat et al., 2010; Schiavon et

20 al., 2006). This activity is explained through a voltage sensor trapping mechanism

21 whereby the peptide binds to the activated position of the DII VSD (both S1-S2 and

22 S3-S4 extracellular loops), locking it in an activated position (Cestele et al., 1998;

23 Pedraza Escalona and Possani, 2013; Zhang et al., 2011). Regions crucial for CssIV

24 binding are found in DI S5-S6, DII S1-S2 and S3-S4 linkers and various residues

25 located in the DIII S5-S6 linker (Cestele et al., 1998). This includes P782 (DII S1-S2)

1 and G845 (DII S3-S4) in $rNa_V1.2$, which when substituted for asparagine result in

2 reduced CssIV binding and in the case of G845, a loss of activity. Within the DIII S5-

3 S6 linker, mutations of N1436, E1438, L1439 and D1445 ($rNa_V1.2$) cause changes in

4 CssIV binding and voltage-sensor trapping activity (**Figure 6 and 11 - 13**) (Zhang et

5 al., 2011).

6 Similarly, the β scorpion toxin Cn2, originally purified as fraction II-10 from the

7 crude venom of *Centruroides noxius Hoffmann,* is particularly notable for its high

8 selectivity for $hNa_V1.6$ (Schiavon et al., 2006). Cn2 causes a robust hyperpolarising

9 shift in activation permitting $Na_V1.6$ opening at usually prohibitive membrane

10 potentials, albeit this effect is only observed during protocols that includes a prepulse

11 to allow toxin binding (Cestele et al., 1998; Schiavon et al., 2006).

12 Intriguingly, Cn2 also enhances resurgent currents in Purkinje neurons (Schiavon et

13 al., 2006), where non-conducting (inactivated) Na_V channels are seen to reopen

14 (resurge) in response to mild repolarization steps from positive potentials (Raman and

15 Bean, 1997). This resurgent current is reduced by 90% in $Na_V1.6$ KO mice leading to

16 the identification of $Na_V1.6$ as the primary channel responsible for carrying his

17 current (Raman et al., 1997). Resurgent currents are thought to allow high frequency

18 firing in neurons and may be involved in clinical disease states such as paroxysmal

19 extreme pain disorder and chemotherapy induced cold allodynia (Jarecki et al., 2010;

20 Sittl et al., 2012) Interestingly, although CssIV has 83% sequence homology with Cn2

21 and is capable of binding $Na_V1.6$, it does not produce a resurgent current (Schiavon et

22 al., 2006). Thus, Cn2 may find future application as a probe to understand the

23 intricacies of resurgent current *in vitro* and *in vivo*.

24 The genus of *Tityus* scorpions, whose habitat ranges from Central to South America,

25 has provided several β-scorpion toxins with interesting pharmacological properties.

1 For example, Ts1 from *T. serrulatus* shifts the voltage dependence of activation of

2 $Na_V1.2$, 1.4 and $Na_V1.6$ and also decreases peak current of $Na_V1.6$, and to a lesser

3 extent $Na_V1.4$ (Peigneur et al., 2015a). Interestingly, this toxin also exerts a full block

4 of $Na_V1.5$ without changes in activation or inactivation (Peigneur et al., 2015a). In

5 contrast, Tz1 from *T. zulianus* also modulates the activity of $Na_V1.4 > Na_V1.6 >$

6 $Na_V1.2$ but not $Na_V1.5$ and $Na_V1.7$ (Leipold et al., 2006). Na_V channel residues

7 involved in Tz1 activity are shown in **Figure 6** and **13**. Highlighting the

8 pharmacological diversity of scorpion toxins, Tf2 from *T. fasciolatus* selectively

9 causes the opening $hNa_V1.3$ at hyperpolarized membrane potentials (Camargos et al.,

10 2015) with no effect (activation or inactivation) on other Na_V channel isoforms. This

11 highly specific activation of a singular Na_V channel isoform may be useful as a tool to

12 delineate the physiological and pathological role of $Na_V1.3$ (Hains et al., 2003; Hains

13 et al., 2005; He et al., 2010).

14

15 *4.1.6. Cone snail venom derived Na_V channel activators*

16 Marine snails of the genus *Conus* have evolved a complex cocktail of venom peptides

17 (conotoxins) for both predatory and defensive behaviour (Dutertre et al., 2013; Lewis

18 et al., 2012). These small peptides are universally cysteine-rich and folded with

19 multiple disulfide bridges (Han et al., 2008; Lewis et al., 2012; Vetter and Lewis,

20 2012). Conotoxins of the μ-, μO-, δ- and ι families target Na_V channels via a variety

21 of mechanisms (Ekberg et al., 2008; Green and Olivera, 2016). However, as μ- and

22 μO-conotoxins are Na_V channel inhibitors this review will focus on δ- and ι-

23 conotoxins.

24

δ-Conotoxins inhibit fast inactivation and modulate the voltage-dependence of activation of Na_V channels (Ekberg et al., 2008; Stevens et al., 2011). The result of this hyperpolarizing shift is prolonged channel opening and thus, persistent firing of excitable cells (Ekberg et al., 2008). In-depth information regarding structure-function relationship between δ-conotoxins and their Na_V channel targets is sparse owing in part to challenges in chemical synthesis and the extreme hydrophobicity of these peptides. Thus, the exact binding site of the δ-conotoxins remains a point of contention. As their pharmacological effects are similar to those of the α-scorpion toxins, site 3 was proposed as a binding site; however, δ-conotoxins do not fully compete with site 3 toxins (Fainzilber et al., 1994; Leipold et al., 2005). Furthermore, site-directed mutagenesis of this region failed to alter all biophysical properties associated with δ-conotoxin binding (Leipold et al., 2005). SVIE (Conus striatus) interacts with a hydrophobic triad of the DIV S3-S4 linker, Y1433–F1434–V1435 ($rNa_V1.4$) **(Figure 9)** (Leipold et al., 2005). Thus, a new binding site (site 6) was suggested, although it remains poorly defined.

While δ-conotoxins are commonly found in vermivorous or piscivorous *Conidae* and activity at Na_V channel homologues of worms and fish might contribute to the use of these peptides in prey capture, they also affect mammalian Na_V channels. For example, δ-EVIA, isolated from *C. ermineus*, inhibits fast inactivation of $rNa_V1.2$, $rNa_V1.3$ and $rNa_V1.6$ without altering $rNa_V1.4$ and $hNa_V1.5$ (Barbier et al., 2004; Volpon et al., 2004). In addition, δ-Am2766 from *C. amadis* inhibits inactivation of $rNa_V1.2$, albeit activity at other subtypes is unknown (Sarma et al., 2005). Interestingly, δ-SuVIA, isolated from the vermivorous *C. suturatus,* is active at low nanomolar concentrations at $hNa_V1.3$, $hNa_V1.4$, $hNa_V1.6$ and $hNa_V1.7$ (Jin et al.,

33

1   2015). This defensive conotoxin causes a hyperpolarizing shift in activation potential

2   at hNa$_V$1.7 but fails to measurably modify inactivation kinetics (Jin et al., 2015). This

3   is at clear odds with other δ-conotoxins studied from fish-hunting Conus and may

4   suggest a different class of conotoxin altogether.

5   The superfamily of I1 conotoxins was initially identified in the venom of *C. radiatus*

6   (Jimenez et al., 2003). These peptides contain a unique cysteine backbone structure

7   with four disulfide bonds (Buczek et al., 2007). ι-conotoxin RXIA shifts the voltage-

8   dependence of activation, without altering inactivation, most potently at rNa$_V$1.6

9   (EC$_{50}$ 2 μM) followed by rNa$_V$1.2 and rNa$_V$1.7 (Fiedler et al., 2008). As ι-RXIA had

10 no effect on other subtypes tested, the ι-conotoxins may provide an additional source

11 of selective agonist peptides.

12 Additionally, tetrodotoxin-sensitive current in DRGs were enhanced by M-

13 superfamily toxin, LtIIIA isolated from C. *litteratus* (Wang et al., 2009). Given that

14 DRGs express a range of Na$_V$ channel isoforms, the selectivity of this peptide is

15 unknown.

16 *4.1.7. Sea anemone derived Na$_V$ channel activators*

17 Sea anemone toxins that associate with Na$_V$ channels are categorised into three

18 groups: Type I, II and III (Frazao et al., 2012; Moran et al., 2009; Norton, 1991).

19 However, in recent years questions about the validity of these broad classifications

20 have arisen as additional peptides are discovered and characterised that diverge from

21 the stringent structural and functional properties proposed originally (Ishida et al.,

22 1997; Moran et al., 2009). Sea anemone toxins bind to site 3 and produce a delay in

23 inactivation at low concentrations as well as a large persistent current at high

1 concentrations (Catterall and Beress, 1978; Moran et al., 2007; Smith and

2 Blumenthal, 2007). ATX-II binds the DIV S3-S4 linker, in particular E1613

3 (rNa$_V$1.2) where charge inversion at this point results in no binding (Rogers et al.,

4 1996). This is direct evidence for the overlap between ATX-II and other site 3 toxins

5 including LqTx described above. Furthermore E1616, V1620 and L1624 alanine

6 substitutions resulted in lower binding affinity of ATX-II but not LqTx (**Figure 9**).

7 Altering the corresponding amino acid of rNa$_V$1.5, D1612, decreased binding affinity

8 for ApB (Anthopleura xanthogrammica) (Benzinger et al., 1998). Taken together, this

9 region of the DIV S3-S4 loop is indeed central to sea anemone toxin binding. The

10 Na$_V$ channel subtype selectivity for mammalian isoforms has been systematically

11 assessed for a limited number of peptides, including ATX-II, AFT-II and Bc-III

12 isolated from *Anemonia sulcata*, *Anthopleura fuscoviridi* and *Bunodosoma*

13 *granulifera*, respectively (Bosmans et al., 2002; Oliveira et al., 2004). Although these

14 toxins display differential effects at Na$_V$1.1-1.6, none are truly selective activators

15 (Table 1) (Oliveira et al., 2004).

16

17 Other Na$_V$ channel activators present in sea anemone venom include CGTX-II and δ-

18 AITX-Bcg1a, from *Bunodosoma cangicum.* CGTX-II interacts with hNa$_V$1.5 over

19 Na$_V$1.6 with minor affects at Na$_V$1.1, 1.2, 1.3, 1.4 and 1.7 (Zaharenko et al., 2012).

20 Interestingly, CGTX-II causes a depolarizing shift in the inactivation curve of only

21 hNa$_V$1.5, whereas the effect on hNa$_V$1.6 includes a large non-inactivating window

22 current (Zaharenko et al., 2012). While the molecular basis of these differential

23 effects remains unclear to date, these pharmacological effects may provide additional

24 insight into Na$_V$ channel gating mechanisms and structural motifs contributing to

25 effects on channel activation and inactivation.

35

1

2 *4.1.8. Dinoflagellate and bacterial Na$_V$ channel activators*

3 Na$_V$ channel activators of marine dinoflagellate origins are the cause of several

4 seafood related food poisoning diseases including ciguatera and "red-tide" shellfish

5 poisoning (Gillespie et al., 1986; Nakanishi, 1985; Swift and Swift, 1993). The

6 causative cyclic polyether toxins, the ciguatoxins (CTX) and brevetoxins (PbTx), are

7 produced by dinoflagellates of the *Gambierdiscus* and *Karenia* families, respectively

8 (Bidard et al., 1984). The pharmacological effects of these site 5 toxins on Na$_V$

9 channels are diverse and include hyperpolarising shifts in activation, a decrease in

10 peak current, as well as effects on the voltage-dependence and time course of channel

11 inactivation (Inserra et al., 2017; Lombet et al., 1987; Trainer et al., 1994). Critical

12 residues for site 5 binding are less well defined, although photoaffinity labelling

13 studies with PbTx highlight DI S6 and most of the DIV S5-S6 linker, including the

14 pore loop with the residues that form part of the sodium selectivity filter (**Figure 4**

15 **and 14**) (Trainer et al., 1994).

16

17 PbTx's are classified as either A or B depending on their backbone structure. The

18 extensively studied PbTx-3 shifted the voltage-dependence of activation of

19 tetrodotoxin-sensitive channels expressed in nodose ganglia (Jeglitsch et al., 1998),

20 inhibited Na$_V$ channel inactivation, and increased channel opening time in single

21 channel recordings (Purkerson et al., 1999). Although PbTx-3 showed some

22 selectivity for the skeletal muscle isoform (Na$_V$1.4) over the cardiac (Na$_V$1.5) and rat

23 brain (Na$_V$1.2) isoforms in a sodium dependent cytotoxicity assays (Bottein

1   Dechraoui and Ramsdell, 2003), selectivity across the full panel of Na_V channels is

2   unknown, and selectivity of other PbTxs has thus far not been studied.

3

4   The ciguatoxins are Na_V channel activator toxins that accumulate in fish through the

5   marine food chain and cause the seafood disease ciguatera in humans. Of the several

6   structurally related congeners, denoted as Pacific, Indian and Caribbean ciguatoxins

7   depending on their origin, P-CTX-1 (Pacific ciguatoxin 1) is the most potent, and the

8   isoform responsible for most symptoms in the Pacific (Lewis, 2006). Although

9   differential effects on tetrodotoxin-resistant and -sensitive isoforms expressed in

10 sensory neurons – including shifts in the voltage-dependence of activation, a decrease

11 in peak current, and effects on recovery from inactivation – were reported several

12 years ago, the relative Na_V channel subtype selectivity of P-CTX-1 has remained

13 unexplored until relatively recently (Strachan et al., 1999). In SH-SY5Y cells, P-

14 CTX-1-induced effects were mediated predominantly through $Na_V1.3$ (Vetter et al.,

15 2012b), consistent with the induction of a small persistent current at this, but not

16 other, Na_V channel isoforms (Inserra et al., 2017). While P-CTX-1 is relatively non-

17 selective for $Na_V1.1$-$Na_V1.9$ overall, as evidenced by a shift in the voltage-

18 dependence of activation at all isoforms, effects on fast inactivation only occurred at

19 $Na_V1.2$, $Na_V1.3$ and $Na_V1.9$.

20

21 Antillatoxin is a Na_V channel activator isolated from the cyanobacteria *Lyngbya*

22 *majuscula* (Orjala et al., 1995) and causes Na^+ influx via $rNa_V1.2$, 1.4 and 1.5 (Cao et

23 al., 2010). Additionally, antillatoxin causes changes in membrane potential resulting

24 from $hNa_V1.7$ activation (Zhao et al., 2016). While there is evidence for allosteric

25 coupling with site 2 and 5, the exact binding site remains unspecified (Cao et al.,

1 2010; Li et al., 2001). Interestingly, most studies assessing the pharmacology of

2 antillatoxin were conducted using fluorescence-based assays, and electrophysiological

3 characterization of its effects on Na_V channel gating, particularly at different isoforms,

4 is sparse.

5

5. Na_V *channel activators as tool compounds*

7

5.1. Na_V channel assays

9 Na_V channels are key therapeutic targets for pain, epilepsy and cardiac arrhythmias;

10 therefore several high-throughput assays have been developed to screen and profile

11 the activity of Na_V channel modulators, including fluorescence-based assays and

12 automated patch-clamp electrophysiology (Dunlop et al., 2008; Yu et al., 2016).

13 Fluorescence-based assays provide an indirect measure of Na_V channel function by

14 detecting changes in membrane potential or intracellular sodium ion concentration,

15 with several fluorescent dyes commercially available (Yu et al., 2016). Whilst the

16 results from fluorescence-based assays do not always correlate well with patch-clamp

17 electrophysiology, which remains the standard for measuring Na_V channel function,

18 they are comparatively cheaper, higher throughput, and do not require significant

19 expertise to perform (Dunlop et al., 2008; Yu et al., 2016).

20 To activate Na_V channels in fluorescence-based assays, the alkaloid veratridine or the

21 pyrethroid deltamethrin are commonly used. Veratridine induces a robust fluorescent

22 response with membrane potential dyes in cells heterologously expressing $Na_V1.1$-

23 1.7, but has negligible effects on $Na_V1.8$ (Deuis et al., 2015; Vickery et al., 2004). In

24 contrast, deltamethrin has a limited effect on the membrane potential of cells

25 heterologously expressing TTXs Na_V channels, but causes a robust fluorescent

1　response in cells heterologously expressing $Na_V1.8$ (Deuis et al., 2016a; Vickery et

2　al., 2004). As Na_V channel activators used in fluorescence-based assays likely

3　stabilise different channel conformations, it is unclear if these assays exhibit bias

4　towards detection of Na_V channel modulators with a specific mechanism of action

5　and/or binding site.

6

7　**5.2. Na_V channel target engagement *in vivo***

8　A key step in the development of any pharmaceutical is to demonstrate target

9　engagement and efficacy *in vivo*. For analgesics, assessment of target-engagement *in*

10　*vivo* is challenging, as most commonly used pre-clinical rodent models of pain, which

11　are based on quantification of pain responses induced by local injection of formalin,

12　carrageenan or Complete Freund's Adjuvant, have a multifactorial pathophysiology.

13　Demonstrating target engagement *in vivo* ensures a sufficient concentration of an

14　analgesic is reached at the site of the intended target, providing crucial information to

15　guide dosing for *in vivo* efficacy studies.

16　$Na_V1.7$ is of particular interest as an analgesic target because loss-of-function

17　mutations in *SCN9A*, the gene encoding $Na_V1.7$, is a cause of congenital insensitivity

18　to pain, an extremely rare condition resulting in the inability to sense pain (Cox et al.,

19　2006; Goldberg et al., 2007). Therefore, selective pharmacological inhibitors of

20　$Na_V1.7$ are being actively pursued as novel analgesics (Sun et al., 2014). The

21　importance of Na_V channels in the initiation and propagation of action potentials in

22　nociceptive sensory neurons is undisputed, with local injection of non-selective Na_V

23　channel activators such as veratridine, ciguatoxin, grayanotoxin and aconitine causing

24　spontaneous pain behaviours in mice, rats and humans (Focken et al., 2016; Gingras

25　et al., 2014; Vetter et al., 2012b). These effects in turn can be reversed by Na_V

1   channel inhibitors, providing convenient *in vivo* target engagement models. For

2   example, reversal of pain behaviours induced by local injection of aconitine was used

3   to demonstrate *in vivo* target engagement of an aryl sulfonamide class of small

4   molecules with $Na_V1.7$ activity (Focken et al., 2016; Gingras et al., 2014). Given that

5   activation of other Na_V channel isoforms, including $Na_V1.1$ or $Na_V1.6$, is sufficient to

6   elicit pain behaviours in rodents (Deuis et al., 2013; Osteen et al., 2016), the

7   usefulness of this approach is particularly apparent for highly subtype-selective Na_V

8   channel activators. Recently, a $Na_V1.7$-specific target-engagement model was

9   developed based on intraplantar injection of the selective $Na_V1.7$ activator OD1

10   (Deuis et al., 2016b). Similar to behaviours elicited by non-selective Na_V channel

11   activators, local administration of OD1 in mice causes rapid development of pain

12   behaviours, including flinching and licking of the injected hind paw. Consistent with

13   the high $Na_V1.7$-selectivity of OD1, these behaviours were significantly attenuated in

14   $Na_V1.7^{-/-}$ mice and by administration of selective $Na_V1.7$ inhibitors GpTx-1 and PF-

15   04856264 (Deuis et al., 2016b). OD1 is therefore a useful pharmacological tool to

16   rapidly profile on-target activity of $Na_V1.7$ inhibitors at sensory nerve endings *in vivo*.

17

18   **5.3. Elucidating pain pathways**

19   Five Na_V channel subtypes ($Na_V1.1$, 1.6, 1.7, 1.8, 1.9) are expressed on adult

20   peripheral sensory neurons (Black et al., 1996; Fukuoka et al., 2008; Fukuoka and

21   Noguchi, 2011). Due to a lack of subtype selective Na_V channel inhibitors,

22   determining the role of Na_V channels in pain pathways has heavily relied on the

23   knockout of Na_V channel genes in mice. While this approach has been partially

24   successful for $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$, global knockout of $Na_V1.1$ and $Na_V1.6$ is

25   lethal as these isoforms are expressed in the central nervous system and $Na_V1.6$ on the

1   nodes of Ranvier in motor neurons (Caldwell et al., 2000; Gingras et al., 2014; Leo et

2   al., 2010; Meisler et al., 2001; Whitaker et al., 2001; Yu et al., 2006). Instead, the role

3   of $Na_V1.1$ and $Na_V1.6$ in specific pain pathways has been elucidated using selective

4   Na_V channel activator toxins.

5   Intraplantar injection of δ-theraphotoxin-Hm1a (5 µM), a selective $Na_V1.1$ activator

6   with low nM EC_{50} (38 nM), causes spontaneous pain behaviours and mechanical

7   allodynia in mice, suggesting a role for $Na_V1.1$ in mechanical pain (Osteen et al.,

8   2016). However, at higher concentrations (>100 nM) Hm1a also inhibits K_V channels,

9   including $K_V2.1$, $K_V2.2$, $K_V4.1$, $K_V4.2$ and $K_V4.3$ (Escoubas et al., 2002). $K_V2.1$ is a

10   delayed rectifier channel that when open, causes outward current of potassium ions,

11   allowing the cell membrane to repolarize after an action potential. It is expressed on

12   peripheral sensory neurons and is important in the regulation of neuronal excitability;

13   therefore, off-target activity at $K_V2.1$ could contribute to spontaneous pain behaviours

14   observed after intraplantar injection of high concentrations of Hm1a (Kim et al., 2002;

15   Tsantoulas et al., 2014). While Hm1a-induced spontaneous pain and mechanical

16   allodynia was partially attenuated in conditional $Na_V1.1$ knockout mice, basal

17   mechanical sensitivity of this mouse line was not reported (Osteen et al., 2016).

18   Therefore, further studies are required to confirm the role of $Na_V1.1$ in mechanical

19   pain and other pain modalities.

20   In peripheral sensory neurons, $Na_V1.6$ is predominately expressed on the nodes of

21   Ranvier, suggesting that it is crucial for the propagation of action potentials in

22   myelinated fibers (Caldwell et al., 2000; Fukuoka et al., 2008). Indeed, intraplantar

23   injection of the β-scorpion Cn2 (1-30 nM), a selective activator of $Na_V1.6$ (EC_{50} 39

24   nM), causes spontaneous pain behaviours, mechanical allodynia and enhances 4-

25   aminopyridine-induced cold allodynia in mice (Deuis et al., 2013). A role for $Na_V1.6$-

1 expressing neurons in pathological cold pain was later confirmed using a combination

2 of Na$_V$ channel knockout mice lines and selective Na$_V$ channel inhibitors in mouse

3 models of oxaliplatin- and ciguatoxin- induced cold allodynia (Deuis et al., 2013;

4 Minett et al., 2014; Vetter et al., 2012b; Zimmermann et al., 2013). While Na$_V$1.6 is

5 predominately expressed on A-fibres, it also expressed at comparatively low levels in

6 C-fibers, where its role is unknown (Fukuoka et al., 2008).

7

5.4. Drug Leads

9 Na$_V$ channel activator toxins may provide useful drug leads for the treatment of

10 conditions caused by loss of Na$_V$ channel function. For example, selective Na$_V$1.1

11 activators might find application as treatment for specific types of epilepsy, such as

12 Dravet syndrome, which is associated with heterozygous loss-of-function mutations in

13 *SCN1A*, the gene encoding Na$_V$1.1 (Claes et al., 2001). Dravet syndrome is

14 characterized by severe seizures that begin during infancy, followed by cognitive

15 impairment, behavioural disorders and motor deficits that develop during early

16 childhood. Potential therapeutics for Dravet syndrome would require exquisite

17 selectivity for Na$_V$1.1 over the other Na$_V$ subtypes, as non-selective activation of Na$_V$

18 channels would result in a limited therapeutic window, with high risk of seizures,

19 cardiac arrhythmias and death. However, given the poor prognosis and limited

20 available treatments for Dravet syndrome, delivery of a Na$_V$1.1 activator by an

21 intrathecal pump that limits systemic exposure is a feasible option to improve the

22 therapeutic window. Although less well-validated, Na$_V$1.4 or Na$_V$1.5 activators might

23 hypothetically treat conditions such as congenital myasthenic syndrome, which is

24 associated with severe muscle weakness; or cardiac arrhythmias and insufficiencies

25 (Jurkat-Rott et al., 2010; Zimmer et al., 2014).

1

6. Conclusions

Na$_V$ channel activators, produced by bacteria, dinoflagellates, algae and many venomous animals including scorpions, sea anemone, wasps, spiders, cone snails and snakes, are a structurally and functionally diverse group of compounds that interact with multiple sites on Na$_V$ channels to enhance function. In practice, the effects elicited by Na$_V$ channel activators are as diverse as their origins, and include enhanced activation, decreased inhibition or altered conductance. However, it is becoming increasingly clear that this remains a simplified view of the pharmacology of Na$_V$ channel activators. For example, enhanced activation can include effects on the voltage-dependence of activation or changes in channel opening kinetics. Similarly, effects on inactivation can manifest as altered voltage- or time-dependence of inactivation; effects on fast or slow inactivation; altered recovery from inactivation, incomplete inactivation or emergence of resurgent or leak currents. Moreover, the assumption that biophysical effects on channel gating are similar across different isoforms clearly does not hold true. In combination with different binding affinities for individual isoforms, we are just beginning to understand the pharmacological complexity of Na$_V$ channel activators. Accordingly, systematic pharmacological characterization of these compounds, in light of better molecular tools and improved crystal structures available to us now and in future, will undoubtedly provide additional insight into the therapeutic and practical applications for Na$_V$ channel activators.

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4

5

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6

Table 1. Potency and selectivity of Na_V activator toxins at $Na_V1.1$-1.9. Where available the EC_{50} values are provided. If not available activity is classified as either sensitive (S) or insensitive (IS) at the concentration stated.

Toxin	$Na_V1.1$	$Na_V1.2$	$Na_V1.3$	$Na_V1.4$	$Na_V1.5$	$Na_V1.6$	$Na_V1.7$	$Na_V1.8$	$Na_V1.9$	Ref
Alkaloid										
Batrachotoxin	NA	S (10 μM)	S (10 μM)	S (10 μM)	S (10 μM)	S (10 μM)	S (10 μM)	S (10 μM)	NA	(Bosmans et al., 2004; Li et al., 2002; Logan et al., 2016)
Spider										
δ-hexatoxin-Mg1a	S (5 μM)	S (5 μM)	S (5 μM)	IS (5 μM)	IS (5 μM)	S (5 μM)	S (5 μM)	IS (5 μM)		(Yamaji et al., 2009)
δ-theraphotoxin-Cg3a	NA	NA	2 μM	> 5 μM	125 nM	NA	> 5 μM	NA	NA	(Huang et al., 2015)
δ-theraphotoxin-Cg1a	NA	870 nM	845 nM	339 nM	335 nM	NA	348 nM	NA	NA	(Tao et al., 2016)
δ-theraphotoxin-Hm1a	38 nM	236 nM	220 nM	IS (100 nM)	IS (100 nM)	IS (100 nM)	IS (100 nM)	IS (100 nM)	IS (100 nM)	(Osteen et al., 2016

										)
Wasp										
β-pompilidotoxin	S (46 μM)	21 μM	S (46 μM)	IS (140 nM)	IS (140 nM)	30 μM	S (46 μM)	NA	NA	(Schiavon et al., 2010)
α-Scorpion										
OD1	NA	5 μM	3 μM	10 nM	4 μM	47 nM	7 nM	IS (1 μM)	NA	(Durek et al., 2013 b; Maertens et al., 2006 a)
Ts2	NA	S (1 μM)	S (1 μM)	IS (1 μM)	S (1 μM)	S (1 μM)	S (1 μM)	IS (1 μM)	NA	(Cologna et al., 2012)
MeuNaTxα-1	NA	S (1 μM)	S (1 μM)	IS (1 μM)	IS (1 μM)	3 μM	S (1 μM)	IS (1 μM)	NA	(Zhu et al., 2012)
MeuNaTxα-2	NA	IS (1 μM)	IS (1 μM)	2 μM	IS (1 μM)	IS (1 μM)	IS (1 μM)	IS (1 μM)	NA	(Zhu et al., 2012)
MeuNaTxα-5	NA	IS (2 μM)	S (2 μM)	S (2 μM)	S (2 μM)	0.79 μM	S (2 μM)	IS (2 μM)	NA	(Zhu et al., 2012)
MeuNaTxα-12	0.91 μM	S (10 μM)	NA	S (10 μM)	IS (10 μM)	S (10 μM)	NA	NA	NA	(Zhu et al., 2013)

81

MeuNaTxα-13	2.5 µM	S (10 µM)	NA	S (10 µM)	IS (10 µM)	S (10 µM)	NA	NA	NA	(Zhu et al., 2013)
β-Scorpion										
Cn2	IS (300 nM)	IS (300 nM)	IS (300 nM)	IS (300 nM)	IS (300 nM)	40 nM	IS (300 nM)	NA	NA	(Schiavon et al., 2006)
Tz1	NA	S (10 µM)	NA	IS (10 µM)	IS (10 µM)	S (10 µM)	IS (10 µM)	NA	NA	(Leipold et al., 2006)
Tf2	IS (1 µM)	IS (1 µM)	S (1 µM)	IS (1 µM)	IS (1 µM)	IS (1 µM)	IS (1 µM)	IS (1 µM)	NA	(Camargos et al., 2015)
Ts1	IS (100 nM)	IS (100 nM)	S (100 nM)	S (100 nM)	S (100 nM)	S (100 nM)	IS (100 nM)	IS (100 nM)	NA	(Peigneur et al., 2015b)
Cone snail										
δ-SuVIA	NA	NA	4 nM	5 nM	1 nM	2.5 nM	NA	NA	NA	(Jin et al., 2015)
ι-RXIA	IS (50 µM)	18 µM	IS (50 µM)	IS (50 µM)	IS (50 µM)	1.8 µM	S (50 µM)	IS (50 µM)	IS (50 µM)	(Fiedler et al., 2008)
Sea anemone										
ATX-II	6 nM	8 nM	759 nM	109 nM	49 nM	~180 nM	1.8 µM	NA	NA	(Oliveira et

										al., 2004; Wanke et al., 2009)
AFT-II	391 nM	2 μM	459 nM	31 nM	63 nM	~300 nM	5.8 μM	NA	NA	(Oliveira et al., 2004; Wanke et al., 2009)
Bc-III	~300 nM	1.5 μM	1.5 μM	821 nM	307 nM	~900 nM	5.7 μM	NA	NA	(Oliveira et al., 2004; Wanke et al., 2009)
CGTX-II	165 nM	> 1 μM	> 1 μM	> 1 μM	105 nM	133 nM	IS (50 nM)	NA	NA	(Zaharenko et al., 2012)
Dinoflagellate										
P-CTX-1	8 nM	8 nM	3 nM	18 nM	10 nM	18 nM	13 nM	2 nM	S (1 nM)	(Inserra et al., 2017)

NA – not assessed

83

Table 2. Summary of experimentally determined Na$_V$ channel interactions with Na$_V$ activator toxins. For simplicity, only the S1-S2 and S3-S4 linkers or the S5 and S6 transmembrane segments (or S5-S6 linker as indicated) are presented for DI, DII, DIII and DIV. The type of experimental evidenced is abbreviated as follows: single-point channel mutations (S); chimeric channels (C); competitive radioligand binding (R); photoaffinity labelling (P). White shading indicates an experimental determined interaction, light grey shading indicates the interaction is unknown, and dark grey shading indicates that it has been experimentally determined that there is no interaction.

	DI				DII				DIII				DIV				Ref
	S1–S2	S3–S4	S5	S6	S1–S2	S3–S4	S5	S6	S1–S2	S3–S4	S5	S6	S1–S2	S3–S4	S5	S6	
Alkaloids																	
Batrachotoxin				S				S				S				S	(Toma et al., 2016; Wang et al., 2001; Wang et al., 2000b; Wang and Wang, 1998, 1999)
Grayanotoxin				S												S	(Ishii et al., 1999; Kimura et al., 2000)
Veratridine				S												S	(Wang et al., 2000a)
Spider																	
δ-hexatoxin-Ar1a			R^{*L}										R^{*}	R^{*}			(Little et al., 1998)
δ-hexatoxin-Hv1a			R^{*L}										R^{*}	R^{*}			(Little et al., 1998)
β-hexatoxin-Mg1a			$R^{\#L}$		$R^{\#}$	$R^{\#}$					$R^{\#L}$			R^{*}			(Corzo et al., 2003)
δ-theraphotoxin-Cg1a														S			(Tao et al., 2016)
δ-theraphotoxin-														S			(Huang et

Cg3a													al., 2015)
δ-theraphotoxin-Hm1a		C			C			C		C	C		(Osteen et al., 2016)
Wasp													
β-pompilidotoxin	C	C	C^L	C	C	C^L	C	C	C	C	S	C^L	(Kinoshita et al., 2001)
α-Scorpion													
LqTx											S		(Rogers et al., 1996)
LqhII			S^L							S	S		(Leipold et al., 2005; Leipold et al., 2004; Wang et al., 2011)
LqhIII											S		(Leipold et al., 2005; Leipold et al., 2004)
β-Scorpion													
CssIV	C		C^L	S	S	C^L	C	C	S^L	C	C	C^L	(Cestele et al., 1998; Cestele et al., 2006; Zhang et al., 2011)
Tz1	C	C	C^L		S				S^L	C	C	C^L	(Leipold et al., 2006)

Cone snail																	
δ-SVIE															S		(Leipold et al., 2005)
Sea anemone																	
ATX-II															S		(Rogers et al., 1996)
ApB															S		(Benzinger et al., 1998)
Dinoflagellate																	
Brevetoxin				P												P^L	(Trainer et al., 1994)
Ciguatoxin				R$^\$$												R$^{\$L}$	(Lombet et al., 1987)

*Competitive radioligand binding with LqhII

$^\#$Competitive radioligand binding with CssIV

$^\$$Competitive radioligand binding with brevetoxin

^{L}S5–S6 linker

1   **Figure legends**

2   **Figure 1. General structural topology of Na$_V$ channels. a)** Na$_V$ channels consist of

3   four homologous domains (domain I, II, III and IV) that are linked *via* large

4   intracellular loops. Each domain contains 6 transmembrane segments (S1 - S6) which

5   can be functionally divided into the voltage-sensing domains (VSD; segments S1-S4)

6   and the pore domains (PD, segments S5-S6). The S4 segments carry several highly

7   conserved positively charged arginine and lysine residues (gating charges) which

8   move across the cell membrane upon changes in the membrane potential and thus

9   function as the voltage sensors of the channel. **b)** Top view of the Na$_V$ channel. The

10   ion-conducting pore of the channel is formed by the four assembled PDs of DI - DIV,

11   which are each shaped by segments S5 and S6 plus their extracellular linker. The

12   arrangement of the voltage-sensing (S1-S4) and pore-forming (S5-S6) segments are

13   offset so that in functional channels, the voltage sensor of each domain is closest to

14   the pore-forming segment of the following domain. Broken lines indicate intracellular

15   linkers and loops; continuous lines indicate extracellular linkers.

16

17   **Figure 2. Simplified gating model of Na$_V$ channels.** Na$_V$ channels exist in at least

18   three, and likely many more, distinct states: resting (closed), open, and inactivated. In

19   the resting or closed state of Na$_V$ channels, all four VSD are presumably in the

20   inactivated state. Movement of the DI-DIII VSDs, which is subsequently transferred

21   to the PDs via the S4 - S5 linkers, changes channel conformation and allows the pore

22   to become permeable to sodium ions. Channel inactivation requires movement of the

23   domain IV VSD, which brings the IFM motif on the third intracellular loop between

24   DIII and DIV close to the intracellular opening of the pore and thus inactivates it.

25

1 **Figure 3. Simplified classification of Na$_V$ channel modulators**

2 In a simple approach, the binding sites of Na$_V$ channel modulators can be divided into

3 the pore region (consisting of all four S5 and S6 segments including their linkers) and

4 the four voltage-sensing domains (consisting of segments S1 to S4 including their

5 extracellular linkers). Further functional classification of activators and inhibitors

6 results in four possible classes of Na$_V$ channel modulators for each binding site. Some

7 Na$_V$ channel modulators belong to more than one class and can have differential

8 functional effects on distinct channel isoforms. Given that binding to the VSDs of

9 different domains likely preferentially affects distinct channel functions, the number

10 of possible classes of Na$_V$ modulators may be even greater.

11

12 **Figure 4. Amino acid sequence alignment of human Na$_V$ isoforms for DI S6**

13 Multiple amino acid sequence alignment of domain I segment 6 and adjacent residues

14 of hNa$_V$ α-subunit isoforms was performed using Clustal Omega 1.2.2 with default

15 parameters. We used the amino acid sequences in the canonical isoform 1 as provided

16 by the UniProtKB database (http:// www.uniprot.org/). UniProtKB entry identifiers:

17 P35498 (SCN1A, hNa$_V$1.1); Q99250 (SCN2A, hNa$_V$1.2); Q9NY46 (SCN3A,

18 hNa$_V$1.3); P35499 (SCN4A, hNa$_V$1.4); Q14524 (SCN5A, hNa$_V$1.5); Q9UQD0

19 (SCN8A, hNa$_V$1.6); Q15858 (SCN9A, hNa$_V$1.7); Q9Y5Y9 (SCN10A, hNa$_V$1.8);

20 Q9UI33 (SCN11A, hNa$_V$1.9). The definition of transmembrane segments and intra-

21 and extracellular domains are as specified by the UniProtKB database. Where dots are

22 shown instead of letters in Na$_V$ isoform sequences, the amino acid is identical to the

23 one in Na$_V$1.1. Consensus symbols: * = positions which have a single, fully conserved

24 residue in all sequences; : = conservation between groups of strongly similar

25 properties in all sequences (STA; NEQK; NHQK; NDEQ; QHRK; MILV; MILF;

89

1 HY; FYW); . = conservation between groups of weakly similar properties in all

2 sequences (CSA; ATV; SAG; STNK; STPA; SGND; SNDEQK; NDEQHK;

3 NEQHRK; FVLIM; HFY). Amino acid residues important for Na$_V$ channel activator

4 toxin binding are in bold. Residues are additionally circled if evidence is based on

5 channel mutagenesis studies and sequence parts are highlighted in rectangles if

6 evidence is based on studies with chimeric channels. As several studies have been

7 carried out on rat Na$_V$ channels, the results are only indicated if the human isoform

8 has the same residues at the corresponding positions. Brevetoxin, ciguatoxin,

9 batrachotoxin, grayanotoxin and veratridine have been shown to bind to residues in

10 the DI S6 region.

11

12 **Figure 5. Amino acid sequence alignment of human Na$_V$ isoforms for DII S6**

13 Multiple sequence alignment of domain II segment 6 and adjacent residues was

14 performed as stated in Figure 4. Batrachotoxin binds to residues in DII S6.

15

16 **Figure 6. Amino acid sequence alignment of human Na$_V$ isoforms for DIII S5 -**

17 **S6 linker**

18 Multiple sequence alignment of domain II S5 - S6 linker and adjacent residues was

19 performed as stated in Figure 4. The shaded conserved lysine and aspartic acid

20 residues are part of the channels sodium selectivity filter in the P-loop of DIII. This

21 linker contains residues important for interactions with batrachotoxin as well as the β-

22 scorpion toxins CssIV and Tz1.

23

24 **Figure 7. Amino acid sequence alignment of human Na$_V$ isoforms for DIII S6**

Multiple sequence alignment of domain III segment 6 and adjacent residues was performed as stated in Figure 4. This segment contains important residues for batrachotoxin binding.

Figure 8. Amino acid sequence alignment of human Na$_V$ isoforms for DIV S6

Multiple sequence alignment of domain IV segment 6 and adjacent residues was performed as stated in Figure 4. DIV S6 contains residues that are important for batrachotoxin and veratridine binding.

Figure 9. Amino acid sequence alignment of human Na$_V$ isoforms for DIV VSD S3-S4 linker

Multiple sequence alignment of domain IV S3 - S4 linker and adjacent residues was performed as stated in Figure 4. The shown region contains important residues for interactions with the δ-spider toxins Cg1a, Cg3a and Hm1a β-pompilidotoxin, the α-scorpion toxins LqTx, LqhII and LqhIII, the δ-conotoxin SVIE, and the sea anemone toxins ATX-II and ApB.

Figure 10. Amino acid sequence alignment of human Na$_V$ isoforms for DIV S1 – S2 linker

Multiple sequence alignment of domain IV S1 – S2 linker and adjacent residues was performed as stated in Figure 4. The DIV S1 – S2 linker contains important residues for LqhII and Hm1a binding.

Figure 11. Amino acid sequence alignment of human Na$_V$ isoforms for DI S5 – S6 linker

1   Multiple sequence alignment of part of the domain I S5 - S6 linker was performed as

2   stated in Figure 4. The vertical lines indicate a break in the sequence. This region

3   contains important residues for CssIV and LqhII binding.

4

5   **Figure 12. Amino acid sequence alignment of human Na$_V$ isoforms for DII S1 –**

6   **S2 linker**

7   Multiple sequence alignment of domain II S1 – S2 linker and adjacent residues was

8   performed as stated in Figure 4. The DII S1 – S2 linker contains a proline important

9   for CssIV binding.

10

11   **Figure 13. Amino acid sequence alignment of human Na$_V$ isoforms for DII S3 –**

12   **S4 linker**

13   Multiple sequence alignment of domain II S3 – S4 linker and adjacent residues was

14   performed as stated in Figure 4. The DII S3 – S4 linker contains important residues

15   for CssIV and Tz1 binding.

16

17   **Figure 14. Amino acid sequence alignment of human Na$_V$ isoforms for DIV S5 –**

18   **S6 linker**

19   Multiple sequence alignment of part of the domain IV S5 – S6 linker was performed

20   as stated in Figure 4. The shaded conserved alanine and aspartic acid residues are part

21   of the channels sodium selectivity filter in the P-loop of DIV. This region contains

22   important residues for interactions with brevetoxin and ciguatoxin.

23

24

DI S6   DI S6 – DII S1 loop

Na$_V$1.1	G K T Y M I F F V L V I F L G S F Y L I N L I L A V V A M A Y E E Q N Q A T L E E A E Q K E A
Na$_V$1.2	. . T Y M I F F V L V I F L G S F Y L I N L I L A V V A M A Y E E Q N Q A T L E E A E Q K . .
Na$_V$1.3	. . . . V . . . .
Na$_V$1.4	. . . . V I . . . . I N . . L . . . . A . . . E . . . A . D K E . . E
Na$_V$1.5	. . I . . . . M . . . . V . . . . I A . T . E . . K
Na$_V$1.6	. . . . V . . . . . . V . . . .
Na$_V$1.7	. . . . V . . . . N I . . . K . . . L
Na$_V$1.8	. . I . . . . V . . . . T . . . . T D . I . A . . K
Na$_V$1.9	. L Y S V F . . I V . . . . T . . . . T . . . . . . K N V A A . I . A . . K
similarity	* . . : : * . * . : . : . * * . * * * * * : . * * . * * * . : . * * * . . * * * : . . * *

Brevetoxin and ciguatoxin

Batrachotoxin, grayanotoxin
and veratridine
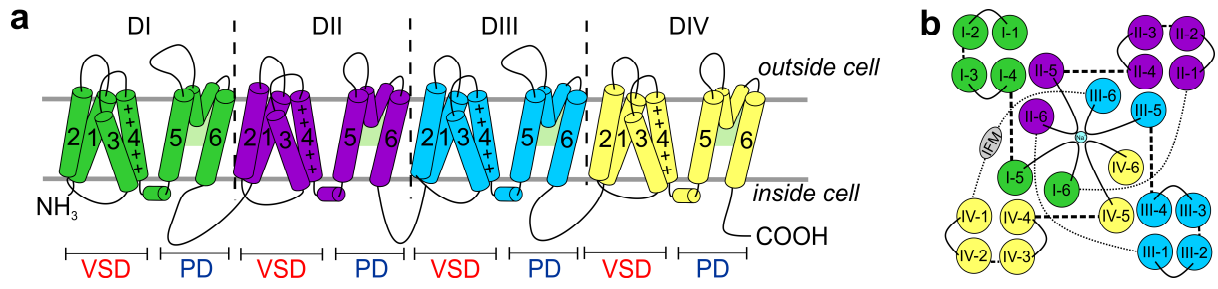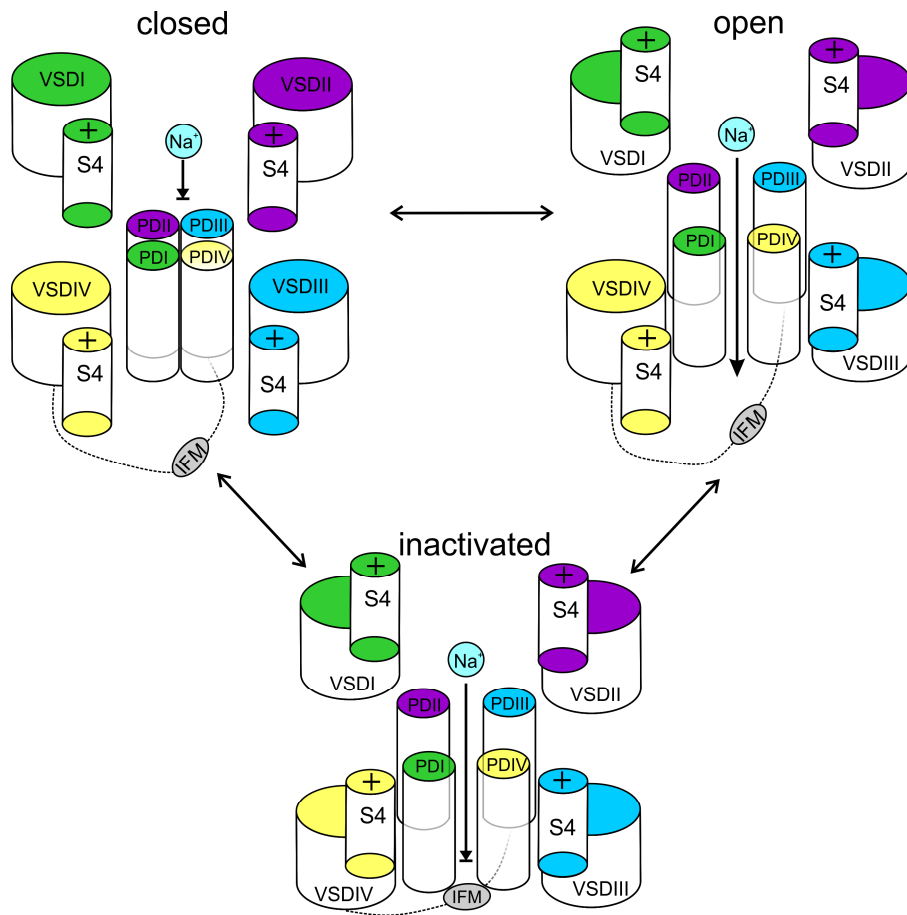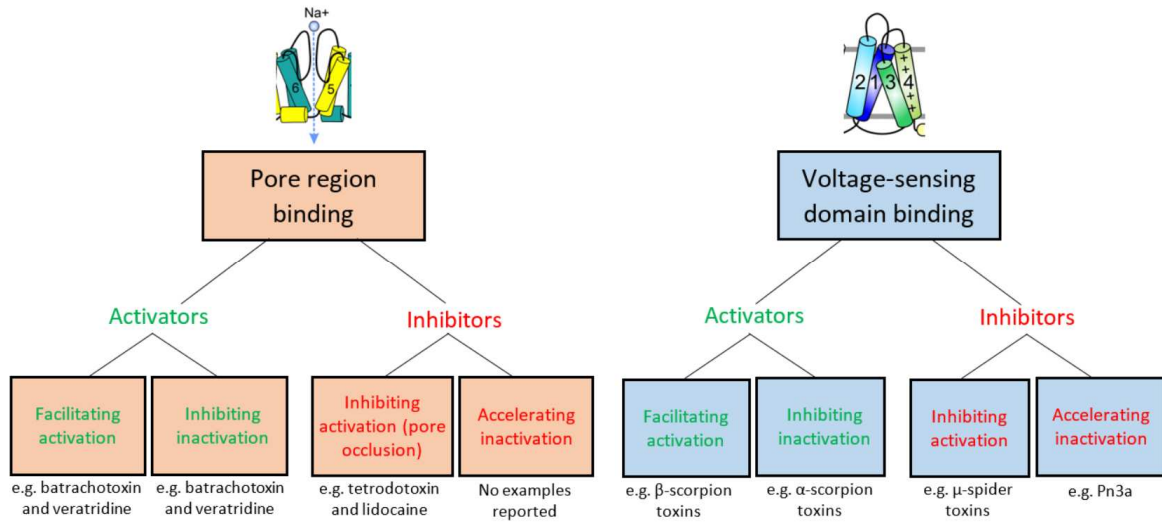
										DII S6																						
Na$_V$1.1	G	Q	A	M	C	L	T	V	F	M	M	V	M	V	I	G	N	L	V	V	L	N	L	F	L	A	L	L	L	S	S	F	S
Na$_V$1.2	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.3	.	.	T	.	.	.	I	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.4	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	Ⓝ	.	.	.	Ⓛ	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.5	.	.	S	L	.	.	L	.	.	L	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.6	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.7	.	.	.	.	.	.	I	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.8	Q	K	S	I	.	.	I	L	.	L	T	.	.	.	L	.	.	.	.	.	.	.	.	.	.	I	.	.	.	N	.	.	.
Na$_V$1.9	S	S	S	L	.	V	I	.	.	I	L	I	T	.	.	.	K	.	.	.	.	.	.	.	.	I	.	.	.	N	.	.	.
similarity		.	:	:	*	:		:	:	:		:				*	:	*	:	*	*	*	*	*	*	*	:	*	*	*	.	*	*	*

Batrachotoxin

										DIII S5-S6 linker																									DIII S6	
Na$_V$1.1	Q	V	A	T	F	K	G	W	M	D	I	M	Y	A	A	V	D	S	R	N	V	E	L	Q	P	K	Y	E	E	S	L	Y	M	Y
Na$_V$1.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	E	L	.	.	.	.	.	D	N	.	.	.	.
Na$_V$1.3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	K	.	.	.	V	.	.	.	N	.	.	.	.
Na$_V$1.4	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	K	.	E	.	.	Q	.	.	V	N	.	.	.	.
Na$_V$1.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	Y	.	E	.	.	Q	W	.	Y	N	.	.	.	.
Na$_V$1.6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	P	D	E	.	.	.	.	.	D	N	I	.	.	.
Na$_V$1.7	.	.	.	.	.	.	.	T	I	.	.	.	.	.	.	.	.	.	V	.	.	D	K	.	.	.	.	.	Y	.	.	.	.	.
Na$_V$1.8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	N	M	.	.	.	W	.	D	N	V	.	.	.
Na$_V$1.9	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	T	E	K	.	Q	.	.	E	F	.	S	N	S	L	G	.
similarity	*	*	*	*	*	*	*	*		*	:	*	*	*	*	*	*				.	*	*	*	:	*		.						*

Batrachotoxin   β-scorpion toxin Tz1   β-scorpion toxin CssIV

				DIII S6																									
Na$_V$1.1	E	E	S	L	Y	M	Y	L	Y	F	V	I	F	I	I	F	G	S	F	F	T	L	N	L	F	I	G	V	I	I	D	N	F
Na$_V$1.2	.	D	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.3	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.4	.	V	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.5	.	Y	N	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.6	.	D	N	I	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.7	.	Y	.	.	.	.	I	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.8	.	D	N	V	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.
Na$_V$1.9	.	S	N	S	L	G	.	I	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
similarity	*		.				*	:	*	*	*	:	*	*	*	*	*	.	*	*	*	*	*	*	:	*	*	*	*	*	*	*	*

Batrachotoxin

								DIV S6																									

$Na_V1.1$ P S V G I F F F V S Y I I I S F L V V V N M Y I A V I L E N F
$Na_V1.2$ . . . .
$Na_V1.3$ . . . .
$Na_V1.4$ . . I . . C . C . . . . F . I . . N . . . . . I . . . . . .
$Na_V1.5$. A . . . L . . T T . . . . I . . . . . I . . . .
$Na_V1.6$ . . . . I . . . . I . . . .
$Na_V1.7$ . . . . . . Y . . . .
$Na_V1.8$. A . . . I . . T T . . . . I M . . . .
$Na_V1.9$. G I A T S Y . . . . I . . . .
similarity * . : . . . : * . : * * * * * * * : : * * * * * : * * * * *

Batrachotoxin and veratridine

	DIV S3	DIV S3-S4 linker	DIV S4
Na_V1.1	S I V G M F L A E L I	E K - - Y F V S P T L F	R V I R L A R I G R

Na_V1.2 | . . . . (F) . . (E) . | (E) . . - - . . (V) . . . (L) | . . . .

Na_V1.3 | . . . . | M | . - -

Na_V1.4 | . . . . L A . S (D) . | Q . . - - (Y) (F) (V) . . . | . . . .

Na_V1.5 | . . . . T V . S (D) I . | Q . . - - . . F . | . . . .

Na_V1.6 | . . . . D I . | . . - - | . . . .

Na_V1.7 | . . . . D . | . T - - | . . . .

Na_V1.8 | . . A S L I F S A I L | K S L Q S Y F . | . . . .

Na_V1.9 | . . . S T M I S T . | E N Q E H I P F P . . | . . I V . . . . . .

similarity | * * . . . . : . : | : . . . . . * * * * | * * : : * * * * * * *

- LqhII
- LqTx, LqhII and ATX-II
- LqhIII
- Cg1a, Cg3a and ApB
- β-pompilidotoxin and ATX-II
- LqhII and SVIE
- ATX-II
- Hm1a
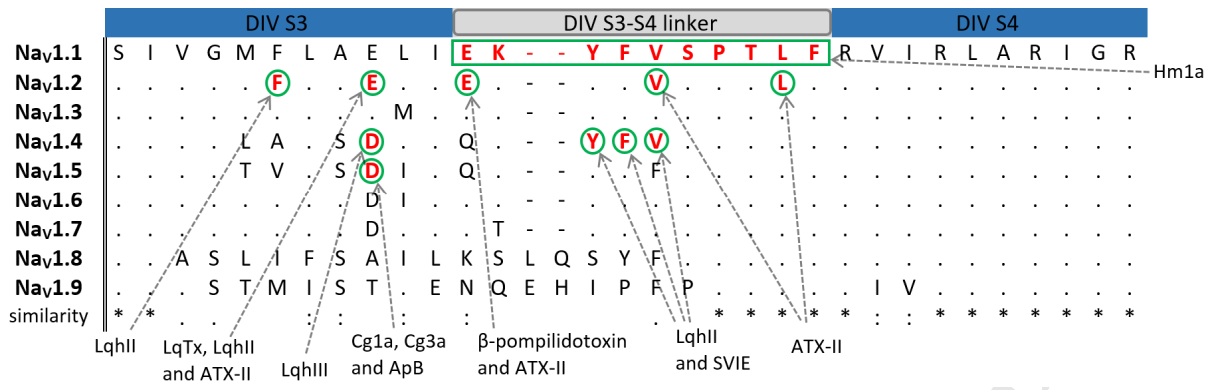
	DIV S1							DIV S1-S2 linker										DIV S2							
Na$_V$1.1	L	N	M	V	T	M	M	V	**E**	**T**	**D**	**D**	**Q**	**S**	**E**	**Y**	**V**	**T**	**T**	I	L	S	R	I	N	L	V	F	I
Na$_V$1.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	E	M	T	N	.	.	Y	W	.	.	.	.	.	.
Na$_V$1.3	.	.	.	.	.	.	.	.	.	.	.	G	K	.	M	.	L	V	.	.	.	.	.	.	.	.	.
Na$_V$1.4	.	.	.	.	.	.	.	N	.	.	Q	L	K	V	D	.	.	Y	N	.	.	M	I	.	.
Na$_V$1.5	.	.	.	.	.	.	.	.	.	.	P	E	K	I	N	.	.	A	K	.	.	.	L	.	V
Na$_V$1.6	.	.	.	.	.	.	.	.	T	.	.	K	Q	M	E	N	.	.	Y	W	.	.	.	.	V
Na$_V$1.7	.	.	.	.	.	.	K	E	G	.	.	Q	H	M	.	E	V	.	Y	W	.	.	V	.	.	.
Na$_V$1.8	.	.	I	.	.	.	.	.	.	.	E	K	.	K	.	.	G	K	.	.	Q	F	.	V
Na$_V$1.9	.	.	I	S	.	.	A	.	S	Y	N	.	P	K	A	M	K	S	.	.	D	H	L	.	W	.	.	V
similarity	*	*	*	:	:	*	*	.	*	.			*							:	*		:	*		.	*	:

LqhII   Hm1a

	DI S5-S6 linker																				DI S5-S6 linker									DI S6	
Na$_V$1.1	D	A	G	Q	C	P	E	G	Y	M	C	V	K	A	G	R	N	P	N	Y	G	Y	T	L	T	L	R	A	A	G	K	T	Y	M	I	F
Na$_V$1.2	.	.	.	Q	C	P	E	G	Y	I	C	V	K	A	G	R	N	P	N	Y	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.3	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.4	.	.	.	H	.	.	.	.	.	E	.	I	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.5	.	.	.	T	.	.	.	.	.	R	.	L	.	.	.	E	.	.	D	H	.	.	.	Q	.	.	.	S	.	.	.	I	.	.	.	.
Na$_V$1.6	.	.	.	.	.	.	.	.	.	Q	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.7	.	S	.	.	.	.	.	.	.	T	.	.	.	I	.	.	.	D	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.8	.	S	.	H	.	.	D	.	.	I	.	L	.	T	S	D	.	.	D	F	N	.	.	Q	.	.	.	T	S	.	.	I	.	.	.	.
Na$_V$1.9	G	N	S	A	.	S	I	Q	.	E	.	K	H	T	K	I	.	.	D	.	N	.	.	Q	.	.	.	T	T	.	L	Y	S	V	F	.
similarity	.	.	.	*	.	.	.	*	.	*	.	.	.	.	.	*	*	.	.	.	*	*		*	*	*	.	:	.	*	.	.	.	.	:	.	*

CssIV

LqhII

		DII S1								DII S1-S2 linker										DII S2										
Na$_V$1.1	L	N	T	L	F	M	A	M	E	H	Y	P	M	T	D	H	F	N	N	V	L	T	V	G	N	L	V	F	T	G
Na$_V$1.2	.	.	.	.	.	.	.	.	.	.	(P)	.	.	E	Q	.	S	S	.	.	S	.	.	.	.	.	.	.	.	.
Na$_V$1.3	.	.	.	.	.	.	.	.	.	.	.	.	.	E	Q	.	S	S	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.4	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.5	.	.	.	.	.	.	L	.	.	.	N	.	.	S	E	.	E	E	M	.	Q	.	.	.	.	.	.	.	.	.
Na$_V$1.6	.	.	.	.	.	.	.	.	.	H	.	.	.	P	Q	.	E	H	.	.	A	.	.	.	.	.	.	.	.	.
Na$_V$1.7	.	.	.	.	.	.	.	.	.	H	.	.	.	E	E	.	K	.	.	.	A	I	.	.	.	.	.	.	.	.
Na$_V$1.8	V	.	I	.	.	.	.	.	.	H	G	.	S	P	T	.	E	A	M	.	Q	I	.	.	I	.	.	.	.	I
Na$_V$1.9	I	.	V	.	L	.	.	.	.	H	K	.	E	A	S	.	E	K	M	.	N	I	.	.	.	.	.	.	.	S
similarity	:	*	*	:	*	:	*	:	*	*	:		*			*	.		:	*		:	*	*	:	*	*	*

CssIV

	DII S3										DII S3-S4 linker					DII S4									
Na$_V$1.1	L	S	L	V	E	L	G	-	-	L	A	N	V	E	G	L	S	V	L	R	S	F	R	L	L	R	V
Na$_V$1.2	.	.	.	M	.	.	.	-	-	.	.	.	.	.	Ⓖ	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.3	.	.	.	M	.	.	.	-	-	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.4	.	.	.	.	.	.	.	-	-	.	.	.	.	Q	Ⓖ	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.5	.	.	.	M	.	.	.	-	-	.	S	R	M	S	N	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.6	.	.	.	M	.	.	S	-	-	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Na$_V$1.7	.	.	.	.	.	F	-	-	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Na$_V$1.8	V	.	.	L	.	.	.	-	-	V	.	K	K	G	S	.	.	.	.	.	.	.	.	.	.	.	
Na$_V$1.9	.	.	F	A	D	V	M	N	C	V	L	Q	K	R	S	W	P	F	.	.	.	.	.	V	.	.	.
similarity	:	*	:		:	:					:				.		*	*	*	*	*	:	*	*	*

CssIV   Tz1

	DIV S5												DIV S5-S6 linker																																					

Nav1.1 A Y V K R E V G I D D M F N F E T F G N S M I C L F Q I T T S A G W D G L L A P I L N S K P P D C D P N K V N
Nav1.2 . . . . . E V G I D D M F N F E T F G N S M I C L F Q I T T S A G W D G L L A P I L N S G P P D C D P D K D H
Nav1.3 . . . . K . A . . . . A . . . . D T I H
Nav1.4 . . . . K . S . . . . I . . . E . . . . N . . . . . G . . . . L E .
Nav1.5 . . . . W . A . . . . Q . . A . . . L . . . . S . . . . T G . . Y . . . T L P .
Nav1.6 . . . . H . A . . . . L . . . . - R . . . . S L D . E H
Nav1.7 . . . . K . D . N . . . . K . . H
Nav1.8 P H . R W . A . . . . Q . . A . . . L . . . . S . . . . T G . . Y . . . . L P .
Nav1.9 S K . N P . S . . . . I . . . K . . A S . . L . . . . . S . . . . . . S . . S . M . R . . E S - . . . . -
similarity   * .   *   * * : * : * * * : * * . . * : :   * * * : * : * * * * * * . * * . * : * .   *

Brevetoxin and ciguatoxin

Highlights

- Voltage-gated sodium channel activator toxins occur in many venoms

- Sodium channel activators are important tool compounds and drug leads

- These compounds modulate voltage-gated sodium channels with high selectivity