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Themed Section: Recent Advances in Targeting Ion Channels to Treat Chronic Pain

REVIEW ARTICLE

Sodium channels and pain: from toxins to therapies

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Voltage-gated sodium channels (Na_V channels) are essential for the initiation and propagation of action potentials that critically influence our ability to respond to a diverse range of stimuli. Physiological and pharmacological studies have linked abnormal function of Na_V channels to many human disorders, including chronic neuropathic pain. These findings, along with the description of the functional properties and expression pattern of Na_V channel subtypes, are helping to uncover subtype specific roles in acute and chronic pain and revealing potential opportunities to target these with selective inhibitors. High-throughput screens and automated electrophysiology platforms have identified natural toxins as a promising group of molecules for the development of target-specific analgesics. In this review, the role of toxins in defining the contribution of Na_V channels in acute and chronic pain states and their potential to be used as analgesic therapies are discussed.

LINKED ARTICLES

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Abbreviations

CIP, congenital insensitivity to pain; DRG, dorsal root ganglion; Na_V, voltage-gated sodium channel; NGF, nerve grow factor; PNS, peripheral nervous system; TTX, tetrodotoxin

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Introduction

Voltage-gated sodium channels (Na_V channels) are key signalling molecules that underlie the action potential upswing in electrically excitable cells as well as being involved in biological processes in non-excitable cells. They are membrane-spanning glycosylated proteins that rapidly activate in response to cell membrane depolarization to generate an influx of Na^+ ions before rapidly inactivating. Na_V channels contribute to the transmission of a broad range of somatosensory signals, including temperature, touch, smell, proprioception and pain, from the periphery to the brain (Vranken, 2012). They also underlie the release of catecholamines by neuroendocrine cells (Yamamoto *et al.*, 1997), angiogenesis (Andrikopoulos *et al.*, 2011), contraction of smooth, skeletal and cardiac muscle (Seda *et al.*, 2007), melanogenesis (Ekmehag *et al.*, 1994; Huh *et al.*, 2010) and the invasion (Carrithers *et al.*, 2009), phagocytosis (Carrithers *et al.*, 2007; Black *et al.*, 2013) and maturation (Zsiros *et al.*, 2009) of immune cells. Thus, Na_V channels are a family of molecules in which physiological roles are broad and vital for normal physiology.

The Na_V family comprises nine homologous α-subunits ($Na_V1.1$–$Na_V1.9$) that form the ion conducting pore plus the auxiliary β-subunits (β1 to β4) that modulate channel gating and trafficking. The overall molecular architecture of the α-subunit is highly conserved amongst Na_V channels (Figure 1). It comprises four domains I–IV containing the transmembrane segments S1 to S6, in which S4 contributes to the voltage sensor and S5 and S6 form the sodium selective

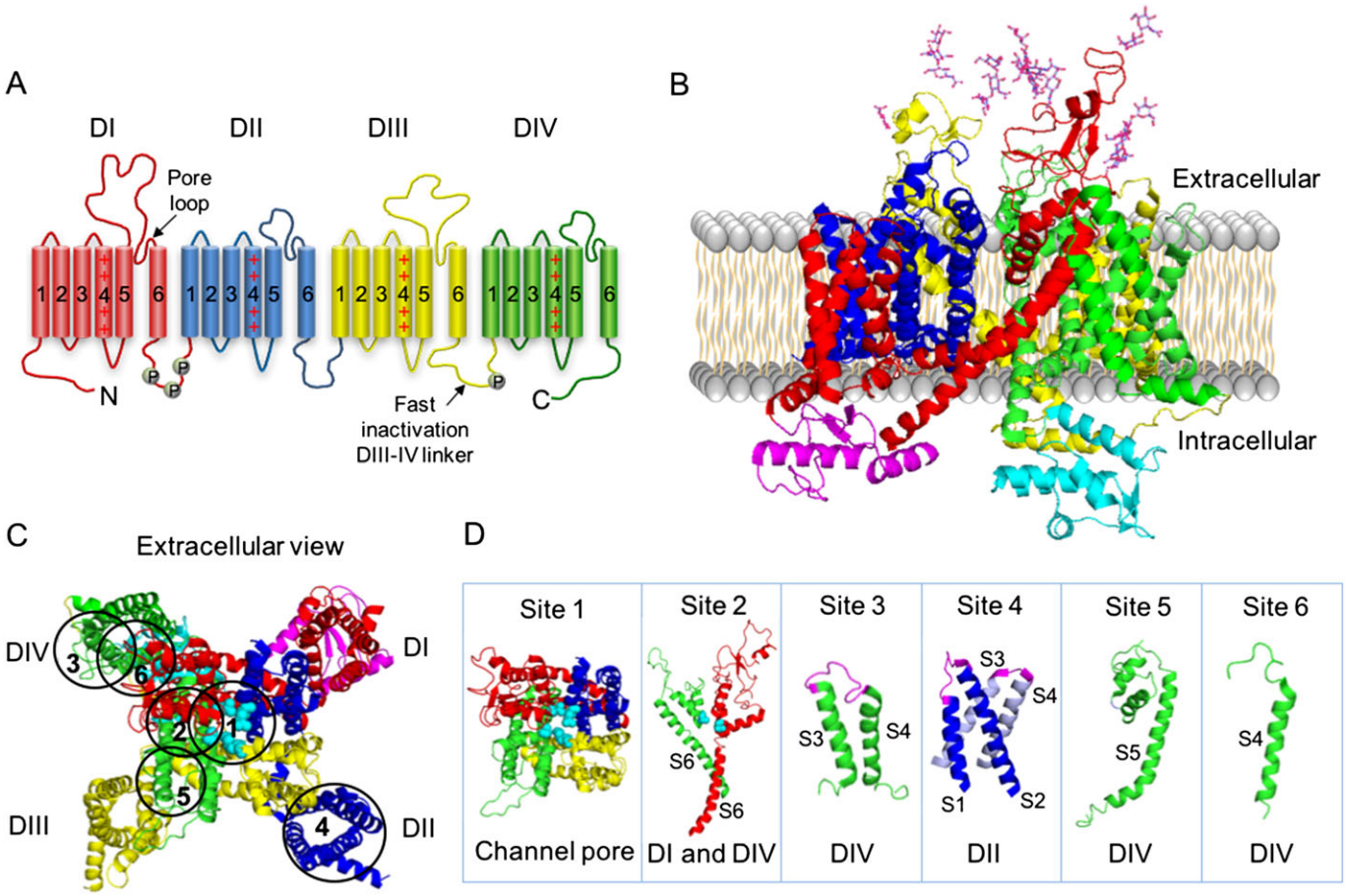


Figure 1

Na_V channel structure. (A) Topology of the eukaryotic Na_V channel α-subunit. Each domain of the tetramer, DI (red), DII (blue), DIII (yellow) and DIV (green), contains six transmembrane helixes, a pore loop and a voltage sensor located in the S4 region, which is positively charged. The pore loops connect S5 and S6 extracellularly, while loops in the intracellular region participate in the regulation of fast inactivation (linker DIII–DIV) and regulation by endogenous molecules, with phosphorylation sites labelled P. (B) Cryo-EM (cryogenic-electron microscopy) structure of the eukaryotic Na_VPaS channel α-subunit (Shen *et al.*, 2017). The Na_V channel domains DI (red), DII (blue), DIII (yellow) and DIV (green) are shown with N- and C-terminal domains coloured in magenta and cyan respectively. Glycosylations located in the extracellular loops S5S6 of DI and DIII are represented by sticks. (C) The extracellular view of the Na_V channel showing the selective Na^+ pore surrounded by the P-loops, with key residues involved in the ion selectivity (asparagine in DI, glutamate in DII, lysine in DIII and alanine in DIV) represented by cyan spheres. Toxins binding sites 1–6 are indicated by circles. (D) Structural features of binding sites 1–6. Site 1 is located within the pore of the channel, site 2 within S6 of DI and DIV, site 3 within the extracellular loop connecting S3–S4 of DIV (magenta), site 4 within the extracellular loops connecting S1–S2 and S3–S4 of DII (magenta), site 5 at S5 of DIV and site 6 at S4 of DIV. Three-dimensional structure was obtained from the RCSB Protein Data Bank and prepared in PyMol (DeLano, 2002).

pore (Stuhmer *et al.*, 1989; Catterall *et al.*, 2005; Alabi *et al.*, 2007; Zhang *et al.*, 2012; Shen *et al.*, 2017) (Figure 1A–C). Cell depolarization alters the electrical field across the membrane resulting in the rapid movement of the S4 segments and conformational changes that opens the ion channel pore, followed by fast inactivation that occurs as channel block by the DIII–DIV intracellular linker. Single and multiple variations in the amino acids sequence of the α-subunit as well as alternative splicing and post-translational modifications such as glycosylation are associated with channel modulation and their *in vivo* physiological signatures (Rizzo *et al.*, 1994; Laedermann *et al.*, 2015). This structural variability and subtype-selective distribution help define channel function in neurons and non-neuronal cells. Table 1 summarizes the distribution of Na_V channels in the central and peripheral nervous system (PNS). **$Na_V1.4$** and **$Na_V1.5$** channels are mostly expressed in skeletal muscle and the heart, respectively, and will not be discussed in this review.

The importance of Na_V channels in therapeutics is highlighted by the clinical use of anticonvulsants,

Table 1

Distribution of voltage-gated sodium channels in the nervous system

Sodium channel	Current type	Tissue expression		Subcellular localization
$Na_V1.1$	TTX-S, fast	CNS	Cerebellum, striatum, hippocampus and thalamus (Brysch *et al.*, 1991; Black *et al.*, 1994)	Neuronal soma and proximal dendrites (Whitaker *et al.*, 2001)
		PNS	DRG large cells, Aδ-fibres and Aαβ-fibres and outer plexiform layer (Black and Waxman, 1996; Mojumder *et al.*, 2007; Fukuoka *et al.*, 2008)	
$Na_V1.2$	TTX-S, fast	CNS	Cerebellum and hippocampus (Gong *et al.*, 1999; Jarnot and Corbett, 2006)	Unmyelinated axons, presynaptic terminals and immature nodes of Ranvier (Westenbroek *et al.*, 1989; Boiko *et al.*, 2001)
		PNS	Cochlear ganglion and nerves, myenteric neurons and outer plexiform layer (Hossain *et al.*, 2005; Bartoo *et al.*, 2006; Mojumder *et al.*, 2007)	
$Na_V1.3$	TTX-S, fast	CNS	Neocortex, hippocampus and dentate gyrus (Westenbroek *et al.*, 1992; Whitaker *et al.*, 2000)	Neuronal soma and proximal dendrites
		PNS	Absent	
$Na_V1.6$	TTX-S, fast	CNS	Cerebellum and hippocampus (Schaller and Caldwell, 2000)	Node of Ranvier and axon initial segment (Caldwell *et al.*, 2000; Boiko *et al.*, 2001)
		PNS	DRG large and medium cells, Aδ-fibres, Aαβ-fibres, myenteric neurons, cochlear ganglion and nerves and outer plexiform layer (Black and Waxman, 1996; Hossain *et al.*, 2005; Bartoo *et al.*, 2006; Mojumder *et al.*, 2007; Fukuoka *et al.*, 2008)	
$Na_V1.7$	TTX-S, fast	CNS	Absent	Axons
		PNS	All DRG cell sizes, C-fibres, Aδ-fibres and Aαβ-fibres (Black and Waxman, 1996; Djouhri *et al.*, 2003b; Fukuoka *et al.*, 2008; Rupasinghe *et al.*, 2012)	
$Na_V1.8$	TTX-R, slow	CNS	Absent	Axons
		PNS	All DRG cells sizes, C-fibres, Aδ-fibres, Aαβ-fibres and trigeminal and nodosal ganglia (Black and Waxman, 1996; Bongenhielm *et al.*, 2000; Coward *et al.*, 2000; Djouhri *et al.*, 2003a; Stirling *et al.*, 2005; Fukuoka *et al.*, 2008)	
$Na_V1.9$	TTX-R, very slow	CNS	Absent	Soma and axons
		PNS	DRG small and medium cells and fibres, enteric neurons and trigeminal ganglia (Dib-Hajj *et al.*, 1998b; Coward *et al.*, 2000; Padilla *et al.*, 2007; Fukuoka *et al.*, 2008)	

antiarrhythmic, local anaesthetics and analgesics that block Na_V channels, usually non-selectively. In addition, human molecular genetics studies have proven that inherited disorders such as cardiac arrhythmias (Wang *et al.*, 1995; Kotta *et al.*, 2010), epilepsy (Wallace *et al.*, 2001; Escayg and Goldin, 2010), paralysis and myotonias (Jurkat-Rott and Lehmann-Horn, 2007; Zhao *et al.*, 2012) and loss and gain of pain sensation, including congenital insensitivity to pain (CIP) (Cox *et al.*, 2006; Danziger and Willer, 2009; Staud *et al.*, 2011; Phatarakijnirund *et al.*, 2016), erythromelalgia (Sheets *et al.*, 2007; Skeik *et al.*, 2012), idiopathic small nerve fibre neuropathy (Faber *et al.*, 2012a) and paroxysmal extreme pain disorder (Fertleman *et al.*, 2007; Choi *et al.*, 2011) can be directly linked to mutations of genes encoding specific sodium channel subtypes. These insights generate singular opportunities for using selective Na_V channel modulators to treat such complex disorders and have attracted considerable attention from both the academic and industry research sectors. Amongst these Na_V channel modulators, natural toxins have shown remarkable modulatory properties by binding at specific sites of these channels (Figure 1C, D) and inducing various pharmacological effects.

In the light of the critical role of Na_V channels in pain processes and the therapeutic potential of Na_V channel modulators, it is essential to understand the distribution and function of these channels to guide the development of novel therapies targeting specific channel family members. This review describes the role of toxins in defining the distribution of these channel subtypes and how their role changes in pathological pain conditions. In addition, the therapeutic potential of natural Na_V-targeting toxins will be discussed.

Alterations in Na_V channels during chronic neuropathic pain

Pain or noxious sensation is a natural defence mechanism associated with a distressing sensory experience due to actual or potential tissue damage. This mechanism comprises primary sensory neurons and axons in the PNS that detect noxious stimuli through ion channels and receptors, with Na_V channels playing a key role in this nociceptive signalling. More recently, research has demonstrated in more detail the participation of some Na_V channel subtypes in pain pathways. The **$Na_V1.1$** was revealed to be involved in mechanical but not thermal pain (Osteen *et al.*, 2016), while **$Na_V1.6$** was found to be involved in multiple peripheral pain pathways including thermal pain (Deuis *et al.*, 2013), and individuals lacking **$Na_V1.7$** or **$Na_V1.9$** function exhibit a congenital insensitivity to pain (CIP), with no other sensory abnormalities apart from anosmia (Cox *et al.*, 2006; Phatarakijnirund *et al.*, 2016). Besides their involvement in normally functioning acute pain pathways, Na_V channels are also implicated in chronic pain disorders.

Pain disorders

Na_V channels are involved in several painful chronic neuropathies as shown in Table 2. These conditions are characterized by sensory neurons membrane remodelling, comprising alterations in expression, trafficking and kinetics of Na_V channel subtypes. Consequently, neurons generate action potentials spontaneously or under subthreshold stimulus, inducing ectopic pain characterized by hyperalgesia, allodynia and spontaneous pain. Chronic pain can be classified in two major types, neuropathic pain (nerve injury pain) and nociceptive pain (tissue injury and inflammatory pain). Although classified into two distinct conditions, there is clearly an overlap in these processes, with nerve injury inducing local inflammation, and inflammation leading to nerve damage.

Neuropathic pain. Neuropathic pain caused by nerve injury has diverse origins. Once the synthesis and transport of Na_V channels is altered, an accumulation and/or displacement of Na_V channels across the axon occurs, leading to membrane remodelling and changes in intrinsic electrical excitability. For example, $Na_V1.7$, **$Na_V1.8$** and $Na_V1.9$ were shown to accumulate in the neuroma endings and in patches of demyelination in both animals and humans with neuropathic pain (Coward *et al.*, 2000; Kretschmer *et al.*, 2002). Interestingly, the remodelling of these channels is often not accompanied by gene expression alterations at the mRNA and protein levels, as is the case for $Na_V1.8$. The expression of this channel is altered in a few conditions such as diabetic neuropathy and post-herpetic neuralgia (Novakovic *et al.*, 1998; Sleeper *et al.*, 2000; Hong *et al.*, 2004; Garry *et al.*, 2005). Nerve injury and demyelination can also markedly alter the gene expression of **$Na_V1.3$**, which is observed in various neuropathy models (Waxman *et al.*, 1994; Dib-Hajj *et al.*, 1999; Kim *et al.*, 2001; Hong *et al.*, 2004; Garry *et al.*, 2005). The fast activation and inactivation kinetics of $Na_V1.3$ associated with its rapid re-priming kinetics and persistent current component is thought to contribute to the ectopic discharges and sustained firing rates in injured sensory neurons (Cummins *et al.*, 2001), although knockout of $Na_V1.3$ produces a mouse with normal pain (Nassar *et al.*, 2006). Table 2 summarizes the alterations in Na_V subtypes in chronic neuropathic pain conditions.

Nociceptive pain. In contrast to nerve injury, nociceptive pain can emerge from the modulation of Na_V channels by inflammatory mediators. Tissue injury results in local inflammation, which induces pain by increasing the excitability of afferent neurons innervating the injured area. Multiple inflammatory mediators can give rise to pain by promoting the release of secondary mediators that exert a direct action on nociceptors.

The inflammatory mediator **PGE_2** sensitizes neurons and decreases pain thresholds in mechanical, thermal and chemical stimuli (Taiwo and Levine, 1991; Aley and Levine, 1999; Fitzgerald *et al.*, 1999), probably through a direct effect on neurons as observed *in vitro* (Khasar *et al.*, 1998). **PKC**, a kinase induced by PGE_2, sensitizes nociceptors by increasing tetrodotoxin-resistant (**TTX**-R) currents (Gold *et al.*, 1998). Such kinases can also be activated by pronociceptive cytokines such as TNF-α and nerve grow factor (**NGF**) that are also associated with hyperalgesia (Lewin *et al.*, 1993; Shu and Mendell, 1999; Jin and Gereau, 2006; Pezet and McMahon, 2006; Fischer *et al.*, 2017). NGF enhances TTX-R currents by up-regulating $Na_V1.8$ expression and increasing excitability (Zhang *et al.*, 2002; Bielefeldt *et al.*, 2003; Lin *et al.*, 2017). $Na_V1.9$ currents are also increased by inflammatory agents,

Table 2

Voltage-gated sodium channels involved in chronic neuropathic pain and their distribution during neuropathy

Sodium channel	Neuropathy type and references	Altered expression, distribution and function
$Na_V1.3$	DRG axotomy (Waxman *et al.*, 1994; Xiao *et al.*, 2002) CCI (Dib-Hajj *et al.*, 1999; Chen *et al.*, 2014) SNL (Boucher *et al.*, 2000; Kim *et al.*, 2001; Yin *et al.*, 2016) Sciatic nerve CCI (Hains *et al.*, 2004) Diabetic neuropathy (Hong *et al.*, 2004) Inflammatory pain (Black *et al.*, 2004) Spared nerve injury (Lindia *et al.*, 2005) Post-herpetic neuralgia (Garry *et al.*, 2005) Trigeminal neuralgia (Xu *et al.*, 2016)	Up-regulation in C-fibres, Aδ-fibres and Aαβ-fibres Up-regulation in DRG and trigeminal ganglion
$Na_V1.6$	Infraorbital nerve injury (Henry *et al.*, 2007) Diabetic neuropathy (Ren *et al.*, 2012) CCI (Tseng *et al.*, 2014) SNL (Xie *et al.*, 2015) Trigeminal neuralgia (Grasso *et al.*, 2016; Tanaka *et al.*, 2016)	Accumulation in nerve injury sites Up-regulation in DRG neurons in diabetic neuropathy Gain of function mutation in trigeminal neuralgia
$Na_V1.7$	Diabetic neuropathy (Hong *et al.*, 2004) Human neuroma (Kretschmer *et al.*, 2002) Inflammatory pain (Black *et al.*, 2004; Nassar *et al.*, 2004; Yeomans *et al.*, 2005) IEM and PEPD (Fertleman *et al.*, 2006; Drenth and Waxman, 2007) CIP (Cox *et al.*, 2006) Trigeminal neuralgia (Xu *et al.*, 2016)	Accumulation in nerve injury sites Up-regulation in DRG neurons in diabetic neuropathy and inflammatory pain Down-regulation in trigeminal ganglia during neuralgia Gain of function mutations in IEM and PEPD, and loss of function in CIP
$Na_V1.8$	Axotomy (Dib-Hajj *et al.*, 1996; Okuse *et al.*, 1997) SNL (Okuse *et al.*, 1997; Boucher *et al.*, 2000) Human neuroma (Kretschmer *et al.*, 2002) Diabetic neuropathy (Hong *et al.*, 2004) Post-herpetic neuralgia (Garry *et al.*, 2005) Mechanical allodynia (Dong *et al.*, 2007) Visceral pain (King *et al.*, 2009) Chronic nerve compression (Frieboes *et al.*, 2010) Inflammatory pain (Zhang *et al.*, 2002; Bielefeldt *et al.*, 2003; Lin *et al.*, 2017) Painful neuropathy (Faber *et al.*, 2012b) Trigeminal neuralgia (Xu *et al.*, 2016)	Accumulation in nerve injury sites Up-regulation in DRG neurons in post-herpetic neuralgia Down-regulation in diabetic neuropathy, axotomy, SNL and in trigeminal ganglia during neuralgia Gain of function mutation in painful neuropathy
$Na_V1.9$	Axotomy (Dib-Hajj *et al.*, 1998a) SNL (Boucher *et al.*, 2000) Inflammatory pain (Priest *et al.*, 2005) CCI (Tseng *et al.*, 2014) Painful neuropathy (Huang *et al.*, 2014) Trigeminal neuralgia (Xu *et al.*, 2016) CIP (Phatarakijnirund *et al.*, 2016)	Accumulation in nerve injury sites Down-regulation in axotomy, SNL and in trigeminal ganglia during neuralgia Gain of function mutation in painful neuropathy and loss of function in CIP

CCI, chronic constriction injury; IEM, erythromelalgia; PEPD, paroxysmal extreme pain disorder; SNL, spinal nerve ligation.

and knockout mice display attenuated inflammatory pain, including diabetic neuropathic pain (Craner *et al.*, 2002; Rush and Waxman, 2004; Baker, 2005; Amaya *et al.*, 2006; Binshtok *et al.*, 2008). The pivotal role of $Na_V1.9$ in chronic visceral pain has been shown in gut-projecting dorsal root ganglion (DRG) neurons, with excitatory abnormal responses mediated by **ATP** and PGE_2 (Hockley *et al.*, 2014; Hockley *et al.*, 2016). Furthermore, both $Na_V1.8$ and $Na_V1.9$ were shown to be up-regulated by **glial-derived neurotrophic factor** (GDNF) in axotomized DRG neurons (Cummins *et al.*, 2000).

Inflammatory mediators also affect TTX-sensitive (TTX-S) currents as an up-regulation of $Na_V1.3$ and $Na_V1.7$ has been demonstrated in a carrageenan-induced inflammatory pain model (Black *et al.*, 2004). Knockdown of $Na_V1.7$ also attenuates inflammatory pain (Nassar *et al.*, 2004; Yeomans *et al.*, 2005). Other mediators such as **adenosine**, **bradykinin**, **5-hydroxytryptamine**, **endothelin-1**, plasma **adrenaline** and the pro-inflammatory cytokine **IL-1β** have also been demonstrated to directly modulate the excitability of sensory neurons through an action on Na_V channels (Cui and Nicol, 1995; Gold *et al.*, 1996; Griswold *et al.*, 1999; Cardenas *et al.*,

2001; Zhou *et al.*, 2002; Binshtok *et al.*, 2008). These inflammatory mediators along with the Na_V subtypes altered in ectopic pain define a group of potential targets for the development of therapeutics.

Natural toxins targeting Na_V channels and applications in pain therapies

Venoms are a rich and complex mixture of biologically active molecules, also called toxins, that have a variety of targets and functions. These have evolved into potent neurotoxins able to induce excitatory and inhibitory effects in the nervous system to disable preys and also to defend against predators (Klint *et al.*, 2012; King and Hardy, 2013). The molecular targets of these neurotoxins include voltage-gated ion channels, with Na_V channels being remarkably modulated (Figures 2 and 3).

To date, Na_V modulators have been characterized from spiders, cone snails, scorpions, wasps, sea anemones, dinoflagellates, frogs, salamanders, puffer fish, octopus and plants such as from genera *Vetrum* and *Aconitum* (Figures 2 and 3; Table 3) (Herzig *et al.*, 2011; Kaas *et al.*, 2012; Pedraza Escalona and Possani, 2013; de O Beleboni *et al.*, 2004; Wanke *et al.*, 2009; Lehane and Lewis, 2000; Moczydlowski, 2013; Daly *et al.*, 1965; Hanifin, 2010; Williams *et al.*, 2011). These molecules display diverse chemical structures and modulatory properties and are capable of distinguishing amongst Na_V subtypes through specific interactions with binding sites in these channels (Figure 1C, D). Their modulatory mechanisms have divided them into two major classes, pore blockers that bind to the ion conduction pore to inhibit Na^+ influx and gating modifiers that interact with the segments S3, S4, S5 and S6 to modulate opening and inactivation.

Natural small molecules toxins

Small molecules were the first group of Na_V modulators described (Figure 2; Table 3). The discovery of TTX, which is produced by microorganisms and bioaccumulated in the food chain by salamanders, frogs, puffer fish and octopus, further classified Na_V channels in TTX-R and TTX-S channels (Hanifin, 2010; Williams *et al.*, 2011; Lago *et al.*, 2015). TTX incorporates a guanidinium group that blocks $Na_V1.1$–$Na_V1.4$ and $Na_V1.6$–$Na_V1.7$ with IC_{50} values in the single nanomolar range (Figure 2B). Similarly, **saxitoxin** (Thottumkara *et al.*, 2014) (Figure 2D), neosaxitoxin (Penzotti *et al.*, 2001) and gonyautoxin (Frace *et al.*, 1986) are potent sodium channel inhibitors produced by aquatic microorganisms and bioaccumulated in the food chain. Guanidinium-based toxins bind to the site 1 of Na_V channels to plug the ion conducting pore (Chen and Chung, 2014). Extensive studies with TTX analogue have revealed a new metabolite 4,9 anhydro-TTX with great selectivity for $Na_V1.6$ (Rosker *et al.*, 2007). Furthermore, TTX has shown *in vivo* efficacy in preclinical pain models of inflammatory and neuropathic pain (Beloeil *et al.*, 2006; Marcil *et al.*, 2006; Alvarez and Levine, 2015; Salas *et al.*, 2015) (Table 4). These results suggest TTX is a potential lead for further development of Na_V-specific blockers.

Another remarkable group of toxins that modulate Na_V channels comprise the ciguatoxins and **batrachotoxin** (Daly *et al.*, 1965; Strachan *et al.*, 1999) (Figure 2). These molecules are capable of activating the influx of Na^+ through interactions with distinct domains of the Na_V channels. Ciguatoxins are a ladder-frame polyether toxin produced by marine dinoflagellates circum-tropically (Figure 2A, C). They are bioaccumulated through the food chain and responsible for the ciguatera caused by the consumption of ciguateric reef fish. Ciguatoxins bind to site 5 of the Na_V channel located on DIV to induce channel opening and increase Na^+ permeability (Lombet *et al.*, 1987). These molecules have played a key role in elucidating the mechanism associated with cold pain, revealing $Na_V1.8$ and TTX-S Na_V channels in specific subsets of nerves containing **TRPA1** as main effectors in ciguatoxin-induced cold allodynia (Vetter *et al.*, 2012c).

Batrachotoxin is a steroidal alkaloid isolated from skin secretions of the frog genus *Phyllobates* present in South and Central America (Figure 2A, E). It is also believed batrachotoxin is bioaccumulated in the food chain, similar to TTX and ciguatoxins. It induces alterations in Na_V channels through its interactions with site 2 located at DI and DIV, leading to channel depolarization and persistent activation at hyperpolarized potentials (Trainer *et al.*, 1994; Trainer *et al.*, 1996). Interestingly, batrachotoxins are also found in the integument of birds from the genus *Pitohui* in New Guinea (Weldon, 2000). Finally, **veratridine** is another Na_V activator alkaloid that is produced by plants of the genus *Veratrum* (Krayer and Acheson, 1946) (Figure 2A, F). It binds to site 2 at DI and DIV and to site 6 at DIV S4 and leads to partial channel activation and stabilization of the open conformation for persistent opening (Wang and Wang, 2003; Yoshinaka-Niitsu *et al.*, 2012). Similarly, **grayanotoxin** produced by plants from the family Ericaceae (Maejima *et al.*, 2002) (Figure 2G), **aconitine** from the plant *Aconitum* (Borcsa *et al.*, 2014) and antillatoxin produced by marine cyanobacterium (Cao *et al.*, 2010) are potent Na_V channel activators. Veratridine and antillatoxin in particular have been used in programmes for the discovery of Na_V channel modulators due to its ability to specifically activate these channels in neuroblastomas and recombinant cells lines and facilitate assay development (Vetter *et al.*, 2012b; Cardoso *et al.*, 2015; Zhao *et al.*, 2016). In addition, veratridine and grayanotoxin have been used in *in vivo* studies of pain mediated by Na_V channels (Gingras *et al.*, 2014; Cardoso *et al.*, 2015).

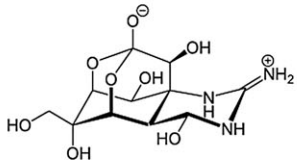
Natural peptide toxins

Peptide toxins often target Na_V channel subtypes selectively, making these attractive starting points to find better research probes with some showing potential as leads to improved pain treatments (Figure 3; Table 3). While finding the most promising amongst the millions of unique disulfide-rich venom peptides, advances in high-throughput screening, transcriptomic and proteomic studies have accelerated the rapid identification and isolation of new activators and inhibitors of Na_V channels (Pineda *et al.*, 2014; Prashanth *et al.*, 2014; Cardoso *et al.*, 2015; Klint *et al.*, 2015; Deuis *et al.*, 2017). Perhaps the most promising are the highly stable disulfide-rich inhibitory cysteine knot (ICK) scaffold peptides common in spider and cone snail venoms, although the more complex disulfide-rich structures are found in scorpions and sea anemones toxins (Figure 3C–F). Those venom peptides showing promising efficacy in reversing chronic pain in

A

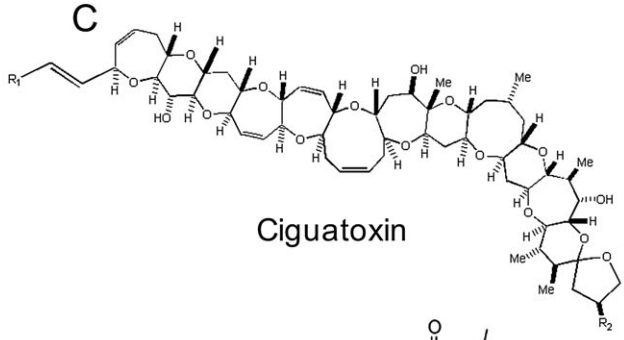
Dinoflagellates	Amphibians	Marine animals	Plants
	   *Phyllobates* *Brachycephalus* *Taricha*	  Tetraodontidae *Hapalochlaema*	   *Vetrum* Ericaceae *Aconitum*
Ciguatoxins Saxitoxins Gonyautoxins	**Batrachotoxin Tetrodotoxin**	**Tetrodotoxin**	**Veratridine Grayanotoxin Aconitine**

B



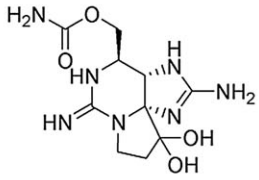
Tetrodotoxin

C



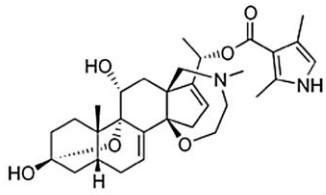
Ciguatoxin

D



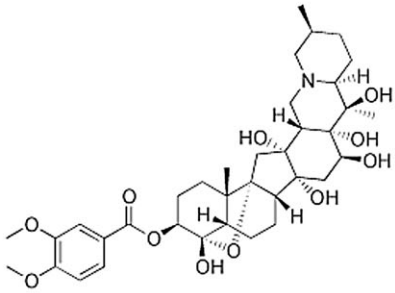
Saxitoxin

E



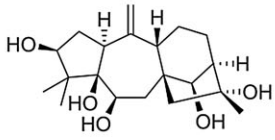
Batrachotoxin

F



Veratridine

G



Grayanotoxin

Figure 2

Natural small molecules toxins targeting Na_V channels. (A) Ciguatoxins are guanidine-based polyether ladder toxins, and saxitoxins and goyautoxins are also guanidine-based toxins produced by dinoflagellates (Frace *et al.*, 1986; Strachan *et al.*, 1999; Thottumkara *et al.*, 2014). Bratrachotoxins are isolated from the skin secretions of frog (Daly *et al.*, 1965), TTX is isolated from salamanders such as of the genus *Taricha*, frogs such as of the genus *Brachycephalus*, puffer fish belonging to order *Tetraodontidae* and octopus of the species *Hapalochlaema lunata* (Hanifin, 2010; Williams *et al.*, 2011; Lago *et al.*, 2015) and veratridine, grayanotoxin and aconitine are isolated from plants of the genus *Veratrum* (Krayer and Acheson, 1946), family *Ericaceae* (Maejima *et al.*, 2002) and genus *Aconitum* (Borcsa *et al.*, 2014) respectively. (B–G) Structure of TTX, ciguatoxins, saxitoxins, batrachotoxin , veratridine and grayanotoxin is shown.

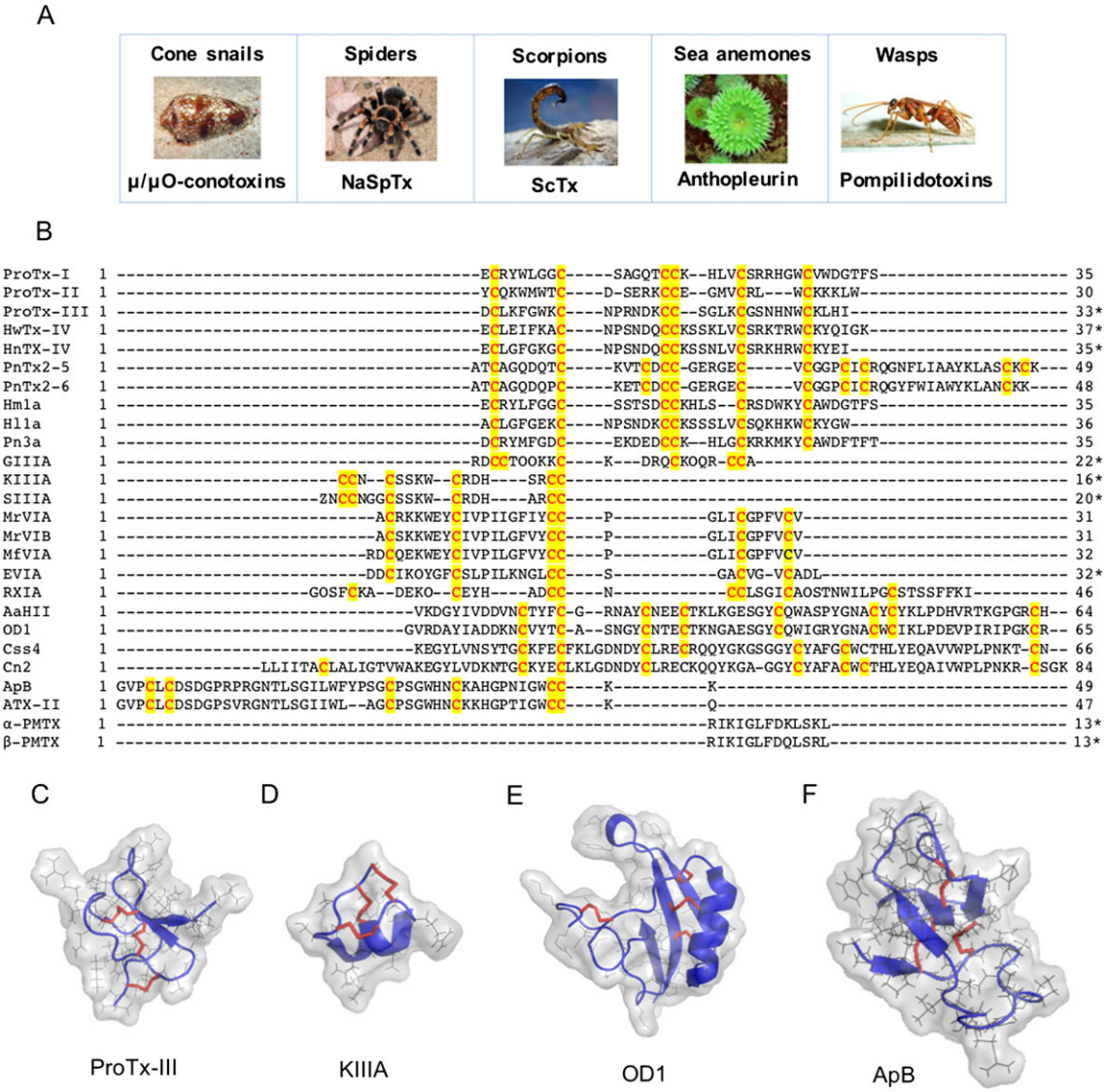


Figure 3

Natural peptide toxins targeting Na_V channels. (A) μ/μO-Conotoxins are a large group of peptidic toxins isolated from cone snails venoms (Lewis *et al.*, 2012), NaSpTx is a large group of peptidic toxins isolated from spider venoms (Klint *et al.*, 2012), ScTx is a group of toxins isolated from the venom of scorpions, anthopleurins are peptidic toxins isolated from sea anemone (Schweitz *et al.*, 1981) and pompilidotoxins are peptides isolated from wasps (Konno *et al.*, 1997; Konno *et al.*, 1998). (B) Amino acid sequences alignment of the peptidic toxins discussed in this review, with highly conserved cysteines highlighted in red and yellow, and C-terminal amidation denoted by an asterisk (*). Sequences were obtained from Arachnoserver, Conoserver and UniProt databases. (C–F) Structure of major classes of natural peptide toxins targeting Na_V channels, including NaSpTx ProTx-III isolated from the spider *T. pruriens* (Cardoso *et al.*, 2015), μ-conotoxin KIIIA isolated from *Conus kinoshitai* (Khoo *et al.*, 2012), ScTx OD1 isolated from the scorpion *Odonthobuthus doriae* (Durek *et al.*, 2013) and anthopleurin B (ApB) isolated from the sea anemone *A. xanthogrammica* (Monks *et al.*, 1995). Disulfide bonds are represented as red sticks, amino acids side chains as grey lines and amino acids core chains as blue cartoon. Three-dimensional structures were obtained from the RCSB Protein Data Bank and prepared in PyMol (DeLano, 2002).

preclinical *in vivo* models of pain are shown in Table 4 and discussed in more detail in this review.

Pore-blocker peptides. Sodium channel toxins present in cone snail venoms comprise a diverse array of small ICK peptides for prey capture and defence. While there are five families (μ-conotoxin, μO-conotoxin, δ-conotoxin and ι-conotoxin), only the μ-conotoxins are pore blockers. μ-Conotoxins bind to site 1 of the Na_V channel and display potent subtype-selective inhibition of **$Na_V1.2$** and/or $Na_V1.4$ as reported for **GIIIA** (Sato *et al.*, 2015), KIIIA (Zhang *et al.*, 2007) and SIIIA (Schroeder *et al.*, 2008). These conotoxins have been used as part of a constellation pharmacology approach to determine the functional sodium channels found in native neuronal tissue such as the DRG (Teichert *et al.*, 2012), as well as in the research for the discovery of new analgesics targeting Na_V channels (Munasinghe and Christie, 2015).

The μ-conotoxins KIIIA and SIIIA have shown promising *in vivo* therapeutic efficacy in preclinical inflammatory pain models (Green *et al.*, 2007; Zhang *et al.*, 2007; Han *et al.*,

Table 3

Natural toxins targeting Na_V channels

Organism	Species	Toxin and references	Na_V channel activity (order of potency)	Mode of action/ potential binding sites
Spider	*T. pruriens*	ProTx-I (Middleton *et al.*, 2002)	$Na_V1.8 > Na_V1.7$ inhibitor	Gating modifier/site 4 or multiple sites
		ProTx-II (Middleton *et al.*, 2002)	$Na_V1.7 > Na_V1.6 > Na_V1.2 > Na_V1.5 > Na_V1.3 > Na_V1.8$ inhibitor	
		ProTx-III (Cardoso *et al.*, 2015)	$Na_V1.7 > Na_V1.6 > Na_V1.2 > Na_V1.1 > Na_V1.3$ inhibitor	
	H. huwenum	HwTx-IV (Xiao *et al.*, 2008a)	$Na_V1.7 > Na_V1.2 > Na_V1.3 > Na_V1.4$ inhibitor (Minassian *et al.*, 2013)	
	H. haianum	HnTX-IV (Liu *et al.*, 2003)	$Na_V1.7 > Na_V1.2 > Na_V1.3$ inhibitor	
	P. nigriventer	PnTx2–5 and PnTx2–6 (Matavel *et al.*, 2002; Matavel *et al.*, 2009)	Na_Vs activator	Gating modifier/site 3
Cone snail	*C. geographus*	GIIIA (Cruz *et al.*, 1985)	$Na_V1.4 > Na_V1.6$ inhibitor	Pore blocker/site 1
	C. kinoshitai	KIIIA (Zhang *et al.*, 2007)	$Na_V1.2 > Na_V1.4 > Na_V1.6 > Na_V1.1 = Na_V1.7$ inhibitor	
	C. striatus	SIIIA (Wang *et al.*, 2006)	$Na_V1.4 > Na_V1.2 = Na_V1.6$ inhibitor	
	C. marmoreus	MrVIA/B (McIntosh *et al.*, 1995)	$Na_V1.7 > Na_V1.4 > Na_V1.2$ inhibitor	Gating modifier/sites 1 and 4
		MrVIB (Ekberg *et al.*, 2006)	$Na_V1.8$ selective inhibitor	
	C. magnificus	MfIVA (Vetter *et al.*, 2012a)	$Na_V1.4$ and $Na_V1.8$ inhibitor	
	C. ermineus	EVIA (Barbier *et al.*, 2004)	$Na_V1.2 = Na_V1.3 = Na_V1.6$ activator	Gating modifier/site 6
	C. radiatus	RXIA (Buczek *et al.*, 2007)	$Na_V1.2 > Na_V1.6 > Na_V1.7$ activator	Not described
Scorpion	*A. australis*	AaHII (Martin and Rochat, 1986)	$Na_V1.7$ activator (Abbas *et al.*, 2013)	Gating modifier/site 3
	O. doriae	OD1 (Jalali *et al.*, 2005)	$Na_V1.7 > Na_V1.4 > Na_V1.6$ activator (Durek *et al.*, 2013)	
	C. suffusus	Css4 (Martin *et al.*, 1987)	Na_V activator (Cestele *et al.*, 1998)	Gating modifier/site 4
	C. noxius	Cn2 (Pintar *et al.*, 1999)	$Na_V1.6$ activator (Schiavon *et al.*, 2006)	
Sea anemone	*A. xanthogramica*	ApB (Schweitz *et al.*, 1981)	Na_V activator	Gating modifier/site 3
	A. sulcata	ATX-II (Romey *et al.*, 1976)	$Na_V1.1 = Na_V1.2 > Na_V1.5 > Na_V1.4 > Na_V1.6$ activator (Oliveira *et al.*, 2004)	
Dinoflagellate	*G. toxicus*	CTX-1 (Strachan *et al.*, 1999)	TTX-S, TTX-R, $Na_V1.2$, and $Na_V1.3$ and $Na_V1.8$ activator (Yamaoka *et al.*, 2009; Zimmermann *et al.*, 2013)	Gating modifier/site 5
	Alexandrium sp.	Saxitoxin (Thottumkara *et al.*, 2014); Neosaxitoxin (Penzotti *et al.*, 2001)	Na_V inhibitors	Pore blocker/site 1
	Gonyaulax sp.	Gonyautoxin (Frace *et al.*, 1986)		
Cyanobacteria	*L. majuscula*	Antillatoxin (Cao *et al.*, 2010)	Na_V activator	Not described
Puffer fish	Family Tetraodontidae		$Na_V1.1$–1.4, $Na_V1.6$–1.7 inhibitor	Pore blocker/site 1
Salamander	Genus *Taricha*	TTX (Hanifin, 2010; Williams *et al.*, 2011; Lago *et al.*, 2015)		
Frog	Genus *Brachycephalus*			
Octopus	*H. lunata*			
Wasp	*A. samariensis*	α-PMTX (Konno *et al.*, 1997)	$Na_V1.1$-$Na_V1.3$, $Na_V1.6$ and $Na_V1.7$ activators (Schiavon *et al.*, 2010)	Not described
	B. maculifrons	β-PMTX (Konno *et al.*, 1998)		
Frog	Genus *Phyllobates*	BTX (Daly *et al.*, 1965)	Na_V activator	Gating modifier/site 2

continues

Table 3 (Continued)

Organism	Species	Toxin and references	Na_V channel activity (order of potency)	Mode of action/ potential binding sites
Plant	Genus *Vetrum*	Veratridine (Krayer and Acheson, 1946)	Na_V activators	Gating modifier/site 2
	Family Ericaceae	Grayanotoxin (Maejima *et al.*, 2002)		
	Genus *Aconitum*	Aconitine (Borcsa *et al.*, 2014)		

The table shows representatives of major venomous species and respective toxins with highest potencies and diversity in modulatory mechanisms. Relative potencies are from references listed in the table, or from the database www.conoserver.org/ for cone snail toxins (Kaas *et al.*, 2012) and from the database www.arachnoserver.org/ for spider toxins (Herzig *et al.*, 2011)

Table 4

Current toxins in *in vivo* preclinical and human clinical trials showing promising therapeutic efficacy for treating chronic pain

Toxin	Organism Species	Na_V subtypes targeted	Preclinical/clinical studies showing therapeutic efficacy	Reference
TTX	Puffer fish Family *Tetraodontidae*	$Na_V1.1$–$Na_V1.4$, $Na_V1.6$ and $Na_V1.7$	Inflammatory and neuropathic muscle mechanical hyperalgesia	Alvarez and Levine (2015)
			Inflammatory thermal and mechanical pain	Beloeil *et al.* (2006)
			Inflammatory, visceral and neuropathic pain	Marcil *et al.* (2006)
			Burn-associated neuropathic pain	Salas *et al.* (2015)
			Chemotherapy-induced neuropathic pain (clinical trials)	Wex Pharmaceutical Inc.
Neosaxitoxin	Dinoflagellates	Na_Vs	Bladder pain syndrome (clinical trials)	Manriquez *et al.* (2015)
Gonyautoxin	Dinoflagellates	Na_Vs	Chronic tension-type headache (clinical trials)	Lattes *et al.* (2009)
ProTx-II	Spider *T. pruriens*	$Na_V1.7$	Painful diabetic neuropathy	Tanaka *et al.* (2015)
			Inflammatory pain	Flinspach *et al.* (2017), Patent US20150099705 A1
HnTX-IV	Spider *H. haianum*	$Na_V1.2$, $Na_V1.3$ and $Na_V1.7$	SNI-induced neuropathic pain and formalin-induced inflammatory pain	Liu *et al.* (2014a)
μ-TRTX-Hl1a	Spider *H. lividium*	$Na_V1.8$	Inflammatory and neuropathic pain	Meng *et al.* (2016)
HwTx-IV	Spider *O. huwena*	$Na_V1.7$	Inflammatory pain and SNI-induced neuropathic pain	Liu *et al.* (2014b)
μ-TRTX-Pn3a	Spider *P. nigricolor*	$Na_V1.7$	Inflammatory pain, co-administrated with opioid	Deuis *et al.* (2017)
μO-MrVIB	Cone snail *C. marmoreus*	$Na_V1.8$	Allodynia and hyperalgesia associate to neuropathic and chronic inflammatory pain	Ekberg *et al.* (2006)
			Post-incision allodynia	Bulaj *et al.* (2006)
μO-MfVIA	Cone snail *C. magnificus*	$Na_V1.4$ and $Na_V1.8$	Inflammatory pain (analogue E5K and E8K MfVIA)	Deuis *et al.* (2016a)
μ-KIIIA	Cone snail *C. kinoshitai*	$Na_V1.2$, $Na_V1.4$, $Na_V1.6$ and $Na_V1.7$	Inflammatory pain	Zhang *et al.* (2007) and Han *et al.* (2009)
μ-SIIIA	Cone snail *C. striatus*	$Na_V1.2$, $Na_V1.4$ and $Na_V1.6$	Inflammatory pain	Green *et al.* (2007)

2009) (Table 4). KIIIA also potently inhibits $Na_V1.6$ and $Na_V1.7$, which are implicated in pain pathways and ectopic pain (Cox *et al.*, 2006; Deuis *et al.*, 2013) (Table 2), and potentially contribute to the analgesia observed. Alanine scan and related analogue studies revealed that K7 in KIIIA was an essential residue for the inhibition of $Na_V1.4$ and $Na_V1.7$, but not the inhibition of $Na_V1.2$ and $Na_V1.6$, with structurally minimized disulfide-deficient KIIIA analogues providing an alternative scaffold for Na_V inhibitors (Han *et al.*, 2009). SIIIA shares 74% identity with KIIIA (Figure 3B), and besides inhibiting $Na_V1.2$ and $Na_V1.4$, it also inhibits $Na_V1.6$ (Wang *et al.*, 2006). Polyethylene glycol (PEG)-SIIIA had improved efficacy compared to SIIIA in inflammatory pain (Green *et al.*, 2007), showing the potential of this peptide class to

be modified to improve pharmacodynamic properties, presumably by reducing renal clearance.

Gating modifier peptides. Gating modifiers are the most diverse group of Na_V modulators venom peptides. They are present in venoms of cone snails, spiders, scorpions and sea anemones (Figure 3; Table 3) and have evolved to modify the gating properties of opening and inactivation of ion channels, causing alterations that either excite or inhibit channel function.

Conotoxins. The family of cone snail toxins that inhibit Na_V channels as gating modifiers are the μO-conotoxins. The toxins MrVIA and MrVIB were the first peptides belonging to this family to be characterized (McIntosh *et al.*, 1995). These μO-conotoxins bind to the Na_V channel pore loop, but only residues present in the DIII seem to the involved in these interactions (Zorn *et al.*, 2006). MrVIA also interacts with site 4 in the DII, sharing similar mechanisms with β-scorpions toxins (Leipold *et al.*, 2007). Curiously, the μO-conotoxins ability to alter Na_V gating properties is strongly regulated by the presence of β auxiliary subunits (Wilson *et al.*, 2011). MrVIB was tested in models of neuropathic and inflammatory pain in rats (Table 4), where i.t. infusions of this peptide reversed neuropathic pain caused by partial ligation of the sciatic nerve and inflammatory pain induced by intraplantar injection of CFA (Ekberg *et al.*, 2006). In a different study, local infusion of MrVIB into the area prior to a surgical incision produced long-lasting reduction of post-incision allodynia in rats (Bulaj *et al.*, 2006), but effects on $Na_V1.4$ may have interfered with the assessment of analgesia. More recently, a μO-MfVIA (E5K and E8K-MfVIA) analogue was developed with improved $Na_V1.8$ activity that reduced inflammatory pain induced by intraplantar injection of **formalin** (Deuis *et al.*, 2016a) (Table 4).

Conotoxins belonging to the pharmacological families δ and ι can induce channel activation through interactions with site 6 in the DIV (Figure 3B; Table 3). EVIA was the first δ-conotoxin described and shows preferential activation of $Na_V1.2$, $Na_V1.3$ and $Na_V1.6$ (Barbier *et al.*, 2004). The structure of EVIA revealed a typical ICK motif from the O superfamily of conotoxins (Volpon *et al.*, 2004). Finally, RXIA is an ι-conotoxin showing preference for activation of $Na_V1.2$, $Na_V1.6$ and $Na_V1.7$ (Buczek *et al.*, 2007). Although still poorly studied, this family of conotoxins revealed the presence of a D-phenylalanine amino acid at position 44 of RXIA, which is essential for its excitatory function (Buczek *et al.*, 2005). While not useful as analgesics, subtype-specific activator toxins can be used to elucidate the role of different Na_V subtypes in driving different pain pathways, as well as for the establishment of Na_V-specific pain models.

Spider toxins. To date, more than 40 000 species of spiders have been described, making this the largest group of venomous animals (Klint *et al.*, 2012). Impressively, these venoms are also the richest source of Na_V modulators, which are classified into 12 distinct families (NaSpTx1–12). NaSpTx produces a diverse array of modulatory effects on Na_V channels. The venom of the spider *Thrixopelma pruriens* contains peptides amongst the most potent Na_V inhibitors described to date, with ProTx-I, **ProTx-II** and ProTx-III showing remarkable potency to inhibit $Na_V1.7$ (Middleton *et al.*, 2002; Priest *et al.*, 2007; Cardoso *et al.*, 2015) (Table 3; Figure 3B, C). Although belonging in the same spider venom, these peptides contain distinct primary structures and are classified into three distinct NaSpTx families. ProTx-I is a NaSpTx family 2 toxin firstly isolated in a $Na_V1.8$ screen (Middleton *et al.*, 2002). ProTx-I also has **Ca_V3** and **K_V** inhibitory activity, highlighting the challenge to find gating modifier toxins that are truly selective for the target of interest. In the same study, ProTx-II, a NaSpTx family 3 toxin, showed similar activity at $Na_V1.8$ and Ca_V3 channels but no activity against K_V channels. The remarkable potency of ProTx-II at $Na_V1.7$ (IC_{50} 0.3 nM) makes it the most potent NaSpTx peptide reported to date (Priest *et al.*, 2007). Mechanism of action studies on ProTx-II revealed interactions with DI, DII and DIV of $Na_V1.2$ and DII and DIV of $Na_V1.7$ that cause a shift in the voltage dependence of activation to more positive potentials and a slowing of fast inactivation to produce a persistent Na^+ current (Bosmans *et al.*, 2008; Xiao *et al.*, 2010). ProTx-III was identified in a $Na_V1.7$-targeting screen and belongs to the NaSpTx family 1. It has shown improved selectivity for Na_V subtypes and a C-terminal amidation that enhances Na_V inhibitory activity by up to ninefold (Cardoso *et al.*, 2015).

The unique potency of ProTx-II for $Na_V1.7$ makes it an attractive lead for the development of new pain therapies. Evaluation in a preclinical model of neuropathic pain showed ProTx-II was able to reverse painful diabetic neuropathy through a reduction of thermal hyperalgesia (Tanaka *et al.*, 2015) (Table 4). ProTx-II analogues studies developed a highly specific and potent $Na_V1.7$ inhibitor with very low affinity for $Na_V1.6$ and no detectable activity for other Na_V subtypes at relevant therapeutic concentrations (Flinspach *et al.*, 2017). This peptide (JNJ63955918) differed from the wild-type ProTx-II at three positions, W7Q, W30L and N-terminal addition of G(−1) and P(0). ProTx-II and JNJ63955918 were evaluated in a formalin model of inflammatory pain, and both produced a significant reduction in phases I and II thermal pain, with no significant effect on motor function. JNJ63955918 and ProTxII bind preferentially to the closed state to inhibit $Na_V1.7$ gating.

HwTx-IV is another NaSpTx that belongs to the family 1. This toxin was isolated from the spider *Haplopelma huwenum* and has preferential inhibition for $Na_V1.7$ and $Na_V1.2$, followed by $Na_V1.3$ and $Na_V1.4$ (Xiao *et al.*, 2008b; Minassian *et al.*, 2013). HwTx-IV binds to DII and traps the S4 voltage sensor in the closed configuration. Consistent with its inhibitory effect on $Na_V1.7$, HwTx-IV reversed hyperalgesia in *in vivo* models of inflammatory and neuropathic pain (Liu *et al.*, 2014b) (Table 4). Mutants of HwTx-IV were developed to enhance its inhibitory activity over $Na_V1.7$, with (E1G, E4G,Y33W)HwTx-IV showing a remarkable improvement in IC_{50} from 27 to 0.4 nM in electrophysiology assays (Revell *et al.*, 2013). More recently, (E1G,E4G,F6W,Y33W)HwTx-IV, a derivative of (E1G,E4G,Y33W)HwTx-IV, was developed to enhance its binding to the lipidic membrane and this increased its potency at $Na_V1.7$ compared with native HwTx-IV from IC_{50} values of 32 to 7.5 nM in fluorescence imaging assays (Agwa *et al.*, 2017).

HnTX-IV isolated from the spider *Haplopelma hainanum* also belongs to the NaSpTx family 1. It has preferential inhibition for $Na_V1.7$, followed by $Na_V1.2$ and $Na_V1.3$ (Liu *et al.*, 2003), binding to DII to trap the S4 voltage sensor in the closed configuration (Cai *et al.*, 2015). Structure–activity relationship studies revealed a mixed positively charged and hydrophobic surface essential for its inhibitory activity (Li *et al.*, 2004; Liu *et al.*, 2012). Consistent with its mode of action, HnTX-IV is efficacious in various preclinical models of pain, alleviating inflammatory pain induced by acetic acid or formalin and allodynia induced by spared nerve injury in a neuropathic pain model (Liu *et al.*, 2014a). In contrast, another likely NaSpTx family 10 toxin, μ-TRTX-Hl1a recently identified in the venom of *Haplopelma lividium*, preferentially inhibited $Na_V1.8$ (Meng *et al.*, 2016). This toxin significantly reversed inflammatory pain induced by **acetic acid** and formalin, with an exceptional reversal of the paw-licking response observed during phase II. Finally, the most recently identified NaSpTx family 2 toxin Pn3a was isolated from in the venom of the spider *Pamphobeteus nigricolor.* Pn3a was found to be a remarkably selective inhibitor of $Na_V1.7$ and reversed pain behaviours in preclinical models of inflammatory pain induced by formalin, carrageenan and Freund's Complete Adjuvant, but only in the presence of subtherapeutic doses of opioids (Deuis *et al.*, 2017).

In addition to the inhibitory NaSpTx described above, a large group of spider toxins preferentially activate Na_V channels through interactions with site 3 in DIV. Classical examples are NaSpTx family 6 toxins PnTx2-5 and PnTx2-6 isolated from the spider *Phoneutria nigriventer* (Matavel *et al.*, 2002), and **Hm1a**, NaSpTx family 2, isolated from the spider *Heteroscodra maculata* (Osteen *et al.*, 2016). These toxins alter the voltage-dependence of activation and inactivation of Na_V channels and probably account for most of the symptoms of envenomation caused by *P. nigriventer* (Matavel *et al.*, 2009). Interestingly, Hm1a displays specific activation of $Na_V1.1$ and has been a unique pharmacological tool to characterize the involvement of $Na_V1.1$ in pain, as previously discussed.

Scorpion toxins. Scorpions peptide toxins (ScTxs) acting on Na_V channels are mostly activators that elicit strong pain. These long-chain peptides are classified into two major groups, α-ScTxs and β-ScTxs. α-ScTxs interact with site 3 at the Na_V α-subunit to slow fast inactivation and prolong channel opening, while β-ScTxs interact with site 4 to alter the voltage-dependence of activation and induce repetitive firing (Bosmans and Tytgat, 2007; Zhang *et al.*, 2011). Within the α-ScTxs, AaHII and OD1 are amongst the best characterized toxins (Figure 3; Table 3). AaHII was isolated from the scorpion *Androctonus australis* and has remarkable preference for $Na_V1.7$ (Martin and Rochat, 1986; Abbas *et al.*, 2013). AaHII alters the voltage-dependence of activation with little effect on the voltage-dependence of inactivation. OD1 is another potent α-ScTx with preference for $Na_V1.4$, $Na_V1.6$ and $Na_V1.7$ (Jalali *et al.*, 2005; Durek *et al.*, 2013). This particular toxin has been used to develop $Na_V1.7$ *in vitro* and *in vivo* assays to rapidly identify and characterize peptides reversing pain behaviours induced by its injection (Cardoso *et al.*, 2015; Deuis *et al.*, 2016b). β-ScTxs elicit similar changes in the Na_V channels as those observed for the α-ScTxs, although with preferential binding to DII loops S1S2 and S3S4. Amongst these toxins, **Css4**, isolated from the scorpion *Centruroides suffusus*, alters Na^+ currents by altering the voltage-dependence of activation and trapping the DII S4 voltage sensor in its outward position (Cestele *et al.*, 1998). Finally, **Cn2**, purified from the venom of *Centruroides noxius*, preferentially activates $Na_V1.6$ (Schiavon *et al.*, 2006) and has helped unravel the role of $Na_V1.6$ channel in different pain pathways (Deuis *et al.*, 2013).

Other peptide Na_V toxins. Sea anemone venoms are another source of Na_V toxins that enhance Na^+ currents by inhibiting channel inactivation and prolonging action potential duration. These toxins share an overlapping binding site with α-ScTxs in the DIV S3S4 loop associated with site 3 (Catterall and Beress, 1978). **ATX-II** from *Anemonia sulcata* (Romey *et al.*, 1976) preferentially activates $Na_V1.1$ and $Na_V1.2$ (Oliveira *et al.*, 2004) and produces persistent and resurgent sodium currents in DRG neurons, but only in the presence of the β4 auxiliary subunit (Klinger *et al.*, 2012). In contrast, anthopleurin B from *Anthopleura xanthogrammica* preferentially activates $Na_V1.5$ (Khera *et al.*, 1995) through an Arg^{12}, Leu^{18} and Lys^{49} pharmacophore essential for activity (Gallagher and Blumenthal, 1994; Dias-Kadambi *et al.*, 1996). Finally, solitary wasps belonging to the family *Pompilidae* produce neurotoxic peptides in their venoms (Konno *et al.*, 1997; Konno *et al.*, 1998). Amongst these peptides, α-PMTX and β-PMTX isolated from *Anopolis samariensis* and *Batozonellus maculifrons*, respectively, are small linear peptides that are structurally distinct from most natural peptidic Na_V toxins (Figure 3B). Interestingly, these peptides activate Na^+ currents by increasing the steady-state currents at $Na_V1.6$ or by slowing $Na_V1.1$, $Na_V1.2$, $Na_V1.3$ and $Na_V1.7$ inactivation currents (Schiavon *et al.*, 2010).

Conclusions and future directions

Ranging from inhibitors to activators of Na_V channels, natural toxins have proven to be a powerful group of molecules for unravelling the role of Na_V channels in pain pathways and as leads towards the development of novel therapies for treating chronic pain. The discovery that $Na_V1.1$, $Na_V1.3$, $Na_V1.6$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ are involved in pain pathways commenced a new era of research for therapeutics targeting these channels. The revelation that humans with mutations causing loss of $Na_V1.7$ function were pain free but otherwise normal (except for loss of olfaction) has led to an expanded effort to find specific inhibitors of this channel with therapeutic potential. TTX has now advanced into human clinical trials for treating chemotherapy-induced neuropathic pain (Wex Pharmaceuticals Inc.), while neosaxitoxin, tested as a blocker of bladder pain syndrome, and gonyautoxin, as blocking chronic tension-type headache, have been shown to be safe and effective in humans (Lattes *et al.*, 2009; Manriquez *et al.*, 2015) (Table 4). In addition, a considerable number of active programmes in pharmaceutical companies have used natural Na_V toxins such as marine toxins (e.g. SiteOne Therapeutics) and spider toxins (e.g. Amgen Inc.) in the development of novel analgesics to treat,

previously untreatable, chronic pain conditions. Gating modifiers are arguably the most promising source of Na_V inhibitors for pain because the binding sites are on the voltage sensors of these channels, which vary amongst Na_V subtypes. In addition to their value in dissecting the role of sodium channels in different types of pain, Na_V modulators have the potential to be a new class of drugs targeting Na_V channels for the treatment of chronic pain.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

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Conflict of interest

The authors declare no conflicts of interest.

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