



**University of
Nottingham**
UK | CHINA | MALAYSIA

Exploring the modulation of the Na_v1.7 voltage-gated sodium channel by a centipede venom peptide



Amy Kerry

BSc

**Thesis submitted to the University of Nottingham
for the degree of Master of Research**

January 2019

Abstract

Voltage gated sodium channels are integral transmembrane proteins, their architecture comprises of pore-forming α -subunits associated with one or more auxiliary β -subunits. $\text{Na}_v1.7$ is a sodium channel subtype expressed in sensory neurones in the dorsal root ganglia and is known to be involved in nociceptive pain. Thus, there is growing interest in animal toxins that target $\text{Na}_v1.7$. Centipedes are among the oldest terrestrial arthropods; minimal clinical significance of stings means they have been overlooked compared to other venomous clades. Encounters with centipedes result in excruciating pain in humans and instant rigid paralysis in prey. Because of the emerging use of animal venoms in novel drug development and as tools to study ion channels, a venom peptide (peptide I) isolated from male *Scolopendra hardwickei* centipede was utilized to investigate the activity against the $\text{Na}_v1.7$ channel. Characterisation of sodium currents expressed by the TE671 cell line using whole-cell patch clamp was initially studied. Sodium currents were completely blocked by tetrodotoxin at a concentration of $1\mu\text{M}$ and with an IC_{50} value of 7.4 nM . To assess the effects of peptide I on $\text{Na}_v1.7$ we analysed sodium current amplitudes at a threshold potential and the voltage dependence of channel activation. Peptide I induced a concentration-dependent hyperpolarising shift in activation, shifting $V_{50,\text{act}}$ from -22.9 ± 3.5 to $-33.9 \pm 0.8\text{ mV}$ at $10\mu\text{M}$. Peptide I isolated from *S. hardwickei* has $\text{Na}_v1.7$ modifying activity resulting in a gain of function of sodium currents. Because of the modulation of the voltage sensitivity of $\text{Na}_v1.7$, it could be speculated that peptide I interacts with the voltage-sensing domain, specifically at the previously characterised site 4 where β -scorpion binds. These results report of a hyperpolarising shift in channel activation, a feature undocumented in centipedes. This novel feature provides a starting point for further investigations into other modulating peptides in centipede venom and the location of their binding sites. Unravelling the mechanism behind venom peptides prompts the discovery of novel drugs and pesticides.

Acknowledgments

A huge thank you to my supervisor Dr Ian Mellor for the unconditional support and patience over the past year. It has been an honour to study at the University of Nottingham, and even more so under his guidance and expertise. I am so grateful for the advice during difficult times, without him this work would not have been possible.

I wish to thank Neville Ngum for acting as a second supervisor. For his patience whilst teaching me the patch clamp technique, and for always replying to my texts when something went wrong. I appreciate all the help he has provided and for paving the way for this research to take place. I wish him the upmost happiness and success.

I wish to thank the electrophysiology lab for spurring me on during countless hours in the lab; Muhamad Yusran Abdul Aziz, Mahmoud Sherif, Maria Koromina and Dr Rohit Patel. I wish you all the best for the future.

Another thank you to my mum, who believed I could do it from day one.

Lastly, I thank my other half for putting up with all the tears and the meltdowns during this time. Thank you for distracting me from sodium channels.

Abstract.....	i
Acknowledgments.....	iv
List of contents	v
List of figures	v

Introduction

1.1 Ion channels.....	1
1.1.1 The action potential : basis for electrical signalling.....	1
1.1.2 Electrophysiology.....	3
1.2 The voltage-gated sodium channel.....	4
1.2.1 Gating.....	7
1.2.2 Voltage-dependence of activation.....	7
1.2.3 Inactivation.....	9
1.2.4 Deactivation.....	12
1.3 Na_v1.7.....	12
1.4 The insect sodium channel.....	12
1.5 Ion channel modulators.....	13
1.5.1 Toxins.....	13
1.5.2 Therapeutic potential of toxins and venom peptides.....	15
1.6 Neurotoxin binding sites.....	16
1.7 The contribution of Na_v1.7 in pain.....	19
1.7.1 Gain-of-function mutations.....	19
1.7.2 Loss-of-function mutations.....	21
1.8 Centipedes.....	21
1.8.1 Clinical importance of centipede stings.....	23
1.8.2 Pharmacological importance of centipede venom.....	24
1.8.3 Centipede venom as an ion channel modulator.....	24
1.9 The Rhabdomyosarcoma TE671 cell line.....	28
1.10 Aims and objectives.....	29

Materials and methods

2.1	Previous research.....	30
2.2	Venom collection and fractionation.....	30
2.3	Peptide identification and structural modelling.....	30
2.4	Cell culture: TE671 human rhabdomyosarcoma cells.....	31
2.5	Electrophysiological measurement of whole-cell patch clamp.....	31
2.6	Application of toxins to TE671 cells.....	33
2.7	Voltage protocols.....	34
2.7.1	Voltage protocol 1: Current-voltage relationship.....	34
2.7.2	Voltage protocol 2: Steady-state inactivation.....	35
2.7.3	Voltage protocol 3: Continuous step depolarisation.....	36
2.8	Data acquisition and analysis.....	36
2.8.1	Analysis of channel activation.....	36
2.8.2	Analysis of channel inactivation.....	38

Results

3.1	Characterisation of voltage-gated sodium channels in TE671 cells.....	39
3.1.1	Activation of sodium channels.....	39
3.1.2	Inactivation of sodium channels.....	41
3.2	Pharmacological characterisation of VGSCs in TE671 cells.....	41
3.2.1	TTX sensitivity.....	42
3.3	Effects of peptide I on Na _v 1.7 whole current kinetics.....	44
3.4	Effects of peptide I on the voltage-dependence of activation in TE671 cells.....	46
3.5	Peptide sequencing, synthesis and structural modelling of peptide I.....	48

Discussion

4.1	Characterisation of TE671 cell VGSCs.....	52
4.2	The effects of peptide I on Na _v 1.7 channel activation.....	53
4.3	Potential applications of peptide I.....	56
4.4	Future work.....	58
References		59

List of figures

Figure 1.1: Schematic representation of the generation of an action potential.....	2
Figure 1.2: Schematic representation of the structure of voltage-gated sodium channel subunits.....	6
Figure 1.3: Architectures of Na _v Pas.....	6
Figure 1.4: Representation of the sliding helix or helical screw model.....	8
Figure 1.5: Representation of the route of gating between activated and inactivated states.....	11
Figure 1.6: Predicted locations of neurotoxin binding sites on the α -subunit.....	16
Figure 1.7: Schematic representation of GMTs mechanism	19
Figure 1.8: Schematic representation of the known mutations of Na _v 1.7	20
Figure 1.9: Illustration of the phylogeny of centipedes.....	22
Figure 1.10: Male venom fractions of <i>S. hardwickei</i> on the voltage-dependence of activation.....	23
Figure 1.11: Female venom fractions of <i>S. hardwickei</i> on the voltage-dependence of activation.....	27
Figure 1.12: Amino acid sequence for peptides identified in M61-72 cocktail.....	27
Figure 1.13: TE671 cells at X200 magnification.....	28
Figure 2.1: Schematic representation of patch-clamp setup.....	32
Figure 2.2: Schematic illustration of whole-cell configuration.....	33
Figure 2.3: TE671 cells showing perfusion tube adjacent to patch-clamped TE671 cell.....	34
Figure 2.4: Schematic illustration of voltage protocol 1.....	35
Figure 2.5: Schematic illustration of voltage protocol 2.....	35
Figure 2.6: Schematic illustration of voltage protocol 3.....	36
Figure 3.1: Characterisation of the voltage-dependence of activation A) Normalised peak current-voltage relationship B) Conductance-voltage relationship C) Family of TE671 sodium currents.....	40
Figure 3.2: Voltage-dependence of inactivation.....	41

Figure 3.3: A family of TE671 sodium currents blocked by TTX.....	42
Figure 3.4: Concentration-inhibition curve for the effect of TTX on TE671 sodium currents.....	43
Figure 3.5: Effects of peptide I on Na _v 1.7 currents.....	44
Figure 3.6: Traces of the effects of peptide I on Na _v 1.7 currents.....	45
Figure 3.7: Effects of peptide I on voltage-dependent activation.....	47
Figure 3.8: Predicted model structure of peptide I.....	50

List of tables

Table 3.1 Mean time-to-peak values ($\pm$ SEM) before and after 10 nM peptide application.....	45
Table 3.2: V _{50.act} values in the absence and presence of peptide I.....	48

Introduction

1.1 Ion Channels

Electrical signalling between cells begins with the uneven distribution of charged ions across an impermeable cell membrane. Successful movement of ions across a membrane is facilitated by ion channels imperative for electrical signalling (Hille, 1992). Voltage-gated and ligand-gated ion channels are major super families of membrane proteins that have key roles in the generation, propagation and regulation of signalling in excitable cells (Keynes, 1994). Voltage-gated ion channels are sensitive to changes in the cell membrane potential and include voltage-gated sodium, calcium and potassium channels that are all essential for neurotransmission (Catterall, 1995). Sodium channels were possibly the first of the ion channels to be well defined in terms of understanding their structure and function (Yu and Catterall, 2003). Ligand-gated ion channels open in response to neurotransmitters released from nerve terminals, for example the nicotinic acetylcholine receptor that is found on the post synaptic membrane at many synapses and neuromuscular junctions. After G protein-coupled receptors, ion channels are the second largest drug targets.

1.1.1 The action potential: basis for electrical signalling

Action potentials are electrical messages carried along the axons of neurones, representing transient changes in the resting membrane potential of neurones. A resting membrane potential (RMP) exists across the membrane before excitation and is largely maintained by the outward concentration gradient of K^+ and inward gradient of small anions. Leak potassium channels are constitutively open so K^+ move out of the cell generating a negative potential inside that cannot be neutralised by outward movement of anions. Ion pumps ensure the correct distribution of ions intracellularly and extracellularly, for example the sodium-potassium pump responsible for re-establishing the driving force of Na^+ and K^+ outside and inside the cell respectively. At rest, the membrane potential of cells ranges between -40 mV to -90 mV. Voltage-gated sodium channels (VGSCs) and voltage-gated potassium channels (VGPCs) are transmembrane proteins involved in action potential generation and propagation.

Neurones must overcome an 'all-or-nothing principle' in order to achieve an action potential. This means sufficient excitation must be achieved to depolarise the membrane, meaning the threshold potential has been reached. With sufficient depolarisation, VGSCs will open and Na^+ will flow into the cell such that – the membrane becomes even less negative. Within 1 ms the VGSCs activate then become inactivated. An efflux of K^+ through the slower activating VGPCs repolarises the cell allowing its return to its resting membrane potential in preparation for this process to repeat. The result is a wave of depolarisation, the action potential that is continued to the nerve terminal, resulting in the activation of voltage-gated calcium channels and the release of a neurotransmitter.

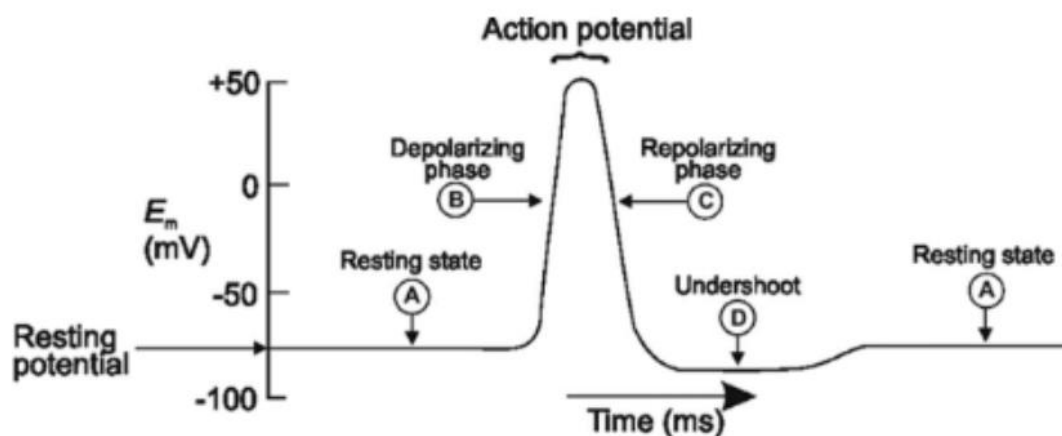


Figure 1.1 Schematic representation of the generation of an action potential. (A) the resting membrane potential is typically about -80mV. If the membrane depolarises to around -50mV, reaching the critical threshold potential, this stimulates VGSCs to open causing an Na^+ influx, responsible for the rising phase of an action potential (B). The inside of the cell becomes more positive causing the inactivation gate of VGSCs to close to terminate the influx of Na^+ . (C) voltage-gated potassium channels open to allow an efflux of K^+ , this contributes to the falling phase of the action potential. (D) potassium channels inactivate, and the membrane returns to its resting membrane potential. Taken from Davies et al (2007).

1.1.2 Electrophysiology

Electrophysiology is concerned with the study of electrical properties of excitable cells. The emerging importance of voltage-gated ion channels has shaped electrophysiology as an essential tool in studying ion channel activity. Hodgkin and Huxley introduced the importance of voltage-gated ion channels and their role in action potential generation in the 1950s. Electrical signals are the result of changes in ion permeability in cellular membranes. Electrophysiological techniques measure the ionic current or the resultant potential difference across the cell membrane of excitable cells (Hille, 2001). The two-electrode voltage clamp (TEVC) technique was improved by Hodgkin, Huxley and Katz on the giant squid axon. One intracellular electrode clamps the membrane potential and the other injects currents to achieve this. However, TEVC is dependent on large enough cells such as *Xenopus* oocytes and the squid giant axons. This is because cells must withstand insertion of two intercellular glass microelectrodes. Neher and Sakmann (1976) developed the patch-clamp technique for studying biological membranes of smaller cells or patches of them. This technique investigates kinetic behaviour of whole-cell and single channel currents. Unlike TEVC, the patch-clamp technique can measure whole cell and single ion channel currents, using a single glass microelectrode placed at the surface of the membrane to clamp membrane potential whilst injecting current. This technique requires direct accessibility of cell membranes for the recording pipette. A single glass microelectrode is filled with an intracellular fluid solution and tightly sealed onto the membrane to isolate the membrane. Measuring whole-cell currents requires applying a small suction pressure to the patched area through the microelectrode in order to break the membrane in the electrode tip. The seal has a high resistance (gigaohms) preventing ion leakages and hence enables much better signal-to-noise ratio. The technique can measure and record ion currents through single ion channels in small patches of cell membranes whilst controlling the transmembrane voltage. The patch clamp technique permits exploration of the physiology of ion channels otherwise inaccessible to voltage-clamp techniques. Current (I) is the measurement of ionic flow of charged ions or electrical charge across a cell membrane in amperes. Following Ohm's law, current is directly proportional to voltage (V) and inversely proportional to

resistance (R). Conductance (G) measures how easily electrical charge flows from A to B.

$$I = V/R$$

Where I is current, V is voltage and R is the membrane resistance. Opening of ion channels lowers the membrane resistance so that for a constant V (clamped membrane potential), I will increase. Electrophysiological experiments grant access to the pharmacological properties of ion channels essential in the understanding of toxins and drugs.

1.2 The voltage-gated sodium channel

Hodgkin and Huxley pioneered the direction of VGSC research in a series of experiments using the squid giant axon. The importance of sodium and potassium ions was recognised in achieving action potential during neurotransmission. In turn, the biphasic nature of an action potential was highlighted by the discovery of sodium channels being key in the rising phase of an action potential (Hodgkin and Huxley 1952a). VGSCs are integral transmembrane proteins that regulate electrical excitability and are responsible for the initiation and propagation of action potentials in excitable neuronal, cardiac, skeletal and smooth muscle tissue. They are composed of a pore-forming α -subunit of approximately 2000 amino acids with associating auxiliary β -subunits of 33 kDa. Affinity column purification of the sodium channel protein using tetrodotoxin and α -scorpion toxin elucidated a 270 kDa protein from the electroplax of the *Electrophorus electricus*, corresponding to a glycosylated α -subunit (Catterall, 2012). This led to Noda et al (1984) revealing the first complete amino acid sequence for the α -subunit using cDNA cloning and sequence analysis for the electric eel electroplax. Deducing the amino acid sequence gave insight into the primary structure of voltage-gated ion channels. The α -subunit comprises four homologous domains (I-IV) each containing six transmembrane helices (S1-S6) and each segment connected by a hydrophilic intervening sequence. Between each S5 and S6 segment is a longer sequence containing a membrane re-entrant loop known as the pore-loop or P-loop. Each segment is responsible for the structural and functional properties of the VGSC. α -subunits form the ion pore and can function in the absence of the modulatory

subunits. More specifically, S5, the P-loop and S6 determine the structure and functional properties of the channel including selecting sodium ions and channel gating. Favre et al (1996) showed that from each domain, the P-loops form the selectivity filter consisting of aspartic acid 400 from domain I, Glutamic acid 755 from domain II, Lysine 1237 from domain III and Alanine 1529 from domain IV (DEKA motif). Segments S1-S4 from each domain form the voltage-sensor that detect changes in voltage across the membrane. The α -subunits in vertebrates are the products of 9 genes leading to the subtypes Nav1.1-1.9. Subtypes Nav1.1, 1.2, 1.3 and 1.6 are dominantly expressed in the central nervous system, Nav1.4 in skeletal muscle, Nav1.5 in cardiac muscle and Nav1.7, 1.8 and 1.9 in the peripheral nervous system. In concordance with this, mutations within the genes encoding these subtypes lead to disorders such as epilepsy (Nav1.1, 1.2, 1.3 and 1.6), muscle weakness/stiffness (Nav1.4), various cardiac disorders (Nav1.5) and sensory disorders including pain (Nav1.7, 1.8 and 1.9). Studies characterising the β -subunits have given a family of four β -subunits (Catterall, 2012). β 1 and β 3 associate non-covalently with the α -subunit whereas the β 2 and β 4 are associated covalently by disulphide bonds to the α -subunit (Vilin and Ruben, 2001). β -subunits affect channel function through modulation of channel and localisation (Catterall, 2000). Despite the importance of both subunits, α -subunits can be functionally expressed in *Xenopus* oocytes resulting in sodium currents that inactivate more slowly than when co-expressed with β -subunits (Deuis et al., 2017). Cryo-electron microscopy has recently determined the first atomic structure of a eukaryotic Nav from the American cockroach (NavPas) (Yan et al., 2017). Shen et al (2017) purified recombinant proteins for a Nav channel NavPas. The determined structure of a eukaryotic Nav channel reveals more complex information about Nav than was previously known. On the other hand, NavPas faces issues when expressed in heterologous systems. The residues essential for fast inactivation of Nav are replaced with Ala/Thr/Asp in NavPas (Yan et al., 2017). What is known about Nav channel is based on the crystal structures of Kv channels, and then bacterial Nav. Structure elucidation has been achieved through the breakthrough of electron microscopy, further insights into the molecular basis of activation, inactivation and toxin binding are now achievable, otherwise hindered by x-ray crystallography.

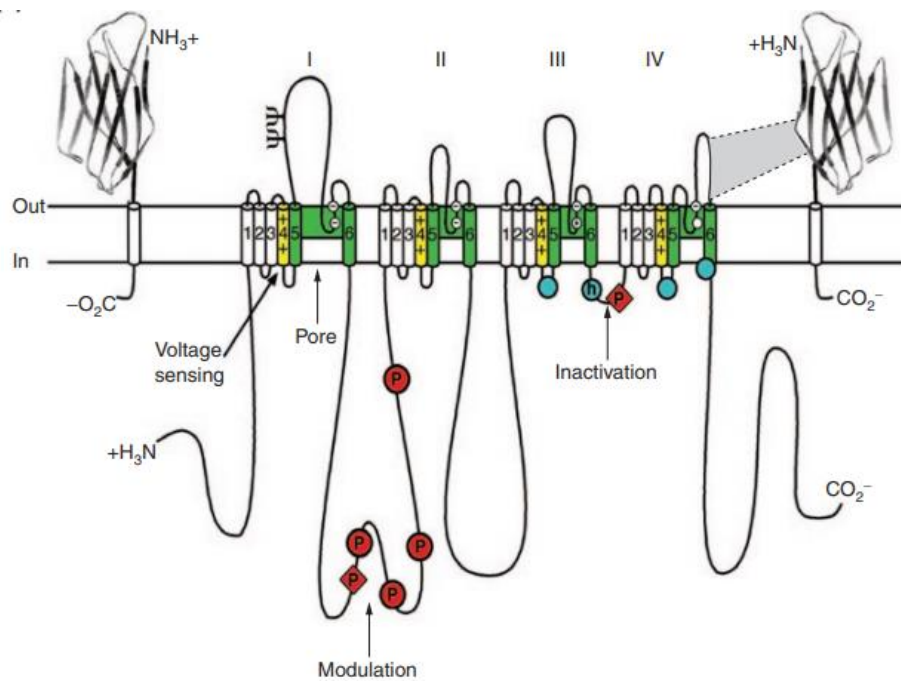
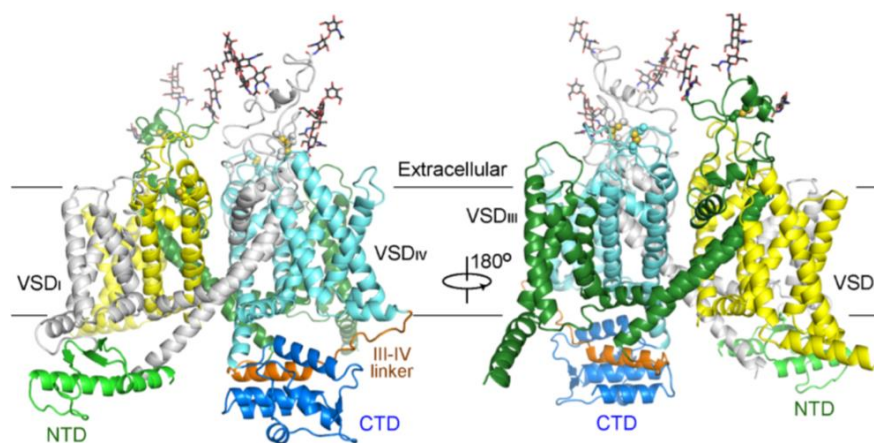


Figure 1.2 Schematic representation of the structure of voltage-gated sodium channel subunits. The extracellular domains of $\beta 1$ and $\beta 2$ subunits are shown as immunoglobulin-like folds which interact with the loops in the α -subunits. I-IV represent the domains in the α -subunits; segments 5 and 6 (in green) line the pore and the S4 helices (yellow) make up the voltage sensors. The intracellular loops of domains III and IV show blue circles which represent the inactivation gate IFM motif and its receptor. Taken from (Yu and Catterall, 2003).

(a)



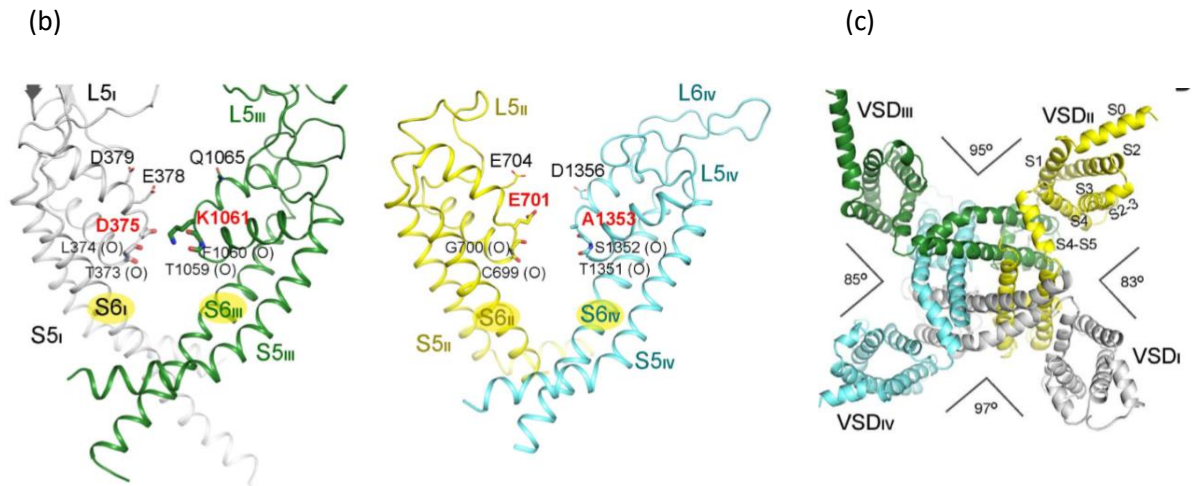


Figure 1.3 (A) Architectures of NavPas. The colours represent the domains, glycosyl and disulfide bonds are shown as sticks and spheres. (B) The selectivity filter of NavPas. The SF vestibule is enclosed by the side groups of Asp/Glu/Lys/Ala (DEKA) and carbonyl oxygen atoms of the two preceding residues in each repeat. The residues that comprise the negative region above the SF vestibule are shown. (C) The four VSDs of NavPas exhibiting distinct conformational states. (Shen et al., 2017)

1.2.1 Gating

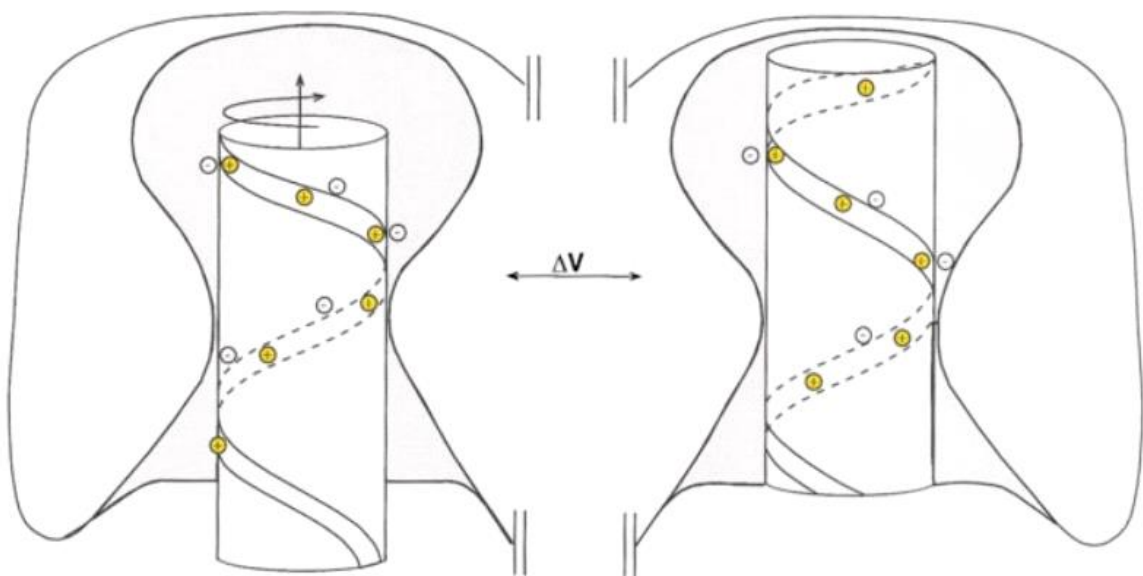
The term gating refers to the conformational changes that alters the functional state of channels resulting in the opening and closing of the channel. Channels exist in three states: resting, activated and inactivated. These states are maintained by the movement of gating charges. During the process of gating VGSCs cycle between conducting and non-conducting states. Gating currents are small transient currents that occur by movement of gating charges.

1.2.2 Voltage-dependent activation

Depolarisation of the membrane permits the influx of sodium ions through the channel. A change in membrane potential to a less negative one activates the channel; during this time the VGSC protein may become permeable to sodium ions. Gating charges mediate the movement of what is termed the "activation gate" into an open state (Armstrong, 2006). The detection of the less negative membrane potential and the mechanism of movement from resting to activated is governed by the S4 voltage sensors. The S4 segment in each domain contains four to eight repeated motifs of a

positively charged amino acid residue (arginine or lysine) followed by two hydrophobic residues (Armstrong, 2006), creating a helix with a high density of positive (gating) charges. These are highly conserved across all Nav subtypes and in voltage-gated potassium and calcium channels. At resting membrane potential, the S4 are held at a relatively intracellular position by the internal negative potential. Upon depolarisation of the membrane potential S4 gating charges move the S4 segment into a more extracellular position. Catterall (1986) proposed the arrangement of these segments are organised on an α -helix as a spiral ribbon of positive charges. The sliding helix or helical screw model (Guy and Seetharamulu, 1986) describes gating as an outward movement of the positively charged residues supported by the spiral arrangement of positive charges. The difference in membrane potential intracellularly and extracellularly pushes the positive charges outward, rotating the S4 helix 60° allowing each positive charge to pair up with fixed negative charges initiating structural changes which opens the pore (Keynes and Elinder, 1999). This model proposes that the gating charges are stabilized by forming ion pairs with negatively charged residues in the surrounding S1-S3 helices. The outward movement of the S4 segment is catalysed by exchange of these ion pair partners (Figure 1.4) (Catterall, 2012).

Figure 1.4 Sliding-helix model of gating. (Left) S4 segment illustrated as an α helix with a ribbon carrying gating charges (Right) positive charges around the cylinder are



neutralized by negative charges in the surrounding transmembrane segments. A change in membrane voltage cause the S4 segment to move so that gating charges exchange ion pair partners. Taken from Catterall (1988).

The movement of S4 helices is proposed to translate into channel opening through rotation of the S4-S5 linkers that form a cuff around the intracellular ends of the S6 helices. At rest the S6 helices converge at the intracellular end of the pore, squeezed together by the S4-S5 cuff. Once the S4-S5 linkers rotate the S6 helices are allowed to move outwards from the pore axis and permitting sodium ions to permeate. Conserved glycine residues at the midpoint of the S6 helices provide a hinge to facilitate this movement (Long et al., 2007). This mechanism of activation is inferred from potassium channel crystal structures.

1.3.3 Inactivation

The process of inactivation closes the channel during depolarisation of the cell membrane, inhibiting sodium conductance and allowing repolarisation of the membrane; only then can the channel recover from inactivation. During this time the channel is in a non-conducting, inactivated state. It is crucial VGSCs inactivate in order to maintain and regulate the excitability of neurones (Cervenka et al., 2018). Several independent components of inactivation have been identified including fast, slow and ultra-slow inactivation. Perhaps the most important component essential for basic functioning and action potential shaping is the fast component. The fast inactivation gate of the sodium channel was a component of the VGSC to be identified experimentally (Catterall, 2000). Hodgkin and Huxley proposed the first model of channel activation and inactivation for the potassium channel. The basis of their model focused on the independent process of the four particles involved in the transition from a resting state to an open state (Bezanilla, 2000). Fast inactivation typically develops within a few milliseconds and lasts for only 10-15 milliseconds, the channel remains inactive during the 'refractory period' a time where the channel is able to recover from the stress of opening. The mechanism underlying inactivation in VGSCs is unique in nature, conserved to only this type of voltage-gated ion channel (Carnevale and Klein, 2017). The underlying molecular mechanism of inactivation involves a structural rearrangement mediated by an intracellular cytoplasmic linker

connecting domains III to IV of the α -subunit. This cytoplasmic linker termed an inactivating blocking particle folds into the channel, forming an inactivation gate at the intracellular mouth of the pore. The structure of the inactivation particle was first described by Rohl et al (1999) on the basis of a 53 amino acid peptide. A pair of glycine residues provide a hinge region allowing the peptide to fold inwards causing a block of the channel during inactivation, known as the “hinged-lid” mechanism (Catterall, 2012). The binding site or receptor for the “hinged-lid” is within domain III and IV between the S4-S5 segments (Cervenka et al., 2018). The ‘hinged-lid’ interacts with the pore and affects ion conductance (Carnevale and Klein, 2017). To maintain the closure of the inactivation gate requires the amino acid motif IFM (Isoleucine, phenylalanine and methionine) (Catterall, 2012). These amino acids are likely to be docking with the internal pore. The idea of the IFM motif is supported by mutagenesis studies; mutating the IFM motif with three glutamine residues completely removes fast inactivation (West et al., 1992). The IFM motif serves as a pore blocker, cysteine substitutional studies have showed glycine and proline could act as hinges to aid in the closure of the inactivation gate (Kellenberger et al., 1997).

Mutations in the S6 segment in domain IV have also been shown to affect inactivation. Cervenka et al (2018) applied cysteine mutagenesis studies to showing how replacing cysteine’s in DIVS6 residues in rNa_v1.4 channels can either increase or decrease the likelihood of entering the fast-inactivated state. Ulbricht (2005) reported DIV-S4 showing a significant effect on inactivation; studies mutating the arginine residues in the S4 segments are known to affect inactivation suggesting strong coupling between activation and inactivation. VGSCs will either rapidly inactivate or inactivate a lot slower upon depolarization, indicating two kinetic classes of inactivation; fast and slow. Fast inactivation occurs within a single action potential and is quickly reversed permitting VGSCs to reactivate (Vilin and Ruben, 2001; Payandeh et al., 2012).

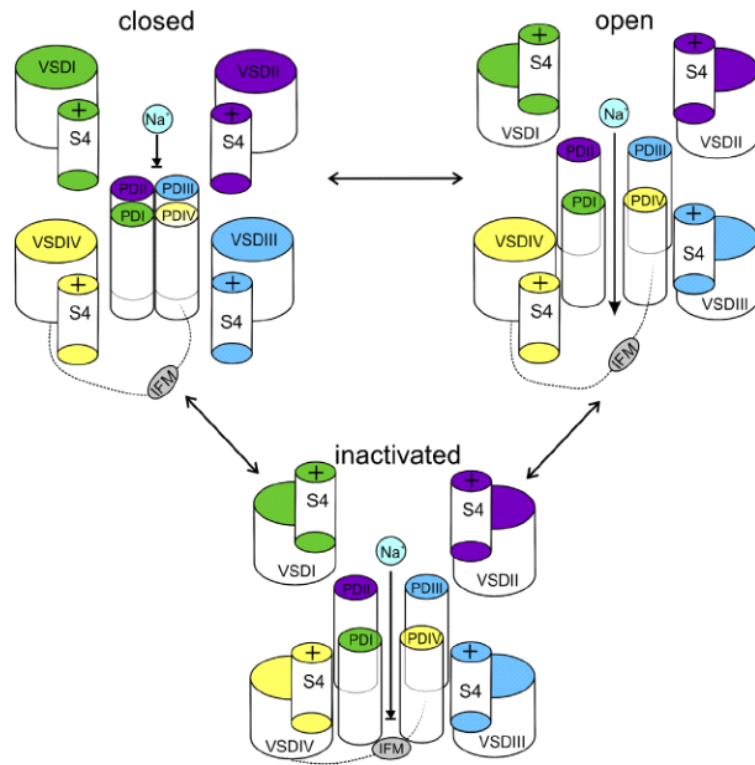


Figure 1.5 Schematic representation of the route of gating between activated and inactivated states. Na_v exist in three distinct states: resting (closed), open and inactivated. All four VSD are in their intracellular position during their resting closed state. Movement of the DI-DIII VSDs changes channel conformation (within the pore domains) and allows the pore to become more permeable to sodium ions. Movement of DIV VSD brings the IFM motif on the third intracellular loop between DIII and DIV, and this inactivates the channel. Taken from Deuis et al (2017).

Another process of inactivation is slow inactivation, distinguishable from fast inactivation as it does not involve the III-IV inactivation particle. Slow inactivation is the result of prolonged (60-90s) depolarisation's which can accumulate over brief depolarizations or over a span of tens of seconds (Vilin and Ruben, 2001). Studies have shown the kinetic differences between fast and slow inactivation; removal of fast inactivation with pronase, trypsin and a chymotrypsin has no effect on slow inactivation (Rudy, 1978; Starkus and Shrager, 1978; Valenzuela and Bennett, 1994). One hypothesis of slow inactivation suggests structural rearrangement of the pore similar to that of C-type inactivation in potassium channels (Goldin, 2003). An even slower process of ultra-slower inactivation, entry into this state of inactivation is

inhibited by binding of the fast inactivation particle. This process has been observed in Na_v1.4 by replacing alanine at position 1529 with aspartate in domain IV P-loop (Goldin, 2003). Mutating the IFM motif removes fast inactivation, leaving slow inactivation intact. The molecular basis of slow inactivation remains unclear, it could be suggested slow inactivation involves conformational rearrangements of the pore region (Vilin and Ruben, 2001).

1.2.4 Deactivation

The process of deactivation occurs once the cell membrane repolarises and involves resetting of the voltage sensors (S4) back to their intracellular location. As inactivation is also voltage-dependent this also is reversed and once complete the VGSC has returned to its resting state. At rest, the 'activation gate' is closed and fully deactivated, so depolarization can now reopen the activation gate.

1.3 Na_v1.7

Na_v1.7 is highly expressed in the dorsal-root ganglion neurones and sympathetic ganglion neurones, although originally cloned from human neuroendocrine cells (Klugbauer et al., 1995). Research has shed much light on the involvement of Na_v1.7 in pain, receiving focus in the development of novel pain therapies. Dysregulated expression of this VGSC subtype is associated with chronic pain and inherited pain disorders. Na_v1.7 produces fast activating and inactivating currents and recovers slowly from fast inactivation, activating in response to low-amplitude depolarizations below the threshold for an action potential to arise (Dib-Hajj et al., 2007). The electrophysiological properties of mutant Na_v1.7 show increased excitability. Mutant Na_v1.7 display a reduced current threshold and increased firing of pain signals, manifesting into pain.

1.4 The insect sodium channel

The genomic sequence of the fruit fly *Drosophila melanogaster* reported two putative sodium channel genes, *DSCI* and *para*. Both are highly homologous to the rat brain sodium channels, for example, *para* has 61% homology to rat brain sodium channel Na_v1.1 and 67% to Na_v1.2 (Dong, 2014; Zlotkin, 1999). Initially *DSC1* was assumed to

encode a sodium channel due to the similarity of the amino acid sequence of this gene with vertebrate VGSCs, but this gene was actually found to encode a calcium channel. The homology in their amino acid residues means that insect and mammalian VGSCs share many pharmacological and structural properties, for example, conservation of the voltage sensors, the selectivity filter (DEKA) and only a minor alteration in the inactivation motif (MFM vs. IFM in vertebrates). The insect genome contains only one functional Na_v channel α -subunit encoding gene (*para*), so diversity is achieved through extensive alternative splicing at 13 sites (in *Drosophila*). The *para* VGSC associates with an auxiliary-regulatory subunit encoded by temperature-induced paralysis (*tipE*) mutation locus. Located on chromosome 3L the *tipE* locus produces a two-domain membrane protein which enhances *para* sodium channel expression and function. *Para* constitutes the crucial residues for activation, inactivation and ion selectivity (Dong 2007). In turn, Zlotkin (1999) reported that co-expression of *para* and *tipE* was found to increase the inactivation rate and current amplitude in *Xenopus* oocytes without affecting voltage dependence of sodium currents.

1.5 Ion channel modulators

1.5.1 Toxins

A neurotoxin is a substance that can have structural and functional consequences on the nervous system. Animals rely on chemical, enzymatic and/or peptidic toxins as defensive or predation strategies often evolving a cocktail of compounds to target prey. Peptide toxins are small in nature, made up of 16-78 amino acid residues, and usually contain multiple disulphide bonds that are imperative for activity (Kwong and Carr, 2015). Prey immobilisation relies on toxins that are able to target multiple voltage-gated and ligand-gated ion channel isoforms. Importantly, toxins can distinguish between ion channel subtype, for example, differentiating between muscular and neuronal sodium channels. Catterall's pharmacological approach identified how neurotoxins modulate VGSCs, altering the functionality of channels through effects on gating and permeability by altering voltage dependence of activation and inactivation or by blocking the pore (Catterall, 2012). The vast majority of toxins that target Na_v modify the process of gating by trapping the channel in a functional state via interactions with one or more VSD. Toxins are imperative tools to

understand the transitions between open and closed states. Pharmacological studies utilizing toxins have provided insight into the structure and function of VGSCs. Neurotoxin binding sites located on VGSCs have helped to classify their effects; Na_v activators mediate activation by reducing the membrane potential at which channels can be activated. Gain of function can also be achieved through prolonging the open state of the channel by inhibiting inactivation. Some toxins are inhibitors of Na_v by inhibiting activation (shifting the activation voltage in the positive direction) or decreasing Na^+ current (Catterall, 2012). Extremely potent toxins can directly block the pore often by inserting a lysine or arginine side chain into the selectivity filter region from the extracellular side of the channel (Gordon, Chen and Chung, 2013). Tetrodotoxin (TTX) is derived from the puffer fish, and completely abolishes sodium currents through occluding the pore, acting from the extracellular side of the membrane and blocking ion conductance. Narahashi (1964) identified TTX as a selective inhibitor of sodium channels, leaving potassium conductance untouched. TTX mimics the state of a hydrated sodium cation and binds electrostatically to the opening of the VGSC pore. Notably, TTX was used to purify VGSC protein that led to its cloning and sequencing, and, along with saxitoxin (STX), helped to identify the selectivity filter and outer pore (Catterall, 2000). TTX is highly selective for CNS ($\text{Na}_v1.1$, 1.2, 1.3 and 1.6) and skeletal muscle ($\text{Na}_v1.4$) VGSC isoforms, but has much less effect on cardiac muscle ($\text{Na}_v1.5$) sodium currents (Baer et al., 1976). In the PNS it is highly selective for $\text{Na}_v1.7$ over $\text{Na}_v1.8$ and 1.9. This has led to the classification of TTX-sensitive (TTX-S; $\text{Na}_v1.1$ -4 and 1.6-7) and TTX-resistant (TTX-R; $\text{Na}_v1.5$ and 1.8-9). Toxins that target the VSD demonstrate greater selectivity over Na_v subtypes than pore blockers (Shen et al., 2018). The understanding of the molecular mechanism of these toxins let down by the lack of structural information. Although bacterial Na_v crystal structures have shed light on mechanical structures, the problem lies with the insensitive nature of homotetrameric prokaryotic orthologs to TTX and STX. This is because the receptor site is only found in eukaryotes (Shen et al., 2018).

Ion channel research has extensively studied the 5S animals (snakes, spiders, scorpions, cone snails and sea anemones) because of their ability to target numerous ion channel subtypes. α -scorpion and β -scorpion toxins are both renowned for their

interaction with VGSCs. Known as gating modifying toxins (GMTs) α and β scorpion toxins contain 60-80 amino acids, linked together by four disulphide bridges. While both toxins cause gain of function, α -scorpion toxins bind at receptor site 3 and inhibit channel inactivation whereas β -scorpion toxins bind to site 4, shifting voltage-dependent activation toward more hyperpolarizing potentials (Deuis et al., 2017). The α -scorpion toxin receptor site between extracellular segments of domains I and IV allows the toxin to associate with the S4 gating charges. It could be said that α -scorpion toxin prevents the coupling of activation gating charge movement with inactivation. Early studies suggested the involvement of the S3-S4 extracellular loop in the binding of GMTs. It is sensible to suggest that GMTs associate with the S1-S2 loop and position within the extracellular facing cavity between the S1 and S4 helices. This mechanism allows the GMT to act as a wedge, locking the voltage-sensor.

Shen et al (2018) recently reported the cryo-EM structure of an insect sodium channel Na_vPaS in complex with Dc1A, a gating modifying toxin from the desert bush spider. Dc1a inserts into the extracellular cavity between the second VSD, interactions between Dc1a and Na_vPaS are polar and hydrophobic in nature, involving the S1-S2 loop, extracellular poor loops and the third repeat of the S5-pore domain helix. Interestingly, this study confirms the lack of interaction with the S3-S4 loop and Dc1a.

1.5.2 Therapeutic potential of toxins and venom peptides

Notably, venomous animal toxins are the forefront of research into therapeutic applications. Over time, venomous animals have evolved a rich and diverse cocktail of toxins to take down prey. In order to be successful, these toxins must efficiently target multiple areas of the nervous system. Selectively targeting neuronal and muscular sodium channels puts venom peptides beyond the capabilities of current sodium channel blockers on the market today. Subtype specificity is the key to develop a drug that induces fewer side effects. Protoxin II (ProTx-II) isolated from the Peruvian green velvet tarantula, *Thrixopelma pruriens*, selectively blocks $\text{Na}_v1.7$ with approximately 100-fold increase in potency over other Na_v subtypes and so could be relevant for analgesia (Kwong and Carr, 2015). At present, the only available peptide toxin on the market is ziconotide (Prialt) developed from ω -conotoxin MVIIA for the treatment of severe chronic pain. It inhibits N-type voltage-gated calcium channels when

administered intrathecally (Kwong and Carr, 2015). Classic biochemistry techniques of bioassay-guided fractionation and sequencing of purified peptides scratches the surface of venom characterization. Advances in cDNA libraries and mass spectrometry make it possible to give an in-depth analysis of the diversity of peptidic toxins. The development of sodium channel blockers is undermined to some extent by the lack of clarity in the crystal structure of eukaryotic sodium channels. But key to the development of therapeutics is the identification of neurotoxins with strong subtype selectivity.

1.6 Neurotoxin binding sites

Sodium channel toxin derived modulators include synthetic derivatives and natural toxins acting on α -subunits extensively across the channel protein.

Neurotoxins act on 6 different sites on VGSCs, either occluding the pore or modifying gating. Toxin classification is based on binding site location on the channel as well as the biophysical modulation that the toxins exhibit (Catterall, 2000).

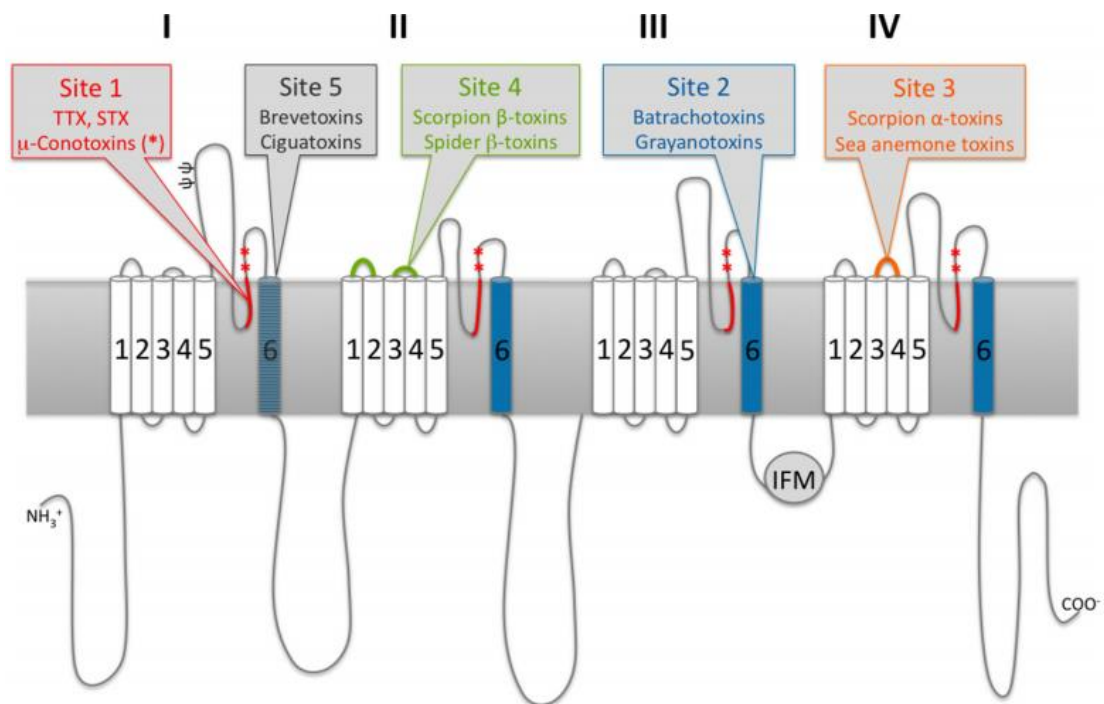


Figure 1.6 Schematic representation of the α -subunit of voltage-gated sodium channels and neurotoxin binding sites. Taken from Stevens, Peigneur and Tytgat (2011).

Site 1 binds the non-peptidic neurotoxins TTX and STX. Site 1 binding site is formed by amino acids on the pore and the selectivity filter located on the extracellular end of the pore (Bagal et al., 2014; Amey et al., 2015). TTX enters the extracellular side of the pore, as well as TTX and STX both toxins generate electrostatic interactions with the residue within the electrostatic region. This interaction attracts cation into the selectivity filter involving the Asp and Glu residues in the DEKA motif. Interactions cease the flow of ions and prevent the conductance of sodium ions and thus abolish sodium currents (Shen et al., 2018). Site directed mutagenesis using brain sodium channels established the composed receptor site as two rings of amino acid residues on the N-terminal side on the P-loops connecting S5 and S6 of all four domains. TTX blockage divides the VGSC family into TTX-S ($\text{Na}_v1.1, 1.2, 1.3, 1.4, 1.6, 1.7$) and TTX-R ($\text{Nav}1.5, 1.8, 1.9$).

μ -conotoxins are unique peptidic toxins derived from marine cone snails, known to target binding site 1 on Na_v because they act as pore blockers. μ -conotoxins have demonstrated higher Na_v subtype specificity than TTX and STX. Pan et al (2019) report the cryo-electron microscopy structures of human $\text{Na}_v1.2$ bound to the μ -conotoxin KIIIA. KIIIA is able to efficiently select $\text{Na}_v1.2$, previously, Zhang et al (2007) showed $\text{Na}_v1.2$ from rats is extensively blocked by KIIIA. Whereas in the presence of $\text{Na}_v1.5$ shows less blockage in the presence of KIIIA. This study reveals KIIIA is complementary to the cavity where the bottom tip of KIIIA plugs into. Further molecular dynamics simulation of a cockroach voltage-gated sodium channel Na_vPas suggests KIIIA occupies the outer pore of the selectivity filter. Lys⁷ constitutes the bottom tip of KIIIA, its side chain extends and interacts with the sodium coordinating Glu⁹⁴⁵. The Lys acts as a cork in the bottleneck and the positively charged amine group preventing the flow of cations.

Site 2 is known for binding lipid-soluble plant alkaloids like veratridine (VTD) and animal toxins like batrachotoxins (BTX) from frogs. These toxins are sodium channel activators that prefer the open state of VGSCs, causing persistent activation. Site 2

toxins shift the voltage-dependency of channel activation toward more negative potentials. Since channels are activated easier and for longer periods of time, channels are unable to inactivate normally or not at all. Du et al (2011) proposes the newest model of BTX binding, within the inner pore with residues in the S6 segments of all four domains.

Site 3 binds sea anemone and α -scorpion toxins. Site 3 was first identified using radiolabelling and site mutagenesis of sea anemone and α -scorpion toxins. Site 3 toxins generally alter voltage-dependent gating by binding to extracellular sites, slowing or inhibiting inactivation. Residues on the extracellular loop between S3 and S4 in DIV constitute the site 3 binding site. α toxins bound to receptor site 3 interfere with the coupling from activated to inactivated states and prevents the conformational change necessary for fast inactivation. The fourth voltage-sensing domain plays a role in fast inactivation, its sensible to suggest that gating modifying toxins modify this process, specifically targeting VSD-IV. Clairfeuille et al (2019) used AaH2, an α -scorpion toxin known to target VSD-IV to provide further insight into fast inactivation. To achieve this, the AaH2 receptor site from Na_v1.7 was engineered onto Na_vPas. The cryo-EM structure of the AaH2 Na_v1.7-Na_vPas complex revealed that AaH2 inserts itself into the extracellular cavity of VSD-IV to form multiple contacts across neurotoxin site 3 and traps the voltage-sensors into a deactivated state.

Site 4 is known to bind β -scorpion toxins, their mode of action includes voltage- sensor trapping hereby trapping the S4 sensors at the S3-S4 loop on the extracellular side of DII in an outward, activated position. The effects of this causes a shift in voltage-dependence of activation to more negative potentials thus enhancing sodium currents (Bagal et al., 2014). Prototoxin (ProTx-II) and huwentoxin (HWTX-IV) are other examples of gating modifying toxins, capable of inhibiting Na_v. These toxins specifically associate with the linker between the S3 and S4 segments in the second VSD of Na_v1.7 to inhibit activation of Na_v (Shen et al., 2019). Recent studies have elucidated the complex structural changes that take place during channel inactivation with the help of these toxins. Shen et al (2019) reported cryo-electron microscopy structures of Na_v1.7 bound to the previously mentioned toxins, highlighting the importance of the S3-S4 linker in the VSD.

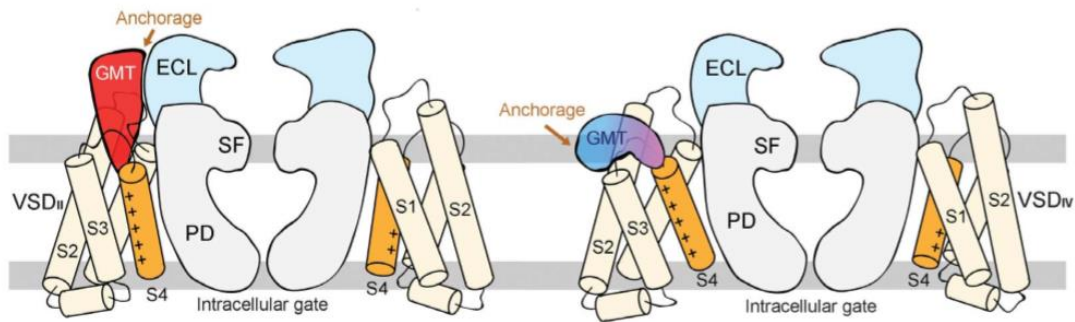


Figure 1.7 Schematic representation of the mechanism for GMTs, NavPas in complex with Dc1a (left) and Na_v1.7 bound to GMTs (right). The pore domain and the surrounding intracellular region could provide support for the GMT to anchor into place and lock the targeted VSD via specific interactions into a functional state. This could be the mode of action for GMTs like ProTx-II and HWTX-IV. Taken from Shen et al., 2019.

Site 5 includes lipophilic marine toxins brevetoxins and ciguatoxins which enhance channel opening by shifting channel activation towards hyperpolarised potentials. (Catterall et al., 2007). Pore helices from DI-S5 and DIV-S6 are proposed as the binding site of brevetoxins (Bagal et al., 2014).

Site 6 binds δ -conotoxin from marine cone snails, slowing the process of inactivation similarly to α -scorpion toxins. The mechanism of this toxin is unclear, however it could be the involvement of the DIV voltage sensor adjacent to site 3 at site 4 (Bagal et al., 2014).

1.7 The contribution of Na_v1.7 in pain

1.7.1 Gain-of-function mutations

The contribution of Na_v1.7 in pain is well documented in various gain-of-function mutations associated with Na_v1.7. Inherited Erythromelalgia (IEM) was the first sodium channelopathy to be identified caused by a missense mutation in the gene *SCN9A*. IEM is characterised by burning pain of the extremities triggered by moderate exercise. Frequency of pain increases with age and episodes of pain can last minutes to hours (Waxman and Dib-Hajj, 2005). The mutations causing IEM demonstrate a gain-of-function leading to peripheral neurone hyperexcitability. Mutated Na_v1.7 in

IEM patients produce a hyperpolarized shift in the voltage-dependence of channel activation (making the channel easier to open), enhancing the response to slow, small depolarisations and slowing deactivation (Yang et al., 2018). Shen et al (2019) demonstrated $\text{Na}_v1.7$ in the presence of $\beta1$ and $\beta2$ subunits to exhibit a hyperpolarising shift in activation. Alterations of these characteristics in $\text{Na}_v1.7$ are consistent with mutants associated with primary erythromelalgia. Another example of a gain-of-function mutation is seen in paroxysmal extreme pain disorder (PEPD). PEPD related mutations impair fast inactivation of VGSCs, increasing excitability of DRG neurones. PEPD features burning pain in the rectal, ocular and mandibular areas and manifestation of skin flushing (Fertleman et al., 2006). The disease locus for PEPD is found at chromosome 2 on *SCN9A*. Patients with PEPD are subjected to treatment with the sodium channel blocker carbamazepine (CBZ), an anti-epileptic drug which alleviates the symptoms of PEPD. Generally patients respond well to this treatment, the pharmacoresponsiveness of PEPD patients to this drug could be a result of the drugs affinity to bind to an open channel (Yang et al., 2018). These channelopathies indicate $\text{Na}_v1.7$ plays a role in the production of pain, IEM and PEPD serve as a potential models for the electrophysiological changes in VGSCs which contribute to pain.

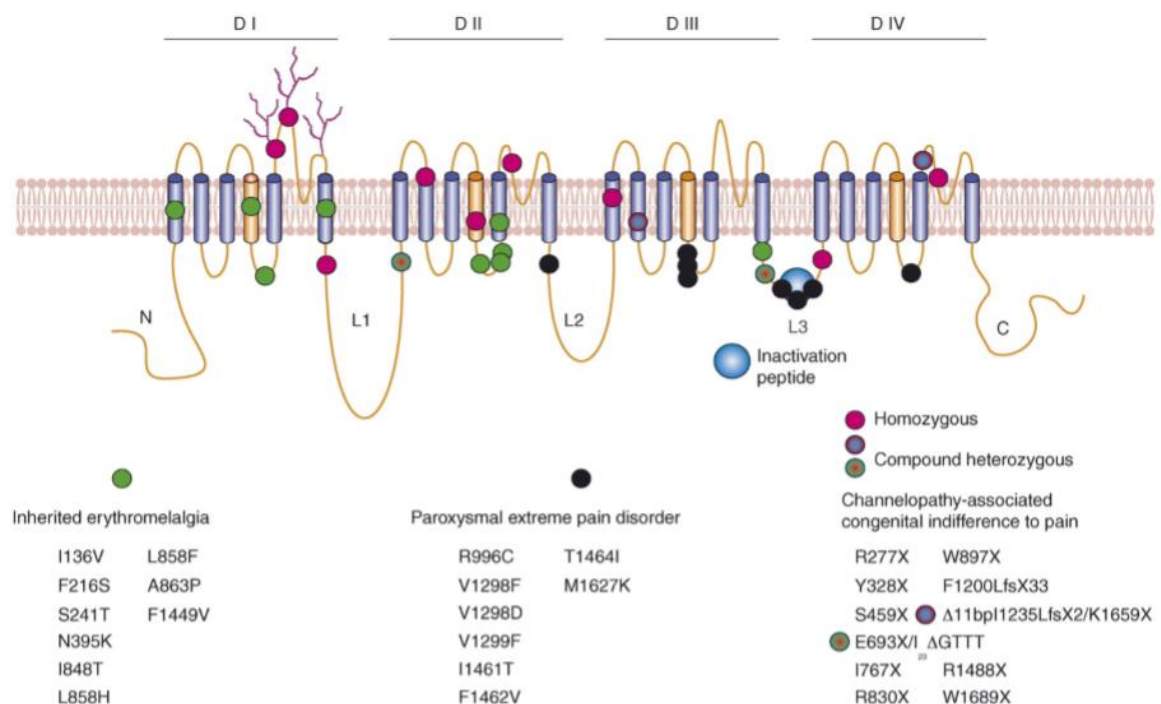


Figure 1.8 A schematic representation of the voltage-gated sodium channel α -subunits showing the locations of the known mutations of $\text{Na}_v1.7$ related inherited pain disorders. IEM (green symbols) and PEPD (black symbols) are dominantly inherited missense substitutions which cause a gain of function. Channelopathy-associated congenital indifference to pain (CIP) is the result of a loss-of-function mutation. Taken from Dib-Hajj et al (2007).

1.7.2 Loss-of-function mutations

Cox et al (2006) studied several families from Northern Pakistan with an inability to experience pain. The condition was reported as a congenital autosomal-recessive trait linked to a region of chromosome 2 containing the gene *SCN9A*. Congenital insensitivity to pain (CIP) is caused by null mutations of $\text{Na}_v1.7$ that introduce premature stop codons leading to a truncated and non-functional protein. Patients do not display any cognitive or motor deficits but feel no pain. Animal studies shed light on the molecular mechanisms and support the role of $\text{Na}_v 1.7$ in pain disorders. Deletion of *SCN9A* decreases responses to induced inflammatory pain (Nassarr et al., 2004). Yang et al (2008) carried out knock-down studies eliminating $\text{Na}_v1.7$ and showed this alleviated pain expressed by inflammation-induced thermal hyperalgesia in mice.

1.8 Centipedes

Centipedes belong to the class Chilopoda, successfully inhabiting all geographical regions from deserts to rainforests, found on every continent except Antarctica. They are organised into the Scutigermorpha (“house centipedes”), Lithobiomorpha (“stone centipedes”), Craterostigmomorpha (only two species), Geophilomorpha (“earth centipedes”), and Scolopendra (the best-known genus).

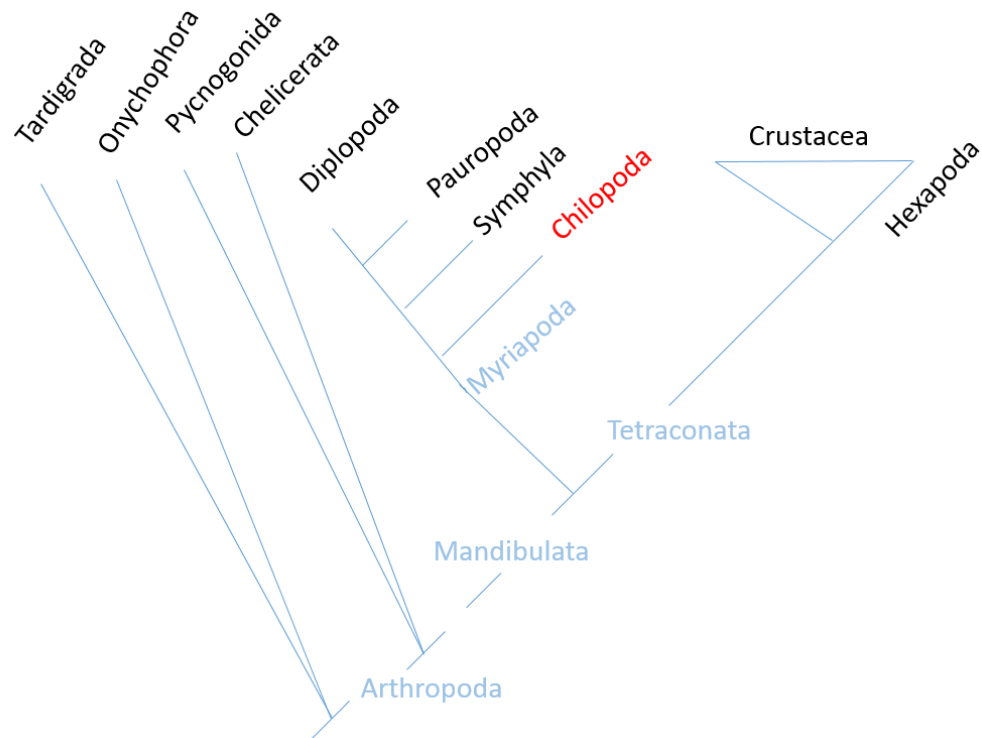


Figure 1.9 Schematic tree of the relationships between the major arthropod groups. Taken from Edgecombe and Giribet (2007).

Centipedes are one of the oldest lineages of terrestrial venomous arthropods, with 3300 species described in these five extant orders (Edgecombe and Giribet, 2007). Ranging from 1-30cm in size, the anatomy of centipedes is divided in an elongated multisegmented thorax with various numbers of segments. Each of the segments bear legs except the first segment, where these legs have been modified into venomous appendages, a feature that unites the members of Chilopoda. These modified parts are known as forcipules, each consisting of four or five segments. Chilopoda are the only carnivorous group belonging to the Myriapoda subphylum. Centipedes are mostly nocturnal, capturing invertebrates such as insects as well as vertebrates – including amphibians, reptiles, small rodents and bats. Some Scolopendra species are among the largest terrestrial invertebrate predators. Centipedes are excellent predators, feeding by ambushing and chasing down their prey. They are also armed with a sophisticated delivery system for their potent venom, secreted from glands connected to the pair of forcipules. The two venom glands are connected via a cuticular duct

which expels venom into a pore located near the tip of the forcipules (Undheim and King, 2011). Chilopoda are the only arthropods to evolve modified claws for the sole purpose of venom injecting (Rong et al., 2015).

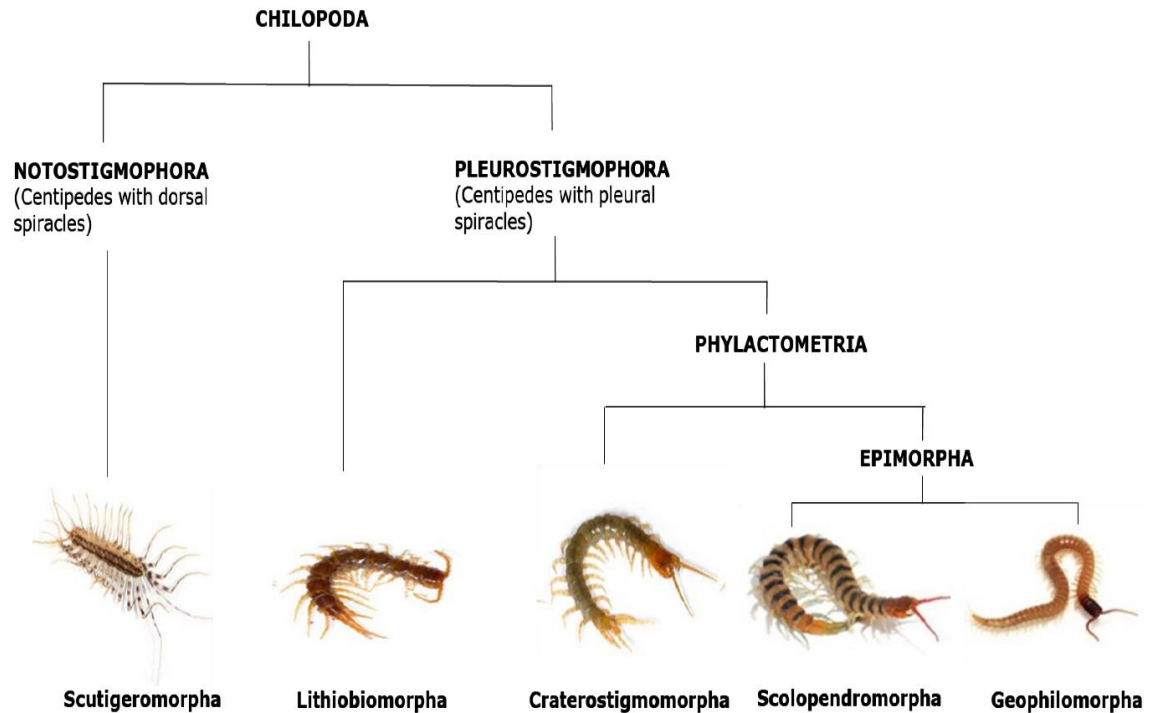


Figure 1.10 Adapted illustration of the phylogeny of centipedes. This is based on the work of Edgecombe and Giribet (2004, 2007) and Undheim et al. (2015). Taken from Ngum (2018).

1.8.1 Clinical importance of centipede stings

Centipede stings are characterised as a local instant extremely sharp pain which can last from hours to days, spreading to other parts of the body. There are reports of a difference in pain severity among taxa. Within the order Scolopendramorpha, the *Ethmostigmus spp* and larger *Scolopendra spp* are able to inflict severely painful stings; this genus is mostly responsible for human encounters, possibly as a reflection of their size and venom yields. Several fatalities have been attributed to centipede envenomation, usually from Scolopendromorph stings. However, fatality is often the result of secondary infections. Localised swelling and erythema are the most common symptoms of centipede envenomations. A hot sensation around the area, dizziness,

headaches, nausea and localized necrosis are associated with centipede stings. Allergy complications have been reported after centipede envenomations such as rashes, itches and anaphylaxis (Supakthanasiri et al., 2004). Severe symptoms from stings have been documented including blood clotting complications (Knysak et al., 1998).

1.8.2 Pharmacological importance of centipede venom

The rapid paralysis of insects induced by centipede envenomations suggests centipede venom comprises a diverse cocktail of neurotoxins. Undheim, Fry and King (2015) report of the enzymatic activity from *Scolopendra* species, suggesting enzymes play a role in centipede venom. Undheim et al. (2014) report metalloproteases to be in abundance in all centipede species. Metalloproteases have been associated with blister formation, inflammation and oedema which are features of centipede stings. Traditional medicine has used centipedes for hundreds of years to treat an array of disorders including epilepsy, tetanus, burns and tuberculosis (Hakim, Yang and Lai, 2015). The use of centipede venom throughout history indicates its potential therapeutic use.

1.8.3 Centipede venom as an ion channel modulator

Centipede venom has become a developing area of interest due to the discovery of disulphide-rich peptides in abundance in these venoms. This discovery has put centipede research forward ahead other venomous animal peptide research.

Characterisation of centipede venom has yet to be extensively studied, research is attempting to identify components of centipede venom with potential pharmacological importance. Unlike centipedes, the S5 animals (snakes, scorpions, spiders, cone snails and sea anemones) have been successfully characterised. The potential of centipede venom has barely been exploited due to difficulty obtaining samples, low venom yields and time consuming venom extraction. The clinical importance of centipede stings are valued as being limited to local and intense pain and redness. Nevertheless, recent studies have highlighted the clinical importance of centipede venom peptides and their pharmacological properties.

Yang et al. (2015) reported 50 components of centipede venom having VGSC, VGKC and VGCC pharmacological activities. Centipede venom is a growing area of interest for drug development with several reports shedding light on the pharmacology of peptides belonging to several centipede families. Mass spectrometry and sequencing of whole venom has given insight into centipede composition which not only determines their mode of action, but also evolutionary convergence.

Undheim, Fry and King (2005) identified disulfide-rich peptides displaying modulating activity in *Scolopendra subspinipes*. Disulphide-rich peptides are widely found in spider venom, these components are less prevalent in centipede venom. However, regardless of the lack of richness of these peptides, centipede venom is outstandingly diverse. From eight species of the genus *Scolopendra*, Undheim, Fry and King (2005) noted 30 peptide families, 24 of these cysteine-rich SPLTX families are especially rich in cysteines. Most venom peptides are highly bridged proteins with multiple pairs of disulphide bonds. The venom composition and strategies between different orders of centipedes has been extensively studied by Undheim, Fry and King (2015). Their focus lies on the venom system of centipede orders, reporting recent discoveries of enzymatic and peptidic activities of several orders of centipedes. In contrast, molecular and electrophysiological studies have only touched the surface of the modulating properties that *Scolopendra* toxins exert on VGSCs.

Yang et al (2013) report venomous peptides of the Chinese red-headed centipede *Scolopendra subspinipes mutilans*. Peptides extracted from this species have been known to have modulating effects on Na_v, Ca_v and K_v. A Na_v1.7 inhibitor was described from *S. subspines mutilans*. Voltage-clamp studies reported a peptide μ -SLPTX-Ssm6a as having more than a 150-fold selectivity for Na_v 1.7 over all other human Na_v subtypes. This molecule has been confirmed as being a more potent analgesic than morphine in rodent models of chemical-induced pain (Hakim, Yang and Lai, 2015).

Yang et al (2012) purified 10 neurotoxic peptides from *S. subspines mutilans* using assay-guided fractionation. Here they report inhibition activity of Na_v in rat DRG neurones and activation of Ca_v and K_v. Most importantly, μ -SLPTX-Ssm1a depressed TTX sensitive Na_v. Clearly Ssm6a has strong analgesic properties, owing to its strong inhibitory effects on Na_v1.7.

The inhibitory effects of Ssm6a on Na_v suggests Ssm6a is a gating modifier that interacts with the voltage-sensing domains of voltage-dependent sodium channels (Hakim, Yang and Lai, 2015).

Hakim, Yang and Lai (2015) reported a peptide in *S. subspinipes mutilans* venom that was a potent activator of TRPV1, a noxious heat sensing ion channel that is sensitive to capsaicin in chilli peppers. This likely explains the powerful burning sensation associated with centipede stings.

Undheim, Fry and King (2015) report a prevalence of β -pore forming toxin-like proteins in the venom of *S. subspinipes*. The pore-forming complex is termed the β -complex domain; toxin monomers assemble and form a β -barrel. A conformational change takes place following insertion into the membrane whereby a pore is formed. Ngum (2018) investigated the venom of *Scolopendra hardwickei*, focussing on effects on the $\text{Na}_v1.7$ channel using a patch-clamp assay with TE671 cells. An interesting initial observation of this work showed that the venom components were differentially expressed between male and female venom. Following fractionation of the venom he found that several fractions from both male and female venom had modulatory effects on $\text{Na}_v1.7$, demonstrating both gain and loss of function (figure 1.10 and 1.11). One male venom fraction was subjected to sequencing resulting in the identification of a small family of homologous peptides (figure 1.12) unique to *S. hardwickei*. In combination they caused a negative shift in the voltage dependence of activation. This further explains why centipede stings can cause excruciating pain. The work described in this thesis focuses on one peptide (peptide I) in this family with the aim of determining more clearly its mechanism of action using the same assay system.

Transcriptome analysis has identified more than 500 peptide sequences in centipede venom. Few peptide toxins have been fully described. Not only snakes, spiders and scorpions but centipede venom could also provide an excellent source for drug development (Hakim, Yang and Lai, 2015). The work of Undheim, Fry and King (2005) confirmed centipede venoms are a rich source of novel toxins.

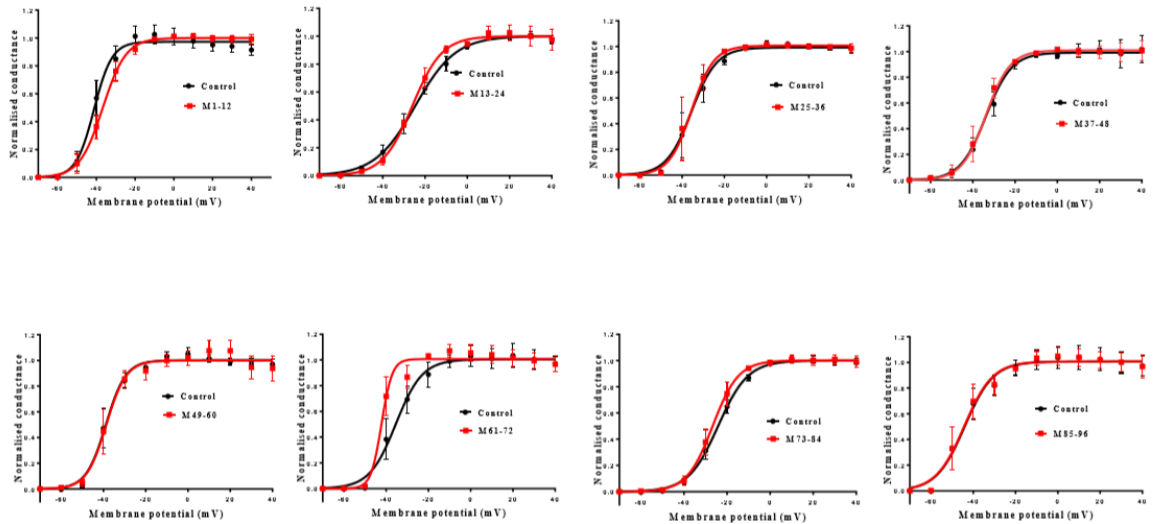


Figure 1.11 The effects of male venom fractions on the voltage dependence of activation. The M61-72 and M73-84 pooled fractions produced significant shifts in the negative direction. On the other hand, the M1-12 cocktail induced a depolarising (positive) shift on the curve. Courtesy of Ngum (2018).

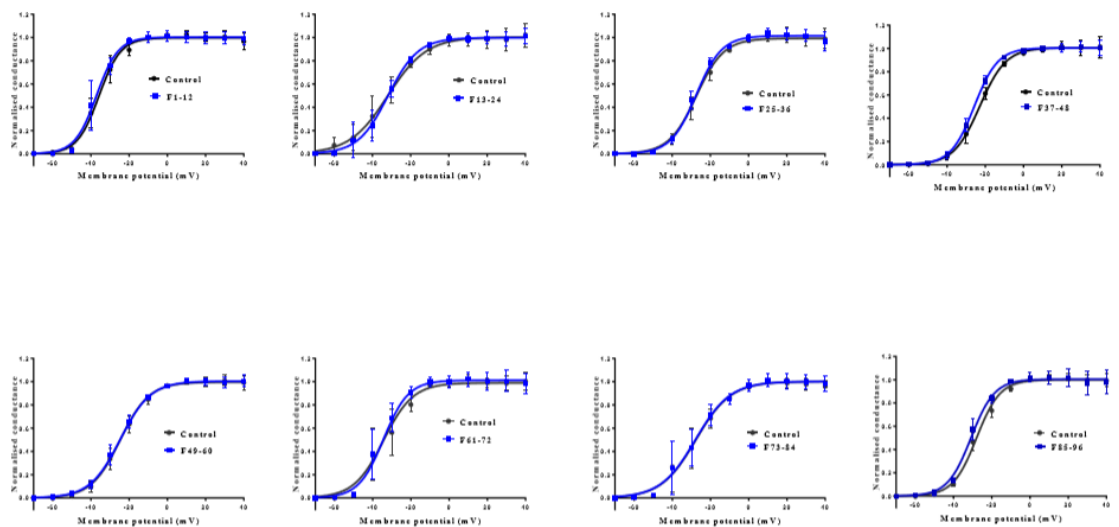


Figure 1.12 The effects of female venom fractions on the voltage dependence of activation. The F37-48 and F85-96 pooled fractions induced a negative shift in activation. Courtesy of Ngum (2018).

characterized the VGSC as being almost entirely of the Nav1.7 subtype through the use of selective toxins and RNA analysis.

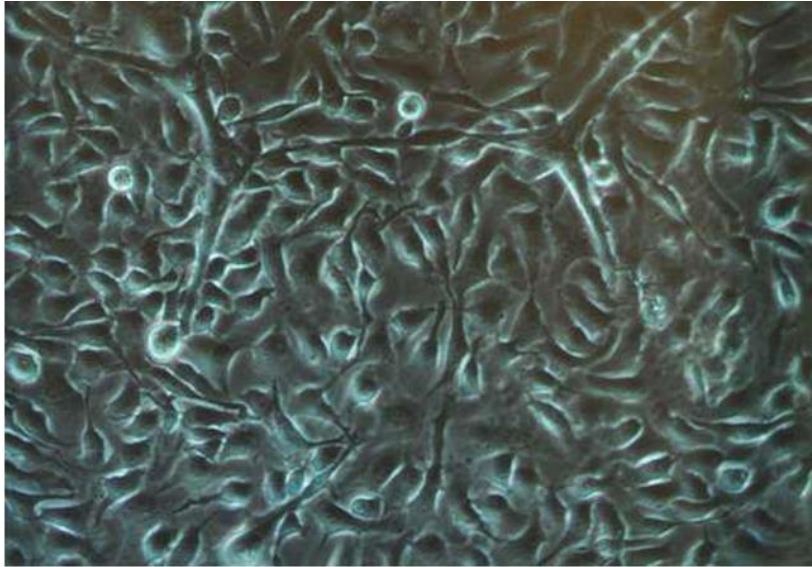


Figure 1.14 TE671 cells at X200 magnification. Courtesy of Ngum (2018).

1.10 Aims and Objectives

In this project the aim is to characterise the modulation of Nav1.7 by peptide I in terms of its mechanism of action using the TE671 cell line. TE671 cells will be patch-clamped and Nav1.7 channels activated through application of depolarising pulses using protocols designed to investigate current amplitudes at specific test potentials or across a range of test potentials to assess the half activation voltage (V_{50}) of the channels in the absence and presence of toxins.

Methods

2.1 Previous research

The work of Neville Ngum assessed the effects of crude venoms, venom cocktails and synthesised venom peptides.

Venom fraction cocktails demonstrating modulating activity of VGSCs were selected; pooled fractions M61-72 demonstrated activity of interest and was therefore selected for further separation, identification and characterisation. From this process, 4 peptides were presented. Characterisation of peptide I (SB5221-1) displayed kinetics which warranted further investigation for this project.

2.2 Venom collection and fractionation

Venom collection took place at the University of Nottingham by Neville Ngum. Adult Indian Tiger centipedes were purchased from Martin Goss company <http://www.martingoss.co.uk/>. The electrostimulation method similar to that described by Peng and colleagues (Peng et al., 2010) was used to collect venom from centipedes. Venom fractionation was done using Reverse Phase-High Performance Liquid Chromatography (rp-HPLC) in the laboratory of Dr David Tooth, School of Life Sciences at University of Nottingham. 96 fractions were separated for screening, so these were pooled into eight cocktails each of twelve fractions to expedite screening. Cocktails of venom displaying activity against Na_v1.7 underwent LC-MS/MS (Liquid chromatography-mass spectrometry) by Dr Eivind Undheim of the University of Queensland, Australia, peptide constituents were sequenced. Peptide sequences were sent to Severn Biotech Ltd, UK for synthesis.

2.3 Peptide identification and structural modelling

The identified peptide sequence was searched against existing peptide sequences using UniProt blast to gather any existing published information about the peptide in question or if any other peptides were structurally similar. UniProt predicted 3D

structure of blasted peptides by arranging the backbone of the sequences to existing templates in the protein database. Modelling was done using the SWISS-MODEL homology modelling server <http://swissmodel.expasy.org/> that searches for templates with BLAST and HHBlits against the SWISS-MODEL template library in the protein database.

2.4 Cell Culture: TE671 Human Rhabdomyosarcoma Cells

TE671 cells were derived from liquid nitrogen stocks and cultured in a 4.5 g/L glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich, UK), 2 mM glutamine (Sigma-Aldrich, UK), 10 IU/mL penicillin and 10ug/mL streptomycin (Sigma-Aldrich, UK).

Cells were maintained in 25 cm² culture flasks (Corning) in 5 mL of medium and passaged once a week. Previously cultured cells that showed confluence were aspirated to remove the culture medium then detached from the culture flask using 2 mL of 0.25% porcine trypsin + 0.02% EDTA. Dissociated cells were added to 10 mL of culture medium and centrifuged at 1000 rpm for 7 minutes. Cells were then suspended in 10 mL of fresh culture medium and 0.5 mL of cell suspension were added to 4.5 mL fresh culture medium in a 25 cm² cell culture flask. To prepare cells for patch-clamp recording, a drop or two of this cell suspension was added to 2 mL fresh culture medium in 35 mm petri dishes containing heat-sterilised 5 x 20 mm glass cover slips. Cells were grown and incubated at 36.5 °C in a humidified 5% CO₂ atmosphere and passaged once a week.

2.5 Electrophysiological measurement of whole-cell patch clamp

Patch-pipettes were made from borosilicate glass capillaries (1B150F-4, World Precision Instruments, USA) using a P-97 Flaming/Brown micropipette puller (Sutter Instrument Co., USA) to give a resistance of ~5-7 MΩ when filled with a caesium pipette solution (in mM): 140 CsCl, 10 NaCl, 1 MgCl₂, 11 EGTA, 5 HEPES, pH 7.2 with CsOH. These were connected to the amplifier via a pipette holder containing a 0.37 mm Teflon coated silver wire with the last 1 cm bared and coated with silver chloride

each experimental day (Advent Research Materials). TE671 cells on glass coverslips were transferred to a perfusion chamber and bathed in extracellular solution containing (in mM): 135 NaCl, 5.4 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES, 10 D-Glucose, pH 7.4 with NaOH.

Cells were observed using a Nikon Eclipse TE200 microscope (Nikon Corporation, Japan) mounted on an anti-vibration table in a Faraday cage. An Axopatch 200A patch-clamp amplifier (Axon Instruments, USA) was used to monitor seal formation with the cell membrane and to measure whole-cell currents. WinWCP V4.5.7 (Dr John Dempster, University of Strathclyde) was used to control the membrane potential and to record output from the patch-clamp amplifier.

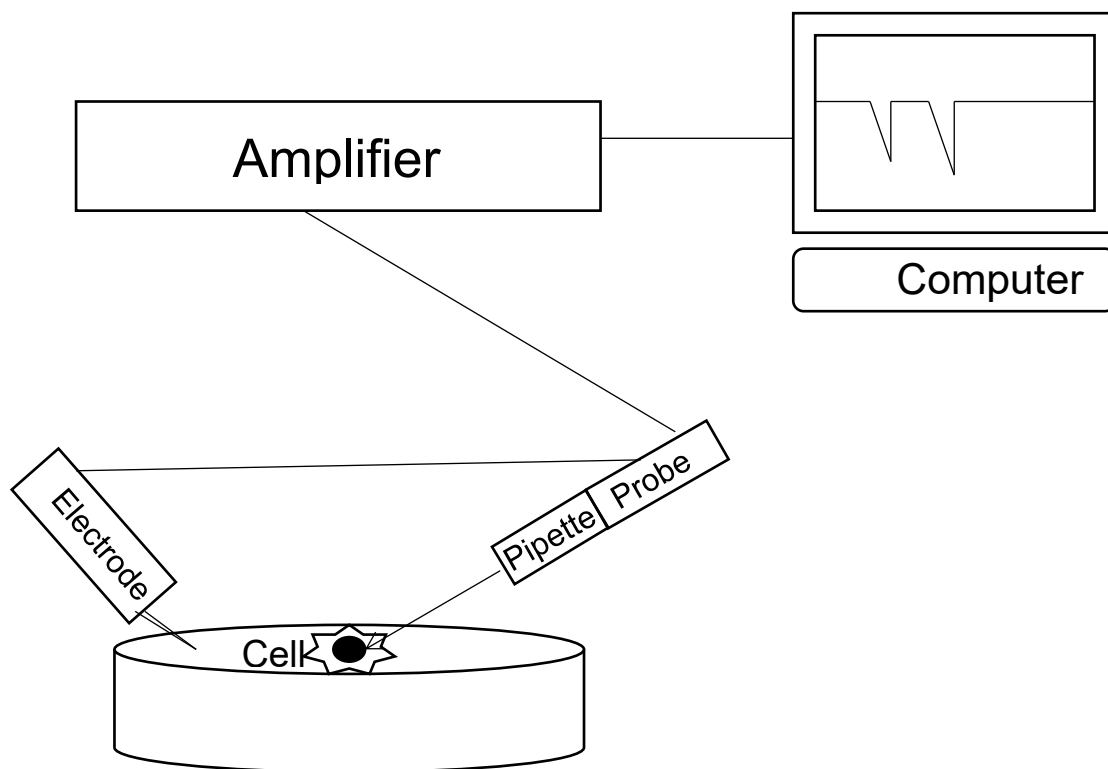


Figure 2.1 Patch clamp schematic representation used in this study. A silver/silver chloride electrode is within the borosilicate micropipette filled with intracellular solution. The pipette is mounted to the probe which is connected to the feedback amplifier. A Teflon/silver chloride coated wire lies within the bath solution

connected to the probe as a ground electrode. TE671 cells were adhered onto the cover slip placed in the bath. Once a whole-cell patch-clamp configuration was achieved, the protocols used to record activity were generated in WinWCP and passed to the amplifier.

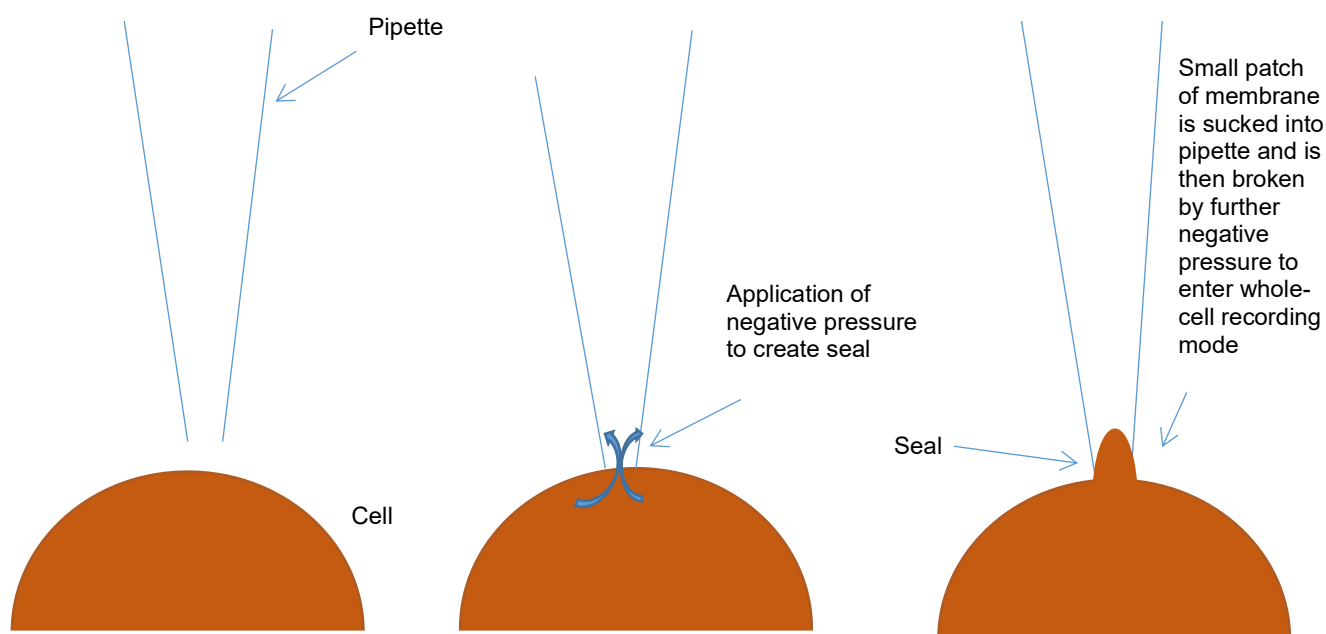


Figure 2.2 Schematic illustration of whole-cell configuration of patch-clamp technique. A syringe attached to the pipette probe is pulled, creating negative pressure to form a high-resistance seal.

2.6 Application of toxins to TE671 cells

Tetrodotoxin (TTX) and peptide I synthesised from *Scolopendra hardwickei* were utilised in this study. Both the peptide and TTX were dissolved in extracellular solution (mammalian ringer) to desired concentrations (TTX at 0.1-10 μM ; Peptide I at 0.01-10 μM). Toxin solutions were applied with a DAD-12 super fusion system fitted with a 100 μm polyamide coated quartz output tube (ALA scientific Instruments, USA). Compressed nitrogen pressurises the drug application super fusion system allowing rapid focal application to single cells. The compounds were perfused onto the cells by

positioning the tip of the perfusion needle facing the cell so the compounds flowed over and around the patched cell. A flush valve containing mammalian ringer flowed through the tip when all other values were turned off to allow the toxin solution to be flushed away.



Figure 2.3 TE671 cells at magnification X200 showing positioning of the perfusion tube adjacent to the patch-clamped TE671 cell.

2.7 Voltage Protocols

A set of three stimulation protocols were used to evoke Nav1.7 currents, to analyse current-voltage relationships, activation and steady state inactivation.

2.7.1 Voltage protocol 1: Current-voltage relationship.

This protocol studied voltage dependence of activation in the absence and presence of toxins. TE671 cells were clamped at -80 mV and subjected to a series of 25 ms step depolarisation test pulse potentials (V_T) from -80 to +60 mV in 10 mV increments at an interval of 1 s.

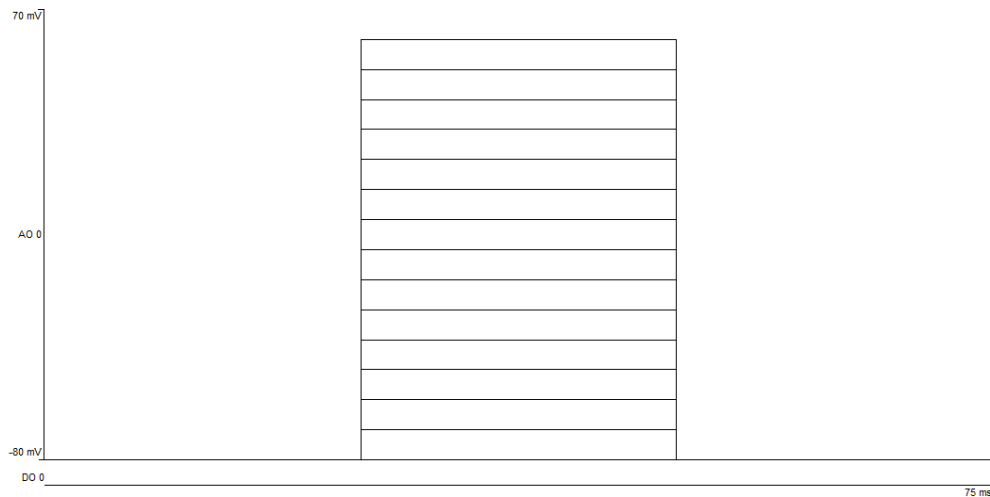


Figure 2.4 Schematic illustration of voltage protocol 1. The holding potential is represented by the baseline; TE671 cells are clamped at -80mV. The lines represent 10 mV incremented step depolarizations (V_T) for 25 ms. The amplitude of current in response to V_T was recorded and plotted to give a current-voltage relationship.

2.7.2 Voltage protocol 2: Steady state inactivation

Voltage protocol 2 measures the voltage dependence of inactivation; cells were clamped at -80mV. Inactivating pre-pulse potentials ranged from -120 mV to 20 mV in 10 mV increments for 200 ms. Each pre-pulse potential was followed by a test pulse of -10 mV for 25 ms to determine the proportion of non-inactivated channels.



Figure 2.5 Illustration of voltage protocol 2. The cells were clamped at -80mV and subjected to pre-pulse voltages ranging between -120 and 20 mV for 200ms before applying a test pulse to -10 mV for 25ms.

2.7.3 Voltage protocol 3: Continuous step depolarisation

The step depolarisation protocol assessed the extent of peak sodium current increase or decrease by TTX and peptide I over time. The cells were subjected to a 25 ms single depolarisation step from a -80 mV holding potential to a -40 mV test potential. Over fifty of these step depolarisations were applied at 2-s intervals.

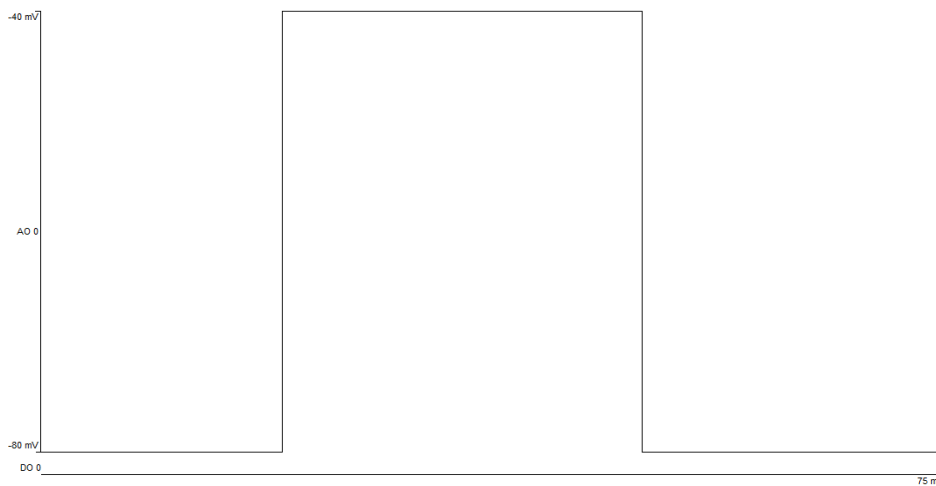


Figure 2.6 TE671 cells were subjected to a continuous series of 50 step depolarizations from -80 mv holding potential to a -40 mV. This protocol assessed the degree to which toxins depressed or enhanced sodium currents.

2.8 Data acquisition and analysis

Electrophysiological data was recorded and measured using WinWCP (Strathclyde Electrophysiology Software). Comparison of parameters determined from a curve fit was done using an extra sum of squares F test in GraphPad Prism 7. All graphs, curves and statistical tests were done using GraphPad Prism 7.

2.8.1 Analysis of channel activation

Voltage protocol 1 recorded TE671 VGSC sodium currents to investigate the current-voltage relationship. Peak current amplitudes recorded at each voltage step were

plotted against test pulse potentials ($V_{T.act}$) to give the current-voltage relationship for channel activation. Peak currents were normalised against the largest negative peak current from each cell. The mean and standard error of the mean for each normalised peak current were computed at each test potential and fitted by a modified Boltzmann equation (Equation 1.1) to produce a current-voltage curve. The current-voltage curve gave two key parameters; the half activation potential ($V_{50.act}$) which is the membrane potential at which half of the number of available VGSCs in the cell are opened, and the reversal potential (V_{rev}) is the membrane potential at which there is no net flow of Na^+ ions across the cell membrane.

$$I_{peak} = G_{max} \left(\frac{V_{T.act} - V_{rev}}{1 + \exp\left(\frac{V_{T.act} - V_{50.act}}{k}\right)} \right)$$

Equation 1.1

Where I_{peak} is the peak sodium current elicited by the step depolarisation test potential ($V_{T.act}$), G_{max} is the maximum sodium conductance, V_{rev} is the reversal potential, $V_{50.act}$ is the test potential for half maximal channel activation and k is the slope factor in mV.

Ohm's law states the conductance (G) from the peak currents for the given test potentials can be calculated using equation 1.2

$$G = \frac{I_{peak}}{V_{T.act} - V_{rev}}$$

Equation 1.2

Using equation 1.1, the maximum conductance value (G_{max}) and the reversal potential (V_{rev}) for each individual cell were calculated. The normalised conductance (G/G_{max}) for each test potential $V_{T.act}$ was then calculated using equation 1.3.

$$\frac{G}{G_{max}} = \frac{\frac{I_{peak}}{V_{T.act} - V_{rev}}}{G_{max}}$$

Equation 1.3

Where I_{peak} is the peak current elicited by the step depolarisation test potential ($V_{T.act}$), G is the conductance at $V_{T.act}$, G_{max} is the maximum sodium ion conductance and V_{rev} is the reversal potential. Normalised conductance (G/G_{max}) for each test potential $V_{T.act}$ and their SEM were calculated and fitted by a Boltzmann sigmoidal Equation (equation 1.4) to produce a normalised conductance-voltage relationship curve and allowed for a more accurate calculation of the $V_{50.act}$ based on ionic conductance.

$$\frac{G}{G_{max}} = Bottom + \frac{Top - Bottom}{1 + \exp\left(\frac{V_{50.act} - V_{T.act}}{k}\right)} \quad \text{Equation 1.4}$$

Where G/G_{max} is normalised conductance at the depolarising step test potential ($V_{T.act}$), and the normalised conductance varies from a minimum value (bottom) to a maximal value (top), $V_{50.act}$ is the half maximal activation potential (at which 50% of the available channel would be activated), and k is the slope factor in mV.

2.8.2 Analysis of channel inactivation

Voltage protocol 2 investigated steady-state inactivation, the peak sodium current amplitude (I_{peak}) elicited at each pre-pulse potential ($V_{T.inact}$) was normalised by dividing the maximum sodium (I_{max}), current for the series of test pulse to -10 mV. The mean normalised currents (I_{peak}/I_{max}) and their SEM were plotted against each pre-pulse voltage and fitted by a Boltzmann sigmoidal equation (Equation 1.5) to produce the half-inactivation voltage ($V_{50.inact}$) which is the voltage at which half the number of VGSC are considered inactivated.

$$\frac{I_{peak}}{I_{max}} = Bottom + \frac{Top - Bottom}{1 + \exp\left(\frac{V_{50.inact} - V_{T.inact}}{k}\right)} \quad \text{Equation 1.5}$$

Where I_{peak}/I_{max} is normalised current given as function of inactivating pre-pulse potentials ($V_{T.inact}$) varying from a minimum Bottom value to a maximal top value, I_{peak} is the peak current sodium given by a single $V_{T.inact}$ pre pulse and I_{max} is the maximum peak current elicited by a series of -10mV pulse and $V_{50.inact}$ is the half-inactivation

potential (at which 50% of the channels would be inactivated), and k is the slope factor in mV.

Results

3.1 Characterisation of voltage-gated sodium channels in TE671 cells

The electrophysiological properties of TE671 VGSCs in this study were characterised and exhibited voltage-dependency, ion selectivity and tetrodotoxin sensitivity. TTX and peptide I from *Scolopendra hardwickei* characterised the pharmacological properties of VGSCs through a series of voltage protocols.

3.1.1 Activation of sodium channels

TE671 cells responded to test pulse potentials ranging from -80 to +60 mV in 10 mV increments with a 25 ms step depolarisation from a -80 mV holding voltage as illustrated in Figure 2.4 (voltage protocol 1). The inward peak current at each test voltage was recorded to produce a peak current-voltage relationship. From this relationship sodium currents were observed to be activated at test pulses around -50 mV and above. The peak currents at each test membrane potential were normalised to the maximum peak current to produce a normalised current voltage-relationship (Figure 3.1A). The current-voltage relationship gave ($V_{50.act}$) of -43.35 ± 1.59 and a (V_{rev}) of 266 ± 62 . The V_{rev} is required to calculate conductance and the $V_{50.act}$ from the conductance-voltage relationship (Figure 3.1B) using the Boltzmann sigmoidal equation. This gave $V_{50.act} = -44.35 \pm 1.59$ mV, $k = 6.7 \pm 1.2$ mV. A series of TE671 sodium currents responding to test pulse potentials ranging from -70 mV to +10mV increments with a 25 ms step depolarisation and from a -80 mV holding voltage is illustrated in (Figure 3.1C).

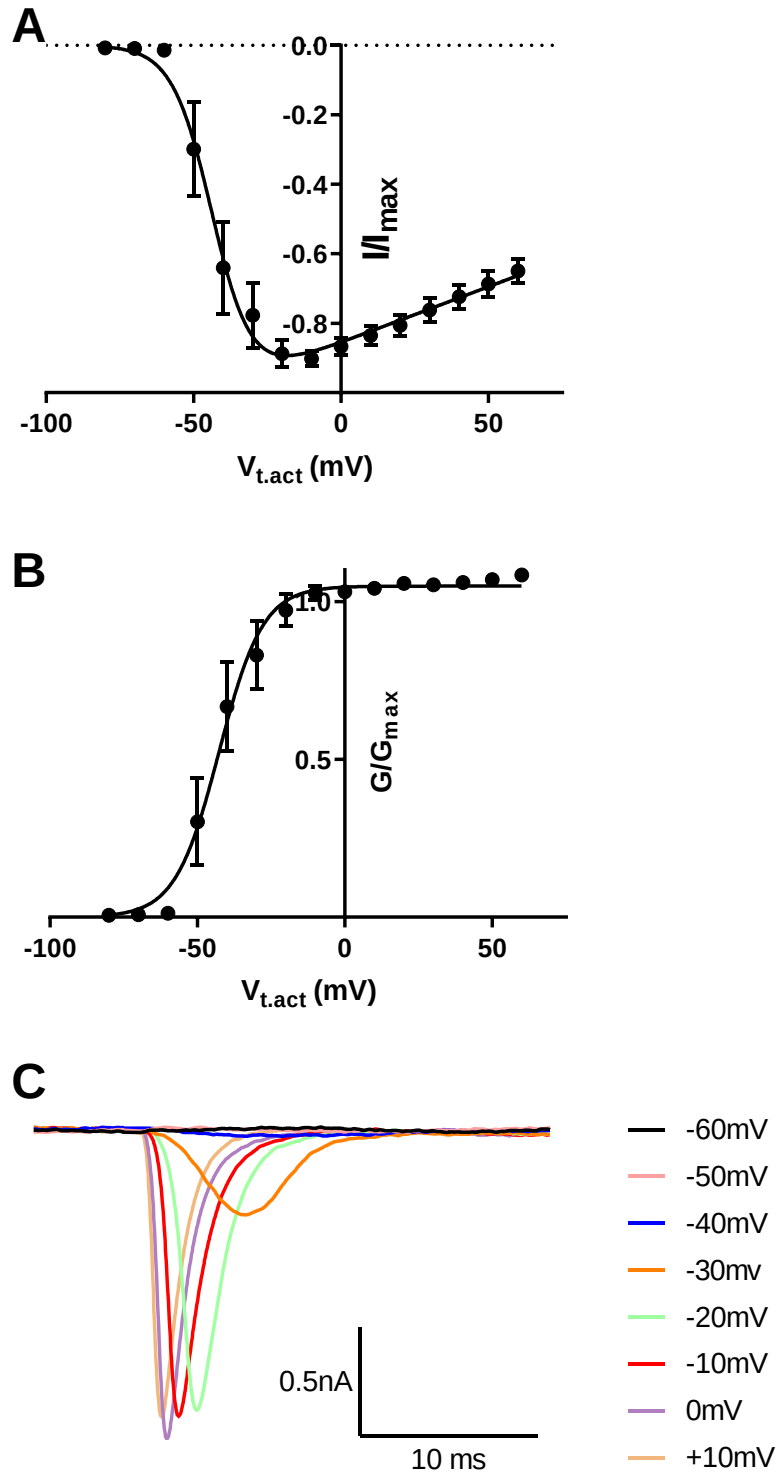


Figure 3.1 A) Normalised peak current-voltage relationship for potentials ranging from -80 mV to +60 mV in 10 increments from a holding potential of -80 mV. B) Conductance-voltage relationship transformed from current-voltage relationship using equation 1.2 and normalised to peak conductance. C) Illustration of sodium

currents from TE671 cells in response to 25 ms depolarising voltage steps of -60 mV to +10 mV in 10 mV increments from a holding voltage of -80 mV, n=15

3.1.2 Inactivation of voltage-gated sodium channels

TE671 cells were subjected to protocol 2 to determine the voltage dependence of inactivation by applying inactivating prepulses (V_{inact}) between -130 and +10 mV then immediately a step depolarisation to -10 mV to estimate the proportion of non-inactivated channels. Figure 3.2 shows the normalised current $V_{\text{T.inact}}$ relationship fitted with the Boltzmann relationship that was applied to determine $V_{50.\text{inact}}$. This gave a $V_{50.\text{inact}}$ of -64.21 ± 0.50 and k of 5.92 ± 0.502 . Inactivation was evident at V_{inact} greater than -80 mV (Figure 3.2).

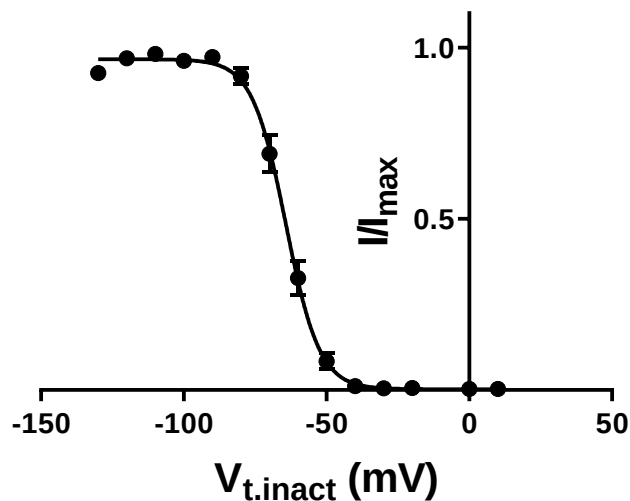


Figure 3.2 Voltage-dependence of inactivation for TE671 VGSCs as recorded using protocol 2. Peak currents at each pre-pulse potential were normalised and fitted into a Boltzmann sigmoidal relationship, n=15.

3.2 Pharmacological characterisation of VGSCs in TE671 cells

For the subtype characterisation of the VGSC in TE671 cells, cells were exposed to Tetrodotoxin (TTX) to assess inhibition of channels and determine the IC_{50} value.

3.2.1 TTX sensitivity

Voltage protocol 3 subjected TE671 VGSCs to 50 step depolarisations to -40 mV with a holding voltage of -80 mV and interval of 2 s.

TE671 VGSCs were TTX sensitive and completely blocked by concentrations higher than 1 μM . Figure 3.3 shows VGSC currents in response to depolarisation potentials before and after TTX application. Plotting % control response against TTX concentration (Figure 3.4) gave a TTX IC_{50} of $7.43 \pm 5.70 \text{ nM}$.

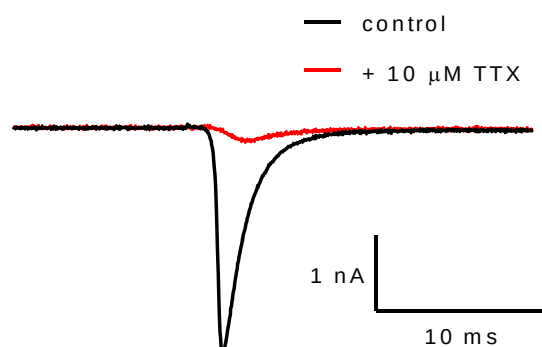
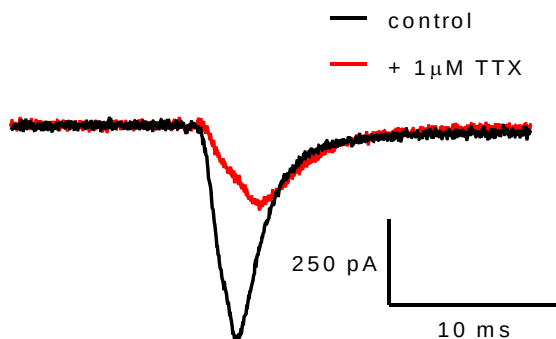
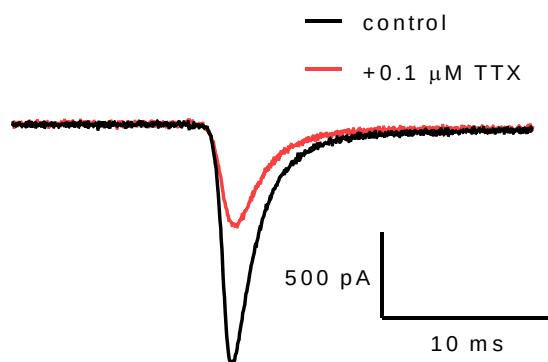


Figure 3.3 TE671 sodium currents were blocked by TTX. Sodium currents were subjected to test pulses to -40 mV from a holding potential of -80 mV, n=3 for each concentration.

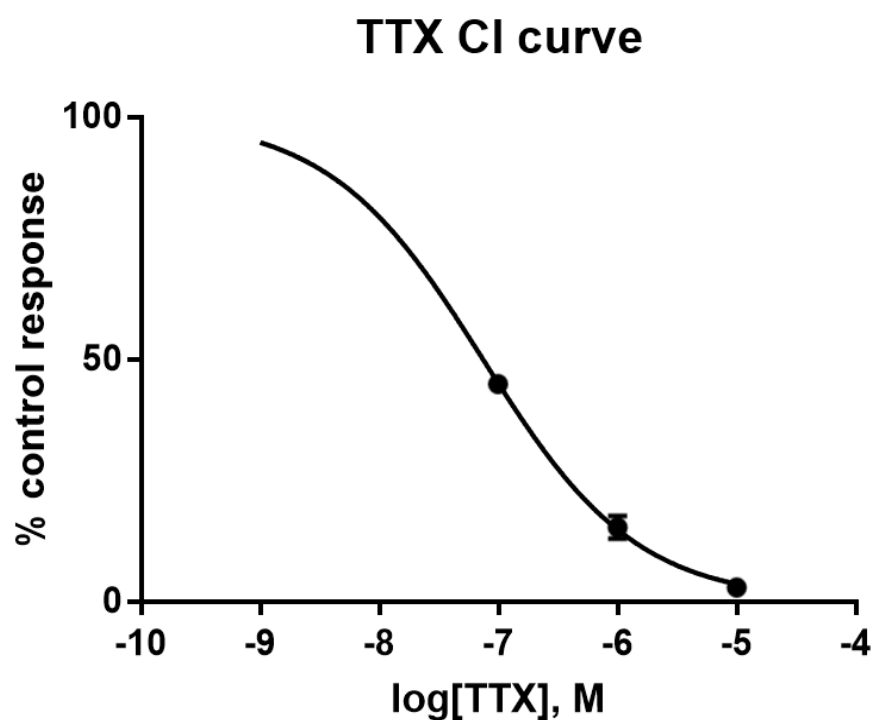


Figure 3.4 Concentration-inhibition curve for TTX block of sodium currents in TE671 cells. Peak currents were normalised as percentages of the control current in the absence of TTX, n=3. The IC₅₀ value was 7.43 ± 5.7 nM.

3.3 Effects of peptide I on Na_v1.7 whole current kinetics

Voltage protocol 3 was developed to continuously subject TE671 cells to 50 depolarising steps to -40 mV from a holding voltage of -80 mV at 2-s intervals. For the first 10 steps TE671 cells did not receive the peptide, during steps 10-50 TE671 cells were perfused with each concentration of peptide I. However, continuous pulsing to -40 mV where sub maximal currents were evoked showed that there is a potentiation of the currents. Figure 3.5 shows plots of mean normalised current over the 50 repeats with peptide 1 being applied after the tenth pulse. Time-to-peak values were analysed during the first twelve pulses before the application of the peptide and the application of the peptide at 10 nM. Table 3.1 shows time-to-peak mean values and reports a significant reduction in time-to-peak in both cells because of the activation of the channel at more negative potentials.

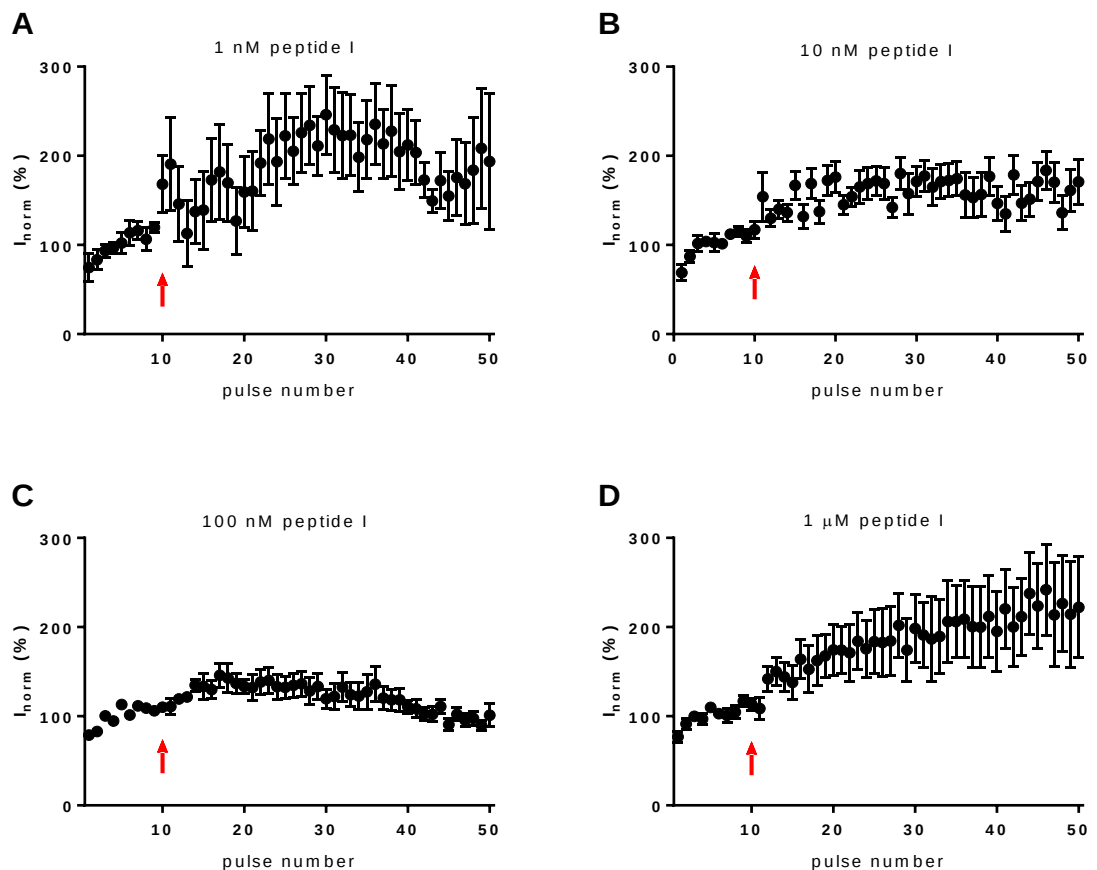


Figure 3.5 Effects of different concentrations of peptide I on Na_v1.7 currents. Currents were evoked by a 25 ms step depolarization to -40 mV from a holding voltage of -80mV every seconds. Each point corresponds a depolarising voltage step every 2 seconds. A gain in function was observed over a period of time. Values plotted as mean $\pm$ SEM. N=8 for each value.

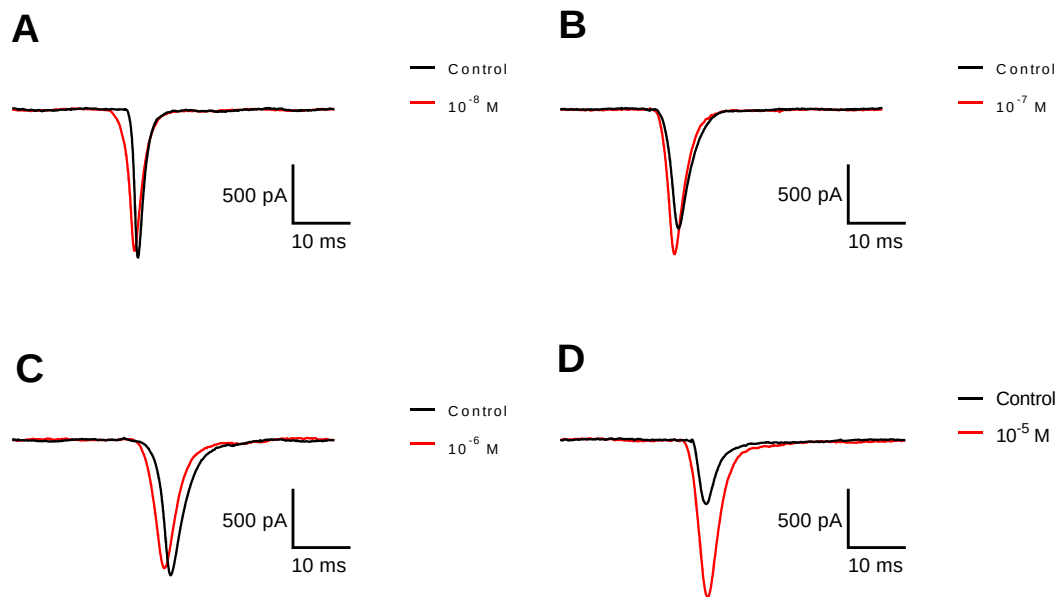


Figure 3.6 Effects of peptide I on Na_v1.7 following the application of voltage protocol 3. A and B show a small potentiating response at -40mV. C shows faster activation by peptide I. D shows a significant gain of function in VGSCs, Peptide I at 10 μ M enhances currents and causes faster activation, n=8.

Time to peak I_{Na} n=2	Control Mean t-t-p ($\pm$ SEM) ms	10^{-7} Mean t-t-p ($\pm$ SEM) ms	Unpaired t-test
Cell 1	6.971 $\pm$ 0.047 ms	6.499 $\pm$ 0.053 ms	p<0.0001
Cell 2	7.463 $\pm$ 0.094 ms	6.341 $\pm$ 0.051 ms	p<0.0001

Table 3.1 Mean time-to-peak values ($\pm$ SEM) before and after 10 nM peptide application, n=2.

3.4 Effects of peptide I on the voltage-dependence of activation in TE671 cells.

Based on the findings from figure 3.6 it was necessary to further investigate the effects of peptide I on channel activation by examining the voltage dependence of activation. Voltage protocol 1 was used to determine the $V_{50.act}$ for channel activation. TE671 cells were recorded before and during the perfusion of peptide I. If cells displayed high and consistent currents, they were perfused with peptide I to assess the functional effects on VGSCs. The $V_{50.act}$ values from the conductance-voltage relationships were analysed and compared in the absence and presence of peptide I. A positive or negative change in $V_{50.act}$ implies a depolarising (inhibitory) or hyperpolarising (excitatory) shift in activation threshold respectively. $V_{50.act}$ was unaffected up to a peptide concentration of 1 μ M but at 10 μ M it caused a -11 mV (hyperpolarising) shift (Table 3.1).

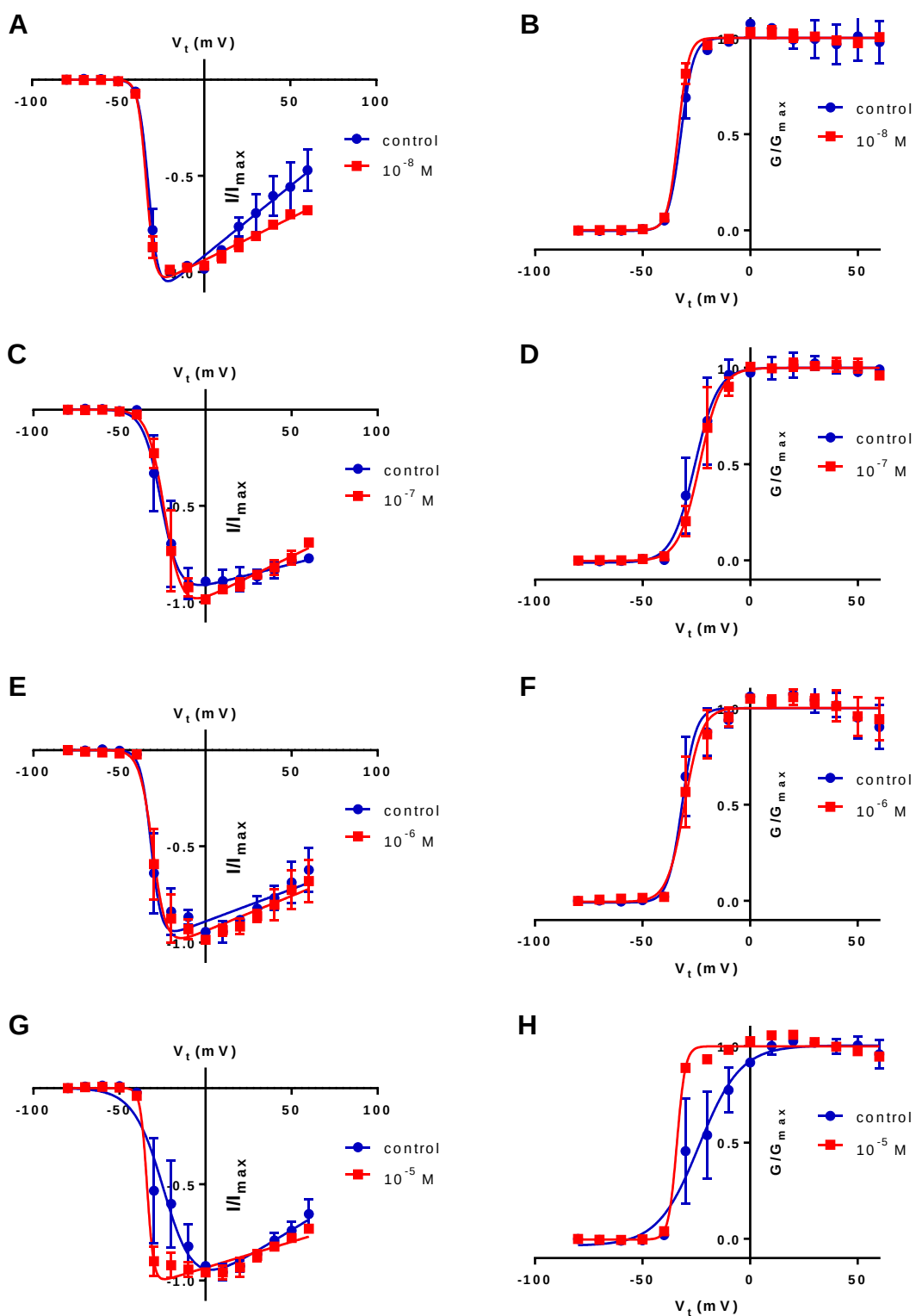


Figure. 3.7 Effects of peptide I on Na_v1.7 currents at concentrations of 10⁻⁸ M (A,B), 10⁻⁷ M (C,D), 10⁻⁶ M (E,F) and 10⁻⁵ M (G, H). **Left:** Normalised peak current-voltage relationship in the absence and presence of peptide I. **Right:** Conductance-voltage relationship for Na_v1.7 in the absence and the presence of peptide I. V_{50.act} and k values are given in Table 3. N=15.

Concentration (M)	V _{50.act} (mV)		k (mV)	
	Control	Peptide I	Control	Peptide I
10 ⁻⁸	- 32.2 ± 0.8	-33.6 ± 0.3	2.86 ± 0.71	2.45 ± 0.19
10 ⁻⁷	-25.6 ± 1.8	-23.5 ± 1.1	5.21 ± 1.30	4.80 ± 0.83
10 ⁻⁶	-31.6 ± 1.1	-30.5 ± 1.1	3.01 ± 1.21	3.86 ± 1.14
10 ⁻⁵	-22.9 ± 3.45	-33.9 ± 0.8	2.20 ± 4.60	1.90 ± 0.35

Table 3.2 Mean V_{50.act} values and slope factors (k) in the absence and presence of peptide I at different concentrations.

3.5 Peptide sequencing, synthesis and structural modelling of peptide I.

Our peptide was identified from the M61-72 cocktail and comprises of 4 peptides. The work of Neville Ngum 2018 describes in detail the sequences belonging to this cocktail (Figure 3.8). Interestingly, 16 of 49 residues are negatively charged compared to only 3 positively charged residues. Peptide I had 23 templates matching its sequence alignment, from which only four had a global model quality estimation (GMQE) score – a quality estimate combining properties from the target-template alignment and the template search – of greater than 0.5. Searches are ranked on a score between 0 and 1. The four templates included κ-SLPTX-Ssm1a (Undheim et al., 2015b), κ-SLPTX3-Sd1a (Sun et al., 2015), μ-SLPTX3-Ssm6a (Undheim et al., 2015b) and U1-agatoxin-Ta1a (Undheim et al., 2015b). κ-SLPTX-Ssm1a showed the highest GMQE score, the model structure of peptide I was built using this peptide sequence. A QMEAN Z-score, an estimator for geometrical properties of the probability of interactions between beta carbon atoms only, the interactions between all atoms, the solvation potential and

the torsion angle potential was -2.08. Each residue of the model was given an individual QMEAN Z-value; values above 0.60 indicate a high quality between the residues in the model and that of peptide I. 34 (80%) of 42 residues scored a QMEAN Z-value more than 0.6. Each residue in the proposed model is plotted as a local quality estimate of similarities to the corresponding residue of the peptide (Figure 3.9). The proposed model is displayed as a heat colouration of the QMEAN values for each residue in the peptide. Low QMEAN Z-score residues appear in orange, indigo to blue for high QMEAN Z-scores. The position of charged amino acid residues are highlighted. The cysteine pattern (C-C-C-CC-C) was identified in all four peptides of the M61-72 cocktail. The cysteine pattern is common to centipede VGPC blockers such as κ -SLPTX-Ssm1a that demonstrates inhibition of potassium channels in DRG neurones (Yang et al., 2012b). The chain of peptide I was 49 residues in length and contained 6 cysteine residues. Figure 3.9E shows four of the negatively charged aspartate and glutamate residues (red) in alignment down one side of one of the α -helices.

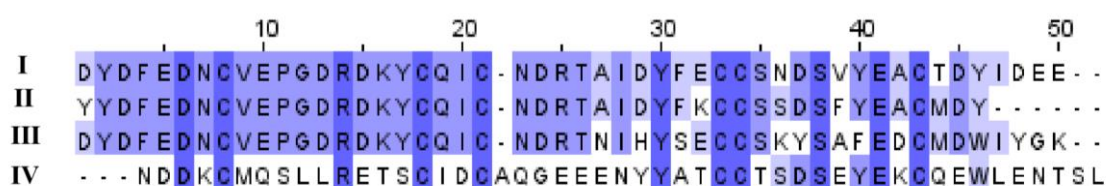


Figure 1.12 Amino acid sequences for the peptides belonging to the M61-72 cocktail. Conserved residues between peptide sequences are blue. Courtesy of Neville Ngum (2018)

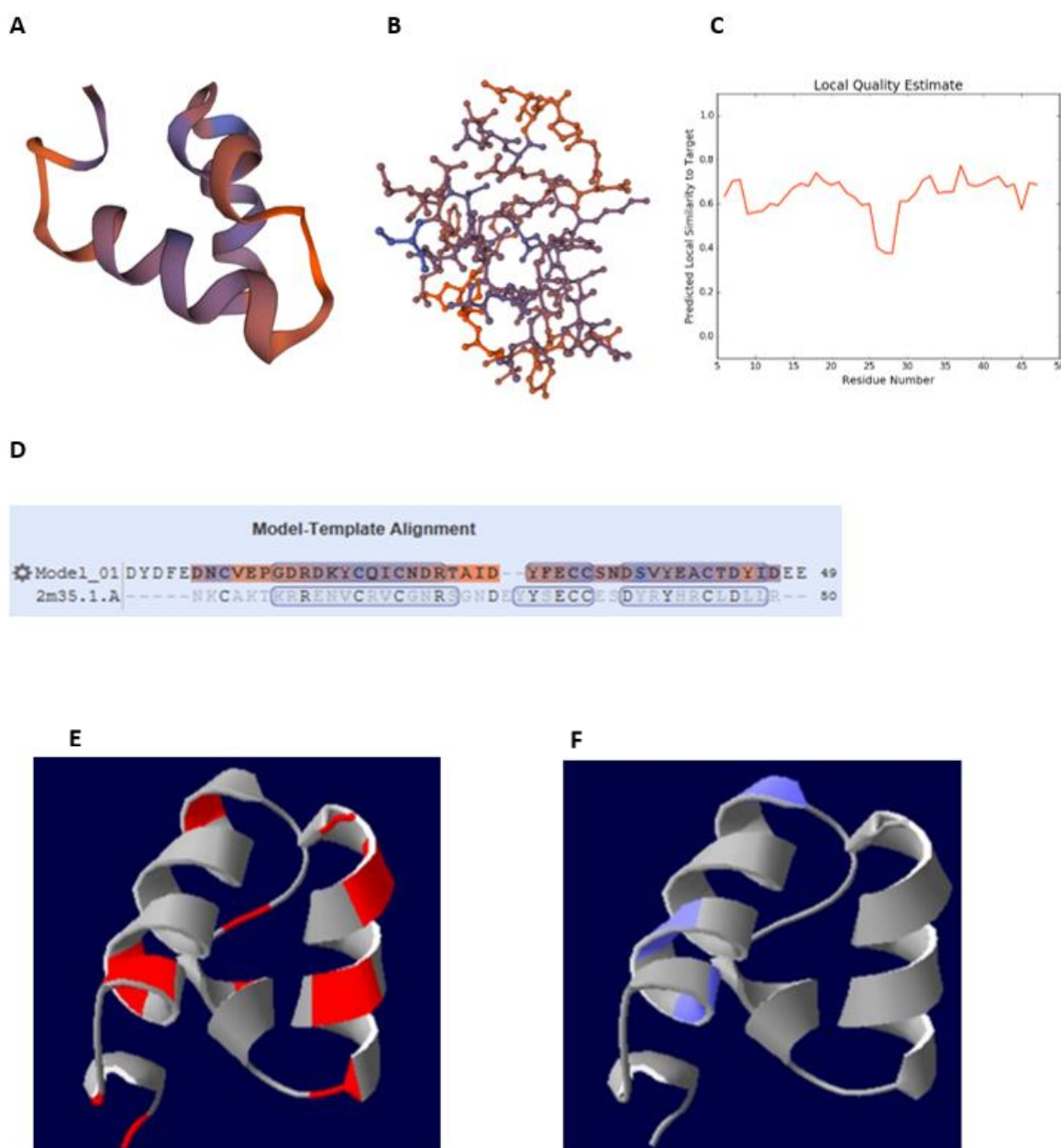


Figure 3.8 The modelled structure of peptide I. A represents the ribbon structure of the peptide backbone. B represents the ball and stick structure for the atoms constituting the different residues. C shows a plot indicating that 80% of the model's residues had QMEAN Z-scores above 0.6. D is the sequence of peptide I (model) aligned with the template sequence κ -SLPTX-Ssm1a. Residues conserved in the template are in bold. Low quality structural similarity is shown as orange and high similarity residues shown from indigo to blue. E represents the position of the

negatively charged amino acids (aspartate and glutamate, in red). F represents the position of positively charged amino acids (lysine and arginine, in blue).

Discussion

4.1 Characterisation of TE671 cell VGSCs

TE671 cell VGSCs were characterised before the application of TTX and peptide I. The TE671 cells were subjected to voltage protocols 1 & 2 to establish activation and inactivation kinetics. Cells exhibited whole-cell voltage-activated currents typical of VGSCs displaying fast activating inward currents followed by a fast current decrease due to inactivation. TE671 cells exhibited activation at potentials around -40mV following depolarisation. Figure 3.1 and 3.2 display the activation and inactivation of TE671 cell VGSCs and the time course of increasing depolarising steps. Similar time course of TE671 VGSC currents was first reported by Gambale and Montal (1990). The mean $V_{50,act}$ reported here -36.8 mV is characteristic of VGSCs. There has been ambiguity among the origin of the TE671 cell line, primary reports classed TE671 as a medulloblasoma cell line (McAllister et al., 1977). Reports later argued for a rhabdomyosarcoma cell line (Stratton et al., 1989b). Gambale and Montal (1990) first characterised the selectivity and voltage-dependence of TE671 cell VGSCs. However, subtype identification of TE671 VGSCs was unclear then and was limited to electrophysiological characterisation only. Molecular profiling of expression levels of VGSC subtypes has revealed that TE671 cells predominately express the $Na_v1.7$ subtype (Abdul-Aziz, unpublished literature, 2018). Because TE671 cells derive from a muscle cell line, one would assume expression of $Na_v1.4$, not $Na_v1.7$. However, it is not unusual for cell lines to exhibit unexpected characteristics in terms of protein expression.

The selective VGSC blocker, TTX, was utilized in this study to pharmacologically characterise the TE671 cell VGSC. Derived from puffer fish venom, TTX is highly neurotoxic and blocks sodium channels via the ion-conducting pore region. Pharmacological studies using TTX aided in the identification and characterisation of VGSCs (Narahashi, 1964). Electrophysiological analysis by Kostyuk et al (1981) first reported TTX-resistant and TTX-sensitive channels in rat DRG neurones. $Na_v1.7$ is TTX-sensitive, as well as $Na_v1.1$ -1.4 and $Na_v1.6$. Figure 3.4 shows sodium currents are completely blocked by 10 μ M TTX. This confirms that all of the voltage-sensitive inward current is mediated by VGSCs and discounts any involvement of, for example,

voltage-gated calcium channels. The IC_{50} obtained in the present work of 7.43 ± 5.70 nM is lower than 24.5 nM reported for $Na_v1.7$ from a human medullary thyroid carcinoma cell line expressing human neuroendocrine sodium channels (Klugbauer et al., 1995). Interestingly, Protoxin-II (ProTx2), a venom peptide from the Peruvian green velvet tarantula shows high selectivity for $Na_v1.7$ with an IC_{50} of 0.3nM (Schmalhofer et al., 2008). This being said, neurotoxins showing such selectivity should be used to establish pharmacology of VGSC subtypes.

Recent VGSC research assesses the involvement of $Na_v1.7$ in pain. $Na_v1.7$ research is usually concerned with DRG neurones where expression of these subtypes is abundant but overlaps with the expression of other Na_v subtypes as well as other voltage-gated ion channels. This study further confirms the importance and the future potential of TE671 cells, exclusively expressing $Na_v1.7$ in a muscle derived cell line is unique and can be easily subjected to other electrophysiological and molecular techniques to study pain. $Na_v1.7$ is a useful experimental model because it easily activates in response to membrane depolarisations. Exploring $Na_v1.7$ in TE671 cells bypasses the need for DRG neurone dissections or cell line transfection; our research shows this cell line can confidently be used to study electrophysiological and molecular properties of $Na_v1.7$ expressed in TE671 cells.

4.2 The effects of peptide I on $Na_v1.7$ channel activation

Initial work from Neville Ngum 2018 explored crude venoms and pooled fractions from male and female *S. hardwickei*. These cocktails demonstrated alterations to activation and inactivation kinetics on TE671 cell VGSCs and were thus subjected to further analysis to identify individual peptides. Peptide I from the M61-72 cocktail was utilized in this thesis; the effects of peptide I on the activation kinetics of TE671 cell VGSCs was at the forefront of investigation in this study based on activity of the corresponding cocktail. Our results confirm that peptide I exhibits a concentration-dependent hyperpolarising shift in the voltage-dependence of activation. 10 μ M peptide I shifted the $V_{50,act}$ by -11 mV (-22.9 ± 3.5 mV to -33.9 ± 0.8 mV; Table 3.1). In a physiological sense this represents a highly significant shift and should exert a major impact on neuronal excitability. The $V_{50,act}$ is closer to the resting membrane potential of the cell suggesting that peptide I is causing VGSCs to open in response to much smaller

depolarisations and enhances activation of sodium currents. Figure 3.5 and 3.6 demonstrates an enhanced response by 10 μ M peptide I, the response is not only enhanced but also activates faster. This is perhaps not surprising when the channel is activated close to the threshold as time to peak reduces greatly when the extent of depolarisation is increased just beyond this point, as seen in Figure 3.5. It can be confirmed peptide I exhibits a gain of function in VGSCs at 10 μ M. The gain of functions was also seen at much lower concentrations when the cells were depolarised continuously to a standard test potential over an increased period of time. This likely indicates a time and use dependent effect and may imply that the peptide preferentially binds to an activated state of the channel. Importantly, a hyperpolarising shift in the voltage-dependence of activation, or any other gain of function mechanism, is not yet reported in centipede venom. On the other hand, this mode of action can be seen in other VGSC activators. The hyperpolarising effects in activation of peptide I act in a similar manner as brevetoxins and ciguatoxins (Trainer et al., 1994). Both marine toxins are produced by certain dinoflagellates targeting VGSC activation and shifting it toward more negative potentials (Dechraoui et al., 1999). These toxins achieve this by binding to site 5 on DIS6 and DIVS5 (Wang, 2003). The negative shift in activation observed by peptide I is also a feature common to the site 2 toxins including batrachotoxin sourced from poison-dart frogs by enhancing and prolonging activation (Dumbacher et al., 2004). These neurotoxins are proposed to bind to site 2 on S6 of domains I-IV in the central pore cavity just above the gate (Wang, 2003). Peptide I would not be expected to bind here because it is a much larger molecule unable to reach this site. A negative shift in $V_{50.act}$ is also characteristic of pyrethroid insecticide activity. These are proposed to bind at a more intracellular pocket bounded by DIIS4-5, DIIS5 and DIIS6 (O'Reilly et al., 2006). Again this site may not be accessible by Peptide I because of space restrictions and also its highly hydrophilic nature; pyrethroids may access the site via the cell membrane because of their highly hydrophobic nature.

Toxins targeting the voltage-sensing domain on α -subunits can move positive charges on the S4 segment extracellularly in response to smaller changes in membrane potential and lock them in an activated position. A mechanism known as voltage-

sensor trapping can be used to explain the mode of action of these toxins, predominantly proposed in scorpion toxins. β -scorpion toxins target site 4 specifically on the extracellular S3-S4 loop in domain II on α -subunits. These toxins trap the S4 sensors and cause a shift in the voltage-dependence of activation to more negative potentials (Cestèle et al., 1998). Figure 3.9 shows predicted models of peptide I with negatively charged aspartate and glutamate residues located on one face of the third helical segment and followed by a further two acidic residues. Upon membrane depolarisation these residues could potentially form a region to attract the positively charged arginine and lysine residues that constitute the S4 voltage sensors of VGSCs. Cohen et al (2004) report a negatively charged residue, Glu-15, from β -scorpion toxin Csx4 involved in voltage sensor trapping. Unlike β -scorpion toxins, α -scorpion toxins bind at site 3 on the extracellular loop connecting the S3 and S4 segments in domain IV on VGSCs (Catterall et al., 2007). The differences in binding site location could explain the difference in mode of actions between these two toxins (Gurevitz, 2012). The voltage-sensor trapping mechanism is proposed in other toxins which also alter voltage-dependent gating of other ion channels including calcium and potassium channels. Modulation of activation kinetics is also observed in δ -Atracotoxins (δ -ACTX) isolated from the venom of Australian funnel-web spider that both shifts voltage-dependent activation in the hyperpolarising direction and slows channel inactivation. Unlike β -scorpion toxins that bind at site 4, δ -ACTX bind at site 3 (Nicholson, Little and Birinyi-Strachan, 2004). Evidence suggests similarity behind the kinetics and areas of binding on the α -subunit of β -scorpion toxins and δ -ACTX, and it would be sensible to suggest that peptide I may share the same site 3 or 4 binding site as these VGSC modulators. We cannot, however, rule out similar binding sites on alternative domains that would also result in enhanced activation by targeting S4. A recent publication has put forth an advanced insight into the inhibition of S4 movement by positively charged residues in Protoxin-II (ProTx2) (Xu et al., 2019). This toxin binds to DIIS3-S4 and inhibits movement of S4 through electrostatic repulsion. Thus, ProTx2 is proposed to trap DIIS4 in a deactivated state by binding to the S3-S4 loop, keeping the channel closed (Schmalhofer et al., 2008). This is similar to our proposed mechanism for peptide I but with a contrasting electrostatic effect.

The potentiating response observed from TE671 cell VGSCs (Figure 3.6) can lead to hyperexcitability and interestingly, insects are observed to display neurotoxicity including twitching body contractions following injection of crude centipede venom (Yang et al., 2012). It could be said enhanced activation may lead to more frequent sodium currents and repetitive action potential firing and result in neuronal hyperexcitability. The hyperpolarising shift in activation could be a contributing factor to the rapid paralysis observed in insects through a gain of function in VGSCs. This feature could be compared to the fast immobilization “lightning strike” induced by δ -conotoxins (Becker and Terlau, 2008). Conotoxins show efficient target site selectivity, μ -conotoxins occlude the pore inhibiting sodium conductance at site 1 whilst δ -conotoxins inhibit fast inactivation, binding to the S3-S4 of domain IV at site 3 (Ekberg, Craik and Adams, 2008). Centipede envenomation in humans causes excruciating burning pain and we can now suggest that a gain of function in human Nav1.7 as demonstrated here may be the cause of this. However, the other constituents of the diverse pools of cocktails within centipede venom must be considered as pain-inflicting too as an agonist of TRPV1, a noxious heat sensing ion channel has also been identified in *S. subspinipes mutilans* (Yang et al., 2015). So these two peptides combined may be responsible for the intensity of the pain. The excruciating pain experienced from centipede encounters and observed rigid paralysis in insects could suggest VGSC activators and inhibitors constitute the cocktail of venom peptides of centipedes. Centipede envenomation in invertebrates is lethal, in humans it can cause extreme pain lasting hours to days. Severe symptoms in humans include itching, fever, chills, haemorrhage and anaphylaxis, suggesting a variety of components are involved in centipede venom action (Yang et al., 2012).

4.3 Potential applications of peptide I

The hyperpolarising shift in the voltage-dependence of activation, as demonstrated here for Peptide I, is a mode of action commonly utilized by insecticides. Scorpion toxins have been exploited for their insecticidal properties; α and β scorpion toxins have been classified based on their toxicity to insects into three groups: i) anti insect alpha toxins; ii) alpha-like toxins; iii) contractive and depressant toxins. The last group mentioned belong to the beta toxins and show the highest insecticidal activity. From

this group, AaH ITI isolated from *Androctonus australis* scorpion venom shows a concentration dependent shift of activation towards more negative potentials observed in axonal sodium currents (De Lima et al., 2007). There is a striking similarity between the induced effects of AaH ITI and peptide I on activation, suggesting peptide I could be a novel source for insecticidal development. Centipede crude venom is lethal to insects and displays more potency than purified neurotoxins (Yang et al., 2012). Crude venom contains many components all acting on various targets, thus demonstrating significant potency over purified neurotoxins (Yang et al., 2012). The insecticidal components of centipede venom have been explored by Yang and colleagues (2012). Lethal activity of crude venom from *S. subspinipes mutilans* is significantly higher than that of purified neurotoxins. Symptoms of injected crude venom in insects included body contractions, twitching and paralysis. The purified peptides from *S. subspinipes mutilans* are ion channel inhibitors. This suggests VGSC inhibitors are not potent enough to cause death in insects. Inhibiting sodium conductance is not a crucial feature of insecticides. Myotoxins, cardiotoxins, cytotoxins and neurotoxins have been found in crude venoms of centipedes, particularly Scolopendramorphs (Undheim and King, 2011).

Pyrethroids are synthetic analogues of chrysanthemic acid (pyrethrins I) and pyrethric acid (pyrethrins II) found in the flowers of *Chrysanthemum cinerareifolium* (Davies et al., 2007). Peptide I employs a mechanism similar to the mode of action behind these insecticides. Pyrethroids induce membrane depolarisation by enhancing and prolonging channel activation and inhibiting the channel from deactivating (Dechraoui et al., 1999). The mode of action between peptide I and pyrethroids is to some extent comparable. However, type II pyrethroids are associated with large tail currents showing channel reopening (Dechraoui et al., 1999). There is no evidence of peptide I producing tail currents in either this study or Ngum (2018). Despite the modifications observed in VGSC activation, peptide I leaves channel deactivation untouched. So in this respect, Peptide I behaves more like type I pyrethroids that exhibit much shorter tail currents. Pyrethroids preferentially bind to the open states of VGSCs and this is something implied here with peptide I (O'Reilly et al., 2006). However, as discussed

earlier it is unlikely that peptide I binds to the same site as pyrethroids. Ideally, an insecticide must employ insect-specific toxins to only modify insect ion channels, not vertebrate. On the other hand, isolating individual peptides from Scolopendramorphs which exhibit a gain of function on VGSCs could exceed current insecticides

4.4 Future work

The presented results show $\text{Na}_v1.7$ modulation from a peptide isolated from *Scolopendra hardwickei* that warrants further characterisation. Excitatory modulation of activation is observed in numerous toxins including δ -ACTX, brevetoxins, ciguatoxins, β -scorpion toxins and can be compared to the “lightning strike” induced by δ -conotoxins. The hyperpolarising shift in the voltage-dependence of activation is a key feature of insecticides such as pyrethroids and insecticidal β -scorpion toxins. A significant shift in V_{50} was observed only by 10 μM peptide I in TE671 cells. It is likely that a lower concentration of peptide I could reproduce such a shift in activation in insect sodium channels, if not larger and display a larger gain of function of potentiating sodium currents. This would elevate interest in this peptide as a starting point for novel insecticides but first it should be screened against a range of insect VGSCs as well as other subtypes of vertebrate VGSCs. Other, highly homologous peptides were also present in the fraction cocktail that contained Peptide I. It is likely that these employ the same target with the same mechanism of action but may be directed towards different animal groups and so warrant similar investigation. It is likely that at least one of these peptides may be insect selective given that insects figure heavily in the diet of *S. hardwickei*. The activity of peptide I against other ion channels such as K_v and Ca_v would be the next step in further characterisation since peptide I and k-SLPTX-Ssm1a are structurally similar based on their cysteine pattern. Further characterisation beyond electrophysiology of this peptide and other modulatory venom peptides from *S. hardwickei* could form the foundation of a novel insecticide. For example, no experiments looking at effects on whole insects have been performed yet.

References

- AMEY, J., O'REILLY, A., BURTON, M., PUINEAN, A., MELLOR, I., DUCE, I., FIELD, L., WALLACE, B., WILLIAMSON, M. AND DAVIES, T. (2015). An evolutionarily-unique heterodimeric voltage-gated cation channel found in aphids. *FEBS Letters*, 589(5), pp.598-607.
- ARMSTRONG, C. (2006). Na channel inactivation from open and closed states. *Proceedings of the National Academy of Sciences*, 103(47), pp.17991-17996.
- BAER M, REUTER H (1976) Voltage-dependent action of tetrodotoxin in mammalian cardiac muscle. *Nature* 263:344-345
- BAGAL, S., CHAPMAN, M., MARRON, B., PRIME, R., STORER, R. AND SWAIN, N. (2014). Recent progress in sodium channel modulators for pain. *Bioorganic & Medicinal Chemistry Letters*, 24(16), pp.3690-3699.
- BECKER, S. AND TERLAU, H. (2008). Toxins from cone snails: properties, applications and biotechnological production. *Applied Microbiology and Biotechnology*, 79(1), pp.1-9.
- BEZANILLA, F. (2000). The Voltage Sensor in Voltage-Dependent Ion Channels. *Physiological Reviews*, 80(2), pp.555-592.
- CARNEVALE, V. AND KLEIN, M. (2017). Small molecule modulation of voltage gated sodium channels. *Current Opinion in Structural Biology*, 43, pp.156-162.
- CATTERALL, W. (1986). Molecular Properties of Voltage-Sensitive Sodium Channels. *Annual Review of Biochemistry*, 55(1), pp.953-985.
- CATTERALL, W. (1988). Structure and function of voltage-sensitive ion channels. *Science*, 242(4875), pp.50-61.
- CATTERALL, W. (1995). Structure and Function of Voltage-Gated Ion Channels. *Annual Review of Biochemistry*, 64(1), pp.493-531.
- CATTERALL, W. (2000). From Ionic Currents to Molecular Mechanisms: The Structure and Function of Voltage-Gated Sodium Channels. *Neuron*, 26(1), pp.13-25.

- CATTERALL, W. (2012). Voltage-gated sodium channels at 60: structure, function and pathophysiology. *The Journal of Physiology*, 590(11), pp.2577-2589.
- CATTERALL, W. A., GOLDIN, A. L. & WAXMAN, S. G. (2005a). International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol Rev*, 57, 397-409
- CATTERALL, W., CESTÈLE, S., YAROV-YAROVY, V., YU, F., KONOKI, K. AND SCHEUER, T. (2007). Voltage-gated ion channels and gating modifier toxins. *Toxicon*, 49(2), pp.124-141.
- CERVENKA, R., LUKACS, P., GAWALI, V., KE, S., KOENIG, X., RUBI, L., ZARRABI, T., HILBER, K., SANDTNER, W., STARY-WEINZINGER, A. AND TODT, H. (2018). Distinct modulation of inactivation by a residue in the pore domain of voltage-gated Na⁺ channels: mechanistic insights from recent crystal structures. *Scientific Reports*, 8(1).
- CESTÈLE, S., QU, Y., ROGERS, J., ROCHAT, H. AND CATTERALL, W. (1998). Voltage Sensor–Trapping: Enhanced Activation of Sodium Channels by β -Scorpion Toxin Bound to the S3–S4 Loop in Domain II. *Neuron*, 21, pp.919-931.
- CHEN, T., DOROTINSKY, C., MACY, M. & HAY, R. 1989. Cell identity resolved. *Nature*, 340, 106.
- CLAIRFEUILLE, T., CLOAKE, A., INFELD, D., LLONGUERAS, J., ARTHUR, C., LI, Z., JIAN, Y., MARTIN-EAUCLAIRE, M., BOUGIS, P., CIFERRI, C., AHERN, C., BOSMANS, F., HACKOS, D., ROHOU, A. AND PAYANDEH, J. (2019). Structural basis of α -scorpion toxin action on Nav channels. *Science*, 363(6433),
- COHEN, L., KARBAT, I., GILLES, N., ILAN, N., BENVENISTE, M., GORDON, D. AND GUREVITZ, M. (2004). Common Features in the Functional Surface of Scorpion β -Toxins and Elements That Confer Specificity for Insect and Mammalian Voltage-gated Sodium Channels. *Journal of Biological Chemistry*, 280(6), pp.5045-5053.
- COX, J., REIMANN, F., NICHOLAS, A., THORNTON, G., ROBERTS, E., SPRINGELL, K., KARBANI, G., JAFRI, H., MANNAN, J., RAASHID, Y., AL-GAZALI, L., HAMAMY, H., VALENTE, E., GORMAN, S., WILLIAMS, R., MCHALE, D., WOOD, J., GRIBBLE, F. AND

- WOODS, C. (2006). An SCN9A channelopathy causes congenital inability to experience pain. *Nature*, 444(7121), pp.894-898.
- DAVIES, T., FIELD, L., USHERWOOD, P. AND WILLIAMSON, M. (2007). DDT, pyrethrins, pyrethroids and insect sodium channels. *IUBMB Life*, 59(3), pp.151-162.
- DE LIMA, M., FIGUEIREDO, S., PIMENTA, A., SANTOS, D., BORGES, M., CORDEIRO, M., RICHARDSON, M., OLIVEIRA, L., STANKIEWICZ, M. AND PELHATE, M. (2007). Peptides of arachnid venoms with insecticidal activity targeting sodium channels. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 146(1-2), pp.264-279.
- DECHRAOUI, M., NAAR, J., PAUILLAC, S. AND LEGRAND, A. (1999). Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels. *Toxicon*, 37(1), pp.125-143.
- DEUIS, J., MUELLER, A., ISRAEL, M. AND VETTER, I. (2017). The pharmacology of voltage-gated sodium channel activators. *Neuropharmacology*, 127, pp.87-108.
- DIB-HAJJ, S., CUMMINS, T., BLACK, J. AND WAXMAN, S. (2007). From genes to pain: Na_v1.7 and human pain disorders. *Trends in Neurosciences*, 30(11), pp.555-563.
- DONG, K. (2007). Insect sodium channels and insecticide resistance. *Invertebrate Neuroscience*, 7(1), pp.17-30.
- DONG, K., DU, Y., RINKEVICH, F., NOMURA, Y., XU, P., WANG, L., SILVER, K. AND ZHOROV, B. (2014). Molecular biology of insect sodium channels and pyrethroid resistance. *Insect Biochemistry and Molecular Biology*, 50, pp.1-17.
- DU, Y., GARDEN, D., KHAMBAY, B., ZHOROV, B. AND DONG, K. (2011). Batrachotoxin, Pyrethroids, and BTG 502 Share Overlapping Binding Sites on Insect Sodium Channels. *Molecular Pharmacology*, 80(3), pp.426-433.
- DUMBACHER, J., WAKO, A., DERRICKSON, S., SAMUELSON, A., SPANDE, T. AND DALY, J. (2004). Melyrid beetles (Choresine): A putative source for the batrachotoxin alkaloids found in poison-dart frogs and toxic passerine birds. *Proceedings of the National Academy of Sciences*, 101(45), pp.15857-15860.

- EDGECOMBE, G. D. & GIRIBET, G. 2007. Evolutionary biology of centipedes (Myriapoda: Chilopoda). *Annu. Rev. Entomol.*, 52, 151-170.
- EKBERG, J., CRAIK, D. AND ADAMS, D. (2008). Conotoxin modulation of voltage-gated sodium channels. *The International Journal of Biochemistry & Cell Biology*, 40(11), pp.2363-2368.
- FAVRE, I., MOCZYDLOWSKI, E. AND SCHILD, L. (1996). On the structural basis for ionic selectivity among Na⁺, K⁺, and Ca²⁺ in the voltage-gated sodium channel. *Biophysical Journal*, 71(6), pp.3110-3125.
- FERTLEMAN, C., BAKER, M., PARKER, K., MOFFATT, S., ELMSLIE, F., ABRAHAMSEN, B., OSTMAN, J., KLUGBAUER, N., WOOD, J., GARDINER, R. AND REES, M. (2006). SCN9A Mutations in Paroxysmal Extreme Pain Disorder: Allelic Variants Underlie Distinct Channel Defects and Phenotypes. *Neuron*, 52(5), pp.767-774.
- GAMBALE, F. & MONTAL, M. 1990. Voltage-gated sodium channels expressed in the human cerebellar medulloblastoma cell line TE671. *Molecular Brain Research*, 7, 123-129.
- GINSBURG, K. AND NARAHASHI, T. (1993). Differential sensitivity of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels to the insecticide allethrin in rat dorsal root ganglion neurons. *Brain Research*, 627(2), pp.239-248.
- GOLDIN, A. (2003). Mechanisms of sodium channel inactivation. *Current Opinion in Neurobiology*, 13(3), pp.284-290.
- GORDON, D., CHEN, R. AND CHUNG, S. (2013). Computational Methods of Studying the Binding of Toxins From Venomous Animals to Biological Ion Channels: Theory and Applications. *Physiological Reviews*, 93(2), pp.767-802.
- GUREVITZ, M. (2012). 172. Mapping the Receptor Sites of Scorpion Toxins at Voltage-gated Sodium Channels. *Toxicon*, 60(2), pp.183-184.
- GUY, H. AND SEETHARAMULU, P. (1986). Molecular model of the action potential sodium channel. *Proceedings of the National Academy of Sciences*, 83(2), pp.508-512.

- HAKIM, M., YANG, S. AND LAI, R. (2015). Centipede Venoms and Their Components: Resources for Potential Therapeutic Applications. *Toxins*, 7(11), pp.4832-4851.
- HILLE, B. (1992). IONIC CHANNELS OF EXCITABLE MEMBRANES, MASS
- HILLE, B. (2001). *Ion channels of excitable membranes*. 3rd ed. Sunderland, Mass.: Sinauer.
- HODGKIN, A. L. & HUXLEY, A. F. (1952a). Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo. *The Journal of Physiology*, 116, 449-472.
- KELLENBERGER, S., WEST, J., SCHEUER, T. AND CATTERALL, W. (1997). Molecular Analysis of the Putative Inactivation Particle in the Inactivation Gate of Brain Type IIA Na⁺Channels. *The Journal of General Physiology*, 109(5), pp.589-605.
- KEYNES, R. (1994). The kinetics of voltage-gated ion channels. *Quarterly Reviews of Biophysics*, 27(04), p.339.
- KEYNES, R. AND ELINDER, F. (1999). The screw-helical voltage gating of ion channels. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 266(1421), pp.843-852.
- KLUGBAUER N, LACINOVA L, FLOCKERZI V, HOFMANN F. (1995). Structure and functional expression of a new member of the tetrodotoxin-sensitive voltage-activated sodium channel family from human neuroendocrine cells. *EMBO J* 14:1084-1090.
- KLUGBAUER, N., LACINOVA, L., FLOCKERZI, V. & HOFMANN, F. 1995. Structure and functional expression of a new member of the tetrodotoxin-sensitive voltage-activated sodium channel family from human neuroendocrine cells. *The EMBO Journal*, 14, 1084-1090.
- KNYSAK, I., MARTINS, R. & BERTIM, C. R. (1998). Epidemiological aspects of centipede (Scolopendromorphae: Chilopoda) bites registered in Greater S. Paulo, SP, Brazil *Revista de saude publica*, 32, 514-518.

Kostyuk PG., Veselovsky NS, Tsyndrenko AY (1981) Ionic currents in the somatic membrane of rat dorsal root ganglion neurons-I. Sodium currents. *Neuroscience* 6:2423-2430. Lee KS, Hume JR, Giles W, Brown AM (1981)

KWONG, K. AND CARR, M. (2015). Voltage-gated sodium channels. *Current Opinion in Pharmacology*, 22, pp.131-139.

LONG, S., TAO, X., CAMPBELL, E. AND MACKINNON, R. (2007). Atomic structure of a voltage-dependent K⁺ channel in a lipid membrane-like environment. *Nature*, 450(7168), pp.376-382.

MCALLISTER, R., ISAACS, H., RONGEY, R., PEER, M., AU, W., SOUKUP, S. & GARDNER, M. (1977). Establishment of a human medulloblastoma cell line. *International journal of cancer*, 20, 206-212.

NARAHASHI, T. (1964). Tetrodotoxin Blockage of Sodium Conductance Increase in Lobster Giant Axons. *The Journal of General Physiology*, 47(5), pp.965-974.

NASSAR, M. A., STIRLING, L. C., FORLANI, G., BAKER, M. D., MATTHEWS, E. A., DICKENSON, A. H. & WOOD, J. N. (2004). Nociceptor-specific gene deletion reveals a major role for Na_v1. 7 (PN1) in acute and inflammatory pain. *Proceedings of the national Academy of Sciences of the United States of America*, 101, 12706-12711.

NEHER, E. and SAKMANN, B. (1976). Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature*, 260(5554), pp.799-802.

NGUM, N. (2018). Investigating the venom components of the Giant Indian Centipede for Na_v1.7 channel modifiers. PhD Thesis, University of Nottingham.

NICHOLSON, G., LITTLE, M. AND BIRINYI-STRACHAN, L. (2004). Structure and function of δ -atractoxins: lethal neurotoxins targeting the voltage-gated sodium channel. *Toxicon*, 43(5), pp.587-599.

NODA, M., SHIMIZU, S., TANABE, T., TAKAI, T., KAYANO, T., IKEDA, T., TAKAHASHI, H., NAKAYAMA, H., KANAOKA, Y., MINAMINO, N., KANGAWA, K., MATSUO, H., RAFTERY, M., HIROSE, T., INAYAMA, S., HAYASHIDA, H., MIYATA, T. AND NUMA, S. (1984). Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. *Nature*, 312(5990), pp.121-127.

O'REILLY, A., KHAMBAY, B., WILLIAMSON, M., FIELD, L., WALLACE, B. AND DAVIES, T. (2006). Modelling insecticide-binding sites in the voltage-gated sodium channel. *Biochemical Journal*, 396(2), pp.255-263.

O'REILLY, A., WILLIAMSON, M., GONZÁLEZ-CABRERA, J., TURBERG, A., FIELD, L., WALLACE, B. AND DAVIES, T. (2013). Predictive 3D modelling of the interactions of pyrethroids with the voltage-gated sodium channels of ticks and mites. *Pest Management Science*, 70(3), pp.369-377.

OSWALD, R. E., PAPKE, R. L. & LUKAS, R. J. (1989). Characterization of nicotinic acetylcholine receptor channels of the TE671 human medulloblastoma clonal line. *Neuroscience letters*, 96, 207-212.

PAN, X., LI, Z., HUANG, X., HUANG, G., GAO, S., SHEN, H., LIU, L., LEI, J. AND YAN, N. (2019). Molecular basis for pore blockade of human Na⁺ channel for Na_v1.2 by the human μ -conotoxin KIIIA. *Science*, 363, pp.1309-1313.

PAYANDEH, J., GAMAL EL-DIN, T., SCHEUER, T., ZHENG, N. AND CATTERALL, W. (2012). Crystal structure of a voltage-gated sodium channel in two potentially inactivated states. *Nature*, 486(7401), pp.135-139.

PAYANDEH, J., SCHEUER, T., ZHENG, N. AND CATTERALL, W. (2011). The crystal structure of a voltage-gated sodium channel. *Nature*, 475(7356), pp.353-358.

PENG, K., KONG, Y., ZHAI, L., WU, X., JIA, P., LIU, J. & YU, H. (2010). Two novel antimicrobial peptides from centipede venoms. *Toxicon*, 55, 274-279.

ROHL, C., BOECKMAN, F., BAKER, C., SCHEUER, T., CATTERALL, W. AND KLEVIT, R. (1999). Solution Structure of the Sodium Channel Inactivation Gate^{†,‡}. *Biochemistry*, 38(3), pp.855-861.

RONG, M., YANG, S., WEN, B., MO, G., KANG, D., LIU, J., LIN, Z., JIANG, W., LI, B., DU, C., YANG, S., JIANG, H., FENG, Q., XU, X., WANG, J. AND LAI, R. (2015). Peptidomics combined with cDNA library unravel the diversity of centipede venom. *Journal of Proteomics*, 114, pp.28-37.

- RUDY, B. (1978). Slow inactivation of the sodium conductance in squid giant axons. Pronase resistance. *The Journal of Physiology*, 283(1), pp.1-21.
- SAEZ, N., SENFF, S., JENSEN, J., ER, S., HERZIG, V., RASH, L. AND KING, G. (2010). Spider-Venom Peptides as Therapeutics. *Toxins*, 2(12), pp.2851-2871.
- SCHMALHOFER, W., CALHOUN, J., BURROWS, R., BAILEY, T., KOHLER, M., WEINGLASS, A., KACZOROWSKI, G., GARCIA, M., KOLTZENBURG, M. AND PRIEST, B. (2008). ProTx-II, a Selective Inhibitor of Nav1.7 Sodium Channels, Blocks Action Potential Propagation in Nociceptors. *Molecular Pharmacology*, 74(5), pp.1476-1484.
- SCHOEPFER, R., LUTHER, M. & LINDSTROM, J. (1988). The human medulloblastoma cell line TE671 expresses a muscle-like acetylcholine receptor Cloning of the α -subunit cDNA. *FEBS letters*, 226, 235-240.
- SHEN, H., LI, Z., JIANG, Y., PAN, X., WU, J., CRISTOFORI-ARMSTRONG, B., SMITH, J., CHIN, Y., LEI, J., ZHOU, Q., KING, G. AND YANG, N. (2018). Structural basis for the modulation of voltage-gated sodium channels by animal toxins. *Sciences*, 362.
- SHEN, H., LIU, D., WU, K., LEI, J. AND YAN, N. (2019). Structures of human Nav1.7 channel in complex with auxiliary subunits and animal toxins. *Sciences*, 363, pp.1303-1308.
- SHEN, H., ZHOU, Q., PAN, X., LI, Z., WU, J. AND YAN, N. (2017). Structure of a eukaryotic voltage-gated sodium channel at near-atomic resolution. *Science*, 355(6328)
- STARKUS, J. AND SHRAGER, P. (1978). Modification of slow sodium inactivation in nerve after internal perfusion with trypsin. *American Journal of Physiology-Cell Physiology*, 235(5), pp.C238-C244.
- STEVENS, M., PEIGNEUR, S. AND TYTGAT, J. (2011). Neurotoxins and Their Binding Areas on Voltage-Gated Sodium Channels. *Frontiers in Pharmacology*, 2.
- STRATTON, M., DARLING, J., PILKINGTON, G., LANTOS, P., REEVES, B. AND COOPER, C. (1989). Characterization of the human cell line TE671. *Carcinogenesis*, 10(5), pp.899-905.

- SUN, P., WU, F., WEN, M., YANG, X., WANG, C., LI, Y., HE, S., ZHANG, L., ZHANG, Y. & TIAN, C. 2015. A distinct three-helix centipede toxin SSD609 inhibits I_k channels by interacting with the KCNE1 auxiliary subunit. *Scientific reports*, 5, 13399.
- SUPAKTHANASIRI, P. (2004). Anaphylaxis to centipede bite*1. *Journal of Allergy and Clinical Immunology*, 113(2), p.S244.
- SYAPIN, P. J., SALVATERRA, P. M. & ENGELHARDT, J. K. (1982). Neuronal-like features of TE671 cells: presence of a functional nicotinic cholinergic receptor. *Brain research*, 231, 365-377
- TRAINER, V., BADEN, D. AND CATTERALL, W. (1994). Identification of Peptide Components of the Brevetoxin Receptor Site of Rat Brain Sodium Channel. *The Journal of Biological Chemistry*, 269(31).
- ULBRICHT, W. (2005). Sodium Channel Inactivation: Molecular Determinants and Modulation. *Physiological Reviews*, 85(4), pp.1271-1301.
- UNDHEIM, E. A., GRIMM, L. L., LOW, C.-F., MORGENSTERN, D., HERZIG, V., ZOBELTHROPP, P., PINEDA, S. S., HABIB, R., DZIEMBOROWICZ, S. & FRY, B. G. (2015b). Weaponization of a hormone: convergent recruitment of hyperglycemic hormone into the venom of arthropod predators. *Structure*, 23, 1283-1292.
- UNDHEIM, E. AND KING, G. (2011). On the venom system of centipedes (Chilopoda), a neglected group of venomous animals. *Toxicon*, 57(4), pp.512-524.
- UNDHEIM, E., FRY, B. AND KING, G. (2015a). Centipede Venom: Recent Discoveries and Current State of Knowledge. *Toxins*, 7(3), pp.679-704.
- UNDHEIM, E., JONES, A., CLAUSER, K., HOLLAND, J., PINEDA, S., KING, G. AND FRY, B. (2014). Clawing through Evolution: Toxin Diversification and Convergence in the Ancient Lineage Chilopoda (Centipedes). *Molecular Biology and Evolution*, 31(8), pp.2124-2148.
- VALENZUELA, C. AND BENNETT, P. (1994). Gating of cardiac Na⁺ channels in excised membrane patches after modification by alpha-chymotrypsin. *Biophysical Journal*, 67(1), pp.161-171.

- VETTER, I., DEUIS, J., MUELLER, A., ISRAEL, M., STAROBOVA, H., ZHANG, A., RASH, L. AND MOBLI, M. (2017). Nav1.7 as a pain target – From gene to pharmacology. *Pharmacology & Therapeutics*, 172, pp.73-100.
- VILIN, Y. AND RUBEN, P. (2001). Slow Inactivation in Voltage-Gated Sodium Channels: Molecular Substrates and Contributions to Channelopathies. *Cell Biochemistry and Biophysics*, 35(2), pp.171-190.
- WANG, S. (2003). Voltage-gated sodium channels as primary targets of diverse lipid-soluble neurotoxins. *Cellular Signalling*, 15(2), pp.151-159.
- WAXMAN, S. AND DIB-HAJJ, S. (2005). Erythromalgia: molecular basis for an inherited pain syndrome. *Trends in Molecular Medicine*, 11(12), pp.555-562.
- WEST, J., PATTON, D., SCHEUER, T., WANG, Y., GOLDIN, A. AND CATTERALL, W. (1992). A cluster of hydrophobic amino acid residues required for fast Na⁽⁺⁾-channel inactivation. *Proceedings of the National Academy of Sciences*, 89(22), pp.10910-10914.
- XU, H., LI, T., ROHOU, A., ARTHUR, C., TZAKONIATI, F., WONG, E., ESTEVEZ, A., KUGEL, C., FRANKE, Y., CHEN, J., CIFERRI, C., HACKOS, D., KOTH, C. AND PAYANDEH, J. (2019). Structural Basis of Nav1.7 Inhibition by a Gating-Modifier Spider Toxin. *Cell*.
- YAN, Z., ZHOU, Q., WANG, L., WU, J., ZHAO, Y., HUANG, G., PENG, W., SHEN, H., LEI, J. AND YAN, N. (2017). Structure of the Na^v 1.4-β1 Complex from Electric Eel. *Cell*, 170(3), pp.470-482.e11.
- YANG, S., LIU, Z., XIAO, Y., LI, Y., RONG, M., LIANG, S., ZHANG, Z., YU, H., KING, G. AND LAI, R. (2012). Chemical Punch Packed in Venoms Makes Centipedes Excellent Predators. *Molecular & Cellular Proteomics*, 11(9), pp.640-650.
- YANG, S., XIAO, Y., KANG, D., LIU, J., LI, Y., UNDHEIM, E., KLINT, J., RONG, M., LAI, R. AND KING, G. (2013). Discovery of a selective Nav1.7 inhibitor from centipede venom with analgesic efficacy exceeding morphine in rodent pain models. *Proceedings of the National Academy of Sciences*, 110(43), pp.17534-17539.

YANG, S., YANG, F., WEI, N., HONG, J., LI, B., LUO, L., RONG, M., YAROV-YAROVY, V., ZHENG, J., WANG, K. AND LAI, R. (2016). A Pain-Inducing Centipede Toxin Targets the Heat Activation Machinery of Nociceptor TRPV1. *Biophysical Journal*, 110(3), p.284a.

YANG, S., YANG, F., WEI, N., HONG, J., LI, B., LUO, L., RONG, M., YAROVYAROVY, V., ZHENG, J. & WANG, K. (2015). A pain-inducing centipede toxin targets the heat activation machinery of nociceptor TRPV1. *Nature communications*, 6.

YANG, Y., MIS, M., ESTACION, M., DIB-HAJJ, S. AND WAXMAN, S. (2018). Na V 1.7 as a Pharmacogenomic Target for Pain: Moving Toward Precision Medicine. *Trends in Pharmacological Sciences*, 39(3), pp.258-275.

YU, F. AND CATTERALL, W. (2003). Overview of the voltage-gated sodium channel family. *Genome Biology*, 4(3).

ZLOTKIN, E. (1999). THE INSECT VOLTAGE-GATED SODIUM CHANNEL AS TARGET OF INSECTICIDES. *Annual Review of Entomology*, 44(1), pp.429