A Pharmacological and Transcriptomic approach to exploring Novel Pain

Targets

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Abstract

Ever since the discovery that mutations in the voltage-gated sodium channel 1.7 protein are responsible for human congenital insensitivity to pain, the voltage-gated sodium channel (Nav) family of ion channels has been the subject of intense research with the hope of discovering novel analgesics. We now know that Na\textsubscript{V}1.7 deletion in select neuronal populations yield different phenotypes, with the deletion of Na\textsubscript{V}1.7 in all sensory neurons being successful at abolishing mechanical and heat-induced pain. However, it is rapidly becoming apparent that a number of pain syndromes are not modulated by Na\textsubscript{V}1.7 at all, such as oxaliplatin-mediated neuropathy. It is particularly interesting to note that the loss of Na\textsubscript{V}1.7 function is also associated with the selective inhibition of pain mediated by specific stimuli, such as that observed in burn-induced pain where Na\textsubscript{V}1.7 gene knockout abolished thermal allodynia but did not affect mechanical allodynia. Accordingly, significant interests exist in delineating the contribution of other Na\textsubscript{V} isoforms in modality-specific pain pathways. Two other isoforms, Na\textsubscript{V}1.6 and Na\textsubscript{V}1.8, are now specifically implicated in some Na\textsubscript{V}1.7-independent conditions such as oxaliplatin-induced cold allodynia. Such selective contributions of specific ion channel isoforms to pain highlight the need to discover other putative protein targets involved in mediating nociception.

The aim of my work is therefore to discover selective molecular inhibitors of Na\textsubscript{V}1.6 and Na\textsubscript{V}1.8, to find useful cell models for peripheral nociceptors, to investigate the roles of Na\textsubscript{V}1.6 and Na\textsubscript{V}1.8 in an animal model of burn-induced pain, and to screen for putative new targets for analgesia in burn-related pain.

Chapter 2 of this thesis describes activity-guided discovery of novel Na\textsubscript{V}1.6 and Na\textsubscript{V}1.8-modulating peptides from crude spider venoms. Crude venom from Poecilotheria metallica successfully reduced Na\textsubscript{V}1.8-mediated voltage changes in HEK293-expressing cells. A new Na\textsubscript{V}1.8-inhibitory peptide was sequenced from the venom and named Pme1a. However, Pme1a exhibited TRPV1 agonist activity and induced nocifensive behaviours, such as paw licking, after injection into the hind paws of mice, indicating the peptide was not a selective Na\textsubscript{V}1.8 modulator and was unsuitable as a tool for Na\textsubscript{V}1.8 investigation.
With the unsuccessful exploration of spider venoms for new specific inhibitors, I then investigated \textit{in vitro} cell models as tools to research specific nociceptor subtypes that express \(\text{Na}_V\text{1.6}\) or \(\text{Na}_V\text{1.8}\). In Chapter 3, I provide the first complete transcriptome of three common \textit{in vitro} neuronal cell lines (SH-SY5Y, F-11, and ND7/23) to examine whether they were appropriate for investigating the functions of \(\text{Na}_V\text{1.6}\) and \(\text{Na}_V\text{1.8}\), and to identify if the cell lines resemble \textit{in vivo} dorsal root ganglion (DRG) neuronal subclasses. The three cell lines examined all expressed proteins belonging to similar pathways and of similar proportions to native DRG neurons. However, they did not express any cellular markers in a fashion that represented any known subclasses of DRG neurons. Therefore, it is unlikely that any of the cell lines examined are appropriate models for \(\text{Na}_V\text{1.6}\) or \(\text{Na}_V\text{1.8}\) function, and highlights the need for careful selection of models in \textit{in vitro} work.

As investigations for selective \(\text{Na}_V\text{1.6}\) and \(\text{Na}_V\text{1.8}\) modulators and appropriate \textit{in vitro} cell models both proved to be unsuccessful, I then turned to animal models to study modality-specific contributions of \(\text{Na}_V\) isoforms. Chapter 4 describes the establishment of a murine model of peripheral burn injury to assess the involvement of \(\text{Na}_V\text{1.6}\) and \(\text{Na}_V\text{1.8}\) in burn-induced pain. The inhibition of \(\text{Na}_V\text{1.6}\) and no other \(\text{Na}_V\) isoforms significantly reduced burn-induced mechanical allodynia, a \(\text{Na}_V\text{1.7}\)-independent pain modality, indicating \(\text{Na}_V\text{1.6}\) plays a vital role in this \(\text{Na}_V\text{1.7}\)-independent condition. However, immunofluorescence studies revealed \(\text{Na}_V\text{1.6}\) expression was not changed in the affected DRGs. I therefore decided to conduct a transcriptomic investigation to examine whether the expression of other pain-related ion channel gene were changed, and to discover if genes differentially expressed in the affected DRG neurons could be potential pharmacological targets for analgesia.

The results of the transcriptomic investigation of DRGs affected by the burn model are discussed in Chapter 5. A total of 30 genes were found to be differentially expressed, including genes known to be involved in nociception (such as neuropeptide Y) as well as genes with no known association to nociception (such as lipase, family member N). It should be noted that none of the \(\text{Na}_V\) isoforms were differentially expressed, indicating that \(\text{Na}_V\) channels may alter pain sensitivity through modulated function rather than expression. Selective inhibitors of the protein products of the genes found to be up-regulated were then tested in the burn-induced pain mouse model. Proglumide, an inhibitor of the cholecystokinin B receptor, exhibited a significant anti-allodynic effect in burn-induced mechanical allodynia and was synergistic with oxycodone. Some genes found to be differentially expressed in burn
injury were also identified in the cell lines covered in Chapter 3, and the cell lines can potentially be used as *in vitro* models to rapidly profile the effects of inhibitors of these proteins in peripheral neurons.


**Declaration by author**

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

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Contributions by others to the thesis

The thesis is a collection of work primarily conducted by me, with contribution from many other co-workers and collaborators as detailed below.

My supervisors, Dr. Irina Vetter, Associate Professor Peter Cabot, and Dr. Fernanda Caldas Cardoso have contributed significantly to the conception and design of many experiments. All crude venoms examined in Chapter 2 were generously provided by Dr. Volker Herzog from the Institute for Molecular Bioscience, and I would like to extend my thanks to Dr. Aihua Jin and Dr. Mu Yu for their provided invaluable support in conducting MS/MS experiments and finalising the sequence of the peptides. Dr. Gregory J. Baillie from the Institute for Molecular Bioscience contributed significantly to bioinformatics data analysis for Chapter 3 and Chapter 5, particularly with gene alignment and mapping. Na\textsubscript{V} inhibitory peptides used in Chapter 4 were synthesised by Mr. Zoltan Dekan from the Institute for Molecular Bioscience. Dr. Jennifer R. Deuis from the Institute for Molecular Bioscience contributed significantly to mechanical and thermal allodynia measurements in mice for Chapter 4 and Chapter 5, and towards the motor assessment of mice (Figures 4.3 and 5.5). Ms Mathilde Israel and Mr. Bryan Tay also contributed significantly to the fluorescence imaging data present in Figures 4.5 and 4.6, assisting with microscope operation and optimisation.

Statement of parts of the thesis submitted to qualify for the award of another degree

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Kathleen Yin
August 2016
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ANZSRC code: 110905 Peripheral Nervous System, 40%
ANZSRC code: 111502 Clinical Pharmacology and Therapeutics, 10%

Fields of Research (FoR) Classification
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FoR code: 1109, Neurosciences, 15%
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>AITC</td>
<td>Allyl isothiocyanate</td>
</tr>
<tr>
<td>AFU</td>
<td>Arbitrary fluorescence unit</td>
</tr>
<tr>
<td>AGRF</td>
<td>Australian Genome Research Facility</td>
</tr>
<tr>
<td>APAF</td>
<td>Australian Proteome Analysis Facility</td>
</tr>
<tr>
<td>ASIC</td>
<td>Acid-sensitive ion channel</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary unit</td>
</tr>
<tr>
<td>BP</td>
<td>Biological processes</td>
</tr>
<tr>
<td>C-LTMR</td>
<td>C low-threshold mechanosensitive receptor</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>Cav</td>
<td>Voltage-sensitive calcium channel</td>
</tr>
<tr>
<td>CCI</td>
<td>Chronic constriction injury</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
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<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-Cyano-4-hydroxycinnamic acid</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Media</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate-buffered saline</td>
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<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>F-3</td>
<td>Coagulation Factor III</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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FLIPR: Fluorescence Imaging Plate Reader

g: Standard gravity x 1

GABA: γ-Aminobutyric acid

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GDNF: Glial cell-derived neurotrophic factor

GO: Gene ontology

GPCR: G Protein-coupled receptor

HAT: Hypoxanthine-aminopterin-thymidine medium

HEK293: Human embryonic kidney cells

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC: High performance liquid chromatograph

IF: Immunofluorescence

i.p: Intraperitoneal injection

i.pl: Intraplantar injection

KCI: Potassium chloride

K_{2P}: Two-pore-domain potassium channels

K_{CA}: Calcium-activated potassium channel

K_{ir}: Inward-rectifier potassium channel

K_{V}: Voltage-gated potassium channel

MALDI: Matrix-assisted laser desorption/ionisation

MgCl$_2$: Magnesium chloride

MRI: Magnetic resonance imaging

MS/MS: Tandem mass spectrometry

N-Cam: Neural cell adhesion molecule

NaCl: Sodium chloride

NaHCO$_3$: Sodium bicarbonate

NaH$_2$PO$_4$: Monosodium phosphate

Na$_V$: Voltage-gated sodium channel

NSAID: Non-steroidal anti-inflammatory drug

O$_2$: Oxygen gas

PAG: Periaqueductal gray

PCR: Polymerase chain reaction

PDL: Poly d-lysine

PNS: Peripheral nervous system
<table>
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<tr>
<td>PSS</td>
<td>Physiological saline solution</td>
</tr>
<tr>
<td>PWF</td>
<td>Paw withdrawal force</td>
</tr>
<tr>
<td>PWT</td>
<td>Paw withdrawal threshold</td>
</tr>
<tr>
<td>RABT</td>
<td>Reference annotation based transcript</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNA-Seq</td>
<td>Ribonucleic acid sequencing</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SNL</td>
<td>Spinal nerve ligation</td>
</tr>
<tr>
<td>TASK</td>
<td>TWIK-Related Acid-Sensitive $K^+$ Channel</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>THIK</td>
<td>Tandem Pore Domain Halothane-Inhibited $K^+$ Channel</td>
</tr>
<tr>
<td>Thy-1</td>
<td>Thy-1 Cell Surface Antigen</td>
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<tr>
<td>TRAAK</td>
<td>TWIK-Related Arachidonic Acid-Stimulated $K^+$ Channel</td>
</tr>
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<td>TWIK-Related $K^+$ Channel</td>
</tr>
<tr>
<td>TRESK</td>
<td>TWIK Related Spinal Cord $K^+$ Channel</td>
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<tr>
<td>TRPA1</td>
<td>Transient Receptor Potential Channel A1</td>
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<tr>
<td>TRPM8</td>
<td>Transient Receptor Potential Channel M8</td>
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<tr>
<td>TRPV1</td>
<td>Transient Receptor Potential Channel V1</td>
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<tr>
<td>TrkA</td>
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<td>TTX</td>
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<td>TTX-s</td>
<td>Tetrodotoxin-sensitive</td>
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<td>TUBA1A</td>
<td>Tubulin Alpha 1a</td>
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<td>TWIK</td>
<td>Potassium Two Pore Domain Channel</td>
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<tr>
<td>V1B</td>
<td>Arginine vasopressin receptor 1B</td>
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<td>VGLUT3</td>
<td>Vesicular glutamate transporter 3</td>
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<tr>
<td>VIM</td>
<td>Vimentin</td>
</tr>
<tr>
<td>WDR</td>
<td>Wide dynamic range neuron</td>
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Chapter 1: Literature review

1.1 Foreword

Pain has been an integral part of the human experience for millennia, yet, it is only recently that we have begun to decipher the neurological roots of this phenomenon. Inefficient analgesia persists across the medical field, particularly in chronic neuropathic pain, conditions in which pain is no longer associated with acute injury but has become a chronic condition. The proportion of Australians who suffer from chronic pain has been estimated to be 20% of the population, and the financial losses endured by Australia due to chronic pain was placed at $7.0 billion of healthcare expenditure and $15.8 billion of non-health financial cost in 2007 alone.1

The expression of a certain voltage-gated sodium channel (Na\textsubscript{\text{V}}) isoform, Na\textsubscript{\text{V}}1.7, on dorsal root ganglia (DRG) neurons, has been found to be associated with the capacity to feel pain and with various neuropathic pain conditions in humans. However, it is becoming increasingly clear that Na\textsubscript{\text{V}}1.7 is not responsible for all pain types. Evidence of other Na\textsubscript{\text{V}} isoforms being associated with neuropathic pain conditions warrants further investigation for these other isoforms, in particular Na\textsubscript{\text{V}}1.6 and Na\textsubscript{\text{V}}1.8, which have been linked to oxaliplatin-induced neuropathic pain2 and inflammatory neuropathic pain conditions3 respectively.

Burn-induced mechanical allodynia is a condition not completely alleviated by the loss of functional Na\textsubscript{\text{V}}1.7. Burn injuries remain a significant burden for the global medical system, with approximately 265,000 burn-related deaths and 11 million burn patients needing medical treatment in 2004.4 Moreover, as burn patients are known to exhibit resistance towards opioids and necessitating opioid doses that are higher than standard for analgesia, there is a real need for the discovery of more efficient drug targets for burn-induced pain5. Na\textsubscript{\text{V}}1.7 knockout mice did not develop burn-induced thermal allodynia, but mechanical allodynia was not affected by the loss of Na\textsubscript{\text{V}}1.7, indicating that burn-induced pain is likely to be mediated by other channels and receptors in sensory neurons in addition to Na\textsubscript{\text{V}}1.7.

This chapter reviews the existing body of literature on the pathophysiology and neurological pathways involved in somatic pain, including the various ion channels and receptors involved
in different levels of pain sensation and transmission. The neuroanatomical pathways of pain signalling, from peripheral DRG neurons to the central spinal and thalamic tracts, are discussed first. The chapter then covers ion channels and receptors responsible for signal transduction, with a focus on transient receptor potential (TRP) channels, acid-sensing ion channels, and voltage-gated ion channels found along the ending and axon of peripheral DRG neurons. The voltage-gated ion channels include voltage-gated calcium (Cav), potassium (Kv), and sodium (Nav) isoforms, all of which are known to contribute to the propagation of the action potential along the sensory neuron.

Finally, this chapter focuses on literature concerning the prominent role of Nav isoforms in pain, covering Nav structure, known modulators, and with a specific focus on the evidence towards the involvement of Nav1.6, 1.7, and 1.8 isoforms in pain, both in physiologically normal and neuropathic pain conditions. The chapter concludes by outlining the hypothesis and aims of the thesis, and leads into Chapter 2 - Discovery of Selective Voltage-gated Sodium Channel Inhibitors from Spider Venoms.

Parts of this chapter have been adopted from a previously published review that arose out of this thesis, published by Biochemical Pharmacology.6

1.2 Pathways of Pain Signal Transmission

1.2.1 Peripheral dorsal root ganglion neurons and subclasses

The perception of pain from external sources starts from the peripheral neurons detecting noxious stimuli, which may be mechanical, chemical, or thermal in nature. Nerve endings near the skin transduce these external stimuli into action potentials, with the resulting electrical signal being transmitted along the DRG neurons and into the spinal cord via the dorsal horn region, specifically through laminae I, II, V, and VI. This nociceptive signal is carried further upstream via the spinoreticular and spinothalamic tracts into the thalamus, where thalamo-cortico projections carry the signal into the forebrain and the cingulate, culminating in the cognition of, and sensory response to, pain.

The neurons responsible for sensing noxious stimuli and initiating our detection of pain are peripheral sensory neurons with cell bodies located in the DRGs near the spinal cord. These
DRG neurons are pseudo-unipolar in structure, with a single axon that splits off in two opposite directions, one branch extending towards the peripheral skin and the other towards - and through - the dorsal horn of the spinal cord⁹. As such, these neurons are uniquely positioned to receive signals from both peripheral nervous system (PNS) stimulation and central innervation, and have the capacity to transcribe proteins for both branches of the axon.

There are two major classes of DRG neurons: those giving rise to myelinated A-fibres (subdivided into Aβ- and Aδ-fibres) and those giving rise to unmyelinated C-fibres. Heavily myelinated Aβ-fibres feature fast conduction velocities (30-100 metres per second), with cell bodies measuring more than 10 µm in diameter⁷. Aδ-fibres, being less myelinated than Aβ-fibres, transmit at a slower speed (12-30 metres per second) and have diameters between 2 and 6 µm. C-fibres, due to their lack of myelination, feature very slow conduction velocities (0.5-2.0 metres per second), with small cells ranging in diameters between 0.4 and 1.2 µm. Aβ-fibres innervate specialised sensory receptors in the skin such as Meissner’s corpuscles, which are responsible for feeling innocuous touch. Traditionally, Aβ-fibres were thought to conduct signals resulting from innocuous touch and thermal stimuli⁸, although emerging evidence suggests that these fibre types can also contribute to aberrant signalling in painful states. Aδ-fibres, on the other hand, appear to mediate modality information in pain experienced by humans, as Aδ-fibre selective block removes the feeling of cold in noxious cold pain and leave patients feeling only pain¹⁰. In general, Aδ nociceptors facilitate rapid transmission of pain signals to the brain to allow reflexive withdrawal actions upon acute injury. As for other physiological noxious signals, C-fibres are considered the primary conductors of nociceptive signals from the nociceptive free nerve endings near the skin to the central nervous system (CNS), though at speeds slower than A-fibre signals.

A-fibres and C-fibres are further classified according to the fibres’ sensitivity towards different stimuli. Evidence exists of two primary Aδ-fibres subtypes, with Type I Aδ-fibres being high threshold heat-sensitive fibres insensitive to capsaicin, which only fire at temperatures higher than 55 °C. In comparison, capsaicin-sensitive Type II Aδ-fibres fire at a lower thermal threshold, activating at approximately 40 °C⁸. There exist other Aδ-fibres, some of which are selectively sensitive to innocuous and noxious heat, cold, or mechanical stimuli¹¹, ¹². Some of these fibres are polymodal, being responsive to multiple types of external stimuli. For example, Aδ-heat-mechano-sensitive fibres respond to noxious heat (> 45 °C) and intense mechanical stimulus, and Aδ-cold-mechano-sensitive fibres respond to
noxious cold (< 15 °C) and mechanical stimulus. The presence of such Aδ-fibres enables discrimination between thermal stimuli of various intensity, differentiating nociceptive responses from those that are innocuous.

Similarly, C-fibres can be divided into two major subclasses of mechano-sensitive and mechano-insensitive neurons. However, as with A-fibres, additional subclasses of C-fibre neurons exist, categorised by sensitivity to a diverse array of stimuli. Some of these C-fibre subclasses are sensitive to a single stimulus type (exclusively responsive to innocuous or noxious heat, cold, or mechanical stimuli\textsuperscript{13-16}), while others are polymodal nociceptors responding to multiple stimuli\textsuperscript{6, 17, 18}. Select C-fibres groups are also activated by noxious chemical stimuli and inflammatory mediators\textsuperscript{19} such as capsaicin\textsuperscript{20} and substance P\textsuperscript{21}, serving as chemo-sensitive nociceptors. Alldynia, the sensation of feeling pain from normally innocuous stimuli, and hyperalgesia, hypersensitisation of tissues towards normally mildly painful stimuli, are thought to be caused by a combination of A- and C-fibre nociceptors, with each fibre type contributing to the exhibited symptoms in distinct ways. The distinction between pain signals conducted by Aδ-fibres and C-fibres can be observed in conditions such as capsaicin-induced pain\textsuperscript{22}. Patients with A-fibre block were able to detect the burning sensation of capsaicin, but experienced a 75%\textsuperscript{23} reduction in pain from mechanical punctate stimuli. A-fibre block also did not affect long-term potentiation following high frequency electrical stimulation\textsuperscript{20}. Spinal nerve ligation in rats caused a reduction in the mechanical threshold of high-threshold mechano-sensitive A-fibres, in addition to an increase in the area of A-fibres’ receptive fields and an increase in maximum instantaneous frequency\textsuperscript{24}. In the same spinal nerve ligation model, however, the high-threshold mechano-sensitive C-fibres only exhibited a reduction in mechanical threshold\textsuperscript{24}. This has led to the conclusion that C-nociceptors predominantly mediate thermal hyperalgesia and allodynia, whereas mechanical hypersensitivity is mediated by A-fibres instead\textsuperscript{25, 26}. Even though this view has been challenged with the emerging evidence of unmyelinated fibres mediating mechanical allodynia in glabrous\textsuperscript{27} and in sunburnt hairy skin\textsuperscript{28}, A-fibres are still postulated to be sensitised to mechanical stimuli following a noxious insult, contributing primarily to secondary mechanical alldynia and hyperalgesia.

Other attempts to classify DRG neurons have separated the neurons into sub-classes according to differences in expression profile\textsuperscript{7, 29, 30}. Trk, a specific family of tyrosine kinase receptors that function as neurotrophin receptors, exhibit selective expression in different
classes of sensory neurons. TrkA is expressed selectively in C-fibres, while TrkC is expressed in A-fibres\textsuperscript{31}. Very little overlap is observed between TrkA-expressing and TrkC-expressing neurons, though TrkB is expressed in both populations\textsuperscript{31}. More recently, the advances of transcriptomic technology have enabled comprehensive analysis of the expression profiles of DRG neurons, allowing for more detailed neuronal subclasses to be discovered. Usoskin et al.\textsuperscript{32} proposed 11 different subclasses of DRG neurons based on unique cellular markers found using bioinformatics analysis, incorporating known cellular markers such as the expression of CGRP on nociceptors\textsuperscript{33}, the expression of VGLUT3 on non-nociceptive low-threshold mechano-sensitive C-fibres\textsuperscript{34, 35}, and the differential expression of Mrgpr proteins in different neuronal subclasses. More recently, Li et al.\textsuperscript{36} further separated DRG neurons into 17 distinct classes, again using bioinformatic methods to analyse the transcriptome of each neuron. Li et al.\textsuperscript{36} also examined the behavioural responses and sensitivities of the 17 subclasses, establishing that conventional classifications according to sensitivity to stimuli does not directly translate to unique protein expression profiles\textsuperscript{36}. However, these studies contradict known evidence in a number of ways, such as purporting that tyrosine hydroxylase (TH) exists in non-nociceptive low-threshold mechano-sensitive C-fibres, despite the established practice of using TH as a sympathetic neuron marker for sympathetic neuron sprouting into DRGs\textsuperscript{37-39}. The number of neurons sorted into the different categories was also concerning. Usoskin et al.\textsuperscript{32} harvested six DRGs each from 18 mice and analysed over 700 individual neurons, yet two of the DRG subclasses discovered only contained 12 neurons per subclass, drawing into question the statistical validity of this classification. What these studies established, however, was that conventional classification according to stimuli sensitivity does not directly reflect specific protein expression profiles in neurons. It is possible that the unique protein expression profiles found are in fact more indicative of the embryonic lineage of the PNS neurons rather than being strictly related to function, and as such, categorisation according to behavioural response and protein expression may not exactly align. Moreover, the process of transcription does not definitively lead to translation, and further studies involving proteomics would be needed to unravel the relationship between transcriptomic data and observed neuronal function.

1.2.2 Central pain pathways and mediators

Following transmission through the DRG neurons, the nociceptive signal is subsequently passed on to spinal cord neurons. Two separate groups of spinal cord neurons are involved in
transmitting peripheral signals to the brain and regulating the perception of and response to pain. Specific nociceptive neurons, those that exclusively respond to painful stimuli, are present in laminae I, II, V, and VI. ‘Classical’ nociceptive fibres, including polymodal C-nociceptive fibres and high-threshold Aδ-nociceptive fibres, affect the neurons in these areas of the spinal cord. The neurons are organised somatotropically, allowing for rapid indication of the location of the signal\textsuperscript{7, 40, 41}. In contrast, wide dynamic range (WDR) neurons are present in laminae I, II, IV, V, VI, X, and in the anterior horn. This group of neurons are essential in the suppression of pain in the Gate Control Theory\textsuperscript{42}, and receives input from both noxious and innocuous sensory neurons, being involved with encoding the intensity of the sensation.

Specific nociceptive neurons and WDR neurons project into various tracts that extend further into CNS structures, transmitting both nociceptive and innocuous sensory information to the brain. The spinothalamic tract, one of the first tracts recognised to mediate pain due to analgesia observed following transection\textsuperscript{43}, projects into various areas of the thalamus. These areas, the lateral complex and the posterior medial complexes, regulate the sensory discrimination of pain and the motivational and emotional aspects of pain sensing respectively. The spinoreticular tract projects into the brain stem, where it is involved in the affective responses to pain, and the activation of CNS areas involved in descending control of pain\textsuperscript{44}. The spinomesencephalic tract projects into the periaqueductal gray (PAG) matter, and is responsible for activating pain signal transmission and initiating central pain inhibitory pathways\textsuperscript{45}. PAG activation by nociceptive signals usually results in excitation followed by inhibition\textsuperscript{45, 46} as well as aversive emotional responses towards pain, suggesting that neurons in the PAG play a role in the affective regulation of pain. Within the CNS, the thalamus is involved in the convergence of the multiple thalamic tracts and has a major role in regulating and gating sensory information. The ventroposterolateral nucleus is the main anatomical structure involved in processing excitatory signals relating to pain, while inhibitory pain circuits arise from the interaction between the ventroposterolateral and ventroposteromedial nuclei\textsuperscript{7, 40, 45, 47}.

Attempts to treat neuropathic pain at the spinal and thalamic stage, however, have been limited by the inability to distinguish between noxious and innocuous sensory signals. At the same time, several congenital pain conditions have been linked to mutations of specific ion channels present in peripheral nociceptive DRG neurons, indicating the importance of the
PNS in pain. Much of the recent efforts in pain research and drug discovery have therefore been directed towards ion channels present in DRG neurons, using gene knockout animal models and channel inhibition studies to decipher the nature of modality-specific pain sensing. The rest of the chapter will therefore discuss the ion channels that initiate and propagate sensory information in peripheral neurons, and illustrate their role in various pain conditions.

1.3 Peripheral ion channels responsible for transducing pain signals

Pain detection involves the process of sensory transduction, which describes the conversion of a thermal, mechanical, or chemical stimulus into depolarisation across the cellular membrane of cutaneous primary sensory nerves. Ion channels that transduce other stimuli into electrical signals are therefore vital in the initiation of the pain signal. The most famous family of ion channels involved in transduction is the transient receptor potential (TRP) channel family, which contains the capsaicin-sensitive channel TRPV1 and the menthol-sensitive channel TRPM8, two channels that have served as the cornerstone of research into stimulus-specific sensory neurons, as outlined below.

1.3.1 Capsaicin- and heat-sensitive TRPV1

Capsaicin-sensitive vanilloid receptor subtype 1 (TRPV1), a non-selective cation channel, was first isolated as an ion channel directly activated by capsaicin and heat\(^48\). The channel has since then been confirmed to be activated by other noxious compounds such as endovanilloids\(^49\) and low pH\(^50\). Such findings suggest that TRPV1 may be responsible for detecting heat-induced pain. TRPV1 is also expressed almost exclusively in C-fibres\(^51\), neurons that are traditionally thought to be responsible for nociceptive and thermal sensation. However, TRPV1 knockout in mice did not completely remove noxious heat-induced neuronal activity\(^52\), nor did it reduce aversive behaviours upon challenge with noxious heat (up to 52 °C)\(^53\). While TRPV1 knockout animals certainly displayed reduced responses to capsaicin, it was evident that TRPV1 is not the only channel responsible for sensing heat and heat-induced pain.

Despite being unable to alleviate noxious heat sensing, global TRPV1 knockout is effective in eliminating carrageenan-induced inflammatory responses in mice, successfully reducing
Inflammatory hyperalgesia\textsuperscript{53, 54}. Inflammatory pain was also related to increased expression of TRPV1 in rat DRG neurons\textsuperscript{55}, indicating inflammatory responses, rather than noxious heat in normal physiological conditions, are more affected by TRPV1. As well as expression on peripheral C-fibres, TRPV1 has also been found to be expressed in the spinal cord and the CNS. TRPV1 block in peripheral neurons significantly reduced long-term potentiation of dorsal horn neurons to pain signals\textsuperscript{20, 56}, and TRPV1 has been found to be involved in neuronal depression within hippocampal interneurons\textsuperscript{57}, indicating that TRPV1 also plays a part in the central regulation and sensitisation of possibly pathological pain\textsuperscript{58}.

1.3.2 Menthol- and cold-sensitive TRPM8

The transient receptor potential melastatin 8 channel (TRPM8)\textsuperscript{59, 60} has significantly advanced our understanding of the processes underlying the transduction of external cold into electrical signals. The channel is essential for the detection of environmental cold, with a series of potassium channels contributing to threshold adjustment and amplification of TRPM8-dependent cold transduction\textsuperscript{18, 61, 62}. TRPM8 knockout mice show drastically reduced responses to innocuous cold\textsuperscript{63-65}. Cultured trigeminal ganglion neurons from TRPM8 knockout mice are also insensitive to menthol or innocuous cold (22 °C), with the number of cold-responsive cutaneous myelinated and unmyelinated nerve endings decreased significantly compared to controls\textsuperscript{63}.

However, as with TRPV1 and heat-related pain, the extent to which TRPM8 is essential for pathological forms of cold pain is controversial, with the results of existing studies often in conflict. TRPM8 knockout mice, mice treated with intrathecal TRPM8 antisense oligonucleotide, and mice with diphtheria toxin-mediated ablation of TRPM8-positive neurons all had reduced innocuous cold sensitivity. The three different strains also all exhibited reduced cold hypersensitivity following Complete Freund’s adjuvant (CFA)-induced inflammation and chronic constriction injury (CCI), as examined by the acetone test\textsuperscript{64, 66, 67}. However, TRPM8 knockout mice retained noxious cold sensitivity, and exhibited nocifensive behaviours similar to those of control mice at temperatures below 10 °C\textsuperscript{63, 65}.

A further confounding factor in studies assessing the contribution of TRPM8 to cold sensing and cold pain is the specificity and selectivity of pharmacological ‘tool’ compounds such as menthol. Many studies equate menthol-induced responses with selective TRPM8 activation.
However, the proto-typical TRPM8 agonist menthol is a promiscuous compound and can elicits responses in TRPM8-negative sensory neurons. In other studies, menthol has been found to inhibit nicotinic acetylcholine receptors\textsuperscript{68}, inactivate voltage-gated calcium currents\textsuperscript{69}, inhibit voltage-gated potassium channels\textsuperscript{18}, and activate and/or desensitise TRPA1, another TRP channel\textsuperscript{70}, demonstrating the varied targets of this compound and the difficulty of using menthol to assess TRPM8 activity.

1.3.3 TRPA1

Transient receptor potential ankyrin 1 (TRPA1) is considered a noxious cold and irritant sensor. The channel mediates, among others, the reaction to the pungent components of mustard, horseradish, and wasabi. TRPA1 is also activated by formaldehyde, bacterial endotoxins and proinflammatory mediators such as bradykinin, methylglyoxal, and prostaglandin E\textsubscript{2}\textsuperscript{71-76}. TRPA1 expression in sensory neuronal populations has little overlap with TRPM8 expression, but TRPA1 is highly co-expressed with TRPV1 and can form functional heteromultimers with TRPV1\textsuperscript{77}, adding to the functional complexity of this channel.

The overwhelming majority of studies assessing TRPA1 activation by cold used cultured rodent DRG neurons, or rodent TRPA1 transfected into mammalian HEK293 cells, CHO cells, or Xenopus oocytes. Many such studies demonstrated TRPA1-mediated intracellular calcium increase or inward current upon exposure to cool temperatures (5–18 °C)\textsuperscript{30, 60, 75, 78-81}. However, species- and tissue-specific differences in the cold sensitivity of TRPA1 soon became becoming apparent\textsuperscript{82}. In fact, while the rodent TRPA1 channel is cold-sensitive, snake and drosophila TRPA1 channels are heat-sensitive\textsuperscript{83-85}, and primate TRPA1 does not respond to cooling in \textit{in vitro} systems\textsuperscript{82}. Furthermore, the standard behavioural cold pain tests in mice are far less sensitive than commonly believed\textsuperscript{86}, as seen in a functional MRI study in mice that showed altered central processing of noxious cold (15 °C) when no measurable aversive behaviour was observed. A number of studies also based their conclusions regarding the cold sensitivity of TRP channels on the perhaps erroneous assumption of the pharmacological specificity of compounds including isothiocyanates, icilin, and menthol\textsuperscript{60, 70, 74, 87-90}, the latter of which has been shown to be promiscuous in the previous section.

Nevertheless, TRPA1 still plays a key role in a human model of ciguatoxin-induced cold allodynia, making it hard to deny a possible role for TRPA1 in cold sensing and cold pain. In
ciguatoxin-induced cold allodynia, de novo cold responses in previously cold-insensitive mouse DRG neurons emerged after treatment with ciguatoxin. This phenomenon was shown to be mediated by TRPA1, and correlated well with a key contribution of TRPA1 to ciguatoxin-induced cold allodynia in mice. A monogenic TRPA1 gain-of-function mutation is also linked to a hereditary disease of episodic debilitating pain, which is triggered by cold. Comparable mechanisms of TRPA1 sensitisation to cold may also apply for cold hypersensitivity in inflammatory conditions, as well as in diabetic neuropathy.

1.3.4 ASIC channels

Acid-sensitive ionic channels (ASICs) is a family of non-voltage-gated cation channels directly activated by protons and pH < 6.9, and is densely expressed in unmyelinated DRG neurons in rats. Three subtypes of ASICs exist, namely ASIC1, ASIC2, and ASIC3. ASIC1 and ASIC3 are activated in vitro at low pH levels comparable to those created via local inflammation. These two isoforms are also expressed on peripheral nociceptors, in the dorsal horn and in the PAG, anatomical areas involved in transmitting and regulation pain signals as described previously.

Interestingly, ASIC isoforms are also present in bone, in joints, and in muscle cells, leading to investigations into the role of ASIC in local inflammatory conditions such as joint inflammation. ASIC expression was increased following arthritis, with ASIC inhibition preventing the development of secondary hyperalgesia, though primary hyperalgesia was unchanged compared to controls. The role of ASICs in mediating inflammation was further consolidated by the finding that non-steroidal anti-inflammatory drugs (NSAIDs) directly bind to ASICs and inhibit neuronal firing, suggesting the involvement of ASICs in general inflammatory responses.

Paradoxically, ASIC1 and ASIC2 knockout animals exhibited exaggerated responses in the formalin test, an inflammatory pain model, suggesting specific pathways may exist for various inflammatory pain conditions. This suggestion is supported by the observation that the mambalgin toxin, extracted from the venom of black mamba snakes and known to inhibit all ASIC channels, provided effective analgesia in carrageenan-induced inflammation. ASIC1 knockout and ASIC3 knockout animals also demonstrated normal responses for
noxious thermal and mechanical stimulation in the absence of inflammation\textsuperscript{95}, indicating the channels do not extensively regulate other non-inflammatory pain modalities.

1.4 Peripheral ion channels responsible for conducting pain signals

Following transduction, pain signals are propagated along the DRG neuron axon to the spinal cord in a process described as conduction. Ion channels on the peripheral axons determine the neuronal resting potential, which in turn dictates the excitability of the sensory neuron and the speed and ease of conduction. These ion channels usually do not exist in the nerve endings close to the skin, but are present throughout the neuron as a mix of voltage-gated (including voltage-gated calcium, sodium, and potassium channels) and voltage-independent (such as background potassium channels) channels involved in conduction. Voltage-gated calcium (Ca\textsubscript{V}) channels and both voltage-gated and voltage-independent potassium channels will be discussed in this section. The large body of literature concerning voltage-gated sodium (Na\textsubscript{V}) channels warrants a section of its own in Section 1.5.

1.4.1 Ca\textsubscript{V} channels

Voltage-gated calcium channels (Ca\textsubscript{V}) are heteromeric channels made of various subunits, including voltage-sensitive α-subunits, as well as β, γ, and α2δ subunits that function to regulate voltage dependencies and gating kinetics\textsuperscript{102}. Ten different α subunits have been identified, including Ca\textsubscript{V}1.1-1.4, Ca\textsubscript{V}2.1-2.3, and Ca\textsubscript{V}3.1-3.3\textsuperscript{103}. The different currents evoked by the activation of distinct Ca\textsubscript{V} isoforms have led these α units to be classified according to their electrical properties, with Ca\textsubscript{V}1.1-1.4 being classified as L-type (for ‘long-lasting), Ca\textsubscript{V}2.1 as P/Q-type (found in Purkinje neurons), Ca\textsubscript{V}2.2 as N-type (intermediate between L- and T-type for voltage dependence), Ca\textsubscript{V}2.3 as R-type (found in cellular granule neurons), and Ca\textsubscript{V}3.1-3.3 as T-type (for ‘transient’)\textsuperscript{102}. Protein purification have determined that Ca\textsubscript{V} isoforms exist physiologically as heteromers consisting of one α subunit, one β subunit, one α2δ subunit, and one γ subunit per functional channel\textsuperscript{104}. While Ca\textsubscript{V} isoforms are widely expressed in skeletal muscle, cardiac muscle, and peripheral and central neurons\textsuperscript{102}, the expression of Ca\textsubscript{V} isoforms in DRG neurons and the spinal cord have been shown to play significant roles in pain sensing, particularly those of the α2δ subunits, and the Ca\textsubscript{V}2.2 and Ca\textsubscript{V}3.2 isoforms.
The Ca_vα2δ subunits are poorly understood in terms of their effects on the regulation of functional Ca_v channels, though these subunits are evidently involved in the conduction of pain signals. Pregabalin and gabapentin, two structurally similar neuropathic pain adjuvant medications that were originally designed to mimic the inhibitory CNS neurotransmitter γ-aminobutyric acid (GABA), are in fact potent ligands for the Ca_vα2δ1 subunit and do not affect GABA pathways. In the spinal nerve ligation (SNL) neuropathic pain model, which produces robust mechanical allodynia, Ca_vα2δ1 was found to be up-regulated in DRG neurons and caused dorsal horn sensitisation. Curiously, transgenic Ca_vα2δ1 over-expressing mice also exhibited dorsal horn neuron sensitisation in the form of increased frequency of excitatory postsynaptic currents, which was reversible upon gabapentin administration. The expression of Ca_v on DRG neurons is also increased with increased transgenic Ca_vα2δ1 expression. These findings suggest the efficacy of drugs that interact with Ca_vα2δ1 work through the regulation of Ca_v present on the plasma membrane of neurons, rather than by controlling the conduction properties of peripheral pain signals.

The spinal analgesic effects of Ca_v inhibitors is further demonstrated by the clinically used Ca_v2.2 N-type Ca_v inhibitor, ziconotide. Ziconotide is a peptide drug derived from the venom of the Conus Magus cone snail species and is administered as an intrathecal infusion, selectively inhibiting N-type Ca_v currents in laminae I and II of the dorsal horn. Despite the analgesic efficacy of ziconotide in post-operative and cancer pain, the drug must be administered intrathecally due to its large molecular mass, making administration difficult and often requiring an implantable pump. Ziconotide also exhibits CNS adverse effects such as sedation, blurred sight, and even psychosis, most likely due to the propagation of N-type Ca_v inhibitory effects throughout the CNS as the peptide diffuses through the spinal fluid and enters the brain, further illustrating the difficulty of dosing molecular targets present in the CNS.

More recently Ca_v3.3, a T-type Ca_v isoform, was found to be exclusively present in C-low-threshold mechanoreceptors (C-LTMR) in the PNS, prompting the inclusion of Ca_v3.3 as a new cellular marker for mechano-sensitive unmyelinated fibres. C-LTMRs also express large amounts of Ca_v3.2, another T-type Ca_v isoform. Ca_v3.2 inhibition has been shown to successfully alleviate cold allodynia (20 °C) in human hairy skin following the induction of muscle pain, prompting speculation about the role of Ca_vS in C-fibre-mediated thermosensation. While Ca_v-selective inhibition in central pain pathways has been
successfully targeted to modulate pain at a systematic level, peripherally expressed T-type CaV isoforms could become a new class of cellular markers that serve to untangle the details of peripheral pain signal conduction prior to transmission into the spinal cord.

1.4.2 Potassium channels

Potassium channels in DRG sensory neurons, unlike their calcium and sodium channel counterparts, serve to hyperpolarise rather than depolarise the neuron. As a result, the presence of potassium channels makes the neuron less excitable. A large variety of potassium channels, encoded by over 40 different genes, are present throughout the body. The four main classes of potassium channels are voltage-gated potassium channels (Kv), which are dependent on voltage for activation; 2-pore potassium channels (K2P), which act as background potassium leaks to regulate the resting potential of the neuron; calcium-activated potassium channels (KCa), which depend upon calcium levels to activate and regulate neuronal synaptic activity; and inward-rectifying potassium channels (Kir), which actively facilitate potassium currents into the neuron to regulate intracellular and extracellular potassium balance.

As potassium channels cause hyperpolarisation in neurons and result in reduced excitability, the presence and activation of Kv serve as a significant regulator of signalling in the PNS. Kv7.2 and Kv7.3, two proteins that form heteromers with each other in both Aδ and C-fibres, are the first potassium channels to become clinical targets for pain. The selective Kv7.2/7.3 channel opener flupirtine is used clinically for pain and has been shown to be effective in animal models of neuropathic injury. Retigabine, a structural analog of flupirtine and also a Kv7 channel opener, has also been found to inhibit spontaneous discharges in damaged neurons. In particular, Kv7 isoforms affect cancer pain, and their suppression is related to the development of bone pain in cancer and dorsal horn hypersensitisation. The expression level of Kv7 channels appear to increase following neuronal inflammation and damage, indicating Kv7 function to be a part of the homeostasis and compensatory system following injury to regain normal sensitivity.

The other main branch of potassium channels, the K2P family, controls the membrane potential of the neuron at the resting state and conducts a voltage-insensitive, ‘leak’ potassium current. A total of 12 functional isoforms of K2P channels exist in DRG neurons,
classified according to their different properties into 6 subfamilies named TASK, TREK, TRESK, TRAAK, TWIK, and THIK. TASK-3 has been revealed to be involved in the sensation of cold, with TASK-3 knockout mice displaying cold hypersensitivity and TASK channels being bilaterally down-regulated following the injection of CFA into the paw. Another prominent K_2P channel, TREK-1, is also involved in temperature sensing and is activated directly by increased temperature, with TREK-1 knockout mice exhibiting marked heat allodynia compared to wild type animals. TRAAK has also been implicated as one of the regulators of thermosensitivity, suggesting other channels in the K_2P family may also have yet-undiscovered roles in the modulation of sensory neurons.

1.5 NaV channels – the ultimate cure?

Out of all the ion channels and receptors present in peripheral sensory neurons, it is the voltage-gated sodium channel (NaV) family that has been pushed into the spotlight of pain research in recent years. The discovery that mutations of the NaV1.7 isoform resulted in human congenital insensitivity to pain, with affected patients being completely insensitive to painful stimuli and exhibiting no physiological abnormalities other than anosmia, was a validation of the indispensable role of NaV channels in pain. Investigations using channel-selective venoms, bioinformatics methods, and in vitro high-throughput assays have all yielded valuable insight to the characteristics and distribution of NaV isoforms. Of all the NaV isoforms present in neurons, NaV1.7 was initially considered to have the most therapeutic potential for pain treatment, although it is now clear that NaV1.7 inhibition does not affect all pain conditions. Two other isoforms distributed throughout the nervous system, NaV1.6 and NaV1.8, have also been subjects of intense research concerning their role in the conduction of pain signals. NaV1.9 had also shown an involvement in pain, however a lack of NaV1.9 assays and NaV1.9 selective modulators have greatly hampered the isoform’s therapeutic potential. Various obstacles also remain in the path towards elucidating the molecular causes of different neuropathic pain conditions, even with the increased understanding of the roles of NaV isoforms. The rest of this review will therefore focus on NaV isoforms NaV1.7, NaV1.6, and NaV1.8, and will discuss existing literature regarding their known properties, and their contribution to various pain conditions.
1.5.1 Structure and properties of \( \text{Na}_V \) channels

\( \text{Na}_V \) channels were first studied by Hodgkin and Huxley in their pioneering work with axonal conduction in the 1950s and 1960s\(^{138} \), showing that the channels mediated a voltage-dependent inward current selective for sodium ions. Apart from neurons, \( \text{Na}_V \) isoforms are also present in other excitable tissues such as muscle cells. The opening of \( \text{Na}_V \) isoforms is responsible for the initiation of action potentials, causing the depolarisation phase of action potentials to occur, followed by rapid inactivation of \( \text{Na}_V \) isoforms and subsequent repolarisation. This rapid voltage-gated closure, together with the activation of \( \text{K}_V \) isoforms, causes the termination of the action potential. Spontaneous firing can therefore be triggered by over-excitatory \( \text{Na}_V \) isoforms, potentially causing the sensation of pain in neuropathic pain conditions.

\( \text{Na}_V \) subunits are separated into \( \alpha \) and \( \beta \) subunits, with \( \alpha \) subunits being the main pore-forming protein (Figure 1). Action potentials can be observed from transfected cells and oocytes expressing \( \alpha \) subunits only, indicating that the \( \alpha \) subunit is the only component required to allow sodium ion movement\(^{139} \). A total of 10 different isoforms of \( \text{Na}_V \) \( \alpha \) subunits have been found to date, including \( \text{Na}_V 1.1-1.9 \) and \( \text{Na}_X \), a voltage-insensitive channel found in glial cells within the CNS\(^{140} \). The \( \text{Na}_V 1.4 \) isofrom is expressed in skeletal muscle cells, with \( \text{Na}_V 1.5 \) present in cardiac muscle cells. \( \text{Na}_V 1.1-1.3 \), as well as \( \text{Na}_V 1.6-1.9 \), are expressed on neurons. \( \text{Na}_V 1.1-1.3 \), 1.6, and 1.7 are known to be expressed abundantly in myelinated neurons, whereas \( \text{Na}_V 1.8 \) and 1.9 are expressed exclusively in unmyelinated neurons, exhibiting the selective expression of different \( \text{Na}_V \) \( \alpha \) subunits in various tissues.
Figure 1.1 Structure of the Na\textsubscript{V} α subunit and toxin binding sites

The domains are labelled by Roman numerals above each domain (I-IV). The segments are labelled by Arabic numbers in each segment (1-6). The voltage sensor in segment 4 of each domain is denoted by ‘+’ signs. The locations of each toxin binding site is shaded with the corresponding colour as noted below the figure. Figure drawn in Adobe Illustrator\textsuperscript{®} C56, version 16.0.3, based on information stated in Catterall et al.\textsuperscript{141} and Stevens et al.\textsuperscript{142}. 
Na\textsubscript{V} \(\alpha\) subunits consist of six \(\alpha\)-helical transmembrane segments (S1-S6), with each functional channel consisting of 4 homologous \(\alpha\) subunits (domains I-IV) surrounding the pore. A loop entering the membrane region between segments S5-S6 creates the outer pore of the channel, selective for the entry of sodium ions\textsuperscript{143}. Other extracellular loops connect the different transmembrane segments, and also attach the S5 and S6 segments to the pore. Large intracellular loops connect the four distinct \(\alpha\) domains, with the N-terminal and C-terminal also being intracellular\textsuperscript{143}. Cryo-electron microscopy has confirmed that on a 3D scale, Na\textsubscript{V} channels exhibit four-fold symmetry of transmembrane structures, with large intracellular and extracellular protein masses. The channel contains numerous inlets and outlets for the pore\textsuperscript{144}, indicating the pore is split into four branches rather than being one simplistic opening.

A voltage sensitive section is present in each of the S4 segments and is composed of one positively charged amino acid followed by two hydrophobic amino acids, repeating in a pattern to achieve a helical arrangement of positive charges in a transmembrane manner\textsuperscript{139}. The voltage-dependent activation of the channels is achieved through the movement of charged amino acids due to the changed membrane potential\textsuperscript{138}. Membrane depolarisation and increased positive intracellular charge results in outward movement of the positive charges in the S4 segment to open up the pore for sodium ion entry\textsuperscript{145, 146}. The rapid inactivation of Na\textsubscript{V} channels is also reliant on the voltage-dependent movement of the inactivation gate between domains III and V, consisting of an intracellular loop that closes and binds to the pore from the inner pore side\textsuperscript{139}.

Acting as regulatory proteins, the \(\beta\) subunits of sodium channels modulate the properties of gating and the interaction between various cells. At least 4 \(\beta\) subunits have been identified so far, named \(\beta1\) to \(\beta4\)\textsuperscript{147}. Co-expression of functional \(\beta\) subunits together with pore-forming \(\alpha\) subunits causes functional channels to activate and inactivate at the appropriate speed and with normal voltage sensitivity, while the expression of only \(\alpha\) subunits in oocytes results in sodium channels with slower kinetics than that observed in physiological tissue\textsuperscript{148}. The similarity between Na\textsubscript{V} \(\beta\) subunits and cell adhesion molecules\textsuperscript{149} also support the observation that the \(\beta2\) subunits bind to the extracellular matrix component tenascin\textsuperscript{150}, suggesting that the presence of \(\beta\) subunits may dictate the interaction of Na\textsubscript{V} isoforms with other neurons and supportive glial cells in the nervous system.
1.5.2 Neurotoxins as selective Na\textsubscript{V} isoform modulators

Much of the structural and functional relationship between the segments and domains of the Na\textsubscript{V} α subunits have been derived from studies using Na\textsubscript{V}-selective neurotoxins purified from the venoms of spiders, scorpions, cone snails, or vertebrates. Biochemical analysis using neurotoxins was one of the key methods used to investigate neuronal channels, playing an invaluable role in the initial discovery and identification of the various Na\textsubscript{V} isoforms\cite{143, 147}. Tetrodotoxin (TTX) and saxitoxin, two drugs that bind to Na\textsubscript{V} isoforms with very high potency, were used to identify the location of the pore and the structures vital for sodium ion selectivity. This has led to the classification of Na\textsubscript{V} isoforms as tetrodotoxin-sensitive (TTX-s) and tetrodotoxin-resistant (TTX-r), two subclasses with distinct differences in distribution and sensitivity to the pore blocking toxin TTX. The TTX-r Na\textsubscript{V} isoforms Na\textsubscript{V}1.5, Na\textsubscript{V}1.8, and Na\textsubscript{V}1.9 are distributed in cardiac muscle (Na\textsubscript{V}1.5) and unmyelinated neurons (Na\textsubscript{V}1.8 and Na\textsubscript{V}1.9), whereas the rest of the Na\textsubscript{V} family are TTX-s and are expressed in skeletal muscle (Na\textsubscript{V}1.4) and throughout the nervous system (Na\textsubscript{V}1.1, 1.2, 1.3, 1.6, and 1.7). Toxins are still used as experimental tools to detect the roles of different Na\textsubscript{V} isoforms and remain some of the most selective inhibitors of Na\textsubscript{V} isoforms, including the Na\textsubscript{V} isoform-selective conotoxins derived from cone toxin venoms\cite{142}.

Specific structures in the Na\textsubscript{V} α subunit are noted to be the binding sites for select toxins, classified by the location of the binding site as well as the effect of the neurotoxin (Table 1.1). The bound neurotoxin may either directly occlude the pore, or alter the kinetics of channel activation and inactivation\cite{142}. Binding site 1, the binding location of TTX and saxitoxin, is a pore-blocking site. Being one of the oldest toxin binding sites identified, binding site 1 was instrumental in identifying the structure of the Na\textsubscript{V} channel pore\cite{151}. Binding site 1 consists of the loops connecting S5 and S6 in all domains, forming part of the pore structure itself. Apart from TTX and saxitoxin, the μ-conotoxin family of cone snail toxins also binds to this site, one example being KIIIA (derived from Conus kinoshitai)\cite{152}. One spider toxin, hainantoxin-1, derived from the spider Ornithoctonus hainana, has also been noted to bind to binding site 1. Most other spider toxins, while having high potency and being selective to Na\textsubscript{V} isoforms, usually bind to other sites on the Na\textsubscript{V} structure.

Binding site 2 functions by regulating channel activation and deactivation rather than interference with the pore. Binding site 2 appears to involve the S6 segment of domains I and
IV\textsuperscript{153}, and is the binding site for many lipid soluble toxins from various sources such as poisonous frogs, alkaloids such as veratridine, toxins found in birds, and antillatoxins found in certain species of cyanobacteria\textsuperscript{154, 155}. The binding of these toxins to binding site 2 results in a voltage-dependence shift towards more negative values, causing the neuron to be more sensitive. Binding site 2 toxins also prolong the activation of the channel by slowing or preventing the closure of the pore, resulting in long-lasting currents. The combined effects cause excessive neuronal firing in the organism, which contributes to the fatal toxicity of many toxins that bind to this site.
<table>
<thead>
<tr>
<th>Binding site</th>
<th>Neurotoxin classes</th>
<th>Examples</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Guanidium molecules, µ-conotoxins, hainantoxin-1</td>
<td>Tetrodotoxin (TTX), saxitoxin, µ-conotoxins such as KIIIA</td>
<td>Pore-blocker, inhibits neuronal firing</td>
</tr>
<tr>
<td>2</td>
<td>Various lipid soluble toxins</td>
<td>Veratridine, toxins found on poisonous frogs and vertebrates, toxins from cyanobacteria</td>
<td>Modulates kinetics of the channel. Channel activators that increase voltage sensitivity and slow inactivation</td>
</tr>
<tr>
<td>3</td>
<td>Scorpion, sea anemone, and spider toxins</td>
<td>α and β scorpion toxins, spider toxins such as Tx4</td>
<td>Slow down and prevent voltage-dependent inactivation, causing prolonged channel opening</td>
</tr>
<tr>
<td>4</td>
<td>β scorpion toxins, spider toxins</td>
<td>µ-agatoxins and β/δ-agatoxins</td>
<td>Maintains voltage sensor segment at its outward position and prolongs pore opening</td>
</tr>
<tr>
<td>5</td>
<td>Toxins produced by marine dinoflagellates</td>
<td>Brevetoxins and ciguatoxins</td>
<td>Brevetoxin inhibits neuronal firing, while ciguatoxin causes membrane potential oscillations</td>
</tr>
<tr>
<td>6</td>
<td>δ-conotoxins</td>
<td>δ-SVIE, TxA, and other δ-conotoxins</td>
<td>Slows NaV channel inactivation without affecting voltage-dependency inactivation</td>
</tr>
</tbody>
</table>

**Table 1.1 Families of neurotoxins that bind to NaV and their binding sites**

A total of six binding sites for neurotoxins have been identified and characterised in NaV α subunits. Distinct classes of neurotoxins bind to separate sites and exert different functions.
Site 1 controls the opening of the pore and directly affects the flow of sodium ions. All other sites affect the voltage-dependent gating of the pore and channel inactivation.
Binding site 3 is located at the extracellular loops between S3 and S4 segments in domain IV. Toxins that bind to this site include α and β scorpion toxins, sea anemone toxins, and selective spider toxins. Studies indicate that the binding of neurotoxins to site 3 causes the S4 voltage sensor segment to become stabilised in its outward position, disabling the movement of the voltage sensor and preventing fast, voltage-dependent inactivation of the channel. The inactivation can be slowed or even prevented entirely, causing prolonged channel opening and slowly-inactivating currents.

Toxin binding site 4 directly interferes with the voltage sensor, holding the voltage sensor segment in its outward position and maintaining the open state of the pore. The binding site consists of the extracellular loops between S1-S2 and S3-S4 in domain II, though the binding of β scorpion toxins are also affected by the pore component of the S5-S6 loop in domain III, as well as involvement of the voltage sensor segment in domain III. A number of spider toxins also bind to binding site 4, with examples including μ-agatoxins and β/δ-agatoxins, extracted from the Agelenopsis family of spiders. Due to the delay of inactivation, many of the toxins that bind to this binding site are considered channel openers, Agatoxins, in particular, shift voltage-dependent activation to more negative voltages as well as slowing inactivation, contributing to channel opener effects.

The location of toxin binding site 5 was determined to be S6 segment of domain I and S5 segment of domain IV, and is the site for photoreactive toxins produced by marine dinoflagellates. These toxins include brevetoxin, produced by algal blooms, and ciguatoxin, produced by marine dinoflagellates and accumulated in carnivorous reef fish. Brevetoxin activates NaV isoforms through shifting voltage-dependent activation to lower thresholds, maintaining channels in the open configuration for longer, and slowing down voltage-dependent inactivation. Ciguatoxin exhibits similar effects to brevetoxin, but also induces neuronal membrane potential oscillations, indicating that the binding site may exert more effects than simple inhibition of inactivation.

Toxin binding site 6, which has been suggested to be the loop connecting S3 and S4 segments in domain IV by investigations using the δ-conotoxin δ-SVIE, remains relatively unexplored compared to other binding sites. To date, only δ-conotoxins, a class of conotoxins that slow down NaV inactivation without changing the voltage-dependency inactivation of the
channel\textsuperscript{161}, have demonstrated evidence of binding to site 6, suggesting the mechanism of inactivation differs from that of other toxins attaching to the other binding sites.

The various toxin binding sites have been instrumental in the discovery of loops and segments important for the functions and properties of the Na\textsubscript{V} channel, such as pore opening and voltage sensitivity. Mutation studies using toxin binding have further pinpointed specific mutations that would cause gain- or loss-of-function changes in the channel. However, despite biochemical evidence that Na\textsubscript{V}1.7 loss-of-function mutations are the main contributor towards congenital insensitivity to pain, Na\textsubscript{V}1.7-selective inhibition and Na\textsubscript{V}1.7 knockout mice do not exhibit a complete lack of pain sensation similar to that observed in human patients, bringing into light potential differences in pain pathways and ion channel distribution between humans and rodents and calling into question the presumed contribution of Na\textsubscript{V}1.7 towards the sensation of pain.

1.5.3 Na\textsubscript{V}1.7

A weighty body of literature concerning Na\textsubscript{V}1.7 has been accumulated since the definitive link between this TTX-s Na\textsubscript{V} isoform with the sensation of pain. Na\textsubscript{V}1.7 is expressed widely throughout the PNS, particularly in DRG neurons\textsuperscript{167, 168} and sympathetic neurons. Na\textsubscript{V}1.7 is also the main Na\textsubscript{V} isoform involved in signal conduction in the olfactory circuits\textsuperscript{169}, corresponding to the lack of smell observed in human congenital insensitivity to pain. In the PNS, Na\textsubscript{V}1.7 is distributed in both A- and C-fibres, with all C-fibres in guinea pig DRG neurons exhibiting positive Na\textsubscript{V}1.7 immunoreactivity\textsuperscript{168}. Not all A-fibres are positive for Na\textsubscript{V}1.7, though 90% of A\textdelta-fibres and 40% of A\textbeta-fibres express Na\textsubscript{V}1.7\textsuperscript{168}. These numbers include nociceptive neurons as well as sensory neurons transmitting innocuous signals. Na\textsubscript{V}1.7 expression can be detected throughout the entire DRG neuron, distributed from the terminal tip of intra-dermal nerve ending in the skin\textsuperscript{170} to the DRG neuronal terminals in the spinal cord\textsuperscript{171}. In myelinated neurons, Na\textsubscript{V}1.7 is preferentially expressed at the nodes of Ranvier, which are small unmyelinated sections of the axon between stretches of myelin to allow for saltatory conduction in myelinated fibres. Na\textsubscript{V}1.7 is therefore involved in conducting pain signals along the entire length of DRG neurons, from the periphery to the spinal cord.
Nav1.7 also exhibits gating kinetics that easily enhance neuronal excitability. Though Nav1.7 is relatively resistant to inactivation from the closed state, the channel has rapid activation and inactivation kinetics, resulting in TTX-s currents that can be evoked by slow depolarisation of the neuron. Compared to other TTX-s Nav isoforms such as Nav1.6, closed and non-refractory Nav1.7 channels inactivate more than seven times slower than the Nav1.6 isoform, suggesting Nav1.7 may amplify sub-threshold potentials and contribute to spontaneous firing. At the same time, Nav1.7 recovered from refractory inactivation almost nine times slower compared to Nav1.6, and the channel may conduct action potentials less frequently. More recently, Nav1.7 was shown to be involved in the initiation and the depolarisation phase of action potentials in nociceptive peripheral neurons. Nav1.7 inhibition stopped spontaneous firing in nociceptors invoked by slow depolarising voltage, demonstrating Nav1.7 could contribute to multiple factors involved in action potential generation.

Studies with Nav1.7 have been promising for select pain conditions and in reproducing pain insensitivity, as seen by the congenital insensitivity to pain exhibited by global Nav1.7 knockout in mice. However, global Nav1.7 knockout is lethal for newborn mice if sufficient husbandry support is not provided, as the lack of smell caused by the lack of Nav1.7 in olfactory circuits result in the neonatal mice being unable to find food and feed. Upon reaching adulthood, the mice exhibited similar symptoms to human Nav1.7 null patients, maintaining normal sensitivity to innocuous stimuli but demonstrating complete insensitivity to noxious thermal, mechanical, and chemical stimuli. The mice did not demonstrate alldynia invoked by inflammatory condition following CFA injection, nor exhibited aversive behaviour following peripheral injection of veratridine and grayanotoxins, both of which are Nav activators that bind to binding site 2. These results confirm that Nav1.7 is involved in conduction of pain signals, and that global Nav1.7 knockout does interrupt normal physiological transmission of pain signals to the CNS.

At the same time, other gene knockout and knockdown studies present conflicting information towards the contribution of Nav1.7 towards pain. Specific deletion of Nav1.7 from Nav1.8-positive peripheral neurons in mice resulted in increased noxious pain thresholds and reduced inflammatory pain invoked by formalin and CFA, confirming the important role Nav1.7 plays in acute and inflammatory pain. However, studies conducted by the same research group also revealed the knockout of Nav1.7 channels in Nav1.8-positive
neurons had no effect in alleviating neuropathic pain after spinal nerve ligation\textsuperscript{177}. Thus, while it has since been found that the presence of \( \text{Na}_\text{V}1.7 \) in peripheral sympathetic neurons\textsuperscript{167} interferes with pain sensations\textsuperscript{178}, the fact remains that \( \text{Na}_\text{V}1.7 \) knockout animals only appear to have reduced acute and inflammatory nociception, and do not demonstrate alleviated pain in neuronal damage models of neuropathic pain.

Biochemical and expression studies concerning \( \text{Na}_\text{V}1.7 \) in neuropathic pain also yield conflicting information concerning \( \text{Na}_\text{V}1.7 \) expression changes following injury. TTX-s currents in unmyelinated C-fibres changed from slow-recovering, as observed with \( \text{Na}_\text{V}1.7 \)-mediated currents, to fast-recovering currents following a nerve injury\textsuperscript{179}, indicating the expression of TTX-s Na\textsubscript{V} isoforms was no longer predominated by \( \text{Na}_\text{V}1.7 \). The expression of \( \text{Na}_\text{V}1.7 \) was also lowered in mice DRG neurons for up to five days following spinal nerve ligation, indicating \( \text{Na}_\text{V}1.7 \) does not contribute to over-excitation of the injured neuron\textsuperscript{180}. The same trend was found in humans, with \( \text{Na}_\text{V}1.7 \) expression found to be decreased after central or peripheral axotomy\textsuperscript{181}. At the same time, \( \text{Na}_\text{V}1.7 \) is accumulated and overexpressed in painful human neuromas\textsuperscript{182}. Inflammation also increased \( \text{Na}_\text{V}1.7 \) expression\textsuperscript{183}, and inflammatory mediators such as nerve growth factors are thought to up-regulate \( \text{Na}_\text{V}1.7 \) expression\textsuperscript{184}. Curiously, a rat model of painful diabetic neuropathy reported increased \( \text{Na}_\text{V}1.7 \) expression\textsuperscript{185}, as opposed to the \( \text{Na}_\text{V}1.7 \) down-regulation found in mice and human nerve injury, leading to the proposition that \( \text{Na}_\text{V}1.7 \) perhaps varies in its importance in pain pathways across species and in different pain conditions.

A plethora of pain conditions have since been identified to be \( \text{Na}_\text{V}1.7 \)-independent. Neuronal injury models, such as chronic constriction injury and spinal nerve transection injury, were only alleviated when \( \text{Na}_\text{V}1.7 \) was knocked out in all DRG neurons or knocked out in sympathetic peripheral neurons, and were not affected in mice with \( \text{Na}_\text{V}1.7 \) knocked out only in \( \text{Na}_\text{V}1.8 \)-positive C-fibres\textsuperscript{186}. At the same time, oxaliplatin-induced neuropathic pain, a condition that exhibits mechanical and cold allodynia, was not alleviated by \( \text{Na}_\text{V}1.7 \) knockout even when \( \text{Na}_\text{V}1.7 \) was also removed from sympathetic neurons\textsuperscript{186}. Cancer-induced bone pain and resultant gait abnormalities were also not significantly affected by \( \text{Na}_\text{V}1.7 \) knockout in all peripheral neurons\textsuperscript{186}. There are also pain conditions that are only partially alleviated by \( \text{Na}_\text{V}1.7 \) knockout in \( \text{Na}_\text{V}1.8 \)-positive neurons, such as allodynia following a burn injury\textsuperscript{187}. Only thermal allodynia was abolished in \( \text{Na}_\text{V}1.7 \) knockouts in a peripheral burn, and mechanical allodynia was unaffected. Symptoms arising from different modalities may
therefore still be mediated by different pain pathways and ion channels, even in pain conditions where Na\textsubscript{V}1.7 plays a recognised role.

Highly potent and selective Na\textsubscript{V}1.7 activators and inhibitors derived from animal venoms have been considered as potential therapy for pain in the clinic. Yet much like the conflicting evidence seen in murine gene knockout models, peptides and small molecules with high potency towards Na\textsubscript{V}1.7 in vitro do not translate directly into analgesia. Na\textsubscript{V}1.7-selective peptides such as ProTX-II, a peptide toxin extracted from the Peruvian green-velvet tarantula spider *Thrixopelma pruriens* and is at least 100 times more selective for Na\textsubscript{V}1.7 than other Na\textsubscript{V} isoforms in vitro, did not alleviate either acute or inflammatory pain in rats following intravenous administration\textsuperscript{188}. However, the Na\textsubscript{V}1.7-selective activator OD1, a toxin extracted from the Iranian yellow scorpion *Odontobuthus doriae*, successfully invoked spontaneous pain behaviours such as licking and flicking of the foot when given peripherally in mice\textsuperscript{189}, indicating the activation of Na\textsubscript{V}1.7 does result in nociceptive behaviour. While behaviours from OD1 injection were reversed by ProTx-III, another toxin highly selective for Na\textsubscript{V}1.7 inhibition\textsuperscript{190}, this evidence still does not reveal the exact mechanisms by which Na\textsubscript{V}1.7 contributes to pain. The need to inhibit Na\textsubscript{V}1.7 activity in sympathetic neurons indicates that Na\textsubscript{V}1.7 may not, in fact, be a vital transmitter in all nociceptive neurons as previously thought, and that it is likely that other Na\textsubscript{V} isoforms are involved in pain signal conduction, especially in pain models not completely abolished by Na\textsubscript{V}1.7 inhibition.

### 1.5.4 Na\textsubscript{V}1.6

Na\textsubscript{V}1.6 is one of the most widely distributed Na\textsubscript{V} isoforms in the peripheral as well as the central nervous system. Similar to Na\textsubscript{V}1.7, Na\textsubscript{V}1.6 is expressed in unmyelinated C-fibres and at the nodes of Ranvier in myelinated A-fibres\textsuperscript{191}. In terms of the extent of expression, Na\textsubscript{V}1.6 is expressed from the dermal terminals and skin receptors to the DRG soma in the peripheral axon, and is expressed up to the dorsal root entry in the spinal cord in the central DRG axon\textsuperscript{191}, as opposed to the expression of Na\textsubscript{V}1.7 up to the neuronal terminal in the spinal cord. Immunostaining studies indicate that Na\textsubscript{V}1.6 is expressed with high density at the nodes of Ranvier, and the lack of immunostaining with other Na\textsubscript{V} α subunit antibodies indicate Na\textsubscript{V}1.6 may be the dominant Na\textsubscript{V} isoform in nodes of Ranvier in myelinated neurons\textsuperscript{192}. The expression of Na\textsubscript{V}1.6 in the CNS is distributed throughout the presynaptic and postsynaptic membranes of the CNS neurons\textsuperscript{192}, suggesting that Na\textsubscript{V}1.6 is heavily
involved in central synaptic transmission as well as peripheral signal transmission. Malfunctions of the Na\textsubscript{V}1.6 isoform are known to lead to central transmission disturbance, with evidence suggesting that Na\textsubscript{V}1.6 is involved in hyperexcitation during epilepsy\textsuperscript{193}.

Na\textsubscript{V}1.6 appears to affect resurgent currents, a peculiar phenomenon observed first in Purkinje neurons in the cerebellum, when prolonged depolarisation followed by mild repolarisation resulted in a TTX-s inward sodium current\textsuperscript{194}. Such currents activate slowly as the membrane is repolarised, followed by deactivation\textsuperscript{194}. Na\textsubscript{V}1.6 knockout mice exhibit disturbed resurgent sodium current in Purkinje neurons\textsuperscript{195}, even though other Na\textsubscript{V} \( \alpha \) subunits such as Na\textsubscript{V}1.1 also mediate resurgent currents\textsuperscript{196}. Compared to the Na\textsubscript{V}1.7 isoform, Na\textsubscript{V}1.6 also developed and recovered from closed-state inactivation much faster\textsuperscript{173}, suggesting Na\textsubscript{V}1.6 is capable of firing at a higher frequency than Na\textsubscript{V}1.7.

Evidence towards the redistribution of Na\textsubscript{V}1.6 following neuronal injury also lends weight to the potential contribution of Na\textsubscript{V}1.6 in abnormal pain sensation. A combined partial axotomy and chronic suture lesion in the infraorbital nerve of the rat found an increased density of Na\textsubscript{V}1.6 expression in myelinated neurons following the injury, together with an increased length of the nodes of Ranvier and increased expression of Na\textsubscript{V}1.6 in the nodes\textsuperscript{197}. Studies with nerve injury and neuronal inflammation rodent models also indicate that Na\textsubscript{V}1.6 could be associated with spontaneous firing following an insult. The spinal nerve ligation model elicited spontaneous activity in myelinated sensory neurons soon after the injury, as well as at the entry of peripheral sympathetic nerve endings into the DRG. Both processes are considered vital in the establishment of neuropathic pain. The injection of small inhibitory Na\textsubscript{V}1.6 RNA into affected DRGs to locally knock down Na\textsubscript{V}1.6 channel expression reduced high frequency and repetitive firing from the nerves in rats, as well as a reduction in mechanical allodynia symptoms\textsuperscript{38}. It is also interesting to note that Na\textsubscript{V}1.6 knockdown reduced sympathetic sprouting into the DRGs, with immunostaining revealed that sympathetic sprouting had a propensity for Na\textsubscript{V}1.6-positive DRG neurons\textsuperscript{38}. Moreover, Na\textsubscript{V}1.6 knockdown did not reduce action potentials evoked by external stimuli, but only dampened spontaneous activity in the affected DRG neurons\textsuperscript{38}, offering intriguing insight into the role Na\textsubscript{V}1.6 plays in action potential transmission.

Experiments involving local Na\textsubscript{V}1.6 knockdown during DRG inflammation yielded similar results. Myelinated nerves which were affected by inflammation and became spontaneously
active expressed more Na\textsubscript{V}1.6 channels compared to other myelinated neurons, with the local injection of small interfering Na\textsubscript{V}1.6 RNA successfully blocking spontaneous activity and reducing aversive behaviour in the rats\textsuperscript{198}. Unmyelinated C-fibres were found to be minimally affected\textsuperscript{198}, suggesting Na\textsubscript{V}1.6 may only play a major role in myelinated sensory neurons. At the same time, a semisection of the rat spinal cord resulted in increased contralateral expression of Na\textsubscript{V}1.6 on unmyelinated DRG neurons\textsuperscript{199}, suggesting Na\textsubscript{V}1.6 could redistribute throughout the organism following neuronal injury. It is also very likely that Na\textsubscript{V}1.6, similar to Na\textsubscript{V}1.7, only affects certain pain conditions and modalities, particularly as Na\textsubscript{V}1.6 expression is decreased in painful diabetic neuropathy in rats, a model in which Na\textsubscript{V}1.7 expression is increased\textsuperscript{185}.

As of now, Na\textsubscript{V}1.6 has already been found to be intimately involved in some clinically relevant pain conditions, one of those being ciguatoxin-induced pain and cold allodynia\textsuperscript{200}. Ciguatoxin is a toxin that binds to toxin binding site 5 in Na\textsubscript{V} channels, and is produced by marine dinoflagellate that accumulate in large reef fish. Human ingestion results in ciguatera, a condition characterised by severe diarrhoea and neurological disturbances such as dysaesthesia and specifically cold allodynia\textsuperscript{201}. Previous studies have indicated that TTX-s Na\textsubscript{V} isoforms as well as Na\textsubscript{V}1.8 mediate ciguatoxin-induced neuronal hyperexcitability through activating cold-specific pathways\textsuperscript{86}. Further research indicates that when GIIIA, a Na\textsubscript{V}1.6-selective inhibitor conotoxin, is peripherally administered together with the selective Na\textsubscript{V}1.8 inhibitor A-803467, a significant decrease in pain behaviours such as flinching was observed in mice with ciguatoxin-induced cold allodynia\textsuperscript{200}. The administration of GIIIA alone also significantly reduced flinching in mice, with the implication that Na\textsubscript{V}1.6 could be the main contributor to the excitatory effects of ciguatoxin in myelinated neurons.

Na\textsubscript{V}1.6 inhibition was also successful at alleviating dysaesthesia caused by oxaliplatin and cisplatin, two platinum-based chemotherapy drugs known to cause neurotoxicity\textsuperscript{133}. Peripherally administered oxaliplatin elicited cold allodynia in mice, resulting in behaviours such as flicking and licking and paw shaking when the mice were exposed to 10 °C one hour after injection\textsuperscript{2}. Examinations using selective inhibitors of all Na\textsubscript{V} isoforms expressed in neurons as well as TRPA1, TRPV1, and TRPM8 blockers and knockout mice indicated that only GIIIA administration was successful at alleviating cold allodynia. Moreover, the co-administration of 4-aminopyridine, a Kv inhibitor, and Cn2, a Na\textsubscript{V}1.6 activator extracted from the venom of the scorpion Centruroides noxius\textsuperscript{202}, successfully replicated cold allodynia.
observed following oxaliplatin administration, concluding that the activation of NaV1.6 and the inhibition of repolarising Kv isoforms forms the backbone of oxaliplatin-induced cold allodynia symptoms. Mechanical allodynia in mice following peripheral administration of cisplatin was also only alleviated by NaV1.6 inhibition. Experimental groups of mice were injected with TTX, the NaV1.1/1.2/1.4 inhibitor TIIIA, the NaV1.7-selective inhibitor ProTx-II, or the NaV1.6-selective inhibitor GIIIA. GIIIA was the only compound that successfully reverted mechanical threshold to normal levels, cementing the notion that NaV1.6 is responsible for certain NaV1.7-independent pain conditions, and that the roles NaV1.6 play in other NaV1.7-independent conditions most certainly warrant further examination.

1.5.5 NaV1.8

NaV1.8 is a TTX-r channel preferentially expressed on C-fibres in peripheral sensory neurons, and has been considered one of the indispensable markers of peripheral nociceptors. As discussed previously, conditional knockout of other NaV isoforms are often conducted on NaV1.8-positive neurons, as this is assumed to include all nociceptive C-fibres. NaV1.8 exhibits slow inactivation kinetics, resulting in long action potentials. At the same time, NaV1.8 also exhibits high firing frequency due to its fast recovery from inactivation, leading to prolonged electrical activity of the neuron. Similar to NaV1.6 and NaV1.7, NaV1.8 also generates resurgent currents, although resurgent currents generated by NaV1.8 activate and inactivate much slower than those generated by NaV1.6. Such properties of NaV1.8 indicate that this isoform could be involved in protracting neuronal firing and making neurons more prone to spontaneous firing.

A vital function of NaV1.8 is the detection of pain in cold temperatures. NaV1.8 is responsible for maintaining the excitability of peripheral nociceptors at noxious cold temperatures (< 10 °C), particularly as TTX-s NaV isoforms are inactivated with decreasing temperature, to which NaV1.8 excitability is completely unaffected. Similar to that seen with other PNS NaV isoforms, NaV1.8 mutations cause neuropathy in human patients. Gain-of-function NaV1.8 mutations further accelerate channel recovery from inactivation and exaggerates channel responses to slow depolarisations, culminating in hyperexcitability of nociceptive C-fibres in patients with idiopathic painful small fibre neuropathy. Transgenic mice with gain-of-function NaV1.8 mutations displayed increased mechanically evoked action potentials in A- and C-fibres, with fibres firing continuously even after the removal of the mechanical
stimulus. However, this did not lead to a decrease in noxious mechanical threshold in the transgenic mice, pointing to a potential role for \( \text{Na}_V1.8 \) in thermal pain sensing but a less vital role in mechanical pain sensing.

Additional studies have investigated the involvement of \( \text{Na}_V1.8 \) using \( \text{Na}_V1.8 \) knockout mice. Neuromas formed by saphenous nerve sectioning exhibited significantly less mechanoexcitability and spontaneous firing in \( \text{Na}_V1.8 \) knockout mice (19% vs 0.4% at 22 days after the injury). While \( \text{Na}_V1.8 \) is responsible for maintaining nociceptive signal transmission in cold temperatures, and \( \text{Na}_V1.8^{-/-} \) mice also failed to show signs of cold alldynia following spared nerve injury, the \( \text{Na}_V1.8 \) knockout mice exhibited no difference in heat sensitivity (up to 56 °C). The expression of \( \text{Na}_V1.8 \) was reduced in the soma of DRG neurons that were injured by a sciatic nerve ligation injury, but was significantly increased in intact and adjacent axons throughout the sciatic nerve. These findings suggest the involvement of \( \text{Na}_V1.8 \) in overall C-fibre hyperexcitability following injuries.

The contribution of \( \text{Na}_V1.8 \) in different pain conditions has also been further assessed, with \( \text{Na}_V1.8 \) appearing to take a more active role in inflammatory and neuropathic pain compared to pain following acute injury or surgery. Mechanical alldynia after CFA administration was significantly reduced with local \( \text{Na}_V1.8 \) antisense oligodeoxynucleotides, and mechanical alldynia following chronic constriction injury was also reduced following local \( \text{Na}_V1.8 \) knockdown. However, pain following skin-incision and vincristine-induced neuropathic pain was not changed by \( \text{Na}_V1.8 \) knockdown, leading to the notion that \( \text{Na}_V1.8 \) may not be involved in acute pain transmission. Conflicting information, however, has emerged regarding the generalisation of such findings, with double \( \text{Na}_V1.7/\text{Na}_V1.8 \) knockout mice exhibiting mechanical alldynia following spinal nerve ligation despite the lack of two major \( \text{Na}_V \) isoforms in nociceptors. Such findings indicate the need to examine the contribution of \( \text{Na}_V \) isoforms specific to each pain condition, as it is now evident that different conditions, even though they result in similar symptoms in animal models and human patients, likely exhibit drastically different molecular changes following the insult.

1.6 Summary

Pain is much more than a measurement of the intensity of the external stimuli. It is a sensation that involves transducing non-electrical stimuli into action potentials at the dermal
nerve endings of DRG neurons, conducting the signal along the DRG neuronal axons to the dorsal horn in the spinal cord, and further transmission into the thalamus and higher cognitive centres. Ion channels expressed in DRG neurons in the PNS are vital in the conduction of action potentials along the axon, as well as controlling the excitability of cellular membranes to modulate the frequency and threshold of action potential generation. \( \text{Na} \), \( \text{Na} \), and \( \text{Na} \) have been the most likely candidates for novel molecular targets for analgesia, due to their selective expression in peripheral sensory neurons as well as their noted role in channelopathies related to pain. It is also now evident that different pain conditions exhibit vastly different molecular changes in nociceptive neurons, and an examination of the contribution of \( \text{Na} \) channels in individual conditions is required to develop specific and successful novel analgesics based on \( \text{Na} \) activity.

1.7 Hypothesis and Aims

Based on the crucial contribution of \( \text{Na} \) isoforms to pain as discussed above, as well as increasing recognition that \( \text{Na} \) and \( \text{Na} \) contribute to modality-specific pain pathways, this thesis will explore the following hypothesis and aims:

1.7.1 Hypothesis

\( \text{Na} \) and \( \text{Na} \) play important roles in \( \text{Na} \)-independent pain conditions.

1.7.2 Aims

1. To discover \( \text{Na} \)- or \( \text{Na} \)-selective channel modulators.
2. To evaluate whether \textit{in vitro} cell models are representative of known DRG neuron subclasses.
3. To investigate the role of \( \text{Na} \) and \( \text{Na} \) in burn-induced mechanical allodynia, a \( \text{Na} \)-independent pain symptom.
4. To investigate transcriptomic changes in DRG neurons during burn-induced pain.
Chapter 2: Discovery of Selective Voltage-gated Sodium Channel Inhibitors from Spider Venoms

2.1 Foreword

Peptides extracted from naturally occurring toxins have offered a wealth of selective ion channel modulators\textsuperscript{208}, as well as being potential drug leads that target nociceptive neurons. Almost all spiders and scorpions are armed with a venom apparatus and secrete a cocktail of various toxins designed to paralyse and kill prey, offering a large number of potential neurotoxins that can be used to explore the mysteries of sensory neurons. I therefore conducted an investigation into the biological activity of a collection of scorpion and spider crude venoms on Na\textsubscript{V}1.6 and Na\textsubscript{V}1.8 isoforms, utilising cells stably transfected with human Na\textsubscript{V}1.6 or Na\textsubscript{V}1.8. An assay-guided fractionation method was used to find putative novel Na\textsubscript{V}1.6 and Na\textsubscript{V}1.8 inhibitors, which may be used as pharmacological tools.

I would like to acknowledge the contributions of the following parties towards this chapter: Dr. Volker Herzog for the supply of crude venoms; the Australian Proteome Analysis Facility (APAF) in Sydney, Australia, for sequence identification of the novel peptide using Edman degradation; and Dr. Aihua Jin and Dr. Mu Yu (Institute of Molecular Biosciences, University of Queensland, Australia) for further analysis of the peptide sequence using tandem mass spectrometry (MS/MS).

2.2 Introduction

Some of the pharmacologically active compounds that have been used experimentally in animal models of pain to affect Na\textsubscript{V}1.6 and Na\textsubscript{V}1.8 channel activities are GIIIA, 4,9-anhydro-TTX, and A-803467. GIIIA is a conotoxin extracted from the venom of cone snails\textsuperscript{130}, 4,9-anhydro-TTX is a metabolite of TTX\textsuperscript{209}, and A-803467 is a small molecule inhibitor\textsuperscript{210}. However, a number of concerns persist for these compounds, as outlined below.

GIIIA, a µ-conotoxin extracted from the venom of the cone snail *Conus geographus*, has known inhibitory effects on the skeletal muscle Na\textsubscript{V} isoform Na\textsubscript{V}1.4\textsuperscript{130}. GIIIA was initially considered to be ineffective at neuronal Na\textsubscript{V} isoforms\textsuperscript{130}. Further studies then determined
GIIIA also inhibits $\text{Na}_V 1.1$, $\text{Na}_V 1.2$, and $\text{Na}_V 1.6$, and is 30-fold more selective for $\text{Na}_V 1.4$ over $\text{Na}_V 1.6$ (GIIIA $\text{IC}_{50} = 0.019 \text{ µM}$ in $\text{Na}_V 1.4$ and $\text{IC}_{50} = 0.68 \text{ µM}$ in $\text{Na}_V 1.6$). GIIIA also exhibits different effects in different species, with the human $\text{Na}_V 1.4$ isoform being resistant to GIIIA-mediated inhibition despite the high potency of the toxin in the rat $\text{Na}_V 1.4$ isoform. In order to examine the effect of $\text{Na}_V 1.6$, GIIIA is administered together with TIIIA, another conotoxin-derived peptide that inhibits $\text{Na}_V 1.1$, 1.2, 1.3, and $\text{Na}_V 1.4$, but not $\text{Na}_V 1.6$. The differential inhibitory activity of the two peptides allows for assessment of the effect of $\text{Na}_V 1.6$ inhibition independent of $\text{Na}_V 1.1$, $\text{Na}_V 1.2$, and $\text{Na}_V 1.4$ inhibition in animal models. An existing peptide with the opposite action to GIIIA is the β-scorpion toxin Cn2, which functions as a potent $\text{Na}_V 1.6$-selective activator. Cn2 injections have been used in animals as a model of local $\text{Na}_V 1.6$ activation. However, as a channel activator, Cn2 cannot be used to investigate the effect of $\text{Na}_V 1.6$ inhibition in clinically relevant pain conditions, necessitating the discovery of inhibitory compounds with better selectivity than GIIIA.

Another compound that has been reported to selectively inhibit $\text{Na}_V 1.6$ is 4,9-anhydro-TTX, a metabolite of tetrodotoxin (TTX). This metabolite is reported to selectively inhibit $\text{Na}_V 1.6$ over other $\text{Na}_V$ isoforms with approximately 50-fold higher selectivity (4,9-anhydro-TTX $\text{IC}_{50} = 7.8 \text{ nM}$ for $\text{Na}_V 1.6$. $\text{Na}_V 1.3$, the $\text{Na}_V$ isoform it inhibits with the second-highest potency, has $\text{IC}_{50} = 341 \text{ nM}$). However, work in my lab has not been able to successfully replicate these findings, drawing into question both the potency and selectivity of the compound. 4,9-anhydro-TTX is also sparingly soluble in aqueous solutions and preferentially dissolves in ethanol, posing a safety concern in animal work. More characterisation studies both in vitro and in animals are therefore required before 4,9-anhydro-TTX can be considered a good selective tool for studies on $\text{Na}_V 1.6$ or as a possible drug lead.

A-803467 is a $\text{Na}_V 1.8$-inhibitory small molecule discovered through structural-activity studies. Prior to its discovery, $\text{Na}_V 1.8$ inhibition could only be achieved through gene knockout or local gene knockdown using interfering RNA and antisense oligonucleotides in animals. A-803467, however, is known to preferentially bind to the inactivated state of the $\text{Na}_V 1.8$ channel rather than the resting state. Furthermore, evidence obtained from global $\text{Na}_V 1.8$ knockout mice, local $\text{Na}_V 1.8$ knockdown mice, and A-803467-mediated $\text{Na}_V 1.8$ inhibition are contradictory in some conditions. $\text{Na}_V 1.8$ global knockout mice maintained robust mechanical allodynia following spinal nerve ligation injury, but
demonstrated significantly prolonged latency to paw withdrawal when challenged with thermal stimuli in the Hargreave's Test, as well as reduced nociceptive behaviours in the formalin inflammatory pain model\textsuperscript{177}. In contrast, antisense oligonucleotides targeting Na\textsubscript{V}1.8 as well as A-803467 administration achieved mechanical allodynia reduction in spinal nerve ligation\textsuperscript{210, 213} as well as in chronic nerve constriction\textsuperscript{3, 210} models. To further complicate the relationship between gene knockout and protein inhibition data, A-803467 did not affect the response of mice in the formalin inflammatory pain model\textsuperscript{210}. The results of these studies suggest Na\textsubscript{V}1.8 expression changes may not be the determinant of neuropathic pain development and maintenance, and functional inhibitors that hinder the activity of Na\textsubscript{V}1.8 channels in the affected neurons would be more appropriate for evaluating the contribution of Na\textsubscript{V}1.8 activity in pain.

One under-explored source of potent and selective Nav modulators is from venoms, in particular from spiders. Crude venoms secreted by spiders contain a mixture of many components, which can be loosely classified into three groups: low molecular mass organic molecules (molecular weight < 1 000 Daltons), polypeptides (molecular weight 3 000 - 10 000 Daltons), and high molecular mass proteins (molecular weight > 10 000 Daltons)\textsuperscript{214}. Other minor components in the crude venom include ions, acids, amino acids, and neurotransmitters such as histamine and dopamine\textsuperscript{214}. While the acids and neurotransmitters contained in spider venoms do affect neurons \textit{in vivo}, the majority of neurotoxins extracted from spider venoms fall into the polypeptide group.

Polypeptides found in many spider venoms share similarities, such as a medium range of molecular weight and structural inflexibility due to the presence of disulfide bridges\textsuperscript{214}. These differences allow polypeptides to be easily separated from other components in the crude venom via methods such as HPLC (high performance liquid chromatography) and MALDI (matrix-assisted laser desorption/ionisation). A large number of peptides have been isolated from spider venoms to date, with the first spider toxin family discovered to act upon Na\textsubscript{V} channels being the \( \mu \)-agatoxins, which are derived from the venom of the highly venomous funnel-web spiders\textsuperscript{215}. Analyses into crude spider venoms using MALDI indicate the presence of two main clusters of polypeptide masses, with one cluster between 3500-4500 Daltons (Da) and another between 6500-7000 Da. It is therefore speculated that two separate groups of polypeptides exists, with one exhibiting moderate length and molecular mass, and another group consisting of long peptides with more than four disulfide bridges\textsuperscript{208}.
Peptides exhibit selectivity between different ion channels, between channels in different tissues, as well as between species, particularly as many spider peptides are selective for insect NaV isoforms compared to mammalian targets. Peptides binding to sites within different NaV domains (see Chapter 1, section 1.5.2) also cause diverse results. Toxins targeting the voltage sensor portions of domains I, II, and III affect voltage-gated opening of the NaV channel, whereas the voltage sensor portion from domain IV dictates NaV inactivation. Curiously, despite the observed promiscuity of many spider toxins, the amino acid sequence homology between the voltage sensor of the various ion channels is low, suggesting that peptides may have evolved to target the general structure of the sensor rather than specific amino acids exhibited in the various channels.

This chapter therefore set out to explore possible polypeptides in crude scorpion and spider venoms that may exhibit inhibitory activity against NaV1.6 or NaV1.8 in order to discover potential inhibitory peptides targeting these ion channels.

2.3 Materials and methods

2.3.1 Materials

All materials were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia) unless otherwise stated. Dr. Volker Herzig (Institute for Molecular Bioscience, University of Queensland) generously provided all venom samples. All crude venom samples, as well as freeze-dried venom fractions and isolated peptide, were reconstituted with 1:1000 BSA (bovine serum albumin, Alfa Aesar, MA, USA; Cat# J65966) dissolved in PSS (physiological saline solution; for composition see Appendix 2.1). Membrane Potential dye and Calcium 4 No-Wash dye stock solution were reconstituted using 10 mL of PSS per vial of powder, and stored at -20 °C for up to 3 months.

2.3.2 Animals

Male wild-type C57BL/6 mice age 6-8 weeks and weighing 20 - 23 grams were used. Ethical approval for in vivo experiments involving live animals was obtained from the University of Queensland animal ethics committee under the ethics approval number
Experiments were conducted in accordance with the Animal Care and Protection Regulation Queensland (2012), the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th edition (2013) and the International Association for the Study of Pain Guidelines for the Use of Animals in Research.

All animals were housed in groups of 2-4 per cage in a stable environment under 12 hour light-dark cycles and had access to standard rodent chow and water *ad libitum*. A red polycarbonate Mouse House (Tecniplast, Italy) and shredded paper nesting material were supplied for enrichment. All animals were obtained from University of Queensland Biological Resources (Brisbane, Australia).

### 2.3.3 Cell culture

HEK293 cells stably expressing human Nav1.6 (hNav1.6) ion channels were obtained from Scottish Biomedical (Glasgow, UK). Cells were grown in MEM (Minimum Essential Media; for composition see Appendix 2.2) with 10% FBS (foetal bovine serum, Life Technologies, Mulgrave, Victoria, Australia) and 2 mM L-glutamine (GlutaMax™, Life Technologies, Mulgrave, Victoria, Australia).

CHO (Chinese hamster ovary cells) cells stably expressing hNav1.8 were obtained from ChanTest (Cleveland, OH, USA). Cells were grown in Ham’s F12 media (Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.3) with 10% FBS. To maintain selective pressure for Nav expression, this culture media was supplemented with blasticidin (10 µg/mL; Life Technologies, Mulgrave, Victoria, Australia), hygromycin (250 µg/mL; Life Technologies, Mulgrave, Victoria, Australia), and zeocin (400 µg/mL; Life Technologies, Mulgrave, Victoria, Australia).

Un-transfected HEK293 cells (American Type Culture Collection, VA, USA) were transfected (to be described in Section 2.3.4) with rat TRPV1 (rTRPV1) ion channels. Cells were grown in DMEM (Dulbecco’s Modified Eagle Medium, Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.4) with 10% FBS and 2 mM L-glutamine. To maintain selective pressure for Nav expression, this culture media was supplemented with hygromycin (50 µg/mL).
All cells were incubated at 37°C and 95% O₂/5% CO₂. Cells were passaged every 3-5 days at a 1:5 dilution ratio or when approximately 90% confluent. All cells were seeded in T75 cm² Corning® (Corning, MA, USA) cell culture flasks unless otherwise stated. In order to passage cells, cells were briefly removed from incubation and maintained in room temperature and growth media was removed from the flask. At least 5 mL/75 cm² of DPBS (Dulbecco’s Phosphate Buffered Saline, Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.5) was added into the flask to briefly wash the cells and was then removed from the flask. 0.25% trypsin/EDTA (0.25% trypsin/ethylenediaminetetraacetic acid in Hank’s Balanced Salt Solution, Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.6) was then added at a concentration of 1 mL/75 cm², and the cells were dislodged by gently tapping the sides of the flask. The dislodged cell suspension was diluted with a further 9 mL of the appropriate growth media. The cells were passaged according to aforementioned ratios in a new T75 cm² flask using new growth media.

### 2.3.4 Transfection

Un-transfected HEK293 cells (American Type Culture Collection, VA, USA) were routinely maintained in DMEM containing 10% FBS and 2 mM L-glutamine. Cells were split every 3-6 days in a ratio of 1:5 using 0.25% trypsin/EDTA. Cells were plated on T75 cm² (Nunc, Denmark) tissue culture flasks 24 hours prior to transfection, and were transfected with rat TRPV1 plasmid DNA (D. Julius, Department of Physiology, University of California, Berkeley, CA, USA) using Lipofectamine™ 2000 (Life Technologies, Mulgrave, Victoria, Australia) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were plated on 384-well plates and used for Ca²⁺ 48 hours after plating.

### 2.3.5 Fluorescent membrane potential and Ca²⁺ assays

All cells were seeded onto 384-well black-walled imaging plates (Corning, MA, USA) at a density of 10,000 - 15,000 cells per well 48 hour prior to membrane potential or calcium imaging experiments, and were incubated with 95% O₂/5% CO₂ for 48 hours. HEK293 cells expressing Naᵥ1.6 or TRPV1 were seeded onto uncoated plates (Corning, Cat No #3542). CHO cells expressing Naᵥ1.8 were seeded onto PDL (poly D-lysine) coated 384-well plates.
Cells were seeded to ensure ~90% confluency on the day of the experiment.

HEK293 cells expressing Na\textsubscript{v}1.6 or TRPV1 were incubated at 37°C prior to imaging experiments in culture media as described in Section 2.3.3. CHO cells expressing Na\textsubscript{v}1.8 were incubated in Ham’s F12 media (Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.3) with 10% FBS. The Na\textsubscript{v}1.8 CHO cells express a tetracycline-inducible vector, therefore the culture media for CHO cells was supplemented with tetracycline (1 µg/mL) from tetracycline stock solution (for composition see Appendix 2.7), freshly added before incubation. CHO cells expressing Na\textsubscript{v}1.8 was incubated at 37°C with 95% O\textsubscript{2}/5% CO\textsubscript{2} for 24 hours following seeding, then incubated at 25°C with 95% O\textsubscript{2}/5% CO\textsubscript{2} for a further 24 hours to induce increased Na\textsubscript{v}1.8 expression.

Membrane Potential dye (Corning, MA, USA) and Calcium 4 No-Wash dye (Corning, MA, USA) stock solutions were further diluted 1:10 in PSS for use in experiments. Cells were incubated in the dye for 30 minutes prior to imaging. Membrane Potential dye was used for all fluorescence experiments except for TRPV1 experiments, for which the Calcium 4 No-Wash dye was used. For CHO cells expressing Na\textsubscript{v}1.8, 1 µg/mL of tetrodotoxin (Sapphire Bioscience, Redfern, New South Wales, Australia) was added into the final volume of the dye to inhibit TTX-s Na\textsubscript{v} calcium responses.

All plates with dye were incubated at 37 °C with 95% O\textsubscript{2}/5% CO\textsubscript{2}. Membrane potential or Ca\textsuperscript{2+} responses were measured using a FLIPR\textsuperscript{TETRA} (Molecular Devices, CA, USA) fluorescent plate reader. Membrane Potential dye assays were exposed to excitation at 510-545 nM and emission at 565-625 nM. Calcium 4 No-Wash dye assays were exposed to excitation at 470-495 nM and emission at 515-575 nM. Camera gain and intensity were adjusted for each plate to yield a minimum of 1000-1500 arbitrary fluorescence units (AFU) baseline fluorescence.

Each assay with Nav1.6-expressing HEK293 cells or Nav1.8-expressing CHO cells consisted of two additions of solutions into the plate. The first addition (10 µL) consists of the compound tested for inhibitory activity (crude venom samples, freeze-dried venom fractions, purified peptide, or buffer solution). All compounds added, apart from the buffer solution, were reconstituted in the buffer solution (1:1000 BSA dissolved in PSS). Prior to the first
addition for HEK293 cells expressing Na\textsubscript{v}1.6, 10 baseline fluorescence readings were taken, followed by fluorescent readings every 2 seconds for 150 reads. Prior to first addition for CHO cells expressing Na\textsubscript{v}1.8, 10 baseline fluorescence readings were taken, followed by fluorescent readings every 2 seconds for 150 reads.

The second addition (10 µL) for Na\textsubscript{v}1.6-expressing HEK293 cells or Na\textsubscript{v}1.8-expressing CHO cells consists of a known agonist to the Na\textsubscript{v} isoform. Veratridine (100 µM; Sapphire Bioscience, New South Wales, Australia) was used as the agonist for assays involving HEK293 cells expressing Na\textsubscript{v}1.6. Deltamethrin (150 µM) was used as the agonist for assays involving CHO cells expressing Na\textsubscript{v}1.8. Both agonists were constituted from powder in DMSO as stock solutions and diluted with PSS. Maximum final DMSO concentration in experimental solutions did not exceed 0.001%. Prior to the second addition for HEK293 cells expressing Na\textsubscript{v}1.6, 10 baseline fluorescence readings were taken, followed by fluorescent readings every 1 seconds for 600 reads. Prior to the second addition for CHO cells expressing Na\textsubscript{v}1.8, 10 baseline fluorescence readings were taken, followed by fluorescent readings every 5 seconds for 450 reads.

For calcium response assays involving capsaicin and activation of TRPV1 using HEK293 cells expressing rTRPV1, only one addition was used. Prior to the addition of potential agonists, 10 baseline fluorescence readings were taken, followed by the addition of capsaicin or Pme1a, after which fluorescent readings were conducted every 1 second for 300 seconds. Capsaicin stock solution was prepared in ethanol and stored at -80 °C. Stock solution of capsaicin was diluted with PSS. Maximum final ethanol concentration in capsaicin solution did not exceed 0.001%. Pme1a was reconstituted with 1:1000 BSA dissolved in PSS.

2.3.6 High Performance Liquid Chromatography (HPLC)

Solvent A (aqueous solvent) was constituted with 0.1% trifluoroacetic acid in Milli-Q water, and Solvent B (organic solvent) was constituted with 0.05% trifluoroacetic acid in acetonitrile (VWR International, Queensland, Australia).

For the fractionation of the *H. hainanum* crude venom, a Vydac 218TP C\textsubscript{18} 300Å 250mm column (Grace, MD, USA; Cat# 218TP54) was used. HPLC solvent B concentration gradient
was as follows: 0-25% over 40 minutes, then 25-50% over 60 minutes, then 50-100% over 10 minutes. The flow rate was 0.70 mL/minute and fractions were collected every minute.

For the fractionation of the *P. metallica* crude venom, a Vydac 218TP C<sub>18</sub> 300Å 250mm column (Grace, MD, USA; Cat# 218TP54) was used. HPLC solvent B concentration gradient was as follows: 0% until minute 5, then 0-50% over 50 minutes, then 50-100% over 20 minutes. The flow rate was 0.70 mL/minute and fractions were collected every minute.

All fractions were then freeze-dried before reconstitution and testing. All experiments were conducted on an UltiMate 3000 HPLC machine (Dionex, Victoria, Australia).

### 2.3.7 Matrix-assisted laser desorption/ionisation (MALDI)

MALDI was conducted to determine the mass of peptides found in fractions. The matrix consists of 10 mg/mL CHCA (α-Cyano-4-hydroxycinnamic acid) dissolved in 50% acetonitrile, 0.1% formic acid (VWR International, Queensland, Australia), and approximately 50% Milli-Q water. 0.5 µL of the matrix suspension was mixed with 0.5 µL of venom fraction (not freeze-dried, with HPLC solvent of the appropriate polarity) and spotted on a MALDI plate (TOF 4700 MALDI sample plate 192-well, Applied Biosystems, Victoria, Australia). Laser intensity was set at 3500 and MALDI analysis was conducted on a 4700 Proteomics Analyser (Applied Biosystems, Victoria, Australia).

### 2.3.8 Sequence identification

Edman’s degradation reaction was performed by APAF (Australian Proteome Analysis Facility, Sydney, New South Wales, Australia) with reduction/alkylation for the purpose of N-terminal sequence identification. The sample was solubilised in ammonium bicarbonate (50 mM) and acetonitrile (35%) then reduced using dithiothreitol (25 mM) at 56 °C for 30 minutes. It was then alkylated using iodoacetamide at room temperature for 30 minutes. The sample mixture was desalted prior to sequence analysis using RP-HPLC with a Zorbax 300SB-C18 column (3 x 150 mm). The target peaks of interest were collected and reduced to minimal volume under vacuum. The sample tube was also washed with a solution of 60% isopropanol containing 0.1% trifluoroacetic acid to extract any adsorbed peptide. The combined extracts were loaded onto a precycled, Biobrene-treated disc and subjected to 35
cycles of Edman N-terminal sequencing. The complete sequence was found using MS/MS (Tandem mass spectrometry) by Dr. Aihua Jin and Dr. Mu Yu (Institute of Molecular Biosciences, University of Queensland, Australia).

2.3.9 Animal behaviour studies

Purified Pme1a peptide was freeze-dried and reconstituted in PSS (for composition see Appendix 2.1) with 1:1000 BSA, and administered by shallow intraplantar injection into the left hind paw of mice (10 µL injection volume) under 3% isoflurane anaesthesia. Mice were allowed to recover in individual polyvinyl circular containers (25 cm in diameter) placed on a plastic surface at room temperature. Spontaneous pain was observed as licking, lifting and/or flinching of the affected hind paw. Mice were allowed to roam freely and spontaneous pain was measured in 5 minute intervals over 10 minutes, counted by a blinded observer. All nocifensive responses during the observation period were recorded.

2.3.10 Data analysis

Data were plotted and analysed by GraphPad Prism, version 6.00. Statistical significance was defined as $P$-value < 0.05 and was determined by unpaired $t$-test assuming equal variance. Data is expressed as the mean ± standard error of the mean (SEM). FLIPR TETRA data was analysed using ScreenWorks, version 3.2 (Molecular Devices, CA, USA). HPLC data was exported from Chromeleon (Dionex, Victoria, Australia).

2.4 Results

2.4.1 Screening of crude spider and scorpion venoms on HEK293 cells expressing human Na\textsubscript{v}1.6 channels

Various species of scorpion and spider crude venoms (6 scorpion and 18 spider species) were tested in cell-based high-throughput assay via the FLIPR TETRA system, using HEK293 cells stably expressing the human Na\textsubscript{v}1.6 (hNa\textsubscript{v}1.6) isoform. Reconstituted venom or control solutions were added as the first addition, and the known Na\textsubscript{v}1.6 activator veratridine was added as the second addition.
All 6 scorpion species tested (Parabuthus schlechteri, Parabuthus granulatus, Hottentotta gentili, Buthus albengai, Parabuthus transvaalicus, and Parabuthus villosus) elicited spontaneous membrane potential elevation when added to hNa\(_V\)_1.6-expressing HEK293 cells (Figure 2.1 A-F). P. schlechteri was the most potent activator, followed by P. transvaalicus (Figure 2.1 A and E). The scorpion toxins that exhibited the least potent agonist activity were H. gentili and P. granulatus (Figure 2.1 C and B). Four out of the six scorpion toxins also exhibited potentiation of veratridine-induced membrane potential elevation in hNa\(_V\)_1.6-expressing HEK293 cells (Figure 2.1 G).

In comparison, only 2 samples out of the 18 spider venoms tested potentiated veratridine-induced membrane potential elevations in hNa\(_V\)_1.6. Macrothele gigas and Poecilotheria fasciata were the spider species that exhibited hNa\(_V\)_1.6 potentiation, amplifying membrane potential elevations by 45% and 46% respectively compared to control cells. All other spider crude venoms examined exhibited inhibitory activity and reduced veratridine-induced membrane potential elevation in hNa\(_V\)_1.6-expressing HEK293 cells. This observation suggests a possible difference between the effects of scorpion venoms, particularly those in the Parabuthus genus, to that of tarantula spider venoms on hNa\(_V\)_1.6 channels, with the scorpion venoms from the Parabuthus genus more likely to exhibit hNa\(_V\)_1.6 agonistic behaviour than tarantula spider venoms (Figure 2.1 G, for potentiation or inhibitory traces from all venoms see Appendix 3.1).

Haplopelma hainanum, a species of tarantula spiders, was found to have marked inhibitory activity. The addition of crude H. hainanum venom in hNa\(_V\)_1.6-expressing HEK293 cells inhibited 85% of veratridine-induced membrane potential elevation compared to control responses (Figure 2.1 G, grey bar; Figure 2.2 A). One other species exhibited greater reduction in hNa\(_V\)_1.6 membrane potential elevations than H. hainanum venom. The sample was the venom from Euathlus species, and veratridine-induced membrane potential increases were reduced by 91% in cells exposed to the venom sample (Figure 2.2 B). However, since only microgram amounts of venom were available for Euathlus species, Haplopelma hainanum venom was chosen for further investigation.
**Figure 2.1 Differential actions of scorpion and spider venoms in hNa\(_{V1.6}\)-expressing HEK293 cells**

The addition of scorpion and spider venoms into wells containing hNa\(_{V1.6}\)-expressing HEK293 cells resulted in different membrane potential changes. Out of the 6 scorpion toxins examined, 4 sample were from the *Parabuthus* genus. All 6 samples of scorpion toxins were found to exhibit spontaneous activation, and 4 samples potentiated veratridine-induced membrane potential increase in hNa\(_{V1.6}\) channels. A-F) All 6 scorpion venoms examined exhibited agonistic behaviour, spontaneously elevating membrane potential in hNa\(_{V1.6}\)-expressing HEK293 cells immediately after addition. Venom from *P. schlechteri* was the most potent activator, followed by venom from *P. transvaalicus*. Traces were normalised to baseline fluorescence readings before the first addition. The X axis indicates time (in seconds) after the first addition. G) Effect of crude venoms on veratridine-induced Na\(_{V1.6}\) responses. Four out of the six scorpion crude venoms (the first six species listed in panel G) exhibited potentiation of veratridine-induced membrane potential responses. In comparison,
only 2 spider venoms potentiated veratridine-induced membrane potential responses. The potentiating spider venoms were from *Macrothele gigas* and *Poecilotheria fasciata*. The spider crude venom with the most inhibitory activity was the sample from *Euathlus* species. Following the addition of venom from *Euathlus* species, 91% of veratridine-induced membrane potential elevation compared to control responses was inhibited in the cells. Addition of venoms from *H. hainanum* resulted in inhibiting 85% of the control response from the cells. The *H. hainanum* sample was coloured in grey in panel G. Traces were normalised to baseline fluorescence readings before the second addition. All responses were normalised to Area Under Curve values in positive-control wells, with 1:1000 BSA dissolved in PSS as the first addition and veratridine (100 µM) as the second addition. The dotted line at 100% indicates the positive-control response.
Figure 2.2 *Haploplema hainanum* and *Euathlus* venoms elicited a large inhibition in veratridine-induced hNav1.6 activity

Crude venom or the control solution was used as the first addition, with veratridine (100 µM) used as the second addition. A) Crude *H. hainanum* venom reduced veratridine-induced membrane potential increase by 85% compared to control cells. B) Crude *Euathlus* venom reduced veratridine-induced membrane potential increase by 91% compared to control cells. All traces were normalised to baseline fluorescence readings before the second addition. All percentages were calculated as Area Under Curve values compared to responses by positive-control wells. Control solution (1:1000 BSA dissolved in PSS) was added as the first addition and veratridine (100 µM) was added as the second addition in positive-control cells. All
traces were normalised to baseline fluorescence readings before the second addition. The X axis indicates time (in seconds) after the first addition.
2.4.2 H. hainanum fractionation and the biological activity of fractions

Crude *H. hainanum* venom (1 mg) was fractionated using a C\textsubscript{18} HPLC column, and the HPLC trace was matched against the biological activity of the fractions in hNav1.6-expressing HEK293 cells (Figure 2.3 A). A total of 70 fractions were tested. An equivalent of 50 µg of crude venom were used in cell experiments. Fraction 24 was found to have the inhibitory effect towards veratridine-induced membrane potential increases. Fraction 24 reduced 87% of the veratridine-induced response (measured as Area Under Curve) compared to positive-control cells (Figure 2.3 B).
Figure 2.3 Biological activity of *H. hainanum* fractions on hNaV1.6 channels

A) The HPLC absorbance and the HPLC solvent B concentration over time were plotted on the same graph. The peak that eluted at minute 22 was associated with potent inhibitory activity in hNaV1.6-expressing HEK293 cells. The peak eluted at 13% B concentration on the HPLC column. The blue line indicates HPLC absorbance values, measured in milli Arbitrary Units (mAU) at the left Y axis. The red dotted line indicates the concentration of Solvent B up to a maximum of 100% at the right Y axis.

B) Sample trace of the inhibitory effect of *H. hainanum* venom Fraction 24 when applied to hNaV1.6-expressing HEK293 cells. Fraction 24 reduced veratradine-induced activation by 87% compared to that observed in control wells. Reconstituted venom fraction or control solution were used as the first addition, and veratradine (100 µM) was used as the second addition. All percentages were calculated as
Area Under Curve values compared to responses by positive-control wells. Control solution (1:1000 BSA dissolved in PSS) was added as the first addition and veratridine (100 µM) was added as the second addition in positive-control cells. All traces were normalised to baseline fluorescence readings before the second addition. The X axis indicates time (in seconds) after the first addition.
2.4.3 Fraction 24 of *H. hainanum* crude venom was dominated by a monoisotopic oxidised mass of 3604.062 Daltons

Matrix-assisted laser desorption/ionisation (MALDI) was conducted on *H. hainanum* venom Fraction 24. Fraction 24 was found to contain a dominant peak with a monoisotopic mass of 3605.062 Da, which fell into the range of potential spider-venom derived polypeptide mass. Due to the ionisation process involved in the MALDI procedure that results in charged peptides, the monoisotopic oxidised mass of the peptide would be one Dalton lower than that observed in MALDI, making the actual monoisotopic oxidised mass of the peptide 3604.062 Da.
Figure 2.4 Fraction 24 from *H. hainanum* crude venom fractionation was dominated by a peak with an observed mass of 3605.062 Da, corresponding to a monoisotopic oxidised mass of 3604.062 Da, likely associated with the known TTX-s Na\textsubscript{V} inhibitor Hainantoxin-I.
MALDI examination of Fraction 24 from the *H. hainanum* venom fractionation. A) MALDI analysis of Fraction 24 from the *H. hainanum* venom fractionation showed the fraction contains a dominant peak of 3605.062 Da. The left Y axis indicates the percentage of compounds present in the sample, normalised to the maximum level of the compound that is most abundant in the sample. The X axis was measured as mass (Da) divided by electrical charge (z). B) The ionisation process during MALDI adds one Dalton to the original mass of the peptide, resulting in an increase of one Dalton in the observed mass, corresponding to a monoisotopic oxidised mass of 3604.062 Da for the peptide. The left Y axis indicates the percentage of compounds present in the sample, normalised to the maximum level of the compound that is most abundant in the sample. The X axis was measured as mass (Da) divided by electrical charge (z).
Mass comparison with other known venom peptides was conducted using the ArachnoServer Database\textsuperscript{217}. The observed monoisotopic oxidised mass in *H. hainanaum* (3604.062 Daltons) was found to be only 1.6 Dalton lower than the monoisotopic oxidised mass of Hainantoxin-I (3605.620 Daltons), a toxin with known inhibition of TTX-s Na\textsubscript{V} channels\textsuperscript{218} already characterised from the venom of *H. hainanum*. Due to the likelihood of having purified this known peptide from *H. hainanum* venom, I decided to investigate the activity of crude venoms in the hNa\textsubscript{V}1.8 isoform as well and explore for potential Na\textsubscript{V}1.8 modulators.

\textbf{2.4.4 Screening of crude spider and scorpion venoms on CHO cells expressing hNa\textsubscript{V}1.8 channels}

The same species of scorpion and tarantula spider crude venoms previously covered in Section 2.4.2 were tested in cell-based high-throughput assays via the FLIPR\textsuperscript{TETRA} system, using CHO cells expressing hNa\textsubscript{V}1.8 and the β3 subunit. Deltamethrin (150 µM) was used as the second addition.

*H. gentili* was the only scorpion venom that exhibited agonistic activity when applied to hNa\textsubscript{V}1.8-expressing CHO cells (Figure 2.5 A-F). The five other scorpion crude venoms tested exhibited a reduction in membrane potential when added into the cells, in contrast to the agonistic activity observed towards hNa\textsubscript{V}1.6. Potentiation of deltamethrin-induced membrane potential elevation was also absent for all scorpion venoms (Figure 2.5 G), which indicate a distinct difference between the actions of the tested scorpion venoms on hNa\textsubscript{V}1.6 and hNa\textsubscript{V}1.8.

None of the 18 spider crude venoms tested exhibited excitatory properties towards hNa\textsubscript{V}1.8, and all exhibited inhibitory activity (Figure 2.5 G, for traces from all venoms see Appendix 3.2). *Poecilotheria metallica* crude venom was found to exhibit the most potent inhibitory activity towards hNa\textsubscript{V}1.8-expressing CHO cells, with 97% inhibition of deltamethrin-induced membrane potential elevation compared to control wells (Figure 2.5 G, grey bar; Figure 2.6 A). Two other species that exhibited potent inhibitory activity comparable to *P. metallica* was *Poecilotheria ornata* and *Poecilotheria regalis*. Cells affected by *P. ornata* and *P. regalis* venoms reduced membrane potential elevation by 97% and 94% respectively (Figure 2.6 B-C). *P. metallica* crude venom exhibited the most potent inhibitory activity (equal potency to *P. ornata*), however it was evident that *P. ornata* did not completely inhibit deltamethrin-
induced response and a small peak was still observed following deltamethrin addition (Figure 2.6 B). Venom from *P. metallica* was therefore chosen as a source of potential hNav1.8 inhibitory peptides for further investigation.
Figure 2.5 Effect of scorpion and spider venoms in hNa\textsubscript{V}1.8-expressing CHO cells

None of the samples tested exhibited potentiation of hNa\textsubscript{V}1.8 responses. A-F) In contrast to the excitatory effect of scorpion crude venoms observed in hNa\textsubscript{V}1.6 channels, the scorpion venom samples did not exhibit excitatory effects towards the hNa\textsubscript{V}1.8 isoform. Only the venom from \textit{H. gentili} exhibited agonistic activity when added to the cells. All other scorpion venom samples exhibited a reduction in membrane potential when added. Traces were normalised to baseline fluorescence readings before the first addition. The X axis indicates time (in seconds) after the first addition. G) Effect of crude venoms on deltamethrin-induced Na\textsubscript{V}1.8 responses. All venom samples tested exhibited inhibitory activity towards deltamethrin-induced membrane potential elevation of the hNa\textsubscript{V}1.8 channel. The samples with the highest inhibitory activity were venoms from \textit{Poecilotheria metallica}, \textit{Poecilotheria ornata}, and \textit{Poecilotheria regalis}. \textit{P. metallica} venom value was coloured grey in panel G and was chosen for further studies. Traces were normalised to baseline fluorescence readings before the second addition. All responses were normalised to Area Under Curve values in
positive-control wells, with 1:1000 BSA dissolved in PSS as the first addition and
deltamethrin (150 µM) as the second addition. The dotted line at 100% indicates the positive-
control response.
A

Response over baseline

Time (seconds)

Buffer
P. metallica venom

B

Response over baseline

Time (seconds)

Buffer
P. ornata venom
Figure 2.6 Venoms from *Poecilotheria metallica*, *Poecilotheria ornata*, and *Poecilotheria regalis* exhibited the highest inhibitory effects in CHO cells expressing hNa\(_{\text{V}1.8}\) channels. Crude venom or the control solution was added into the cells as the first addition. Deltamethrin, a known Na\(_{\text{V}1.8}\) activator, was added as the second addition. A) Crude *P. metallica* venom reduced deltamethrin-induced membrane potential increase in hNa\(_{\text{V}1.8}\)-expressing CHO cells by 97%. B) Crude *P. ornata* venom reduced deltamethrin-induced membrane potential increase in hNa\(_{\text{V}1.8}\)-expressing CHO cells by 97%. C) Crude *P. regalis* venom reduced deltamethrin-induced membrane potential increase in hNa\(_{\text{V}1.8}\)-expressing CHO cells by 94%. All percentages were calculated as Area Under Curve values compared to responses by positive-control wells. Control solution (1:1000 BSA dissolved in PSS) was added as the first addition and deltamethrin (150 µM) was added as the second addition in positive-control cells. All traces were normalised to baseline fluorescence readings before the second addition. The X axis indicates time (in seconds) after the first addition.
2.4.5 P. metallica venom fractionation and the biological activity of fractions

Crude *P. metallica* venom (1 mg) was fractionated using a C$_{18}$ HPLC column, and the biological activity of the resultant fractions were tested on hNa$_{v}$1.8-expressing CHO cells. An equivalent of 50 µg of crude venom were used in cell experiments. Fraction 40 was observed to exhibit inhibition of deltamethrin-induced hNa$_{v}$1.8 activation. This fraction elutes at 35% Solvent B concentration and is associated with a peak in the chromatogram (Figure 2.7 A). Cells incubated with Fraction 40 exhibited a reduction in response, inhibiting deltamethrin-induced activation by 68% (measured as Area Under Curve) compared to positive-control cells (Figure 2.7 B).
Figure 2.7 Biological activity of *P. metallica* fractions

Inhibitory activity of deltamethrin-induced hNa\(_V\)1.8 activation was found in Fraction 40 of the *P. metallica* venom. A) The HPLC absorbance and the HPLC Solvent B concentration were plotted with each other. The most potent inhibitory activity towards deltamethrin-induced activation in hNa\(_V\)1.8-expressing CHO cells was found in Fraction 40, which was associated with a peak in the chromatogram as indicated by the black arrow. This fraction eluted at 35% Solvent B concentration. The blue line indicates HPLC absorbance values, measured in milli Arbitrary Units (mAU) at the left Y axis. The red dotted line indicates the concentration of Solvent B up to a maximum of 100% at the right Y axis. B) Sample trace of the inhibitory effect of Fraction 40 from *P. metallica* venom when applied to hNa\(_V\)1.8-expressing CHO cells in membrane potential assays. The fraction inhibited deltamethrin-
induced activation by 68% compared to membrane potential changes observed in control wells. Reconstituted venom fraction or control solution was used as the first addition, and deltamethrin was used as the second addition. All percentages were calculated as Area Under Curve values compared to responses by positive-control wells. Control solution (1:1000 BSA dissolved in PSS) was added as the first addition and deltamethrin (150 µM) was added as the second addition in positive-control cells. All traces were normalised to baseline fluorescence readings before the second addition. The X axis indicates time (in seconds) after the first addition.
2.4.6 Fraction 40 of *P. metallica* venom is dominated by a single mass of 3910.171 Da

Fraction 40 was analysed for the presence of peptide masses using MALDI. Fraction 40 presented with a dominant peak with a mass of 3911.171 Da (Figure 2.8 A and B). A smaller peak with a mass of 1955.992 Dalton was also observed in the sample (Figure 2.8 C). As the MALDI assay detects the mass of the peptide as mass divided by electric charge, the 1955.992 Dalton peak would be a double-charged variant of the dominant peak. Indeed, the mass of the smaller peaks within the single-charged peak with 3911.171 Da increased stepwise by one Dalton per peak, due to the increase in the number of deuterium atoms by one per peak. In contrast, the mass of the smaller peaks within the peak with 1955.992 Da increased stepwise by 0.5 Dalton per peak, consistent with a doubly charged ion. The monoisotopic oxidised mass of the peptide observed is therefore 3910.171 Da.
Figure 2.8 Fraction 40 was dominated by a peak with a monoisotopic oxidised mass of 3910.171 Da

MALDI analysis of Fraction 40 from *P. metallica* was conducted. A) MALDI analysis of Fraction 40 was dominated by a peak of 3911.171 Da. A smaller peak with half the intensity of the dominant peak, and with a mass that is half of the mass seen for the dominant peak, was also found in the sample. The left Y axis indicates the percentage of compounds present in the sample, normalised to the maximum level of the compound that is most abundant in the sample. The X axis was measured as mass (Da) divided by electrical charge (z). B) Increased zoom on the peak at 3911.171 Da show stepwise increase of mass by one Dalton per peak for the smaller peaks. The ionisation process of MALDI adds one Dalton to the original mass of the peptide, indicating the true monoisotopic oxidised mass of the peptide to be 3910.171 Da. The left Y axis indicates the percentage of compounds present in the sample, normalised to the maximum level of the compound that is most abundant in the sample. The X axis was measured as mass (Da) divided by electrical charge (z). C) Increased zoom on the peak at 1955.992 Da show stepwise increase of mass by 0.5 Dalton per peak for the smaller peaks, indicating that while the number of deuterium increases by one per peak, the peptide has a charge of two and is a double-charged variant of the dominant mass. The left Y axis indicates the percentage of compounds present in the sample, normalised to the maximum level of the compound that is most abundant in the sample. The X axis was measured as mass (Da) divided by electrical charge (z).
2.4.7 A novel peptide, Pme1a, was isolated in *P. metallica* venom

Fraction 40 from the *P. metallica* venom was subjected to Edman sequencing to elucidate the sequence of the peptide present in the fraction. A novel peptide, named Pme1a, was found to be present in the fraction.

The complete sequence for the peptide was finalised using tandem mass spectrometry (MS/MS). The novel peptide was successfully recovered in sufficient amounts following MS/MS. It was isolated with high purity (>90%) and was named Pme1a. The mass of Pme1a is determined to be 3910.268 Da, confirming that the mass observed in the Fraction 40 in the MALDI analyses was indeed that of Pme1a.

Sequence comparison was conducted using the ArachnoServer Database and the sequence of Pme1a was found to be related to the vanillotoxin family (Figure 2.9 A). The vanillotoxin with the highest similarity to Pme1a is vanillotoxin 2 (80% similarity), followed by vanillotoxin 1 (72% similarity). Both vanillotoxins have known agonistic activity towards TRPV1 channels, and produce inflammatory pain when injected into wild type mice. The sequence of Pme1a is also similar to jingzhaotoxins, a family of tarantula spiders peptides with known inhibitory activity towards Nav and Kv channels. However, the jingzhaotoxins found to be similar to Pme1a were not characterised pharmacologically and their functions and molecular targets remain unknown (72% similarity with jingzhaotoxin-37; 66% similarity with jingzhaotoxin-36; 66% similarity with jingzhaotoxin-29). The isolated peptide was tested using MALDI to check for peptide mass, and showed the same single-charged and double-charged peaks that were observed in Fraction 40 (Figure 2.9 B). Increased zoom into the MALDI data indicate the mass of the peptide to be 3910.298 (Figure 2.9 C) and confirms the purified peptide to be that of the mass found in Fraction 40.
A

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<td>Pmela</td>
<td>GCCXYPFLGCSTHSSCUGCRCGLNYCAXSTF</td>
<td>Na_{1}.8</td>
</tr>
<tr>
<td>Vanillotoxin2</td>
<td>GACGFLGCSTHSSCUGCRCGLNYCAXSTF</td>
<td>TRPV1</td>
</tr>
<tr>
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<td>SCGSPMGGGSTLCCGSCMGLYYCAXSTF</td>
<td>TRPV1</td>
</tr>
<tr>
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<td>SKMGCGSKHECCAGLCKTFNYCAXGSF-</td>
<td>Unknown</td>
</tr>
<tr>
<td>Jingzhaoxin-36</td>
<td>SKMGCGSKHECCAGLCKTFNYCAXGSF-</td>
<td>Unknown</td>
</tr>
<tr>
<td>Jingzhaoxin-29</td>
<td>SKMGCGSKHECCAGLCKTFNYCAXGSF-</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

B

![Mass spectrum](image)

C

![Mass spectrum](image)
**Figure 2.9 Isolation and sequencing of Pme1a**

One novel peptide was isolated from Fraction 40 of the *P. metallica* venom. The peptide was named Pme1a. A) The structural similarity of Pme1a to known peptides was compared using BLAST analysis. Pme1a was found to be most similar to vanillotoxin 2 and vanillotoxin 1, two peptides with known TRPV1 agonistic activity, followed by three uncharacterised venoms from the jingzhao toxin family. Cysteine residues are aligned within red boxes. Hydrophobic amino acid residues are highlighted in yellow. Positively charged amino acid residues are highlighted in fuchsia. Negatively charged amino acid residues are highlighted in green. B) MALDI analysis of the purified and sequenced peptide. The sample exhibited the same peaks as that observed in Fraction 40, with a main peak exhibiting 3911.298 Da and a double-charged peak with 1957.113 Da. The left Y axis indicates the percentage of compounds present in the sample, normalised to the maximum level of the compound that is most abundant in the sample. The X axis was measured as mass (Da) divided by electrical charge (z). C) Increased zoom on the main peak show the monoisotopic mass of the peptide was 3911.298. The ionisation process of the MALDI method adds one Dalton to the original mass of the peptide, indicating the true monoisotopic oxidised mass of the peptide to be 3910.298 Da. This is identical to the mass found in Fraction 40. The left Y axis indicates the percentage of compounds present in the sample, normalised to the maximum level of the compound that is most abundant in the sample. The X axis was measured as mass (Da) divided by electrical charge (z).
2.4.8 Pme1a was found to inhibit NaV1.8 with an IC50 of 3 µM

The concentration-response curve between the concentration of purified (> 90%) Pme1a added to hNaV1.8-expressing CHO cells and the membrane potential elevation response was established to determine the potency of Pme1a. Cells were incubated with various concentrations of Pme1a, ranging from 10 µM to 100 nM (Figure 2.10 A). Pme1a effectively reduced membrane potential elevation elicited by deltamethrin (150 µM) throughout the range of concentrations tested. The IC50 of Pme1a for NaV1.8 was determined to be 3 µM.

Representative traces of membrane potential assays following incubation with various concentrations of Pme1a indicate that the peptide reduced the membrane potential elevations elicited by deltamethrin in a concentration-dependent manner (Figure 2.10 B). The venom did not delay the initiation of deltamethrin-induced membrane potential elevations, suggesting Pme1a may not affect hNaV1.8 activation kinetics (Figure 2.10 B).
Figure 2.10 Purified Pme1a was found to inhibit \( \text{Na}_V\)1.8 with an IC\(_{50}\) of 3 µM

Purified Pme1a inhibited deltamethrin-induced membrane potential elevations in h\( \text{Na}_V\)1.8-expressing CHO cells in a concentration-dependent manner, with an IC\(_{50}\) of 3 µM. A) Purified Pme1a was tested in h\( \text{Na}_V\)1.8-expressing CHO cells at concentrations between 10 µM and 100 nM, and exhibited inhibitory activity throughout the range tested. The IC\(_{50}\) for Na\( \text{V}_1.8\) was determined to be 3 µM. Data was normalised to the average of maximum Area.
Under Curve values from positive control wells. All points were from an average of at least 3 individual experiments. Error bars were represented as ± SEM. B) Representative traces of deltamethrin-induced membrane potential changes following incubation with various concentrations of Pme1a. Increased Pme1a concentration is linked to a concentration-dependent reduction of the Area Under Curve value elicited by deltamethrin. Increased Pme1a concentration did not delay the time taken to initiate deltamethrin-induced membrane potential responses of hNaV1.8-expressing CHO cells. Each trace was representative of at least 9 wells over at least 3 individual experiments. Reconstituted peptide or control solution was used as the first addition, and deltamethrin was used as the second addition. Control solution (1:1000 BSA dissolved in PSS) was added as the first addition and deltamethrin (150 µM) was added as the second addition in positive-control cells. All traces were normalised to baseline fluorescence readings before the second addition. The X axis indicates time (in seconds) after the first addition.
2.4.9 Pme1a exhibits agonistic activity towards TRPV1 and induces nociceptive behaviours in mild-type mice

Pme1a was tested on TRPV1-expressing HEK293 cells following the discovery that Pme1a shares sequence similarities with vanillotoxins, a group of peptides with known agonistic activity towards the TRPV1 channel (Figure 2.11 A). Consistent with the structural similarities observed between Pme1a and vanillotoxins, Pme1a was found to exhibit TRPV1 agonist activity at 30 µM. While Pme1a possesses low potency as a TRPV1 agonist, it is not a selective Na\textsubscript{v}1.8 inhibitor and exhibits off-target activity towards TRPV1.

As the activation of TRPV1 by vanillotoxin is associated with spontaneous pain\textsuperscript{219}, the physiological effects of Pme1a was therefore suspected to be nociceptive rather than antinociceptive, contrary to what may be expected for a selective Na\textsubscript{v}1.8 inhibitor. Pme1a was injected locally into the hind paws of wild type C57BL/6 mice at the IC\textsubscript{50} concentration (3 µM) observed in membrane potential assays. Mice injected with Pme1a exhibited significantly more spontaneous nociceptive behaviours such as licking and lifting of the injected hind paw, and the effect of Pme1a peripheral administration was nociceptive instead of analgesic (Figure 2.11 B).
Figure 2.11 Pme1a has TRPV1 agonist activity and elicits nociceptive behaviours in mice

Pme1a was tested in TRPV1-expressing HEK293 cells and in wild-type animals. A) Pme1a was tested in TRPV1-expressing HEK293 cells and elicited calcium influx responses at 30 µM. The known TRPV1 agonist, capsaicin, was used as the positive control. All data points were normalised to baseline Area Under Curve values in negative control wells. All data points were the mean values from at least 3 experiments. Error bars are shown as ± SEM. B) Peripheral injection of Pme1a into the hind paws of mice elicited significantly more nocifensive spontaneous responses, such as flicking and licking of the injected paw, compared to control animals injected with the control solution. Each animal was observed for
10 minutes following the injection of Pme1a or control solution, and all nocifensive responses during the observation period was recorded. Pme1a was freeze-dried and reconstituted in PSS with 1:1000 BSA. Control solution injected into the control cohort consisted of PSS with 1:1000 BSA. All animal groups were allocated with n = 3. * $P$-value < 0.05. Error bars are shown as ± SEM.
2.5 Discussion

This chapter describes activity-guided isolation of a novel peptide from the crude venom of the tarantula spider species *Poecilotheria metallica*. The novel peptide, named Pme1a, exhibits hNa\(_{\text{V}}\)1.8-inhibitory activity and activates TRPV1. Pme1a is a 35-amino-acid long peptide with 6 cysteine residues, and is the first peptide to be isolated from the venom of *Poecilotheria metallica*. Compared to A-803467, the small molecule currently used experimentally to inhibit Na\(_{\text{V}}\)1.8, Pme1a exhibits weak potency as a Na\(_{\text{V}}\)1.8 inhibitor (IC\(_{50}\) = 3 µM). Surprisingly, instead of exhibiting structural similarities with other Na\(_{\text{V}}\)-inhibitory spider peptides, the structure of Pme1a was related closely to vanillotoxins, a class of spider peptides associated with TRPV1 channel opening, leading to my examination of Pme1a in TRPV1 channels.

Vanillotoxins, a class of toxins isolated from the tarantula *Psalmopoeus cambridgei*, was discovered via screening of spider and scorpion toxins for agonistic activity towards the capsaicin-sensitive TRPV1 channel\(^{219}\). Three vanillotoxins (VaTx) with varying potencies as TRPV1 agonists were discovered within the crude venom of *P. cambridgei*. Out of the three vanillotoxins, VaTx3 was the most potent TRPV1 activator (EC\(_{50}\) = 0.45 ± 0.04 µM), followed by VaTx2 (EC\(_{50}\) = 1.35 ± 0.15 µM) and VaTx1 (EC\(_{50}\) = 9.9 ± 0.97 µM). All vanillotoxins belong to the inhibitory cysteine knot (ICK) family of peptides, which includes spider toxins as well as scorpion, cone snail, and fungal toxins\(^{222}\). Many ICK proteins exhibit some form of protein inhibition, such as plant protease inhibitors and various cone snail and spider peptides\(^{222-224}\). On a three-dimensional scale, the ICK motif consists of three anti-parallel β-sheets held together via a highly stable cysteine knot\(^{222}\), which is constructed with two disulphide bonds forming a ring and connected vertically by a third disulphide bond\(^ {223}\). The ICK motif was first described due to the discovery of this three-dimensional cysteine structure in growth factors\(^{225}\). Its presence in spider and scorpion venoms have now become a classification distinct from the cysteine knots in animal, plant, or fungal proteins\(^{222}\). ICK toxins are now recognised as common inhibitors and activators of cationic channels, and are found in many spider and scorpion venom peptides.

The two-dimensional structure of peptides that exhibit the ICK motif also contain properties that apply to vanillotoxins as well as Pme1a. A typical spider toxin with the ICK motif contains 30 to 40 amino acids and at least 6 cysteine residues\(^{224}\). The two-dimensional
sequence for such toxins are observed to follow a pattern with various numbers of other amino acids interspersed between the six cysteine residues, arranged in the following configuration: \(CX(3\sim7)CX(3\sim8)CX(0\sim7)CX(1\sim4)CX(4\sim13)C\). Both the vanillotoxins and Pme1a fit into this molecular arrangement. In particular, vanillotoxins exhibit a hyper-variable \(\beta\)-loop protruding out of the cysteine loop, which is observed two-dimensionally as the six amino acid residues between C5 and C6\(^{219}\). Pme1a, VaTx1, and VaTx2 are identical at five of the six residues between C5 and C6 (Figure 2.10 A)\(^{219}\). However, VaTx3, the vanillotoxin that is most potent as a TRPV1 channel agonist, exhibited significant deviations from the amino acids present in this section compared to the other two vanillotoxins\(^{219}\). This section is considered to be crucial in the effect of vanillotoxins on TRPV1\(^{219}\), which could explain the differences in potency between VaTx3 and the other two vanillotoxins, rendering VaTx1 and VaTx2 less potent TRPV1 agonists. The similarities observed in this section between Pme1a and the vanillotoxins could be the reason behind the off-target activity of Pme1a at TRPV1. It is however unknown whether vanillotoxins affect the Na\(_V\)1.8 channel, as the toxins have not been tested for activity in Na\(_V\) isoforms.

Injection of vanillotoxins into the hind paws of wild-type mice elicited nociceptive behaviours such as licking and flinching of the foot, much like that observed after injection of capsaicin, a known potent TRPV1 activator\(^{219}\). However, apart from agonistic activities at TRPV1 channels, vanillotoxins also exhibit inhibitory activities towards the K\(_V\)2.1 channel\(^{219}\), which is responsible for inhibitory repolarisations in sensory neurons. Interestingly, the inhibitory activity of vanillotoxin at K\(_V\)2.1 channels is inversely related to the potency of individual vanillotoxins as TRPV1 agonists. VaTx1, the least potent TRPV1 activator, was the most potent inhibitor of K\(_V\)2.1, with VaTx1 exhibiting similar EC\(_{50}\) values for TRPV1 activation (EC\(_{50}\) = 9.9 ± 0.97 µM) and K\(_V\)2.1 inhibition (IC\(_{50}\) = 7.4 ± 1.9 µM). VaTx2, the second-most potent TRPV1 activator, inhibited K\(_V\)2.1 at IC\(_{50}\) values seven-fold higher than its EC\(_{50}\) value for TRPV1. The most potent TRPV1 activator from the family, VaTx3, exhibited very little K\(_V\)2.1 inhibition even at concentrations 100-fold higher than its EC\(_{50}\) as a TRPV1 activator\(^{219}\). Pme1a exhibits high structural similarity with VaTx1 and VaTx2, but low similarities with VaTx3. Therefore, while it is possible that the nociceptive effects of Pme1a observed in animals is from TRPV1 activation, it is also plausible that Pme1a exhibits inhibitory activities in K\(_V\) channels, a dual function that may also apply to vanillotoxins. This possibility is further supported by the observation that Pme1a only activated TRPV1 in transfected cells at a concentration of 30 µM or higher, yet the injection
of 3 µM of Pme1a locally into mice elicited significant increase in nocifensive behaviours. Further examinations into the selectivity of Pme1a in other ion channel families would be needed before the peptide can be considered a potential pharmacological tool.

Pme1a also may display off-target activity in NaV channels other than NaV1.8. While vanillotoxins have not been examined for their efficacy in NaV channels, crude venom from P. metallica was observed to exhibit inhibitory effects when added to hNaV1.6-expressing HEK293 cells (Figure 2.1 G). It is therefore probable that Pme1a also exhibits inhibitory effects towards hNaV1.6. Future studies should be conducted to investigate the activity of Pme1a in other NaV isoforms, however time and material constrictions during the candidature did not permit the completion of such experiments.

The tarantula spider species, Haplochelma hainanum, produced the venom in which I found potent hNaV1.6 inhibition and is the source of a family of venom peptides, named hainantoxins. The spider species H. hainanum, firstly described as Selenocosmia hainana, yielded 5 characterised hainantoxins and more than 190 venom peptides sequences. All five characterised hainantoxins are known to inhibit select NaV isoforms, and exhibit the ICK motif seen in Pme1a and vanillotoxins. While all members of the hainantoxin family have been characterised on NaV1.1, NaV1.2, NaV1.4, NaV1.5, and NaV1.7 channels, there have been limited exploration to the effect of the toxins in other NaV isoforms and other ion channels families. Hainantoxin-I, initially described as an inhibitor of NaV channels, was discovered to be ineffective in CaV channels and activates calcium-activated potassium (KCA) channels. Meanwhile hainantoxin-II, a toxin known to cause paralysis and death when injected into cockroaches and mice, has not been pharmacologically characterised and its neuronal target remains unknown. Hainantoxin-V, the hainantoxin discovered most recently, has also not been characterised in detail, and is simply known to inhibit TTX-s NaV channels without altering the channels' activation kinetics.

More information is available for hainantoxin-III and hainantoxin-IV, the other two members of the family. However, a complete analysis of their effects in NaV channels, let alone in other ion channel families, has not been conducted. Hainantoxin-III is a potent TTX-s NaV inhibitor (IC50 = 1.1 nM) with no effect in TTX-r NaV channels. The toxin preferentially inhibits rat NaV1.7 and also inhibits NaV1.1, 1.2, and 1.3 to a smaller degree. However, its efficacy at NaV1.6 was not examined. Similarly, hainantoxin-IV was found to
inhibit TTX-s rat NaV isoforms and was shown to inhibit NaV1.1, 1.2, and 1.7, while having no effect over NaV1.4 and 1.5. The efficacy of hainantoxin-IV in other TTX-s rat NaV isoforms, such as NaV1.3 and NaV1.6, were not tested. The binding site and the binding surface of hainantoxins is also poorly defined, with some suggesting hainantoxin-I may bind to binding site 1 on NaV channels, while evidence based on kinetics changes following exposure to hainantoxin-IV indicate binding via other sites that regulate inactivation kinetics. More characterisation would need to be conducted regarding hainantoxins before the properties and molecular targets of this family can be defined.

Future studies concerning Pme1a would also need to be carried out before a full understanding of the novel peptide’s properties can be reached. It would be of interest to investigate the effect of Pme1a on other NaV isoforms, and also to examine whether it affects activation or inactivation kinetics of NaV1.8. Given the similarities between the structure of Pme1a and that of vanillotoxins, and the known activity of vanillotoxins at KV channels, it would also be insightful to explore the effect of Pme1a in KV channel isoforms to fully comprehend the physiological effects of this novel peptide.

2.6 Conclusions

Spider venoms offer a treasure of unexplored neurotoxins, many of which have the potential to become powerful tools in pharmacological studies as ion channel inhibitors. This chapter presents an exploration into spider venoms in search of potential NaV1.6 and NaV1.8 selective inhibitors, as the existing options are either not selective or do not corroborate well with knockout or gene mutation data. While a novel spider peptide, named Pme1a, was successfully isolated with promising inhibitory activity towards Na1.8, sequence homology with known activators of the TRPV1 channel indicated off-target activity. Injection of Pme1a into wild-type mice resulted in nocifensive rather than analgesic behaviour, highlighting the time-consuming and unpredictable nature of isolating novel peptides from crude venoms in search of NaV modulators. I therefore investigated other models that can be used to better study the role of NaV1.6 and NaV1.8 channels in sensory neurons, such as in vitro cell models that have been employed commonly as a substitute for native in vivo DRG sensory neurons. To better assess the appropriateness of these models as tools to investigate peripheral neurons expressing NaV isoforms, I conducted bioinformatics analysis of three common in vitro cell lines derived from DRG neurons, to be presented in the next chapter.
Chapter 3 - Neuronal cell lines as model dorsal root ganglion neurons: a transcriptomic comparison

3.1 Foreword

Specific channel modulators, discovered through activity-guided purification and isolation, are good tools to study the contribution of specific ion channels in the pain mechanism of various conditions, and may lead towards the development of new analgesics. However, Chapter 2 described the unexpected difficulty of that approach due to off-target activity. One of the alternative tools to investigate the roles of $\text{Na}_V1.6$ and $\text{Na}_V1.8$ in nociceptive neurons and to screen for selective modulators would be to use \textit{in vitro} DRG neuron-derived cell models, which have been used for research in lieu of native DRG neurons. A remaining challenge is that the gene expression of the cell lines have not been thoroughly classified and have not been compared to known subtypes of \textit{in vivo} DRG neurons. I therefore examined the expression profile of three common \textit{in vitro} cell lines, that of SH-SY5Y, F-11, and ND7/23 cells. A bioinformatics analysis was conducted, as well as comparison between the transcriptome of these cell lines with native sensory neuron subtypes, particularly regarding markers known to be expressed in specific neuronal subtypes. I set out to explore how appropriate these cell lines would be as tools to study the function of various sensory neuron subtypes, and whether they could be used to profile and discover novel modulators of $\text{Na}_V1.6$ and $\text{Na}_V1.8$.

Data presented in this chapter have been published in Molecular Pain\textsuperscript{234}. Compared to the publication, the contents have been modified and adopted to fit the flow and format of the thesis.

I would like to thank Dr. Gregory J Baillie from the Institute for Molecular Bioscience Sequencing Facility for his assistance with bioinformatics sequencing and alignment.

3.2 Introduction

As outlined in Chapter 1, Section 1.2.1, utilising bioinformatics methods to associate specific DRG neuron functional subclasses with unique cellular markers and expression profiles is an
increasingly common research method. It is generally accepted that many specific proteins do indeed denote specific functional neuron subclasses, with examples including the transient receptor potential vanilloid 1 channels (TRPV1) serving as an indication of capsaicin-and-heat-sensitive DRG neurons, or the CaV isoform CaV3.3 working as a selective marker for C-low-threshold mechanosensitive receptors (C-LTMR). Further studies have divided neurons present in whole DRGs into as many as 17 different subclasses based on combined expression and electrophysiological data, illustrating the molecular differences between neurons with varied sensitivity to different stimuli, and the need to separate desired DRG subclasses when investigating the effect of compounds in peripheral neurons.

However, while primary DRG neurons are used extensively to study the pharmacological mechanisms involved in sensory neuron functions such as pain and itch, this approach has several drawbacks. The isolation and culture of DRG neurons is time-consuming, highly technical, and requires the use of animals. Importantly, not all neurons survive this process. Removal of DRG neurons from the in vivo environment and axotomy of the peripheral processes also lead to adaptive changes such as down-regulation of the potassium channel subunit Kv9.1, over-expression of the nerve growth factor receptor tropomyosin receptor kinase A (TrkA), and increased neuronal excitability. In addition, as DRG neurons are heterogeneous, only a subset of cells is likely to express the target of interest, and a substantial proportion of cultured cells include non-neuronal cell types such as fibroblasts and glial cells.

Accordingly, researchers have turned to DRG-derived immortal cell lines as substitutes for intact DRG neurons when investigating endogenous receptors. Common candidates are the DRG-hybridoma cell lines F-11 and ND7/23, which were derived from embryonic or neonatal rat DRG neurons by fusion with the mouse neuroblastoma cell line N18Tg2 and display neuron-like properties including excitable membranes and sensory neuronal cell surface markers. Similarly, it was shown that the neuroblastoma cell line SH-SY5Y, which is commonly used as a model of adrenergic or dopaminergic neurons, exhibits traits of peripheral sensory neurons such as functional expression of the sensory neuron-specific sodium channel isoform NaV1.7. However, although these cell lines are often considered suitable models to study mechanisms of nociceptor activation, and individual investigations of specific protein families have been conducted, a systematic analysis of the expression profile of these cell lines in comparison with DRG neurons is lacking.
Therefore, RNA-Seq analysis with particular emphasis on ion channel and G protein-coupled receptor (GPCR) expression in DRG neuron-derived cell lines including the human neuroblastoma cells SH-SY5Y\textsuperscript{246, 247}, the mouse neuroblastoma/rat embryonic DRG neuron hybrid cell line F-11\textsuperscript{242}, and the mouse neuroblastoma/rat neonatal DRG neuron hybrid cell line ND7/23\textsuperscript{241}, was conducted and presented in this chapter. Data from cell lines were compared to RNA-Seq analysis conducted on whole murine DRGs. Functional expression of select targets was confirmed by Ca\textsuperscript{2+} imaging.

3.3 Materials and Methods

3.3.1. Materials

All materials were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia) unless otherwise stated. Oxytocin and vasopressin were a kind gift from Professor Paul Alewood (Institute for Molecular Bioscience, University of Queensland). Capsaicin stock solution was prepared in ethanol and stored at -80 °C. Maximum final ethanol concentration from capsaicin stock did not exceed 0.001%. Menthol, AITC (allyl isothiocyanate), vasopressin, and oxytoxin stock solutions were prepared in DPBS (Dulbecco’s Phosphate Buffered Saline, Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.5) and stored at -4 °C. Calcium 4 No-Wash dye stock solution was reconstituted using 10 mL of PSS (for PSS composition see Appendix 2.1) per vial of powder, and stored at -20 °C for up to 3 months.

3.3.2 Animals

Male wild-type C57BL/6 mice age 6-8 weeks and weighing 20 - 23 grams were used in the study. Ethical approval for experiments involving animal tissues was obtained from the University of Queensland animal ethics committee under the ethics approval number TRI/IMB/091/14/NHMRC. Experiments were conducted in accordance with the Animal Care and Protection Regulation Queensland (2012), the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8\textsuperscript{th} edition (2013) and the International Association for the Study of Pain Guidelines for the Use of Animals in Research.
All animals were housed in groups of 2–4 per cage in a stable environment under 12 hour light-dark cycles and had access to standard rodent chow and water *ad libitum*. A red polycarbonate Mouse House (Tecniplast, Italy) and shredded paper nesting material were supplied for enrichment. All animals were obtained from University of Queensland Biological Resources (Brisbane, Australia).

### 3.3.3 Cell culture

Human neuroblastoma SH-SY5Y cells (The European Collection of Cell Cultures) were grown in RPMI (Roswell Park Memorial Institute medium, Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.8) with 15% FBS and 2 mM L-glutamine.

Mouse neuroblastoma/rat embryonic DRG neuron hybrid F-11 cells (The European Collection of Cell Cultures) were grown in Ham’s F12 media (Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.3) with 10% FBS and 2% hypoxanthine-aminopterin-thymidine (HAT) media supplement (50x) Hybri-Max™ (for composition see Appendix 2.9).

Mouse neuroblastoma/rat DRG neuron hybrid ND7/23 cells (The European Collection of Cell Cultures) were grown in DMEM (Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.4) with 10% FBS, 2 mM L-glutamine, pyridoxine, and 110 mg/ml sodium pyruvate.

All three cell lines were incubated at 37°C and in 95% O₂/5% CO₂ air mixture. SH-SY5Y and ND7/23 cells were passaged every 3–5 days at a 1:5 dilution ratio or when approximately 90% confluent. F-12 cells were passaged every 2–3 days at a 1:5 dilution ratio or when approximately 80% confluent. All cells were seeded in T75 cm² Corning® (Corning, MA, USA) cell culture flasks. In order to passage cells, the cells were briefly removed from incubation and maintained in room temperature and growth media was removed from the flask. At least 5 mL/75 cm² of DPBS was added into the flask to briefly wash the cells. The DPBS was then removed from the flask. 0.25% trypsin/EDTA (Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.6) was then added at a concentration of 1 mL/75 cm², and the cells were dislodged by gently tapping the sides of the flask. The
dislodged cell suspension was diluted with a further 9 mL of the appropriate growth media (as mentioned above) and passaged according to aforementioned ratios in a new T75 cm² flask using new growth media.

3.3.4 DRG extraction and dissociation

Mice were sedated via inhalation of 50% O₂/50% CO₂ gas inhalation and euthanised via asphyxiation using 100% CO₂, followed by cervical dislocation. The spine was immediately removed using surgical equipment, the dorsal side of the vertebra bones removed, and the tissue immersed in ice-cold DMEM. The spinal cord was then removed, and thoracic and lumbar DRGs (T6-L6 inclusive) were dissected with forceps under a dissection microscope and placed in fresh DMEM within a 35mm-diameter petri dish (Nunclon™ Delta treated, Nunc, Denmark), followed by trimming of excess myelin. Dissected DRGs were then placed into fresh DMEM with 1 mg/mL collagenase and 0.5 mg/mL protease, and incubated at 37°C with 95% O₂/5% CO₂ for 45 minutes within a 15 mL conical centrifuge tube (Falcon™, Corning, MA, USA) to allow for dissociation. Following incubation, the cell suspension was centrifuged at 20,000 rpm (revolutions per minute) for 3 minutes, and excess DMEM was removed. The cell pellet was then washed in approximately 2 mL of DPBS, centrifuged briefly, and excess DPBS was removed. Cell samples derived from the whole DRGs of 2 mice were pooled together for RNA extraction.

3.3.5 Cell dissociation and preparation

Cells were brought up from frozen stocks and allowed to recover for at least 3-5 passages. RNA was extracted from SH-SY5Y cells at passage 29, from F-11 cells at passage 9, and from ND7/23 cells at passage 28. All cell lines were allowed to reach ~90% confluence in a T75 cm² flask before harvesting. Cells were initially dissociated as described in Section 3.3.3 with DPBS and 0.25% trypsin/EDTA. The dislodged cell suspension was diluted with a further 9 mL of the appropriate growth media, removed from the flask, and centrifuged at 14,000 rpm for 5 minutes to form a pellet. Excess media was removed and the cell pellet was left intact for RNA extraction.
3.3.6 RNA extraction

RNA was extracted using the RNeasy Mini Kit (Qiagen, Melbourne, Australia) according to the manufacturer’s instructions, including on-column DNase digestion. Briefly, 600 µL of Buffer RLT (with 1% β-mercaptoethanol) were added to the cells, and the mixture was vortexed to disrupt the tissue and homogenise the lysate. The lysate was then centrifuged at full speed for 3 minutes and the supernatant removed, with an equivalent volume of 70% ethanol in Milli-Q water. Up to 700 µL of the mixture was centrifuged in a RNeasy spin column for 15 seconds at 8000 x g, and the flow-through was discarded. Samples in excess of 700 µL were centrifuged in the same RNeasy spin column until all of the lysate have been centrifuged. Buffer RW1 (350 µL; Qiagen, Melbourne, Australia) was then added to the RNeasy spin column and centrifuged for 15 seconds at 8000 x g, and the flow-through was discarded. DNase I stock solution (Qiagen, Melbourne, Australia; for composition see Appendix 2.10) was added (10 µL) to 70 µL Buffer RDD (Qiagen, Melbourne, Australia) in a separate PCR tube (Corning, MA, USA). The two solutions were mixed by inverting the tube and centrifuged briefly, and the entirety of the mixture was applied to the RNeasy spin column membrane and left to incubate in room temperature for 15 minutes. At the end of the incubation, a further 350 µL of Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 seconds at 8000 x g, and the flow-through was discarded. To wash the column, 500 µL of Buffer RPE (pre-diluted with 4 equivalent volumes of 100% ethanol; Qiagen, Melbourne, Australia) was added to the RNeasy spin column and centrifuged for 15 seconds at 8000 x g, and the flow-through was discarded. This was followed by a further wash step with the addition of 500 µL of Buffer RPE (pre-diluted with 4 equivalent volumes of 100% ethanol) to the RNeasy spin column and centrifuged for 2 minutes at 8000 x g, and the flow-through was discarded. The RNeasy spin column was then removed from the collection tube and placed into a new 2 mL collection tube. 50 µL of RNAse-free water (Qiagen, Melbourne, Australia) was added to the column, and the column was centrifuged for 1 minute at 8000 x g. The concentration of RNA in the final flow-through was determined using a NanoDrop Spectrophotometer (ND-1000, NanoDrop, DE, USA), with RNAse-free water as the negative control.
3.3.7 RNA-Seq and bioinformatics analysis

Library construction and bioinformatics analysis were conducted by AGRF (the Australian Genome Research Facility, Melbourne, Australia), which determined the RNA Integrity Number (RIN) was above 8.5 for all samples. RNA-Seq was conducted on an Illumina HiSeq-2000 machine as a 100-nucleotide single-end run, and primary .fastq data was generated with the CASAVA 1.8.2 pipeline (Illumina, CA, USA). Reads were screened for the presence of any adaptor or overrepresented sequences, ambiguous characters were clipped where required, and sequence bias was corrected. Alignment was performed with Tophat with default analysis parameters in single-end mode. The number of mismatches were restricted to no more than 2 bases. Reads from SH-SY5Y cells were mapped against the human genome (hg19), DRG reads were mapped against the mouse genome (mm9), and F-11 and ND7/23 reads were mapped twice, against both the mouse genome (mm9) and rat genome (rn5). Transcripts assembly was performed by Cufflinks with the RABT (reference annotation-based assembly) option using the UCSC (University of California, Santa Cruz) annotation, and multi-reads correction was used. Gene counts were then compiled using the HTSeq software.

3.3.8 Accession number and gene ontology (GO) analysis

Raw bioinformatics data in the form of .fastq files and count tables generated with HTSeq are both available at the Gene Expression Omnibus database under the accession number GSE75811. Gene ontology (GO) analysis was conducted with the PANTHER database with the Gene List Analysis tool. Genes with >100 reads in each sample were compiled and submitted to Gene List Analysis and analysed according to Biological Processes (BP) terms. Genes from whole DRGs were assigned according to the Mus musculus genome, the SH-SY5Y sample was assigned to the Homo sapiens genome, and F-11 and ND7/23 samples were assigned to both Mus musculus and Rattus norvegicus genomes each.

3.3.9 Transfection

HEK293 cells (American Type Culture Collection, VA, USA) were routinely maintained in DMEM containing 10% FBS and 2 mM L-glutamine. Cells were split every 3-6 days in a ratio of 1:5 using 0.25% trypsin/EDTA. Cells were seeded on T75 cm² (Nunc, Denmark) tissue culture flasks 24 hours prior to transfection and transfected with the plasmid DNA of
rat TRPV1 (D. Julius, Department of Physiology, University of California, Berkeley, CA, USA), mouse TRPA1 (A. Patapoutian, The Scripps Research Institute, La Jolla, CA, USA), rat TRPM8 (K. Zimmermann, Department of Anesthesiology, Friedrich-Alexander-University, Erlangen-Nuremberg, Erlangen, Germany), human AVPR1B (arginine vasopressin receptor 1B; OriGene Technologies, MD, USA), or human oxytocin receptor (OriGene Technologies, MD, USA) using Lipofectamine™ 2000 (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were seeded on 384-well black-walled imaging plates (Corning, MA, USA) and used for Ca²⁺ experiments 24 hours after plating.

3.3.10 Fluorescent Ca²⁺ assays

Cells were prepared as previously reported. Briefly, SH-SY5Y, F-11, and ND7/23 cells were seeded onto 384-well black-walled imaging plates (Corning, MA, USA) at a density of 30 000 - 50 000 cells per well 48 hour prior to calcium imaging experiments and incubated at 37°C with 95% O₂/5% CO₂. SH-SY5Y cells were placed onto uncoated plates (Corning, Cat No #3542), while F-11 and ND7/23 cells were placed onto CellBIND® (Corning, Cat no #3683) plates. Transfected HEK293 cells were plated 24 hours prior to calcium imaging assays as previously stated. Cells were seeded to ensure ~90% confluency on the day of the experiment.

Calcium 4 No-Wash dye (Corning, MA, USA) was diluted 1:10 in PSS from stock solution, and was loaded into cells 30 minutes prior to imaging. The plates with loaded dye were incubated at 37 °C with 95% O₂/5% CO₂. Ca²⁺ responses were measured using a FLIPRTETRA (Molecular Devices, CA, USA) fluorescent plate reader with excitation at 470-495 nM and emission at 515-575 nM. Camera gain and intensity were adjusted for each plate to yield a minimum of 1000-1500 arbitrary fluorescence units (AFU) baseline fluorescence. Only one addition was used. Prior to addition of agonists (capsaicin, menthol, AITC, vasopressin, or oxytocin), 10 baseline fluorescence readings were taken, followed by fluorescent readings every 0.5 seconds for 300 reads.
3.3.11 Data analysis

Unless stated otherwise, all data are presented as mean ± standard error of the mean (SEM). Statistical significance was determined using an unpaired, two-tailed Student’s \( t \)-test unless otherwise stated, with statistical significance defined as \( P \)-value < 0.05.

For heatmap generation, data was compiled into .csv format and heatmaps were created in RStudios with R version 3.2.2 with the gplots, pheatmap, and RColorBrewer libraries.

GO term charts were generated using the online PANTHER database.

Pie graphs were generated using default pie graph parameters within Microsoft Excel®.

\( \text{FLIPR}^{\text{TETRA}} \) \( \text{Ca}^{2+} \) response data was analysed using Screenworks 3.2 (Molecular Devices, CA, USA), and were plotted and its statistical parameters determined using GraphPad Prism™ (Version 6.00).

To establish dose-response curves, the maximum response in fluorescence over the baseline after the addition of the agonist was fitted to a 4-parameter Hill equation with variable slope using GraphPad Prism™ (Version 6.00).

Sample sizes of each experiment are detailed in the figure legends of the corresponding figure.

3.4 Results

3.4.1 TUBA1A was selected to normalise reads

As data normalisation relies upon housekeeping genes expressed in similar levels across all samples, I examined the expression of various housekeeping genes due to the recognised issue of differential expression levels for standard housekeeping genes \(^{252}\) and found TUBA1A to be one of the only candidates expressed at a similar global level (0.02-0.08% of all aligned reads) across all samples in my data. In contrast, other popular housekeeping genes commonly used in data normalisation, such as GAPDH and \( \beta \)-actin, were not expressed with similar intensity across all my samples. For instance, 64 reads aligned to GAPDH in the DRG
sample, while 12,475 reads aligned to GAPDH in the SH-SY5Y sample. I thus normalised all reads to the highly-expressed α-tubulin gene, with the intensities of gene expression presented as normalised gene ratios.

3.4.2 F-11 and ND7/23 cells were aligned against mouse and rat genomes

The two hybrid cell lines examined, F-11 and ND7/23, are known to contain both rat and mouse chromosomes (F-11)\textsuperscript{242} and to express rat surface markers after fusion with mouse neuroblastoma cells (ND7/23)\textsuperscript{241}. It was not clear which genes are transcribed from the rat genome and which are from the mouse genome in these cell lines. It was therefore decided to align F-11 and ND7/23 reads twice, once against the mouse genome and once against the rat. I found that qualitatively, expression profiles largely mirrored each other between mouse and rat alignments in these two cell lines, varying predominantly in intensity.
Figure 3.1 Neuronal markers and gene ontology analysis on RNA samples

The SH-SY5Y, F-11, and ND7/23 cell lines do not fit into existing categories of peripheral neurons, yet gene ontology analysis demonstrates the cell lines’ transcriptomes retain similar ratios of enriched biological processes compared to whole DRGs. A) Expression of markers commonly used to identify specific peripheral neuron sub-populations, microglia, and non-neuronal cells in SH-SY5Y, F-11, and ND7/23 cell lines. The majority of the genes exhibited very low expression levels across all samples. B) Gene ontology analysis of whole DRGs. C) Gene ontology analysis of SH-SY5Y cells. D) Gene ontology analysis of F-11 cells (aligned to the mouse genome). E) Gene ontology analysis of F-11 cells (aligned to the rat genome). F) Gene ontology analysis of ND7/23 cells (aligned to the mouse genome). G) Gene ontology analysis of ND7/23 cells (aligned to the rat genome).
3.4.3 Cell lines do not share expression profiles with known DRG neuron subclasses

Cell line expression data was initially analysed against a wide range of known markers for specific neuronal types (Figure 3.1 A). Some of these include the peptidergic nociceptor marker TrkA (NTRK1), the mechanosensor marker PIEZO2/FAM38B, the myelinated neuronal marker CNTNAP2 (contactin associated protein-like 2), and unmyelinated C-LTMR marker Cav3.2 (CACNA1H). Out of the 43 neuronal markers investigated (B2M and VIM are non-neuronal markers), transcripts of 9 markers were found in all cell lines. These 9 genes included the nociceptor markers Na\textsubscript{v}1.9 (SCN11A) and ASIC1, both of which are mentioned in Chapter 1; TLX3, a transcription factor that regulates the expression of several sensory channels and receptors implicated in sensing pain, itch and temperature; PLXNC1, a marker for unmyelinated nonpeptidergic nociceptors and thermosensors; RET, a marker for myelinated and unmyelinated neurons; the C-LTMR marker TH; RUNX3, a transcription factor that regulates the development of proprioceptive DRG neurons; the unmyelinated C-fibre marker peripherin (PRPH); and LDHB, a marker of myelinated neurons (Figure 3.1 A). However, the co-expression of some of these markers in individual cell lines was surprising given their expression profiles do not overlap significantly in subtypes of DRG neurons. Specifically, some markers are known to be selectively expressed in unmyelinated C-fibre neurons (such as PRPH and TH) while others are indicative of myelinated A-fibres (such as LDHB) and would thus not be expected to be present together in a single sensory neuron type. Many sensory neuron genes were also absent across all cell line samples, including nociceptive markers such as TRPV1, TRPA1, CGRP, and Na\textsubscript{v}1.8 (SCN10A); mechanoreceptor markers such as PIEZO2 and VGLUT3/SLC17A8; and markers for neurons responsible for itch such as par2 (F2RL1). This suggests that while the cell lines are known to express receptors found on DRG neurons, they do not mimic the entire repertoire of proteins expressed by functional in vivo DRG neuron classes.

3.4.4 SH-SY5Y cells express conflicting molecular markers for multiple DRG neuron subtypes

In SH-SY5Y cells, the most enriched marker was RET, the glial cell line-derived neurotrophic factor (GDNF) receptor tyrosine kinase, which was consistent with previous reports that these cells respond to GDNF with increased neurite outgrowth. SH-SY5Y cells expressed markers of A-fibres, including LDHB, CNTNAP2, and neurofilament heavy
polypeptide (NEFH). However, TrkA (NTRK1), a C-fibre marker, was also expressed, while the A-fibre marker TrkC (NTRK3) was completely absent. SH-SY5Y also lacked the nociceptor markers TRPV1, MAS-Related GPR Member D (MRGPRD), the peptidergic neuron markers CGRP, substance P (TAC1), the itch receptor marker somatostatin (SST), the mechanoreceptor marker TAFA4/FAM19A4, and the proprioceptor marker parvalbumin (PVALB). Overall, SH-SY5Y cells expressed a range of genes associated with different subtypes of sensory neurons and could not be classified according to known DRG neuron subclasses, including those defined by Usoskin et al.\textsuperscript{32}, Li et al.\textsuperscript{36}, or simpler categorisation based on expression of established markers relating to functional aspects, such as CGRP, IB4 and NF200. Although further classification of sensory neurons was recently reported by Li et al.\textsuperscript{36}, DRG classification criteria based on transcriptional markers remain relatively broad and as yet do not unambiguously encompass the possible 17 or more different fibre types that have been proposed based on responsiveness to mechanical and thermal stimuli as well as conduction velocity and rate of adaptation\textsuperscript{29}. Thus, while these cells express genes - as well as their respective protein products\textsuperscript{243, 244} - known to be involved in key sensory neuron functions, they should not be regarded as a model for DRG neurons.

3.4.5 F-11 and ND7/23 cells also express molecular markers for multiple DRG neuron subtypes

F-11 and ND7/23 cells were both created from a fusion of mouse neuroblastoma cells and rat embryonic or neonatal DRG neurons, respectively\textsuperscript{241, 242}. Accordingly, the expression profile of neuronal markers was similar in both cell lines, and included a number of markers for A-fibre mechanosensors and proprioceptors, including LDHB, NEFH and TrkC (Figure 3.1 A). As with SH-SY5Y cells, markers for C-fibres were also present in both cell lines, including P2RX3, a purinergic ion channel expressed in C-fibre non-peptidergic neurons; plexin C1 (PLXNC1), TH, PRPH, RET, and Cav\textsubscript{3.3} (CACNA1I). ND7/23 cells expressed comparatively higher levels of TLX3, SCN11A, TrkC, and TrkA than F-11 cells, while ASIC1, PLXNC1, TH, PRPH, NEFH, CNTNAP2, LDHB, CACNA1H, and CACNA1I had reduced expression in ND7/23 cells compared with F-11 cells. TrkB was expressed at very low levels in both cell lines. The C-fibre marker TrkA was enriched approximately 20-fold in ND7/23 cells compared with F-11 cells, while TH was elevated approximately 10-fold in F-11 cells compared to ND7/23 cells. Such findings established that, similar to SH-SY5Y cells, both F-
11 and ND7/23 cells expressed a range of cellular markers that are usually found in distinct sensory neuron subtypes and a specific neuronal origin can thus not be definitely assigned.

### 3.4.6 Whole DRGs and cell lines expressed similar proportions of reads towards gene ontology terms

All samples showed a similar distribution of genes expressed according to Biological Processes (BP) terms following global gene ontology (GO) analysis. The BP term with the most genes associated to it was ‘metabolic process’ across all samples, followed by the terms ‘cellular process’ and ‘biological regulation’. Fourteen different BP terms were found to be present in all samples, with whole DRGs being the only sample that contained one extra term, that of ‘rhythmic process’ (Figure 3.1 B-G). The proportions of reads aligned to each BP term were not significantly different between each sample, indicating that while the cell lines do not exhibit expression profiles that fit the profile of endogenous DRG neurons, the cell lines maintain the physiological proportion of transcripts for various cellular functions. F-11 and ND7/23 samples both exhibited reduced numbers of genes associated with each BP term when analysed against the rat genome, however the distribution of associations across different terms remains very similar across the mouse and rat genomes for both samples.

### 3.4.7 Whole DRGs expressed significantly more Cav, Kv, and Nav reads than the cell lines

The expression of ion channels in whole DRGs and the three cell lines were analysed next. The expression of ion channels and GPCRs with putative therapeutic importance was assessed based on classification obtained from IUPHAR (http://www.guidetopharmacology.org).

There were 261 ion channel genes detected in whole DRGs (≥ 1 read aligned to the gene), 217 in SH-SY5Y cells, 223 in F-11 (mouse), 201 in F-11 (rat), 214 in ND7/23 (mouse), and 189 genes in ND7/23 (rat). Twenty-one families of ion channels were expressed by all samples, including several considered as therapeutic targets for pain such as ASIC receptors, GABA_A receptors, and voltage-gated sodium, potassium, and calcium channels (Figure 3.2 A). Whole DRGs had the highest number of ion channel transcripts among the samples, with 494,237 reads aligned to ion channel genes. The level of ion channel expression was also at least three times higher in whole DRGs (1.42% of total aligned reads) than in the cell lines.
examined. 0.53% of total aligned reads in SH-SY5Y cells consisted of ion channel reads, while ion channel reads made up 0.43% of total aligned reads in F-11 (mouse), 0.48% of total aligned reads in F-11 (rat), 0.42% of total aligned reads in ND7/23 (mouse), and 0.45% of total aligned reads in ND7/23 (rat). 58 ion channels were found to be uniquely expressed in whole DRGs, while 8 uniquely expressed ion channel genes were found in SH-SY5Y, 3 in F-11, and 4 in ND7/23 (Table 3.1).

The most striking difference in the distribution of ion channel transcripts between DRGs and cell lines lies in the percentage of voltage-gated sodium, potassium, and calcium channels. Together, Na\textsubscript{V}, K\textsubscript{V}, and Ca\textsubscript{V} consisted of 59% of all reads aligned to ion channels in whole DRG, a trend that was reflected in the three cell lines. Na\textsubscript{V}, K\textsubscript{V}, and Ca\textsubscript{V} constituted 43% of all ion channel transcripts in SH-SY5Y cells, 40% in F-11 cells (mouse and rat), 35% in ND7/23 cells (mouse), and 38% in ND7/23 cells (rat) counts. However, much of the Ca\textsubscript{V} expression in the cell lines was contributed by Ca\textsubscript{V} auxiliary units rather than the channel-forming Ca\textsubscript{V} α units, and this was especially true for SH-SY5Y cells. SH-SY5Y cells also abundantly expressed cholinergic nicotinic receptors, amounting to 27% of all ion channel transcripts. In contrast, cholinergic nicotinic receptors consisted of 15% or less ion channel transcripts in all other samples (whole DRG 2%, F-11 mouse 3%, F-11 rat 2%, ND7/23 mouse 14%, and ND7/23 rat 11%) (Figure 3.2 B-G).
Figure 3.2 Whole DRGs and cell lines have unique expression patterns of ion channels families

Sections of the pie chart without an annotated percentage constituted ≤ 0.5% of total ion channel expression in the sample. A) Compilation of the ion channel genes with the highest

106
expression levels for each sample. B) Distribution of ion channel expression in whole DRGs. C) Distribution of ion channel expression in SH-SY5Y cells. D) Distribution of ion channel expression in ND7/23 cells (aligned to the rat genome). E) Distribution of ion channel expression in ND7/23 cells (aligned to the mouse genome). F) Distribution of ion channel expression in F-11 cells (aligned to the rat genome). G) Distribution of ion channel expression in F-11 cells (aligned to the mouse genome).
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Comparison of ion channels and GPCRs detected in whole DRGs and cell lines reveals that large number of genes are absent from the cell lines. A gene is considered to be uniquely expressed if >100 reads are detected only in one sample.

**Table 3.1 Ion channels and GPCRs uniquely expressed in whole DRGs and cell lines**

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<th>Gene</th>
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<td>P2RY12</td>
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<td>PTGDR</td>
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<td>PTGER3</td>
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<td>S1PR1</td>
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A compilation of the 30 ion channels with the highest expression in each sample revealed significant differences as well as similarities between the cell lines and compared with whole DRGs (Figure 3.2 A). The ion channels with the highest expression in whole DRGs were Na\textsubscript{v}1.8 (SCN10A), K\textsubscript{v}1.1 (KCNA1), and the Na\textsubscript{v} β4 subunit (SCN4B). Other ion channels highly expressed in whole DRGs, but expressed at very low levels in the cell lines, included Na\textsubscript{v}1.9, aquaporin 1, the Na\textsubscript{v} β1 subunit, K\textsubscript{v}4.1 (KCND1), and glycine receptor β unit (GLRB). In addition, transcripts for Na\textsubscript{v}1.1 (SCN1A), ionotropic glutamate receptor 1 (GRIK1), and K\textsubscript{v}4.3 (KCND3) were found at lower levels in DRGs but were virtually absent in the three cell lines. Transcripts for SCN8A (Na\textsubscript{v}1.6) and SCN9A (Na\textsubscript{v}1.7), channels responsible for signal transduction and known to have important roles in pain, were present in all cell lines as well as being highly expressed in whole DRGs. F-11 and ND7/23 cells in particular expressed Na\textsubscript{v}1.6 and Na\textsubscript{v}1.7 at higher levels than SH-SY5Y cells, with F-11 cells expressing more Na\textsubscript{v}1.6 than ND7/23, and ND7/23 cells expressing more Na\textsubscript{v}1.7 than F-11 cells.

The nicotinic cholinergic receptor α3 (CHRNA3) was the ion channel with the highest expression in SH-SY5Y and ND7/253 cells, but few transcripts were found in whole DRGs and F-11 cells. The second-most enriched ion channel in SH-SY5Y cells, the Ca\textsubscript{v}α2δ subunit 2 (CACNA2D2), also had very little expression in the other samples. Other ion channels highly expressed in SH-SY5Y cells included Ca\textsubscript{v} auxiliary units such as CACNG4 and CACNA2D1, and voltage-gated chloride channels such as CLCN3 (Figure 3.2 A).

While the overall gene expression profiles of F-11 and ND7/23 cells were similar, there was notable disparity within ion channel expression. F-11 cells expressed a large amount of transcripts for K\textsubscript{v}11.1 (KCNH2, otherwise known as hERG) and 5-HT receptor 3a (HTR3A), genes that were expressed at much lower level in ND7/23 cells (Figure 3.2 A). In contrast, CHRNA3 was the most highly expressed ion channel transcript in ND7/23 cells, but was only expressed at low levels in F-11 cells. Apart from these exceptions, many ion channel genes where expressed at similar levels in F-11 and ND7/23 cells. Several of these, including ion channels with recognised roles in nociception such as ASIC1, Ca\textsubscript{v}3.2 (CACNA1H), and K\textsubscript{v}7.2 (KCNQ2), were highly expressed in F-11 and ND7/23 cells as well as in whole DRGs and SH-SY5Y cells (Figure 3.2 A). Similarly, P2RX3, Ca\textsubscript{v}3.3 (CACNA1I), TRPV2, and
Ca\textsubscript{v}2.2 (CACNA1B) were enriched in F-11 and ND7/23 cells to a lesser extent compared with DRGs and SH-SY5Y cells. For all of these ion channels, transcript numbers were higher in F-11 cells compared with ND7/23 cells. In contrast, TRPM2, CLCN2, KCNK3, CLCN5, GRIA2, TPCN1, KCNC3, KCNC1, and KCNT1 were expressed at higher levels in ND7/23 cells than F-11 cells.

3.4.9 The GPCR genes with the highest expression in whole DRGs are GABA\textsubscript{B} receptor genes

Many GPCRs initiate intracellular changes after activation from external chemicals, making this family of receptors ideal pharmacological targets. A number of GPCRs are already established analgesic targets, such as the opioid receptors, while others have recognised roles in inflammation and nociception, such as bradykinin and protease-activated receptors\textsuperscript{262-264}.

A total of 300 different GPCR genes from the IUPHAR list were expressed in whole DRGs, 218 genes in SH-SY5Y cells, 215 in F-11 (mouse), 188 in F-11 (rat), 225 in ND7/23 (mouse), and 187 in ND7/23 cells (rat) (Figure 3.3 A-G). Whole DRGs contained the most GPCR transcripts, with 185,707 reads aligned to GPCR genes accounting for 0.54% of all aligned reads. SH-SY5Y cells had the second-most abundant GPCR expression (0.38% of all aligned reads), followed by ND7/23 mouse (0.35%), ND7/23 rat (0.33%), and F-11 rat and mouse (both 0.26%). Similar to the trend seen for ion channels, whole DRGs had both the highest GPCR expression level and the highest number of uniquely expressed GPCRs, with 46 GPCRs uniquely expressed in whole DRGs. SH-SY5Y cells expressed 8 unique GPCRs, F-11 cells expressed 1, and ND7/23 cells expressed 10 (Table 3.1).

Forty-five families of GPCRs were expressed by all four samples. Amongst these, the adhesion class GPCR family had the highest expression levels in all samples, constituting 42% of all GPCR transcripts from SH-SY5Y cells, 35% in ND7/23 cells (rat), 32% in F-11 cells (rat), 29.5% in ND7/23 (mouse), 28.3% in F-11 (mouse), and 17% in whole DRGs.

The GPCR family with the second highest expression in whole DRGs were the GABA\textsubscript{B} receptors GABBR1 and GABBR2 (16% of all GPCR reads), a trend that was not observed in the cell lines where GABA\textsubscript{B} receptors only consisted of 3% of GPCR reads in F-11 and ND7/23 (rat), and 2% in ND7/23 (mouse) and SH-SY5Y cells. However, expression of approximately equivalent transcript numbers for both the R1 and R2 subunit of the GABA\textsubscript{B}
receptor was only observed in DRG neurons, suggesting that functional GABA_B receptors do not form in the neuronal cell lines (Figure 3.3 B).

Other GPCRs with high expression in whole DRGs include sphingosine-1-phosphate receptor 3 (S1PR3) and the orphan GPCRs GPR158 and GPR56. These genes, together with the prostaglandin F receptor (PTGFR), par3 (F2RL2), 5-HT receptor 1D (HTR1D), prostaglandin E receptor 3 (PTGER3), and adenosine A1 receptor (ADORA1), were expressed at very low levels in neuronal cell lines. Two exceptions in this group include the orphan GPCRs with unknown function, GPR137 and GPR153, which were expressed in all samples (Figure 3.3 A).
Figure 3.3 Whole DRGs and cell lines have unique expression patterns of GPCRs

Sections of the pie chart without an annotated percentage constituted ≤ 0.5% of total ion channel expression in the sample. A) Compilation of the GPCR genes with the highest

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expression levels for each sample. B) Distribution of GPCR expression in whole DRGs. C) Distribution of GPCR expression in SH-SY5Y cells. D) Distribution of GPCR expression in ND7/23 cells (aligned to the rat genome). E) Distribution of GPCR expression in ND7/23 cells (aligned to the mouse genome). F) Distribution of GPCR expression in F-11 cells (aligned to the rat genome). G) Distribution of GPCR expression in F-11 cells (aligned to the mouse genome).
3.4.10 GPCR genes enriched in cell lines

The GPCR with the highest level of expression in SH-SY5Y cells was latrophilin 2 (LPHN2). Latrophilin 2 is an adhesion GPCR that plays a role in exocytosis and is closely related to latrophilin 1 (LPHN1), the receptor for latrotoxin, the active peptide in black widow spider venom. Interestingly, latrophilin 1 was the GPCR with the highest expression in F-11 cells and the second highest expressed in ND7/23 cells, and was also present in whole DRGs. Many genes enriched in SH-SY5Y cells, such as smoothened (SMO) and par1 (F2R), were also expressed in other samples. However, a small group of genes, including the muscarinic acetylcholine receptor M3 (CHRM3) and prostaglandin E2 receptor (PTGER2), were enriched in SH-SY5Y cells but largely absent in other samples (Figure 3.3).

In F-11 cells, the prostaglandin I2 receptor (PTGIR), par1 (F2R), and LPHN1 were the most highly expressed GPCRs. In ND7/23 cells the GPCRs with the highest expression were the cannabinoid receptor 1 (CNR1), LPHN1, and pituitary adenylate cyclase-activating polypeptide type I receptor (ADCYAP1R1). The majority of GPCR genes were expressed at similar levels in F-11 and ND7/23 cells. GPCRs enriched in F-11 and ND7/23 tend to be expressed at very low levels in whole DRGs, whereas a group of GPCRs highly expressed in DRGs, including CELSR3 and BAI2, were enriched in SH-SY5Y cells. Of note, GPR123 and PTGER4 were enriched exclusively in F-11 cells, and CD97 and FZD2 were enriched only in ND7/23 cells. When aligned to the mouse genome, the top 30 GPCRs expressed for F-11 and ND7/23 cells resembled each other very closely. Interestingly, F-11 cells expressed mouse but not rat transcripts of PTGIR, while the same was the case for ADCYAP1R in ND7/23 cells (Figure 3.3 A).
Figure 3.4 Calcium response to oxytocin and vasopressin addition in DRG neurons and cell lines

Functional oxytocin receptors are expressed in SH-SY5Y cells, but not in F-11 or ND7/23 cells. Functional vasopressin receptors are absent from all three cell lines. This confirms the findings regarding oxytocin and vasopressin receptors in the transcriptome. A) Calcium influx responses of SH-SY5Y, F-11, and ND7/23 cell lines upon exposure to oxytocin. B) Calcium response of HEK293 cells transfected with oxytocin receptor (OXTR) upon exposure to oxytocin. C) Calcium influx responses of SH-SY5Y, F-11, and ND7/23 cell lines upon exposure to vasopressin. D) Calcium response of HEK293 cells transfected with the vasopressin V1B receptor (AVPR1B) upon exposure to vasopressin.
3.4.11 Functional vasopressin receptors were absent from all cell lines, while the oxytocin receptor was present in SH-SY5Y cells and absent in the other cell lines

Both arginine vasopressin receptors and oxytocin receptors, while not traditionally considered important targets in pain pathways and are not previously thought to be present in peripheral DRG neurons, are now observed to have an emerging functional role in pain. The vasopressin receptor has been linked to strain-dependent pain sensitivity to formalin and capsaicin266 and oxytocin activation ameliorates visceral pain in particular267.

Consistent with a specific role of these GPCRs in pain, both vasopressin receptor 1B and vasopressin receptor 2 were expressed at very low level or absent in DRGs. In contrast, all three cell lines expressed oxytocin receptors, but vasopressin receptor 1A and 1B were absent. Functional Ca$^{2+}$ imaging via the FLIPR$^\text{TETRA}$ was therefore used to verify these findings. Transcriptomic data indicated the oxytocin receptor was expressed in SH-SY5Y cells, absent in F-11 cells, and only aligned to three reads in ND7/23 cells. Accordingly, only SH-SY5Y responded to oxytocin at concentrations that elicit Ca$^{2+}$ responses in HEK293 cells transfected with the oxytocin receptor, confirming the presence of functional oxytocin receptors in SH-SY5Y cells and its absence in F-11 and ND7/23 cells (Figure 3.4 A-B). Moreover, consistent with transcriptomic results, vasopressin did not elicit an increase in intracellular Ca$^{2+}$ in any of the cell lines tested, albeit robust Ca$^{2+}$ responses were elicited in HEK293 cells transiently transfected with arginine vasopressin receptor 1B (Figure 3.4 C-D).

3.4.12 $\text{Ca}_V$ expression in the cell lines is consistent with previous experimental data

The expression profile of $\text{Ca}_V$ transcripts in SH-SY5Y cells was entirely consistent with that previously reported245, with $\text{Ca}_V$2.1, $\text{Ca}_V$2.3, $\text{Ca}_V$3.2, and $\text{Ca}_V$1.1 being completely absent in SH-SY5Y cells. $\text{Ca}_V$3.1 had the largest number of reads in SH-SY5Y cells, followed by $\text{Ca}_V$2.2 and $\text{Ca}_V$1.3. SH-SY5Y cells also expressed several $\text{Ca}_V$ auxiliary units at high levels, a trend that was also observed in F-11 and ND7/23 cell lines. In contrast, the order of expression of $\text{Ca}_V$ alpha units in DRGs was observed as $\text{Ca}_V$2.2 > $\text{Ca}_V$2.1 > $\text{Ca}_V$3.2 > $\text{Ca}_V$1.2 > $\text{Ca}_V$3.3 > $\text{Ca}_V$1.3 > $\text{Ca}_V$2.3 > $\text{Ca}_V$3.1 > $\text{Ca}_V$1.1 > $\text{Ca}_V$1.4, with all alpha isoforms being present (for detailed data see Appendix 3.3). This was mirrored by the F-11 and the ND7/23 cells, both of which expressed all $\text{Ca}_V$ isoforms with a similar pattern. F-11 and ND7/23
expressed most CaV3.2 out of all CaV alpha subunits, closely followed by CaV2.2, CaV1.2, and CaV3.3.

3.4.13 K\textsubscript{\textit{V}}11.1 was the \textit{K\textsubscript{\textit{V}}} isoform with the highest expression in all cell lines, and all cell lines lacked K\textsubscript{\textit{V}}1.1

The expression of \textit{KCNQ}2 and \textit{KCNQ}3, which are the genes encoding the K\textsubscript{\textit{V}}7.2 and K\textsubscript{\textit{V}}7.3 isoforms, was consistent with previous results indicating the presence of this channel in DRGs\textsuperscript{18}, F-11 cells\textsuperscript{268}, and SH-SY5Y cells\textsuperscript{269}. \textit{KCNQ}3, however, was absent in ND7/23 cells. K\textsubscript{\textit{V}}1.1, a key K\textsubscript{\textit{V}} channel regulating excitability in DRG neurons, was found to be virtually absent in all cell lines, whereas the K\textsubscript{\textit{V}}11 subtype family was expressed most abundantly (55.06\% of all K\textsubscript{\textit{V}} transcripts in SH-SY5Y, 58.56\% in F-11, and 38.30\% in ND7/23), and K\textsubscript{\textit{V}}1 family expression was at least 30-fold lower in all cell line samples compared to whole DRGs. K\textsubscript{\textit{V}}11.1 (\textit{KCNH}2), commonly known as the human ether-a-go-go-related gene (hERG), was the most abundant K\textsubscript{\textit{V}} subtype channel in all three cell line samples. These findings are consistent with previous studies reporting absence of K\textsubscript{\textit{V}}1.1, and endogenous expression of hERG, in these cell lines\textsuperscript{270-272}.
Figure 3.5 Calcium responses to TRP activators for cell lines and transfected HEK293 cells

Calcium responses to the application of selective TRP activators for cell lines and HEK293 cells transiently expressing TRPA1, TRPM8, or TRPV1. None of the cell lines responded to AITC, menthol, or capsaicin, confirming the absence of TRPA1, TRPM8, and TRPV1 expression in the three cell lines examined. (A) Calcium responses of the cell lines to AITC. (B) Calcium responses of HEK293 cells transiently expressing rat TRPA1 channels to AITC. (C) Calcium responses of the cell lines to menthol. (D) Calcium responses of HEK293 cells transiently expressing rat TRPM8 channels to menthol. (E) Calcium responses of the cell lines to capsaicin. (F) Calcium responses of HEK293 cells transiently expressing rat TRPV1 channels to capsaicin.
3.4.14 Very low amounts of TRPA1, TRPM8, and TRPV1 were expressed in all cell lines

The expression profile of TRP channel transcripts was similar across all cell lines. TRPA1 and TRPV1, the TRP channels receptive to the noxious stimuli mustard oil and capsaicin respectively, were largely missing from the three cell lines. Very small amounts of TRPV1 were present in the three cell lines and TRPA1 was completely absent. TRPM8, the menthol and cold receptor, was also expressed at very low levels in all cell lines.

The recently-identified cold-sensitive channel TRPC5 was revealed to be present in whole DRGs and in ND7/523 cells, with very low expression in SH-SY5Y and F-11 cells. TRPM3, another novel channel suggested to be a potential nociceptor and thermoceptor, was present in whole DRGs, F-11 cells, and ND7/23 cells, with very low expression levels in SH-SY5Y cells (for detailed data see Appendix 3.3).

These findings were confirmed with fluorescent intracellular calcium measurements after the application of selective TRP channel agonists (Figure 3.5). The TRPA1 activator, AITC, did not elicit a response from any of the cell lines despite activating transfected HEK293 cells transiently expressing rat TRPA1 at the same concentrations (Figure 3.5 A-B). The application of the TRPM8 agonist menthol also elicited minimal responses from all of the three cell lines, while robust Ca\textsuperscript{2+} responses were observed in transfected HEK293 cells (Figure 3.5 C-D). Similarly, capsaicin, known to activate rat, mouse, and human TRPV1, elicited no responses in the cell lines, but robustly activated TRPV1-transfected HEK293 cells (Figure 3.5 E-F). The relatively lack of expression of these key thermosensitive TRP channels suggests that none of the three cell line examined should be used as model nociceptive or thermosensitive neurons.

3.5 Discussion

Immortalised neuronal cell lines as models of primary sensory neurons represent a comfortable compromise for the investigation of the neuropharmacological mechanisms underlying pain. These neuroblastoma-derived cell lines often express molecular targets consistent with those found in sensory neurons, bypassing the need for excision and primary culture of tissue while – presumably – retaining endogenous protein expression profiles. Indeed, one advantage of using immortalised neuronal cell lines over heterologously
transfected cells is the co-expression of relevant auxiliary subunits as well as interacting proteins, and the constellation of channels and receptors that would form the 'signalosome' in a native neuron, providing a more holistic insight to nociceptive pharmacology and signalling. However, these properties are often based on assumptions about the gene expression profile of such cell lines, and the lack of comprehensive analyses of receptor expression in these cell lines limits their applicability.

Sensory neurons are a heterogeneous group of neurons with distinct functional characteristics. Since cell lines can be considered monoclonal and originate from a single cell colony, the absence of genes enriched in specific subgroups of neurons could be expected – it is plausible that the gene expression profile of ND7/23 and F-11 cells would resemble only one subset of the many different types of sensory neurons. Indeed, both ND7/23 and F-11 cells are often considered a model for small nociceptive neurons that give rise to unmyelinated C-fibres. However, the precise type of sensory neuron that these cell lines may have arisen from, or that they most closely resemble, is difficult to assess currently, not least because the precise number of individual neuronal subtypes found in DRG are unclear. Based on functional characteristics of the fibres innervating target organs including skin, at least 16 subclasses of purely sensory neurons should exist. In recent years, significant efforts have been made towards identifying the gene expression profile of these individual neuronal classes, as it is becoming increasingly clear that it is the constellation of ion channels, GPCRs, auxiliary subunits and interacting proteins expressed in a given neuron that defines its functional properties. For example, cold sensitivity in trigeminal neurons is defined by co-expression of Kv1.1/1.2 channels with TRPM861, while in sensory neurons innervating the skin a similar functional relationship was observed between TRPM8 and Kv7.2/7.3 channels18. There is also existing evidence towards the presence of multiple neuron types within F-11 cell lines277, indicating that while neuron-derived cell lines are supposedly clones of the parent cell, a myriad of neuronal subtypes exists within one population.

This chapter thus set out to assess the gene expression profile of the model sensory neuron cell lines ND7/23 and F-11 as well as the neuroblastoma cell line SH-SY5Y, which has previously been reported to express genes and ion channels normally restricted to peripheral sensory neurons such as Na\textsubscript{v}1.7244. Moreover, if any of the cell lines are found to resemble specific classes of peripheral sensory neurons with known expression of Na\textsubscript{v}1.6 or Na\textsubscript{v}1.8,
the cell lines could be used as *in vitro* models for high-throughput investigation of compounds that may modulate the two Na\textsubscript{v} isoforms.

When compared with the gene expression profile of whole DRGs, I found the expression of many individual genes known to be expressed in, or play an important role in the function of, sensory neurons. These include *RET, TH, PRPH*, and *LDHB*. At the same time, many sensory neuron-enriched genes were also absent from the cell lines, such as *TRPV1* and *SCN10A*. As an assessment of markers between PNS and CNS, the absence of central nervous system microglial markers such as *CCL4* and *GPR183*\textsuperscript{278} in the neuronal cell lines was expected, given that all three cell lines derive from peripheral neurons.

Based on a comprehensive study assessing the gene expression profiles of sensory neurons using single-cell RNA sequencing, Usoskin et al.\textsuperscript{32} defined four basic PNS sensory neuron subtypes, characterised by expression of *TAC1, NTRK1* and *CALCA* (peptidergic cluster); *MRGPRD* and *P2RX3* (non-peptidergic cluster); *TH* (tyrosine hydroxylase containing cluster); *NEFH* and *PVALB* (neurofilament-containing cluster); as well as eleven subgroups by correlating both old and novel markers to physiological function in peripheral nerves. Li et al.\textsuperscript{36} also examined the types of neurons present in DRGs in a separate study, and classified mouse DRG neurons into 10 main types and 14 subordinate subtypes based on molecular markers, functional annotations and transcriptional patterns. The resulting clusters matched the classification by Usoskin et al. to some extent, but also introduced additional transcriptional markers defining subgroups of sensory neurons. Importantly, the gene expression profile of F-11, ND7/23 and SH-SY5Y cells resembled none of these classifications closely, with all cell lines expressing markers from several different clusters, as well as markers of non-neuronal cells including *B2M* and *VIM*\textsuperscript{32}. Indeed, F-11 cells expressed high levels of *P2RX3, TH* and *NEFH*, thus simultaneously incorporating markers of non-peptidergic, tyrosine hydroxylase-containing, and neurofilament-expressing neurons based on the classification proposed by Usoskin et al.\textsuperscript{32}. ND7/23 cells also expressed high levels of the peptidergic neuron marker *NTRK1*, the non-peptidergic marker *P2RX3, TH*, and the neurofilament-expressing marker *NEFH*. The cell lines also expressed markers that belong to separate clusters based on the classifications by Li et al.\textsuperscript{36}, including black (*TH*), turquoise (*P2RX3, NTRK1*) and blue (*NEFH*)\textsuperscript{36}. 

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Similar discrepancies were also observed when comparing the gene expression profile of the cell lines to the transcriptome of neurons expressing Na\textsubscript{v}1.8, which are typically considered nociceptive and express high levels of \textit{ANO3}, \textit{TRPA1} and \textit{CHRNA6}\textsuperscript{131}, none of which were present in the immortalised neuronal cell lines examined. These discrepancies are perhaps less surprising given none of the cell lines expressed more than one read of Na\textsubscript{v}1.8 transcripts in the entire sample. Indeed, my results showed that allocation of neuronal cell lines to any single meaningful subtype of sensory neuron is hardly possible, as they expressed a mixture of cellular markers that are typically found in non-overlapping cell populations, including markers for myelinated neurons as well as for unmyelinated neurons. Some examples include the presence of TrkA, TrkB, and TrkC in all cell line samples, suggesting a mixed heritage of the cell lines. Thermo-sensitive proteins responsible for temperature sensing such as \textit{TRPV1}, \textit{TRPA1}, and \textit{TRPM8} were all virtually absent in the cell lines, making them unlikely to have been derived from thermosensors. The complete lack of \textit{PIEZO2} also indicate the cell lines are not to be used to investigate stretch- and mechano-sensitive neurons in their native state\textsuperscript{279}. Other subclasses I can rule out are subsets of pruriceptors due to the lack of par2 and somatostatin in the cell lines.

Despite the discrepancies in cellular markers, global GO analysis of all samples according to BP terms revealed similar functional distribution of genes between all samples, suggesting that these neuronal cell lines maintain broad functional similarities with DRG neurons regardless of the poor fit into the existing framework of neuron classification. It is possible that while gene markers are not expressed in the cell lines, other genes with similar roles are expressed instead through compensatory mechanisms to maintain functional similarity with \textit{in vivo} peripheral neurons.

While the chapter assessed gene expression profiles in undifferentiated ND7/23, F-11 and SH-SY5Y cells, all three cell lines have the capacity and plasticity to develop into mature, terminally differentiated neurons upon exposure to various reagents such as retinoic acid or forskolin\textsuperscript{269, 280}. These agents can be used to drive the cells towards various phenotypes away from their native form. This is certainly observed in SH-SY5Y cells, which can express different receptors based on the method used to induce differentiation (for a brief review see\textsuperscript{281}). It is thus possible that differentiated cell lines may resemble certain neuronal subtypes more closely, and a transcriptomic analysis on differentiated cells could provide additional insight into processes involved in neuronal maturation.
A drawback of transcriptomic studies lies in difficulties reconciling gene expression with expression of functional proteins. However, the presence of N-methyl-D-aspartate receptors (GRIN1), HCN2 channels, Na\textsubscript{v}1.7 (SCN9A), Ca\textsubscript{v}2.2 and opioid receptors in all cell lines have been confirmed, consistent with previous findings\textsuperscript{243, 244}. I have additionally confirmed the observed pattern of expression in the cell lines for selected targets using a pharmacological approach where sufficiently selective agonists were readily available.

3.6 Conclusions

This chapter presents the first comprehensive transcriptomic analysis of the gene expression profile in F-11 and ND7/23 cell lines in comparison with whole mouse DRGs and the human neuroblastoma-derived cell line SH-SY5Y. I found that all cell lines expressed a multitude of receptors and ion channels with relevance to nociceptive signalling. However, although the cell lines investigated express molecular targets found in native DRGs, they are not representative of specific subclasses of peripheral sensory neurons and do not fit into existing peripheral neuron categories. Therefore, despite being derived from DRG neurons, these in vitro cell models do not represent native DRG neuron subtypes such as nociceptors, making it difficult to use these cells to investigate for potential modulators of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 channels, or to investigate nociceptors in general. The next chapter therefore turns to an animal model of a Na\textsubscript{v}1.7-independent pain modality, that of burn-induced pain, to study the role of selective Na\textsubscript{v} isoforms in animal pain pathways and to discern other potential novel therapeutic targets and clinically effective new drugs for burn-induced pain.
Chapter 4 – Behavioural and pharmacological analysis of a burn-induced pain model in mice

4.1 Foreword

Using bioactive compounds to isolate modulators of the Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 isoforms, as well as using neuron-derived \textit{in vitro} cell models to simulate the effect of modulators in nociceptive neurons, have been covered in chapters 2 and 3 of this thesis. However, both methods have challenges and issues that the previous chapters have brought to light. Using bioactive venoms to screen for selective modulators resulted in a lead peptide, but one that has off-target activity. This concern was only revealed after extensive chemical purification and the identification of the amino acid sequence, making the method time-consuming and with a high rate of failure. At the same time, chapter 3 analysed the transcriptome profile of three common \textit{in vitro} cell lines and found none of them to be representative of native DRG neuron subtypes, and are not appropriate as tools to investigate Na\textsubscript{v}-expressing nociceptors. I therefore utilised an animal model instead in this chapter, capitalising on the clinical relevance of animal models of pain and using known channel modulators to study the role of selective Na\textsubscript{v} channels in burn-induced pain, a condition that is known to be only partially affected by the absence of Na\textsubscript{v}1.7.

Some data presented in this chapter have been accepted for publication by Molecular Pain\textsuperscript{282}. The content has been edited and additional data has been included to fit the structure of a thesis chapter.

4.2 Introduction

Burn injury is one of the most common injuries around the world, causing an estimated 265,000 deaths annually world-wide and necessitating medical treatment of nearly 11 million people in 2004\textsuperscript{4}. Out of the three potential causes of a thermal burn - scald, flame, and contact burns - burns from a flame is consistently responsible for approximately 40% of all cases across patients of all ages, with the exception of young children under 5 years of age, who primarily suffer from contact burns\textsuperscript{283}. Electric and chemical burns, two non-thermal causes of burn injuries, only make up less than 10% of all burn incidences when combined. Pain is
present in most patients following a burn injury, particularly as the patient recovers and scar tissue develops. Burn pain is a complex symptom consisting of multiple components, including spontaneous or non-evoked pain as well as hypersensitivity to mechanical and thermal stimuli that can transition from an acute, self-limiting nociceptive response to chronic pain with neuropathic components \(^{284}\).

Procedural pain, which is pain associated with surgical interventions including debridement, grafting, and dressing changes, is short-lasting but severe in comparison to background pain \(^{285}\), and often requires additional intensive analgesic treatment in addition to the patient's existing regimen. Pain intensity can also fluctuate unpredictably over time and requires constant dose monitoring. The degree of pain appears to be independent of existing analgesic dose and burn severity \(^{286}\). Moreover, pain can last long after the burn injury has healed, and evidence of chronic pain with neuropathic features has been observed in patients irrespective of burn intensity \(^{287}\), making pain a difficult-to-manage and a frequently undertreated consequence of burn injury.

While opioids remain a central approach for analgesia, especially in the acute phase \(^{5}\), concerns remain regarding opioid dependence, withdrawal, and side effects in burn patients. In addition, the fluid and unpredictable nature of burn pain results in frequent dose adjustments and pain control that is often unsatisfactory. Accordingly, adjuvant analgesics such as antidepressants, anticonvulsants, and non-steroidal anti-inflammatory drugs are used extensively in burn pain \(^{288}\), but often with conflicting results \(^{289, 290}\).

Burn-induced pain is one of the conditions that do not fully respond to Na\(_{\text{V}}\)1.7 inhibition. While burn-induced thermal allodynia was completely alleviated in Na\(_{\text{V}}\)1.7 knockout mice, mechanical allodynia was not affected by Na\(_{\text{V}}\)1.7 gene knockout, suggesting molecular mechanisms amongst neuropathic pain symptoms differ in a modality-dependent manner even if these symptoms are induced by the same injury. The efficacy of selective Na\(_{\text{V}}\) isoform inhibition in abolishing mechanical allodynia was then examined using venom-derived Na\(_{\text{V}}\) inhibitors, and Na\(_{\text{V}}\)1.6 inhibition was shown to be the only treatment that significantly modulated mechanical allodynia. The burn-induced pain model also successfully replicated the efficacy of Na\(_{\text{V}}\)1.7 inhibition in burn-induced thermal allodynia. Examination of Na\(_{\text{V}}\)1.6 expression in affected DRG neurons, however, indicate Na\(_{\text{V}}\)1.6 expression was not changed.
following the burn injury, prompting an in-depth bioinformatic analysis of injured DRG neurons, to be described in Chapter 5.

4.3 Materials and Methods

4.3.1 Materials

All materials were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia) unless otherwise stated. Meloxicam was obtained from Abcam (Melbourne, Victoria, Australia). Oxycodone was obtained from Mundipharma Pty Ltd (Sydney, New South Wales, Australia). The \( \mu \)-conotoxins GIIIA and TIIIA were synthesised by Boc chemistry using methods described previously\(^{291} \) by Mr. Zoltan Dekan (Institute for Molecular Biosciences, University of Queensland), who also synthesised Pnc1.

4.3.2 Animals

Male wild-type C57BL/6 mice age 6 - 8 weeks and weighing 20 - 23 grams were used in the study. Ethical approval for experiments involving live animals was obtained from the University of Queensland animal ethics committee under the ethics approval number PHARM/074/13/RAMACIOTTI. Experiments were conducted in accordance with the Animal Care and Protection Regulation Queensland (2012), the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th edition (2013) and the International Association for the Study of Pain Guidelines for the Use of Animals in Research.

All animals were housed in groups of 2 - 4 per cage in a stable environment under 12-hour light-dark cycles and had access to standard rodent chow and water \textit{ad libitum}. A red polycarbonate Mouse House (Tecniplast, Italy) and shredded paper nesting material were supplied for enrichment. All animals were obtained from University of Queensland Biological Resources (Brisbane, Australia).

Sample sizes of each experiment are detailed in the figure legends of the corresponding figure.
4.3.3 Induction of burn injury

To induce a mild burn injury, the plantar skin of the left hind paw of mice was applied with firm pressure to a Peltier plate (Hot/Cold Plate, Ugo Basile, Comerio, Italy) set at 52.5 °C for 25 seconds under 3% isoflurane anaesthesia. A pair of tweezers, with the tips wrapped in tissue paper, was used to press the plantar portion of the paw onto the Peltier plate. Sham control animals underwent the same procedure with the plate set at 22 °C (room temperature). Behavioural assessment was performed at the time points indicated and the same animals were used for each of the behavioural tests.

4.3.4 Mechanical allodynia testing

Mechanical allodynia was assessed using electronic von Frey (MouseMet Electronic von Frey, TopCat Metrology, United Kingdom). All measurements were conducted by a blinded observer unaware of treatments. Mice were habituated in the von Frey apparatus for at least 10 minutes prior to testing. The von Frey filament was placed against the plantar skin of the hind paw and the pressure increased at a rate of 1 gram per second through rotation of the device. The MouseMet Software automatically recorded the force at which paw withdrawal occurred. The paw withdrawal force (PWF) was determined by the average of three tests, separated by at least 2 minutes.

4.3.5 Thermal allodynia testing

Thermal allodynia was assessed using the Hargreaves Test (Plantar Analgesia Meter, IITC, CA, USA) for model establishment and clinical drug treatment, and the thermal threshold probe (MouseMet Thermal, TopCat Metrology, United Kingdom) for selective Nav inhibitors. All measurements were conducted by a blinded observer unaware of treatments.

For the Hargreaves Test, mice were habituated in the Hargreaves apparatus in individual polyvinyl boxes (10 x 10 x 10 cm) placed on glass heated to 25 °C for at least 30 minutes prior to testing. A radiant light heat source (50 °C) was focused on the plantar skin of the hind paw, and the latency to a withdrawal response was recorded. The mean time to withdrawal was determined from the average of three tests, separated by at least 2 minutes. A cut-off time of 20 seconds was used to avoid tissue damage.
For the thermal threshold probe, mice were tested according to methods described previously\textsuperscript{292}. Briefly, mice were habituated in individual mouse runs for at least 5 min prior to testing. The thermal probe (MouseMet Thermal, TopCat Metrology, United Kingdom) was lightly placed against the plantar surface of the mouse paw and initiated heating of the probe while in contact with the mouse’s paw. The probe was preheated to \(~37\,^\circ C\) before coming in contact with the paw, then once in contact heats at a rate of 2.5 °C per second, with a cut-off set at 60 °C to prevent tissue damage. Removal of the probe from the paw terminates heating and displays the withdrawal temperature. The temperature that elicited a paw withdrawal, known as the paw withdrawal temperature (PWT), was determined by a single test.

4.3.6 Paw thickness

Paw thickness was measured along the distal-proximal axis at the metatarsal level using a digital vernier caliper (Kincrome, Queensland, Australia) whilst the mouse was under 3% isoflurane anaesthesia. The paw thickness of the left (ipsilateral) paw was normalised to the value of the contralateral paw for each animal.

4.3.7 Gait Analysis

Gait analysis was assessed using the CatWalk XT analysis system (Noldus Information Technology, The Netherlands) as previously described\textsuperscript{293}. Mice were placed individually at one end of the elevated glass walkway and allowed to walk freely to the other end until three successful runs were recorded. Mice received no prior training. Runs that took longer than 10 seconds or with a speed variance > 90% were deemed unsuccessful and discarded. The green intensity of the walkway background was set at 0.10 and camera gain at 20.00. Recordings were analysed using the CatWalk XT software with the parameters described in Appendix 3.4.

4.3.8 Drug Treatment

The analgesic efficacy of clinical compounds was assessed 3 days after the induction of burn injury or the sham procedure. All compounds were administered by intraperitoneal injection (i.p) with a drug concentration of 10 µL/g using a 30-gauge needle. Animals were randomised to receive either meloxicam (5 mg/kg) once daily, gabapentin (100 mg/kg) three
times daily, amitriptyline (3 mg/kg) once daily, or matched vehicle control, with the first dose administered at the time of the burn injury. Oxycodone (1, 3, and 10 mg/kg) was administered once only on the day of testing. All compounds were diluted in DPBS (Dulbecco’s Phosphate Buffered Saline, Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.5), except meloxicam, which was diluted in DPBS with 10% dimethyl sulfoxide (DMSO). Behavioural assessment was performed at least 30 minutes after the final dose of all compounds by an investigator unaware of the treatments received by each individual animal.

4.3.9 Motor Assessment

Motor performance was assessed 3 days after the induction of burn injury or the sham procedure using the Parallel Rod Floor Apparatus with ANY-Maze Software (Stoelting Co., Wood Dale, USA). A single dose of oxycodone (10 mg/kg), gabapentin (100 mg/kg), or amitriptyline (3 mg/kg) was administered by i.p injection 30 minutes prior to motor assessment. Animals were placed in the Parallel Rod Floor Apparatus, and the ANY-Maze software recorded the distance travelled and number of foot slips over a 1-minute period. The ataxia index was calculated as the ratio of the total number of foot slips to the total distance (in metres) travelled.

4.3.10 Analgesic efficacy of selective NaV inhibitors

Experiments were conducted 3 days after the induction of burn injury or the sham procedure. All compounds were administered by shallow intraplantar injection (i.pl) into the left hind paw of mice under 3% isoflurane anaesthesia (for location see Appendix 3.5). Tetrodotoxin (TTX; Cayman Chemical, MI, USA) was diluted in DPBS. TIIIA, GIIIA, and Pnc1a were reconstituted in PSS with 1:1000 BSA (Alfa Aesar, MA, USA; Cat# J65966). ICA-121431 and A-803467 were diluted in DPBS with 10% DMSO. Behavioural assessment was performed at least 5 minutes after the dose by an investigator unaware of the treatments received by each individual animal. All i.pl injections had a volume of 10 µL.
4.3.11 PDL-coating of coverslips for cell culture

PDL (Poly D-lysine) powder was reconstituted with sterile Milli-Q water at a concentration of 200 µg/mL and separated into aliquots. Round coverslips (Thermo Fisher Scientific, Victoria, Australia) were sterilised via autoclave and were placed into sterile 24-well tissue culture plates (Nunclon™ Delta treated, Nunc, Denmark), with one round coverslip occupying one well. Each coverslip had 40 µL of the PDL solution placed on the centre and was left in a sterile environment for 60 minutes to coat the coverslip surface with PDL to facilitate better cell adhesion. The PDL solution was removed from the coverslip at the end of the 60-minute incubation.

4.3.12 Cell culture

HEK293 cells stably expressing human Na\textsubscript{V}1.1 (hNa\textsubscript{V}1.1) ion channels were obtained from Scottish Biomedical (SB cells, Glasgow, UK). Cells were grown in MEM (Minimum Essential Media; for composition see Appendix 2.2) with 10% FBS (foetal bovine serum, Life Technologies, Mulgrave, Victoria, Australia) and 2 mM L-glutamine (GlutaMax™, Life Technologies, Mulgrave, Victoria, Australia). To maintain selective pressure for Na\textsubscript{V} expression, this culture media was supplemented with blasticidin (2 µg/mL; Life Technologies, Mulgrave, Victoria, Australia) and geneticin (300 µg/mL; Life Technologies, Mulgrave, Victoria, Australia).

HEK293 cells stably expressing human Na\textsubscript{V}1.2 (hNa\textsubscript{V}1.2) ion channels were obtained from Scottish Biomedical (SB cells, Glasgow, UK). Cells were grown in MEM with 10% FBS and 2 mM L-glutamine. To maintain selective pressure for Na\textsubscript{V} expression, this culture media was supplemented with blasticidin (4 µg/mL) and geneticin (600 µg/mL). Cells were seeded in T75 cm\textsuperscript{2} Corning® (Corning, MA, USA) cell culture flasks with CellBIND® surface.

HEK293 cells stably expressing human Na\textsubscript{V}1.3 (hNa\textsubscript{V}1.3) ion channels were obtained from Scottish Biomedical (SB cells, Glasgow, UK). Cells were grown in MEM with 10% FBS and 2 mM L-glutamine. To maintain selective pressure for Na\textsubscript{V} expression, this culture media was supplemented with blasticidin (2 µg/mL) and geneticin (600 µg/mL).
HEK293 cells stably expressing human Na\textsubscript{v}1.4 (hNa\textsubscript{v}1.4) ion channels were obtained from Scottish Biomedical (SB cells, Glasgow, UK). Cells were grown in MEM with 10% FBS and 2 mM L-glutamine. To maintain selective pressure for Na\textsubscript{v} expression, this culture media was supplemented with blasticidin (2 μg/mL), geneticin (600 μg/mL), and zeocin (500 μg/mL; Life Technologies, Mulgrave, Victoria, Australia). Cells were seeded in T75 cm\textsuperscript{2} Corning® (Corning, MA, USA) cell culture flasks with CellBIND® surface.

HEK293 cells stably expressing human Na\textsubscript{v}1.5 (hNa\textsubscript{v}1.5) ion channels were obtained from Scottish Biomedical (SB cells, Glasgow, UK). Cells were grown in MEM with 10% FBS and 2 mM L-glutamine. To maintain selective pressure for Na\textsubscript{v} expression, this culture media was supplemented with blasticidin (2 μg/mL) and geneticin (600 μg/mL). Cells were seeded in T75 cm\textsuperscript{2} Corning® (Corning, MA, USA) cell culture flasks with CellBIND® surface.

HEK293 cells stably expressing human Na\textsubscript{v}1.6 (hNa\textsubscript{v}1.6) ion channels were obtained from Scottish Biomedical (SB cells, Glasgow, UK). Cells were grown in MEM with 10% FBS and 2 mM L-glutamine.

HEK293 cells stably expressing human Na\textsubscript{v}1.7 (hNa\textsubscript{v}1.7) ion channels were obtained from Scottish Biomedical (SB cells, Glasgow, UK). Cells were grown in MEM with 10% FBS and 2 mM L-glutamine. To maintain selective pressure for Na\textsubscript{v} expression, this culture media was supplemented with blasticidin (4 μg/mL) and geneticin (600 μg/mL).

HEK293 cells stably expressing human Na\textsubscript{v}1.8 (hNa\textsubscript{v}1.8) were obtained from Scottish Biomedical (SB cells, Glasgow, UK). Cells were grown in MEM with 10% FBS and 2 mM L-glutamine. To maintain selective pressure for Na\textsubscript{v} expression, this culture media was supplemented with blasticidin (2 μg/mL) and geneticin (600 μg/mL). Cells were seeded in T75 cm\textsuperscript{2} Corning® (Corning, MA, USA) cell culture flasks with CellBIND® surface.

All cells were incubated at 37°C and 95% O\textsubscript{2}/5% CO\textsubscript{2}. Cells were passaged every 3-5 days at a 1:5 dilution ratio or when approximately 90% confluent. All cells were seeded in T75 cm\textsuperscript{2} Corning® (Corning, MA, USA) cell culture flasks unless previously stated. In order to passage cells, the cells were briefly removed from incubation and maintained in room temperature and had growth media removed from the flask. At least 5 mL/75 cm\textsuperscript{2} of DPBS was added into the flask to briefly wash the cells and was then removed from the flask.
0.25% Trypsin/EDTA (0.25% trypsin/ethylenediaminetetraacetic acid in Hank’s Balanced Salt Solution, Life Technologies, Mulgrave, Victoria, Australia; for composition see Appendix 2.6) was then added at a concentration of 1 mL/75 cm$^2$, and the cells were dislodged by gently tapping the sides of the flask. The dislodged cell suspension were diluted with a further 9 mL of the appropriate growth media (as mentioned above) and passaged in 1:10 dilution ratio in a new T75 cm$^2$ flask and using new growth media.

**4.3.13 Immunofluorescence antibody validation**

HEK293 cells stably expressing human Na$_V$ (hNa$_V$) isoforms hNa$_V$1.1-1.8 were grown in conditions as described in Section 4.3.12. Following passaging of the cells, ~100 µL of the cell suspension was placed into the wells of the 24-well tissue culture plate with PDL-coated coverslips within them. Approximately 500 µL of the appropriate culture media was then added into individual wells. The 24-well tissue culture plate was then incubated at 37 °C and 95% O$_2$/5% CO$_2$ for 24 hours.

After 24 hours, media was removed from the 24-well plate and the coverslips were washed with 500 µL of fresh DPBS for 5 times. The cells growing on the coverslip were fixed for immunofluorescence for 10 minutes using 100 µL of 4% formaldehyde (Thermo Fisher Scientific, Victoria, Australia) diluted in DPBS, followed by washing with 500 µL of fresh DPBS for 5 times. The cells were further blocked using 3% donkey serum, 3% BSA, and 0.1% Triton™ X-100 dissolved in DPBS for 10 minutes, followed by washing with 500 µL of fresh DPBS for 5 times.

Two primary antibodies for Na$_V$1.6, produced by Abcam (Abcam, Melbourne, Victoria, Australia; rabbit anti-mouse antibody, Cat #: ab65166) and Alomone (Alomone, Jerusalem, Israel; rabbit anti-mouse antibody, Cat #: ASC-009), were reconstituted in sterile Milli-Q water as per manufacturers’ instructions. Both antibodies were diluted 1:300 in the blocking solution and 100 µL of the diluted solution were placed on the coverslips and incubated at 4 °C overnight. The solution was removed at the end of the incubation and the coverslips were washed with 500 µL of fresh DPBS for 5 times.

The fluorescent secondary antibody (Abcam, Melbourne, Victoria, Australia; Donkey Anti-Rabbit IgG Alexa Fluor® 488, Cat #: ab150073) were then diluted 1:1000 in the blocking
solution and 100 µL of the diluted solution were placed on the coverslips and incubated at 4 °C overnight. The solution was removed at the end of the incubation and the coverslips were washed with 500 µL of fresh DPBS for 5 times.

DAPI (4',6-diamidino-2-phenylindole) was then diluted 1:1000 in DPBS and sufficient amounts of the diluted solution were placed on the coverslips for 5 minutes. The solution was removed at the end of the incubation and the coverslips were washed with 500 µL of fresh DPBS for 5 times.

The glass coverslips were carefully removed from the 24-well plate using tweezers, and were mounted on glass slides (Superfrost, Menzel-Glaser, Thermo Fisher Scientific, Queensland, Australia) using 10 µL of fluorescent mounting media (Dako, Sydney, New South Wales, Australia) per coverslip, and kept at 4 °C for a minimum of 2 hours to allow the mounting media to set.

The slides were then imaged with an Olympus BX-51 upright fluorescent microscope (Olympus, NY, USA), equipped with an Olympus DP-71 12Mp colour camera (Olympus, NY, USA) and an Exfo Xcite 120 lamp (Excelitas, MA, USA). Images were taken using the DP Capture software (Olympus, NY, USA) when examined through the GFP-4050A filter using the 100x/1.4 Plan Apo Oil lens (Nikon Instruments, NY, USA) with immersion oil (Carl Zeiss Immersol™ Immersion Oil, Carl Zeiss, New South Wales, Australia).

4.3.14 DRG neuron immunofluorescence experiments

At 3 days after the burn injury or the sham procedure, mice were sedated via inhalation of 50% O2/50% CO2 gas inhalation and euthanised via asphyxiation using 100% CO2, followed by cervical dislocation. The spine was immediately removed using surgical equipment, the dorsal side of the vertebra bones removed, and the tissue immersed in ice-cold DMEM. The spinal cord was then removed, and lumbar DRGs (L3-L5 inclusive) were dissected with forceps under a dissection microscope and placed in fresh DPBS within a 33mm-diameter petri dish (Nunclon™ Delta treated, Nunc, Denmark), followed by trimming of excess myelin. At least 3 mice were sacrificed per separate sample in the control and burn cohorts.
DRGs were then placed into 4% formaldehyde diluted in DPBS for 15 minutes, then rinsed in fresh DPBS. The DRGs were then placed for 1 hour into 10% sucrose (Chem-supply, South Australia, Australia) dissolved in DPBS, followed by immersion into 20% sucrose solution for approximately 6 hours, then immersion into 30% sucrose solution overnight.

Individual DRGs were then placed separately on parafilm (Labtek, Queensland, Australia) with one drop of Tissue-Tek® O.C.T. media (ProSciTech, Queensland, Australia) on top of each DRG. The DRG blocks were then transferred to a Leica cryostat (Leica CM3050 S, Leica Biosystems, New South Wales, Australia) and cut at a thickness of 14 µM per slice with object temperature and chamber temperature both set at -20 °C. Slices were transferred to positively-charged glass slides (Superfrost Plus, Menzel-Glaser, Thermo Fisher Scientific, Queensland, Australia) and left in room temperature for approximately 30 minutes to dry.

A fluid-resistant barrier was drawn using a PAP-Pen (Aqua hold pap pen, ProSciTech, Queensland, Australia) around each sample on the glass slide. The samples were then blocked using 3% donkey serum, 3% BSA, and 0.1% Triton™ X-100 dissolved in DPBS for 10 minutes, followed by washing with 500 µL of fresh DPBS for 5 times.

Primary antibody for Na⁺V¹.6 (Abcam, Melbourne, Victoria, Australia; rabbit anti-mouse antibody, Cat #: ab65166) was reconstituted in sterile Milli-Q water as per manufacturers’ instructions. The antibody was diluted 1:300 in the blocking solution and 25 µL of the diluted solution were placed on the sample. Each sample were then covered with coverslips and incubated at 4 °C overnight. Coverslips were then carefully taken off the sample and the solution removed at the end of the incubation, and the slides were washed with 500 µL of fresh DPBS for 5 times.

The fluorescent secondary antibody (Abcam, Melbourne, Victoria, Australia; Donkey Anti-Rabbit IgG Alexa Fluor® 488, Cat #: ab150073) were then diluted 1:1000 in the blocking solution and 25 µL of the diluted solution were placed on the sample. Each sample were then covered with coverslips and incubated at 4 °C overnight. Coverslips were then carefully taken off the sample and the solution removed at the end of the incubation, and the slides were washed with 500 µL of fresh DPBS for 5 times.
Samples were mounted using 10 µL of fluorescent mounting media (Dako, Sydney, New South Wales, Australia) per sample. Individual coverslips were placed carefully over each sample and kept at 4 °C for a minimum of 2 hours to allow the mounting media to set.

The slides were then imaged with an Olympus BX-51 upright fluorescent microscope (Olympus, NY, USA), equipped with an Olympus DP-71 12Mp colour camera (Olympus, NY, USA) and an Exfo Xcite 120 lamp (Excelitas, MA, USA). Images were taken using the DP Capture software (Olympus, NY, USA) when examined through the GFP-4050A filter using the 20x/0.75 Plan Apo lens (Nikon Instruments, NY, USA).

4.3.15 Data Analysis

Data were plotted and analysed by GraphPad Prism, version 6.00. Statistical significance was defined as $P$-value < 0.05 and was determined by unpaired $t$-test assuming equal variance. Data is expressed as the mean ± standard error of the mean (SEM). CatWalk data was extracted from the CatWalk XT software (Noldus, The Netherlands) and plotted in GraphPad Prism. Relative optical density calculation and composite immunofluorescence image compilation were done using ImageJ (Public Domain software, obtained from imagej.nih.gov). Figures were assembled using Adobe Illustrator® C56, version 16.0.3.

4.4 Results

4.4.1 Mice in the burn injury model exhibited robust mechanical allodynia, thermal allodynia, and inflammation of the injured paw

A peripheral mild burn injury was produced in wild-type male C57BL/6 mice based on methods used in a previous mouse model$^{187}$ and in rat models$^{294-296}$. Exposure of the left hind paw in mice to a metal surface set at a temperature of 52.5°C for 25 seconds resulted in a first-degree or superficial burn (for detailed location see Appendix 3.5), as evidenced by the immediate development of localised redness and swelling, without the presence of blisters. Mild spontaneous pain was present immediately after burn injury (9.8 ± 2.7 pain behaviours / 5 minutes) and persisted for up to 6 hours, precluding behavioural assessment of mechanical and thermal alldynia. Once spontaneous pain subsided, a significant reduction in mechanical and thermal thresholds was observed compared to sham control animals, which was observed
within 6 hours of the burn injury and persisted for at least 4 days post burn injury (Figure 4.1 A-B). Inflammation, as evidenced by a statistically significant increase in the thickness of the affected paw of mice compared to the mice's contralateral paw, developed rapidly, peaking immediately after the burn injury (Paw thickness 6 hours: 156 ± 5 %), and persisted for at least 4 days (Figure 4.1 C).
Figure 4.1 Establishment of a mouse model of burn-induced pain after a unilateral hind paw mild heat injury

Model establishment measurements for the mouse burn-induced pain model. A) Time course of the development of mechanical allodynia assessed using the electronic von Frey apparatus. B) Time course of the development of thermal allodynia assessed using the Hargreaves method. C) Time course of development of inflammation, as measured by paw thickness normalised to the contralateral paw. Statistical significance was determined using t-test, * P-value < 0.05 compared to sham control. All data are represented as mean ± SEM; n = 3 for sham control group and n = 18 for burn group.
4.4.2 Gait abnormalities following the injury were examined holistically using the CatWalk apparatus

While gait analysis has previously been utilised to quantify pain-related behaviours in other rodent models of inflammatory and neuropathic pain\textsuperscript{297, 298}, gait abnormalities have not previously been assessed in rodent models of burn injury. Here, the chapter provides for the first time a detailed characterisation of gait analysis following a unilateral burn-injury in mice using the CatWalk XT. All paw pressure parameters (mean intensity, mean intensity of the 15 most intense pixels, max intensity) were significantly reduced in the ipsilateral hind paw compared to the contralateral hind paw (Figure 4.2 A-C), signifying a difference in weight bearing between the hind paws. Paw print area parameters (print area, max contact area) were minimally affected, perhaps confounded by the presence of swelling in the ipsilateral hind paw (Figure 4.2 D-E). Some differences in dynamic paw parameters (stand, swing, duty cycle) were detected in the first 24 hours, but lost statistical significance after 48 hours (Figure 4.2 F-H). Interlimb coordination, as measured by the regularity index, remained unchanged from sham controls, indicating that burn-injured mice followed a normal step sequence (Figure 4.2 I). As the paw pressure parameter ‘mean intensity of the 15 most intense pixels’ (Figure 4.2 B) was the most sensitive parameter to detect differences between ipsilateral and contralateral hind paws in burn-injured mice, this parameter was used to assess the effects of clinical compounds on weight bearing behaviour. Descriptions of all assessed measurements can be found in Appendix 3.4.
Figure 4.2 Characterisation of gait abnormalities in a unilateral mouse model of burn injury using selected parameters from the CatWalk XT

Time course of changes in (A-C) paw pressure, (D, E) paw print area, and (F-H) dynamic paw parameters in the ipsilateral and contralateral hind paw in burn injured animals. (I) No changes in interlimb coordination as measured by the regularity index were detected between burn and sham controls. Statistical significance was determined using t-test. * P-value < 0.05 compared to contralateral paw or sham control as indicated. All data is presented as mean ± SEM with 5-10 mice per group.
4.4.3 Analgesic efficacy of clinically used analgesics in the mouse model

To further characterise the model, the analgesic efficacy of a range of clinically used compounds were assessed in the model, as systematic assessment of clinically used analgesics has not previously been reported in burn-induced pain. Oxycodone (3 and 10 mg/kg), meloxicam, and gabapentin all significantly alleviated mechanical allodynia compared to vehicle control, however PWF was not returned to baseline values (PWF: oxycodone (3 mg/kg), 2.1 ± 0.4 g; oxycodone (10 mg/kg), 2.5 ± 0.5 g; meloxicam (5 mg/kg), 2.2 ± 0.4 g; gabapentin (100 mg/kg), 2.7 ± 0.3 g; vehicle control, 1.1 ± 0.5 g; \( P \)-value < 0.05; Figure 4.3 A), while amitriptyline and oxycodone (1 mg/kg) had no significant effect. Both oxycodone (10 mg/kg) and amitriptyline abolished thermal allodynia, significantly increasing the time of withdrawal to a radiant light stimulus compared to vehicle control (time to withdrawal: oxycodone (10 mg/kg), 12.1 ± 3.5 seconds; amitriptyline (3 mg/kg), 15.3 ± 2.0 seconds; vehicle control, 6.7 ± 1.1 seconds; * \( P \)-value < 0.05; Figure 4.3 B), while meloxicam, gabapentin and oxycodone at doses less than 3 mg/kg had no significant effect. Weight bearing on the ipsilateral hind paw was significantly increased by oxycodone (10 mg/kg) and amitriptyline (ratio to control: oxycodone (10 mg/kg), 1.27 ± 5 %; amitriptyline (3 mg/kg), 1.18 ± 7 %; vehicle control, 0.99 ± 6 %; * \( P \)-value < 0.05; Figure 4.3 C), but not by meloxicam, gabapentin or doses of oxycodone less than 3 mg/kg. The observed anti-allodynic effects of oxycodone and amitriptyline were not due to motor impairment, as only gabapentin (100 mg/kg) significantly increased the ataxia index compared with vehicle control (ataxia index: vehicle control, 2.8 ± 0.4; gabapentin, 9.6 ± 0.9. * \( P \)-value < 0.05; Figure 4.3 D).
Figure 4.3 Activity of clinically used analgesic compounds in the burn-induced pain mouse model

The analgesic efficacy of drugs clinically used to treat burn pain was examined in the mouse burn-induced pain model. A) Oxycodone (3 and 10 mg/kg), meloxicam, gabapentin, and amitriptyline partially but significantly alleviated mechanical allodynia as assessed by electronic von Frey. B) Only oxycodone (10 mg/kg) and amitriptyline significantly reversed thermal allodynia assessed by the Hargreaves method. C) Oxycodone (10 mg/kg) and amitriptyline led to a significant increase in weight bearing of the ipsilateral hind paw, as detected by the ‘Mean Intensity of the 15 most intense pixels’ parameter using the Catwalk XT. D) Only gabapentin (100 mg/kg) had a significant effect on motor impairment, significantly increasing the ataxia index (calculated as the ratio of the total number of foot slips to the total metres travelled; all numbers are ratios and are therefore without units) compared to vehicle control. Statistical significance was determined using t-test. * P-value <
0.05 compared to vehicle control. Dotted lines represent the mean value from sham control mice that did not undergo the burn injury. All data are represented as mean ± SEM with 5-17 mice per group.
4.4.4 Analgesic effect of selective Na\textsubscript{V} inhibitors in different burn-induced pain modalities

The model was then used to ascertain the analgesic efficacy of various selective Na\textsubscript{V} inhibitors administered peripherally via i.pl injection (injection into the left paw; for exact location see Appendix 3.5), both to validate the previous finding that Na\textsubscript{V}1.7 affects burn-induced thermal allodynia and to investigate the involvement of various Na\textsubscript{V} isoforms in burn-induced mechanical allodynia. Intraplantar injection was chosen over intraperitoneal injection due to a variety of factors, including limited availability of the compound (Pnc1a in particular) and systemic side effects following systemic administration of other compounds (such as TTX). Moreover, I sought to understand the effect of local Na\textsubscript{V} inhibition in neurons directly affected by the burn injury, as opposed to general inhibition of both affected and unaffected neurons.

Thermal allodynia was examined with the thermal threshold probe instead of the Hargreave’s apparatus. The Hargreave’s apparatus required mice to be re-habituated in the cages following drug injection for an average of 1 hour. However, the rapid onset and offset of Na\textsubscript{V}-inhibitory effects of the peptides when administered i.pl (effects are observed to completely disappear at an average of 10-30 minutes following injections) makes it unpractical to test the effect of the peptides with the Hargreave’s apparatus, as the analgesic effect is likely to have disappeared after the re-habitation period and could not be assessed.

Thermal allodynia was significantly alleviated with TTX (1 µM, i.pl), which conformed to the previous observation that the inhibition of Na\textsubscript{V}1.7, a TTX-s isoform, significantly diminished burn-induced thermal allodynia\textsuperscript{187} (Paw withdrawal temperature: TTX, 46.1 ± 1.4 °C; vehicle control 43.3 ± 0.6 °C; * P-value < 0.05; Figure 4.4 A). The inhibition of TTX-resistant Na\textsubscript{V}1.8 using A-803467 (10 µM, i.pl.) did not revert thermal alldyinia (Paw withdrawal temperature: A-803467, 44.9 ± 0.8 °C; vehicle control, 43.3 ± 0.6 °C; Figure 4.4 A). Injection of Pnc1a (1 µM, i.pl), a selective Na\textsubscript{V}1.7 peptide inhibitor extracted from the venom of Pamphobeteus nigricolor and characterised extensively in-house (data not shown), successfully alleviated thermal alldyinia, which concurred with data obtained from Na\textsubscript{V}1.7-knockout mice\textsuperscript{187} (Paw withdrawal temperature: Pnc1a, 47.3 ± 1.0 °C; vehicle control 43.3 ± 0.6 °C; * P-value < 0.05; Figure 4.4 A). GIIIA (10 µM, i.pl), a venom peptide that is inhibitory for Na\textsubscript{V}1.1, Na\textsubscript{V}1.2, Na\textsubscript{V}1.4, and Na\textsubscript{V}1.6, did not significantly alter the symptoms of thermal allodynia, suggesting none of those Na\textsubscript{V} isoforms contribute substantially towards
the neuronal activity responsible for burn-induced thermal alldynia (Paw withdrawal temperature: GIIIA, 43.3 ± 1.2 °C; vehicle control, 43.3 ± 0.6 °C; Figure 4.4 A).

Mechanical alldynia from burn injury was examined using the electrical von Frey system. TTX (1 µM, i.pl) was first tested to ascertain whether TTX-s or TTX-r Na\textsubscript{v} isoforms predominantly affect mechanical alldynia in burn injury. Since TTX significantly reverted symptoms of mechanical alldynia symptom (PWF: TTX, 2.2 ± 0.3 g; vehicle control, 1.2 ± 0.1 g; * P-value < 0.05; Figure 4.4 B), TTX-s Na\textsubscript{v} isoforms were determined to play a major role in burn-induced mechanical alldynia. To confirm that TTX-r Na\textsubscript{v} isoforms do not contribute to burn-induced mechanical alldynia, A-803467 (10 µM, i.pl.) was used to inhibit Na\textsubscript{v}1.8 and was indeed found to be ineffective (PWF: A-803467, 1.2 ± 0.2 g; vehicle control, 1.2 ± 0.1 g; Figure 4.4 B). Pnc1a (1 µM, i.pl.) was therefore tested due to the known efficacy of Na\textsubscript{v}1.7 inhibition in burn-induced thermal alldynia, and also to validate the observation in knockout mice that Na\textsubscript{v}1.7 gene knockout did not alleviate burn-induced mechanical alldynia. Pnc1a was not effective in modifying mechanical alldynia (PWF: Pnc1a, 0.8 ± 0.2 g; vehicle control, 1.2 ± 0.1 g; Figure 4.4 B), suggesting that Na\textsubscript{v}1.7 inhibition does not contribute to TTX-s Na\textsubscript{v} isoform-mediated reversal in burn-induced mechanical alldynia. GIIIA (10 µM, i.pl.) was then tested to examine the efficacy of inhibiting Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.4, and Na\textsubscript{v}1.6, and significantly alleviated paw withdrawal threshold to levels comparable to TTX administration (PWF: GIIIA, 2.0 ± 0.3 g; TTX, 2.2 ± 0.3 g; vehicle control, 1.2 ± 0.1 g; * P-value < 0.05; Figure 4.4 B). To better elucidate the contribution of various Na\textsubscript{v} isoforms inhibited by GIIIA, TIIIA (10 µM, i.pl.), a conotoxin with known inhibitory activity for Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, and Na\textsubscript{v}1.4\textsuperscript{212}, was administered and did not demonstrate a significant difference in PWF compared to vehicle control (PWF: TIIIA, 1.0 ± 0.2 g; vehicle control, 1.2 ± 0.1 g; Figure 4.4 B). Thus, these results suggest that the inhibition of Na\textsubscript{v}1.6 in the periphery caused a significant reduction in burn-induced mechanical alldynia in the mouse model, but did not affect burn-induced thermal alldynia, and the opposite was observed for Na\textsubscript{v}1.7 inhibition. Lastly, weight bearing was not alleviated from the local administration of GIIIA or TTX, suggesting that while TTX-s Na\textsubscript{v} isoforms are vital in mediating mechanical and thermal alldynia, these channels were not effective in modulating weight bearing.
Figure 4.4 Na\textsubscript{V}1.6 inhibition alleviated burn-induced mechanical allodynia but was not effective in burn-induced thermal allodynia

The analgesic effect of Na\textsubscript{V} inhibitors were used to examine the contribution of Na\textsubscript{V} channels in different modalities of burn-induced pain. Peripheral Na\textsubscript{V}1.6 inhibition significantly reduced mechanical allodynia and did not affect thermal allodynia, and the opposite was observed in peripheral Na\textsubscript{V}1.7 inhibition. A) Both TTX and Pnc1a significantly reverted mechanical allodynia as assessed by the electronic von Frey system. B) TTX and GIIIA significantly reversed thermal allodynia as measured by the thermal probe method. The involvement of Na\textsubscript{V}1.6 was determined by utilising TIIIa to inhibit other Na\textsubscript{V} isoforms (Na\textsubscript{V}1.1, Na\textsubscript{V}1.2, and Na\textsubscript{V}1.4) also affected by GIIIA. C) TTX-s Na\textsubscript{V} isoforms do not revert gait abnormalities of the injured mice as observed in the CatWalk. Statistical significance was determined using \textit{t}-test. * \textit{P}-value < 0.05 compared to vehicle control. Dotted lines represent the mean value from sham control mice that did not undergo the burn injury. All data are represented as mean ± SEM with 4-15 mice per group.
4.4.5 Validation of Na\textsubscript{V}1.6 antibodies for selectivity in immunofluorescence

Since pharmacological characterisation of the model suggested the involvement of Na\textsubscript{V}1.6 in mechanical allodynia, immunofluorescence (IF) was used to examine Na\textsubscript{V}1.6 expression in DRG neurons following the burn injury. Two antibodies, manufactured by Abcam (category number: ab65166) and Alomone (category number: ASC-009), were validated in HEK293 cells expressing hNa\textsubscript{V}1.1-1.8 (Figure 4.5) for immunofluorescence.

The anti-Na\textsubscript{V}1.6 antibody produced by Abcam selectively labelled HEK293 cells expressing Na\textsubscript{V}1.6 over cells expressing Na\textsubscript{V}1.1-1.5, 1.7, and 1.8 (Figure 4.5 A). In comparison, the Na\textsubscript{V}1.6 antibody produced by Alomone exhibited significant labelling for HEK293 cells expressing Na\textsubscript{V}1.1-1.4 as well as cells expressing Na\textsubscript{V}1.6 (Figure 4.5 B), indicating a lack of Na\textsubscript{V}1.6 selectivity. The Abcam Na\textsubscript{V}1.6 antibody was therefore selected for immunofluorescence studies in native tissues for Na\textsubscript{V}1.6 expression changes following the burn injury.
Figure 4.5 Validation of commercially available Na\textsubscript{\textit{V}}1.6 antibodies

Two commercially available antibodies for Na\textsubscript{\textit{V}}1.6, produced by Abcam (category number: ab65166) and Alomone (category number: ASC-009), were tested for Na\textsubscript{\textit{V}}1.6 selectivity in HEK293 cells expressing Na\textsubscript{\textit{V}}1.1-1.8. The antibody produced by Abcam exhibited more Na\textsubscript{\textit{V}}1.6 selectivity than the Alomone antibody and was chosen to stain native tissues from the burn injury model. A) Selectivity of the Abcam Na\textsubscript{\textit{V}}1.6 antibody in HEK293 cells expressing Na\textsubscript{\textit{V}}1.1-1.8 channels. Marked fluorescence was observed only in HEK293 cells expressing Na\textsubscript{\textit{V}}1.6. B) Selectivity of the Alomone Na\textsubscript{\textit{V}}1.6 antibody in HEK293 cells expressing Na\textsubscript{\textit{V}}1.1-1.8 channels. Marked fluorescence was observed in cells expressing Na\textsubscript{\textit{V}}1.1, 1.2, 1.3, and 1.6 isoforms. Binding was also observed in cells expressing Na\textsubscript{\textit{V}}1.4, 1.5, and 1.7 to a lesser degree. As the antibody exhibits binding to Na\textsubscript{\textit{V}} isoforms other than Na\textsubscript{\textit{V}}1.6, the antibody produced by Alomone was deemed to be inappropriate for use in future studies and the Abcam antibody was chosen to study the expression of Na\textsubscript{\textit{V}}1.6 in native tissues.
4.4.6 The expression of Na\textsubscript{V}1.6 in DRG neurons affected by the burn injury

Cryo-sections of DRGs extracted from sham control mice and mice with the burn injury were compared using IF staining, with the hypothesis that as Na\textsubscript{V}1.6 inhibition successfully alleviated burn-induced mechanical allodynia, Na\textsubscript{V}1.6 expression could have become up-regulated in DRG neurons following the injury. However, IF studies did not indicate an observable difference in Na\textsubscript{V}1.6 expression in L3, L4, and L5 DRG neurons following the burn injury (Figure 4.6 A-B). Analysis of the relative optical density of the IF images between the sham cohort and the burn injury cohort showed no significant differences between the two groups (Relative Optical Density: Burn group, 1244 ± 84.9; Control group (Normal), 1421 ± 119.4; Figure 4.6 C. Images were representative of 6 control samples and 13 burn injury samples over 2 separate experiments), indicating that the expression of Na\textsubscript{V}1.6 in DRG neurons was not altered significantly despite the efficacy of Na\textsubscript{V}1.6 inhibition 3 days following the burn injury.
A Control sample
B Burn sample

C

Relative optical density (ROD)

Burn  Normal
Figure 4.6 Immunofluorescence imaging was used to examine the expression of Na\(\text{\textgreek{v}}\)1.6 in DRG neurons before and after the peripheral burn injury

A) Representative image of control DRG slices from animals that did not undergo the burn injury (6 images from 2 separate experiments). B) Representative image of DRG slices at 3 days following the burn injury, without the administration of any compounds to the animal (13 images from 2 separate experiments). C) Comparison of average relative optical density (ROD) of immunofluorescence images indicate no significant differential expression of Na\(\text{\textgreek{v}}\)1.6 was detected in DRG neurons following the burn injury. Image analysis was conducted using Image J. Bar graph is presented as mean ± SEM.
4.5 Discussion

This chapter established and characterised a mouse model of burn injury-induced pain using behavioural and pharmacological approaches. This model appears suitable for analgesic efficacy profiling of novel treatments that may provide improved management approaches for this under-treated and difficult-to-manage condition. In contrast to previously reported mouse models\textsuperscript{187}, this model causes a superficial or first-degree burn, without the presence of blisters or broken skin, while still producing robust mechanical and thermal alldynia as well as detectable differences in weight bearing behaviour.

Mechanical and thermal alldynia are both abnormal stimulus-induced sensations that may mimic elevated pain experienced by patients during procedures such as dressing changes\textsuperscript{285}. However, stimulus-evoked alldynia may be poorly reflective of spontaneous or background pain and I thus evaluated the ability of the CatWalk XT system to detect burn-induced gait abnormalities as a surrogate for non-stimulus evoked pain. Consistent with previous studies, I found a good correlation between development of altered CatWalk XT parameters, specifically those relating to paw contact with the glass walkway, with mechanical and thermal alldynia detected by more traditional behavioural assessment. However, gait abnormalities were only alleviated by high dose oxycodone and amitriptyline, suggesting that these parameters are not simply a reflection of mechanical alldynia but represent a distinct pain modality. It is possible that this discrepancy arises from interference of local inflammation and paw swelling with normal gait. Accordingly, it is currently unclear whether analgesic effects detected using the CatWalk XT system can be translated more readily to clinical efficacy in human burn patients than effects detected using conventional tests such as the von Frey or Hargreaves apparatus. The often poor predictive power of conventional assessment of evoked nociceptive behaviours suggests that effects on gait should be assessed routinely, as these parameters may reflect clinically meaningful measures of pain and analgesia.

Mild burn injuries affecting less than 10% of total body surface area represent the overwhelming majority of burn injuries in the clinical setting and account for approximately 80% of all hospital or emergency service burn admissions\textsuperscript{299}, making this model relevant to many patients. Clinically used analgesics and adjuvants known to have some efficacy in burn-induced pain, such as oxycodone and gabapentin\textsuperscript{300, 301}, reduced mechanical and thermal
allodynia as well as gait abnormalities, supporting the translational validity of my model. Specifically, gabapentin and amitriptyline, two drugs used as chronic pain adjuvant treatments, were effective at alleviating mechanical and thermal allodynia respectively. However, both drugs were only partially anti-allodynic, consistent with effects seen in the clinic.

A small human study indicated the administration of a single 1200 mg dose of gabapentin effectively alleviated mechanical allodynia immediately following burn injuries, but lacked efficacy in acute pain post-burn as an adjunct analgesic in a randomised control trial involving 53 patients. Similarly, amitriptyline is widely used for management of chronic pain, and intraplantar administration of amitriptyline also produced thermal antihyperalgesia in a rat model of local mild burn. My model identified both gabapentin and amitriptyline as potentially effective analgesic compounds in burn-induced pain, although systematic clinical trials assessing efficacy of adjuvants in human burn patients are lacking.

Opioids form the mainstay of therapy for burn-induced pain, albeit the specific drug of choice varies. Oxycodone, a commonly used µ opioid receptor agonist, successfully reversed mechanical allodynia, thermal allodynia, as well as weight bearing parameters. Notably, burn injury-induced pain behaviours were relatively opioid-resistant compared with doses required to elicit analgesia in simple nociceptive models (1-2 mg/kg). A requirement for higher opioid doses to achieve effective analgesia has also been noted for human burn patients, suggesting that the pathophysiological mechanisms underpinning burn-injury induced pain incorporate elements that lead to decreased efficacy of opioids. This phenomenon can either lead to ineffective analgesia due the clinician’s unwillingness to prescribe beyond standard opioid doses, or place patients at higher risk of opioid adverse effects.

As NaV1.7 knockout animals did not develop burn-induced thermal allodynia but exhibited robust burn-induced mechanical allodynia, this chapter further explored the contribution of various NaV isoforms in burn-induced thermal and mechanical allodynia. TTX was examined first in thermal alldonya and was found to be effective, suggesting that TTX-s NaV isoforms are involved in burn-induced thermal allodynia. NaV1.8 inhibition subsequently demonstrated no effect on thermal alldonya, reiterating that TTX-s NaV channels are the mediators of the nociceptive signals in this modality. The finding also concurred with the previous finding that NaV1.8 and NaV1.9 knockout mice displayed no differences in burn-induced thermal
allodynia compared to wild-type mice\textsuperscript{187}. The inhibitor used for Na\textsubscript{\text{V}}1.8, the small molecule A-803467, exhibits high potency for Na\textsubscript{\text{V}}1.8 (IC\textsubscript{50} = 8 nM) with more than a-hundred-fold selectivity for Na\textsubscript{\text{V}}1.8 compared to Na\textsubscript{\text{V}}1.2, Na\textsubscript{\text{V}}1.3, and Na\textsubscript{\text{V}}1.7\textsuperscript{210}, and is widely used in animal models and in electrophysiology for Na\textsubscript{\text{V}}1.8 inhibition\textsuperscript{134,174,204}. Na\textsubscript{\text{V}}1.7 inhibition via Pnc1a i.pl injection resulted in the highest paw withdrawal temperature among all experimental cohorts tested, but Na\textsubscript{\text{V}}1.7 inhibition did not revert the paw withdrawal temperature to that observed in uninjured mice (dotted line, average of 49.6 °C, n = 15, Figure 4.4 A). In comparison, Shields et al.\textsuperscript{187} observed a complete reversal of thermal allodynia in Na\textsubscript{\text{V}}1.7 knockout animals 3 days after the injury. This discrepancy could be due to physiological differences between global gene knockout and local functional inhibition. Treatment with GIIIA, a peptide that inhibits Na\textsubscript{\text{V}}1.1, 1.2, and 1.6, did not alleviate thermal allodynia in the mice, indicating these isoforms are also unrelated to burn-induced thermal allodynia.

The study conducted by Shields et al.\textsuperscript{187} showed that Na\textsubscript{\text{V}}1.7 knockout mice demonstrated no behavioural differences compared to control wild-type mice in burn-induced mechanical allodynia, suggesting a lack of Na\textsubscript{\text{V}}1.7 involvement in that pain modality. At the same time, the study conducted by Salas et al.\textsuperscript{306} found subcutaneous injections of TTX to successfully alleviate mechanical allodynia in rats with a peripheral burn injury. Such evidence would suggest TTX-s Na\textsubscript{\text{V}} isoforms apart from Na\textsubscript{\text{V}}1.7 are responsible for burn-induced mechanical allodynia. In my burn injury model, the peripheral administration of TTX was found to be effective in abolishing mechanical allodynia, confirming the contribution of TTX-s Na\textsubscript{\text{V}} isoforms. Further, the administration of the highly selective Na\textsubscript{\text{V}}1.7 inhibitor Pnc1a elicited no change in mechanical withdrawal thresholds in injured mice compared to control mice. I therefore confirmed that Na\textsubscript{\text{V}}1.7 does not affect burn-induced mechanical allodynia. TTX-r Na\textsubscript{\text{V}} isoforms were shown to be ineffective at reversing burn-induced mechanical allodynia, seen both in my model and in Na\textsubscript{\text{V}}1.8 and Na\textsubscript{\text{V}}1.9 knockout mice in the publication by Shields et al.\textsuperscript{187}.

The peripheral administration of GIIIA, however, restored mechanical pain threshold to levels comparable to that achieved by TTX. The conotoxin GIIIA inhibits Na\textsubscript{\text{V}}1.1, Na\textsubscript{\text{V}}1.2, Na\textsubscript{\text{V}}1.4, and Na\textsubscript{\text{V}}1.6\textsuperscript{211}. In order to further dissect the contribution of different Na\textsubscript{\text{V}} isoforms in burn-induced mechanical allodynia, TIII A was also administered in the mice model and was found to be ineffective towards mechanical allodynia. TIII A inhibits Na\textsubscript{\text{V}}1.1, Na\textsubscript{\text{V}}1.2,
and \( \text{Na}_V \, 1.4^{211,212} \). As \( \text{Na}_V \, 1.4 \) is a muscle \( \text{Na}_V \) isoform not present in neurons, the discrepancy between the results for GIIIA and TIIIA implies that \( \text{Na}_V \, 1.6 \) plays a pivotal role in burn-induced mechanical allodynia. Such findings make \( \text{Na}_V \, 1.6 \) a potential pharmacological target for burn-induced mechanical allodynia, further adding to the roles \( \text{Na}_V \, 1.6 \) already play in various \( \text{Na}_V \, 1.7 \)-independent pain states\(^2,133\).

Examples of \( \text{Na}_V \, 1.6 \) contributing to \( \text{Na}_V \, 1.7 \)-independent pain states include neuropathies caused by oxaliplatin and cisplatin\(^{133}\), two platinum-based chemotherapy drugs. For example, only GIIIA administration was successful at alleviating oxaliplatin-induced cold allodynia\(^2\), whereas selective inhibitors for all \( \text{Na}_V \) isoforms as well as inhibitors of TRPA1, TRPV1, and TRPM8 all proved to have no efficacy. In particular, oxaliplatin-induced cold allodynia was reproduced by the combined administrations of the \( \text{K}_V \) inhibitor 4-aminopyridine and the \( \text{Na}_V \, 1.6 \) activator Cn2\(^{2,202}\). Cisplatin-induced mechanical allodynia in mice following peripheral administration of cisplatin was also only alleviated by GIIIA, with TIIIA being ineffective at affecting mice symptoms\(^{133}\). Both oxaliplatin and cisplatin-induced neuropathies are \( \text{Na}_V \, 1.7 \)-independent pain conditions, much the same as burn-induced mechanical allodynia.

The different mechanisms between burn-induced thermal and mechanical allodynia could be due to the involvement of different peripheral fibres. \( \text{Na}_V \, 1.7 \) is diffusely expressed in C-fibres and in the nodes of Ranvier of a portion of A\( \delta \)-fibres within the sciatic nerve and dorsal root\(^{171}\), while \( \text{Na}_V \, 1.6 \) is expressed diffusely in C-fibres as well as in the nodes of Ranvier of all A-fibres\(^{191}\). A\( \delta \)-fibres are known to conduct fast pain signals\(^{10}\) and allow for protective reflexes away from painful stimuli, and selective A\( \delta \)-fibre block in humans causes a loss in the perception of pain modality information, such as noxious heat or cold, and leaves subjects feeling only pain\(^{10}\). A\( \delta \)-fibres are further divided into high-threshold and low-threshold heat sensors\(^{8}\), as well as various polymodal sensors\(^{11,12}\). Different contributions of A and C-fibres towards mechanical and thermal allodynia have also been noted in other pain conditions. A-fibre block in capsaicin-induced pain resulted in a 75% reduction of mechanical allodynia, but retained the burning sensation felt from capsaicin\(^{22,23}\). At the same time, spinal nerve ligation in rats resulted in increased excitability of mechano-sensitive A-fibres, as well as an increase in the size of these fibres' receptive fields and maximum firing frequency\(^{24}\). Such findings have led towards the preposition that A-fibres contribute more towards mechanical allodynia, while C-fibres are responsible for thermal hyperalgesia. In light of this chapter's
findings, which indicate \( \text{Na}_V1.7 \) mediates burn-induced thermal allodynia but not mechanical alldynia, these data align with the hypothesis that A-fibres contribute more towards burn-induced mechanical alldynia and C-fibres play a greater role in burn-induced thermal alldynia.

I therefore wanted to examine if the expression of \( \text{Na}_V1.6 \) in DRG neurons of the mice with the burn injury was elevated. However, the expression of \( \text{Na}_V1.6 \) in L3-L5 DRG neurons was not changed following the injury. While the overall expression of \( \text{Na}_V1.6 \) was unchanged in DRG neuron somas, the presence of \( \text{Na}_V1.6 \) along the sensory neuron axons could have been altered following the burn injury due to altered trafficking. There is certainly existing evidence that \( \text{Na}_V1.6 \) distribution is changed along the axons in neuronal injury models\(^{197,199} \), and future studies investigating the function and distribution of \( \text{Na}_V \) channels in the axon would provide useful insight in selective \( \text{Na}_V \) isoform trafficking in various pain conditions.

At the same time, the quality of the commercially available \( \text{Na}_V1.6 \) antibodies was also concerning, as seen in Figure 4.5. While the Abcam \( \text{Na}_V1.6 \) antibody was superior to the \( \text{Na}_V1.6 \) antibody produced by Alomone, the selectivity of the Abcam antibody remains mediocre. Additionally, more precise measurements to further investigate the expression levels of \( \text{Na}_V1.6 \) such as RNA expression analysis and PCR (polymerase chain reaction) are also warranted to assess expression changes quantitatively. It also should not be overlooked that other proteins, not necessarily \( \text{Na}_V \) channels, could be differentially expressed following the burn injury. I therefore decided to conduct a RNA sequence analysis on the ipsilateral DRGs of mice from the burn-induced pain model, to be described in the next chapter, and analyse in-depth the expression of genes following the injury.

### 4.6 Conclusions

This chapter describes a mouse model of burn-induced pain associated with mechanical allodynia, thermal allodynia, inflammation, and gait abnormalities, which all developed within a day of the thermal insult and lasted for at least four days. This chapter also presents, for the first time, indications that \( \text{Na}_V1.6 \) is involved in mediating burn-induced mechanical alldynia, a condition previously established as being independent of \( \text{Na}_V1.7 \) involvement. However, immunofluorescence analysis of affected DRG neurons indicate a lack of change in the expression of \( \text{Na}_V1.6 \), and highlight the unreliability of available commercial \( \text{Na}_V1.6 \)
antibodies. Therefore, I conducted a RNA sequence (RNA-seq) transcriptomic analysis of L3-L5 ipsilateral DRGs to better investigate the changes in the landscape of gene expression following the burn injury, to be presented in the next chapter of this thesis.
Chapter 5 – Bioinformatic investigation of the mice burn injury model and identification of putative novel pharmacological targets for burn-induced pain

5.1 Foreword

The previous chapter described the successful establishment of a burn-induced pain model in mice, and how it was used to effectively evaluate the contribution of various Na\textsubscript{V} isoforms in different modalities of burn-induced pain. However, the subsequent attempt to analyse potential expression changes of Na\textsubscript{V}1.6 using immunofluorescence methods indicated a lack of Na\textsubscript{V}1.6 expression changes in ipsilateral DRG neurons following the burn injury, despite the efficacy of Na\textsubscript{V}1.6 inhibition in alleviating burn-induced mechanical allodynia. In order to quantitatively analyse all gene expression changes following the burn injury, I have conducted a RNA-seq analysis on the ipsilateral DRG neurons from the burn pain model, and to research whether known modulators of the protein products of genes differentially expressed may be used as putative therapeutic leads for analgesia.

Some data presented in this chapter have been accepted for publication by Molecular Pain\textsuperscript{282}. The content has been edited and additional data has been added to enhance the flow of the thesis.

5.2 Introduction

The development of rapid RNA sequencing techniques have revolutionised the field of neuropharmacology, with sequencing being employed increasingly frequently to discern changes in transcription following a nerve injury\textsuperscript{307, 308}, genetic mutations that cause neuronal disorders\textsuperscript{127}, or selective protein expression that could be related to functional neuron subclasses\textsuperscript{32, 131, 309}. The expression changes exhibited by neurons during chronic pain conditions have been particularly exciting, as it can be hypothesised that an up-regulation of certain genes could indicate these genes are actively related to the establishment and maintenance of neuropathic pain, while the opposite can be hypothesised of genes down-
regulated, presenting researchers with a novel way to examine the molecular causes of neuropathic pain.

The burn-induced pain mouse model, described in Chapter 4, demonstrated that burn-induced allodynia was alleviated after local inhibition of Na\textsubscript{v} isoforms. While previous transcriptomic studies have examined burnt skin, muscle, and circulating leukocytes of burn patients\textsuperscript{310-312}, there was no published transcriptomic analysis of murine DRG neurons affected by a burn injury. Bioinformatic analysis of skeletal muscle directly affected by a burn injury and muscle distant from the burn site have both been assessed in mice, revealing the differential expression of genes involved with inflammation, defence mechanisms, and chemokines\textsuperscript{310}. Similarly, the transcriptome from circulating leukocytes following severe burn injuries in humans found that genes involved in innate immunity were significantly up-regulated\textsuperscript{311}. In comparison, a study of chemical burn injury on the porcine skin indicated genes involved in cellular movement and signalling were differentially expressed, suggesting that various types of burn injuries affect different genes.

This chapter thus presents the first transcriptomic study of murine DRGs following a peripheral burn injury, utilising the burn-injury model established in Chapter 4. The data this chapter present give a view into the neuronal molecular changes in burn-induced pain, as well as pointing towards potential molecular targets that can be investigated to try to alleviate burn-related pain. Gene ontology was also analysed based on differentially expressed genes. Proglumide, a known inhibitor the of cholecystokinin B receptor, which was found to be up-regulated at the gene level following the burn injury, was tested for analgesic efficacy in the mouse model. Cell models investigated in Chapter 3 were also re-evaluated to assess their appropriateness in testing novel targets found from the bioinformatic analysis of DRGs affected by the burn injury.

5.3 Materials and Methods

5.3.1 Chemicals

All materials were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia) unless otherwise stated. Oxycodone was obtained from Mundipharma Pty Ltd (Sydney, New South Wales, Australia).
5.3.2 Animals

Male wild-type C57BL/6 mice age 6 - 8 weeks and weighing 20 - 23 grams were used in the study. Ethical approval for experiments involving live animals and animal tissues was obtained from the University of Queensland animal ethics committee under the ethics approval number PHARM/074/13/RAMACIOTTI. Experiments were conducted in accordance with the Animal Care and Protection Regulation Queensland (2012), the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th edition (2013) and the International Association for the Study of Pain Guidelines for the Use of Animals in Research.

All animals were housed in groups of 2 - 4 per cage in a stable environment under 12 hour light-dark cycles and had access to standard rodent chow and water ad libitum. A red polycarbonate Mouse House (Tecniplast, Italy) and shredded paper nesting material were supplied for enrichment. All animals were obtained from University of Queensland Biological Resources (Brisbane, Australia).

Sample sizes of each experiment are detailed in the figure legends of the corresponding figure.

5.3.3 Induction of burn injury

The burn injury was induced with modifications from that described in Section 4.3.3, using temperature and burn durations according to a previous mouse model\textsuperscript{187}. A pair of tweezers, with the tips wrapped in tissue paper, was used to press the plantar portion of the paw onto the Peltier plate. Sham control animals underwent the same procedure with the plate set at 22 °C (room temperature). Behavioural assessment was performed at the time points indicated and the same animals were used for each of the behavioural tests.

5.3.4 RNA extraction

On the third day following burn or sham injury, animals were euthanised with carbon dioxide followed by cervical dislocation. The spine was immediately removed and the dorsal side of
the vertebra dissected to expose the spinal cord. The entire spine was then immersed in ice-cold Dulbecco’s Modified Eagle’s Medium (DMEM). The spinal cord was then removed, and L3, L4, and L5 DRGs were extracted from the ipsilateral side of the injury with forceps. The DRGs were immediately placed into RNALater (Thermo Fisher Scientific, Scoresby, Victoria, Australia) and excess myelin was trimmed. DRGs were then cut into pieces in RNALater, and RNA was extracted using the RNeasy Mini Kit (Qiagen, Melbourne, Australia) according to manufacturer’s instructions (as stated in Section 3.3.6), including on-column DNase digestion. Samples from 4 individual animals were pooled together for one biological replicate. Three biological control replicates and three burn injured biological replicates were analysed to ensure statistical significance.

5.3.5 RNA-Seq

Library construction and bioinformatics analysis were conducted by the Institute for Molecular Bioscience Sequencing Facility (The University of Queensland, Brisbane, Australia). RNA-Seq was conducted on the Illumina NextSeq 500 platform as 75-nucleotide single-end runs, with libraries prepared using TruSeq stranded total RNA library preparation. Reads were mapped using the STAR aligner (version star/2.3.0e) and samtools. Reads were mapped to the Ensembl Mus musculus mm10 genome (version GRCm38). Count tables were generated using HTSeq_count from HTSeq package, and differential expression analysed using the R (version 3.2.2) package DESeq2.

5.3.6 Bioinformatics analysis

Plots were generated using the R packages gplots, ggplots2, and pheatmap. Differentially expressed genes from DESeq2 analysis were ranked by adjusted \( P \)-value. Genes with adjusted \( P \)-value < 0.05 were used for gene ontology (GO) analysis, which was conducted using Cytoscape 3.2.1 with the ClueGO 2.1.7 plugin. ClueGO was used with the following settings: Ontologies/Pathways used Mus musculus GO (Biological Processes, version from 30 April 2015). Evidence: all. GO tree internal 3-8 levels. GO term restrictions: 3 minimum genes and 1% of genes. Network connectivity (Kappa score) = 0.4. STRING diagrams were generated using STRING v10. Following alignment, count output from HTSeq was analysed using the DESeq2 statistical package.
5.3.7 Drug treatment

The analgesic efficacy of compounds was assessed 3 days after the induction of burn injury or the sham procedure. All compounds were administered by intraperitoneal injection (i.p) with compound concentration of 10 μL/g using a 30-gauge needle. Animals were randomised to receive either proglumide (30 mg/kg) once daily or matched vehicle control, with the first dose administered at the time of the burn injury. Oxycodone (1 mg/kg) was administered once only on the day of testing. All compounds were diluted in DPBS (Life Technologies, Mulgrave, Victoria, Australia). Behavioural assessment was performed at least 30 minutes after the final dose of all compounds by an investigator unaware of the treatments received by each individual animal.

5.3.8 Measurement of mechanical allodynia

Mechanical allodynia was assessed as described in Section 4.3.4.

5.3.9 Measurement of thermal allodynia

Thermal allodynia was assessed as described in Section 4.3.5. Only the Hargreave’s apparatus was used for results presented in Chapter 5.

5.3.10 Measurement of gait abnormalities

Gait analysis was assessed using the CatWalk XT analysis system (Noldus Information Technology, The Netherlands) as described in Section 4.3.7.

5.3.11 Motor Assessment

Motor performance was assessed as described in Section 4.3.9.

5.3.12 Heatmap generation

Genes found to be differentially expressed in burn samples were analysed in the cell lines examined in Chapter 3. For heatmap generation, data was compiled into .csv format and
heatmaps were created in RStudios with R 3.2.2 with the gplots, pheatmap, and RColorBrewer libraries.

5.4 Results

5.4.1 All samples were aligned to the mouse genome

All samples were uploaded and available for the public under the Gene Expression Omnibus accession number GSE75691. Data includes raw .fastq files and count tables produced with HTSeq as .txt files.

The transcriptomic analysis of gene expression changes in whole DRGs was conducted using Illumina NextSeq 500 RNA-seq. A total of 4,302 billion reads, 72% of which were uniquely mapped to the *Mus musculus* genome, were generated from all samples. All samples were aligned with the mouse genome (mm10). Three separate samples were extracted from the control cohort and from mice with the burn injury. All RNA samples were extracted from L3, L4, and L5 whole DRGs.
<table>
<thead>
<tr>
<th></th>
<th>Control #1</th>
<th>Control #2</th>
<th>Control #3</th>
<th>Burn #1</th>
<th>Burn #2</th>
<th>Burn #3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total reads</strong></td>
<td>60256073</td>
<td>29770261</td>
<td>138943352</td>
<td>127060392</td>
<td>39879029</td>
<td>48647489</td>
</tr>
<tr>
<td><strong>Aligned reads</strong></td>
<td>46413203</td>
<td>21967696</td>
<td>107534778</td>
<td>95718380</td>
<td>28751886</td>
<td>36988499</td>
</tr>
<tr>
<td></td>
<td>(77.03%)</td>
<td>(73.79%)</td>
<td>(77.39%)</td>
<td>(75.33%)</td>
<td>(72.10%)</td>
<td>(76.03%)</td>
</tr>
<tr>
<td><strong>Reads with no feature</strong></td>
<td>10196437</td>
<td>4576618</td>
<td>25087845</td>
<td>25266646</td>
<td>6585482</td>
<td>6728193</td>
</tr>
<tr>
<td></td>
<td>(16.92%)</td>
<td>(15.37%)</td>
<td>(18.06%)</td>
<td>(19.89%)</td>
<td>(16.51%)</td>
<td>(13.83%)</td>
</tr>
<tr>
<td><strong>Ambiguous reads</strong></td>
<td>225655</td>
<td>117170</td>
<td>478791</td>
<td>449063</td>
<td>142839</td>
<td>185837</td>
</tr>
<tr>
<td></td>
<td>(0.37%)</td>
<td>(0.39%)</td>
<td>(0.34%)</td>
<td>(0.35%)</td>
<td>(0.36%)</td>
<td>(0.38%)</td>
</tr>
</tbody>
</table>

**Table 5.1 Read numbers of RNA samples**

RNA samples were obtained from DRGs from burn-injured animals and control animals that underwent a sham procedure. The samples were aligned to the mouse genome (mm10). ‘Total reads’ show the amount of reads in each alignment. 'Aligned reads' indicate the number of reads matched to one unique position in the genome. ‘Reads with no feature’ indicate the number of reads that did not match to any position in the genome. 'Ambiguous reads' indicate the number of reads mapped to more than one position in the genome. All percentage values are based on the number of total reads.
5.4.2 Thirty genes were found to be differentially expressed in the burn injury model

A total of 30 genes were differentially expressed at $P$-value < 0.05 in whole DRGs after the burn injury (Table 5.2, for full list see Appendix 3.6), with the level of gene expression ranging from at least log2FoldChange 0.46 (ANXA1O) to 2.59 (ATF3). Six of these genes are associated with a known function in pain\textsuperscript{319}. These genes include neuropeptides with a known role in nociceptive signalling (Table 5.2), specifically neuropeptide Y (NPY), neurotensin precursor (NTS), and galanin (GAL); as well as the gastrin and cholecystokinin receptor (CCKBR), the ionotropic N-Methyl D-Aspartate 2B receptor (GRIN2B), and glutamic acid decarboxylase 2 (GAD2). ATF3, the most up-regulated gene, codes for a protein associated with neuronal injury, supporting the hypothesis that at least some sensory projections are directly injured in the burn\textsuperscript{187}. 


<table>
<thead>
<tr>
<th>Gene</th>
<th>log2FoldChange</th>
<th>P-value</th>
<th>Adjusted P-value</th>
<th>P-value</th>
<th>Role in Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATF3</td>
<td>2.59</td>
<td>4.12E-84</td>
<td>8.73E-80</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>GPR151</td>
<td>2.00</td>
<td>1.05E-36</td>
<td>1.11E-32</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>NPY</td>
<td>1.42</td>
<td>4.82E-20</td>
<td>3.41E-16</td>
<td>Known analgesic effect ³²⁰</td>
<td></td>
</tr>
<tr>
<td>LIPN</td>
<td>1.20</td>
<td>1.53E-15</td>
<td>8.11E-12</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>NTS</td>
<td>1.16</td>
<td>3.93E-15</td>
<td>1.66E-11</td>
<td>Involved in visceral nociception and stress-related analgesia ³²¹</td>
<td></td>
</tr>
<tr>
<td>ECEL1</td>
<td>1.17</td>
<td>2.26E-13</td>
<td>7.99E-10</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>GAL</td>
<td>1.09</td>
<td>1.69E-12</td>
<td>5.12E-09</td>
<td>Knockout animals show reduced nociceptive behaviours ³²² and chronic administration causes pain hypersensitivity ³²³</td>
<td></td>
</tr>
<tr>
<td>CCKBR</td>
<td>1.05</td>
<td>3.72E-12</td>
<td>9.87E-09</td>
<td>Knockout animals show reduced sensitivity in neuropathic pain ³²⁴</td>
<td></td>
</tr>
<tr>
<td>TMEM88B</td>
<td>1.03</td>
<td>1.15E-10</td>
<td>2.71E-07</td>
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<td></td>
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<tr>
<td>SEMA6A</td>
<td>0.83</td>
<td>4.64E-09</td>
<td>9.84E-06</td>
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<td></td>
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<td>SEZ6L</td>
<td>0.88</td>
<td>1.61E-08</td>
<td>3.11E-05</td>
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<td></td>
</tr>
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<td>STAR</td>
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<td>1.93E-08</td>
<td>3.40E-05</td>
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</tr>
<tr>
<td>FLRT3</td>
<td>0.67</td>
<td>4.25E-07</td>
<td>0.00069</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>STMN4</td>
<td>0.62</td>
<td>7.42E-07</td>
<td>0.00112</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>GRIN2B</td>
<td>0.77</td>
<td>9.27E-07</td>
<td>0.00130</td>
<td>Overexpression enhanced inflammatory pain ³²⁵</td>
<td></td>
</tr>
</tbody>
</table>
Knockouts enhanced acute nociceptive responses.\(^{326}\)

Table 5.2 Table of genes found to be differentially expressed in DRG neurons affected by a peripheral burn injury

Differentially expressed genes are arranged according to adjusted \(P\)-value (in-build false discovery rate control in the DESeq2 package, using the Benjamini-Hockberg method). A gene is considered to be statistically significant if \(P\)-value < 0.05.

<table>
<thead>
<tr>
<th>Gene</th>
<th>(F)</th>
<th>(ES)</th>
<th>(P)-value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAPPA</td>
<td>0.55</td>
<td>9.84E-07</td>
<td>0.00130</td>
<td></td>
</tr>
<tr>
<td>FST</td>
<td>0.66</td>
<td>1.43E-06</td>
<td>0.00178</td>
<td></td>
</tr>
<tr>
<td>FGF3</td>
<td>0.64</td>
<td>2.66E-06</td>
<td>0.00314</td>
<td></td>
</tr>
<tr>
<td>UCN</td>
<td>0.55</td>
<td>5.96E-06</td>
<td>0.00665</td>
<td></td>
</tr>
<tr>
<td>SERPINB1A</td>
<td>0.50</td>
<td>9.75E-06</td>
<td>0.01034</td>
<td></td>
</tr>
<tr>
<td>PLXNA4</td>
<td>0.46</td>
<td>1.80E-05</td>
<td>0.01738</td>
<td></td>
</tr>
<tr>
<td>TUBB6</td>
<td>0.63</td>
<td>1.78E-05</td>
<td>0.01738</td>
<td></td>
</tr>
<tr>
<td>GM5152</td>
<td>0.47</td>
<td>3.22E-05</td>
<td>0.02848</td>
<td></td>
</tr>
<tr>
<td>MMP16</td>
<td>0.57</td>
<td>3.19E-05</td>
<td>0.02848</td>
<td></td>
</tr>
<tr>
<td>GAD2</td>
<td>0.62</td>
<td>3.59E-05</td>
<td>0.03045</td>
<td>Knockout animals showed sensitised pain behaviours.(^{327})</td>
</tr>
<tr>
<td>CCL8</td>
<td>0.63</td>
<td>5.23E-05</td>
<td>0.03961</td>
<td></td>
</tr>
<tr>
<td>GADD45A</td>
<td>0.48</td>
<td>5.02E-05</td>
<td>0.03961</td>
<td></td>
</tr>
<tr>
<td>WFDC3</td>
<td>0.64</td>
<td>5.20E-05</td>
<td>0.03961</td>
<td></td>
</tr>
<tr>
<td>SLC4A8</td>
<td>0.518428</td>
<td>5.72E-05</td>
<td>0.041841</td>
<td></td>
</tr>
<tr>
<td>ANXA10</td>
<td>0.47941</td>
<td>7.05E-05</td>
<td>0.049852</td>
<td></td>
</tr>
</tbody>
</table>
5.4.3 Volcano plot revealed all differentially expressed cells were up-regulated

A volcano plot revealed significant up-, but no down-regulation of genes in comparison with control DRGs (Figure 5.1). It is interesting that *ATF3*, a cellular marker for inflammation and injury, was also previously found to be significantly up-regulated in another murine burn-induced pain model\(^\text{187}\), which suggests the mechanism of DRG neuron injury may be similar across different peripheral burn injury models.
Figure 5.1 Volcano plot of differentially expressed genes in the burn samples

Volcano plot comparing log$_2$(fold change) to -log$_{10}$(adjusted $P$-value) reveals the identity of differentially expressed genes. Genes with adjusted $P$-value < 0.05 are coloured red, and genes with the lowest $P$-value are annotated with gene names. All differentially expressed genes were found to be up-regulated.
5.4.4 No ion channels were found to be differentially expressed in DRG neurons following the burn injury

The expression of ion channels was examined specifically in the transcriptome, especially as the inhibition of NaV isoforms alleviated symptoms of the burn-induced pain, as indicated in Chapter 4 and in existing literature\textsuperscript{187, 306}. However, it was revealed that no ion channels were differentially expressed in DRG neurons following the peripheral burn injury. A volcano plot comparing log\textsubscript{2}(fold change) to -log\textsubscript{10}(adjusted P-value) was constructed (Figure 5.2), with -log\textsubscript{10}(adjusted P-value) = 0 indicating no change (P-value = 0.99). The cut-off for statistical significance is -log\textsubscript{10}(adjusted P-value) = 1.3, which indicates a P-value of 0.05.

The volcano plot demonstrated that KCNN4, the gene encoding the calcium-activated potassium channels KC\textsubscript{A}3.1, was the ion channel with the smallest P-value in all ion channel genes. However the adjusted P-value for KCNN4 was only 0.069, which renders the down-regulation observed to be non-statistically significant.

The only other ion channel genes with P-values lower than 0.99 were GABRA5 (0.1136), KCNK16 (0.4112), and KCNQ3 (0.8737). GABRA5 encodes the $\gamma$-aminobutyric acid (GABA) A receptor, alpha 5, a subtype of inhibitory GABA receptors. KCNK16 encodes the background potassium channel TALK-1, otherwise known as K\textsubscript{2p}16.1. KCNQ3 encodes the potassium voltage-gated channel Kv7.3, which is the molecular target of flupirtine and retigabine, two clinical drugs already used as adjuvants for neuropathic pain. However, the P-value associated with all three genes were higher than 0.10, indicating a non-statistically significant change in expression.
Figure 5.2 Analysis of the expression changes of ion channels in DRGs following the burn injury

The ion channel with the lowest adjusted $P$-value was $KCNN4$, followed by $GABRA5$, $KCNK16$, and $KCNO3$. However, $P$-values for the expression changes of all genes fell above 0.05, and were not statistically significant. Therefore, it can be concluded that none of the ion channel were found to be differentially expressed following the mild heat injury.
5.4.5 Gene ontology analysis of differentially expressed genes

Gene Ontology (GO) analysis was conducted to further decipher the processes that were enhanced following the burn injury. Analysis of all 30 genes found to be differentially expressed identified ‘Behavioural Defense Response’, ‘Neuropeptide Signalling Pathway’, ‘Axon Guidance’ and ‘Response to Extracellular Stimulus’ as the four main functional clusters of altered gene expression (Figure 5.3 A-B). Additional biological process terms found to be involved were glutamatergic synaptic transmission, associative learning, associative learning, and acid secretion (Figure 5.3 B).
Figure 5.3 Gene ontology analysis of genes differentially expressed in burn samples

Functional categories were assigned to genes within Cytoscape according to the Biological Processes terms, version 30 April 2015. Visualisations were drawn with the Cytoscape plugin ClueGO. A) Genes were clustered into one main functional group and three smaller groups. Overview pie chart was representative of the number of gene ontology interactions in each functional group. B) GO Biological Processes pathway terms for the differentially expressed genes. Numbers after the bar indicate the number of genes associated with each term. The X-axis indicates the percentage of associated genes for each term. Asterisks indicate the P-value
of the association after Bonferroni stepdown adjustment; * = $P$-value < 0.05, ** = $P$-value < 0.01.
5.4.6 Network analysis of differentially expressed genes

To gain additional insight into the molecular mechanisms contributing to altered neuronal function and pain after burn injury, I also conducted a network analysis with STRING (Version 10). Glutamatergic transmission and neuronal secretion formed distinctive networks and clusters (Figure 5.4), confirming the findings of GO analysis. *GRIN2B* and *GAD2* were functionally linked to *NPY, GAL, NTS*, and *CCKBR*, consistent with the established roles of these genes in pain and nociception. A second functional cluster linked *ATF3, ECEL1* and *TUBB6*. Several other genes significantly up-regulated after the burn injury could not be linked to functional networks, reflecting the poorly defined physiological roles of genes such as *TMEM88B, SEZL6, LIPN, ANXA10, CCL8*, and *GPR151* in sensory neurons.
Figure 5.4 Network analysis of up-regulated genes in burn samples

STRING diagram showing interactions between differentially expressed genes, generated with STRING version 10 and with the ‘More’ option (add 10 more gene partners) utilised once only. The diagram is presented in confidence view, and the thickness of edges connecting genes is determined by the strength of associations based on evidence.
5.4.7 Proglumide is effective in alleviating mechanical allodynia and synergistically increase the analgesic effect of opioids

The cholecystokinin 2 receptor (CCKBR), a G protein-coupled receptor known as a pain target, was found to be up-regulated in the transcriptome following burn injury. I therefore assessed the analgesic effects of the cholecystokinin receptor (CCK) antagonist proglumide, which is already available in the clinic. Proglumide was tested alone and in combination with a sub-therapeutic dose of oxycodone (1 mg/kg) in the burn injury, as CCK receptor antagonists are known to augment the analgesic effects of opioids in humans\textsuperscript{328, 329}. This could lead to solid clinical benefit due to the findings in Chapter 4 that oxycodone was not effective at alleviating burn-induced allodynia at standard doses in the mouse model, and a high dose is required for appropriate pain relief.

Proglumide alone significantly alleviated mechanical allodynia compared to vehicle control, albeit only partial analgesia was achieved (PWF: proglumide (30 mg/kg), 2.3 ± 0.3 g; vehicle control, 1.2 ± 0.1 g; \(P\)-value < 0.05; Figure 5.5 A). Co-administration of proglumide with oxycodone significantly reversed mechanical allodynia further (PWF: proglumide (30 mg/kg) + oxycodone (1 mg/kg), 2.3 ± 0.3 g), although the combination was not statistically significant compared to proglumide alone (\(P\)-value = 0.13). Proglumide had no significant effect on thermal allodynia, however in combination with oxycodone, the time to withdrawal in response to a radiant light heat source was significantly increased (time to withdrawal: proglumide (30 mg/kg) + oxycodone (1 mg/kg), 8.8 ± 2.1 s; vehicle control, 3.0 ± 0.5 s; \(P\)-value < 0.05; Figure 5.5 B). Interestingly, neither proglumide alone nor proglumide in combination with oxycodone significantly increased weight bearing in the ipsilateral hind paw (ratio to control: proglumide (30 mg/kg), 1.09 ± 4 %; proglumide (30 mg/kg) + oxycodone (1 mg/kg), 1.07 ± 4 %; vehicle control, 1.00 ± 3 %; \(P\)-value > 0.05; Figure 5.5 C). Proglumide had no significant effect on the ataxia index compared to vehicle control (ataxia index: vehicle control, 3.3 ± 0.6; proglumide (30 mg/kg), 3.1 ± 1.0; \(P\)-value > 0.05; Figure 5.5 D), indicating proglumide does not result in drowsiness or motor deficiencies.
**Figure 5.5 Analgesic activity of the cholecystokinin receptor antagonist proglumide in burn-induced pain, both alone and in combination with a low dose opioid**

A) Both proglumide (30 mg/kg) administered on its own and proglumide (30 mg/kg) in combination with oxycodone (1 mg/kg) significantly alleviated burn-induced mechanical allodynia. B) Proglumide (30 mg/kg) alone had no significant effect on burn-induced thermal allodynia, however thermal allodynia was significantly reversed when administered in combination with oxycodone (1 mg/kg). C) Neither proglumide (30 mg/kg) alone nor proglumide (30 mg/kg) in combination with oxycodone (1 mg/kg) significantly improved weight bearing behaviour of the ipsilateral hind paw, as detected by the ‘Mean Intensity of the 15 most intense pixels’ parameter using the Catwalk XT. D) Proglumide (30 mg/kg) had no significant adverse effect on motor behaviour as assessed by the parallel rod floor test. Statistical significance was determined using t-test. * P-value < 0.05 compared to vehicle control. Dotted lines represent the mean value for the contralateral paw. All data are represented as mean ± SEM with 4-8 mice per group.
5.4.8 Assessment of the expression of genes differentially expressed in the burn model in in vitro cell lines examined in Chapter 3

The in vitro cell lines examined in Chapter 3 were re-analysed in regard to the expression of the 30 genes found to be differentially expressed in the burn-injury model (Figure 5.6). The 30 genes were examined with the hypothesis that if any genes are found to be expressed abundantly in the cell lines, the cell lines could be used as potential in vitro models for assessing inhibitors of these genes.

Genes differentially expressed following the burn injury are expressed at low levels in physiologically normal DRGs, with the exception of ATF3 and PLXNA4, genes associated with transcription and cytoskeleton arrangement. NPY, PLXNA4, and TUBB6 are all expressed in the three cell lines investigated, with F-11 expressing an abundant amount of NPY and TUBB6. SEZ6L, ECEL1, and SLC4A8 were also expressed in F-11 and ND7/23 cells. Interesting, GAL was expressed abundantly only in SH-SY5Y cells, and was largely absent in DRGs and the other cell lines.
The expression of genes found to be differentially expressed in the burn samples were examined in whole DRG and the cell lines studied in Chapter 3. Normalised expression of each gene (normalised to TUBA1A) are shown in the middle of each cell. Genes differentially expressed following the burn injury are mostly expressed at low levels in physiologically normal DRGs. *NPY*, *PLXNA4*, and *TUBB6* are expressed in all three cell lines. F-11 expressed large amounts of *NPY. GAL* was only expressed in large amounts in SH-SY5Y. *TUBB6, SEZ6L, ECEL1*, and *SLC4A8* were expressed in F-11 and ND7/23 cells.

**Figure 5.6 Analysis of the expression of genes up-regulated in the burn injury in cell lines**

<table>
<thead>
<tr>
<th></th>
<th>DRG</th>
<th>SH-SY5Y</th>
<th>F-11 mouse</th>
<th>F-11 rat</th>
<th>ND7/23 mouse</th>
<th>ND7/23 rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atf3</td>
<td>0.259</td>
<td>0.065</td>
<td>0.034</td>
<td>0.014</td>
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<td>Gpr151</td>
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<td>0.000</td>
</tr>
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5.5 Discussion

In this study, I have used RNA-seq analysis to explore gene expression changes that could provide insight into altered sensory neuron functions after a burn injury. Although transcriptomic analyses have been widely used to comprehensively analyse differential gene expression, global gene expression changes that occur in DRGs after a mild peripheral burn have not been previously explored. However, changes in expression of cytokines and inflammation-related genes that occur after burn injuries have been assessed in cultured keratocytes from burn patients\(^{330}\), and RNA from circulating leukocytes of patients critically injured in burn injuries have also been studied\(^{311}\). None of the differentially expressed genes identified in these studies overlapped with those identified in my DRG transcriptome, as expected based on the different tissue types studied. Consequently, gene ontology terms enriched in these other studies were also distinct from mine. However, compared with a study which analysed whole porcine skin following a bromine-induced chemical burn\(^{312}\), my GO analysis yielded similar pathways and biological functions. These included the terms 'axonal guidance' and 'cell signalling', suggesting convergent molecular changes in both burn models. While 'cancer' was a biological function highly enriched in the porcine skin, this was not the case in my study, possibly due to the nature of the bromine burn. Similar term enrichment was also found in a transcriptomic analysis of skeletal muscle both at the burn site and distant from the injury in mice\(^{310}\), which - similar to my findings - also identified the enriched terms 'stress response' and 'defense response'.

Overall, I detected 30 genes that were differentially up-regulated in DRGs following a first-degree burn. The small number of genes is likely a reflection of the relatively mild injury induced. The biological processes enriched in my dataset exhibited an inflammatory and regenerative profile consistent with peripheral nerve injury and regeneration, as well as synaptic signalling and neuropeptide secretion indicative of functional sensitisation. Up-regulation of genes associated with neuronal damage, including *ATF3*\(^{331}\), *ECEL1*\(^{332}\) and *STAR*\(^{333}\), support the view that at least some sensory projections are directly injured during the burn\(^{187}\). Interestingly, although Na\(_v\) channels have previously been identified as crucial for burn-induced thermal alldynia at the behavioural level in Chapter 4 and in existing literature\(^{187}\), gene expression levels of all Na\(_v\) isoforms were unchanged. This perhaps indicate Na\(_v\) channels affect neuropathic pain through functional rather than transcriptomic
alterations, necessitating possible future live-cell functional studies to better understand the mechanism behind the involvement of $\text{Na}_V$ isoforms in burn injury.

When the expression of all ion channels were analysed, none of the ion channels were found to be significantly altered in gene expression. The ion channel that had the highest statistical significance for expression change was the $\text{KCNN4}$ gene, a calcium-activated potassium channel known to be preferentially expressed in small diameter neurons$^{334}$. The product of the $\text{KCNN4}$ gene, $\text{K}_{\text{CA3.1}}$, also has a definite role in CNS inflammation, with its distribution and activity positively related to the presence of neuronal and microglial inflammation$^{335}$. $\text{K}_{\text{CA3.1}}$ inhibition speeds up motor recovery in mice models of spinal cord injury and protects neurons from death$^{336}$, making the channel a putative pharmaceutical target for analgesia. However, the high $P$-value of contributed to $\text{KCNN4}$ expression changes ($P$-value = 0.069) makes this a doubtful find compared to the 30 significantly up-regulated genes the study did yield. Future studies could certainly be conducted to assess the contribution of the $\text{K}_{\text{CA3.1}}$ channel towards burn-induced pain, particularly as burn injury results in significant local inflammation that may be related to the known roles of $\text{K}_{\text{CA3.1}}$ in inflammation.

Potential pain-related genes identified from the 30 genes found to be significantly upregulated included $\text{NPY}$, $\text{NTS}$, and $\text{GAL}$. All three genes are well-known to be involved in nociceptive signalling pathways. Up-regulation of $\text{NPY}$ has been consistently observed in inflammatory and neuropathic pain models and appears to play an important role in spinal nociceptive transmission, as well as recovery from hyperalgesia$^{337, 338}$. $\text{NPY}$ receptor inhibitions has also been reported to lead to reduction in pain behaviours, particularly in rats following intraplantar carrageenan injection$^{339}$. In contrast, the role of $\text{GAL}$ and $\text{NTS}$ in pain is more complex, with both peptides reported to mediate pro-algesic as well as analgesic effects$^{340-342}$. Galanin is required developmentally for the survival of small diameter, unmyelinated neurons$^{343}$, which are commonly considered nociceptors$^{341}$. Galanin knockout mice do not exhibit allodynia and hyperalgesia behaviours following neuronal injury$^{343}$, which suggest galanin mediates nociceptive signals in the organism. At the same time, the excessive expression of galanin also reduces pain from partial sciatic nerve injury, making it likely that a 'nociceptive' range of galanin exists, outside of which analgesia may arise. With neurotensin, knockout mice paradoxically exhibit both a deficiency in nociception as well as a deficiency in stress-induced analgesia. It is
possible that NTS also exhibits a 'nociceptive' range of concentrations that allows for pain, and neurotensin levels outside of the concentration range would result in analgesia.

Other major genes which are significantly up-regulated in the burn injury transcriptome include the ionotropic N-Methyl D-Aspartate (NMDA) 2B receptor \( (GRIN2B) \), the glutamic acid decarboxylase 2 \( (GAD2) \), and the gastrin and cholecystokinin receptor \( (CCKBR) \). NMDA receptors are widespread throughout the nervous system, with NMDA2B implicated in glutamate-mediated hyperalgesia\(^{344} \) as well as use-dependent spinal sensitisation and long-term potentiation\(^{345} \). Ketamine, a non-selective NMDA receptor antagonist, has also been used in burn pain with some success\(^{288, 294} \). However, NMDA antagonists are often associated with hyper-excitability and excitatory CNS side effects.

The enzyme glutamic acid decarboxylase (GAD) converts glutamate to the inhibitory neurotransmitter GABA and altered expression of \( GAD2 \), a subtype of GAD, is likely to result in dysregulation of inhibitory neuronal neurotransmission. Accordingly, epigenetic suppression of \( GAD2 \) expression in central pain pathways has been linked to the development of persistent pain\(^{327} \), while peripheral overexpression of \( GAD2 \) led to attenuation of neuropathic pain behaviours\(^{346} \).

Up-regulation of the gene encoding the cholecystokinin 2 receptor \( (CCK-2 \text{ or the gene} \ CCKBR \) ), a gastrin and cholecystokinin receptor, was particularly interesting in light of my observation that high opioid doses are required to elicit analgesia in the burn pain model. Overexpression of the CCK-2 receptor is known to be associated with enhanced nociception and neuropathic pain\(^{324, 347} \) and its activation interferes with opioid-induced analgesia\(^{263, 348} \). I thus investigated the analgesic and opioid-sparing effect of the CCK inhibitor proglumide on the basis that this compound is already in clinical use with standard doses, and any efficacy in the model could be directly translated to improved management of burn-induced pain in the clinic. Indeed, proglumide was efficacious in mechanical allodynia when administered as monotherapy, or together with oxycodone at opioid doses that did not produce analgesic effects \( (1 \text{ mg/kg}) \). This corroborates with existing evidence that proglumide interacts synergistically with opioids to increase analgesic efficacy in other pain conditions\(^{328, 329} \). It is also interesting to note that co-administration with oxycodone did not statistically further increase PWF values compared to proglumide monotherapy, suggesting that proglumide itself could be a potent analgesic and more than an adjuvant drug in burn pain.
Co-administration of proglumide and oxycodone also alleviated burn-induced thermal allodynia, although proglumide alone had no significant effect on thermal withdrawal thresholds or gait, suggesting that CCK-2 receptor-mediated analgesia is modality-specific. Proglumide also did not induce any motor deficits in the animals, suggesting that cholecystokinin receptor antagonists do not cause drowsiness or CNS disturbances.

Proglumide was selected to investigate the effect of CCK-2 inhibition as the compound is already a clinical drug with an established dose and safety profile. While proglumide is not an inhibitor of both CCK-1 and CCK-2 channels, it was chosen for the ease with which the finding can be translated into the clinic. Future studies with more selective CCK-2 antagonists could be conducted to investigate the specific involvement of CCK-2, as well as becoming potential drug leads in drug discovery.

While the in vitro cell lines were deemed as not representative of any of the known native DRG neuron subtypes, these cell lines nonetheless can serve as models to screen potential modulators of proteins that the cell lines do express. The presence of galanin in SH-SY5Y cells and the abundant expression of neuropeptide Y in F-11 cells indicate the two cell lines can be used as tools to screen for compounds that may interact with these two neuropeptides, stressing the need for better understanding of the strengths and weaknesses of individual in vitro models. Many of the genes up-regulated in the burn-injury transcriptome warrants further studies, as the majority of the genes transcribe proteins that researchers have little or no knowledge of, such as the orphan receptor GPR151, or were previously considered to be unrelated to pain or inflammation. Such findings open up more options for investigating putative drug targets for pain, and broaden the list of proteins that could contribute to the complex molecular changes in peripheral neurons following thermal injuries.

5.6 Conclusions

This chapter described the first transcriptomic analysis of DRG neurons affected by a peripheral burn injury, based on the mouse burn-induced pain model described in Chapter 4. Despite evidence that the inhibition of NaV isoforms reduced burn-induced allodynia behaviours in the mice, no ion channels were found to be differentially expressed in the neurons following the injury. Instead, 30 non-ion-channel genes were found to be
significantly up-regulated. The *CCKBR* gene was up-regulated in DRG neurons following the burn injury, an interesting find given the known role of CCK-2, the protein product of *CCKBR*, in opioid analgesia. Proglumide, an inhibitor of CCK receptors, was found to significantly alleviate mechanical allodynia in the burn injury model, offering a clinically relevant discovery towards more efficient analgesia in burn-induced pain in patients. Many of the genes found to be differentially expressed in the transcriptome have not previously been linked to pain or injury, offering a breadth of novel putative drug targets for burn-induced pain that awaits exploration in greater detail.
Chapter 6 - Overall Conclusions

Pain remains an enigmatic condition with no clear answers for many patients in the clinic, even after the pivotal discovery that the loss of functional Na\(_V\)1.7 channels causes human congenital insensitivity to pain\(^{127}\). While global Na\(_V\)1.7 knockout in mice was shown to be non-responsive to a range of neuropathic pain conditions and demonstrated deficiencies in acute pain sensing\(^{175}\), it is now evident that Na\(_V\)1.7 is not responsible for all that is painful\(^{2, 178, 186}\), dashing the hopes that the Na\(_V\)1.7 channel may be a panacea for all pain. The subsequent surging interest in the role of Na\(_V\) channels in pain have revealed that other Na\(_V\) isoforms, in particular Na\(_V\)1.6 and Na\(_V\)1.8, also play a major part in noxious sensing\(^{2, 186, 197, 198, 205, 207, 349, 350}\), suggesting that further understanding of Na\(_V\)1.6 and Na\(_V\)1.8 may help us to decipher the pathophysiological mechanisms of pain.

In this thesis, I set out in pursuit of better, more optimised tools and models that could be used to investigate the mechanistic roles of Na\(_V\)1.6 and Na\(_V\)1.8 in nociception. Due to the diffuse distribution of Na\(_V\) channels in the CNS as well as in the PNS, an ideal Na\(_V\) inhibitor with clinical potential should be one that is highly selective for the desired Na\(_V\) isoform and does not cross the blood-brain barrier, avoiding significant CNS side effects. Peptides derived from spider venoms are historically known to yield highly potent and selective modulators of ion channels\(^{188, 219, 351}\). These peptides also have large molecular masses between 3 000 and 10 000 Daltons\(^{214}\), which restrict distribution in vivo and often lead to an inability to penetrate into the CNS, making peptides good candidates as Na\(_V\) inhibitors. However, peptide administration becomes complicated and inconvenient when central analgesic effects are needed. The best example would be the peptide-derived analgesic ziconotide, which requires administration as an intrathecal infusion to function as a centrally acting analgesic\(^{112-114}\). Moreover, as shown in this thesis, the rapid process of finding promising peptides from spider venoms cannot guarantee the selectivity of the compounds. Extensive off-target activity analysis is required for peptides discovered based on activity-guided isolation. For the compound Pme1a outlined in this thesis, off-target activity for TRPV1 was found after sequence identification. Structure-activity guided modification of the peptide sequence, a lengthy process involving the chemical synthesis of many analogues, would be required to eliminate the off-target activities. Lastly, peptides have inherent disadvantages when considered as clinical leads, such as extremely poor oral bioavailability and difficulties with
chemical synthesis, which pose limitations on the efficiency of extracting Na\textsubscript{V} channel modulators from crude spider venoms.

An alternative means to rapidly assess potential Na\textsubscript{V}1.6 and Na\textsubscript{V}1.8 inhibitors would be to use cell models to examine the efficacy of possible modulators, which drove me to examine in vitro cell models commonly used in lieu of DRG neurons. The Na\textsubscript{V} \(\alpha\) subunits do not function alone in the native neuron. The pore-forming Na\textsubscript{V} \(\alpha\) subunit exists together with \(\beta\) subunits as well as second messenger pathways, all of which are often missing in cells transfected with only Na\textsubscript{V} \(\alpha\) subunits. The absence of \(\beta\) subunits are known to alter the kinetics and voltage sensitivity of the channels, with single \(\alpha\) subunits exhibiting slower kinetics than that observed in Na\textsubscript{V} channels on native neurons\textsuperscript{148}, arguing that neuron-derived cell lines better resemble in vivo conditions due to the retention of intracellular second-messenger pathways and auxiliary units. The three cell lines I examined in this thesis can be considered sufficient tools to study proteins that the cell lines do express, however they should not be used broadly as representatives of peripheral sensory neurons subclasses, as seen in the lack of resemblance in expression profile towards all known DRG neuron subclasses.

I also developed a wild-type mouse model of burn-induced mechanical allodynia, a known Na\textsubscript{V}1.7-independent pain condition, instead of using global Na\textsubscript{V}1.6 or Na\textsubscript{V}1.8 knockout mice. Global knockout of Na\textsubscript{V}1.6 is lethal in mice, making it impossible to conduct research using these animals. A recent report of the disturbance of opioid analgesic pathways in human patients with Na\textsubscript{V}1.7 loss-of-function mutations also indicate that global knockout of Na\textsubscript{V} \(\alpha\) subunits can lead to compensatory changes in other neurological pathways\textsuperscript{352}, implying that results observed in global gene knockout animals may not necessarily reflect physiological responses. Therefore, I used wild-type mice to establish the burn-induced injury model in this thesis, and used known inhibitors of various Na\textsubscript{V} isoforms to elucidate the roles of the isoforms in burn-induced pain.

The contribution of individual Na\textsubscript{V} isoforms in pain are indeed not as clean-cut as previously expected, particularly as my investigations in burn-induced pain revealed a functional contribution of Na\textsubscript{V}1.6 to mechanical allodynia, yet no up-regulation of Na\textsubscript{V} isoforms were found on the gene level. It is possible that Na\textsubscript{V}1.6, as well as other Na\textsubscript{V} isoforms, exhibits functional increases in activity in neuropathic pain that is not reflected in gene expression
levels. It is plausible that Na\textsubscript{V}1.6 may demonstrate kinetic property changes following the injury independent of \(\alpha\) or \(\beta\) subunit gene expression changes, resulting in an alteration in voltage-dependency and action potential firing rate for the axon. It is also possible that Na\textsubscript{V} isoforms could function synergistically with other, yet un-explored proteins, as seen in the relationship between opioid analgesia and cholecystokinin receptors\textsuperscript{328, 347, 348}, despite the previous presumption that cholecystokinin functions exclusively in the digestive system. Alternatively, analgesic effects of Na\textsubscript{V} inhibitors could simply reflect enhanced neuronal excitability in pain states that is normalised by functional inhibition or reduction of action potential conduction.

Na\textsubscript{V}1.6 and Na\textsubscript{V}1.8 undoubtedly function as important modulators in physiological pain pathways, as seen in the altered pain responses following local gene knockout\textsuperscript{38, 178, 198, 205} or functional inhibition\textsuperscript{2}. However, these channels are likely to be potential drug targets only for selective pain conditions with proven relationship with Na\textsubscript{V}1.6 or Na\textsubscript{V}1.8, such as oxaliplatin- or ciguatoxin-induced cold allodynia. It would also be desirable for potential Na\textsubscript{V}1.6 or Na\textsubscript{V}1.8 clinical inhibitors to remain in the periphery and do not cross the blood-brain barrier due to the widespread expression of Na\textsubscript{V}1.6 in the CNS, especially in the forebrain. Given such considerations, it would be perhaps be preferable for future clinical Na\textsubscript{V} inhibitors to be administered via local injections with slow distribution, potentially subcutaneously, that allows for the functional inhibition of Na\textsubscript{V} isoforms in the local area for a long period of time. While it remains unclear what conditions will benefit from Na\textsubscript{V}1.6 and Na\textsubscript{V}1.8 inhibition, these ion channels are still promising targets for specific neuropathic pain conditions that remain poorly treated throughout the world.
References

13. LaMotte RH and Thalhammer JG. Response Properties of High-Threshold Cutaneous Cold Receptors in the Primate. Brain Res. 1982; 244.

15. Lynn B and Carpenter SE. Primary afferent units from the hairy skin of the rat hind limb. *Brain Res.* 1982; 238: 29-43.


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88. Munns C, Al Qatari M and Koltzenburg M. Many cold sensitive peripheral neurons of the mouse do not express TRPM8 or TRPA1. *Cell Calcium*. 2007; 41: 331-42.


137. Leo S, D’Hooge R and Meert T. Exploring the role of nociceptor-specific sodium channels in pain transmission using Nav1.8 and Nav1.9 knockout mice. *Behav Brain Res.* 2010; 208: 149-57.


155. Cao Z, Gerwick WH and Murray TF. Antillatoxin is a sodium channel activator that displays unique efficacy in heterologously expressed rNav1.2, rNav1.4 and rNav1.5 alpha subunits. *BMC Neurosci*. 2010; 11.


165. Schreibmayer W and Jeglitsch G. The sodium channel activator Brevetoxin-3 uncovers a multiplicity of different open states of the cardiac sodium channel *Biochim Biophys Acta*. 1992; 1104: 233-42.


249. Anders S, Pyl PT and Huber W. HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxf)*. 2014.


293. Parvathy SS and Masocha W. Gait analysis of C57BL/6 mice with complete Freund's adjuvant-induced arthritis using the CatWalk system. *BMC Musculoskelet Disord*. 2013; 14.


## Appendix 1 – Alphabetical List of Materials

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Appendix 2 – Buffer composition

2.1 Physiological Saline Solution (PSS) (Sigma-Aldrich)

140 mM NaCl
11.5 mM glucose
5.9 mM KCl
1.4 mM MgCl₂
1.2 mM NaH₂PO₄
5 mM NaHCO₃
1.8 mM CaCl₂
10 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid); pH 7.4

2.2 Minimum Essential Medium (MEM) (Sigma-Aldrich)

0.2 g/L CaCl₂
0.10 g/L MgSO₄ (anhydrous)
0.4 g/L KCl
2.2 g/L NaHCO₃
6.8 g/L NaCl
0.122 g/L NaH₂PO₄ (anhydrous)
8.9 x 10⁻³ g/L L-alanine
0.13 g/L L-arginine • HCl
0.015 g/L L-Asparagine • H₂O
0.013 g/L L-aspartic acid
0.031 g/L L-Cystine • 2HCl
0.015 g/L L-glutamic acid
7.5 x 10⁻³ g/L glycine
0.042 g/L L-Histidine • HCl • H₂O
0.052 g/L L-isoleucine
0.052 g/L L-leucine
0.073 g/L L-lysine • HCl
0.015 g/L L-methionine
0.032 g/L L-phenylalanine
0.012 g/L L-proline
0.011 g/L L-serine
0.048 g/L L-threonine
0.01 g/L L-tryptophan
0.052 g/L L-tyrosine • 2Na • 2H₂O
0.046 g/L L-valine
1.0 x 10⁻³ g/L choline chloride
1.0 x 10⁻³ g/L folic acid
2.0 x 10⁻³ g/L myo-inositol
1.0 x 10⁻³ g/L niacinamide
1.0 x 10⁻³ g/L D-pantothenic acid (hemicalcium)
1.0 x 10⁻³ g/L pyridoxal • HCl
1.0 x 10⁻⁴ g/L riboflavin
1.0 x 10⁻³ g/L thiamine • HCl
1.0 g/L glucose
0.011 g/L Phenol Red • HCl

2.3 Ham’s F12 media (Invitrogen)

0.30 mM CaCl₂
1.0 x 10⁻⁶ mM CuSO₄ • 5H₂O
3.0 x 10⁻³ mM FeSO₄ • 7H₂O
0.60 mM MgCl₂
3.0 mM KCl
14.0 mM NaHCO₃
131.0 mM NaCl
1.0 mM Na₂HPO₄ (anhydrous)
3.0 x 10⁻³ mM ZnSO₄ • 7H₂O
0.10 mM Glycine
0.010 mM L-alanine
1.0 mM L-arginine hydrochloride
0.10 mM L-asparagine • H₂O
0.10 mM L-aspartic acid
0.20 mM L-cysteine hydrochloride • H₂O
0.10 mM L-glutamic acid
1.0 mM L-glutamine
0.10 mM L-histidine hydrochloride • H₂O
0.031 mM L-isoleucine
0.10 mM L-leucine
0.20 mM L-lysine hydrochloride
0.030 mM L-methionine
0.030 mM L-phenylalanine
0.30 mM L-proline
0.10 mM L-serine
0.10 mM L-threonine
0.010 mM L-tryptophan
0.030 mM L-tyrosine disodium salt dihydrate
0.10 mM L-valine
3.0 x10⁻⁵ mM biotin
0.10 mM choline chloride
1.0 x10⁻³ D-calcium pantothenate
2.9 x10⁻³ mM folic acid
3.0 x10⁻⁴ mM niacinamide
2.9 x10⁻⁴ mM pyridoxine hydrochloride
9.8 x10⁻⁵ mM riboflavin
8.9 x10⁻⁴ mM thiamine hydrochloride
1.0 x10⁻³ mM vitamin B12
0.10 mM i-Inositol
10 mM D-glucose
0.03 mM hypoxanthine sodium
3.0 x10⁻⁴ mM linoleic acid
1.0 x10⁻³ mM lipoic acid
3.2 x10⁻³ mM Phenol Red
1.0 x10⁻³ mM putrescine 2HCl
1.0 mM sodium pyruvate
2.9 x10⁻³ mM thymidine
2.4 Dulbecco's Modified Eagle’s Medium (DMEM) (Sigma-Aldrich)

0.20 g/L CaCl₂
1.0 x 10⁻⁴ g/L Ferric Nitrate • 9H₂O
0.098 g/L MgSO₄ (anhydrous)
0.40 g/L KCl
3.7 g/L NaHCO₃
6.4 g/L NaCl
0.11 g/L NaH₂PO₄ (anhydrous)
0.084 g/L L-arginine • HCl
0.063 g/L L-cystine • 2HCl
0.58 g/L L-glutamine
0.03 g/L glycine
0.042 g/L L-histidine • HCl • H₂O
0.11 g/L L-isoleucine
0.11 g/L L-leucine
0.15 g/L L-lysine• HCl
0.030 g/L L-methionine
0.066 g/L L-phenylalanine
0.042 g/L L-serine
0.095 g/L L-threonine
0.016 g/L L-tryptophan
0.10 g/L L-tyrosine • 2Na • 2H₂O
0.094 g/L L-valine
0.0040 g/L choline chloride
0.0040 g/L folic acid
0.0072 g/L myo-inositol
0.0040 g/L niacinamide
0.0040 g/L D-pantothenic acid (hemicalcium)
0.0040 g/L pyridoxine • HCl
0.00040 g/L riboflavin
0.0040 g/L thiamine • HCl
4.5 g/L D-glucose
0.016 g/L Phenol Red • Na
0.11 g/L Pyruvic Acid • Na

2.5 Dulbecco’s Phosphate Buffered Saline (DPBS) (Sigma-Aldrich)

0.20 g/L KCl
0.20 g/L KH₂PO₄
8.0 g/L NaCl
1.15 g/L Na₂HPO₄ (anhydrous)

2.6 0.25% trypsin/EDTA solution (Invitrogen)

2.5 g/L porcine trypsin
0.38 g/L EDTA • 4Na • 2H₂O
Dissolved in Hank’s Balanced Salt Solution:
0.40 g/L KCl
0.060 g/L KH₃PO₄
0.35 g/L NaHCO₃
8.0 g/L NaCl
0.090 g/L Na₂HPO₄ • 7H₂O
1.0 g/L D-glucose
0.010 g/L Phenol Red

2.7 Tetracycline solution

1 mg/mL tetracycline dissolved in DPBS

2.8 Roswell Park Memorial Institute medium (RPMI) (Invitrogen)

0.10 g/L Ca(NO₃)₂ • 4H₂O
0.049 g/L MgSO₄ (anhydrous)
0.40 g/L KCl
2.0 g/L NaHCO₃
6.0 g/L NaCl
0.80 g/L Na₂HPO₄ (anhydrous)
0.010 g/L glycine
0.20 g/L L-arginine
0.050 g/L L-asparagine
0.020 g/L L-aspartic acid
0.065 g/L L-cystine • 2HCl
0.020 g/L L-glutamic acid
0.30 g/L L-glutamine
0.015 g/L L-histidine
0.020 g/L L-hydroxyproline
0.050 g/L L-isoleucine
0.050 g/L L-leucine
0.040 g/L L-lysine hydrochloride
0.015 g/L L-methionine
0.015 g/L L-phenylalanine
0.020 g/L L-proline
0.030 g/L L-serine
0.020 g/L L-threonine
0.0050 g/L L-tryptophan
0.029 g/L L-tyrosine disodium salt dehydrate
0.020 g/L L-valine
2.0 x 10⁻⁴ g/L biotin
3.0 x 10⁻³ g/L choline chloride
2.5 x 10⁻⁴ g/L D-calcium pantothenate
1.0 x 10⁻³ g/L folic acid
1.0 x 10⁻³ g/L niacinamide
1.0 x 10⁻³ g/L para-aminobenzoic acid
1.0 x 10⁻³ g/L pyridoxine hydrochloride
2.0 x 10⁻⁴ g/L riboflavin
1.0 x 10⁻³ g/L thiamine hydrochloride
5.0 x 10⁻⁶ g/L vitamin B12
0.035 g/L i-inositol
2.0 g/L D-glucose
1.0 x 10⁻³ g/L glutathione (reduced)
5.0 x10^{-3} \text{ g/L Phenol Red}

2.9 Hypoxanthine-aminopterin-thymidine (HAT) media supplement (50x) Hybri-Max™ (Sigma-Aldrich)

Supplied as powder, reconstituted in DPBS to 10 mL, which is then added to 500 mL of culture media.

Final concentrations in 10 mL reconstituted:
5.0 x10^{-3} \text{ M hypoxanthine}
2.0 x10^{-5} \text{ M aminopterin}
8.0 x10^{-4} \text{ M thymidine}

2.10 DNase I stock solution (Qiagen)

Lyophilised DNase I (1500 Kunitz units) was dissolved in 550 \mu\text{L of RNase-free water and divided into 10 \mu\text{L aliquots.}}
Appendix 3 - Supplementary data

![Graph showing response over baseline for Buffer and Ancylometes sp venom over time (seconds).]

![Graph showing response over baseline for Buffer and B. albengai venom over time (seconds).]
Graphs showing the response over baseline for different times:

- **Buffer** vs. **P. atrichomatus venom**
- **Buffer** vs. **P. fasciata venom**
- **Buffer** vs. **P. fera venom**
Appendix Figure 3.1 Veratridine-induced responses in Na$_v$1.6-expressing HEK293 cells exposed to scorpion and spider venoms

Crude venom or the buffer solution was added into the cells as the first addition. Veratridine, a known Na$_v$1.6 activator, was added as the second addition. All experiments were conducted using membrane potential dye. The buffer solution was composed of PSS with 1:1000 BSA. Traces were normalised to baseline fluorescence readings before the second addition. The X axis indicates time (in seconds) after the first addition.
Response over baseline

Time (seconds)

Buffer
P. regalis venom

Response over baseline

Time (seconds)

Buffer
P. rufilata venom

Response over baseline

Time (seconds)

Buffer
P. schlechteri venom

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Appendix Figure 3.2 Deltamethrin-induced responses in Na$_\text{v}$_1.8-expressing CHO cells exposed to scorpion and spider venoms
Crude venom or the buffer solution was added into the cells as the first addition. Deltamethrin, a known Na\textsubscript{v}1.8 activator, was added as the second addition. All experiments were conducted using membrane potential dye. The buffer solution was composed of PSS with 1:1000 BSA. Traces were normalised to baseline fluorescence readings before the second addition. The X axis indicates time (in seconds) after the first addition.
Appendix Figure 3.3 Heat map of Ion channels and GPCRs
The heat map of all ion channel and GPCR genes can be found from
https://www.dropbox.com/s/btb8gi5b2k8s26v/Appendix%20Figure%203.1.xlsx?dl=0

Appendix Figure 3.4 Description of CatWalk XT parameters used to analyse burn-induced gait abnormalities

<table>
<thead>
<tr>
<th>Paw Pressure Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean intensity</td>
</tr>
<tr>
<td>Mean intensity of the 15 most intense pixels</td>
</tr>
<tr>
<td>Max intensity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Paw Print Area Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Print area</td>
</tr>
<tr>
<td>Maximum contact area</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dynamic Paw Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stand</td>
</tr>
<tr>
<td>Swing</td>
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<tr>
<td>Duty Cycle</td>
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<table>
<thead>
<tr>
<th>Interlimb Coordination</th>
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<tbody>
<tr>
<td>Regularity index</td>
</tr>
</tbody>
</table>
In the establishment of the murine model of burn-induced pain, a section of the mouse’s left hind paw (labelled ‘Location of burn injury’ in the picture) was placed on a heated thermal plate to induce the injury. A pair of tweezers, with the tips wrapped in tissue paper, was used to press the plantar portion of the paw onto the Peltier plate. Subsequent allodynia was tested using electronic von Frey fibres and the thermal threshold probe to assess for secondary allodynia at the site labelled ‘Location of allodynia testing and compound injection’. Intraplantar injections with clinical compounds, Na_v isoform inhibitors, and inhibitors of novel pharmacological targets (detailed in chapter 5) were also injected into the site labeled ‘Location of allodynia testing and compound injection’.

**Appendix Figure 3.6 Full list of burn transcriptome**

The full list of differential expression data in the burn transcriptome for all genes can be found from

[link](https://www.dropbox.com/s/s71y56prrg3dpwv/Appendix%20Figure%203.4.xlsx?dl=0)