Identification and Characterisation of Sodium Channel Nav1.7 Protein-Protein Interactions Using an Epitope-Tagged Gene-Targeted Mouse

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Declaration

I, Alexandros Kanellopoulos confirm that the work presented in this thesis is my own. Where information has been derived from other sources or people, I confirm that this has been appropriately indicated in the thesis.
Abstract

Voltage-gated sodium channels are fundamental to the electrical properties of neurons. In mammals, ten isoforms of sodium channel are known and have been characterised (Nav1.1-1.9 and NaX). One particular subtype Nav1.7, plays a critical role in pain pathways and has a large number of functions including the control of neurotransmitter release, synaptic integration and the conduction of nociceptive signals. Humans born with loss of function mutations in this channel are unable to feel pain. This heritable disease known as congenital insensitivity to pain (CIP) can be recapitulated in transgenic mouse models and has made Nav1.7 a promising target for the development of analgesic drugs. However, this phenotype cannot be recapitulated with even the most specific of Nav1.7 channel blockers, possibly indicating that this channel’s role in pain signalling is more complex than simply signal transmission. In this thesis, we aimed to gain a greater understanding of Nav1.7 and its role in pain. Firstly, to further explore the function of this channel, we used an epitope-tagged Nav1.7 mouse that showed normal pain behaviour to identify and study the proteins that interact with this channel. The high-affinity epitope-tag consisted of a HAT-tag in tandem with a 3X FLAG tag separated by a Tobacco Etch Virus (TEV) cleavage site to enable two rounds of protein purification. Nav1.7 distribution throughout the central and peripheral nervous system was determined using Tap-tagged Nav1.7 mice. A Nav1.7 complex affinity-purified under native conditions by mass spectrometry revealed over 300 proteins associated with Nav1.7 in vivo, including previously known and novel interacting proteins. We conducted both validatory and functional assessments on these proteins. Previously known interactors included β-subunits as well as other signalling and synaptic proteins. We also showed an association with the Colapsin Response Mediator Protein 2 (CRMP2) and conducted a functional validation of this protein with regards to it’s trafficking role of Nav1.7. Furthermore, we identified this protein as one of the targets of the analgesic drug Lacosamide. We show that lacosamide acts through Nav1.7 in both a direct manner and through CRMP2 to reduce Nav1.7 channel current density. We also found and validated a number of synaptic proteins that interact with Nav1.7 such as Snap25 and
synaptotagmin I and II. Moreover, we validated a number of interesting Nav1.7 interactions including L-type amino acid transporter 1 (Lat1), transmembrane P24 trafficking protein 10 (Tmed10), Neurofascin, A-kinase anchoring protein 12 (AKAP12) and G-protein regulated inducer of neurite outgrowth 1 (GPRIN1) (a µ-opioid receptor-binding protein), demonstrating a physical and functional link between Nav1.7 and opioid signalling. We further studied this link between Nav1.7 and opioid signalling. Type-A GPCRs are known to be regulated through a specific sodium binding site the occupancy of which diminishes agonist binding. We used an electrophysiological assay of Protein Kinase A activity to examine the role of intracellular sodium on opioid signalling. Phosphorylation of sodium channel Nav1.8 by activation of Protein Kinase A with db-cAMP is unaffected by altered intracellular sodium. By contrast, there is a dose-dependent inhibition of fentanyl action on Nav1.8 currents when intracellular sodium is increased from 0mM to 20mM. Fentanyl shows a 50% loss of activity and 80-fold increase in EC50 with 20mM intracellular sodium. These data suggest an effect of altered intracellular sodium levels on opioid receptors, where it might play a role in the modulation of opioid receptor signalling.
Impact Statement

When considering the impact of this thesis, it is important to first consider the impact pain has on our society. Over the past 20 years, inconsistencies in the methodologies employed to determine the economic and social consequences of pain make it hard to determine its true impact. According to a European wide survey, chronic pain in severe or moderate forms with the ability to seriously affect daily activities, social and working conditions occurs in approximately 19% of adult Europeans (Breivik et al. 2005). The indirect (productivity) cost of pain in the UK is estimated at between £10 and £20 billion a year and a cost to national health services of around £1 billion (Breivik et al. 2005). Importantly, 40% of those suffering from pain report a lack in the efficacy of their medication, depicting the true inadequacies in currently available therapies to treat pain and chronic pain conditions. Furthermore, as well as a lack of efficacy, many of the currently available analgesics are associated with alarming side effects and have the potential for substance abuse. I believe the findings outlined in this thesis have an impact on both the research of voltage-gated sodium channels and more broadly on pain research as a whole. The realisation that selectively blocking Nav1.7 lacked analgesic effect means that other more subtle mechanisms are at play. Through our mapping of Nav1.7 protein interactions, we have provided an important resource of information that will surely be useful in further characterising these mechanisms and in the development of novel drugs used to target Nav1.7. By highlighting the link between Nav1.7 and the opioid system we add to the existing evidence pointing to the need for a synergistic approach to targeting Nav1.7 and that the current approach of channel blockade is not sufficient to create potent analgesia. Furthermore, our results outlining the use of Lacosamide to target Nav1.7 also adds to the increasing evidence pointing to the value of this drug in treating pain. Therefore, with regards to the scientific community, we were able to give a clearer perspective of how Nav1.7 functions, thereby offering new opportunities for the development of drugs that target this channel in the pursuit of novel more efficient pain medication. With regards to the direct benefit of this work on society as a whole, like most medical research, the aim is always to ultimately enhance the
human condition through a greater understanding of how genetic and environmental factors generate disease and translate this acquired knowledge into novel therapeutics. Through the work accomplished in this thesis, I believe we have participated in advancing our understanding of how pain systems functions which, I am confident, will play a part in ultimately addressing the problem of pain highlighted above.
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List of Abbreviations

AAV Adenoassociated virus
ASIC Acid sensing ion channel
ATP Adenosine triphosphate
BDNF Brain derived neurotrophic factor
cAMP Cyclic adenosine monophosphate
CFA Complete Freund’s adjuvant
CGRP Calcitonin gene related peptide
CHO Chinese hamster ovary
CNS Central nervous system
CO-IP Co-immunoprecipitation
COX Cyclo-oxygenase
Cre Cyclization recombinase protein
dNTPs Deoxyribonucleotide triphosphate
DRG Dorsal root ganglia
ELISA Enzyme linked immunosorbent assay
ES Embryonic stem cells
GDNF Glial cell derived neurotrophic factor
GFP Green fluorescent protein
GPCR G protein coupled receptor
HEK Human embryonic kidney cell
IA Intermediate adapting current
IB4 Isolectin B4
ICH Immunohistochemistry
KO Knockout
LC-MS Liquid chromatography mass spectrometry
LTMR Low threshold mechanoreceptor
MS Mass spectroscopy
mRNA Messenger RNA
Nav1.7 Voltage-Gated Sodium Channel 1.7
NF200 Neurofilament 200
Neo Neomycin resistance cassette
NGF Nerve growth factor
NSAIDs Non-steroidal anti-inflammatory drugs
PEPD Paroxysomal extreme pain disorder
PENK Pro-enkephalin
PCR Polymerase chain reaction
PKA/C Protein Kinase A/C
PLC Phospholipase C
PWT Paw withdrawal threshold
qPCR Quantitative real time PCR
RA Rapidly adaptive current
SA Slowly adapting current
SBP Sodium binding protein
SNI Spared nerve injury
SNL Spinal nerve ligation
TG Trigeminal ganglia
TRP Channel transient receptor potential channel
TTX Tetrodotoxin
VGCC Voltage gated calcium channel
VGSC Voltage gated sodium channel
WB Western blot
WT Wild-type
Chapter 1

General Introduction

1.1 Pain

With few exceptions, pain is experienced by all people. As a sensory tool, it has an undisputed importance in our ability to navigate the world and avoid potential or actual harm. The neurologist Charles Sherrington defined pain in a textbook on physiology published in 1900 as “the psychical adjunct of a protective reflex”, highlighting the dual nature of pain (Levine 2007); incorporating both a higher order cognitive component - “the psychical adjunct” - and a dynamic sympathetic component - “the protective reflex”. Over the past century, this definition has been tortuously expanded in an attempt to integrate more components of both the physical and cognitive aspects of pain. The world’s leading organisation on pain, The International Association for the Study of Pain (IASP), defines pain as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. As a highly complex and adaptive phenomenon, the primary function of pain is one of physical avoidance to danger and promoting recovery from injury. However, pain can become maladaptive, taking the form of a disease that persists beyond recovery. This form of pain dramatically reduces the quality and well being of a sufferer’s life. Despite complex definitions, the word “pain” echoes ambiguity and is often better described using visual or experience based analogies, which range from a purely physical nature to something wholly psychological. In normal circumstances, pain is a conscious experience influenced by memories, emotions and a wide range of environmental and contextual factors that is specifically evoked by potential or actual noxious stimuli caused by tissue damage. Nociception, a term not to be taken as synonymous with pain, is the physiological and neurological mechanism of detecting, transmitting and processing of noxious
stimuli of the peripheral and central nervous system.

1.2 Overview of the Peripheral Sensory Nervous System and Nociception

The nervous system can be anatomically subdivided into the central nervous system (CNS), comprised of the spinal cord and brain, and the peripheral nervous system (PNS), comprised of all tissues outside these organs. In mammals, pathways responsible for pain processing operate at multiple levels of both the peripheral and central nervous system, under both voluntary and involuntary control and involve various different cell types, both neuronal and not (Schaible 2006)(e.g. immune cells). Functionally, the PNS has two subdivisions; the sensory nervous system, which uses primary afferent neurons to convey information from the periphery to the CNS and the motor nervous system, which controls bodily functions and movement via information conveyed from the CNS to the periphery via efferent neurons. The contribution of both these systems and their neuronal components to the perception of pain, is examined below. Noxious stimuli come in multiple forms and can be classified as mechanical, temperature and chemical. Exposure to one of these stimuli, activate the “free nerve endings” of finely tuned primary sensory neurons in the skin and deep tissue known as nociceptors, which in turn relay information to the spinal cord dorsal horn.

1.2.1 Peripheral Nervous System: Primary Afferent Neurons

Primary afferent neurons of the somatosensory system convey mechanical, thermal and chemical sensory information from the peripheral and visceral tissues to the spinal cord. These afferents somatise into ganglia in the intervertebral foramina referred to as the dorsal root ganglia (DRG) and in the apex of the petrous part of the temporal bone known as the trigeminal ganglia (TG). These peripheral fibres are classified on the basis of morphology, degree of myelination and conduction velocity into C-, Aδ, and Aα/β -fibres (cutaneous and visceral afferents) (see table 1.1). These anatomical divisions are also tied to specific sensory modalities. The majority of large (>40µm) cutaneous afferents, classified as Aα/β -fibres, are fast conducting (70-120m/s and 40-70m/s), heavily myelinated, low-threshold mechanoreceptors, responding to non-noxious mechanical stimulation (i.e light touch) (Julius et al. 2001). In contrast, the lightly myelinated, medium diameter Aδ (30-40µ m), mediate “first/fast pain” at a velocity of 3-25m/s. Whilst unmyelinated, small diameter C-fibres
(<30\mu), have slower conduction velocities of 0.5-2m/s and convey the poorly localised second phase “slow” pain (Julius et al. 2001).

<table>
<thead>
<tr>
<th>Fibre</th>
<th>Myelination</th>
<th>Diameter</th>
<th>Conduction Velocity</th>
<th>Modality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aα</td>
<td>Heavy</td>
<td>&gt;35\mu</td>
<td>70-120 m/sec</td>
<td>Proprioception, Low Threshold Mechanical</td>
</tr>
<tr>
<td>Aβ</td>
<td>Heavy</td>
<td>&gt;35\mu</td>
<td>10-30 m/sec</td>
<td>Proprioception</td>
</tr>
<tr>
<td>Aδ</td>
<td>Light</td>
<td>25-35\mu</td>
<td>3-25 m/sec</td>
<td>Low Threshold mechanical, Thermal</td>
</tr>
<tr>
<td>C</td>
<td>None</td>
<td>&lt;25\mu</td>
<td>0.5-2 m/sec</td>
<td>Noxious Mechanical, Thermal, Chemical</td>
</tr>
</tbody>
</table>

**Table 1.1**: Primary sensory neuron classification. C-, Aδ, and Aα /β -fibres are classified according to size, morphology, degree of myelination and conduction speed.

It is therefore generally accepted that neurons of the dorsal root ganglia can be divided into nociceptors and non-nociceptors. The former, conveying noxious stimuli and the latter innocuous stimuli associated with mechanosensory organs, as well as the roots of various hair types in non-glabrous skin (Li et al. 2011). Nociceptors are pseudo unipolar (Bessou 1969) and have a high threshold of activation needed to transduce and encode noxious stimuli. Historically, DRG sensory neuronal subtypes have further been delineated based on the expression of unique molecular markers. A classification based on expression of receptors that are crucial for cell survival, peripheral innervation of appropriate targets, expression of ion channel subtypes and other molecular properties defining the functional characteristics of the different types of sensory neurons. Lallemend & Ernfors classify DRG neurons according to expression of neurotrophic factor receptor expression, tropomyosin-receptor-kinase A (TrkA), TrkB, TrkC, Met and Ret receptor tyrosine kinase, which serve as a receptor for the neurotrophins; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophic-3 (NT3), hepatocyte growth factor and glial-derived neurotrophic (GDNF) family ligands (Lallemend et al. 2012) (see figure 1.1).
Glutamate is the predominant excitatory neurotransmitter in all nociceptors. However, according to specific peptide markers, nociceptors can be divided into peptidergic and non-peptidergic populations, which express distinct families of receptors and ion channels (Snider et al. 1998). All nociceptors derive from stem cells of the neural crest during late neurogenesis (Woolf et al. 2007) and express the receptor TrkA (Marmigère et al. 2007). Peptidergic nociceptors, which contain the neuropeptide substance P and calcitonin gene related peptide (CGRP), retain their expression of TrkA. Non-peptidergic nociceptors do not express these neuropeptides but instead can be identified through their expression of a surface carbohydrate group that selectively binds to a plant lectin known as isolectin B4 (IB4) (Silverman et al. 1990). During the perinatal and postnatal period IB4-positive cells suppress the expression of TrkA and switch on expression of Ret, the transmembrane singling component of the GDNF receptor. The classification of sensory neurons based on unique expression profiles has been further expanded thanks to advancements of new comprehensive transcriptomic tools (Usoskin et al. 2015). Based on the idea that functional cell-type identity is reflected in the gene expression profiles of individual cells, de novo cell type discovery is possible by unbiased single-cell sampling and the identification of individual cellular
expression profiles. Through the use of single cell transcriptomics and unsupervised grouping of similar expression profiles through principal component analysis to reveal cell populations, Usoskin et al. (2015), have proposed a new unbiased classification of dorsal root ganglion neurons. In their model, they have integrated old classifications with new molecular identifiers. They classify all dorsal root ganglia neurons into eleven subtypes marked by expression of NF1 to NF5 (neurofilament containing 1-5), NP1 to NP3 (non-peptidergic 1-3), PEP1, PEP2 (peptidergic nociceptors 1 and 2) and TH (tyrosine hydroxide containing).

Further specific, novel and already known identifiers complement the expression profile based classification (see figure 1.2). These taxonomic methods also functionally reveal three distinct groups of low-threshold mechanoreceptive neurons (LTMRs), two groups of proprioceptive, and six principal types of thermosensitive, itch sensitive, type C low-threshold mechanosensitive and nociceptive neurons with markedly different molecular and operational properties (see figure 1.2) (Usoskin et al. 2015).

<table>
<thead>
<tr>
<th>NF1</th>
<th>NF2</th>
<th>NF3</th>
<th>NF4</th>
<th>NF5</th>
<th>NP1</th>
<th>NP2</th>
<th>NP3</th>
<th>PEP1</th>
<th>PEP2</th>
<th>TH</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDHB</td>
<td>CACNA1H</td>
<td>TRKB&lt;sup&gt;Thr&lt;/sup&gt;</td>
<td>CACNA1H</td>
<td>RET</td>
<td>PLXNC1&lt;sup&gt;Thr&lt;/sup&gt;</td>
<td>P2X3</td>
<td>GFRα2</td>
<td>MGPRD</td>
<td>RET</td>
<td>PIEZO2&lt;sup&gt;Thr&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDHB</td>
<td>TRKC&lt;sup&gt;Thr&lt;/sup&gt;</td>
<td>PV</td>
<td>CNTNAP2</td>
<td>RET</td>
<td>TRKA</td>
<td>CGRP</td>
<td>MGPRD</td>
<td>RET</td>
<td>CNTNAP2</td>
<td>GFRα2</td>
</tr>
<tr>
<td>LDHB</td>
<td>TRKC&lt;sup&gt;NK&lt;/sup&gt;</td>
<td>RET</td>
<td>CNTNAP2</td>
<td>RET</td>
<td>SST</td>
<td>RET</td>
<td>RET</td>
<td>RET</td>
<td>RET</td>
<td>RET</td>
</tr>
<tr>
<td>LDHB</td>
<td>TRKD&lt;sup&gt;Thr&lt;/sup&gt;</td>
<td>PV</td>
<td>CNTNAP2</td>
<td>RET</td>
<td>TRKA</td>
<td>CGRP</td>
<td>MGPRD</td>
<td>RET</td>
<td>CNTNAP2</td>
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</table>

**Figure 1.2:** New sensory neuronal classification proposed by Usoskin et al., (2015) based on unbiased full RNA transcriptome analysis. Gene products noted at the top are suggested identifiers of neuronal subtypes. Subsequent markers in red are newly identified; in black are selected previously used markers. The gene products at the bottom refer to the distribution of gene products commonly studied in the field. Gene names are as follows: LDHB (lactate dehydrogenase B), CACNA1H (calcium channel, voltage-dependent, T type, alpha 1H), TRKB (Tropomyosin receptor kinase B; high and low refer to expression level), NECAB2 (N-terminal EF-hand calcium binding protein 2), NEFH (neurofilament heavy chain), CALB1 (calbindin 1), RET (receptor for the glia cell-line derived family of neurotrophic factors), FAM19A1 (family with sequence similarity 19, member A1), PV (parvalbumin encoded by Pvalb), SPP1 (secreted phosphoprotein 1), CNTNAP2 (contactin associated protein-like 2), ASIC1 (acid-sensing ion channel 1), RUNX3 (runt related transcription factor 3), PLXNC1 (plexin C1), P2X3 (purinergic receptor P2X ligand-gated ion channel 3), GFRA2 (GDNF family receptor alpha-2), MRGPRD (Mas-related G-protein coupled receptor member D, TRPA1 (Transient receptor potential cation channel subfamily A member 1), TRPV1 (Transient receptor potential cation channel subfamily V member 1), TRPC3 (short transient receptor potential channel 3), Nav1.7/9 (voltage gated sodium channel 1.1-1.9), SST (somatostatin), TRKA (Tropomyosin receptor kinase A), CGRP (calcitonin gene-related peptide), KIT (Photoreceptor c-Met tyrosine kinase), TAC1 (Preprotachykinin-1), PIEZO2 (piezo-type mechanosensitive ion channel component 2), VGLUT3 (vesicular glutamate transporter 3). Taken from Usoskin et al., (2015).
1.2.2 Central Sensory Processing: Transmission to the Spinal Cord

The spinal cord is the lowest level of the central processing. Sensory information from primary afferents that innervate the skin and viscera send sensory information to the first point of relay in the spinal cord. Primary afferents project into the dorsal horn of the spinal cord where they have determined patterns of distribution according to sensory modality and region of the body they innervate (Todd 2010). The spinal cord exhibits visually recognisable patterns of lamination where distinct classes of afferents terminate and are organised (see figure 1.3). In general, non-noxious information from myelinated low-threshold mechanoreceptive afferents (i.e proprioceptive muscle afferents) project to laminae II-V of the dorsal horn. Nociceptive input from A\(\delta\) and C fibres, project mainly to lamina I and II, with some scarcer projections innervating lamina V (Sugiura 1996). Within each laminae primary afferent neurons synapse with second order projection neurons. These second order projection neurons can be either nociceptor-specific, which respond solely to stimuli of high intensity and or these primary afferents can synapse to wide dynamic range (WDR) neurons that respond to a variety of stimuli intensities with graded responses. Nociceptor specific projections are usually confined to the outer laminae (I&II) of the spinal cord, whereas wide dynamic range neurons are found in deeper laminae (V) (D’Mello et al. 2008). The spinal cord is also the first location of nociceptive processing, where input from primary afferents is under both excitatory and inhibitory control from local inhibitory interneurons and descending control from the brainstem (Todd 2010). Additionally nociceptive information can also be conveyed to the ventral horn of the spinal cord and contributes to spinally-mediated nociceptive reflexes (Sivilotti et al. 1994). As a site of neural relay, the spinal cord neurons also play a crucial role in the development of central sensitisation. Central sensitisation can be described as an enhancement in neuron and neuronal circuitry function in nociceptive pathways. This is caused by an increase in membrane excitability and synaptic efficacy of central nociceptive pathways. Phenotypically, central sensitisation is manifested by and increased response to noxious stimulation, the lowering of pain thresholds at the site of sensitisation and an expansion of the receptive pain field. The molecular machinery that creates central sensitisation is complex. These changes in spinal nociceptive processing involve both pre- and postsynaptic mechanisms. Firstly, peripheral inflammation causes the release of spinal mediators such as, substance P, neurokinin A, CGRP and glutamate (Schaible 2006). This causes the sensitisation of spinal neurons due to the activation of N-methyl-D-aspartate receptors (NMDA) (see figure 1.4). In addition, this process is also supported by the
activation of NK1 and CGRP receptors (Schaible 2006). This abnormal activation of NMDA receptors causes an increase in calcium influx into spinal neurons, which in turn activates calcium dependent kinases that phosphorylate the NMDA receptors. Secondly, these events provoke an up-regulation of NMDA and glutamate receptor subunits (NR1, NR2B and GluR1) thus, enhancing glutamatergic transmission (Schaible 2006). This chain of events also causes an increase in expression of genes that code for neuropeptides, in particular prostaglandins and cytokines that modify central hyperexcitability, which are known to cause increased transmitter release (Schaible 2006).
primary afferent sensory neurons in the spinal cord. Nociceptive afferents project to the dorsal horn of the spinal cord, while low-threshold mechanoreceptive afferents project to the ventral horn of the spinal cord. Neurons in the dorsal horn of the spinal cord receive tracts that convey afferent signals from the periphery. The dorsal horn contains several layers, each with distinct functions. Layer I is rich in peptidergic C-fibers, which are involved in nociception. Layer II contains non-peptidergic C-fibers and Aδ fibers, which are involved in nociception and temperature sensation. Layer III contains Aβ fibers, which are involved in low-threshold mechanoreception. Layer IV contains non-peptidergic Aδ fibers, which are involved in nociception and temperature sensation. Layer V contains peptidergic C-fibers, which are involved in nociception. Layer VI contains non-peptidergic Aδ fibers, which are involved in nociception and temperature sensation. Layer VII contains Aβ fibers, which are involved in low-threshold mechanoreception. Layer VIII contains non-peptidergic Aδ fibers, which are involved in nociception and temperature sensation. Layer IX contains peptidergic C-fibers, which are involved in nociception. Layer X contains non-peptidergic Aδ fibers, which are involved in nociception and temperature sensation. Layer XI contains Aβ fibers, which are involved in low-threshold mechanoreception. Proprioceptive information from muscle spindles terminate in the ventral horn of the spinal cord, where they can directly synapse onto motor neurons to mediate reflexes.
1.3 Molecular Nociception and Pain Mechanisms

Nociceptors require a fine tuning to respond to high threshold noxious stimuli (Plaghki et al. 2010). Noxious stimuli fall into three broad categories: noxious thermal, noxious mechanical and noxious chemical (Woolf et al. 2007). Like all neurons, nociceptors encode and process electrophysiological stimuli. It is therefore only the inherently unique array of modality specific molecular elements (see figure 1.4) that define the neurochemistry of nociceptors and allow their fine tuning to detect and transmit noxious stimuli to the CNS (Bessou 1969). Other than the morphological characteristics previously discussed, determinants of neuronal activation thresholds and conduction characteristics are for the most part determined by the expression of a diverse array of ion channel and receptor subtypes. The following section will describe the mechanisms and molecular elements involved in the detection (transduction) and propagation (transmission) of nociceptive stimuli.

1.3.0.1 Transduction

Sensory transduction is the conversion of a stimulus into an electrical potential within a neuron (i.e action potential). By using a repertoire of specific high threshold ion channels (see figure 1.4), nociceptors have the ability to change noxious stimuli (heat, chemical and mechanical) into changes in neuronal electrical potentials. One of the largest and most important family of noxious detectors is the TRP channel family (Caterina et al. 1997). Discovered in *Drosophila melanogaster* in the late 70’s by mutating the TRP-gene, transient receptor potential (TRP) channels are responsible for photoreceptor electrophysiological responses changes from transient to continuous when exposed to light (Minke 1977). There are now approximately 30 known TRP channels that respond to somatosensory stimuli and are expressed within dorsal root ganglia neurons (Hjerling-Leffler et al. 2007).

1.3.1 TRPV1

The first member of the TRP channel family to be characterised as a pain transducer is TRPV1. TRPV1 is a non-selective cation channel with high permeability to \( \text{Ca}^{2+} \), expressed in approximately 50% of all DRG and TG neurons (Caterina et al. 1997). TRPV1 consists of six transmembrane domains and has the ability to assemble as both a homo- and heterotetrameric complex (Moiseenkova-Bell et al. 2009). The channel responds to capsaicin and is a detector of physical noxious heat (>43°C)(Caterina et al. 1997). This channel is also

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Figure 1.4: Schematic showing major receptor and voltage-gated ion channels involved in nociceptive transduction and transmission. Left circled panel shows afferent terminal with all known channels and receptors involved in nociceptive transduction. Centre circular panel illustrates presence of voltage-gated sodium channels. Right circular panel indicates presence of voltage-gated sodium channels at fibre terminal, synapsing on to second order neurons in the dorsal horn spinal cord.
activated by decreased pH (tissue acidosis during inflammation), and lipids such as anandamide (Palazzo et al. 2010; Caterina et al. 1997; Obata et al. 2005). TRPV1 channel function is known to be modulated by phosphatidylinositol-4, 5-biphosphate (PIP$_2$), such modulation is likely to be physiologically important since PIP$_2$ regulation is a major component of downstream G-protein activation (Stein et al. 2006). Animal studies have confirmed TRPV1 to be a primary target for various compounds generated during inflammation (Docherty et al. 1996). The compounds present in the “inflammatory soup”, such as Bradykinin, NGF and PGI$_2$, have the ability to activate downstream kinases PKC and PKA which in-turn determine the phosphorylation of TRPV1 thereby modulating the channel (Docherty et al. 1996; Michael et al. 1999; Meents et al. 2010; Ji et al. 2002; Moriyama et al. 2005). TRPV1 knockout mice (Trp$^{-/-}$) exhibit decreased responses to acute thermal stimuli and develop milder thermal hyperalgesia caused by inflammation (Kasama et al. 2007). In neuropathic pain conditions, an up-regulation of TRPV1 takes place in both C- and A-fibres. This was shown by Hudson et al., (2001) who measured the expression of TRPV1 in rodents in nerve injury models, using fluorescent retrograde tracers to identify damaged and undamaged neurons. This group found a persistence in TRPV1 expression following partial sciatic nerve transection and spinal nerve ligation. They found an increase in expression of nearly three fold in A-fibre somata and a much greater increase in C-fibres. Furthermore, peripheral nerve injury also up-regulates the channel in spinal terminals of both injured and non-injured neurons. This evidence suggests that changes in TRPV1 expression patterns following injury may account in part for changes in underlying allodynia (Hudson et al. 2001).

### 1.3.2 TRPA1

Originally named ankyrin transmembrane protein 1 (ANKTM1), TRPA1 was discovered in 1999 and was the only known member of the TRP ankyrin family to be found in humans (Jaquemar et al. 1999). TRPA1 strongly co-localises with TRPV1 (Story et al. 2003) and is present in neurons expressing substance P and preferentially expressed in IB4 neurons (Barabas et al. 2012) as well as in non-neuronal cell types (Kim et al. 2009; Nozawa et al. 2009). Activated by capsaicin-like compound (capsiate) (Kenji et al. 2011), TRPA1 can be further activated by a large number of irritant chemical compounds including, isothiocyanates, cinnamaldehyde, acrolein and allicin as well as active ingredients in horseradish, mustard, cinnamon and garlic (Bandell et al. 2004; Bautista et al. 2006). TRPA1 (Trpa1$^{-/-}$) knockout mice are known to be insensitive to these chemicals and also show
strong deficits in somatosensory chemosensation (Bautista et al. 2006). Several lines of evidence have also suggested that TRPA1 channels can be activated by noxious cold (Story et al. 2003) however, this role as a noxious hot and cold sensor still remains unresolved.

### 1.3.3 TRPM8

TRP metastatic 8 receptor (TRPM8) was first discovered and characterised as a cooling and menthol sensor in 2002 (McKemy et al. 2002). This channel is expressed in small and medium DRG and TG neurons. In-vitro experiments have shown that TRPM8 is activated by drops in temperature below 26°C over a range of both noxious and innocuous cold temperatures (McKemy et al. 2002). Genetic and pharmacological ablation of TRPM8 provides evidence for it’s role as a non-redundant cold sensor (McKemy et al. 2002). A role for this channel in injury-induced hypersensitivity has also been suggested. Colburn and colleagues showed that TRPM8 is required for the development of cold hypersensitivity induced by injury, where TRPM8−/− mice exhibit almost complete loss of acute cold sensation (Colburn et al. 2007). Furthermore, cold induced analgesia is also mediated by TRPM8 (Dhaka et al. 2007). TRPM8 knockout mice, however, still remain responsive to noxious heat and mechanical stimuli (Colburn et al. 2007; Proudfoot et al. 2006).

### 1.3.4 ASICs (Acid-Sensing Ion Channels)

Acid evoked currents were first observed in neurons in the 1980’s (Gruol et al. 1980). However, it was not until 1997 that the first acid sensing protein was cloned (Waldmann et al. 1997). There are four subtypes of acid-sensing ion channels (ASICs) (ASIC1-4) which are part of the DEG/ENaC superfamily. Generally, these channels tend to be highly expressed in the cell bodies and peripheral terminals of nociceptors, where they can assemble in both homo- and heteromeric complexes with distinct pH sensitivity and expression profiles (Waldmann et al. 1997; Alvarez et al. 2003). ASICs are voltage-independent, proton-gated, permeable cation channels that are activated by extracellular acidosis (i.e changes in pH) generally caused by inflammation or endo/exogenous irritants (Reeh et al. 1996; Price et al. 2001; Deval et al. 2008).

### 1.3.5 Transmission

Following the sensing of a noxious stimulus by nociceptive transducers, creating an initial change in membrane potential, the job of relaying painful information to the central nervous
system is down to voltage-gated ion channels (VGICs). More specifically, voltage-gated sodium and potassium channels (VGSCs, KVs). Membrane channels play a key role in determining neuronal excitability and action potential propagation. Technological advances in genome sequencing and research into heritable pain syndromes have significantly contributed to much of our understanding of the conduction of sensory and nociceptive inputs. It has highlighted the importance of voltage-gated sodium channels (VGSCs) in the generation of pain and pain related diseases. The following section describes the structure, function, distribution and role of VGSCs in pain, with a special focus on the VGSC subtype Nav1.7.

1.4 Voltage-Gated Sodium Channels: Structure, Subtype and Cellular Distribution

Voltage-gated sodium channels are members of the ion channel superfamily that also include potassium and calcium channels. Structurally, VGSCs are heteromeric transmembrane protein complexes formed of α and β subunits. Nine homologous members make up the VGSC family whose α subunits are encoded by genes SCN1A–11A with all genes for these channels found clustered within chromosomes 2, 3, 11 and 15 in humans (George et al. 1992; H Yu et al. 2003). These pore forming α subunits, each composed of a long polypeptide chain (1700–2000 amino acids), comprise four homologous domains (DI–DIV) with each domain broken down into six transmembrane α helical segments (S1–S6). The ion selective pore of the channel is formed by S5–S6 and the voltage sensor is located at S4. Domains are linked by three intracellular loops (L1–L3). Both N- and C- terminals of VGSC proteins are intracellular (see figure 1.8). Vertebrate VGSC α-subunit genes have greater than 50% amino acid sequence similarity (H Yu et al. 2003). All VGSC subtypes are encoded by at least 20 exons each. An evolutionary analysis of these channels in vertebrates show that the genetic sequences of these channels can fall into four groups. There are four paralogous chromosome segments that contain α-subunits. Genes for Nav1.1, 1.2, 1.3 and 1.7 are located on chromosome 2 (Plummer et al. 1999) in both humans and mouse and have the highest amount of sequence similarities and biophysical characteristics (can be blocked by nanomolar concentrations of TTX) (Goldin et al. 2000). The second cluster of VGSC genes encoding Nav1.5, 1.8 and 1.9 are located on chromosome 3 in both mouse and human (Goldin et al. 2000). Although, this group closely resembles the group found on chromosome 2, these channels confer varying degrees of TTX resistance (H Yu et al. 2003). Four of these
voltage-gated sodium channels have restricted distribution in nociceptors, these are Nav1.3, Nav1.7, Nav1.8 and Nav1.9. The properties and expression patterns of these four subtypes alter in animal models of pain (Berta et al. 2008). Nav1.3 is a TTX sensitive channel and is more highly expressed during embryonic development than in adult neurons. Interestingly, during inflammation following axotomy, Nav1.3 transcript levels are increased in sensory neurons (Waxman et al. 1994). The rapid recovery from inactivation of Nav1.3 suggests that it could play an important role in rapid sustained firing or bursts of action potentials in chronic pain condition. This has been verified in knockdown experiments of Nav1.3 using antisense oligonucleotides and knockout mice (Hains et al. 2004). Some evidence showing an up-regulation of Nav1.3 in rodent dorsal root ganglia (DRG) neurons following nerve injury has also suggested a potential role for this channel in pain (Waxman et al. 2014) but will not be addressed further in this thesis. Nav1.7 is expressed in peripheral sympathetic and sensory neurons as well as olfactory epithelia (Djouhri et al. 2003). Nav1.7 channel inactivates slowly at negative membrane potentials this contributes to the large currents during slow ramp depolarisations, making Nav1.7 an important determinant in setting action potential generation threshold (Momin et al. 2008). Nav1.8 has an essential role in maintaining the excitability of nociceptors at low physiological temperatures where it acts as the sole impulse generator (Zimmermann et al. 2007). This capacity for transducing cold was found in behavioural studies of Nav1.8 knockout mice , which show negligible responses to noxious cold (Zimmermann et al. 2007). Nav1.8 is a major contributor to action potential upstroke in sensory neurons. nav1.8 voltage dependence of activation and inactivation is more depolarised for Nav1.8 which reprimes more rapidly than Nav1.9. Nav1.9 activates at potentials almost identical to resting membrane potentials (-70mV) and is responsible for producing a persistent currents essentially setting membrane resting potential (Momin et al. 2008) (see figure 1.5).

Although it is possible to create a fully functional voltage gated sodium channel from only a pore forming α subunit, such as in the case of the electric eel (Namadurai et al. 2015) all mammalian VGSC α subunits associate with β subunits. There are four β subunit genes (Scn1b-4b) encoding proteins β1-4 respectively. As later shown with α subunits, β subunits are also expressed with distinct tissue specificities (Patino et al. 2010) (see table 1.2 for expression of beta subunits in various nervous tissues). All β subunits are type 1 intrinsic membrane proteins (see figure 1.6). They comprise an extracellular amino-terminal region containing a single V-type immunoglobulin domain (Namadurai et al. 2015). This immunoglobulin domain is in turn connected to a single α-helical transmembrane domains as well as a carboxy-terminal region located intracellularly (Namadurai et al. 2015). In terms of
sequence similarities, $\beta_1$ and $\beta_3$ are more closely related than any other subunit. $\beta$ subunits 1 and 3 interact in a non-covalent manner with $\alpha$ subunits, whereas $\beta_2$ and 4 attach to the pore forming subunit in a covalent manner via inter-subunit disulfide bonds (Namadurai et al. 2015; Buffington et al. 2013; Chen et al. 2012). X-ray structure of the human $\beta_3$ immunoglobulin domain has been solved and because of the sequence similarities between $\beta_3$ and $\beta_1$ this evidence has informed the structure of the latter. Super resolution imaging has detected a trimeric full length $\beta_3$ subunits as a major species in HEK293 cells (Namadurai et al. 2014). The detection of this primer is consistent with previous evidence showing that when expressed in cells, $\beta_3$ subunits self-associate with the immunoglobulin domain being necessary for this interaction (Namadurai et al. 2014).

Nav1.7 is expressed in large and small diameter dorsal root ganglion neurons. Nav1.7 was first found in somatosensory and sympathetic neurons (Toledo-Aral et al. 1997) and has since been detected in myenteric neurons, smooth myocytes, olfactory neurons and visceral sensory neurons. In the CNS, Nav1.7 has been reported in the hypothalamus (Black et al. 2013), as well as in free nerve endings within superficial laminae of the dorsal horn in the spinal cord (see figure 4.1). Previously, the expression of Nav1.8 was thought to be exclusively confined to small and medium diameter nociceptive subsets of sensory neurons of the DRG (Akopian et al. 1996; Benn et al. 2001). However it has subsequently been shown that 75% of dorsal
Figure 1.6: Cartoon structure of human voltage-gated sodium channel β3 subunit as determined by Namadurai et al., 2015. Diagram shows the arrangement of the β3 trimer and immunoglobulin domain (IG) (Taken from Namadurai et al., 2015)

<table>
<thead>
<tr>
<th>Tissue-subcellular domain</th>
<th>β1</th>
<th>β1B</th>
<th>β2</th>
<th>β3</th>
<th>β4</th>
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<tbody>
<tr>
<td>Hippocampus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Cortex</td>
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<tr>
<td>Basal ganglia</td>
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<td>Retinal ganglia</td>
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<td>Optic nerve</td>
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<td>Astrocytes</td>
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<td>DRG</td>
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<td>Ventral horn</td>
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<td>Radial glia</td>
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<td>Cerebellar Purkinje</td>
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<tr>
<td>Cerebellar granule</td>
<td>+</td>
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<td>Deep cerebellar nuclei</td>
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<tr>
<td>Bregman glia</td>
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<tr>
<td>Peripheral nerves</td>
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<tr>
<td>Sciatic nerve</td>
<td>+</td>
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<tr>
<td>Schwann cells</td>
<td>+</td>
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Table 1.2: A summary of all known voltage-gated sodium β subunits channels and their distribution in tissues of the central and peripheral nervous system (adapted from Patino et al., 2010)

root ganglion cells express Nav1.8 with 90% of these expressing nociceptive markers (Shields et al. 2012). Evidence also suggests the presence of Nav1.8 in approximately 40% of myelinated A fibres (Shields et al. 2012). Furthermore, Nav1.8 has been detected in intracardiac ganglia possibly exerting its effects on cardiac electrophysiological properties
Nav1.9 was first identified in the soma and central terminals of functionally identified nociceptors of the DRG and trigeminal ganglia (Dib-Hajj et al. 1998) and is preferentially expressed in small-diameter DRG neurons, trigeminal ganglia neurons and in intrinsic myenteric neurons (Dib-Hajj et al. 2002). The differing properties and cellular localizations of these channels endow different cell types with much of their electrophysiological and functional properties. Nav1.7 produces a rapidly activating and inactivating tetrodotoxin (TTX) sensitive current (see figure 1.5). This type of channel is well suited to low-frequency firing in C-fibres due to its slow repriming nature (Cummins et al. 1998; Herzog et al. 2003) and is considered a threshold channel due to its ability to boost subthreshold stimuli and thereby increase action potential firing frequency (Rush et al. 2007; Dib-Hajj et al. 2007). The TTX resistant current produced by Nav1.8 acts as a major contributor to the upstroke of action potentials (Akopian et al. 1999; Renganathan et al. 2001) (see figure 1.5). Under cold conditions, where slow inactivation is enhanced in TTX sensitive channels, Nav1.8 is the sole channel able to generate electrical impulses acting to maintain nociceptor excitability (Zimmermann et al. 2007). Nav1.9 has remarkably unique biophysical properties (see figure 1.5). This channel generates a persistent TTX-resistant current that has very slow gating kinetics and can be activated at potentials close to resting membrane potential (Dib-Hajj et al. 2002). Activation kinetics of this nature are unable to contribute to the up-stroke of action potentials and instead act as modulators of membrane excitability through persistent inward currents and the ability to activate at a strategic range that is negative to, and overlaps with, the voltage thresholds of other transient sodium channels.

1.4.1 Voltage-Gated Sodium Channel Structure: Clues From Bacteria

The difficulty in isolating, expressing, purifying and crystalising human membrane proteins has meant that most of the crystallographic information we have on the structure/function of human voltage-gated sodium channel alpha subunits comes from the a bacterial crystal structure deduced by Payandeh et., al (2011). Due to some degree of homology between prokaryotic and eukaryotic VGSC functional subunits, the analysis of the *Arcobacter butzleri* voltage-gated sodium channel, has offered some insight into the biophysical functionality of human VGSCs (Payandeh et al. 2011) including: voltage-dependent activation, slow inactivation, ion selectivity, and drug block (Payandeh 2015). These shared functional characteristics would imply a certain amount of robust structural evolutionary conservation.
The BacNav have approximately 275 residues making them 1/8th the size of the 2000 residue eukaryotic Nav pore forming subunit. Furthermore, instead of the 24 transmembrane segment architecture of Navs, BacNavs are comprised of 6 transmembrane segments that comprise a voltage sensing domain and a pore forming domain that assemble into homotetramers at the cell membrane (Mio et al. 2010; Shaya et al. 2011; McCusker et al. 2011; Payandeh et al. 2011; Payandeh et al. 2012; Zhang et al. 2012). The BacNav also shares similar ion selectivity profiles of Cavs and Navs (Ren et al. 2001; Durell et al. 2001; Shaya et al. 2014). As illustrated in figure 1.8, the bacterial structure deciphered by Payandeh and colleagues is composed of four separate discontinuous, yet homologous, protein domains; in contrast to the continuous protein sub-domains of human VGSCs (see bottom panel of figure 1.8) (Payandeh 2015), however functionality within these subunits are somewhat conserved. Like all VGIC, the BacNav structure cements the concept that all known voltage-gated sodium channels share a conserved architecture (Payandeh et al. 2011; Payandeh et al. 2012; Zhang et al. 2012; Shaya et al. 2014; Tsai et al. 2013; McCusker et al. 2012; Bagneris et al. 2014).

Transmembrane segments S1-4 form the voltage-sensing domain and S5-6 are responsible for forming the pore of the channel that houses the ion selectivity filter. Furthermore, the voltage-sensing unit is reliant on a highly conserved arginine residues located at S4, within the electric field of the membrane and undergoes an outward movement upon depolarisation (Kuzmenkin et al. 2004; DeCaen et al. 2009; Chahine et al. 2004; Blanchet et al. 2007; Yarov-Yarovoy et al. 2012; Armstrong et al. 1973; Bezanilla et al. 1974). The BacNav structure also shows further homology with human Nav1.7 in terms of pore forming subunits, where the S5-S6 are connected through a helix-loop-helix motif which could give further clues to the ion selectivity filters of VGICs and better define the structure of the extracellular vestibule (Payandeh et al. 2011; Payandeh 2015).

Despite the plethora of natural and synthetic molecules that target Navs (Moreau et al. 2014; Catterall et al. 2007; Knapp et al. 2012; Nardi et al. 2012; Klint et al. 2012; Thottummkara et al. 2014; Quintero-Hernández et al. 2013) mechanism of action and selectivity are still somewhat opaque. BacNav homology with human Navs has provided accurate templates to understand the physical interaction of Nav modulators. Molecules that affect Nav function have modes of action that fall inline with their complex functional states that are mirrored by the shared homology of both eukaryotic and prokaryotic Navs including key characteristics including pore block, open state stabilisation of the pore and altering voltage-sensing characteristics. Despite the limitations of the bacterial Nav structure, the mirroring of functionalities and the efficacy of certain eukaryotic Nav blockers on BacNav provides the
most accurate template for predictive models of pharmacological drug binding (see figure 1.7) (Payandeh et al. 2011; Payandeh 2015). The extracellular vestibule is a key site for Nav modulation by protons, divalent cations and small toxins (see figure 1.7) (Sun et al. 1997; Jones et al. 2014). The BacNav structure suggests that these modulators may neutralise key sidechains required to coordinate and conduct Na-H2O complexes that destabilise the selectivity filter. Tetrodotoxin is one such molecule that binds directly to the selectivity filter of eukaryotic Navs with nanomolar affinity. Predictive binding models generally point to the guanidinium group of TTX interacting with the glutamate side-chain from the DEKA motif of the selectivity filter (Lipkind et al. 1994; Penzotti et al. 1998; Walker et al. 2012; Tikhonov et al. 2012; Chen et al. 2014). This however was not recapitulated in BacNavs, where TTX has no effect on channel block (Ren et al. 2001). One the other hand, BacNav structures provide an excellent template to study channel blockers used for epilepsy or analgesia that act in the central cavity of Navs. These drugs act by blocking opened or inactivated Nav channels (Ragsdale et al. 1998). Two highly conserved aromatic residues on S6 of domain IV of eukaryotic Navs are major determinants of central cavity mediated block (Ragsdale et al. 1991; Ragsdale et al. 1996). Equivalent residues have been found in bacterial BacNavs that line the central cavity (Hockerman et al. 1997; Yarov-Yarovoy et al. 2001; Yarov-Yarovoy et al. 2002). BacNavs have also provided further confirmation regarding the pharmaceutical targeting of Nav voltage sensing domains. Small ”gating modifying” neurotoxins are classically understood to act on the voltage sensing domain present on domain II or domain IV (see figure 1.8) and enhance or suppress the activation properties of the channel, which
VGSCs are integral membrane proteins that organise into four homologous domains (DI-IV) each consisting of 6 transmembrane segments (SI-VI). The sodium ion sodium selective pore is formed by re-entrant loops between helices SV and SVI and is embedded into the transmembrane region of the channel to form a narrow ion selective extracellular filter at the top of the pore. The voltage sensitive section of the channel is found at SIV. Top left: Side view of crystal structure of bacterial NavAb showing voltage sensor and side view of the pore. Top right: Top view looking down into the crystal structure of NavAb sodium channel, clearly showing the four domains and the formation of the ion selective pore. Lower diagram: Shows cartoon of a generic voltage-gated sodium channel, with IV domains. Voltage sensitive helix is indicated by "+" sign. Crystal structure illustrations in this figure where obtained from the RCSB Protein Data Bank and were based on the structure of a bacterial voltage-gated sodium channel from *Arcobacter butzleri* that was deduced by Payandeh et al., 2011. Interactive version of this model can be viewed at https://www.rcsb.org/3d-view/3RW0. Resolution = 2.95 Å. "Rainbow" style colouring represent the different portions on the protein helix. At the N-terminal portion the colour of the helix is blue and runs through the colour spectrum until it reaches red which is the C-terminal of the protein.

have been confirmed through comparisons with the NavBac structure. Further information regarding the current state of pharmacological targeting of Nav1.7 is outlined towards the end of this chapter (see section 1.5.0.7 of this chapter).

### 1.4.2 Nav1.7(SCN9A) Gene, Channel, Distribution and Regulation

Nav1.7 is preferentially expressed in a number of peripheral and central tissues including, DRG, trigeminal ganglia, olfactory epithelia and sympathetic neurons (Toledo-Aral et al. 1997; Weiss et al. 2011). Nav1.7 expression has been recently reported in the hypothalamus (Black et al. 2013), as well as in free nerve endings within superficial lamina of the dorsal horn in the spinal cord. Additionally, Nav1.7 is expressed in visceral sensory neurons, nodose ganglion neurons (Muroi et al. 2011), smooth myocytes (Holm et al. 2002; Jo et al. 2004), myenteric neurons (Sage et al. 2007) as well as excitable cells including immune cells (Kis-Toth et al. 2008).
et al. 2011). Human progenitor cells (Hoffman et al. 2004) and pancreatic islet β-cells, where Nav1.7 is thought to potentially be involved in the triggering of glucose-stimulated insulin secretion (Ernst et al. 2009). Furthermore, an increased expression of Nav1.7 has been found in human cells derived from prostate and breast cancer patients (Diss et al. 2005).

1.4.2.1 Cellular Distribution of Nav1.7

Immunohistochemical methods show that Nav1.7 is most highly expressed in unmyelinated small diameter C-fibre neurons. Using peripherin-staining of DRG neurons to mark small diameter DRG neurons (<30µm), Black et al., (2012), show that 63% of these neurons are immunoreactive to Nav1.7. In contrast, Neurofilament marked A fibres, medium diameter neurons (30–40µm) show relatively low immunoreactivity to Nav1.7 (15%) (Black et al. 2012) and large diameter neurofilament-positive neurons rarely exhibit Nav1.7 expression (Black et al. 2012). Furthermore, both populations of peptidergic and non-petidergic C-fibres display robust expression of Nav1.7 (Black et al. 2012). Evidence from Usoskin et al., (2015), show that SCN9A is highly expressed in unmyelinated small low mechanothreshold C-fibre neurons, unmyelinated peptidergic and non-peptidergic neurons, as well as myelinated neurons with expression in the NF5 subtype of large proprioceptors (Usoskin et al. 2015). DRG neurons send two processes from their soma, one branch to peripheral targets (sciatic nerve) and one branch centrally to terminate in the spinal cord (central projection). Nav1.7 is continuously expressed in 27% of unmyelinated peripherin expressing fibres of the sciatic nerve (Black et al. 2012). Additionally, although expression is low, Nav1.7 can be found at the nodes of Ranvier of small diameter myelinated Aδ-fibers. Intraepidermal nerve fibres (IENF) in the skin branch perpendicular from C and Aδ-fibers and run subjacent and parallel to the dermis/epidermis boundary following which they ascend layers of the epidermis. Nav1.7 can be found in subepidermal nerve bundles from both peptidergic and non-peptidergic neurons. Furthermore, Nav1.7 can be found at the branching point of IENFs as well as in the tips of the fibres (Black et al. 2012). Co-immunostaining for Nav1.7 with the pre-synaptic synaptophysin in the spinal cord dorsal horn, indicates a robust expression of the channel in pre-synaptic terminals in laminae I and II, with no visible expression postsynaptically (Black et al. 2012). Central projections of DRG neurons which terminate in the spinal cord dorsal horn highly express Nav1.7 in peripherin positive unmyelinated neurons. Similar to sciatic projections, Nav1.7 is also expressed in nodal regions of myelinated A fibres (Black et al. 2012).
1.4.2.2 SCN9A: Transcriptional Regulation of Nav1.7

Alternative splicing of VGSC genes can alter the biophysical properties, kinetics, pharmacological sensitivities and tissue distribution of the channels (Sarao et al. 1991; Dietrich et al. 1998; Tan et al. 2002). Four alternative splice variants of the Nav1.7 gene SCN9A are expressed in human dorsal root ganglion neurons. Two variants are found to differ in exon 5, an exon which encodes an extracellular linker and voltage sensor. These variants differ by two amino acids in the S3 segment of domain I and denoted as exon 5A and 5N (Chatelier et al. 2008; Raymond et al. 2004). Two other positions for alternative splicing exist in exon 11, an exon that encodes the cytoplasmic loop connecting domains I and II. These variants are denoted 11L and 11S are characterised by the presence or absence of an eleven amino acid sequence and is a region regulated by PKA (Raymond et al. 2004). Functionally, alternative splicing at exon 5 affects ramp current properties of the channels and may shift ramp current activation to more hyperpolarised potentials in certain disease states mediated by Nav1.7 (Jarecki et al. 2009). Studies looking at the effect of cAMP levels on channel regulation indicate that a site on exon 11 is subject to phosphorylation by 8Br-cAMP, which causes a negative shift of the activation curve of variants containing 11S, whereas isoforms containing the 11L variant were unchanged after 8Br-cAMP exposure (Chatelier et al. 2008). Furthermore, alternative splicing of the channel at both exon 5 and 11 have an effect on the modulation of Nav1.7 by β1, where when in the presence of β1, exon 5 splice variants have an effect on voltage-dependent activation of the channel and exon 1 variants affecting voltage dependence of steady state inactivation of the channel (Farmer et al. 2012).

1.4.2.3 Post-Translational Modification of Nav1.7

Akin to most proteins, many physiological properties of voltage-gated sodium channels are regulated through processes of post-translational modifications and channel availability through intracellular trafficking. Our current understanding of these processes with regards to VGSCs as a whole is limited and in particular when it comes to Nav1.7. This following section will present and discuss the current information regarding post-translational modification and intracellular trafficking of VGSCs through protein-protein interactions.

1.4.2.4 Glycosylation of Nav1.7

Extensive protein glycosylation takes place in the Golgi body, after the protein is synthesised and before it is packaged and exported to it’s final destination, which in the case of VGSCs, is
the plasma membrane. Glycosylation can account for up to 30% of a protein’s molecular weight (MW) (Rossie et al. 1987). This form of post-translational modification is crucial to many biological processes, such as cell adhesion, cell signaling and immunity (Moremen et al. 2012). In the case of VGSC α-subunits, glycosylation modifies the gating properties of the channel by interfering with the electric field near the gating sensor (Cronin et al. 2005; Bennett et al. 1997; Ednie et al. 2012). Furthermore, it is in the Golgi body that VGSC α-subunits associate with auxiliary β-subunits which are known to impact gating properties and enhance trafficking. There are four β-subunits known to interact with VGSCs denoted β1-4. In the case of Nav1.7, this modulation by β-subunits is in part achieved by manipulating channel glycosylation patterns. Leaderman et al., (2013) found that under experimental conditions, when Nav1.7 was expressed alone, the channel is present in two forms: a fully glycosylated state (MW of 280 kDa) and a core-glycosylated state (MW 250 kDa). When co-transfected with β1 and β3 subunits Nav1.7 current density is significantly increased while kinetic properties of the channel are only slightly altered, indicating an increase in Nav1.7 presence at the membrane. The model proposed by these researchers is one where in the Golgi, the Nav1.7 α-subunit assembles with β1 and β3 subunits and mediate a specifically glycosylated form of Nav1.7. β1 co-expression enhances Nav1.7 surface expression of a fully glycosylated form and β3 of a core glycosylated form. Other β-subunits (i.e β2 and β4) do not affect channel kinetics or current density, as it is thought that these subunits only interact with the channel just before insertion into the plasma membrane (Rossie et al. 1987; Laedermann et al. 2013).

### 1.4.2.5 Phosphorylation

The phosphorylation of a protein is a reversible post-translational modification that affects up to 30% of proteins in vivo and mediates a broad range of protein functions, from structural conformation to biochemical activity (Kreegipuu et al. 1999). The phosphate group is usually added to a serine, histidine, tyrosine and threonine in eukaryotic proteins. Protein kinases are known to have a varied and complex effect on protein function due to the extensive number of phosphorylation sites and the large number of different protein kinases found in eukaryotic neurons.
1.4.2.6 MAPK/ERK1/2

Mitogen-activated protein kinases (MAPKs) are an important family of kinases that play a crucial role in mammalian cell signaling. Three members of MAPK exist: ERK, p38, and c-JUN. Each kinase activates a specific intracellular pathway (Widmann et al. 1999). These kinases have been implicated in various human diseases including various forms of pathological pain states (Obata et al. 2004). In transected axons of experimental neuromas, ERK1/2 accumulates with Nav1.7 at the site of injury. (Stamboulian et al. 2010). Following activation of MAPKs, by pro-inflammatory cytokines released during nerve injury, Nav1.7 accumulates and co-localizes with phosphorylated ERK1/2. This enables the phosphorylation of Nav1.7 at the site of injury, altering the channels biophysical properties and rendering it more easily activated in response to stimuli, effectively enhancing nociceptive firing (Ji et al. 2009; Persson et al. 2011). Enhanced trigeminal excitability caused by ERK1/2 mediated Nav1.7 phosphorylation, induced by IL-6, has also been proposed as a mechanism of migraine related pain (Yan et al. 2012). Another member of the MAPK family, p38, is reported to be increased in animal models of neuropathic pain and is thought to underlie the upregulation of TTX-resistant sodium channels in sensory neurons (Obata et al. 2004; Jin et al. 2006). TNF-α activates p38, resulting in the modification and slow inactivation of Nav1.8/Nav1.9, which in turn increases the TTX-resistant inward current and enhances nociceptive hyperexcitability (Gudes et al. 2015).

1.4.2.7 Protein Kinase A and C

Protein kinase A (PKA) is an important modulator of ion channel activity under both normal and pathological conditions (see figure 1.9). PKA activation is mediated by upstream levels of cAMP and AKAP15. Additionally, some PKA subunits such as, RIIβ, are primarily expressed in peripheral nociceptive neurons and are activated by some inflammatory agents (Isensee et al. 2014). Following tissue injury or inflammation, the resulting immediate increase in nociceptive neuronal excitability can be in part attributed to the increased activation of G-protein coupled receptors and the resulting intracellular signaling pathways (Woolf et al. 1999). Stimulatory G-protein subunit (Gs) activation leads to increased cAMP production by adenylate cyclase, ultimately activating PKA. In animal models, triggering this pathway by direct cAMP application is sufficient to elicit hyperalgesic behaviour (Taiwo et al. 1989; Kress et al. 1996; Hucho et al. 2007). Furthermore, PKA activation plays a role in the maintenance of inflammatory pain (Woolf et al. 1999) through pathways that are known to be activated by
PGE\(_2\). PGE\(_2\) binds to the prostaglandin E2 receptor and increases cAMP in sensory neurons leading to the activation of PKA, inducing hyperalgesic states (Pitchford et al. 1991; Pierre et al. 2009; England et al. 1996). PKA modulates the biophysical properties of multiple VGSCs. For example, the phosphorylation of Nav1.2 by this kinase has the ability to decrease its peak current by up to 50% (Gershon et al. 1992). The effects of PKA mediated phosphorylation have also been found in Nav1.1, Nav1.6 and Nav1.7 (Gershon et al. 1992; Cantrell et al. 1997; Zhou et al. 2000; Vijayaragavan et al. 2004). Interestingly, Vijayaragavan et al., (2004) reported that in Xenopus oocytes PKA activation decreases Nav1.7 peak current density while leaving channel kinetics unaffected (Vijayaragavan et al. 2004). However, PKA regulation of Nav1.7 in inflammatory states could be isoform dependent. As mentioned previously Nav1.7 can be alternatively spliced at exon 11 into long 11L and short 11S isoforms. While most splice variants have no impact on Nav1.7 kinetics, expression of the 11S isoform while in the presence of PKA lowers the activation threshold of Nav1.7. Coupling the increase of this splice variant with these effects of PKA could be an important mechanism for inducing the neuronal hyperexcitability observed in pathological pain states. In the case of TTX-resistant VGSC subtypes, PKA sharply increases sodium peak currents in DRG neurons, an effect thought to be partially due to the presence of PGE\(_2\) (England et al. 1996). Furthermore, PKA activation in pathological conditions also mediates nociceptive hyperexcitability through increased membrane trafficking of Nav1.8 (Liu et al. 2010).

Figure 1.9: Cartoon of VGSC showing main phosphorylation sites of PKA: four sites on longest intracellular loop between domains I and II. PKC: One main site close to voltage inactivation gate on intracellular loop between domains III and IV.

PKC pathways of peripheral sensitisation and the generation of nociceptive states has been extensively studied (Rang et al. 1988; Schepelmann et al. 1993; Souza et al. 2002). In models
of diabetic neuropathy, inhibition of PKC decreases states of hyperalgesia. As in the case of PKA, a number of inflammatory mediators such as PGE\(_2\) and bradykinin are also able to activate PKC pathways (Cesare et al. 1996; Ferreira et al. 2005). Similar to PKA, PKC is also able to phosphorylate Nav1.2, and has the ability to reduce VGSC currents by up to 80% (Numann et al. 1991). The reduction is in part due to a positive shift in the voltage-dependence of activation of the channel (Dascal et al. 1991). Furthermore, studies in Xenopus oocytes have shown that PKC down-regulates the skeletal muscle sodium channel Nav1.4 and the cardiac channel Nav1.5 and pain channel isoforms Nav1.7 and Nav1.8. PKC activation is also associated with a decrease in Nav1.7 current. However, this discrepancy could again be related to splice variant expression and cellular background (Vijayaragavan et al. 2004). Indeed, blocking PKC phosphorylation is associated with a decrease in Nav1.7 protein expression following continuous opioid administration in a model of diabetic neuropathy (Chattopadhyay et al. 2008). Chattopadhyay et al., (2008) examined the effect of transgene mediated increased enkphalin expression on changes in pain behaviour and the molecular and biochemical corelates present in DRG neurons. In a model of diabetic neuropathy, persistent vector mediated expression of enkephalin in vivo prevented the increase in Nav1.7 in dorsal root ganglia that normally characterises this pain model. Moreover, this effect correlated with the inhibition of p38 MAPK phosphorylation and PKC. This effect was also seen in painful conditions, where the inhibition of specific PKC isoforms in mice diminishes thermal hyperalgesia (Ohsawa et al. 1997; Ohsawa et al. 1999). These findings are also consistent with rodent models where increased PKC activity correlates with increased MAPK p38 activity, which as previously mentioned leads to the up-regulation of Nav1.7 in dorsal root ganglion neurons (Chattopadhyay et al. 2008; Hong et al. 2004; Jin et al. 2006).

1.4.2.8 Calmodulin (CaM) and Cam Kinase II (CaMKII)

Calmodulin is a small calcium sensor that drives rapid responses to changes in intracellular calcium concentrations and is known to be involved in the regulation of Ca\(^{2+}\)-dependent neuronal plasticity (Solà et al. 2001). The IQ motif at the C-terminal of VGSCs is an interaction site for CaM and calcium sensing proteins (Solà et al. 2001; Mori et al. 2000). The exact functional nature of this binding remains elusive, however there are a few studies reporting the regulation of VGSC currents by CaM. Interestingly, this regulation seems to be isoform specific despite the IQ motif being conserved throughout VGSCs (Deschênes et al. 2002; Young et al. 2005; Biswas et al. 2008). This is exemplified by the more potent
regulation of Nav1.4 as compared to Nav1.5, and the binding of CaM having a lower affinity for Nav1.7 compared to Nav1.6 (Herzog et al. 2003). Ca\(^{2+}\)-dependent activation of CaM leads to the activation of singaling molecules including CaM kinase II. CaM is a multifunctional serine/threonine kinase that mediates a broad range of cellular functions including membrane excitability and ion channel biophysical properties (Frankland et al. 2001). It is also an important regulatory unit for VGSC clustering and organisation in the membrane (Hund et al. 2010) CaMKII is expressed in nociceptive neurons and has been intimately involved in pain transmission (Hiruma et al. 1999; Brüggemann et al. 2000). CaMKII is thought to be one of the elements responsible for the transition from acute to chronic pain by inducing hyperalgesic priming, a process involving the activation of PKC isoform activation PKCe. Furthermore, in rodent neuropathic and inflammatory pain models, the inhibition of CaMKII reverses mechanical allodynia (Chen et al. 2009; Luo et al. 2008). Since the effects of CaM of VGSC regulation is thought to be due to the recruitment and subsequent phosphorylation of VGSCs by CaMKII, it is likely that the activation of this kinase in chronic pain states is a contributing factor responsible for the VGSC mediated hyperexcitability.

### 1.4.2.9 Ubiquitylation

Through complex cellular mechanisms, newly synthesised VGSCs undergo a number of quality controls in the endoplasmic reticulum before being packaged for export to the plasma membrane. Often these processes involve specific regulatory motifs present on the protein. For example, following synthesis in the ER, VGSCs Nav1.5 and Nav1.8 contain an RXR motif found in the intracellular loop between domains I and II (Allouis et al. 2006; Zhang et al. 2008), which retains the channels until they are associated with the correct \(\beta\)-subunit. Association with this protein masks the RXR motif and allows export of the channel (Zhang et al. 2008). Another important form of post-translational protein regulation is Ubiquitylation. Ubiquitylation is responsible for the negative regulation of many different cell surface plasma membrane proteins. This process is important for both the degradation of faulty proteins and the continuous turnover and membrane expression of proteins (Staub et al. 2006). Ubiquitin is a small conserved polypeptide of 76 amino acids that is covalently attached to the lysine residues of the target protein. Once ubiquitylated, proteins are internalised and either degraded or recycled (Abriel et al. 2005; Ciechanover 2005; Shih et al. 2000). Three successive enzymatic steps are required for the ubiquitination of a protein. Firstly, E1 enzyme activates ubiquitin in an ATP-dependent manner, following which,
ubiquitin is transferred to a ubiquitin-conjugating enzyme E2 via a thioester bond, and finally, this complex interacts with the E3 ubiquitin ligase enzyme that ubiquitylates the substrate protein (Pickart 2001). The mechanisms of ubiquitylation are best-described in the case of the epithelial sodium channel ENaC (Abriel et al. 1999; Rossier et al. 2002). A short proline rich protein interaction site, known as a PY motif, is the site used for channel ubiquitylation, leading to channel internalisation and degradation. Mutations in this motif, leading to an abnormal increase in ENaC channel function is sufficient to cause a hypertensive phenotype characterised by high blood pressure and abnormal kidney function (Firsov et al. 1996; Schild et al. 1996). Nedd4 and Nedd4-2 proteins bind to the channel’s PY motif on large members of the E3 ubiquitin ligase family, leading to membrane protein internalisation (Abriel et al. 1999; Metzger et al. 2012). Most VGSCs posses a PY motif at the C-terminal of their α-subunit enabling Nedd4-2 dependent ubiquitylation of the channel (Abriel et al. 2000; Laedermann et al. 2014). Both, Nav1.7 and Nav1.8 possess a PY motif and have been shown to be negatively regulated by Nedd4-2 in experimental conditions (Laedermann et al. 2013). The significance of Nedd4-2 regulation of these sodium channels can be further demonstrated in chronic pain syndromes. In an animal model of neuropathic pain, ubiquitin ligase expression was shown to be dramatically reduced in rodents and consequently causes an up-regulation of both Nav1.7 and Nav1.8 in nociceptive neurons (Abriel et al. 2000; Laedermann et al. 2013). The regulation of VGSCs by Nedd4-2 seems to be restricted to Nav1.7, Nav1.8 and possibly Nav1.6, with Nav1.9 being exempted due to the lack of the PY motif.

1.4.2.10 Palmitylation and Lipid Rafts

Lipid rafts are subdomains of the plasma membrane that are rich in glycosphingolipids, cholesterol, gangliosides and a number of cytoskeletal regulators. These dynamic micro-domains are suggested to have an important regulatory role in cytoskeletal rearrangement, modulation of signalling molecules and the clustering of membrane proteins (Echarri et al. 2007; Gowrishankar et al. 2012). Certain VGSCs such as Nav1.5, cluster in a type of lipid raft rich in caveolin, a cholesterol binding protein (Head et al. 2011; Yarbrough et al. 2002). Furthermore, reports of Nav1.8 clustering in lipid rafts along the sciatic nerve and in DRG neurons have been reported (Pristerà et al. 2012). Palmitylation is the covalent binding of palmitic acid to cysteine residues to newly synthesised membrane proteins within the ER and Golgi. By targeting certain VGSCs to the plasma membrane this reversible modification plays a role in cellular signalling, protein trafficking and membrane protein
functionality (Iwanaga et al. 2009). Important palmitylation sites have been identified within Nav1.2 at the intracellular loop between domains II and III. Mutations in this site (C1182A) lead to changes in sensitivity of the channel, making it more sensitive to PauTtx3 and altered gating properties.

1.4.3 Nav1.7 Protein-Protein Interactions

Along with the aforementioned proteins involved in the post-translational modification of VGSCs other interacting partners have been identified. These include transcriptional regulators, growth factor hormones, intracellular traffickers and other regulatory proteins that directly mediate channel biophysical properties. These proteins reveal not only the mechanisms that modulate VGSCs but also offer insights into the roles of these channels in cellular processes. This is crucial knowledge when considering the importance of VGSCs in pain related disorders. The following proteins have been identified to be associated with VGSC α-subunits.

1.4.3.1 β-Subunits

In vivo, most VGSCs associate with one or multiple auxiliary β-subunits (Meadows et al. 2005). Four β-subunits (β1-4) and two splice variants (β1A, β1B) have been identified that modulate many of the functions and subcellular localisation of VGSCs. Together with α-subunits they form heteromeric complexes on the plasma membrane where they fine tune the functional properties of the channel. In experimental conditions, co-expression of β1 with Nav1.7 in Xenopus oocytes increases channel current kinetics and produces a steady-state-inactivation (Vijayaragavan et al. 2001). Furthermore, co-expression of Nav1.7 with both β1 and β3 in HEK293 cells strongly increase sodium current density, thereby indicating a role for these subunits in the localisation of the channel at the plasma membrane (Laedermann et al. 2013). In situ hybridisation studies have shown that β3 mRNA is highly expressed in nociceptive type small and medium diameter DRG neurons. This cellular distribution overlaps with many of the pain related sodium channels (Nav1.7-1.9) (Shah et al. 2000). This subunit appears to play a role in the development of neuropathic pain. In chronic constriction injury and sciatic nerve axotomy models, increased β3 mRNA and protein levels increase in parallel with TTX-sensitive VGSC levels (Cummins et al. 1997).
1.4.3.2 P11 (Annexin A2 Light Chain)

Also known as annexing A2 light chain, p11 belongs to the S100 family of small, dimeric EF hand-type Ca\(^{2+}\)-binding proteins, despite being a calcium insensitive isoform. P11 forms heterotetrameric complexes with the calcium sensitive membrane associated protein annexing A2 (Hayes et al. 2004). P11 is known to interact with a number of channels including a number of ASICS and serotonin receptor 1B (Donier et al. 2005; Girard et al. 2002; Okuse et al. 2002; Svenningsson et al. 2006). With regards to VGSCs, p11/annexin A2 complex is known to bind to the cytosolic N-terminal domain of Nav1.8 and targets it to the plasma membrane (Okuse et al. 2002). Co-overexpression of p11 with Nav1.8 in CHO cells showed increased Nav1.8 functional activity at the plasma membrane (Okuse et al. 2002). Knocking out p11 in the sensory neurons of mice leads to decreased Nav1.8 current density in these neurons (Foulkes et al. 2006; Okuse et al. 2002). However, the absence of p11 in these nociceptive neurons does not change inflammatory pain thresholds but decreases neuropathic pain behaviour in p11\(^{-/-}\) mice. (Foulkes et al. 2006; Okuse et al. 2002). Together this evidence suggests that p11 is important but not essential to the intracellular trafficking of Nav1.8 (Foulkes et al. 2006).

1.4.3.3 Ankyrin

Ankyrins are scaffolding proteins important in the anchoring and localisation of structurally diverse membrane-associated proteins. Three ankyrin isoforms are known as Ankyrin-R, -B, -G (Mohler et al. 2007). The membrane binding domain of these proteins bind to a 9-residue motif located in the intracellular loop between domains II and III of VGSCs (Lemaillet et al. 2003). This motif has been shown to be necessary for the localisation of Nav1.5 at the plasma membrane in the heart (Garrido et al. 2003; Mohler et al. 2004). Furthermore, the ankyrin-G subtype is important for the clustering of Nav1.2 and Nav1.6 at nodes of Ranvier (Garrido et al. 2003; Jenkins et al. 2001). The exact mechanisms through which ankyrin traffics VGSCs is still unclear. However, ankyrin-mediated targeting may require spectrin, a cytoskeletal protein that simultaneously binds cytosolic proteins, integral membrane proteins and phospholipids, effectively creating multifunctional scaffolds (De Matteis et al. 2000; Kosaka et al. 2008). Furthermore, spectrin is thought to play a significant role in the early secretory pathway, at the ER-Golgi interface (Watabe et al. 2008). Therefore, it is probable that an ankyrin-spectrin system is involved in the trafficking of VGSCs from the ER to the Golgi body and the subsequent incorporation of the channel into the plasma membrane as a complex.
Section 1.4.3.4 Syntrophin

Syntrophins exist as three subtypes $\alpha$, $\beta$, $\gamma$. Functionally, syntrophins are adaptor proteins that bind and localise signaling proteins to the plasma membrane. They can interact simultaneously with multiple proteins at PDZ domains, two pleckstrin homology domains and a conserved syntrophin unique region. The PDZ domain binds the last three residues of the C-terminus of Nav1.4 and Nav1.5 (Haenggi et al. 2006). In the case of Nav1.5, the heart syntrophins are found in a complex with dystrophin and are both important for the membrane stabilisation of Nav1.5 (Gavillet et al. 2006). A similar PDZ binding motif has been found on the C-terminus of Nav1.8, but an association and functional modulation of this channel by syntrophin is yet to be determined.

Section 1.4.3.5 Papin

Plakophilin-related armadillo-repeat protein-interacting protein (papin), was first discovered and cloned from rat brain and human prostate cancer tissue (Deguchi et al. 2000). Consisting of 2766 amino acids, papin has four N-terminal and two C-terminal PDZ domains. A number of papin binding proteins have been identified, including Nav1.8, which binds to two C-terminal PDZ domains through a site on the channel’s intracellular loop between domains II and III (Malik-Hall et al. 2003). Other conserved PDZ binding motifs exist on similar channel loops of Nav1.2, Nav1.3 and importantly Nav1.7. Due to its structure and multiple binding motifs, papin most probably acts as a scaffolding protein and plays a role in plasma membrane localisation and trafficking of VGSCs (Malik-Hall et al. 2003). In normal conditions papin is found in the basal cell layer of prostate tissue, but becomes widely expressed in prostatic intraepithelial neoplasia and malignant glands (Chaib et al. 2001), indicating that papin might be closely associated with initiation or early promotion of prostate cancer. Interestingly, the two known proteins that bind to papin and are functionally associated with cancer metastasis are $\delta$-catenin and Nav1.7 (Diss et al. 2005; Nakajima et al. 2009). In prostate cancer cells, an increase in $\delta$-catenin is synchronous with a down regulation and redistribution of major cell junction proteins of E-cadherin and p120ctn. It is possible that papin acts as a scaffold, bridging Nav1.7 and the E-cadherin-catenin system.

Section 1.4.3.6 CRMP2

Collapsin response mediator protein 2 (CRMP2) was initially identified as being important in axonal outgrowth through it’s role in mediating growth cone collapse (Inagaki et al. 2001).
Interestingly, CRMP2 has also been found to also be important in the modulation of ion channel trafficking (Bertin et al. 2016; Chi et al. 2009). CRMP2 has been linked to the trafficking of Nav1.7. In a recent study conducted in CAD cells and sensory neurons, Dustrude et al., (2013) found that the modification of CRMP2, by a small ubiquitin-like modifier (SUMO), could affect Nav1.7 trafficking. They found that CRMP2 undergoes reversible SUMOtylation at a number of lysine residues. To be more precise, Dustrude et al., expressed a CRMP2 SUMOylation-incompetent mutant in neuronal catecholamine A differentiated cells. Mutating these sites caused a significant decrease in membrane expression of Nav1.7. Furthermore, CRMP2 has also been implicated in a number of pain-related disorders attributed to the dysregulation of trafficking of sodium channel by this protein being a key mechanism in the etiology of neuropathic pain. In a spared nerve injury (SNI) model in rodents, Moutal et al., (2018) found an increase in expression of a cyclin dependent kinase 5 (Cdk5)-phosphorylated form of the CRMP2 protein in the dorsal horn of the spinal cord and in DRG. Furthermore, through biochemical fractionation of the SNI spinal cord revealed that this increase in Cdk5-mediated CRMP2 phosphorylation was enriched to pre-synaptic sites. By using siRNA knockdown of CRMP2, the group were also able to reverse the SNI-induced pain phenotype (Moutal et al. 2018). These results clearly outline the necessity of Cdk5-mediated CRMP2 phosphorylation in the creation of the peripheral neuropathic pain phenotype. Moreover, the important role of CRMP2 as an ion channel trafficker is not only restricted to voltage-gated sodium channels, but is also understood to traffic certain voltage-gated calcium channels (Moutal et al. 2016). Indeed, the link between the phosphorylation of CRMP2 by Cdk5 in SNI models has been further evaluated with relation to voltage gated calcium channels, particularly Cav2.2 (Moutal et al. 2016; Brittain et al. 2012). The phosphorylation of CRMP2 by Cdk5 dynamically regulates the interaction of CRMP2 with Cav2.2 (Brittain et al. 2012). Interestingly, the antiepileptic drug (2R)-2-(acetylamino)-N-benzyl-3-methoxypropanamide (Lacosamide), binds to CRMP2 and arrests axonal outgrowth by inhibiting the Cdk5-mediated phosphorylation of CRMP2. Furthermore a number of biophysical studies and in silico docking models determined a binding pocket for lacosamide on CRMP2 (Moutal et al. 2016). Furthermore, while the activation of Cdk5 increases CRMP2 expression in rat DRG and underlines several apin phenotypes, the disruption of the Cav2.2-CRMP2 interaction by Lacosamide inhibits depolarisation-evoked calcium currents. This provides a solid example illustrating the role of CRMP2 in the trafficking of voltage gated ion channels and validates the possibility that this protein traffics Nav1.7 (Errington et al. 2008).
1.4.3.7 Synaptotagmin

As previously mentioned, aside from the generation and propagation of action potentials in sensory neurons, further functional roles for Nav1.7 have been uncovered such as, the involvement of Nav1.7 in itch (Devigili et al. 2014; Lee et al. 2014) and neurotransmitter release (Alexandrou et al. 2016; Minett et al. 2012a; Lee et al. 2014; Weiss et al. 2011; Lee et al. 2014; Devigili et al. 2014). In the olfactory system, deleting Nav1.7 in all olfactory sensory neurons leads to an absence of postsynaptic responses and currents in olfactory bulb projection neurons. However, when under electrophysiological recording these neurons produce normal action potentials when depolarized via current injection through the patch pipette, thereby suggesting an involvement of Nav1.7 in neurotransmitter release (Raouf et al. 2012). Furthermore, Sampo et al., (2000) showed that the calcium regulated synaptic vesicle protein, synaptotagmin, displayed direct high affinity interaction with neuronal sodium channels (Sampo et al. 2000). By methods of co-immunoprecipitation, solubilized [3H]saxitoxin-prelabeled sodium channels were found to pull-down with synaptotagmin I. Furthermore, by mutating specific intracellular regions of Nav1.2 they found the binding site of synaptotagmin I to be located within the cytoplasmic loop between domains I and II (Sampo et al. 2000). There is however, little evidence regarding the functionality of this interaction.

1.4.3.8 ERM Proteins

The cytoskeletal erin/radixin/moesin (ERM) protein family share a high level of homology and are known to be involved in mechanisms of cellular motility and morphology. ERM proteins directly associate with actin filaments to mediate the link between the plasma membrane and the cytoskeleton (Louvet-Vallée 2000). Protein interaction yeast-two hybrid assays show binding of moesin to the C-terminal domain of Nav1.8, in a region that is highly conserved among the VGSCs (Malik-Hall et al. 2003). Although ERM proteins are known to facilitate intracellular trafficking and membrane clustering, the nature of this interaction between ERM proteins and VGSCs has not yet been characterised. It has been suggested that moesin might influence Nav1.8 gating properties through cytoskeletal reorganisation (Shumilina et al. 2003).
1.4.3.9 Nav1.7 Pain and Disease

Functional mutations in SCN9A are known to be the underlying cause of a number of heritable disorders such as congenital insensitivity to pain (CIP) (Cox et al. 2006), inherited primary erythromelalgia (IEM) (Yang 2004), paroxysmal extreme pain disorder (PEPD) (Fertleman et al. 2006) and small fibre neuropathy (SFN) (Faber et al. 2012). Small fibre neuropathy is a painful sensory nervous system disorder characterized by damage to small diameter unmyelinated C-fibres and lightly myelinated Aδ fibres (Han et al. 2012). Small fibre neuropathy is usually evaluated by either skin biopsy, quantitative sensory testing, nerve conduction studies and screening genomic DNA for possible variants in the Nav1.7 gene SCN9A. Symptoms associated with SFN include burning, short bursts of pain and loss of sensation along with a number of autonomic symptoms (Han et al. 2012). Reports of the involvement of Nav1.7 variants that impair slow-inactivation in DRG neurons can render these cells hyperexcitable and contributing to the painful phenotype characterised in SFN patients (Han et al. 2012). Over the past decade several mouse studies have demonstrated the importance of Nav1.7 in pain sensation, as the conditional knockout of Nav1.7 in Nav1.8 positive nociceptors shows a loss of acute noxious mechanosensation and inflammatory pain (Nassar et al. 2004). Consistent with human findings, mice that lack functional Nav1.7 display marked insensitivity to pain and anosmia but are otherwise phenotypically normal (Toledo-Aral et al. 1997; Weiss et al. 2011; Minett et al. 2012a). These mice show no defects in mechanical sensitivity and supraspinal thermal sensitivity (Hargreaves’ test). In addition, Nav1.7 knockout mice do not develop formalin-induced inflammatory pain or complete Freund’s adjuvant (CFA)-induced thermal hyperalgesia (see table 1.4). Nav1.7 expression in different sets of mouse sensory and sympathetic neurons are essential for distinct types of pain sensation (Faber et al. 2012; Minett et al. 2012a). Deletion of Nav1.7 in all sensory neurons leads to an additional loss of noxious thermosensation (Minett et al. 2012a) (see table 1.4). Furthermore, although responses to the hotplate, as well as neuropathic pain are unaffected when SCN9A is deleted in all sensory neurons, in mice the ablation of Nav1.7 in all DRG sensory neurons and sympathetic neurons, shows a significant reduction in thermal sensitivity in the hotplate test and mechanical hypersensitivity in a surgical neuropathic pain model (Minett et al. 2012a). Furthermore, it has also been reported that electrical stimulation of isolated sciatic nerve roots failed to induce increased substance P release in the dorsal horn of conditional Nav1.7 KO mice where Nav1.7 was deleted in all sensory neurons (Minett et al. 2012a). These studies alone indicate the importance of Nav1.7 in pain sensation. Over the
past few decades, Nav1.7 has become an increasingly attractive target for the development of analgesic drugs. The topic of using VGSCs and in particular Nav1.7 in drug development is further discussed in a later section of this introduction.
<table>
<thead>
<tr>
<th>Channel</th>
<th>Gene</th>
<th>Tetrodotoxin Sensitive</th>
<th>Expression</th>
<th>Disease Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nav1.1</td>
<td>SCN1A</td>
<td>+</td>
<td>CNS: hippocampus, cerebellum, spinal cord, brainstem, PNS: spinal cord, DRG, nodes of ranvier, Heart</td>
<td>Epilepsy, Migraine, Autism</td>
</tr>
<tr>
<td>Nav1.2</td>
<td>SCN2A</td>
<td>+</td>
<td>CNS: cortex, thalamus, globus pallidus, hippocampus, cerebellum, PNS: DRG, spinal cord</td>
<td>Epilepsy, Autism, Episodic ataxia</td>
</tr>
<tr>
<td>Nav1.3</td>
<td>SCN3A</td>
<td>+</td>
<td>CNS: cortex, hippocampus, corpus callosum, trigeminal neurons, PNS: DRG, spinal cord</td>
<td>Epilepsy</td>
</tr>
<tr>
<td>Nav1.4</td>
<td>SCN4A</td>
<td>+</td>
<td>Skeletal muscle</td>
<td>Hyperkalaemic periodic paralysis, Paramyotonia congenital, Hypokalaemic periodic paralysis</td>
</tr>
<tr>
<td>Nav1.5</td>
<td>SCN5A</td>
<td>-</td>
<td>Cardiac muscle</td>
<td>Brugada syndrome, Long QT syndrome 3, Atrial fibrillation</td>
</tr>
<tr>
<td>Nav1.6</td>
<td>SCN8A</td>
<td>+</td>
<td>CNS: hippocampus, Purkinje cells, cortex, cerebellum, PNS: DRG, spinal cord, Smooth muscle</td>
<td>Mental retardation, Pancerebellar atrophy, Ataxia, Infantile Experimental encephalopathy</td>
</tr>
<tr>
<td>Nav1.7</td>
<td>SCN9A</td>
<td>+</td>
<td>PNS: spinal cord, DRG, olfactory neurons</td>
<td>Congenital insensitivity to pain, Inherited primary erythromalgia, Paroxysmal extreme pain disorder, Small fibre neuropathy, anosmia</td>
</tr>
<tr>
<td>Nav1.8</td>
<td>SCN210A</td>
<td>-</td>
<td>PNS: trigeminal ganglia, DRG, spinal cord, heart</td>
<td>Small fibre neuropathy</td>
</tr>
<tr>
<td>Nav1.9</td>
<td>SCN11A</td>
<td>-</td>
<td>PNS: DRG, spinal cord, trigeminal ganglia,</td>
<td>Congenital insensitivity to pain, Painful peripheral neuropathy, Familial episodic pain syndrome</td>
</tr>
<tr>
<td>NaX</td>
<td>SCN7A</td>
<td>+</td>
<td>Circumventricular organ</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

**Table 1.3:** A summary of all known voltage-gated sodium channels, their distribution, tetrodotoxin sensitivity (+/-) and disease association. CNS refers to the central nervous system, PNS refers to the peripheral nervous system.
<table>
<thead>
<tr>
<th>Transgenic mice</th>
<th>Acute pain</th>
<th>Acute pain</th>
<th>Neuropathic pain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RS vF HG Hot plate Cold plate Acetone</td>
<td>F CFA C Other</td>
<td>SNT CCI SNI</td>
</tr>
<tr>
<td>Nav1.7KO</td>
<td>- 0 - -</td>
<td>- - (HG)</td>
<td></td>
</tr>
<tr>
<td>Nav1.7Nav1.8</td>
<td>- 0 0/- 0 0 0</td>
<td>- - (HG) (HG, vF)</td>
<td></td>
</tr>
<tr>
<td>Nav1.7Advillin</td>
<td>- 0 - 0 0 -</td>
<td>- - (HG) (HG, vF)</td>
<td>0(vF)</td>
</tr>
<tr>
<td>Nav1.7Wnt1</td>
<td>- 0 - - 0 -</td>
<td>- - (HG) (HG, vF)</td>
<td>-(vF)</td>
</tr>
<tr>
<td>Nav1.8KO</td>
<td>- 0 0/- 0 0</td>
<td>0 - 0/(HG) (HG), 0 (vF)</td>
<td>0(vF)</td>
</tr>
<tr>
<td>Nav1.8DTA</td>
<td>- 0 0 0 0 -</td>
<td>0/- -(vF, HG) (Hot plate, warm water, WB), 0 (vF, acetone)</td>
<td>0(vF,HG)</td>
</tr>
<tr>
<td>Nav1.9KO</td>
<td>0 0 0 0 0</td>
<td>- -(vF, plate, warm water, WB), 0 (vF, acetone)</td>
<td>0(vF,WB), 0(vF, acetone)</td>
</tr>
</tbody>
</table>

Table 1.4
Table 1.4: A summary of pain behaviour in Nav1.7, Nav1.8 and Nav1.9 KO mice (0: no change, -: reduced responses) Nav1.7 Nav1.8: Nav1.7 floxed Nav1.8-Cre (Nav1.7 is deleted in Nav1.8-positive sensory neurons), Nav1.7Advillin: Nav1.7 floxed Advillin-Cre (Nav1.7 is deleted in all sensory neurons), Nav1.7Wnt1: Nav1.7 floxed Wnt1-Cre (Nav1.7 is deleted in all sensory neurons and sympathetic neurons), Nav1.8DTA: floxed stop DTA Nav1.8-Cre (Nav1.8-positive neurons are ablated with diphtheria toxin). Mechanical pain: Randall–Selitto test (RS), light touch: Von Frey (vF), thermal spinal reflex: Hargreaves’ test (HG), supraspinal thermal: hot plate test, noxious cold: cold plate test, noxious cooling: acetone test, weight distribution to each hindpaw: weight-bearing test (WB). SNT, spinal nerve transection; CCI, chronic constriction injury; SNI, spared nerve injury. F: formalin test, C: carrageenan.

1.4.3.10 Nav1.8 (SCN10A)

Nav1.8 is expressed in small diameter unmyelinated nociceptive sensory neurons (Akopian et al. 1996). The predominant sodium conductance generated by Nav1.8 in small neurons (18–25µm diameter) is sensitive to TTX and slower than in many larger DRG neurons (44–50µm diameter), whereas sodium conductance in the large neurons is kinetically faster and TTX-resistant (Rizzo et al. 1994). This TTX-resistant sodium channel (IC50 = 60µM) (Akopian et al. 1999; Nassar et al. 2004) is the main contributor to the upstroke phase of action potentials in nociceptive neurons (Akopian et al. 1999; Renganathan et al. 2001). Nav1.8 also mediates the excitability of nociceptors at low temperatures, and is therefore an essential component in the propagation of cold stimuli (Zimmermann et al. 2007). In mice, deleting Nav1.8 in sensory neurons reduces the sensitivity to noxious mechanical stimuli, thermal stimuli and causes insensitivity to noxious cold (Abrahamsen et al. 2008; Dib-Hajj et al. 2007; Renganathan et al. 2001). Engineered gain-of-function mutations in SCN10A in mice increase sensitivity to cold stimuli by enhancing Nav1.8 sodium currents, as well as mechanically evoked action potential firing in subclasses of Aβ, Aδ and C- fibres (Blasius et al. 2011; Garrison et al. 2014). This evidence cumulatively supports the role of Nav1.8 in cold stimuli and painful neuropathies in human studies. Multiple studies also support the role of Nav1.8 in inflammatory pain; Nav1.8 KO mice show reduced CFA-induced heat hypersensitivity (Leo et al. 2010); and deletion of Nav1.8-expression neurons in sensory ganglia using diphtheria toxin reduces mechanical and heat hypersensitivity in carrageenan and CFA models (Abrahamsen et al. 2008). Furthermore, the administration of antisense oligodeoxynucleotides in rats leads to a decrease in prostaglandin E2 (PGE2)-induced hyperalgesia (Khasar et al. 1998) and reverses (CFA)-induced heat and mechanical hypersensitivity (Yu et al. 2011). In contrast, the role of Nav1.8 in neuropathic pain is unclear. In some antisense studies, knockdown of Nav1.8 attenuates the development and
maintenance of neuropathic pain in rats (Joshi et al. 2006; Lai et al. 2002). On the other hand, Nav1.8 knockout mice, as well as Nav1.7 and Nav1.8 double knockout mice, show normal behaviour in neuropathic pain models (Lai et al. 2002; Kerr et al. 2001; Leo et al. 2010) (see table 1.5). In humans, gain-of-function of mutations in Nav1.8 patients with small fibre neuropathies underlie mechanical hypersensitivity (Alexandrou et al. 2016; Emery et al. 2015). Electrophysiology studies using Nav1.8-null mouse DRG neurons, transfected with either Nav1.8 WT or mutant constructs, have demonstrated that these mutations cause increased excitability of small DRG neurons characterized as nociceptors. These neurons have reduced current threshold, increased firing frequency and increased spontaneous firing (Alexandrou et al. 2016; Emery et al. 2015). A recent electrophysiological study has provided novel distinctions between properties of the human and rodent Nav1.8 orthologues, including slower inactivation kinetics, larger persistent and ramp currents, as well as longer-lasting action potentials and increased firing frequency in human Nav1.8 channels (Han et al. 2015).

1.4.3.11 Nav1.9 (SCN11A)

Nav1.9 is expressed in DRG, trigeminal ganglia and motor neurons (Dib-Hajj et al. 1998). SCN11A encodes a 1765 amino acid protein that shows the least degree of homology to other members of the neuronal VGSC family (Dib-Hajj et al. 1999). Because this TTX-resistant channel activates at more hyperpolarizing voltages in comparison with other VGSCs, which produces its characteristic persistent current, Nav1.9 regulates resting membrane potentials and prolongs the depolarization response to subthreshold stimuli (Herzog et al. 2001; Cummins et al. 1999) that lower the threshold for single action potentials and increasing repetitive firing (Baker et al. 2003). Nav1.9 plays an important role in the generation of inflammatory pain (see table 1.5). Rodent DRG neurons treated with inflammatory mediators, including interleukin-1B, bradykinin and PGE2, show increased current density of the channel, lowering the threshold for action potential generation in these neurons and ultimately enhancing excitability (Binshtok et al. 2008; Petho et al. 2012; Maingret et al. 2008). Nav1.9 knockout mice show reduced inflammatory responses compared with wild type (Leo et al. 2010; Priest et al. 2005; Amaya et al. 2006; Lolignier et al. 2011) 1.4. These mice do not develop thermal hyperalgesia after CFA (Priest et al. 2005; Amaya et al. 2006; Lolignier et al. 2011) or carrageenan injection (Priest et al. 2005) (see table 1.4). Mechanical hypersensitivity induced by CFA- and formalin-induced inflammation is also diminished in Nav1.9 knockouts (Leo et al. 2010; Priest et al. 2005; Amaya et al. 2006; Lolignier et al. 2011)
A number of rare mutations in the SCN11A gene have been reported to underlie pain disorders. Familial episodic pain (Zhang et al. 2013) is caused by two missense mutations in Nav1.9 that reduce the threshold for action potential generation and increase firing frequency without changing the resting membrane potential. Furthermore, a mutation in the domain II S4–S5 linker region of the channel (G699R) is associated with small fibre neuropathy. By causing a hyperpolarization in channel activation and a depolarization in steady-state fast inactivation along with enhancing ramp responses of Nav1.9, this mutation leads to a hyperexcitability in dorsal root ganglion neurons via the maintenance of a depolarized resting membrane potential (Han et al. 2015). Other gain-of-function mutations in this channel include small fibre neuropathy with large fibre involvement (Huang et al. 2014) and heritable pain insensitivity (Leipold et al. 2013).

### 1.5 Human Heritable Sodium Channelopathies

#### 1.5.0.1 Inherited Primary Erythromelalgia (Nav1.7)

Mutations in SCN9A underlie inherited erythromelalgia (IEM). Symptoms of this gain-of-function disease include symmetrical burning pain accompanied by redness, increased skin temperature, oedema and erythema. Symptoms usually appear in childhood or adolescence and the phenotype can vary from mild to severe, even within the same family. The first IEM-related mutations were identified in a Chinese family in 2004 (Yang 2004) and more have been reported since (Ahn et al. 2010; Dib-Hajj et al. 2010). Electrophysiological characterizations of IEM-related mutations in SCN9A demonstrate a hyperpolarizing shift in the voltage-dependence activation and increased persistent current of the channel (Dib-Hajj et al. 2010). Recent studies have also suggested that these mutations lead to a significant hyperpolarized shift in voltage-dependent activation (Stadler et al. 2015), and a persistent current, which in turn, leads to a reduced current threshold and enhanced action potential firing probability (Vasylyev et al. 2014). Furthermore, along with the notable expression of Nav1.7 in sensory neurons, sympathetic ganglion neurons, and olfactory neurons, expression of Nav1.7 in smooth muscle cells of cutaneous arterioles, arteriole–venule shunts and endothelial cells in the skin may also account for the skin redness seen in IEM patients (Rice et al. 2015). However, despite the evidence supporting SCN9A mutations in IEM, only 10% of IEM families are proven to have SCN9A mutations, implying that some other genetic mutations may be linked to this disorder (Goldberg et al. 2012).
1.5.0.2 Paroxysmal Extreme Pain Disorder (Nav1.7)

PEPD (originally termed familial rectal pain syndrome) is a dominant heritable condition with the most common symptom involving severe burning pain in the rectal, ocular and submandibular areas, with episodes lasting up to several hours. Pain attacks can also be accompanied by flushing of the skin, legs, eyelid and buttocks (Fertleman et al. 2006; Goldberg et al. 2012). In 2006 Fertleman and colleagues described the first mutations associated with PEPD. The eight disease causing mutations in SCN9A were mapped in 11 families and 2 sporadic cases (Fertleman et al. 2006). Electrophysiological analyses showed that gain-of-function mutations in SCN9A are linked to this disorder. Although, both IEM and PEPD are caused by gain-of-function mutations in SCN9A, their electrophysiological profiles are distinct. In contrast with IEM, certain PEPD mutations shift the voltage dependency of steady-state fast-inactivation in a depolarizing direction, thereby increasing the probability of incomplete inactivation and resulting in a persistent current after activation. Interestingly, one SCN9A mutation is associated with a mixed clinical phenotype, displaying characteristics of both IEM and PEPD (Estacion et al. 2008).

1.5.0.3 Pain Insensitivity (Nav1.7 & Nav1.9)

Two genes encoding VGSCs have been associated with pain insensitivity, namely SCN9A and SCN11A. The first description of homozygous nonsense mutations in SCN9A causing a CIP phenotype was described in a number of patients originating from consanguineous families in northern Pakistan (Cox et al. 2006). Since this discovery, the same loss-of-function mutations in SCN9A causing pain insensitivity have been reported in other families (Cox et al. 2010; Goldberg et al. 2007). However, a recent electrophysiological study has revealed that three CIP-associated mutations in Nav1.7 retained some channel functions but that all mutations demonstrated a significant reduction in peak current following activation and changes in activation and/or inactivation properties. Two C-terminal mutations (W1775R and L1831X) showed a depolarizing shift in channel activation; the other mutation (A1236E, location: D3/S2) and one of the C-terminal mutations (L1831X) resulted in a hyperpolarizing shift in steady-state fast inactivation (Emery et al. 2015). Further studies in vivo are needed to investigate the link between these mutations and the CIP phenotype, but it seems likely that these mutations lead to a loss of function in vivo. Another phenotype caused by loss-of-function mutations in exon 22 of SCN9A was described in 2013 in two Japanese families (Yuan et al. 2013). In contrast with the Pakistani families, these patients demonstrated
complete loss of temperature sensation, autonomic nervous dysfunctions, hearing loss and hyposmia in addition to adolescent onset loss of pain. The reason for the underlying differences in the sensory phenotypes in these individuals remains elusive. Interestingly, pain insensitivity phenotypes are not solely generated by mutations confined to SCN9A. In 2013, a de novo missense mutation in SCN11A was found in two patients exhibiting a CIP phenotype (Leipold et al. 2013). These individuals displayed signs of mild muscular weakness, delayed motor development, slightly reduced motor and sensory nerve conduction velocities with normal amplitudes, no intellectual disability and a prominent hyperhidrosis together with gastrointestinal dysfunction. A mutation changing a highly conserved amino acid within the D2/S6 was identified in these patients and an investigation into the electrical properties of mouse DRG neurons suggested a gain-of-function mutation in this channel is the underlying cause of this pain insensitive phenotype. Loss of functional Nav1.9 channel in the mice had a minor effect on the electrical activity of DRG neurons (Leipold et al. 2013).
Figure 1.10: Schematic of generic voltage gated sodium channel along with location of pain associated mutations in Nav1.7, Nav1.8 and Nav1.9. Numbers correspond to "No. in figure" column in table 1.5. (taken from Kanellopoulos et al., 2014)

<table>
<thead>
<tr>
<th>No.</th>
<th>VGSC</th>
<th>Protein Mutation</th>
<th>Pain Disease</th>
<th>Functional Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nav1.7</td>
<td>S459X</td>
<td>CIP</td>
<td>Loss</td>
<td>Black et al., (2013)</td>
</tr>
<tr>
<td>2</td>
<td>Nav1.7</td>
<td>I767X</td>
<td>CIP</td>
<td>Loss</td>
<td>Black et al., (2013)</td>
</tr>
<tr>
<td>3</td>
<td>Nav1.7</td>
<td>W897</td>
<td>CIP</td>
<td>Loss</td>
<td>Black et al., (2013)</td>
</tr>
<tr>
<td>4</td>
<td>Nav1.7</td>
<td>L1331P</td>
<td>CIP</td>
<td>Loss</td>
<td>Joshi et al., (2006)</td>
</tr>
<tr>
<td>5</td>
<td>Nav1.7</td>
<td>I848T/L858H</td>
<td>IEM</td>
<td>Gain</td>
<td>Akopian et al., (1996)</td>
</tr>
<tr>
<td>6</td>
<td>Nav1.7</td>
<td>M1627K</td>
<td>PEPD</td>
<td>Gain</td>
<td>Benn et al., (2001)</td>
</tr>
<tr>
<td>7</td>
<td>Nav1.7</td>
<td>I1461T/T1464I</td>
<td>PEPD</td>
<td>Gain</td>
<td>Benn et al., (2001)</td>
</tr>
<tr>
<td>8</td>
<td>Nav1.7</td>
<td>A1632E</td>
<td>IEM/PEPD</td>
<td>Gain</td>
<td>Blasius et al., (2011)</td>
</tr>
<tr>
<td>9</td>
<td>Nav1.8</td>
<td>L544P</td>
<td>SFN</td>
<td>Gain</td>
<td>Zimmermann et al., (2007)</td>
</tr>
<tr>
<td>10</td>
<td>Nav1.8</td>
<td>A1304T</td>
<td>SFN</td>
<td>Gain</td>
<td>Zimmermann et al., (2007)</td>
</tr>
<tr>
<td>12</td>
<td>Nav1.9</td>
<td>R255C</td>
<td>FEP</td>
<td>Gain</td>
<td>Huang et al., (2013)</td>
</tr>
<tr>
<td>13</td>
<td>Nav1.9</td>
<td>A808G</td>
<td>FEP</td>
<td>Gain</td>
<td>Huang et al., (2013)</td>
</tr>
<tr>
<td>14</td>
<td>Nav1.9</td>
<td>I381T</td>
<td>PPN</td>
<td>Gain</td>
<td>Nassar et al., (2005)</td>
</tr>
<tr>
<td>15</td>
<td>Nav1.9</td>
<td>L1158P</td>
<td>PPN</td>
<td>Gain</td>
<td>Nassar et al., (2005)</td>
</tr>
<tr>
<td>16</td>
<td>Nav1.9</td>
<td>G699R</td>
<td>SFN</td>
<td>Gain</td>
<td>Binshtok et al., (2008)</td>
</tr>
</tbody>
</table>

Table 1.5: Table summarising currently known mutations in Nav1.7, Nav1.8 and Nav1.9 that produce altered pain phenotypes. ‘No.’ column corresponds to the approximate location of each mutation illustrated in figure 1.10. In the column labelled ‘Functional Effect’ Gain, refers to gain-of-function mutation; Loss, refers to loss-of-function-mutation. Figure is adapted from Kanellopoulos et al., 2016
1.5.0.4 Small Fibre Neuropathies/Painful Peripheral Neuropathies (Nav1.7, Nav1.8 & Nav1.9)

Adult-onset SFN affects, often caused by endocrine and metabolic disorders, unmyelinated and thin myelinated axons and leads to a reduced intraepidermal nerve fibre density. This disorder is often characterized by burning pain, allodynia and hyperesthesia. Large diameter axons are not damaged by SFN, resulting in normal tendon reflexes and vibration sense, and preservation of normal nerve conduction. The exact causes of SFN onset are not clear, however a number of both genetic and environmental factors may contribute to the onset of the disease in early adulthood. Diabetes and prediabetes are frequently associated with SFN. Furthermore, reports have shown that the largest contributing environmental factor to SFN development if hyperlipidemia (Vincent et al. 2009; Wiggin et al. 2009). Other conditions associated with acquired small fibre neuropathy are: HIV, celiac disease and hepatitis C (Hovaguimian et al. 2011). Diagnosis of SFN can be done often using a combination of several different methods. These include, quantitative sensory testing where thermal and vibratory sensation is measured using a QST study. A diagnosis can also be made by skin biopsy, where a 3mm dermatologic punch biopsy can be analysed for morphological abnormalities of nerve fibres typical of SFN. Electromyography and nerve-conduction studies can also be used to assess the integrity of neurons (Hovaguimian et al. 2011). Genetically, SCN9A, SCN10A, SCN11A have been identified as the three VGSC encoding genes which correlate with SFN. Gain-of-function missense variants in SCN9A have been reported in approximately one-third of individuals with small fibre neuropathies (Faber et al. 2012). The phenotype of these individuals with SCN9A mutations is different from IEM. For instance, SFN patients have reported experiencing pain throughout the body, whereas in IEM, pain tends to localize to the extremities. Furthermore, neither heat nor cold trigger symptoms in SFN patients, in contrast to patients with IEM (Faber et al. 2012). Functional profiling of the SFN mutant Nav1.7 channels showed impaired slow inactivation, depolarized slow and fast inactivation and increased resurgent currents. However, the hyperpolarizing shift in voltage dependence of activation and enhanced ramp responses that normally characterize IEM were not observed. In addition, the SFN mutant Nav1.7 channels did not demonstrate the incomplete fast inactivation often found in PEPD (Faber et al. 2012). Gain-of-function mutations in Nav1.8 have also been identified in patients with painful SFN (Huang et al. 2013). These mutations caused enhanced ramp responses, recovery from inactivation and activation, and led to hyperexcitability of small neurons in DRG, characterized by reduced current threshold,
increased firing frequency and an increase in spontaneous activity. More recently, mutations in SCN11A have been identified as a cause of painful peripheral neuropathy (PPN) (Huang et al. 2014). From a cohort of 393 patients, diagnosed with SFN, the gene SCN11A was sequenced from patients of this cohort who did not have mutations in SCN9A and SCN10A. From this cohort of 345 patients, eight variants were found in 12 patients. Functional analysis showed the mutations depolarize resting membrane potential of DRG, enhance spontaneous firing and increase evoked firing, indicating that gain-of-function mutations in Nav1.9 can cause PPN.

1.5.0.5 Familial Episodic Pain Syndrome (Nav1.9)

Familial episodic pain syndrome (FEP) is a Mendelian heritable trait resulting in severe pain and is triggered by conditions such as fatigue, fasting and cold. Three distinct types of this disease have so far been documented. Type I, characterized by pain localized predominantly to the upper body, is linked to a gain-of-function missense mutation in TRPA1 (Kremeyer et al. 2010). This is in contrast with the autosomal dominant type III disease form, first identified in two Chinese families, which is characterized by pain in the distal parts of the body, more specifically localized to the hands and feet (Zhang et al. 2013). In these patients, pain attacks usually occur late in the day with pain expanding simultaneously in different localizations, often triggered by intercurrent illness and fatigue after exercise. A combination of linkage analysis and whole-exome sequencing in both Chinese families has revealed missense mutations in SCN11A. Electrophysiological analysis has shown these mutations cause hyperexcitability of DRG neurons with increased peak current densities and enhanced action potential firing after current injection.

1.5.0.6 Nav1.7 and Cancer

Aside from the characterised functions of VGSCs in excitable cells, there have been multiple reports of functional expression of these channels in a multitude of non-excitable cells including macroglia (Sontheimer et al. 1992), oligodendrocytes (Tong et al. 2009), macrophages (Carrithers et al. 2007; Carrithers et al. 2009), T-lymphocytes (DeCoursey et al. 1985; Fraser et al. 2004), osteoblasts, odontoblasts (Allard et al. 2006), keratinocytes (Zhao et al. 2008), and fibroblasts (Chatelier et al. 2008). Most of the physiological roles of these channels have not yet clearly been demonstrated but have been proposed to regulate cellular functions such as survival, proliferation, cell migration, cell differentiation, endosome acidification, phagocytosis and podosome formation (Black et al. 2012). As such, the
physiological importance of VGSCs in non-excitable cells may still be underestimated. Additionally, a more interesting role for these channels in non-excitable cells, is their altered expression patterns in cancer cells. VGSCs were first suggested to play a role in cancer in the late 1980’s, where TTX sensitive voltage dependent sodium currents were recorded in human leukaemia cells and small-cell lung cancer cells (Pancrazio et al. 1989; Yamashita et al. 1989). Since then, abnormal overexpression of voltage-gated sodium channels have been found in a multitude of carcinomas of different cell types. Carcinomas, which originate from epithelial tissues, such as, lung, breast, prostate, cervix and colon, are responsible for most of the disease’s mortality (“Global cancer statistics, 2002.”). Because plasma membrane ion channels are key components in the regulation of homeostasis, membrane potential and intracellular singaling events, their abnormal expression has been found to regulate several cancer cell biological processes, such as, proliferation and apoptosis resistance (Litan et al. 2015; Schwab et al. 2014). A number of recent studies have highlighted an important role for Nav1.7 as being a particular VGSC subtype to be aberrantly regulating cell processes in cancer cells. For example, functional expression of Nav1.7 in non-small cell lung carcinoma cells (NSCLC) promotes cancerous invasion mechanisms (Campbell et al. 2013). Furthermore, inhibition of Nav1.7 activity, using small interfering RNA, reduces cell invasion by up to 50%. Campbell et al., (2013) also found that epidermal growth factor receptor (EGFR), an important receptor in the cell lineage determination and oncogenesis, EGFR-mediated up regulation of Nav1.7 is necessary for invasive cell behaviour. Nav1.7 has also been shown to be the most aberrantly expressed VGSC in gastric cancer cell tissue along with two experimental gastric cancer cell lines (Xia et al. 2016). Aberrant expression in these cells was correlated with the Na\(^+\)/H\(^+\) exchanger-1 (NHE1) and oncoprotein metastasis-associated in colon cancer (MACC1), two proteins intimately linked to cancer disease progression. Nav1.7 suppression in these cells reduced voltage currents and decreased expression of NHE1 and decreased intracellular pH, ultimately reducing invasion and proliferation behaviours. Mechanistically, the link to Nav1.7 is thought to be through NF-kB p65 nuclear translocation via p38, reducing MACC1 expression. Following which, the down regulation of MACC1 decreases c-Jun phosphorylation and subsequently reduces NHE1 expression. Although mechanistically the link between Nav1.7 and cancer cell behaviour is still very much unresolved, Nav1.7 seems to play a clear role in cancer metastasis through the manipulation of local protein signalling pathways.
Nav1.7 plays an integral part in the electrical excitability of nociceptive neurons. It’s selective role in nociception and the lack of cognitive and cardiac adverse effects in individuals with non-functional Nav1.7 (CIP and Nav1.7 knockout mouse models (Nassar et al. 2005; Cox et al. 2006)) has fuelled efforts to develop Nav1.7 specific blockers for the treatment of pain. However, despite some reports outlining restricted efficacy of Nav1.7 channels blockers in animal models of pain (Chowdhury et al. 2011; Williams et al. 2007; London et al. 2008), in vitro experiments (Cao et al. 2016), and the occasional IEM patient responding to monotherapy using pan-sodium channel blockers (Choi et al. 2009; Fischer et al. 2009), progress in this field of therapeutic development has been slow with marginal progress (England et al. 2009). The lack of potent analgesia from efficient Nav1.7 blockers suggests a more complex involvement for this channel in the generation of pain aside from electrical conduction. Indeed, in a recent study, the deletion of Nav1.7 in mouse sensory neurons of the DRG was shown to provoke an important dysregulation in a number of genes (194 genes >1.5-fold dysregulated) including an up-regulation in the gene encoding the pro-enkephalin Penk, a well characterized endogenous opioid (Minett et al. 2015; Chu Sin Chung et al. 2013). Furthermore, the administration of naloxone into a human CIP individual with two mutations in SCN9A, and injection into Nav1.7-null mutant mice, reversed endogenous analgesia (Minett et al. 2015) This change in expression of endogenous opioid peptides could explain the CIP phenotype in Nav1.7 null mutant mice and non-functional Nav1.7 in humans, also introducing a level of complexity in the role of Nav1.7 and/or sodium, associated with transcriptional regulation. Indeed, the association between transcription and ion channels is well documented in the case of the closely related VGSCs (Abrahamsen et al. 2008; Leo et al. 2010). A recent study focusing on the natural antisense transcript (NAT) for SCN9A (Koenig et al. 2015), that is conserved in humans and mice, showed it can down-regulate Nav1.7 mRNA and protein levels and reduce Nav1.7 peak sodium currents in human embryonic kidney cells (HEK293A) and human neuroblastoma (SH-SY5Y) cell lines. These results suggest that the SCN9A-NAT can attenuate native sodium currents and regulate SCN9A post-transcriptionally, potentially altering pain thresholds, making it a potential candidate for therapeutic development.
1.5.1 Structure and Physiology of Voltage Gated Calcium Channels

Like voltage gated sodium channels, calcium channel alpha subunits consist of one single polypeptide chain organised into four repeated domains (see figure 1.11). The recent determination of the high resolution structure of CaV1.1 inform the general structure of voltage gated calcium channel alpha subunit (Wu et al. 2016) and the channel complexes as a whole previously determined by low resolution structural modelling (obtained more than a decade ago (Fallon et al. 2009). The old modelling provided structural interpretation that was mostly based on the crystal structures of Kv channels (Long et al. 2005; Long et al. 2007; Long et al. 2009), bacterial homologs of Nav channels NavAb7 and Navrh8 and a NavAb variant that was engineered to become CaV selective (Tang et al. 2013; Tang et al. 2014). However, the formation of these channels by four polypeptide chains cannot support reliable modelling of the pseudo-tetrameric CaV and Nav channels, nor were they able to clarify the molecular basis for the interactions between alpha 1 and the auxillary subunits. The overall structure of voltage gated calcium channels is approximately 170Å in height and 100Å in width (Tang et al. 2013; Tang et al. 2014). New more refined detailed architecture of a eukaryotic CaV channel in complex with its auxiliary subunits provides important framework regarding the intersubunit interfaces identified in the complex structure and inform the modulatory effects of auxillary subunits across all voltage gated ion channels.

![Figure 1.11: Schematic showing the overall structure of a voltage gated calcium channel. Similarly to VGSCs, VGCCs are composed of four domains each consisting of six segments with the voltage sensitive segment at SIV. Voltage gated calcium channels typically have three major subunits β, γ and α2δ.](image-url)
1.5.2 N-Type Voltage-Gated Calcium Channels and Neurotransmitter Release

N-type voltage-gated calcium channels are almost exclusively confined to neuronal tissue (Dolphin 2009; Dolphin 2013) and enriched in primary afferent fibre terminals localised to the dorsal horn of the spinal cord. By virtue of their biochemical coupling to vesicle docking and release machinery, these channels are intimately involved in mediating the depolarization-evoked release of neurotransmitters. This includes glutamate, CGRP and substance P (Takasusuki et al. 2011). Through the creation of a high concentration (10-50µM) transient calcium microdomain within the active zone of the synapse, they work as an essential trigger for the fusion of presynaptic vesicles with the plasma membrane (Long et al. 2008). Like many voltage gated ion channels, the pore forming alpha subunit of the N-type calcium channel, namely CaV2.2, undergoes a degree of alternate splicing at different loci (Castiglioni et al. 2006; Weiss 2007). Of particular interest to pain research, are the contrasting functional roles of two alpha subunit variants generated from a splice site present at exon 37, which gives rise to two variants known as 37a and 37b16. These variants differ by only 14 amino acids at the cytoplasmic COOH-terminal tail of the channel. Interestingly, channels composed of the 37a variant are almost exclusively confined to Nav1.7 positive nociceptive neurons. These two variants contrast greatly in their contribution to nociceptive neurotransmission. In transient expression systems, the electrophysiological profile of channels containing the 37a exon results in increased whole cell current density, a shift in half life voltage and altered regulation of channel activity by G-proteins (Castiglioni et al. 2006; Weiss 2007). In vivo siRNA knockdown experiments also suggest that channels containing the 37a variant mediate basal nociception and inflammatory pain. Studies in transgenic mice, exclusively expressing the 37a variant, also uncovered an increased sensitivity to morphine compared to 37b exclusive mice. However, following nerve injury, there is an equal contribution of both variants to tactile allodynia. This is in sharp contrast with thermal hyperalgesia which seems to be predominantly dependent on the 37a variant of the channel (Castiglioni et al. 2006; Weiss 2007). In a recent issue of the journal of Human Molecular Genetics, Groen and colleagues have reported a congenital mutation in CaV2.2 present in a family with myoclonus-dystonia. Using exome sequencing along with linkage analysis in three generations of this family they identified a missense mutation in the gene encoding CaV2.2 (CACNA1B), resulting in the substitution of an arginine residue at position 1389 to a histidine. This mutation present in the pore-forming loop between segments S5 and S6 of the
third transmembrane domain of the channel alpha subunit is essential for calcium conductivity. This in turn leads to the involuntary movement disorders seen in individuals suffering from myoclonus-dystonia, a disease associated with Complex Region Pain Disorder (Groen et al. 2014). Interestingly, this is the only case in which a mutation in the alpha subunit of an N-type calcium channel has been linked to a genetic disorder with no evidence of such mutations leading to sensory deficits or heritable pain diseases. In contrast, engineered knockouts of these channel subtypes have revealed distinctly altered hyposensitive pain phenotypes (Hatakeyama et al. 2001; Kim 2001; Saegusa et al. 2001; Saegusa et al. 2002), which include lowered inflammatory and neuropathic pain and differential responses to general anaesthetic agents such as halothane and propofol. Sensitivity to the cone snail peptide \(\omega\)-conotoxin GVIA is one of the distinguishing characteristics of N-type calcium channels (Bourinet et al. 2014). The formation of three disulphide bonds, give this 27-amino acid peptide a rigid backbone, which blocks calcium current flow by occluding the outer vestibule of the alpha subunit pore (Bourinet et al. 2014). When administered intrathecally into mice, this peptide elicits the selective inhibition of N-type channels activity leading to decreased neurotransmission and consequent potent analgesia (Atanassoff et al. 2000; Miljanich 2004; Staats et al. 2004; Wallace et al. 2006). In humans, a structurally related peptide isolated from the Conus magus also causes potent suppression of pain and has been approved by regulatory bodies in both Europe and the USA for use in the treatment of pain. This peptide, known as Zicontide, sold under the trade name Prialt, is impermeable to the blood brain barrier, requiring the drug to be delivered intrathernically. In addition, the reported side effects of Prialt, which include dizziness, blurred vision, hypotension and memory problems, give the drug a narrow therapeutic window. Interestingly, this is in sharp contrast with total CaV2.2 ablation, which leads to total analgesia with no indication of such side effects. Together, this evidence strongly reinforces the developments in targeting such channels for the development of novel therapeutics (Staats et al. 2004; Wallace et al. 2006).

1.5.3 L-Type Voltage-Gated Calcium Channels and Transcriptional Regulation

While L-type channels hold crucial and well-documented electromechanistic/synaptic roles as well as being intimately involved in processed such as hormone secretion, more recent evidence suggest a important implication of L-type voltage-gated calcium channels in the regulation of gene transcription. Local changes in intracellular calcium permit the regulation
of many cellular events, including cell division, synaptic transmission and gene regulation (Bourinet et al. 2014). L-type voltage-gated calcium channels, which are encoded by the CaV1.2 and CaV1.3 pore forming subunit, are preferentially and densely localised in neuronal cell bodies and proximal dendrites (Bourinet et al. 2014). Calcium influx through L-type channels activates transcription factors such as CREB, MEF and NAFT (Gomez-Ospina et al. 2006; Tian et al. 2010; Lu et al. 2015). It stands to reason that because of this somatic localisation, L-type channels along with the calcium that flows through them, hold a preferential localisation with respect to transcriptional regulation, facilitating the modulation of transcription partners by extracellular calcium. Studies using selective calcium buffers indicate that specifically bound calcium dependent regulatory proteins respond by an up-regulation following local calcium increases (Lu et al. 2015). Calmodulin is famously a calcium dependent regulator of CaV1 channels and through the binding of the proximal carboxy terminal domain of CaV1.2, holds a crucial role in the transcriptional regulation of neurons. A proposed transcriptional mechanism involves changing to an activated form and translocating to the nucleus where it interacts with other factors to regulate transcription. Additionally, the interaction between calcineurin and the distal end of CaV1 channels has been proposed to regulate transcription through the dephosphorylation of certain key regulatory proteins. In hippocampal neurons, the dephosphorylation of NFAT5c4 by calcineurin bound to the distal end of CaV1.2 channels, prompts its dissociation and subsequent translocation to the nucleus (Tian et al. 2010; Lu et al. 2015). In a more recent study, conducted by Ospina and colleagues, the carboxy terminal of L-type voltage gated calcium channels has itself been proposed as an endogenous transcription factor (Gomez-Ospina et al. 2006). The proteolitic cleavage of the relatively large carboxy-terminal domain of Cav1.1 and Cav1.2, produces a noncovalant 300 amino acid long associated distal protein which previously was linked to a mechanism of channel autoregulation (Satin et al. 2011). This proteolitic processing requires extracellular calcium influx in neurons and can be blocked using calpain inhibitors (De Jongh et al. 1994; Hell et al. 1996). This carboxy terminal has been detected in the nucleus of certain neuronal subtypes in the developing brain and in cultured neurons. This proteolitic product binds to nuclear proteins and associates with an endogenous promoter to regulate the expression of a wide variety of genes associated with neurotransmission and neuronal excitability. In cardiac myocytes, this cleaved transcription factor can regulate the transcription of a substantial number of other genes including the gene for the CaV1.2 channel itself (Satin et al. 2009). Indeed, this regulatory mechanism allows for the necessary selective autoregulation of CaV1.2. However, the reason
for the differential effect of regulating channel activity directly versus transcriptionally is unknown. In neurons, the evidence points to a small proportion of the cleaved C-terminal segment migrating to the nucleus, therefore it seems the majority of channel regulation might be carried out locally. According to Gomez-Ospina et al., (2006) other major proteins found to be regulated by the cleaved CaV1.2 peptide include several gap-junction associated genes, a glutamate receptor, several potassium channels, a sodium calcium exchanger and some signalling proteins such as RGS5, Formin and Nitric Oxide Synthase. Evidence suggesting a role for L-type calcium channels in afferent pain pathways is slim. Knockout mice lacking CaV1.3 display normal pain behaviour (Jeon et al. 2010; Bernstein et al. 1995; Verma et al. 2009). However some reports of an upregulation of CaV1.2 channels in spinal cord neurons following the onset of chronic pain has suggested a possible role for these channels in pain. Furthermore, this upregulation seems to be regulated by miR-103, a single microRNA which simultaneously regulates the expression of all three subunits which form CaV1.2. This regulation is bidirectional since the knocking-down of miR-103 in rats upregulates the level of CaV1.2 expression leading to pain hypersensitivity in these animals (Favereaux et al. 2011). Region specific knockouts of this channel in the anterior cingulate cortex can also been seen to alter pain responses and to modulate the effects of morphine. The chronic treatment of rodents with morphine elevates L-type calcium channel expression in the spinal cord (Bourinet et al. 2014).

1.5.4 Auxiliary Subunit of Voltage-Gated Calcium Channels and Pain

Despite poor membrane expression and erratic channel kinetics, the exclusive expression of a pore forming voltage gated calcium channel α subunit is sufficient to produce a functional calcium channel (Bourinet et al. 2014). Three auxiliary subunits, α2δ, β, and γ, are known to associate with the α subunit of Cav channel. These subunits enhance channel expression and trafficking to the membrane as well as confer channel kinetics and gating properties (Tran-Van-Minh et al. 2010; Yasuda et al. 2004). However, the modulation of certain auxiliary subunits are also involved in the generation of chronic pain. Following peripheral nerve injury (Nieto-Rostro et al. 2014) the resulting increased expression of α2δ is understood to be a key factor in the generation of altered pain states. Since the accidental discovery that the analgesic effects of Gabapentin have been linked to the α2δ subunit of the Cav complex, there has been increasing interest in targeting Cav auxiliary subunits for the development of
therapeutic analgesics (Dolphin 2009; Hendrich et al. 2008). By reducing excitatory transmission in the spinal cord, two isoforms of this subunit hold the feature of binding the anti-epileptic and anti-allodynic drugs gabapentin and pregabalin and confer their mechanism of action (Hendrich et al. 2008). The chronic application of gabapentin markedly reduces cell surface localisation of the $\alpha_{2}\delta$ and $\alpha_{1}$ subunits and hence decreases calcium currents (Hendrich et al. 2008). A suggested mechanism of action of gabapentoid is through the disruption of the interaction between $\alpha_{2}\delta$-subunits and sorting proteins thus inhibiting post-golgi forward trafficking of the $\alpha_{2}\delta$ subunit. The increased trafficking of the calcium channel subunit $\alpha_{2}\delta$-1 to presynaptic terminals in neuropathic pain is inhibited by the $\alpha_{2}\delta$ ligand pregabalin (Hendrich et al. 2008). This protein is derived from the same gene and later modified post-translationally into two segments $\alpha_{1}$ and $\delta$ which associate and are anchored to the membrane through GPI anchoring in the C-terminal of the $\delta$-subunit (Kadurin et al. 2016). Proteolitic cleavage occurs after ala960 resulting in the N-terminal $\alpha$-subunit and the C-terminal $\delta$-subunit. Mechanisms of drug action, the key feature of the $\alpha_{2}\delta$ subunits is that it binds to the antiepileptic drug category known as gabapentinoids and which includes gabapentin and pregabalin.

### 1.6 Opioid Activation

Opioid receptors are widely distributed throughout the human body and are involved in numerous physiological processes, including pain signaling, respiration, growth, reproduction and immune response. Opioid receptors are divided into three major categories, $\mu$ (mu, MOR), $\delta$ (delta, DOR), and $\kappa$ (kappa, KOR), encoded by the genes OPRM1, OPRD1 and OPRK1, respectively. All three opioid receptors were discovered, cloned and initially characterised and categorised in the 1990s. All three receptors belong to a the family of G protein-coupled receptors and are integral membrane proteins, coupled to heteromeric inhibitory G$i/o$ proteins. The general structure of opioid receptors consists of seven hydrophobic transmembrane domains (I-VII), three intracellular and three extracellular inter-membrane loops, and extracellular N-terminal and intracellular C-terminal, both of which are important targets of glycosylation. Activation of these receptors by an endogenous or exogenous ligand follow a typical pattern of G-protein singling and subsequent transduction. Each G-protein consists of three subunits: $\alpha$, $\beta$ and $\gamma$. Opioid receptors are all coupled to the inhibitory G$i/o$ subunit. Following ligand binding all three subunits dissociate resulting in the activation of various pathways of signal transduction. The canonical pathway
of opioid-receptor signaling pathway involves the dissociation of the heterotrimeric G-protein into Goi and Gβγ. Goi has multiple targets but importantly interacts with the cAMP/PKA pathway by inhibiting adenylyl cyclase activity thereby decreasing cAMP levels (Taussig et al. 1993). Another important target of opioid activation is the effect both subunits have in modulating potassium and calcium ion channels. Although, it was previously thought that Goi was responsible for the activation and potentiation of G-protein activated potassium channels (GIRK) such as Kir3, a mounting body of evidence has more recently shown a direct interaction between with the Gβγ subunit in mediating potassium channel activation (Logothetis et al. 1987; Lüscher et al. 2010). It is also clear that the activation of all three opioid receptor subtypes has a dramatic effect on both Ca²⁺ currents mediated through Gβγ subunits and P/Q, N- and L-type calcium channels (Dunlap et al. 1978; Rusin et al. 1997). This inhibition is characterised by a slowing of the current activation kinetics, thought to be due to a time and depolarisation-dependent recovery from voltage dependent-inhibition (Bean 1989). Furthermore, evidence suggests that acute opioid administration reduces Ca²⁺ levels in synaptic vesicles and synaptosomes. Other effectors are linked to Gβγ, including GPCR kinase 2/3, phosphatidylinositol-3-kinase (PI3K) and phospholipase Cβ (PLCβ) (Dupré et al. 2009; Piros et al. 1996). Following effector activation, through the hydrolysis of GTP to GDP, Go loses its potent activity and returns to its resting state by rejoining Gβγ. This recovered complex is then inactive and can be further activated by the binding of a new ligand to the opioid receptor. Many other intracellular proteins such as calmodulin, calnexin, periplakin and importantly β-arrestin, are known to interact and modulate the opioid receptors. β-arrestins are versatile adapter proteins that
associate with most G protein-coupled receptors following agonist binding and phosphorylation of G-protein coupled receptor kinases (GRKs) and play a central role in the process of receptor desensitisation and sequestration and therefore terminating the activated receptor (Ferguson 2001; Freedman et al. 1996). Once recruited β-arrestin binds to the GPCR and both uncouples receptors from heteromeric G protein targets and targets them to clathrin-coated pits for endocytosis. Furthermore, more recent evidence suggests β-arrestins could act as further signal transducers for GPCRs. Interestingly, they interact and form complexes with signaling proteins from several signaling pathways including Src family tyrosine kinases and components of the ERK1/2 and JNK3 MAP kinase pathways. The recruitment of these kinases to agonist-bound/activated GPCRs mediates a number of signaling activities ultimately leading to the activation of these signaling cascades and the endocytosis of the GPCR.

1.6.1 Sodium, Nav1.7 and the Regulation of Opioid Receptors

The idea of sodium modulating ligand binding affinity of GPCRs was first suggested at the end of the 1970s (Pert et al. 1974). First proposed by Snyder and colleagues, through experimental radioimmunoassay binding assays. Through the use of radioligand binding assays, they found that incubating membrane fractions from homogenised rat brains, in varying concentrations of sodium (0-100nM) could affect the binding affinity of agonist/antagonists to the receptor. More precisely, high sodium concentrations would negatively affect the binding affinity of agonist 3H-dihydromorphine and more readily bind antagonist 3H-naloxone. This indicated a clear ability of opioid receptors to be modulated by sodium ions. Furthermore, studies examining the effects of sodium binding, through N-methyl-D-glucamine exchange, in intact 7315c and NG108-15 cells revealed the main allosteric site for sodium binding to be intracellular (Puttfarcken et al. 1986). More recently, the elucidation of the crystal structure of the δ-opioid receptor (Fenalti et al. 2014) and other GPCRs have confirmed and refined these initial results. Indeed site directed mutagenesis studies revealed a sodium mediated allosteric site in the δ-opioid receptor coordinated by five oxygen atoms located on the side chains of three amino acids (Asp 95, Ser 135 and Asn131). Moreover, high resolution structural studies of reengineered human adenosine receptors, further revealed an allosteric sodium binding location on intracellular loop three of the receptor, at a highly conserved aspartate residue (Liu et al. 2012). These later studies confirm the presence of intracellular binding sites on GPCRs and their importance in the modulation
of receptor ligand affinity.
1.7 Thesis Aims

The principal aim of this thesis was to investigate the network of proteins that regulate and are regulated by voltage-gated sodium channel 1.7 and gain a greater understanding of the role this sodium channel plays in pain. The following are the concise aims that I set out in this thesis:

- **CHAPTER 3** Using a genetically engineered mouse containing a tandem affinity tagged endogenous Nav1.7 along with mass spectrometry, I aimed to decipher and analyse all important Nav1.7-protein interactors involved in all aspects of the channel’s function, from transport to kinetic regulation. By adapting a method of co-immunoprecipitation I aimed to experimentally isolate all interacting proteins in order to analyse and determine how they may interact and mediate the functions of Nav1.7. In this chapter I also aimed to use this epitope tag to better determine the expression profile of Nav1.7 in the mouse central and peripheral nervous system. Additionally, in this chapter I aimed to begin work aimed at determining the crystal structure of Nav1.7. For this I determined as strategy to purify and crystallise Nav1.7.

- **CHAPTER 4** In this chapter I sought to use computational methods to class and organise the proteins identified as Nav1.7 interacting proteins (obtained in chapter 3). I also aimed to validate and confirm the interaction of Nav1.7 with a number of proteins from the list that I selected based on a criteria of interest relating to the function of Nav1.7 in pain signalling. Furthermore, because of previous interest in the function of CRMP2 towards Nav1.7, I aimed to further characterise this interaction using electrophysiological techniques.

- **CHAPTER 5** In this chapter I aimed to better understand the link between Nav1.7 and the opioidergic system. I aimed to use a modified electrophysiological assay to determine the effect of changing amounts of intracellular sodium on the efficiency of the endogenous opioidergic system. Furthermore, I sought to use and immunosorbent based assays, to confirm the link between sodium/Nav1.7 based regulation of opioid receptors as well as endogenously expressed enkephalins. Finally, I generated a novel tool in the form of a transflectable construct containing an exogenous gene which coded for a sodium chelating protein originally found in a species of bacillus firmus. I aimed to develop and confirm the functionality of this protein in an *in vitro* context.
Chapter 2

Materials and Methods

2.0.1 Immunohistochemistry Cell

Immunohistochemistry was performed on the TAP-tagged Nav1.7-HEK293 cell line that was previously generated by members of the Molecular Nociception lab. Cells were plated on poly-D-lysine coated coverslips in 24-well plates. All cells were manipulated under sterile fume hoods and cultured in sterile incubators at recommended optimal conditions of 37°C and 5% CO2 in DMEM medium to which 10% foetal bovine serum (Life Technologies), 50 U/ml penicillin, 50 µg/ml streptomycin and 0.2 mg/ml G418 was added. Antibiotic resistance was conferred by the Nav1.7 containing plasmid and used for selection of TAP-Tagged Nav1.7 cells (Importantly, we aimed to only use cells with a relatively low passage number (between 5-20), when cells reached beyond this passage number, the integrity of the cells and the expression of TAP-tagged Nav1.7 was checked by immunohistochemistry and RT-PCR). Cells were then fixed in cooled methanol -20°C for a period of 10 minutes, and then permeabilised with -20°C acetone for 1 minute. The cells were then washed 3 times with 1x PBS and incubated with blocking buffer specific to the FLAG-Tag antibody at room temperature for 30 minutes. The blocking buffer consisted of 1x PBS, 0.3% Triton X-100 and 10% goat serum. The fixed cells were then incubated with the anti-FLAG antibody at a concentration of 1:500 in blocking buffer (FLAG-tag antibody, F1804, Sigma) at 4°C overnight. following primary antibody incubation, the cells were washed three times with 1x PBS and incubated with secondary antibody. The secondary antibody was a goat anti-mouse IgG conjugated with an Alexa Fluor 488 (A11017, Invitrogen). Secondary antibody was done at room temperature for 2 hours. The coverslips were then washed 3 times in 1x PBS and mounted with VECTASHIELD HardSet Antifade Mounting Medium with DAPI (H-1400, Vectorlabs). The cells could then be visualised using a fluorescence Leica microscope. Aside from antibody type
and the concentration at which they were used, this immunohistochemistry protocol was the standard protocol used in all other instances in this thesis. The protocol is a standard protocol used by the Molecular Nociception lab and can be modified to obtain optimal results.

2.0.2 Immunocytochemistry Tissue and perfusion fixing of tissue

Because of the difficulties I experienced in the staining for Tap-Tag Nav1.7 in specific tissue types including skin and DRG, a number of modifications in the concentration of PFA and other fixing methods were made in an attempt to obtain a better signal to noise ratio in these experiments (please see chapter 3 for more details). For a better understanding of the modifications made in these experiments, any changes to this method have been outlined in the results chapters. Therefore, the following method should be seen as a general guideline for how perfusion/fixing/immunohistochemistry took place, but for further details on what modifications were made to this method, please see relevant experiments in chapter 3.

Animals were anaesthetised using isoflurane gas 4% and and euthanised using pentobarbatol. Animals were then transcardially perfused with 2% (w/v) paraformaldehyde in PBS. Following perfusion whole brains, spinal cord, DRG and skin were dissected and directly placed in fixative for 4 hours at 4°C and then transferred to a solution containing 30% sucrose in PBS for 2 days at 4°C. For the purpose of slicing, tissues was embedded in O.C.T. (Tissue-Tek) and snap-frozen in a dry-ice/2-methylbutane bath. Using a cryostate, the tissues could then be sliced at a thickness of (20 µm) and collected on glass slides (Superfrost Plus, Polyscience) and stored at -80°C until stained. For immunohistochemistry with FLAG-tag antibody, sections were first incubated in a standard blocking solution which consisted of 4% horse serum and 0.3% Triton X-100 in PBS, for a period of 1 hr at room temperature. Following blocking the primary antibody (mouse FLAG-Tag, F-1804, Sigma) was prepared at a concentration of 1:200 in blocking solution and left to incubate for 2 days at 4°C. Following incubation, the tissues were washed three times in PBS buffer following which, the secondary antibody, either an Alexa 488 or 594 conjugated goat anti mouse secondary antibody (1:800, Invitrogen), was added. The IB4 staining in tissues such as the spinal chord has been previously described and a comparison with previous litterature was made of IB4 staining to check the quality of our work (Zhao et al., 2010). A Nav1.7 antibody was also used to identify the protein without the use of the FLAG-tag in the spinal cord (Neuromab, 1:500). In order to facilitate the identification of certain brain regions, a number of anatomical stains were used including stains targeted at the bregma levels, these nuclei were stained using Hoechst
33342 (1:10,000, Invitrogen). These sections were also mounted with a specific fluorescence mounting medium (DAKO). The brain slices were visualised under a fluorescence microscope and were acquired on an epifluorescence microscope (BX61 attached to a DP71 camera, Olympus) or captured using a confocal laser scanning microscope (LSM 780, Zeiss). Images were of high quality and therefore very minimal adjustments in contrast and brightness was needed to identify staining, however any slight modifications could be performed using Adobe Photoshop Elements 10. The Bregma levels and other key brain regions could be identified by refering to the stereotaxic coordinates in “The Mouse Brain” atlas by Paxinos and Franklin (2001).

2.0.3 Animals

All animal experiments were approved by the United Kingdom Home Office Animals Scientific Procedures Act 1986. Experiments were conducted using both female and male mice (*mus musculus*). Animals were housed as a maximum of 6 per cage, with *ad libitum* food and water, on a 12hour day and night cycle. For experiments using transgenic mice, wild-type littermate animals were used as controls. All strains of mice used for procedures were of C57Bl/6 background. All mice used in the experiments were male and at least 6 weeks old.

2.0.4 ARRIVE Guidelines

All experiments on animals were conducted according to the ARRIVE guidelines. In all experiments the maximum precautions were taken to minimise suffering to animals and animal numbers were reduced to as few animals as possible. Furthermore, when possible *in vitro* cell lines or other non-animal methods were used instead of animals.

2.0.5 Behaviour

All behavioural tests were approved by the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. All animals were used for behavioural experiments were age between 6-12 weeks. All experimenters were blind to the genetic status of test animals. All replicates (i.e n numbers) were biological replicates. Due to the subjective nature of animal behavioural testing and to avoid inconsistencies between experimenters, in our lab it was common practice to perform all behavioural experiments in pairs and all post-testing data analysis was performed independently by each experimenter and then compared to make sure there were no
large discrepancies between the results. All experiments were conducted in collaboration with Queensta Millet from the Molecular Nociception lab. All data are presented as mean ± SEM. For all behavioural testing sample size was based on a power calculation used in order to determine the number of animals necessary for each behavioural assay. I used the MINITAB software to calculate the sample size for each assays. This software calculates the sample size needed and takes into account Type1 errors, effect size, standard deviation, power of the study and which statistical test is used and the direction of the test. Sample size equation is:

\[ \text{Sample size} = \frac{\text{SD}^2 (Z_{\alpha/2}^2 + Z_\beta^2)}{d^2} \]

where standard deviation (SD) is taken from previous pilot studies, \(Z_{\alpha/2} = \) is percentage of type 1 errors (0.5%), \(Z_\beta = \) power (set at 80 %), \(d = \) effect size.

**Formalin Test:** The Formalin test is used as a model of continuous, moderate pain (Hunskaar et al., 1985). Before beginning the test, animals were acclimatised to a Perspex box for a minimum of 1 hour prior to testing. Following acclimatisation, a 20µl intraplantar injection of 5% Formalin was administered into the hind paw. Animals were then observed and recorded for the next 60 minutes following injection. The time spent licking, shaking or biting the affected paw was then measured in blocks of 5 minutes. The test is often described as involving an early acute phase, which is believed to involve direct activation of C fibres via TRPA1 (Tjolsen et al., 1992, McNamara et al., 2007) and occurs shortly after injection at around 5-10 minutes. The second phase, characterised as an inflammatory phase, begins around 20 minutes after injection and is thought to result from the action of prostaglandins and other inflammatory mediators as well as changes in pain sensitivity that are spinally mediated (Dubuisson and Dennis, 1977, Hunskaar and Hole, 1987, Tjolsen et al., 1992).

**Rotarod:** The rotarod test was used to measure of motor coordination in the animals (Jones and Roberts 1967). Mice were placed onto a ”rotarod machine” turning at a speed of 6 rpm. This speed was maintained for a period of 30 seconds to allow the mice to acclimatise. Following acclimatisation, the time spent on the rotarod was recorded, at which point the speed was increased from 6rpm to 40 rpm on a 3 minute ramp with a cut-off of 5 minutes after the start of the acceleration. The time at which the animals fell from the rotarod or made 3 full rotations within a 10 second period was recorded. The rotarod test was repeated 3 times per animal.

**Randall Selitto:** The Randall Selitto test is most commonly used to assess the noxious mechanical pain thresholds of animals and in the case of mice could be used on the tail and
paw of the animals. The test designed by Randall and Selitto (1957, as cited in Minett (2011)) and modified by Takesue et al. (1969) requires the application of a 89 blunt probe to a chosen surface (paw or tail) and increasing the pressure until the animal exhibits a painful behavioural response. This response could include struggling, withdrawal or vocalisation. We repeated the test 3 times for each animal. When testing the tail, the animals were carefully restrained in a clear Perspex plastic tube and acclimatised for a period of 5 minutes. For testing the paw, animals were restrained by scruffing of the neck in such a way that a behavioural response could be observed without difficulty.

**Hargreaves:** Thermal nociceptive thresholds were measured using the Hargreaves tests by observing hindpaw-withdrawal latencies following exposure to nociceptive heat stimuli (Hargreaves, Dubner et al. 1988)(50 & 55°C)(Eddy and Leimbach 1953).

**Von Frey:** The Von Fray test is a behavioural paradigm designed to investigate sensitivity to an innocuous mechanical stimuli. A selection of of 20 nylon hairs with designated weights ranging from 0.008g — 300g were used to apply designated pressure to the plantar surface of the hind paw. A hair of 0.4g was selected and used first in all experiments. The widley used up-down method of Von Fray testing was used (Chaplan et al., 1994) to determine the 50% paw withdrawal threshold. This method involved applying a Von Frey filament to the paw; if a positive response was obtained then a smaller filament of lesser force was applied next. If no response was seen, a thicker filament of greater strength was applied. This was up-down process was continued until 6 responses were obtained with a positive response consisting of lifting, flinching or licking of the tested paw during the stimulus application. Animals were kept in a mesh bottomed enclosure in which they were acclimatised for 1 hour prior to testing.

### 2.0.6 Tandem-Affinity Tag Purification

Tandem affinity tag (TAP) purification is a method that allows the rapid purification of proteins under native conditions. One particular strength to this method is that prior knowledge of the protein complex composition or function is not required and the detection of the protein can be done even when expressed at their natural level. This powerful method, firstly requires the fusion of a tandem affinity tag to either the C- of N- terminus of the target protein. This genetic manipulation can be performed in both *in vitro* (cell lines) and *in vivo* (genetically modified model organisms such as rodents). The TAP-tag itself consists of two IgG binding domains, in our case a polyhistidine-tag followed by a FLAG-tag composed or
three consecutive repeating sequences consisting of aspartic acid, tyrosine and lysine. Both
tags are separated by a protease cleavage site which in our case is a tobbaco-etch virus (TEV)
site. The following section will give a detailed outline of the purification procedure using the
tandem affinity tag.

2.0.7 Single-Step and Tandem Affinity Purification

Sample preparation was undertaken with the up most care and attention. For each attempted
single-step and tandem purification the following purification, sample tissues were collected
and pooled: DRG, olfactory bulbs, spinal cord and hypothalamus. These tissues were chosen
based on prior knowledge of them containing Nav1.7 (see chapter 1 discussion for further
information regarding Nav1.7 tissue expression). Tissue samples were broken up and
homogenized using Precellys Tissue Homogenizer and Precellys lysis kit (Precellys ceramic kit
1.4 mm, Order no. 91-PCS-CK14, Peqlab). Homogenisation was performed according to kit
instructions for a period of 60 seconds. All purifications were done in 1% CHAPS lysis buffer
( composed of 30 mM Tris-HCl pH 7.5; 150 mM NaCl, 1% CHAPS, 1 complete EDTA free
Protease inhibitor cocktail (Roche) in 10 ml of CHAPS lysis buffer). After mechanical
breakdown, the lysates were left to incubated on a slightly shaking horizontal plate on ice for
a period of 1 hour. Following incubation, the sample was clarified by centrifugation at 14,000
g for 8 min at 4°C. Throughout the preparation, the sample was always maintained at 4°C in
order to avoid excessive protein breakdown. Protein concentrations were then measured using
the Pierce BCA Protein Assay Kit (Product no. 23225, Thermofisher, all procedures were
done according to kit instructions). A starting concentration of 10 mg of protein containing
supernatant was incubated with magnetic M-270 Epoxy Dynabeads (14311D, Invitrogen)
which were already covalently coupled to mouse Anti-Flag M2 (F-1804, Sigma). The sample
was incubated with the beads and left for 2 hours in a cold room at 4°C and kept in moving
using an end-over-end shaker. Movement was necessary during the incubation to avoid
aggregation of the magnetic beads, which would lead to a decrease in coupling. The magnetic
Dynabeads were collected on a DynaMAG rack (Invitrogen). Washing of the beads could then
be carried out carefully to remove any excess proteins that did not bind or aggregate onto the
beads. Three washes were carried out using 1% CHAPS Buffer. Following the wash steps the
protein complex could then be separated from the beads by incubating the sample in 1x
AcTEV protease cleavage buffer ( containing 50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM
DTT). The incubation with the protease released the protein complex from the Dynabeads.
due to the cleavage at the TEV protease site (AcTEV protease 12575015, Invitrogen).

Protease cleavage was done at 30°C for 3 hours, the separated sample was then collected and stored at -80°C. In the case of tandem purification, eluted protein samples were collected after AcTEV cleavage and 15x diluted in protein binding buffer (composed of 50 mM Sodium phosphate, 300 mM NaCl, 10 mM imidazole, 0.01% Tween 20; pH 8.0). The complex was then incubated with Ni-NTA beads (Cat no. 36111, Qiagen) which are targeted to the polyhistidine tag. Samples were incubated with Ni-NTA beads on an end-over-end shaker overnight at 4°C. TAP-tagged Nav1.7 protein complexes could then be released from the Ni-NTA beads by heat elution at 95°C for 30s in 1x SDS protein sample buffer.

2.0.8 Western Blot

Proteins for Western blot were isolated from freshly excised different tissues, such as DRG, spinal cord, sciatic nerve, olfactory bulb, cortex, hypothalamus, cerebellum, skin, lung, heart and pancreas taken from TAP-tagged Nav1.7 mice and littermate control mice. The protein samples were prepared as the same as described in the section of Single-step and tandem affinity purification in Material and Methods. Briefly, proteins were extracted from different tissues were homogenized in 1% CHAPS lysis buffer. Centrifugation for 15 min at 14,000 rpm removed the nuclear fraction and cell debris were removed by centrifugation at 14,000 rpm for 15 minutes at 4°C. Protein concentrations were determined with Pierce BCA protein assay kit, and then samples of 40 µg were separated on SDS-PAGE gel in Bio-rad Mini-PROTEAN Vertical Electrophoresis Cell System and blotted to the Immobilin-P membrane (IPVH00010, Millipore) in transfer buffer (25 mm Tris-HCl, pH 8.3, 192 mm glycine, 0.1% SDS, and 20% methanol) for 1 h at 100 V with a Bio-Rad transfer cell system. The membrane was blocked in Blocking buffer (5% nonfat milk in PBS-Tween buffer (0.1% Tween 20 in 1x PBS)) for 1 h at room temperature and then incubated with primary antibody anti-FLAG (1:1000; Sigma, catalogue F1804) and anti-HAT (1:400; LSBio, LS-C51508) in Blocking buffer overnight at 4°C. The membrane was washed three times with TBS-Tween (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) and then incubated with secondary antibody goat anti-mouse or goat anti-rabbit IgG-HRP (1:4000, Jackson ImmunoResearch Laboratories) in TBS-Tween at room temperature for 2 hours. Detection was performed using a Western Lightning Chemiluminescence Reagent (Super Signal Western Dura, Thermo Scientific, 34075) and exposed to BioMax film (Kodak). Other primary antibodies used for Western blotting were ankyrin-3 (Santa Cruz, SC-12719), neurotrimin (Santa Cruz, SC-390941), Kif5b (Santa Cruz, SC-79 Alexandros Kanellopoulos
SC-28538), PEBP1 (Thermo fisher, 36-0700), neurofascin (Abcam, ab31457), neuroligin (Abcam, ab177107) and AKAP12 (Abcam, ab49849). Concentrations were applied as suggested by the manufacturers.

2.0.8.1 Silver Staining

All silver stain methods were done according to the Pierce Silver Stain Kit (cat number: 24612). Standard SDS-page Bio-Rad gels were used for silver staining (Mini-Protean TGX Stain-Free Precast Gels). Gels were scanned using a conventional computer canning device.

2.0.9 Plasmids, Cloning and Primers

A number of plasmids used in these experiments were obtained from the Addgene plasmid databank (Cambridge, MA). These include: Tmed10 (TMED-BIO-HIS, 51852), Lat1 (pEMS1229, 238146). Gprin1 plasmid was obtained from OriGene (RC207340). Other plasmids were cloned by myself using both classic restriction enzyme cloning and Gibson assembly. To validate the interaction between Synaptotagmin-2 and Nav1.7, I first had to clone the human gene for insertion into a mammalian expression plasmid. Synaptotagmin-2 gene was cloned from human dorsal root ganglion neuron tissue. To clone this gene, whole mRNA from human DRG was first reverse transcribed into a cDNA library to then be used as a template for amplification of the Synaptotagmin-2 gene by PCR. Due to some difficulties in the amplification process, mainly involving the design of primers targeted to the gene, a wider portion of the gene was amplified and inserted into a TOPO vector, which could then be used as a template to create the Synaptotagmin-2 insert. From the TOPO vector the Synaptotagmin-2 gene was successfully cloned into the high expression vector, pcDNA3.1 IRES-AcGFP plasmid by using the Gibson assembly method. The Collapsin Response Mediator Protein 2 (Crmp2) gene was also cloned but with relatively little difficulty from human dorsal root ganglia neuron mRNA and inserted into pcDNA3.1 plasmid also using Gibson assembly. The Scn3b mammalian expressing vector was used to investigate loss-of-function of Nav1.7 (Cox et al., 2006). The HA-tagged CaV2.2 construct was a gift from Prof. Annette Dolphin, and was co-expressed with the auxiliary subunits, α2δ-1 and β-1 that were also gifted to us by the Dolphin lab.
2.0.10 Co-Immunoprecipitation (Co-IP)

All tissues obtained from dissected mouse and used in immunoprecipitation experiments were selected on the basis of already known Nav1.7 expression (Black et al. 2012). The following tissues were selected: Olfactory bulb, hypothalamus, spinal cord, dorsal root ganglia and sciatic nerve. Depending on experiments, these tissues could either be pooled or treated as individual samples. Following extraction, tissues were flash frozen using dry-ice and stored at -80°C to avoid any degradation of the protein. In the case of HEK293 cells that stably expressed TAP-tagged Nav1.7, cells were harvested by gentle trypsinisation followed by centrifugation at 800 rpm for 5 minutes to aggregate all living cells. The cell pellets could then be immediately stored at -80°C to avoid protein degradation if not directly used for experiments. For the purpose of immunoprecipitation, both cell lines and tissue samples were first lysed in a 1% CHAPS lysis buffer containing a protease inhibitor cocktail (invitrogen) and then mechanically homogenised using ceramic zirconium oxide mix beads of 1.4 mm and 2.8 mm lysing kit (Precellys). When incubated, a total of 10-25mg of protein was incubated with M2 Magnetic FLAG coupled beads (Sigma-Aldrich) for 2 hours at 4°C. The M2 Magnetic beads were bought with the FLAG targeted antibody already attached to the bead as this provided greater consistency in our experiments. Following incubation, the bead-protein complex was then washed with three cycles of 5 resin volumes of 1% CHAPS buffer and once with the TEV-protease buffer (Invitrogen) that was later used for the elution process - the TEV protease buffer did not contain the protease enzyme at this point. The tagged protein as well as the accompanying complex was then cleaved from the beads by the addition of TEV protease enzyme (Invitrogen) to the buffer and incubated for 3 hours at 37°C to elute the protein complex. The sample eluate - protein complex - was then separated from the beads using a magnetic rack and stored at -80°C. For cell line co-immunoprecipitation, in HEK293 cells were previously transfected with a construct containing the gene of interest to be expressed alongside Tap-tag Nav1.7. I performed the transfection using a standard lipofectamine protocol described by the manufacturer (Lipofectamine 200, Invitrogen, 52887). All cells were left 48 hours after transfection in order for optimal expression of the construct before cells were harvested and co-immunoprecipitation was undertaken.

Nav1.7 interacting protein candidates were selected by two criteria: a) Present in at least two knock-in biological experiments but absent from wild type experiments. b) Present in more than three knock-in and more than one wild type experiments, the ratio of average abundance is more than 1.5 fold increased in knock-in experiments as compared with wild type.
experiments. Further cellular component and function classification were performed on PANTHER Classification System (11.0). Ingenuity pathway analysis (IPA) (QIAGEN) was used to elucidate pathways and protein interaction networks using candidate proteins.

2.0.11 Culture and Transfection of Dorsal Root Ganglia

For the purpose of transfection, DRG tissue was extracted from adult 6-12 week mice. Mice were euthanised using a common CO2 chamber. Immediately following euthanasia, DRG tissue was extracted from the mouse and placed in Ca2+ and Mg2+ free Hanks’ balanced salt solution (HBSS) containing 5 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 10 mM glucose, 5 mg/ml collagenase type XI and 10 mg/ml dispase, for a period of 35 minutes at 37°C for digestion of the collagenase tissue surrounding the ganglia. Following digestion DRG were very gently titurated in Dulbecco’s modified Eafle’s medium (DMEM) that contained 10% qualified foetal bovine serum (FBS) that had been previously sterilised by filtration. Tituration was done with tree decreasing diameter fired glass Pasteur Pipettes. Two methods for transfection were used in DRG depending in the transfection rate of a plasmid. For small easily transfectable plasmids, the lipofectamie transfection method was used (described previously). In instances where lipofectamine transfection was not successful, we used a standard electroporator to transfect these plasmids. In this cases, neurons were electroporated using the Neon Transfection System (Life Technologies) according to the supplier’s recommendations, in 10 µl tips and with 0.6 µg plasmid per reaction, applying 2 x 20 ms pulses of 1100 V. Following transfection, cells were plated on dishes coated with laminin and poly-lysine in DMED containing 10% FBS and 125 ng/ml nerve growth factor (NGF). Neurons were kept at 37°C in 5% CO2 and used for patch clamp or other studies within the following two days of transfection.

2.0.12 Electrophysiology and Patch Clamp Recordings

Developed in the late 1970’s, patch clamping allows for the electrophysiological measurements of currents through ion channels in the cell membrane. In involves using a conducting electrode to measure changes in electrical potential in living cells. Whole-cell patch clamp recordings were conducted at room temperature (21°C) using an AxoPatch 200B amplifier and a Digidata 1322A digitiser (Axon Instruments), controlled by Clampex software (version 10, Molecular Devices). Filamented borosilicate microelectrodes (GC150TF-10, Harvard Apparatus) were pulled on a Model P-97 Flaming/Brown micropipette puller (Sutter
Instruments) and fire-polished to a resistance of between 2.5 and 4 MOhm. Standard pipette intracellular solution contained: 10 mM NaCl, 140 mM CsF, 1.1 mM EGTA, 1 mM MgCl2 and 10 mM HEPES. The standard bathing extracellular solution contained: 140 mM NaCl, 1 mM MgCl2, 3 mM KCl, 1 mM CaCl2 and 10 mM HEPES. Both intracellular and extracellular solutions were adjusted to a physiological pH of 7.3. The amplifier’s offset potential was zeroed when the electrode was placed in the solution. After a gigaseal was obtained, short suction was used to establish whole-cell recording configuration. Errors in series resistance were compensated by 70–75%. Cells were briefly washed with extracellular solution before a final 2 ml of solution was transferred to the dish. Cells were held at 100 mV for 2 min before experimental protocols were initiated. Currents were elicited by 50-ms depolarisation steps from 80 mV to +80 mV in 5 mV increments. Compounds were added and mixed at the desired concentrations in extracellular solution before being added to the bath. Following addition of the compound, protocols were repeated on previously unrecorded cells. All currents were leak-subtracted using a p/4 protocol. The following compounds were used in electrophysiology experiments: lacosamide ((R)-2-acetamido-N-benzyl-3-methoxypropionamide) was obtained from Toronto Research Chemicals Inc (L098500) and tetrodotoxin was obtained from Sigma-Aldrich (T8024). Incubation with lacosamide was done for 5 h prior to recording.

Voltage-clamp experiments were analysed using pCLAMP software and Origin (OriginLab Corp., Northampton, MA) software programs. Current density–voltage analysis was carried out by measuring peak currents at different applied voltage steps and normalised to cell capacitance (pA/pF). Voltage-dependent activation data were fitted to a Boltzmann equation \[ y = \frac{(A_2 + (A_1 \cdot A_2)/(1 + \exp((V_h - x)/k))) \cdot x \cdot V_{rev}}{A_1} \], where A1 is the maximal amplitude, Vh is the potential of half-maximal activation, x is the clamped membrane potential, Vrev is the reversal potential and k is a constant. All Boltzmann equations were fitted using ORIGIN software.

2.0.13 Nav1.7 Interaction Protein Selection and Functional Analysis

Candidate proteins of potential interactors with Nav1.7 were selected by two criteria: a) The protein must be present in at least two knock-in biological experiments but absent from wild-type experiments (when referring to table 3.2 these proteins are designated as "KI Only" under the "Ratio" column). b) Proteins are present in more than three knock-in and more than one wild type experiment, therefore the the ratio of average abundance is greater or equal...
to 1.5 fold increased in knock-in experiments as compared with wild-type experiments. For the purpose of this thesis several other candidates were also included in this list based on previous knowledge of Nav1.7 interaction. If a protein was previously known to interact with Nav1.7 and appeared on our list but did not exceed the 1.5 threshold, I felt it appropriate to include this protein in the list of possible interactors (where this is the case a clear indication of the protein has been made in the relevant results section). Further cellular component and function classification were performed on PANTHER Classification System (11.0) (performed by Honglei Hunag at Oxford University). Ingenuity pathway analysis (IPA) (QIAGEN) was used to automatically elucidate pathways and protein interaction networks using candidate proteins. The algorithms used by these two pieces of software are outlined in discussion and results section of chapter 3. For further information regarding the function of these protein analysis algorithms please visit https://string-db.org/.

2.0.14 Enzyme-Linked Immunosorbent Assay (ELISA)

Dorsal root ganglion neurons were cultured according to normal standards previously mentioned. For the purpose of immunosorbent assays, DRGs were plated onto a 48 well plate coated with polylysine and laminin. The neurons were cultured for 48 hours before use in experiment. Stimulation with all agonists was performed for a duration of 2 minutes in both DRG and cultured cell lines. Working concentrations used for agonists: PGE2 5µM, Fentanyl 10µM, Metaclopramine 10µM, Rolipram 10µM, Forskolin 10µM. Stimulation was performed in 200ml extracellular solutions (as described in electrophysiology methods section) with sodium concentrations adapted to either 2 and 20 mM. Following stimulation solution was removed and immediately cells were lysed in the ELISA assay buffer containing 0.1M HCL 1% TritonX. Samples were frozen at -80 degrees and stored until used. ELISA assay was performed according to the provided protocol (abacom: ab133051, Cyclic AMP COMPLETE ELISA KIT). The plate was read using a Multiscan FC Microplate Photometer (Thermofisher) plate reader at 405nM. Data from the absorbance reading provided %bound cyclic AMP. A standard curve was created using cAMP standards for each assay and used to convert values to concentrations of cAMP pmol/ml.

2.0.15 Statistical Analysis

Statistical analysis was performed using either repeated-measures ANOVA with Bonferroni post hoc testing or Unpaired Student’s t-test as described in the results or figure legends.
Both these statistical tests measure the statistical significance of the results obtained in the experiment. ANOVA can be one-way or two-way, which refers to the number of independent variables in the Analysis of Variance test. The one-way ANOVA test was used in situations where I sought to compare two means from two independent groups, whereas the two-way ANOVA was used when assessing changes in two variables. The Bonferroni test is a type of multiple comparison test that adjusts the p value when several dependent or independent tests are performed simultaneously on a single set of data. It works by dividing the p value by number of comparisons made, the statistical power of the study is then based on this modified p value. I used this type of correction throughout this thesis to reduce the chance of obtaining any false-positive results when making multiple pairwise tests. In chapters where False Discovery Rate was mentioned (FDR), this is defined as the proportion of false positives among all significant results (i.e possible type I errors). Sample size calculation was conducted by doing a power analysis for each test, sample size calculations were done using GraphPad Prism 6.0. Method for calculating sample size can be found at Charan et al., (2013). The GraphPad Prism 6.0 was used to perform the statistical analysis. All n numbers referred to biological replicates unless otherwise mentioned. All data are presented as mean ± SEM and significance was determined at p < 0.05.

2.0.16 Cell Line Culture and Maintenance

Cells were cultured in sterile incubators at recommended optimal conditions of 37°C and 5% CO2. For cell splitting, cell culture medium was removed and trypsin added (GIBCO, Life Technologies) for 2-3 minutes. Trypsin was aspirated and cells re-suspended in 10 ml of culture media. The cell suspension was transferred into 15ml tubes and centrifuged 5 min at 800rpm. The supernatant was aspirated and the cell pellets were re-suspended in 10 ml stable cell media. Cells were split into 2ml dishes or a flask. Best transfection results were obtained with cells at around 90% confluence on the day of transfection.

2.0.16.1 Cell Counting

In all instances cells were counted using a a standard cell hemocytometer. Using a pipette, 100 µL of Trypan Blue-treated cell suspension was applied on the hemocytometer. When counting viable cells, care was taken to avoid counting dead cells or other types of cells (i.e. glial cells when counting DRG neurons).
Chapter 2

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293/NDC/N2A</td>
<td>DMEM (GIBCO), 10% FBS, 1% Pen/Strep</td>
</tr>
<tr>
<td>HEK 293 Tap-Tag Nav1.7 stable cell line</td>
<td>DMEM (GIBCO), 10% FBS, 1% Pen/Strep, 0.5% G418 (Sigma)</td>
</tr>
<tr>
<td>HEK293 stable cell line</td>
<td>DMEM (Sigma), 2mM L-Glutamine (Sigma), 10% FBS (Invitrogen), 2 (\mu) g/ml Blastocycin (calbiochem), 0.6mg/ml Geneticin (Invitrogen), 10ml/L Penicillin – streptomycin (Sigma)</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Ham’s F12: DMEM (1:1) 2 mM glutamine (Life technologies) 1% non essential amino acids and 10% FBS (Life technologies)</td>
</tr>
</tbody>
</table>

Table 2.1: Medium used for cell culture

### 2.0.17 RNA Quantification

Dorsal root ganglion neurons from lumbar segments L4, L5 and L6 were extracted from 6 week old wild-type mice and pooled. RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. If experiments were conducted on cultured DRG neurons, the neurons were dissociated and cultured as previously mentioned. Changes in intracellular concentration of the neurons were performed by incubating the neurons in 0.5 \(\mu\)M Monensin (Sigma) for a chosen period (see figure 5.1). Monensin is a polyether antibiotic first isolated from *Streptomyces cinnamonensis*. It is an ionophore with a preference in forming complexes with monovalent cations such as Na, Li, K, RB and Ti. Monensin transports these cations across the lipid membranes of cells in a non-depolarising exchange, thereby playing a role as a Na+/H+ antiporter (Pinkerton et al. 1970). DRG neurons were then harvested and reverse traand retranscription was performed using iSript Reverse Transcription Supermix for reverse transcriptase, real time PCR following a protocol outlined by the supplier (Bio-Rad). DNA PCR amplification was measured using a Bio-Rad CFX Connect Real-Time PCR Detection System thermocycler. All expression levels for genes of interest, in this case Pro-Enkephalase (PENK), was normalised to the
The 2-\( \Delta \) Ct equation was used to determine fold changes in PENK expression. Wild-type littermate DRG was designated as the calibrator in these calculations. The data expressed in figure 5.1 are given as the mean of the fold changes. Statistical comparisons were done using a standard one-way ANOVA (see statistical analysis section of this methods section).

Primers used for qPCR:
- Gapdh forward: TGCGACTTCAACAGCAACTC
- reverse: CTTGCTCAGTGTCCTTGCTG;
- Penk forward: TTCAGCAGATCGGAGGAGTTG;
- reverse: GAAGCGAACGGAGGAGGAGAT.
Chapter 3

Mapping Protein Interactions of Sodium Channel Nav1.7 using Epitope-Tagged Gene-Targeted Mice

3.1 Abstract

Voltage-gated sodium channel Nav1.7 plays a critical role in pain pathways and has a large number of functions including control of neurotransmitter release, synaptic integration and the conduction of nociceptive signals. Humans born with loss of function mutations in this channel are unable to feel pain. However, this phenotype cannot be recapitulated with even the most specific of Nav1.7 channel blockers, possibly indicating that this channel’s role in pain signalling is more complex than simply signal transmission. To further explore the function of this channel, members of the molecular nociception group previously generated an epitope-tagged Nav1.7 mouse that showed normal pain behaviour to identify channel-interacting proteins. The high affinity epitope-tag consisted of a HAT-tag in tandem with a 3X FLAG tag separated by a Tobacco Etch Virus (TEV) cleavage site to enable two rounds of protein purification. Nav1.7 distribution throughout the central and peripheral nervous system was determined. Analysis of Nav1.7 complexes affinity-purified under native conditions by mass spectrometry revealed close to 300 proteins associated with Nav1.7 in vivo, including previously known and novel interacting proteins. Furthermore, an attempt to use this new affinity tag to purify and crystallise Nav1.7 was undertaken with no success. An account of an attempt to purify this protein is outlined at the end of the chapter and further details regarding how I aimed to purify and crystallise this protein are also laid out at the end
of this chapter.

## 3.2 Introduction

Voltage-gated sodium channels are essential for electrogenesis in excitable cells. Nine isoforms of α-subunits have been identified each consisting of a large pore-forming α-subunit (\( \sim 300 \) kDa). These α-subunits form heteromeric transmembrane complexes by associating with auxiliary β-subunits (33-36 kDa). Encoded by the gene SCN9A, Nav1.7 is an important component of action potential upstroke in primary afferent nociceptive neurons (Klugbauer et al. 1995). In humans, Nav1.7 is known to be selectively expressed peripherally, in dorsal root ganglion (DRG), trigeminal ganglia and sympathetic neurons (Black et al. 2012; Toledo-Aral et al. 1997); as well as in the central nervous system, in the spinal cord dorsal horn, olfactory bulb and hypothalamus, where it mediates a number of processes from neuronal transmission to synaptic integration (Branco et al. 2016; Weiss et al. 2011). Over the past decade, the apparent role of Nav1.7 in pain has fuelled the development of selective Nav1.7 blockers as therapeutic compounds. The presumption being that blocking the channel would decrease global nociceptor activity and possibly inhibit any other functionalities the channel may mediate. Highly selective blockers could be used as potential novel analgesic compounds for the relief of pain related disorders. However, aside from some reports outlining restricted efficacy of Nav1.7 channels blockers in animal models of pain (Williams et al. 2007; London et al. 2008; Chowdhury et al. 2011), *in vitro* experiments (Cao et al. 2016) and the occasional inherited erythromelalgia (IEM) patient responding to monotherapy using pan-sodium channel blockers (Choi et al. 2009; Fischer et al. 2009), the use of Nav1.7 blockers as therapeutic agents has been disappointing (England et al. 2009). This lack of potent analgesia from even the most efficient blockers suggests a more complex involvement for this channel in the generation of nociceptive signals aside from electrical conduction, most probably mediated through interactions with other proteins. Tandem affinity purification (TAP) is a generic two-step affinity purification protocol for isolating and precipitating proteins and their associated partners. This high-throughput strategy involves the modification of the endogenous protein by fusing a tandem affinity tag onto the N- or C-terminus of the protein. This can either be done *in vitro* (cell line) or through the use of modern mouse genetics *in vivo*. This method coupled with the power of mass spectroscopy is a reliable method for large scale protein interaction studies (Fernández et al. 2009). Since it’s conception, Tap-tag purification has permitted the identification of individual protein
components of cellular mechanisms in a number of different organisms such as, multiprotein
binding events, signalling cascades and cellular protein networks (Angrand et al. 2006;
it has been successfully adapted and optimised for use in mammals. All isoforms of neuronal
voltage-gated sodium channels (Nav1.1-1.9) share a high percentage sequence homology
(∼50%) (Catterall 2000; Catterall et al. 2005), making the analysis of Nav1.7 distribution in
vivo challenging through generic immunohistochemical methods. Because of the inherent
importance that Nav1.7 holds in pain signalling and the previously mentioned lack of potent
analgesia produced by even the most efficient of channels blockers, the identification of the
cellular protein networks operating the core functionalities of this channel would be
profoundly beneficial to the understanding of the molecular nociceptive machinery.
Furthermore, it would allow for the identification of potential novel drug targets, thereby
indirectly targeting Nav1.7. Although, some previous evidence by Black et al., (2012) have
undertaken some studies of Tap-tag Nav1.7 expression we have been unable to reproduce
these results (outlined in this chapter) and find that the antibodies available and used for
immunohistochemical and western blot detection of Nav1.7 are poor and lack specificity. The
insertion of a high affinity exogenous tag circumvents this problem. Through the generation
of an epitope-tagged Nav1.7 mouse and the use of affinity tagged purification techniques
coupled with LC-mass spectroscopy, the aim of this study was to provide new knowledge into
the macromolecular network of proteins that interact with Nav1.7. The rational behind the
use of "Tap-tagging" over other methods of protein-protein interaction analysis is further
explained in the discussion of this chapter but in brief, relates to the scope of proteins I
wished to identify. Here I obtained a broad picture of all Nav1.7 protein interactors.
Information about the proteomic network that regulates Nav1.7 and the further cellular
processes regulated by the channel will offer valuable insight into the role of this channel in
mediating pain. Moreover, as outlined in the aims of this chapter, obtaining information
regarding the Nav1.7 proteomic network will also provide insight into how this channel
propagates action potentials and its role in mediating pain signalling pathways and
neurotransmitter release. The methods used in determining the proteomic network
surrounding Nav1.7 are outlined in the methods chapter. Because some I chose to discuss the
validity and efficiency of certain methods, these have also been outlined in the discussion of
this chapter. For the sake of completeness, certain experiments in the following results section
that have been performed in collaboration with, or entirely by, other members of the
Molecular Nociception group have been included in the appendix section of this thesis and
can be referred to if needed. Furthermore, where this is the case, the name of the individual who has conducted the experiment or provided the data has been appropriately noted.
3.3 Results

3.3.1 Electrophysiological Characterisation of the Nav1.7 Tap-Tag Cell Line

Considering the possible functional disruptions that can be caused by inserting the tag onto the C-terminus of Nav1.7, prior to generating the TAP-tagged Nav1.7 knock-in mouse, an electrophysiological assessment of the channel in HEK293 cells was performed (please see methods section 2.0.12 for details on how the electrophysiological analysis was conducted). This was done by first establishing a HEK293 cell line stably expressing TAP-tagged Nav1.7. A human clone of Nav1.7 was used for the creation of the stable cell line (see appendix figure A.1 for details on construct). The tag used was identical to the one used for the mouse line and consisted of a 5-kDa TAP-tag comprised of a poly-histidine affinity tag (HAT) and a 3x FLAG tag in tandem, separated by a unique TEV-protease cleavage site, which was fused to the C-terminus of Nav1.7 (see figure 3.1). The expression of TAP-tagged Nav1.7 was detected by immunocytochemistry using an anti-FLAG and anti-HAT antibody (see figure 3.1).

Following the establishment of the cell line, electrophysiological characteristics of TAP-tagged Nav1.7 were tested by measuring activation and inactivation properties. Results of this analysis revealed normal kinetic function of all Nav1.7 currents (see figure 3.1). Activation and fast inactivation kinetics were identical to wild-type Nav1.7, demonstrating that the TAP-tag does not affect channel function. (Creation of the TAP-tagged Nav1.7 HEK293 cell line was done by Dr. Jing Zhao of the Molecular Nociception group, electrophysiological recordings and analysis were performed by myself. Further information regarding the generation of the tap-tag stable cell line can be found in the appendix section of this thesis (A.1).
Chapter 3

(a) 

(b) 

(c) 

Electrophysiological Current Traces

(d) 

Voltage (mV)

I(pA/pF)

Current Trace

Simultaneous imaging of hNav1.7

Alexandros Kanellopoulos
Figure 3.1: Characterisation of TAP-tagged Nav1.7 in HEK293 cells. (a) TAP-tagged human Nav1.7 construct used to establish the stably expressing TAP-tagged Nav1.7 HEK293 cell line. A sequence encoding a TAP-tag was cloned immediately upstream of the stop codon of Nav1.7 in the human SCN9A mammalian expression construct FLB. TAP-tagged Nav1.7 expression is driven by a CMV promoter. (b) Representative result of immunohistochemistry in TAP-tagged Nav1.7 HEK293 cells using an anti-FLAG antibody (green). Cell nuclei are stained with DAPI (blue). All the cells express TAP-tagged Nav1.7. Scale bar = 25 µm. (c) Current traces recorded from HEK293 cells and HEK293 cells stably transfected with the wild-type human Nav1.7 or the TAP-tagged human Nav1.7 clone in response to depolarisation steps from -100 to 40 mV. (d) I(V) fitted curves obtained in Nav1.7 and TAP-tagged Nav1.7 expressing cells using the same protocol as in (c) and showing no significant difference in voltage of half-maximal activation (V1/2) (n = 10, p > 0.05) and reversal potential (Vrev) (n = 10, p > 0.05). (e) & (f) show voltage dependence of fast activation and inactivation, assessed by submitting the cells to a 500 ms prepulse from -130 to -10 mV prior to depolarization at -10 mV. No significant difference in the voltage of half activation or inactivation was observed between the two cell lines (n = 10, p > 0.05) (all cells were patched in the same day). All error bars represent standard error of the mean (± SEM). Adaptations of these figures can be found in Kanellopoulos et al., 2018. All immunohistochemical analysis and electrophysiological recordings were performed by myself.

3.3.2 Generation of the Nav1.7 Tap-tag Mouse Line

Previous to my starting in the lab, members of the Molecular Nociception team had already begun working on a genetically modified Tap-tagged Nav1.7 mouse. Upon starting in the lab I took over all responsibility of breeding and crossing the animals to generate the mice numbers needed for the pulldown experiments. Further details regarding how the mouse was generated by Dr. Jing Zhao and Dr. Yuri D. Bogdanov of the Molecular Nociception group can be found in the appendix section of this thesis (see figure A.1) or alternatively details of the mouse generation were also published in Kanellopoulos et al., 2018.

3.3.3 Nav1.7TAP Mice Display Normal Pain Behaviour

The homozygous Nav1.7TAP knock-in mice (KI) were healthy, fertile and apparently normal. Age (6-12 weeks)–matched and sex-mixed KI mice (n = 7) and littermate wild-type (WT) controls (n = 7) were weighed and used for acute pain behaviour studies. The average weight was 18.77 ± 0.81 g (WT) and 20.13 ± 1.38 g (KI) (see figure 3.2). Motor function of the mice was examined using the Rotarod test. The average time that animals stayed on the rod was 50.57 ± 8.43 seconds (sec) (WT) and 52.72 ± 8.57 sec (KI) (see figure 3.2). There were no significant differences between the two groups either in weight (p = 0.4134, Student’s t-test)
or in the time spent on the rod \((p = 0.8619, \text{ Student’s t-test})\) (see figure 3.2). Response to mechanical stimulation was tested using the Von Frey test. 50% threshold of paw withdrawal in response was not significantly different between two groups \((0.73 \pm 0.04 \text{ g (WT)} \text{ and } 0.74 \pm 0.05 \text{ g (KI); } p = 0.9237, \text{ Student’s t-test})\) (see figure 3.2). The responses of animals in thermal and mechanical pain models were also examined. Acute thermal thresholds were measured using the Hargreaves’ test (see figure 3.2). The mean latency of withdrawal of the hind-paw from a thermal stimulus was similar between test groups \((5.80 \pm 0.13 \text{ (sec) (WT)} \text{ and } 6.14 \pm 0.29 \text{ sec (KI); } p = 0.3038, \text{ Student’s t-test})\). The response to noxious mechanical pressure was tested with the Randall-Selitto apparatus (Figure 1.4e). The mean weight that resulted in a withdrawal response was \(197.30 \pm 19.92 \text{ g (WT)} \text{ and } 188.48 \pm 12.18 \text{ g (KI). } \) No significant difference in weight applied to the tail was found \((p = 0.7124, \text{ Student’s t-test})\). The response to inflammatory pain of the animals was assessed with the Formalin test (see figure 3.2). The results showed that Nav1.7TAP mice had similar behavioural responses in both time courses \((p = 0.2226, \text{ F } = 1.36, \text{ two-way ANOVA with repeated measures and Bonferroni’s post-tests})\) and two phases \((p = 0.1690, \text{ Phase I; } p = 0.5047, \text{ Phase II; } \text{ Student’s t-test})\) (see figure 3.2), indicating no acute or formalin-induced inflammatory pain deficits in Nav1.7TAP mice. A basic power calculation was used to determine the number of mice needed for these experiments. For all details on how behavioural testing and power calculations were conducted please refer to section 2.0.5 of the methods section of this thesis.
Figure 3.2: Acute pain behaviour tests. (a) Animal weight showed no alterations in weight at P21 (n=10, WT; n=8, KI). (b) Rotarod test showed no motor deficits in TAP-tagged Nav1.7 animals (n=7, WT; n=7, KI; P = 0.9237, Student’s t-test). (c) Hargreaves’ apparatus demonstrated identical latencies of response to thermal stimulation (n=7, WT; n=7, KI; P = 0.3038, Student’s t-test). (d) Responses to low-threshold mechanical stimulation by von Frey filaments were normal in the KI mice (n=7, WT; n=7, KI; P = 0.9237, Student’s t-test). (e) Acute mechanical pressure applied with the Randall-Selitto apparatus demonstrated identical behaviour in KI and WT mice (n=7, WT; n=7, KI; P = 0.7124, Student’s t-test). (f) Formalin test. Licking/biting response to acute peripheral inflammation induced by intraplantar injection of 5% formalin in hind-paw was recorded. (g) the time course of development of the response of KI (blue) and WT littermate controls (red) showed similar response patterns (P = 0.29, two-way ANOVA). (f) the early (0 - 10 minutes) and late (10 - 45 minutes) phases of the formalin response in KI (n=7) and WT (n=10) mice showed similar responses ((P = 0.1690, first phase; P = 0.5017, second phase; Student’s t-test) between KI and WT mice. N numbers refer to biological replicates. All replicates are biological replicates (i.e different animals). All animals used were male. Because of the limited breeding capacity of Tap-Tagged Nav1.7 animals in order to get an adequate sample size, these experiments were done over a period of a few months. All error bars represent ± SEM. Details on statistical tests and how sample size was determined can be found in methods section 2.0.5.
3.3.4 Expression of Tap-tag Nav1.7 in the Nervous System

To examine protein expression and distribution of Nav1.7, whole protein lysates were obtained from a series of Nav1.7^{TAP/TAP} mouse tissues. These consisted of olfactory bulb, cerebellum, hypothalamus, cerebral cortex, spinal cord, dorsal root ganglia, sciatic nerve, skin, pancreas, heart and lung. The presence of Nav1.7 in these samples was detected by western blot using an anti-FLAG antibody. Consistent with previous studies (Weiss et al. 2011), Nav1.7 was found to be present in the olfactory bulb, hypothalamus, spinal cord, dorsal root ganglia and sciatic nerve. Nav1.7 was not detected in the cerebellum, cortex, skin, pancreas, heart or lung (see figure 3.6). Tap-tagged Nav1.7 could also be selectively immunoprecipitated and detected from individual tissue samples using single step purification (ss-AP). Nav1.7 was selectively immunoprecipitated from hypothalamus, sciatic nerve, spinal cord, olfactory bulb and dorsal root ganglia tissue (see figure 3.6).
Figure 3.3: Immunohistochemistry of FLAG-tag expression in the central nervous system (CNS). A-D In the main olfactory bulb (MOB), FLAG-tag expression (in green) is visible in the olfactory nerve layer (ONL) and in the glomerular layer (GL) in TAP-tagged Nav1.7 knock-in mice (KI) but not in the littermate wild-type controls (WT). In the posterior olfactory bulb, staining is also evident in the (C) accessory olfactory bulb (AOB). Staining is absent in the (D) MOB and AOB of wild-type control mice. E-F FLAG-tag expression is present in the medial habenula (MHb, arrow), the anterodorsal thalamic nucleus (AD, arrow), the laterodorsal thalamic nucleus (LD, dotted line), and (F) in the subfornical organ (SFO, arrow) located in the roof of the dorsal third ventricle. G-I FLAG-tag expression is present in neurons of the posteroventral aspect of the medial amygdala (MePD, arrow, dotted line) and in the hypothalamus in neurons of the arcuate nucleus (Arc, arrow, dotted line). J-L FLAG-tag expression is present (J) in neurons of the substantia nigra reticular part (SNR) and (K) the red nucleus magnocellular part (RMC) of the midbrain, and (L) in neurons of the pontine nuclei (Pn) located in the hindbrain. cp, cerebral peduncle; CTX, cortex; DM, dorsomedial hypothalamic nucleus; EPL, external plexiform layer; ME, median eminence; opt, optic tract; sm, stria medullaris; TH, thalamus; VMH, ventromedial hypothalamic nucleus; 3V, third ventricle. M-N The cross section of lumbar spinal cord (L4) is labelled with anti-FLAG (in red). FLAG tag expression is present in laminae I, II and III in spinal cord of KI mice (N) but not in spinal cord of WT mice (M). (O) The cross sections of spinal cord of KI mice were co-stained with Laminae II marker IB4 (o, in green). Sketches on the left illustrate the CNS regions and bregma levels (in mm) of the fluorescence images shown on the right. Scale bars: 500 µm (A, C, D, G); 250 µm (E, J, K, M-O); 100 µm (B, F, I, L); 50 µm (H). (All brain immunohistochemistry (figures A, B, C, D, E, F, G, H, I, J, K) present in this figure was performed by Frank Zufall and colleagues at Saarland University, however all tissue dissection and preparation was done by myself. All spinal cord staining was performed by myself.)
Next, we used immunohistochemistry to examine the expression pattern of Tap-tagged Nav1.7 throughout the nervous system of Nav1.7\textsuperscript{TAP} mice. Using a standard immunohistochemical protocol, Tap-tagged Nav1.7 could be detected, for the most part, in many of the tissues that had been previously examined by western blot. Consistent with previous results, FLAG-Tag staining was detected in the olfactory bulb, with strong staining visible in the olfactory nerve layer, the glomerular layer and in the accessory olfactory bulbs (see figure 3.3). In the brain, the FLAG-tag expression was present in the medial habenula, the anterodorsal thalamic nucleus, the laterodorsal thalamic nucleus and the subfornical organ that is located in the roof of the dorsal third ventricle.

![Figure 3.4](image)

Figure 3.4: Figure showing the attempted Nav1.7 staining of a spinal cord slice from a wild type animal. Left panel was stained for Nav1.7, left panel is the control image for this experiment. No clear signal can be detected in the spinal cord. According to the results from our tap-tag staining (see above figure 3.3) and previous evidence from by Black and colleagues (2012) there should be clear detection of Nav1.7 within the I and II laminae of the spinal cord dorsal horn. Antibody used was mouse Nav1.7 antibody (Neuromab) 1:500, secondary donkey anti-mouse 488. scale bar = 100\mu M.

The FLAG-tag was also present in neurons of the posterodorsal aspect of the medial amygdala and in the hypothalamus in neurons of the arcuate nucleus. A clear staining could also be seen in neurons of the substantia nigra reticular region and the red nucleus magnocellular region of the midbrain, along with neurons of the pontine nuclei located in the hindbrain. Staining of the spinal cord revealed strong FLAG-tag expression in the superficial layers of the spinal cord dorsal horn. Spinal cord samples were co-stained with IB4 to better distinguish anatomical layers of the dorsal horn laminae. Tap-tagged Nav1.7 was detected in laminae I, II and III. Despite the efficacy of FLAG and HAT antibodies, detection of peripheral expression of Tap-tagged Nav1.7 was difficult. Contrary to our western blot results (see figure 3.6), neither FLAG nor HAT staining could be found in the dorsal root ganglia, sciatic nerve or skin. A series of alternate immunohistochemical strategies were adopted in the hope of increasing signal/noise ratios thereby obtaining convincing TAP-tagged Nav1.7 staining in these tissues. Unfortunately, none of the following methods provided clear immunohistochemical detection of TAP-Tagged Nav1.7 in DRG, sciatic nerve or skin (see figure 3.5).
Figure 3.5: Immunohistochemistry of flag-tag expression in dorsal root ganglia of Tap-tag Nav1.7 mice (a), sciatic nerve (b) and skin (c) in mice. FLAG (F1804 sigma) antibody was used to target flag-tagged Nav1.7, neurofilament marker NF200 was used as an anatomical marker. No difference in signal can be seen between WT and KI mouse in any of the three selected tissues. White arrows in (b) point to individual fibres in the sciatic nerve, white dotted lines in (c) delineate the dermis from the epidermis layer of the skin, all scale bars = 50µm. Attempted immunohistochemistry of flag-tag expression in dorsal root ganglia (a & b) and skin (c). Various different approaches were used in an attempt to obtain a clear signal from the tandem affinity tag linked to Nav1.7. Left top and bottom panels in (d) show results from TSA amplification stain using the second part of the tag (HAT), with no difference in signal between KI and WT. Centre top and bottom panels show immunohistochemistry from non-amplified HAT staining with no difference in signal between KI and WT. Right top and bottom panels in (d) show TSA amplification stain using FLAG-tag antibody, no difference in signal was detected between KI and WT. (e) Attempted immunohistochemistry of flag-tag expression in dorsal root ganglia using different fixation and antigen retrieval methods. Left top and bottom panels: 1% PFA with + overnight incubation with primary antibody. Centre top and bottom panels: Fresh frozen (acetone fixation) tissue + citrate antigen retrieval buffer pH6. Right panel top and bottom: Perfusion 2% PFA + boiled for 20 mins (100°C) in tris-EDTA buffer pH 9. (f) Attempted immunohistochemistry of flag-tag expression in skin. Top and bottom panel: Fresh frozen (acetone fixation) + citrate antigen retrieval buffer pH6. All scale bars = 150µm
3.3.5 Purification of Tap-Tag Nav1.7

The identification of Nav1.7 interacting proteins is a critical step in understanding it’s function. While many techniques have been developed to characterise protein-protein interactions (please see table 4.6 for summary of current available methods of studying protein interactions), the one strategy that offers high-quality and high-throughput information is affinity-purification coupled to mass spectrometry (AP-MS). Our Nav1.7\textsuperscript{TAP} generated mouse, offered the possibility to undertake this strategy. Before spectroscopic analysis could be undertaken, Tap-tagged Nav1.7 had to be immunoprecipitated from selected Nav1.7\textsuperscript{TAP} tissues using FLAG antibodies. In the interest of obtaining a sample containing both weak and strong binding interacting partners, a single purification step was performed. In this process, all tissues having been identified as containing Tap-tagged Nav1.7 were pooled (olfactory bulb, DRG, Sciatic nerve, spinal cord and hypothalamus) and mechanically homogenised to obtain a crude tissue lysate. The crude lysate could then be immunoprecipitated using the 3X FLAG tag. This step relied on the unique specificity of the 3X FLAG epitope to precipitate only tagged proteins. The now bound protein complex could be washed and eluted using the TEV cleavage site (see figure 3.6 for summary of the protocol). The use of the TEV cleavage site again increases the specificity of the sample to proteins interacting with Nav1.7, as only the interacting complex was eluted. This is in contrast to heat elution methods and can result in sample contamination as with heat elution all proteins bound to the precipitation column, including non-specific proteins, are eluted (Dunham et al. 2012). 1% CHAPS buffer was experimentally evaluated (see table 3.1 for buffer comparison) as the most suitable detergent for all immunopurification steps due to it’s non-denaturing zwitterionic properties, which preserves both weak and strong protein interactions. Furthermore 1% CHAPS is highly compatible with downstream mass spectrometry (see figure 3.6). Six samples from both Nav1.7\textsuperscript{TAP} and wild type animals were processed in this manner for subsequent mass spectrometry analysis. In the interest of optimising the immunoprecipitation, a series of different buffers containing various detergents were tested (see figure 3.6). CHAPS was ultimately chosen as the best detergent due to it’s non-denaturing zwitterionic properties, high solubilising capacity and general compatibility with the immunoprecipitation protocol. The result showed that TAP-tagged Nav1.7 from DRG and olfactory bulbs was clearly solubilized and precipitated by both purifications - AP and tandem affinity purification in 1% CHAPS buffer (see figure 3.6). The result from AP showed that TAP-tagged Nav1.7 could be immunoprecipitated from hypothalamus, sciatic
nerve, spinal cord, olfactory bulb and DRG of KI mice, but not WT control mice.

Figure 3.6: (a) Tissue expression of TAP-tagged Nav1.7. Whole cell lysis protein sampled were extracted from different tissues in both KI and WT littermate control mice and anti-FLAG used to detect TAP-tagged Nav1.7 using western blotting. All tissue is extracted from one mouse per group (i.e. one Tap-Tag mouse and one wild type). Anti β-Tubulin was used as a loading control. Panel (a) shows western blot results for KI. Panel (b) shows western results for WT. (c) Panel of tissues used for co-immunoprecipitation using single-step affinity purification. Panel shows individual tissue specific immunoprecipitations. For LC-MS all tissues including hypothalamus, sciatic nerve, spinal cord, olfactory bulbs and DRG from KI mice, and control pooled tissues from WT mice were extracted in 1% CHAPS lysis buffer. After single-step affinity purification, TAP-tagged Nav1.7 was detected using western blotting with anti-HAT antibody. (d) Optimisation purification panel of single step purified tap-tagged Nav1.7 in DRG tissue using three different buffers. 1% CHAPS was chosen as the optimal purification buffer due to high yields of protein detected by western blot using anti-HAT antibody (see table 3.1 for more information on buffer composition). (e)Top panel: A schematic illustrating the affinity purification (single step affinity purification and Tandem step) procedure by using tandem affinity tags separated by the proteolytic TEV cleavage site. The proteins from DRG and olfactory bulbs were extracted in 1% CHAPS lysis buffer. After single-step and tandem affinity purification, TAP-tagged Nav1.7 were detected using western blotting with anti-HAT antibody. Bottom western blot shows detection levels of tap-tagged Nav1.7 following the purification procedure.
<table>
<thead>
<tr>
<th>Detergent</th>
<th>Buffer Composition</th>
<th>CO-IP Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIPA</td>
<td>20 mM Tris-HCl (pH 7.5); 150 mM NaCl; 1 mM Na2EDTA; 1 mM EGTA; 1% NP-40 1% sodium deoxycholate; 2.5 mM sodium pyrophosphate; 1 mM complete EDTA free Protease inhibitor cocktail (Roche); 0.1% SDS</td>
<td>RIPA buffer can be quite harsh and although I was able to detect Tap-Tag Nav1.7 in WB the about of detection was inconsistent between trials. The properties of RIPA buffer also mean the detergent disrupts weak interactions between proteins.</td>
</tr>
<tr>
<td>CHAPS</td>
<td>30 mM Tris-HCl (pH 7.5); 150 mM NaCl, 1% CHAPS; 1 complete EDTA free Protease inhibitor cocktail (Roche)</td>
<td>Clear and consistent detection of Tap-Tag Nav1.7. The zwitterionic properties of CHAPS means that the detergent does not denature proteins and does not disrupt interactions between proteins</td>
</tr>
<tr>
<td>DDM</td>
<td>20 mM Tris-HCL (pH 7.5), 150 mM NaCl, and 0.03% DDM; 1 complete EDTA free Protease inhibitor cocktail (Roche)</td>
<td>Although DDM is often used to solubilise large membrane lipid molecules such as cholesterol, it did not show consistent strong solubilisation of Tap-Tag Nav1.7</td>
</tr>
</tbody>
</table>

Table 3.1: Table showing a summary of the three detergents tested for Co-Immunoprecipitation experiments of Tap-Tag Nav1.7. Although the choice of 1% CHAPS buffer was my first choice based previous literature (Phizicky et al. 1995) and the zwitterionic properties of the buffer being favourable to maintaining protein-protein interactions during Co-IP studies, I still sought to provide some comparison with other buffers that were commonly used for Co-IP experiments in the lab. However, as expected, CHAPS buffer did provide the best results compared to RIPA and DDM, as outlined in figure 3.6(d).
3.3.6 Identification of Nav1.7 Interacting Proteins

We next identified the components of Nav1.7 complexes from ss-AP by Liquid Chromatography-Mass Spectrometry (LC-MS/MS). Analysis of the LC-MS data was conducted entirely by Dr Honglei Huang at Oxford university and methods for this analysis can be found in Appendix A of this thesis. In brief, a total 189,606 spectra were acquired from 12 samples. Each group (Nav1.7\textsuperscript{TAP} and WT) contained 6 biological replicate samples, with each sample originating from one mouse. A total of 1,252 proteins were identified which had a false discovery rate (FDR) of 0.96%. Using the Proteowizard v.2.1.2476 software, all the raw mass spec data was processed to generate MGF files (200 most intense peaks). The identification of proteins was performed using these MGF files and the central proteomics facilities pipeline (Trudgian et al. 2010). The UniProtKB Mus musculus database was used in the CPF Proteomics pipeline for data analysis. This pipeline functions by combining data from three search engines: Mascot, OMSSA and X! tandem k-score. The false discovery rate was calculated using the peptide/proteinprophet software or estimated empirically from decoy hits, all identified proteins were filtered to above 1% FDR. Proteins were clustered and annotated with the use of STRING, PHANTER, IPA (Quigen) and UniProt software (further information regarding the software used for the analysis of the identified proteins can be found in appendix section A.1). STRING algorithm is outlined in section 4.3.1). These are high throughput, genome wide analysis tools for proteomic and expression studies. Group data analysis of wildtype (n=6) versus tap-tagged Nav1.7 (n=6) samples, determined a minimum of 1.5 fold increase of 267 proteins in the tagged sample (see table 3.2). Our collaborator Dr. Honglei Huang performed the statistical analysis that determined the cut off of 1.5 fold increase for the protein list. The detection of specific protein abundance is a fundamentally difficult task in Mass-Spectrometry. In this experiment, our collaborators used the standard method of using a standard 2-sample t-test to compare WT vs KI samples in order to outline possible statistically significant increases in the KI sample. However, although this is standard practice, it is important to consider that in this analysis there were only 6 samples per group and therefore it is important to mention that there could be some uncertainty in our numbers due to sample variability. Kammers et al., (2015) outlines this method of determining fold change significance between groups with the following 2-sample t-test analysis (identical to the analysis done by our collaborators):

In order to detect differential expression of proteins in a balanced proteomic experiment, with n samples, in this case KI Tap-Tag Nav1.7 (log2 of the relative abundances X1p, \ldots, Xnp for
protein p) and n control samples, in this case WT samples (log 2 relative abundances \(Y_{1p}, \ldots, Y_{np}\)), inference is based on a standard 2-sample t-test for each protein, with the following test statistic

\[
t_p = \frac{\text{estimated log fold change}}{\text{estimated standard error}} = \frac{\bar{X}_p - \bar{Y}_p}{s_p \sqrt{2/n}},
\]

where mean of \(\bar{X}\) and \(\bar{Y}\) are the group mean log2 of relative abundances of the proteins, and

\[
s_p = \sqrt{\frac{\sum_i (X_{ip} - \bar{X}_p)^2 + \sum_i (Y_{ip} - \bar{Y}_p)^2}{2n-2}}
\]

is the standard deviation within the group test sample. Using this method a p-value is then calculated for each protein which refers to the test statistic \(t_p\) to a t-distribution with \(df = 2Xn - 2\) degrees of freedom as the null distribution. For the above calculations the log2 of relative abundances are assumed to have a normal distribution with equal variance within each group to be normally distributed with equal variance in each group. Kammers et al., (2015) do point out that although t-test are a reliable and somewhat robust method of detecting significance between groups, there remains the issue, relevant to this thesis, of small sample sizes that should be taken as a possible caveat when interpreting the following data. Furthermore, contaminating proteins also have to be considered in these co-IP experiments, as the assay itself can in some circumstances pick up contaminants that may be detected in further mass spectrometry analysis (please see further experiments section of this chapter for an analysis of how contaminants can be considered in these experiments).

All proteins that were subsequently analysed in the following chapter or flagged as proteins of interest have been highlighted in yellow in table 3.2. In order to avoid the appearance of "cherry picking" and to avoid damaging the integrity of the mass-spec list I wish to point out a discrepancy that might appear between this mass spec list and the choice of protein candidates that are studied in the following chapter. In the following chapter, certain proteins of interest that were chosen as candidates for further validation experiments did not meet the statistically set threshold of 1.5 fold increase. These proteins include CRMP2, TMED10 and Cav2.2. These proteins did appear on my list of interacting proteins but at the lower value of 1.2 fold increase. The reasons for studying these proteins are outlined extensively throughout the following chapter and generally relates to their implication in pain signalling. However, it is important to take into consideration that their fold increase did not meet the statistically set cutoff point. More information regarding how the cutoff point was determined as well as
all other information regarding the mass spectrometry method undertaken by our collaborator at Oxford can be found in appendix section A.1 of this thesis.

STRING cellular component analysis of these 267 proteins revealed multiple different cellular components. These proteins were further classified into 22 groups based on their function, including 12 membrane trafficking proteins, 23 enzyme modulators and 4 transcription factors (Mass spectrometry was performed by Honglei Huang at Oxford university, post analysis of the protein network was performed by myself). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org/) via the PRIDE partner repository with the dataset identifier PXD004926.

<table>
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<th>MGI Symbols</th>
<th>Protein Name</th>
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<th>AveWT</th>
<th>KI/WT Ratio</th>
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| Atp2a2 | ATPase H+ Transporting ATPase Subunit 2 | O55143 | 5.99E-07 | 2.21E-07 | 2.71 |
| Atp2b1 | Plasma Membrane Calcium-Transporting ATPase | G5E829 | 2.73E-07 | 5.29E-08 | 1.55 |
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Alexandros Kanellopoulos
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Cellular Processes
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/Mitochondria

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**Membrane Transporters/Trafficking**

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**Ion Channel/Receptor/Ion Transport**

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| Slc7a5 | Large Neutral Amino Acids Transporter Small Subunit 1 | Q9Z127 | 1.66E-07    | 3.11E-08  | 5.33        |
### Chapter 3

**Table 3.2:** List of gene names (MGI Symbols) of all the proteins identified by LS-Mass Spectrometry from immunoprecipitation experiments of Tap-tag Nav1.7 with a KI/WT ratio of larger than 1.5. In ‘KI/WT Ratio’ column ‘KI only’ indicates the protein was identified only in samples co-immunoprecipitated from tissue containing the Tap-tagged Nav1.7, all other numbers indicate ratios between KI and WT samples. Some proteins can be found under different names than the ones shown here (refer to UniProt accession number). Proteins were classed according to general function using the PANTHER algorithm analysis. The present list corresponds to the list published in Kanellopoulos et al., (2018) and therefore represents pooled data obtained by myself and Dr. Jennifer Koenig.

#### 3.4 Crystal Structure of Nav1.7

As part of a wider project, I aimed to use the tandem affinity tags present on Nav1.7 to attempt the purification and crystallisation of Nav1.7. Despite my efforts to purify Nav1.7 within our lab, we did not have the necessary resources to optimise the expression of Nav1.7 within a cell line and subsequently grow the necessary quantities needed for crystallisation. I therefore sought assistance from the team at the Oxford Protein Production Facility (OPPF-UK) who gladly agreed to assist me. However, because of their own limits on time and resources, the team at the OPPF would only proceed with the purification and crystallisation steps if we were first able to successfully express the Nav1.7 construct within
Figure 3.7: Figure showing an example of a silver stained gel run in parallel with a western blot immunostained for Nav1.7. Both gels are running the same pulldown sample (tandem pulldown of Nav1.7 from our Tap-tag Nav1.7 cell line) and were run in exactly the same manner. Using the western blot gel as a reference, we were able to pinpoint approximately to which silver stained band Nav1.7 corresponds. The silver stained gel also shows a number of other bands which correspond to other proteins, which in the process of protein purification are seen as impurities. Antibody used for the staining of Nav1.7 was targeted at the histidine portion of the Tap-Tag (anti-HAT (1:400; LSBio, LS-C51508).

their optimised cell lines. Unfortunately, we were not able to express the necessary clones at the OPPF. Upon failure to do so, the OPPF was unfortunately forced to withdraw my access to its facilities and abandon our collaboration. Before approaching the OPPF I set about assembling a basic outline of the experiments I wished to undertake and the possible problems I would have to address during the process. I have outlined all hypothetical future experiments in the discussion of this chapter. The following section, however, contains the results from the initial purifications done in the lab along with details of the expression clones that were assembled at the OPPF.

3.4.1 Methodology for the Purification of Nav1.7

Using our lab’s successful expression of Nav1.7 in HEK293 cells as a reference, the aim was to first begin by attempting to purify the protein using the anti-FLAG antibody binding, the TEV-protease cleavage, and the HAT-Ni-NTA and/or Talon columns capture in our lab. One of the conditions required for OPPF assistance was to first show a successful pull-down attempt at purifying Nav1.7. Using silver stain analysis in parallel with western blot, I was able to show a clear and distinct band on the silver stained gel that according to size, most probably corresponded to Nav1.7 (see figure 3.7. The method used for the pulldown purification was identical to that described in methods section 2.0.7). Because I realised the challenge of producing large amounts of purified protein expressed in mammalian cells, I applied for, and was granted access to the Oxford Protein Production Facility (OPPF-UK) to increase the yields/purity should I manage to obtain a sufficiently pure sample of Nav1.7. The aim here was first to simply see the degree of purification of Nav1.7 using our standards CHAPS buffer and tandem purification process. Following this initial attempt at purification I was expecting to begin screening and optimising detergents at the OPPF with the assistance
of the OPPF team who would have advised me on detergent selection.

### 3.4.2 Expression of Tap-Tag Nav1.7 in pOPINE-3C-eGFP

The most important requirement for the OPPF to move forward in assisting me in purifying Tap-Tag Nav1.7 was that the tap-tagged gene could be efficiently expressed in their optimised vector, pOPINE-3C-eGFP. A selection of different voltage gated sodium channels were given to the OPPF team by myself, including Tap-tag Nav1.7, Nav1.8 and Nav1.9, which they then used to clone each sodium channel into their own vectors (pOPINE-3C-eGFP). I personally sequenced all constructs cloned by the OPPF and all sequences were correct. Next I aimed to determine which sodium channel showed better expression in an HEK293 cell line. It is important to note that the OPPF requested I use their own cell lines and vectors in all experiments and dismissed the idea of using the stable cell line that had been previously generated by Jing Zhao in our lab (see table 3.3. For further information regarding the exact sequence of the clones, please see https://www.addgene.org/41125/).

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<tr>
<td>F1</td>
<td>Nav1.7</td>
<td>N/A</td>
<td>pTAP</td>
<td>C-CBP-FLAG</td>
</tr>
<tr>
<td>G1</td>
<td>Control</td>
<td>OPPF12807</td>
<td>pOPINE-FLAG</td>
<td>C-3C-FLAG-His8</td>
</tr>
<tr>
<td>H1</td>
<td>eGFP Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.3: Table showing the voltage-gated sodium channel or control construct used in the transfection screen in HEK293 cells. The constructs were provided by the OPPF directly except for the Nav1.7, Nav1.8, Nav1.9 and Nav1.7-TAP insert which was provided from our lab. Nav1.7-TAP insert is the same gene that was used in the generation of our stable cell line (see appendix additional methods for further clarification). The column described as "Vector" indicates the type of construct the sodium channel gene was cloned into. The ID number relates to figure 3.8. All voltage gated sodium channel subtypes were of human origin.

Unfortunately, neither myself nor skilled members of the OPPF team with which I was collaborating, were able to successfully express the plasmids listed in table 3.3 in the OPPF’s HEK293 cell line. Both lipofectamnie and electroporation (see cell transfection in methods section of this thesis) were attempted. Figure 3.9 and 3.8 illustrates the attempted expression of the plasmids in HEK293 cells. Figure 3.7 is an example of a silver stained gel obtained in our lab at UCL following purification of Tap-tag Nav1.7. Right panel is the a western blot...
Chapter 3

Figure 3.8: Figure showing attempt to express voltage-gated sodium channel subtypes in HEK293 cells. Cells containing the sodium channels inserted into OPPF designed vectors described in table 3.3 which have been plated in an 8 well plate. Left column from top to bottom wells correspond to A1, B1, C1, D1 from table 3.3. Right column from top to bottom correspond to E1, F1, G1, H1 in table 3.3. Wells were viewed under a fluorescence microscope to detect GFP tags in the expressed vectors in an attempt to identify successful expression of a voltage-gated sodium channel in this cell line. As illustrated only control wells E1 and H1 contain fluorescence. Close analysis of these wells can be found in the two magnifications found on the right of the figure. Top right panel is a magnification of well E, bottom right panel is a picture of Well F. Analysis of the protein. Right panel is the corresponding silver stained gel. As seen in figure 3.8 no fluorescence could be found from the eGFP tags that would have been expressed with the sodium channels (A1, B1, C1, D1, E1, F1, G1), whereas clear expression of the tag can be seen in the control well (H1). These same results can be seen in the western blot analysis of the proteins in figure 3.9 Please see Further Experiments section of this chapter for further information regarding these experiments.

Figure 3.9: Shows gel immunofluorescence of samples collected from the wells in figure 3.3. As illustrated fluorescence could only be achieved in control samples D1, E1 and H1 and no florescence could be found in any of the sodium channel containing wells, indicating the lack of expression of these plasmids in these cells. Left panel gel: Following harvesting of the cell and treatment with SDS I ran both the totality of the sample (i.e both membranes and cytoplasm of the cell) and soluble components which would have excluded membrane portions of the cells. Right panel gel: Gel immunofluorescence following the transient transfection of the Tap-Tag Nav1.7 construct in lane F1. Lane G1 contains control plasmid. (Please refer to figure 3.3 for information regarding samples and in which lane the samples were run)
3.5 Discussion

Protein-protein interactions constitute the molecular machinery responsible for the trafficking, distribution, modulation and stability of ion channels (Catterall 2010; Bao et al. 2015; Chen-Izu et al. 2015; Laedermann et al. 2013; Shao et al. 2009; Leterrier et al. 2010). The lack of protein interaction evidence regarding Nav1.7 has impeded our understanding of the underlying role this channel plays in the generation of nociceptive signals. Here, we mapped the protein-interaction network of Nav1.7 using an AP-MS proteomic approach with an epitope-tagged Nav1.7 knock-in mouse line. 267 proteins were identified as possible interactors of Nav1.7. Furthermore, we were able to use this tagged channel to immunohistochemically determine its expression and distribution throughout the central and peripheral nervous system. AP-MS requires specific high-affinity antibodies against the proteins (Wildburger et al. 2015). However, the binding of antibodies to endogenous antigens on the protein of interest may compete with normal protein-protein interactions. In order to overcome this limitation, epitopes in the form of robustly characterised tags were introduced into the target protein sequence. The coupling of this technique with tandem affinity purification has been widely applied in protein-protein interaction studies (Wildburger et al. 2015; Fernández et al. 2009). This technique is high throughput and produces low background and low levels of sample contamination, making it superior to the more traditional Yeast-2-Hybrid technique, at least in my case where I wished to identify as many interactors in one experiment as possible. Coupled with the use of highly sensitive mass spectrometry and powerful quantitative proteomic analysis methods, this Tap-Tag technique has been employed in the past to identify both transient and stable protein-protein interactors (Keilhauer et al. 2015; Oeffinger 2012). These tools provided us with the perfect platform to study the proteomic network surrounding Nav1.7. When considering my aim in conducting this experiment, I made some fundamental choices which would determine the scope of my results. Firstly, my source material was a pooled selection of Nav1.7 expressing tissues. I chose a pooled sample as my aim was to gather a global picture of what interacts with Nav1.7 without considering particular tissues or how the channel's role may differ contextually in different areas of the nervous system. Although, I felt this would also be the best way of initially determining the validity of the assay (please see conclusion and further experiments for more details) I do feel that this may have carried a number of limitations, especially when considering the different roles Nav1.7 may potentially play in different tissue types. Secondly, I chose to only perform a single purification step despite having the option of a tandem purification. I felt that by
performing only a single step we would gather a broader set of Nav1.7 interactors which would contain both strongly and more weakly binding proteins. Again, this experimental specificity was chosen to maximise the scope of our results. Using our experimental paradigm, a stable and detailed network of 267 proteins was isolated. Along with already known Nav1.7 interactors, this complex contained a vast range of important trafficking, regulatory and transcriptional proteins most of which have never before been identified as interactors.

VGSCs are known to exist in macromolecular complexes with a number of different β subunits (Meadows et al. 2005). Important in the regulation of Nav1.7, β subunits are also members of the immunoglobulin (Ig) domain family of cell-adhesion molecules (CAM). As well as sodium channel α subunits, the β subunits bind to a variety of cell adhesion molecules such as Neurofascin, Contactin, Tenascins, and NrCAMs through an extracellular Ig cell adhesion domain (Srinivasan et al. 1998; McEwen et al. 2004; Ratcliffe et al. 2001; Namadurai et al. 2015; Cusdin et al. 2008). Our data confirmed the co-precipitation of Nav1.7 with sodium channel β3 subunit along with CAM family proteins, Ncam1 and Neurofascin (see table 3.2). With the crystal structure of the human β3 subunit recently solved, evidence suggests that the β3 subunit Ig-domain assembles as a trimer in the crystal asymmetric unit (Namadurai et al. 2015). This implies that trimeric β3 subunits binding to Nav1.7 α subunit(s) most probably form a large complex in the plasma membrane together with other sodium channels, as well as CAMs and other identified cytoskeletal proteins such as KIF5B, AnkyrinG and Neurofascin (Namadurai et al. 2015) identified in our study, important new information regarding how the sodium channel is regulated at the membrane.

Prior evidence by Minett et al., (2015) showing a complete abolishment of neurotransmitter release in the spinal cord of Nav1.7 knockout mice, alluding to a role for Nav1.7 in the release of neurotransmitters and perhaps, similar to VGCC (Catterall 2015), a vital component in synaptic architecture. We were intrigued to find that a number of key synaptic proteins co-immunoprecipitate with Nav1.7. We identified two isoforms of synaptotagmin (I & II) along with other SNARE complex proteins such as syntaxin-12 and SNAP25.

Synaptotagmin’s primary role as a vesicle fusion component activated by calcium binding at the synapse is well established (Chapman 2002). However, Sampo et al., (2000) showed a direct physical binding of synaptotagmin-1 with Nav1.2 at a site which is highly conserved across all voltage-gated sodium channels (Sampo et al. 2000) suggesting that the synaptotagmin family can associate with other VGSCs, and inferring a fundamental role for Nav1.7 in mediating neurotransmitter release. Prior to our study, a number of Nav1.7 interacting proteins have previously been identified. Our study provided concuring evidence
for some of these candidates while also uncovering novel interactors. One previously established interactor is Calmodulin (Cam1). Cam1 has previously been shown to bind and regulate steady state inactivation of Nav1.4, Nav1.6, Nav1.7 and voltage-gated calcium channels (Herzog et al. 2003; Biswas et al. 2008). Despite distinct functional differences between calcium and sodium ion channels, vestigial evolutionary motif homology present on the C-terminal tail of these channels have been suggested as the binding site for calmodulin in both sodium and calcium channels. This conserved C-terminal motif is also present in Nav1.7 and suggests a potential location for the binding of Cam1 to the channel (Ben-Johny et al. 2014; Chagot et al. 2009; Leon et al. 1995; Miloushev et al. 2009). Nav1.7 has also been linked to opioid peptide expression, and enhanced activity of opioid receptors is found in Nav1.7 null mutant mice (Minett et al. 2015). In Minett et al., (2015) the authors found an increase in the expression of proenkephalins in genetically engineered Nav1.7 deficient mice. Interestingly, a number of opioid regulatory proteins appeared as Nav1.7 interactors. One such protein is the G-protein regulated inducer of neuron outgrowth 1 (Gprin1, also known under the name GRIN1). Gprin1, has previously been implicated in the tethering of opioid receptors as well as other GPCRs within lipid rafts (Ge et al. 2009). The evidence linking the regulation of opioid receptors by Nav1.7 is further explored in Chapter 3 of this thesis. However, for the purpose of this discussion it is important to mention that the evidence linking the regulation of opioid receptors by entities involving sodium, dates back to the 1980’s (Puttfarcken et al. 1986) and has recently found new light in Minett et al., (2015); as outlined earlier. This link is important when considering the contradiction reported in the literature that genetic loss of Nav1.7 causes congenital insensitivity to pain in mice and humans (Cox et al. 2006; Gingras et al. 2014; Nassar et al. 2004), yet the potent and selective pharmacological block of the channel does not. This contradiction could be resolved if Nav1.7 is found to not only contribute to neuronal electrogenesis, but also regulate a number of other cellular processes, contributing to analgesia in perhaps a more non-canonical fashion. These concurring pathways are beginning to be uncovered. For example, Isensee et al., (2016) found that mice lacking Nav1.7 have alterations beyond acute depolarisation events, where a loss of this channel results in altered pro-nociceptive GPCR signaling, more specifically a lack of Nav1.7 causes an increase in opioid receptor efficacy within sensory neurons (Isensee et al. 2016). The exact mechanisms at play in Nav1.7−/− remain to be elucidated, yet uncovering the physical link between Nav1.7 and opioid signalling could be the first step in understanding how CIP phenotypes arise. Interestingly, a few previously known positive Nav1.7 interactors were not revealed by our assay. Shao et al., (2009) demonstrated that Pdzd2 binds to the
second intracellular loop of Nav1.7 by a GST pull-down assay in an in vitro system (Shao et al. 2009). Furthermore, we were unable to find the previously reported Nav1.7 interactor Nedd4-2, a crucial regulator of sodium channel internalisation. This could be due to the transient nature of their interaction with Nav1.7. For example, in a recent study by Yang et al., (2017), the group provided clear evidence for an interaction between Nav1.7 and intracellular fibroblast growth factor 13 (FGF13). This protein stabilises the channel on the plasma membrane in a heat dependent process, providing a mechanism for noxious heat-sensing in DRG neurons (Yang et al. 2017). This lack of detection may be because of the transient and context based interaction between these proteins and Nav1.7, where binding of Nedd4-2 has only been shown in neuropathic pain conditions and Pdzd2 shows strong binding in vitro but not in vivo. Moreover, as previously mentioned, the interaction with FGF13 was primarily found in DRG neurons in the presence of noxious heat where this protein stabilises the Nav1.7 channel on the plasma membrane increasing neuronal excitability, effectively acting as a heat sensing complex (Yang et al. 2017). Further study of these context dependent interactions will surely provide insight into the role of Nav1.7 in various types of neuropathic pain conditions. Our Tap-tagged Nav1.7 provides the tools necessary to do so.

The broad role of Nav1.7 is best illustrated by the number of intracellular signalling pathways that mediate and are mediated by this sodium channel. We uncovered a direct interaction between Nav1.7 and mitogen-activated protein kinase 7, a member of the ERK1/2 MAPK pathway. The inhibition of pERK1/2 regulates rising membrane potential and firing properties of DRG neurons through the phosphorylation of Nav1.7 at a site within intracellular loop I of the channel. The inhibition of pERK1/2 causes a depolarising shift of activation and fast inactivation of Nav1.7 without altering current density (Stamboulian et al. 2010). Furthermore, elements of the serine/threonine kinase mammalian target of rapamycin (mTOR) pathway, important in the regulation of cell growth, maintenance and synaptic plasticity were present in our MS list. Multiple mTOR complexes (mTORC1 and mTORC2), that differ in activation and cellular localisation are essential in the control of local protein synthesis and translation in neuronal cells via the phosphorylation of transcriptional initiation factors (eIFs) and ribosomal kinases. A number of eIFs were also identified in our list (eIF3a, 3b, 3c, 3d, 3e, 3i, 3l). Chronic pain states are characterised by increased excitability of primary afferents and by short and long term changes in protein expression, leading to central and peripheral sensitisation. Coincidentally, TTX-sensitive voltage gated sodium channels including Nav1.7 are unregulated during acute and chronic pain states (Matzner et al. 1994; Omana-Zapata et al. 1997). A study by Wada et al., (2004) found that the inhibition of
mTOR in chromaffin cells increases the number of voltage gated sodium channels localised at the plasma membrane via increased trafficking of VGSCs from the trans-golgi (Wada 2004). The suppression of this pathway by intrathecal injection or direct application of rapamycin in animal models of neuropathic pain, was able to attenuate mechanical hypersensitivity (Asante et al. 2009; Liang et al. 2013). This suggests an important role for the mTOR pathway in mediating neuropathic pain conditions and may do so through the regulation of Nav1.7. The Tap-tagged Nav1.7 also enabled a more succinct analysis of the distribution of Nav1.7 throughout the nervous system. By using the FLAG tag we were able to use immunohistochemistry and western blot analysis to successfully detect the presence of Nav1.7 in the different tissues of the CNS and PNS. Previous reports by Black et al., (2012) showed a clear expression of Nav1.7 in the mouse peripheral nervous system. We found very similar expression patterns to Black and colleagues in lamina I and II of the dorsal horn spinal cord which co-localised with IB4, which is consistent with the belief that this expression arises from the pre-synaptic central terminals of nociceptive DRG neurons. However, disparity between our study and that of Black and colleagues was apparent when were were not able to obtain clear detection of Tap-Tag Nav1.7 in glaborous skin, DRG or the sciatic nerve. According to the study by Black and colleagues, we should have detected Nav1.7 in the somata of virtually all small diameter DRG neurons, which despite multiple attempts we were not able to achieve. Furthermore, Black et al., described strong colocalisation of Nav1.7 in numerous peripherin stained fibres of the sciatic nerve, which again we could not confirm in our study. The reason for this inability to replicate what had been previously illustrated in the litterature remains speculative. Our ability to co-immunoprecipitate from DRG suggests that the FLAG-tag is present in these cell bodies, excluding the possibility that the tag may be cleaved off before or after the protein is synthesised. This leads to the conclusion that it might be due to the location of the tag on the C-terminus of the channel that may be covered by tissue specific interactions between Nav1.7 and other proteins, essentially masking the tag. Excitingly, however, our immunohistochemical analysis found that Nav1.7 is more widely distributed in the CNS than previously thought. Clear detection of the channel was found in the arcuate nucleus but also in other regions of the brain such as the medial amygdala, medial habenula, anterodorsal thalamic nucleus, laterodorsal thalamic nucleus, throughout the hypothalamus, in the subfornical organ, substantia nigra, reticular part and the red nucleus magnocellular part of the midbrain, and in neurons of the pontine nuclei located in the hindbrain. Recent findings have started to decipher the role of Nav1.7 in the central nervous system. In a study by Branco and colleagues (2016), they reported that Nav1.7 in hypothalamic neurons plays an
important role in body weight control (Branco et al. 2016). They found that synaptic
integration mechanisms in the hypothalamus are dependent on Nav1.7. This selectively
extends excitatory synaptic potentials in several hypothalamic neuronal populations and is
critical for maintaining energy homeostasis and determining weight gain in mice. Further
localisation of Nav1.7 and functional correlations in the brain have yet to be determined.

Despite not having a vast amount of data to discuss regarding the purification and
crystalisation attempt of Nav1.7, I believe it is important to point out a few possible reasons
for the failure of expression of the OPPF assembled Nav1.7 constructs. Considering our
relative success in generating a Tap-Tag Nav1.7 stable cell line in our lab at UCL using the
Nav inserts mentioned in table 3.3, the failure to express these channels using the optimised
OPPF vectors is puzzling. We avoided any codon bias by pairing the human Nav genes with
HEK293 cells, that are also of human origin. And all kozak sequences were also checked and
optimised for expression. I was also informed by the OPPF that the vectors had previously
been used by members of the lab to express human proteins in HEK293. As mentioned in the
result, all clones were sequenced and checked by both myself and members of the OPPF and
we in no way defective. I initially posited that one possible reason for this failure in
expression may arise from the size of the inserts. When combining the length of both the
vector backbone and the sodium channel, the full size of the vector was in excess of 1.3 Kbp.
This is a relatively large plasmid, which can be difficult to transfect using lipofectamine or
electroporation. However, despite there being certain difficulties, members of our lab have, in
the past, successfully transfected sodium channels into HEK293 cells, as demonstrated in
figure 3.1. In hindsight I believe the problem was mostly likely due to the cell culture
conditions after transfection. As highlighted in the cell culture methods of this thesis, as
standard practice following transfection, cells were returned to standard incubator conditions
at 37\(^{\circ}\)C and then left to incubate for 48 hours before assessed for expression by fluorescence. I
believe that due to the large size and complexity of the protein both in its expression and
post translational modifications these conditions might not have been sufficiently optimal to
obtain detectable expression in such a short period of time. To circumvent this problem, a
protocol outlined by Senatore et al., 2011 has suggested a longer incubation of 3-7 days at
28\(^{\circ}\)C, in normal culture medium, can in effect increase the expression of transiently
transfected ion channels. The thermodynamic properties of folding and unfolding reactions of
large globular proteins are known accurately to be a function of temperature where lower
temperatures slow down hydrophobic interactions which can favour correct protein folding
and avoid proteins from aggregating (Baldwin 1986) which would possibly lead to high
expression of Navs and more efficient insertion of the protein into the plasma membrane. Overall, the present findings provide new insights into the interactome of Nav1.7 and advances our understanding of Nav1.7 function. Our data also show that the ss-AP coupled LC-MS/MS is a sensitive, reliable and high-throughput approach for identifying protein-protein interactors of membrane ion channels using epitope-tagged gene targeted mice.

3.6 Further Thoughts and Experiments

In this chapter we first discussed a newly developed transgenic mouse line altered to express endogenous tandem affinity tagged Nav1.7 that was developed by members of the Molecular Nociception Lab at UCL. Using this tool I was able to purify Nav1.7 under physiological conditions and identify a complex of proteins that associate and regulate aspects of the functioning of this sodium channel. This study provides the first comprehensive large-scale analysis of the Nav1.7 protein interactome. I believe this resource of proteins will be a crucial source of information for future study of this sodium channel and the identification of targets for the development of analgesic drugs. Furthermore, this list offers insight into the regulation of sodium channels as a whole, from post-translational modification to trafficking and kinetic regulation. Although much was achieved in this study the tap-tag tool has a vast scope of exciting experiments that could be undertaken in future work. Firstly, considering the importance of Nav1.7 in the generation of various pain states, performing a comparison study of the Nav1.7 interactome in different rodent pain models could provide crucial insight into the causal modification of such disorders. Recent studies conducted by Yang and colleagues (Yang et al. 2017) have shown that a coupling of the fibroblast growth factor (FGF13) to Nav1.7 in noxious heat conditions mediates the conduction of nociceptive signals.

Furthermore, we aim to understand the independently selective roles of that Nav1.7 might be having in different tissues. Evidence of such varied roles within the CNS have been highlighted by Branco et al., where Nav1.7 is shown to be an important synaptic integrator in the hypothalamic neurons that mediate regulate body weight (Branco et al. 2016). Our study shows that this channel is more widely distributed within the central nervous system than previously thought. By identifying Nav1.7 interacting proteins in selective tissues this could lead to further functional insights mediated by this channel. One caveat to using affinity purification to identify interactors is the possibility of false positive proteins, or contaminating proteins to be falsely identified as interactors. Although, careful attention was used when
selecting proteins from the list to be further analysed (see yellow highlighted proteins in table 3.2) it is important to mention that these proteins could also be considered as possible contaminants. By using the Contaminant Repository for Affinity Purification (CRAPOME) I was able to gain some information regarding the probability that these proteins were simply contaminants instead of true Nav1.7 interactors. The CRAPOME is a contaminant repository that contains the lists of proteins identified from negative control experiments collected using affinity purification followed by mass spectrometry. Using this database, I queried the Nav1.7 interactors that I had chosen. The query then searches the control experiment database to determine if it is a possible contaminant by analysing how prevalent this particular protein is in control samples by determining its spectral index. The following table (table 3.4) outlines the results of that query.

<table>
<thead>
<tr>
<th>Gene</th>
<th>N of Experiments</th>
<th>Average SC</th>
<th>% Frequency of Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scn9a</td>
<td>2/411</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Scn3b</td>
<td>0/411</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Syt1</td>
<td>0/411</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Syt2</td>
<td>0/411</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Kif5b</td>
<td>97/411</td>
<td>8.3</td>
<td>65</td>
</tr>
<tr>
<td>Akap12</td>
<td>53/411</td>
<td>6.2</td>
<td>25</td>
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<td>15/411</td>
<td>1.7</td>
<td>2</td>
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<td>Ntm</td>
<td>0/411</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Slc7a5</td>
<td>35/411</td>
<td>3.4</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3.4: Table showing the summary of the CRAPOME analysis of the chosen protein candidates that are highlighted in the generated mass spectrometry list. These proteins are further characterised in the next chapter of this thesis. Column of the table headed ”N of Experiments” refers to the number of control experiments available in the CRAPOME database used in the query. Different types of magnetic beads and tags can be used in the immunoprecipitation. The CRAPOME database takes this into account and when possible I referred only to the the experiments in the database that used a FLAG-tag and M2 magnetic beads.

Conclusions that we can draw from the CRAPOME analysis are that there are a few candidates that have a high spectral index and high % frequency of occurrence including, Kif5b and Akap12. It is therefore appropriate to consider that contamination of these proteins in our mass spectroscopy sample could be due to contamination. Occurrence of 65% and an average spectral index of 8.3 in the case of Kif5b can be considered as particularly high. However, due to the previous connection between this protein and Nav1.7 (outlined in the following chapter) I believe I is still possible to determine that this protein is of particular interest with relation to Nav1.7 In this chapter I also attempted to purify Nav1.7 with the
prospect of crystallising the protein and determining its subsequent structure. Unfortunately, as outlined in this chapter I was unable to express the sodium channel in a compatible cell line. The following is an outline of the subsequent steps I would have undertaken to purify and crystalise the protein. I wish to disclose this information as a future experiment in the hope that it may be of some assistance to any individual attempting to determine the crystal structure of Nav1.7 in the future. Electron Microscopy: Samples, Data Collection and Structure Analysis – One concern with a project such as this is that it is dependent on the ability to produce high resolution crystals and solve their structures, so we also considered alternatives to traditional crystallography. Taking into account of the recent developments in high resolution single particle cryo-EM to examine the structure of large proteins and complexes (Liao et al. 2013), I considered this as an alternative method for structure determination. Major advantages are that electron microscopy does not require crystals and would require samples with concentrations of 0.1-0.3 mg/ml (10-100 fold lower than for crystallography). The full length human Nav1.7 is at the lower end of the size of structures that have been successfully solved using this method, but previous studies on negatively-stained electric eel VGSCs (Charalambous et al. 2011) suggest they are globular and well-defined structures (with generally similar morphology to TRPV1 channels (Liao et al. 2013)) and should be suitable for such studies. Issues to be considered in using this technique are that the protein must be highly pure (likely to be higher stringency than for crystallisation), and that the protein should be in a single conformational state. Once purified using the tandem tags, further purification and preparation of the protein could have preferentially been undertaken using chromatography, which also enables desalting, detergent exchange and identification of monodisperse monomeric species. Further purification might also of required ion exchange chromatography. Additional GFP tags would also further have been added for ease of monitoring purification steps. The protein identity would then be established by mass spectroscopy analysis. No methods beyond these descriptions are provided as our failure to express Nav1.7 in the OPPF cell lines resulted in the termination of our collaboration with the teams and the loss of access to their facilities, therefore, all further mention of any methods are hypothetical approaches to conducting the purification of Nav1.7 with sadly no data. Figure 3.7 provides the negative data of the pulldown and silverstain analysis of Nav1.7 that was part of the preliminary attempt of purifying Nav1.7 (method section 2.0.8.1 provides details on the silver stain and western blot analysis method). I aimed to optimise channel extraction procedures with detergent solubilisation screening.
Medium-scale detergent solubilisations would have used a panel of detergents with different head groups, hydrophobic chain lengths, critical micelle concentrations (CMCs) that I would have tested with the Nav1.7 protein. Detergents would have been tested for both efficiency of extraction and for selectivity of extraction – the former to improve yield, whilst the latter is to improve purification procedures. The solubilised material from each clonal cell line would have been assessed for membrane protein expression via anti-fusion tag (either anti-FLAG or anti-HAT) western blotting.

Large Scale Growth of Nav1.7 Protein– Once a suitable procedure for expression/purification would have been identified, cells would have been grown in medium (approximately 1-3 litre spinner flasks) and/or large (10-liter cellbag bioreactor) suspension cultures. I would have used a serum-free medium formulation designed for the suspension cell cultures (Chaudhary et al. 2012). Pluronic F-68 would have been used in the media as it has been shown to both reduce cell adherence and protect cells against high levels of shear stress introduced by sparging and stirring in the suspension culture (Tharmalingam et al. 2008). This would have permitted us to grow the TAP-tagged NaV1.7-HEK293 cells as a suspension culture. The large-scale growth experiment would have been done using the resources at the OPPF.

Crystallisation– The purified protein would have been screened for detergents suitable for crystallisation, including criteria such as: protein monodispersity, stability (both thermal and temporal), minimisation of micelle size, CMCs, and effects of additives (including cholesterol, amongst others), using CD spectroscopic methods, dynamic light scattering, and thermofluor analyses. It is important to note that detergents most suitable for extraction and purification are often not the most suitable ones for crystallisation. We would have also examined the effects of including different detergent and lipid head group and fatty acid chains and amphiphiles on crystallisability. The protein and protein/ligand complexes used for crystallisation and biophysical characterisations would have included, in addition to native full length protein, designed constructs based initially on bioinformatics examination of disordered regions, electrostatics, and potential stabilising mutations which may be more suitable for crystallisation. It may have ultimately been advantageous to look at complexes of the channel with either ligands or established Nav1.7 macromolecular partners to improve the stability and native form of the protein.

Biophysical Characterisation– The structures of the isolated proteins and the effects of the above noted parameters for crystallisation would have been monitored using several techniques, including:
• 1 Circular dichroism spectroscopy, for integrity of folding in different detergents, the effects of binding ligands (including drugs and toxins) and temporal and thermal stability.

• 2 Fluorescence thermostability monitoring, in conjunction with construct modifications such as alanine scanning, for stability studies.

• 3 Surface plasmon resonance for characterising the binding of drugs/toxins.

• 4 Dynamic light scattering for monodispersity.

• 5 Electrophysiological measurements on isolated proteins (in comparison to in whole cells), to demonstrate integrity of function, effects of lipids and detergents, and ligand binding.

Crystallographic Structure Determination—This can be an arduous process for membrane proteins. Initial crystals often do not diffract to high resolution, so improvements can be achieved by changing the protein (including proteolytic products and designed mutants and constructs), protein concentrations, additives and detergent screens. Improvements in diffraction characteristics can be enabled by a variety of techniques such as dehydration and annealing protocols. Data collection would have been at the synchrotron OPPF Diamond facility and would have been undertaken by experts at the institute. Standard methods for structure determination, phase improvement, model building and refinement would also have been done by experts at the OPPF. To examine complexes with inhibitors and toxins, we would have used both co-crystallisation and soak techniques. Unfortunately, due to the lack of success in expressing the channel, I was not able address the procedure for crystalising and determining the structure of this protein.
Chapter 4

Nav1.7 Interacting Proteins Regulate Channel Activity and Nociception

4.1 Abstract

Functional analysis of the proteomic interaction network that modulates and regulates Nav1.7 is key to understanding the contribution of this channel to the generation of nociceptive signals. Multiple known and previously uncharacterised Nav1.7 interactions were identified by LC-MS coupled with the Tap-Tag Nav1.7 mouse. Bioinformatic analysis of the of Nav1.7 interactors, enabled a functional and component based categorisation that will be useful for future functional analysis of Nav1.7 modulators. This new data revealed that Nav1.7 forms multimeric membrane complexes with $\beta3$ (SCN3B) rather than the $\beta1$ subunit. Furthermore, in this chapter we demonstrate a physical interaction between collapsing-response mediator protein (CRMP2) and Nav1.7, which along with the sodium channel is the target of the analgesic drug lacosamide. We show that lacosamide acts through Nav1.7 in both a direct manner and through CRMP2 to reduce Nav1.7 channel current density. We also found and validated a number of synaptic proteins that interact with Nav1.7 such as Snap25 and synaptotagmin I and II. Moreover, we validated a number of interesting Nav1.7 interactions including L-type amino acid transporter 1 (Lat1), and transmembrane P24 trafficking protein 10 (Tmed10), Neurofascin, AKAP12 and GPRIN1 (a $\mu$-opioid receptor-binding protein), demonstrating a physical and functional link between Nav1.7 and opioid signalling.
4.2 Introduction

Assigning biological functions to proteins is an important task in the post-genomic era. Protein-protein interactions (PPIs) mediate a wide range of biological processes, including intracellular signaling cascades, membrane excitability, protein trafficking and gene regulation. It is estimated that over 90% of all proteins exist as part of multi-protein complexes, composed of adhesion molecules, cytoskeletal adaptors, transport proteins and many others (Okuse et al. 2002; Leterrier et al. 2010). Ion channels are highly dynamic and intrinsically unstable macromolecules, requiring external proteins to ensure kinetic tuning and location. Following the synthesis of Nav1.7, cellular pathways mediate the folding, transport and anchoring of the channel in specialised cellular domains (Hartl et al. 2011). Membrane anchoring and integration along with all modulation of Nav1.7 is mediated by direct and indirect, transient and stable interactions, with a wide number of functional proteins (Salzer 2003; Leterrier et al. 2010; Leterrier et al. 2011). β-subunits are the most studied and understood of the Nav1.7 interactors (Laedermann et al. 2013). However, little functional evidence exists regarding other Nav1.7 interactors. In the last decade, small molecule PPI inhibitors have been used as therapeutic strategies to address a number of diseases and offered new targets to pathways that were previously thought of as “un-druggable” (Andrei et al. 2017). One success story using a protein-protein disruption strategy has arisen where disrupting PPIs have been used to induce apoptosis in cancer cells (Vassilev et al. 2004). Furthermore, PPI disruption has also been employed as a strategy to address pain diseases such as fibromyalgia. The accidental discovery that Gabapentin (first developed as a GABA mimicking compound for the treatment of epilepsy) produced potent analgesia by disrupting the association of VGCC Cav2.2 α and α2δ subunits, thereby decreasing synaptic transmission, has firmly illustrated the potential for using this strategy in developing novel pain therapies (Arkin et al. 2014; Kuenemann et al. 2015). Furthermore, it illustrates how disrupting ion channel PPI can influence channel kinetics and therefore neural conductivity and synaptic transmission (Hendrich et al. 2008; Dolphin 2009; Bauer et al. 2010). Employing this strategy to modulate Nav1.7 could offer novel analgesic solutions to target this nociceptive protein in a novel manner. However, this strategy requires in depth analysis of the functionalities and nature of each PPI. Currently, little evidence is available regarding the functional roles of Nav1.7 interactions. After having identified the interactome that surrounds Nav1.7 (Chapter 3) we now aimed to select interesting candidates and characterise their function towards Nav1.7. However, the question of what a protein does inside a living cell is
not a simple one to answer. *In silico* analysis and predictive studies of proteins can be helpful in categorising and identifying the pathways that might be at play. However, predictive studies alone do not provide sufficient functional validation and further experimental techniques are necessary. Here, we used a number of both *in silico* and experimental methods to validate and explore the relative functions of a selection of the Nav1.7 interactome. First, using the STRING algorithm (outlined in section 4.4.1 of this chapter) we assigned and classified all identified proteins according to function and location. Next, using co-immunoprecipitation, we validated Nav1.7 interactors in sensory neural tissue and using an *in vitro* system we validated the direct physical interaction between a selection of candidate proteins of interest and Nav1.7. Criteria for the selection of the candidates of interest are discussed in the subsequent results section and discussion section of this chapter.

Furthermore, a table summarising the reasons for the selection of these proteins as candidates of interest can be found in section 4.3.2 of this chapter (see table 4.5) However, these included two isoforms of the synaptic neurotransmitter release proteins synaptotagmin (chosen due to prior knowledge of decreased neurotransmitter release in Nav1.7KO mice (Minett et al. 2012b), transporter protein TMED10 (chosen due to its function in protein transport), protein kinase AKAP12 (chosen due to its function as an anchoring protein and could be involved in the anchoring of Nav1.7 at the cell membrane), Kinesin associated protein KIF5B (chosen as it is known to be involved in the forward axonal transport of Nav1.8 (Yuan et al. 2013)) and CRMP2 (chosen as it is a protein known to be the target of the analgesic drug lacosamide (Jo et al. 2017; Wilson et al. 2015a). We also sought to identify a possible interaction with neurotransmitter mediator Cav2.2 through co-immunoprecipitation.

Following the validation of these candidates, we electrophysiologically characterised the role of collapsing response mediator protein 2 on Nav1.7 and further characterised the nature of the analgesic effect of lacosamide on the Nav1.7/CRMP2 interaction.

### 4.3 Results

#### 4.3.1 In Silico Analysis of Nav1.7 Interaction Network

Many bioinformatic tools are available for the *in silico* analysis of protein interaction data obtained from mass spectroscopy. To explore the functional organisation of the Nav1.7 complexes, we used a protein functional analysis from the high-quality STRING database. This data is derived from multiple sources and databases that provide (1) known experimental
interactions (Orchard et al. 2012), (2) pathway knowledge from manually curated databases (Kerrien et al. 2012; Schaefer et al. 2012; Licata et al. 2012; Chatr-Aryamontri et al. 2017) (3) automated text-mining (literature) is used to uncover statistical and/or semantic links between proteins, (4) some associations are predicted de novo by a number of algorithms using genomic information and co-expression analysis (Valencia et al. 2002; Huynen et al. 2003; Lewis et al. 2010) and (5) functionalities and interactions that are observed in one organism are systematically transferred to other organisms via pre-computed orthodoxy relations (for more information on STRING see (Szklarczyk et al. 2015)). Using this algorithm all Nav1.7 interacting proteins were categorised. The first of these tables categorises Nav1.7 interacting proteins according to functionality/protein functional class (see table 4.2). The second of these tables categorises these proteins according to the cellular pathways in which they are known to play a role (see table 4.4). I thought it necessary to include both functionality and localisation of these proteins as these two classifications provide valuable insight into how Nav1.7 may be regulated and where. As the aim of generating this list of proteins is, in part, to provide information for the further development of new drugs targeting regulators of Nav1.7, knowing where these proteins are localised is just as important as the pathways in which they play a role. The candidates of interest that are further validated in this chapter are highlighted in yellow in both subsequent lists.

![Pie chart depicting the distribution of identified Nav1.7 protein interactome by functionality.](image)

**Figure 4.1:** Pie chart depicting the distribution of identified Nav1.7 protein interactome by functionality. Labels correspond to protein function, percentages correspond to fraction of the proteins corresponding to particular function (fraction representing G protein associated proteins was less than 1% and not noted). (All functions correspond to main functionality of the protein, as illustrated in tables 4.3.1 and 4.4 most proteins have multiple functions and belong to multiple pathways.)
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GO.0000166 nucleotide binding $1.67^{\pm 13}$
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GO.0017111 nucleoside-triphosphatase activity $5.77^{\pm 13}$
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GO.0035639 purine ribonucleoside triphosphate binding $1^{\pm 11}$
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GO.0043168 anion binding 1.63e-11
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GO.0032550 purine ribonucleoside binding 2.79e-11
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GO.0097367 carbohydrate derivative binding 2.79e-11
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GO.00032555 purine ribonucleotide binding 4.98e-11
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Chapter 4

GO.1901363 heterocyclic compound binding

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GO.0004298 threonine-type endopeptidase activity

Psma1, Psma4, Psma5, Psma7, Psmb2, Psmb4, Psmb5, Psmb6

GO.0097159 organic cyclic compound binding

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GO.0008092 cytoskeletal protein binding

Ablim1, Bin1, Camasp3, Capz2, Cct5, Clasp2, Dctn2, Des, Det, Flna, Fmr1, Gsn, Homer2, Hsp1, Kif5b, Mapk8ip3, Mtap1a, Mtap2, Mtap6, Myo6, Pascin1, Prnp, Sclal, Sclal, Snap25, Tin1, Tmem2. Ywhag

GO.0032403 protein complex binding

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- **Eef1a1, Eef1a2, Eef1b2, Eef1d, Eef1g, Eef2, Tufm** Atpla1, Atpla3, Atpla1b, Atp5c1, Atp5d, Atp5j, Atp5j2, Atp5k, Atp5p, Atp6v1g2, Dync1h1, Hsp90aa1, Hspap5, Kif5b, Nsf, Psmc2, Psmc3, Psmc4, Psmc5, Vep
- **Aco2, Aqp4, Aldoa, Ampd2, Arsgl1, Atpla1, Atpla3, Atpla1b, Atp2b1, Atp5c1, Atp5d, Atp5j, Atp5j2, Atp5k, Atp5p, Atp6v1g2, Dynl2, Hsp90aa1, Hspap5, Kif5b, Nsf, Psmc2, Psmc3, Psmc4, Psmc5, Vep
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GO.0022892 substrate-specific transporter activity 5.32e-06
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GO.0043167 ion binding 5.32e-06
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GO.0008324 cation transmembrane transporter activity 1.13e-05
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Table 4.2: List of all protein names identified by LC-Mass Spectrometry from immunoprecipitation experiments of Tap-tag Nav1.7 with a KI/WT ratio of larger than 1.2. All proteins were classified according to roles and contributions to cellular pathways. A large majority of the uncovered proteins contribute to multiple cellular pathways. The Gene ontology (GO) and the functional enrichment was performed using DAVID and KEGG pathway analysis. Lists were constructed using STRING application with minimum required interaction score of 0.7 (high confidence). The false discovery rate (FDR) is the rate of type I errors in null hypothesis testing when conducting multiple comparisons.
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Chapter 4

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Syt1, Tnl1, Tnl2, Tmocl2, Uchl1, Vim, Ywhae

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Chapter 4

GO.0043226 organelle 7.54\(^{-20}\)

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GO.0030054 cell junction 1.7\(^{-19}\)

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GO.0044456 synapse 8.25\(^{-19}\)

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GO.0000502 proteasome complex $2.2^{e-15}$
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- Acly, Aco2, Atp6a1, Atp6c1, Atp5d, Bcap31, Cct7, Ckn1, Ctc, Cnp, Cox4i1, Coxa5, Cyc1, Dls, Dps1, Echs1, Fasn, Gpd1, Glul, Got2, Gpx4, Hnrnpk, Hsp90a1, Hsp90ab1, Hspa5, Hspa9, Immt, Ldha, Ldhb, Mec2, Mdh1, Mdh2, Mtfrd1, Ndufa10, Ndufa7, Ndufa9, Ndufb10, Ndufb7, Ndufs7, Ndufs8, Ndufv3, Nehf, Oxtc1, Pebb, Pebp1, Pih2, Pkm, Ppplcc, Prdx1, Prdx2, Prnp, Sfn3, Sclaa3, Scl2a3, Scl2a4, Sxbp1, Tuflm, Uprox2, Umsg5, Vdac1, Vdac3, Ywhe, Ywhe, Ywhe, Ywhe, mt-Co2

GO.0005739 mitochondrion $3.23^{e-15}$
- Acly, Aco2, Atp5a1, Atp5c1, Atp5d, Bcap31, Cct7, Ckn1, Ctc, Cnp, Cox4i1, Coxa5, Cyc1, Dls, Dps1, Echs1, Fasn, Glul, Got2, Gpx4, Hnrnpk, Hsp90a1, Hsp90ab1, Hspa5, Hspa9, Immt, Ldha, Ldhb, Mec2, Mdh1, Mdh2, Mtfrd1, Ndufa10, Ndufa7, Ndufa9, Ndufb10, Ndufb7, Ndufs7, Ndufs8, Ndufv3, Nehf, Oxtc1, Pebb, Pebp1, Phb2, Pkm, Ppplcc, Prdx1, Prdx2, Prnp, Sfn3, Sclaa3, Scl2a3, Scl2a4, Sxbp1, Tuflm, Uprox2, Umsg5, Vdac1, Vdac3, Ywhe, Ywhe, Ywhe, Ywhe, mt-Co2

GO.0030424 axon $6.98^{e-15}$
- Ank3, Ap3d1, Bin1, Calb2, Dst, Ebp4, Glul, Hnrnpk, Homer1, Igsf8, Klcl1, Mapkip3, Myo6, Napa, Nehf, Nefm, Omp, Pascin1, Pebp1, Pph, Ruy3, Sep7, Scl17a7, Sclaa2, Scl2a1, Snap25, Sxbp1, Sy2a, Uch1, Ywhe
- Bin1, Camsp3, Cct2, Cct3, Cct4, Cct5, Cct6a, Cct7, Cct8, Clasp2, Cnp, Dtn1, Dtn2, Dnm1, Dps1, Dst, Dynch1, Dynll2, Ki55b, Kl5c, Klcl1, Mtap1a, Mtap2, Mtap6, Tp1, Tub4a, Tub2b, Tub5b, Tub6b, Anxa2, Atp6a1, Atp6v1g2, Cct4, Ctc, Cnp, Fasn, Flot1, Hsp90a1, Hsp90ab1, Hspa5, Prdx1, Rab1, Sec22b, Sclaa1, Ywhe, Ywhe, Ywhe, Ywhe

GO.0005874 microtubule $5.2^{e-14}$
- Actr2, Akap12, Arpc2, Calr, Camsp3, Cap1, Cdh13, Cdh2, Cfl1, Ctc, Ctt, Ddr2, Des, Dsp, Dst, Flna, Flot1, Gsp, Hnrnpk, Hspa5, Hspa9, Marcks, Ppia, Ppplce, Tn1, Tn2, Vim, Ywhe, Ywhe, Ywhe, Ywhe

GO.0005912 adherens junction $1.98^{e-13}$
- Actr2, Akap12, Arpc2, Calr, Camsp3, Cap1, Cdh13, Cdh2, Cfl1, Ctc, Ctt, Ddr2, Des, Dsp, Dst, Flna, Flot1, Gsp, Hnrnpk, Hspa5, Hspa9, Marcks, Ppia, Ppplce, Tn1, Tn2, Vim, Ywhe, Ywhe, Ywhe, Ywhe, Ywhe

GO.0005832 chaperonin-containing T-complex $2.33^{e-13}$
- Cct2, Cct3, Cct4, Cct5, Cct6a, Cct7, Cct8, Tp1

GO.0005740 mitochondrial envelope $2.86^{e-13}$
- Atp5a1, Atp5c1, Atp5d, Ckn1, Ctc, Cnp, Cox4i1, Coxa5, Cyc1, Gpd1, Got2, Gpx4, Hadha, Immt, Mdh2, Ndufa10, Ndufa7, Ndufa9, Ndufb10, Ndufb7, Ndufs7, Ndufs8, Ndufv3, Pebp1, Phb2, Prnp, Sfn3, Sclaa3, Scl2a3, Scl2a4, Tuflm, Uprox2, Umsg5, Vdac1, Vdac3, mt-Co2

GO.0005838 proteasome regulatory particle $3.42^{e-13}$
- Psmc2, Psmc3, Psmc4, Psmc5, Psmc11, Psmd12, Psmd13, Psmd2, Psmd3, Psmd4

GO.0005925 focal adhesion $7.21^{e-13}$
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GO.0015630 microtubule cytoskeleton $1.07^{e-12}$
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Chapter 4

GO.0005875 microtubule associated complex 3.4e-07 Actr1a, Actr1b, Dctn1, Dctn4, Dynl2, Kif5b.
GO.0005747 mitochondrial respiratory chain complex I 6.59e-07 Actr1a, Actr1b, Dynl2, Kif5c, Mtap2, Ywhae, Ndufa8, Ndufa9, Ndufa10, Nduf5, Nduf7, Nduf8, Nduf9, Nduf10, Nduf11, Nduf12, Nduf13, Nduf15, Nduf16, Nduf21, Nduf22.
GO.0042383 sarcolemma 9.09e-07 Ank2, Ank3, Anxa2, Atpl1a1, Atpl1a3, Atpl1b1, Bin1, Des, Dst, Flot1, Slc8a1.
GO.0008540 proteasome regulatory particle, base subcomplex 1.33e-06 Psmc2, Psmc3, Psmc4, Psmc5, Psmd4.
GO.0031595 nuclear proteasome complex 5.96e-05 Ank2, Ank3, Anxa2, Atp1a1, Atp1a3, Atp1b1, Bin1, Des, Dst, Flot1, Slc8a1.
GO.0005815 microtubule organizing center 3e-05 Actr1a, Actr1b, Cct4, Cct5, Cct8, Cnl, Dynl1, Dynl2, Eif4a, Flot1, Kif5a, Marcks, Mdh1, Psm1, Psm5, Rp2h, Tcp1, Tnn.
GO.0006455 growth cone 3.11e-05 Basp1, Calm1, Dctn2, Dpysl2, Dpysl3, Gprin1, Klc1, Mab, Snap25, Tmod2.
GO.0008021 synaptic vesicle 5.96e-05 Amph, Atp6v1g2, Dmxl2, Slc17a7, Slc32a1, Sv2a, Syt1, Syt2, Wdr7.
GO.0008539 proteasome core complex, alpha-subunit complex 2.7e-05 Amph, Atp6v1g2, Dmxl2, Pebp1, Ppfia3, Slc17a7, Slc32a1, Sv2a, Syt1, Syt2, Wdr7.
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GO.0044433 synaptic vesicle membrane 0.000376 Amph, Atp6v1g2, Dmxl2, Pebp1, Ppfia3, Slc17a7, Slc32a1, Sv2a, Syt1, Syt2, Wdr7.
GO.0005753 mitochondrial proton transporting ATP synthase complex 7.3e-05 Psmb2, Psmb4, Psmb5, Psmb6.
GO.0005839 proteasome core complex, alpha-subunit complex 0.000238 Actr1a, Actr1b, Cct4, Cct5, Cct8, Cnl, Dynl1, Dynl2, Eif4a, Flot1, Kif5a, Marcks, Mdh1, Psm1, Psm5, Rp2h, Tcp1, Tnn.
GO.0070044 synaptobrevin 2-SNAP-25-synaptin-1a complex 0.000251 Amph, Atp6v1g2, Dmxl2, Pebp1, Ppfia3, Slc17a7, Slc32a1, Sv2a, Syt1, Syt2, Wdr7.
GO.0005813 centrosome 0.000238 Actr1a, Actr1b, Cct4, Cct5, Cct8, Cnl, Dynl1, Dynl2, Eif4a, Flot1, Kif5a, Marcks, Mdh1, Psm1, Psm5, Rp2h, Tcp1, Tnn.
GO.0005577 fibrinogen complex 0.000476 Fga, Fgb, Fgg.
GO.0005913 cell-cell adherens junction 0.000516 Cama2, Cdh2, Des, Dsp, Flot1, Tnn2, Anxa2, Aqp4, Calr, Cdh13, F3, Fga, Fgb, Fgg.
GO.0009986 cell surface 0.000522 Cama2, Cdh2, Des, Dsp, Flot1, Tnn2, Anxa2, Aqp4, Calr, Cdh13, F3, Fga, Fgb, Fgg, Got2, Hsp90ab1, Hsp90aa, Lsamp, Nlg2, Ntm.
GO.0031253 cell projection membrane 0.000555 Amph, Atp6v1g2, Dmxl2, Pebp1, Ppfia3, Slc17a7, Slc32a1, Sv2a, Syt1, Syt2, Wdr7.
GO.0030425 dendrite 0.000654 Amph, Atp6v1g2, Dmxl2, Pebp1, Ppfia3, Slc17a7, Slc32a1, Sv2a, Syt1, Syt2, Wdr7.
GO.0030672 synaptic vesicle membrane 0.000688 Amph, Atp6v1g2, Dmxl2, Pebp1, Ppfia3, Slc17a7, Slc32a1, Sv2a, Syt1, Syt2, Wdr7.
GO.0005882 intermediate filament 0.000809 Amph, Atp6v1g2, Dmxl2, Pebp1, Ppfia3, Slc17a7, Slc32a1, Sv2a, Syt1, Syt2, Wdr7.
GO.0045177 apical part of cell 0.000919 Amph, Atp6v1g2, Dmxl2, Pebp1, Ppfia3, Slc17a7, Slc32a1, Sv2a, Syt1, Syt2, Wdr7.

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**Chapter 4**

Alexandros Kanellopoulos
Table 4.4: List of all protein names identified by LC-Mass Spectrometry from immunoprecipitation experiments of Tap-tag Nav1.7 with a KI/WT ratio of larger than 1.2. All proteins were classified according to cellular component. A large majority of the uncovered proteins contribute to multiple cellular pathways. The Gene ontology (GO) and the functional enrichment was performed using DAVID and KEGG pathway analysis. Lists were constructed using STRING application with minimum required interaction score of 0.7 (high confidence). The false discovery rate (FDR) is the rate of type I errors in null hypothesis testing when conducting multiple comparisons.
Co-immunoprecipitation enables the direct physical validation of interactions between proteins in experimental conditions. Using LC-Mass spectroscopy analysis, the physical interactions between Nav1.7 and carefully selected protein candidates were assessed using both *in vivo* and *in vitro* co-immunoprecipitation methods and western blot analysis. The criteria for the selection of these proteins was based on the possibility of these proteins contributing to the function of Nav1.7 in the conduction of pain signalling. The validation of direct physical interactions between identified proteins and Nav1.7 brought validity to our Tap-tag/LC-MS method. A number of candidates of interest were chosen on the basis of prior knowledge of their function and in some cases prior evidence involving mechanisms and cellular processes associated with Nav1.7, sodium channels and pain. Other newly identified partners were also chosen based on functionality (please see table 4.5 that outlines the reasons for choosing these proteins for further validation).

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<th>Gene name</th>
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<td>SCN9A</td>
<td>275kDa</td>
<td>Voltage-gated sodium channel Nav1.7 – protein of interest of this assay</td>
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<tr>
<td>SCN3B</td>
<td>32kDa</td>
<td>Voltage-gated sodium channel subunit that is known to commonly associate with Nav1.7 at the cell membrane and modulates the kinetic activity of the sodium channel (Laedermann et al. 2013).</td>
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<tr>
<td>SYT1</td>
<td>44kDa</td>
<td>Synaptic protein that associates with the SNARE complex and is a key determinant of neurotransmitter release. SYT1 has been previously shown to colocalise with a number of other synaptic proteins such as synaptophysin (Black et al. 2012). Furthermore, in Nav1.7 knockout studies, substance P release into the dorsal horn of the spinal cord is completely abolished, leading to a loss of wind-up and centrally mediated pain sensitization</td>
</tr>
<tr>
<td>SYT2</td>
<td>44kDa</td>
<td>Synaptic protein that associates with the SNARE complex and is a key determinant of neurotransmitter release. SYT2 has been shown to directly interact with Nav1.2 (Sampo et al. 2000). Nav1.7 has been previously shown to colocalise with a number of other synaptic proteins such as synaptophysin (Black et al. 2012). Furthermore, in Nav1.7 knockout studies, substance P release into the dorsal horn of the spinal cord is completely abolished, leading to a loss of wind-up and centrally mediated pain sensitization</td>
</tr>
<tr>
<td>Lat1</td>
<td>57kDa</td>
<td>Amino acid transporter, though to possibly mediate the transport of the pain drug GABA</td>
</tr>
<tr>
<td>Tmed10</td>
<td>21kDa</td>
<td>Involved in the transport of correctly synthesised/folded proteins from the ER to the Golgi (Kaiser 2000) and is known to be involved in the trafficking of other membrane proteins such as amyloid-β (Vetrivel et al. 2007).</td>
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</table>
Gprin1 110kDa

Gprin1 has previously been shown to tether opioid receptors with the G-protein alpha subunit and subsequently regulate receptor distribution within lipid rafts (Ge et al. 2013).

CRMP2 70kDa

A direct relationship between CRMP2 and Nav1.7 has previously been established (Dustrude et al. 2013). A study conducted by Dustrude et al., (2013) illustrates how CRMP2 trafficking of Nav1.7 is controlled by a SUMOylation site within CRMP2. The binding of CRMP2 and Nav1.7 is enhanced by conjugation of CRMP2 with a small ubiquitin-like modifier and further controlled by the phosphorylation of CRMP2.

AKAP12 191kDa

Protein known to bind to the regulatory subunit of PKA and PKC and has been shown to interact with VGSCs in rat brain preparations. Furthermore, in hippocampal neurons, the PKA-dependent dopamine D1 receptor mediated decrease of sodium current can be abolished by disrupting PKA association with AKAP15 using competing peptides such as HT31 (Tibbs et al. 1998; Westenbroek et al. 1999).

Nfasc 138kDa

Important in the clustering and anchoring of voltage gated sodium channels at the nodes of Ranvier (Komada et al. 2002)

Ntm 38kDa

Neurotrimin is an immunoglobulin domain-containing glycosylphosphatidylinositol (GPI)-anchored cell adhesion molecule and Ntm is closely linked to a related family member, opioid binding protein/cell adhesion molecule-like (OPCML), on chromosome 11 (Struyk et al. 1995).

Kif5b 110kDa

This protein is a microtubule-dependent motor kinesin responsible for anterograde axonal transport and is abundantly expressed in dorsal root ganglia and has previously been shown to promote the transport of Nav1.8 to the plasma membrane (Su et al. 2013)

Ank3 243kDa

Important in the clustering and anchoring of voltage gated sodium channels at the nodes of Ranvier (Zhou et al. 1998)

Pebp1 23kDa

PEBP regulates GPCR signalling through both the Raf/MEK/ERK pathway and G protein-coupled receptor kinase 2 (GRK2), a major feedback inhibition of GPCRs which has been implicated in a number of neurological disorders including chronic pain (Sedivy 2011).

Table 4.5: Table summarising the main reasons for having chosen these protein candidates for further validation with Nav1.7. This table contains a summary and definition of the function of each candidate either towards Nav1.7 or involvement in pain signalling. These proteins are further discussed in the discussion section of this thesis.

The physical interactions between Nav1.7 and interacting protein candidates were assessed using co-IP with the DRG tissues from TAP-tagged Nav1.7 mice. A number of candidates of interest, such as Scn3b, Syt2, Lat1, Tmed10, Gprin1, CRMP2, Isoform 2 of A-kinase anchor protein 12 (Akap12), Neurofascin (Nfasc), Neurotrimin (Ntm), Kinesin-1 heavy chain (Kif5b), Ankyrin-3 (Ank3) and Phosphatidylethanolamine-binding protein 1 (Pebp1) were chosen from the Nav1.7 associated protein list previously identified by MS. After single step affinity
purification (ss-AP), the Nav1.7 complexes were separated in the SDS-PAGE gel and the protein interactors of Nav1.7 were detected by Western blotting. Our results show that all 12 candidates were detected by highly specific antibodies (see figure 4.2a). In an effort to further validate certain candidates to a human Nav1.7, we also validated 6 candidates (Scn3b, Syt2, CRMP2, Gprin1, Lat1 and Tmed10) using co-IP in an in vitro system by co-expressing the candidates in a TAP-tagged Nav1.7 stable cell line (see figure 4.2(b)). The mammalian expression vectors carrying cDNAs of these candidates were transfected into a TAP-tagged Nav1.7 stable HEK293 cell line. 48 hours post-transfection, the proteins in the transfected cells were extracted. The TAP-tagged Nav1.7 multiprotein complexes were then immunoprecipitated with anti-FLAG antibody and analysed with western blot using different specific antibodies against those selected candidates. The western blot showed that all these 6 candidates were detected with the expected sizes on the blot (see figure 4.2), confirming that these candidates are in a complex with Nav1.7. In summary, all these potential interacting proteins selected for further validation were confirmed as interactors by co-IP, giving confidence in the Nav1.7TAP mass spectrometry list. However, for the purpose of validating these proteins as interactors I used the immunoprecipitation of Nav1.7 as the bait to immunoprecipitate other proteins. It is important to state that for further, more robust, validation I could have attempted the reverse co-immunoprecipitation using the protein of interest as the bait for the co-IP and testing whether I could immunoprecipitate Nav1.7. The way that I would have produced this reverse Co-IP is by simply cloning a different tag to the ones used in the Tap-Tag Nav1.7 (i.e. not HAT or FLAG) onto the end of each candidate protein of interest and performed an identical immunoprecipitation to that used for Tap-Tag Nav1.7. This would have provided further validation to the interaction of Nav1.7 with these proteins. Furthermore, it is important to state that CO-IP is not the only method for validating protein-protein interactions. In the Further Experiments section of this chapter, I have provided a summary table (taken from Rao et al., 2014) of all the currently available methods for identifying, validating and characterising protein-protein interactions, all these methods can also be considered as future methods for validating Nav1.7-protein interactions that were performed in this thesis (see table 4.6).
Selected Nav1.7 protein-interactor candidates were validated using Nav1.7 endogenous expressing DRG tissue. Tissues were extracted from DRG and lysed in 1% CHAPS lysis buffer as outlined in the previous chapter. Following this step, Nav1.7 complexes were immunoprecipitated using anti-FLAG M2 Magnetic beads. Thirteen Nav1.7 interactor candidates which included Scn3b (32 kDa), Syt2 (44 kDa), Syt1 (44kDa), CRMP2 (70 kDa), Gprin1 (110 kDa), Lat1 (57 kDa), Tmed10 (21 kDa), Akap12 (191 kDa), Nfasc (138 kDa), Ntm (38 kDa), Kif5b (110 kDa), Ank3 (243 kDa) and Pebp1 (23 kDa) were chosen and detected using specific targeted antibodies to these proteins in a western blotting assay. The interaction between TAP-tagged Nav1.7 and protein interactors including Scn3b, Syt2, CRMP2, Gprin1, Lat1 and Tmed10 were validated using the standard co-immunoprecipitation in vitro system. Expression vectors containing the genes of the chosen interacting proteins were transfected into a HEK293 cell line that stably expressed TAP-tagged Nav1.7. Following transfection, TAP-tagged Nav1.7 complexes were immunoprecipitated with anti-FLAG antibody and the candidates were subsequently detected using their specific antibody (via western blot). The results indicated the correct expected sizes of Scn3b (32 kDa), Syt2 (44 kDa), Syt1 (44kDa), CRMP2 (70 kDa), Gprin1 (110 kDa), Lat1 (57 kDa), Tmed10 (21 kDa), Akap12 (191 kDa), Nfasc (138 kDa), Ntm (38 kDa), Kif5b (110 kDa), Ank3 (243 kDa) and Pebp1 (23 kDa) were chosen and detected using specific targeted antibodies to these proteins in a western blotting assay. (b) The interaction between TAP-tagged Nav1.7 and protein interactors including Scn3b, Syt2, CRMP2, Gprin1, Lat1 and Tmed10 were validated using the standard co-immunoprecipitation in vitro system. Expression vectors containing the genes of the chosen interacting proteins were transfected into a HEK293 cell line that stably expressed TAP-tagged Nav1.7. Following transfection, TAP-tagged Nav1.7 complexes were immunoprecipitated with anti-FLAG antibody and the candidates were subsequently detected using their specific antibody (via western blot). The results indicated the correct expected sizes of Scn3b (32 kDa), Syt2 (44 kDa), Syt1 (44kDa), CRMP2 (70 kDa), Gprin1 (110 kDa), Lat1 (57 kDa), Tmed10 (21 kDa), Akap12 (191 kDa), Nfasc (138 kDa), Ntm (38 kDa), Kif5b (110 kDa), Ank3 (243 kDa) and Pebp1 (23 kDa) were chosen and detected using specific targeted antibodies to these proteins in a western blotting assay. (c) The interaction between TAP-tagged Nav1.7 and protein interactors including Scn3b, Syt2, CRMP2, Gprin1, Lat1 and Tmed10 were validated using the standard co-immunoprecipitation in vitro system. Expression vectors containing the genes of the chosen interacting proteins were transfected into a HEK293 cell line that stably expressed TAP-tagged Nav1.7. Following transfection, TAP-tagged Nav1.7 complexes were immunoprecipitated with anti-FLAG antibody and the candidates were subsequently detected using their specific antibody (via western blot). The results indicated the correct expected sizes of Scn3b (32 kDa), Syt2 (44 kDa), Syt1 (44kDa), CRMP2 (70 kDa), Gprin1 (110 kDa), Lat1 (57 kDa), Tmed10 (21 kDa), Akap12 (191 kDa), Nfasc (138 kDa), Ntm (38 kDa), Kif5b (110 kDa), Ank3 (243 kDa) and Pebp1 (23 kDa) were chosen and detected using specific targeted antibodies to these proteins in a western blotting assay. (b) The interaction between TAP-tagged Nav1.7 and protein interactors including Scn3b, Syt2, CRMP2, Gprin1, Lat1 and Tmed10 were validated using the standard co-immunoprecipitation in vitro system. Expression vectors containing the genes of the chosen interacting proteins were transfected into a HEK293 cell line that stably expressed TAP-tagged Nav1.7. Following transfection, TAP-tagged Nav1.7 complexes were immunoprecipitated with anti-FLAG antibody and the candidates were subsequently detected using their specific antibody (via western blot). The results indicated the correct expected sizes of Scn3b (32 kDa), Syt2 (44 kDa), Syt1 (44kDa), CRMP2 (70 kDa), Gprin1 (110 kDa), Lat1 (57 kDa), Tmed10 (21 kDa), Akap12 (191 kDa), Nfasc (138 kDa), Ntm (38 kDa), Kif5b (110 kDa), Ank3 (243 kDa) and Pebp1 (23 kDa) were chosen and detected using specific targeted antibodies to these proteins in a western blotting assay.

Figure 4.2: (a) Selected Nav1.7 protein-interactor candidates were validated using Nav1.7 endogenous expressing DRG tissue. Tissues were extracted from DRG and lysed in 1% CHAPS lysis buffer as outlined in the previous chapter. Following this step, Nav1.7 complexes were immunoprecipitated using anti-FLAG M2 Magnetic beads. Thirteen Nav1.7 interactor candidates which included Scn3b (32 kDa), Syt2 (44 kDa), Syt1 (44kDa), CRMP2 (70 kDa), Gprin1 (110 kDa), Lat1 (57 kDa), Tmed10 (21 kDa), Akap12 (191 kDa), Nfasc (138 kDa), Ntm (38 kDa), Kif5b (110 kDa), Ank3 (243 kDa) and Pebp1 (23 kDa) were chosen and detected using specific targeted antibodies to these proteins in a western blotting assay. (b) The interaction between TAP-tagged Nav1.7 and protein interactors including Scn3b, Syt2, CRMP2, Gprin1, Lat1 and Tmed10 were validated using the standard co-immunoprecipitation in vitro system. Expression vectors containing the genes of the chosen interacting proteins were transfected into a HEK293 cell line that stably expressed TAP-tagged Nav1.7. Following transfection, TAP-tagged Nav1.7 complexes were immunoprecipitated with anti-FLAG antibody and the candidates were subsequently detected using their specific antibody (via western blot). The results indicated the correct expected sizes of Scn3b (32 kDa), Syt2 (44 kDa), Syt1 (44kDa), CRMP2 (70 kDa), Gprin1 (110 kDa), Lat1 (57 kDa), Tmed10 (21 kDa), Akap12 (191 kDa), Nfasc (138 kDa), Ntm (38 kDa), Kif5b (110 kDa), Ank3 (243 kDa) and Pebp1 (23 kDa) were chosen and detected using specific targeted antibodies to these proteins in a western blotting assay. (c) This figure shows the co-immunoprecipitation of Nav1.7 with voltage gated calcium channel Cav2.2. Left panel shows a negative western blot result from a pull-down from cells transiently transfected with HA-tagged CaV2.2 from TAP-tagged Nav1.7 complex (HAT antibody for detection) (performed in TAP-tagged Nav1.7 HEK293 stable cell line). Centre panel shows the negative control western blot results for the pull-down of Cav2.2 by Tap-tag Nav1.7 in a transgenic Cav2.2-HA/Nav1.7-Tap mouse dorsal root ganglia tissue. The right panel shows a control western blot from whole cell lysate of HA-tagged CaV2.2 and TAP-tagged Nav1.7. An adapted version of this figure can be found in Kanellopoulos et al., 2018.
4.4 Functional Characterisation of Collapsin Response Mediator Protein 2

The electrophysiological nature of ion channels offers an added advantage when assessing the functional nature of a protein-protein interaction as in many instances modification to the channel by PPIs has dramatic effects on channel kinetics or current density. Both these changes can be studied in high resolution using patch clamp techniques (Hamill et al. 1981; Calhoun et al. 2014). Collapsin Response Mediator Protein 2 has previously been implicated in the trafficking of, ligand and voltage-gated, calcium and sodium channels (Chi et al. 2009; Brittain et al. 2009; Brittain et al. 2011; Bertin et al. 2016; Dustrude et al. 2013). However, a direct interaction between this protein and voltage-gated ion channels has not been previously demonstrated. In addition to these suggested protein targets, CRMP2 is the presumed target of the anti-epileptic drug Lacosamide (Wilson et al. 2015b). We sought to evaluate the possible electrophysiological effects of CRMP2 on Tap-tag Nav1.7 and in addition understand the relevance of CRMP2 to the action of lacosamide on Nav1.7 current density. A human CRMP2 construct in which a reporter GFP reporter gene was inserted, was transfected into our Tap-tag Nav1.7 expressing stable HEK293 cell line. Electrophysiological assessment, 48 hours post-transfection, of the presence of CRMP2 on Nav1.7 was assessed. The transfection of CRMP2 into the human Tap-tag Nav1.7 stable cell line revealed a nearly two-fold increase in sodium current density compared to untransfected, complying with previous evidence suggesting that CRMP2 acts as a trafficking partner for Nav1.7 (Dustrude et al. 2013). Next, we sought to investigate the effect of Lacosamide on sodium currents. Due to the proposed mode of action of Lacosamide, in all experiments cells were chronically incubated with 100µM lacosamide (Jo et al. 2017) for a minimum of 5 hours before recordings. Un-transfected Nav1.7 currents displayed a slight decrease in current density following chronic exposure to Lacosamide. Interestingly, following incubation with Lacosamide in CRMP2 transfected cells, a large decrease was observed where the previous increase caused by the transfection of CRMP2 was reversed (see figure 4.3).
Nav1.7

Nav1.7 + 100µM LCM

Nav1.7 + CRMP2

Nav1.7 + CRMP2 + 100µM LCM

Tap-tag Nav1.7 Current

Voltage (mV)

I (pA/pF)

Tap-tag Nav1.7

+100µM LCM

Control HEK

Tap-tag Nav1.7 Current

Voltage (mV)

I (pA/pF)

Tap-tag Nav1.7

+100µM LCM

Control HEK
Figure 4.3: Electrophysiological characterisation of Nav1.7 in HEK293 cells following transfection with CRMP2 and incubation with Lacosamide (LCM). Representative raw current traces of human Nav1.7 stably expressed in HEK293 cells in response to the activation pulse protocol shown. Each trace shows a different condition: (a) Is the raw Tap-tag Nav1.7 trace from stable cell line. (b) is Tap-tag Nav1.7 with the addition of 100µm Lacosamide. (c) represents Nav1.7 current in cell co-transfected with CRMP2. (d) demonstrates the typical raw current trace of Nav1.7 following the transfection of CRMP2 and following incubation with 100µm Lacosamide. (e) IV plot of Tap-tag Nav1.7 current density in the absence and presence of 100µm Lacosamide. Compared with Tap-tag Nav1.7 basal currents, Lacosamide incubation had no significant effect on sodium channel density (n = 12). (f) IV plot of Tap-tag Nav1.7 in HEK293 cells in the presence and absence of CRMP2 transfection. Compared to Tap-tag Nav1.7 basal currents, CRMP2 transfection caused a significant increase in Tap-tag Nav1.7 current density (n = 16, p<0.05) (one-way ANOVA with Bartlett’s post hoc test). (g) IV plot showing current density of Tap-tag Nav1.7 following transfection of CRMP2 and incubation with 100µm Lacosamide (n = 10). Incubation with Lacosamide reversed the CRMP2-mediated current increase (n = 9, p>0.05) (one-way ANOVA with Bartlett’s post hoc test).

4.5 Discussion

Protein-protein interactions are functionally complex and mediate every aspect of Nav1.7. Here, we sought to begin to understand the nature of these interactions by using the data we obtained through our LC-MS experiment. We used bioinformatic tools to organise and categorise Nav1.7 protein interactors into groups by highlighting their function and cellular component. Following this we chose to validate a number of interesting proteins that potentially mediate crucial components involved in the function of Nav1.7 and could potentially be new targets for therapeutic development. Finally, through electrophysiological analysis, we found that CRMP2 is a positive mediator of Nav1.7 current density and is most probably one of the targets of the analgesic drug Lacosamide. Protein-protein interactions occur with varying degrees of affinity and specificity. Validating these interactions is complicated by the fact that proteins themselves are chemically distinct entities with charges, different secondary and tertiary structures and have a variety of post-translational modifications. Therefore, many parameters may influence their interactions including protein concentration, counter ion type, ionic strength, environmental pH, dissociation constants and oligomeric states (Phizicky et al. 1995; Nooren et al. 2003; Om et al. 2004; Marchesseau et al.)
This also means that the experimental method of validation as well as the environment used to discern interactions will influence detection. The use of the Tap-tag enabled the indiscriminate high throughput detection of proteins that interact with Nav1.7. Furthermore, in our study we took certain precautions in order to ensure detection of genuine interactions including the use of flash frozen primary sensory neuronal tissue in our co-IP experiments in order to conserve the initial native environment of the proteins avoiding nonspecific aggregation of randomly denaturated proteins (Phizicky et al. 1995) and our choice of the 1% CHAPS detergent providing a relatively soft environment, favouring the detection of even weakly interacting partners. The success of this method also provided us with a way of validating certain Nav1.7 interactors further, using co-IP coupled with western blot for detection. We successfully validated the interactions between a handful of interesting interactors including two isoforms of the synaptic neurotransmitter release proteins synaptotagmin (I&II), transporter protein TMED10, protein kinase AKAP12, Kinesin associate protein KIF5B and CRMP2. The validation of β3 provided confirmation of the efficacy of the epitope tag as a tool for the study of proteomic interactions. The β3 subunit is a small ~ 40kDa transmembrane protein that links non-covalently to Nav1.7 and is a key regulator of channel functional activity (Laedermann et al. 2013). β3 assembles into trimers in the plasma membrane and is known to facilitate interactions with various signalling molecules and other ion channels (Shah et al. 2000; Shah et al. 2001). β3 and β1 also enhance glycosylation of Nav1.7 through an anticipated interaction in the Golgi apparatus compared to β2 and β4 which associate post-maturation at the cell membrane (Laedermann et al. 2013). This association affects channel kinetics of voltage gated sodium channels in general, confirming that Nav1.7 interacts with β3 in the plasma membrane. Association with β3 increases channel current density, shifts the V1/2 of steady-state inactivation toward depolarised potentials and shifts the V1/2 of activation toward more hyperpolarised potentials compared to all other known β-subunits (Laedermann et al. 2013), allowing for a slow repriming and endowing C-fibres with characteristic low frequency firing. The phosphorylation of Nav1.7 is also an important channel modulator and previous sites of phosphorylation have been identified on multiple voltage-gated sodium channels (Gershon et al. 1992; Cantrell et al. 1997; Zhou et al. 2000; Vijayaragavan et al. 2004). The A-kinase anchor protein 12 (AKAP12) is part of a structurally diverse group of proteins that have the common function of binding to the regulatory subunit of PKA and PKC to confer its location and function within the cell. Previously, AKAP15 has been shown to associate with voltage-gated sodium channels in purified rat brain preparations (Tibbs et al. 1998). Furthermore, in hippocampal
neurons, the PKA-dependent dopamine D1 receptor mediated decrease of sodium current can be abolished by disrupting PKA association with AKAP15 using competing peptides such as HT31 (Tibbs et al. 1998; Cantrell et al. 1999). It’s validation as a Nav1.7 interactor suggests this isoform of AKAP provides the membrane anchoring and regulation of Nav1.7 phosphorylation, which could be used for future manipulation of Nav1.7 phosphorylation.

The transport of correctly synthesised/folded proteins is an essential part of the intracellular secretory pathway. Transmembrane emp24 domain (TMED) or p24 protein is an important regulator of protein-cargo selection and vesicular bidirectional transport from the ER to the Golgi apparatus (Kaiser 2000). Previous studies have highlighted that Tmed10 plays an important role as a cargo receptor in the trafficking of various membrane proteins. For example, Tmed10 has been observed to modulate the transport and trafficking of amyloid-β precursor protein (Vetrivel et al. 2007), endogenous glycosylphosphatidylinositol (GPI)-anchored proteins CD59 and folate receptor alpha (Bonnon et al. 2010), and several G protein-coupled receptors (GPCRs) (Luo et al. 2011). TMED family members are small (24kDa) type I transmembrane proteins primarily found in COP-I and COP-II coated vesicles in the Golgi apparatus, the ER and the plasma membrane of secretary granules in pancreatic B cells and is an interesting validated Nav1.7 interactor. Structurally, these proteins are able to exist as monomers, dimers, oligomers and heterooligomeric complexes (Liaunardy-Jopeace et al. 2014). Other members of the p24 family do not associate with the cellular plasma membrane. However, the interacting domain of TMED10 is known to be responsible for its efficient trafficking to the cell surface. Understanding mechanisms by which Nav1.7 is trafficked offers important potential therapeutic targets. Furthermore, other components of the trafficking mechanism were validated in our study. Transport of VGSCs via vesicles to the nerve terminals along long-distance axons involves several components, including motor and scaffold proteins. KIF5B is a microtubule-dependent motor kinesin responsible for anterograde axonal transport. Both KIF5A and KIF5B are abundantly expressed in dorsal root ganglia neurons and have previously been shown to promote the transport of Nav1.8 to the plasma membrane. Furthermore, in a model of peripheral inflammation, an increase in KIF5B and Nav1.8 in the sciatic nerve have previously been reported, possibly involving this transport protein in mediating inflammatory neuronal hyperexitability (Su et al. 2013).

Although, there exists substantial evidence for a KIF5B/Nav1.8 interaction, Nav1.7 has never before been reported to interact with this protein. KIF5B was revealed in our LC-MS list as a strong possible Nav1.7-interacting protein (present in KI only), and was therefore chosen and validated by CO-IP. This validation sheds light on the mechanisms of regulation of subcellular
distribution of Nav1.7 in primary sensory neurons. The clustering and anchoring of voltage gated sodium channels at nodes of Ranvier and axon initial segment is reliant on Ankyrin-G and Neurofascin, two major components of the membrane cytoskeleton (Zhou et al. 1998; Srinivasan et al. 1998; Kordeli et al. 1991; Komada et al. 2002). Early intermediate developing nodes of Ranvier in the optic nerve axon are defined by Ankyrin-G, which subsequently recruits other components such as neurofascin, β-spectrin and importantly voltage gated sodium channels (previously shown to recruit Nav1.2 and Nav1.6) (Jenkins et al. 2002). Their validated interaction with Nav1.7 suggests these proteins are also responsible for the recruitment and anchoring of Nav1.7 to the nodes of Ranvier. Within nerve terminals, Synaptotagmin I&II associate with the SNARE complex and other factors (Vrljic et al. 2010) to catalyse the Ca\(^{2+}\)-triggered fusion of vesicles with the plasma membrane (Zhou et al. 2015). Multiple pharmacological and biochemical studies have shown an enrichment of sodium channels at nerve endings and evidence pointing to a functional role for Nav1.7 at pre-synaptic terminals has highlighted the possible involvement of this channel in the process of neurotransmitter release. Nav1.7 has been found to co-localise with a number of synaptic proteins such as synaptophysin, a marker of pre-synaptic terminals (Black et al. 2012) and Snap25 a t-SNARE protein associated with vesicle-membrane fusion (Rizo et al. 2002). Furthermore, in Nav1.7 knockout studies, substance P release into the dorsal horn of the spinal cord is completely abolished, leading to a loss of wind-up and centrally mediated pain sensitization (Minett et al. 2012a). The only direct physical evidence suggesting a link between voltage-gated sodium channels and synaptic proteins comes from a study by Sampo et al., (2000). This study showed a direct physical association of synaptotagmin I with Nav1.2 at a site located on the second intracellular loop of the channel, a site which is highly conserved across all voltage-gated sodium channels (Sampo et al. 2000). Knowledge of how Nav1.7 acts at synaptic terminals provides great insight into the role of this channel in synaptic vesicle release and therefore could provide targets for novel drugs. Like the action of gabapentin, inhibiting neurotransmitter release in nociceptive neurons has already proven to be a promising mechanism for the development of novel therapeutics. This is best exemplified by the action of the epilepsy and neuropathic pain drug Gabapentin. Gabapentin has previously been identified as a ligand to the α2δ subunit of voltage gated calcium channels (Hendrich et al. 2008). Mechanistically it is thought to disrupt the trafficking of voltage gated calcium channel Cav2.2 (Hendrich et al. 2008) and is proposed to function therapeutically by blocking new synapse formation (Eroglu et al. 2009) thereby, reducing channel activity and neurotransmitter release in DRG neurons. Because of the branched chain amino acid like
structure of gabapentin it’s entry across the blood brain barrier has been linked, in a number
of studies, to amino acid transporters. The transport of gabapentin, can be blocked by
inhibiting the amino acid l-phenylalanine, suggesting the involvement of a particular L-alpha
amino acid transporter, LAT1 (Su et al. 1995). Surprisingly we found Nav1.7 to interact with
Lat1 in our LC-MS list as a possible interactor to Nav1.7. We validated the interaction of
Nav1.7 with the Lat1 however the functional nature of this interaction is puzzling. Because
the important involvement of both Nav1.7 and Cav2.2 in neurotransmitter release and the
overlap in synaptic localisation, we sought to explore a possible link between these channels.
Although, neither Cav2.2 nor any of it’s subunits co-localised with Nav1.7 we sought explore
the idea of a functional clustering of the channels at the synapse. However, Nav1.7 and Cav2.2
did not co-precipitate (see figure 4.2), excluding a direct physical link between the channels.

There is one major caveat to these co-immunoprecipitation studies that I wish to points out,
and that is that I did not conduct any reverse CO-IP on any of the proteins of interest. This
would have involved co-precipitating Nav1.7 using a tagged protein of interest. By performing
the reverse CO-IP with these proteins if interest would have provided a more robust
confirmation of protein-protein interaction. G protein-regulated inducer of neural outgrowth 1
(Gprin1) and Phosphatidylethanolamine-binding protein (PEBP) are both intricately
involved in the regulation and anchoring of opioid receptors (Ge et al. 2009; Edwards et al.
2016). Gprin1 has previously been shown to tether µ-opioid receptors with the G-protein
alpha subunit and subsequently regulate receptor distribution within lipid rafts. PEBP
regulates GPCR signalling through both the Raf/MEK/ERK pathway and G protein-coupled
receptor kinase 2 (GRK2), a major feedback inhibition of GPCRs which has been implicated
in a number of neurological disorders including chronic pain. The validation of these proteins
provides support to the recent claim depicting the mechanisms underlying complete
insensitivity to pain phenotype, providing a physical link between this sodium channel and
opioid receptors. Collapsin Response mediator protein 2 specifies axon/dendrite fate and
axonal outgrowth (Inagaki et al. 2001). Further experiments specifying the location of the
interactions must be undertaken if these are to be useful in using these interactions as drug
targets (see conclusions and further work for more details). A study conducted by Dustrude
et al., (2013) illustrates how CRMP2 trafficking of Nav1.7 is controlled by a SUMOylation
site within CRMP2. The binding of CRMP2 and Nav1.7 is enhanced by conjugation of
CRMP2 with a small ubiquitin-like modifier and further controlled by the phosphorylation of
CRMP2(Dustrude et al. 2016). Furthermore, the loss of SUMOylation of CRMP2 triggers
Nav1.7 internalisation in a Cathrin-dependent manner involving a known Nav1.7 interaction
Nedd4-2. Although, this clear evidence sheds light on the regulatory machinery responsible for the normal activity of Nav1.7, we have shown and validated the first direct interaction between the proteins. This protein has also been linked to pain due to its ability to bind the anti-epileptic/analgesic drug Lacosamide, a compound whose mechanisms is still unclear but is, in part, thought to act by stabilising sodium channels in the slow inactivating state, thereby reducing the pathological activity of hyperexcitable neurons typified by prolonged depolarization (Errington et al. 2008; Wang et al. 2010). We demonstrated that transient over-expression of CRMP2 can up-regulate Nav1.7 current density in stably expressing Nav1.7 HEK293 cells and this up-regulation can be reversed by applying Lacosamide. This suggests a slight contradiction to the mainstream understanding of the action of Lacosamide, as we suggest a complete indirect action on Nav1.7 through CRMP2. We propose an alternative mechanism for the analgesic properties of Lacosamide. We suggest that the drug may also work by inhibiting the interaction between CRMP2 and Nav1.7. As in silico predictions of Lacosamide binding sites have been suggested within the active components of CRMP2 (Wang et al. 2010). Taken together, this information concerning the physical and functional validation of these proteins provides valid new therapeutic targets for the indirect targeting of Nav1.7, essentially bypassing the “channel blocker” approach of targeting Nav1.7 to provide potent analgesia. However, more functional experimentation will be needed in the future to fully understand the true nature of their interaction with Nav1.7.

Overall, the present findings provide new insights into the the possible functionalities of the proteins that interact with Nav1.7. I have provided a classification according to cellular component and functionality of these proteins, which outlines the complexity and variability in function of the proteins that potentially interact with Nav1.7. Furthermore, in this chapter I have selected and part-validated a number of candidates of interest that may play a role in modulating Nav1.7. Finally, I have added to the already existing suspicion that the protein CRMP2 may play an integral part in modulating Nav1.7.

4.6 Further Thoughts and Experiments

In this chapter I performed an in silico analysis of the identified Nav1.7 interacting proteins and validated a selection of proteins that were known to associate with Nav1.7 and unknown novel interactors. I provided some sorting of proteins in terms of what cellular process the proteins were involved in as well as what cellular component they belonged to. Using co-immunoprecipitation coupled with Western blotting, from dorsal root ganglion mouse
tissue, I validated the Nav1.7 interactions with β-3, synaptotagmin I & II, CRMP2, GPRIN1 LAT1, TMED10, AKAP12, Neurofascin, Neurotrimin, KIF5B, Ankyrin G and PEBP. Using an in vitro setup, I co-immunoprecipitated Nav1.7 with a number of exogenously expressed proteins and was able to validate interactions between Nav1.7 β-3, Synaptotagmin I, CRMP2, GPRIN1, LAT1 and TMED10. We also functionally validated the trafficking role of colapsin response mediator protein 2, showing that the overexpression of this protein increased Nav1.7 mediated current density in HEK293 cells. Furthermore, I characterised the mechanism of the analgesic drug Lacosamide, demonstrating that the drug is most probably acting indirectly on Nav1.7 through CRMP2 to inhibit current density. Future experiments would primarily involved the further identification of false positive proteins and the further functional characterisation of interacting proteins. My in silico analysis could also be further explored. It is important to keep in mind that these proteins exist in sub interactomes compartmentalised in within cellular structures. An interesting further piece of analysis would be to create an architectural breakdown of the interactomes themselves, thereby understanding the intricate subnetworks of proteins from which a detailed pathway of how Nav1.7 is trafficked and regulated. When considering the use of this list of interactors as possible drug targets a vital piece of information needed to develop a successful drug is to understand exactly where these proteins are binding. Although predictive models can be used for this task, they can often be inaccurate. One approach would be to construct glutathione-S-transferase (GST) fusion proteins with fragmented pieces of a candidate of interest. Through the selective pulldown of each fragment this would enable the identification of interaction sites. Functional validation could be more difficult and will have to be developed on a case by case basis. In our case, to look at CRMP2 we were fortunate that appropriate electrophysiological assays were available to perform such a functional assay. However, associative functionalities are not always as simple to characterise and may have to rely on more structural and predictive studies. However, an alternative approach to deriving the functionality of a protein to Nav1.7 might be to impede the interaction between this protein in vitro. To study the modulatory role between voltage gated calcium channel transmembrane pore forming subunits and accessory cytoplasmic proteins, Findeisen and colleagues (Findeisen et al. 2017) developed meta-xylyl stapled peptides that target a prototypic high affinity protein-protein interaction, in their case the interaction between the CaV pore-forming α-subunit and the cytoplasmic β-subunit. Following the identification of interaction sites this approach could be used study the role of these interactions in vitro.
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<td>Protein-fragment complementation assays (PCAs) can be used to detect PPI between proteins of any molecular weight and expressed at their endogenous levels</td>
</tr>
<tr>
<td>In vitro</td>
<td>Phage display (H)</td>
<td>Phage-display approach originated in the incorporation of the protein and genetic components into a single phage particle</td>
</tr>
<tr>
<td>In vitro</td>
<td>X-ray crystallography</td>
<td>X-ray crystallography enables visualization of protein structures at the atomic level and enhances the understanding of protein interaction and function</td>
</tr>
<tr>
<td>In vitro</td>
<td>NMR spectroscopy</td>
<td>NMR spectroscopy can even detect weak protein-protein interactions</td>
</tr>
<tr>
<td>In vivo</td>
<td>Yeast 2 hybrid (Y2H) (H)</td>
<td>Yeast two-hybrid is typically carried out by screening a protein of interest against a random library of potential protein partners</td>
</tr>
<tr>
<td>In vivo</td>
<td>Synthetic lethality</td>
<td>Synthetic lethality is based on functional interactions rather than physical interaction</td>
</tr>
<tr>
<td>In silico</td>
<td>Ortholog-based sequence approach</td>
<td>Ortholog-based sequence approach based on the homologous nature of the query protein in the annotated protein databases using pairwise local sequence algorithm</td>
</tr>
<tr>
<td>In silico</td>
<td>Domain-pairs-based sequence approach</td>
<td>Domain-pairs-based approach predicts protein interactions based on domain-domain interactions</td>
</tr>
<tr>
<td>In silico</td>
<td>Structure-based approaches</td>
<td>Structure-based approaches predict protein-protein interaction if two proteins have a similar structure (primary, secondary, or tertiary)</td>
</tr>
<tr>
<td>In silico</td>
<td>Gene neighborhood</td>
<td>If the gene neighborhood is conserved across multiple genomes, then there is a potential possibility of the functional linkage among the proteins encoded by the related genes</td>
</tr>
<tr>
<td>In silico</td>
<td>Gene fusion</td>
<td>Gene fusion, which is often called as Rosetta stone method, is based on the concept that some of the single-domain containing proteins in one organism can fuse to form a multidomain protein in other organisms</td>
</tr>
<tr>
<td>In silico</td>
<td>In silico 2 hybrid (I2H)</td>
<td>The I2H method is based on the assumption that interacting proteins should undergo coevolution in order to keep the protein function reliable</td>
</tr>
<tr>
<td>In silico</td>
<td>Phylogenetic tree</td>
<td>The phylogenetic tree method predicts the protein-protein interaction based on the evolution history of the protein</td>
</tr>
<tr>
<td>In silico</td>
<td>Phylogenetic profile</td>
<td>The phylogenetic profile predicts the interaction between two proteins if they share the same phylogenetic profile</td>
</tr>
</tbody>
</table>
In silico Gene expression

The gene expression predicts interaction based on the idea that proteins from the genes belonging to the common expression-profiling clusters are more likely to interact with each other than proteins from the genes belonging to different clusters.

**Table 4.6:** Table summarising the current methods available to study and validate protein-protein interactions (taken directly from Rao et al., 2014).
Chapter 5

Congenital Insensitivity to Pain: Nav1.7, Sodium and Opioids

5.1 Abstract

Experiments measuring the modulation of activated PKA subtypes have shown that opioid receptor activity is significantly increased in sensory neurons from pain-free mice lacking the voltage–gated sodium channel Nav1.7 (Isensee et al. 2014). Type-A GPCRs are known to be regulated through a specific sodium binding site the occupancy of which diminishes agonist binding. We have used an electrophysiological assay of Protein Kinase A activity to examine the role of intracellular sodium on opioid signalling. Phosphorylation of sodium channel Nav1.8 by activation of Protein Kinase A with db-cAMP is unaffected by altered intracellular sodium. By contrast, there is a dose-dependent inhibition of fentanyl action on Nav1.8 currents when intracellular sodium is increased from 0mM to 20mM. Fentanyl shows a 50% loss of activity and 80-fold increase in EC50 with 20mM intracellular sodium. These data suggest an effect of altered intracellular sodium levels on opioid receptors, where it might play a role in the modulation of opioid receptor signalling.

5.2 Introduction

The classical opioid receptors $\mu$, $\kappa$ and $\delta$ are G-protein-coupled receptors essential for the regulation of nociception, mood and awareness (Pasternak 2014). All opioid GPCRs are activated by endogenous peptides (endorphins, enkephalins, dynorphins, nociceptin) and an expanding number of exogenous natural and synthetic molecules. Most of these agonists
interact with the orthosteric site located in the extracellular portion of the seven transmembrane bundle. Despite and increased understanding of GPCR activation through the determination of receptor crystal structure (Katritch et al. 2013), the underlying molecular mechanisms responsible for many of the signal transduction and allosteric modulation remain elusive (Wootten et al. 2013; Rosenbaum et al. 2009). Pert and Snyder first showed the influence of sodium on opioid receptor activity in 1974 by demonstrating through the use of radioligand binding assays, that increased sodium concentrations caused diminished agonist binding (Pert et al. 1974), albeit by unknown mechanisms (Pert et al. 1974). Forty years later, the binding site for sodium on the δ-opioid receptor was identified by Fenalti and colleagues (Fenalti et al. 2014). Opioid receptors are members of the Class-A GPCR family that comprises about 700 members. A sodium binding site has been identified in adenosine A2A, β-1 adrenergic and PAR-1 receptors using ultra high resolution crystallography and a related site is present in almost all Class-A GPCRs (Liu et al. 2012; Zhang et al. 2012; Miller-Gallacher et al. 2014). Modelling studies of the A2A receptor have suggested that sodium may access receptors via a water-filled passage that links extracellular and intracellular sides of the receptor (Katritch et al. 2014). Extracellular sodium is maintained at about 145mM, whilst intracellular sodium is much lower, in the range of 5mM for sympathetic neurons (Galvan et al. 1984) to 15mM in lobster nerves (Walker et al. 1977). Nav1.7 is central for the sensitivity and activation of nociceptive neurons in response to pain initiation stimuli and loss of function in this channel results in lifelong absence of pain (Cox et al. 2006). The mechanism responsible for pain insensitivity due to loss of Nav1.7 are incompletely understood (Waxman et al. 2014). However, a link between sodium channels and opioid modulation may be at the root of this phenotype. In mice, Nav1.7 deficiency results in increased expression of the endogenous opioid encoding the PENK gene (Minett et al. 2015) (See figure 5.1, figure was recreated from a figure in Minett et al., 2015. This figure along (along with the data gathered to produce this figure) was contributed by myself). Furthermore, in these animals and humans the blocking of opioid receptors with the antagonist naloxone partially restores the ability to perceive pain (Minett et al. 2015).
In these individuals, there exists an imbalance in pro- and anti-nociceptive signaling mediated by an increase in activity of the opioid mediated $G_{\alpha I}$, an effect first shown by Pert and Snyder to be mediated by sodium. Could this be the mechanism responsible for Nav1.7 mediated CIP? Is sodium coming through this channel and homeostatically modulating the efficiency of opioid receptors? Hypothetically this could mean that the lack in presence or functionality of this channel could create potent tonic analgesia. In contrast to voltage gated ion channels, the complexity of GPCR intracellular signalling is inherently difficult to directly measure. In order to uncover the possible link between Nav1.7 and opioid receptor regulation, here we looked at the effect of changing intracellular sodium concentrations on the activity of $\mu$-opioid receptors in individual mouse sensory neurons using a sensitive electrophysiological assay. We also sought to further confirm our findings using an alternative means of measuring this effect involving changing cAMP levels in response to $G_i/G_s$ activity as a measure of receptor efficacy. Furthermore, we developed a novel tool, a sodium chelating protein isolated from a plasma membrane $\text{Na}^+/\text{H}^+$ exchanger from *Basilius firmus* and can be readily used in both sensory neurons and HEK293 cell lines. This tool could be used as a useful tool in future for studying the role of sodium on the allosteric modulation of GPCRs and the role of intracellular sodium more generally.
5.3 Results

5.3.1 An Electrophysiological Method to Measure GPCR Activity

Protein Kinase A (PKA) is known to phosphorylate five serine residues in the first intracellular loop of Nav1.8, a tetrodotoxin-resistant voltage gated (TTXr) sodium channel that is uniquely expressed in sensory neurons (Fitzgerald et al. 1999). This results in a large increase in TTXr sodium channel activity that can be quantified by electrophysiological recordings (see fig 5.2). Fentanyl, acting through $\mu$-opioid receptors and $G_i$ proteins can suppress the activity of PKA and diminish the level of this current (Fitzgerald et al. 1999; Villiger et al. 1983; Isensee et al. 2017). These observations provide us with a simple assay system for measuring opioid action in intact dorsal root ganglia neurons, allowing us to vary the level of intracellular sodium and examine the consequences. Importantly, intracellular sodium levels can be effectively altered in intact cells by perfusion from a patch-clamp recording electrode (Fenwick et al. 1982). Because of the inherent variability and complexity of the assay a number of critical criteria for sample inclusion enhanced the robustness of our assay. The criteria for cell recording is outlined in methods section 2.0.12. The standard criteria that were respected in this assay were identical to all electrophysiological testing and included obtaining a strong gigaseal for each recording and a maintenance of the seal throughout the recording process to avoid any cell drifting, as drifting would falsify my results by showing a decrease in recorded current that might have been interpreted as an effect of fentanyl, when in fact it could have been due to a deterioration of the patch seal. However, because of the nature of this assay there were some additional difficulties that had to be taken into account. Because of the difficulties in cellular recording quality, approximately 90% of all recorded cells had to be discarded. The difficulties came mainly from the deterioration of patch quality over the time span of the recording, falsifying the results. This deteriorating quality was made worse by the movement of the patch bath following the addition of fentanyl. Furthermore, there is always a tendency for the cell recording to drift slightly due to degradation of the seal or the deterioration of the health of the cell during the experiment. This factor could be taken into account and used to rectify each recording however, once fentanyl was applied to the bath, we were unable to determine whether a change in membrane potential was due to the action of the drug or a disruption in the quality of the recording. The variability of each cell also meant a considerable number of DGR cells were needed for each group. All these caveats to experimental paradigm were taken into account and used to
carefully implement a strong sample selection criteria. Having considered this problems, first, we investigated the effects of fentanyl on Nav1.8-mediated TTXr sodium currents in sensory neurons (Isensee et al. 2017) for which we found that there was a concentration-dependent inhibition of fentanyl-evoked TTXr currents with increasing intracellular sodium (see figure 5.3). Next we manipulated intracellular sodium concentrations using 20mM and sodium free (0mM) intracellular medium in the patch pipette, in order to measure the allosteric effects of sodium on opioid receptors. Once equilibrium was reached, TTXr baseline currents were determined. Following which, 100µM fentanyl was added to the dish and the subsequent response measured 5.3. Interestingly, we found that 20mM sodium substantially lowered the inhibitory activity of fentanyl on TTXr current density. In contrast with nominal (0mM) sodium concentrations which, we found to strongly potentiate inhibitory activity. All experiments were performed in WT mouse DRG neurons.

**Figure 5.2:** TTX-resistant currents were recorded using whole cell patch clamp recordings in dorsal root ganglion neurons, permitting the manipulation of intracellular sodium concentrations. TTX-sensitive currents were blocked using TTX. Opioid receptor-mediated G\textsubscript{i} activity causes an inhibition of adenyl cyclase leading to decreased PKA activity and lower levels of Nav1.8 phosphorylation, thereby decreasing sodium currents entering through Nav1.8. An accurate recording of opioid receptor activity following the exposure to fentanyl is acquired through measurement of TTX-resistant currents (Nav1.8). The illustration present in this figure was produced by myself.
We next tested whether the downstream activity of PKA on Nav1.8 was modified by altered sodium levels (see fig 5.3). Using the highly selective PKA activator db-cAMP, we found that the level of TTX resistant Nav1.8 channel activity was upregulated by 53.4% in the presence of nominal 0mM intracellular sodium and by 52.8% in the presence of 20mM sodium and therefore determined that there was no difference in db-cAMP-mediated effects on TTX-r currents in 0mM or 20mM sodium. Thus, downstream sensitisation of Nav1.8 by PKA is independent of intracellular sodium concentrations. Next, we compared the activity of fentanyl on neurons expressing Nav1.8 with either 0mM or 20mM intracellular sodium. The maximal effect of fentanyl on Nav1.8 activity was lowered by 50% in the presence of 20mM intracellular sodium. In addition, the EC50 for fentanyl action changed from 2nM in 0mM sodium to 126nM in the presence of 20mM sodium. Strikingly, these data recapitulate early studies of sodium effects on opioid binding carried out by Kosterlitz and colleagues on whole brain membrane preparations where a 40-fold loss of fentanyl activity was noted at high sodium conditions (Kosterlitz et al. 1978) compared to a 60 fold change in the functional assays described here. The simplest explanation of these data is that the activity of the µ-opioid receptor is regulated by intracellular sodium.
Figure 5.3: (a, b, c and d) Electrophysiological sample traces of TTX-resistant Nav1.8 currents following exposure to 100nM fentanyl on TTXr currents following varying changes in intracellular sodium. Top row illustrates electrophysiological examples current traces of TTX-resistant Nav1.8 currents from dorsal root ganglia neurons, baseline (red) and following the addition of fentanyl (blue) at different intracellular concentrations of sodium. Corresponding dot plot of TTX-resistant Nav1.8 currents following exposure to 100nM fentanyl at different sodium concentrations. (a) 0mM sodium, n=14. (b) 5mM sodium, n=10. (c) 10mM sodium, n=14, (d) 20mM, n=6. All currents are normalised and compared to baseline. Student’s t test, 0mM p<0.001, 5mM p<0.001, 10mM p<0.001 and 20mM p<0.05 (e) Data from (a, b c and d) plotted as normalised peak currents compared to sodium concentration. Data represents ±SEM. (f) Electrophysiological recordings of TTX-resistant Nav1.8 currents in dorsal root ganglia neurons following exposure to db-cAMP in 0 mM and 20 mM intracellular concentrations of sodium. WT 0mM vs db-cAMP 0 mM, ***p<0.0002, change from baseline ∆=52.3 pA/pF. WT 20 mM vs db-cAMP 20 mM, ***p<0.0006, change from baseline ∆=59.8 pA/pF. No significant change between db-cAMP 0 mM vs db-cAMP 20 mM.
Interestingly, we also found that the activity of PKA on Nav1.8 currents was dramatically diminished by fentanyl in Nav1.7 null mutant mice compared to wild type controls when intracellular sodium was held at 10mM (see figure 5.4). These mice are pain free, and there have been earlier reports suggesting that this phenotype is mediated by an increase in opioid drive (Minett et al. 2015). In addition, the effectiveness of fentanyl in blocking PKA is dramatically enhanced in Nav1.7 null mutant neurons using an immunohistochemical single cell assay of PKA phosphorylation (Isensee et al. 2017). This would suggest that sodium entering through Nav1.7 ion channels regulates opioid signalling mechanisms. We tested the effect of fentanyl on wild type and Nav1.7 null mutant mice using 0mM concentrations of intracellular sodium. At 0mM sodium, the two strains showed identical maximal responses to fentanyl (see figure 5.4).

![Figure 5.4](image)

**Figure 5.4:** Maximal inhibition of PKA (65%) by fentanyl with 0mM intracellular sodium occurs in both wild type and Nav1.7 null mutant sensory neurons. Responses are normalised to controls without fentanyl. Nav1.7 KO cells in Nav1.7−/− group were from mouse where Nav1.7 had been knocked out in all sensory neurons using advillin Cre mouse (Minett et al. 2015).
5.3.2 Measuring the Effect of Sodium on G-protein Coupled Receptor Efficacy

Because of the inherent complexity and variability of our electrophysiological assay we sought to use an alternative method to analyse GPCR function to verify and support our claim. Downstream signal transduction of GPCRs is dependent on the type of G-protein subunit that associates with the receptor. There are three main G-protein signalling pathways mediated by four sub-classes of G-proteins: $G_{\alpha_s}$, $G_{\alpha_i/o}$, $G_{\alpha_q/11}$ and $G_{\alpha_{12/13}}$. The effector of both $G_{\alpha_s}$, and $G_{\alpha_i}$ is the cyclic-adenosine monophosphate (cAMP) generating enzyme adenylate cyclase. Activation of $G_{\alpha_s}$, which associates with 5-Hydroxytryptamine receptor 4 and Prostaglandin E2 receptors, increases cAMP levels, compared to $G_{\alpha_i}$ that associates with all three known opioid receptor subtypes, which reduces levels of cAMP produced by the enzyme adenylate cyclase. By using the ionophore monensin to manipulate intracellular concentrations of sodium, we used an enzyme-like immunosorbent assay based method for measuring the changes in GPCR activity in 2mM and 20mM intracellular sodium in intact cells (2mM of sodium was used instead of 0mM as we sought to not completely compromise any intracellular signalling pathways or other cellular factors that may be sensitive to sodium concentrations). Initially, in order to correlate our previous electrophysiological findings, this experiment was performed on primary cultured DRG neurons. As most absorbance based assays are optimised for use with cell lines, we first optimised this assay for primary neuronal cultures (see fig 5.5).
Chapter 5

Control

Fentanyl

Metoclopramide

Metoclopramide + Fentanyl

Forskolin

Forskolin + Fentanyl

Figure 5.5: Enzyme-like immunosorbent assay using mouse primary sensory DRG cultured neurons. (a) Shows the approximate quantity of neurons needed to obtain detectable amounts of cAMP. All cells were pooled and distributed equally amongst conditions. Cell numbers were counted using a conventional hemocytometer (2.0.16). (b) ELISA assay performed in cells preincubated in either 0mM or 20mM sodium extracellular medium with monensin. Cells are then stimulated with appropriate G-protein agonist or antagonist. Forskolin was used as a G-protein independent regulator of cAMP. Fentanyl was used as an agonist to µ-opioid receptor. Metoclopramide was used agonist to 5HT4 receptor to upregulate Gs. n = 6 for each data point, each, replicates are technical.

However, even in the most efficient culturing conditions, an excessive number of DRGs were necessary in order to provide sufficient numbers of DRG neurons for each data point to be within detectable range. In the most efficient of culture conditions we obtained an average of around 15000 viable DRG neurons per culture originating from all pooled DRGs from one adult mouse. Interestingly, when those numbers were halved (i.e 7500 cultures neurons were used) we did not get a halving of the response. We attributed this factor to the health of the culture diminishing when less densely packed in the dish, leading to a greater number of cell deaths during the assay in these already unhealthy cultures. However, considering the quantity of animals necessary, we chose an average of 7500 DRG neurons per data point for our assay. In order to increase basal cAMP levels for the assay we used the 5HT4 specific agonist Metaclopramide, which stimulates the Gs coupled receptor thereby increasing cAMP levels through adenylyl cyclase. Forskolin was used as a sodium independent control between the 2mM and 20mM sodium. Fentanyl was again chosen as the opioid agonist to measure opioid efficiency through Gi activity. Despite multiple assay repetitions we found there to be
high variability between the groups and were not able to determine a robust and clear effect of sodium variability on opioid signalling in dorsal root ganglia (see fig 5.5). One major caveat to using DRG neurons was the presence of contaminant non-neuronal cells in the cultures. Culturing conditions of DRG neurons are such that non-neuronal cells, in this glial cells, are an important contaminant to the assay. Furthermore, reports in the literature have shown that glial cells respond to Forskolin, PGE2, Monensin and opioids (Hutchinson et al. 2009; Watkins et al. 2009) and therefore fluctuating cAMP levels could have drastically falsified the ELISA assay. Methods, such as adding enzymes that prevent glial proliferation, such as DNA topoisomerase, have been previously suggested as solutions to this problem (Andersen et al. 2003), however, the fragility of the DRG neurons and possible other unknown repercussions to exposing the neurons to such harsh treatments could have affected the viability of our cultures. Because of the high inherent variability and the high number of animals necessary to perform this assay using dorsal root ganglion neurons we sought to find an alternative, more efficient system to determine the effects of sodium on opioid receptor efficacy. We chose three candidate cell lines based on expression profile of both Gs and Gi coupled GPCRs. The prostaglandin E2 (PGE2) receptor was chosen as the Gs component and δ-/μ- receptors as the opioid Gi components. The cell lines chosen were SH-SY5Y (Alique et al. 2007; Rasmuson et al. 2012; Caputi et al. 2014), NDC and N2A (Tamiji et al. 2010). To our knowledge previous expression of these receptors was known for SH-SY5Y and N2A but not for NDC, we therefore screened each cell line for responses to PGE2, Forskolin, PGE2/Forskolin, Forskolin/Fentanyl and Fentanyl to determine which cell line would be used for subsequent experiments (see fig 5.6). We found that the SH-SY5Y cell line responded the most efficiently and consistently to all agonists and gave responses in the correct detectable range for the assay. Therefore, SH-SY5Y cells were chosen for this assay (see fig 5.6). Next, we determined the effect of low (2mM) and high (20mM) sodium on the efficiency of GPCR signalling. Identical incubation times and other parameters for monensin were used as in previous sensory neuron experiments (Minett et al. 2015). We found there to be an important effect of PGE2 which did not differ significantly between 2mM and 20mM sodium. However, Fentanyl responses were dramatically and significantly higher in 2mM sodium indicating a possible increased effect of opioid signalling. Forskolin was chosen as a positive control between the groups. Interestingly, we found that there was a clear effect of differing sodium concentrations on the Forskolin mediated cAMP increase, which acts via an independent pathway by directly binding to adenylyl cyclase. This assay was performed multiple times (n=4) and the data was pooled. Interestingly, all responses to Fentanyl and PGE2 were consistent throughout all three assays. The discrepancies found
amongst the Forskolin data was variable between the assays and was not consistent enough to
draw any stern conclusions from these experiments.
Figure 5.6: Enzyme-like immunosorbent assay in primary cell lines. The data plotted is the relative change in cAMP level in a selection of cell lines following exposure to a selection of agonists (n = 6 per column in all experiments, all replicates are biological).

(a) Preliminary tests for cell selection. Cells previously known to express PGE2 and opioid receptors were selected, tested and compared. Cells tested as alternatives to DRG were: SH-SY5Y, N2A and NDC. SH-SY5Y was chosen as the most consistent and accurate cell line due to high levels of expression of cAMP in detectable range of the assay. SH-SY5Y cells also responded robustly to all agonists. (b) ELISA assay performed using the cell line SH-SY5Y. SH-SY5Y cells were preincubated in either 0 mM or 20 mM sodium in an extracellular medium that contained monensin. Cells were stimulated with appropriate agonist to detect effect of variable sodium on G-protein reaction. Forskolin was used as a G-protein independent regulator of cAMP. Fentanyl was used as an agonist to µ-opioid receptor and fentanyl responses were significantly higher between groups showing and increase efficacy of the drug in 2 mM sodium. However, changes in forskolin responses between groups indicates probable other sources of variation as forskolin is an independent modulator of cAMP which should therefore remain constant under differing sodium concentrations. PGE2 was used as agonist to prostaglandin E2 receptor to upregulate Gs. n = 6 for each data point, each, replicates are technical.
Studying the physiological effects of intracellular ion concentrations is challenging. The main caveat to these studies are the methods used to manipulate the intracellular sodium concentrations which often require non-physiological methods involving either ionophores, such as monensin or the pharmacological manipulation of ion pumps and ion channels. These methods are harsh and can have various other effects on the cell such as changes in pH. We therefore sought to develop an alternative tool to manipulate intracellular sodium concentrations. We devised an easily transfectable construct containing part of an Na\(^+\)/H\(^+\) anti-porter gene known to contain a sodium binding component from the alkalophilic prokaryotic Bacillus firmus characterised in a 1991 patent by Krulwich and colleagues (Krulwich et al. 1994). Na\(^+\)/H\(^+\) antiporters are ubiquitous in living cells and have been assigned a large variety of important functions (Boron et al. 1983). The sequence of the chelating protein was obtained from the patent and synthesised externally by Genscript gene synthesis services (see figure 5.7). The gene arrived in a cloning vector and was then cloned into a pIRES AcGFP1 construct with a (CMV) promoter and a reporter gene (Tomato on IRES). In silico analysis of the AcGFP1 coding sequence revealed a 68 amino acid peptide of approximately 7000 daltons with high hydrophobicity (see figure 5.7) indicating the high probability of it being membrane associated. We were able to successfully transfect this construct into a HEK293 cell line stably expressing Nav1.7. Next, using a ratiometric sodium imaging set-up we determined the effect of transiently expressing this construct in HEK293 cell line stably expressing Nav1.7. We found there to be a sharp decrease (approximately 50%) in sodium concentrations in the transfected cell line compared to cells transfected with the empty vector. In order to certify that the changes resulted from a real change in free intracellular sodium and that this protein was not chelating other ions, we did control experiments using a calcium sensitive dye (Fura) which showed no difference in calcium concentrations after transfection of the construct compared to tomato empty vector (see figure 5.8 in the appendix). Interestingly, we also found that in the SBP transfected cells Tomato intensity never reached the same levels as in the control cells perhaps indicating that over expression of this construct is lethal due to over-chelation of intracellular sodium thereby disrupting normal cellular functions. This evidence suggests this construct can be used for future study of intracellular sodium concentrations in sensory neurons to study the effect of modulating intracellular sodium and its effects on opioid receptor affinity. We sought to confirm these results in sensory neurons. However, the transfection of dorsal root ganglion neurons was difficult using conventional less damaging methods such as lipofectamine transfection which, produced around a 2% transfections rate. Furthermore, using an
electroporation approached this killed a large proportion of the cells and in those that were positively transfected the high degree of transfection seemed to be lethal to the neurons (please see conclusions and future experiments for further analysis).
Figure 5.7: Nucleotide and peptide sequences used for the identification and cloning of sodium binding protein (NahS). This nucleotide sequence corresponds to a truncated version of the full sequence cloned into the pIRES-AcTOM plasmid to create SBP. The sequence shows the entire length of the protein translated segment. The full DNA sequence can be found in appendix section A.3. Top shows the bacterial sequence from Bacillus firmus Na+ /H+ antiporter gene Orf4, accession number: U61539.1 synthesised and cloned into pIRES-AcTOM plasmid, translation of the NahS in red above the sequence. Bottom shows peptide sequence of translated NahS (SBP) sequence from Bacillus firmus NahS, accession number AAC45433.1. Residues are coloured according to properties: acidic, basic, polar uncharged and hydrophobic nonpolar.
Chapter 5

(a) Sodium Standard Curve

\[ y = 0.729x + 0.5731, \quad R^2 = 0.93885 \]

(b) Empty Vector

\[ R^2 = 0.00802 \]
Chapter 5

Tomato Intensity

Na\(^{+}\) concentration (mM)

SBP

\(R^2 = 0.12012\)

(c)

Empty Vector

Ratio (340/380)

(d)
Figure 5.8: Sodium and calcium imaging of HEK293 cells stably expressing Nav1.7 and transiently transfected with SBP vector or an empty tomato vector. (a) represents the standard curve created to plot sodium concentrations corresponding to ratiometric recordings. (b) Sodium concentration measurement plot of control cells transfected with an empty vector construct containing a tomato reporter gene alone. (c) Sodium concentration measurement plot of cells transiently transfected with vector containing the sodium chelating protein gene. Regression lines were plotted in (b) and (c) determine changes in intracellular sodium concentration in accordance with increased tomato expression. With increased tomato expression in (c) there was a decrease in intracellular sodium compared to (b) where sodium concentrations did not change in function of tomato expression. (d) Calcium imaging of HEK293 cells stably expressing NaV1.7 and transiently transfected with (d) SBP vector or (e) an empty tomato vector. Calcium concentration measurement plot of cells transiently transfected with a vector containing the sodium chelating protein gene versus an empty reporter vector. Ratiometric readings did not show any changes in calcium concentrations in SBP transfected cells compared to empty vector transfected cells.
5.4 Discussion

The discovery that the lack of functional Nav1.7 generates pain free phenotypes has made this voltage gated sodium channel one of the main targets for the development of novel analgesic drugs over the last decade. Despite sustained efforts to exploit this pharmaceutical target, many Nav1.7 drug development programs have had limited success (Emery et al. 2016). Even the most potent, selective and stable antagonists, developed and tested in humans, show debatable and limited efficacy in treating pain. Furthermore, most claims to some efficacy using neutralising monoclonal antibodies towards Nav1.7 can not be replicated (Lee et al. 2014). In fact, it seems that the more selective the inhibitor (e.g. protoxin II), the less potent the analgesia (Emery et al. 2016). Whilst less selective pan sodium channel blockers (e.g. CNV-1014802 and lidocaine) are very effective analgesics (Emery et al. 2016). The mechanisms behind how Nav1.7 mutations mediate CIP phenotypes are far more complex than initially thought and the disease cannot be solely reliant on decreased neuronal excitability (Emery et al. 2016). Indeed, early work from the pre-genomic era, on patients with what is known as probable Nav1.7 null mutant mediated CIP, had already provided evidence that the endogenous opioid system had a substantial contribution to the pain free phenotype (Dehen et al. 1978). In recent years, further work from our lab has found there to be a major role for enhanced opioid signalling in creating the analgesia associated with Nav1.7 null mutant CIP mice and humans, as the vast majority of analgesia (80%) in the CIP tested patients was naloxone reversible (Minett et al. 2015). We found that a loss of Nav1.7 expression is linked to a transcriptional up regulation of the precursor of met-enkephalin (Penk) (please see figure 5.1 in appendix chapter 3). This evidence provided a link to an intimate interplay between both the opioid and sodium systems in nociceptive neurons. But is this the only role sodium may have in regulating the opioid system? Building on robust pharmacology from the late 70’s outlining a clear allosteric effect of sodium on opioid receptors, we aimed to expand the investigation into this relationship. Early investigations into the effects of sodium on the binding of both opioid agonists and antagonists provided the first evidence of sodium mediated GPCR activity (Pert et al. 1974). Now we have shown that in physiological conditions, this sodium modulation is vital to the correct functioning of opioid receptors and perhaps even all class A GPCRs. The first hint that Nav1.7 might interact with opioid receptors was the physical proximity between the channel and the receptors, evidence of which originated from our Tap-tag Co-IP experiments (see mass spec table in Chapter 1). When epitope-tagged Nav1.7 is immunoprecipitated with channel-associated proteins from
mouse DRG neurons, proteins that are known to bind to \(\mu\)-opioid receptors also associate with Nav1.7, as judged by mass spectrometry. One such protein, GRIN1, that appears in our mass spec list is known to associate with \(\mu\)-opioid receptors within lipid rafts. GRIN1 mediates \(\mu\)-opioid receptor clustering within lipid rafts (Ge et al. 2009). Moreover, this is one of the proteins whose association was further validated \textit{in vitro}. Previous studies of Nav1.8 KO mice have shown that there is no effect on \(\mu\)-opioid receptor activity as measured with a PKA assay following the deletion of this sodium channel gene (Isensee et al. 2017) suggesting that this is a solely Nav1.7 mediate regulation. Moreover, the physical apposition of Nav1.7 and \(\mu\)-opioid receptors could explain the specific significance of sodium flux through Nav1.7 for the regulation of fentanyl signalling. Persistent currents associated with Nav1.7 have also been reported and these may have effects on local sodium concentrations near the ion channel at the membrane (Branco et al. 2016). If sodium is acting as a second messenger linking Nav1.7 activity with opioid GPCR signalling, is it possible that other cation channels could be linked to other class A GPCR receptor regulation in an analogous way. For example, the regulation of adrenergic receptors by Nav1.8 through PKA mediated phosphorylation (Gu et al. 2015). In the case of Nav1.7, we may speculate that intense stimulation associated with extreme tissue damage could lead to the attenuation of opioid signalling and an amplification of nociceptor activity that could transiently potentiate avoidance behaviour. As a consequence of the discovery of a highly conserved sodium binding site within opioid receptors and all class A GPCRs (Pert et al. 1974), a model of the involvement of sodium on receptor activation has previously been proposed where within the ligand free receptor, sodium ions gain access to the allosteric pocket where it forms a network of ionic and polar interactions as part of a sodium water cluster. Following GPCR agonist activation changes in the conformation of the protein causes a momentary collapse of the allosteric site, leading to a displacement of the sodium ion and it’s release into the intracellular environment (Katritch et al. 2014). This activation collapse of the sodium pocket implicates a specific role for sodium in the transduction mechanism. Our findings also point to a major role for sodium in the regulation of the opioid receptor and that the source of this sodium most probably comes from Nav1.7 local influx. From this we can also deduce a probable mechanism by which anomalies in Nav1.7 can cause a reduction in local sodium concentrations, thereby decreasing allosteric inhibition and increasing the efficacy of the opioid receptor. It is also important to note that the regulation of cellular processes by ion channels has already firmly been established in the case of calcium channels. The localisation of L-type voltage gated calcium channels CaV1.2 and CaV1.3 at neuronal cell bodies puts them in proximity to calcium activated transcription
factors such as CREB, MEF and NAFT (Bourinet et al. 2014; Gomez-Ospina et al. 2006; Tian et al. 2010; Lu et al. 2015) enabling the calcium coming through these channels to act as secondary messengers in gene regulation. Furthermore, the presence of N-type calcium channels at the synapse is the main catalysing entity for neurotransmitter release (Takasusuki et al. 2011). Therefore, location dependent role must also be true for Nav1.7. Previous evidence by Minett et al., (2015) found that the lack of Nav1.7 presence caused an increase in the transcription of the enkephalin precursor PENK. Indeed, a number of transcription factors, such as NFAT5 are sensitive to sodium concentrations (Berry et al. 2017) and could be the main factors acting to regulate the transcription of opioid ligands.

The results that we have obtained allude to this role of sodium in the regulation of opioid receptors. Because of the difficulty in measuring GPCR activity we were not able to fully confirm beyond reasonable doubt the validity of this claim. However, we showed through our electrophysiological experiments that the efficacy of fentanyl was much greater in low intracellular sodium compared to higher 20mM sodium, despite the caveats contained in the assay itself the main caveat being the patching process itself. Because our experimental paradigm involved the indirect measurement of Nav1.8 phosphorylation provoked by intracellular signalling mechanisms activated by the µ-opioid receptor, key intracellular proteins involved in the cascade leading to Nav1.8 phosphorylation could be affected by changes in the gradients of these proteins due to the high volume in the patch pipette or other disruptions which could arise following the perforation of a cell (see conclusions and further experiments for explanation on how to solve this problem). However, despite these caveats and difficulties in dealing with variabilities in the assay, our results are still robust enough to suggest sodium having an allosteric effect in regulating opioid receptors. This information is an important factor in creating new therapies to combat pain disorders (Minett et al. 2015). Indeed, as previously mentioned by Minett et al., (2015) through the inhibition of sodium currents using selective sodium channel blockers this could increase opioid receptor efficiency thereby increasing the efficacy of existing opioid drugs which could be given at a fraction of the dose with equal efficacy for pain relief. Of course, more work still needs to be done to further understand and ultimately harness this physiological effect to potentially create novel synergistic approaches to developing analgesics. The development of our sodium chelating protein could be and interesting tool to further investigate the role of sodium in opioid receptor modulation. Our present findings however, are preliminary and the building of a clearer picture of this sodium/Nav1.7/opioid story will need further attention in the future. However, my work clearly establishes the grounds for a story worth expanding. I believe, the
most important task for further developments in this area is the establishment of a consistent and robust assay for the measurement of G-protein coupled receptors. Interestingly, some alternative solutions already exists. Kroeze and colleagues (2015) established a robust assay by which measurement of G protein–independent -arrestin recruitment provides a feasible and universal assay platform to measure GPCR activity (Kroeze et al. 2015). Because nearly all GPCRs can induce arrestin translocation, by engineering a luciferase based reporter directly linked to -arrestin recruitment, this group can with great precision observe changes in GPCR activity. This assay is open source, and with the combination of our sodium binding protein, we could develop a robust assay to measure changes in µ-opioid receptors mediated by sodium with relative ease.

5.5 Further Thoughts and Experiments

In this chapter I developed and performed an electrophysiological assay to characterise the relationship between the sodium channel Nav1.7 in the modulation of the opioid system. Through this electrophysiological assay by which we monitored Protein Kinase A activity on Nav1.8, we showed that there is a dose-dependent inhibition of fentanyl action on Nav1.8 currents when intracellular sodium is increased from 0mM to 20mM. Fentanyl shows a 50% loss of activity and an 80-fold increase in EC50 with 20mM intracellular sodium. These data suggest an effect of altered intracellular sodium levels on opioid receptors, suggesting that it plays a role in the modulation of opioid receptor signalling. As previously mentioned the monitoring of GPCR activity is challenging and our experimental paradigm held many caveats which increased the variability of our assay. There are however, a few alterations to our existing model that may enhance the credibility of our assay. Firstly, the volume of intracellular fluid present within the patch pipette was a concern as our measurement of Nav1.8 phosphorylation was dependent on the correct functioning of a series of intracellular components notably PKA and cAMP. In order to limit the diffusion from the pipette a perforated patch approach could be used. However, I fundamentally believe that in order to progress with confirming the preliminary data obtained through this electrophysiological assay, an alternate approach must be taken. The most convincing method for the direct monitoring of GPCR activation is an assay described by Kroeze and colleagues using using the endogenous cellular signalling mechanism for the inactivation of GPCRs to monitor their activity coined the PRESTO-TANGO assay (see figure 5.9).

All necessary components of the assay are available including the modified transfectable...
construct as well as the luciferase and β-arrestin/TEV protease expressing cell lines. In combination with the transfection of our sodium chelating construct (SBP) it would be possible to develop a more physiological and accurate assay to monitor the sodium mediated changes in opioid activity. Moreover, there are also important steps that need to be taken to optimise the sodium binding protein we described in chapter 3. Indeed, despite the promising results showing decreased free sodium within SBP transfected cells more needs to be done to optimise this tool. Our failed attempts in reproducing the sodium imaging results in dorsal root ganglion neurons was due to difficulties in transfecting the neurons with the construct.

This problem can be circumvented using an viral transfection approach (AAV). Following this I believe further characterisation of the protein can also be performed. By using a Na22 based radioactive binding assay, we could in future better characterise the binding qualities of this protein thereby clarifying its potential for further use in manipulating intracellular sodium concentrations. In conclusion, this thesis has provided both resource and insight into the role of Nav1.7 in pain signalling. Through an understanding of it’s component parts, the mechanisms by which pain is generated will provide for the innovation and development of
much needed therapeutic development in this field.
Appendix A

Appendix

A.1 Additional Methods

A.1.1 LC-MS/MS Analysis

Proteins cleaved from Ni-NTA beads after affinity purification were tryptic digested following a FASP protocol (Wisniewski et al., 2009). In brief, proteins were loaded to 30 KDa filters (Millipore), then filter units were centrifuged at 14,000g for 15 min to remove other detergents. Two hundred µl of urea buffer (10 mM dithiothreitol 8M urea (Sigma) in 0.1 M Tris/HCl pH 8.5) were added to the filters and left at room temperature for 1 hour to reduce proteins. The filters were centrifuged to remove dithiothreitol. Two hundred µl of 50 mM iodoacetamide in urea buffer were added to filters and left 30 min in the dark. The filters were centrifuged as before to remove IAA. Then the samples were buffer exchanged twice using 200 µll of urea buffer, and one more time using 200 µl of 50 mM NH4HCO3 in water. 40µl of 50 ng/µl trypsin in 50 mM NH4HCO3 were added to filter, filters were vortexed briefly and proteins were digested at 37°C for overnight. After tryptic digestion, the filters were transferred to new collection tubes, and the peptides collected by placing the filter upside down and spinning. The samples were acidified with CF3COOH and desalted with C18 cartridge (Waters). The pure peptides were dried by Spedvac (Millipore) and resuspended with 20 µl of 2% ACN, 0.1% FA. Five µl of samples were injected into Orbitrap velos mass spectrometry (Thermo) coupled to a UPLC (Waters) (Thézénas et al., 2013). LC-MS/MS analysis was carried out by nano-ultra performance liquid chromatography tandem MS (nano-UPLC-MS/MS) using a 75 µm-inner diameter x 25 cm C18 nanoAcquity UPLCTM column (1.7-µm particle size, Waters) with a 180 min gradient of 3 – 40% solvent B (solvent A: 99.9% H2O, 0.1% formic acid; solvent B: 99.9% ACN, 0.1% Formic acid). The
Waters nanoAcquity UPLC system (final flow rate, 250 nl/min) was coupled to a LTQ Orbitrap Velos (Thermo Scientific, USA) run in positive ion mode. The MS survey scan was performed in the FT cell recording a window between 300 and 2000 m/z. The resolution was set to 30,000. Maximum of 20 MS/MS scans were triggered per MS scan. The lock mass option was enabled and Polysiloxane (m/z 371.10124) was used for internal recalibration of the mass spectra. CID was done with a target value of 30,000 in the linear ion trap. The samples were measured with the MS setting charge state rejection enabled and only more than 1 charges procures ions selected for fragmentation. All raw MS data were processed to generate MGF files (200 most intense peaks) using the Proteowizard v.2.1.2476 software. The identification of proteins was performed using MGF files with the central proteomics facilities pipeline. Mus musculus (Mouse) database containing entries from UniProtKB was used in CPF Proteomics pipeline for data analysis. This pipeline combines database search results from three search engines (Mascot, OMSSA and X!tandem k-score). The search was carried out using the following parameters. Trypsin was the enzyme used for the digestion of the proteins and only one missed cleavage was allowed. The accepted tolerance for the precursor was 20 ppm and 0.5 Da for the fragment. The search encompassed 1+, 2+ and 3+ charge state, fixed modification for cysteine carbamidomethyl and variable modification for asparagine and glutamine deamidation, and methionine oxidation. All trypsin fragments were added to an exclusion list. False discovery rate was calculated by peptide/proteinprophet or estimated empirically from decoy hits, identified proteins were filtered to an estimated 1% FDR. The label-free analysis was carried out using the normalized spectral index (SINQ) (Trudgian et al., 2011). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org/) via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD004926. (performed by Dr. Honglei Huang and his team at Oxford University)

(methods have been taken directly from Kanellopoulos et al., 2018)

A.1.2 Southern Blot Analysis

The genomic DNA was extracted from either ES cells or tails of mice following the procedures as described (Sambrook and Russell, 2001). The probes for Southern blot were amplified by PCR using mouse genomic DNA isolated from C57BL/6 as a template and purified with a Qiagen Gel Purification Kit. The restriction enzymes StuI, BspHI and PsiI were used to digest genomic DNA for either wild-type and knock-in bands. The sizes of wild-type and
knock-in bands is shown in Fig 2B. The primers used to create probes (5’ external probe: 768 bp; 3’ external probe: 629 bp) included:

5’PF (5’ probe, forward) - ACCAAGCTTTTGATATCACCATCAT
5’PR (5’ probe, reverse) - CAACCTGAGAACAGGTAAGACATGACAGTG
3’PF (3’ probe, forward) - TTGAAGCTTCTGCCCCTATTCCTGCT
3’PR (5’ probe, reverse) – TTAGGATCCATGCACACTACTGACCTTGCTTATAGGT

(methods have been taken directly from Kanellopoulos et al., 2018)

The gene targeting vector was generated using a BAC homologous recombineering-based method (Liu et al., 2003). Four steps were involved in this procedure (Fig 2 – figure supplement 1). Step 1, two short homology arms (HA) HA3 and HA4 corresponding to 509 bp and 589 bp sequences within intron 26 and after exon 27 of Nav1.7, respectively, were amplified by PCR using a BAC bMQ277g11 (Source Bioscience, Cambridge UK) DNA as a template, and then inserted into a retrieval vector pTargeter (Fernandez et al., 2009) (a gift from Dr Seth GN Grant) by subcloning. Step 2, a 9.1 kb genomic DNA fragment (3.4 kb plus 5.8 kb) was retrieved through homologous recombineering by transforming the KpnI-linearized pTargeter-HA3-HA4 vector into EL250 E. coli cells containing BAC bMQ277g11. Step 3, short homology arms HA1 and HA2 corresponding to 550 bp (before stop codon of Nav1.7) and 509 bp (starting from stop codon of Nav1.7) respectively were amplified by PCR, and then cloned into pneoflox vector (Fernandez et al., 2009) (a gift from Dr Seth GN Grant) containing TAP tag, leaving in between the TAP tag sequence, 2 LoxP sites, PGK and EM7 promoters, the G418r gene and a SV40 polyadenylation site. Step 4, the cassette flanked by two homology arms (HA1 and HA2) was excised by XhoI and BglII digestion and transformed into recombination-competent EL250 cells containing pTargeter-HA3-HA4 plasmid. Then, the TAG tag cassette was inserted into the pTargeter-HA3-HA4 vector by recombination in EL250 E. coli cells. The correct recombination and insertion of the targeting cassette were confirmed by restriction mapping and DNA sequencing. The complete gene targeting vector containing 5'-end homologous Nav1.7 sequence of 3.4 kb and a 3'-end homology arm of 5.8 kb was linearized with PmeI digestion for ES cell electroporation. All the homology arms HA1, HA2, HA3 and HA4 were amplified with NEB Phusion PCR Kit using bMQ277g11 BAC clone DNA as a template. Primers used to create the recombination arms included:

HA1XhoIF (HA1, forward) - acaacctcagAGGCCAAAACAAAGTCCAGCT
HA1XbaIR (HA1, reverse) - tgttcttacATTTTCTGCTTCTTCT
HA2Acc65IF (HA2, forward) - tgggtacctagAGCTTCCGGTTTGTGATACACT
To generate TAP tagged Nav1.7 mice, the linearized targeting vector was transfected into 129/Sv ES cells. Cells resistant to G418 were selected by culturing for 9 days. Recombined ES cell clones were identified using Southern blot-based screening. Three Clones that were confirmed to be correct using Southern blot were injected into C57BL/6 blastocysts at the Transgenic Mouse Core facility of the Institute of Child Heath (ICH). The chimeric animals were crossed to C57BL/6 and the germline transmission was confirmed by Southern blot. The neomycin cassette was removed by crossing with global Cre mice. The correct removal of the neomycin cassette and TAP tag insertion was confirmed by Southern blot, genotyping (PCR) and RT-PCR. The genomic DNA was extracted from ear punch and PCR genotyping was performed as described previously (Dicer 2008). Primers used for PCR included:

5aF1 (forward) - ACAGCCTCTACCATCTCTCCACC
3aR4 (reverse) - AACACGAGTGAGTCACCTTCGC

The wild-type Nav1.7 allele and TAP tagged Nav1.7 allele gave a 170bp band and a 411bp band, respectively. The TAP tagged Nav1.7 mRNA was confirmed by RT-PCR. Briefly, the total RNA was extracted from dorsal root ganglia with Qiagen RNEasy kit (Qiagen) and 1.0 µg of total RNA was used to synthesize cDNA using Biorad cDNA script-II synthesize kit with oligo-dT primer. The following primers were used to detect mRNA of Nav1.7:

E25-26-F (forward) - CCGAGGCCAGGGAACAAATTCC
3’UTR-R (reverse) - GCCTGCGAAGGTGACTCACTCGTG

The wild-type and TAP tagged Nav1.7 alleles gave a 1521 bp band and a 1723 bp band, respectively. (methods have been taken directly from Kanellopoulos et al., 2018)

A.1.3 Generation of tap-tagged NaV1.7 mouse

A conventional gene targeting approach to generate an epitope-tagged Nav1.7 mouse was used. The gene targeting vector was constructed using an Escherichia coli recombineering based method (Catterall 2000; Lee et al. 2001) (see figure A.1). A TAP-tag, which contains a HAT domain, a TEV cleavage site and 3x Flag-tags, was inserted into the open reading frame
at the 3’-end prior to the stop codon in exon 27 of SCN9A (NCBI Reference: NM_001290675) (see figure A.1). The final targeting vector construct containing a 5’ homology arm (3.4 kb), a TAP tag, a neomycin cassette and a 3’ homology arm (5.8 kb) was transfected into the 129/Sv embryonic stem (ES) cells. 12 colonies with the expected integration (targeting efficiency was 3.5%) were detected using Southern blot (see figure A.1). Germline transmission and intact TAP tag insertion after removal of the neomycin cassette was confirmed by Southern blot, PCR and RT-PCR. The mouse line generated is henceforth referred to as Nav1.7\textsuperscript{TAP} or Tap-tagged Nav1.7. Breeding and line crossing of heterozygous Nav1.7\textsuperscript{TAP/+} mice revealed no abnormalities in transmission frequency in the offspring of Nav1.7\textsuperscript{TAP/TAP} intercrosses. To verify mRNA expression of Nav1.7\textsuperscript{TAP/TAP}, whole mRNA was extracted from DRG and reverse transcribed into cDNA and PCR genotyped using a standard forward Nav1.7 primer and specific Tap-Tag reverse primer(see figure A.1). Tap-tag mouse was generated by Dr. Jing Zhao and Dr. Yuri D. Bogdanov of the Molecular Nociception group. (methods have been taken directly from Kanellopoulos et al., 2018)
Chapter A
Figure A.1: Generation of TAP-tagged Nav1.7 knock-in mice. (a) The location of TAP tag in the Nav1.7 locus. A sequence encoding a TAP-tag peptide comprised of a HAT domain, TEV cleavage site and 3 FLAG-tags was inserted immediately prior to the stop codon (indicated by the small black square) at the extreme C-terminus of Nav1.7. (b) Schematic diagrams of the targeting strategy. White boxes represent Nav1.7 exons (exon numbers are indicated on the box), grey box represents TAP-tag and black boxes represent homologous arms, respectively. The positions of the external probes used for Southern blotting are indicated below the diagram. Floxed neomycin (neo), DTA expression cassettes and restriction sites are also indicated. Expected fragment sizes for Southern screening are indicted by the arrows and the small triangle box represents the single loxP site. (c) Southern blot analysis of genomic DNA from Founder 1 mice (TAP-tagged Nav1.7 carrying the neo cassette). Genomic DNA was digested with Stul and was then hybridized with either 5’ or 3’ external probe. Wild-type (WT) and mutant alleles were detected as 13.8 kb and 6.6 kb (5’)/7.3 kb (3’) fragments, respectively. (d) Southern blot analysis of the neo deleted TAP-tagged Nav1.7 after Cre recombination. Genomic DNA was digested either with BspHI (5’) or PsfI (3’) and was then hybridized with either the 5’ or 3’ external probe. Wild-type alleles were detected as 5.8 kb (5’) and 8.4 kb (3’), respectively. The neo-deleted TAP-tagged Nav1.7 alleles were detected as 4.5 kb (5’) and 6.1 kb (3’) fragments, respectively. (e) Genotyping analysis by PCR. Representative result of the PCR screening of Nav1.7 TAP mice showing the 411 bp band (knock-in allele) and the 170 bp band (wild-type allele). The primers used for PCR are indicated with black arrows. (f) TAP-tagged Nav1.7 expression analysis with RT-PCR. Total RNA was isolated from DRG of Nav1.7 TAP mice and cDNA synthesis was primed using oligo-dT. PCR was performed with the primers indicated with black arrows. A 1.5 kb wild-type band and a 1.8 kb band were detected from either littermate control animals or Nav1.7 TAP knock-in mice, respectively (performed by Dr. Jing Zhao and colleagues). (methods have been taken directly from Kanellopoulos et al., 2018)
A.2 Generation of tap-tagged NaV1.7 stable cell line

A HEK293 cell line stably expressing TAP tagged Nav1.7 was established as previously described (Koenig et al., 2015). Briefly, a sequence encoding a TAP tag (peptide: SRK DHL IHN VHK EEH AHA HNK IEN LYF QGE LPT AAD YKD HDG DYK DHD IDY KDD) was inserted immediately prior to the stop codon of Nav1.7 in the SCN9A mammalian expression construct FLB (Cox et al., 2006). The TAP tag at the extreme C-terminus of Nav1.7 comprises a HAT domain and 3 FLAG tags, enabling immunodetection with either anti-HAT or anti-FLAG antibodies. The function and expression of TAP tagged Nav1.7 in this HEK293 cell line were characterized with both immunocytochemistry and electrophysiological patch clamp analysis (performed by Dr. Jing Zhao and colleagues).

(methods have been taken directly from Kanellopoulos et al., 2018)

Figure A.2: Schematic diagrams showing the steps for constructing a TAP-tagged NaV1.7 gene targeting vector using BAC homologous recombineering method. Step (1), two short homology arms HA3 and HA4 were amplified by PCR, and then inserted into a retrieval vector pTargeter. Step (2), a 9.1 kb genomic DNA fragment (3.4 kb plus 5.8 kb) was retrieved from BAC clone bMQ277g11 through homologous recombineering. Step (3), Homology arms HA1 and HA2 were amplified and subcloned into pneofox vector. Step (4), the excised TAG tag cassette was inserted into the pTargeter-HA3-HA4 vector by homologous recombineering in EL250 cells. The targeting vector was linearized with PmeI restriction enzyme and was used to generate TAP-tagged NaV1.7 mouse. (This construct was designed and assembled by Dr. Jing Zhao in the molecular nociception lab UCL) (methods have been taken directly from Kanellopoulos et al., 2018)
A.3 Full Length Sodium Binding Protein

![DNA sequence]

**Figure A.3:** This figure corresponds to figure 5.7 in chapter 5. However, the figure in chapter 5 corresponds to a truncated version of the nucleotide sequence. The DNA sequence above corresponds to the complete sequence which was cloned into the pRES-AcTOM plasmid to created the sodium binding protein.
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