Visualisation of the

Native N-type Calcium Channel

Krishma Hanisha Ramgoolam

Doctor of Philosophy

UCL

2019

Department of Neuroscience, Physiology and Pharmacology

Division of Biosciences

Andrew Huxley Building

University College London

Gower Street

London WC1E 6BT
Declaration

I, Krishma Hanisha Ramgoolam confirm that the work presented in this thesis is my own. Where information is derived from other sources, I confirm that this has been indicated in this thesis.

Krishma Hanisha Ramgoolam
Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor Professor Annette Dolphin for giving me the opportunity to work on this research project and for her unwavering support throughout my PhD. Her encouragement, guidance and scientific advice have provided me with a wealth of knowledge and insight into the subject. I could not imagine a better mentor for my PhD study.

I would also like to thank the past and present members of the Dolphin lab. This thesis would not have been possible without the help, support and friendship of Dr Shehrazade Dahimene, Dr Otto Meyer, Dr Manuela Nieto-Rostro, Dr Laurent Ferron, Dr Ivan Kadurin, Dr Karen Page, Kanchan Chaggar, Wendy Pratt and Kjara Pilch. I am particularly grateful to Dr Manuela Nieto-Rostro and Dr Laurent Ferron who have been generous with their time, advice and unsurpassed knowledge which have formed the foundation of my research. I would also especially like to thank Dr Shehrazade Dahimene and Dr Otto Meyer for proofreading my thesis.

Finally, I would like to extend my gratitude to my family. I am grateful for the time that I had with my uncle Vikash, who I know would have been proud to see how far I have come. I would like to unequivocally thank my aunt Teermala and two of the most special people in the world to me, Priyanka and Khushal. Thank you for keeping me sane and never failing to make me laugh. Most of all, I am incredibly grateful to my mum and dad to whom I am indebted for all that I am. I would like to thank them for their constant loving support, all the sacrifices that they have made for me and for always encouraging me to aim higher in every aspect of life. I am and will always remain grateful. This accomplishment would not have been possible without them.

Krishma Hanisha Ramgoolam
Publications

Abstract

N-type calcium channels (Ca\textsubscript{V}2.2) are important for neurotransmitter release in the central and peripheral nervous system. Immunohistochemical detection of native Ca\textsubscript{V}2.2 has not been possible until now due to the low expression of these channels and lack of suitable antibodies. The present study utilises the recently developed constitutive knock-in (KI) transgenic mouse, expressing Ca\textsubscript{V}2.2 with an epitope tag (2 x haemagglutinin; HA) inserted in the extracellular loop between S3 and S4 of domain II (Ca\textsubscript{V}2.2\textsuperscript{HA\textsuperscript{KI}}). The tag does not affect the function of the channel when expressed \textit{in vitro} (Cassidy et al., 2014). In the somatosensory nervous system, the data show that Ca\textsubscript{V}2.2\textsubscript{HA} is expressed on the cell surface of dorsal root ganglion (DRG) neurons. In the spinal cord, Ca\textsubscript{V}2.2\textsubscript{HA} is predominantly in the superficial laminae LI and LII of the dorsal horn, mainly in the primary afferent terminals, since there is a reduction in Ca\textsubscript{V}2.2\textsubscript{HA} staining following rhizotomy. Co-cultures between DRG and spinal cord neurons permit the study of Ca\textsubscript{V}2.2\textsubscript{HA} at the presynaptic terminal. Super-resolution images of the synapses formed by these co-cultures revealed the regulation of Ca\textsubscript{V}2.2\textsubscript{HA} expression at the presynaptic terminal over time in culture. Preliminary studies of TRPV1 activation by capsaicin on cell surface Ca\textsubscript{V}2.2\textsubscript{HA} was tested on cultured DRG neurons. Short incubations (20 s) with capsaicin increases Ca\textsubscript{V}2.2\textsubscript{HA} expression at the cell membrane of small, medium and large-diameter neurons. However, following longer incubations (2 to 4 min) with capsaicin, there is a substantial decrease of Ca\textsubscript{V}2.2\textsubscript{HA} immunoreactivity at the membrane. Nevertheless, further studies are required to determine whether this is mediated by a TRPV1-dependent or -independent mechanism. The Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KI}} mice will be instrumental in future studies to enhance the understanding of the presynaptic role of endogenous N-type calcium channels in physiological and pathological states.
Impact Statement

Chronic pain is a debilitating condition associated with a wide variety of disease states. There is a high unmet medical need for new analgesics which requires not only a better understanding of how existing analgesics work, but also the discovery of novel therapeutic targets. Ion channels are key determinants of neuronal excitability and are a major target for analgesic drug discovery. Voltage-gated calcium channels (VGCCs) are integral components of all excitable cells. These channels play an essential role in the cellular signal transduction which regulate, to name but a few, gene expression and synaptic transmission. The importance of VGCCs is highlighted in a multitude of pathologies, such as neuropathic pain, where their function is dysregulated. VGCCs are multi-subunit complexes composed of the central pore-forming $\alpha_1$ and auxiliary subunits, $\alpha_2\delta$ and $\beta$. A significant role for $\alpha_2\delta$-1 has been established in chronic neuropathic pain. As such, neuropathic pain treatments include the use of the gabapentinoids, gabapentin and the newer derivative pregabalin, which act on the $\alpha_2\delta$-1 subunit. However, current data indicate that gabapentinoids are not effective in relieving on-going pain and pain not associated with nerve injury. Ziconotide, a synthetic version of a $\text{Ca}_V2.2$ channel blocker, has also recently been licensed for chronic pain. But due to the narrow therapeutic window and numerous side effects, ziconotide is only used as a last resort if patients have failed to respond to other treatments. The work presented in this thesis will contribute to the field of VGCCs by examining the native cell surface expression of $\text{Ca}_V2.2$ in the nociceptive pathway. Finally, this thesis reports a substantial advance as it uses a novel mouse model to identify, for the first time, endogenous $\text{Ca}_V2.2$ channels at the plasma membrane. These mice will expand the biochemical tools available to study disease-associated changes in the subcellular distribution of $\text{Ca}_V2.2$ channels in the pain pathway and other neurons with high sensitivity and specificity. This can eventually help answer the still unresolved question of whether $\text{Ca}_V2.2$ is a suitable target for novel analgesic therapies.
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### Abbreviations

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<tbody>
<tr>
<td>AF</td>
<td>Alexa Fluor</td>
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<tr>
<td>AID</td>
<td>Alpha interacting domain</td>
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<td>AKAP</td>
<td>A kinase anchoring protein</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>AP</td>
<td>Action potential</td>
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<td>ara-C</td>
<td>Cytarabine</td>
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<td>Dulbecco’s Modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal Root Ganglia</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EGTA</td>
<td>egtazic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic-reticulum-associated protein degradation</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal–regulated kinases</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMRP</td>
<td>Fragile X mental retardation protein</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-amino-butryic acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GFL</td>
<td>GDNF family of ligands</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GFR</td>
<td>GDNF family receptors</td>
</tr>
<tr>
<td>G&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Conductance</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks' Balanced Salt Solution</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Horse serum</td>
</tr>
<tr>
<td>HVA</td>
<td>High voltage-activated</td>
</tr>
<tr>
<td>IB4</td>
<td>Isolecin B4</td>
</tr>
<tr>
<td>IQ</td>
<td>Isoleucine-glutamine</td>
</tr>
<tr>
<td>KI</td>
<td>Knock-in</td>
</tr>
<tr>
<td>KRH</td>
<td>Krebs–Ringer–Hepes</td>
</tr>
<tr>
<td>KV</td>
<td>Voltage-gated potassium channel</td>
</tr>
<tr>
<td>L15</td>
<td>Leibovitz's</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>Levo-DOPA</td>
</tr>
<tr>
<td>LC</td>
<td>Light chain</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>Levo-DOPA</td>
</tr>
<tr>
<td>LVA</td>
<td>Low voltage-activated</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>mEPSC</td>
<td>Miniature post-synaptic current</td>
</tr>
<tr>
<td>MFC</td>
<td>Microfluidic chamber</td>
</tr>
<tr>
<td>MOM</td>
<td>Mouse-on-mouse</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MT</td>
<td>Melatonin receptor</td>
</tr>
<tr>
<td>NaV</td>
<td>Voltage-gated sodium channel</td>
</tr>
<tr>
<td>NF200</td>
<td>Neurofilament 200</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NK</td>
<td>Neurokinin</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide sensitive fusion</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>ORL</td>
<td>Opioid receptor-like</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase-chain reaction</td>
</tr>
<tr>
<td>PDVF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>PF</td>
<td>Post-fix</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>PP1</td>
<td>Protein phosphatase 1</td>
</tr>
<tr>
<td>PP2</td>
<td>Protein phosphatase 1/calcineurin</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>PtFPs</td>
<td>Phototransformable fluorescent proteins</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>Rab</td>
<td>Ras associated binding proteins</td>
</tr>
<tr>
<td>Ret</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RFP</td>
<td>RING-finger protein</td>
</tr>
<tr>
<td>RIM</td>
<td>Rab3-interacting molecule</td>
</tr>
<tr>
<td>RIM-BP</td>
<td>RIM-binding protein</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SM</td>
<td>Sec1/Munc18</td>
</tr>
<tr>
<td>SNAP</td>
<td>Synaptosomal associated protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble NSF Attachment Receptor</td>
</tr>
<tr>
<td>Synprint</td>
<td>Synaptic protein interaction</td>
</tr>
<tr>
<td>TAT</td>
<td>Trans-activator of transcription</td>
</tr>
<tr>
<td>TG</td>
<td>Trigeminal ganglia</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>trkA</td>
<td>Tyrosine kinases receptor A</td>
</tr>
<tr>
<td>TRPA</td>
<td>Transient receptor potential ankyrin</td>
</tr>
<tr>
<td>TRPM</td>
<td>Transient receptor potential melastatin</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential vanilloid</td>
</tr>
<tr>
<td>TSA</td>
<td>Tyramide Signal Amplification</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle associated membrane protein</td>
</tr>
<tr>
<td>VDI</td>
<td>Voltage-dependent inactivation</td>
</tr>
<tr>
<td>vGluT</td>
<td>Vesicular glutamate transporter</td>
</tr>
<tr>
<td>vGpH</td>
<td>vGluT phluorin</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>$V_{rev}$</td>
<td>Reversal potential</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1 Background

1.1.1 Plasma Membrane

In mammalian cells, the plasma membrane provides a barrier between intracellular spaces and the exterior environment. The plasma membrane is composed of a phospholipid bilayer which is partially permeable. Phospholipids are amphipathic lipids that consist of hydrophilic ‘head’ and hydrophobic ‘tail’ groups. The phospholipid bilayer forms the foundation for the plasma membrane structure and permits the diffusion of non-polar molecules such as O$_2$ and CO$_2$ whilst remaining impermeable to ions. Ions can cross the plasma membrane through pumps, transporters and ion channels. These processes maintain a difference in ionic concentrations when comparing the inside with the outside of the cell. This generates and maintains an electrochemical gradient across the phospholipid bilayer which creates a membrane potential.

1.1.2 Resting Membrane Potential

The resting membrane potential is determined by the difference in concentration of ions across the plasma membrane of the cell. The predominant ions involved in generating the potential difference across the cell membrane are Sodium (Na$^+$), Potassium (K$^+$), Calcium (Ca$^{2+}$), Chlo-
ride (Cl⁻) and Bicarbonate (HCO₃⁻) ions (approximate concentrations shown in Figure 1.1A).

The Nernst equation (Figure 1.1B) can be used to determine the equilibrium potential for each ion shown in Figure 1.1A. The resting membrane potential varies but in neurons ranges from -60 mV to -90 mV. The K⁺ ion is the closest to being in electrochemical equilibrium when the cell is at rest. This implies that the resting membrane is more permeable to the K⁺ ion and that this permeability is the source of the resting potential. The maintenance of the resting membrane potential is determined by the Na⁺-K⁺ adenosine triphosphatase (ATPase) pump (Brodie et al., 1987; Pivovarov et al., 2019). The Na⁺-K⁺ pumps utilise ATP as an energy source to transport two K⁺ into and three Na⁺ ions out of the cell. This process is essential for maintaining the resting membrane potential in neurons and other cells.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intracellular</td>
</tr>
<tr>
<td>K⁺</td>
<td>140</td>
</tr>
<tr>
<td>Na⁺</td>
<td>5-15</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>4-30</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Figure 1.1: Typical ion concentrations for mammalian cells and Nernst equation
(A) Table of extracellular and intracellular ion concentrations of mammalian neurons under physiological conditions (B) Nernst Equation for calculating the potential of a cell: $E_{cell} = E^0 - \frac{RT}{nF} \ln Q$
1.1.3 Ion Channels

The neuronal plasma membrane contains ion channels which are transmembrane proteins that permit the highly selective transport of ions across the phospholipid bilayer. The movement of ions through their respective ion channels controls neuronal excitability. Ion channels have an aqueous pore which is accessible to ions following a conformational change in the channel protein structure causing it to open. The conductance of ions through their respective channels is extremely fast as ions pass down their electrochemical gradient. The archetypal ion channel is governed by a ‘gate’ which can open or close in response to stimuli. There are mainly two types of ion channels: ligand-gated and voltage-gated ion channels. Ligand-gated ion channels are opened by the binding of a ligand such as a hormone or neurotransmitter whereas voltage-gated ion channels respond to changes in local membrane potentials. Ion channels are essential in establishing the resting membrane potential and shaping the action potentials of neurons.

1.1.4 Action Potentials

The Hodgkin-Huxley model describes the fundamental mechanism for the propagation of action potentials through which information is carried throughout the nervous system. Work by Hodgkin and Huxley on the giant squid axon established the time- and voltage- dependence of inward Na\(^+\) and outward K\(^+\) current (Hodgkin and Huxley, 1952\(a,b,c\)). This work was used to propose a model which accurately predicts the shape of the action potential (Hodgkin and Huxley, 1952\(d\)). The work by Hodgkin and Huxley established a framework in which later studies further detailed the coordinated activity of ion channels during the generation of action potentials. Indeed, later it was found that the voltage-gated sodium (\(N_{aV}\)) channels fully accounted for the increase in Na\(^+\) conductance upon depolarisation of the neuron which was
blocked by tetrodotoxin, a \( \text{Na}_V \) channel blocker, whilst \( \text{K}^+ \) conductance remained intact (Narahashi et al., 1964).

An action potential is generated when the membrane of an excitable cell is sufficiently depolarised from a negative to a more positive potential. During depolarisation, \( \text{Na}_V \) channels open allowing an influx of \( \text{Na}^+ \) down its electrochemical gradient which changes the charge inside the cell from net negative to positive. An action potential is generated only if a voltage-threshold (\( \sim -55 \text{ mV} \)) is reached; as such action potentials are described as an ‘all or nothing’ response. During the depolarisation stage of the action potential, the influx of \( \text{Na}^+ \) causes the membrane potential to increase to \( \sim +40 \text{ mV} \), after which the \( \text{Na}_V \) channels inactivate. The raised positive charge inside the cell activates voltage-gated potassium (\( \text{K}_V \)) channels which open to allow \( \text{K}^+ \) to move down its electrochemical gradient. As \( \text{K}^+ \) moves out of the cell, the membrane potential repolarises. Normally, repolarisation overshoots the resting membrane potential resulting in hyperpolarisation. After every action potential there is a refractory period in which \( \text{Na}_V \) and \( \text{K}_V \) channels remain inactive and cannot be re-opened. Thus, no further action potentials can be generated. It is important to note that the amplitude of the action potential is constant for any depolarising stimulus. As such, the frequency and not the size of action potentials encodes the information sent. Action potentials are propagated along the neuron via local currents which depolarise the adjacent axonal membrane. The action potential only travels in one direction as the areas of membrane which have been recently depolarised will not depolarise again due to the refractory period (Figure 1.2).
At rest, the neuronal plasma membrane has a membrane potential around -70 mV (resting membrane potential). Stimulation of the neuron depolarises the membrane. When the stimulus is large enough, the membrane potential rises above -55 mV and the influx of Na\(^{+}\) ions takes the membrane potential to +40 mV. At this potential, K\(_{V}\) channels open, allowing and influx of K\(^{+}\) ions resulting in repolarisation of the membrane potential. Following hyperpolarisation of the membrane the membrane potential returns to the resting potential.

### 1.1.5 Neurons

Neurons are specialised cells which carry information throughout the central and peripheral nervous system (CNS and PNS). In the human nervous system, there are approximately 86 billion neurons (Herculano-Houzel, 2009). One of the many ways in which neurons are adapted to best suit their role is through their differential expression of ion channels on the neuronal plasma membrane. The rich repertoire of ion channels encodes a wide range of action potential frequencies and patterns (Bean, 2007).
There are three basic parts to a neuron: the cell body (soma), axon and dendrites (Figure 1.3). The axon is a generally thin and long structure which projects from the cell body. Once an action potential has reached the end of an axon, the information is transmitted across the synaptic gap to the dendrite of the adjacent neuron. Dendrites extend from the soma and contain neurotransmitter receptors permitting the communication between neurons (Arimura and Kaibuchi, 2007).

Figure 1.3: Schematic diagram of the neuron

Generic diagram of the pre- and postsynaptic neuron. Neurons showing cell body containing a nucleus and dendritic projections from the cell body. Axons are surrounded by insulating myelin sheath. The presynaptic neuron connects with a postsynaptic neuron via a synapse for signal transmission.

1.1.6 Synapses: Chemical and Electrical

There are two main modalities of synaptic transmission: chemical and electrical. Chemical synapses mediate the functional interaction between neurons or neurons and target tissue through neurotransmitters. The arrival of an action potential at the presynaptic terminal depolarises the membrane, triggering the influx of Ca\(^{2+}\) through voltage-gated calcium channels (VGCCs). Neurotransmitters are released upon Ca\(^{2+}\)-mediated synaptic vesicle exocytosis into the synaptic cleft where they bind and activate receptors on the postsynaptic membrane (Figure 1.4B). The importance of depolarisation-mediated Ca\(^{2+}\) influx in this fundamental mechanism was first studied in the neuromuscular junction by Katz and Miledi (1965), prior...
to the identification of VGCCs. Additionally, electrophysiological studies on neurotransmitter release have shown that the presynaptic action potential is tightly coupled to Ca\(^{2+}\) entry and synaptic vesicle fusion. Under physiological conditions, Ca\(^{2+}\) influx orchestrates the fusion of neurotransmitter vesicles in a few hundred microseconds (Sabatini and Regehr, 1999). Neurotransmission occurs at the active zone. The active zone is a specialised area of presynaptic membrane characterised by the presence of docked synaptic vesicles and release machinery which is aligned with the postsynaptic density (reviewed in Südhof (2012); Michel et al. (2015)).

Electrical synapses, although they are a discrete minority, are found throughout the nervous system. At the electrical synapse, the membranes of communicating neurons are associated with each other through intercellular specialisations called gap junctions. Gap junctions are composed of aligned paired channels in the plasma membrane of the pre- and postsynaptic neuron. The precise alignment of each pair of channel forms a pore (Figure 1.4A). The pore of a gap junction is large enough to allow the diffusion of ions and high molecular weight molecules from the cytoplasm of pre- and postsynaptic neurons. Thus, electrical synapses work by permitting the passive flow of ionic current through gap junctions. The passive current flow across gap junctions allow for the virtually instantaneous communication between neurons which can occur without the characteristic delay of chemical synapses.
1.1.7 Neurotransmitter release

The components of the presynaptic active zone are integrally involved in a multitude of events which govern synaptic vesicle release and will be described here (Figure 1.5). Soluble NSF Attachment REceptor (SNARE) and Sec1/Munc18-like (SM) proteins undergo a cycle of assembly and disassembly mediating synaptic vesicular fusion events. The vesicular SNARE proteins consist of synaptobrevin (also known as VAMP – vesicle associated membrane protein) and synaptotagmin. Prior to membrane fusion, synaptic vesicles dock at the membrane of the presynaptic active zone through the formation of a trans-SNARE complex. The trans-SNARE complex is composed of synaptobrevin and the presynaptic membrane SNAREs: syntaxin-1 and Synaptosomal Associated Protein-25 (SNAP-25) (Söllner et al., 1993). The SM protein attaches to syntaxin-1 in the assembling SNARE complex (Hata et al., 1993; Dulubova et al.,
Following the partial assembly of the trans-SNARE complex, synaptotagmin binds to syntaxin-1 and the SNARE complexes to mediate fusion-pore opening (Bennett et al., 1992). All synaptotagmin-mediated fusion is dependent on complexin which acts as a cofactor to complete synaptic vesicle priming (Reim et al., 2001). The complex is now “super-primed” and awaits Ca\(^{2+}\) to bind to synaptotagmin to trigger fusion-pore opening (McMahon et al., 1995). Ca\(^{2+}\)-triggered fusion-pore opening involves the zipping of the four-helical SNARE proteins embedded in the fusing membranes in an N- to C-terminal direction. This forces the synaptic vesicle and presynaptic membrane to come into close proximity with one another, destabilising their hydrophilic surfaces (Hanson et al., 1997). During fusion-pore expansion, N-ethylmaleimide sensitive fusion (NSF) and SNAP proteins bind to form the fully assembled cis-SNARE complex. Following completion of fusion-pore opening, the cis-SNARE complexes are disassembled by the NSF/SNAP ATPases and the components of the complex are recycled (Söllner et al., 1993).
Figure 1.5: Schematic diagram of synaptic machinery mediating docking, priming and fusion of synaptic vesicles (taken from Südhof., 2013)

Assembly and disassembly of SNARE and SM proteins. Vesicular SNARE proteins synaptobrevin and synaptotagmin bind presynaptic SNARES, syntaxin-1 and SNAP-25. The SM protein Munc-18 and complexin joins the SNARE complex. \( \text{Ca}^{2+} \) enters through VGCCs, which binds to synaptotagmin triggering fusion-pore opening. Disassembly of synaptic machinery occurs through ATPase with the proteins recycled for future fusion events.

1.1.8 Spatial Organisation of Synaptic Release Machinery

Fast neurotransmitter release can only be achieved by tethering VGCCs to docked and primed synaptic vesicles at the presynaptic active zone. Rab3-interacting molecule (RIM), RIM-Binding Protein (RIM-BP) and Munc13 complexes mediate docking and priming of synaptic vesicles and recruit VGCCs to these vesicles (Kaesen et al., 2011). RIM docks synaptic vesicles by binding to Ras associated binding proteins (Rabs) such as Rab3 and Rab27 GTP-binding proteins which are situated on these vesicles (Gracheva et al., 2008; Fernández-Busnadiego et al., 2013). RIM also binds and activates Munc13 (Deng et al., 2011). Munc13
acts as a priming factor as it catalyses the conformational change of syntaxin-1 from a close to an open state, promoting SNARE complex assembly (Ma et al., 2013). RIM also binds to RIM-BP and VGCCs (Hibino, 2002). The importance of RIM and RIM-BP is highlighted by RIM knockout mice and the deletion of RIM-BP in Drosophila (Kaeser et al., 2011; Liu et al., 2011). These studies showed that the loss of either RIM or RIM-BP resulted in the loss of VGCCs and VGCC-mediated Ca^{2+} influx at presynaptic active zones (Kaeser et al., 2011). These data demonstrate that RIM and RIM-BP work collectively to recruit VGCCs to the presynaptic release site. It is essential for VGCCs to be localised adjacent to docked and primed synaptic vesicles to ensure the fast coupling of the arrival of an action potential with Ca^{2+}-triggered exocytosis. Typically, VGCCs are positioned less than 100 nm from docked vesicles (Eggermann et al., 2012).
1.2 Voltage-Gated Calcium Channels

VGCCs are essential in the physiology of all excitable cells such as endocrine, skeletal, heart, muscle and neuronal cells. Under normal physiological conditions, the concentration of neuronal intracellular Ca\(^{2+}\) lies at \(\sim 100\) nM whilst extracellularly it is \(\sim 1\) mM, thus providing a large concentration gradient across the plasma membrane. An influx of Ca\(^{2+}\) can trigger a cascade of cellular events such as the regulation of cell growth and gene transcription (Simmons, 1988; Simms and Zamponi, 2014). At presynaptic nerve terminals, Ca\(^{2+}\) entry (through VGCCs) mediates Ca\(^{2+}\)-dependent neurotransmitter release from synaptic vesicles (Dunlap et al., 1995). The low intracellular Ca\(^{2+}\) concentrations allow for small Ca\(^{2+}\) influxes to produce large and quick changes in local Ca\(^{2+}\) concentrations. Nevertheless, intracellular Ca\(^{2+}\) concentrations must be tightly regulated as prolonged elevation can lead to cytotoxicity (Stanika et al., 2012). The dysregulation of Ca\(^{2+}\) homeostasis levels can, therefore, lead to neurological disorders such as pain and epilepsy (Cain and Snutch, 2011). The specific function of individual VGCCs is dependent on their expression, distribution and subcellular localisation within cells.

1.2.1 Voltage-Gated Calcium Channels

VGCCs are composed of a central pore-forming \(\alpha_1\) subunit encoded by the \textit{CACNA1x} genes. To date, 10 isoforms of the \(\alpha_1\) subunit have been identified in the mammalian genome (Ertel et al., 2000). VGCCs can be further divided into two categories: high-voltage activated (HVA) channels which respond to large changes in membrane potentials and have variable rates of inactivation; the second, low-voltage activated (LVA) channels are activated by small membrane depolarisations and rapidly inactivate (Carbone and Lux, 1984; Fedulova et al., 1985). The \(\alpha_1\) subunit, which is the key determinant of VGCC properties, can be categorised into
three major families – Ca\textsubscript{V}1, Ca\textsubscript{V}2 (HVA family) and Ca\textsubscript{V}3 (LVA family) (Figure 1.6).

Figure 1.6: Dendrogram of the different subclasses of $\alpha_1$ subunits
The VGCC family can be divided into three subclasses according to their amino acid sequence. The Ca\textsubscript{V}1 and Ca\textsubscript{V}2 classes are loosely termed high voltage activated (HVA) channels. HVA channels are associated with $\beta$ and $\alpha_2\delta$ subunits. The Ca\textsubscript{V}3 $\alpha_1$ all form low voltage activated (LVA) channels. The original names (shown in blue) and Ca\textsubscript{V} nomenclature (shown in red).

The HVA currents can be further divided into dihydropyridine (DHP)-sensitive and -insensitive (Hess et al., 1984; Stanley and Atrakchi, 1990). A subset of HVA currents are relatively ‘long’ lasting, resulting in a subgroup of VGCCs being termed ‘L-type’ channels. Ca\textsubscript{V}1 (L-type) channels are ubiquitously found in skeletal, smooth and cardiac muscle and are selectively blocked by DHP agonists (Randall and Tsien, 1995).

To identify the channels responsible for the remaining DHP-insensitive HVA currents, various peptide neurotoxins were used (Olivera et al., 1984, 1991). This allowed for the identification of the Ca\textsubscript{V}2 family which is responsible for neurotransmitter release at synapses and neuromuscular junctions. The P-type channels were termed after their identification in Purkinje neurons. P-type calcium channels are selectively blocked by, a funnel web spider neurotoxin, $\omega$-agatoxin IVA which binds to extracellular elements of the channel and influences the voltage sensors (Hillman et al., 1991; Mintz et al., 1992). The Q-type channel exhibits less sensitivity
to \(\omega\)-agatoxin IVA and was identified in cerebellar granule cells. However, the Q-type channel is attributed to an alternatively spliced variant of the P-type channel, as such, the channel is now referred to as P/Q-type (Randall and Tsien, 1995; Bourinet et al., 1999). \(\text{Ca}_V^{2.2}\) encodes the ‘Neuronal’, N-type, channels which are inhibited by \(\omega\)-conotoxins GVIA and MVIIA, derived from the cone snail \textit{Conus geographus} and \textit{magus}, respectively (Nowycky et al., 1985; McCleskey et al., 1986; Olivera et al., 1987). When all HVA current components are blocked the “residual” current can be attributed to the R-type calcium channel which is blocked by SNX-482 (Niidome et al., 1992; Newcomb et al., 1998).

Finally, the LVA currents inactivate quickly resulting in ‘transient’ currents, which led to these VGCCs being termed T-type channels. \(\text{Ca}_V^{3}\) (T-type) channels have a pace-making responsibility in cardiac muscle and neuronal cells. Blockers of the T-type channel include mibefradil, carbamazepine, phenytoin and ethosuximide. Moreover, \(\text{Ca}_V^{3}\) channels can be discriminated by their sensitivity to \(\text{Ni}^{2+}\) (Perez-Reyes et al., 1998). Tissue distribution and specific blockers of different VGCCs are summarised in Figure1.7.
1.2.2 Distribution of VGCCs in the Nervous System

The different $\alpha_1$ subtypes are all present, in varying degrees, throughout the nervous system (summarised in Figure 1.7). In the majority of synapses in the rat hippocampus, Cav1 channels are found postsynaptically rather than presynaptically (Zhang et al., 2005; Tippens et al., 2008). The L-type calcium channels are typically found in the somatodendritic compartment of central neurons where they regulate membrane excitability (Morgans, 2000). In the PNS, Cav1 channels are involved in the somatic release of substance P from isolated dorsal root ganglion (DRG) neurons (Harding et al., 1999).

<table>
<thead>
<tr>
<th>Type</th>
<th>VGCC</th>
<th>Tissues</th>
<th>Blockers</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>Cav1.1</td>
<td>Skeletal muscle</td>
<td>DHPs</td>
<td>Excitation-contraction coupling, Calcium homeostasis, Gene regulation</td>
</tr>
<tr>
<td></td>
<td>Cav1.2</td>
<td>Cardiac muscle, Endocrine cells, Neurons</td>
<td>DHPs</td>
<td>Excitation-contraction Coupling, Calcium homeostasis, Gene regulation</td>
</tr>
<tr>
<td></td>
<td>Cav1.3</td>
<td>Endocrine Cells, Neurons</td>
<td>DHPs</td>
<td>Hormone secretion, Gene regulation</td>
</tr>
<tr>
<td></td>
<td>Cav1.4</td>
<td>Retina</td>
<td>DHPs</td>
<td>Tonic neurotransmitter release</td>
</tr>
<tr>
<td>P/Q</td>
<td>Cav2.1</td>
<td>Nerve terminals, Dendrites</td>
<td>W-Agatoxin, IVA</td>
<td>Neurotransmitter release, Dendritic Ca$^{2+}$ transients</td>
</tr>
<tr>
<td>N</td>
<td>Cav2.2</td>
<td>Nerve terminal Dendrites</td>
<td>W-Conotoxin, GVIA</td>
<td>Neurotransmitter release, Dendric Ca$^{2+}$ transients</td>
</tr>
<tr>
<td>R</td>
<td>Cav2.3</td>
<td>Cell bodies, Nerve terminals, dendrites</td>
<td>SNX-482</td>
<td>Neurotransmitter release</td>
</tr>
<tr>
<td>T</td>
<td>Cav3.1</td>
<td>Cardiac muscle, Skeletal muscle, Neurons</td>
<td>TTA-A2, TTA-P2 block for all Cav3</td>
<td>Pacemaking and repetitive firing action potential</td>
</tr>
<tr>
<td></td>
<td>Cav3.2</td>
<td>Cardiac muscle, Neurons</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cav3.3</td>
<td>Neurons</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>
There is increasing evidence that Cav3 channels are implicated in neurotransmitter release in the retina, olfactory bulb and in hippocampal interneurons (Pan et al., 2001; Egger et al., 2003; Tang et al., 2011). In the PNS, T-type calcium channels are responsible for the trans-glial and neuron-glial communication within the DRG (Rozanski et al., 2013). The biophysical characteristics of T-type channels, specifically their low voltage dependence of activation and inactivation, supports their role in low threshold exocytosis.

In the CNS and PNS, VGCCs are essential mediators of depolarisation-evoked release of neurotransmitters. As mentioned above, to ensure efficient Ca\(^{2+}\) coupling to synaptic vesicle release, VGCCs are localised at the active zones of presynaptic terminals. Most synapses in the nervous system rely on Cav2.1 and Cav2.2 for fast synaptic transmission (Wheeler et al., 1994). Cav2.3 channels can also mediate neurotransmitter release, however, this channel possesses different syntaxin structural determinants (Davies et al., 2011). The above underscores the importance of how the differential expression of VGCCs and interacting partners in neurons (Figure 1.8) is tailored to specific neurophysiological functions.

**Figure 1.8: Summary of VGCCs subcellular distribution and function in neurons**

Distribution and function of VGCCs in neurons of the (A) CNS and (B) PNS (adapted from Dolphin., 2012)
1.2.3 Structure and Composition of VGCCs

The discovery of the first HVA channels was made using biochemical preparations of transverse tubule membranes of skeletal muscles. The skeletal muscle is highly enriched in $\text{Ca}_V 1.1$ channels which bind with high affinity to the channel blockers of the DHP class, including nifedipine (Curtis and Catterall, 1984). The purification of the $\text{Ca}_V 1.1$ revealed the channel complex to contain five components: $\alpha_1$ (175 kDa), auxiliary $\alpha_2$ (143 kDa), $\beta$ (54 kDa), $\gamma$ (30 kDa) and $\delta$ (24-27 kDa) subunits (Takahashi et al., 1987).

1.2.4 Structure of the $\alpha_1$ Subunit

VGCCs are closely related to $\text{Na}_V$ channels reflecting their shared ancestry (Catterall, 1996; French and Zamponi, 2005). Structure-function studies of bacterial $\text{Na}_V$ channels have enhanced our understanding of VGCCs (Ren et al., 2001; Koishi et al., 2004). However, more recent structural information about VGCCs has come from cryo-electron microscopy studies of $\text{Ca}_V 1.1$ (Wu et al., 2016). All ten $\text{Ca}_V \alpha_1$ subunits share a common topology. The $\alpha_1$ subunit contains the channel pore, voltage sensor and selectivity filter. The topology of $\alpha_1$ consists of four homologous transmembrane domains each containing six membrane-spanning $\alpha$ helices (S1-S6) (Figure 1.9) (Dolphin, 2006; Catterall, 2011).
The VGCC $\alpha_1$ subunit is organised into four homologous repeats (I-IV). Each domain repeat is composed of six transmembrane segments (S1-S6). The fourth transmembrane segment of each repeat (S4; shown in blue) is the voltage-sensing domain of the channel. The six transmembrane units are connected by loop regions (pink). Between S5 and S6 there is a re-entrant P-loop (shown in red). The $\beta$ subunit binds to the AID sequence of the domain I-II linker (shown in green) to promote the refolding of the loop and channel trafficking. $\alpha_2\delta$ is anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI)-linker and is located on the extracellular side of the plasma membrane.

1.2.5 Voltage Sensor of the $\alpha_1$ Subunit

The gating process of VGCCs determines the opening and closing of the channel, which allows or denies $\text{Ca}^{2+}$ influx, in response to changes in membrane potential. VGCC gating involves movement of the voltage-sensing domains as well as the opening and closing of the S6 gate. The S5 and S6 helices form the activation gate at the intracellular end of the transmembrane domain. In the resting state, the ends of the S6 helices converge at the intracellular side of VGCCs, occluding the pore which denies $\text{Ca}^{2+}$ entry (Wu et al., 2016).

The S4 segments of each homologous domain serve as the voltage sensor. The voltage sensor includes positively charged amino acids. At rest, the S4 sensor sits in the ‘down-state’ below the charge transfer centre (CTC). The CTC is composed of highly conserved negative or polar residues as well as a highly conserved hydrophobic residue on S2 (Wu
et al., 2016). Depolarisation of the membrane causes the S4 domain to move to an ‘activated-up’ position whereby the sensor moves outward and rotates under the influence of the electric field, initiating a conformational change of the S6 gates opening the channel pore (Domene et al., 2005). Upon membrane repolarisation, the S4 domains enter the ‘down-state’ and permit the S6 units to obstruct and close the VGCC (Domene et al., 2005; Hering et al., 2018).

1.2.6 Channel Pore of the $\alpha_1$ Subunit

The central pore of the $\alpha_1$ subunit is lined by the transmembrane helices of S5 and S6 and the re-entrant P-loop motif which together form the permeation pathway in VGCCs. Each P-loop region contains highly conserved negative residues (glutamate for HVA channels) which form a pore that is selective for permeant cations such as Ca$^{2+}$, barium (Ba$^{2+}$) and strontium (Sr$^{2+}$) (Ellinor et al., 1995; Tang et al., 2014; Bourinet et al., 1996).

The selectivity of VGCCs is particularly important as under normal physiological conditions Ca$^{2+}$ concentrations are lower (50-100 fold) than other cations such as Na$^+$ and K$^+$. Using crystallographic analyses of the Ca$^{2+}$ selectivity filter constructed into the bacterial Na$_V$ channel, Tang et al. (2014) suggest that there are three consecutive Ca$^{2+}$ binding sites (sites 1-3) which permit two interchangeable functional states of the selectivity filter. These authors show that the conserved glutamate residues allow for high affinity binding of Ca$^{2+}$ (site 1 and 2), but only site 2 mediates divalent cation block. The electrostatic repulsion between Ca$^{2+}$ ions make it energetically unfavourable to bind adjacent sites simultaneously. Tang et al. (2014) propose that in state 1, site 1 and 3 are occupied by Ca$^{2+}$ ions. In state 2, a single Ca$^{2+}$ ion occupies site 2. The switch between these two states occurs when Ca$^{2+}$ jumps from site 1 or 3 to site 2. In high extracellular Ca$^{2+}$ concentrations, Ca$^{2+}$ will prefer to enter site 1 causing Ca$^{2+}$ bound weakly at site 3 to move into the intracellular solution. This results in the ‘knock-off’ mecha-
nism of ion permeation permitting the unidirectional flow of \( \text{Ca}^{2+} \) into the cell through VGCCs (Figure 1.10).

![Figure 1.10: Catalytic cycle for \( \text{Ca}^{2+} \) conductance by VGCCs](image)

An ionic occupancy state diagram of a VGCC pore showing two proposed low energy states and the potential transitions that connect them. Each state of the selectivity filter is represented by a three-box rectangle with Sites 1–3 going from left to right. Green circles represent \( \text{Ca}^{2+} \) ions. Note that transitions in the inner circle potentially lead to ion repulsion, which might facilitate conduction. These transitions in the inner circle are more probable than those in the outer circle, as denoted by the bold arrows (taken from Tang et al., 2014)
1.3 Regulations of VGCCs

VGCCs have evolved as a crucial player in Ca\(^{2+}\) entry permitting a diverse number of cellular functions. In turn, the fine-tuning of VGCC activity is tightly regulated at translational and post-translational levels as well as through protein-protein interactions. This section explores the different regulatory pathways controlling the function of VGCCs.

1.3.1 G protein Regulation of VGCCs

VGCC activity is tightly regulated by G protein coupled receptors (GPCRs), including those activated by dopamine, opioids and glutamate. GPCRs are seven-transmembrane domain receptors, binding to which triggers intracellular signalling pathways by governing the activity of heterotrimeric G proteins. Heterotrimeric G proteins are composed of a G\(\alpha\) subunit which binds and hydrolyses guanosine triphosphate (GTP) to guanosine diphosphate (GDP), and the G\(\beta\) and G\(\gamma\) subunits (which form the G\(\beta\gamma\) dimer) (Wettschureck and Offermanns, 2005).

In the absence of ligand-binding, G\(\alpha\) is bound to GDP and the G\(\beta\gamma\) dimer, and is found in complex with the receptor. The binding of an extracellular ligand induces a conformational change in the GPCR. This promotes the exchange of GDP to the GTP-bound G\(\alpha\) subunit. Following this, both the GTP-bound G\(\alpha\) subunit and G\(\beta\gamma\) dimer dissociates from the receptor. The GTP-bound G\(\alpha\) subunit and G\(\beta\gamma\) dimer mediates intracellular signalling pathways and can regulate the activity of VGCCs. G\(\alpha\)-GTP activates adenylyl cyclase which catalyses the conversion of adenosine triphosphate (ATP) to the second messenger, 3'-5'-cyclic AMP (cAMP) which activates Protein Kinase A (PKA). In turn, PKA can phosphorylate VGCCs (Arnot et al., 2000). After the hydrolysis of GTP by the G\(\alpha\) subunit, the GDP-bound G\(\alpha\) subunit re-associates with the G\(\beta\gamma\) dimer, terminating G protein signalling.

Inhibition of VGCCs involves the direct binding of G\(\beta\gamma\) dimers onto various structural determi-
nants of the $\alpha_1$ subunit (Boland and Bean, 1993; Wu and Saggau, 1997). The binding of $G_{\beta\gamma}$ to the $\alpha_1$ subunit stabilises the close conformational state of VGCCs. $G_{\beta\gamma}$-mediated inhibition of VGCCs is less pronounced at depolarised potentials and this modulation was thus named voltage-dependent inhibition (VDI) (Bean, 1989). In $\text{Ca}_V2$ channels, it is thought that $G_{\beta\gamma}$ dimer dissociation from the $\alpha_1$ subunit is not directly due to the change in membrane potential but the conformation changes during the opening of $\text{Ca}_V2$ channels, thus making the modulation intrinsically channel opening-dependent rather than voltage-dependent (Weiss et al., 2006).

A plethora of studies have reported the physical coupling of VGCCs and GPCRs, either indirectly via G proteins, or a direct physical interaction. In particular, the $G_{\beta\gamma}$ dimer regulates $\text{Ca}_V2.2$ channels through binding to the I-II linker and N-terminal of the $\alpha_{1B}$ subunit (Canti et al., 1999). In DRG neurons, prolonged exposure to the ORL1 receptor agonist, nociception, triggers internalisation of the N-type calcium channels. This effect is dependent on G-proteins which produces a down-regulation of $\text{Ca}^{2+}$ entry which parallels the selective removal of N-type calcium channels from the plasma membrane (Beedle et al., 2004; Altier et al., 2006). Furthermore, the dopamine (D)1 and D2, melatonin 1 (MT1) and gamma-amino-butyric acid (GABA$_B$) receptors have all been reported to interact directly with $\text{Ca}_V2.2$ to regulate channel inhibition (Kisilevsky and Zamponi, 2008; Laviv et al., 2011; Benleulmi-Chaachoua et al., 2016). A direct physical association of the C-terminal of $\text{Cav}_2.1$ with metabotropic glutamate receptors (mGluR1) has also been reported in cerebellar Purkinje neurons (Kitano et al., 2003). The physical interaction of GPCRs with $\text{Ca}_V2$ controls the activity of the channel, thus providing an extra level of $\text{Ca}^{2+}$ influx modulation.
1.3.2 \( \text{Ca}^{2+}\)-dependent Inactivation of VGCCs

\( \text{Ca}^{2+}\)-dependent inactivation (CDI) mechanisms provide a crucial negative feedback system to prevent excessive \( \text{Ca}^{2+}\) entry after VGCC activation. Experiments in the Paramecium by Brehm and Eckert (1978) found that inactivation was faster in solutions containing \( \text{Ca}^{2+}\) rather than \( \text{Ba}^{2+}\). Similarly, these authors found that \( \text{Ca}^{2+}\) buffers reduced inactivation of VGCCs, thus, demonstrating CDI of \( \text{Ca}_\text{V} \) channels.

The calmodulin (CaM) \( \text{Ca}^{2+}\) sensor has been found to be critical in CDI (Lee et al., 1999; Peterson et al., 1999; Qin et al., 1999). At rest, CaM is pre-associated with the C-terminal of certain \( \alpha_1 \) subunits such that the C and N-terminal lobes of CaM interact with the IQ (isoleucine-glutamine) motif and upstream EF-hand region of the \( \alpha_1 \) subunit, respectively (Johny et al., 2013; Liu et al., 2010) (Figure 1.11A). Upon an increase in intracellular \( \text{Ca}^{2+}\) concentrations, VGCC-anchored-CaM binds \( \text{Ca}^{2+}\) which promotes a conformational change in the C-terminus/CaM complex which in turn gives rise to CDI (Peterson et al., 2000; Ben-Johny and Yue, 2014). It has been suggested that the EF hand region also contributes to CDI through transducing \( \text{Ca}^{2+}\) signals from \( \text{Ca}^{2+}/\text{CaM}\)-binding domains rather than direct \( \text{Ca}^{2+}\) binding (Peterson et al., 2000; Anderson, 2001).

CDI was first thought to only occur with L-type calcium channels as no \( \text{Ca}^{2+}\)-induced current decay was detected with N-, R- or T-type channels. It was postulated that this was due to whole-cell recordings being performed with 10 mM EGTA in the patch pipette which is a known \( \text{Ca}^{2+}\) chelating agent. However, with less stringent intracellular \( \text{Ca}^{2+}\) buffering using 0.5 mM EGTA, electrophysiological studies revealed CDI of the \( \text{Ca}_\text{V} \) channels (Liang et al., 2003), particularly \( \text{Ca}_\text{V}2.1 \). This demonstrates that CDI differs between different VGCCs. CDI of L-type calcium channels is mediated by the local increase in \( \text{Ca}^{2+}\) through the channel itself (Zamponi, 2003). In contrast, CDI of \( \text{Ca}_\text{V} \) channels is supported by the global increase of
intracellular Ca\textsuperscript{2+} (Dick et al., 2008) (Figure 1.11B and 1.11C). However, in the case of the Ca\textsubscript{V}3 family no CDI has been observed due to the lack of Ca\textsuperscript{2+}-binding and CaM binding domains (Budde et al., 2002). The differential sensitivity to local and global Ca\textsuperscript{2+} rises shows that CDI is a tunable mechanism which provides quick feedback inhibition in response to an increase in intracellular Ca\textsuperscript{2+} levels.

Figure 1.11: Schematic of CDI induced by CaM in Ca\textsubscript{V} channels

(A) CDI involves the interaction of CaM with VGCCs. The left panel shows the relative positions of the EF hands (orange square) and the IQ-binding domains (pink square) of VGCCs. Ca\textsuperscript{2+} binding sites are indicated by white circles in the absence of Ca\textsuperscript{2+} and its occupation by Ca\textsuperscript{2+} is indicated by red circles. At rest (left panel), the N-terminal lobe of CaM is tethered to the EF region of the α\textsubscript{1} subunit and the C-terminal lobe of CaM is bound to the IQ motif. Following the influx of Ca\textsuperscript{2+} (middle panel), the Ca\textsuperscript{2+} -binding sites of the C-terminal lobe of CaM becomes occupied which causes a repositioning of CaM on the C-terminus of the α\textsubscript{1} subunit. The additional interactions of the C-terminal lobe of CaM with the N-terminus of the α\textsubscript{1} subunit leads to an inactivated conformation of the calcium channel. (B) Local Ca\textsuperscript{2+} influx through a Ca\textsubscript{V}1 channel is sufficient in activating CaM which triggers CDI. (C) For Ca\textsubscript{V}2 channels, multiple channels are required to create a large enough microdomain of Ca\textsuperscript{2+} which activates CaM to trigger CDI (adapted from Simms and Zamponi., 2014)

1.3.3 Post-translational Modification of VGCCs

The regulation of VGCCs occurs via a myriad of pathways which fine-tunes their expression and activity. Post-translational modifications (PTMs) play a crucial regulatory role on VGCCs.
PTMs includes phosphorylation, glycosylation, ubiquitination and S-palmitoylation and these have been recognised to play an important role in the maturation, trafficking and functional expression of VGCCs.
1.3.3.1 Phosphorylation and Dephosphorylation of VGCCs

In mammalian neurons, there is an antagonistic relationship between protein phosphorylation and dephosphorylation which control VGCCs or their interactors’ activity (Chad and Eckert, 1986; Kalman et al., 1988; Armstrong, 1989). Adrenaline increases Ca\(^{2+}\) currents in the mammalian myocardium (Reuter, 1967; Pappano and Carmeliet, 1979). This causes an elevation in cardiac action potentials which can be mimicked by the injection of cAMP into myocardial fibres (Tsien, 1973). The L-type calcium channels are modulated by the \(\beta\)-adrenergic receptor/ cAMP signalling. Yue et al. (1990) found that PKA-dependent phosphorylation of cardiac \(\text{Ca}_V1.2\) channels produced a marked increase in whole-cell current density. Activation of this pathway in myocytes increases \(\text{Ca}_V1.2\) currents through PKA-mediated phosphorylation of the L-type calcium channel and/or associated proteins (Reuter and Scholz, 1977; Osterrieder et al., 1982). PKA phosphorylation of \(\text{Ca}_V1\) channels is dependent on PKA anchoring to the \(\alpha_1\) subunit by an A kinase anchoring protein (AKAP) (Johnson and Byerly, 1994). AKAP binds to the C-terminal domain of \(\text{Ca}_V1.1\) and \(\text{Ca}_V1.2\) channels via a modified leucine zipper interaction near the PKA phosphorylation sites. Moreover, the block of the AKAP interaction prevents PKA modulation of \(\text{Ca}_V1\) channels in skeletal and cardiac myocytes (Hulme et al., 2002, 2003).

As mentioned in section 1.3.1, VGCCs can be regulated by GPCRs. This is exemplified by the regulation of \(G_{\beta\gamma}\) inhibition of N-type channels by PKC. In peripheral neurons, interference of G protein-induced inhibition is due to PKC phosphorylation of the G protein interaction site on \(\text{Ca}_V2\) channels (Swartz, 1993; Barrett and Rittenhouse, 2000). PKC phosphorylates a threonine residue within the putative domain I-II linker interaction site which destabilises binding of the \(G_{\beta\gamma}\) peptides to N-type calcium channels (Zamponi et al., 1997). This observation suggests that the activation of Gq-coupled receptors can regulate \(G_{\beta\gamma}\) signalling which in turn modulates N-type channel activity (Hamid et al., 1999; Cooper et al., 2000).
As mentioned in Section 1.3.2, CDI provides crucial negative feedback in a neuronal setting. One of the mechanisms behind CDI is the dephosphorylation of VGCCs. Early findings in the molluscan neuron revealed that promoting dephosphorylation enhanced the rate of inactivation of VGCCs (Chad and Eckert, 1986). Further studies in mammalian neurons showed that there was an antagonistic relationship between PKA and protein phosphatase calcineurin (PP2B). Calcineurin is a ubiquitous protein phosphatase which is activated by CaV and CaM (Chad and Eckert, 1986; Burley and Sihra, 2000). Protein phosphatase 1 (PP1) is indirectly regulated by CaV through calcineurin. Calcineurin activates PP1 through dephosphorylation of the PP1 inhibitor. In the case of L-type calcium channels, PP1 induces CDI through dephosphorylating CaV1, thereby inducing CDI (Armstrong, 1989). There are also other phosphatases such as serine/threonine phosphatase PP1 and PP2A that directly dephosphorylate CaV1.2 channels which decreases CaV currents through these channels (Ono and Fozzard, 1993; Davare et al., 2000). These findings show that dephosphorylation is a potent mediator of CDI of VGCCs in the mammalian nervous system.

It has also been proposed by Su et al. (2012) that phosphorylation of the C-terminus of CaV2.2 by cyclin dependent kinase 5 (CDK5) results in an increase in current density and open channel probability. These effects were thought to be mediated by an increased interaction between RIM1 and CaV2.2 resulting in the tethering of CaV2.2 to the presynaptic active zone. Furthermore, Kim and Ryan (2013) showed that cdk5 and calcineurin regulate the size of the synaptic vesicle pool. However, these authors found that in contrast to Su et al. (2012), a removal of calcineurin activity enhances VGCC function and the suppression of cdk5 potentiates synaptic activity (Kim and Ryan., 2013). Although these studies contradict each other, it is possible that the integration of the kinase and phosphatase activity regulate VGCCs. Moreover, in DRG neurons, calcineurin can be activated by Ca2+ entry through the transient receptor potential vanilloid receptor-1 (TRPV1). Upon stimulation of the TRPV1 channel, calcineurin has been reported to downregulate the N-type calcium channel (Wu et al., 2005). Altogether
these data show the importance of the reciprocal relationship between phosphorylation and dephosphorylation which provides several crucial mechanisms in the regulation of VGCCs.

### 1.3.3.2 Glycosylation of VGCCs

Glycosylation is a form of co-translational and post-translational modification to proteins which occurs in the endoplasmic reticulum (ER) and Golgi apparatus. Asparagine (N) linked-glycosylation involves the building of an oligosaccharide tree to an asparagine residue within a N-X-S/T (X being any amino acid) consensus motif of a target protein. N-linked glycosylation has emerged as a crucial regulator of ion channels, which provides an additional level of modulation over the expression and function of ion channels (Aebi, 2013). The canonical N-linked-glycosylation sites are present in all ten mammalian Ca\textsubscript{V}\textsubscript{\alpha\textsubscript{1}} pore-forming subunit of VGCCs (Lazniewska and Weiss, 2017).

The Ca\textsubscript{V}1.2 cardiac/ neuronal channel has four potential N-glycosylation sites located in domain I (N124 and N299) and IV (N1359 and N1410). The disruption of the N-glycosylation sites (N124Q/ N299Q/ N1359Q/ N1410Q) results in the substantial decrease of channel surface expression and current density (Wang et al., 2004; Park et al., 2015). This shows that N-linked-glycosylation of Ca\textsubscript{V}1.2 channels controls the expression and gating properties of the channel.

N-linked-glycosylation has also been reported in T-type channels. Biochemical analysis of Ca\textsubscript{V}3.1 and Ca\textsubscript{V}3.3 channels, in mouse forebrain and cerebellum, revealed differences in the molecular mass of these proteins (Yunker et al., 2003). Further studies demonstrated that there are four potential N-glycosylation motifs present on Ca\textsubscript{V}3.2 located in the first (N192 and N271), third (N1466) and fourth (N1710) domains. Enzymatic de-glycosylation of Ca\textsubscript{V}3.2 in heterologous cell lines resulted in a significant reduction in T-type mediated currents. Fur-
thermore, site directed mutagenesis revealed that disruption of the asparagine residues N271 and N1710 caused a dramatic loss of CaV3.2 channel expression and stability (Weiss et al., 2013). On the other hand, N-linked-glycosylation at N192 and N1466 was found to be essential in regulating the permeability of the CaV3.2 channel (Ondacova et al., 2016). These data demonstrate that the specific location of N-linked glycosylation tightly regulates the expression and functioning of VGCCs.

### 1.3.3.3 Ubiquitination of VGCCs

Ubiquitination is an essential post-translational modification which regulates the expression and function of ion channels (Lazniewska and Weiss, 2014). The ubiquitin-proteasome system (UPS) plays an important role in the modulation of VGCC expression. Ubiquitination is the process by which ubiquitin is activated by a ubiquitin-activating enzyme (E1), then transferred to a ubiquitin-conjugating enzyme (E2). Subsequently, this complex then interacts with ubiquitin-protein ligase (E3) which ubiquitinates the target protein.

The auxiliary \(\beta\) subunit promotes CaV1 and CaV2 cell surface expression through protection from proteasomal degradation (Altier et al., 2011; Waithe et al., 2011). In the absence of the \(\beta\) subunit, CaV1.2 channels are robustly ubiquitinated by the RING-finger E3 ubiquitin ligase (RFP2) and interacts with the endoplasmic-reticulum-associated protein degradation (ERAD) complex proteins, derlin-1 and p97. This facilitates the targeting of the channel to the proteasome for degradation. However, co-expression of CaV1\(\beta\) disrupts ubiquitination and targeting of the channel to the ERAD complex which promotes the trafficking of the channel from the ER to the cell surface (Altier et al., 2011). In agreement with this study, it was also found that the ancillary \(\beta\) subunit protects CaV2.2 from proteasomal degradation (Waithe et al., 2011). These authors showed through site mutagenesis (W391A) of the I-II linker of CaV2.2 channels, the \(\beta\) subunit was not able to bind and prevent degradation compared to its wildtype
counterpart. Altogether these data reveal the crucial role of the UPS in the expression of functional VGCCs.

1.3.3.4 Palmitoylation of VGCCs

Another mechanism which underlies the regulation of VGCCs is palmitoylation. Palmitoylation is a reversible lipid modification which involves the addition of a long chain fatty acid to a target protein via a thioester bond to a cysteine residue (Resh, 1999). Mutagenesis studies have revealed that palmitoylation of cysteine residues on the $\beta_2$ subunit is essential for decreasing inhibition of R-type-mediated calcium currents (Qin et al., 1998). Additionally, co-expression of $\alpha_{1B}$ along with $\beta_{2a}$ and $\alpha_2\delta$ subunits in tsA 201 cells revealed that the $\beta_{2a}$ subunit is palmitoylated at two N-terminal cysteine residues. These authors found, through the inhibition of palmitoylation, using tunicamycin, there was an increase in steady-state inactivation of the N-type calcium channel (Hurley et al., 2000). These results suggest that VGCCs containing the $\beta_2$ subunits may be modulated by dynamic palmitoylation.
1.4 N-Type Calcium Channels

The relationship between VGCC expression and function was previously described in sections 1.2.1 and 1.2.2. This next section will focus on the distribution and function of the N-type calcium channel (also referred to as CaV2.2).

1.4.1 Composition of the N-Type Calcium Channel

N-type calcium channels are encoded by the CACNA1B gene which is extensively expressed in the CNS and PNS. CaV2.2 channels are HVA channels formed by α1B and auxiliary α2δ and β subunits. The N-type currents are characterised by their intermediate activation at potentials more depolarised than -20 mV in 5 mM extracellular CaCl2 (Bleakman et al., 1995). Furthermore, it was found that presynaptic terminals favour intermediate-conductance CaV2.2 channels over high conductance CaV1 channels for Ca²⁺-triggered synaptic vesicle fusion (Weber et al., 2010). These authors demonstrated the standard CaV1>CaV2>CaV3 conductance hierarchy was based on recordings using non-physiological divalent cation concentrations. However, using physiological Ca²⁺ gradients the hierarchy of conductance was in fact that N-type channel conductance was higher than both L- and T-type channels and CaV2.2-mediated Ca²⁺ influx was sufficient for activating Ca²⁺-fusion sensors situated on synaptic vesicles (Weber et al., 2010).

ω-Conotoxin-GVIA and -MVIIA are members of the large family of peptide toxins derived from the cone snail C. geographus and C. magus, respectively, which binds with high affinity to S5-S6 of domain III of CaV2.2 (Olivera et al., 1987; Ellinor et al., 1994). Experiments have employed the use of ω-Conotoxins to demonstrate the physiological importance of CaV2.2 channels in synaptic transmission (Hirning et al., 1988; Luebke et al., 1993; Turner et al., 1993; Snutch, 2005).
1.4.2 Distribution of N-Type Calcium Channels in Neurons

A site-directed anti-peptide antibody against an intracellular epitope on the II-III loop of \( \alpha_{1B} \) (referred to as II-III loop antibody for simplicity) was developed by Westenbroek et al. (1992). Using this antibody, these authors found that the N-type calcium channels were located at the large terminals of mossy fibres of the dentate gyrus granule neurons. Furthermore, \( \text{CaV}^{2.2} \) immunolabelling was also detected, at low levels, in the cell bodies of pyramidal cells in layers II, III and V of the dorsal cortex, Purkinje cells and other cell bodies in the brain. These results showed that the N-type calcium channel was expressed in the dendrites, nerve terminals and cell bodies of many central neurons. A later study, using the anti-II-III loop antibody, found smooth and punctate \( \text{CaV}^{2.2} \) immunolabelling in the cell bodies and nerve terminals of the rat spinal cord (Westenbroek et al., 1998). These results suggested an important role for N-type calcium channels in synapses that carry nociceptive information (Westenbroek et al., 1998).

Electrophysiological experiments have also confirmed the cell body and presynaptic expression of \( \text{CaV}^{2.2} \) in the nervous system. \( \text{CaV}^{2.2} \) is the dominant presynaptic VGCC in sympathetic and sensory neurons (Hirning et al., 1988; Mori et al., 2002; Nowycky et al., 1985; Fox et al., 1987; Murakami et al., 2001). \( \text{CaV}^{2.2} \) channels have also been identified at the cell surface of non-neuronal cells such as non-excitable microglia (Saegusa and Tanabe, 2014). However, the mechanism behind activation of N-type calcium channels in microglia remains unclear and this work will focus on neuronal \( \text{CaV}^{2.2} \) expression.

1.4.3 Role of N-Type Calcium Channels

\( \text{CaV}^{2} \) channels are enriched in neurons. At the presynaptic terminal, \( \text{CaV}^{2.1} \) and \( \text{CaV}^{2.2} \) channels play essential roles in mediating fast \( \text{Ca}^{2+} \)-neurotransmitter release coupling (Takahashi and Momiyama, 1993; Luebke et al., 1993; Wheeler et al., 1994). The coupling distance
between VGCCs and Ca\(^{2+}\) sensors triggering synaptic vesicle release is an essential regulator of release probability (Bucurenciu et al., 2008). In the mature brain, tight coupling has been reported for enhancing the release probability for reliable synaptic signalling whereas loose coupling is attributed to plastic synapses (Eggermann et al., 2012; Vyleta and Jonas, 2014). During the development of excitatory synapses, the coupling tightens and the number of VGCCs gating vesicular release decreases (Nakamura et al., 2015; Kusch et al., 2018).

In the neocortical layer 5 pyramidal neurons, release probability is ensured by the EGTA-sensitive Ca\(^{2+}\)-microdomains generated by the N- and P/Q-type channels. However, these synapses undergo a developmental re-organisation resulting in the high probability release sites being governed by Ca\(^{2+}\)-nanodomains formed by EGTA-resistant P/Q-type channels (Bornschein et al., 2019). This shows the importance of developmental re-organisation in forming functional synaptic diversity.

The molecular identity of VGCC designated slots at the presynaptic terminal is unknown. Nevertheless, it seems that some slot preferences are dependent on synapse type, as some slots have a Ca\(_{\text{V}2.2}\) preference and others are specific to Ca\(_{\text{V}2.1}\) (Cao and Tsien, 2010; Ariel et al., 2013). Lübbert et al. (2019) overexpressed Ca\(_{\text{V}2.1}\) and Ca\(_{\text{V}2.2}\) \(\alpha_1\) subunits at both immature and mature calyx of Held and showed that Ca\(_{\text{V}2.1}\) currents, channels and synaptic strength increased. This suggests that the presynaptic active zone showed preferential expression of Ca\(_{\text{V}2.1}\) to Ca\(_{\text{V}2.2}\) at both developmental stages. It was therefore proposed that Ca\(_{\text{V}2.1}\) channel slots are not saturated and synaptic strength could be regulated by enhancing Ca\(_{\text{V}2.1}\) levels.

However, at central synapses, upon presynaptic membrane depolarisation, Ca\(_{\text{V}2.2}\) channels have also been shown to be critically involved in the release of glutamate from rat hippocampal slices (Luebke et al., 1993). In rat striatal tissue, the N-type calcium channel is also involved in the regulation of GABA, acetylcholine and dopamine release at nerve terminals (Turner et al.,
In the PNS, the N-type calcium channel plays a dominant role in the stimulus-secretion coupling of norepinephrine from rat sympathetic neurons (Hirning et al., 1988). Furthermore, at the presynaptic terminal of DRG neurons, neurotransmitter release is predominantly mediated by CaV2.2 (Fox et al., 1987; Gross and Macdonald, 1987; Scroggs and Fox, 1992). A more recent study showed that a significant proportion of total current recorded from mouse DRG neurons was in fact mediated through N-type calcium channels (Murali et al., 2014).

Ca\textsuperscript{2+} influx also regulates smooth muscle excitation-transcription coupling (Berridge, 2008). A study revealed that Ca\textsuperscript{2+} influx mediated by CaV2 channels, couple membrane depolarisation to cAMP response element binding (CREB) phosphorylation and gene expression. Nevertheless, Ca\textsuperscript{2+} influx through CaV2 is less effective than that mediated through CaV1 channels in signalling to the nucleus. Furthermore, CaV2 mediated Ca\textsuperscript{2+} influx is buffered by uptake into the ER and mitochondria which buffer the spatial spread of Ca\textsuperscript{2+}, restricting CaV2-regulated gene transcription (Wheeler et al., 2012).

### 1.4.4 N-Type Calcium Channel Splice Variants

CaV2.2 is encoded by 46 exons and RNA editing such as alternative splicing results in multiple splice isoforms with differing expression, trafficking and functionality (Lipscombe et al., 2002). These variants can exhibit different biophysical properties, for example alternative splicing of exon 31a of CaV2.2 modulates channel kinetics. The exon 31a-containing CaV2.2 splice variants, which insert glutamate and threonine (ET) residues in the S3-S4 loop of domain IV, are restricted to peripheral neuronal expression. The ET insertion results in a hyperpolarising shift which slows the gating kinetics of CaV2.2. This alternative splice site is conserved evolutionarily among the CaV\textsubscript{\alpha 1} genes from Drosophila to human (Lin et al., 1997, 1999, 2004).

Splice variants of CaV2.2 can also affect channel trafficking. Alternative splice variants of
Ca_{2.2} lacking parts of the cytoplasmic II-III loop (R756-L1139 and K737-A1001) including the synprint interaction domain were identified by Szabo et al. (2006). These authors demonstrated that the loss of this region almost abolished the synaptic targeting of Ca_{2.2} suggesting that splice variants lacking in the synprint site distinguish the ability of these calcium channels from mediating fast synaptic neurotransmitter release (Szabo et al., 2006).

Alternative splicing of exon 37, situated in the C-terminus of the α_{1B} subunit, gives rise to either exon 37a (e37a) or exon 37b (e37b) (Lipscombe et al., 2002; Bell et al., 2004). This introduces a small change in the amino acid sequence, however, Ca_{2.2}e37a has larger and longer lasting currents compared to those of Ca_{2.2}e37b channels (Bell et al., 2004; Castiglioni et al., 2006; Raingo et al., 2007; Marangoudakis et al., 2012). Ca_{2.2}e37a is preferentially expressed in a subset of nociceptive DRG neurons (Bell et al., 2004). Furthermore, small interfering RNA silencing Ca_{2.2}e37a shows that, in vivo, this splice variant is specifically required for the development of thermal and mechanical hyperalgesia during inflammatory and neuropathic pain (Altier et al., 2007).

The preferential inclusion of exon 37a in the rat Cacna1b gene in nociceptors generates, de novo, a C-terminal module that regulates voltage-independent inhibition. This form of inhibition is mediated by tyrosine kinase but not G_{\beta\gamma}. A tyrosine residue encoded within exon 37a, which is absent in e37b, is essential for voltage-independent inhibition of Ca_{2.2}e37a channels (Raingo et al., 2007). Ca_{2.2}e37a channels also contain canonical adaptor protein (AP-1) binding motifs which enhance trafficking of the channel from the trans-Golgi network to the axon and plasma membrane of DRG neurons (Macabuag and Dolphin, 2015). On the other hand, the elements in Ca_{2.2}e37b are predisposed to its downregulation by the UPS which reduces Ca_{2.2} current density (Marangoudakis et al., 2012). These studies demonstrate that cell-specific alternative splicing of the mRNA encoding Ca_{2.2} serves as a molecular switch regulating the sensitivity of N-type calcium channels to neurotransmitter
release and tailor its specific role during the development of neuropathic pain.

1.4.5 Mutations of N-Type Calcium Channels

$\text{Ca}_V2.2$ channels essentially contribute to synaptic activity and neuronal communication. As such, channel mutations cause disruption to neuronal function that can lead to a neurodevelopmental disorder with seizures and nonepileptic hyperkinetic movements (NEDNEH). One disease state is Myoclonus-dystonia (M-D) syndrome which is a rare hyperkinetic movement disorder. Using exome sequencing and linkage analysis in a three-generation family with M-D, Groen et al. (2015) identified a missense mutation in $\text{CACNA1B}$ where there was a substitution of an arginine residue at position 1389 to histidine (R1389H). This mutation is in the pore-forming loop (S5 and S6) which is essential for $\text{Ca}^{2+}$ conductivity of the channel. Whole-cell and single-cell patch clamping revealed a decrease in current when patching tsA201 cells transfected with the mutant ($\text{Ca}_V2.2^{R1389H}$) compared to the wild-type channel. Groen and colleagues proposed that the $\text{Ca}_V2.2^{R1389H}$ mutation increased the opening duration of channels by decreasing the G-protein-mediated inhibitory pathway. These functional changes could be a potential mechanism for the observed hyperexcitability characteristic in M-D.

Additionally, Gorman et al. (2019) report a bi-allelic loss of function $\text{CACNA1B}$ variants in six children from unrelated families who have Progressive Epilepsy-Dyskinesia. The mutations, which were found by exome sequencing and confirmed by Sanger sequencing, segregated in the families from whom parental DNA was available. All affected individuals presented with epileptic encephalopathy, severe neurodevelopmental delay (often with regression), and a hyperkinetic movement disorder. Bi-allelic loss-of-function variants in $\text{CACNA1B}$ are predicted to cause disruption of $\text{Ca}^{2+}$ influx, leading to impaired synaptic neurotransmission. The resultant effect on neuronal function is likely to be important in the development of involuntary movements and epilepsy. This loss of function mutation sheds light on the importance of
the correct functioning of Ca\textsubscript{V}2.2 and this needs to be considered with developing therapeutics to target this channel during neuropathic pain. As the aberrant targeting of the normal functioning Ca\textsubscript{V}2.2 channels during neuropathic pain may cause serious side effects.

1.4.6 Interactors of N-Type Calcium Channels

1.4.6.1 Auxiliary $\alpha_2\delta$ Subunit

The HVA channels are associated with the membrane anchored $\alpha_2\delta$ subunit (Dolphin, 2013). There are four mammalian $\alpha_2\delta$ subtypes, $\alpha_2\delta$-1-4, encoded by the CACNA2D1-4 genes, respectively. The auxiliary $\alpha_2\delta$ subunit is expressed from a single gene and the $\alpha_2$ and $\delta$ polypeptides are cleaved post-translationally but are disulphide-bonded under native conditions. The canonical role of $\alpha_2\delta$ subunits is to enhance trafficking of Ca\textsubscript{V}1 and Ca\textsubscript{V}2 channels to the plasma membrane of heterologous cells and neurons (Patel et al., 2013; Cassidy et al., 2014). Over expression of $\alpha_2\delta$ in neuronal systems produces a 5-10-fold increase in whole-cell Ca\textsubscript{V} currents (Canti et al., 2005; Hendrich et al., 2008; Davies et al., 2010; Hoppa et al., 2012).

An early study showed that when co-expressing $\alpha_2\delta$-1 with $\alpha_1$B and $\beta_1$b in Xenopus oocytes, $\alpha_2\delta$-1 has little effect on unitary Ca\textsubscript{V}2.2 properties (Wakamori et al., 1999). However, $\alpha_2\delta$-1 decreased the opening-channel probability whilst reducing the number of null traces recorded (Wakamori et al., 1999). These data demonstrate that the $\alpha_2\delta$ subunit has a limited effect on single channel conductance and therefore the increase in Ca\textsubscript{V} current is likely to represent, at least in part, an increase in Ca\textsubscript{V} channel trafficking.

$\alpha_2\delta$-1 also has a well-established role in neuropathic pain and was identified as the therapeutic target for the drugs gabapentin and pregabalin (Brown et al., 1998; Field et al., 2006). Gabapentin produces a chronic reduction of Ca\textsuperscript{2+} currents through disruption of the total number of $\alpha_2\delta$ and $\alpha_1$ subunits at the plasma membrane of heterologous expression systems and
DRG neurons (Hendrich et al., 2008). The mechanism by which gabapentin induces a reduction in Ca\(^{2+}\) currents is thought to be through the prevention of the recycling of \(\alpha_2\delta\) from Rab11-positive recycling endosomes to the plasma membrane (Tran-Van-Minh and Dolphin, 2010). A plethora of studies have also implicated \(\alpha_2\delta-1\) in the development of neuropathic pain (Newton et al., 2001; Lin et al., 2004; Bauer et al., 2009; Patel et al., 2013), and this will be discussed further in section 1.5.7.

1.4.6.2 Auxiliary \(\beta\) Subunit

The Ca\(_V\beta\) subunits have a marked effect on the biophysical properties and trafficking of functional HVA channels. The \(\beta\) subunit enhances the macroscopic current density through hyperpolarising the voltage dependence of activation and increasing the maximum open-channel probability (Meir et al., 2000; Neely et al., 2004).

The \(\beta\) subunit has also been found to increase the density of Ca\(_V\) channels at the plasma membrane. It was postulated that \(\beta\)-mediated increase in Ca\(_V\) channel expression was due to masking of an ER retention signal in \(\alpha_1\) subunits (Bichet et al., 2000; Cornet et al., 2002). Nevertheless, Waithe et al. (2011) found that Ca\(_V\)2.2 containing a mutation (W391A), prevented the binding of \(\beta\) subunits to the I-II linker. There was no evidence of Ca\(_V\)2.2 (W391A) mutants accumulating in the ER of the soma or growth cones which points to the \(\beta\) subunit providing protection from proteasomal degradation rather than acting as an ER retention signal.

1.4.6.3 Other Interactors of N-Type Calcium Channels

VGCCs are associated with several interactors which regulate channel localisation. Collapsin response mediator proteins (CRMPs) induce signal transduction of neuronal outgrowth during neuronal development. In DRG neurons CRMP-2 interacts with N-type calcium channels.
and these two co-localise in immature synapses and growth cones (Chi et al., 2009). Over-expression of CRMP-2 in sensory neurons significantly increase CaV2.2 current density and CaV2.2-mediated synaptic transmission compared with cells expressing EGFP alone. These findings support an important role for CRMP-2 in regulating N-type calcium channels.

It has also been reported that presynaptic CaV2.2 localisation is influenced through its interaction with light chain (LC) 2 of microtubule-associated protein (MAP1A). Deletion of the 23 amino acid residues, mediating the binding within the C-terminus of CaV2.2 to MAP1A, results in a dramatic decrease of CaV2.2 synaptic distribution (Leenders et al., 2008). On the other hand, interaction of LC1 of MAP1B with splice variant CaV2.2e37a and CaV2.2e37b regulates internalisation of the channel via a dynamin and clathrin-dependent pathway. Furthermore, the CaV2.2/MAP1B-LC1 complex was suggested to interact with E2 conjugase of the UPS UBE2L3, mediating UBE2L3 channel ubiquitination (Gandini et al., 2014). Thus, demonstrating a crucial role of cytomatrix components in anchoring CaV2.2 to the actin cytoskeleton of the presynaptic terminal.

CaV2.2 can be regulated by the structural proteins, caveolins. The isoform caveolin-1 is responsible for remodelling the plasma membrane as it binds and transports newly synthesised cholesterol from the ER to the cell membrane. N-type current density is attenuated in NG108-15 cells expressing caveolin-1 (Toselli et al., 2005). These authors suggested that caveolin-1 could potentially modulate CaV2.2 membrane expression through indirectly regulating the cholesterol content of the plasma membrane (Toselli et al., 2005). Altogether these data by no means present an exhaustive list of methods in which CaV2.2 can be regulated but provide insight to the multitude of ways in which CaV2.2 expression can be modulated to parallel its function.
1.5 Neuropathic pain

Pain has been defined as a “complex constellation of unpleasant sensory, emotional and cognitive experiences provoked by real or perceived tissue damage and manifested by certain autonomic, psychological, and behavioural reactions” (Colloca et al., 2017). Although pain is an unpleasant experience it has evolved as a protective mechanism warning us of potential tissue damage (Hucho and Levine, 2007). However, neuropathic pain is usually chronic and does not necessarily provide useful feedback. Neuropathic pain has been defined as “pain caused by a lesion or disease of the somatosensory system” by the International Association for the Study of Pain (Jensen et al., 2011). Neuropathy is a common and debilitating complication of diseases in the PNS such as diabetic neuropathy, postherpetic neuralgia, trigeminal neuralgia and painful radiculopathy (Colloca et al., 2017).

Chronic neuropathic pain affects 7-10% of the general population and severely impairs the quality of life of patients with neuropathic pain (Torrance et al., 2006; Bouhassira et al., 2008). It was estimated that the national cost of pain in the United States to range from $560 to $635 billion in 2010 (Gaskin and Richard, 2012). This total was estimated from care and annual costs of low worker productivity attributable to pain. This study suggested that research in pain treatment, management and prevention was essential due to the economic toll on society.

1.5.1 Organisation of the Pain Pathway

First-order neurons in the somatosensory system transduce information from the periphery and form synapses with second-order neurons the spinal cord. Somatosensory information from the body and head is conveyed by the DRG and trigeminal ganglia (TG) neurons, respectively, to the CNS. Primary afferent neurons are pseudo-unipolar, with an axon that branches into two: a peripheral (projects to the periphery) and central branch (terminates in the CNS).
Figure 1.12: Organisation of the Pain Pathway
Schematic diagram of the organisation of the pain pathway showing sensory neurons with an axon that branches into two: a peripheral (projects to peripheral targets) and central branch (terminates in the CNS). The peripheral axon of sensory neurons arrives from the peripheral targets. The dorsal root ganglia contain the cell bodies of sensory neurons such as mechanoreceptors, nociceptors and proprioceptors. The central axon of sensory neurons branches from the dorsal root ganglia to specific laminae in the dorsal horn of the spinal cord.

DRG neurons are comprised of a heterogenous population of neurons with distinct properties which allow for the discrimination of a wide variety of sensory modalities. Nociceptors are a subset of DRG neurons specialised for the detection of harmful stimuli, including noxious mechanical, chemical and thermal stimuli (Burgess and Perl, 1967; Bessou and Perl, 1969). A comprehensive classification of DRG neurons is essential for determining the cell types involved in neuropathic pain. DRG neurons can be classified based on myelination, cell body (soma) size, molecular composition and dorsal horn projections (Price, 1985; Lawson et al., 1996; Jackman and Fitzgerald, 2000; Priestley et al., 2002). These parameters have provided criteria for DRG neuronal classification which allow for the study of different primary afferent functions.
1.5.2 Classification of Nociceptors

DRG neurons can be classified based on their degree of myelination and separated into Aβ, Aδ and C-fibres. In rats, Aβ-fibres are heavily myelinated and fast-conducting with a conduction velocity of > 14 ms⁻¹. Aδ-fibres are thinly myelinated with a conduction rate ranging from 2.2-8 ms⁻¹ whilst C-fibres conduct more slowly (< 1.4 ms⁻¹) (Harper and Lawson, 1985). The differences in conduction velocities have also been associated with discrete functions. The initial fast onset of pain is mediated by Aδ-fibre nociceptors and provide precise localisation of pain as they have small receptive fields. C-fibres are activated by a variety of high-intensity stimuli and carry information from polymodal nociceptors (McCarthy and Lawson, 1990; Lawson et al., 1996). The receptive field of C-fibres are large and, therefore, less precise for pain localisation.

Detailed studies have also analysed the relationship between DRG neuronal soma size and conduction velocity (Harper and Lawson, 1985; Lawson and Waddell, 1991). In general, cell size positively correlates with conduction velocity. Proprioceptors and mechanoreceptors are associated with Aβ conduction velocities and mediated by DRG neurons with large cell bodies (Angel and Alston, 1964; Appelberg et al., 1966; Knibestöl, 1973). Large neurons can be distinguished by their expression of phosphorylated heavy-chain (200 kDa) neurofilament (Lawson and Waddell, 1991). On the other hand, slow-conducting Aδ- and C-fibres are associated with small neurons (Lawson and Waddell, 1991). Nociceptors have small cell bodies and, by interference, conduct through Aδ- and C-fibres. Furthermore, the C-fibre neurons are neurofilament poor (Lawson et al., 1993).
1.5.2.1 Neuropeptides and Neurotrophic Growth Factors

Nociceptors are commonly divided by their neurochemical signatures. A multitude of histochemical markers have been used to classify sensory neurons. These markers include receptors and peptides which allows nociceptors to detect distinct sensory modalities (Lawson et al., 1993). The different populations of nociceptors express an array of molecular markers, including ion channels and receptors for specific neurotrophic growth factors.

C-fibres can be divided into peptidergic and non-peptidergic nociceptors which allow for further DRG neuron classification. Neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) are currently used to demarcate the peptidergic class of nociceptors (Price, 1985; Lawson et al., 1996, 2002). On the other hand, isolectin-B4 (IB4) from the plant *Griffonia simplicifolia* binds to α-D-galactose carbohydrate residues on the cell membrane and is used to identify non-peptidergic DRG neurons (Silverman and Kruger, 1990). IB4-binding neurons also express P2X3 and somatostatin (Vulchanova et al., 1996; Bennett et al., 1998).

Nociceptor growth, maintenance and survival is supported by neurotrophic growth factors such as nerve growth factor (NGF) and glial cell lined-derived factor (GDNF) (Ernsberger, 2008, 2009). NGF is essential for the development and phenotypic maintenance of sympathetic, sensory nervous system and cells derived from the neural crest (Cowan, 2001). This neurotrophic factor is synthesized in target areas of NGF-responsive neurons. NGF synthesis and secretion has been shown (*in vitro*) to occur in non-neuronal cells including astroglial cells, fibroblast cells and Schwann cells (Furukawa and Furukawa, 1990). NGF is also a target derived growth factor as it is synthesised by basal keratinocytes in the skin and retrogradely transported by DRG neurons following binding to the trkA receptor (Ascano et al., 2009).

Additionally, in the CNS, NGF is produced in the cortex, hippocampus and pituitary gland. In the CNS, NGF plays a crucial role in the survival of cholinergic neurons of the basal forebrain.
complex which is responsible for functions including motivation, memory and consciousness (McAllister, 2007).

NGF exerts its biological action via the tyrosine kinase receptor (trkA) (Lewin and Mendell, 1994; Harrison et al., 2014). The major cellular signaling cascades activated by NGF include mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)-Akt and phospholipase C (PLC) – γ (Klesse and Parada, 1999). NGF can also bind to the p75-pan-neurotrophin receptor (p75NTR) which is a transmembrane glycoprotein and regulates signaling through trkA (Friedman and Greene, 1999).

The expression of CGRP is dependent on target-derived NGF as axotomy results in a decrease of CGRP levels and treatment with exogenous NGF prevents a loss of CGRP expression in DRG neurons (Verge et al., 1995; Fjell et al., 1999). Thus, there is an extensive overlap between trkA-positive and CGRP expressing DRG neurons (Averill et al., 1995). As NGF regulates the expression of a multitude of neuropeptides in DRG neurons, it can also modulate the sensitivity of neurons to stimuli such as capsaicin or protons (Winter et al., 1995). In new-born rats, treatment with NGF results in acute sensitisation leading to thermal hyperalgesia (Lewin and Mendell, 1994). Moreover, high pharmacological doses of exogenous NGF can produce pain in humans (Dyck et al., 1997). To determine whether changes in the physiological levels of endogenous NGF-concentrations can modulate the function of primary afferents, a study used trkA-IgG fusion molecules to neutralize endogenous NGF. This study showed following NGF sequestration, adult rats experienced a reduction in sensitivity to bradykinin-induced inflammation and noxious temperatures (Bennett et al., 1998). These data demonstrate that endogenous NGF can modulate the sensitivity of primary afferent nociceptors to thermal and chemical stimuli \textit{in vivo}.

NGF causes rapid sensitization of nociceptors to painful thermal stimuli due to its action on the TRPV1 channels. Lewin et al. (1993) showed in rats that the rapid sensitization to noxious
thermal stimuli occurs within minutes of an injection of NGF into the hind paw. There is a
consensus that changes in expression of proteins involved in nociception are responsible for
maintaining long-term hyperalgesia (Lewin et al., 1993). Furthermore, Bonnington and Mc-
Naughton (2003) found that following the application of capsaicin on mouse DRG neurons
there was a rise in [Ca^{2+}], and this increased by 37% when NGF was applied to capsaicin-
sensitive neurons. This was found to be mediated via the PI3K pathway as Wortmannin -a
specific inhibitor of PI3K- totally abolished the effect of NGF. Pharmacological block of PKC or
CAMKII also prevented NGF-induced sensitization. The activation of trkA receptors recruits
many signaling molecules to activate different pathways. These results suggest that PI3K,
PKC and CAMKII are involved in NGF-mediated sensitization of TRPV1. These authors sug-
gest that the potential order for the players in the pathway leading to TRPV1 phosphorylation
may be PI3K → CAMKII → PKC → TRPV1.

During the development of neuropathic pain in a rat model of chronic constriction injury (CCI),
Guo et al. (2019) found an increase in TRPV1 and CAMKII protein expression in the spinal
dorsal horn compared to the sham group which is consistent with previous findings (Kanai
et al., 2005). Intrathecal delivery of TRPV1 siRNA resulted in the down-regulation of spinal
TRPV1 expression which was paralleled by a significant increase in the threshold of the me-
chanical and thermal stimuli. Furthermore, elevated levels of pro-inflammatory cytokines such
as TNFα, IL-1β and IL-6 have been found in DRG neurons of CCI animals. Malek et al. (2015)
propose that the increased expression of interleukins and kinases are responsible for TRPV1
channel sensitization. Dependent on the model and strain of animal used an increase in
TRPV1 mRNA or protein levels have been observed (Ji et al., 2002; Wu et al., 2013). This
upregulation of TRPV1 is believed to mediate both inflammatory and neuropathic pain (Pat-
apoutian et al., 2009).

Additionally, clinical trials have explored the analgesic efficacy of NGF blockade on osteoarthri-
ngs (OA) pain. One of the monoclonal antibody-based NGF antagonists include tanezumab which has shown tremendous potential for palliating OA pain. Kan et al. (2016) performed a meta-analysis to appraise the efficacy of tanezumab and showed that inhibiting NGF substantially alleviated pain symptoms of patients with knee OA. Furthermore, in studies where tanezumab therapy alone was used rather than with non-steroidal anti-inflammatory drugs (NSAIDs) or opiates, tanezumab was significantly more effective in palliating pain (Schnitzer et al., 2015). Nevertheless, there are some safety concerns to the use of anti-NGF treatment including progressive OA and osteonecrosis involving the joints without known OA. The risk of developing progressive OA is increased using tanezumab with NSAIDs, compared to tanezumab monotherapy (Hochberg et al., 2016). Notwithstanding the risks, anti-NGF therapy has also been shown to be cost-effective as even if 10% of patients develop progressive OA, this would not nullify the overall improvement in quality of life (Losina et al., 2016). Furthermore, NGF sequestering antibodies have also been administered to a mouse model of bone cancer pain and this produces a profound reduction in both ongoing and movement-induced bone cancer pain-related behaviours. The anti-NGF therapy has also been shown to decrease several neurochemical characteristics associated with central and peripheral sensitisation in the DRG and spinal cord as NGF modulates expression and function of a wide variety of molecules and proteins (Sevcik et al., 2005). However, future mechanistic studies are required for improving the targeting of NGF-therapy (Miller et al., 2017).

NGF is also a vital factor involved in the survival of nociceptors. Mice lacking a functional NGF (NGF−/−) gene have a large reduction in the normal complement of sensory neurons. When NGF−/− mice are crossed with mice overexpressing NGF in the periphery driven by the human K14-keratin promoter, the transgenic expression of NGF restores a number of sensory neurons (Harrison et al., 2004). This study revealed the importance of the production of NGF from peripheral targets in providing an essential influence in the development of the PNS.
In mice and rats, approximately 80% of DRG neurons require NGF for survival during embryonic development (Ruit et al., 1992; Silos-Santiago et al., 1995). Shortly after neurogenesis, most DRG neurons initiate the expression of trkA and are responsive to NGF (White et al., 1996). The proapoptotic BCL2 homolog BAX has been shown to regulate peripheral neuron survival via NGF. Patel et al. (2000) show using mice that are double null for BAX and NGF, BAX deficient DRG neurons survive in the absence of NGF/trkA signalling, in vivo. However, these neurons failed to express the biochemical markers characteristic of nociceptors. These findings further confirm the role of NGF/trkA signalling in the survival and phenotypic differentiation of sensory neurons.

During the late embryonic period a sub-population of neurons switch their dependency from NGF to GDNF (Bennett et al., 1996; Molliver and Snider, 1997). The selective down-regulation of trkA occurs in a sub-population of neurons that do not express neuropeptides but are labelled by IB4. Most members of the GDNF family of ligands (GFL) are synthesised by Schwann cells and satellite glial cells around DRG neurons (Oh-hashi et al., 2009). After birth, GDNF supports the growth and survival of the non-peptidergic population of DRG neurons (Kotzbauer et al., 1996; Matheson et al., 1997). GDNF exerts its effect via a receptor complex composed of RET, a tyrosine kinase receptor, and one of four accessory GDNF family receptors (GFRα1, GFRα2, GFRα3 and GFRα4) which acts as a ligand binding domain (Lindsay and Yancopoulos, 1996).

NGF controls the expression of RET and its co-receptors GFRα1 and GFRα2. Subsequently, RET signalling promotes the expression of GFRα1 and GFRα2 whilst down-regulating trkA. Several transcription factors have been shown to control nociceptor development (Chen et al., 2006; Kramer et al., 2006; Yoshikawa et al., 2007; Samad et al., 2010). Runx1, the Runt domain transcription factor, is critical for nociceptor differentiation (Chen et al., 2006). These authors show that NGF regulates the expression of Runx1 which in turn controls the expres-
sion of Ret and GFRs. Moreover, NGF regulates the expression of the characteristics of non-peptidergic neurons mediated through a RET-independent pathway (Luo et al., 2007). These authors show that ablation of Runx1 results in the failure of trkA-positive immature neurons to adopt a non-peptidergic neuronal phenotype (Figure 1.13).

Figure 1.13: A model of development of non-peptidergic and peptidergic DRG neurons
NGF, Runx1 and Ret function hierarchically to instruct the phenotypic diversification and maturation of non-peptidergic neurons. NGF is essential for the maintenance of Runx1 expression in cells destined to become non-peptidergic neurons. Runx1 regulates the expression of genes characteristic of non-peptidergic neurons through Ret-dependent and –independent pathways. The red and black lines depict positive and negative regulation, respectively.

GDNF has also been reported to produce an analgesic effect following nerve injury. In rats, following partial sciatic nerve ligation, intrathecal infusion of GDNF produces a strong analgesic effect through reversing injury-induced plasticity of sodium channel subunits (Boucher et al., 2000). Furthermore, following sciatic nerve axotomy, GDNF application can prevent the loss of DRG neurons (Matheson et al., 1997). Chronic GDNF application, after axotomy,
also causes the sensory axons to regenerate back into the spinal cord and form functional connections which rescues sensory function (Ramer et al., 2000). These results also show that, in contrast to NGF, GDNF does not affect pain sensation in normal animals.

It is important to note that even though the majority of C-fibre nociceptors can be distinguished by IB4-binding and CGRP expression, there are also a subset of neurons which show an overlap between the two markers. Using immunohistochemical methods, it has been shown that rat DRG neurons expressing GFRα3 are positive for trkA, CGRP and IB4 (Orozco et al., 2001). Furthermore, in adult rat TG and DRG neurons, there is significant co-localisation of CGRP and substance P with IB4 (Price and Flores, 2007). Furthermore, early studies have also revealed a subpopulation of tyrosine hydroxylase (TH)-positive neurons which are CGRP- and IB4-negative in mouse DRG neurons (Brumovsky et al., 2006). TH is an enzyme which catalyses the synthesis of levo-DOPA (L-DOPA), a precursor of dopamine, adrenaline and noradrenaline, from the amino acid tyrosine. Following carrageenan-induced inflammatory pain in rats, a dopamine D2 receptor agonist was found to produce anti-hyperalgesic effects whereas a dopamine D1 receptor antagonist induced anti-hyperalgesia in the inflamed hindpaws (Gao et al., 2001). These findings suggest that the dopaminergic system in the spinal cord is implicated in the modulation of inflammatory hyperalgesia which is induced by the different dopamine receptors. Altogether these data indicate that prudence is required when extrapolating experimental conclusions about neuronal classification of sensory neurons.

Growth factors have also been reported to play an essential role in the regulation of ion channel expression. Following sciatic nerve transection in rats there is an attenuation of tetrodotoxin-resistant (TTX-R) Na⁺ current with a concomitant increase in TTX-sensitive (TTX-S) Na⁺ currents in small DRG neurons. Infusion of NGF partially restored TTX-R Na⁺ currents, whereas GDNF treatment resulted in a complete restoration of TTX-R Na⁺ currents to near normal levels (Fjell et al., 1999). These findings show that both NGF and GDNF can differen-
tially regulate TTX-R Na⁺ channels following nerve injury. Furthermore, during inflammation there is an increase in levels of NGF and GDNF which results in an increase in TRPV1 expression in DRG neurons. Neutralizing the action of the augmented NGF and GDNF using anti-NGF and -GDNF antibodies, respectively, prevented the increase in TRPV1 expression (Amaya et al., 2004). These results suggest that inflammation enhances levels of NGF and GDNF which facilitates the expression of TRPV1, resulting in the induction of thermal hyperalgesia. Together, these data demonstrate the crucial role of neurotrophic factors in differentially regulating ion channel expression.

1.5.2.2 C-low threshold mechanoreceptors (C-LTMRs)

The somatosensory system is devoted to deciphering between harmful and non-harmful stimuli. The majority of C fibres are nociceptors and respond to noxious stimuli. However, a large subset of these fibres is involved in the perception of innocuous mechanical stimuli referred to as C-low threshold mechanoreceptors (C-LTMRs) (Brown and Iggo, 1967; Iggo and Kornhuber, 1977). C-LTMRs are exclusively found in hairy skin and respond to gentle touch. The physiological properties of C-LTMRs have been characterised using ex vivo skin-nerve preparations (McIlwrath et al., 2007). Intracellular recordings performed on cutaneous C fibre neurons have shown the classically defined properties of C-LTMRs such as slow conduction velocities (0.58 m/sec) and trains of spikes in response to the application of light mechanical force (1-5 mN) (Bessou et al., 1971; Seal et al., 2009).

C-LTMRs also have a diverse molecular composition. One of the defining features of C-LTMRs is the expression of TH. To visualise these neurons, Li et al. (2011) knocked-in CreER into the TH locus which allowed for the further characterisation of the anatomical features of mouse C-LTMRs. This revealed that the axonal branches of C-LTMRs for longitudinal lanceolate endings which are associated with the hair follicles. C-LTMRs also uniquely express
vGluT3. vGluT3 knock out mice have impaired mechanical pain sensation (Seal et al., 2009). In addition, Mas-related G-protein coupled receptor member B4 (MrgprB4) is also expressed in C-LTMRs and are implicated in pleasant touch. Thus, a variety of C-LTMRs subtypes are involved in the perception of light touch.

1.5.3 The Spinal Cord

The dorsal horn of the spinal cord receives sensory information from primary afferents that innervate the body and respond to noxious and non-noxious stimuli. The central axons of DRG neurons enter the spinal cord via the dorsal root and branch out and innervate the spinal cord laminae. The primary afferents terminate in the dorsal horn in a lamina-specific pattern that can be classified by their sensory modalities (Figure 1.14). The information from the periphery is processed by circuits including excitatory and inhibitory interneurons and transmitted to projection neurons for relay to different brain areas. Additionally, nociceptive information is relayed to the ventral horn and mediates nocifensive reflexes (Sivilotti and Woolf, 1994).
Primary afferents arborise within the dorsal horn in a highly ordered manner: laminar termination pattern is based on primary afferent diameter and function. Nociceptive primary afferents terminate in Lamina (L) I-II. Peptidergic afferents (purple) terminate in LI and LII outer (LIIo) and non-peptidergic (orange) fibres terminate in LII inner (LIIi). The afferents of mechanoreceptors (green) terminate in LIII-LIV and afferents of proprioceptors (blue) terminate in LV-VI.

The primary afferents can be classified by their peripheral targets, neurochemical phenotypes, response properties and conduction velocities. These features are interrelated as the majority of large myelinated Aβ-fibres are low threshold mechanoreceptors, whereas most finely Aδ and unmyelinated C afferents are nociceptors and thermoreceptors. In general, Aβ-fibres arborise in Lamina II inner-VI (LIIi-VI) whereas the nociceptive and thermoreceptive Aδ- and C-fibres terminate in LI and II (Millan, 1999; Basbaum et al., 2009). Additionally, the Aδ hair follicle afferent arborize on either side of lamina II/III border. The Aδ nociceptors, on the other hand, terminate in lamina I with some branching to lamina V and X (Abraira and Ginty, 2013; Light et al., 1979).

In the spinal cord, peptidergic and non-peptidergic fibres terminates in distinct laminae in the dorsal horn (Figure 1.14). Peptidergic neurons project to LI and the outer region of LII (LIIo) of the spinal cord whereas IB4-positive neurons project principally in the interior LII (LIIi), which suggests that the information conveyed by these two subpopulations may be processed.
differently in the CNS (Hunt and Rossi, 1985; Molliver et al., 1995).

The primary afferents release glutamate as their major fast neurotransmitter and exert an excitatory action on their postsynaptic targets (Basbaum et al., 2009). At the presynaptic nerve terminal, glutamate is stored in synaptic vesicles and released by exocytosis. The identification of different vesicle glutamate transporters (vGluT) has allowed for the study of the diversity of glutamatergic synapses in the spinal cord. The synaptic vesicles of the spinal cord are enriched in vGluT1 and vGluT2 (Takamori et al., 2001; Alvarez et al., 2004). Using confocal and electron microscopy, vGluT1 localisation was found to be most abundant in LIII-LIV. This suggests that vGluT1 is responsible for glutamatergic neurotransmission from cutaneous and muscle mechanoreceptors. On the other hand, vGluT2 immunolabelling was most prominent in LII, suggesting that vGluT2 is accountable for glutamatergic transmission from small-calibre nociceptors (Alvarez et al., 2004). The synaptic arrangements of nociceptive fibres form synaptic glomeruli in the spinal cord, in which the afferent axons form axodendritic and axoaxonic synapses (Light et al., 1982; Ribeiro-da Silva et al., 1985).

1.5.4 Ion Channel Expression and Pain

The effective and safe treatment of pain remains an immense clinical challenge. One of the therapeutic avenues includes modulation of ion channels. To date, a large proportion of research has focussed on targeting ion channels along the pain axis; dorsal root ganglion (DRG), spinal dorsal horn, brainstem and pain-associated areas of the brain. Of the 215 ion channels within the human genome, 85 have been linked to multiple pain types (Skerratt and West, 2015). Figure 1.15 highlights the common ion channels targeting drugs for pain.
Ion channels play an essential role along the pain pathway including the detection of noxious stimuli, propagation and transmission of impulses to the CNS at the level of the spinal cord. The distinct properties and diverse population of ion channels allows excitability of nociceptors to be modulated throughout the pain pathway. Ion channels expressed exclusively in sensory DRG neurons are therefore key candidates for therapeutic targeting during neuropathic pain without affecting the CNS.

**Figure 1.15: Common Ion Channel-targeting drugs for pain** (adapted from Skerratt and West., 2015)
1.5.4.1 TRPV1

The TRPV1 ion channel belongs to the transient receptor potential (TRP) ion channel superfamily. TRPV1 is a homotetrameric, non-selective cation channel with a preference for Ca\(^{2+}\) (Chung et al., 2008). The TRPV1 channel has six transmembrane domains and a short pore-forming hydrophobic stretch between the fifth and sixth transmembrane domain. This channel is activated by low pH, noxious heat (\(\geq 42^\circ C\)) and endogenous agonist vanilloids or exogenous ligands such as capsaicin (Cortright and Szallasi, 2004) (Figure 1.16). TRPV1 localisation studies show that this channel is mainly expressed in small- and medium-sized rat DRG neurons which are negative for neurofilament immunoreactivity (Tominaga et al., 1998; Michael and Priestley, 1999). In rats, TRPV1 is expressed by the majority of IB4 and CGRP/trkA positive neurons (Michael and Priestley, 1999; Popovich et al., 1999). However, in mice TRPV1 is localised to peptidergic subgroup of nociceptors (Guo et al., 1999; Zwick et al., 2002; Dirajlal et al., 2003; Woodbury et al., 2004).

Figure 1.16: A schematic diagram of a TRPV1 subunit at the cell membrane
The subunit has six transmembrane domains (green segments) and a pore loop. Functional TRPV1 forms a tetramer. The red circles mark the residues of the capsaicin binding sites. Yellow circles mark the functionally important residues of proton binding.

Electrophysiological recordings, using outside-out patches, were performed on TRPV1 mRNA-injected oocytes and showed that the binding of at least two capsaicin molecules is required
for complete activation of TRPV1 channels. In experiments where an impermeant form of capsaicin is applied extracellularly, TRPV1 remains inactive, suggesting that capsaicin binds to intracellular sites on TRPV1 (Jung et al., 1999). Multiple intracellular sites have been proposed to be the binding site of capsaicin (Figure 1.16). In the rodent forms of TRPV1, amino acids arginine 114 and glutamate 761 in the intracellular N- and C-termini, respectively, have been identified as the interaction sites of capsaicin (Jung et al., 2002). Furthermore, two additional residues tyrosine 511 and serine 512 in the second extracellular loop and tyrosine 550 of the fifth transmembrane domain have also been suggested to interact with capsaicin (Jordt and Julius, 2002; Gavva et al., 2004). Activation of the TRPV1 channel with capsaicin results in the influx of Ca\textsuperscript{2+} in cultured rat DRG neurons (Jung et al., 1999). Ca\textsuperscript{2+} influx through TRPV1 causes membrane depolarisation, leading to the activation of Na\textsubscript{V} channels and the generation of an action potential.

In the case of noxious thermal stimuli, the application of temperature ramps to TRPV1-transfected HEK 293 cells shows that TRPV1 channels act as molecular thermometers. These electrophysiological experiments have shown that at temperatures > 43°C the inward current through TRPV1 abruptly increases (Tominaga et al., 1998). Additionally, single-channel openings elicited by heat are observed in inside-out patches of HEK 293 cells expressing TRPV1 (Numazaki and Tominaga, 2004). These data suggest that there are temperature sensor domains within TRPV1 channels. By using chimeras between the heat receptor TRPV1 and cold receptor TRPM8, the distal half of the C-terminal of TRPV1 was shown to be involved in thermal sensitivity (Brauchi et al., 2006).

During inflammation and ischemia, a reduction in tissue pH can enhance pain sensation experienced by mammals (Kwon et al., 2014). It has been found that the addition of protons to the extracellular solution activates TRPV1 channels. This suggests that proton binding sites are on the extracellular part of TRPV1 channels (Jordt et al., 2000). Notably, a growing list of
inflammatory mediators of TRPV1 have been identified such as NGF and bradykinin (Szallasi et al., 2007). The phosphorylation of TRPV1 by PKA (Bhave et al., 2002) and PKC (Premkumar and Ahern, 2000) sensitises the channel whilst dephosphorylation of TRPV1 desensitises the protein (Mohapatra and Nau, 2005). Analgesics work by targeting these pathways. For example, morphine inhibits TRPV1 sensitisation by blocking its phosphorylation by PKA (Vetter et al., 2006). As such, it is important to note that within the TRPV1 channel there are not only binding sites of activators but also gating sites that lead to allosteric changes within the protein. For example, studies have shown that low concentrations of capsaicin, which fail to activate the receptor, sensitise TRPV1 to protons and noxious heat. This is likely to occur due to the decrease in free energy of the closed state, allowing these stimuli to be closer to the open states (Hui et al., 2003; Ryu et al., 2003). These studies demonstrate that TRPV1 is a multi-state ion channel whose activity varies between the rate-limiting closed and open states which may depend on voltage, temperature and a variety of agonists.

The roles of TRPV1 have been confirmed by studies on TRPV1 knockout mice which present with no TRPV1 evoked pain and a marked reduction in thermal hypersensitivity. Interestingly, following nerve injury TRPV1 knockout mice develop thermal hypersensitivity, indicating that there are probably more receptors, as well as TRPV1, which are stimulated by noxious heat (Caterina et al., 2000; Davis et al., 2000).

1.5.5 Role of CaV2.2 in Neuropathic Pain

The CaV2.2 channels have been widely accepted to play a crucial role in neuropathic pain following nerve injury (Snutch, 2005; Yaksh, 2006; Gribkoff, 2006). Many lines of evidence indicate the involvement of the N-type calcium channel in neuropathic pain including studies of CaV2.2 subcellular distribution, knockout mice, and inhibitors and modulators of the channel.
Extensive studies have reported the presence of Ca\textsubscript{V}2.2 throughout the pain pathway, in DRG cell bodies and their central terminals in the dorsal horn of the spinal cord (Wu and Pan, 2004; Westenbroek et al., 1998; Gohil et al., 1994). The block of substance P, CGRP and glutamate release by N-type channel antagonists further confirms the essential role of Ca\textsubscript{V}2.2 in regulating synaptic transmission in nociceptive primary afferents (Maggi et al., 1990; Santicioli et al., 1992; Evans et al., 1996). It has been reported that in rats, following sciatic nerve ligation, there is a prominent upregulation of Ca\textsubscript{V}2.2 protein but not mRNA expression in LII of the dorsal horn (Cizkova et al., 2002). This suggests that blocking synaptic transmission mediated by N-type calcium channels could serve as a prime therapeutic target during neuropathic pain.

Further insight into the role of Ca\textsubscript{V}2.2 has been gained from studying Ca\textsubscript{V}2.2 knockout (Ca\textsubscript{V}2.2\textsuperscript{−/−}) mice. However, different acute nociceptive responses have been reported depending on the different mutant lines (summarized in table 1.1).

<table>
<thead>
<tr>
<th>Paper</th>
<th>Method of generation of (\alpha_{1B}) knockout line</th>
</tr>
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<tbody>
<tr>
<td>Saegusa et al. (2001)</td>
<td>Part of exon 1 was deleted and (n\text{LacZ}) encoding \textit{Escherichia coli} (\beta)-galactosidase with a nuclear localization signal was inserted in frame in its place</td>
</tr>
<tr>
<td>Ino et al. (2001)</td>
<td>The exon encoding cytoplasmic loop between repeat II and III of (\alpha_{1B}) was disrupted by the insertion of neomycin resistant gene (neo\textsuperscript{R})</td>
</tr>
<tr>
<td>Kim et al. (2001)</td>
<td>A green fluorescent protein-neo\textsuperscript{R} cassette was inserted in the exon coding for S2 of repeat 1 of (\alpha_{1B})</td>
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Table 1.1: \(\alpha_{1B}\) knockout lines

A summary of the phenotypes observed from the different Ca\textsubscript{V}2.2 knockout lines can be seen in table 1.2. The discrepancies observed may be due to the genetic background of these mice which may affect the behavior of the mice.
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<tbody>
<tr>
<td>Von Frey</td>
<td>Normal</td>
<td>Not determined</td>
<td>Increased threshold</td>
<td></td>
</tr>
<tr>
<td>Tail pinch</td>
<td>Normal</td>
<td>Normal</td>
<td>Increased threshold</td>
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<tr>
<td>Paw-flick</td>
<td>Normal</td>
<td>Not determined</td>
<td>Prolonged latency</td>
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<tr>
<td>Tail-flick</td>
<td>Prolonged latency</td>
<td>Not determined</td>
<td>Prolonged latency</td>
<td></td>
</tr>
<tr>
<td>Hot-plate</td>
<td>Normal</td>
<td>Prolonged latency</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Formalin</td>
<td>Reduced licking time</td>
<td>Reduced licking time</td>
<td>Reduced licking time</td>
<td></td>
</tr>
<tr>
<td>Acetic acid writhing</td>
<td>Normal</td>
<td>Not determined</td>
<td>Reduced number of writhing</td>
<td></td>
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</table>

Table 1.2: Comparison of phenotypes of CaV2.2 knockout mice in several pain-related behavioural tests

Despite the difference in behaviours observed, all mutant lines show a reduced response to the formalin test which suggests that CaV2.2 plays an important role in inflammatory pain. Furthermore, following spinal nerve ligation there is a marked reduction in neuropathic pain symptoms (Saegusa et al., 2002). These data suggest that blocking the CaV2.2 channel can possibly lead to a therapeutic method for neuropathic pain.

The use of highly specific and high affinity conotoxin peptides have also been used to dissect the role of CaV2.2 in nociceptive transmission. Studies have shown that ω-conotoxin GVIA can irreversibly modulate the gating properties of CaV2.2 by destabilising the open state and alter gating states of the N-type calcium channel (Yarotskyy and Elmslie, 2009). In contrast, ω-conotoxin MVIIA reversibly blocks all gating states of CaV2.2 and reduces the release of pain-inducing neurotransmitters in the dorsal horn (Feng et al., 2003). A role for the N-type calcium channel in neuropathic pain has also been solidified by reports that show that spinal delivery of CaV2.2 antagonist, such as ω-conotoxin GVIA, can block nerve-injury induced tactile allodynia and dorsal horn responses (Chaplan et al., 1994; Matthews and Dickenson, 2001).

Additionally, N-type calcium channels can be inhibited through GPCRs associated with noci-
ception. The G-protein $\beta\gamma$ subunit, released from the G$\alpha$ subunit upon activation of $\mu$-opioid receptors, has been shown to inhibit the N-type calcium channels (Seward et al., 1991; Bourinet et al., 1996). Similarly, activation of the ORL1 receptors results in the inhibition of CaV2.2 mediated through a G-protein (Beedle et al., 2004). Altogether, these data highlight the significant role of the N-type calcium channel in the pain pathway and reaffirm the importance of targeting CaV2.2 for neuropathic pain management.

1.5.6 CaV2.2 as a Therapeutic Target for Neuropathic Pain

Meta-analyses have revealed that within the general population, neuropathic pain has a prevalence of 7-10% (Van Hecke et al., 2014). Unfortunately, the majority of neuropathic pain patients fail to achieve adequate analgesic relief from current treatments (Finnerup et al., 2010). Furthermore, the prolonged use of common treatments such as opioid-based medications, including codeine and oxycodone results in addiction, as well as side effects including nausea, vomiting and constipation (Furlan et al., 2006). These studies highlight the importance of developing novel therapeutics and better utilisation of current treatments.

The drug ziconotide (Prialt™) is a synthetic version of the CaV2.2 channel blocker $\omega$-conotoxin MVIIA and is licensed for chronic pain. Ziconotide is only administered intrathecally to patients who have severe unremitting pain (Sanford, 2013). Due to the peptidergic nature of this drug, it is only approved for intrathecal administration which requires hospitalisation of the patient. Ziconotide can reversibly block CaV2.2 currents with high potency (Nadasdi et al., 1995; Lewis et al., 2000). Pre-clinical studies have shown that blocking N-type calcium channels with ziconotide leads to a reduction in neurotransmitter release in the spinal cord during neuropathic pain states (Chaplan et al., 1994; Wang et al., 2000; Sluka, 1998).

Clinical trials have also shown ziconotide reduces pain scores of patients who have failed
to respond to opioid treatment. The concomitant use of ziconotide and opioids further enabled patients to manage their pain (Mathur, 2000). Furthermore, patients suffering with chronic cancer/ AIDS-related pain treated with ziconotide had reduced pain intensity scores (Wallace, 2006). However, the limited usage of this drug is attributable to the route of administration. Additionally, the severe neurological side effects of ziconotide include sedation, dizziness, headache, confusion, memory impairment etc, which limits its use to exceptional circumstances (Penn and Paice, 2000).

Ion channels, such as Ca\textsubscript{V}2.2, are key determinants of neuronal excitability. As such, Ca\textsubscript{V}2.2 channels are potential drug targets for analgesics to combat neuropathic pain. However, it is important that the analgesics alleviate aberrant pain but do not affect normal sensory transduction. Additionally, Ca\textsubscript{V}2.2 is expressed in the brain (Westenbroek et al., 1992; Luebke et al., 1993; Takahashi and Momiyama, 1993; Wheeler et al., 1994). It is therefore essential for novel therapeutics to be targeted to the site at which Ca\textsubscript{V}2.2 channels have been modulated during neuropathic pain rather than to brain regions where it may affect normally functioning synaptic connectivity. Thus, novel analgesics must exhibit an activation state-dependent block of Ca\textsubscript{V}2.2 channels to circumvent the side effects of state-independent Ca\textsubscript{V}2.2 channel blockers.

As well as N-type calcium channel blockers being used for the management of pain, new approaches targeting VGCC trafficking have also been investigated. The regulatory protein CRMP-2 has been reported to enhance Ca\textsubscript{V}2.2 trafficking to axonal growth cones of DRG neurons whereas knockdown of CRMP-2 reduces Ca\textsuperscript{2+} current and peptide release mediated by Ca\textsubscript{V}2.2 (Chi et al., 2009). Ca\textsubscript{V}2.2 membrane trafficking can be disrupted by the Ca\textsuperscript{2+} channel-binding domain 3 of CRMP-2 fused to a HIV-1 trans-activator of transcription (TAT) peptide (forming TAT-CBD3), which interferes with the interaction between Ca\textsubscript{V}2.2 and CRMP-2 (François-Moutal et al., 2015). Electrophysiological studies in rat spinal cord slices demon-
strate that perfusion with TAT-CBD3 peptides decreased VGCC-mediated release of CGRP (Brittain et al., 2011). TAT-CBD3 was also effective in markedly attenuating carrageenan-induced thermal hypersensitivity as well as reversing mechanical hypersensitivity associated with chronic inflammatory pain (Wilson et al., 2011; François-Moutal et al., 2015). Collectively, these data suggest that TAT-CBD3 could be a potential anti-nociceptive drug used to alleviate neuropathic pain.

The ubiquitous expression and plethora of roles regulated by VGCCs complicates the use of channel blockers for neuropathic pain. However, due to the important role of the N-type calcium channel in transmitter release, \( \text{Ca}_{\text{V}}2.2 \) channels are regarded as a potential therapeutic target for modulating sensory neurotransmission. Further studies are, therefore, required to improve efficacies and side effect profiles of current neuropathic pain medications.

### 1.5.7 Gabapentinoid Drugs and \( \text{Ca}_{\text{V}}2.2 \)

The Gabapentinoids, gabapentin and pregabalin, are one of the most frequently prescribed calcium channel modulators for neuropathic pain. Gabapentin and pregabalin were originally designed as analogues of the neurotransmitter GABA. However, gabapentin was identified as a ligand for the auxiliary subunit \( \alpha_{2\delta}-1 \) and \( \alpha_{2\delta}-2 \) rather than GABA receptors (Gee et al., 1996).

A strong link between \( \alpha_{2\delta}-1 \) and neuropathic pain has been established using animal models of neuropathic pain and transgenic mice. It has been reported that there is an upregulation of \( \alpha_{2\delta}-1 \) mRNA expression in DRG neurons following partial sciatic nerve ligation in rats (Newton et al., 2001). Additionally, studies have found an increase in \( \alpha_{2\delta}-1 \) protein levels in DRG neurons and spinal cord dorsal horn (Li et al., 2004; Bauer et al., 2009). To investigate whether spinal dorsal horn \( \alpha_{2\delta}-1 \), derived from the upregulation of the auxiliary subunit in the DRG neu-
rons plays a crucial role in neuropathic pain, Li et al. (2004) examined the expression of spinal dorsal horn $\alpha_2\delta$-1 in spinal nerve-ligated rats following rhizotomy. Li and colleagues found that following spinal nerve ligation there was a time-dependent upregulation of $\alpha_2\delta$-1 in the spinal dorsal horn which correlated with the development and maintenance of neuropathic allodynia. Dorsal rhizotomy reduced the basal expression of $\alpha_2\delta$-1 and blocked the upregulation of injury-induced in the spinal dorsal horn. This resulted in a reversal of injury-induced tactile allodynia. Furthermore, the delivery of intrathecal antisense $\alpha_2\delta$-1 oligonucleotides also blocked the injury-induced increase of spinal dorsal horn $\alpha_2\delta$-1 and decreased the tactile allodynia. These findings suggest that $\alpha_2\delta$-1 basal expression occurs both pre and post-synaptically in the spinal dorsal horn. Spinal nerve ligation mainly causes an increase in $\alpha_2\delta$-1 in the injured presynaptic DRG neuron which contributes to injury-induced spinal neuroplasticity and central sensitisation that underlies the development and maintenance of neuropathic pain.

Chronic pregabalin treatment of sciatic nerve ligated animals show a profound decrease in the elevation of $\alpha_2\delta$-1 in the spinal cord and ascending axon tracts. In vitro, gabapentinoids have been shown to inhibit axonal trafficking of $\alpha_2\delta$ and the recycling of calcium channel complexes between intracellular compartments and the synaptic terminals (Tran-Van-Minh and Dolphin, 2010). This results in a decrease of synaptic transmission from the DRG neurons to the superficial lamina of the dorsal horn (Shimoyama et al., 2000; Fehrenbacher et al., 2003; Hendrich et al., 2012). The reduction in synaptic transmission is thought to be through a long-term down-downregulation of N-type calcium channel surface expression which results in the analgesic effects of gabapentin (Vega-Hernández and Felix, 2002).

Behavioural assays, after partial sciatic nerve injury in mice, have also shown that both systemic (intraperitoneal) and local (intracerebroventricular or intrathecal) injections of pregabalin reduced thermal and mechanical hypersensitivity (Takeuchi et al., 2007). These authors showed that the supraspinal analgesic effects of pregabalin activates the noradrenergic sys-
tem to alleviate neuropathic pain. The antinociceptive effects of gabapentinoids are through its interaction with $\alpha_2\delta$-1. This is strongly supported by mice expressing a mutated form of $\alpha_2\delta$-1 which led to a substantial reduction in the binding affinity of pregabalin to $\alpha_2\delta$-1 (Field et al., 2006). Additionally, $\alpha_2\delta$-1 null mice following partial sciatic nerve injury, were insensitive to pregabalin-mediated analgesia (Patel et al., 2013).

Gabapentin and pregabalin are widely prescribed to neuropathic patients, however, gabapentinoids also show adverse effects such as nausea, dizziness and ataxia. There is also little evidence of gabapentinoids being effective in acute conditions and in chronic conditions where nerve damage is not the primary source of pain such as arthritis (Moore et al., 2014). Exploratory analysis also suggests that gabapentin use is associated with an increased risk of suicidal acts and violent deaths (Patorno et al., 2010). As such, there is still a high unmet need for further investigation into novel therapies to tackle neuropathic pain.
1.6 The Need for Studying Ca\textsubscript{V}2.2 Surface Expression

For Ca\textsubscript{V}2.2 to be functional it must be expressed at the cell surface. At the presynaptic membrane, Ca\textsubscript{V}2.2 mediates Ca\textsuperscript{2+} entry which regulates neurotransmission by controlling the neurotransmitter release machinery. Ca\textsuperscript{2+} influx is controlled by several factors, some of which include the biophysical properties and surface density of Ca\textsubscript{V}2.2 channels.

The magnitude of Ca\textsuperscript{2+} influx in response to membrane depolarisation is regulated by the number of Ca\textsubscript{V}2.2 channels at the cell surface. There is a plethora of modulatory mechanisms which control the insertion and internalisation of Ca\textsubscript{V}2.2 at the cell surface. Moreover, the positioning of Ca\textsubscript{V}2.2 close to the synaptic vesicle release machinery is mediated by a multitude of presynaptic proteins. This is essential in the efficient coupling between action potentials and neurotransmitter release. The biophysical properties of Ca\textsubscript{V}2.2 have been extensively studied, as such, this work will focus on examining the cell surface expression of Ca\textsubscript{V}2.2.

1.6.1 Exofacially tagged Ca\textsubscript{V}2.2

Currently, mRNA and protein expression and distribution of Ca\textsubscript{V}2.2 has been studied by a variety of methods. Electrophysiology can reveal information about Ca\textsubscript{V}2.2 by measuring current density. Using specific channel blockers of Ca\textsubscript{V}2.2, such as ω-conotoxin GVIA, the proportion of Ca\textsuperscript{2+} current mediated through Ca\textsubscript{V}2.2 can be determined. However, this method does not disclose information about the distribution of the channel at the cell surface. This results in ambiguity over the precise location of Ca\textsubscript{V}2.2 channels at the plasma membrane.

A common method for assessing Ca\textsubscript{V}2.2 in overexpression systems is through using Ca\textsubscript{V}2.2 tagged to fluorophores such as green fluorescent protein (GFP). Overexpression systems
expressing GFP-CaV2.2 have been used to examine CaV2.2 distribution within heterologous cell lines (Raghib et al., 2001; Viard et al., 2004). Antibodies against intracellular epitopes of CaV2.2 have also been used to label fixed and permeabilised cells. This method weakly labels endogenous CaV2.2 channels making them difficult to use in tissue samples. However, both these methods are unable to distinguish, with any accuracy, between cell surface and intracellular CaV2.2 expression.

Reverse transcription PCR allows for the quantification of CaV2.2 mRNA levels in neurons and neuronal tissue. However, it is important to note that mRNA expression does not necessarily correlate with mature protein expression. Additionally, mRNA levels do not provide information about the amount of protein at the cell surface or its distribution within neurons.

Other groups have attempted to insert epitopes into exofacial loops of different VGCCs but these all result in channels with compromised function and expression (Dubel et al., 2004; Watschinger et al., 2008; Di Biase et al., 2011). Cassidy et al. (2014) recently developed a construct expressing CaV2.2 with a double haemagglutinin (HA) tag in the second extracellular loop of domain II (CaV2.2_HA). In vitro characterisation of this construct showed that the extracellular HA-tag did not affect channel function and could be used to study CaV2.2_HA trafficking in neurons (Cassidy et al., 2014). Since then, a knock-in (KI) mouse line (for simplicity, mice are referred to as CaV2.2_HA^{KIK}) mice in which the native CaV2.2 channels are HA-tagged was generated.
1.7 Aims

The principle aim of this study is to examine the expression of endogenous CaV2.2_HA both in vivo and in vitro. This thesis can be divided into three methodological aims:

1. The first aim is to characterise the CaV2.2_HA^KIKI mice in comparison to CaV2.2_WTW mice, to ensure that the insertion of the HA-tag does not disrupt CaV2.2 function and expression. In particular, this study will examine α1B mRNA expression and CaV2.2_HA protein expression in the brain and spinal cord. The present study also aims to develop an effective protocol to immunolabel CaV2.2_HA at the cell surface of DRG neurons.

2. The functional importance of CaV2.2 in the pain pathway has been alluded to in previous CaV2.2 knockout studies (Saegusa et al., 2001; Hatakeyama et al., 2001; Kim et al., 2001). The present study aims to examine the differential cell surface expression of CaV2.2_HA in different subpopulations of DRG neurons and how this may change under the influence of different neurotrophic growth factors. The present study also aims to examine the expression of CaV2.2_HA in the spinal cord dorsal horn and determine the origin of CaV2.2_HA in this region, by performing rhizotomy.

3. The final aim is to develop and utilise a reliable method for the co-culture of mouse DRG and dorsal horn neurons to help characterise CaV2.2_HA at presynaptic terminals. The present study will then go on to investigate the effects of capsaicin on CaV2.2_HA in DRG neurons.
Chapter 2

Material and Methods

This chapter contains details of all the materials (given with source and catalogue number in Table 2.4) and protocols required to repeat the experiments in this thesis. Unless stated otherwise all reagents were from Sigma Aldrich (United Kingdom).

2.1 Generation of Cav2.2_HA Epitope-Tagged Knock-in Mice

The Cav2.2_HA mouse line was generated by Taconic Artemis in the C57BL/6 background by homologous recombination with the targeting vector, which included the genomic region around exon 13 of the Cacna1b gene from clones of a C57BL/6J RPCIB-731 BAC library into which the sequence coding for the tandem HA-tag was cloned. The targeting vector also carried the puromycin resistance gene (PuroR) as a positive-selection marker in intron 13 between two Flipper recombination sites and the negative-selection marker thymidine kinase outside the homologous regions. The targeting vector was linearized and transfected into embryonic stem cells. The homologous recombinant clones were isolated by positive and negative selection and injected into blastocysts from BALB/c mice. Highly chimeric mice were crossed with C57BL/6, and transmission to the germ line was confirmed by black offspring.
The positive selective marker was removed by Flipper recombinase after crossing the first generation of knock-in mice with Flp deleter transgenic mice. Subsequent backcrossing with wild-type C57BL/6 mice allowed us to select mice without the Flipper transgene and only the tandem HA-tag insertion in exon 13. Genotyping PCR was performed with the primers forward, 5'-CACACCAGCATACATGCTCG-3' and reverse, 5'-TCCAGCCTCACATGCTGC-3', that bind to the intronic sequences just before and after exon 13 to generate amplicons of 279 and 345 for the wild-type and knock-in allele, respectively. The CaV2.2_HA^KIKI mice showed no difference compared with CaV2.2^WTWT mice with respect to body weights (Table 2.1).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Male body weight (g), (n)</th>
<th>Female body weight (g), (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaV2.2^WTWT</td>
<td>28.4 ± 0.7 (19)</td>
<td>21.2 ± 0.5 (16)</td>
</tr>
<tr>
<td>CaV2.2_HA^KIKI</td>
<td>27.2 ± 0.5 (22)</td>
<td>22.6 ± 0.6 (15)</td>
</tr>
</tbody>
</table>

Table 2.1: Body weights of mice of different genotypes
The body weights of mice (9-11 weeks old), measured prior to experiments in this study.

Mice were housed in groups of no more than five on a 12-h:12-h light:dark cycle; food and water were available *ad libitum*. All experimental procedures were covered by UK Home Office licenses, had local ethical approval by University College London (UCL) Bloomsbury Animal Welfare and Ethical Review Body, and followed the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

### 2.2 Quantitative PCR

CaV2.2_HA^KIKI and CaV2.2_HA^KIKI mice were euthanized by CO₂ exposure and decapitated, according to Schedule 1 guidelines (Home Office Animals (Scientific procedures) Act 1986, UK). Brains were dissected as described previously in Schlick et al. (2010). Tissue samples were disrupted using a rotor-stator homogeniser (Disperser T10, IKA, Staufen, Germany). Total RNA was extracted from one hemisphere of homogenised brain tissue using the RNeasy
Lipid Tissue Kit including an on-column DNase step. Reverse transcription was performed on 5 μg of total RNA using the High capacity RNA-to-cDNA kit. The RT mix was incubated for 60 min at 37°C and the reaction was stopped by heating to 95°C for 5 min and held at 4°C. TaqMan qRT-PCR (40 cycles) of sample triplicates was used to determine the relative abundance of the CaV2.2 α1 subunit, performed with an Applied Biosystems 7500/7500 Fast Real-Time PCR system. The following TaqMan assays with TaqMan Gene Expression Master Mix were used in accordance to the manufacturer's protocol (gene name: assay ID: Cat No. 4331182): Hypoxanthine phosphoribosyltransferase (Hprt1): Mm00446968_m1; Cacna1b: Mm01333678_m1; Glyceraldehyde 3-phosphate dehydrogenase (gapdh): Mm99999915_g1.

The CACNA1B probe targets exon 1 and therefore with detect all the splice variants.

A standard curve was generated using different standard dilutions for Cacna1b and the housekeeping Gapdh and Hprt1 probes (Figure 2.1A). The optimal threshold was chosen automatically (by the applied Biosystems 7500 Real-Time PCR Software) and used to determine the cycle number (C_T) (Figure 2.1B). The C_T values were plotted against the logarithm of the input amount of standard material (Figure 2.1C). From the slope, the efficiency was calculated using the equation shown in Figure 2.1D. Ideally the efficiency of a PCR should be 100% which would mean that the PCR product doubles each cycle. But an acceptable range is 90-110% for assay validation.

The comparative C_T (2^-ΔΔCT) method was used for relative quantification of fold differences (given as the mean ± s.e.m) of CaV2.2 mRNA levels in the brain of CaV2.2_HA^{KIKI} and CaV2.2^{WT/WT} mice aged 1, 2, 3 and 10 weeks of age. To determine the relative CaV2.2 mRNA expression in CaV2.2_HA^{KIKI} and CaV2.2^{WT/WT} mice, ΔΔC_T was calculated using (C_T, α1B - C_T, housekeeping)_{KIKI} - (C_T, α1B - C_T, housekeeping)_{WT/WT}. Fold change in gene expression relative to the CaV2.2_HA^{KIKI} control was calculated using 2^-ΔΔCT. To calculate the relative CaV2.2 mRNA expression throughout development, ΔΔC_T was calculated using (C_T, α1B -
Table 1. Known concentrations of PCR product (ng/μl)

<table>
<thead>
<tr>
<th>Concentration (ng/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>0.8</td>
</tr>
</tbody>
</table>

Figure 2.1: Generation of standard curve for RT-qPCR

(A) Table of standard series dilution of PCR products (ng/μl) (B) Typical amplification plot showing increase in relative fluorescence vs cycle number. The Rn value is calculated as the ratio of the fluorescence of the reporter divided by the quencher dye. ΔRn is the Rn value minus the baseline (C) Example of a standard curve used to evaluate the efficiency of the RT-PCR through calculating the slope of the line. CT value plotted against the log amount of standard concentration (D) Equation to calculate PCR efficiency

\[ \text{PCR Efficiency} = (10^{(-1/\text{Slope})}) - 1 \times 100 \]

\[ \text{Slope} = \frac{Y_2 - Y_1}{X_2 - X_1} \]

CT, housekeeping)Time \( x = (\text{CT, } \alpha_{1B} - \text{CT, housekeeping}) \) week of age. Time \( x \) is either the time point 2, 3 or 10 weeks of age. Fold change in gene expression relative to the \( \alpha_{1B} \) mRNA expression at 1 week of age was calculated using \( 2^{-\Delta \Delta CT} \).

The \( \Delta \Delta CT \) method (previously described in Livak and Schmittgen (2001)) was also used to determine Cav2.2 mRNA levels in DRG neurons of P0 and 11-week-old Cav2.2_HA\text{KIKI} and Cav2.2\text{WTWT} mice. To ensure a high enough concentration of RNA was extracted per preparation, DRG tissue was collected from three mice (aged P0/P1) of each genotype. Hprt1 was mainly used to normalise the data (when calculating \( \Delta CT \)) as its expression is stable in different tissue and over development. Measurements were performed on at least three independent RNA preparations from each age.
2.3 Protein Biochemistry

2.3.1 Synaptosome preparation

Synaptosomal fractions were prepared by differential centrifugation (Kato et al., 2007; Ferron et al., 2014). Spinal cord and brain from Cav2.2_HA^KIKI and Cav2.2^WTWT mice were homogenised in buffer I on ice (0.32 M sucrose, 3 mM HEPES-Na, pH 7.4, 0.25 mM DTT containing protease cocktail inhibitor [Roche Diagnostics, Lewes, UK]). The homogenate was centrifuged at 1,000 x g for 10 min at 4°C to produce a pellet (P1) and a supernatant (S1). The pellet P1 was resuspended in buffer I and centrifuged at 1,000 x g for 10 min at 4°C. This produced a pellet (P1') and a supernatant (S1'). S1'and S1 were combined and centrifuged at 12,000 x g for 15 min to produce pellet P2 and supernatant S2. P2 was resuspended in buffer I and centrifuged for 15 min at 13,000 x g to yield the crude synaptosomal fraction (P2'). P2'was solubilised in 50 mM Tris HCl pH7.4, 150 mM NaCl, 0.5% NaDeoxycholate, 0.1% SDS, 1% Igepal for 45 min on ice. The solubilised mixture was clarified by centrifugation for 30 min at 12,000 x g. This produced a supernatant termed the synaptosomal fraction which was subject to Western-blotting. The supernatant was transferred to a fresh Eppendorf and the protein concentration was determined by the Bradford assay (Bio-Rad).

2.3.2 Western-blotting

All water (H₂O) used in Western blotting was purified by Milli-Q (Millipore) water purification system. Samples were adjusted to the same concentration (2 μg/μl) using 5X Loading Buffer (containing 10% SDS, 0.5 M DTT, 0.1% Bromophenol Blue) in H₂O. Samples were then denatured by heating at 55°C for 10 min. 60 μg per sample was loaded onto 3-8% NuPage Tris/acetate gels and proteins were resolved by SDS-polyacrylamide gel electrophoresis.
(SDS-PAGE). Gels were run for 65 min at 150 mV, 50 mA in Running Buffer (760 ml of H\textsubscript{2}O, 40 ml of 20X NuPage Running Buffer). The Full Range Rainbow Marker (GE Life Sciences) was used as a protein marker ladder to allow for the determination of the weight of the protein bands. Following the separation of proteins, gels were soaked in Gel Soaking Buffer (2.5 ml of 20X NuPAGE Transfer Buffer, 22.5 ml of H\textsubscript{2}O, 20 \mu l of 20% SDS) for 10 min at RT. The polyvinylidene difluoride (PDVF) membrane and Whatmann filter paper were soaked in Transfer Buffer (80 ml of H\textsubscript{2}O, 10 ml of methanol, 10 ml of 20X NuPAGE Transfer Buffer). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes with a Transblot SemiDry Transfer Cell (Bio-Rad) for 30 min at 25 mV, 1mA.

After the transfer, the membrane was blocked using 10 mM Tris pH7.4, 500 mM NaCl, 0.5% Igepal, 3% BSA for 1 h at RT. Blots were then probed with rat monoclonal anti-HA (1:500; Roche) and mouse monoclonal anti-GAPDH (1:25000; Ambion) antibodies at 4\textdegree C overnight. The protein-antibody complexes were then labelled with a horseradish-peroxidase-conjugated secondary antibody (1:2000) for 1 h at room temperature (RT). Bands were detected using the enhanced ECL Plus reagent visualized with a Typhoon 9410 scanner (GE Healthcare) and analysed using ImageJ.

### 2.4 Neuronal Cultures

#### 2.4.1 DRG Neuronal Cultures

DRGs were removed from the entire spine of 10 weeks old Ca\textsubscript{v2.2\_HA\textsuperscript{KIKI}} and Ca\textsubscript{v2.2\textsuperscript{WTWT}} mice. DRG neuronal cultures were obtained using a method adapted from Hendrich et al. (2008) using enzymatic and mechanical dispersion. The DRG tissue was vigorously shaken for 18 min at 37\textdegree C in Hank’s basal salt solution (HBSS) containing DRG Enzyme (1000 U/ml DNAse 1, 3.75 mg/ml dispase and 0.8 mg/ml collagenase type 1A). The partially digested
tissue was washed and trituated in growth medium (DMEM/F12 with 10% fetal bovine serum, 1 mM GlutaMAX and 100 U penicillin-100 μg streptomycin/ml). For immunocytochemistry experiments, DRG neurons were then plated on coverslips coated with poly-L-lysine and laminin in growth medium containing 0.5 μg/ml nerve growth factor. DRG neurons were cultured in 5% CO₂ at 37°C for 24 h before using cultures for immunocytochemistry and electrophysiological experiments.

For electrophysiological experiments, laminin was not used as a substrate when plating DRG neuronal cultures. To test the effects of growth factors on cell surface Cav2.2_HA expression, DRG neurons were incubated with either 0.5 μg/ml glial cell-derived neurotrophic factor (GDNF) or 0.5 μg/ml NGF or both for 24 h before fixing and staining.

2.4.2 DRG and Spinal Cord Neuronal Co-Culture

To guarantee that only presynaptic Cav2.2_HA was examined, DRG neurons from Cav2.2_HA<sup>KIKI</sup> mice were cultured with spinal cord neurons from Cav2.2<sup>WTWT</sup> P0/1 mice. DRG and spinal cords were extracted in ice cold dissection medium (Leibovitz’s L15 Medium without supplements). The spinal cord was dissociated in 200 μl of 2.5% trypsin and 100 μl of 1200 U/ml DNase I for 23 min at 37°C. The spinal cord was washed three times with warm culture medium (culture medium I; 10% fetal bovine serum, 100 U penicillin-100 μg streptomycin/ml and DMEM). Following this, the spinal cord was triturated twice gently with fire polished glass pipettes. Spinal cord neurons were plated onto glass coverslips which were coated with poly-L-lysine and laminin.

The DRGs were dissociated in 1 ml DRG enzyme solution for 21 min at 37°C. Culture medium I was added to the dissociated DRG which were then transferred to a 1.5ml Eppendorf tube and centrifuged at 1000 rpm for 5 min. The supernatant was removed, and the pellet was
triturated three times with glass pipettes in culture medium I. The supernatant was removed by centrifuging at 1000 rpm for 5 min. Culture medium I was added to the pellet before the DRG neurons were plated on top of the spinal cord neurons. The dishes were flooded with warm culture medium I 1 h after plating. The following day, culture medium I was replaced with culture medium II (DMEM supplemented with 100 μg/ml insulin, 100 μg/ml transferrin, 5% horse serum, 2% B27 supplement and 2 mM GlutaMAX™, 0.5 μl/ml NGF and 100 U penicillin-100 μg streptomycin/ml). Half of the growth medium was replaced every 3-4 days with culture medium II. After 2 and 4 days in vitro (DIV), 5 μM cytosine arabinoside (ara-C) was applied to co-cultures to inhibit the proliferation of non-neuronal cells.

For live cell imaging experiments, DRG neurons were transfected before plating. After dissociation, DRG neurons were washed with 1 ml of warm HBSS and centrifuged at 1000 rpm for 5 min. For DRG transfection, the supernatant was discarded, and the cells were resuspended in 100 μl Nucleofector™ transfection reagent and transferred to a fresh tube containing 2 μg of cDNA mix (synaptophysin-GCaMP6f and mCherry = 3:1). Following the electroporation protocol in the Nucleofector cuvette, 500 μl of RPMI supplemented with 10% FBS was added to the neurons in the cuvette. The DRG neuronal suspension was then transferred into a 1.5 ml Eppendorf tube and incubated in a 5% CO₂ incubator at 37°C for 5 min. The cells were plated on top of the plated spinal cord neurons. The wells were flooded with warm medium 1 h after the transfected DRG neurons were plated.

### 2.5 Electrophysiology

Ca²⁺ channel currents in DRG neurons were investigated by whole-cell patch-clamp (essentially as described by Hamill et al. (1981)) recording (after 1 DIV). The patch pipette solution contained (in mM): 140 Cs-aspartate, 5 EGTA, 2 MgCl₂, 0.1 CaCl₂, 2 K₂ATP, 20 HEPES, pH 7.2 with 3 M CsOH, 310 mOsm. The external solution for recording Ba²⁺ currents contained (in
mM): 150 tetraethyl-ammonium Br, 3 KCl, 1.0 NaHCO₃, 1.0 MgCl₂, 10 HEPES, 4 D-glucose, 2 BaCl₂, 0.001 TTX, pH 7.4 with Sigma 7-9 trisbase buffer, 320 mOsm. Electrophysiology was performed using an Axopatch 200B amplifier with pClamp 10.2 (Axon Instruments, Molecular Devices). Pipettes of resistance 2-4 MΩ were used. Data were filtered at 1-2 kHz and digitized at 5-10 kHz. To record current-voltage (IV) relationships, cells were held at −80 mV, and a 100 ms step potential was applied to between −60 and +50 mV in 10 mV increments every 10 s. Analysis of data was performed using pClamp 10.7 and Origin Pro 2017 (Originlab). Mean I-V relationships were fitted with a modified Boltzmann equation: 

\[ I = G_{\text{max}} \frac{(V-V_{\text{rev}})}{(1+\exp[-(V-V_{50,\text{act}})/k])} \]

where \( G_{\text{max}} \) is the maximum conductance, \( V_{\text{rev}} \) is the reversal potential, \( k \) is the slope factor and \( V_{50,\text{act}} \) is the voltage for 50% current activation.

### 2.6 Dorsal Rhizotomy

Three male mice were anesthetised with 2.5% isoflurane and the lumbar spinal cord exposed by laminectomy. Two (L4 and L5, \( n=1 \)) or three (L3-L5, \( n=2 \)) dorsal roots were sectioned on one side, midway between the DRG and the dorsal root entry zone. The surrounding muscle was closed with absorbable 6-0 vicryl sutures (Ethicon, VetTech, UK), and the skin closed with surgical wound clips. After surgery the mice were allowed to recover for 7-10 days, before perfusion fixation for immunohistochemistry. All rhizotomy surgeries were performed by Professor Maria Fitzgerald, UCL.

### 2.7 Immunohistochemistry

For immunohistochemistry, mice were deeply anaesthetized with an intraperitoneal injection of pentobarbitone (Euthatal, Merial Animal Health, Harlow, UK; 600 mg/kg), perfused transcardially with saline containing heparin, followed by perfusion with 4% paraformaldehyde in
0.1 M phosphate buffer (PB, pH 7.4) at a flow rate of 2.5 ml/min for 4 min. All perfusions were performed by Dr Manuela Nieto-Rostro, in our laboratory, under Home Office licence PPL 70/8156, PIL I11E5C6BD. The spinal cord was dissected out and post-fixed for 2 h. Tissue was washed with PB, cryoprotected by incubation in PB with 15% sucrose overnight, and finally mounted in Optimal cutting temperature (OCT) compound (VWR) before storing at -80°C until sectioning with a cryostat. The spinal cord was sectioned at 20 μm using a cryostat, placing the sections sequentially in series of 6 slides, so the distance between any section and the next in any slide is 120 μm. Slides were stored at -80°C until processed.

For all spinal cord immunohistochemistry, blocking and antibody solution was made using 5% goat serum containing 0.3% Triton X-100 in PBS. For primary and secondary antibody dilutions, see Table 2.2 and 2.3.

2.7.1 CGRP, vGluT2 and NPY labelling

Spinal cord sections were first incubated with blocking solution at RT for 1 h. For CGRP, vGluT2 and NPY immunolabelling was performed using the mouse anti-CGRP, guinea pig anti-vGluT2 and rabbit anti-NPY antibody, respectively. Spinal cord sections were incubated in primary antibody for 2-3 days at 4°C. Following this, sections were incubated using the corresponding secondary antibodies (anti-mouse AF 488, anti-guinea pig AF 633 and anti-rabbit AF 594, respectively) for each marker for 2-3 days at 4°C.

2.7.2 CaV2.2_HA and α2δ-1 labelling

It has been shown that fixation with formaldehyde can lead to extensive cross-linking of proteins (Grizzle, 2009). This reduces the penetration of the primary antibody to its antigen target which may result in a substantial decrease in immunorecognition for some antibodies.
Such was the case for the HA-tag and α2δ-1, therefore, sections used to identify CaV2.2_HA and α2δ-1 underwent an extra step to retrieve visualisation of the respective epitopes. To improve CaV2.2_HA immunoreactivity the perfusion time was adjusted to minimise over-fixation. Additionally, following incubation with primary antibodies the samples were subject to either post-fixation, to avoid the gradual disappearance of the signal, or tyramide-based signal amplification (TSA; PerkinElmer) was used.

For the post-fixation method, spinal cord sections were first incubated in blocking solution for 1 h. For CaV2.2_HA labelling, tissue samples were incubated with rat anti-HA antibody for 2-3 days at 4°C. Following extensive washes, spinal cord samples were incubated for 30 min at RT with 4% paraformaldehyde in PBS, washed thoroughly and then incubated with anti-rat AF 488 antibody for 2-3 days at 4°C.

The TSA system is an enzyme-mediated detection method that uses horseradish peroxidase (HRP) to generate high-density labelling of the CaV2.2_HA (Figure 2.2). The activation and covalent binding of TSA reagent is catalysed using peroxidase. Initially, to quench endogenous peroxidases, samples were incubated in 3% hydrogen peroxide. Sections were then incubated with rat anti-HA antibody for 2 days at 4°C. After thorough washes, the samples were incubated with goat anti-rat HRP (which catalyses the amplification system) and incubated for 2 days at 4°C. The TSA reagent was applied to samples and left for 3 min, followed by thorough washes with PBS supplemented with 0.1% Triton X-100. The HRP converts the labelled TSA reagent into highly reactive free radicals that form covalent bonds with tyrosine residues immediately proximal to the enzyme. The free radicals have a short half-life and will form dimers that are washed away if not used, ensuring that the signal is localised to the target. This results in more fluorophores present where only one antibody existed before and the signal has been amplified (Figure 2.2).
Figure 2.2: Schematic diagram of the TSA reaction pathway
To enhance $\alpha_2\delta-1$ staining, heat-induced antigen retrieval (10 mM citrate buffer, pH 6.0, 0.05% Tween 20, 98°C for 10 min) was used. Following this, the sections were first blocked in 10% goat serum in the presence of 0.2% Triton X-100 in PBS for 1 h. To detect $\alpha_2\delta-1$, endogenous IgGs were further blocked with the unconjugated goat Fab anti-mouse IgG (1:100; Jackson ImmunoResearch) for 1 h to reduce the background signal as a mouse antibody was used on mouse tissue. Following this, the sections were incubated with the primary mouse anti-dihydropyridine receptor ($\alpha_2$-1) antibody for 2 days at 4°C. After extensive washing, the samples were incubated (for 1 day at 4°C) with biotin-conjugated goat Fab fragment anti-mouse antibody (1:500; Jackson ImmunoResearch). Subsequently, samples were washed and incubated with Streptavidin-Alexa Fluor-488 (Molecular Probes) for 1 day at 4°C.

Following all immunohistochemical protocols, spinal cord sections were extensively washed and treated with the nuclear stain DAPI and mounted in VectaShield (Vector Laboratories).

### 2.8 Immunocytochemistry

#### 2.8.1 Immunolabelling of DRG neurons

Cell surface $\text{Ca}_V2.2\_HA$ staining was investigated in DRG neurons by immunocytochemistry (after 1 day in vitro (DIV)), unless stated otherwise. For all immunolabelling of cell surface $\text{Ca}_V2.2\_HA$ of DRG neurons presented in Chapter 3 and 4, neurons were fixed and post-fixed using 4% PFA and 4% sucrose in PBS. Furthermore, unless stated otherwise, blocking of neuronal cultures was performed using blocking buffer (20% goat serum, 4% BSA in PBS) and antibodies were diluted in antibody solution (10% goat serum, 2% BSA in PBS). All primary and secondary antibodies used are listed in Table 2.2 and 2.3, respectively.
2.8.1.1 Cell surface Ca\textsubscript{v}2.2_HA labelling

In Chapter 3 section 3.5.1, the optimal protocol for labelling cell surface Ca\textsubscript{v}2.2_HA was as follows: DRG neurons were first fixed for 5 min and incubated with blocking solution for 1 h at RT. For Ca\textsubscript{v}2.2_HA immunolabelling neurons were incubated with rat anti-HA antibody overnight at 4°C. Unbound primary antibody was washed off using PBS and cells were post fixed for another 5 min at RT. Following washes, neurons were incubated with anti-rat Alexa Fluor (AF) 488 for 1.5 h at RT.

In Chapter 3 section 3.5.2, in order to improve cell surface labelling of Ca\textsubscript{v}2.2_HA, the biotin–streptavidin amplification system (BSAS) and tyramide signal amplification (TSA) was tested, separately. For the BSAS, neurons were first fixed, blocked and labelled with rat anti-HA antibody overnight at 4°C, followed by a biotinylated secondary antibody for 1 h at RT. Following this, neuronal cultures were incubated with Streptavidin Alexa Fluor 594 for 1 h at RT.

For the TSA protocol, to quench endogenous peroxidases, DRG neurons were incubated with 3% hydrogen peroxidase for 5 min at RT. To label Ca\textsubscript{v}2.2_HA, neurons were incubated with rat anti-HA antibody overnight at 4°C. Following this, samples were incubated with secondary antibody conjugated to Horseradish peroxidase (HRP) for 1 h at RT. The TSA reagent was applied to samples and left for 3 min, followed by thorough washes with PBS.

2.8.1.2 Cell Membrane labelling

To label the cell membrane, non-permeabilised DRG neurons were incubated in 0.5 μl/ml of Cell Tracker\textsuperscript{TM} CM-Dil (Thermofisher) prior to blocking at RT for 20 min.
2.8.1.3 Cell surface CaV2.2_HA, IB4 and CGRP labelling

For IB4 staining, live DRG neuronal cultures were incubated with 1:100 IB4 conjugated to fluorescein isothiocyanate (IB4-FITC) for 10 min at 37°C, and then washed with Krebs Ringer Hepes (KRH) buffer (in mM; 125 NaCl, 5 KCl, 1.1 MgCl₂, 1.2 KH₂PO₄, 2 CaCl₂, 6 Glucose, 25 HEPES, 1 NaHCO₃). After this, neurons were fixed and incubated with blocking solution for 1 h at RT. To detect surface CaV2.2_HA staining, neurons were incubated with rat anti-HA antibody overnight at 4°C. Following post-fixation, neurons were then incubated with anti-rat AF 594 antibody for 1 h at RT. Following this, DRG neurons were permeabilized with 0.1% Triton X-100 for 10 min; and to reduce the background signal resulting from the use of a mouse Ab on mouse DRG neuronal cultures, endogenous IgGs were blocked by incubation with the unconjugated Fab anti-mouse IgG (H+L) (0.1 mg/ml in PBS, Jackson ImmunoResearch Laboratories) for 1 h at RT. To detect CGRP, DRG neurons were incubated with mouse anti-CGRP antibody overnight at 4°C followed by 1 h at RT with an anti-mouse AF 633 antibody.

2.8.1.4 NF200 labelling

After surface labelling of CaV2.2_HA (as described in section 2.6.1.1), DRG neurons were permeabilized with 0.1% Triton X-100 for 10 min. To detect NF200, DRG cultures were incubated with rabbit anti-NF200 overnight at 4°C. Following this, neurons were incubated with anti-rabbit AF 488 antibody for 1 h at RT.

2.8.1.5 Capsaicin application and immunocytochemical protocol

1 μM capsaicin diluted in KRH was applied to DRG neurons after 1 DIV for 0, 20, 120 and 240 s at 37°C. As a control, neurons were also incubated in KRH (without capsaicin) for the same time periods. After incubation with capsaicin, DRG cultures were washed with KRH.
and fixed for 5 min with 4% PFA and 4% sucrose in PBS at RT. DRG neurons were blocked using 20% horse serum in PBS for 1 h at RT. For cell surface CaV2.2_HA labelling, neurons were incubated with rat anti-HA antibody diluted in 10% horse serum in PBS overnight at 4°C. Following this, neurons were post-fixed (as described in section 2.6.1.1) and incubated with anti-rat AF 594 antibody for 1.5 h at RT. After this, for TRPV1 immunolabelling, DRG neurons were incubated with goat anti-TRPV1 antibody diluted in 10% horse serum in PBS overnight at 4°C. DRG cultures incubated with anti-goat AF488 antibody 1.5 h at RT.

Following all DRG immunocytochemical protocols, nuclei were stained with DAPI (500 nM) before mounting on slides using Vectashield (Vector Laboratories) to reduce photobleaching.

2.8.2 Immunolabelling of DRG-Spinal Cord Co-Cultures

All co-cultures were fixed and post-fixed for 5 min using 4% PFA and 4% sucrose in PBS. All immunocytochemistry experiments in co-cultures were performed in permeabilised conditions. Co-cultures were incubated in blocking solution composed of 20% goat serum and 0.3% Triton X-100. All antibodies (Table 2.2 and 2.3) were diluted in 10% goat serum and 0.3% Triton X-100 (unless stated otherwise).

2.8.2.1 CaV2.2_HA, Homer and vGluT2 labelling

DRG and spinal cord co-cultures were fixed at DIV 1, 7, 15, 22 and 28 and blocked for 1 h at RT. First, for CaV2.2_HA immunolabelling, cultures were incubated with rat anti-HA antibody overnight at 4°C. Following post-fixation, neurons were incubated with anti-rat AF 488 antibody for 1 h at RT. Next, for Homer immunolabelling, cultures were incubated with rabbit anti-Homer antibody overnight at 4°C. Subsequently, cultures were incubated with anti-rabbit Homer AF 594 antibody. Finally, for vGluT2 immunolabelling, neurons were incubated with
guinea pig anti-vGluT2 antibody overnight at 4°C. the next day, secondary anti-guinea pig AF 633 antibody was applied to neurons for 1.5 h at RT. All primary and secondary antibodies were applied separately and consecutively to one another to prevent the cross-linking of antibodies.

### 2.8.2.2 Ca\textsubscript{V}2.2_HA and II-III loop labelling

For double labelling of Ca\textsubscript{V}2.2_HA and II-III loop in co-cultures, cultures were fixed at DIV15 and blocked for 1 h at RT. Cultures were first labelled for cell surface Ca\textsubscript{V}2.2_HA in non-permeabilised conditions with rat anti-HA antibody overnight at 4°C. Following post-fixation, cultures were incubated with anti-rat AF488 antibody for 1.5 h at RT. II-III loop labelling was performed in permeabilised conditions. Neurons were incubated with rabbit anti-II-III loop antibody diluted in 10% goat serum and 0.1% Triton X-100 overnight at 4°C. Following this, cultures were incubated with anti-rabbit AF 594 antibody diluted in 10% goat serum and 0.1% Triton X-100 for 1.5 h at RT.

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**Table 2.2: Primary antibodies used in experiments**
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Table 2.3: Secondary antibodies used in experiments

2.9 Imaging

2.9.1 Confocal Imaging and Image Analysis

Immunostaining was visualized using a LSM 780 (Zeiss) confocal microscope equipped with a Plan-Apochromat 63x/1.4 oil immersion objective lens, in 16-bit mode. The laser powers, gains and acquisition settings were kept constant for images that were used subsequently for quantification. Confocal images were imported and analysed in ImageJ (National Institutes of...
2.9.1.1 DRG neurons

Images were either single optical sections or Z-stacks of 0.7 μm optical sections. DRG neuron analysis was performed using images with temporarily enhanced brightness and contrast, solely to aid visualization of the circumference of even dimly stained cells. The first ROI measured was of the cell membrane immunolabelling of IB4 and CaV2.2_HA. A 10-pixel wide line (0.9 μm) was drawn following the perimeter of the cell from which the length was recorded as an estimation of the size of the cell (small <61 μm, medium 61-94 μm or large >94 μm) (Sommer et al., 1985), and the mean membrane intensity. The line width was chosen for practical reasons to make sure that the membrane was covered, as it can be quite convoluted, but comparing a subset of measurements with a 5 μm line did not alter the results obtained. Following this, a second ROI was drawn inside the first to measure intracellular intensity of either CGRP, NF200, CaV2.2 II-III loop antibody or TRPV1. The second ROI excluded the plasma membrane and nucleus. The intracellular ROIs were used to categorise neurons into CGRP, NF200, II-III loop or TRPV1-positive or negative, based on levels of staining observed.

The relative IB4 intensity was calculated by averaging the IB4 fluorescence intensity of DRG neurons and using the mean IB4 intensity to normalise the IB4 staining of CaV2.2_HA-positive neurons. The normalised IB4 values were multiplied by 100 to give the percentage relative immuno-intensity. DRG neurons that were judged as IB4-positive by the observer consistently had intensities >20%, whereas those considered as IB4-negative consistently had intensities <20%. Therefore, the borderline between IB4-positive and IB4-negative in this study was taken as 20% intensity (Fang et al., 2006). All data were normalized to the appropriate average intensity.
2.9.1.2 Spinal Cord

Spinal cord sections were imaged at low magnification using a 20 x 0.8 NA objective (5 μm optical section), acquiring tile images covering the whole section, which were stitched with Zen software. For all immunohistochemical protocols performed, a total of 15 spinal cord sections (5 sections from each mouse) were used for analysis (except where noted). For analysis of spinal cords of mice that underwent rhizotomy, using the Zen software, the mean intensity was recorded from a profile scan of a rectangular ROI of 50 x 300 μm placed approximately perpendicular to the pial surface of the superficial layers of the lateral, central and medial part of each dorsal horn (Figure 2.3). The CaV2.2_HA, CGRP, NPY, α2δ-1 and vGluT2 data from different experiments were pooled according to genotype and presented as the mean ± s.e.m.

Figure 2.3: Schematic of ROIs placed on the spinal cord for analysis
ROIs in the dorsal horn, ipsilateral (ipsi; red) and contralateral (contra; black) to rhizotomy.

For high magnification examination of spinal cord, a 63 x 1.4 NA objective was used in conventional confocal mode (0.7 μm optical sections) for CaV2.2_HA, with CGRP and vGluT2.

2.9.2 Airyscan Imaging and Image Analysis

For better resolution of co-cultured DRG cell bodies and synapses, high magnification images were acquired with a 63 x 1.4 NA oil immersion objective in super resolution mode (0.2 μm
optical sections). All super-resolution images underwent pixel reassignment and Airyscan processing (7x) using Zen software. The laser powers, gains and acquisition settings were kept constant for images that were used subsequently for quantification.

For samples with a very weak signal, the airyscan image drastically improves signal-to-noise (SNR) ratio in order to retrieve high resolution information. The resolution of a confocal microscope is restricted by the diffraction of light which produced a point spread function (PSF). The classic confocal microscope works by placing a pinhole to an image conjugated plane. This pinhole narrows the detection PSF which increases the contribution of the signal with higher localisation precision. On a confocal microscope, the smaller the pinhole, the higher the resolution. However, often the gain in resolution by closing the pinhole compromises the SNR as the pinhole rejects the out of focus light. The Airyscan uses a point detector array as the detector element (32 sub-elements). Each element is 0.2 AU each and in combination they cover 1.25 AU in diameter. The Airyscan allows the experimenter to scan with each element and each individual element collects less signal. But whilst a single element provides less signal the resolution is also smaller. The Airyscan collects the signal from all the of the elements. All the signals acquired from the 32 different elements are mapped, recorded and combined which allows for a better resolution and sensitivity. Additionally, a deconvolution step can also be applied where information about the scanning probe is made relative to the detection which increases resolution. Therefore, the combination of point-source excitation and array detection of the Airyscan was used to increase resolution and SNR when imaging synapses.

2.9.2.1 DRG neuronal cell bodies

Images of CaV2.2_HA, and vGluT2 at the cell body were acquired at a resolution of 2048 x 2048 pixels. Airyscan images were imported and analysed in ImageJ (National Institutes of
Health). To quantify Cav2.2_HA expression in DRG neuronal cell bodies, a 5-pixel wide line (0.25 μm) was drawn following the perimeter of the cell from which the length was recorded as an estimation of the size of the cell and mean membrane intensity, as described in section 2.9.1.1. Following this, a second ROI was drawn inside the first to measure intracellular intensity of vGluT2. Cav2.2_HA and vGluT2 intensity measured from individual neurons were normalised to the average intensity of each respective marker.

2.9.2.2 Synapses

For imaging of synapses, 0.2 μm optical sections in Z-stacks (optical section thickness was 0.1 μm) were acquired at a resolution of 2048 x 2048 pixels for Cav2.2_HA, Homer and vGluT2 puncta. To avoid bias, every identifiable synaptic punctum was imaged. Synaptic puncta images were analysed using the Zen Blue software. For puncta analysis, ROIs of 2 μm diameter were selected on the basis of Homer-positive immunoreactivity. The average intensity of Cav2.2_HA, Homer and vGluT2 was measured. Individual puncta intensities of Cav2.2_HA, Homer and vGluT2 were normalised to the average respective intensities.
2.10 Live Cell Imaging and Analysis

DRG neurons of co-cultures transfected with synaptophysin-GCaMP6f (syn-GCaMP6f) and mcherry were imaged after 19-22 DIV. Coverslips were mounted in a laminar flow perfusion and field stimulation chamber (Warner Instruments) on the stage of an LSM 780 (Zeiss) confocal microscope. Fluorescence was collected over a 512 x 512-pixel area at 100 Hz. Cells were imaged in KRH solution. Neurons were stimulated by passing 1 ms current pulses through the field stimulation chamber via platinum electrodes. Neurons expressing mCherry were selected for imaging. Neurons expressing syn-GCaMP6f were identified by stimulating the preparation at 33 Hz for 180 ms every 4 s. For analysis, ROIs of 2 μm diameter were selected according to their responsiveness to the 33Hz stimulation by their increased fluorescence. To analyse recordings, a maximum of 6 ROIs were placed at the cell body of DRG neurons and for neurites, on average, 15-25 ROIs were analysed per field of view. Changes in GCaMP6f fluorescence were normalized to the baseline fluorescence before stimulation. Analysis was performed with ImageJ (http://rsb.info.nih.gov/ij), using a custom-written plugin (http://rsb.info.nih.gov/ij/plugins/time-series.html).
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**Table 2.4: Materials used in this thesis**
Chapter 3

Characterisation of the Ca\textsubscript{V}2.2_{HA\textsuperscript{KIKI}} mouse

3.1 Introduction

Ca\textsubscript{V}2.2 channels are expressed in functional complexes at the plasma membrane in the central (Westenbroek et al., 1992) and peripheral nervous system (Gohil et al., 1994). This channel permits the entry of extracellular Ca\textsuperscript{2+} when the plasma membrane is depolarised. At the active zone of presynaptic terminals, N-type calcium channels efficiently couple Ca\textsuperscript{2+} influx to neurotransmitter release machinery. Synaptic transmission is a multi-step process, mediated by a series of biophysical mechanisms and protein-protein interactions. Key to this process is the correct localisation of VGCCs. Within the somatosensory nervous system, Cav2.2 is the main VGCC involved in transmission of nociceptive signals from DRG neurons (McCallum et al., 2011; Murali et al., 2014). The importance of this channel in nociception is highlighted by the therapeutic effectiveness of N-type calcium channel blockers in neuropathic pain (Snutch, 2005; Park and Luo, 2010). Additionally, several studies have highlighted VGCC regulators as potential therapeutic targets in alleviating neuropathic pain (Yu et al., 2019; Moutal et al.,...
2019; Khanna et al., 2019). However, to effectively target cell surface Ca\textsubscript{V}2.2, it is essential to characterise and understand plasma membrane expression and trafficking of this channel.

Previous investigations have used selective ligand (Mills et al., 1994; Jones et al., 1997) and radioligand (Filloux et al., 1994) binding of Ca\textsubscript{V}2.2 to study its expression in the rat brain. However, limits to this method include being unable to ascertain 100\% channel occupancy and function after the binding of labelled conotoxin. Additionally, molecular tools for locating VGCCs have been generated and used in various overexpression systems (Altier et al., 2006; Di Biase et al., 2011). However, not all of these resulted in fully functional VGCCs and it remains uncertain whether partial or complete loss of function results in altered channel trafficking. More recently, Cassidy et al. (2014) developed a Ca\textsubscript{V}2.2 construct with an extracellular tandem hemagglutinin (HA)-tag (Ca\textsubscript{V}2.2\_HA), which is fully functional and can be used to study channel trafficking in neurons. The extracellular HA-tag allows for the selective labelling of channels inserted into the plasma membrane and quantification of channel density. In order to accurately model Ca\textsubscript{V}2.2 behaviour it was essential that this tool be fully functional to ensure the preservation of normal physiological processes.

\textit{In vitro} characterisation of Ca\textsubscript{V}2.2\_HA by Cassidy et al. (2014) suggested that the extracellular HA-tag did not affect channel function. This led to the generation of a knock-in (KI) mouse line (for simplicity mice referred to as Ca\textsubscript{V}2.2\_HA\textsuperscript{KIKI} mice). A tandem HA-tag was inserted in constitutive exon 13, equivalent to that developed by Cassidy et al. (2014), of the \textit{Cacna1b} gene of C57BL/6 mice. This resulted in endogenous Ca\textsubscript{V}2.2 containing a double-HA tag in the second extracellular loop of domain II, a position established not to affect channel function \textit{in vitro} (Figure 3.1).
Figure 3.1: Schematic of CaV2.2_HA^KIKI structure

(A) Strategy for generation of CaV2.2_HA^KIKI mice. Mouse knock-in construct consisted of exon 13 with the HA-tag (red rectangle). See Material and Methods for further detail. (B) Schematic diagram of CaV2.2 with auxiliary subunits α2δ-1 and β1b, location of the tag site (HA) identified. HA-tag of 9 amino acid residues; (red circle) was introduced into exon 13 of the mouse CaV2.2 gene. (C) Cell surface localisation of CaV2.2_HA (red) in TSA cells with DAPI (blue). Cells transfected with CaV2.2_HA, β1b and α2δ-1 (Scale bar 50 μm). (D) Mean IV plots for CaV2.2_HA/β1b/α2δ-1 (red triangles) and CaV2.2/β1b/α2δ-1 (black squares). Individual IV relationships were fit by a modified Boltzmann function. Mean Gmax, V50 act, Vrev and k values showed no significant differences. (C and D taken from Cassidy et al. (2014))

The purpose of this study was to characterise the CaV2.2_HA^KIKI mice in comparison to CaV2.2^WTWT mice. RT-qPCR, synaptosomal blots, electrophysiology, immunolabelling and confocal microscopy were used to confirm the cell surface expression and function of CaV2.2_HA.
3.2 \( \alpha_{1B} \) mRNA expression in the Ca\( V \)\( 2.2 \_HA^{KIKI} \) mouse brain

3.2.1 Generation of standard curves

PCR amplification efficiency is the rate at which a PCR amplicon is generated. If a PCR amplicon doubles in quantity during the geometric phase of its PCR amplification, then the PCR assay has 100% efficiency. The slope of a standard curve is commonly used to estimate the PCR amplification efficiency of a real-time (RT) PCR reaction (Taylor et al., 2019). Standard curves were generated using PCR products of known concentration (see Material and Methods, Section 2.2) for \( \alpha_{1B} \) and the housekeeping GAPDH and HPRT probes. The optimal threshold was chosen automatically and used to read the cycle number (C\( T \)) (see Material and Methods, Section 2.2). Average linear regressions were then calculated for the combined results of all standard curve replicates. Figure 3.2 shows the amplification plots and standard curve of the \( \alpha_{1B} \), GAPDH and HPRT probes. Slope and correlation coefficient values were used to provide information about the performance of the reaction. The value of the slope for the standard curves were -3.5 \( \pm \) 0.06, -3.4 \( \pm \) 0.02 and -3.4 \( \pm \) 0.06 for \( \alpha_{1B} \), GAPDH and HPRT probes, respectively (Figure 3.2). From the slope, the efficiency was calculated using the equation shown in the Material and Methods, Section 2.2. The calculated efficiency of the \( \alpha_{1B} \) probe was 93\%, and 96\% for both GAPDH and HPRT. This confirmed that the GAPDH and HPRT probes could be further used as internal controls to characterise the Ca\( V \)\( 2.2 \_HA^{KIKI} \) mouse at the mRNA level.
3.2.2 α1B mRNA expression in the brains of CaV2.2_HA<sup>KIKI</sup> and CaV2.2<sup>WTWT</sup> mice

Previous studies have demonstrated the presence of α1B mRNA and protein in the mammalian brain (Catterall, 2000; Arikkath and Campbell, 2003). The present study attempted to determine whether the mRNA expression of CaV2.2 was affected by the insertion of the HA–tag via quantitative RT-PCR. Here, CaV2.2 mRNA expression was compared between the brains of CaV2.2_HA<sup>KIKI</sup> and CaV2.2<sup>WTWT</sup> 10-week-old mice. The amplification plots (Figure 3.3A)
represent the accumulation of product over 40 cycles. As expected, neither cDNA extracted from Ca\textsubscript{V}2.2\textsuperscript{HA\textsuperscript{KIKI}} nor Ca\textsubscript{V}2.2\textsuperscript{WTWT} mice presented a difference in threshold cycles. The dot plot shows the averaged data from 3 mice at 10 weeks of age normalised to either GAPDH (Figure 3.3B) or HPRT (Figure 3.3C). Relative fold expression of Ca\textsubscript{V}2.2 was calculated with respect to Ca\textsubscript{V}2.2\textsuperscript{WTWT} mice for both house-keeping genes. Normalised Ca\textsubscript{V}2.2 C\textsubscript{T} values to GAPDH had a relative fold expression of 1.00 ± 0.09 and 1.18 ± 0.1 for Ca\textsubscript{V}2.2\textsuperscript{WTWT} and Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KIKI}} mice, respectively. Additionally, C\textsubscript{T} values normalised to HPRT had a relative fold expression of 1.00 ± 0.09 compared to 0.85 ± 0.1 in Ca\textsubscript{V}2.2\textsuperscript{WTWT} and Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KIKI}} mice, respectively. The dot plots show that Ca\textsubscript{V}2.2 C\textsubscript{T} values compared to either house–keeping genes showed no significant difference in relative fold expression between the Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KIKI}} and Ca\textsubscript{V}2.2\textsuperscript{WTWT} mice. This data not only confirms the expression of Ca\textsubscript{V}2.2 in the brain but also ascertains that, at the transcription level, Ca\textsubscript{V}2.2 mRNA expression is not disturbed by the presence of the HA–tag.
Figure 3.3: Similar CaV2.2 mRNA levels in the brains of CaV2.2_HA<sub>KIKI</sub> and CaV2.2<sub>WTWT</sub> mice

(A) Example amplification plot (relative fluorescence (ΔRn) vs cycle number) of cDNA from brains of CaV2.2_HA<sub>KIKI</sub> (red) and CaV2.2<sub>WTWT</sub> (black) at 10 weeks of age. Threshold (pink) set at 0.1. qPCR results for CaV2.2 mRNA levels in brains from CaV2.2_HA<sub>KIKI</sub> (red) and CaV2.2<sub>WTWT</sub> (black) mice at 10 weeks postnatally (n=3 mice per condition, each assayed in triplicates). Data normalised to house-keeping gene (B) GAPDH (0.3026 and 1.704 p and F value, respectively) and (C) HPRT (0.2118 and 1.110 p and F value, respectively). Mean ± SEM is shown. ns, not significant, unpaired t test.

3.2.3 CaV2.2 mRNA expression over development

VGCCs have previously been demonstrated to be important for neurogenesis, migration and maturation (Pravettoni et al., 2000; Splawski et al., 2004; Chi et al., 2009). As such, this study sought to examine whether and how CaV2.2 mRNA expression changed during development. RNA was extracted from brains of 1, 2, 3 and 10-week-old CaV2.2_HA<sub>KIKI</sub> and CaV2.2<sub>WTWT</sub> mice. RT–PCR was used to generate expression profiles and relative fold expression of CaV2.2 was made to 1-week old mice (Figure 3.4). In line with these profiles, there was no calculated differences in CaV2.2 expression between CaV2.2_HA<sub>KIKI</sub> and CaV2.2<sub>WTWT</sub> mice throughout development (Figure 3.4A; only data normalised to HPRT shown).
Data from Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KIKI}} and Ca\textsubscript{V}2.2\textsuperscript{WTWT} mice were analysed separately (Figure 3.4B and 3.4C). In both mice, there was an overall decrease in relative fold expression of \(\alpha_1B\) over the different time points. The highest expression observed was at 1 week, 1.00 ± 0.05 and 1.00 ± 0.08 for Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KIKI}} and Ca\textsubscript{V}2.2\textsuperscript{WTWT} mice, respectively. Following this, there was a gradual decrease of Ca\textsubscript{V}2.2 relative expression in the brain as the mice aged. The lowest relative expression was seen in 3-week-old mice at 0.54 ± 0.04 and 0.52 ± 0.03 corresponding to the Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KIKI}} and Ca\textsubscript{V}2.2\textsuperscript{WTWT} mice. Similar reductions were also seen at the last time point (10 weeks) where relative fold expression was 0.56 ± 0.05 and 0.49 ± 0.04 Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KIKI}} and Ca\textsubscript{V}2.2\textsuperscript{WTWT} mice, respectively. These findings agree with previous reports where a similar decrease in Ca\textsubscript{V}2.2 transcript in the brain could be seen (Tanaka et al., 1995; Schlick et al., 2010).
Figure 3.4: Cav2.2 mRNA levels in the brain decreases over development in Cav2.2_HA<sup>KIKI</sup> and Cav2.2<sup>WTWT</sup> mice

(A) qPCR results for Cav2.2 mRNA levels in brains from Cav2.2_HA<sup>KIKI</sup> (red) and Cav2.2<sup>WTWT</sup> (black) mice at 1, 2, 3 and 10 weeks postnatally (n = 3 mice per condition, each assayed in triplicates). C<sub>T</sub> values of α<sub>1B</sub> normalised to HPRT data shown only. Mean ± SEM is shown. ns, not significant, paired t test. p and F values (respectively) are as follows for 1, 2, 3 and 10 weeks: (1 week = 0.312 and 1.201, 2 week = 0.256 and 1.406, 3 week = 0.306 and 1.656 and 10 week = 0.2118 and 1.110). (B) Relative fold expression of α<sub>1B</sub> from brains of Cav2.2<sup>WTWT</sup> mice (black) (p<0.0001 and 20.47, p and F value, respectively) and (C) Cav2.2_HA<sup>KIKI</sup> (red) mice (p = 0.0002 and F = 17.85). Statistical analysis as follows: one–way ANOVA with Bonferroni multiple comparison as post hoc test. ***p<0.001, **p<0.01.
3.3 Protein expression of Ca\textsubscript{2.2}\_HA in the spinal cord and brain

Synapses are structures that permit neurons to communicate with one another. Ca\textsubscript{2.2} channels have a major presynaptic role in the regulation of transmitter release (Turner et al., 1993). In order to study this in depth synaptosomal fractions can be used. Synaptosomal fractionation consists of isolating the presynaptic and postsynaptic terminals, including mitochondria and synaptic vesicles, from the other cell structures (Rai et al., 2014). As such, synaptosomal preparations enriched in synaptic particles were used to determine the presence of Ca\textsubscript{2.2}\_HA.

The HA-tag expression was determined using spinal cord and brain synaptosomes from both Ca\textsubscript{2.2}\_HA\textsuperscript{KIKI} and Ca\textsubscript{2.2}\_HA\textsuperscript{WTWT} mice. Synaptosome fractions were prepared by differential centrifugation from brain and spinal cord tissue (Kato et al., 2007; Ferron et al., 2014). This protein fraction was then used in western blots. Endogenous Ca\textsubscript{2.2}\_HA from synaptosome extracts were probed using a rat anti-HA antibody. Figure 3.5A and 3.5B show immunoblots of spinal cord and brain, respectively and GAPDH was used as the loading control. Enrichment of Ca\textsubscript{2.2}\_HA can be seen in both brain and spinal cord synaptosomal fractions from Ca\textsubscript{2.2}\_HA\textsuperscript{KIKI} mice which is absent in Ca\textsubscript{2.2}\_HA\textsuperscript{WTWT} mice. In order to estimate the molecular weight of the Ca\textsubscript{2.2}\_HA band, a standard curve (Figure 3.5C) was constructed using the logarithm of known molecular weights from the protein ladder and plotted against Rf values and used for protein weight estimation. The molecular mass of the Ca\textsubscript{2.2}\_HA band was calculated to be $261 \pm 1.2$ kDa which corresponds to the expected Ca\textsubscript{2.2} molecular weight.
Figure 3.5: Confirmation of Ca\textsubscript{v}2.2_HA expression in synaptosomes

Immunoblot of (A) spinal cord and (B) brain synaptosomes from Ca\textsubscript{v}2.2\textsubscript{HA\textsuperscript{KIKI}} (left lane) and Ca\textsubscript{v}2.2\textsubscript{WTWT} (right lane) mice confirming the expression of Ca\textsubscript{v}2.2_HA at the expected size (red arrow). GAPDH (lower) is the loading control. (C) Standard curve constructed from known molecular weights of the protein ladder. Representative of three independent experiments from different mice. The molecular mass of Ca\textsubscript{v}2.2_HA is 261.0 ± 1.2 kDa.
3.4 Functionality of Ca\textsubscript{\textgreek{v}}\textsubscript{2.2}\_HA validated in DRG neurons

The extant literature suggests that DRG neurons primarily used Ca\textsubscript{\textgreek{v}}2.2 channels to facilitate the Ca\textsuperscript{2+} influx used for neurotransmission (Fox et al., 1987; Gross and Macdonald, 1987). Therefore, once expression of Ca\textsubscript{\textgreek{v}}2.2\_HA was determined by Western blot, the conductance of VGCCs was investigated. DRG neurons were cultured, for 1 DIV, from 10 to 12-week-old Ca\textsubscript{\textgreek{v}}2.2\_HA\textsuperscript{KIKI} and Ca\textsubscript{\textgreek{v}}2.2\textsuperscript{WTWT} mice. To examine the possibility of the HA–tag disrupting the function of the calcium channels, current density–voltage relationships were measured using whole–cell patch clamping, in the voltage configuration. Barium (Ba\textsuperscript{2+}) was chosen as the charge carrier in these experiments to avoid Ca\textsuperscript{2+}-mediated inactivation (Hagiwara and Nakajima, 1966; Kostyuk and Krishtal, 1977; Eckert and Chad, 1984). Voltage steps were applied from -60 mV to +50 mV in increments of 10 mV from a holding potential of -80 mV (Figure 3.6A). Example traces (Figure 3.6B) show no difference in currents recorded from Ca\textsubscript{\textgreek{v}}2.2\_HA\textsuperscript{KIKI} and Ca\textsubscript{\textgreek{v}}2.2\textsuperscript{WTWT} DRG neurons. The current density–voltage relationships (Figure 3.6C) of Ca\textsubscript{\textgreek{v}}2.2\_HA\textsuperscript{KIKI} and Ca\textsubscript{\textgreek{v}}2.2\textsuperscript{WTWT} mice show no observable differences. These channels conduct Ba\textsuperscript{2+} currents as well as the untagged channel and this suggests that the insertion of the tag did not affect the function of VGCCs. As such the channels were able to traffic to the cell surface. The results were consistent with that demonstrated by Cassidy et al. (2014) where the HA–tag was found to not significantly disrupt the biophysical properties of the channel in overexpression systems.
Figure 3.6: Comparable current densities recorded from CaV2.2_HA^KIKI and CaV2.2^WTWT DRG neurons

(A) Protocol used to measure Ba^{2+} currents. Currents were recorded at 10 mV intervals from -60 to +50 mV. (B) Representative calcium channel currents recorded from CaV2.2^WTWT (black traces) and CaV2.2_HA^KIKI (red traces) DRG neurons in culture (1 DIV). Capacitance transients have been cropped. (C) Current–voltage (IV) relationships (mean ± SEM) from I_{Ba} from CaV2.2_HA^KIKI (red circles; n = 39 cells from 4 mice) and CaV2.2^WTWT (black squares; n = 37 from 4 mice). The data were fit with a modified Boltzmann relationship: I = G_{max}(V-V_{rev})/(1+exp[-(V-V_{50,act})/k]), where G_{max} is the maximum conductance, V_{rev} is the reversal potential, k is the slope factor and V_{50,act} is the voltage for 50% current activation. For CaV2.2_HA^KIKI and CaV2.2^WTWT, the parameters for the illustrated fits are V_{50,act} = -10.69 and -8.00 mV; G_{max} 1.34 and 1.42 nS.pF^{-1}; and V_{rev} +45.9 and +44.7 mV, respectively. For the individual data for CaV2.2_HA^KIKI and CaV2.2^WTWT, V_{50,act} was -9.87 ± 0.62 and -8.3 ± 0.48 mV; G_{max} was 1.31 ± 0.15 and 1.43 ± 0.09 nS.pF^{-1}; and V_{rev} was +44.0 ± 1.5 and +44.3 ± 1.5 mV, respectively. None of the parameters show any statistical difference (Student’s t test). p values: G_{max} = 0.553, V_{rev} = 0.906, V_{50} = 0.062, K = 0.534.
3.5 Ca$_{\text{V}2.2}$_HA staining in DRG neurons

Previous studies have developed molecular tools to explore VGCC cell surface expression (Altier et al., 2006; Di Biase et al., 2011). However, these constructs were not fully functional. The patch clamp experiments (section 3.4) determined that the insertion of the HA–tag did not affect the function and thus trafficking of Ca$_{\text{V}2.2}$ channels. Taking advantage of the exofacial HA-tag, I next determined cell surface labelling of Ca$_{\text{V}2.2}$_HA in DRG neurons.

3.5.1 Optimisation of Ca$_{\text{V}2.2}$_HA staining in DRG neurons

DRG neurons were cultured from 10–12-week-old Ca$_{\text{V}2.2}$_HA$^{\text{KIKI}}$ and Ca$_{\text{V}2.2}$WTWT mice. After three days in vitro (DIV) cells were fixed for either 5 or 20 min (Figure 3.7). Following this, neurons were incubated with rat anti–HA antibody in either permeabilised (using 0.1% Triton X-100) or non–permeabilised conditions. DRG neurons cultured from Ca$_{\text{V}2.2}$WTWT mice were used as a control for non-specific labelling. Figure 3.7Ai, shows weak anti–HA immunolabelling of neurons fixed for 5 min in non–permeabilised conditions. This immunolabelling, at the cell surface, was not evident in either permeabilised conditions or Ca$_{\text{V}2.2}$WTWT neurons (Figure 3.7Aii, Bi and Bii respectively). Next, neurons were fixed for 20 min and post fixed after primary antibody incubation for 5 min. No obvious Ca$_{\text{V}2.2}$_HA cell surface immunolabelling could be observed in either non-permeabilised (Figure 3.7Aiii), permeabilised (Figure 3.7Aiv) or Ca$_{\text{V}2.2}$WTWT (Figure 3.7Biii and 3.7iv) conditions. As the most promising Ca$_{\text{V}2.2}$_HA staining could be seen with 5 min fixation, neurons were fixed for 5 min and following rat anti–HA incubation, were further fixed for 5 min. Figure 3.7Av shows Ca$_{\text{V}2.2}$_HA immunoreactivity in non–permeabilised neurons which can also be seen, to a lesser extent, in permealised cells (Figure 3.7Avi). As expected, this staining was not observed in DRG neurons cultured from Ca$_{\text{V}2.2}$WTWT mice (Figure 3.7Bv and vi). In all protocols (Figure 3.7 i to
vi), high levels of autofluorescence was detected. Autofluorescence is defined as the fluorescence emission observed when cell molecules are excited by relevant wavelengths (Pampillo and Babwah, 2010). To reduce autofluorescence, glycine washes were used to quench potential C=C bonds created during fixation. However, as shown in Figure 3.7vii, no visible changes in auto-fluorescence levels were noticed. The failure of the glycine washes to reduce autofluorescence was also confirmed by the negative control (Figure 3.7viii). In the negative control condition, cells were not incubated in primary antibody, but did receive glycine washes and exhibited similar levels of autofluorescence. Nevertheless, this data confirms that the HA epitope is recognised by the rat anti–HA antibody, as such Cav2.2_HA channels can be observed at the plasma membrane. These data confirm the electrophysiology data suggesting that Cav2.2_HA is appropriately trafficked to the cell membrane. However, access to the HA epitope is determined by fixation time with paraformaldehyde (PFA).

In summary, to assess Cav2.2_HA at the membrane the following protocol shown in a schematic diagram (Figure 3.8) was established. DRG neurons were first fixed for 5 min and labelled with rat anti–HA antibody overnight at 4°C. Washes were next performed at room temperature. The anti–HA labelled neurons were then post–fixed to allow for the stabilisation of the primary antibody to its epitope. Following washes and incubation of the secondary antibodies, the cells were then imaged to assess cell surface Cav2.2_HA.
Figure 3.7: 5 mins post-fixation following anti-HA antibody incubation is required for optimal Ca\textsubscript{v}2.2_HA staining in DRG neurons

Representative confocal images showing Ca\textsubscript{v}2.2_HA staining and merge representative (left to right) of DRG neurons (DIV 3) from (A) Ca\textsubscript{v}2.2_HA\textsuperscript{KIKI} and (B) Ca\textsubscript{v}2.2\textsuperscript{WTWT} mice after fixing neurons in different conditions. From top to bottom: (i) Neurons fixed for 5 min only (non-permeabilised). (ii) Neurons fixed for 5 min only (permeabilised). (iii) Neurons fixed for 20 min and 5 min post-fixed (non-permeabilised). (iv) Neurons fixed for 20 min and 5 min post-fixed (permeabilised). (v) Neurons fixed for 5 min and 5 min post-fixed (non-permeabilised). (vi) Neurons fixed for 5 min and 5 min post-fixed (permeabilised). Neurons fixed for 5 min and washed with glycine (non-permeabilised) (vii) incubation with anti-HA (viii) Not incubated with anti-HA (negative control; NCT). (Scale bars: 5 \mu m.) Ca\textsubscript{v}2.2_HA staining (green) and DAPI (blue). Labelling at the plasma membrane is indicated by yellow arrow.
DRG neurons cultured from either CaV2.2_HA<sup>Kiti</sup> and CaV2.2<sup>WTWT</sup> mice were fixed for 5 min. Cells were then incubated in rat anti–HA antibody overnight at 4°C. Unbound primary antibody was washed off at room temperature and neurons were post-fixed for another 5 min. Following washes, neurons were incubated in secondary antibody for 1.5 h at RT.

3.5.2 Autofluorescence in DRG neurons

The presence of autofluorescence can be distinguished from fluorescence obtained using exogenous markers. Nevertheless, autofluorescence presents complications when acquiring images using confocal microscopy due to the high noise to signal ratio (Spitzer et al., 2011). As autofluorescence (shown in Figure 3.7) was visible when excited by the 488 nm wavelength, further optimisation to the immunolabelling protocol was required.

Here, cultured DRG neurons were either post-fixed and immunolabelled using a different secondary antibody or fixed and labelled using an amplification system (Figure 3.9). Figure 3.9Ai shows CaV2.2_HA at the cell surface using the secondary antibody Alexa Fluor 594. This membrane staining was absent in DRG neurons cultured from CaV2.2<sup>WTWT</sup> mice (Figure 3.9Bi). More importantly, a dramatic loss of autofluorescence was observed when exciting the sample using the 594 nm excitation wavelength.

In a further attempt to maximise the potential for successful detection of cell surface CaV2.2_HA, the biotin–streptavidin amplification system (BSAS) was used for signal ampli-
fication, shown in Figure 3.9Aii. Fixed cells were first labelled with a rat anti-HA antibody, followed by a biotinylated secondary antibody. This signal was then amplified through incubation with biotinylated antibody with complexes made of streptavidin conjugated to Alexa Fluor 594. \( \text{Cav}_{2.2}\_\text{HA} \) could be seen using BSAS and there was a marked decrease in autofluorescence. However, due to the presence of endogenous biotin in neuronal cultures, non-specific labelling could be seen in \( \text{Cav}_{2.2}^{\text{WTWT}} \) neurons, rendering this protocol ineffective (Figure 3.9Bii). Finally, the tyramide signal amplification (TSA) was tested, in an attempt to improve the sensitivity of \( \text{Cav}_{2.2}\_\text{HA} \) immunoreactivity. DRG neurons were incubated in Horeseradish peroxidase (HRP) conjugated secondary antibodies following rat anti–HA antibody application and washes. Detection was ultimately achieved once tyramide (bound to fluorophores) served as HRP substrate and formed covalent bonds with tyrosine residues adjacent to the HA epitope. In this experiment, even though autofluorescence could not be detected, immunoreactivity could be visualised in both the \( \text{Cav}_{2.2}\_\text{HA}^{\text{KIKI}} \) (Figure 3.9Aiii) and \( \text{Cav}_{2.2}^{\text{WTWT}} \) (Figure 3.9Biii) DRG neurons. These results show that due to the presence of endogenous substrates in DRG neuronal cultures, the amplification systems produced non-specific labelling and therefore could not be used to improve \( \text{Cav}_{2.2}\_\text{HA} \) immunoreactivity sensitivity. Nevertheless, the secondary antibody Alexa Fluor 594 could be used to reduce autofluorescence and provide insight about \( \text{Cav}_{2.2}\_\text{HA} \) expression in DRG neurons. It was next important to confirm that \( \text{Cav}_{2.2}\_\text{HA} \) was in fact expressed at the cell surface.
Figure 3.9: Secondary antibody α-rat-AF 594 is optimal for identifying Cav2.2_HA and reducing autofluorescence

Confocal images of Cav2.2_HA staining and merge representative (left to right; in non-permeabilised conditions) of DRG neurons (DIV 3) from (A) Cav2.2_HA<sup>KIKI</sup> and (B) Cav2.2<sup>WTWT</sup> mice using different secondary antibodies. From left to right Cav2.2_HA staining using: (i; left) α-Rat Alexa Fluor 594 (α-Rat AF 594), (i; right) schematic of staining protocol using secondary antibody Alexa Fluor 594. (ii; left) α-Rat-Biotin Streptavidin Alexa Fluor 594 (α-Rat_BioSt_AF 594), (ii; right) schematic of staining protocol using biotin–streptavidin system, after fixation cells incubated in Biotinylated anti-rat IgG. Streptavidin conjugated secondary antibody used to bind to biotin. (iii; left) tyramide signal amplification, (iii; right) schematic of Tyramide Signal Amplification (TSA). After fixation, neurons incubated in horseradish peroxidase (HRP) conjugated secondary antibody. When inactive tyramide is applied it reacts with HRP. Multiple fluorescent molecules are deposited and covalently bind to adjacent tyrosine molecules. (Scale bars: 5 μm.)

3.5.3 Cav2.2_HA at the cell membrane

The HA–tag, as previously described (Figure 3.1), was inserted into an extracellular loop of Cav2.2 which provided an exofacial tag for cell surface Cav2.2 to be studied. Although the images acquired previously (Figure 3.7 and 3.9), in non-permeabilised conditions, showed what could be a plasma membrane ring it was next important to confirm that the Cav2.2_HA fluorescence intensity was in fact at the cell surface. To ensure Cav2.2_HA was expressed at the plasma membrane, the fluorescent lipophilic dye, CM-Dil, was used to label the cell membrane. This marker was applied prior to primary antibody incubation. Figure 3.10 shows
DRG neurons post–fixed and stained using either secondary antibody Alexa Fluor 488 (Figure 3.10Bi) or 594 (Figure 3.10Bii). Cav\(_{2.2}\)_HA could be detected at the plasma membrane and this was not detected in Cav\(_{2.2}^{WTWT}\) DRG neurons (Figure 3.10C). This confirmed that the channel is a useful tool for investigating the cell surface expression of Cav\(_{2.2}\).

![Figure 3.10: Optimisation of Cav\(_{2.2}\)_HA staining at the cell membrane in DRG neurons](image)

(A) Schematic of Cav\(_{2.2}\)_HA staining in non–permeabilised conditions. Confocal images showing Cav\(_{2.2}\)_HA, CM-DiI and merge representative of DRG neurons. (DIV 3) from (B) Cav\(_{2.2}\)_HA\(^{KIKI}\) and (C) Cav\(_{2.2}\)_HA\(^{WTWT}\) mice using different secondary \(\alpha\)Rat antibodies. (i) \(\alpha\)Rat Alexa Fluor 594 (\(\alpha\)Rat AF 594). (ii) \(\alpha\)Rat-Biotin Streptavidin Alexa Fluor 594 (\(\alpha\)Rat_BioSt_AF 594). (Scale bars: 5 \(\mu\)m.)

### 3.5.3.1 Cav\(_{2.2}\)_HA at the cell membrane; Confocal vs Airyscan

One of the crucial aspects of examining Cav\(_{2.2}\)_HA relates to its distribution at the cell surface. For a better understanding of this, Airyscan microscopy, which has been found to enhance spatial resolution and signal to noise ratio over confocal microscopy, was used. DRG neurons were immunolabeled for CM-Dil and Cav\(_{2.2}\)_HA (described in section 3.5.3). CM–Dil and Cav\(_{2.2}\)_HA intensity was measured from three regions of interest (ROI; 2 \(\mu\)m x 1\(\mu\)m) placed at the cell surface to 2 \(\mu\)m into the cell (Figure 3.11A). Using both Confocal and Airyscan microscopy, Cav\(_{2.2}\)_HA was seen to to share a similar distribution to CM–Dil (Figure 3.11B and F). Surprisingly, when using Airyscan microscopy there was a marked decrease in observable autofluorescence. Quantitative analysis revealed that both Cav\(_{2.2}\)_HA
and CM–Dil had comparable peaks. Although this data is not definitive, the spatial overlap of these two probes indicates that Cav2.2_HA is at the cell membrane. These results offer significant advances in the detection of colocalised lipid membrane marker and Cav2.2_HA at the membrane of DRG neurons.
Figure 3.11: Cell surface expression of CaV2.2_HA in DRG neurons
(A) CM–DiI and CaV2.2_HA intensity was measured from three regions of interest (ROI; 2 μm x 1μm) placed at the cell surface to 2 μm into the cell. Confocal (Bi and Bii) and Super resolution (Fi and Fii) (images of cultured DRG neurons (DIV 1) from CaV2.2_HA KIKI and CaV2.2 WT WT mice showing (from left to right) CaV2.2_HA, CM-Dil staining (in non-permeabilised conditions) and merged for representative CaV2.2_HA KIKI and CaV2.2 WT WT cells. (Scale bars: 5 μm.) Plot profile of CaV2.2_HA and CM-Dil intensity (mean ± SEM) in ROIs. (C and G) Normalised intensity of CaV2.2_HA KIKI (red circles) and CM-Dil (blue circles). (D and H) Normalised intensity of CaV2.2 WT WT (black circles) and CM-Dil (blue circles). (E and I) Normalised intensity of CaV2.2_HA KIKI (red circles) and CaV2.2 WT WT (black circles). Individual data points represent the mean data from 27 ROIs. Three ROIs were measured from three DRG neurons from three separate experiments.
3.6 Summary and Discussion

In this study, the expression and functionality of Ca\textsubscript{v}2.2_HA was assessed to determine whether the Ca\textsubscript{v}2.2_HA\textsuperscript{KIKI} mouse model could expand the biochemical tools available to study cell surface expression and trafficking of Ca\textsubscript{v}2.2. To achieve this, Ca\textsubscript{v}2.2_HA\textsuperscript{KIKI} mice were first characterised. Quantitative RT–PCR was used to show that the insertion of the HA–tag did not disrupt the expression profile of Ca\textsubscript{v}2.2 (Figure 3.3). Furthermore, changes in Ca\textsubscript{v}2.2 mRNA expression was observed during development. Notably, a large decrease in relative Ca\textsubscript{v}2.2 mRNA expression was observed in 3-week-old mice when compared to 1-week old mice, falling by 48% and 46% in Ca\textsubscript{v}2.2\textsuperscript{WTWT} and Ca\textsubscript{v}2.2_HA\textsuperscript{KIKI} mice, respectively. This percentage reduction remained constant in adult mice, relative to 1-week old mice, falling by 51% and 44% in Ca\textsubscript{v}2.2\textsuperscript{WTWT} and Ca\textsubscript{v}2.2_HA\textsuperscript{KIKI} mice, respectively (Figure 3.4). Next, to determine Ca\textsubscript{v}2.2_HA expression at the presynaptic terminal, synaptosomal fractions were used. Ca\textsubscript{v}2.2_HA expression could be verified in synaptosome preparations of brain and spinal cord. In both tissue types the molecular weight of Ca\textsubscript{v}2.2_HA was calculated to be 261 ± 1.2 kDa (Figure 3.5). Through electrophysiological assessment, the tagged channel did not show any disruption to its function as shown by the unchanged current density and biophysical properties of the currents in DRG neurons (Figure 3.6). To ascertain Ca\textsubscript{v}2.2_HA immunoreactivity, immunolabelling protocols were optimised and confocal microscopy was used to assess staining. The immunolabelling protocol shown in Figure 3.8 was chosen to best visualise Ca\textsubscript{v}2.2_HA expression in DRG neurons. Figure 3.10 further demonstrates the presence of Ca\textsubscript{v}2.2_HA at the cell membrane with the use of CM–Dil, a plasma membrane marker. Overall, these data suggest that the insertion of the HA–tag did not affect the expression and function of Ca\textsubscript{v}2.2.

As mentioned before, the study of Ca\textsubscript{v}2.2 has been impeded by the paucity of antibodies to cell surface accessible epitopes. This channel allows Ca\textsuperscript{2+} into the cell which contributes to
both the physiology and biochemistry of the organism. Ca\(^{2+}\) plays a critical role in neurotransmitter release from neurons. As a result, the insertion of any tag into Ca\(_{\text{V}}\)2.2 could alter the biophysical properties of this channel rendering it impractical when studying its expression and trafficking. Despite the potential disruption of channel function, many successful epitope tags have been inserted into exofacial positions of ion channels (Poteser et al., 2006; Maue, 2007). Previous attempts have been made to engineer surface tags on VGCCs. These tagged channels led to a reduction (Di Biase et al., 2011) or complete loss of channel function (Al-tier et al., 2006)-personal communication to Professor Annette Dolphin. Insufficient labelling of Ca\(_{\text{V}}\)2.2 curtails our ability to answer questions about channel expression, regulation and interaction with different proteins. Fortunately, Cassidy et al. (2014) designed and assessed a HA–tagged Ca\(_{\text{V}}\)2.2 which retained surface expression and functionality. This construct was used to further characterised Ca\(_{\text{V}}\)2.2_HA association with \(\alpha_2\delta\)-1 at the plasma membrane. The Ca\(_{\text{V}}\)2.2_HA construct has also been used in Ferron et al. (2014) to investigate how fragile X mental retardation protein (FMRP) regulates the functional expression of Cav2.2. Other experiments published from this group include Dahimene et al. (2018), where the Ca\(_{\text{V}}\)2.2_HA construct was used to compare the effects of \(\alpha_2\delta\)-1 and Cachd1 on the N–type channel distribution. These studies, and many others, have revealed the necessity and utility of the HA–tag on Ca\(_{\text{V}}\)2.2 to determine its interactions, trafficking and function. The present study utilises the fully functionally Ca\(_{\text{V}}\)2.2_HA previously described in Cassidy et al. (2014) to generate a knock-in mouse line expressing the HA–tag in the Cacna1b gene. Before using this mouse, it was important to assess Ca\(_{\text{V}}\)2.2_HA functionality and expression.

RT–qPCR is a potent tool for both the amplification, detection and quantification of mRNA. As such this provided a useful method of investigating Cacna1b gene expression in the brain. GAPDH and HPRT have previously been used as internal controls to study mRNA expression during development (Schlick et al., 2010). GAPDH and HPRT were chosen as internal controls as the standard curves showed that their expression levels were stable and both ef-
ficiency values were over 90%. RT–qPCR confirmed similar Cacna1b transcriptional profiles of Cav2.2_HA^KIKI and Cav2.2^WTWT mice brains. Cav2.2 mRNA expression profile is tightly correlated to its expression in different brain regions (Chin, 1998; Latour et al., 2003). The present study uses RNA from whole brain extracts for RT–qPCR experiments. As such this data reflects a sum of Cav2.2 expression patterns in a variety of brain regions. Unpublished data from the lab shows that when dividing the brain into cortex, hippocampus, cerebellum and brain stem, a similar down–regulation in α1B mRNA expression is observed when comparing P0 to adult mice. Generally, this agrees with Schlick et al. (2010), where a decline in Cav2.2 transcript levels is observed in the cortex and hippocampus. The data from the present study could potentially support the idea of the developmental switch from N to P/Q type channels which has been reported to occur in both excitatory and inhibitory synapses (Scholz and Miller, 1995; Miki et al., 2013). Additional evidence supporting the developmental switch is demonstrated by α1B-deficient mice showing no major neurophysiological defects (Ino et al., 2001) whereas ablation of P/Q–type channels led to numerous neurological defects (Jung et al., 2002). In contrast, Vance et al. (1998), using radioligand binding, showed that there was an increase in spatiotemporal protein expression of Cav2.2 during rat brain ontogeny. However, our lab has not been able to replicate the detection method used in this study. As such the relationship between transcription and translation of Cav2.2 remains to be resolved. 

Ca^2+ entry and VGCCs have been implicated in sculpting neuronal development (Kater and Mills, 1991; Moorman and Hume, 1993). As such, the Cav2.2_HA^KIKI mouse model is ideal for studying native N–type channel expression over development. Further experiments would need to be performed to investigate the concomitant expression of N- and P/Q-type channels and whether this switch during ontogeny leads to functional redundancy of Cav2.2 channels.

Synaptosomes are cell fractions of isolated synapses (Evans, 2015). This includes various subcellular membranes and organelles such as synaptic vesicles, synaptic membranes, presynaptic proteins and postsynaptic densities (Bai and Witzmann, 2007). Cav2.2 channels
play a prominent role in neurotransmitter release at the presynaptic terminal (Jahn et al., 2003; Mochida, 2018). The current study confirms Ca\textsubscript{V}2.2\textsubscript{HA} expression in synaptosomes as the 261 kDa band (the expected molecular mass of Ca\textsubscript{V}2.2) is recognised by anti–HA antibodies in Western blots of brain and spinal cord tissue (Figure 3.5). Previous studies have also demonstrated similar data using the anti–Ca\textsubscript{V}2.2 antibody in synaptosomes from rat brains (Ferron et al., 2014). To further validate this data, proteins levels of Ca\textsubscript{V}2.2 in both mice could have been assessed using the anti–Ca\textsubscript{V}2.2 antibody. This commercial antibody is to an epitope of the intracellular loop between domain II and III and would provide detail about the total Ca\textsubscript{V}2.2 protein levels. Nevertheless, the data established the presynaptic expression of Ca\textsubscript{V}2.2\textsubscript{HA} in brain and spinal cord synaptosomes.

Native VGCCs have been classified in DRG neurons. The availability of specific pharmacological agents has helped to elucidate their physiological contribution (Doering and Zamponi, 2003; Sanford, 2013; Bourinet and Zamponi, 2017). VGCCs are organised in a subpopulation selective manner in DRG neurons, although all have been shown to express N-type channels, as well as other calcium channels in differing proportions (Yusaf et al., 2001; Murali et al., 2014). In the present study, Ba\textsuperscript{2+} currents were recorded from small to medium-sized DRG neurons. VGCC currents recorded from DRG neurons cultured from Ca\textsubscript{V}2.2\textsubscript{HA}\textsuperscript{Kiki} and Ca\textsubscript{V}2.2\textsubscript{WTWT} mice (Figure 3.6) were like those of previous studies (McCallum et al., 2011; Murali et al., 2014). To further dissect these VGCC currents, \omega–conotoxin GIVA, an N-type calcium channel blocker, could be used to reveal what proportion of currents recorded are Ca\textsubscript{V}2.2 currents. Nevertheless, this experiment verified that the addition of the HA–tag did not affect the disrupt the function of VGCCs, similar to Cassidy et al. (2014).

N–type calcium channels are highly concentrated in the cell bodies of DRG neurons and primary afferents terminating in the spinal cord (Gohil et al., 1994). These primary afferents (C and A\textsubscript{δ} fibres) are implicated in the pain pathway. Ca\textsubscript{V}2.2 channels are the principal source of
Ca\textsuperscript{2+} entry to trigger neurotransmission (Bao et al., 1998; Heinke et al., 2004; Rycroft et al., 2007). As such, the present study investigated Ca\textsubscript{V}2.2_HA expression in DRG neurons. The advantage of the extracellularly tagged \(\alpha_{1B}\) subunit is the ability to distinguish surface and intracellular Ca\textsubscript{V}2.2_HA channels. Ca\textsubscript{V}2.2_HA staining in non-permeabilised DRG neurons resulted in distinct patterning showing fine clusters along the neuronal surface of the soma. Further optimisation of the staining protocol would be to ultimately detect similar patterns using a live cell immunolabelling protocol. However, previous studies in the lab (data not shown) have demonstrated surprisingly that little Ca\textsubscript{V}2.2_HA could be observed by this protocol. Nevertheless, the staining protocol defined in Figure 3.8 was determined as the most reliable and reproducible to show extracellular Ca\textsubscript{V}2.2_HA channels.

Native autofluorescence is due, in large part, to substances like lipofuscins which are prominent in the cytoplasm of aged neuronal cells. Lipofuscins are found throughout the neuraxis (Schnell et al., 1999). Their broad excitation and emission spectra complicate the interpretation of fluorescence microscopy (Dowson, 1982). Another source of autofluorescence could be due to the aldehyde fixative reacting with amines and proteins to generate fluorescent products. For this reason, I examined several potential immunolabelling methods to reduce or eliminate autofluorescence whilst retaining immunoreactivity to Ca\textsubscript{V}2.2_HA. When using either secondary antibody Alexa Fluor 594, BSAS or TSA, autofluorescence was eliminated (Figure 10). Nevertheless, BSAS and TSA surprisingly revealed immunoreactivity in Ca\textsubscript{V}2.2\textsubscript{WTWT} DRG neurons. BSAS is based on the ability of streptavidin conjugated secondary antibody to bind to biotin and amplifying immunofluorescence four-fold. However, preceding studies have described the presence of endogenous biotin in neuronal cells (Wang and Pevsner, 1999; McKay et al., 2004). This could potentially result in the misrepresentation of the results in Ca\textsubscript{V}2.2\textsubscript{WTWT} DRG neurons. Additionally, when using TSA, it is essential to quench native peroxidase. However, the immunoreactivity observed in Figure 3.9 may be due to the insufficient quenching of endogenous peroxidase, leading to tyramide activation. Further incubation times
with H$_2$O$_2$ would be required to establish the TSA protocol in neuronal cells. Nevertheless, the immunolabelling protocol (Figure 3.8) whereby neurons are post–fixed following anti–HA incubation and using either secondary antibody Alexa Fluor 488 or 594 revealed Ca$_V$2.2_HA at the membrane. This method was determined to best illustrate Ca$_V$2.2_HA at the membrane only in the Ca$_V$2.2_HA$^{KIKI}$ DRG neurons.

Interference of autofluorescence is a major shortcoming of confocal microscopy. Traditional confocal microscopy uses a combination of pinhole and single detectors whereas Airyscan is made of 32 detectors that collects a pinhole-plane image at every scan position. Each detector functions as a small pinhole which blocks out-of-focus light and gives a high-resolution image. As a result (Figure 3.11) shows autofluorescence is eliminated allowing for the clear detection of Ca$_V$2.2_HA at the cell membrane of DRG neurons.

CM–DiI is a lipophilic dye which stains cellular membranes (Jensen and Berg, 2016). Clustered structures of Ca$_V$2.2_HA can be seen co–localised with this membrane marker. This analysis method chosen here focuses on the distribution of fluorescent signal from CM–DiI and Ca$_V$2.2_HA. However, further analysis concentrating on pixel overlap could be used to distinguish co–localisation between the two probes (Costes et al., 2004; Dunn et al., 2011). This would further define limits on pixel intensity and distance between the two probes and this combined data would increase the information available about Ca$_V$2.2_HA at the membrane. There are multiple further optimisation steps that could be performed when establishing an immunolabelling protocol for Ca$_V$2.2_HA. Based on the above experiments, I found that post–fixing after anti–HA antibody application was sufficient for visualising Ca$_V$2.2_HA at the membrane of DRG neurons.

The advantage of examining the distribution of Ca$_V$2.2_HA in vitro is two-fold. Firstly, it allows for direct correlation between immunocytochemical evaluation and electrophysiological assessment. Second, having a better understanding of endogenous cell membrane expre-
sion will allow for more targeted experiments. These findings confirm those found in Cassidy et al. (2014) which showed robust and stable expression of overexpressed CaV2.2_HA in heterologous cell lines and neurons. Despite these advantages, it is important to compare and potentially validate these findings to evaluations performed in vivo from CaV2.2_HA^{KIKI} mice. Interestingly, here the in vitro data reported are, in general, comparable to in vivo data shown in Nieto-Rostro et al. (2018). The experimental evidence shown above demonstrates that the HA-tagged CaV2.2 is a robust and reliable molecular tool to assess endogenous cell surface CaV2.2.
Chapter 4

Ca\textsubscript{v}2.2-HA expression in DRG neurons and spinal cord tissue

4.1 Introduction

4.1.1 Ca\textsubscript{v}2.2 expression in DRG neurons

VGCCs govern a multitude of biological functions including mediating signal transduction, gene expression and neurotransmission. Thus, a large body of work has explored the existence and properties of different VGCCs in the nociceptive pathway (Westenbroek et al., 1998; Wilson et al., 2000). Using the whole-cell and cell-attached patch clamp technique, previous studies have distinguished the presence of N-type calcium channels from other VGCCs in the cell bodies of DRG neurons (Nowycky et al., 1985; Fox et al., 1987; Wilson et al., 2000). It has previously been demonstrated that the bulk of Ca\textsuperscript{2+} influx in most DRG neurons is conducted through N-type calcium channels (Regan et al., 1991; Cardenas et al., 1995; Wu and Pan, 2004). A comprehensive study found that 40% of calcium currents recorded from mouse small DRG cell bodies, with a diameter less than 30 \( \mu \text{m} \), were composed of N-type currents (Murali
et al., 2014). Furthermore, these authors found that only 20% of calcium currents recorded from large DRG cell bodies, with a diameter greater than 30 μm, consisted of N-type currents. Despite these electrophysiological studies highlighting the importance of Ca\textsubscript{2.2}, the scarcity of molecular tools to examine cell surface Ca\textsubscript{2.2} has hindered answering basic questions with regards to its expression in DRG neurons.

DRG neurons reside in the ganglia of the dorsal roots of the spinal cord and have afferent axons transmitting sensory stimuli to the CNS. Numerous subcategories of DRG neurons are present which respond to different sensory modalities. It is unquestionable that cultured neurons do not fully represent neuronal patterning in vivo (McGuigan and Javaherian, 2016). Thus, understanding cell surface expression of ion channels is a challenge owing to the existence of a multitude of specialised cell subtypes and morphology which may not be accurately represented in vitro. Nociceptive DRG neurons mainly have thinly myelinated A\textsubscript{δ} and unmyelinated C-fibres and can be broadly divided into peptidergic and non-peptidergic neurons. A central axiom in determining cell fate is the interaction between growth factors and cell-intrinsic information. Neurotrophins such as NGF promote neuronal survival and expression of ion channels on peptidergic nociceptive sensory neurons (Patel et al., 2000; Chen et al., 2006). In contrast, non-peptidergic neuron growth is activated by GDNF signalling. The ion channel expression profiles in these sensory neurons diversify their response to different stimuli (Raouf et al., 2010). Therefore, when culturing DRG neurons, the application of different growth factors is essential for sensory neuron specification, cell survival and phenotypic maturation (Mamet et al., 2003; Zhu and Oxford, 2011). Cell surface expression of Ca\textsubscript{2.2} remains insufficiently defined; in this chapter, the novel Ca\textsubscript{2.2}_HA\textsuperscript{KIKI} mouse line was used to decipher Ca\textsubscript{2.2}_HA organisation in primary sensory neurons.
4.1.2 Ca\textsubscript{V}2.2 expression in mouse spinal cord

The superficial dorsal horn of the spinal cord is involved in the processing of sensory information and forms the site of the first synapses in the pain pathway. Afferents terminate in the dorsal horn with a laminated distribution pattern that is generally determined by their sensory modality. Lamina I-II contain most of the termination zones of nociceptive afferents, small diameter myelinated A\textdagger and unmyelinated C-fibres (Christensen and Perl, 1970; Craig et al., 2001; Morris et al., 2004). At the presynaptic site of primary afferents, neurotransmission is mediated by Ca\textsuperscript{2+} influx through VGCCs (Park and Luo, 2010). Through measuring miniature and evoked EPSCs, previous reports have demonstrated that Ca\textsubscript{V}2.2 is critically involved in excitatory neurotransmission from primary afferent terminals with a lesser contribution from P/Q type channels (Bao et al., 1998; Heinke et al., 2004; Rycroft et al., 2007).

Westenbroek et al. (1992) developed antibodies to an intracellular epitope of Ca\textsubscript{V}2.2 which corresponded to residues in the cytoplasmic loop between domain II and III (for simplicity referred to as the anti-II-III loop antibody). Westenbroek et al. (1998), using the anti II-III loop antibody, showed immunoreactivity to Ca\textsubscript{V}2.2 in nerve terminals and cell bodies in the dorsal horn. Double labelling experiments using the anti-II-III loop antibody with either anti-Syntaxin (presynaptic marker) or anti-substance P (peptidergic marker) antibodies revealed a substantial overlap of these probes in the nerve terminals of the dorsal horn. Thus, showing that the N-type calcium channel is most abundant in lamina I and II of the dorsal horn. Nevertheless, the antibody developed by Westenbroek et al. (1992) and commercially available antibodies (Chi et al., 2009; Yang et al., 2018) cannot discriminate between Cav2.2 at the membrane from those localised intracellularly. Thus, complicating the interpretation of these data in the context of Ca\textsubscript{V}2.2 expression at the cell surface.
4.1.3 Importance of Ca\textsubscript{v}2.2 in the pain pathway

VGCCs are multi-subunit complexes composed of the central pore-forming $\alpha_1$ and auxiliary subunits $\alpha_2\delta$ and $\beta$. A significant role for $\alpha_2\delta$-1 has been established in chronic neuropathic pain. Significant upregulation of the calcium channel accessory subunit $\alpha_2\delta$-1 mRNA has been observed in DRG neurons following partial sciatic nerve ligation in rats (Newton et al., 2001). Additionally, peripheral nerve injury in rats increases $\alpha_2\delta$-1 protein expression, in both DRG neurons and the spinal cord dorsal horn (Li et al., 2004). Furthermore, mice with a deletion of the $\alpha_2\delta$-1 gene, display a delay in mechanical hypersensitivity (Patel et al., 2013). The $\alpha_2\delta$-1 ligands pregabalin and gabapentin are also effective in the treatment of a range of chronic neuropathic conditions (Moore et al., 2009, 2014). These studies suggest that $\alpha_2\delta$-1 plays a key role in the development of neuropathic pain following nerve injury. $\alpha_2\delta$-1 has also been found to increase Ca\textsuperscript{2+} currents through N-type channels following heterologous expression in cell lines, and in neurons (Patel et al., 2013; Cassidy et al., 2014). There is little evidence that $\alpha_2\delta$s influence single-channel properties of Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 (Wakamori et al., 1999). As such $\alpha_2\delta$-enhanced currents have been accredited to an upregulation of VGCC trafficking and insertion into the plasma membrane (Wakamori et al., 1999; Davies et al., 2010; Cassidy et al., 2014).

The therapeutic importance of the N-type calcium channel has been highlighted using Ca\textsubscript{v}2.2 knockout mouse models. Ca\textsubscript{v}2.2 knockout mice reveal a dramatic reduction in neuropathic pain symptoms (Saegusa et al., 2001; Hatakeyama et al., 2001; Kim et al., 2001). Thus, suggesting that Ca\textsubscript{v}2.2 is essential for the development of neuropathic pain following nerve injury. The compelling nature of this evidence suggests that Ca\textsubscript{v}2.2 trafficking could present as a therapeutic target for treatment of neuropathic pain. In an attempt to address this, ziconotide, a synthetic version of the reversible Ca\textsubscript{v}2.2 channel blocker, $\omega$-conotoxin MVIIA, was developed for chronic pain (Staats et al., 2004). Nevertheless, therapeutics for neuropathic pain still...
have a high unmet need as ziconotide is unsatisfactory due to severe neurological side-effects and impracticalities of intrathecal delivery (Sanford, 2013; Patel et al., 2018). Considering the essential role for Ca\textsubscript{V}2.2 in the pain pathway, this channel and its regulators still present as promising therapeutic targets.

Despite the myriad of studies highlighting the importance of Ca\textsubscript{V}2.2 in the pain pathway, endogenous cell surface Ca\textsubscript{V}2.2 expression has not yet been studied due to the lack of available molecular tools. The aim of the present study was to use the novel Ca\textsubscript{V}2.2_HA\textsuperscript{KIKI} mouse model, detailed in Chapter 3, to examine the distribution of cell surface Ca\textsubscript{V}2.2_HA in DRG neurons and primary afferents terminating in the spinal cord.
4.2 Optimisation of DRG neuronal markers

Nociceptors generally have either unmyelinated C-fibres or thinly myelinated Aδ-fibres. C-fibres can be broadly classified into two subgroups. One is a peptidergic population that contains neuropeptides such as substance P and calcitonin gene related peptide (CGRP). The other is referred to as a non-peptidergic population that binds isolectin-B4 (IB4) (Snider and McMahon, 1998). To examine the expression of Cav2.2_HA in different subtypes of DRG neurons, it was first necessary to test and optimise the immunolabelling of the peptidergic, CGRP-positive, and non-peptidergic, IB4-positive, markers in DRG neuron cultures.

4.2.1 Optimisation of IB4_FITC immunolabelling in DRG neurons

IB4 is a plant lectin which binds to α-D-galactose carbohydrate epitopes on sensory ganglion neurons and is commonly used to define the non-peptidergic population of DRG neurons (Silverman and Kruger, 1990). To identify this subpopulation, IB4 conjugated to FITC (IB4_FITC) was used for DRG neuron labelling. Previous studies have successfully examined IB4_FITC staining in both fixed and live neurons (Molliver and Snider, 1997; Barabas et al., 2012). As such, an immunolabelling protocol was first established in non-permeabilised DRG neurons cultured from 10–12-week-old Cav2.2_HA[KIKI] mice (Figure 4.1). Figure 4.1Ai shows fixed DRG neurons incubated with IB4_FITC for one hour. IB4 immunolabelling revealed a complete ring of FITC around the soma perimeter. However, high levels of autofluorescence could be detected within the cell body.

Preceding studies have also live-labelled sensory neurons to identify non-peptidergic populations (Stucky and Lewin, 1999; Molliver and Snider, 1997). Considering this, DRG neurons were live labelled with 10 μg/ml of IB4_FITC at 37°C for either 10, 20 or 60 min. Incubating DRG neuron cultures for either 20 or 60 min did indeed reveal IB4_FITC immunoreactivity.
at the cell membrane. However, the staining observed was slightly diffuse, with observable signal within the neuron. As this protocol involves live application of IB4_FITC, it is possible that intracellular staining could be due to endocytosis of the marker (Figure 4.1Aiii and iv). The clearest IB4_FITC staining was in neurons incubated for 10 min (Figure 4.1Aii) and this was established as the optimal time for IB4_FITC live-labelling for further studies.

![Figure 4.1: 10 minutes live labelling with IB4_FITC is optimal for identifying IB4-positive DRG neurons](image)

(A) Images of cultured DRG neurons from CaV2.2_HA<sup>KIKI</sup> showing (i) IB4_FITC staining after fixation and live labelling with IB4_FITC for (ii) 10 min (iii) 20 min (iv) 60 min (first column). (B; second column) of merged representative image with DAPI in blue. (C; third column) of negative control (NCT) where IB4_FITC was not applied (Scale bars: 5 μm.)
4.2.2 Optimisation of CGRP immunolabelling in DRG neurons

In the present study, CGRP was used to identify the peptidergic population of DRG neurons. We obtained two anti-CGRP antibodies raised in different species, rabbit and mouse. As such, assays were conducted to evaluate which antibody would best identify CGRP-positive DRG neurons. DRG neurons were cultured from 10 to 12-week-old Cav2.2_HA^KIKI mice and fixed after 1 DIV. Neurons were then permeabilised and incubated with either mouse or rabbit anti-CGRP antibody overnight at 4°C. Following this, neurons were incubated with the corresponding secondary antibody (anti-mouse Alexa Fluor 633 or anti-rabbit Alexa Fluor 647). Figure 4.2Ai shows very weak CGRP staining when using the rabbit anti-CGRP antibody. However, when using the mouse anti-CGRP antibody, although the staining is somewhat diffuse, CGRP immunoreactivity can be seen within the DRG neuron (Figure 4.2Aii). Nevertheless, the inability of the anti-mouse secondary antibody to distinguish between endogenous mouse IgG and mouse anti-CGRP results in a high background signal. This would obfuscate results and potentially lead to DRG neurons being misidentified. To address this problem, mouse-on-mouse (MOM) blocking reagent was used to bind and saturate endogenous mouse antibodies. When using the additional MOM blocking step, CGRP could be seen in compact structures concentrated in perinuclear regions and throughout the cytoplasm (Figure 4.2Aiii). CGRP immunolabelling in DRG cultures was similar to that observed in previous studies (Zhang et al., 1995; Belanger et al., 2002).
Figure 4.2: The mouse anti-CGRP antibody and MOM are required for identifying CGRP-positive DRG neurons

(A) Images of cultured DRG neurons from Ca\textsubscript{\textgamma}2.2_\textsubscript{HA}\textsuperscript{KIKI} showing (i) rabbit anti-CGRP antibody immunolabelling, (ii) mouse anti-CGRP antibody immunolabelling, (iii) mouse anti-CGRP antibody immunolabelling with added MOM blocking (B) Merged representative image with DAPI in blue (C) Negative control (NCT) no primary antibody (Scale bars: 5 μm.)
4.3 Characterisation of Ca$_V$2.2_HA in different subpopulations of DRG neurons

4.3.1 Cell surface Ca$_V$2.2_HA is predominantly expressed in small and medium-sized IB4-negative DRG neurons

To study the distribution of cell surface Ca$_V$2.2_HA in DRG neurons, cultures were prepared from Ca$_V$2.2_HA$^{KIKI}$ mice and live-labelled with IB4_FITC. To ensure that only cell surface Ca$_V$2.2_HA was examined, neurons were fixed in non-permeabilised conditions and labelled with rat anti-HA antibody. Confocal images were taken of all Ca$_V$2.2_HA-positive neurons. These images were next subdivided based on whether neurons were IB4-positive or negative. Figure 4.3 shows DRG neurons stained for Ca$_V$2.2_HA and IB4 in a ring-like fashion at the cell membrane. As expected, Ca$_V$2.2_HA staining was not seen in DRG neurons cultured from Ca$_V$2.2_HA$^{WTWT}$ mice (Figure 4.3Aiii). Only 20% ± 3% of DRG neurons positive for Ca$_V$2.2_HA surface staining were also positive for IB4. Cell surface Ca$_V$2.2_HA was present, to a much greater extent (80% ± 3%) on IB4-negative neurons (Figure 4.3B).

Another criterion to distinguish different types of DRG neurons is soma size. Cell surface Ca$_V$2.2_HA intensity was measured and averaged from all Ca$_V$2.2_HA-positive neurons. The mean fluorescence intensity value of cell surface Ca$_V$2.2_HA was then used to normalise cell surface Ca$_V$2.2_HA intensity of individual neurons. Comparisons between cell surface Ca$_V$2.2_HA expressed in IB4-positive and negative DRG neurons were made with respect to cell size. The perimeter measured from these DRG neurons was used as an estimation of neuron size (small <61 μm, medium 61-94 μm and large >94 μm; Sommer et al. (1985)). Within the small-sized DRG population, Ca$_V$2.2_HA immunoreactivity was significantly higher by 56% in IB4-negative compared to IB4-positive neurons (normalised values were 1.44 ± 0.09 and 0.92 ± 0.07, respectively, Figure 4.3C). Ca$_V$2.2_HA immunolabelling was found 18%...
higher in medium-sized IB4-negative DRG neurons compared to its IB4-positive counterpart (normalised values were 1.04 ± 0.10 and 0.87 ± 0.06, respectively, Figure 4.3C). Both IB4-positive and negative large-diameter neurons presented with the lowest CaV2.2_HA staining at 0.76 ± 0.08 and 0.74 ± 0.07, respectively (Figure 4.3C). The overall relationship of CaV2.2_HA to IB4 staining shows that they both have distinct distributions. These data suggest that CaV2.2_HA is preferentially expressed in small and medium-sized IB4-negative DRG neurons.
Figure 4.3: Cell surface Cav2.2_HA is predominantly expressed in small and medium-sized IB4-negative DRG neurons

(A) Images of cultured DRG neurons from Cav2.2_HA^{KIKI} (i and ii) and Cav2.2\textsuperscript{WTWT} mice (iii), showing (left to right) Cav2.2_HA staining (prior to permeabilisation), IB4_FITC and merged for representative IB4-positive (i and iii; top and bottom) and IB4-negative (ii; middle) neurons. (Scale bars: 5 μm). (B) Quantification of the percentage of neurons with cell surface Cav2.2_HA that were also positive (green circles) or negative (open black circles) for IB4. Individual data points represent the mean data from three separate experiments and a total of 267 DRG neurons. Mean ± SEM of the three experiments is superimposed. ***p<0.001 (unpaired t test) and F value = 1.281 (C) Normalised cell surface Cav2.2_HA intensity with respect to cell size: small (s), medium (m) and large (l) DRG neurons that are either IB4-positive (green circles) or IB4-negative (open black circles). Cell surface Cav2.2_HA intensity was measured and averaged from all Cav2.2_HA-positive neurons. The mean fluorescence intensity value was then used to normalise cell surface Cav2.2_HA intensity of individual neurons. Individual data points represent normalised Cav2.2_HA intensity measured from all Cav2.2_HA-positive neurons from three separate experiments and a total of 16, 27, 8, 72, 89 and 55 DRG neurons, from left to right on x-axis. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; ***p<0.001, ns = not significant, F value = 9.336.
4.3.2 Cell surface CaV2.2_HA is predominantly expressed in small and medium-sized CGRP-positive DRG neurons

CaV2.2_HA expression was next studied in CGRP-positive peptidergic neurons. DRG neuron cultures were first fixed and incubated with rat anti-HA antibody in non-permeabilised conditions. Following this, neurons were permeabilised and using the immunolabelling protocol established in section 4.2.2, CGRP immunoreactivity was achieved. Strong immunoreactivity to CaV2.2_HA can be seen at the cell membrane of DRG neurons cultured from CaV2.2_HA<sup>KIKI</sup> mice. Confocal microscopy showed that CGRP can be seen within these neurons in confined regions (Figure 4.4A). CaV2.2_HA can also be seen in neurons negative for CGRP and cell surface CaV2.2_HA staining is not observed in DRG neurons cultured from CaV2.2<sup>WTWT</sup> mice (Figure 4.4Aiii). The majority of CaV2.2_HA-positive neurons (66 ± 5%) were CGRP-positive whilst 34% ± 5% were negative for CGRP. Figure 4.4B clearly shows that the highest cell surface CaV2.2_HA intensity was recorded in CGRP-positive neurons.

Previous studies have shown that CGRP is mainly expressed in small to medium-sized DRG neurons (Zhang et al., 1995; Kestell et al., 2015). As such, neurons were next categorised by size as most nociceptors have small diameter unmyelinated axons (C-fibres) (Djouhri and Lawson, 2004). CaV2.2_HA intensity was measured and averaged from all CaV2.2_HA-positive neurons. The mean fluorescence intensity value of cell surface CaV2.2_HA was then used to normalise cell surface CaV2.2_HA intensity of individual neurons. Comparison of CaV2.2_HA immunoreactivity in CGRP-positive and negative neurons were then made with respect to cell size. In small DRG neurons, plasma membrane CaV2.2_HA labelling was strikingly higher by 92% in CGRP-positive compared to CGRP-negative neurons (normalised values were 1.33 ± 0.15 to 0.69 ± 0.11, respectively, Figure 4.4C). Within the medium-sized subpopulation, plasma membrane CaV2.2_HA was 23% higher in CGRP-positive compared to CGRP-negative neurons (normalised values were 1.03 ± 0.13 to 0.83 ± 0.15, respectively, Fig-
ure 4.4C). However, no statistically significant difference was detected between large CGRP-positive and negative neurons (1.02 ± 0.26 and 1.21 ± 0.50, respectively, Figure 4.4C). This study showed that, at the cell surface, Cav2.2_HA was preferentially expressed in small and medium-sized CGRP-positive nociceptors.

![Figure 4.4: Cell surface Cav2.2_HA is predominantly expressed in small and medium-sized CGRP-positive DRG neurons](image)

**Figure 4.4: Cell surface Cav2.2_HA is predominantly expressed in small and medium-sized CGRP-positive DRG neurons**

(A) Images of cultured DRG neurons from Cav2.2_HA^KIKI (i and ii) and Cav2.2_WWTWt mice (iii) showing (left to right) Cav2.2_HA staining (prior to permeabilisation), CGRP (following permeabilisation) and merged for representative CGRP-positive (i and iii; top and bottom) and CGRP-negative (ii; middle) neurons. (Scale bars: 5 μm). (B) Quantification of the percentage of neurons with cell surface Cav2.2_HA that were also positive (purple circles) or negative (open black circles) for CGRP. Individual data points represent the mean data from three separate experiments and a total of 237 DRG neurons. Mean ± SEM of the three experiments is superimposed. *p value = 0.0123, F value = 1.000 (unpaired t test) (C) Normalised cell surface Cav2.2_HA intensity with respect to cell size: small (s), medium (m) and large (l) DRG neurons that are either CGRP-positive (purple circles) or CGRP-negative (open black circles). Cell surface Cav2.2_HA intensity was measured and averaged from all Cav2.2_HA-positive neurons. The mean fluorescence intensity value was then used to normalise cell surface Cav2.2_HA intensity of individual neurons. Individual data points represent normalised Cav2.2_HA intensity measured from all Cav2.2_HA-positive neurons from three separate experiments and a total of 81, 62, 20, 37, 33 and 4 DRG neurons, from left to right on x-axis. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one-way ANOVA with Bonferroni’s selected comparison test as post hoc test; ***p<0.001, ns = not significant, F value = 6.873.
4.3.3 Co-expression of Ca\textsubscript{V}2.2\_HA, CGRP and IB4-binding glycoprotein in DRG neurons

Literature on DRG neuron characterisation has reported the co-expression of CGRP with IB4-binding glycoproteins (Wang et al., 1994; Barabas et al., 2012; Harrison et al., 2014). To further explore the patterning of Ca\textsubscript{V}2.2\_HA expression in DRG neurons, cultures were triple labelled with Ca\textsubscript{V}2.2\_HA, CGRP and IB4. Neurons were first live labelled with IB4\_FITC, fixed, stained for Ca\textsubscript{V}2.2\_HA in non-permeabilised conditions, then permeabilised and labelled for CGRP (Figure 4.5A). As expected, confocal imaging revealed that the majority of Ca\textsubscript{V}2.2\_HA-positive neurons also expressed CGRP (56% ± 6%). This was significantly different from Ca\textsubscript{V}2.2\_HA immunoreactive neurons positive for IB4 (11% ± 1.6%). Surprisingly, amongst the Ca\textsubscript{V}2.2\_HA-positive neurons there was a population which expressed both CGRP and IB4 (13% ± 7%). Furthermore, 21% ± 4% of Ca\textsubscript{V}2.2\_HA expressing neurons were not positive for either marker (Figure 4.5B). This study suggests that cell surface Ca\textsubscript{V}2.2\_HA immunoreactivity can be observed predominantly in peptidergic neurons. However, a statistically significant smaller proportion of Ca\textsubscript{V}2.2\_HA immunoreactive neurons can be identified in neurons expressing both IB4-binding glycoproteins and CGRP.
Figure 4.5: Co-expression of Ca\textsubscript{v}2.2_HA, CGRP and IB4-binding glycoproteins in DRG neurons

(A) Images of cultured DRG neurons from Ca\textsubscript{v}2.2_HA\textsuperscript{KIKI} mice showing (left to right) Ca\textsubscript{v}2.2_HA and IB4 staining before permeabilization, CGRP staining following permeabilization and merged for representative CGRP-positive (i; top), IB4-positive (ii; middle) and CGRP/IB4-positive neurons. (Scale bars: 5 \textmu m). (B) Quantification of the percentage of neurons with cell surface Ca\textsubscript{v}2.2_HA that were either positive for IB4 (green circles), CGRP (purple circles), CGRP and IB4 (grey circles), or neither marker (red circles). Individual data points represent the mean data from three separate experiments and a total of 206 DRG neurons. Mean ± SEM of the three experiments is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s multiple comparison test as post hoc test; **p<0.01, *p<0.05, F value = 15.69.
4.3.4 Cell surface Cav2.2_HA is predominantly expressed in neurofilament 200-negative DRG neurons

It is well established that DRG neurons can be divided into distinct populations based on size (Lawson and Waddell, 1991). Somata of C-fibres are restricted to the size range of small neurons (where the diameter is <19 μm) (Harper and Lawson, 1985). In the present study, Cav2.2_HA was labelled prior to permeabilization. Then, anti-neurofilament 200 (NF200) antibody was used to label large diameter neurons. Figure 4.6A shows NF200 labelling in large-sized DRG neurons and Cav2.2_HA labelling of the neuronal plasma membrane in neurons negative for NF200. 77% ± 2% of Cav2.2_HA immunoreactive neurons were negative for NF200 (Figure 4.6B). As expected, Cav2.2_HA was not detected in DRG neurons cultured from Cav2.2WTWT mice (Figure 4.6Aiiii).

Cav2.2_HA intensity was measured and averaged from all Cav2.2_HA-positive neurons. The mean fluorescence intensity value of cell surface Cav2.2_HA was then used to normalise cell surface Cav2.2_HA intensity of individual neurons and categorised based on cell size (Figure 4.6C). DRG neurons express low (68 kDa), medium (155 kDa) and high (200 kDa) molecular weight neurofilament protein. The anti-NF200 antibody recognises both the phosphorylated and non-phosphorylated forms of NF200. Large-diameter DRG neurons predominantly express NF200 in both phosphorylation states, however, a few small-sized DRG neurons also express the phosphorylated forms of NF200 (Perry et al., 1991). As such, a few small-diameter DRG neurons were labelled positive for NF200. Analysis of the NF200-positive population showed cell surface Cav2.2_HA was 44% higher in small compared to medium-sized DRG neurons, and 66% higher in small compared to large-sized DRG neurons (normalised values were s: 1.37 ± 0.35, m: 0.95 ± 0.15, l: 0.82 ± 0.12, Figure 4.6C). A similar pattern in cell surface Cav2.2_HA expression was observed in NF200-negative neurons. Within this sub-population, cell surface Cav2.2_HA was 44% higher in small compared to medium-sized...
DRG neurons, and 228% higher in small compared to large-sized DRG neurons (normalised values were s: 1.41 ± 0.10, m: 0.97 ± 0.09, l: 0.43 ± 0.19, Figure 4.6C). This data suggests that Ca\textsubscript{v}2.2\_HA is predominantly expressed in small and medium-sized DRG neurons.

Figure 4.6: Cell surface Ca\textsubscript{v}2.2\_HA is predominantly expressed in NF200-negative DRG neurons

(A) Images of cultured DRG neurons from Ca\textsubscript{v}2.2\_HA\textsuperscript{KIKI} (i and ii) and Ca\textsubscript{v}2.2\_HA\textsuperscript{WTWT} mice (iii) showing (left to right) Ca\textsubscript{v}2.2\_HA staining (prior to permeabilisation), NF200 (following permeabilisation) and merged for representative NF200-positive (i and iii; top and bottom) and NF200-negative (ii; middle) neurons. (Scale bars: 5 \( \mu \)m). (B) Quantification of the percentage of neurons with cell surface Ca\textsubscript{v}2.2\_HA that were also positive (green circles) or negative (open black circles) for NF200. Individual data points represent the mean data from three separate experiments and a total of 402 DRG neurons. Mean ± SEM of the three experiments is superimposed. ***\( p < 0.001, \) F value = 1.000 (unpaired t test) (C) Normalised cell surface Ca\textsubscript{v}2.2\_HA intensity with respect to cell size: small (s), medium (m) and large (l) DRG neurons that are either NF200-positive (green circles) or NF200-negative (open black circles). Cell surface Ca\textsubscript{v}2.2\_HA intensity was measured and averaged from all Ca\textsubscript{v}2.2\_HA-positive neurons. The mean fluorescence intensity value was then used to normalise cell surface Ca\textsubscript{v}2.2\_HA intensity of individual neurons. Individual data points represent normalised Ca\textsubscript{v}2.2\_HA intensity measured from all Ca\textsubscript{v}2.2\_HA-positive neurons from three separate experiments and a total of 15, 37, 54, 181, 112, and 3 DRG neurons, from left to right on x-axis. Mean ± SEM of the total number of DRG neurons is superimposed. #: Note very few large DRG neurons are NF200-negative. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; ***\( p < 0.001, \) F value = 8.472
4.4 Influence of growth factors on \( \text{Ca}_{2.2} \text{HA} \) expression in DRG neuronal cultures

The use of dissociated DRG neurons, in contrast to acute or slice preparations means that growth signalling mechanisms can be more easily studied. The environment of isolated neurons can be modified \textit{in vitro} to reflect \textit{in vivo} states. Previous studies have shown that growth factors such as NGF and GDNF are involved in survival and neuronal subtype specification (Krames, 2014). Small nociceptive neurons can be divided into NGF- and GDNF-sensitive subpopulations (Snider and McMahon, 1998). These neuronal subtypes express the high affinity NGF receptor tyrosine kinase (trkA) (Averill et al., 1995) or GDNF receptor, Ret (Molliver and Snider, 1997). It has been reported that these two populations can be identified by immunoreactivity for CGRP and the binding of IB4 to distinguish NGF and GDNF sensitive neurons, respectively. Furthermore, ion channel expression has also been reported to be regulated by growth factors (Aoki et al., 2005; Ernsberger, 2009). This next section aims to examine the dependence of cell surface \( \text{Ca}_{2.2} \text{HA} \) expression on neurotrophic factors.

4.4.1 The effect of NGF on cell surface \( \text{Ca}_{2.2} \text{HA} \) expression

To investigate the effects of NGF on cell surface \( \text{Ca}_{2.2} \text{HA} \) expression, DRG neurons were cultured from \( \text{Ca}_{2.2} \text{HA}^{\text{KIKI}} \) mice. Neurons were plated with media containing 50 ng/ml NGF and cultured \textit{in vitro} for 24 h. Immunolabelling was performed as previously described in section 4.3.3. Results were similar to those observed in section 4.3 where NGF was applied. Figure 4.7 shows that the largest proportion of \( \text{Ca}_{2.2} \text{HA} \) expressing neurons were also positive for CGRP (47% ± 9%) whereas a significantly smaller percentage of neurons were identified as non-peptidergic (9% ± 2%). A proportion of neurons were also positive for both IB4 and CGRP (22% ± 9%) or for neither marker (\( \text{Ca}_{2.2} \text{HA} \) only; 22% ± 2%; Figure 4.7B).
Next, neurons positive for cell surface \( \text{Ca}_{V}^{2.2} \text{_HA} \) and positive for either IB4, CGRP, both markers or \( \text{Ca}_{V}^{2.2} \text{_HA} \) only were analysed with respect to neuron size. Cell surface \( \text{Ca}_{V}^{2.2} \text{_HA} \) intensity was measured and averaged from all \( \text{Ca}_{V}^{2.2} \text{_HA} \)-positive neurons. This mean fluorescence intensity value was then used to normalise cell surface \( \text{Ca}_{V}^{2.2} \text{_HA} \) fluorescence intensity of individual neurons. \( \text{Ca}_{V}^{2.2} \text{_HA} \) immunoreactivity was significantly higher in CGRP-positive neurons compared to the IB4-positive subpopulation. Mean plasma membrane \( \text{Ca}_{V}^{2.2} \text{_HA} \) immunoreactivity was 137%, 107% and 265% higher in small, medium and large-sized CGRP-positive neurons, respectively, compared to their respective IB4-positive counterparts (Figure 4.7C). A smaller proportion of neurons was identified as positive for both CGRP and IB4. In this subpopulation, cell surface \( \text{Ca}_{V}^{2.2} \text{_HA} \) was 21% higher in small compared to medium-sized DRG neurons, and 39% higher in small compared to large-sized DRG neurons (normalised values were s: 1.20 ± 0.26, m: 1.00 ± 0.19, l: 0.87 ± 0.14, Figure 4.7C).

Further analysis also revealed a group of neurons negative for CGRP and IB4. Within this sub-group, cell surface \( \text{Ca}_{V}^{2.2} \text{_HA} \) was 39% higher in small relative to large-diameter DRG neurons, and 66% higher in medium relative to large-diameter DRG neurons (normalised values were s: 0.81 ± 0.15, m: 0.97 ± 0.16, l: 0.58 ± 0.13, Figure 4.7C). These data suggest that under the influence of NGF, \( \text{Ca}_{V}^{2.2} \text{_HA} \) is preferentially expressed at the cell surface of small CGRP-positive DRG neurons.
Figure 4.7: Cell surface Ca\textsubscript{V}2.2_HA is predominantly expressed on CGRP-positive DRG neurons following NGF incubation

(A) Images of cultured DRG neurons from Ca\textsubscript{V}2.2_HA\textsuperscript{KIKI} mice showing (left to right) Ca\textsubscript{V}2.2_HA (prior to permeabilisation), live labelling with IB4_FITC, CGRP staining (following permeabilisation) and merged representative image. (Scale bars: 5 \(\mu\)m). (B) Quantification of the percentage of neurons with cell surface Ca\textsubscript{V}2.2_HA that were either positive for IB4 (green circles), CGRP (purple circles), CGRP and IB4 (grey circles), or neither marker (red circles). Individual data points represent the mean data from three separate experiments and a total of 192 DRG neurons. Mean ± SEM of the three experiments is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s multiple comparison test as post hoc test; *\(p<0.05\), F value = 15.69. (C) Normalised cell surface Ca\textsubscript{V}2.2_HA intensity with respect to cell size: small (s), medium (m) and large (l) DRG neurons that were either positive for IB4 (green circles), CGRP (purple circles), CGRP and IB4 (grey circles), or neither marker (red circles). Cell surface Ca\textsubscript{V}2.2_HA intensity was measured and averaged from all Ca\textsubscript{V}2.2_HA-positive neurons. The mean fluorescence intensity value was then used to normalise cell surface Ca\textsubscript{V}2.2_HA intensity of individual neurons. Individual data points represent normalised Ca\textsubscript{V}2.2_HA intensity measured from all Ca\textsubscript{V}2.2_HA-positive neurons from three separate experiments and a total of 48, 29, 12, 12, 9, 5, 13, 15, 2, 21, 23 and 3 DRG neurons from left to right on x-axis. Mean ± SEM of the respective total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; ***\(p<0.001\), *\(p<0.05\), F value = 4.542.
4.4.2 The effect of GDNF on cell surface Ca\textsubscript{V}2.2_HA expression

To explore whether GDNF supported Ca\textsubscript{V}2.2_HA expression in a subpopulation of sensory neurons, 50 ng/ml GDNF was applied to dissociated murine DRG neurons for 24 h. GDNF has been reported to favour the growth of IB4-positive neurons (Matheson et al., 1997; Snider and McMahon, 1998). In the present study, the addition of GDNF increased the overall proportion of IB4-positive neurons, in turn 31% ± 1% of Ca\textsubscript{V}2.2_HA expressing neurons were positive for IB4-binding and 36% ± 2% of Ca\textsubscript{V}2.2_HA expressing neurons were distinguished as CGRP-positive. A significantly smaller proportion of Ca\textsubscript{V}2.2_HA expressing neurons were positive for both markers (16% ± 2%) and 17% ± 2% of Ca\textsubscript{V}2.2_HA-positive neurons expressed neither CGRP nor IB4-binding glycoproteins (Figure 4.8B).

Subsequently, cell surface Ca\textsubscript{V}2.2_HA of neurons cultured with GDNF was analysed with respect to cell size and divided into four groups: IB4-, CGRP-, both IB4 and CGRP, and Ca\textsubscript{V}2.2_HA only-positive neurons. Cell surface Ca\textsubscript{V}2.2_HA fluorescence intensity was measured from all Ca\textsubscript{V}2.2_HA-positive neurons and averaged. This mean fluorescence intensity value was used to normalise cell surface Ca\textsubscript{V}2.2_HA of individual neurons. Despite the increase in number of IB4-positive neurons, when analysed with respect to size, Ca\textsubscript{V}2.2_HA expression was higher in CGRP expressing neurons. In the presence of GDNF, cell surface Ca\textsubscript{V}2.2_HA immunolabelling was higher by 42%, 46% and 39% in small, medium and large CGRP-positive neurons, respectively, compared to their corresponding subgroups of IB4-positive neurons (Figure 4.8C). In neurons expressing CGRP and IB4-binding glycoproteins, plasma membrane Ca\textsubscript{V}2.2_HA was 48% higher in small compared to medium-sized DRG neuron, and 30% higher in small compared to large-sized DRG neurons (normalised values were s: 1.42 ± 0.15, m: 0.96 ± 0.07, l: 1.10 ± 0.25, Figure 4.8C). A small population of Ca\textsubscript{V}2.2_HA-positive neurons was not positive for either CGRP expression or IB4-binding. In this sub-group, cell surface Ca\textsubscript{V}2.2_HA was 68% higher in small relative to medium-diameter
DRG neurons, and 53% higher in small relative to large-diameter DRG neurons (normalised values were s: 1.39 ± 0.25, m: 0.82 ± 0.10, l: 0.91 ± 0.14, Figure 4.8C). These results suggest that GDNF supports the growth of IB4-positive neurons. Nonetheless, cell surface CaV2.2_HA is found to be preferentially expressed in small peptidergic neurons.
Figure 4.8: Cell surface Ca\textsubscript{v}2.2\_HA is predominantly expressed on CGRP-positive DRG neurons following incubation with GDNF

(A) Images of cultured DRG neurons from Ca\textsubscript{v}2.2\_HA\textsuperscript{KIKI} mice showing (left to right) Ca\textsubscript{v}2.2\_HA (prior to permeabilisation), live labelling with IB4\_FITC, CGRP staining (following permeabilisation) and merged representative image. (Scale bars: 5 \(\mu\)m). (B) Quantification of the percentage of neurons with cell surface Ca\textsubscript{v}2.2\_HA that were either positive for IB4 (green circles), CGRP (purple circles), CGRP and IB4 (grey circles), or neither marker (red circles). Individual data points represent the mean data from three separate experiments and a total of 302 DRG neurons. Mean ± SEM of the three experiments is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s multiple comparison test as post hoc test; ***\(p<0.001\), **\(p<0.01\), ns = not significant, F value = 4.201. (C) Normalised cell surface Ca\textsubscript{v}2.2\_HA intensity with respect to cell size: small (s), medium (m) and large (l) DRG neurons were either positive for IB4 (green circles), CGRP (purple circles), CGRP and IB4 (grey circles), or neither marker (red circles). Cell surface Ca\textsubscript{v}2.2\_HA fluorescence intensity was measured from all Ca\textsubscript{v}2.2\_HA-positive neurons and averaged. This mean fluorescence intensity value was used to normalise cell surface Ca\textsubscript{v}2.2\_HA of individual neurons. Individual data points represent normalised Ca\textsubscript{v}2.2\_HA intensity measured from all Ca\textsubscript{v}2.2\_HA-positive neurons from three separate experiments and a total of 27, 52, 40, 12, 55, 5, 29, 37, 9, 3, 20 and 13 DRG neurons from left to right on x-axis. Mean ± SEM of the respective total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; ***\(p<0.001\), **\(p<0.01\), *\(p<0.05\), F value = 41.16
4.4.3 The effect of both NGF and GDNF on cell surface Ca\textsubscript{V}2.2\_HA expression

Following the application of both NGF and GDNF on DRG cultures, cell surface Ca\textsubscript{V}2.2\_HA expression was investigated. Figure 4.9 shows that despite both growth factors being present, a statistically significant proportion of Ca\textsubscript{V}2.2\_HA-positive neurons was CGRP-positive (43% ± 3%) in comparison to IB4-binding neurons (17% ± 1%). As shown in previous experiments, a statistically significant proportion of Ca\textsubscript{V}2.2\_HA-positive neurons was positive for both markers (21% ± 1%), and 19% ± 2% of neurons were positive for neither (Figure 4.9B).

Next, cell surface Ca\textsubscript{V}2.2\_HA fluorescence intensity of neurons cultured in both NGF and GDNF was measured and averaged. The mean cell surface Ca\textsubscript{V}2.2\_HA fluorescence intensity was used to normalise the cell surface Ca\textsubscript{V}2.2\_HA intensity of individual Ca\textsubscript{V}2.2\_HA-positive neurons. Surprisingly, analysis of cell surface Ca\textsubscript{V}2.2\_HA with respect to size showed that Ca\textsubscript{V}2.2\_HA immunoreactivity did not differ significantly between IB4 and CGRP labelled neurons (Figure 4.9C). Application of both GDNF and NGF in cultured DRG neurons allows the direct comparison of cell surface Ca\textsubscript{V}2.2\_HA expression in peptidergic and non-peptidergic neurons. These results suggest that although the majority of Ca\textsubscript{V}2.2\_HA is expressed in CGRP-positive DRG neurons, there is no statistically significant difference in cell surface Ca\textsubscript{V}2.2\_HA expression between peptidergic and non-peptidergic subpopulations under these conditions.
Figure 4.9: Cell surface Ca_{v}2.2_HA is predominantly expressed on CGRP-positive DRG neurons following incubation with both NGF and GDNF.

(A) Images of cultured DRG neurons from Ca_{v}2.2_HA^{KIKI} mice showing (left to right) Ca_{v}2.2_HA (prior to permeabilisation), live labelling with IB4_FITC, CGRP staining (following permeabilisation) and merged representative. (Scale bars: 5 μm). (B) Quantification of the percentage of neurons with cell surface Ca_{v}2.2_HA that were either positive for IB4 (green circles), CGRP (purple circles), CGRP and IB4 (grey circles), or neither marker (red circles). Individual data points represent the mean data from three separate experiments and a total of 367 DRG neurons. Mean ± SEM of the three experiments is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s multiple comparison test as post hoc test; ***p<0.001, F value = 32.63. (C) Normalised cell surface Ca_{v}2.2_HA intensity with respect to cell size: small (s), medium (m) and large (l) DRG neurons were either positive for IB4 (green circles), CGRP (purple circles), CGRP and IB4 (grey circles), or neither marker (red circles). Cell surface Ca_{v}2.2_HA fluorescence intensity of neurons cultured in both NGF and GDNF was measured and averaged. The mean cell surface Ca_{v}2.2_HA fluorescence intensity was used to normalise the cell surface Ca_{v}2.2_HA intensity of individual Ca_{v}2.2_HA-positive neurons. Individual data points represent normalised Ca_{v}2.2_HA intensity measured from all Ca_{v}2.2_HA-positive neurons from three separate experiments and a total of 33, 76, 61, 5, 35, 13, 20, 46, 11, 4, 32 and 31 DRG neurons from left to right on x-axis. Data from three separate experiments. Mean ± SEM of the respective total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; data not significant (ns), p value = 0.0645, F value = 1.734.
4.4.4 Cell surface Cav2.2_HA expression is highest in DRG neurons cultured with NGF and GDNF

To examine the dependence of cell surface Cav2.2_HA expression on neurotrophic factors, analysis of cell surface Cav2.2_HA was performed across culture conditions with respect to cell size. Cell surface Cav2.2_HA intensity from DRG neurons incubated with NGF was averaged. This mean intensity value was used to normalise individual cell surface Cav2.2_HA intensity measured from neurons cultured in NGF, GDNF and both growth factors. Figure 4.10A shows that within the small-sized subpopulation, cell surface Cav2.2_HA expression was 102% higher in neurons cultured with GDNF compared to NGF, and 116% higher in neurons cultured in both growth factors compared to NGF (normalised values were NGF: 1.05 ± 0.09, GDNF: 2.11 ± 0.26, NGF and GDNF: 2.26 ± 0.22, Figure 4.10A). Within the medium-sized DRG neuron sub-group, plasma membrane Cav2.2_HA expression was 83% higher in neurons cultured with GDNF compared to NGF, and 138% higher in neurons cultured in both growth factors compared to NGF (normalised values were NGF: 0.89 ± 0.10, GDNF: 1.64 ± 0.13, NGF and GDNF: 2.13 ± 0.19, Figure 4.10B). Interestingly, no statistically significant difference in cell surface Cav2.2_HA intensity was observed in the large-sized subpopulation of DRG neurons (Figure 4.10C). These data suggest that cell surface Cav2.2_HA is most highly expressed in DRG neurons cultured with both NGF and GDNF concurrently, as this culture condition favours the survival both of peptidergic and non-peptidergic neurons.
Figure 4.10: GDNF has a stronger effect on cell surface $\text{Ca}_2.2\_\text{HA}$ than NGF in small and medium but not large DRG neurons

Normalised cell surface $\text{Ca}_2.2\_\text{HA}$ intensity of DRG neurons cultured in NGF (green circles), GDNF (blue circles) and NGF and GDNF (orange circles), neurons separated into (A) small, $p<0.001$, $F$ value = 13.78, (B) medium, $p<0.001$, $F$ value = 19.34 and (C) large neurons, $p=0.0977$, $F$ value = 2.344. Cell surface $\text{Ca}_2.2\_\text{HA}$ intensity from DRG neurons incubated with NGF was averaged. This mean intensity value was used to normalise individual cell surface $\text{Ca}_2.2\_\text{HA}$ intensity measured from neurons cultured in NGF, GDNF and both growth factors. Individual data points represent normalised $\text{Ca}_2.2\_\text{HA}$ intensity measured from small DRG neurons (NGF = 94, GDNF = 71 and NGF and GDNF = 62), medium DRG neurons (NGF = 80, GDNF = 164 and NGF and GDNF = 189) and large DRG neurons (NGF = 18, GDNF = 67 and NGF and GDNF = 116). Data from three separate experiments. Mean ± SEM of the three experiments is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; ***$p<0.001$, not significant (ns)
4.5  \( \text{Ca}_v2.2\text{-HA} \) expression in the spinal cord

4.5.1  Optimisation of \( \text{Ca}_v2.2\text{-HA} \) immunoreactivity in the spinal cord

\( \text{Ca}_v2.2 \) is implicated in the sensory processing pathway and concentrated at different neuronal levels. The dorsal horn is innervated by primary afferents that terminate in a highly ordered lamina-specific pattern (Light et al., 1979). Next, \( \text{Ca}_v2.2\text{-HA} \) expression in the dorsal horn of the spinal cord was explored. However, \( \text{Ca}_v2.2\text{-HA} \) immunoreactivity within this region was first optimised to allow for a more accurate assessment of \( \text{Ca}_v2.2 \) expression in the spinal cord. \( \text{Ca}_v2.2\text{-HA}^{\text{KIKI}} \) mice were first perfused (as described in Methods) and spinal cord extracted. Figure 4.11A shows spinal cord tissue incubated with rat anti–HA antibody for two days followed by secondary anti-rat Alexa Fluor 488 antibody for a day. Weak \( \text{Ca}_v2.2\text{-HA} \) can be seen in the superficial laminae of the dorsal horn which cannot be detected in \( \text{Ca}_v2.2\text{-WT} \) spinal cord (Figure 4.11A(ii)). However, high levels of background fluorescence were observed in both \( \text{Ca}_v2.2\text{-HA}^{\text{KIKI}} \) and \( \text{Ca}_v2.2\text{-WT} \) spinal cord sections. To evaluate \( \text{Ca}_v2.2\text{-HA} \) expression, its intensity was measured along a 50 x 300 \( \mu \)m region of interest (ROI) placed at the pial surface extending into the dorsal horn (white rectangle on Figure 11A, B and C). Figure 4.11Aiii shows the profile plots of \( \text{Ca}_v2.2\text{-HA} \) intensity throughout the laminae of \( \text{Ca}_v2.2\text{-HA}^{\text{KIKI}} \) and \( \text{Ca}_v2.2\text{-WT} \) mice which also reflects the high levels of background observed in Figure 4.11Ai and ii.

Subsequently, to increase the signal to noise ratio, spinal cord tissue was post-fixed after primary antibody incubation. Confocal images reveal strong \( \text{Ca}_v2.2\text{-HA} \) immunoreactivity in the dorsal horn which could not be detected in the \( \text{Ca}_v2.2\text{-WT} \) spinal cord (Figure 4.11Bi and ii). Furthermore, profile plots of \( \text{Ca}_v2.2\text{-HA} \) intensity confirmed that the highest levels of expression were confined to LI, LIII and LIIo (Figure 4.11Biii).
Finally, given the low CaV2.2_HA immunoreactivity under a standard immunohistochemical protocol and the sensitivity of the HA epitope to fixation, tyramide signal amplification (TSA) was assessed. Spinal cord immunohistochemistry showed that TSA enhanced CaV2.2_HA signal-to-noise ratio (Figure 4.11Ci and ii). Nevertheless, the staining is comparatively diffused to that observed in Figure 4.11Bi. Figure 4.11Ciii shows once again that CaV2.2_HA is restricted mainly to the superficial laminae of the dorsal horn which cannot be seen in the CaV2.2WTWT mouse spinal cord.

The CaV2.2_HA distribution pattern in the spinal cord was similar to that previously described by Westenbroek et al. (1998). Confocal images show a dense plexus of CaV2.2_HA labelled fibres in the superficial layers of the spinal cord. These results suggest that examining CaV2.2_HA using a standard immunohistochemical protocol produced high levels of background which may interfere with the analysis of CaV2.2_HA expressed in the dorsal horn. The TSA protocol augments fluorescence by HRP reacting with inactive tyramide and depositing active tyramide near the target protein. Although this method increases detection sensitivity compared to conventional immunohistochemical procedures, the free diffusion of radical intermediates results in inferior localisation of the signal. As such, although TSA augments CaV2.2_HA intensity, post-fixation depicts a more accurate distribution of CaV2.2_HA and can be used in further assessment of CaV2.2_HA at the dorsal horn.
4.5.2 **Ca\textsubscript{V}2.2\_HA is expressed in the superficial laminae of the dorsal horn**

Next, Ca\textsubscript{V}2.2\_HA distribution was investigated in the superficial laminae of the dorsal horn. Here, CGRP is normally used to define the primary afferents terminating in LI and LII outer (LIIo). Figure 4.12A shows strong Ca\textsubscript{V}2.2\_HA immunoreactivity in the superficial laminae. Using the CGRP marker, the immunohistochemistry suggests that Ca\textsubscript{V}2.2\_HA is expressed in LI and LIIo as these markers can be seen to colocalise in this region. However, Figure 4.12B shows that Ca\textsubscript{V}2.2\_HA expression extends into LII inner (LIIi) and, to a lesser extent, into deeper layers which does not correlate with CGRP expression. Nevertheless, these results
agree with Westenbroek et al. (1998) where double-immunolabelling using anti-II-III loop and anti-CGRP antibodies determined co-localisation of these two proteins. In the present study, Ca\textsubscript{v}2.2\_HA immunoreactivity can be seen in LI, LIIo and LIII and the substantial overlap between CGRP and Ca\textsubscript{v}2.2\_HA immunoreactivities indicate that CGRP is located in terminals which have Ca\textsubscript{v}2.2 channels.
Figure 4.12: Comparison of CaV2.2_HA and CGRP distribution in the dorsal horn

(A) Image of (left to right) CaV2.2_HA, CGRP and merge representative at the dorsal horn of the spinal cord. ROI depicted in merge panel as white rectangle. Scale bar 200 μm. (B) Profile plots of normalised CaV2.2_HA and CGRP fluorescence intensity (mean ± SEM of 9 sections, 3 sections per mice, CaV2.2_HA intensity normalised to the average intensity between 10 to 50 μm; CGRP intensity normalised to the average intensity between 4 to 24 μm).
4.6 Rhizotomy

A large body of research exploring the localisation of N-type calcium channels derives from pharmacological evidence (Gohil et al., 1994). Section 4.4 has shown that cell surface CaV2.2_HA is strongly expressed in the peptidergic and to a lesser extent, non-peptidergic subpopulations of small sensory neurons. In addition to Westenbroek et al. (1998), section 4.5 confirms that CaV2.2_HA is distributed in a lamina-specific pattern in the dorsal horn. However, this does not determine whether CaV2.2_HA immunoreactivity is due to its enrichment in terminals of the primary afferents or from within the dorsal horn. This poses the question of where the primary origin of CaV2.2 resides. To determine the origin of CaV2.2_HA, a rhizotomy procedure was performed, wherein the dorsal roots between the DRG and the dorsal horn were cut (Figure 4.13A). If the source of CaV2.2_HA was from the dorsal horn, then there would be no expected decrease in the staining intensity in the spinal cord. However, if the origin was in fact from the terminals of primary afferents, then a decrease in CaV2.2_HA immunoreactivity would be expected. Following unilateral dorsal rhizotomy, CaV2.2_HA mice were perfused, and spinal cord extracted. Spinal cord sections were probed with several markers to identify the effects of rhizotomy and to help characterise CaV2.2_HA. When analysing these data, to ensure changes across the dorsal horn were taken into consideration, the dorsal horn was divided into three regions of interest (ROIs). Figure 4.13B shows the ROIs labelled as lateral, central and medial; ipsilateral changes in these ROIs were compared to the contralateral dorsal horn. To further examine where, if any, changes occurred, the dorsal horn was divided into its constituent laminae; the superficial layer is considered here to comprise LI to LII, and the deeper layers refer to LIII to LV.
4.6.1 Rhizotomy results in loss of CGRP and vGluT2, but not NPY immunolabelling

Rhizotomy induced a marked reduction in the density of CGRP immunoreactive primary afferent nerve fibres. This can be seen in Figure 4.14A showing that in all ROIs a statistically significant loss of CGRP can be observed in the dorsal horn. Analysis of these data revealed that within the superficial layers of the lateral, central and medial ROIs there was a substantial decrease of 52%, 48% and 44%, respectively, in CGRP signal intensity. A statistically significant reduction (by an average of 48%) was also observed in the deeper layers of the dorsal horn (Figure 4.14D, E and Fii, left). These data are consistent with previous results (Gibson et al., 1984; Traub et al., 1990), where a marked loss of CGRP-immunoreactive fibres was observed from the dorsal horn of the spinal cord of cats and rats following rhizotomy.

Spinal cord sections were also labelled for Neuropeptide Y (NPY; Figure 4.14B). NPY is a modulator of neurotransmission and is enriched in a subset of inhibitory interneurons in laminae I-III of the dorsal horn (Sasek and Elde, 1985; Rowan et al., 1993). Unilateral rhizotomy did not produce a significant depletion of NPY labelling in the superficial layers of the ipsilateral dorsal horn (Figure 4.14D, E and Fii, middle). There was no detectable change in NPY immunoreactivity following rhizotomy, which suggests that NPY may have an intrinsic origin.
within the spinal cord, agreeing with previous reports (Gibson et al., 1984).

Glutamate is the major excitatory neurotransmitter in the spinal cord. The vesicular glutamate transporter 1 and 2 (vGluT1 and 2) are found associated with synaptic vesicles in excitatory terminals (Takamori et al., 2000, 2001). Like vGluT1, vGluT2 is associated with glutamate containing synaptic vesicles in a subset of primary afferents. Extensive immunolabelling and in situ hybridisation experiments have concluded that vGluT2 varicosities are most densely distributed throughout L1 and LII (Alvarez et al., 2004). In the present study, following rhizotomy, vGluT2 immunoreactivity showed a small depletion in the superficial but not deeper layers of the spinal cord (Figure 4.14C). This decrease was recorded in the lateral, central and medial ROIs (by 19%, 15% and 9%, respectively; Figure 4.14D, E and Fii, right). These results concur with previous reports where only a small decrease in vGluT2 immunoreactivity is observed after rhizotomy (Alvarez et al., 2004). These results suggest that vGluT2 expression can be regulated both centrally and peripherally.
Figure 4.14: Unilateral Rhizotomy reduces CGRP and vGlut2 but not NPY immunoreactivity in the spinal cord
Images of (A) CGRP (green), (B) NPY (red) and (C) vGlut2 (magenta) at the dorsal horn of a Cav2.2_HA<sup>KIKI</sup> mouse following rhizotomy (asterisks). Plot profile (i) of CGRP (left), NPY (middle) and vGlut2 (right) of the lateral (D), central (E) and medial (F) ROI. Fluorescence intensity (mean ± SEM of 15 sections, 3 animals, 5 sections per animal, normalized to the average contralateral intensity between either 4 and 24 μm (CGRP) or 10 to 50 μm (NPY and vGlut2), contralateral (black) and ipsilateral (red) to rhizotomy. Scatter plots (ii) of CGRP (left), NPY (middle) and vGlut2 (right) in the lateral (D), central (E) and medial (F) ROI. Intensity (mean ± SEM) for data from i, in superficial laminae I and II and in deeper laminae III to V, contralateral (black circles) and ipsilateral (red circles) to rhizotomy. ****p<0.0001, *p<0.05, ns = not significant (paired t test).

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Table 4.1: p values of Rhizotomy data in Figure 4.14

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Table 4.2: F values of Rhizotomy data in Figure 4.14

4.6.2 Rhizotomy reduces Cav2.2_HA in the dorsal horn of the spinal cord

In Cav2.2_HA<sup>KIKI</sup> mice, unilateral rhizotomy induced a statistically significant loss of Cav2.2_HA immunoreactive fibres from the dorsal horn (Figure 4.15). Given the difficulty of obtaining Cav2.2_HA immunoreactivity, both TSA and post-fixation was used to visualise Cav2.2_HA. The TSA method revealed a dramatic decrease in Cav2.2_HA immunolabelling in all ROIs (Figure 4.15). Within the superficial lamina there was a reduction of 43%, 53%
and 50% of Cav2.2_HA in the lateral, central and medial ROIs, respectively. A further 44% decrease was observed in the deeper layers of the central ROI and this was not seen in either the lateral or medial ROI (Figure 4.15D, E and Fii, left). As the major origin of CGRP in the dorsal spinal cord is extrinsic and from afferent fibres derived from the DRG (Gibson et al., 1984), spinal cord sections were also labelled for CGRP. A concomitant reduction was observed in the superficial layers of the lateral, central and medial ROIs of 21%, 53% and 39%, respectively. A pronounced decrease was also seen in the deeper layers of the central and medial ROIs (by 58.6% and 12%, respectively; Figure 4.15D, E and Fii, right).
Figure 4.15: Unilateral Rhizotomy reduces Ca\textsubscript{V}2.2_HA (using TSA) and CGRP immunoreactivity in the spinal cord

Images of (A) Ca\textsubscript{V}2.2_HA (TSA, green), (B) CGRP (red) and (C) merge representative at the dorsal horn of a Ca\textsubscript{V}2.2_HA\textsuperscript{KIKI} mouse following rhizotomy (asterisks). Plot profile (i) of Ca\textsubscript{V}2.2_HA (left) and CGRP (right) of the lateral (D), central (E) and medial (F) ROI. Fluorescence intensity (mean ± SEM of 15 sections, 3 animals, 5 sections per animal, normalized to the average contralateral intensity between either 10 to 50 μm (Ca\textsubscript{V}2.2_HA) or 4 and 24 μm (CGRP), contralateral (black) and ipsilateral (red) to rhizotomy. Scatter plots (ii) of Ca\textsubscript{V}2.2_HA (TSA, left) and CGRP (right) in the lateral (D), central (E) and medial (F) ROI. Intensity (mean ± SEM) for data from i, in superficial laminae I and II and in deeper laminae III to V, contralateral (black circles) and ipsilateral (red circles) to rhizotomy. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns = not significant (paired t test).

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Table 4.3: p values of Rhizotomy data in Figure 4.15

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Table 4.4: F values of Rhizotomy data in Figure 4.15

As mentioned in section 4.5.1, TSA enhances fluorescence intensity but results in more diffuse Ca\textsubscript{V}2.2_HA staining in the dorsal horn of the spinal cord when compared with conventional immunohistochemical methods. Post-fixation of spinal cord tissue was used instead to help stabilise the primary antibody when investigating Ca\textsubscript{V}2.2_HA immunofluorescence. Similar to that observed using the TSA approach, a statistically significant attenuation of Ca\textsubscript{V}2.2_HA immunoreactivity can be seen in the ipsilateral dorsal horn (Figure 4.16). In all three ROIs there was a substantial decrease of 26%, 34% and 44% corresponding to the lateral, central and medial ROIs, respectively. No statistically significant reduction was observed in the deeper layers (Figure 4.16D, E and Fii, left). Once again, the primary afferent marker, CGRP, was
used to confirm successful disappearance of significant proportions of presynaptic peptider-
gic afferents (Figure 4.16). CGRP-immunolabelling showed comparable reductions of staining
following rhizotomy. Figure 4.16D, E and Fii (right) show that in the superficial layers of the
ipsilateral lateral, central and medial ROIs there was a loss of 46%, 42% and 30%, respec-
tively. The correspondence between Ca\textsubscript{V}2.2\_HA and CGRP, whose source is exclusively from
presynaptic afferents, suggests that the origins of Ca\textsubscript{V}2.2\_HA is predominantly from the DRG
neurons.
Figure 4.16: Unilateral Rhizotomy reduces \( \text{Cav}_2.2\text{HA} \) (using post-fixation, PF) and CGRP immunoreactivity in the spinal cord

Images of (A) \( \text{Cav}_2.2\text{HA} \) (post-fixed, green), (B) CGRP (red) and (C) merge representative at the dorsal horn of a \( \text{Cav}_2.2\text{HA}_{KIKI} \) mouse following rhizotomy (asterisks). Plot profile (i) of \( \text{Cav}_2.2\text{HA} \) (left) and CGRP (right) of the lateral (D), central (E) and medial (F) ROI. Fluorescence intensity (mean ± SEM of 10 sections, 2 animals, 5 sections per animal, normalized to the average contralateral intensity between either 10 to 50 \( \mu \text{m} \) (\( \text{Cav}_2.2\text{HA} \)) or 4 and 24 \( \mu \text{m} \) (CGRP), contralateral (black) and ipsilateral (red) to rhizotomy. Scatter plots (ii) of \( \text{Cav}_2.2\text{HA} \) (post-fixed, left) and CGRP (right) in the lateral (D), central (E) and medial (F) ROI. Intensity (mean ± SEM) for data from i, in superficial laminae I and II and in deeper laminae III to V, contralateral (black circles) and ipsilateral (red circles) to rhizotomy. ****\( p<0.0001 \), **\( p<0.01 \), *\( p<0.05 \), ns = not significant (paired t test).

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Table 4.5: \( p \) values of Rhizotomy data in Figure 4.16

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<td>HA_PF</td>
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<td>CGRP</td>
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Table 4.6: \( F \) values of Rhizotomy data in Figure 4.16

The post-fixation method of spinal cord tissue allowed for superior localisation of \( \text{Cav}_2.2\text{HA} \) at the dorsal horn. Although the signal intensity was lower than when using the TSA protocol, the increase in spatial resolution provided by post-fixation permitted the acquisition of higher magnification (x63) images. When comparing high magnification (x63) images of contralateral and ipsilateral dorsal horns, a clear depletion of \( \text{Cav}_2.2\text{HA} \), CGRP and vGluT2 puncta is visible (Figure 4.17Aii and 17Bii). Furthermore, there are discernible \( \text{Cav}_2.2\text{HA} \) ring-like structure (Figure 4.17Aii and 17Bii; arrow) which are present both in the contra- and ipsilateral sides. These ring-like structures do not appear positive for either CGRP or vGluT2. Figure 4.17 confirms that the distribution of \( \text{Cav}_2.2\text{HA} \) was distinctly reduced in
the dorsal horn, following rhizotomy. CaV2.2_HA patterning in the contralateral dorsal horn revealed that CaV2.2_HA puncta were present in the superficial laminae in rosette-like structures. CaV2.2_HA often surrounded a vGluT2-positive core and was associated with CGRP; these structures are similar to glomerular synapses (Ribeiro-da Silva et al., 1985). Following rhizotomy, CaV2.2_HA intensity was markedly reduced, particularly in these glomerular-like structures (Figure 4.17Ciii and Diii). Within these structures, there was a concomitant decrease in CGRP immunoreactivity along with a reduction, but not complete loss, of vGluT2. These results support the idea that CaV2.2_HA is mainly located presynaptically in L1 and L2.

Figure 4.17: High–magnification images of CaV2.2_HA puncta at the dorsal horn in the contra and ipsi side of rhizotomy mice
(i) Low magnification images (x20) (left panels, from top to bottom) of CaV2.2_HA (post-fixed, green), CGRP (red), vGluT2 (magenta) and merged Scale bar 200 μm. (ii) High magnification (x63) images of the central ROI of (A) contra and (B) ipsi sides of the dorsal horn of CaV2.2_HA<sup>KIKI</sup> mice following rhizotomy. Immunolabelling of CaV2.2_HA (post-fixed, green), CGRP (red), vGluT2 (magenta) and merged representative image (from top to bottom). Scale bar 10 μm. (iii) High magnification (x63) images of the ROI depicted as a white square in A and B now referred to as (C) contra and (D) ipsi sides of the dorsal horn of CaV2.2_HA<sup>KIKI</sup> mice following rhizotomy for CaV2.2_HA (post-fixed, green), CGRP (red), vGluT2 (magenta) and merged representative immunolabelling in the dorsal horn of CaV2.2_HA<sup>KIKI</sup> mice after rhizotomy (from top to bottom). Scale bar 2 μm.
4.6.3 Rhizotomy reduces $\alpha_2\delta$-1 expression in the dorsal horn

The $\alpha_2\delta$ subunits are important for the functional assembly and expression of $\text{Ca}_V2.2$ (Gurnett et al., 1996; Cassidy et al., 2014; Kadurin et al., 2016). In this study, I also examined the distribution of $\alpha_2\delta$-1 in the dorsal horn following rhizotomy. Figure 4.18 shows that there was a considerable reduction of $\alpha_2\delta$-1 expression in the dorsal horn following unilateral dorsal rhizotomy. $\alpha_2\delta$-1 immunoreactivity significantly diminished in the superficial layers of the lateral, central and medial ROIs by 18%, 21% and 22%, respectively (Figure 4.18C). However, this depletion was not observed in the deeper layers of the dorsal horn. These findings indicate that $\alpha_2\delta$-1 basal expression occurs both in presynaptic primary afferents and is also intrinsic to the spinal cord, in agreement with findings by Li et al. (2004).
Figure 4.18: Unilateral Rhizotomy reduces $\alpha_2\delta-1$ immunoreactivity in the spinal cord

(A) Image of $\alpha_2\delta-1$ at the dorsal horn of a CaV2.2_HA$^{KIK}$ mouse following rhizotomy (asterisks) showing (from left to right) the lateral, central and medial ROI. (B) Plot profile of $\alpha_2\delta-1$ (from left to right, lateral, central and medial ROI) fluorescence intensity (mean ± SEM of 15 sections, 3 animals, 5 section per animal, normalized to the average contralateral intensity between 10 and 50 μm), contralateral (black) and ipsilateral (red) to rhizotomy. (C) Scatter plots of $\alpha_2\delta-1$ intensity (mean ± SEM) for data from B for respective lateral, central and medial ROIs, in superficial laminae I and II and in deeper laminae III to V, contralateral (black circles) and ipsilateral (red circles) to rhizotomy. ***$p<0.001$, **$p<0.01$, ns = not significant (paired t test)

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<tr>
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<td>$p = 0.0005$</td>
<td>$p = 0.2175$</td>
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Table 4.7: p values of Rhizotomy data in Figure 4.18

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<td>$F = 3.543$</td>
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Table 4.8: F values of Rhizotomy data in Figure 4.18
Naïve mice are those not subjected to rhizotomy and were used to ensure that the immuno-
labelling of CaV2.2_HA, CGRP, NPY, vGluT2, and α2δ-1 (shown in Figure 4.14 to 4.18) were
due to the rhizotomy procedure. In naïve mice, no difference was observed between left and
right dorsal horn levels of CGRP (Figure 4.19A), NPY (Figure 4.19B), vGluT2 (Figure 4.19C),
CaV2.2_HA (Figure 4.20 and 4.21), or α2δ-1 (Figure 4.22). There was no evidence of statisti-
cally significant difference in either the superficial or deeper layers of the lateral, central and
medial ROIs when comparing the two sides of the dorsal horns.
Figure 4.19: CGRP, NPY and vGlut2 immunoreactivity in naïve mice
Images of (A) CGRP (green), (B) NPY (red) and (C) vGlut2 (magenta) at the dorsal horn from of a CaV2.2_HA<sup>Kki</sup> naïve mouse (left and right dorsal horn marked on images). Plot profile (i) of CGRP, NPY and vGlut2 of the lateral (D), central (E) and medial (F) ROI. Fluorescence intensity (mean ± SEM of 5 sections, normalized to the average left intensity between either 4 and 24 μm (CGRP) or 10 to 50 μm (NPY and vGlut2), left (black) and right (red) to rhizotomy. Scatter plots (ii) of CGRP, NPY and vGlut2 in the lateral (D), central (E) and medial (F) ROI. Intensity (mean ± SEM) for data from i, in superficial laminae I and II and in deeper laminae III to V, left (black circles) and right (red circles) to rhizotomy. ns = not significant (paired t test).

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<tr>
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<td>NPY</td>
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<td>vGlut2</td>
<td>p = 0.4319</td>
<td>p = 0.9140</td>
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Table 4.9: p values of Rhizotomy data in Figure 4.19

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<td>CGRP</td>
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<td>NPY</td>
<td>F = 1.612</td>
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<td>F = 1.221</td>
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Table 4.10: F values of Rhizotomy data in Figure 4.19
Figure 4.20: Ca$_{v}$2.2_HA TSA and CGRP immunoreactivity in naïve mice
Images of (A) Ca$_{v}$2.2_HA (TSA, green), (B) CGRP (right; red) and (C) merge representative at the dorsal horn of a Ca$_{v}$2.2_HA$^{Kki}$ naïve mouse (left and right dorsal horn marked on images). Plot profile (i) of Ca$_{v}$2.2_HA (left) and CGRP (right) of the lateral (D), central (E) and medial (F) ROI. Fluorescence intensity (mean ± SEM of 5 sections, normalized to the average left intensity between either 10 to 50 μm (Ca$_{v}$2.2_HA) or 4 and 24 μm (CGRP), left (black) and right (red) to rhizotomy. Scatter plots (ii) of Ca$_{v}$2.2_HA (TSA, left) and CGRP (right) in the lateral (D), central (E) and medial (F) ROI. Intensity (mean ± SEM) for data from i, in superficial laminae I and II and in deeper laminae III to V, left (black circles) and right (red circles) to rhizotomy. ns = not significant (paired $t$ test).

### Table 4.11: $p$ values of Rhizotomy data in Figure 4.20

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### Table 4.12: $F$ values of Rhizotomy data in Figure 4.20

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<td>CGRP</td>
<td>$F = 2.106$</td>
<td>$F = 2.109$</td>
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Figure 4.21: \( \text{Ca}_v2.2\_\text{HA PF and CGRP immunoreactivity in naive mice} \)

Images of (A) \( \text{Ca}_v2.2\_\text{HA} \) (post-fixed, green), (B) CGRP (red) and (C) merge representative at the dorsal horn of a \( \text{Ca}_v2.2\_\text{HA}^{\text{KIKI}} \) naive mouse (left and right dorsal horn marked on images). Plot profile (i) of \( \text{Ca}_v2.2\_\text{HA} \) (left) and CGRP (right) of the lateral (D), central (E) and medial (F) ROI. Fluorescence intensity (mean ± SEM of 5 sections, normalized to the average left intensity between either 10 to 50 \( \mu \text{m} \) \( \text{Ca}_v2.2\_\text{HA} \) or 4 and 24 \( \mu \text{m} \) CGRP), left (black) and right (red) to rhizotomy. Scatter plots (ii) of \( \text{Ca}_v2.2\_\text{HA PF} \) (left) and CGRP (right) in the lateral (D), central (E) and medial (F) ROI. Intensity (mean ± SEM) for data from i, in superficial laminae I and II and in deeper laminae III to V, left (black circles) and right (red circles) to rhizotomy. ns = not significant (paired \( t \) test).

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Table 4.13: \( p \) values of Rhizotomy data in Figure 4.21

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</tr>
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<td>( \text{HA PF} )</td>
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<td>( F = 3.553 )</td>
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<td>( \text{CGRP} )</td>
<td>( F = 3.504 )</td>
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Table 4.14: \( F \) values of Rhizotomy data in Figure 4.21
Figure 4.22: α2δ-1 immunoreactivity in naïve mice
Image of (A) α2δ-1 (green) at the dorsal horn of a Cav2.2_HA<sup>KIKI</sup> naïve mouse (left and right dorsal horn marked on images). Plot profile (i) of α2δ-1 of the lateral (B), central (C) and medial (D) ROI. Fluorescence intensity (mean ± SEM of 5 sections, normalized to the average left intensity between either 10 to 50 μm (α2δ-1), left (black) and right (red) to rhizotomy. Scatter plots (ii) of α2δ-1 in the lateral (B), central (C) and medial (D) ROI. Intensity (mean ± SEM) for data from i, in superficial laminae I and II and in deeper laminae III to V, left (black circles) and right (red circles) to rhizotomy. ns = not significant (paired t test).

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<td>α2δ-1</td>
<td>p = 0.3981</td>
<td>p = 0.3996</td>
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Table 4.15: p values of Rhizotomy data in Figure 4.22

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<tr>
<td>α2δ-1</td>
<td>F = 7.798</td>
<td>F = 1.400</td>
<td>F = 7.752</td>
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Table 4.16: F values of Rhizotomy data in Figure 4.22
4.7 Summary and Discussion

In the present study, CaV2.2_HA expression has been studied in the primary afferent neuronal pathway because of its importance in synaptic transmission. This work uses the CaV2.2_HA^KiKi mouse model characterised in Chapter 3 to examine endogenous cell surface CaV2.2_HA. Native CaV2.2_HA expression was assessed in DRG neurons, the dorsal horn of the spinal cord, and thereafter the source of its expression was examined by analysing the effect of rhizotomy. This study found that CaV2.2_HA is preferentially expressed on small and medium-sized DRG neurons negative for NF200. Additionally, the majority of cell surface CaV2.2_HA was found in small and medium-sized DRG neurons positive for CGRP (Figure 4.4). The present study also examined cell surface CaV2.2_HA expression on DRG neurons incubated with NGF and GDNF. These data showed that both NGF and GDNF differentially regulated CaV2.2_HA expression (Figures 4.7-4.10). The localisation of CaV2.2_HA in DRG neurons is paralleled by its conspicuous expression in the dorsal horn of the spinal cord. In the dorsal horn, CaV2.2_HA expression is most abundant in LI and LII (Figure 4.12). Finally, unilateral rhizotomy was used to ablate presynaptic input from the primary afferents and this confirmed that CaV2.2_HA expression was predominantly pre-synaptic in origin (Figure 4.15 and 4.16).

4.7.1 CaV2.2_HA expression in DRG neurons

The DRG comprises of a diverse population of neurons with distinct neurochemical characteristics (Hunt and Rossi, 1985). C-fibres can be broadly classified into peptidergic and non-peptidergic by their expression of CGRP and binding to IB4, respectively. DRG neurons cultured from CaV2.2_HA^KiKi mice were analysed for immunoreactivity to the neurochemical markers IB4, CGRP and NF200 with respect to cell surface CaV2.2_HA expression.
4.7.1.1 Ca\textsubscript{v}2.2_HA expression in peptidergic neurons

In the present study, of the total Ca\textsubscript{v}2.2\_HA-positive neurons, 66% ± 5% were CGRP-positive (Figure 4.4). Additionally, the highest Ca\textsubscript{v}2.2\_HA expression was observed in small–sized DRG neurons (Figure 4.4). This demonstrates that cell surface Ca\textsubscript{v}2.2\_HA was preferentially expressed in small peptidergic DRG neurons in culture. Visualising cultured DRG neurons permitted the straightforward assessment of channel distribution. Despite this advantage, it is important to compare and potentially validate these findings in relation to evaluations performed \textit{in vivo}. Interestingly, here the \textit{in vitro} data reported are generally comparable to \textit{in vivo} data shown in Nieto-Rostro et al. (2018) where Ca\textsubscript{v}2.2\_HA is enriched in small CGRP-positive nociceptors. Previous studies have critically explored the close relationship between Ca\textsubscript{v}2.2 and CGRP, showing that the blockade of N-type calcium channels attenuates evoked-induced CGRP release from rat DRG and trigeminal neurons (Evans et al., 1996; Xiao et al., 2008). Furthermore, overexpression and knockdown of collapsin response mediator protein-2 (CRMP-2), a protein known to increase Ca\textsubscript{v}2.2 currents, results in the significant enhancement and reduction, respectively, of Ca\textsubscript{v}2.2-mediated CGRP release (Chi et al., 2009). Taken together with the data from the present study, it appears that the N-type calcium channel is closely associated with CGRP-positive DRG neurons.

4.7.1.2 Ca\textsubscript{v}2.2\_HA expression in non-peptidergic neurons

Data from the present study suggest that 80% ± 3% of Ca\textsubscript{v}2.2\_HA is expressed on the cell surface of IB4-negative neurons whereas only 20% ± 3% of Ca\textsubscript{v}2.2\_HA immunoreactivity can be found on the cell surface of IB4-positive neurons (Figure 4.3). However, this has not been consistently demonstrated. mRNA expression patterns of \textit{Cacna1b} show similar profiles in IB4-positive and negative nociceptors (Chiu et al., 2014). This agrees with the high intracellular Ca\textsubscript{v}2.2\_HA immunoreactivity observed in both CGRP-positive and negative small DRG neurons (Nieto-Rostro et al., 2018). Additionally, Wu and Pan (2004) suggest
that in rats there is a higher density of N-type calcium channels in IB4-positive neurons. This
dichotomy may be explained in several ways. First, the neurons selected for recording were
labelled as small if their diameter was 15-30 μm (Wu and Pan, 2004). However, in the present
study, neurons were considered as small with a diameter of <19 μm. Furthermore, although
not statistically significant, medium sized (19-30 μm in diameter) IB4-positive neurons had
the highest CaV2.2_HA expression within this subpopulation (Figure 4.3). This may explain
why Wu and Pan (2004) found that IB4-positive neurons have a significantly higher density of
CaV2.2 channel currents. It is also important to note that this study used rat DRG neurons,
therefore, calcium current differences may arise from species differences. Furthermore, the
Pan group also explored the distinct inhibition of different VGCCs by δ-opioid agonists and
found a notable inhibition of N-type currents in IB4-negative DRG neurons. This conflicting
evidence suggests that the increased inhibition could result from a higher density of CaV2.2
channels present in IB4-negative neurons. Nevertheless, a comprehensive study by Murali
et al. (2014) found that the N-type channel made up a large proportion of total calcium current
in mouse DRG neurons.

The differential expression of cell surface CaV2.2_HA may also be explained by the role of
neurotrophic factors in culture. Data shown from section 4.3.3 (Figure 4.5) were from DRG
neurons cultured with NGF. The NGF receptor tyrosine kinase, TrkA, is extensively colocalised
with CGRP (Averill et al., 1995) and supports the growth of peptidergic neurons (Molliver and
Snider, 1997). Many studies have explored N-type calcium currents in cultured DRG neurons
not supplemented with growth factors (Wu and Pan, 2004; Murali et al., 2014), resulting in
the almost equal CaV2.2 expression within different subpopulations (discussed further in sec-
tion 4.7.2) This may account for the discrepancies reported here where CaV2.2 cell surface
expression is found to be highest in CGRP-positive neurons.
4.7.1.3 Co-expression of Ca\textsubscript{v}2.2_HA, CGRP and IB4 binding epitopes

This study reports 11% ± 7% of cell surface Ca\textsubscript{v}2.2_HA expressing neurons to be positive for both CGRP and IB4 binding. Many studies have also reported that CGRP-positive mouse neurons bind IB4, showing that these markers mostly but not always exclusively label the peptidergic and non-peptidergic subpopulations (Wang et al., 1994; Barabas et al., 2012; Harrison et al., 2014). Previous studies have also shown that 5% to 13% of C-fibre neurons express both trkA receptors and shown IB4-binding properties (Averill et al., 1995; Molliver et al., 1995). To validate these findings \textit{in vivo}, triple immunolabelling with trkA and the GDNF receptor, Ret, markers would help confirm whether a significant proportion of cell surface Ca\textsubscript{v}2.2_HA is present in this subset of neurons. Nonetheless, these data highlight the importance of external influences such as growth factors in DRG neuronal cultures (discussed in section 4.7.2). The addition of growth factors must be taken into consideration when representing the normal, constitutive state of DRG neurons in culture.

4.7.1.4 Ca\textsubscript{v}2.2_HA only expressing neurons

A substantial proportion (21% ± 4%) of Ca\textsubscript{v}2.2_HA-positive neurons were also found to be negative for both CGRP expression and IB4-binding (Figure 4.5). The majority of non-peptidergic neurons exhibit IB4-binding. However, other markers such as P2X3 can also be used to identify this subpopulation. Pan et al. (2012) have reported a small proportion of neurons which do not show colocalization of P2X3 and IB4 binding. As such, neurons identified in the present study as only positive for Ca\textsubscript{v}2.2_HA may also show immunoreactivity for P2X3.

Many small-diameter peptidergic DRG neurons express neuropeptides such as CGRP and substance P. However, a small subset of these neurons lack CGRP and show substance P immunoreactivity (Kestell et al., 2015). To further examine Ca\textsubscript{v}2.2_HA-positive neurons, anti-substance P antibodies can be used to identify this neuronal subpopulation and further
classify the cell surface expression of Ca\textsubscript{V}2.2_HA on different peptidergic DRG neurons.

The expression of tyrosine hydroxylase (TH) has also been studied in mouse DRG neurons. TH is expressed in a subpopulation of small DRG neurons negative for both CGRP and IB4-binding (Brumovsky et al., 2006). TH catalyses the production of levo-DOPA from tyrosine, which is a precursor to dopamine (Levitt et al., 1965). In rats, following intrathecal administration of substance P, dopamine has been found to reduce nociceptive behaviour (Shimizu et al., 2004). Further studies would be required to explore whether Ca\textsubscript{V}2.2_HA expression is found within the nociceptors positive for TH.

4.7.1.5 Ca\textsubscript{V}2.2_HA expression in NF200-negative DRG neurons

Another criterion for categorising DRG neurons is the presence of NF200. Double labelling of DRG neurons with Ca\textsubscript{V}2.2_HA and NF200 revealed that 77\% ± 2\% of Ca\textsubscript{V}2.2_HA-positive neurons were negative for NF200. The majority of large diameter neurons do not convey noxious input and can be distinguished by their content of phosphorylated and non-phosphorylated heavy-chain NF200 (Perry et al., 1991). Preceding studies have shown that the intensity of NF200 immunolabelling in the soma correlated with conduction velocity. Slower conducting fibres, A\textsubscript{δ} and C-fibres have low NF200 immunoreactivity (Lawson and Waddell, 1991). Additionally, co-labelling studies have reported that the preponderance of CGRP rich neurons are neurofilament poor (Lawson et al., 1993). These data along with Figure 4.3 and 4.4 again suggest that cell surface Ca\textsubscript{V}2.2_HA is preferentially expressed in small peptidergic DRG neurons.
4.7.2 Growth factors and Ca\textsubscript{v}2.2_HA expression

Cultured DRG neurons allow for the examination of cell surface Ca\textsubscript{v}2.2_HA in a more controlled environment to an in vivo setting. Despite this advantage, it is important to note that neuronal function and phenotype are subject to change depending on environmental influence. A large body of work has shown that growth factors play a pivotal role in ion channel expression (Ernsberger, 2008, 2009).

DRG neurons are highly sensitive to NGF during development and express the NGF receptor, trkA (Bennett et al., 1996). Manipulation of NGF signalling by the administration or removal of the neurotrophic factor or its receptors has highlighted the importance of neurotrophin signalling in the survival and differentiation of DRG neurons (Silos-Santiago et al., 1995; Amaya et al., 2004).

In the present study, when DRG neurons were cultured with NGF, 47% ± 2% of total Ca\textsubscript{v}2.2_HA neurons were CGRP-positive whereas significantly fewer neurons were IB4-positive, at 9% ± 2%. NGF has been shown to support the growth of peptidergic neurons, as 92% of small trkA-positive neurons co-express CGRP (Averill et al., 1995). This may explain why the highest proportion of Ca\textsubscript{v}2.2_HA expressing neurons were positive for CGRP. Moreover, Amaya et al. (2004) has shown, using anti-NGF that this neurotrophin can differentially regulate ion channel expression such as TRPV1 in rat DRG neurons. This method could be used to further explore the relationship between cell surface Ca\textsubscript{v}2.2_HA and NGF.

GDNF promotes the growth of Ret receptor expressing neurons (Molliver and Snider, 1997). There is a large overlap between Ret expression and IB4-binding (Bennett et al., 1998). In rat DRG neurons, GDNF has been shown to regulate ion channel expression. For example, in Ret knockout mice TRPA1 mRNA expression is almost absent from DRG sections (Luo et al., 2007). Furthermore, Guo et al. (2001) has shown that there is a 60% overlap between Ret
and TRPV1 immunoreactivity in rat DRG neurons. This evidence suggests that Ret controls the expression of a subset of genes characteristics of non-peptidergic neurons. In the present study, application of GDNF to DRG cultures promotes the survival of IB4-positive neurons, on which cell surface \( \text{Ca}_v2.2\_\text{HA} \) expression was identified (Figure 4.8). However, to further investigate this, Ret mutants could be used to help unravel the relationship between GDNF and \( \text{Ca}_v2.2\_\text{HA} \) expression.

During development, there is an IB4-positive population of DRG neurons that is lost in trkA knockout animals (Silos-Santiago et al., 1995), suggesting that even though IB4-positive neurons do not express trkA they are indeed dependent on NGF during development. To study population-specific expression of \( \text{Ca}_v2.2\_\text{HA} \), both GDNF and NGF were added to DRG neuronal cultures. Surprisingly, in this case, the present study found that although the highest proportion of \( \text{Ca}_v2.2\_\text{HA} \) expressing neurons were CGRP-positive (43 ± 3%), cell surface \( \text{Ca}_v2.2\_\text{HA} \) expression did not significantly differ between the IB4 and CGRP-positive neurons (Figure 4.9). Although there is a strong correlation between the growth factors NGF and GDNF with CGRP expression and IB4-binding, respectively, further studies are required to clarify whether \( \text{Ca}_v2.2\_\text{HA} \) expression correlates with either trkA, Ret or both receptors. Non-neuronal cells in these cultures may also synthesise and secrete survival and differential growth-promoting factors. Therefore, additional experiments not applying factors and minimising non-neuronal cells would provide better controls for determining the relationship between growth factors and cell surface \( \text{Ca}_v2.2\_\text{HA} \) expression.

The data here by no means presents a conclusive body of work with regards to growth factors and \( \text{Ca}_v2.2\_\text{HA} \) cell surface expression in different subpopulations of DRG neurons. This highlights the importance of tailoring culture conditions to experimental design. However, the data along with Nieto-Rostro et al. (2018) indicate that cell surface \( \text{Ca}_v2.2\_\text{HA} \) is preferentially expressed in small CGRP-positive neurons.
4.7.3 Ca\textsubscript{V}2.2_HA expression in the spinal cord

A\textdelta and C-fibres are associated with first order neurons. Laminae I and II of the dorsal horn of the spinal cord receive inputs from A\textdelta and C-fibres (Pinto et al., 2010). The expression of Ca\textsubscript{V}2.2_HA in DRG neurons is paralleled by its conspicuous localisation in the dorsal horn of the spinal cord in lamina I and II. Here, presynaptic Ca\textsubscript{V}2.2_HA puncta are associated in part with the primary afferent marker CGRP in LI and LIIo (Figure 4.12) as previously described by Westenbroek et al. (1998). Previous studies have explored the relationship between CGRP and N-type calcium channels in the spinal cord (Maggi et al., 1990; Santicioli et al., 1992). These studies provide evidence of the fundamental role of Ca\textsubscript{V}2.2 in evoked CGRP release in the dorsal horn spinal cord. Nieto-Rostro et al. (2018) have further shown that Ca\textsubscript{V}2.2_HA also shares topographic distribution with IB4-positive, non-peptidergic primary afferents terminating in LIII.

4.7.4 Ca\textsubscript{V}2.2_HA expression originates in the DRG

To elucidate the origin of Ca\textsubscript{V}2.2_HA in the spinal cord, rhizotomies were performed on Ca\textsubscript{V}2.2_HA\textsuperscript{KIKI} mice. Statistically significant reductions in CGRP and vGluT2 immunoreactivity established that rhizotomies were indeed successful (Figure 4.14). However, it is important to note that rhizotomy is generally found to be incomplete, as it has been reported that primary afferents can ascend or descend 5 to 8 segments before terminating in the dorsal horn (Chung et al., 1988).

The presynaptic localisation of Ca\textsubscript{V}2.2_HA in the primary afferents is confirmed through its loss following unilateral dorsal rhizotomy. The observed decrease in Ca\textsubscript{V}2.2_HA was most prominent in LI and LII which are known to receive primary sensory afferents (Figure 4.15 and 4.16). Since Ca\textsubscript{V}2.2_HA is present in many nociceptive DRG neurons (Figure 4.3 and 4.4; Nieto-Rostro et al. (2018)) this suggests that a high proportion of Ca\textsubscript{V}2.2 is derived from DRG
neurons and that these dorsal roots transport Ca\textsubscript{V}2.2_HA to the terminals.

Inspection of the mice after rhizotomy indicated that the operations seemed successful and neither the spinal cord nor ventral roots were affected. The source of remaining Ca\textsubscript{V}2.2_HA immunoreactivity in the ipsilateral dorsal horn was postulated to be from interneurons or projection neurons or primary afferents that were not cut. To further validate this, intraspinal colchicine could reveal a population of Ca\textsubscript{V}2.2_HA immunoreactive interneurons in the dorsal horn. Additionally, Gibson et al. (1984) proposed that the residual CGRP comes from the ascending pathway of supraspinal regions which may also be the case for Ca\textsubscript{V}2.2_HA

Following rhizotomy there was a statistically significant decrease but not complete loss of the auxiliary subunit $\alpha_2\delta$-1 expression. This suggests that, like Ca\textsubscript{V}2.2_HA, $\alpha_2\delta$-1 may be present both in primary afferents, intrinsic spinal cord neurons and unaffected terminals in the dorsal horn. Similar results with regards to $\alpha_2\delta$-1 following rhizotomy have been found by Luo et al. (2001); Li et al. (2004).

In conclusion, Ca\textsubscript{V}2.2_HA immunoreactivity has a widespread distribution in different levels of the sensory pathway. The most salient finding of this study was that CGRP-positive DRG neurons are endowed with cell surface Ca\textsubscript{V}2.2_HA. Ca\textsubscript{V}2.2_HA immunolabelling in DRG neurons and its concentration in the superficial laminae of the dorsal horn encourages further work on the role of Ca\textsubscript{V}2.2 in the modulation of sensory transmission. Moreover, the cell surface expression of Ca\textsubscript{V}2.2_HA on DRG neurons and native Ca\textsubscript{V}2.2_HA expression in spinal cord tissue demonstrates that the Ca\textsubscript{V}2.2_HA\textsuperscript{Kiki} mice are a suitable model for studying Ca\textsubscript{V}2.2 trafficking.
Chapter 5

CaV2.2_HA expression in DRG and spinal cord co-cultures

5.1 Introduction

At the presynaptic terminal, Ca\(^{2+}\) enters through VGCCs to trigger neurotransmission. Within the primary afferents, CaV2.2 plays a vital role as it is the main presynaptic VGCC in DRG neurons (Altier et al., 2007; Murali et al., 2014). The N-type calcium channel is a key mediator of nociceptive transmission and its importance is highlighted by its use as a therapeutic target (Snutch, 2005; Patel et al., 2018). Examining surface expression of CaV2.2 at the presynaptic site is critical in understanding the development of neuropathic pain and enables therapeutic targeting of this channel.

The transient receptor potential vanilloid type 1 (TRPV1) is a critical detector of noxious stimuli in DRG neurons. TRPV1 channels are activated by noxious heat (≥ 42°C), low pH, endogenous vanilloids and exogenous ligands such as capsaicin. The activation of TRPV1 channels triggers the release of substance P, CGRP and glutamate (Tognetto et al., 2001; Marvizón et al., 2003; Labrakakis and MacDermott, 2003). TRPV1 is a non-selective cation channel with a preference for Ca\(^{2+}\). It is located at the plasma membrane of a distinct subpopulation
of DRG neurons and plays an essential role in nociception (Szallasi et al., 2007; Wong and Gavva, 2009). Ca$^{2+}$ influx through TRPV1 initiates a cascade of events, one of them being changes in VGCC expression (Wu et al., 2005).

The effects of capsaicin on either the up- or down-regulation of VGCC expression in different cell types remain unclear. Capsaicin has been found to indirectly reduce Ca$^{2+}$ entry through VGCCs in rat trigeminal and hippocampal neurons, and gastric smooth muscle (Kopanitsa et al., 1995; Sim et al., 2001). Similarly, in rat sensory neurons, TRPV1 activation, through capsaicin, mediates an overall reduction in N-type current (Wu et al., 2005; Hagenacker et al., 2005). In contrast, it has also been shown, using DRG neurons from guinea pigs, that capsaicin shifts the VGCC current-voltage relationship to more hyperpolarised potentials which indicates facilitated activation of VGCCs in response to smaller depolarisations. Furthermore, Cheng et al. (2004) demonstrated that within myocytes, application of capsaicin results in an increase in VGCC currents. However, the mechanisms behind the functional interaction of TRPV1 and VGCCs in primary nociceptors remains poorly understood.

Co-cultures between DRG and spinal cord neurons are an ideal model system for the study of synaptic transmission at primary afferent synapses (Medvedeva et al., 2008; Hendrich et al., 2012). Although DRG neurons provide an appropriate environment to study Ca$_V^{2.2}$-HA, they differ from central neuronal cultures as they do not form synapses between each other. The co-culture system has been previously used to study the synapses between DRG and spinal cord neurons (Gu and MacDermott, 1997; Lee et al., 2004). In preceding studies, the DRG-spinal cord co-culture system was used to explore the mechanistic link between TRPV1 and Ca$^{2+}$ signalling (Medvedeva et al., 2008). This study demonstrated that a brief stimulation of TRPV1 with capsaicin resulted in glutamate release, independent of N-type calcium channels. It was postulated that Ca$^{2+}$ influx through TRPV1 mediated a Ca$^{2+}$/ calcineurin-dependent inhibition of Ca$_V^{2.2}$ (Wu et al., 2005). In this study, a polyclonal antibody raised against
an intracellular epitope in the II-III loop of Ca\textsubscript{V}2.2 was used to monitor the internalisation of the N-type calcium channel in rat DRG neurons (Wu et al., 2005). However, since this antibody cannot distinguish cell surface and intracellular Ca\textsubscript{V}2.2 expression, Wu et al. (2005) cannot conclusively determine the internalisation of Ca\textsubscript{V}2.2 using these immunocytochemical methods.

Using the novel Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KIKI}} and Ca\textsubscript{V}2.2\textsubscript{WTWT} mice, several combinations of neurons can be cultured, expanding the tools available to study presynaptic Ca\textsubscript{V}2.2\_HA. The co-cultures used in the present study consist of DRG neurons cultured from Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KIKI}} with spinal cord neurons from Ca\textsubscript{V}2.2\textsubscript{WTWT} mice. This will ensure that the events being investigated are only those occurring at the presynaptic terminal and these co-cultures will provide a unique approach to studying Ca\textsubscript{V}2.2\_HA at the presynaptic membrane. In the previous chapter, Ca\textsubscript{V}2.2\textsubscript{HA\textsuperscript{KIKI}} mice were used to answer fundamental questions about cell surface Ca\textsubscript{V}2.2\_HA expression. Ca\textsubscript{V}2.2 is found at the presynaptic membrane of neurons \textit{in vivo}. As such, this chapter will first characterise Ca\textsubscript{V}2.2\_HA in co-cultures, and then investigate the effects of capsaicin on Ca\textsubscript{V}2.2\_HA in DRG neurons and discuss how to study this in co-cultures.
5.2 Establishing DRG and spinal cord co-cultures

In the present study, a co-culture system was devised to offer optimal conditions for neuron maturation and synapse formation. DRG neurons were first cultured with spinal cord neurons using P0 or P1 CaV2.2WTWT mice in a co-culture system. DRG glial cells and spinal cord astrocytes served as substrates to the co-cultured neurons. DRG neurites have shown a preferential growth in vitro into dorsal cord spinal explants (Peterson and Crain, 1981). Under these co-culture conditions, settled DRG and dorsal horn neurons were able to form synapses, as shown in Figure 5.1.

Figure 5.1 shows co-cultures fixed and permeabilised after 15 DIV. The class III beta tubulin is a neuron-specific marker (Moskowitz et al., 1993) and was used to identify neurons within the co-culture. The initial structural sign of synapse formation is the clustering of synaptic vesicles around the presynaptic active zone (Dyson and Jones, 1980; Amaral and Dent, 1981; Blue and Parnavelas, 1983; Vaughn, 1989). The vesicular glutamate transport 2 (vGluT2) accumulates glutamate into synaptic vesicles and has been previously used as a presynaptic marker (Prange et al., 2004; Graf et al., 2004). All primary afferents use glutamate as their major neurotransmitter, thus having an excitatory effect on their postsynaptic targets (Yoshimura and Jessell, 1990; Pan et al., 2002). It has been reported that anti-Homer antibodies can be used to reveal a great majority of glutamatergic synapses (Gutierrez-Mecinas et al., 2016). Therefore, Homer was used to classify the excitatory postsynaptic site. Figure 5.1A (arrow) shows class III β tubulin staining in the DRG cell body and processes. vGluT2 can also be seen along the processes of this neuron. Next, I assessed the co-localisation of vGluT2 and Homer as evidence of synapse formation. Confocal microscopy confirmed the presence of synapses as Homer and vGluT2 puncta can be seen opposed to each other (Figure 5.1B). These results confirmed the healthy state of neurons and synapses in these co-cultures.
Figure 5.1: DRG and spinal cord mice co-cultures
Confocal images of DRG and spinal cord co-cultures fixed and stained at DIV 15 showing (A; top row) the cell body and processes (white arrows) of a DRG labelled with βIII tubulin (left; green), vGlut2 (middle, magenta) and merge representative image with DAPI in blue, (B; bottom row) Homer puncta (left; green), vGlut2 puncta (middle; magenta) and merge representative image (right). (Scale bars: 5 μm)
5.3 The HA-tagged $\text{Ca}_V2.2$ does not perturb $\text{Ca}^{2+}$ dynamics in DRG neurons of co-cultures

$\text{Ca}_V2.2$ channels play a major role in synaptic transmission at the presynaptic terminal. In Chapter 3, I show, using whole-cell patch clamping, that the insertion of the HA-tag does not affect VGCC currents measured from the cell body of DRG neurons. Here, to determine the physiological impact of the insertion of the HA-tag in $\text{Ca}_V2.2$ in DRG processes, $\text{Ca}^{2+}$ signalling was assessed. Co-cultures were prepared using DRG neurons from $\text{Ca}_V2.2_{\text{HA}^{\text{KIKI}}}$ and spinal cord neurons from $\text{Ca}_V2.2_{\text{WTWT}}$ mice and compared to co-cultures prepared solely from $\text{Ca}_V2.2_{\text{WTWT}}$ or $\text{Ca}_V2.2_{\text{HA}^{\text{KIKI}}}$ mice.

$\text{GCaMP}$ is a fluorescent $\text{Ca}^{2+}$ sensor, composed of a green fluorescent protein (GFP), calmodulin (CaM) and M13, a peptide chain from myosin light chain kinase (Nakai et al., 2001). At rest, the N-terminus of GFP is connected to M13 (a target sequence of CaM) and the C-terminus of GFP is bound to CaM. Upon $\text{Ca}^{2+}$ influx in response to a stimulus, $\text{Ca}^{2+}$ binds to CaM inducing a conformational change in the GCaMP complex such that there is an increase in fluorescence intensity of GFP (Nakai et al., 2001). Changes to the GCaMP complex have been made to improve signal-to-noise ratio and response kinetics resulting in the development of other GCaMPs including GCaMP6f (Nakai et al., 2001; Tallini et al., 2006; Tian et al., 2009; Akerboom et al., 2012; Chen et al., 2013). In the present study, synaptophysin, a presynaptic marker, was fused to this genetically encoded $\text{Ca}^{2+}$ indicator to form synaptophysin-GCaMP6f and used as an optical reporter to measure $\text{Ca}^{2+}$ transients.

At the time of plating, P0/P1 DRG neurons were transfected with synaptophysin-GCaMP6f and free mCherry (transfection marker). Subsequently, these neurons were plated with untransfected spinal cord neurons. DRG neurons were imaged after 19-22 DIV. The change in fluorescence of synaptophysin-GCaMP6f was monitored in response to 6 action potentials (AP) at 33 Hz (Figure 5.2A). To analyse recordings, regions of interest (ROIs) with a diameter...
of 2 μm were placed along neurites and 6 ROIs were placed at the cell body (Figure 5.2B). The change in fluorescence measured from each ROI was normalised to the fluorescence obtained at rest (Figure 5.2C). Figure 5.2G and 5.2H show the changes in fluorescence measured from the cell bodies and neurites of all three co-cultures following a stimulation of 6 AP. As expected, all three co-cultures produced a similar response to stimulation at the cell body (Figure 5.2D-H). The similar change in fluorescence in the cell body agrees with the electrophysiology data presented in Chapter 3 and confirms that the insertion of the HA-tag does not disrupt Ca\textsuperscript{2+} influx in DRG neurons (Figure 5.2G). Figure 5.2G and H show that there is a difference in the amplitude in the change in fluorescence between the cell body and neurites. One explanation for this could be because synaptophysin is a presynaptic marker and not ideal for measuring somatic Ca\textsuperscript{2+} influx. Altogether, these data suggest that the combination of DRG neurons from Cav2.2\textsubscript{HA\textsuperscript{KIKI}} mice co-cultured with spinal cord neurons from Cav2.2\textsubscript{WT\textsuperscript{WT}} mice does not perturb Ca\textsuperscript{2+} signalling of these neurons. However, further work is required to delineate the differences in Ca\textsuperscript{2+} transients observed in neurites.
Figure 5.2: Ca\textsuperscript{2+} signalling is unperturbed in co-cultures of DRG neurons from Ca\textsubscript{\textit{V}}\textsubscript{2.2\_HA\_KIKI} mice and spinal cord from Ca\textsubscript{\textit{V}}\textsubscript{2.2\_WTWT} mice

(A) Schematic diagram of stimulation protocol. Co-cultures were stimulated at 33 Hz over 180 ms resulting in 6APs delivered every 4 seconds. (B) To analyse change in fluorescence (\(\Delta F\)), 2 \(\mu\)m ROIs were placed along the neurite and 6 ROIs were placed at the cell body. (C) Change in fluorescence (\(\Delta F\)) was then normalised to baseline fluorescence (F0). Representative images of fluorescence changes (\(\Delta F\)) of synaptophysin-GCaMP6f in the cell body and neurites of DRG neurons from (D) Ca\textsubscript{\textit{V}}\textsubscript{2.2\_WTWT}, (E) Ca\textsubscript{\textit{V}}\textsubscript{2.2\_HA\_KIKI} and (F) DRG neurons from Ca\textsubscript{\textit{V}}\textsubscript{2.2\_HA\_KIKI} and spinal cord from Ca\textsubscript{\textit{V}}\textsubscript{2.2\_WTWT} co-cultures at rest (top panel) and in response to electrical stimulation of 6 AP (bottom panel). Pseudocolour scale is shown on the top right of last bottom panel. Scale bar = 5 \(\mu\)m. Average normalised synaptophysin-GCaMP6f responses at the (G) cell body and (H) neurites of Ca\textsubscript{\textit{V}}\textsubscript{2.2\_WTWT} (black), Ca\textsubscript{\textit{V}}\textsubscript{2.2\_HA\_KIKI} (red) and Ca\textsubscript{\textit{V}}\textsubscript{2.2\_HA\_KIKI} DRG Ca\textsubscript{\textit{V}}\textsubscript{2.2\_WTWT} spinal cord (green). Each peak in (G) and (H) correspond to the response to 6APs. For the cell body, individual data points are the average of a total 54 ROIs from 3 neurons from 3 separate cultures for each co-culture type. For neurites individual data points are the average of a total of 150 ROIs from 3 neurons from 3 separate cultures for each co-culture type. Mean ± SEM of the ROIs is superimposed. Statistical analysis was as follows: two–way repeated measures ANOVA with Bonferroni’s multiple comparison test as post hoc test; p and F values given in table in Figure.
5.4 Ca\textsubscript{v}2.2_HA at the cell body of DRG neurons in co-cultures

To guarantee that only presynaptic Ca\textsubscript{v}2.2_HA was examined, DRG and spinal cord neurons were cultured from Ca\textsubscript{v}2.2_HA\textsuperscript{KIKI} and Ca\textsubscript{v}2.2\textsuperscript{WTWT} mice, respectively. Permeabilised conditions were used to study both Ca\textsubscript{v}2.2_HA at the cell body and synapse. Ca\textsubscript{v}2.2_HA was examined in cultures fixed at different time points (DIV 1, 7, 15, 22 and 28). To ensure that there was no non-specific binding of secondary antibodies, cultures were first incubated with rat anti-HA antibody overnight followed by anti-rat Alexa Fluor 488. Subsequently, neurons were incubated with guinea pig anti-vGluT2 antibody overnight prior to incubation with secondary anti-guinea pig Alexa Fluor 633, and cultures were then imaged. Ca\textsubscript{v}2.2_HA was first assessed at the cell body of DRG neurons with respect to cell size. The perimeter measured from these DRG neurons was used as an estimation of cell size. Ca\textsubscript{v}2.2_HA and vGluT2 intensities were measured from DRG neurons at DIV 1 and averaged. The respective mean fluorescence intensity values were then used to normalise Ca\textsubscript{v}2.2_HA and vGluT2 intensity of individual neurons at different days in vitro (7, 15, 22 and 28).

Figure 5.3 shows a ring-like pattern of Ca\textsubscript{v}2.2_HA in small-sized DRG neurons which appears to diminish as the cultures are left for longer in vitro. Further analysis revealed that in comparison to DIV 1, there was a significant consistent decrease of 25%, 50%, 55% and 43% of Ca\textsubscript{v}2.2_HA immunolabelling at DIV 7, 15, 22 and 28, respectively (Figure 5.3B). In contrast, a significant increase of 47% in vGluT2 immunolabelling was observed from DIV 1 to 7. This increase in vGluT2 immunoreactivity was consistent at 39%, 41% and 35% in DIV 15, 22 and 28 cultures, respectively, compared to DIV 1 (Figure 5.3C).
Figure 5.3: Cav2.2_HA expression at the cell body of small-sized DRG neurons decreases over time in co-cultures

(A) Small-sized DRG neurons from co-cultures composed of DRG neurons from Cav2.2_HA\textsuperscript{KIKI} and spinal cord from Cav2.2\textsuperscript{WTWT} mice. Airyscan images (from left to right) of (i) Cav2.2_HA, (ii) vGluT2 and (iii) merged representative immunolabelling of small-sized DRG neurons. From top to bottom; co-cultures fixed at DIV 1, 7, 15, 22 and 28. (Scale bars: 5 \( \mu \text{m} \)). Cell surface Cav2.2_HA and total vGluT2 intensity was measured and averaged from all Cav2.2_HA-positive neurons at DIV 1. The mean fluorescence intensity values were then used to normalise cell surface Cav2.2_HA and total vGluT2 intensity of individual neurons. (B) Normalised cell surface Cav2.2_HA and (C) total vGluT2 intensity measured from cell bodies of DRG neurons from co-cultures fixed at DIV 1, 7, 15, 22 and 28. Individual data points represent normalised Cav2.2_HA and vGluT2 intensity measured from three separate cultures and a total of 167, 203, 98, 103 and 122 neurons from DIV 1, 7, 15, 22 and 28, respectively. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; **** \( p < 0.0001 \), *** \( p < 0.001 \), ** \( p < 0.01 \). \( F_{\text{Cav2.2_HA}} \) value = 5.110, \( F_{\text{vGluT2}} \) value = 1.1020

Cav2.2_HA immunolabelling was next assessed in medium-sized DRG neurons (Figure 5.4A). When compared to Cav2.2_HA expression at DIV 1, there was a decrease of 25%, 53%, 44% and 33% at DIV 7, 15, 22 and 28, respectively (Figure 5.4B). Similar to small-sized DRG neurons there was a significant increase in vGluT2 immunoreactivity in medium-sized DRG neurons (Figure 5.4C). Once again, vGluT2 labelling at DIV 1 was compared to DIV
7, 15, 22 and 28 and an augmentation of 63%, 36%, 55% and 40% in vGluT2 expression was measured, respectively (Figure 5.4C). The data shown in Figure 5.5 are the preliminary results for Cav2.2_HA and vGluT2 immunolabelling in large-diameter DRG neurons. However, before drawing a conclusion from these data, the experiment needs to be repeated due to the difference in cell numbers across the different days in vitro.

![Figure 5.4](image)

**Figure 5.4: Cav2.2_HA expression at the cell body of medium-sized DRG neurons decreases over time in co-cultures**

(A) Medium-sized DRG neurons from co-cultures composed of DRG neurons from Cav2.2_HA^KIKI and spinal cord from Cav2.2_WTWT mice. Airyscan images (from left to right) of (i) Cav2.2_HA, (ii) vGluT2 and (iii) merged representative immunolabelling of medium-sized DRG neurons. From top to bottom; co-cultures fixed at DIV 1, 7, 15, 22 and 28. (Scale bars: 5 μm). Cell surface Cav2.2_HA and total vGluT2 intensity was measured and averaged from all Cav2.2_HA-positive neurons at DIV 1. The mean fluorescence intensity values were then used to normalise cell surface Cav2.2_HA and total vGluT2 intensity of individual neurons. (B) Normalised cell surface Cav2.2_HA and (C) total vGluT2 intensity measured from cell bodies of DRG neurons from co-cultures fixed at DIV 1, 7, 15, 22 and 28. Individual data points represent normalised Cav2.2_HA and vGluT2 intensity measured from three separate cultures and a total of 83, 119, 164, 262 and 359 neurons from DIV 1, 7, 15, 22 and 28, respectively. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; ****p<0.0001, ***p<0.001, *p<0.05. F_{Cav2.2_HA} value = 3.115, F_{vGluT2} value = 6.051
Figure 5.5: Ca\(_{\text{V}2.2}\) \textit{HA} expression at the cell body of large-sized DRG neurons over time in co-cultures

(A) Large-sized DRG neurons from co-cultures composed of DRG neurons from Ca\(_{\text{V}2.2}\) \textit{HA}\textsuperscript{KIKI} and spinal cord from Ca\(_{\text{V}2.2}\) \textit{WTWT} mice. Airyscan images (from left to right) of (i) Ca\(_{\text{V}2.2}\) \textit{HA}, (ii) vGluT2 and (iii) merged representative immunolabelling of large-sized DRG neurons. From top to bottom; co-cultures fixed at DIV 1, 7, 15, 22 and 28. (Scale bars: 5 \(\mu\)m). Cell surface Ca\(_{\text{V}2.2}\) \textit{HA} and total vGluT2 intensity was measured and averaged from all Ca\(_{\text{V}2.2}\) \textit{HA}-positive neurons at DIV 1. The mean fluorescence intensity values were then used to normalise cell surface Ca\(_{\text{V}2.2}\) \textit{HA} and total vGluT2 intensity of individual neurons.

(B) Normalised cell surface Ca\(_{\text{V}2.2}\) \textit{HA} and (C) total vGluT2 intensity at DIV 1, 7, 15, 22 and 28. Individual data points represent normalised Ca\(_{\text{V}2.2}\) \textit{HA} and vGluT2 intensity measured from three separate cultures and a total of 5, 13, 10, 53 and 53 neurons from DIV 1, 7, 15, 22 and 28, respectively. Mean ± SEM of the total number of DRG neurons is superimposed.
5.5 Ca\textsubscript{\textit{V}}2.2 mRNA expression in DRG neurons

RT qPCR was next used to determine whether the decrease in Ca\textsubscript{\textit{V}}2.2_HA expression, seen with increasing time in culture resulted from a decrease in Ca\textsubscript{\textit{V}}2.2 mRNA levels in DRG neurons. Prior to this, Ca\textsubscript{\textit{V}}2.2 mRNA levels were first assessed in DRG tissue extracted from Ca\textsubscript{\textit{V}}2.2\textsubscript{HA\textsuperscript{KIKI}} and Ca\textsubscript{\textit{V}}2.2\textsuperscript{WTWT} mice. To ensure that a high enough concentration of RNA was extracted, DRG tissue was collected from three mice (aged P0/P1) of each genotype. Figure 5.6A shows the dot plot of the average data from 3 experiments normalised to the housekeeping, \textit{Hprt}, gene. Relative fold expression was calculated with respect to Ca\textsubscript{\textit{V}}2.2\textsuperscript{WTWT} mice. Normalised Ca\textsubscript{\textit{V}}2.2 C\textsubscript{\textit{T}} values had a relative fold expression of 1.02 ± 0.13 and 0.98 ± 0.10 for Ca\textsubscript{\textit{V}}2.2\textsuperscript{WTWT} and Ca\textsubscript{\textit{V}}2.2\textsubscript{HA\textsuperscript{KIKI}} mice, respectively. Figure 5.6A shows that there is no significant difference between Ca\textsubscript{\textit{V}}2.2 expression in DRG tissue between the Ca\textsubscript{\textit{V}}2.2\textsubscript{HA\textsuperscript{KIKI}} and Ca\textsubscript{\textit{V}}2.2\textsuperscript{WTWT} mice.

Next, this study sought to assess whether Ca\textsubscript{\textit{V}}2.2 mRNA expression in DRG tissue changed over development. As no significant difference in Ca\textsubscript{\textit{V}}2.2 mRNA expression was observed between Ca\textsubscript{\textit{V}}2.2\textsubscript{HA\textsuperscript{KIKI}} and Ca\textsubscript{\textit{V}}2.2\textsuperscript{WTWT} mice (Figure 5.6A), DRG tissue from Ca\textsubscript{\textit{V}}2.2\textsubscript{HA\textsuperscript{KIKI}} mice only were next used. RNA was extracted from DRG tissue of 11-week-old and P1 Ca\textsubscript{\textit{V}}2.2\textsubscript{HA\textsuperscript{KIKI}} mice. C\textsubscript{\textit{T}} values were normalised to \textit{Hprt} and relative fold expression of Ca\textsubscript{\textit{V}}2.2 was made with respect to P1 mice. Figure 5.6B shows a significant decrease of 44% in Ca\textsubscript{\textit{V}}2.2 transcript in 11-week-old mice when compared to P1 mice (normalised data were P1: 1.01 ± 0.10 and 11-week-old: 0.57 ± 0.01, Figure 5.6B). These data are consistent with previous reports which have shown a similar age dependent decrease in VGCC mRNA expression in the rodent brain (Chang et al., 2007; Schlick et al., 2010). The results from the present study suggest that there is a reduction in Ca\textsubscript{\textit{V}}2.2 mRNA expression in DRG tissue during development.
Figure 5.6: Cav2.2 mRNA expression in DRG tissue decreases over development
(A) RT qPCR results for Cav2.2 mRNA levels in DRG tissue from Cav2.2WTWT (black) and Cav2.2_HAHAHAHAHAHA (red) P0/P1 mice (RNA extracted from DRG tissue collected from 3 mice and this was repeated 3 times, each assayed in triplicates). Data were normalised to housekeeping gene Hprt. Mean ± SEM of three experiments is shown. ns, not significant, paired t test, $p = 0.8599$, F value = 1.588. (B) Relative fold expression of Cav2.2 in DRG tissue from P1 and 11-week-old Cav2.2_HAHAHAHAHAHA mice (red) mice. $C_T$ values of Cav2.2 normalised to Hprt and relative fold expression of Cav2.2 was made with respect to P1 mice. Mean ± SEM of three experiments is shown. Statistical analysis as follows: unpaired t test. *$p<0.05$, ns = not significant. $p = 0.0128$, F value = 11.33
5.6 Comparison of Ca\textsubscript{V}2.2\textunderscore HA and II-III loop immunolabelling at the cell body

The polyclonal II-III loop antibody corresponds to an intracellular epitope found in the loop between domains II and III of Ca\textsubscript{V}2.2. However, this antibody is unable to differentiate between cell surface and intracellular Ca\textsubscript{V}2.2 immunoreactivity. The following experiment aimed to show how the exofacially HA-tagged Ca\textsubscript{V}2.2 could be used in combination with the II-III loop antibody to demarcate cell surface and intracellular Ca\textsubscript{V}2.2 expression, respectively. First, DIV 15 co-cultures were incubated with rat anti-HA antibody followed by secondary anti-rat Alexa Fluor 488 in non-permeabilised conditions. Neurons were then permeabilised and incubated with rabbit anti-II-III loop antibody overnight prior to incubation with secondary anti-rabbit Alexa Fluor 594 antibody and imaged. Cell surface Ca\textsubscript{V}2.2\textunderscore HA and total Ca\textsubscript{V}2.2 fluorescence intensity was measured from three regions of interest (ROI; 1 \( \mu \text{m} \times 9 \mu \text{m} \)) placed at the cell surface to 9 \( \mu \text{m} \) into the neuron (Figure 5.7). Figure 5.7A shows Ca\textsubscript{V}2.2\textunderscore HA immunoreactivity at the cell membrane and this endogenous pattern of immunolabelling could not be detected with the anti II-III loop antibody. Quantitative analysis revealed that Ca\textsubscript{V}2.2\textunderscore HA and II-III loop immunolabelling did not have comparable peaks (Figure 7B).
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Figure 5.7: Comparison of Ca\_v2.2 expression using anti-HA and anti II-III loop antibodies in DRG cell bodies

(A) Confocal images of cultured DRG neurons (DIV 15) from DRG and spinal cord co-cultures from Ca\_v2.2\textsubscript{HA\textsuperscript{KIKI}} and Ca\_v2.2\textsubscript{WTWT} mice (respectively) showing (from left to right) Ca\_v2.2\textsubscript{HA} (prior to permeabilisation; green), total Ca\_v2.2 (following permeabilisation; red) and merged representative image. Scale bar 5\,\mu m. (B) Plot profile of cell surface Ca\_v2.2\textsubscript{HA} (green) and total Ca\_v2.2 intensity (red) (mean ± SEM) in ROIs from the cell surface to 9\,\mu m into the neuron. Individual data points represent the mean data from 27 ROIs. Three ROIs were measured from three DRG neurons from three separate experiments.

Comparisons between cell surface Ca\_v2.2\textsubscript{HA} and total Ca\_v2.2 intensity were next made with respect to cell size. Cell surface Ca\_v2.2\textsubscript{HA} and total Ca\_v2.2 intensity was measured and averaged from all Ca\_v2.2\textsubscript{HA}-positive neurons. The respective mean fluorescence intensity values were then used to normalise cell surface Ca\_v2.2\textsubscript{HA} and total Ca\_v2.2 intensity of individual neurons. Figure 5.8 shows that cell surface Ca\_v2.2\textsubscript{HA} was 34% higher in small compared to large-sized DRG neurons and 38% higher in medium compared to large-sized DRG neurons (normalised values were s: 0.97 ± 0.06, m: 1.00 ± 0.03, l: 0.72 ± 0.05, Figure 5.8B). Comparable expression patterns were observed using the anti-II-III loop antibody. The total Ca\_v2.2 intensity was 49% higher in small compared to large-diameter DRG neurons and
57% higher in medium compared to large-diameter DRG neurons (normalised values were s: 0.97 ± 0.05, m: 1.02 ± 0.03, l: 0.65 ± 0.04, Figure 5.8C). These results confirm that the anti-HA antibody recognises endogenous CaV2.2 in the same DRG neuron subpopulations as the anti-II-III loop antibody. However, it is possible the II-III loop antibody cannot detect CaV2.2 at the plasma membrane due to the epitope of the antibody being occluded when CaV2.2 channels are at the cell surface.
Figure 5.8: Comparison of \( \text{Ca}_{\text{V}} 2.2 \) expression using anti-HA and anti II-III loop antibodies in DRG cell bodies with respect to cell size

(A) Images of DRG neurons (DIV 15) from DRG and spinal cord co–cultures from \( \text{Ca}_{\text{V}} 2.2 \)\textsubscript{HA}\textsuperscript{KIKI} and \( \text{Ca}_{\text{V}} 2.2 \text{WTWT} \) mice (respectively) showing (from left to right) \( \text{Ca}_{\text{V}} 2.2 \)\textsubscript{HA} (prior to permeabilisation; green), total \( \text{Ca}_{\text{V}} 2.2 \) (following permeabilisation; red) and merged representative image. Scale bar 10\( \mu \)m. Cell surface \( \text{Ca}_{\text{V}} 2.2 \)\textsubscript{HA} and total \( \text{Ca}_{\text{V}} 2.2 \) intensity was measured and averaged from all \( \text{Ca}_{\text{V}} 2.2 \)\textsubscript{HA}-positive neurons. The mean fluorescence intensity values were then used to normalise cell surface \( \text{Ca}_{\text{V}} 2.2 \)\textsubscript{HA} and total \( \text{Ca}_{\text{V}} 2.2 \) intensity of individual neurons. \( p = 0.0012, \ p < 0.001, \ F \text{ value} = 6.850 \). Normalised (B) cell surface \( \text{Ca}_{\text{V}} 2.2 \)\textsubscript{HA} and (C) total \( \text{Ca}_{\text{V}} 2.2 \) intensity with respect to cell size: small (s: 88), medium (m: 271) and large (l: 42) DRG neurons. Individual data points from three separate cultures. Mean $\pm$ SEM of the total DRG neurons from three experiments is superimposed. Statistical analysis was as follows: one–way ANOVA with post hoc Bonferroni’s multiple comparison test; $***p<0.001$, $F$ value $= 15.16$. 

\( \text{Ca}_{\text{V}} 2.2 \)\textsubscript{HA}
5.7 Confocal vs Airyscan microscopy of synaptic markers in co-cultures

As discussed in Chapter 3, Airyscan microscopy uses a detector concept that considerably improves signal-to-noise ratio to provide high resolution images. As such, this method was compared to normal confocal microscopy methods to ameliorate spatial resolution when imaging synapses. DIV 15 co-cultures were fixed, permeabilised and immunolabelled for pre- and postsynaptic markers, vGluT2 and Homer, respectively. Figure 5.9A and B show a side by side comparison of the same synapses imaged using confocal and Airyscan microscopy. Both Figure 5.9A and B show the distribution of Homer immunoreactive puncta also positive for vGluT2 (Figure 5.9) which is similar to that observed in spinal cord tissue (Gutierrez-Mecinas et al., 2016). Nevertheless, Airyscan images show a substantial increase in image resolution and signal-to-noise ratio in comparison to confocal images (Figure 5.9). These results show that Airyscan processing further improved the image resolution by pixel reassignment and further experiments studying synapses of co-cultures were imaged with the Airyscan modality.
Figure 5.9: Comparison of synaptic puncta imaged using confocal and Airyscan microscopy

Images of single optical sections of synaptic puncta from DIV 15 co-cultures from Cav2.2<sup>WT WT</sup> mice taken using (A) Confocal and (B) Airyscan microscopy. Showing from left to right: (i) Homer, (ii) vGluT2 and (iii) merge representative synaptic puncta. Scale bar 2 μm. (iv) 2 x 2 μm close up view of synaptic puncta marked with a yellow arrow on merge panel. From top to bottom; Homer, vGluT2 and merged representative image.
5.8 Ca\textsubscript{V}2.2_HA at excitatory synapses of co-cultures

Next, to study the development of presynaptic Ca\textsubscript{V}2.2_HA expression, DRG and spinal cord neurons were cultured from Ca\textsubscript{V}2.2_HA\textsuperscript{KIKI} and Ca\textsubscript{V}2.2\textsuperscript{WTWT} mice, respectively. The time course for functional synapse formation between DRG neurons with their dorsal horn partners has been reported to commence at DIV 5 (Joseph et al., 2010). Additionally, previous studies have investigated synaptogenesis between DRG and dorsal horn neurons co-cultured for up to 4 weeks (Gu and MacDermott, 1997). In the present study, to examine presynaptic Ca\textsubscript{V}2.2_HA expression, co-cultures were fixed at DIV 7, 15, 22 and 28. Immunolabelling with anti-vGluT2 and Homer antibodies were used to identify synapses. A previous study showed that Homer immunostaining reveals the majority of excitatory synapses (Gutierrez-Mecinas et al., 2016). As such, boutons were chosen based on the presence of Homer immunoreactivity and classified into either being positive or negative for both presynaptic Ca\textsubscript{V}2.2_HA and vGluT2, (puncta were also classified as negative if Homer was apposed to only Ca\textsubscript{V}2.2_HA or vGluT2). Figure 5.10A shows, in immature cultures, that there was weak Ca\textsubscript{V}2.2_HA immunostaining in puncta varying in size and intensity at DIV 7. 43% ± 2% of Homer-positive puncta were positive for both Ca\textsubscript{V}2.2_HA and vGluT2. However, a significantly higher proportion of Homer-positive puncta were negative for both presynaptic Ca\textsubscript{V}2.2_HA and vGluT2 (57% ± 2%; Figure 5.10B).
Figure 5.10: Both CaV2.2_HA and vGluT2 are not predominantly expressed in Homer-positive synaptic puncta at DIV 7

(A) Airyscan images of single optical sections (0.3 μm thick) of synaptic puncta from DIV 7 co-cultures (DRG from CaV2.2_HA^KI and spinal cord from CaV2.2^WTWT mice). Top panels from left to right of CaV2.2_HA (green) and vGluT2 (magenta) and bottom panels from left to right Homer (red) and merged representative image. Scale bar = 2 μm. Yellow arrows on merge panels show examples of Homer-positive puncta selected for quantification. (B) Quantification of the percentage of Homer-positive puncta (n = 148) that were also positive (n = 63/148; green circles) or negative (n = 85/148; black circles) for both CaV2.2_HA and vGluT2. Mean ± SEM of the three experiments is superimposed. Statistical analysis as follows: unpaired t test. *p = 0.0256, F value = 1.000

At DIV 15, CaV2.2_HA immunoreactivity appeared more puncta-like compared to that observed at DIV 7 (Figure 5.11A). Both CaV2.2_HA and vGluT2 puncta were closely associated with Homer. Moreover, no significant difference was detected between the number of Homer-positive boutons that were either positive or negative for both CaV2.2_HA and vGluT2 (Figure 5.11B). In contrast, in more mature co-cultures (DIV 22), CaV2.2_HA staining profile was strikingly different, with the appearance of intense punctate staining along neuritic processes (Figure 5.12A). Many Homer-positive puncta were found to be associated with both CaV2.2_HA and vGluT2 (78% ± 1.5%) whereas only a small number of boutons were CaV2.2_HA and vGluT2-negative (22% ± 1.5%; Figure 5.12B).
Figure 5.11: Comparable expression of both Ca\textsubscript{V2.2_HA} and \textit{vGluT2} in Homer-positive and -negative synaptic puncta at DIV 15

(A) Airyscan images of single optical sections (0.3 \(\mu\)m thick) of synaptic puncta from DIV 15 cocultures (DRG from Ca\textsubscript{V2.2_HA\textsuperscript{KIKI}} and spinal cord from Ca\textsubscript{V2.2\textsuperscript{WTWT}} mice). Top panels from left to right of Ca\textsubscript{V2.2_HA} (green) and \textit{vGluT2} (magenta) and bottom panels from left to right Homer (red) and merged representative image. Scale bar = 2 \(\mu\)m. Yellow arrows on merge panels show examples Homer-positive puncta selected for quantification. (B) Quantification of the percentage of Homer-positive puncta (\(n = 232\)) that were also positive (\(n = 98/232\); green circles) or negative (\(n = 134/232\); black circles) for both Ca\textsubscript{V2.2_HA} and \textit{vGluT2}. Mean \(\pm\) SEM of the three experiments is superimposed. Statistical analysis as follows: unpaired \(t\) test. \(p = 0.9063\), F value = 1.000.
Figure 5.12: Both Ca\textsubscript{v}2.2\_HA and vGluT2 are predominantly expressed in Homer-positive synaptic puncta at DIV 22

(A) Airyscan images of single optical sections (0.3 μm thick) of synaptic puncta from DIV 22 cocultures (DRG from Ca\textsubscript{v}2.2\_HA\textsuperscript{KIKI} and spinal cord from Ca\textsubscript{v}2.2\_WT\textsuperscript{WT} mice). Top panels from left to right of Ca\textsubscript{v}2.2\_HA (green) and vGluT2 (magenta) and bottom panels from left to right Homer (red) and merged representative image. Scale bar = 2 μm. Yellow arrows on merge panels show examples Homer-positive puncta selected for quantification. (B) Quantification of the percentage of Homer-positive puncta (n = 1182) that were also positive (n = 926/1182; green circles) or negative (n = 256/1182; black circles) for both Ca\textsubscript{v}2.2\_HA and vGluT2. Mean ± SEM of the three experiments is superimposed. Statistical analysis as follows: unpaired t test. ***p<0.001

At DIV 28, the appearance of distinct mature Ca\textsubscript{v}2.2\_HA and vGluT2 puncta can be seen which are also apposed to Homer (Figure 5.13). In addition to punctate structures, Ca\textsubscript{v}2.2\_HA can also be seen distributed around a central core of vGluT2 associated with Homer (see arrows on Figure 5.13), resembling glomerular synapses (Ribeiro-da Silva et al., 1985). The rosette-shaped clusters of Ca\textsubscript{v}2.2\_HA were comprised of up to five puncta. These patterns of immunoreactivity are consistent with our \textit{in vivo} study (Nieto-Rostro et al., 2018). Figure 5.13B shows that 78% ± 5.3% of Homer-positive puncta were found in association with both Ca\textsubscript{v}2.2\_HA and vGluT2. Taken together these data suggest that the expression pattern on Ca\textsubscript{v}2.2\_HA is developmentally regulated.
Figure 5.13: Both \( \text{Ca}_2.2 \text{HA} \) and \( \text{vGluT2} \) are predominantly expressed in Homer-positive synaptic puncta at DIV 28
(A) Airyscan images of single optical sections (0.3 \( \mu \)m thick) of synaptic puncta from DIV 28 co-cultures (DRG from \( \text{Ca}_2.2 \text{HA}^{\text{KIKI}} \) and spinal cord from \( \text{Ca}_2.2 \text{WTWT} \) mice). Top panels from left to right of \( \text{Ca}_2.2 \text{HA} \) (green) and \( \text{vGluT2} \) (magenta) and bottom panels from left to right Homer (red) and merged representative image. Scale bar = 2 \( \mu \)m. Yellow arrows on merge panels show examples Homer-positive puncta selected for quantification. (B) Quantification of the percentage of Homer-positive puncta (n = 1782) that were also positive (n = 1379/1782; green circles) or negative (n = 403/1782; black circles) for both \( \text{Ca}_2.2 \text{HA} \) and \( \text{vGluT2} \). Mean ± SEM of the three experiments is superimposed. Statistical analysis as follows: unpaired \( t \) test. **\( p = 0.0017 \)

To further study \( \text{Ca}_2.2 \text{HA} \) expression at the synapse, I developed a simple method of analysis with regards to \( \text{Ca}_2.2 \text{HA} \) immunoreactivity. Synaptic boutons have been reported to be 1–2 \( \mu \)m in diameter (Yeow and Peterson, 1991; Knodel et al., 2014; Chéreau et al., 2017). As such, a region of interest (ROI) with a diameter of 2 \( \mu \)m was placed on Homer-positive boutons. \( \text{Ca}_2.2 \text{HA} \), \( \text{vGluT2} \) and Homer intensity was measured within the ROIs (Figure 5.14A) of co-cultures fixed at DIV 7, 15, 22 and 28. \( \text{Ca}_2.2 \text{HA} \), \( \text{vGluT2} \) and Homer intensities were normalised to their respective averages measured at DIV 7.

Figure 5.14B highlights the change in \( \text{Ca}_2.2 \text{HA} \) immunoreactivity in co-cultures over development. At DIV 7 and 15, \( \text{Ca}_2.2 \text{HA} \) puncta have a diffuse appearance around Homer
and vGluT2 puncta. However, at DIV 22 and 28 CaV2.2_HA-positive puncta appear clearly defined in apposition to Homer and vGluT2 puncta. Furthermore, as shown in Figure 5.14B, glomerular like structures can also be identified in 4-week-old co-cultures. In contrast to the decrease in CaV2.2_HA immunolabelling observed at the cell body, an increase in CaV2.2_HA fluorescence intensity in synapses was observed as a function of time in culture. CaV2.2_HA immunoreactivity increased by 127%, 191% and 491% in DIV 15, 22 and 28 co-cultures, respectively, compared to that measured at DIV 7 (normalised values were DIV 7: 1.00 ± 0.07, DIV 15: 2.27 ± 0.10, DIV 22: 2.92 ± 0.17, DIV 28: 5.91 ± 0.40, Figure 5.14C). The increase in CaV2.2_HA immunolabelling was still observed between older cultures as there was a significant increase of 102% in CaV2.2_HA staining in DIV 28 compared to DIV 22 co-cultures (Figure 5.14C). The striking increase of CaV2.2_HA in DIV 28 cultures may be explained by the organisation of CaV2.2_HA in glomeruli.

A similar increase in vGluT2 staining was observed in co-cultures over development (Figure 5.14B). Once again, sharp increases of 45%, 89% and 223% of vGluT2 immunoreactivity was detected at DIV 15, 22 and 28, respectively, when compared to staining at DIV 7 (normalised values were DIV 7: 1.00 ± 0.06, DIV 15: 1.45 ± 0.09, DIV 22: 1.89 ± 0.09, DIV 28: 3.23 ± 0.14, Figure 5.14D). This increase was consistent as vGluT2 augmented by 30% in DIV 22 compared to DIV 15 co-cultures and 123% in DIV 28 compared to DIV 15 co-cultures. Furthermore, in mature cultures, a further increase of 71% was revealed in vGluT2 expression in DIV 28 compared to DIV 22 co-cultures (Figure 5.14D).

A comparable significant increase in Homer immunoreactivity can also be seen in Figure 5.14E. When compared to DIV 7 cultures, a striking increase of 73%, 132% and 247% in Homer immunolabelling was observed at DIV 15, 22 and 28, respectively (normalised values were DIV 7: 1.00 ± 0.05, DIV 15: 1.72 ± 0.08, DIV 22: 2.32 ± 0.13, DIV 28: 3.47 ± 0.15, Figure 5.14E). Once again, a corresponding increase was also found in older cultures.
Homer staining was 34% higher in DIV 22 compared to DIV 15 co-cultures and 101% higher in DIV 28 compared to DIV 15 co-cultures (Figure 5.14E). Previous studies have also shown that in co-cultures, MAP2, a dendritic marker, and vGluT2 puncta increased with time in culture which overlapped with the formation of functional synapses (Joseph et al., 2010). In the present study, triple labelling with CaV2.2_HA and pre- and postsynaptic markers vGluT2 and Homer, respectively, revealed the developmental regulation of presynaptic CaV2.2_HA in co-cultures. Taken together, these data suggest that co-cultures are able to form functional synapses and have direct application for studies on factors controlling the development of presynaptic CaV2.2_HA at nociceptive DRG/dorsal horn synapses.
Figure 5.14: Ca\textsubscript{v}2.2_HA expression at the presynaptic membrane over time in culture

(A) Schematic diagram of synaptic puncta analysis. ROI with a diameter 2 μm was placed over Homer-positive puncta (red) associated with Ca\textsubscript{v}2.2_HA (green) and vGluT2 (magenta) puncta. Intensity of each puncta was then measured. (B) Images of single optical sections of synaptic puncta. 2 x 2 μm representative image showing from left to right Ca\textsubscript{v}2.2_HA (green), vGluT2 (magenta), Homer (red) and merged panels. Synaptic puncta from co-cultures fixed (from top to bottom) at DIV 7, 15, 22 and 28. (C) Ca\textsubscript{v}2.2_HA, F value = 80.69 (D) vGluT2, F value = 92.85 and (E) Homer puncta intensity, F value = 87.37, at DIV 7, 15, 22 and 28 normalised to the mean fluorescence intensity of Ca\textsubscript{v}2.2_HA, vGluT2 and Homer at DIV7, respectively. Individual data points represent normalised Ca\textsubscript{v}2.2_HA, vGluT2 and Homer intensity measured from Homer-positive puncta from three separate cultures and a total of 150 puncta from DIV 7, 15, 22 and 28 were measured. Mean ± SEM of the three experiments is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s multiple comparison test as post hoc test; ***p<0.001, **p<0.01, *p<0.05.
5.9 Comparison of Ca\textsubscript{\textsuperscript{2.2}}_HA and II-III loop immunolabelling in DRG neurites of co-cultures

The anti-II-III loop and anti-HA antibodies were next used in co-cultures to assess cell surface and total Ca\textsubscript{\textsuperscript{2.2}} expression. Figure 5.15A shows clear cell surface Ca\textsubscript{\textsuperscript{2.2}}_HA at the plasma membrane of DRG neurons which cannot be detected using the anti-II-III loop antibody. However, II-III loop immunolabelling can be found within the cell body distributed in the perinuclear region. Along the DRG neuritic process Ca\textsubscript{\textsuperscript{2.2}}_HA puncta can be seen (Figure 5.15A, arrows) which appear to co-localise with II-III loop immunoreactivity (Figure 5.15B). These results suggest that the anti-HA and anti II-III loop antibody share similar immunoreactivity to Ca\textsubscript{\textsuperscript{2.2}} in neurites.

![Figure 5.15: Comparison of Ca\textsubscript{\textsuperscript{2.2}} expression using anti-HA and anti II-III loop antibodies in DRG processes](image)

(A) Confocal images of cultured DRG neurons (DIV 22) from DRG and spinal cord co-cultures from Ca\textsubscript{\textsuperscript{2.2}}_HA\textsuperscript{KIKI} and Ca\textsubscript{\textsuperscript{2.2}}_WTWT mice (respectively) showing (from left to right) Ca\textsubscript{\textsuperscript{2.2}}_HA (green; using anti-HA antibody), total Ca\textsubscript{\textsuperscript{2.2}} (red; using anti II-III loop antibody) and merged representative. Both antibodies applied after permeabilization. Scale bar 10\mu m. (B) 2 x 2 \mu m ROIs showing Ca\textsubscript{\textsuperscript{2.2}} puncta indicated by yellow arrow on merge panel. From top to bottom Ca\textsubscript{\textsuperscript{2.2}}_HA (green), Ca\textsubscript{\textsuperscript{2.2}} (red) and merge panel.
5.10 Cell surface $\text{Ca}_V\text{2.2}_{-\text{HA}}$ is preferentially expressed in TRPV1-positive DRG neurons

Capsaicin sensitivity is a principal pharmacological trait of a major subset of nociceptive sensory neurons which mediates its effects through TRPV1 (Caterina et al., 1997; Tominaga et al., 1998). As described above, capsaicin has been reported to profoundly inhibit VGCC currents in DRG neurons (Docherty et al., 1991). In order to understand their association in DRG populations, DRG neurons cultured from $\text{Ca}_V\text{2.2}_{-\text{HA}}^{\text{KIKI}}$ mice were immunolabelled for both $\text{Ca}_V\text{2.2}_{-\text{HA}}$ and TRPV1. To ensure only cell surface $\text{Ca}_V\text{2.2}_{-\text{HA}}$ was examined neurons were first fixed and incubated with rat anti-HA antibody followed by secondary anti-rat Alexa Fluor 594 antibody in non-permeabilised conditions. Neurons were then permeabilised and incubated with goat anti-TRPV1 antibody prior to secondary anti-goat Alexa Fluor 488 incubation. Confocal images show a clear ring of $\text{Ca}_V\text{2.2}_{-\text{HA}}$ at the plasma membrane of neurons positive for TRPV1. Cell surface $\text{Ca}_V\text{2.2}_{-\text{HA}}$ immunoreactivity can also be seen, to a lesser extent, on TRPV1-negative neurons (Figure 5.16A). As expected, $\text{Ca}_V\text{2.2}_{-\text{HA}}$ immunoreactivity is not observed in wildtype neurons. Figure 5.16B shows that 66% ± 6% of $\text{Ca}_V\text{2.2}_{-\text{HA}}$-positive neurons express TRPV1 whereas only 34% ± 6% are TRPV1-negative.

Next, cell surface $\text{Ca}_V\text{2.2}_{-\text{HA}}$ intensity was measured and averaged from all $\text{Ca}_V\text{2.2}_{-\text{HA}}$-positive neurons. This mean fluorescence intensity value was then used to normalise cell surface $\text{Ca}_V\text{2.2}_{-\text{HA}}$ fluorescence of individual neurons. DRG neurons were further subdivided based on size. In this study, TRPV1 expression was found to be highest in small- and medium-sized DRG neurons which has similarly been shown by previous studies (Caterina et al., 2000; Kobayashi et al., 2005). Cell surface $\text{Ca}_V\text{2.2}_{-\text{HA}}$ was 29%, 45% and 51% higher in small, medium and large TRPV1-positive neurons, respectively, compared to their TRPV1-negative counterparts (Figure 5.16C). These data suggest that $\text{Ca}_V\text{2.2}_{-\text{HA}}$ is preferentially expressed in TRPV1-positive DRG neurons.
Figure 5.16: Cell surface $\text{Ca}_V2.2_{\text{HA}}$ is preferentially expressed in TRPV1-positive DRG neurons

(A) Images of cultured DRG neurons from $\text{Ca}_V2.2_{\text{HA}}^{\text{KIKI}}$ and $\text{Ca}_V2.2^{\text{WTWT}}$ mice showing (top to bottom) $\text{Ca}_V2.2_{\text{HA}}$ staining before permeabilisation, TRPV1 staining following permeabilisation (middle row) and merged for three representative $\text{Ca}_V2.2_{\text{HA}}^{\text{KIKI}}$ and $\text{Ca}_V2.2^{\text{WTWT}}$ DRG neurons (bottom row). (B) Quantification of the percentage of neurons with cell surface $\text{Ca}_V2.2_{\text{HA}}$ that were also positive (red circles) or negative (black circles) for TRPV1. Individual data points represent the mean data from three separate experiments and a total of 311 DRG neurons. Mean ± SEM of the three experiments is superimposed. $p = 0.0209$, F value = 1.000 (unpaired t test). For normalisation, cell surface $\text{Ca}_V2.2_{\text{HA}}$ intensity was measured and averaged from all $\text{Ca}_V2.2_{\text{HA}}$-positive neurons. This mean fluorescence intensity value was then used to normalise cell surface $\text{Ca}_V2.2_{\text{HA}}$ fluorescence of individual neurons. (C) Normalised cell surface $\text{Ca}_V2.2_{\text{HA}}$ intensity with respect to cell size: small (s), medium (m) and large (l) DRG neurons that are either TRPV1-positive (red circles) or TRPV1-negative (black circles). Individual data points represent normalised $\text{Ca}_V2.2_{\text{HA}}$ intensity measured from all $\text{Ca}_V2.2_{\text{HA}}$-positive neurons from three separate experiments and a total of 85, 104, 10, 18, 70 and 24 DRG neurons from left to right on x-axis. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison as post hoc test; **$p<0.001$, *$p<0.01$, *$p<0.05$, F value = 5.075.
5.11 Capsaicin modulates cell surface Ca\textsubscript{2.2}_HA expression

To study the effects of capsaicin (CAP) on cell surface Ca\textsubscript{2.2}_HA expression, live DRG neurons cultured from Ca\textsubscript{2.2}_HA\textsuperscript{KIKI} mice were incubated with 1 \(\mu\)M CAP for either 0, 20, 120 or 240 seconds (s) at 37\(^{o}\)C. Immunolabelling for cell surface Ca\textsubscript{2.2}_HA and TRPV1 were conducted as described in section 5.10. Furthermore, neurons were subdivided into size categories and TRPV1-positive and -negative neurons were analysed separately.

5.11.1 Cell surface Ca\textsubscript{2.2}_HA expression in TRPV1-positive neurons following capsaicin application

As a control, neurons were treated in Krebs-Ringer Bicarbonate (KRH) buffer solution for 0, 20, 120 or 240 s at 37\(^{o}\)C. Cell surface Ca\textsubscript{2.2}_HA fluorescence intensity was measured from all TRPV1-positive neurons and the perimeter of these DRG neurons was used as an estimation of neuron size, the size-groups were analysed independently. In all experiments, the fluorescence intensity of each neuron was normalised against the mean fluorescence of control DRG neurons (KRH, 0 seconds) from their respective size-groups. Figure 5.17 shows that when incubating neurons in control solution for either 0, 20, 120 or 240 s, there was no visible change in plasma membrane labelling of Ca\textsubscript{2.2}_HA in either small, medium or large-sized DRG neurons (Figure 5.17Ai, ii and iii).
Figure 5.17: Control solution (KRH) does not modify cell surface \( \text{Ca}_{\text{V}}2.2_{\text{HA}} \) expression in TRPV1-positive neurons

(A) Images of DRG neurons cultured from \( \text{Ca}_{\text{V}}2.2_{\text{HA}}^{\text{KIKI}} \) mice, showing (from left to right) cell surface \( \text{Ca}_{\text{V}}2.2_{\text{HA}} \) (red), TRPV1 (green) and merge representative image. DRG neurons incubated with KRH for (from top to bottom) 0, 20, 120 and 240 s. Normalised cell surface \( \text{Ca}_{\text{V}}2.2_{\text{HA}} \) of DRG neurons divided into (i) small, \( p = 0.8151, \) \( F \) value = 1.421, (ii) medium, \( p = 0.9547, \) \( F \) value = 1.910 and (iii) large, \( p = 0.6246, \) \( F \) value = 1.857 subgroups incubated with KRH for 0, 20, 120, 240 s. For normalisation, the fluorescence intensity of each neuron was normalised against the mean fluorescence of control DRG neurons (KRH, 0 s) from their respective size-groups. Individual data points represent normalised \( \text{Ca}_{\text{V}}2.2_{\text{HA}} \) intensity (red circles) measured from all TRPV1-positive neurons from three separate experiments. Total of 50, 59, 26 and 38 small-sized neurons; 77, 76, 81 and 44 medium neurons; 19, 34, 33 and 33 large-sized neurons incubated with KRH for 0, 20, 120 and 240 s, respectively. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s multiple comparison test as post hoc test; ns = not significant

Following this, cell surface \( \text{Ca}_{\text{V}}2.2_{\text{HA}} \) of small-sized DRG neurons incubated with CAP for 0, 20, 120 and 240 s were normalised against the mean fluorescence of small-sized control DRG neurons (KRH, 0 s). In previous studies, the effect of CAP on glutamate release has been explored through measuring spontaneous excitatory postsynaptic potentials (sEPSPs) in DRG/dorsal horn co-cultures (Medvedeva et al., 2008; Hendrich et al., 2012). Both these studies found that CAP application increased the sEPSP frequency within the first 20 s of stimulation with CAP. Indeed, a 20% increase of cell surface \( \text{Ca}_{\text{V}}2.2_{\text{HA}} \) expression was
observed after 20 s incubation with CAP compared to control conditions (normalised values were CAP 20 s: $1.21 \pm 0.08$ and KRH 0 s: $1.00 \pm 0.07$, Figure 5.18B). After 120 s of incubation with CAP, cell surface CaV$_{2.2\_HA}$ immunoreactivity decreased back to comparable levels in control conditions (normalised values were CAP 120 s: $1.02 \pm 0.08$ and KRH 0 s: $1.00 \pm 0.07$, Figure 5.18B). Plasma membrane CaV$_{2.2\_HA}$ immunolabelling also reduced by 24% after 240 s CAP incubation relative to small-diameter neurons incubated in control solution (normalised values were CAP 240 s: $0.76 \pm 0.05$ and KRH 0 s: $1.00 \pm 0.07$, Figure 5.18B).

Figure 5.18: Capsaicin modulates cell surface CaV$_{2.2\_HA}$ expression in small-sized TRPV1-positive neurons

(A) Images of small-sized DRG neurons cultured from CaV$_{2.2\_HA}^{Ka/Kb}$ mice, showing (from left to right) cell surface CaV$_{2.2\_HA}$ (red), TRPV1 (green) and merge representative image. DRG neurons incubated with capsaicin (CAP) for (from top to bottom) 0, 20, 120 and 240 s. (B) Normalised cell surface CaV$_{2.2\_HA}$ intensity (red circles) of small-sized DRG neurons incubated with KRH for 0 s and CAP for 0, 20, 120 and 240 s. For normalisation, the fluorescence intensity of each neuron was normalised against the mean fluorescence of small-sized control DRG neurons (KRH, 0 s). Individual data points represent normalised CaV$_{2.2\_HA}$ intensity measured from all TRPV1-positive neurons from three separate experiments. Total of 72, 81, 87 and 77 small-sized neurons incubated with CAP for 0, 20, 120 and 240 s, respectively. Mean ± SEM of total number of DRG neurons is superimposed. Statistical analysis was as follows: one-way ANOVA with Bonferroni’s selected comparison test as post hoc test; ***$p<0.001$, ns = not significant, F value = 4.99
Medium-sized DRG neurons similarly demonstrated a transient increase followed by a gradual decrease in Ca\textsubscript{V}2.2\_HA cell surface staining the longer neurons were incubated with CAP (Figure 5.19A). Further analysis showed an increase of 33% in cell surface Ca\textsubscript{V}2.2\_HA expression in medium-sized neurons incubated with CAP for 20 s compared to the control (normalised values were CAP 20 s: 1.32 ± 0.05 and KRH 0 s: 0.99 ± 0.05, Figure 5.19B). Plasma membrane Ca\textsubscript{V}2.2\_HA then decreased to similar levels as the control condition following incubation with CAP for 120 s (normalised values were CAP 120 s: 0.99 ± 0.04 and KRH 0 s: 0.99 ± 0.05, Figure 5.19B). Nonetheless, cell surface Ca\textsubscript{V}2.2\_HA expression decreased by 20% after neurons were incubated with CAP for 240 s (normalised values were CAP 240 s: 0.79 ± 0.04 and KRH 0 s: 0.99 ± 0.05, Figure 5.19B). These data indicate that similar to small-sized DRG neurons, a concomitant decrease in cell surface Ca\textsubscript{V}2.2\_HA of medium-sized neurons was observed when neurons were incubated with CAP for longer than 20 s.
Figure 5.19: Capsaicin modulates cell surface Ca\textsubscript{v}2.2_HA expression in medium-sized TRPV1-positive neurons

(A) Images of medium-sized DRG neurons cultured from Ca\textsubscript{v}2.2_HA\textsuperscript{KIKI} mice, showing (from left to right) cell surface Ca\textsubscript{v}2.2_HA (red), TRPV1 (green) and merge representative image. DRG neurons incubated with capsaicin (CAP) for (from top to bottom) 0, 20, 120 and 240 s.

(B) Normalised cell surface Ca\textsubscript{v}2.2_HA intensity (red circles) of medium-sized DRG neurons incubated with KRH for 0 s and CAP for 0, 20, 120 and 240 s. For normalisation, the fluorescence intensity of each neuron was normalised against the mean fluorescence of medium-sized control DRG neurons (KRH, 0 s). Individual data points represent normalised Ca\textsubscript{v}2.2_HA intensity measured from all TRPV1-positive neurons from three separate experiments. Total of 115, 139, 174 and 122 medium-sized neurons incubated with CAP for 0, 20, 120 and 240 s, respectively. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one-way ANOVA with Bonferroni’s selected comparison test as post hoc test; ***p<0.001, ns = not significant, F value = 12.81

Astonishingly, in large-sized DRG neurons an increase in Ca\textsubscript{v}2.2_HA expression was observed, the longer neurons were treated with CAP (Figure 5.20). Relative to the control condition, cell surface Ca\textsubscript{v}2.2_HA increased by 30% and 73% after incubation with CAP for 120 and 240 s, respectively (normalised values were KRH 0 s: 0.99 ± 0.11, CAP 120 s: 1.29 ± 0.09, CAP 240 s: 1.71 ± 0.14, Figure 5.20B). These data suggest that in large-sized TRPV1 DRG neurons, application of CAP results in an increase in cell surface Ca\textsubscript{v}2.2_HA.
Figure 5.20: Capsaicin modulates cell surface Ca\textsubscript{v}2.2_HA expression in large-sized TRPV1-positive neurons

(A) Images of large-sized DRG neurons cultured from Ca\textsubscript{v}2.2_HA\textsuperscript{KIKI} mice, showing (from left to right) cell surface Ca\textsubscript{v}2.2_HA (red), TRPV1 (green) and merge representative image. DRG neurons incubated with capsaicin (CAP) for (from top to bottom) 0, 20, 120 and 240 seconds. (B) Normalised cell surface Ca\textsubscript{v}2.2_HA intensity (red circles) of large-sized DRG neurons incubated with KRH for 0 s and CAP for 0, 20, 120 and 240 s. For normalisation, the fluorescence intensity of each neuron was normalised against the mean fluorescence of large-sized control DRG neurons (KRH, 0 s). Individual data points represent normalised Ca\textsubscript{v}2.2_HA intensity measured from all TRPV1-positive neurons from three separate experiments. Total of 40, 59, 57 and 51 large-sized neurons incubated with capsaicin (CAP) for 0, 20, 120 and 240 s, respectively. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; ***p<0.001, ns = not significant, F value 8.504

5.11.2 Cell surface Ca\textsubscript{v}2.2_HA expression in TRPV1-negative neurons following capsaicin application

Next, cell surface Ca\textsubscript{v}2.2_HA expression was examined in TRPV1-negative neurons following incubation with CAP for 0, 20, 120 and 240 s. Surprisingly, in small-sized DRG neurons an overall decrease can once again be seen in cell surface Ca\textsubscript{v}2.2_HA staining. Relative to the control condition, plasma membrane Ca\textsubscript{v}2.2_HA immunoreactivity reduced by 30% and 48% in small-sized neurons incubated with CAP for 120 and 240 s, respectively (normalised values
were KRH 0 s: 1.01 ± 0.11, CAP 120 s: 0.71 ± 0.09, CAP 240 s: 0.52 ± 0.07, Figure 5.21B). A similar reduction was also detected in medium-sized DRG neurons. A loss of 37% and 59% in cell surface Ca\textsubscript{V\textsubscript{2.2_HA}} was found in medium-diameter neurons treated for 120 and 240 s with CAP, respectively, relative to control conditions (normalised values were KRH 0 s: 0.98 ± 0.09, CAP 120 s: 0.61 ± 0.05, CAP 240 s: 0.40 ± 0.06, Figure 5.22B). Nevertheless, unlike TRPV1-positive neurons, within the large-sized TRPV1-negative DRG neurons no change in cell surface Ca\textsubscript{V\textsubscript{2.2_HA}} was observed (Figure 5.23). These surprising results may relate to the difficulty in determining whether neurons are definitely TRPV1-negative or may relate to indirect effects of mediators release by CAP from neighbouring TRPV1-positive neurons.

![Figure 5.21: Capsaicin modulates cell surface Ca\textsubscript{V\textsubscript{2.2_HA}} expression in small-sized TRPV1-negative neurons](image)

(A) Images of small-sized DRG neurons cultured from Ca\textsubscript{V\textsubscript{2.2_HA}}\textsuperscript{K\textsubscript{K}} mice, showing (from left to right) cell surface Ca\textsubscript{V\textsubscript{2.2_HA}} (red), TRPV1 (green) and merge representative image. DRG neurons incubated with capsaicin (CAP) for (from top to bottom) 0, 20, 120 and 240 s. (B) Normalised cell surface Ca\textsubscript{V\textsubscript{2.2_HA}} intensity (red circles) of small-sized DRG neurons incubated with KRH for 0 s and CAP for 0, 20, 120 and 240 s. For normalisation, the fluorescence intensity of each neuron was normalised against the mean fluorescence of small-sized control DRG neurons (KRH, 0 s). Individual data points represent normalised Ca\textsubscript{V\textsubscript{2.2_HA}} intensity measured from all TRPV1-negative neurons from three separate experiments. Total of 4, 21, 20 and 23 small-sized neurons incubated with CAP for 0, 20, 120 and 240 s, respectively. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; **p<0.01, F value = 3.549.
Figure 5.22: Capsaicin modulates cell surface $\text{Ca}_\text{V}2.2\text{HA}$ expression in medium-sized TRPV1-negative neurons

(A) Images of medium-sized DRG neurons cultured from $\text{Ca}_\text{V}2.2\text{HA}^{\text{KlKl}}$ mice, showing (from left to right) cell surface $\text{Ca}_\text{V}2.2\text{HA}$ (red), TRPV1 (green) and merge representative image. DRG neurons incubated with capsaicin (CAP) for (from top to bottom) 0, 20, 120 and 240 s.

(B) Normalised cell surface $\text{Ca}_\text{V}2.2\text{HA}$ intensity (red circles) of medium-sized DRG neurons incubated with KRH for 0 s and CAP for 0, 20, 120 and 240 s. For normalisation, the fluorescence intensity of each neuron was normalised against the mean fluorescence of medium-sized control DRG neurons (KRH, 0 s). Individual data points represent normalised $\text{Ca}_\text{V}2.2\text{HA}$ intensity measured from all TRPV1-negative neurons from three separate experiments. Total of 41, 53, 59 and 36 medium-sized neurons incubated with CAP for 0, 20, 120 and 240 s, respectively. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; **$p<0.001$, F value = 6.380.
Figure 5.23: Capsaicin modulates cell surface Cav2.2_HA expression in large-sized TRPV1-negative neurons

(A) Images of large-sized DRG neurons cultured from Cav2.2_HA^{KIKI} mice, showing (from left to right) cell surface Cav2.2_HA (red), TRPV1 (green) and merge representative image. DRG neurons incubated with capsaicin (CAP) for (from top to bottom) 0, 20, 120 and 240 s. (B) Normalised cell surface Cav2.2_HA intensity (red circles) of large-sized DRG neurons incubated with KRH for 0 s and CAP for 0, 20, 120 and 240 s. For normalisation, the fluorescence intensity of each neuron was normalised against the mean fluorescence of large-sized control DRG neurons (KRH, 0 s). Individual data points represent normalised Cav2.2_HA intensity measured from all TRPV1-negative neurons from three separate experiments. Total of 26, 42, 38 and 19 medium-sized neurons incubated with CAP for 0, 20, 120 and 240 s, respectively. Mean ± SEM of the total number of DRG neurons is superimposed. Statistical analysis was as follows: one–way ANOVA with Bonferroni’s selected comparison test as post hoc test; ns = not significant, F value = 1.625.
5.12 Summary and Discussion

In this study, CaV2.2_HA expression was studied in co-cultures of DRG and spinal cord neurons. To achieve this, healthy cultures were first established using P0/P1 CaV2.2<sup>WTWT</sup> mice. Here, DRG neurons could be distinguished based on cell body size and pre- and postsynaptic sites were easily detected by vGluT2 and Homer, respectively (Figure 5.1). To study CaV2.2_HA expression at the presynaptic site, DRG neurons cultured from CaV2.2_HA<sup>KIKI</sup> mice were plated with spinal cord neurons from CaV2.2<sup>WTWT</sup> mice. Ca<sup>2+</sup> signalling within these cultures showed no significant differences compared to co-cultures from exclusively CaV2.2_HA<sup>KIKI</sup> and CaV2.2<sup>WTWT</sup> mice (Figure 5.2). CaV2.2_HA expression was next examined, with respect to cell size, in cell bodies of DRG neurons from co-cultures fixed between DIV 1 to 28. A reduction in CaV2.2_HA immunoreactivity at the cell body of small- and medium-sized neurons relative to DIV 1 was observed (Figure 5.3 and 5.4). Further investigation of CaV2.2 transcript levels in DRG neurons revealed a 44% reduction in CaV2.2 relative fold expression of 11-week-old mice compared to P0/P1 mice (Figure 5.6). Cell surface CaV2.2_HA was next distinguished from total CaV2.2 expression using the anti-HA and II-III loop antibodies, respectively. The anti II-III loop antibody was unable to detect endogenous cell surface CaV2.2 (Figure 5.7). Nevertheless, a similar distribution of CaV2.2 expression among different size groups was found when using either the anti-HA or II-III loop antibodies (Figure 5.8). Following this, CaV2.2_HA immunolabelling was studied at the presynaptic site of co-cultures from DIV 7 to DIV 28. This study found that, in immature cultures, of the total Homer-positive puncta only 43% ± 2% were positive for CaV2.2_HA and vGluT2 (Figure 5.10). However, in mature cultures (DIV 22 and 28) a greater proportion (78%) of Homer puncta was found in association with CaV2.2_HA and vGluT2 (Figure 5.12 and 5.13). Further analysis of CaV2.2_HA, vGluT2 and Homer immunolabelling revealed a concomitant increase of immunoreactivity of all three markers from DIV 7 to DIV 28 (Figure 5.14). These data suggest
that 

The impact of CAP on cell surface 

The present study found that cell surface labelling of 

Nevertheless, after 120 or 240 s of CAP application, a significant decrease of expression at the plasma membrane was found in small- and medium-sized TRPV1-positive DRG neurons (Figure 5.18 and 5.19). In contrast, in large-sized DRG TRPV1-positive neurons a striking increase in cell surface CaV2.2_HA was detected (Figure 5.20). Surprisingly, in TRPV1-negative neurons a decrease in cell surface CaV2.2_HA can also be detected in small- and medium-sized neurons (Figure 5.21 and 5.22). These results suggest that co-cultures can be used to successfully examine presynaptic CaV2.2_HA. Additionally, CAP may regulate CaV2.2_HA expression. However, it is yet unclear as to whether this is dependent or independently mediated through TRPV1.

5.12.1 Developmental regulation of CaV2.2_HA expression in DRG neuronal cell bodies of co-cultures

Co-culture preparations of dissociated DRG and spinal cord neurons have been used as a model system to characterise the functional properties of the first sensory synapse (Medvedeva et al., 2008; Hendrich et al., 2012). The present study demonstrates the use of this in vitro model to study CaV2.2_HA at the cell body and presynaptic sites. This work shows the differentiation of CaV2.2_HA in co-cultures over 28 days after plating and synapse formation in a time-dependent manner.

The present study shows a significant decrease in cell surface expression of CaV2.2_HA at
the cell body of small- and medium-sized DRG neurons in co-cultures over time (Figure 5.3 and 5.4). In contrast a significant increase of Cav2.2_HA expression was observed at the DRG presynaptic terminals within co-cultures (Figure 5.10-5.13). It is postulated that neurons mainly introduce new proteins to the axon terminal through transporting them from the cell body. After protein synthesis in the neuronal soma, several ways have been proposed to mediate the delivery of membrane proteins to the axons. These include direct transport after modification at the Golgi apparatus, non-polarised delivery to somatic membranes followed by transcytosis and transport in axons (Horton and Ehlers, 2003; Winckler and Mellman, 2010). Transcytosis is where newly synthesised membrane proteins are inserted into the somatic compartment followed by endocytosis and anterogradely transported to axonal terminals. It has been shown that trkA receptors are transported to axons from the soma by transcytosis during development of sympathetic neurons (Ascaño et al., 2009). The decrease in Cav2.2_HA at the plasma membrane may be due to transcytosis of the channel in developing neurons. To further study transcytosis of Cav2.2_HA, long-term time-lapse microscopy could be used to monitor any dynamic movement in DRG neuronal axons.

Another method of synapse formation is through the local synthesis of proteins at axonal sites (Kim and Jung, 2015). To screen for axonally synthesised proteins, future studies would use microfluidic chambers (MFCs) to physically separate DRG cell bodies from their axons. DRG neurons would be plated in the proximal chamber and over a sufficient period of time, axons would grow through microgrooves into the distal chamber. MFCs would allow for the isolation of axonal mRNA and provide evidence for the presence of presynaptic mRNAs encoding presynaptic proteins as shown by Taylor et al. (2005).

To determine whether Cav2.2 is trafficked to the presynaptic terminal or synthesised locally, future studies could employ the use of phototransformable fluorescent proteins (PtFPs). PtFPs can change their fluorescence excitation and emission spectra after irradiation by a unique
wavelength of light. The use of PtFPs fused to Ca\textsubscript{V}2.2 would allow for the study of the trafficking of newly synthesised Ca\textsubscript{V}2.2 proteins *en route* to their destination.

In the case of vGluT2, a significant increase in total vGluT2 in small and medium-sized DRG neurons was observed over time in culture (Figure 5.3 and 5.4). As glutamate is the most abundant excitatory neurotransmitter in DRG neurons, the machinery involved in glutamate production is present in DRG neurons (Miller et al., 2011). Once glutamate is synthesised it is incorporated into synaptic vesicles via vGluTs. In adult mice DRG neurons, vGluT2 is predominantly expressed in the cell body of peptidergic and non-peptidergic nociceptors (Oliveira et al., 2003; Brumovsky et al., 2007). It has been suggested that vGluT2 may be involved in somatic glutamate release which may be involved in intra-ganglionic cross excitation (Amir and Devor, 1999). The increasing vGluT2 expression observed in the present study is in agreement with previous studies showing abundant expression of vGluT2 in DRG neurons (Alvarez et al., 2004; Brumovsky et al., 2007). Synthesised vGluT2 in DRG neurons undergoes central axonal transport, as shown by the abundance of vGluT2-positive primary afferents in the dorsal horn of the spinal cord (Todd et al., 2003; Li et al., 2003). These data agrees with the data shown in Figure 5.14 where similarly at the synaptic terminal a significant increase in vGluT2 immunoreactivity was detected over time in culture.

While the co-culture system offers many advantages in examining cell bodies and synapses, there are several limitations worth noting. Although there is a decrease of Ca\textsubscript{V}2.2_HA in DRG cell bodies, it is possible that non-neuronal peripheral components are different *in vivo* and may be lacking in dissociated neuronal cultures. The 3D organisation, architecture and signalling milieu have inherent limitations of artificial growth and are potentially lost *in vitro*. Additionally, the temporal regulation of developmental programmes may be altered in comparison to *in vivo* systems. This underscores the importance of corroborating results from cultured neurons with acute preparations of DRG and spinal cord tissue of Ca\textsubscript{V}2.2_HA\textsuperscript{KIKI}
mice at different ages.

5.12.2 Developmental regulation of CaV2.2_HA expression at the first sensory synapse

In this study, triple labelling was used to identify synapses that included presynaptic DRG terminals and postsynaptic dorsal horn dendrites. As vGluT2 is also expressed by dorsal horn neurons (Alvarez et al., 2004), synapses first were characterised by both the expression of CaV2.2_HA and vGluT2 apposed to Homer. For quantification, CaV2.2_HA/vGluT2 and Homer clusters were counted, and Figures 5.10 to 5.13 shows that the number of synaptic boutons increased with time in culture starting at DIV 7 to 28. Additionally, immunoreactivity for CaV2.2_HA assumed a punctate profile in DIV 22 cultures and many of these puncta were preferentially localised in apposition to Homer (Figure 5.12). This pattern of increased synapse formation agrees with studies using various neuronal cultures (Scheiffele, 2003; McAllister, 2007).

In future studies, anti-Tau and MAP2 antibodies could be used to identify DRG axons and spinal cord dendrites, respectively. The additional use of these markers would help characterise presynaptic CaV2.2_HA on the axons of DRG neurons which contacted dendrites of dorsal horn neurons. Furthermore, to determine whether the synaptic boutons are functional, conventional whole cell patch recording of dorsal horn neurons could be performed. The frequency of mEPSCs would reveal whether the increase of CaV2.2_HA puncta corresponds to the formation of functional synapses. Furthermore, live-cell imaging, using the optical reporter vGluT-phluorin (vGpH), could be used to confirm whether the co-cultures have functional synapses. pHluorins are pH-sensitive green fluorescent protein-based sensors and can be fused to presynaptic markers such as vGluT. Synaptic vesicles maintain an acidic lumen with a pH $\sim$ 5.6 at rest. Following an action potential, the vesicular membrane and plasma
membrane of the presynaptic terminal fuse which rapidly raises the pH in the proximity of vGpH to \( \sim 7.6 \). vGpH can detect changes in pH resulting in a change of fluorescence which reflect synaptic vesicle fusion and transmitter release. In future experiments, DRG neurons of co-cultures could be transfected with vGpH and upon stimulation, an increase in vGpH fluorescence would be measured. Additionally, the use of the specific \( \text{Ca}_{\text{v}}\text{V}_{2.2} \) channel blocker (\( \omega \)-conotoxin GVIA) in future electrophysiological and live-cell imaging experiments would determine the proportion of functional synapses attributed to \( \text{Ca}_{\text{v}}\text{V}_{2.2} \).

In the present study, \( \text{Ca}_{\text{v}}2.2\text{HA} \) immunoreactivity in synaptic boutons was measured by analysing the intensity of \( \text{Ca}_{\text{v}}2.2\text{HA} \) immunolabelling in a 2 \( \mu \text{m} \) ROI. However, there are some limitations to this simple form of analysis. Firstly, this methodology requires the experimenter to place ROIs on what they determine to be Homer-positive puncta. Whilst this approach may be accurate, it is subject to human error and bias. There are several commercial software programmes available which uses multiple thresholding, segmentation approaches and proximity to dendrites for synaptic analysis (Ippolito and Eroglu, 2010; Schätzle et al., 2012). However, endogenous \( \text{Ca}_{\text{v}}2.2\text{HA} \) puncta expression throughout development has yet to be characterised. As such, \( \text{Ca}_{\text{v}}2.2\text{HA} \) puncta intensity may occupy a broad grayscale range over development, therefore, simple thresholds would alter its size, shape and intensity. Future studies would therefore require finding optimal thresholds for the present data.

Furthermore, a previous study has used Imaris software to reconstruct dendrites in 3D to study synapse formation and maturation following the loss of \( \alpha_2\delta-1 \) (Risher et al., 2018). For the present data, analysis would ideally be performed using z-stacks of synapses which would allow for 3D reconstruction of \( \text{Ca}_{\text{v}}2.2\text{HA} \)-positive synapses. The results from the present study show that, with improvement to synaptic analysis methods, the co-culture system represents a model suitable for immunocytochemical studies to explore the role of \( \text{Ca}_{\text{v}}2.2\text{HA} \) at the presynaptic site.
5.12.3 TRPV1 and Ca\textsubscript{v}2.2_HA expression

The results show that upon DRG neuron incubation with capsaicin for 20, 120 and 240 s, there are two different changes in cell surface Ca\textsubscript{v}2.2_HA expression. The first is a significant increase in cell surface Ca\textsubscript{v}2.2_HA in response to incubation with capsaicin for 20 s in small and medium TRPV1-positive DRG neurons (Figure 5.18 and 5.19).

These results suggest that Ca\textsubscript{v}2.2_HA channels may potentially be docked below the membrane of the DRG neuron so that they are primed and ready for membrane insertion upon stimulation. The cell bodies of DRG neurons generally are good surrogates for functional protein expression in axons and nerve endings based on consistency of responses to selective pharmacological agents obtained in cell bodies, nerve fibres, nerve endings, and behavioural studies in vivo with wild-type and knockout mice (Caterina et al., 2000). At the presynaptic membrane, RIM and RIM-BPs is required for tethering Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 channels to the active zone (Kaeser et al., 2011; Kiyonaka et al., 2007). Future experiments are required to shed light on the distribution of Ca\textsubscript{v}2.2_HA within the DRG neuron.

Extensive research has shed light on the trafficking of functional aquaporin-8 water channels in isolated rat hepatocytes. Reports have shown that these channels are located primarily in intracellular vesicles and its relocalisation to the plasma membranes can be induced using cAMP (Gradilone et al., 2003). Gradilone and colleagues found that after hepatocytes were incubated in glucagon there was a significant increase in osmotic water transport within 10 s compared to DMSO controls. Therefore, the fast increase in cell surface Ca\textsubscript{v}2.2_HA expression observed after 20 s incubation with capsaicin may occur through similar mechanisms regulating aquaporin-8 channels.

Furthermore, the fast insertion of channel proteins into the membrane of adipocytes has also been reported for the glucose transporter 4 (GLUT4) (Tengholm and Meyer, 2002). These authors found that GLUT4 resided in perinuclear membrane as well as in vesicles near the cell periphery and in the cytoplasm. Stimulation with insulin resulted in a decrease in vesic-
ular GLUT4 distribution and an increase (within seconds) in plasma membrane fluorescence intensity. It was suggested that the application of insulin stimulated the PI3K pathway which induced GLUT4 insertion. Such mechanisms may also explain the fast insertion (20 s) of CaV2.2_HA in the cell surface of DRG neurons.

The present study shows for the first time that a significant decline in cell surface CaV2.2_HA can be detected as a response to incubation with capsaicin for 120 and 240 s in small- and medium-sized TRPV1-positive neurons (Figure 5.18 and 5.19). Capsaicin binds to the TRPV1 receptor in nociceptive neurons. Receptor occupancy triggers Ca\textsuperscript{2+} influx. The entry of Ca\textsuperscript{2+} initiates a cascade of events within the neurons. Findings from previous studies revealed that stimulation of TRPV1 by capsaicin down-regulated N-type calcium channel currents in rat DRG neurons (Wu et al., 2005; Hagenacker et al., 2005). Wu et al. (2005) found that this was by dephosphorylation of CaV2.2 through Ca\textsuperscript{2+} dependent activation of calcineurin in rat DRG neurons (Figure 5.24). The role of calcineurin in Ca\textsuperscript{2+}-dependent regulation of Ca\textsuperscript{2+} influx has previously been examined in NG108-15 cells (Burley and Sihra, 2000). These authors demonstrated a decrease in high-voltage activated (HVA) current when calcineurin was overexpressed which was reversed by intracellular FK506 (calcineurin inhibitor), attesting to calcineurin dependency. Furthermore, the addition of intracellular BAPTA highlights that this is in fact a Ca\textsuperscript{2+}-dependent mechanism. The importance of calcineurin in shaping cellular responses to Ca\textsuperscript{2+} entry is reinforced by the overexpression of calcineurin mediating a large Ca\textsuperscript{2+} entry (Burley and Sihra, 2000).
Figure 5.24: Schematic diagram of the potential mechanism behind TRPV1-dependent regulation of CaV2.2

Schematic diagram showing Ca\textsuperscript{2+} entry through TRPV1. (A) Influx of Ca\textsuperscript{2+} causes the Ca\textsuperscript{2+}-dependent activation of calcineurin which de-phosphorylates cell surface CaV2.2 mediating its down-regulation from the plasma membrane. (B) Influx of Ca\textsuperscript{2+} causes the Ca\textsuperscript{2+}-dependent activation of calcineurin which de-phosphorylates cytoskeletal proteins. This may cause the down-regulation of CaV2.2 from the plasma membrane. The red arrow and question marks on A and B indicate other mechanisms which may regulate the down-regulation of CaV2.2.

Several studies investigating the release of neurotransmitter from central nervous systems-derived nerve terminals have implicated calcineurin as a potential modulator by acting on many putative target sites (Sihra et al., 1995; Kim and Ryan, 2013). It was initially demonstrated that CaV2.2 could be phosphorylated by the cyclin-dependent kinase 5 (CDK5) in mice hippocampal cultures which significantly increase Ca\textsuperscript{2+}-current density and channel open probability. Additionally, expression of CaV2.2 in which these phosphorylation sites are mutated reduces synaptic performance compared to the wildtype channel (Su et al., 2012). It has been shown that CDK5 and the Ca\textsuperscript{2+}-dependent phosphatase calcineurin regulate the size of the synaptic vesicle pool involved in neurotransmission (Kim and Ryan, 2010). Therefore, the release probability may be regulated by the integration of the kinase and phosphatase activi-
ties on VGCCs. Although in direct contradiction to results from Su et al. (2012), Kim and Ryan (2013) show that a removal of calcineurin activity reduces AP-induced Ca\textsuperscript{2+} influx whereas inhibiting CDK5 activity results in a large potentiation of Ca\textsuperscript{2+} influx. The most parsimonious reason for the discrepancies observed in these two studies may be that although CDK5 can phosphorylate Ca\textsubscript{v}2.2, this may be overridden by other factors controlled by CDK5 and calcineurin that regulate Ca\textsuperscript{2+} influx. The work by Su et al. (2012) identified 8 serine residues in Ca\textsubscript{v}2.2 to be essential for synaptic function through mutation studies. Nevertheless, it is important to note that these mutants may not have trafficked correctly resulting in a loss of synaptic function. Nevertheless, the data reported by (Kim and Ryan, 2013) suggest that the tight regulation of these enzymes has an impact on action potential-driven exocytosis of neurotransmitters mediated through Ca\textsubscript{v}2.2.

There is also evidence to indicate that cytoskeleton proteins such as microtubules and microfilaments play a crucial role in the compartmentalisation, anchoring and regulation of receptors and ion channels (Fukuda et al., 1981). Johnson and Byerly (1994) suggest that the microtubules modulates VGCCs in rat hippocampal pyramidal neurons. In addition to the direct dephosphorylation of VGCCs postulated by Wu et al. (2005), it is also possible that the phosphorylation and dephosphorylation of cytoskeleton proteins may indirectly affect VGCC activity (Unno et al., 1999).

Surprisingly, an increase in plasma membrane Ca\textsubscript{v}2.2_HA was observed in large-sized DRG neurons following TRPV1 activation (Figure 5.20). Yu et al. (2008) found that, upon intraplantar injection of complete Freund’s adjuvant (CFA), there is an increase of TRPV1 expression in large-sized DRG neurons. The shift in TRPV1 expression to larger neurons has also been reported in diabetic neuropathy and bone cancer pain animal models (Amaya et al., 2003; Hong and Wiley, 2005; Niiyama et al., 2007). Although it is difficult to make direct comparisons between in vivo and in vitro data, these studies indicate that nerve injury may cause changes
in cell surface protein expression in different size DRG neurons. However, further studies are required to understand the mechanisms behind capsaicin-induced increase in cell surface Ca\textsubscript{V}2.2_HA expression in large-sized DRG neurons.

Surprisingly, a decrease in cell surface Ca\textsubscript{V}2.2_HA can also be detected in TRPV1-negative neurons. It has been found that the suppression of N-type calcium currents following capsaicin application was restricted to the TRPV1-positive DRG subpopulation (Wu et al., 2005). These data suggest that within the present study the identification of TRPV1-negative neurons must be stringently re-assessed. Wu et al. (2005) used the highly specific TRPV1 antagonist, iodoresiniferatoxin, which eliminated the effect of capsaicin-induced inhibition of VGCC currents. These authors also use IB4 to distinguish TRPV1-positive neurons as TRPV1 is predominantly expressed in IB4-positive rat DRG neurons (Guo et al., 1999). As such, future studies would require a TRPV1 intensity thresholds to be established and IB4 to help demarcate TRPV1-positive and -negative neurons.

On the other hand, there is contrasting evidence suggesting that there are also TRPV1-independent pharmacological effects of capsaicin. A previous study postulated that capsaicin causes the release of substance P from sensory neurons which acts on NK1 receptors on non-neuronal cells. The activation of neurokinin (NK1) receptors results in an increase in NGF production. NGF then acts on trkA receptors activating the intracellular ERK pathway. In the rat sciatic nerve and DRG, the NK1 receptor antagonist, SR140333, was found to inhibit capsaicin-mediated ERK phosphorylation (Donnerer and Liebmann, 2006). Previous studies have shown the importance of the presence of putative ERK consensus phosphorylation sites within the intracellular loop between domain I and II of Ca\textsubscript{V}2.2. These sites are essential for ERK-dependent modulation of N-type calcium channels. Mutations of the phosphorylation sites results in a decrease in Ca\textsubscript{V}2.2 current (Martin et al., 2006). However, further experiments are required to investigate the potential TRPV1-independent mechanisms (outlined in
Figure 5.25: Schematic diagram of the potential mechanism behind TRPV1-independent regulation of Ca$_{V2.2}$

Schematic diagram showing Ca$^{2+}$ entry through TRPV1. Ca$^{2+}$ influx causes release of substance P which activates NK1 receptors on non-neuronal cells. This causes an up-regulation of NGF which is secreted and acts on its trkA receptors. This activates the ERK pathway which modulates the cell surface expression of Ca$_{V2.2}$.

However, cultured DRG neurons from TRPV1 knockout mice exhibit a deficit in Ca$^{2+}$ influx following treatment with capsaicin (Caterina et al., 2000). Considering the contradictory nature of this evidence, future studies are required to help determine the pathway by which N-type calcium channel expression is modulated by capsaicin.

At the point of synaptic transmission between DRG and dorsal horn (DH), capsaicin has been shown to increase EPSC frequency (Medvedeva et al., 2008; Hendrich et al., 2012). Therefore, there are at least two routes for Ca$^{2+}$ entry which mediate neurotransmission either through TRPV1 or VGCCs. Heinke et al. (2004) found that the presynaptic N-type calcium channels are responsible for evoked EPSCs in lamina I of the rat dorsal horn. However, Medvedeva et al. (2008), using DRG-DH co-cultures, show that Ca$_{V2.2}$ contributes very little to the presynaptic Ca$^{2+}$ influx and glutamate release following the application of capsaicin. These data agree with earlier findings demonstrating that capsaicin-induced Ca$^{2+}$ influx results in glutamatergic synaptic transmission in the brain independent of VGCCs (Marinelli et al., 2002; Jin et al., 2004). It was postulated that this was due to calcineurin-dependent inhibition of the N-type calcium channel (Wu et al., 2005).
On the other hand, the gabapentinoid class of drugs has proven effective in the treatment of pain, as their effects are mediated through $\alpha_2\delta-1$ (Brown et al., 1998; Field et al., 2006). The accessory subunit, $\alpha_2\delta-1$, has been shown to promote the trafficking of $\text{Ca}_V2.2$ (Canti et al., 2005; Tran-Van-Minh and Dolphin, 2010; Cassidy et al., 2014) which is reduced with the chronic application of gabapentin (Hendrich et al., 2008). Indeed, chronic pregabalin treatment was found to attenuate the capsaicin-induced increase in EPSC frequency in DH neurons (Hendrich et al., 2012). It is possible that chronic pregabalin reduces neurotransmitter release, as such decreases capsaicin-induced transmitter release, bypassing VGCCs. Nevertheless, further studies are required to delineate the mechanisms contributing to capsaicin-induced changes in $\text{Ca}_V2.2$ channel expression.

DRG neurons from rat embryonic co-cultures have been previously characterised as nociceptor-like as they express TRPV1, trkA, CGRP and substance P (Joseph et al., 2010). However, it is important to note the inconsistencies of immunoreactive profiles of these markers from intact adult rat ganglia (Molliver et al., 1995; Averill et al., 1995). These results suggest that there may be differential expression of nociceptive markers within DRG neurons cultured from either embryonic or postnatal animals (Goldstein et al., 1996). As such it is important to consider and characterise phenotypic maturation of DRG neurons before examining capsaicin on presynaptic $\text{Ca}_V2.2\_HA$ in co-cultures. Furthermore, as shown in Chapter 4, culture conditions can easily be manipulated to enhance the survival of nociceptive-like DRG neurons. As such the tight regulation of culture conditions must be considered for future experiments.

In the present study a significant decline in cell surface $\text{Ca}_V2.2\_HA$ can be detected as a response to long (120-240 s) incubation with capsaicin. It is possible that when studying capsaicin on co-cultures, these extracellular cues will impinge on axon terminals and influence the long-range anterograde transport of $\text{Ca}_V2.2\_HA$ from neuronal cell bodies to axon termi-
nals. As such further studies are required to explore the role of capsaicin on Ca\textsubscript{v}2.2_HA at the presynaptic site of DRG-spinal cord co-cultures.
Chapter 6

General Discussion and Future Work

6.1 Characterisation of the CaV2.2_HA^KIKI mouse

CaV2.2 plays an essential role in mediating neurotransmitter release in many neurons. Despite the growing interest in CaV2.2 and its modulators as therapeutic targets, the study of cell surface CaV2.2 expression has been impeded by a lack of molecular tools available. The N-type calcium channel can only function at the cell surface, therefore, the mechanisms behind its membrane targeting are critical in understanding its role in neurotransmission and neuropathic pain.

Electrophysiology is one of the most commonly used techniques when examining surface expression of functional VGCCs in neurons. A large body of work has used pharmacological agents in combination with patch clamping to provide the current understanding of function and regulation of VGCCs. However, it is important to note that this approach is limited by many factors. Firstly, the patch clamping of neurons is generally restricted to the soma, particularly for calcium channels. As such, this limits the use of patch clamping in unravelling the role of CaV2.2 at the presynaptic terminal, except in large accessible terminals. Secondly, this technique does not provide detail about the spatial resolution and homogeneity of distribution of the channels at the cell surface. Finally, due to the presence of several types of VGCCs
in neurons, sequential pharmacological block is required to dissect which VGCC is mediating Ca\(^{2+}\) influx, however, this analysis is limited because Ca\(^{2+}\) from multiple channels acts cooperatively to produce release at most synapses. Furthermore, the blockade of several types of calcium channels results in the production of an unnatural state in which cells are being observed. This augments the likelihood of encountering artefacts when demarcating the differential expression of VGCCs. In this regard, the cell surface HA-tagged Ca\(_{\text{V}}\)\(2.2\) represents an essential tool in shedding light on the spatial and dynamic expression of Ca\(_{\text{V}}\)\(2.2\) at the plasma membrane.

Artificial peptide sequences specifically recognised by antibodies have been inserted into extracellular positions of VGCCs. Extracellular accessible epitope tags enable the quantification of cell surface channel labelling (Dubel et al., 2004), to study membrane turnover (Altier et al., 2006) and to distinguish cell surface expression from intracellular pools (Obermair et al., 2004). However, tag insertions have disturbed channel expression in neurons (Watschinger et al., 2008) and either reduced functionality (Di Biase et al., 2011) or shown complete loss of function (Altier et al., 2006)-personal communication. This makes experimental results obtained with these constructs difficult to interpret. It is important to note that Ca\(^{2+}\) influx through VGCCs plays many roles within the cell and Ca\(^{2+}\) disruption of homeostasis could lead to complications. This highlights the importance of functionality in the tagged calcium channel, to ensure that the physiological role of the channel is unperturbed.

In Chapter 3 of this thesis, the characterisation of the functionally exofacially HA-tagged Ca\(_{\text{V}}\)\(2.2\) knock-in mouse is described. The tandem HA-tag was inserted into the second extracellular loop of domain II. As expected, the insertion of the tandem HA-tag did not perturb endogenous VGCC function and it could be efficiently detected at the cell surface of DRG neurons. The results were consistent with that demonstrated by Cassidy et al. (2014), where the HA–tag did not disrupt the biophysical properties of expressed Ca\(_{\text{V}}\)\(2.2\). Current approaches
in studying CaV2.2 localisation often utilise the overexpression of this channel in neurons. However, there are many caveats in interpreting data from these studies. One caveat is that overexpression systems often result in aberrant protein trafficking and localisation. Nevertheless, these studies show that both exogenous and endogenous CaV2.2_HA channels are well expressed at the cell surface of DRG neurons which confirms the utility of this molecular tool in experiments focused on the role of CaV2.2 at the presynaptic terminal. These results confirm that the CaV2.2_HAKiki mice can be used to study the surface expression of CaV2.2.

6.2 CaV2.2_HA Kiki expression in DRG neurons and spinal cord tissue

Immunohistochemical localisation studies are an important prerequisite to revealing the expression of CaV2.2_HA in various tissue types. In Chapter 4, the HA-tagged CaV2.2 channel permitted the direct investigation of cell surface CaV2.2 expression in different DRG neuronal subpopulations. These findings confirmed that CaV2.2_HA is strongly expressed at the cell surface of CGRP-positive neurons. It is highly likely that even short-termed cultured DRG neurons do not fully represent the in vivo situation, and that rapid changes occur in cell-surface expression of channels when cells are enzymatically dissociated and maintained in culture, allowing neurite outgrowth (Emery et al., 2016). Since evoked synaptic currents in laminae I and II are 74% N-type (Bao et al., 1998), there is likely to be a differential synaptic localisation of these channels in vivo. However, the cell surface expression of CaV2.2_HA could also be recapitulated in DRG neurons in vivo (Nieto-Rostro et al., 2018). Cell surface CaV2.2_HA could also be seen to a lesser extent IB4-positive small DRG neurons in vitro. In contrast, a previous study used transcriptional profiling of DRG neurons and found similar levels of Cacna1b mRNA in IB4-positive and -negative neurons, the latter including CGRP-positive DRG neurons (Chiu et al., 2014). This would agree with in vivo data from Nieto-Rostro et al. (2018) which showed high intracellular levels of CaV2.2_HA in both CGRP-positive and -negative
DRG neurons.

The results from Chapter 4 also highlight the importance of the strict regulation of neuronal culture conditions when attempting to recapitulate in vivo environmental settings. The addition of NGF and GDNF in DRG cultures promotes the expression of peptidergic and non-peptidergic neurons, respectively (Ernsberger, 2008, 2009). In the presence of GDNF alone, cell surface Cav2.2_HA was higher in small- and medium-sized DRG neurons compared to neuronal cultures only including NGF. As GDNF supports the survival of IB4-binding neurons (Kotzbauer et al., 1996; Matheson et al., 1997), unlike NGF, an increase in cell surface Cav2.2_HA level was observed. The current study also shows that when NGF and GDNF are both included in the culture media as opposed to either factor alone, there was an increase in cell surface Cav2.2_HA on DRG neurons. Plasma membrane Cav2.2_HA expression may have synergistically increased due to the survival of both peptidergic and non-peptidergic neurons. As such, culture conditions must be carefully taken into consideration when studying Cav2.2_HA expression in DRG neurons.

The localisation of Cav2.2_HA in DRG neurons is paralleled by its predominantly laminar expression pattern in LI and LII of the dorsal horn of the spinal cord. In the current study, Cav2.2_HA can be seen associated with the primary afferent marker, CGRP, in LI and LIIo as described by Westenbroek et al. (1998). Nieto-Rostro et al. (2018) also showed Cav2.2_HA puncta in close association with IB4 and vGlut2 in LIII, present in the glomerular primary afferent terminals as described previously by Ribeiro-da Silva et al. (1985). Nieto-Rostro et al. (2018) further characterised Cav2.2_HA expression in the spinal cord using super-resolution Airyscan imaging. These images showed the localisation of Cav2.2_HA puncta in the active zones of individual glomerular terminals where presynaptic Cav2.2_HA puncta were adjacent to the postsynaptic density protein Homer. Furthermore, from the high-resolution immunoelectron-microscopic localisation of Cav2.2_HA, Nieto-Rostro et al., also
confirmed that these rosette structure formed by the Cav2.2_HA puncta are likely to represent Cav2.2_HA in active zones of individual glomerular terminals. Altogether these data show the laminar pattern of Cav2.2_HA in LII and LIII and reveal the excitatory targets of Cav2.2_HA in the spinal cord dorsal horn.

It has been suggested that Cav2.2 in the dorsal horn of the spinal cord is mainly of primary afferent origin (Bao et al., 1998; Heinke et al., 2004; Rycroft et al., 2007). In the present study, following dorsal rhizotomy, a significant reduction of Cav2.2_HA immunoreactivity was observed in the spinal cord dorsal horn. This revealed that the majority of Cav2.2_HA immunolabelling had extrinsic origins confirming its presynaptic localisation in primary afferents. However, since weak Cav2.2_HA immunoreactivity could still be detected in the spinal cord following rhizotomy, an origin solely extrinsic to the spinal cord cannot be assumed. These data highlight the importance of Cav2.2 in synaptic transmission in the primary afferent neuronal pathway and its therapeutic importance as a drug target (Chaplan et al., 1994; Bowersox et al., 1996). However, further studies are required to confirm Cav2.2_HA immunolabelling in spinal cord interneurons.

The auxiliary subunit αδ-1 has been shown to increase the surface density of Cav2.2_HA in expression systems (Cassidy et al., 2014). Furthermore, the importance of the αδ-1 subunit has been highlighted in its critical role in the development of neuropathic mechanical hypersensitivity (Luo et al., 2001; Bauer et al., 2009; Patel et al., 2013) and overexpression of αδ-1 mimics features of neuropathic injury (Li et al., 2006). In rats, the highest expression of αδ-1 has been found in small DRG neurons and this has been confirmed by Nieto-Rostro et al. (2018) where a similar αδ-1 distribution was also observed in mice. However, it had not been possible to examine the role of αδ-1 on the trafficking of the N-type calcium channels in vivo. Characterisation of the Cav2.2_HA<sup>ΔΔ</sup> mouse model was therefore first necessary to elucidate the cellular localisation of endogenous Cav2.2_HA in the pain pathway. Nieto-Rostro
et al. (2018) crossed Cav2.2_HA^KIKI and α2δ^-1^KOKO mice, in which α2δ^-1 was globally ablated. This study highlighted the critical role of α2δ^-1 in plasma membrane targeting of Cav2.2_HA in DRG neurons and its presynaptic targeting at the dorsal horn of the spinal cord. But the loss of α2δ^-1 did not affect the distribution of other spinal cord synaptic markers.

In conclusion, the present study shows that Cav2.2_HA has a widespread distribution in the primary afferent pain pathway. Cell surface Cav2.2_HA was highly expressed in the small and medium-sized DRG peptidergic neurons. Furthermore, the addition of NGF and GDNF in culture significantly increases cell surface Cav2.2_HA expression in subpopulations of DRG neurons. The localisation of Cav2.2_HA in DRG neurons is paralleled by its expression in predominantly LI and LII of the spinal cord dorsal horn. The presynaptic localisation of Cav2.2_HA is confirmed through its ablation by unilateral dorsal rhizotomy. These current findings encourage further work on the putative role of Cav2.2 in sensory neurotransmission using the Cav2.2_HA^KIKI mice to study its endogenous expression.

### 6.3 Cav2.2_HA expression in DRG and spinal cord cocultures

In Chapter 5, a co-culture system between DRG and spinal cord neurons was used to examine Cav2.2_HA at the presynaptic terminal of the first synapse in the pain pathway. To ensure that Cav2.2_HA at the presynaptic terminal was studied, DRG and spinal cord neurons were cultured from Cav2.2_HA^KIKI and Cav2.2^WTWT mice, respectively. Action potential-induced Ca^{2+} entry was unperturbed in co-cultures comprised of DRG neurons from Cav2.2_HA^KIKI and spinal cord from Cav2.2^WTWT mice compared to co-cultures from exclusively Cav2.2_HA^KIKI and Cav2.2^WTWT mice. These data suggest that the insertion of the HA-tag does not disturb Ca^{2+} influx in DRG neurons of these co-cultures. Furthermore, confocal imaging of co-cultures from exclusively Cav2.2^WTWT mice revealed vGluT2 and Homer apposed to each
other, which confirmed synapse formation within these co-cultures. Altogether these data demonstrated that the DRG/spinal cord co-cultures were ideal for visualising primary afferent synaptic terminals and can also be used as a model system for examining \( \text{CaV}_{2.2} \_\text{HA} \) during synaptogenesis.

Subsequently, \( \text{CaV}_{2.2} \_\text{HA} \) was examined at the cell body and presynaptic terminal. Super-resolution imaging revealed a marked decrease in \( \text{CaV}_{2.2} \_\text{HA} \) immunoreactivity at the cell body of small and medium-diameter DRG neurons as a function of time in culture. A similar decrease in \( \text{CaV}_{2.2} \) transcript levels in DRG neurons was found in adult compared to early postnatal mice. On the other hand, a concomitant increase of \( \text{CaV}_{2.2} \_\text{HA}, \) vGluT2 and Homer-positive puncta was observed in co-cultures over time. Although further work is required, these preliminary results suggest that newly synthesised \( \text{CaV}_{2.2} \_\text{HA} \) may be inserted into the somatic membrane and during development the channel is endocytosed and anterogradely transported to the axonal terminals.

In the present study, the pattern of increased synaptic bouton formation in DRG/ dorsal horn co-cultures agreed with Joseph et al. (2010). These authors also characterised the expression of the nociceptive markers TRPV1, TrkA, peripherin, CGRP and substance P in co-culture assays. Joseph et al. (2010) reported that the dorsal horn neurons of co-cultures are comprised of heterogenous subpopulations. Dorsal horn neurons were characterised by a previous study by immunocytochemistry and revealed subpopulations of excitatory and inhibitory neurons (Albuquerque et al., 1999). On the other hand, the DRG neurons in co-cultures appeared to be homogenous in terms of nociceptive function (Joseph et al., 2010). It is accepted that cultured neurons do not fully represent the \textit{in vivo} situation. Nonetheless, the co-culture system offers an \textit{in vitro} model of synaptic connections between DRG and spinal cord neurons where environmental conditions can be modulated to study the development of nociceptive synapses.
Previous studies have explored the mechanistic link between TRPV1 and Ca\textsuperscript{2+} signalling in DRG/dorsal horn co-cultures (Medvedeva et al., 2008). This study found that a brief stimulation of the TRPV1 receptor with capsaicin caused the release of glutamate which was independent of Ca\textsubscript{V}2.2 channels. The current study investigated the effects of capsaicin on cell surface Ca\textsubscript{V}2.2_HA of DRG neurons cultured from Ca\textsubscript{V}2.2_HA\textsuperscript{KIKI} mice. Capsaicin application has been shown to mediate its effects within the first 20 s of stimulation (Medvedeva et al., 2008; Hendrich et al., 2012). Indeed, the findings from Chapter 5 suggest that after short incubations (20 s) with capsaicin, cell surface Ca\textsubscript{V}2.2_HA expression increases in small and medium-sized TRPV1-positive DRG neurons.

Following longer incubations (2 to 4 min) with capsaicin, Ca\textsubscript{V}2.2_HA plasma membrane labelling significantly decreased in small and medium-sized TRPV1-positive neurons. Wu et al. (2005) found a similar decrease in Ca\textsubscript{V}2.2 currents following incubation with capsaicin. These authors attributed this to Ca\textsuperscript{2+} influx through TRPV1 mediating a Ca\textsuperscript{2+}/calcineurin-dependent inhibition of Ca\textsubscript{V}2.2. Burley and Sihra (2000) found that overexpression of calcineurin in NG108-15 cells increased the Ca\textsuperscript{2+}-dependent inhibition of VGCCs, hence implicating the phosphatase in the negative-feedback modulation of Ca\textsuperscript{2+} entry through protein dephosphorylation. In addition to direct dephosphorylation of VGCCs, kinases and phosphatases can phosphorylate and dephosphorylate, respectively, cytoskeletal proteins resulting in structural changes and internalisation of the channel protein (Unno et al., 1999). Internalisation of VGCCs appears to be an essential mechanism responsible for capsaicin-induced loss of Ca\textsubscript{V}2.2_HA cell surface density. However, further studies are warranted to identify the exact substrates dephosphorylated by calcineurin in DRG neurons. Such a control mechanism may be crucial in the physiology of neurotransmission and in pathophysiological states.

Surprisingly, in large-diameter TRPV1-positive DRG neurons, following incubation with capsaicin, a concomitant increase of Ca\textsubscript{V}2.2_HA surface density was detected. In agreement
with other data (Yu et al., 2008), this suggests that large DRG neurons may mediate mechanical hypersensitivity. Previous studies have also shown that TRPV1 expression is differentially increased in large DRG neurons in diabetic neuropathy and bone cancer pain animal models (Amaya et al., 2003; Hong and Wiley, 2005; Niiyama et al., 2007). Nevertheless, further studies are required to explore the mechanisms behind capsaicin-induced increase in cell surface CaV2.2_HA expression in large-sized DRG neurons.

In TRPV1-negative neurons, an unexpected decrease in CaV2.2_HA immunoreactivity was also observed in small and medium-sized DRG neurons, but no changes were found in large-diameter neurons. Capsaicin has been shown to produce a rapid and robust Ca\(^{2+}\) influx in DRG neurons. However, in neurons cultured from TRPV1-null mice, the application of capsaicin failed to exhibit a response (Caterina et al., 2000). It is therefore possible that the subpopulation identified as TRPV1-negative here may in fact include low-expressing TRPV1-positive neurons. As such, TRPV1 may be mediating Ca\(^{2+}\)/ calcineurin-dependent inhibition of CaV2.2, following capsaicin application. On the other hand, there is a growing body of evidence which suggests that following nerve injury, the network of non-neuronal cells surrounding DRG neurons release different factors which may contribute to the multitude of changes in protein expression levels within each cell type (Martin et al., 2019). This may account for the TRPV1-independent mechanisms regulating CaV2.2_HA cell surface expression. Nevertheless, further studies are required to determine the Ca\(^{2+}\)-dependent and -independent mechanisms behind CaV2.2_HA cell surface expression changes following capsaicin application.
6.4 Future Work

Previous studies have utilised overexpression of CaV2.2_HA in cell lines and cultured neurons to improve our understanding of this channel. Future work with the CaV2.2_HA KIKI mice will therefore improve our understanding of the basic mechanisms underlying the surface expression and trafficking regulation of endogenous CaV2.2.

6.4.1 CaV2.2_HA expression in the brain

Based on biophysical and pharmacological characteristics, several VGCCs have been identified at central synapses. N-type calcium channels have been shown to play an important role in supporting neurotransmission in central synapses (Williams et al., 1992; Takahashi and Momiyama, 1993; Wheeler et al., 1994; Reuter, 1995). Using an anti-II-III loop antibody, the N-type calcium channel has been identified at cellular and subcellular levels in the rat brain (Westenbroek et al., 1992). Furthermore, the N-type calcium channel blocker, ω-conotoxin GVIA, abolished inhibitory postsynaptic potentials recorded from the stratum radiatum of brain slices from 5-day old rats (Poncer et al., 1997). In hippocampal neurons, synapses were found to vary greatly in their dependence of on N and/or P/Q type calcium channels for neurotransmission (Ariel et al., 2013). These studies all suggest an important presynaptic role for CaV2.2 in the brain. As such, future studies should use the CaV2.2_HA KIKI mice to examine cell surface CaV2.2 expression in the brain.

6.4.2 CaV2.2_HA expression following the induction of neuropathic pain

Neuropathic pain has been defined by the International Association for the Study of Pain (IASP) as a pain that arises as a direct consequence of a lesion or disease affecting the somatosensory system. It has been characterised by unpleasant symptoms, such as shooting
pain, numbness and altered sensation. In attempt to address this, ziconotide, a synthetic version of the \( \text{Ca}_{2+} \text{V}_{2.2} \) channel blocker \( \omega \)-conotoxin MVIIA, was developed and licensed for intrathecal use in chronic pain (Staats et al., 2004; Miljanich, 2004). An upregulation of \( \text{Ca}_{2+} \text{V}_{2.2} \) at the dorsal horn of the spinal cord have previously been reported to play a crucial role in the initiation and maintenance of pain states after peripheral nerve injury in rats (Cizkova et al., 2002). The data from Nieto-Rostro et al. (2018) leaves many questions unanswered with regards to the role of \( \text{Ca}_{2+} \text{V}_{2.2} \_ \text{HA} \) in neuropathic pain. How does native cell surface \( \text{Ca}_{2+} \text{V}_{2.2} \_ \text{HA} \) expression differ in different DRG neuronal populations and primary afferent terminals following peripheral nerve injury? How do the current N-type calcium channel blockers alter cell surface \( \text{Ca}_{2+} \text{V}_{2.2} \) expression in neuropathic pain models? As such the \( \text{Ca}_{2+} \text{V}_{2.2} \_ \text{HA}^{\text{KIKI}} \) mice will permit the direct visualisation of native \( \text{Ca}_{2+} \text{V}_{2.2} \) in DRG cell bodies and nerve terminals during the development of neuropathic pain.

The data from Nieto-Rostro et al. (2018) has confirmed the importance of \( \alpha_{2\delta}-1 \) in regulating the cell surface expression of \( \text{Ca}_{2+} \text{V}_{2.2} \_ \text{HA} \). Furthermore, \( \alpha_{2\delta}-1 \) has been shown to have an important role in the development of neuropathic pain (Luo et al., 2001; Bauer et al., 2009; Boroujerdi et al., 2011). As such, the \( \text{Ca}_{2+} \text{V}_{2.2} \_ \text{HA}^{\text{KIKI}} \times \alpha_{2\delta}-1^{\text{KOKO}} \) mice can be used in future studies to examine \( \text{Ca}_{2+} \text{V}_{2.2} \_ \text{HA} \) expression after the onset of neuropathic pain following nerve injury, in the absence of \( \alpha_{2\delta}-1 \).

New approaches to target \( \text{Ca}_{2+} \text{V}_{2.2} \) trafficking have also been explored. The regulatory protein, CRMP-2, has been shown to enhance \( \text{Ca}_{2+} \text{V}_{2.2} \) trafficking to the presynaptic membrane. On the other hand, CRMP-2 knockdown reduced calcium currents and transmitter release, which suggests that \( \text{Ca}_{2+} \text{V}_{2.2} \) is endogenously regulated by CRMP-2 (Chi et al., 2009). A peptide (CBD3) has been shown to block the binding of \( \text{Ca}_{2+} \text{V}_{2.2} \) to CRMP-2 (François-Moutal et al., 2015). Electrophysiological studies in rat spinal cord slices show that CBD3 diminished \( \text{Ca}_{2+} \text{V}_{2.2} \) mediated CGRP release (Brittain et al., 2011). Furthermore, the delivery of CBD3 into
lumbar 4 and 5 DRG neurons of rats following tibial nerve injury was found to attenuate pain
behaviour (Yu et al., 2019). Taking this into account, the novel Ca\textsubscript{V}2.2\textsubscript{HA}\textsuperscript{KIKI} mice could be
used in the future to study the regulators of cell surface Ca\textsubscript{V}2.2\textsubscript{HA} and their use as potential
therapeutic targets.

### 6.4.3 Ca\textsubscript{V}2.2\textsubscript{HA} trafficking

The insertion of the HA-tag into extracellular epitopes also allows for the tracking of
Ca\textsubscript{V}2.2\textsubscript{HA} with quantum dot (Qdot) technology. Qdots are semiconductor nanocrystals that
exhibit strong fluorescence. The major advantage of using Qdots is their remarkable photosta-
bility which allows the investigation of a single Qdot over several minutes (Groc et al., 2007).
Single particle tracking has been used to investigate the surface mobility of the \(\alpha_2\delta-1\) subunit
at the cell surface (Voigt et al., 2016). The use of Qdots could advance our understanding
of plasma membrane targeting of Ca\textsubscript{V}2.2\textsubscript{HA} and the mechanisms controlling its cell surface
movement.

To study the role of Ca\textsuperscript{2+} influx on Ca\textsubscript{V}2.2\textsubscript{HA} membrane dynamics, a combination of
GCaMPs and Qdots could be used to unravel the interplay between the two processes.
GCaMP is a genetically encoded calcium indicator and has been used to examine Ca\textsuperscript{2+} sig-
nalling. To study Ca\textsuperscript{2+} influx at the presynaptic site of co-cultures, GCaMP constructs could
be transfected into DRG neurons cultured from Ca\textsubscript{V}2.2\textsubscript{HA}\textsuperscript{KIKI} mice. Following this, Qdots
specific to the HA-tag could be used in combination with Ca\textsuperscript{2+} imaging to study the dynamic
organisation of Ca\textsubscript{V}2.2 in presynaptic terminals. Understanding how Ca\textsubscript{V}2.2 is functionally
organised opens new avenues of research for innovative therapeutic strategies in which a
balance of the dynamics and distribution of Ca\textsubscript{V}2.2 can be explored.
6.4.4 Axonal targeting of Ca\textsubscript{V}2.2_HA in co-cultures

Axonal targeting of Ca\textsubscript{V}2.2_HA is critical for neurotransmission, yet the underlying trafficking mechanisms remain unclear. The use of compartmentalised cultures using microfluidic chambers will allow for the study of Ca\textsubscript{V}2.2_HA expression from the neuronal soma to the distal axon. Future studies could also use time-lapse imaging of axonal transport to study the transcytosis of Ca\textsubscript{V}2.2_HA from the cell body to axonal compartments, a method previously described by Ascaño et al. (2009).

6.4.5 Ca\textsubscript{V}2.2_HA and TRPV1

A valuable investigation to unravel the role of TRPV1 activation in cell surface Ca\textsubscript{V}2.2_HA expression would be to knockdown the expression of TRPV1 using CRISPR-cas9 gene editing technology. The knockdown of TRPV1 would shed light on TRPV1-positive and -negative mechanisms behind changes in Ca\textsubscript{V}2.2_HA cell surface expression. The data from Chapter 5 suggest that there may be Ca\textsuperscript{2+}-dependent and -independent mechanisms governing cell surface expression of Ca\textsubscript{V}2.2_HA. As such, further work is warranted to identify the pathways behind Ca\textsubscript{V}2.2_HA internalisation. This future work will enhance our understanding of the molecular mechanisms behind analgesic action produced by capsaicin and its related analogues.
6.5 Conclusion

Chapter 3 presents the first published functional exofacially HA-tagged Cav2.2 knock-in mouse model. The Cav2.2_HA<sup>KIKI</sup> mouse line will improve our understanding of endogenous Cav2.2 channels with regards to surface expression in primary neuronal cultures and in vivo studies. This mouse model can also be used to study the native expression of Cav2.2 during the development of neuropathic pain.

Chapter 4 charts the investigation of Cav2.2_HA expression in DRG neurons and spinal cord tissue. The findings from this work have confirmed the predominant expression of cell surface Cav2.2_HA on small and medium peptidergic DRG neurons. This work demonstrates the laminar expression pattern of Cav2.2_HA in L1 and LII of the dorsal horn of the spinal cord. The presynaptic localisation of Cav2.2_HA in primary afferents is confirmed through its ablation following dorsal rhizotomy.

Finally, Chapter 5 shows that the Cav2.2_HA<sup>KIKI</sup> mouse model can be used in combination with Cav2.2<sup>WTWT</sup> mice to prepare co-cultures in which Cav2.2_HA can be studied at the presynaptic terminals of primary afferent terminals. Preliminary studies examining the cell surface expression of Cav2.2_HA in DRG neurons following capsaicin application, suggest that capsaicin may have a modulatory effect on N-type channel expression. However, further studies are required to unravel the mechanisms governing capsaicin-induced Cav2.2_HA expression.

The findings described in this thesis provide insight into the cell surface expression of Cav2.2_HA. However, this merely scratches the surface in disentangling the complex mechanisms governing cell surface Cav2.2_HA expression. In conclusion, the Cav2.2_HA<sup>KIKI</sup> mouse model will provide an excellent opportunity to better understand the regulation of Cav2.2 in health and disease in the future.
Bibliography


Bornschein, G., Eilers, J. and Schmidt, H. (2019), ‘Neocortical high probability release sites are formed by distinct ca 2+ channel to release sensor topographies during development’, Available at SSRN 3353704.


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Davare, M. A., Horne, M. C. and Hell, J. W. (2000), ‘Protein phosphatase 2a is associated with class c l-type calcium channels (cav1. 2) and antagonizes channel phosphorylation by camp-dependent protein kinase’, *Journal of Biological Chemistry* 275(50), 39710–39717.


Hibino, H. (2002), ‘Pironkova r, onwumere o, vologodskai m, hudspeth aj, and lesage f’, *RIM binding proteins (RBPs) couple Rab3-interacting molecules (RIMs) to voltage-gated Ca 2*, 411–423.


Hui, K., Liu, B. and Qin, F. (2003), ‘Capsaicin activation of the pain receptor, vr1: multiple open states from both partial and full binding’, *Biophysical journal* 84(5), 2957–2968.


Obermair, G. J., Szabo, Z., Bourinet, E. and Flucher, B. E. (2004), ‘Differential targeting of the \( \text{L} \)-type \( \text{Ca}^{2+} \) channel \( \alpha 1c \) (cav1.2) to synaptic and extrasynaptic compartments in hippocampal neurons’, *European Journal of Neuroscience* **19**(8), 2109–2122.


Stanley, E. F. and Atrakchi, A. H. (1990), ‘Calcium currents recorded from a vertebrate presynaptic nerve terminal are resistant to the dihydropyridine nifedipine’, Proceedings of the National Academy of Sciences 87(24), 9683–9687.


Vyleta, N. P. and Jonas, P. (2014), ‘Loose coupling between ca2+ channels and release sen-
sors at a plastic hippocampal synapse’, Science 343(6171), 665–670.

Waithe, D., Ferron, L., Page, K. M., Chaggar, K. and Dolphin, A. C. (2011), ‘β-subunits pro-
mote the expression of cav2. 2 channels by reducing their proteasomal degradation’, Journal of Biological Chemistry 286(11), 9598–9611.

Wakamori, M., Mikala, G. and Mori, Y. (1999), ‘Auxiliary subunits operate as a molecular
switch in determining gating behaviour of the unitary n-type ca2+ channel current in xen-

chronic pain’, Expert review of neurotherapeutics 6(10), 1423–1428.

functions in the hippocampus and implications for its use in avidin-biotin technology’, Cell and tissue research 296(3), 511–516.

and binding of the isolectin b4 from griffonia simplicifolia i in rat primary sensory neurons’, Neuroscience 62(2), 539–551.

‘The three-dimensional structure of the cardiac l-type voltage-gated calcium channel com-
parison with the skeletal muscle form reveals a common architectural motif’, Journal of Biological Chemistry 279(8), 7159–7168.

cca2+ channels carry the largest current: implications for nanodomains and
transmitter release’, Nature neuroscience 13(11), 1348.

protein dissociation to the characteristic modifications of n-type calcium channel activity’, Neuroscience research 56(3), 332–343.

sion and function of cav3.2 t-type calcium channels are controlled by asparagine-linked glycosylation’, Pflügers Archiv-European Journal of Physiology 465(8), 1159–1170.

(1992), ‘Biochemical properties and subcellular distribution of an n-type calcium channel α1
subunit’, Neuron 9(6), 1099–1115.

subtypes on rat spinal motor neurons, interneurons, and nerve terminals’, Journal of Neuro-
science 18(16), 6319–6330.


