Exploring the Role of CASPR2 in Neuropathic Pain

Mandy Tseng
Green Templeton College
University of Oxford

A thesis submitted for the degree of
Doctor of Philosophy
Hilary Term 2023

Declarations

I declare that the work within this thesis is my own unless otherwise stated. Acknowledgements are found at the end of each data chapter.

Significant contributions were made by:

- A/ Prof J. Dawes, with whom I performed most of the rodent surgeries in Chapter 2.
- Dr A. Farah generated the electrophysiology data in Chapter 2, Figure 5.
- Dr S. Ramanathan performed the patient characterisations for Chapter 3.
- Dr A. Davies collected the patient sera-binding data from human iPS-derived neurons in Chapter 3, Figure 3.
- Dr B. Sun and Prof S. Irani provided the patient characterisations and the monoclonal antibodies used in Chapter 4.

Acknowledgements

I would like to thank my supervisors, Prof David Bennett, A/ Prof John Dawes, and Prof Sarosh Irani, for their mentorship. Their insightful scientific input and constant support have guided me throughout my DPhil studies. I am incredibly grateful to Prof Bennett and A/ Prof Dawes for welcoming me into the Neural Injury Group four years ago and for their support and encouragement ever since. I couldn't have imagined a better environment to grow as a scientist.

I would like to thank the members of the Neural Injury Group, who have been excellent colleagues and even better friends. The lab has been an incredible place to learn, and I am thankful to Prof Bennett for fostering a welcoming environment with brilliant scientists and- above all- amazing people. I have had a wonderful time in (and outside!) the lab and will remember these times fondly.

I would also like to thank my friends and former colleagues at ABC, who taught me everything I knew prior to my DPhil. I am appreciative of the scientific foundation that was built and of the encouragement that led me to undertake further studies.

I would like to thank the directors of the OXION programme, Prof Stephen Tucker and Dame Fran Ashcroft, for accepting me into this prestigious programme and the Wellcome Trust for their generosity in funding this research.

Above all, I would like to thank my family and friends, who have been immensely supportive throughout this journey. Without them, this work would not have been possible.

Abstract

Neuropathic pain is caused by a lesion or disease affecting the somatosensory nervous system affecting 7-10% of the general population. Neuropathic pain is characterised by pathophysiological changes within the nervous system that trigger disordered transmission of sensory signals. There is a critical need for targeted therapeutics for neuropathic pain, as current treatments are challenged by limited efficacy and poor tolerance. Understanding the induction and mechanism of neuropathic pain is crucial for the development of novel, efficacious therapies for its treatment.

Preclinical models of neuropathic pain illustrate that voltage-gated potassium channels (VGKCs) are essential in limiting neuronal excitability and disruption of these channels has been shown to contribute to neuropathic pain. Contactin-associated protein-like 2 (CASPR2) is a transmembrane protein part of the VGKC complex. CASPR2 is a known target of autoantibodies in patients with neuropathic pain, and CASPR2 autoantibodies have been shown to cause neuropathic pain by disrupting VGKC channel distribution in sensory neurons. This disruption caused increased neuron excitability and elicited pain-like behaviour in mice. Conversely, increased expression of CASPR2 in cultured peripheral sensory neurons led to reduced excitability- suggesting that modulation of CASPR2 expression could be a viable therapeutic approach for neuropathic pain.

This thesis endeavours to understand the effect of CASPR2 overexpression on pain sensibility and to elucidate the pathogenic mechanisms of patient CASPR2 autoantibodies. We investigate the effect of CASPR2 overexpression and explore its therapeutic potential using preclinical models. The overexpression of CASPR2 in nociceptors and sensory neurons of mice did not significantly alter mechanical or thermal pain sensitivity but reduced capsaicin-induced acute pain. This suggests that CASPR2 may modulate other proteins and regulate the excitability of specific neuronal populations. Future experiments are required to establish whether CASPR2 overexpression is sufficient to increase the surface expression of K_v1 channels or other proteins that may alter capsaicin sensitivity. The characterisation of the CASPR2 autoantibody-binding using cultured rodent neurons revealed differential binding of CASPR2 IgG from patients with and without neuropathic pain. Internalisation assays showed that CASPR2-IgG could internalise in sensory neurons in an antigen-specific manner. Taken together, these assays demonstrate the value of thorough patient IgG screening in strengthening clinical-serological correlations. Further elucidation of the pathogenic mechanisms of autoantibodies will likely promote the discovery and use of targeted therapies for neuropathic pain.

Abbreviations

Ab	antibody
ADAM22/23	a disintegrin and mettaloproteinase domain 22/23
AIE	autoimmunie encephalitis
AIS	axon initial segment
AP	action potential
ASD	autism spectrum disorder
ATF3	activating transcription factor 3
CASPR2	contactin-associated protein-like 2
CBA	cell-based assay
CCI	chronic constriction injury
CGRP	calcitonin gene-related peptide
CHO	chinese hamster ovary
CIPN	chemotherapy-induced peripheral neuropathy
CNTN	contactin
CNS	central nervous system
CRPS	complex regional pain syndrome
CSF	cerebrospinal fluid
DIV	days *in vitro*
DLS	dynamic light scattering
DRG	dorsal root ganglion
DTX	dendrotoxin
Fab	fragment antigen-binding
FMS	fibromyalgia syndrome
GDNF	glial cell-derived neurotrophic factor
GPI	glycosylphosphatidylinositol
HLA	human leucocyte antigen
HEK	human embryonic kidney
IB4	isolectin B4

IgG	Immunoglobulin G
iPSC	induced pluripotent stem cell
iPSN	induced pluripotent stem cell-derived sensory neurons
IV-IG	intravenous immunoglobulin
JXP	juxtaparanode
KO	knockout
Kv	voltage-gated potassium channel
LE	limbic encephalitis
LGI1	leucine-rich glioma inactivated
LTMR	low threshold mechanoreceptor
MoS	morvan syndrome
NeuPSIG	neuropathic pain special interest group
NF155	neurofascin-155
NF200	neurofilament 200
NGF	nerve growth factor
NMDA	n-methyl-D-aspartate
NMO	neuromyelitis optica
NMT	neuromyotonia
NOD	non-obses diabetic
Npy2r	neuropeptide Y
NS-EM	negative staining electron microscopy
ODN	oligodeoxynucleotide
PFA	paraformaldehyde
PN	paranode
PNS	peripheral nervous system
PSD-95	post-synaptic density protein 95
PSN	peripheral sensory neurons
RIA	radioimmunoprecipitation assay
S100β	s100 calcium-binding protein B
SGC	satellite glia cells
SNI	spared nerve injury

SNL	spinal nerve ligation
STZ	streptozotocin
TAG-1	transient axonal glycoprotein 2
TBA	tissue-based assay
TH	tyrosine hydroxylase
TrkA	tropomyosin kinase A
TRP	transient receptor potential
TRPA1	transient receptor potential ankyrin 1
TRPV1	transient receptor potential vanilloid 1
VF	von Frey
VGKC	voltage-gated potassium channel
VGKCC	voltage-gated potassium channel complex
VGSC	voltage-gated sodium channel
WT	wild type

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Word count: 32,133 (excluding figure legends, references and appendices)

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General Introduction

1. The Somatosensory System

The ability to detect environmental changes and avoid potentially injurious stimuli is a crucial part of survival for all organisms. The somatosensory system allows the detection of a range of innocuous or noxious sensory inputs such as temperature, pain, touch, and proprioception. The perception of these sensory modalities originates from somatosensory nerves innervating the skin, muscles, fascia, viscera or joints. These sensory neurons detect and transduce stimuli into electrical signals that are relayed to higher brain centres for processing (Colloca et al., 2017; Emery et al., 2018a)

Primary afferents mediate the initial detection of somatosensory information from the periphery. Primary afferents are sensory neurons in the peripheral nervous system that transduce information about mechanical, thermal, and chemical stimuli to the central nervous system (CNS). These sensory neurons are pseudounipolar, with their cell bodies located within the dorsal root ganglia (DRG) and a single axon that bifurcates into two branches: the peripheral afferent branch that innervates target tissue and the central process that synapses with second-order neurons in the dorsal horn of the spinal cord. The dorsal horn neurons, in turn, transmit messages to higher brain centres and ultimately the cerebral cortex (Delhaye et al., 2018; Dubin & Patapoutian, 2010).

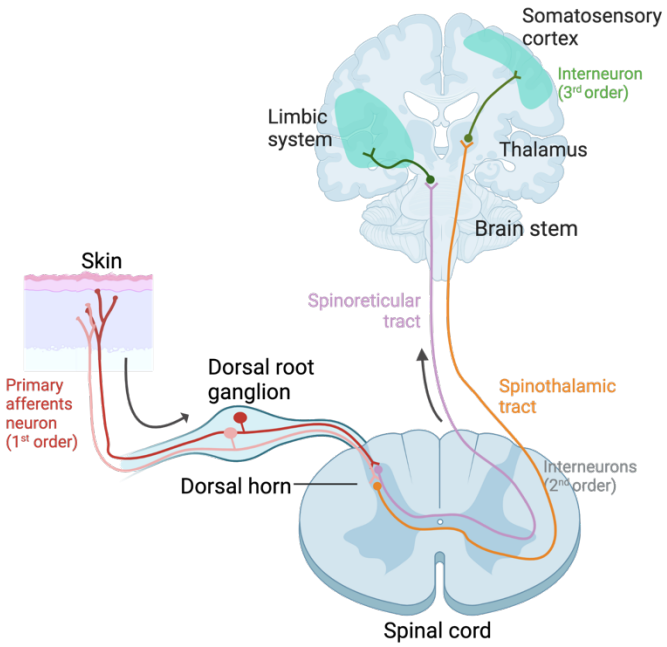

Figure 1. Anatomy of the ascending pain pathway

Simplified schematic of the ascending pathway. Noxious stimuli are detected by nociceptors and transmitted to the dorsal horn via primary afferent fibres. The primary afferent central processes synapse with second order interneurons in the dorsal horn of the spinal cord. The second order interneurons ascend the spinothalamic or spinoreticular tracts to the thalamus and brain stem, respectively. Third order interneurons then relays information to cortical structures. Figure generated using BioRender based on figures by von Hehn et al., 2012 and Finnerup et al., 2021.

1.1. Nociception and Nociceptors

Nociception- the process by which organisms detect aversive and potentially damaging stimuli- serves as a "detect and protect" mechanism that shields organisms from injury. Nociceptors are highly specialised primary afferents that respond to various noxious stimuli and provide information about perceived stimuli to higher processing centres (Basbaum et al., 2009a; St. John Smith, 2018; Tracey, 2017). The activation of peripheral terminals requires a high threshold stimulus of sufficient amplitude and duration to trigger the depolarisation of the nociceptor. This threshold requirement confers the specific tuning of nociceptors to noxious stimuli, while the distinct receptor and ion

channel repertoires expressed by nociceptors mediate the discrimination of sensory modalities (Dubin & Patapoutian, 2010; Julius & Basbaum, 2001; St. John Smith, 2018).

Nociceptors detect and transmit information to the CNS in spatially distinct subcellular compartments. Signal transduction begins at the sensory receptor terminals, which express various receptors and ion channels activated by various stimuli. These channels open upon the high-threshold stimuli, and the cation influx through these ion channels produces a local depolarisation in the membrane (sensor potential), which in turn triggers the voltage-gated sodium channels to open upon sufficient depolarisation, and action potentials are initiated with the aid of Ca^{2+} channels along the axon. The action potentials are propagated down the axon toward the central terminals. Due to the pseudounipolar structure of nociceptors, the cell soma is not in the line of action potential propagation. Transmission ultimately occurs at the central terminals, where signalling molecules such as glutamate or neuropeptides are released in response to calcium influx from VGCCs. (Harriott & Gold, 2009; Middleton et al., 2021)

Nociceptors can be classified by the type of sensory modalities they detect. Mechanical nociceptors detect high threshold mechanical stimuli and sufficient activation would lead to sharp, pricking pain. Thermal and mechano-thermal nociceptors respond to noxious heat or cold and polymodal nociceptors detect mechanical, thermal, and chemical stimuli (Dubin & Patapoutian, 2010; St. John Smith, 2018; Tracey, 2017). The sensory specificity of nociceptors is conferred by the heterogenous expression of channels and receptors that respond with high specificity and threshold to the mechanical, thermal or chemical stimuli (Basbaum et al., 2009b; St. John Smith, 2018). These channels and protein markers further delineate the heterogenous population of nociceptors and sensory neurons in the

peripheral nervous system. These populations of primary afferents are described briefly below.

1.2. The Dorsal Root Ganglia: heterogenous population of cells

The DRG houses the cell bodies of a heterogenous population of primary sensory neurons. These DRG neurons can be characterised by morphological characteristics such as soma size, myelination, protein expression (molecular markers), and electrophysiological properties like AP width and conduction velocity (Emery et al., 2018b; Haberberger et al., 2019). Recent studies using single-cell and single-nuclear sequencing technologies have revealed novel discrete populations (Li et al., 2016; Tavares-Ferreira et al., 2022; Usoskin et al., 2015).

DRG neuron sizes range from 20- 100 μm, and their somas sizes can be categorised as small (<25 μm in diameter), medium (25– 35 μm), or large (>35 μm) (Haberberger et al., 2019). Their sizes are related to the myelination status and thus, conduction velocity. Neurons with large and medium diameter soma are moderately myelinated Aβ fibres and thinly myelinated Aδ fibres, respectively. Myelinated A-fibres conduct action potentials at a higher velocity (1.2 – 40 m/s), while the small-diameter C-fibers are unmyelinated and therefore have a lower conduction velocity than those of A-fibres (0.3 – 1.2 m/s) (Cain et al., 2001; E. S. J. Smith & Lewin, 2009).

The myelinated Aβ fibres are thought to detect innocuous stimuli and do not contribute to pain (Julius & Basbaum, 2001). However, some studies have demonstrated that Aβ nociceptors exist (Djouhri & Lawson, 2004). The thinly myelinated Aδ fibres can be subdivided into Aδ nociceptors that detect high-intensity stimuli and Aδ low-threshold mechanoreceptors (LTMRs), also known as

D-hairs. Aδ nociceptors express markers tropomyosin kinase A (TrkA), the receptor for nerve growth factor (NGF); voltage-gated sodium channel 1.8 ($Na_v1.8$); NF200; and S100β. Some Aδ-nociceptors also express calcitonin gene-related peptide (CGRP) and neuropeptide Y (Npy2r) (Arcourt et al., 2017; Emery et al., 2018b).

The unmyelinated C-fibre nociceptors can be divided into peptidergic nociceptors and non-peptidergic nociceptors. As the name indicates, peptidergic C-fibre nociceptors express neuropeptides such as CGRP or substance P. They also express TrkA and therefore respond to NGF. These nociceptors express TRPV1 and are able to detect noxious heat or capsaicin. In contrast, non-peptidergic nociceptors do not express neuropeptides substance P or CGRP, or TrkA but express the receptor for glial cell line-derived neurotrophic factor receptor (GDNF), c-Ret (Bennett et al., 1998). These nociceptors also express the MrgprD and P2X3 receptors and can be identified with Isolectin B4 (IB4) labelling (Emery et al., 2018a; Julius & Basbaum, 2001). Most of the C-fibre nociceptors are polymodal and are responsive to noxious stimuli such as mechanical, heat and chemical stimuli. However, there is a subset of C-fibres (C-LTMRs) that are activated by innocuous stimuli and proposed to mediate pleasant touch in humans (Smith & Lewin, 2009).

The DRG also contains other cell types, such as satellite glia cells (SGCs) which form a sheath around neuronal soma. The SGCs express receptors for substances released from neurons, which enables them to communicate with adjacent neurons. Although the exact role of SGCs in the DRG is unclear, their close association with DRG neurons suggests a crucial functional relationship between the two cell types. There is increasing evidence of their role in chronic pain, where SGCs are activated by nerve injury and undergo structural and functional alterations (Hanani & Spray, 2020). Additionally, SGCs have recently been

reported to be targets of autoantibodies in fibromyalgia (Goebel et al., 2021; Krock et al., 2022)

A population of innate and adaptive immune cells such as macrophages, T-lymphocytes, and B-lymphocytes also reside in the DRG. Studies have shown that immune cells can interact with sensory neuroma soma via neuropeptides and cytokines (Pinho-Ribeiro et al., 2017). Flow cytometry analysis of cells in the naïve DRG showed that fourteen types of immune cells reside in the DRG. Although these immune cells exist in low abundance (0.03 to 0.5% of total cells), their numbers have been observed to increase in nerve injury- suggesting their involvement in neuropathic pain (Liu et al., 2014; Woolf & Ma, 2007).

In the context of neuropathic pain, primary afferent input, SGCs, and immune cells of the DRG all play a role in the initiation and maintenance of pain (Harriott & Gold, 2009; von Hehn et al., 2012).

2. Neuropathic Pain

Chronic pain, moderate to severe pain lasting at least three months, is a global affliction of significant economic and societal burden. Approximately one in five adults in Europe suffers from chronic pain, and as many as 100 million Americans are affected by persistent pain (Breivik et al., 2006; Gaskin & Richard, 2012). The economic impact of the loss of productivity or direct medical expenditures, which amount to more than $600 billion per year in the United States alone, is substantial (McCarberg & Billington, 2006; McDermott et al., 2006).

Neuropathic pain is a type of chronic pain condition that affects 7-10% of the general population (Van Hecke et al., 2014). The Neuropathic Pain Special Interest Group (NeuPSIG) guidelines define it as ‘pain arising as a direct

consequence of a lesion or disease affecting the somatosensory system' (Haanpää et al., 2011). Neuropathic pain is distinguished from other pain conditions, such as inflammatory or nociceptive pain, which are conditions in which pain originates from nonneural tissues (Campbell & Meyer, 2006). Neuropathic pain is often caused by injury to nerves in the peripheral or central nervous systems (Jensen et al., 2011; Loeser & Treede, 2008). The injury may be caused by mechanical trauma, metabolic disease, viral infections, or neurodegenerative condition, which may lead to pathophysiological changes within the PNS and CNS and cause disordered transmission of sensory signals (Costigan et al., 2009; Woolf & Mannion, 1999).

Neuropathic pain manifests in heterogeneous symptoms and pain distributions. It is characterised by spontaneous (pain in the absence of a painful stimulus) and evoked pain, the latter of which includes allodynia (pain elicited by a non-noxious stimulus) and hyperalgesia (increased, prolonged pain response to a noxious stimulus). Other common symptoms include dysesthesia and paraesthesia (pins and needles or a burning sensation) (Baron, 2006). The area of pain may be widespread or limited to single or several nerves. Neuropathic pain patients often suffer from comorbidities such as sleep disturbances, anxiety, and depression. In a survey by Attal *et al.*, patients with chronic neuropathic pain reported more impaired quality of life than those with chronic non-neuropathic pain (Attal et al., 2011).

There is a critical need for targeted therapeutics for neuropathic pain as most patients do not respond to current therapies, such as non-steroidal anti-inflammatory drugs and opioids. Management of neuropathic pain is further challenged by adverse effects, such as dependence and tolerance, that limit their use at effective doses (Finnerup et al., 2015; Labianca et al., 2012). Understanding the induction and mechanism of neuropathic pain is crucial for the development

of novel, targeted therapies necessary for the efficacious treatment of neuropathic pain.

2.1. Mechanisms of Neuropathic Pain

Neuropathic injury results in maladaptive plasticity within the nervous system (Costigan et al., 2009). Experimental and clinical evidence indicates that both peripheral and central changes contribute to the neuronal hyperexcitability that may lead to the onset, development, and maintenance of neuropathic pain (Campbell & Meyer, 2006; von Hehn et al., 2012). I focus on changes in the peripheral nervous system that are thought to be the main contributors to neuropathic pain.

2.1.1. Peripheral sensitisation

Nerve injury leads to increased spontaneous activity of primary afferents, which causes altered transmission of sensory signals, resulting in an abnormal perception of pain. The importance of primary afferent input in maintaining neuropathic pain is supported by studies using selective nerve blockade or antisense oligodeoxynucleotides (ODNs) directed against channels expressed in afferents (Haroutounian et al., 2014; Porreca et al., 1999). Pain sensations are usually caused by activity in unmyelinated (C-) and thinly myelinated (Aδ-) primary afferent neurons. These neurons are usually silent unless triggered by a noxious stimulus (Baron, 2006). However, pathological spontaneous activity can develop after nerve injury; this can be attributed to ectopic impulse generation or reduction of the threshold of peripheral neurons. This increase in spontaneous firing in the afferent neurons linked to the injury site has been termed ectopic discharge (Wall & Gutnick, 1974). Clinical observations suggest that ectopic

activity is responsible for ongoing pain in patients (Gracely et al., 1992). Many studies have shown that molecular and cellular changes at the primary afferent neurons may underpin ectopic activity.

2.1.2. Alteration in ion channel expression

Insult to peripheral nerves induces the dysregulation of membrane proteins. Alterations in the expression levels and locations of ion channels likely contribute to the various pain symptoms experienced by neuropathic pain patients. Key ion channels, such as voltage-gated sodium channels (VGSC), voltage-gated potassium channels (VGKC), and transient receptor potential (TRP) channels, have been reported to have altered membrane expression after nerve injury. The upregulation of excitatory ion channels after nerve injury is posited to be a driver of increased neuronal excitability and ectopic firing in primary sensory neurons (Finnerup et al., 2021). VGSCs, which are crucial for action potential generation, have been shown to be upregulated after nerve injury at the site of lesion (Wood et al., 2004; Woolf & Mannion, 1999). The increased expression and clustering of these sodium channels might contribute to lowered action potential threshold and consequent hyperactivity in injured neurons (Lai et al., 2003).

On the other hand, alterations to potassium currents- which mainly serve to repolarize the neuronal membrane- also contribute to the altered excitability of these neurons. Several studies demonstrate that the function or expression of K+ channels is diminished after nerve injury (Smith, 2020). Moreover, it has been shown that the downregulation of K^+ channels at the membrane can lead to primary afferent excitability (Dawes et al., 2018).

Studies have shown that damage to primary afferents resulted in the downregulation of TRPV1 in many injured afferents but induced the expression of TRPV1 in uninjured C- and A- fibres. There was also an upregulation of TRPV1 in

medium and large injured DRG cells and uninjured DRG neurons after nerve injury (Hudson et al., 2001; Ma et al., 2005). These changes in TRPV1 expression are thought to contribute to heat hyperalgesia symptoms (Baron, 2006). These altered expression profiles of ion channels in different afferent populations likely bestow the varied symptoms and sensitivities of neuropathic pain patients.

2.1.3. Central sensitisation

In addition to peripheral mechanisms, central sensitisation has also been demonstrated to be an important mechanism in neuropathic pain. Central sensitisation is the phenomenon in which hyperexcitability in the central nervous system leads to enhanced nociceptive responses to noxious stimuli (Basbaum et al., 2009b; Woolf, 2011). The amplification of neural signalling within the CNS could be caused by increases in membrane excitability, synaptic efficacy, or reduced inhibition (Latremoliere & Woolf, 2009). After nerve injury, damaged afferents can generate spontaneous action potentials (ectopic input) that can initiate activity-dependent central sensitisation in the dorsal horn. Structural changes within the dorsal horn, activation a of glial cell, and infiltration of peripheral immune-competent cells, as well as alterations in GABAergic and Glucinergic inhibition, all contribute to the occurrence of central sensitisation (Latremoliere & Woolf, 2009; Woolf, 2011).

Recent work from Gangadharan *et al*. shows the integration of peripheral structural changes and central disinhibition of spinal circuits underlies a novel mechanism of neuropathic pain. They revealed that nociceptors mis-targeting mechanosensory end organs during re-innervation after nerve injury, together with central disinhibition from altered spinal circuitry, resulted in re-innervation-induced neuropathic pain (Gangadharan et al., 2022).

2.1.4. Neuroimmune Interactions

While immune cells were initially thought to be relevant only to inflammatory pain disorders, there is increasing evidence that the immune system plays a role in neuropathic pain in the periphery (Finnerup et al., 2021; Thacker et al., 2007). The neuroimmune crosstalk is essential in the regulation of inflammation and pain. However, dysregulation of this interaction can lead to altered neuronal excitability and inflammatory disease. After nerve injury, the resident immune cells in the nerve and DRG secrete inflammatory mediators that recruit more immune cells to the site of injury. Schwann cells proliferate, and macrophages are recruited to remove dead cells and aid axon regeneration. These cells release inflammatory factors that can sensitise injured and adjacent intact fibres (Calvo et al., 2012; Ji et al., 2016). Receptors of immune cell-derived factors expressed on nociceptive afferents are activated and mediate the downstream signalling cascades within the neuron; this can lead to altered gating properties of key ion channels, ultimately resulting in increased neuronal firing (Pinho-Ribeiro et al., 2017).

The communication between immune cells and neurons is bidirectional. The transduction of nociceptive signals to the terminals triggers the calcium influx and causes neurons to release neuropeptides and neurotransmitters from their peripheral terminals. These neuropeptides can bind to the receptors of innate and adaptive immune cells and activate downstream pathways that stimulate the secretion of cytokines and chemokines (Pinho-Ribeiro et al., 2017). Neurons can also recruit immune cells to the damaged axons. For example, the NKG2D ligand, RAE-1, is expressed on injured, intact axons and recruits Natural killer (NK) cells for the degeneration of injured neurons (Davies et al., 2019).

2.1.5. Autoantibodies and pain

Autoantibodies are another component of neuroimmune interactions that can promote pain conditions. Pain is a common symptom of autoimmune diseases, and in autoantibody disorders such as VGKCC autoimmunity, it can be the only presenting symptom in some patients (Klein et al., 2012). Furthermore, treatments that reduce autoantibodies have been shown to reduce neuropathic pain, further adding to the evidence of autoantibodies as drivers of pain (Klein et al., 2012). The pathogenic potential of autoantibodies has been validated in several studies. For instance, autoantibodies from rheumatoid arthritis patients injected into mice induced increased pain-like behaviour without inflammation, suggesting that autoantibodies can contribute to pain without inflammation (Wigerblad et al., 2016). Passive transfer models in other painful diseases further support this notion. The passive transfer of IgG from patients with anti-CASPR2 antibodies in mice also induced mechanical hypersensitivity without inflammation or neuronal damage (Dawes et al., 2018).

Of all the antibody isotypes (IgA, IgD, IgE, IgM, IgG), Immunoglobulin G (IgG) is the most abundant in human serum, accounting for approximately 10-20% of plasma protein. IgG antibodies can be further divided into subclasses IgG1, IgG2, IgG3, and, IgG4; which differ primarily in their invariant 'constant' fragment crystallizable Fc domains. Functionally, the different subclasses of antibodies vary in their ability to activate complement, bind Fc gamma receptors (FcγR), recruit and trigger effector cells, and the bivalent binding of antigens (Vidarsson et al., 2014). Therefore, the subclasses of autoantibodies dictate their mechanisms of action.

The ways by which autoantibodies can mediate their pathogenic effects include receptor internalisation; alterations in protein-protein interaction; changes

in receptor signalling; complement activation, and antibody-dependent cell cytotoxicity (**Figure 2**). Autoantibodies can act across the neuraxis and cause nociceptor hyperexcitability leading to chronic pain. Antibodies in the skin and joints can activate the complement system or recruit macrophages that secrete inflammatory factors that lead to tissue damage (Gill & Venkatesan, 2022; Lacagnina et al., 2021b). At the peripheral nerve, autoantibodies can target components of the VGKC complex, leading to reduced surface expression of VGKCs and neuronal hyperexcitability (Dawes & Bennett, 2018).

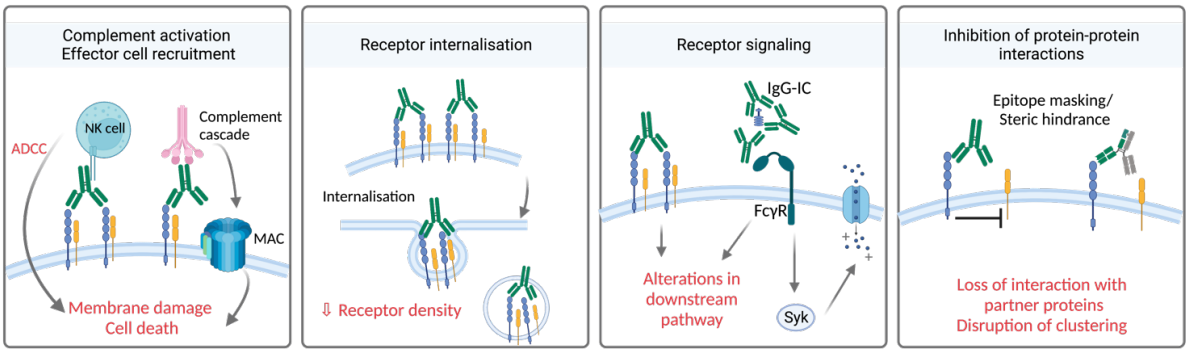

Figure 2. Potential mechanisms of autoantibodies
Schematics of potential mechanisms of autoantibodies. **A)** Autoantibodies can recruit complement proteins and other immune cells with effector function that mediate ADCC (antibody dependent cell cytotoxicity). **B)** Autoantibodies can bind to antigens and cause the crosslinking and internalization of membrane proteins. **C)** Autoantibodies with agonist or antagonist activity can bind to membrane receptors that alter downstream signaling pathways. IgG-immune complexes (IgG-IC) can also bind to Fc receptors on neurons and activate the Syk signaling pathway. **D)** Autoantibody binding to surface antigens can also block the binding of partner proteins. Figure generated using BioRender based on a figure from Lacagnina et al., 2021a

In summary, autoantibodies can influence neuron excitability in the periphery via various mechanisms. Although significant advances have been made in elucidating the targets and mechanisms of autoantibodies, the lapse in the B cell tolerance pathway that leads to the generation of autoantibodies is still unclear.

2.2. Rodent models of neuropathic pain

Preclinical animal models of pain have been indispensable in understanding the aetiology and pathophysiology underlying pain conditions. Rodent models, in particular, have been widely utilised in investigating neuropathic pain at the cellular and molecular level, owing to the relative ease and efficiency of engineering transgenic lines (Burma et al., 2017; Colleoni & Sacerdote, 2009; Mogil, 2009). Several models of neuropathic pain in rodents have been developed to simulate the aspects of human neuropathic pain. These models generally involve physical damage to the nerve or pharmacological manipulations to mimic disease states (Decosterd & Woolf, 2000; Kim & Chung, 1992; Mogil, 2009).

2.2.1. Nerve injury models

Peripheral nerve injury models are commonly used as a model of neuropathic pain (**Figure 3**). Generally, these models injure the nerves through surgical means that partially transect or constrict the nerves (Bauder & Ferguson, 2012; Bennett & Xie, 1988; Decosterd & Woolf, 2000). The complete sciatic nerve transection (axotomy) model is the oldest model of neuropathic pain that results in the development of a neuroma at the transected nerve (Wall et al., 1979). This model causes autotomy (self-mutilation of the digits) and motor weakness, which precludes accurate results in evoked response outcomes. The human syndrome of phantom limb pain modelled by this assay is rare. Thus, more recent models induce partial nerve injury to better reflect the more common nerve injuries in humans (Colleoni & Sacerdote, 2009; Stemkowski & Smith, 2011).

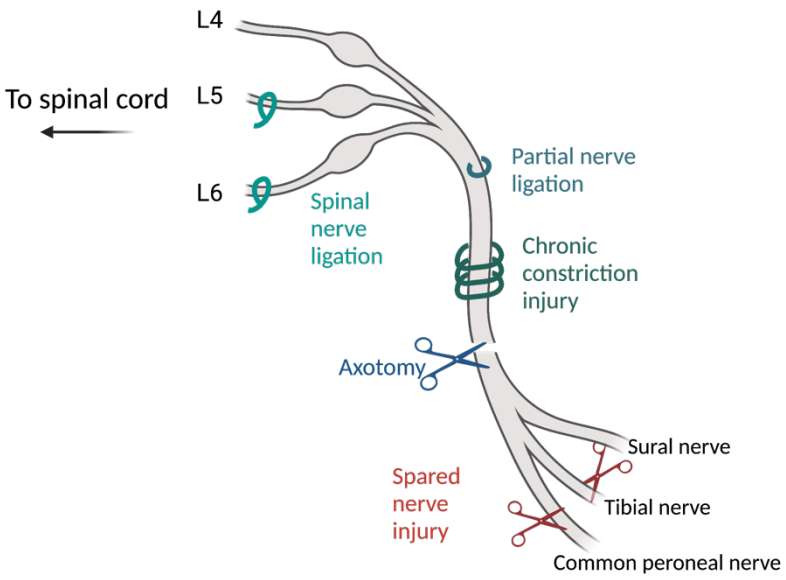

Figure 3. Nerve injury models in rodents.
Figure generated using BioRender, adapted from a review from Campbell & Meyer, 2006.

In the chronic constriction injury (CCI) model, loose constrictive ligatures of chromic gut are tied around the sciatic nerve, creating a mixture of intact and injured fibres and resulting in spontaneous pain behaviours, allodynia, and hyperalgesia (Bennett & Xie, 1988). The catgut sutures cause inflammation at the nerve, adding an immune component to the model (Tandrup et al., 2000). A partial nerve ligation model was developed, which involves the unilateral ligation of approximately half of the sciatic nerve (Seltzer et al., 1990). This model also produces rapid onset of pain-related behaviours and mimics spontaneous burning pain in humans (Stemkowski & Smith, 2011). The spinal nerve ligation (SNL) model ligates an entire spinal segmental nerve (L5 & L6) distal to the DRG and causes the complete transection of all axons. This model offers the opportunity of studying injured and uninjured afferents (Kim & Chung, 1992). The spared nerve injury model (SNI) lesions two of the three terminal branches of the sciatic nerve (usually the tibial and the common peroneal nerve) while sparing the remaining

(sural nerve); this produces pain-related behaviours such as cold and mechanical allodynia in the non-injured nerve territories (Decosterd & Woolf, 2000).

2.2.2. Painful Disease Assays

Rodent models that simulate clinical pain syndromes have been developed to study diseases such as diabetic neuropathy, cancer-associated, or chemotherapeutic agent-induced neuropathic pain. Neuropathic pain related to diabetes is most commonly induced in rodents by injection of the beta-islet cell cytotoxin streptozotocin (STZ), which causes hyperglycaemia and results in tactile allodynia and hyperalgesia, similar to symptoms seen in human neuropathy (Jakobsen & Lundbæk, 1976; Murakami et al., 2013). However, it has been observed that STZ can directly stimulate TRPA1 on sensory neurons, complicating the outcomes of STZ-induced neuropathy (Andersson et al., 2015). Other murine diabetic models, such as the non-obese diabetic (NOD) or leptin-deficient mouse, may be suitable alternatives to studying diabetic neuropathy (Drel et al., 2006; Gabra & Sirois, 2005).

Neurotoxicity is a common complication of chemotherapy treatment; it often manifests as peripheral neuropathy and is a major cause of ongoing pain in cancer survivors (Höke & Ray, 2014; Starobova & Vetter, 2017). Chemotherapeutic agent-induced models of pain are well-established in rodents. The administration of various chemotherapeutic agents such as paclitaxel, oxaliplatin, cisplatin, and vincristine have been described to produce neuropathic pain in rodents (Aley et al., 1996; Anand et al., 1995; Toma et al., 2017). Chemotherapy-induced peripheral neuropathy (CIPN) is most commonly characterised by paraesthesias, dysesthesia, numbness, and burning pain (Park et al., 2013). Nevertheless, measuring the numbness or tingling is difficult in rodents as most studies rely on evoked pain as an output measure. Cancer-associated pain can be caused by nerve compression or

the release of immune and pronociceptive substances from tumours (Pacharinsak & Beitz, 2008). Rodent models of bone pain using implantation of cancer cells result in allodynia and thermal hyperalgesia have been reported (Mouedden & Meert, 2005; Schwei et al., 1999).

Studies on the roles of autoantibodies in painful diseases have applied the passive transfer model in rodents. Recently, Goebel *et al*. demonstrated that the passive transfer of purified IgG from fibromyalgia syndrome (FMS) patients caused hypersensitivity to noxious mechanical and cold stimuli in mice (Goebel et al., 2021). Similarly, the passive transfer of CASPR2 autoantibodies from neuropathic pain patients resulted in mechanical pain-related hypersensitivity in mice (Dawes et al., 2018). The passive transfer model is particularly useful for elucidating the pathophysiology of autoantibodies in pain conditions.

2.2.3. Measuring pain-like responses

Since animals cannot self-report their severity of pain, the development of objective assays for pain-like behaviour in rodent models is vital. The most commonly used behavioural tests measure the withdrawal latency to a noxious stimulus (mechanical, thermal, chemical). These evoked withdrawal responses are helpful in the measure of hypersensitivity (allodynia and hyperalgesia). Additionally, tactile allodynia in rodent models mirrors the symptoms of mechanical hypersensitivity observed in patients (Costigan et al., 2009; Koltzenburg et al., 1994). The evoked withdrawal responses have been suggested to be spinal reflexes or simple innate behaviours, but others have shown that measurement of mechanical allodynia is a reliable surrogate for human mechanical pain hypersensitivity (Mogil, 2009; Reitz et al., 2016).

While many neuropathic pain patients experience hypersensitivity and allodynia, the most prevalent symptom is non-evoked spontaneous pain (Backonja

& Stacey, 2004). Non-evoked behavioural assays such as the grimace scale, home-cage monitoring, weight-bearing, free-choice, and operant assays have been developed and widely utilised to model spontaneous pain in rodents (Klinck et al., 2017; Mogil, 2009). The grimace scale grades the orofacial features in rodents that correlate to pain using a standardised behavioural coding system (Langford et al., 2010; Sotocinal et al., 2011). The grimace scale is similar to the one used in humans and is more pertinent to the symptoms in human patients than evoked measures of hypersensitivity. Unlike evoked measures that require the withdrawal of a body part from the applied stimulus, the grimace scale is generally not affected by functional motor deficits caused by nerve injury (Mogil et al., 2020). Efforts in measuring the naturalistic behaviours that may be suppressed after nerve injury have produced significant advances in home-cage monitoring systems that allow the tracking and analysis of multiple animals in the same cage. These systems reduce the confound of stress-induced analgesia and pain-masking behaviours often observed in rodents in the presence of experimenters (Bains et al., 2016; Burma et al., 2017; Zentrich et al., 2021)

Operant conditioning assays such as conditioned place preference/aversion are also used to measure ongoing and affective components of pain (King et al., 2009; Labuda & Fuchs, 2000). The conditioned place preference assay is based on Pavlovian conditioning, where analgesics or pain treatments are paired with a chamber in a two-chamber apparatus. Measurements of the change in the time spent in the treatment-associated chambers before and after conditioning quantify the animal's preference and indirectly assess the effectiveness of the applied treatment (Bannister et al., 2017; Griggs et al., 2015; King et al., 2009).

Models of spontaneous and on-going pain in rodents are invaluable in contributing to our understanding of the human pain condition. However,

compared to evoked-behaviour assays, spontaneous pain assays are much lower throughput, and extensive animal/experimenter training is required. Although some assays can be semi- or fully automated, as in the case of home-cage monitoring, analysis of a large amount of data is an enormous undertaking. Fortunately, advancements in high-speed videography and machine learning have been pivotal in developing more efficient, unbiased, and standardised analyses of animal behaviour.

2.2.4. The automated future

The development of animal tracking and pose approximation using machine learning and deep neural networks has led to the advent of automated behavioural assays. Platforms such as DeepLabCut and SLEAP can track multiple animals and can identify body parts of the animals (Lauer et al., 2022; Mathis et al., 2018; Pereira et al., 2022). These platforms are beneficial in home-cage monitoring and the automated measurement of pain-like behaviour (Bains et al., 2016; Wotton et al., 2020). A semi-automated pipeline has also been established for the automated grading of the facial grimace scale (Andresen et al., 2020). Recently, a free-moving behavioural assessment system ("Black box") has been described to objectively measure the naturalistic behaviour of rodents by combining weight-bearing and whole-body pose imaging. Assessment of pain hypersensitivity in this system is unbiased and observer-independent (Zhang et al., 2022).

Machine learning and neural networks can also be applied to evoked pain assays. High-speed videography and pose approximation software have resulted in reliable assays to quantify pain-related behaviours (Wotton et al., 2020). The development of software that quantifies and combines behavioural features into a univariate pain score obviates experimenter bias and allows the standardisation of analysis across research institutions (Jones et al., 2020). Despite significant

improvements in preclinical pain models, there is still a dearth of effective therapeutics due to the high rate of translational failure from animal models to human patients (Burma et al., 2017). The use of evoked and spontaneous pain assays in synergy with machine learning platforms will hopefully further our understanding of neuropathic pain and ultimately lead to the development of effective pain therapies.

3. CASPR2: Contactin-associated protein-like 2

Contactin-associated protein-like 2, CASPR2, was first described by Poliak *et al*. in 1999. It is a type I transmembrane protein widely expressed in the nervous system. It has a large multi-domain extracellular region with one transmembrane domain and a short cytoplasmic domain. CASPR2 is colocalized with shaker-type potassium channels in juxtaparanodes of myelinated neurons (Poliak et al., 1999)

3.1. Neurexin and CASPR Family

CASPR2 is a part of the Neurexin family of cell adhesion proteins, which play roles in synapse formation and function (Graf et al., 2004; Poliak et al., 1999). The neurexin family proteins are essential in mediating cell-cell interactions. They bind to postsynaptic cell-adhesion proteins (LRRTMs) and can induce excitatory or inhibitory synapses via interaction with their postsynaptic partners (Gokce & Südhof, 2013; Graf et al., 2004). CASPR2 belongs to the NCP subgroup (Nrx-IV/Caspr/Paranodin) of the neurexin superfamily, which also has the discoidin/neuropilin- and fibrinogen-like domains. CASPR2 contains epidermal growth factor repeats and laminin G domains like other neurexin proteins. Mutations in the neurexin proteins have been implicated in autism, schizophrenia,

and learning disabilities (Ahmad & Missler, 2009; Reichelt et al., 2012; Südhof, 2008).

CASPR2 is closely related to other CASPR proteins (CASPR1, CASPR3-5) in the CASPR protein family. These CASPR proteins are transmembrane proteins with similar structures, each with their functional specificity (Zou et al., 2017). The most notable member of the CASPR family is CASPR1, located at the paranodes. It has 45% amino acid identity to CASPR2 and also has a large extracellular domain and a 4.1B binding site. CASPR1 is located at the paranodes and binds with partner proteins Contactin-1 and Neurofascin-155 (NF155) to form a barrier between the nodes of Ranvier and internodes (Zou et al., 2017).

3.2. CASPR2 Gene

CASPR2 is encoded by the CNTNAP2 gene located in chromosomal region 7q35 (Poot, 2017). It is one of the largest genes in the human genome, with over 2.0Mb of DNA, and comprises 24 exons (Alarcón et al., 2008; Nakabayashi & Scherer, 2001; Poot, 2015). CNTNAP2 encodes five transcripts, 3 of which (Ensembl: CNTNAP2-201, 203, 207) are translated (Cunningham et al., 2022; Poot, 2015). The CNTNAP2-201 transcript encodes the 1331 amino acid residue full-length CASPR2 protein. The CNTNAP2-203 and CNTNAP-207 transcripts encode truncated proteins of 390 aa and 108 aa residues, respectively. The 108 aa residue protein (Uniprot: Q9UHC6-2) contains a short portion of the laminin-like G4 domain and the cytoplasmic region. The 390 aa residue protein (Uniprot: B7Z1Y6) contains the EGF-like 2 domain, laminin-like G4, and the transmembrane and intracellular domains (Poot, 2015). The CNTNAP2 gene contains binding sites to transcription factors: TCF4, STOX1A, and FOXP2 (Poot, 2017). FOXP2, a transcription factor related to speech and language development, has been shown

to bind and drastically downregulate CNTNAP2 (Vernes et al., 2008). There is 87% coding DNA homology and 94% amino acid homology between human and mouse CNTNAP2/CASPR2 (Poot, 2015).

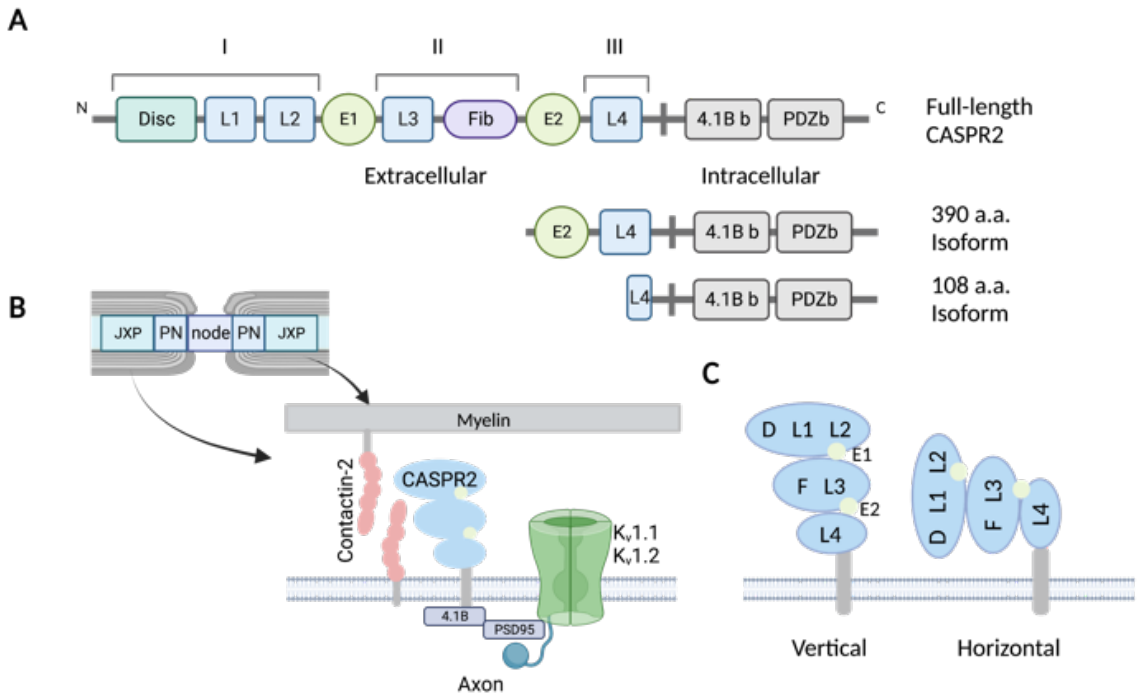

Figure 4. Structure and domain organisation of CASPR2
A) Schematic of the secondary structure of full-length CASPR2 and the two isoforms. **B)** CASPR2 is expressed in the juxtaranode (JXP) of myelinated axons, where it associates with partner proteins contactin-2 and shaker-type potassium channels. **C)** Schematic of the putative tertiary structure of CASPR2. CASPR2 is comprised of three lobes with molecular hinges that coincide with EGF-like repeats. CASPR2 can be present in the vertical or horizontal orientation. Figure is based on structures by Lu et al., 2016; Rubio-Marrero et al., 2016. Figure generated by BioRender.

3.3. Structure of CASPR2

3.3.1. Secondary Structure

CASPR2 is a type I transmembrane protein. The N-terminal extracellular domain is comprised of a signal peptide and eight subdomains. Starting from the most N-terminal domain: there is an F58C discoidin/neuropilin domain (Disc), laminin G-like G binding domains 1 and 2 (L1 and L2), an EGF-like domain (E1), a

fibrinogen-like region (F), a third laminin G-like domain (L3), the second EGF-like domain (E2), and finally the final laminin G-like domain (L4). There is a small transmembrane domain and a short, 48-residue intracellular domain containing the C-terminus (Poliak et al., 1999) (**Figure 4**). The intracellular domain contains a protein-4.1B binding motif (4.1Bb) and a PSD95/Disc large/Zona occludens-1 (PDZ) binding domain. The 4.1Bb motif mediates the binding of CASPR2 to 4.1B proteins, which link molecules to the actin/spectrin cytoskeleton (Poliak et al., 1999; Traka et al., 2003)

3.3.2. Tertiary Structure

Biochemical studies using size exclusion chromatography and dynamic light scattering (DLS) have shown the purified extracellular domain of CASPR2 (CASPR2-1261) as a monomeric protein (Lu et al., 2016; Rubio-Marrero et al., 2016). These studies estimate CASPR2 to be 145 Å long, 90 Å wide, and 50 Å thick, small enough that it can fit in the synapse in the vertical and horizontal orientation (Lu et al., 2016). CASPR2 is also heavily glycosylated as it contains 12 N-glycosylation sites (Poliak et al., 1999; Rubio-Marrero et al., 2016). Computation predictions suggest that there are 36 cysteine residues forming 18 disulfide bonds, which may affect its tertiary structure and binding to partner proteins (Lu et al., 2016; Poliak et al., 1999; Rubio-Marrero et al., 2016).

The architecture of CASPR2 was explored using negative staining electron microscopy (NS-EM) and showed an 'F-shaped' structure with three compact globular domains (Lu et al., 2016; Rubio-Marrero et al., 2016). Using nanogold labelling (Ni-NTA tagged nanogold binds to C-terminal histidine tag), antibody labelling (K67/25 binds to L4 region), and expression of domain fragments, Lu *et al.* were able to determine the domain organization within CASPR2. They showed that the large lobe contains F58C, L1, and L2; the medium lobe contains the

fibrinogen-like and L3 domains; and the small lobe contains the C-terminal L4 domains. Similar to Rubio-Marrero et al., their model also showed that the molecular hinges between the three lobes coincide with EGF-like repeats (Lu et al., 2016).

3.4. Isoforms and binding partners of CASPR2

3.4.1. Isoforms

Immunoprecipitation of cortex and hippocampal extracts from CASPR2 KO mice showed the presence of a shorter CASPR2 variant (isoform 2). This isoform corresponds to the C-terminal region of the canonical isoform 1 and is also predicted to be present in humans (Chen et al., 2015). It is the 108 aa protein encoded by transcript CNTNAP2-203 (Cunningham et al., 2022; Poot, 2015). Isoform 2 can interact with a subset of the typical CASPR2 interactors (LGI1, K_vB2, and ADM22). Interactions between isoform 2 and these partners likely depend on intracellular domains 4.1Bb and PDZb. This full-length CASPR2 is approximately ten times more abundant than the truncated isoform 2 (Chen et al., 2015). There is also an isoform of 390 aa, encoded by the CNTNAP2-203 transcript (Poot, 2015). Recently, a soluble, "shed", extracellular domain of CASPR2 has been identified. It is found in the cerebral spinal fluid (CSF) of mice and humans, with a size consistent with the cleaved extracellular domain of CASPR2 (Martín-de-Saavedra et al., 2022).

3.4.2. Binding Partners

Immunoprecipitation experiments have shown CASPR2 to interact with multiple proteins, including Contactin-2 (TAG-1/CNTN2), the ADAM proteins (ADAM22, 23, 11), LGI1, K_v1 channels, and membrane-associated guanylate kinase

proteins (MAGUKs) in mouse hippocampal lysates (Chen et al., 2015). The intracellular 4.1B-binding region of CASPR2 associates with the 4.1B proteins, which anchor the axonal CASPR2 to actin-based cytoskeletons (Denisenko-Nehrbass et al., 2003; Horresh et al., 2010). The association of the 4.1B protein has been demonstrated to be crucial for properly positioning CASPR2 and K_v channels in the juxtaparanode but is not responsible for the interaction between the two proteins (Horresh et al., 2010). The PDZ domain of CASPR2 binds to the scaffolding protein CASK and polarity protein PAR3 (Gao et al., 2018, 2020)

The main protein associated with CASPR2 is contactin-2 (also known as TAG-1, CNTN-2), a GPI-linked immunoglobulin-like cell adhesion molecule expressed in myelinated Schwann cells and oligodendrocytes (Traka et al., 2003). In the PNS, TAG-1 is expressed by DRG neurons and myelinating Schwann cells, allowing the interaction of with CASPR2 in both cis (expressed on the axon) or trans (expressed in the myelinating cell) orientations (Saint-Martin et al., 2019; Traka et al., 2003). CASPR2 and contactin-2 knockout experiments show that the localisation of the two proteins is interdependent and is necessary for the clustering of K+ channels at the juxtaparanode (JXP) (Poliak et al., 1999, 2003; Traka et al., 2003). Domain deletion experiments showed L4 as the domain associated with contactin-2 (Saint-Martin et al., 2019).

While many studies have shown the indirect interaction of CASPR2 and K_v channels, the exact mechanism of their association is currently unknown (Chen et al., 2015; Horresh et al., 2008; Poliak et al., 1999). It was previously thought that this interaction was mediated through the PDZ domains of PSD95; since the intracellular region of CASPPR2 contains a PDZ-binding domain, and K_v1.1 and 1.2 channels have been shown to bind to the PDZ domains of PSD95 (Poliak et al., 1999). However, it was shown that the PDZb domain of CASPR2 is not necessary

for the association with K_v channels. Furthermore, CASPR2 and K_v channels remain colocalized in PSD-95-null mice (Horresh et al., 2008).

3.5. Expression of CASPR2

CASPR2 is predominantly expressed in the nervous system. Northern blot analysis of CNTNAP2 mRNA expression in human tissues showed the presence of CNTNAP2 mRNA in the brain and spinal cord. Low but detectable CNTNAP mRNA was also found in the ovary and prostate (Poliak et al., 1999). In the adult mouse brain, CASPR2 was detected in many brain areas involved in sensory processing in all senses. The expression of CASPR2 is exceptionally strong in the cortex, hippocampus, substantia nigra, interpeduncle nucleus, pontine nucleus, and medial mammillary nucleus (Gordon et al., 2016). Comparisons of the CNTNAP2 transcript between humans and rodents revealed marked expression differences in the cortical regions, with high enrichment of CNTNAP2 in the anterior cortex in the human that is not found in the mouse or rats. These areas are involved in diverse higher cognitive processes, such as language, suggesting the importance of CASPR2 in cognitive functions (Abrahams et al., 2007). In the PNS, CASPR2 was detected in the footpad, whiskers, DRG, and dorsal part of the spinal cord of E15 mice (Gordon et al., 2016)

At the cellular level, CASPR2 is expressed primarily in neurons (Poliak et al., 1999). CASPR2 is highly expressed in myelinated nerve fibres (Dawes et al., 2018; Poliak et al., 2003). In the DRG, CASPR2 expression was found to be highest in the NF200-positive population (myelinated Aβ and Aδ afferents); expression in small diameter afferents was lower than in myelinated afferents, and very low levels of expression were seen in C-LTMRs (TH-positive, non-nociceptive C-fibres) (Dawes et al., 2018). CASPR2 can be differentially expressed in neuronal structures

at the subcellular level, including the soma, dendrites, or myelinated axons (Poliak et al., 1999). In the adult rat brain, it is expressed in the dendrite and cell bodies of pyramidal neurons of the hippocampus and cortex (Poliak et al., 1999). It is also found in regions with high concentrations of ion channels, namely the axon initial segment (AIS) in the CNS and the juxtaparanode region in the nodes of Ranvier (NOR) in myelinated neurons in the CNS and PNS (Inda et al., 2006; Poliak et al., 1999).

The NOR of myelinated neurons is crucial in effective saltatory conduction, and CASPR2 plays a vital role in the accumulation of K_v channels at the JXP (Horresh et al., 2010; Poliak et al., 2003). These channels are essential for membrane repolarization following an action potential and stabilize AP conduction by regulating repetitive firing (Lai & Jan, 2006). CASPR2 is also required for the positioning of contactin-2 and PSD-95, serving an important role in establishing axoglial contacts. (Horresh et al., 2008; Traka et al., 2003).

The AIS is the region separating the somatodendritic and axonal compartments in the neuron. It is highly enriched in Na^+ and K^+ channels and is where action potentials are generated (Leterrier, 2018). In human neocortical pyramidal cells, CASPR2 was localised to the distal domain of the AIS with $K_v1.2$ (Inda et al., 2006). However, in cultured hippocampal neurons, CASPR2 was not colocalized with K_v1 or contactin-2 in the AIS but was evenly distributed across the axon (Pinatel et al., 2017). Unlike in the JXP, CASPR2 is not required to localise K_v1 channels to the AIS (Ogawa et al., 2008). Whilst the AIS and JXP share common components of the voltage-gated potassium channel complex (VGKCC), the molecular mechanisms underpinning their organisations are distinct.

CASPR2 is also expressed in both inhibitory and excitatory synapses. Studies found it to be highly enriched in the synaptic subfractions of mouse

hippocampal lysates. Immunoblotting of synaptic fractions showed that CASPR2 is abundant in the lipid raft, synaptosome and synaptic membrane but is absent in the PSD fraction, suggesting that it is mainly located outside the PSD (Chen et al., 2015).

3.6. Syndromes associated with CASPR2

Given the widespread expression of CASPR2 in the CNS and PNS, mutations of CASPR2 have been linked to neurodevelopmental disorders such as autism spectrum disorder (ASD) and intellectual disability (Poot, 2015, 2017; Rodenas-Cuadrado et al., 2014). Patients with homozygous deletions of CNTNAP2 have severe intellectual disability, epilepsy, language impairments, and features of ASD (Rodenas-Cuadrado et al., 2014). Most mutations of CNTNAP2 are heterozygous, suggesting that the loss of a single allele is sufficient to disrupt protein function (Poot, 2017; Rodenas-Cuadrado et al., 2014). Genetic knock-out of CNTNAP2 in mice resulted in seizures and the development of autism traits (Peñagarikano et al., 2011).

CASPR2 antibodies have also been linked to neurodevelopmental disorders. Maternal-to-foetal transfer experiments in mice using CASPR2 antibodies from a mother of a child with ASD resulted in ASD-like behaviour in the injected mice. These mice also had altered brain morphology, like reductions in the number of inhibitory neurons in the hippocampus (Brimberg et al., 2016). Diseases that result from the alterations of CASPR2 highlight the importance of functional CASPR2 in neuronal development and connectivity in both the CNS and PNS.

4. CASPR2 Autoantibodies

4.1. Anti-Voltage-gated potassium channel complex antibodies

Voltage-gated potassium channels are crucial in modulating peripheral neuron excitability (Lai & Jan, 2006; Rasband et al., 2001). Autoantibodies toward the voltage-gated potassium channel complex have been identified in patients with neuronal hyperexcitability disorders such as limbic encephalitis (LE) and neuromyotonia (NMT) (Hart et al., 1997; Irani et al., 2010). While many patients with anti-VGKC complex antibodies experience pain with other neurological symptoms, pain had not been the focus of the manifestation of VGKCC autoimmunity until the early 2010s (Irani et al., 2010; Klein et al., 2012). Of 316 patients with anti-VGKCC antibodies evaluated neurologically at the Mayo Clinic, 159 (50%) had pain, indicating the involvement of the hyperexcitability of the nociceptive pathway. Furthermore, 80% of patients treated with immunotherapies showed improvements in their pain, suggesting that these autoantibodies may be causative to pain (Klein et al., 2012).

Historically, the anti-VGKC antibodies were measured using the radioimmunoprecipitation assay (RIA) using mammalian brain lysates labelled with ^{125}I-α-dendrotoxin (Hart et al., 1997; Shillito et al., 1995). However, most of these antibodies failed to bind to cells transfected with VKGCs. Instead, the antibodies bound to synaptic and axonal neuronal proteins associated with the complex that co-precipitate with detergent-solubilised VGKCs. The main autoantigens identified were CASPR2 and LGI-1 (Irani et al., 2010). Anti-VGKC complex antibodies that do not bind to CASPR2 and LGI-1 were classified as 'double-negative' antibodies and, unlike antibodies toward CASPR2 and LGI, do not have well-delineated clinical syndromes (Michael et al., 2020). The 'double-negative' antibodies did not bind to live hippocampal neurons and were shown

instead to target intracellular epitopes of VGKCs, and therefore are thought to lack pathogenic potential (Lang et al., 2017).

4.2. Detection of Autoantibodies

The current gold-standard detection method for autoantibodies in diagnostic testing is the cell-based assay (CBA). HEK293 (human embryonic kidney) cells are transfected with the antigen of interest, and patient serum or CSF is applied to the transfected cells at varying concentrations (Irani et al., 2012; Lang & Prüss, 2017; Michael et al., 2020). The binding of autoantibodies is measured using a fluorescent antibody detected by microscopy or flow cytometry. Transfection in a mammalian cell line allows the expression of the protein in the native conformation with mammalian post-translational modifications (Irani et al., 2012; Miller et al., 2021; van Coevorden-Hameete et al., 2016). The CBA also offers the option of detecting intracellular antigens by fixation and permeabilization of the transfected cells. However, fixation of cells comes with the caveat of potentially altering native epitopes (Lang & Prüss, 2017; Miller et al., 2021). Similarly, serum or CSF samples can be added to rodent primary neuronal cultures (e.g. hippocampal or DRG) for assessment of binding in a more native environment in the presence of all neuronal accessory proteins (Michael et al., 2020; Ramanathan et al., 2021). These assays are more time- and resource-intensive, as primary cells need to be isolated from rodent tissue and cultured for up to a few weeks before assay (Lang et al., 2017; Pinatel et al., 2015; Ramanathan et al., 2021).

Immunohistochemistry or tissue-based assays (TBA) can be performed to assess the binding of patient serum or CSF on primate or rodent neural tissue sections. Tissues such as brain, spinal cord, nerve, or DRG are frozen and cryosectioned before the application of patient samples. This method offers insight

into binding of autoantibodies to different regions or particular cell types in the brain or the (Miller et al., 2021; van Coevorden-Hameete et al., 2016). For example, sera from Morvan's syndrome patients have been shown to bind to orexin and vasopressin neurons in the hypothalamus (Irani et al., 2012).

Radio Immunoprecipitation Assays (RIA) were commonly used to detect antibodies toward protein and synaptic complexes. In these assays, radioactively labelled toxins with high affinity for their receptor proteins are incubated with brain lysates. Patient serum or CSF is incubated with the labelled lysates, and bound antibodies are precipitated with anti-human IgG sepharose. The bound autoantibodies are then measured with a gamma counter and quantified using a standard curve (Irani et al., 2010; Lang et al., 2017; Pozo-Rosich et al., 2003). These assays are highly sensitive due to the detection of radioactivity. However, unlike the CBA, the RIA captures antibodies against protein complexes instead of single antigens. Subsequent assays are required to determine the specific antigen (Miller et al., 2021). Additionally, detergent solubilisation of brain tissues may lead to misfolding of the target proteins, altering the epitopes and affecting antibody binding (van Coevorden-Hameete et al., 2016).

4.3. Anti-CASPR2 antibodies and clinical syndromes

Anti-CASPR2 antibodies are associated with a spectrum of clinical syndromes. Limbic encephalitis or Morvan's syndrome (MoS) are the most common syndromes associated with patients with anti-CASPR2 antibodies. Morvan's syndrome is a rare constellation of symptoms, consisting of peripheral nerve hyperexcitability (neuromyotonia), encephalopathy, insomnia (Irani et al., 2012). The prime symptom of limbic encephalitis is severe impairment of short-term memory that is often accompanied by seizures. Other symptoms include,

confusion, loss of consciousness. The symptoms of these syndromes overlap significantly and may include cerebral or cerebellar symptoms, muscle stiffness and twitching, and neuropathic pain. Studies have shown that antibodies toward CASPR2 are more common in patients with pain than those without pain (Irani et al., 2010; Klein et al., 2012; Ramanathan et al., 2021).

The most common clinical features associated with anti-CASPR2 antibodies are limbic encephalitis (18-51%); seizures (55%); cognitive impairment (55-79%); and peripheral nerve hyperexcitability (13%) (Gadoth et al., 2017; Muñiz-Castrillo et al., 2020; Van Sonderen et al., 2016a). 46-61% of these patients reported having pain (Gadoth et al., 2017; Irani et al., 2010; Van Sonderen et al., 2016a). Thymomas were also seen in many patients with Morvan's disease (Joubert et al., 2016; Muñiz-Castrillo et al., 2020; Vincent & Irani, 2010). Treatment of CASPR2 autoantibody patients include steroids, intravenous immunoglobulin (IV-IG), and second-line treatments rituximab or cyclophosphamide (Van Sonderen et al., 2016b).

Rodent studies have demonstrated the pathogenic potential of CASPR2 autoantibodies. The disruption of CASPR2 by autoantibodies has been shown to reduce $K_v1.1$ expression in DRG neuron cell bodies and cause neuronal hyperexcitability in primary afferents. The passive transfer of anti-CASPR2 antibodies in mice also induced mechanical hypersensitivity and increased pain-like behaviours, implicating that CASPR2 antibodies may be causative to increased pain sensitivity in patients (Dawes et al., 2018). Administration of CASPR2 antibodies in conjunction with disruption of the blood-brain barrier in mice resulted in cognitive and behaviour changes such as reductions in short- and long-term memories, altered social interactions, and reduced non-social behaviours (Giannoccaro et al., 2019). The observed behavioural changes are similar to clinical features in patients with CASPR autoantibody who experience central and

peripheral symptoms (Giannoccaro et al., 2019; Van Sonderen et al., 2016a). These rodent studies highlight the pathogenic effects that anti-CASPR2 antibodies may exert on the CNS and PNS in patients.

4.4. Epidemiology and Properties of CASPR2 Autoantibodies

4.4.1. Epidemiology and genetic association

Patients with CASPR2 antibodies are predominantly older males with a median age of symptom onset at around age 60 (Irani et al., 2012; Van Sonderen et al., 2016a). Rare paediatric cases have been reported in children as young as two years of age (Syrbe et al., 2020). The female: male ratio in younger patients is 6:8, in contrast with older patients with strong male dominance (Irani et al., 2012; Jensen et al., 2011). A comparative analysis of human leucocyte antigen (HLA) and CASPR2-autoantibody mediated diseases showed a strong HLA-DRB1*11:01 association in CASPR2-antibody patients (Binks et al., 2018; Ramanathan et al., 2021). The HLA-DRB1*11:01 allele was found to be carried more frequently only in patients with limbic encephalitis. Conversely, this association was absent in patients with peripheral nerve excitability (Muñiz-Castrillo et al., 2020).

4.4.2. Properties of CASPR2 antibodies

In patients with CASPR2 autoantibodies, the antibody titres in the serum are higher than in the CSF (Bien et al., 2017; Joubert et al., 2016). Differences exist in the clinical manifestations of patients with antibodies in their serum or CSF. A retrospective cohort analysis of patients with anti-CASPR2 antibodies exclusively in their CSF showed an association with a subtype of autoimmune encephalitis and seizures. The high antibody index in the CSF of these patients indicates intrathecal synthesis of autoantibodies. In contrast, patients with NMT or MoS had anti-

CASPR2 antibodies only in their serum, indicative of the synthesis of autoantibodies outside of the CNS (Joubert et al., 2016). Antibody levels also differ in patients. Anti-CASPR2 antibody titres in the serum range from 1:80 – 1:52000 (Joubert et al., 2016; Ramanathan et al., 2021). CASPR2-antibody levels were the highest in patients with limbic encephalitis (Muñiz-Castrillo et al., 2020). Several studies have reported IgG4 and IgG1 as the most predominant antibody subclasses in CASPR2 antibody patients (Joubert et al., 2016; Patterson et al., 2018; Van Sonderen et al., 2016a). These subclasses are thought to influence their mechanisms of action (Patterson et al., 2018; Vidarsson et al., 2014).

CASPR2 autoantibodies have a wide variety of epitopes toward all extracellular regions. Using domain deletion constructs, Olsen *et al*. revealed that CASPR2 antibodies in patient sera target multiple epitopes in the extracellular domain, and some of these epitopes are not glycosylation or tertiary structure-dependent (Olsen et al., 2015). The N-terminal discoidin and Laminin G1 domains are the most common epitopes, and most patients with CASPR2 autoantibodies have antibodies toward these two domains (Joubert et al., 2016; Olsen et al., 2015; Saint-Martin et al., 2018). The common epitopes in all patients suggest the potential link between CASPR2-Ab epitopes and disease genesis. Overall, the antibody titre, location of antibody production, and antibody subtype and epitope all play a role in the multifaceted clinical presentations in patients with anti-CASPR2 antibodies.

4.5. Mechanisms of action

The potential mechanisms of action of autoantibodies described in the section 'Autoantibody mechanisms' have been elucidated and described in diseases such as neuromyelitis optica (NMO) or autoimmune encephalitis (AIE) (Hinson et al., 2017; Masdeu et al., 2016). The pathogenic mechanism of CASPR2 antibodies is

currently unclear. Studies have shown that CASPR2 antibodies may block the interaction of CASPR2 and Contactin-2 (Patterson et al., 2018; Pinatel et al., 2015). Patterson et al. showed that patient sera applied on immobilised CASPR2 inhibited the association of Contactin-2 in a solid phase binding assay. However, these studies were done on heterologously expressed CASPR2 or using soluble antigens, and the direct inhibition of binding of contactin-2 to CASPR2 has not been shown in neurons thus far (Pinatel et al., 2015).

Current studies of CASPR2 autoantibodies have focused on the binding and effects of patient sera on hippocampal neurons (Giannoccaro et al., 2019; Joubert et al., 2022; Lang et al., 2017; Saint-Martin et al., 2019). A few of these studies have detected the internalisation of CASPR2 in hippocampal neurons, contrary to what Patterson *et al*. have observed (Giannoccaro et al., 2019; Joubert et al., 2022; Patterson et al., 2018). Given that most CASPR2 autoantibody patients have IgG4 as the most prevalent subclass of antibody in serum, it is unexpected to see internalisation *in vivo*. IgG4 antibodies undergo Fab arm exchange and are functionally monovalent (Vidarsson et al., 2014). However, Fab fragments from LGI1 antibodies have been demonstrated to be internalised; it is possible that fab arm exchange could generate bivalent antibodies that target the same antigen (Jentzer et al., 2022; Ramberger et al., 2020). Current published studies utilised polyclonal patient sera, and there may be other mechanisms in disease pathogenicity that can be further elucidated by examining a larger patient pool. Given the diversity of epitope and affinity of these patient antibodies, it is likely that numerous mechanisms may occur concurrently in disease aetiology.

5. Aims

Previous work using genetic and immune disruption of CASPR2 has demonstrated the role of CASPR2 in regulating pain hypersensitivity. Autoantibodies to CASPR2 caused increased neuron excitability by disruption of K_v1 channel expression and elicited pain-like behaviour in mice. In a CASPR2 knock-out mouse model, loss of K_v1 membrane expression and hyperexcitability in sensory neurons and neuropathic pain-like behaviour was observed. Following these results, increased expression of CASPR2 in PSNs led to reduced sensory neuron excitability *in vitro*- Implying that in the context of neuropathic pain (i.e. hyperexcitable PSNs), modulating CASPR2 expression could be a viable therapeutic approach (Dawes et al., 2018). Therefore, this project endeavours to understand the effect of CASPR2 overexpression on neuropathic pain using preclinical models and explore whether increasing CASPR2 levels in sensory neurons is a feasible treatment for neuropathic pain.

While there are studies of the mechanisms of CASPR2 Autoantibodies in the CNS, our understanding of their roles in the PNS is still lacking. Hence, I aim to determine the mechanism of action CASPR2-Abs in the pathophysiology of neuropathic pain. I utilise rodent peripheral neurons and examine the binding and effects of polyclonal and monoclonal Abs from CASPR2-IgG patients *in vitro.*

6. Acknowledgements

Figures were generated with BioRender. Figure 1 was adapted from and based on figures by von Hehn et al., 2012 and Finnerup et al., 2021. Figure 2 was based on a figure from Lacagnina et al., 2021a. Figure 3 was adapted from a review from Campbell & Meyer, 2006.

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2

CASPR2 Overexpression

1. Introduction

Neuropathic pain is a widespread chronic pain condition that affects 7-10% of the general population (Van Hecke et al., 2014). There is an urgent need for targeted therapeutics for neuropathic pain, as most patients do not respond to current therapies. Management of neuropathic pain is further challenged by adverse effects, such as dependence and tolerance, that limit their use at effective doses (Finnerup et al., 2015; Labianca et al., 2012). Understanding the induction and mechanism of neuropathic pain is crucial for further developing novel, targeted therapies necessary for the efficacious treatment of neuropathic pain.

Studies have shown that hyperexcitability in primary sensory neurons is a significant contributor to neuropathic pain conditions (Tsantoulas & McMahon, 2014; Woolf & Mannion, 1999). Voltage-gated potassium channels (VGKCs), K_v1 channels in particular, are essential in limiting neuronal excitability (D'adamo et al., 2020; Tsantoulas & McMahon, 2014) and the disruption of these channels have been shown to contribute to neuropathic pain in preclinical models (Kim et al., 2002; Zhao et al., 2013).

1.1. K_v1 channels and neuropathic pain

$K_v1.1$ and $K_v1.2$ are shaker-type potassium channels that are delayed rectifiers activated by membrane depolarisations. In the peripheral nervous system, these channels are found in the soma and juxtaparanodes of medium- to large-sized DRG neurons (Rasband et al., 2001; Tsantoulas & McMahon, 2014). K_v1 channels influence the threshold and frequency of action potential firings in primary sensory neurons and thus play a role in regulating neuronal excitability (Busserolles et al., 2016). Nerve injury leads to increased spontaneous activity of

afferents, and the alteration of ion channel expression following nerve injury is thought to be a key driver in the genesis of neuropathic pain (Finnerup et al., 2021; Wood et al., 2004; Woolf & Mannion, 1999).

Studies have shown the reduction of K_v1 channels at the protein or transcript levels after nerve injury in rodent models, suggesting that multiple mechanisms may be occurring transcriptionally or post-translationally in the regulation of K_v1 channel expression (Calvo et al., 2016; Kim et al., 2001, 2002; Rasband et al., 2001). Using a spinal nerve ligation model of nerve injury, Rasband *et al*. showed a substantial decrease of $K_v1.1$ and $K_v1.2$ protein in the ipsilateral DRG after injury (Rasband et al., 2001). Others have corroborated the decrease in K_v1 channel mRNA after injury (Kim et al., 2001, 2002; Yang et al., 2004). The attenuation of K_v channel function after nerve injury was supported by a study by Abdulla and Smith, where they demonstrated that axotomy caused a significant reduction of K^+ current in DRG neurons, suggestive of a correlation between this decrease in K^+ current and the observed nerve injury-induced increase in excitability (Abdulla & Smith, 2001). The decreased expression of K_v1 channels in the DRG has also been shown to contribute to neuropathic pain genesis. The downregulation of $K_v1.2$ after nerve injury produced neuronal hyperexcitability in the DRG and pain-like behaviour in neuropathic rats, but the rescue of $K_v1.2$ expression attenuated the nociceptive phenotype (Fan et al., 2014).

Significant efforts have been made to elucidate the mechanisms underlying the reduction of K_v1 channels after nerve injury. Epigenetic mechanisms, in particular, have been well-studied. DNA methylation represses gene transcription by physically restricting access of transcription factors or acting as docking sites for transcriptional repressors. The DNA methyltransferases DNMT1 and DNMT3a are upregulated via parallel and independent pathways after spinal nerve ligation

injury. These upregulated DNA methyltransferases cause the downregulation of the K_v1.2 transcript, *Kcna2*, by hypermethylation in promoter regions, leading to reduced K_v1.2 expression in the DRG (Mo et al., 2018; Sun et al., 2019; Zhao et al., 2017). Notably, the blockade of DNA methyltransferases reduced hyperexcitability in DRG neurons and attenuated pain hypersensitivity in neuropathic rodents, while the upregulation of activators of DNA methyltransferases led to increased pain sensitivities in naïve mice (Sun et al., 2019; Zhao et al., 2017).

Other epigenetic mechanisms involving histone modification have been described to contribute to reducing K_v1 channel expression after nerve injury. The Euchromatic histone-lysine N-methyltransferase 2, G9a, was found to be upregulated in DRG neurons after axotomy. G9a methylates histone H3 and consequently causes chromatin condensation and transcriptional repression of genes, including *Kcna2*. Blocking the increase of G9a in the DRG after nerve injury rescued K_v1.2 expression and attenuated neuropathic pain. Conversely, the overexpression of G9a induced neuropathic pain-like behaviours (Liang et al., 2016). Histone deacetylases, HDAC1 and HDAC2, have also been found to be upregulated after nerve injury, and the inhibition of HDACs rescued the decreased K_v1.2 expression and mechanical and thermal hypersensitivity in neuropathic rats (Li et al., 2019).

Non-coding RNAs have been implicated in the downregulation of K_v1.2 after nerve injury. The non-coding miniature RNA, miR-137, was discovered to be upregulated concurrently with K_v1.2 downregulation in the DRG after chronic constriction injury. The inhibition of miR-137 rescued K_v1.2 expression, reduced DRG neuronal hyperexcitability, and alleviated mechanical allodynia and thermal hyperalgesia in neuropathic rats (Zhang et al., 2021). Long non-coding RNAs (lncRNAs) have also been described to contribute to neuropathic pain by

regulating the gene expression of K_v1.2. Zhao *et al*. identified a *Kcna2* antisense RNA upregulated in medium-large DRG neurons after nerve injury. This lncRNA reduced the expression of K_v1.2 by inhibiting *Kcna2* mRNA and increased the excitability in DRG neurons. Blocking the upregulation of the *Kcna2* antisense RNA attenuated neuropathic pain (Zhao et al., 2013).

Post-translational processes may also play a role in the injury-induced downregulation of K_v1 channels. A recent study uncovered a role of a cap-dependent translation repressor, eIF4G2, in neuropathic pain. Increased expression of eIF4G2 was found in the DRG after spinal nerve ligation, which likely contributes to nerve injury-induced pain hypersensitivity by repressing K_v1.2 mRNA translation in injured DRGs. Reduction of eIF4G2 by siRNA in injured DRGs attenuated the downregulation of K_v1.2 after nerve injury and reduced nociceptive hypersensitivities (Zhang et al., 2021). The expression and localisation of K_v1 channels can also be regulated by post-translational phosphorylation of the channels. Tyrosine phosphorylation of the K_v1.2 channel at the N-terminus facilitated the endocytosis of the channel and suppressed its ionic current (Nesti et al., 2004). A study by Yang *et al*. also disclosed that K_v1.2 channel trafficking is regulated by a cluster of C-terminal phosphorylation sites. They found that phosphorylation of the S440/441 sites is restricted to the cell-surface population of K_v1.2 channels, indicating that the phosphorylation status may alter the membrane expression of the channel (Yang et al., 2007). These studies suggest that alterations to the activity of protein kinase and phosphatases may modulate the surface expression levels of K_v1.2.

Multiple mechanisms at the transcriptional and post-translational levels contribute to the downregulation of K_v channels after nerve injury. Therefore, efforts to attenuate the decrease in K_v channel expression can be applied at many

levels. For instance, the delivery of K_v1.2 sense RNA into injured DRG neurons blocked the lncRNA and attenuated neuropathic pain (Zhao et al., 2013). Moreover, the direct over-expression of K_v1.2 RNA in a nerve injury model blocked the development and maintenance of neuropathic pain, lending support to K_v1 upregulation as a potential therapeutic (Fan et al., 2014).

1.2. CASPR2 and neuropathic pain

CASPR2 is a transmembrane protein in the VGKC complex and has been shown to interact with K_v1 channels. The association of CASPR2 with contactin-2 is necessary for the clustering of VGKCs at the juxtaparanode (Poliak et al., 1999, 2003). The importance of CASPR2 in regulating neuron excitability is underscored by a study in which the genetic ablation of CASPR2 in mice caused a reduction in K_v1 membrane expression, resulting in neuron hyperexcitability and neuropathic pain-like behaviour. Conversely, *in vitro* overexpression of CASPR2 in sensory neurons led to neuron hypoexcitability through increased K_v1 channel activity (Dawes et al., 2018). Furthermore, autoantibodies toward CASPR2 have been identified in patients with neuropathic pain (Irani et al., 2010; Ramanathan et al., 2021) and these CASPR2 autoantibodies can cause pain by disrupting K_v1 channel function in sensory neurons, further adding to the evidence of CASPR2-disruption contributing to neuropathic pain (Dawes et al., 2018).

In a spared-nerve injury model of neuropathic pain in mice, CASPR2 mRNA was significantly down-regulated in PSNs at time points coincident with pain-related behaviour (unpublished data). This downregulation of CASPR2 mRNA was pronounced in the ATF3-positive, nerve-injured neuron population, which is crucial in the development and maintenance of neuropathic pain (Tsujino et al., 2000). The disruption of CASPR2 after nerve injury may alter K_v1 channel

expression and contribute to neuron hyperexcitability. The known lncRNA, *rno-Cntnap2*, was determined to be down-regulated after nerve injury, although the effects of this downregulation of Cntnap2 lncRNA are unclear (Mao et al., 2018). These findings suggest CASPR2 is a potential regulator of neuronal excitability through its association with K_v1 channels. The introduction of CASPR2 in HEK cells stably expressing $K_v1.2$ increased $K_v1.2$ channel expression on the cell surface (Saint-Martin et al., 2019). Hence, increasing CASPR2 expression in sensory neurons could be a viable treatment for neuropathic pain.

The widespread expression of K_v channels in cell types other than primary afferents precludes the use of K^+ modulators as safe therapeutics. The delivery of $K_v1.2$ RNA, or rescue of K_v1 channels by blockade of Kcna2 antisense RNA have been demonstrated in nerve injury models, and proven efficacious in reducing neuropathic pain (Fan et al., 2014; Zhao et al., 2013). The close association of CASPR2 with K_v1 channels in cell populations and subcellular locations crucial in regulating neuronal excitability may offer advantages in a targeted approach to modulating K_v1 channels. This targeted overexpression in physiologically relevant environments may mitigate any unwanted off-target effects of widespread channel expression in other cell populations.

1.3. Aims

In this study, I investigate the effects of CASPR2 overexpression *in vivo* and evaluate CASPR2 as a potential therapeutic target for modulating neuron hyperexcitability. I utilised transgenic mouse models that overexpresses CASPR2 in nociceptors ($Na_v1.8$ Cre: $R26^{LSL:hCNTNAP2(+/+)}$) or in DRG neurons (Hoxb8 Cre: $R26^{LSL:hCNTNAP2(+/+)}$). First, I validate and confirm the transgenic lines for overexpression of CASPR2. Second, we assess the thermal and mechanical sensitivity in naïve and nerve-injured mice and evaluate the effect of CASPR2

overexpression in nerve injury models. Finally, I explore the therapeutic potential of adenoviral vectors to deliver recombinant CASPR2 *in vivo*.

2. Methods

2.1. Mouse lines and animal care

2.1.1. Animal Care

All procedures were carried out in accordance with UK home office regulations and the Animals Scientific Procedures Act 1986 at a licensed facility within the University of Oxford. Animals were group housed in IVC cages on a 12-hour light-dark cycle in temperature and humidity-controlled rooms, with food and water available *ad libitum*.

The ARRIVE guidelines were consulted and followed for all animal experiments. Study design, sample sizes, randomisation and blinding were determined in accordance with the ARRIVE guidelines. Sample sizes were chosen based on a power calculation using historical data relating to mechanical and thermal threshold responses (alpha error of 0.05 and a power of 80%). Calculations showed that groups of 8 would be needed using the assumption that an effect size of 25% would be biologically meaningful.

Both male and female mice were used in this study. Mice were tested at a consistent time of day in the same designated room. No animal was excluded in this experiment. Prior to behavioural tests, mice were acclimatised to their testing environment and equipment for a minimum of 30 minutes. The experimenter was blind to animal genotype prior to testing and until after behavioural analysis was complete.

2.1.2. Generation of transgenic lines

All mice were maintained on a C57Bl/6J background. The transgenic mouse line $R26^{LSL:hCNTNAP2(+/+)}$ was made in collaboration with Dr Ben Davies (Wellcome Trust Centre for Human Genetics). The transgenic mouse line contains a floxed stop codon and an exogenous human (hu) CASPR2 placed in the Rosa26 locus under the control of the neuronal CAG promoter. The Cre-dependent removal of the stop codon upstream of huCASPR2 will drive the overexpression of huCASPR2 in specific neuron populations expressing the Cre recombinase.

The $R26^{LSL:hCNTNAP2(+/+)}$ mouse line was crossed with a $Nav1.8^{Cre}$ line provided by J Wood (UCL) to overexpress CASPR2 specifically in nociceptors ($Na_v1.8^{Cre}$:$R26^{LSL:hCNTNAP2(+/+)}$). Heterozygous Cre^+ mice ($Nav1.8^{Cre(-/+)}$:$R26^{LSL:hCNTNAP2(+/+)}$) was bred with Cre^- mice ($Nav1.8^{Cre(-/-)}$:$R26^{LSL:hCNTNAP2}$) to ensure that all offspring are experimentally relevant (i.e. 50% Cre^+, 50% Cre^-).

Similarly, to obtain overexpression in all neurons in the dorsal root ganglion (caudal to C4 spinal segment), the $R26^{LSL:hCNTNAP2(+/+)}$ mouse line was crossed with the $Hoxb8^{Cre}$ line (U Zeilhofer, ETH). To overexpress huCASPR2 in all sensory neurons I crossed the $R26^{LSL:hCNTNAP2(+/+)}$ mice with the tamoxifen-inducible $Advillin^{CreERT2}$ mice (J Wood, UCL) to create the $Advillin^{CreERT2}$:$R26^{LSL:hCNTNAP2(+/+)}$ transgenic mouse line, which will overexpress huCASPR2 upon tamoxifen treatment.

Cre recombinase in all transgenic mice was detected by PCR of genomic DNA. Primers for Cre (Forward 5′-agcctgttttgcacgttcacc-3′, Reverse 5′-ggtttcccgcagaacctgaa-3′) and PCR control primers (Forward 5′-cctagcacccacccaaagagctg-3′, Reverse 5′-ggtcctcactggcagcagctgca-3′) (Liput, 2018).

2.1.3. Spared nerve injury

Mice were anaesthetised using 2% isoflurane and prepared for surgery by shaving and sterilising the thigh. A small incision was made to the thigh skin parallel to the thigh bone. The sciatic nerve was identified through separation of muscle and connective tissues. The point where the sciatic nerve trifurcates was identified, and sutures were tied around the nerves for transection. Non-absorbable sutures were used for the nerve ligation. For the spared sural nerve injury model, the common peroneal and tibial nerves were ligated, and the nerves were transected while removing ~1 mm of the transected nerve. For the spared tibial nerve injury model, the sural and common peroneal nerves were ligated separately and transected while removing ~1 mm of the transected nerves to prevent regeneration. The incision site was closed with an absorbable suture and the ski was closed surgical staples. Appropriate post-operative pain medications care given with a local injection of 2 mg/kg Marcain (AstraZeneca), and a systemic injection of 5 mg/kg Rimadyl (Pfizer). Mice were monitored daily for changes to wound healing, autotomy, and body weight.

2.1.4. Adenoviral transduction *in vivo*

Adenoviruses containing CASPR2-EGFP (AAV C2-EGFP) or EGFP (AAV EGFP) vectors were delivered by subcutaneous injection at the nape of the neck of neonatal (P5) mice C57Bl/6 mice. 10 μl of virus (2.2×10^{13} vector genomes/mL) was injected in each mouse. Mice were tattooed on their forepaws for identification of virus injected. Mice were placed in their home cage with their mothers. The injected mice were tested for behaviour at 8 weeks of age.

2.2. Behavioural tests

2.2.1. Mechanical sensory testing

von Frey hairs

The same designated room was used for all behavioural studies and testing was performed at a consistent time of day. Mechanical sensitivity was assayed using von Frey filaments. Mice were randomly assigned and acclimatized to a test box (5x5x10cm) elevated on a wire mesh base. Mice were tested on their plantar hind paws using calibrated von Frey hairs (Linton Instrumentation) using the 'up-down' method (Chapman et al., n.d.; Dixon, 1980) to evaluate their 50% paw withdrawal thresholds. Averages were taken from three measurements per paw on at least three different days.

Pinprick

Pinprick test assesses the response to noxious mechanical stimulus. Mice were randomly assigned and acclimatized to a test box (5x5x10cm) elevated on a wire mesh base. Mice were then tested on their plantar hind paws using a dissection pin attached to a 1g calibrated von Frey filament (Arcourt et al., 2017). The latency to withdrawal was recorded using an iPhone XS (Apple) at 240 fps (4.14 ms / frame) and analysed using Avidemux 2.7.2. Responses from left and right paws were averaged on three different days.

Dynamic brush

Mice were randomly assigned and acclimatized to a test box (5x5x10cm) elevated on a wire mesh base. A small paintbrush (5-0, the Art Shop) was brushed on the plantar surface of the mouse hindpaw from the heel to the toes at approximately 2 cm/s. Responses are scored on a scale from 0 – 3. 0: no response or moving or lifting of the paw for less than 1 second. 1: Sustained lifting of the paw or a single flinch. 2: quick withdrawal and latera paw lift above the level of the body 3: Intense, quick

withdrawal of the paw accompanied by flinching and licking or biting of the paw. Averages were taken from four measurements per paw on at least three different days.

2.2.2. Thermal sensory testing

Hargreaves Test

Thermal sensitivity was assessed using the Hargreaves method. Mice were randomly assigned a test box (5x5x10cm) elevated on glass floor and were acclimatised for 30-60mins. Mice were then tested on the plantar surface of their hind paws using the Basile Plantar test apparatus (Ugo Basile), which provides a radiant laser heat source. The latency for mice to withdraw their hind paws was recorded and taken as a measure of nociceptive threshold to radiant heat. Averages were taken from three measurements per paw on three different days.

50/53°C Hotplate

Mice were placed onto a Perspex enclosed Hotplate (Ugo Basile) set at 50 or 53°C and were observed until mice displayed pain behaviours on their hind paws (lifting, flicking, licking of the hind paw). The latency to respond was recorded and mice were tested on 3 different days to obtain average baseline value. The experiment is set at cut off at 20 s to prevent tissue damage.

Dry Ice Assay

Mice were placed into a randomly assigned test box elevated on a glass floor and acclimatized for 30 – 50 mins. A 3 ml syringe was filled with dry ice by removing the tip of the syringe and packing the syringe with powdered dry ice. A cold stimulus is delivered by applying a dry iced-filled syringe to the glass underneath the paw. The latency to withdrawal was recorded and averages were taken from three measurements per paw on three different days.

Thermal Gradient

Mice were able to freely explore for 60 minutes on a thermal gradient apparatus (Ugo Basile) where a metal platform was heated in a gradient from 6°C to 54°C. Their activity (time spent in each temperature zone, number of entries) was monitored and tracked using a camera using the ANYMaze software. (Stoelting Co.)

Cold place preference:

Mice were acclimatised to the cold place preference apparatus (Ugo Basile) before baseline testing. The apparatus comprises of two Perspex-enclosed chambers on top of cold plates connected by a small corridor. Mice were allowed to explore freely between the two chambers (both at room temperature) during habituation. During testing, the floors of the chambers were set at 16°C and room temperature (20-21°C). Chamber temperatures and order of testing of mice were randomised on test days. During testing, mice were allowed to freely explore over a 10-minute period and the time in each chamber is recorded using the ANYMaze software (Stoelting Co.). Baseline values before nerve injury were established from two tests. One test was performed for each timepoint after nerve injury.

2.2.3. Chemical algogen assays

Capsaicin assay

Mice received an intraplantar injection of 1.5μg of capsaicin (Sigma-Aldrich) diluted in sterile saline with 1% ethanol and 0.5% tween-20 (Sigma-Aldrich) in a volume of 10 μl. Mice were placed in a Perspex cylinder and the duration of pain-related behaviour (biting, licking, flinching, or paw lifting) was recorded over a 5 minute period.

Formalin assay

Mice received an intraplantar injection of 4% formalin. The nocifensive behaviours of the injected hindpaw (lifting, licking, flinching, shaking) was recorded (live

timed) every 5 mins for 60 mins. The formalin assay was also analysed in 2 phases with the early phase spanning the first five minutes, and the late phase lasting 20 – 60 min post-injection.

2.2.4. Motor testing

Beam walk

The apparatus is a round wooden beam of about 1 m in length and 2 cm in diameter elevated from the bench surface (made in-house). Mice were briefly acclimatised to the wooden beam apparatus before testing. The time of crossing and the percentage of correct steps was recorded; The percentage of missteps was calculated by counting the number of missed steps as a percentage of total steps taken to cross the beam. An average was taken from three testing days.

Rotarod

Mice were acclimatised and briefly trained on the Rotarod apparatus (Ugo Basile) until all mice were able to complete a minimum of 30 seconds at 28 RPM. The latency to fall on the 32 RPM was recorded on three separate days and averaged. The maximum testing time for all mice is 180 seconds.

Open field

Mice were not acclimatised to the open field apparatus as this is a novelty and exploratory test. Mice were placed in a black box with a grid system on the box floor, and the number of boxes the mouse entered during a three-minute period was recorded. An average was obtained from two tests.

2.2.5. Conditioned place preference (CPP)

The conditioned place preference protocol was based on a study by (Griggs et al., 2015). Mice were acclimatised to the CPP apparatus (76-0278, Harvard apparatus) for 15 minutes a day before testing. The CPP apparatus consists of two large chambers and a small connecting chamber. The two chambers are distinguished

using visual (spots or stripes), scent (vanilla or strawberry), and tactile (smooth or ridged floors).

On the baseline testing day, mice were assigned a random order. On day 1, the baseline time spent in each chamber was measured. Mice were allowed to freely explore the two chambers for 30 minutes and the time spent in each chamber was tracked and recorded using the ANYMaze software (Stoelting Co.). Mice were assigned chambers for drug pairing randomly, whilst balancing the average time spent in each chamber as a cohort. Conditioning using gabapentin (ApexBio) was performed on days 2, 3, and 4. In the morning, mice were given an intraperitoneal injection of saline, returned to their home cages for 5 minutes, then transferred to their assigned pair chamber for 30 minutes. Four hours after the saline injection, mice were injected with gabapentin (30 mg/kg, Apexbio), returned to their home cages for 5 minutes, then transferred to the alternate chamber for 30 minutes. Day 5 is the post-conditioning testing day, when mice are allowed to freely explore all chambers for 30 minutes. The time spent in each chamber was tracked and recorded using ANYmaze. The preference for a paired chamber was calculated by subtracting the post-conditioning time spent from the pre-conditioning time (baseline).

2.3. Quantitative Real Time PCR

RNA was isolated from fresh-frozen mouse DRGs. RNA was isolated using a combination of TriPure (Roche) and a High Pure RNA tissue kit (Roche). Briefly, tissue was homogenised in Tripure using a handheld homogeniser (Cole-Parmer), treated with chloroform and then column-purified and eluted in RNase-free water. cDNA synthesis was carried out using Transcriptor reverse transcriptase (Roche), random hexamers (Invitrogen) and dNTPs (Roche).

Gene expression was quantified by detecting amplified material using LightCycler SYBR Green Master Mix (Roche) on the LightCycler 480 II system (Roche). Three technical replicates were included for each sample. Results were normalized to three reference genes controls (18s, GAPDH and HPRT1) using the $\Delta\Delta C_T$ method.

2.4. Western Blot

DRGs and sciatic nerves (~1cm) were isolated and homogenised on ice in RIPA buffer (Sigma) with protease inhibitors (Roche). Insoluble cell debris was removed by centrifugation (12,000 RPM, 15 min). Protein concentration of the extract was quantified using the BCA assay (Pierce). Extracts were separated by SDS-PAGE (4-20% gel, Bio-Rad) then transferred onto nitrocellulose membranes. Membranes were blocked with PBS-milk and incubated overnight with primary antibodies: anti-CASPR2 antibody (1:200 dilution, abcam), anti-EGFP antibody (1:1000 dilution abcam), or an anti-GAPDH antibody (1:5,000 diluton, abcam) as a loading control. The blots were washed twice with PBS-Tween, then probed with HRP-labelled secondary antibodies. The blots were washed again and processed for chemiluminescence detection (ECL, Amersham). For fluorescent detection, Alexa 488, Alexa 546, or Dylight 680-conjugated secondary antibodies were used at a 1:3,000 dilution. Immunoblots were imaged using the ChemiDoc imager (Bio-rad). Analysis was performed using the Image Studio software (Li-Cor).

2.5. Histology

2.5.1. Tissue preparation

Mice were overdosed with pentobarbital and transcardially perfused with saline followed by 4% paraformaldehyde (PFA, 0.1M Phosphate buffer (PB)). Once

dissected, the sciatic nerves were post-fixed in 4%PFA for 0.5 hours and DRG for 2 hours at RT. All tissue was dehydrated for cyroprotection in 30% sucrose (0.1M PB) at 4°C for 24 hours. Tissue was then embedded in optimal cutting temperature (OCT) medium (Tissue-Tek). Tissue was sectioned onto Superfrost plus slides (VWR) using a cryostat. Sciatic nerve and DRG sections were cut at 12 µm. The slides were then stored at -80.

2.5.2. Immunohistochemistry (IHC)

Tissue sections were washed once in PBS and then blocked for 1 hour before being incubated overnight at RT with primary antibody diluted in PBS triton-X (0.3%). Primary antibodies were washed off in PBS triton-X and tissue was then incubated with secondary antibodies at RT for 2 hours. Immunostaining was visualized using a confocal microscope (Zeiss) and images acquired using the Zen black software.

2.6. Primary DRG Neuron Culture

Adult mice were sacrificed in a CO_2 chamber. The spinal column was removed and bisected, and DRGs were taken at all levels and placed into Hanks' Balanced Salt Solution (HBSS, without Ca^{2+} and Mg^{2+}, Invitrogen). DRG were digested enzymatically at 37°C for 1.5 hrs in Collagenase II (4 mg/mL, Gibco) and Dispase II (4.7 mg/mL, Roche) diluted in HBSS. DRG were mechanically dissociated by gentle trituration with a fire-polished glass pipette and washed in HBSS. Dissociated cells were suspended in culture medium (Neurobasal medium with 2% B27 (v/v) and 1% glutaMAX (v/v), Gibco) supplemented with mouse NGF (50 ng/µl, Peprotech) and GDNF (10 ng/µL, Peprotech) and plated in laminin / poly-D Lysine-coated 96-well plates (Greiner) or coverslips. Cultured cells were incubated at 37°C with 5% CO_2 until assay.

2.6.1. Live Cell Staining

Monoclonal antibodies were diluted in DRG culture medium (Neurobasal + B27 + GlutaMAX + mNGF, GDNF). 1/4th of the cultured cells' medium was removed, replaced with diluted antibodies, and incubated for one hour at 37°C. The subsequent steps were all completed at room temperature. The medium was removed, and cells were washed twice and then incubated with Alexa 488-goat anti-human IgG (H+L) for an hour. The cells were washed twice, then fixed with 4% PFA (paraformaldehyde) and permeabilised with 0.3% Triton-X diluted in PBS. The neuronal marker, Alexa 546-rabbit anti-βIII tubulin antibody (Abcam), was added and incubated with cells for one hour. After an hour of incubation, the cells were washed and mounted on slides with Vectashield mounting medium (Vector Labs). Slides were imaged using the Zeiss LSM 700 using 405nm, 488nm, and 546nm diode lasers. Images were processed using FIJI (ImageJ).

2.6.2. Analysis of IgG binding on cell membranes

Analysis of IgG binding to transfected HEK or muDRG neurons was performed using FIJI (Image J). Briefly, the cell membranes of transfected cells or neurons were identified by thresholding or manually delineating the EGFP fluorescence or tubulin marker, respectively. The cells or neurons were then added as regions of interest (ROIs). The 'Enlarge' and 'Make band' tools were used to create a band surrounding the membrane, which should identify the extracellular membrane binding of IgGs. Then, the mean intensities of the identified bands were measured. To facilitate the analysis of large numbers of images, an ImageJ macro (unpublished) was written for the ease for data analysis. The mean intensity data was exported to Excel or GraphPad Prism9 for further analysis.

2.7. Calcium imaging

Cultured DRG neurons were loaded with 2 μM Fura-2 AM (ThermoFisher) dye for 30 minutes and returned to the incubator. Immediately prior to imaging, the medium was replaced with extracellular fluid (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM d-glucose, 2 mM CaCl2 and 1 mM $MgCl_2$, pH 7.4). Cells were excited intermittently with 340 and 380 nm light with one image captured per second to monitor the relative changes in intracellular calcium. Extracellular fluid was perfused with the addition of chemical algogens by a gravity-driven application system with remote-controlled pinch valves. We acquired a baseline reading for 60 seconds before the addition 50 nM capsaicin, followed by 1 μM capsaicin, 10 μM ATP, and a final depolarising 50 mM KCl. A washout of 120 s was performed between each stimulus, and each stimulus was applied for 30s. Regions of interest (ROIs) were identified in brightfield images using the free hand selection tool. The raw data was background subtracted and normalised using the RatioPlus plug-in and the 340/380 ratios were analysed using FIJI (NIH). Cells with $Ca2^+$ transients >0.2 $\Delta F/F$ were identified as responding cells.

2.8. Electrophysiology

Data were low-pass filtered at 2 kHz and sampled at 10 kHz. Series resistance was compensated 70%–80% to reduce voltage errors. Patch pipettes (2–4MΩ) were pulled from filamental borosilicate glass capillaries (1.5 mm OD, 0.84 mm ID; World Precision Instruments) Patch pipettes were filled with internal solution containing (mM): 130 KCl, 1 MgCl2, 5 MgATP, 10 HEPES, and 0.5 EGTA; pH was adjusted to 7.3 with KOH and osmolarity set to 305 mOsm. Extracellular solution contained (mM): 140 NaCl, 4.7 KCl, 1.2 MgCl2, 2.5 CaCl2, 10 HEPES and 10 glucose;

pH was adjusted to 7.3 with NaOH and osmolarity was set to 315 mOsm. Resting membrane potential was assessed in bridge mode, while firing properties were assessed in current clamp mode. Input resistance was calculated from the voltage deflections caused by increasing (Δ20 pA) hyperpolarising current pulses. To determine rheobase, cells were depolarised from a holding potential of -60 mV by current steps (50 ms) of increasing magnitude (Δ25 pA) until an action potential was generated. Repetitive firing was assessed by 500 ms depolarising current steps of increasing magnitude (50pA). Data were analysed by Clampfit 10 software (Molecular Devices)

2.9. Statistical analyses

Student's t-test was used to compare the mean of two groups. In experimental groups where multiple comparisons were made, one-way or two-way analysis of variance (ANOVA) tests with appropriate post-hoc tests were performed. Fisher exact test was used to compare discrete binary variables. All data is represented as mean ± the standard error of the mean (SEM) unless otherwise stated. Statistical significance for all experiments was placed at $p < 0.05$. Statistical significance is indicated as follows * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The statistical tests used are reported in the appropriate figure legend. All statistical tests were carried out with GraphPad Prism 9.

3. Results

3.1. Overexpression of CASPR2 in nociceptors

In unpublished data (Appendix Figure 1), we have shown that CASPR2 mRNA is downregulated in injured neurons in nerve-injured mice. This

downregulation of CASPR2 mRNA was shown to be coincident with increased mechanical hypersensitivity. This data supports the notion that CASPR2 expression may be important in regulating pain sensitivity after nerve injury and that increasing the expression of CASPR2 in neurons may reduce cell excitability.

To evaluate the effect of CASPR2 overexpression in a preclinical model, we generated transgenic mouse lines that overexpress CASPR2 in sensory neurons (made in collaboration with Dr Ben Davies, Wellcome Trust Centre for Human Genetics). The transgenic mouse line $R26^{LSL:hCNTNAP2(+/+)}$ has an exogenous human (hu) CASPR2 placed in the Rosa26 locus under the control of the neuronal CAG promoter, which will drive CASPR2 overexpression in specific neuron populations upon Cre-dependent removal of a floxed stop codon (**Figure 1A**). This allows the control of overexpression of CASPR2 in targeted neuronal populations when crossed with specific Cre-driver lines. This $R26^{LSL:hCNTNAP2(+/+)}$ mouse line was crossed with a $Na_v1.8^{Cre}$ line (Stirling et al., 2005) to overexpress CASPR2 specifically in nociceptors ($Nav1.8^{Cre}$:$R26^{LSL:hCNTNAP2(+/+)}$). Voltage-gated sodium channel Nav1.8 is expressed in over 75% of DRG neurons and in over 85% of nociceptors, small diameter, unmyelinated afferents that are specialized for the detection of noxious stimuli (Shields et al., 2012; Stirling et al., 2005).

I examined the DRG and sciatic nerve for the overexpression of the CASPR2 protein using western blot (**Figure 1B**). CASPR2 was significantly overexpressed in the sciatic nerve of $Na_v1.8$ Cre^+ mice (**Figure 1B-i**). Surprisingly, I did not observe significant overexpression of CASPR2 protein in the DRG (**Figure 1B-ii**). Nonetheless, I detected the overexpression of CASPR2 transcript in the DRG by qPCR (**Figure 1B-iii**) and showed an over three-fold increase in transcript levels compared to control Cre^- mice. I also confirmed the overexpression of CASPR2 on the surface of $Na_v1.8$ Cre^+ DRG neurons by live-cell staining using an extracellular-

binding antibody. **Figure 1C** shows the quantification of CASPR2 antibody intensity on the different-sized neurons. Surface expression of CASPR2 was significantly higher in Nav1.8 Cre + DRG neurons compared to the Cre – control neurons. The increased CASPR2 surface expression was restricted to small cells (< 25μm in diameter). **Figure 1D** shows example images of preferential upregulated CASPR2 expression in small-diameter cells. Previous work has also demonstrated the expression of Nav1.8 in mainly small and medium-diameter nociceptive neurons, and this result further supports that CASPR2 overexpression is targeted to nociceptors (Djouhri et al., 2003b).

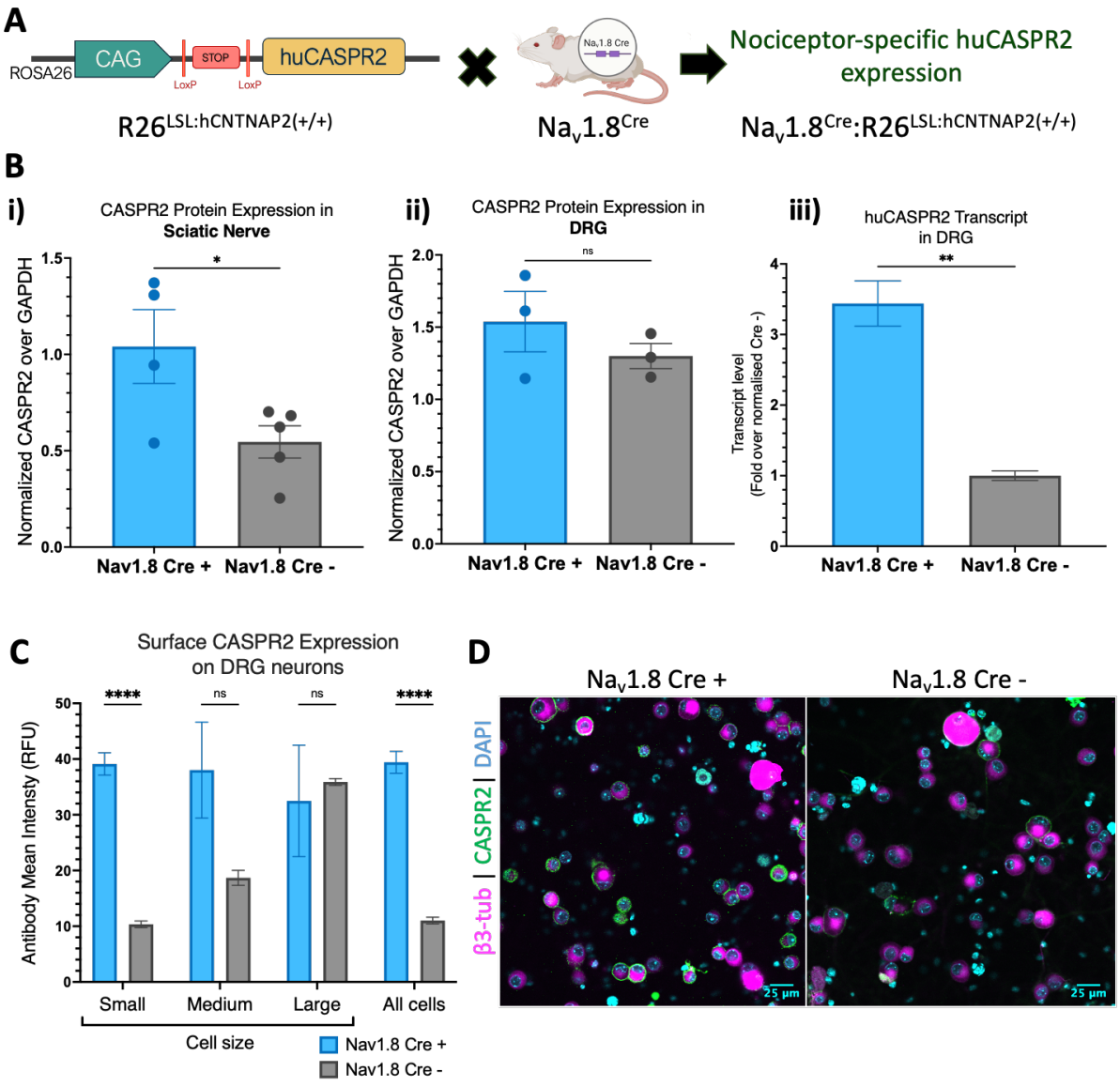


Figure 1. CASPR2 overexpression in nociceptors of Nav1.8 Cre : R26$^{LSL:hCNTNAP2(+/+)}$ mouse line

A) Design of R26$^{LSL:hCNTNAP2(+/+)}$ mouse line with exogenous human CASPR2 (untagged) placed in the Rosa26 locus with a floxed stop cassette to prevent overexpression. The CAG promoter drives the overexpression of huCASPR2 Following the Cre-dependent removal of this cassette. The R26$^{LSL:hCNTNAP2(+/+)}$ mouse line was crossed with a Nav1.8^{Cre} line to induce nociceptor-specific overexpression of CASPR2. **B) i)** Quantification of CASPR2 protein in the sciatic nerve **ii)** and DRG. CASPR2 protein is overexpressed in the sciatic nerve, but not DRG. Full-sized blots shown in Appendix Figure 10. **iii)** Quantification of mRNA transcript measured by qPCR in the whole DRG. CASPR2 transcript was significantly higher in the DRG of $Na_v1.8$ Cre $^+$ than Nav1.8 Cre$^-$ mice. Two-tailed unpaired t-test, * $p<0.05$, **$p<0.01$, n=3-4) **C)** Quantification of CASPR2 expression on the surface of DRG neurons, separated by sizes small (<25 μm in diameter), medium (25 -35 μm), and large (>35 μm). CASPR2 stained with an antibody targeting an extracellular epitope on live neurons. DRG neurons pooled from 2 animals/genotype. Number of neurons: Small Cre$^+$ n= 260, Cre $^-$ n=190; Medium Cre $^+$ n = 49, Cre $^-$ n= 40; Large Cre $^+$ n= 12, Cre $^-$ n=9. CASPR2 is overexpressed in small-sized $Na_v1.8$ Cre+ neurons compared to Cre$^-$ control neurons. Two-way ANOVA with Šídák's multiple comparisons test, ****$p<0.0001$ **D)** Representative images of CASPR2 expression (green) on Nav1.8 Cre + (left) and Nav1.8 Cre – (right) DRG neurons. DAPI (cyan) and βIII-tubulin (magenta). Scale bar 25 μm.

3.2. CASPR2 overexpression in nociceptors does not affect thermal or mechanical sensitivity

Sensory testing was performed in adult mice to determine if overexpression of CASPR2 in nociceptors affects mechanical or thermal sensitivities. Mechanical sensitivity was assessed by von Frey hairs, dynamic brush, and pinprick test (**Figure 2A**). These mechanical assays disclosed no significant difference between the $Na_v1.8$ Cre$^+$ and control Cre$^-$ mice. I then assessed thermal sensitivity by Hargreaves test, 53°C hot plate, and the dry ice assay (also known as the cold plantar assay) (**Figure 2B**). There was no difference between $Na_v1.8$ Cre$^+$ and control Cre $^-$ mice in heat sensitivity, as measured by the paw withdrawal latency in the Hargreaves test or 53°C hot plate. There was also no difference in cold detection between the genotypes in the dry ice assay. Overall, no difference was detected in all mechanical and thermal sensitivities between Nav1.8 Cre$^+$ or Nav1.8 Cre$^-$ genotypes. These initial results suggest that overexpression of CASPR2 in nociceptors likely does not affect thermal or mechanical sensory sensitivity. To evaluate whether there are sex-specific differences in sensory sensitivity, I analysed

the male and female mice separately in **Appendix Figure 2** and showed that there is no difference in mechanical or thermal sensitivity between male and female mice of either genotype.

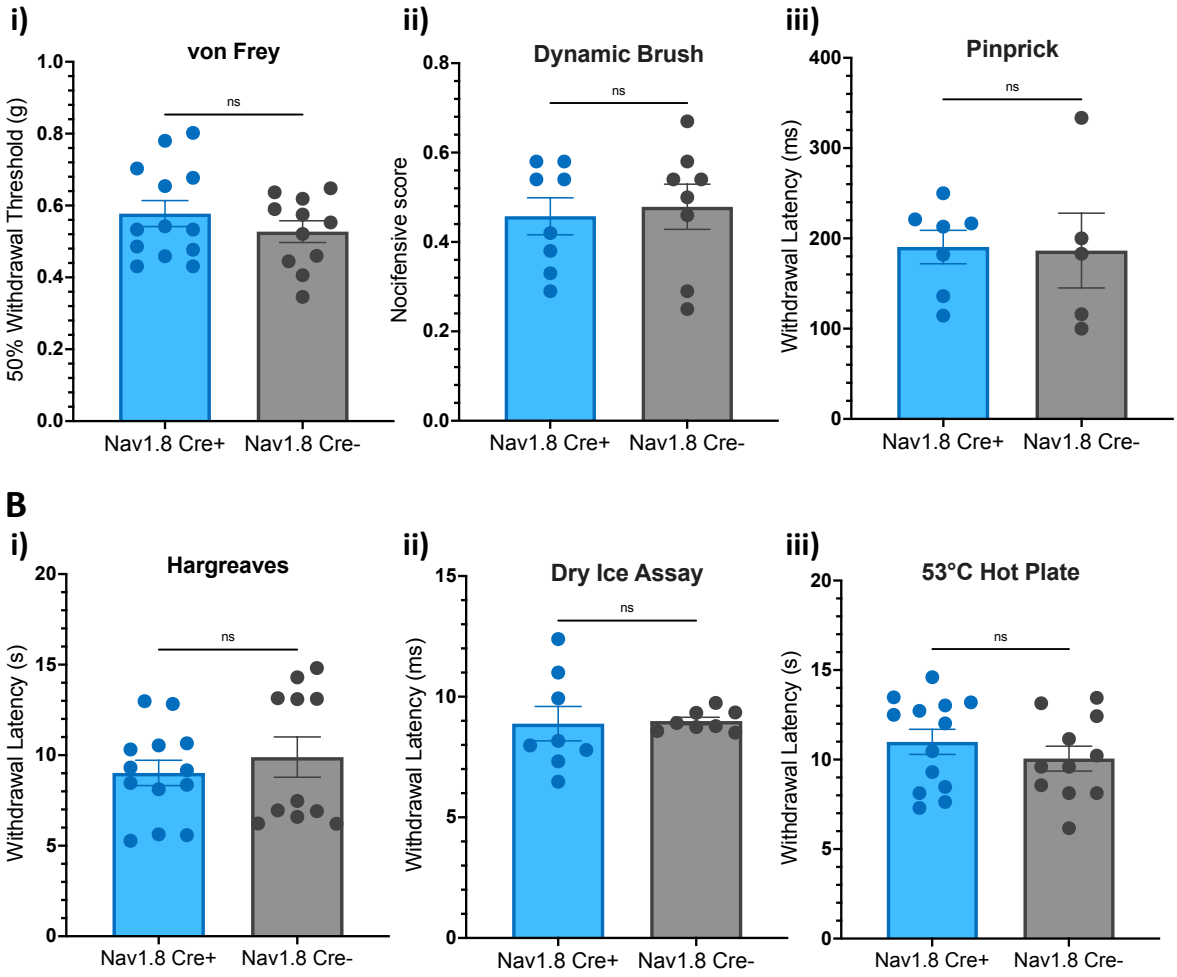


Figure 2. Mechanical and thermal sensory testing in mice overexpressing CASPR2 in nociceptor

A) There were no significant differences in mechanical sensitivity detected between $Na_v1.8$ Cre^+ and $Na_v1.8$ Cre^- control littermates measured by **i)** von Frey hairs, **ii)** dynamic brush, or **iii)** pinprick test. Two-tailed unpaired t-test. $Na_v1.8$ Cre^+, *n*=7-13; $Na_v1.8$ Cre^-, *n*=5-11, *ns*. **B)** There were no significant differences in thermal sensitivity detected between $Na_v1.8$ Cre^+ and $Na_v1.8$ Cre^- control littermates measured by **i)** Hargreaves **ii)** dry ice assay, or **iii)** 53°C hot plate. Two-tailed unpaired t-test. $Na_v1.8$ Cre^+, n=7-13; $Na_v1.8$ Cre, n=5-11, ns.

3.3. CASPR2 overexpression in nociceptors reduces capsaicin-induced pain-like behaviours

It has been reported that the genetic ablation of CASPR2 in mice resulted in hypersensitivity to capsaicin and increased overt pain behaviour (Dawes et al., 2018; Xing et al., 2020). I applied the same capsaicin treatment to determine if the pain-like behaviour is altered in CASPR2-overexpressing mice in response to a more acute noxious stimulus. The injection of capsaicin (3 μg / paw) into the plantar skin of the hindpaw elicited the rapid onset of robust nocifensive responses that consisted of licking and shaking of the treated paw. **Figure 3A** shows a significant reduction in nocifensive behaviour in $Na_v1.8$ Cre^+ mice compared to $Na_v1.8$ Cre^- control littermates. I also utilised the formalin test, a common model for inflammatory and tissue injury pain, to evaluate the role of CASPR2 in behavioural responses to inflammation. Injection of formalin into the plantar surface of the hindpaw elicited the well-described biphasic pain response, with an early phase occurring during the first five minutes and a late phase following 20 – 60 minutes post-injection. There was no difference in total nocifensive duration between the CASPR2-overexpressing Nav1.8 Cre^+ or control Nav1.8 Cre- mice at any timepoint (**Figure 3B**) or in either phase (**Figure 3C**).

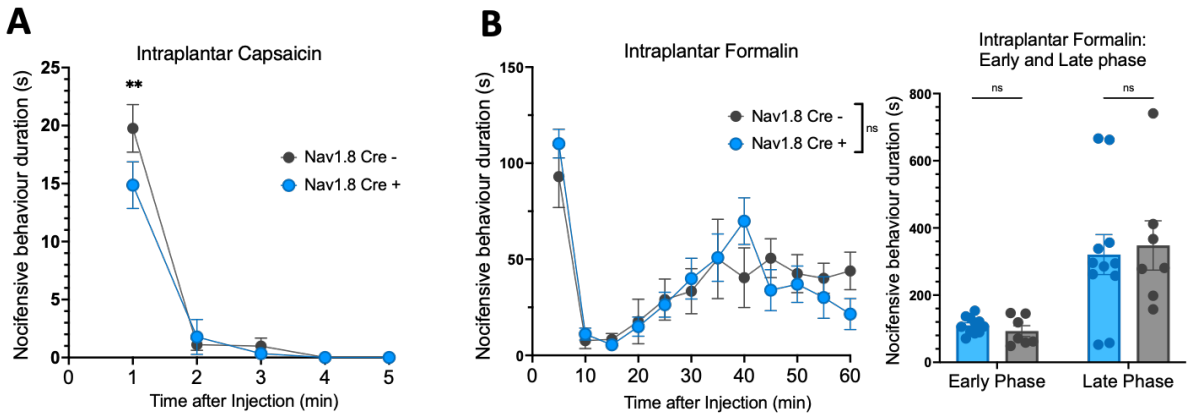


Figure 3. Response to chemical algogens in mice overexpressing CASPR2 in nociceptors

A) $Na_v1.8$ Cre^+ mice exhibited reduced nocifensive behaviour in the first minute after injection, compared to Cre- littermates. Two-way RM ANOVA with Šídák's multiple comparisons test, **p<0.01, $Na_v1.8$ Cre^+ n=18, $Na_v1.8$ Cre^- n=17. **B)** Left, time-course of nocifensive behaviour after intraplantar injection of 5% formalin measured in five-minute bins. There was no difference in nocifensive behaviour duration at any timepoint, between $Na_v1.8$ Cre^+ and $Na_v1.8$ Cre^- mice. Two-way RM ANOVA with Šídák's multiple comparisons test, ns. Right, there was also no difference in total nocifensive behaviour displayed during the early phase (0-5 minutes) and late phase (20-60 minutes) of the assay. Two-way ANOVA with Šídák's multiple comparisons test, ns. $Na_v1.8$ Cre^+, n=11; $Na_v1.8$ Cre^-, n=7

3.4. CASPR2 overexpression in nociceptors does not affect neuropathic pain-related behaviours

To assess if CASPR2 overexpression has an impact on pain-like behaviours in the context of neuropathic pain, I utilised the spared nerve injury model and compared the pain sensitivity between Nav1.8 Cre^+ and Cre^- mice. **Figure 4A** shows the schematic of the spared sural nerve injury model, which results in hypersensitivity in the area innervated by the sural nerve. I tested for changes to punctate and dynamic mechanical pain sensitivity using von Frey hairs (**Figure 4B**) and dynamic brush (**Figure 4C**). While there was a marked decrease in withdrawal threshold in the ipsilateral paw, indicative of hypersensitivity after surgery, no difference was detected between the $Na_v1.8$ Cre^+ and Cre^- mice in either punctate or dynamic allodynia.

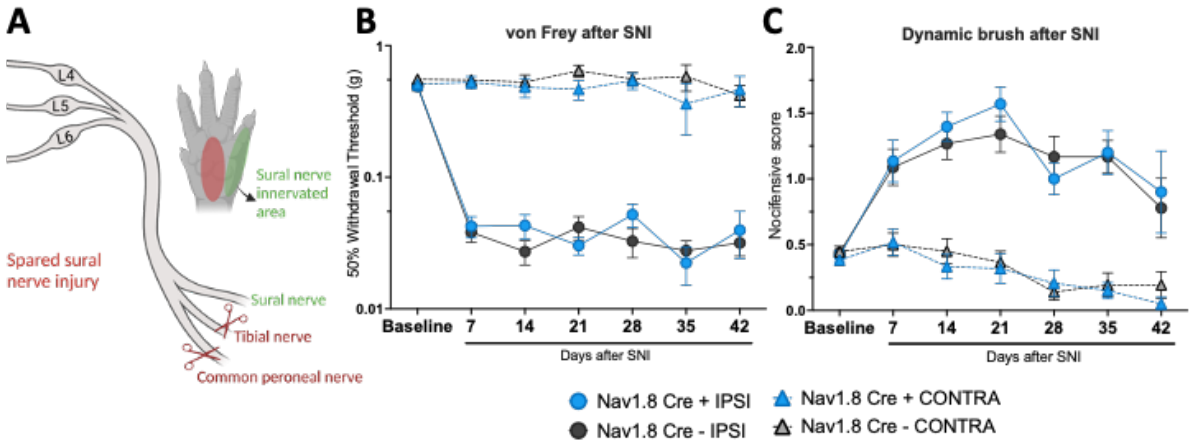


Figure 4. Nerve injury model in mice overexpressing CASPR2 in nociceptors
A) Schematic of the spared nerve injury (SNI) model used (left). The tibial and common peroneal nerves were ligated then transected, sparing the sural nerve. All testing was done in

the lateral area innervated by the sural nerve (right, in green). **B)** Spared nerve injury caused sustained mechanical hypersensitivity in the ipsilateral hindpaw in all mice. There was no difference in punctate mechanical hypersensitivity, measured by von Frey hairs, between the $Na_v1.8$ Cre^+ and $Na_v1.8$ Cre^- control littermates after nerve injury. **C)** There was also no difference in dynamic allodynia between the two genotypes after injury. Two-way RM ANOVA with Šídák's multiple comparisons test, ns. $Na_v1.8$ Cre^+, n=15; $Na_v1.8$ Cre^-, n=17

I also examined whether CASPR2 overexpression in nociceptors had any effect in cold pain-sensitivity after nerve injury using the dry ice assay (**Appendix Figure 3**). However, there was no increased sensitivity to dry ice in the ipisilateral paw in both Cre^+ and Cre^- mice after nerve injury.

To determine whether transcript levels are altered after nerve injury, I also examined the transcript levels of voltage-gated potassium complex genes (*Cntnap2*, *Cntn2*, *Kcna1*, and *Kcna2*) and the injury marker, Atf3, by qPCR (**Appendix Figure 4**), and observed no significant differences in the expression levels of VGKCC genes. Atf3 transcript was significantly upregulated in the ipsilateral DRG, with a slight trend of decrease in Nav1.8 Cre^- mice.

3.5. CASPR2 overexpression in nociceptors does not alter neuron excitability

We examined neuron excitability in DRG neurons to determine whether CASPR2 overexpression has any effect in regulating excitability in vitro. The CASPR2 is untagged, so we focused on the small- and medium-sized neurons to best capture the nociceptor neurons. There was no difference observed in the rheobase (**Figure 5A)** or repetitive firing (**Figure 5B**) between $Na_v1.8$ Cre^+ and $Na_v1.8$ Cre- neurons. However, the current injections utilised to elicit repetitive impulse activity in medium cells may not be sufficient, as we did not inject current that exceeds the rheobase for medium-sized neurons in this experiment.

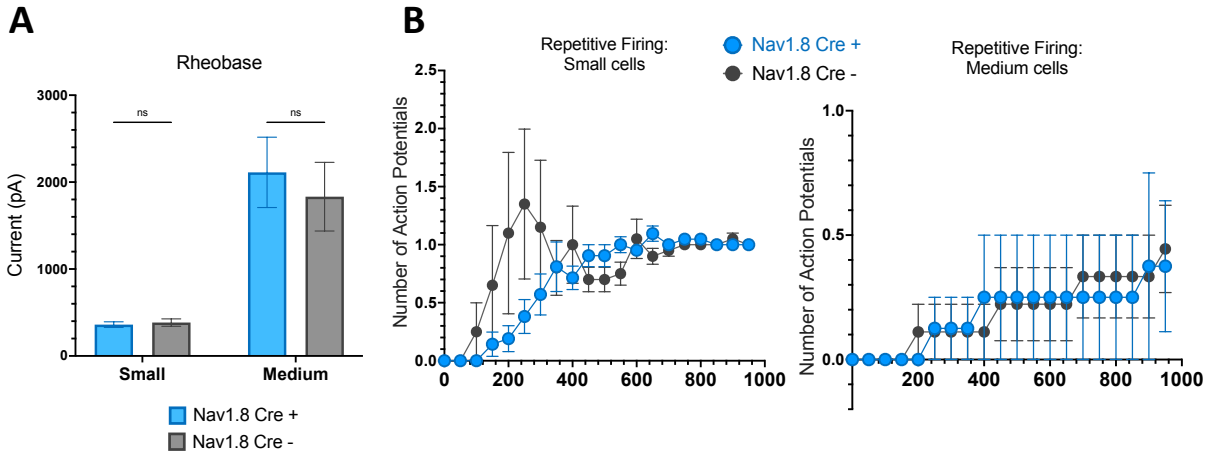


Figure 5. Electrophysiological characterisations of CASPR2 overexpression in nociceptors

A) No significant difference was detected in the rheobase in small and medium-sized DRG neurons from $Na_v1.8$ Cre^+ and $Na_v1.8$ Cre^- mice. **B)** There was also no difference between $Na_v1.8$ Cre^+ and cre^- DRG neurons in repetitive firing. Two-way RM ANOVA with Šídák's multiple comparisons test, ns. $Na_v1.8$ cre^+ small n=21, medium n=8 ; $Na_v1.8$ cre^- small n=20, medium n=9.

3.6. Overexpression of CASPR2 in the Hoxb8^{Cre}: R26$^{LSL:huCNTNAP2(+/+)}$ mouse line

The Nav1.8^{Cre}: R26$^{LSL:hCNTNAP2(+/+)}$ mouse line data suggest that the overexpression of CASPR2 in nociceptors is insufficient to effect changes in neuronal excitability and pain sensitivity. Therefore, I utilised another transgenic mouse line, Advilin^{CreERT2}: R26$^{LSL:hCNTNAP2(+/+)}$, to drive overexpression in a wider population of peripheral sensory neurons (**Appendix Figure 5**). Tamoxifen-induction of the Advilin^{CreERT2} line successfully induced the overexpression of CASPR2 in Advilin Cre $^+$ mice (**Appendix Figure 5B & 5C**). Mechanical and thermal sensitivities were assessed 4 weeks after tamoxifen administration. Although there was no difference in mechanical or thermal sensitivity observed between the genotypes, there was a significant reduction in response to capsaicin-induced acute pain in the Advilin Cre$^+$ mice (**Appendix Figure 5D & 5E**). Unfortunately, we observed unexpected mortality in the Advilin^{CreERT2}: R26$^{LSL:hCNTNAP2(+/+)}$ mouse line and determined that this mouse line was not viable. Therefore, we turned to the Hoxb8^{Cre} line (**Figure 6A**), which will induce the

overexpression of CASPR2 in all DRG and spinal cord neurons caudal to the C4 spinal segment (Witschi et al., 2010).

I confirmed the overexpression of CASPR2 protein in the DRG and sciatic nerve but not in the brain of Hoxb8 Cre+ mice (**Figure 6B**). There was also significantly higher CASPR2 expression on the surface of DRG neurons of all sizes, as quantified in **Figure 6C**. **Figure 6D** shows the marked increase of CASPR2 antibody signal (green) on the surface of DRG neurons from Hoxb8 Cre^{+} mice, compared to those of Hoxb8 Cre^{-} mice.

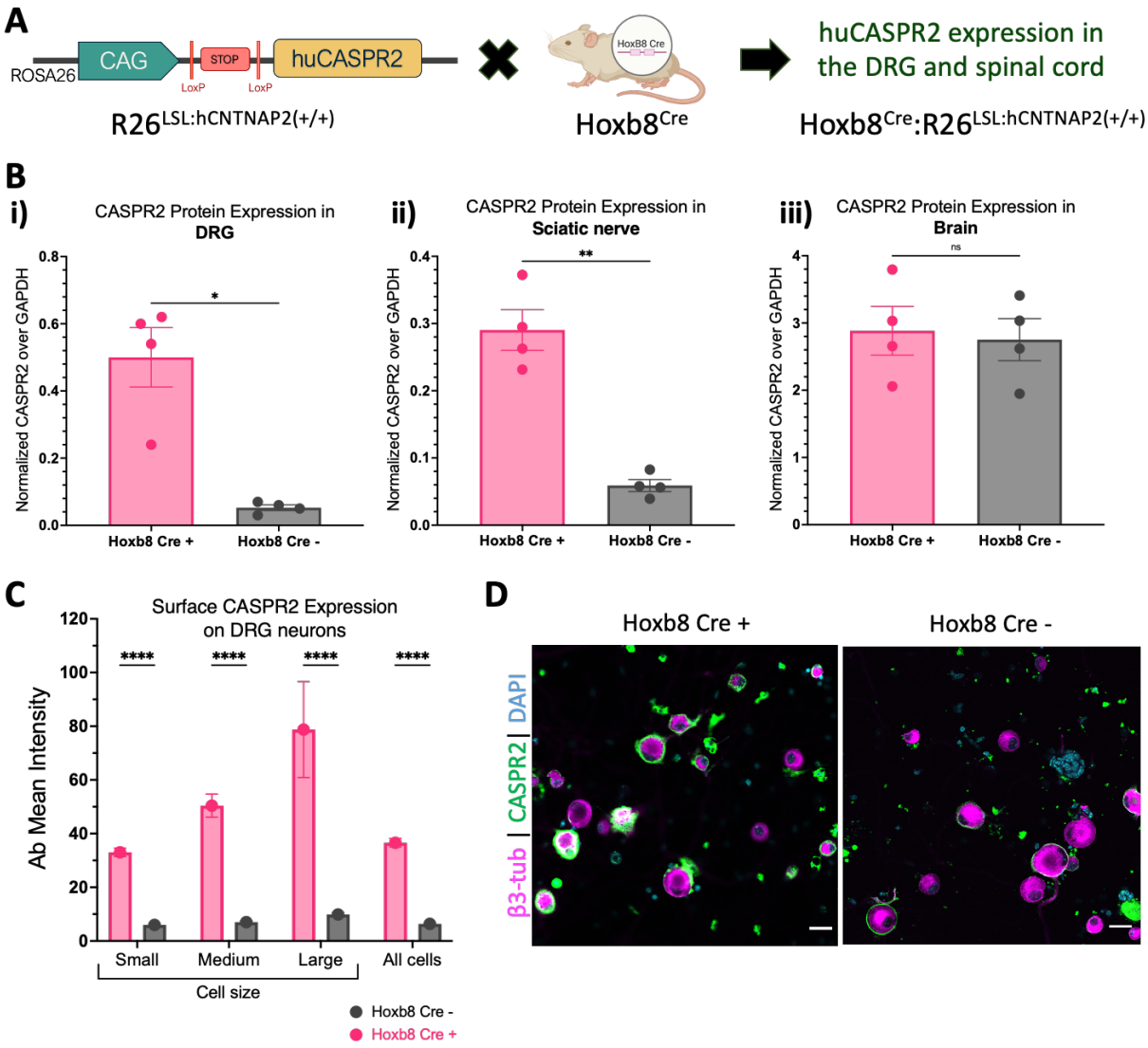


Figure 6. CASPR2 overexpression in the PNS of Hoxb8 Cre : R26$^{LSL:hCNTNAP2(+/+)}$ mouse line

A) The $R26^{LSL:hCNTNAP2(+/+)}$ mouse line was crossed with the $Hoxb8^{Cre}$ line to induce overexpression of CASPR2 in the DRG and spinal cord, while sparing the brain. **B) i)** Quantification of CASPR2 protein in the DRG, **ii)** sciatic nerve, and **iii)** brain. CASPR2 protein is overexpressed in the sciatic nerve and DRG in Hoxb8 Cre^+ mice compared to control Cre^- littermates. **iii)** CASPR2 is not overexpressed in the brain of Hoxb8 Cre + mice. Two-tailed upaired t test, * $p<0.05$, ** $p<0.01$, ns, n=4) **C)** Quantification of CASPR2 expression on the surface of DRG neurons, separated by sizes small (<25 μm in diameter), medium (25 -35 μm), and large (>35 μm). CASPR2 stained with an antibody targeting an extracellular epitope on live neurons. DRG neurons pooled from 2 animals/genotype. CASPR2 is overexpressed in neurons of all sizes in Hoxb Cre^+ neurons compared to Cre^- control. Two-way ANOVA with Šídák's multiple comparisons test, **** $p<0.0001$ **D)** Representative images of CASPR2 expression (green) on Hoxb8 Cre^+ (left) and Hoxb8 Cre^- (right) DRG neurons. DAPI (cyan) and βIII-tubulin (magenta). Scale bar 25 μm.

3.7. CASPR2 overexpression in the DRG does not affect acute pain sensitivity but reduces capsaicin-induced pain-like behaviour

Sensory testing was done to assess whether the overexpression of CASPR2 in the Hoxb8 Cre^+ mouse line affected acute pain-related behaviours. **Figure 7A** shows no difference detected in mechanical sensitivity by von Frey hairs or dynamic brush. I also assessed thermal sensitivity using the Hargreaves assay, 50°C and 53°C hot plate, and the dry ice assay and observed no differences between the two genotypes (**Figure 7B**). Similar to the result shown in mice overexpressing CASPR2 in nociceptors, the capsaicin assay revealed a significant reduction in nocifensive behaviour duration in Hoxb8 Cre^+ mice compared to the control Hoxb8 Cre^- littermates. Again, suggesting a role of CASPR2 in regulating capsaicin-induced pain. The increased reduction in response to capsaicin injection in Hoxb8 Cre^+ mice compared to Nav1.8 Cre^+ mice is likely due to the increased expression of CASPR2 in the Hoxb8 Cre^+ mice.

The Hoxb8 Cre^+ mice display normal motor activity, as measured by rotarod and beam walk (**Appendix Figure 6A**), compared to Cre^- littermates. There was also no difference in thermal preference between the two genotypes as measured by the thermal gradient assay (**Appendix Figure 6B**).

In order to determine if there are sex-specific difference between the Hoxb8 Cre^{+} and Cre^{-} mice, I analysed the mechanical and thermal sensory testing data separated by sex. There was no difference detected between male and female mice of both genotypes in any of the sensory tests (**Appendix Figure 7**).

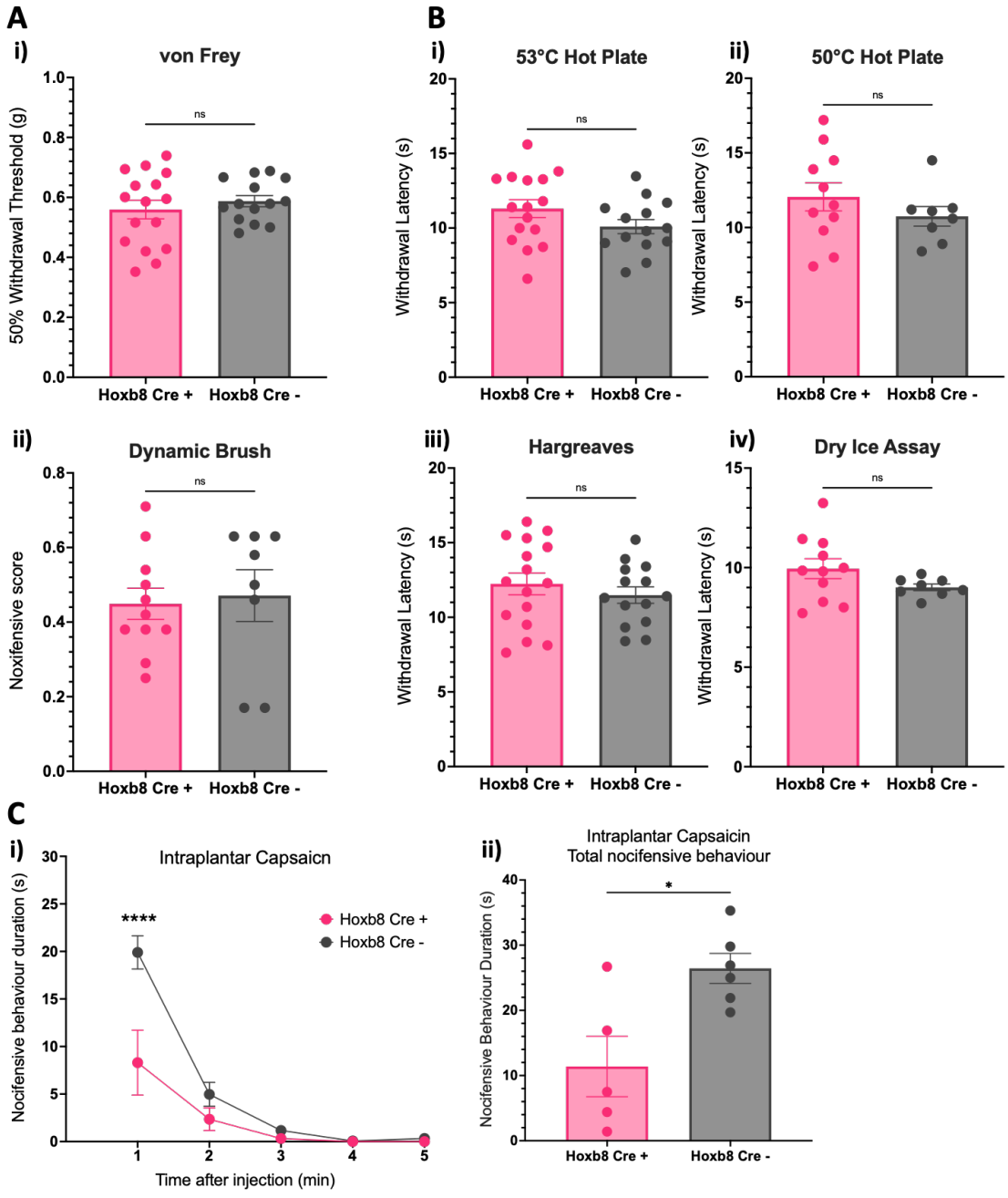


Figure 7. Sensory testing and capsaicin assay of Hoxb8^{Cre}: R26$^{LSL:hCNTNAP2(+/+)}$ mouse line
A) There were no significant differences in mechanical sensitivity detected between Hoxb8 Cre^{+} and Hoxb8 Cre^{-} control littermates measured by **i)** von Frey hairs, **ii)** dynamic brush. Two-tailed unpaired t-test. Hoxb8 Cre^{+}, n=11-16; Hoxb8 Cre^{-}, n=9-14, *ns*. **B)** There were no significant differences in thermal sensitivity between Hoxb8 Cre^{+} and Hoxb8 Cre^{-} control littermates measured by **i)** 53°C or **ii)** 50°C hot plate, **iii)** Hargreaves test, or **iv)** dry ice assay.

Two-tailed unpaired t-test. $Na_v1.8$ Cre^+, *n*=7-13; $Na_v1.8$ Cre^-, n=5-11, ns. **C)** Hoxb8 Cre^+ mice exhibited significant reduced nocifensive behaviour after intraplantar capsaicin injection **i)** in the first minute. and **ii)** in total nocifensive behaviour over 5 minutes, compared to Cre- littermates. i) Two-way RM ANOVA with Šídák's multiple comparisons test, ***p<0.001 ii_Unpaired two-way t-test, *p<0.05 Hoxb8 Cre^+ n=5, Hoxb8 Cre^- n=6.

3.8. CASPR2 overexpression may reduce neuropathic pain-related behaviours in male mice

I used an alternative version of the spared nerve injury model to investigate the effects of CASPR2 overexpression in the Hoxb8 Cre mouse line. In the spared tibial nerve injury model, the tibial nerve- which contains sensory and motor neurons- was spared, while the common peroneal and sural nerves were transected. This resulted in hypersensitivity in the tibial nerve-innervated areas after nerve injury (**Figure 8A**). The spared tibial nerve injury did not result in the drastic reduction in the withdrawal threshold observed in the spared sural nerve injury. Nonetheless, the paw withdrawal threshold was reduced in all mice post-injury (**Figure 8B**). There was no significant difference in mechanical pain sensitivity between the genotypes post-injury. However, there seemed to be a trend for recovery in the ipsilateral paw of Hoxb8 Cre^+ mice at day 21 (**Figure 8B-i**). To examine this trend, I separated the cohort by sex, and indeed, there was a significant increase in the ipsilateral paw withdrawal threshold in male Hoxb8 Cre^+ mice on day 21, compared to the Hoxb8 Cre^- littermates (**Figure 8B-ii**). There was also a reduction in the nocifensive score in the dynamic brush assay in the ipsilateral paw of Hoxb8 Cre+ mice, but this was not significant (**Figure 8C-ii**).

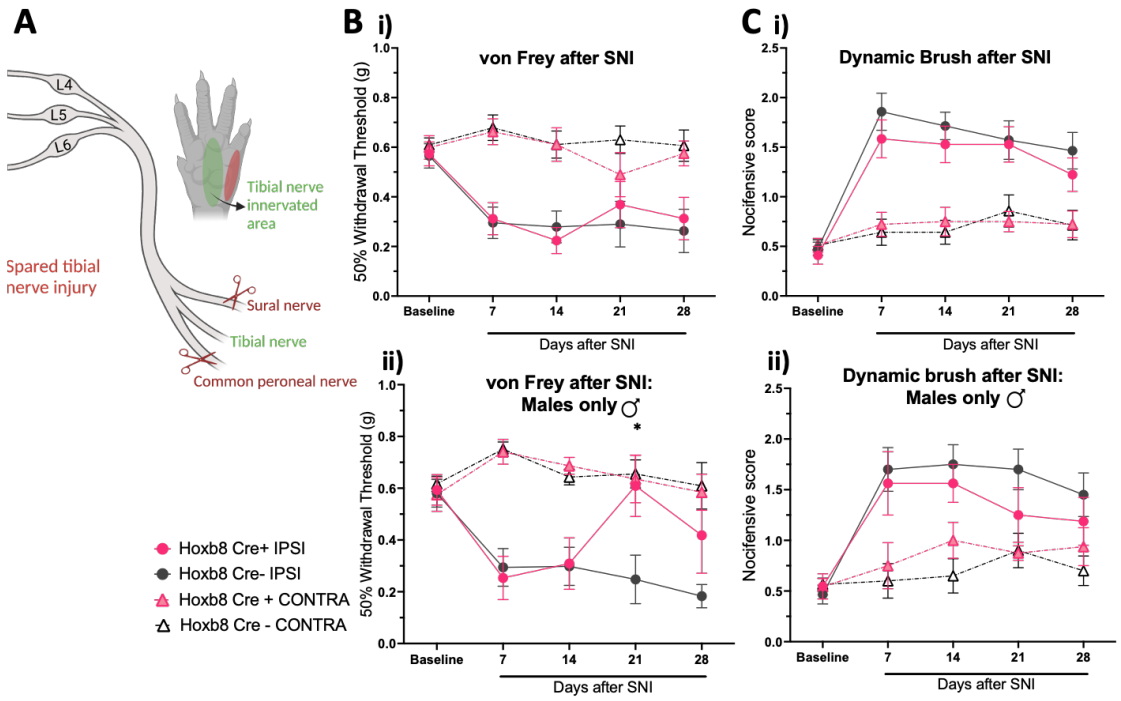


Figure 8. Nerve injury model in Hoxb8^{Cre}: R26$^{LSL:hCNTNAP2(+/+)}$ mice
A) Schematic of the spared nerve injury (SNI) model used (left). The sural and common peroneal nerves were ligated then transected, sparing the tibial nerve. All testing was done in the centre area of the plantar surface innervated by the tibial nerve (right, in green). **B)** Spared nerve injury caused sustained mechanical hypersensitivity in the ipsilateral hindpaw in all mice. **i)** There was no difference in punctate mechanical hypersensitivity, measured by von Frey hairs, between the Hoxb8 Cre^{+} and Hoxb8 Cre^{-} control littermates after nerve injury. **ii)** However, there is a difference in male mice on Day 21, when the withdrawal threshold of the Hoxb8 Cre + ipsilateral paw is significantly increased compared to that of the Cre $^{-}$ mice. Two-way RM ANOVA with Šídák's multiple comparisons test, ns. Male Hoxb8 Cre + vs Male Hoxb8 Cre – IPSI *p<0.05. **C)** There was no difference in dynamic allodynia between the two genotypes after injury in i) both sexes combine or ii) in male mice. Two-way RM ANOVA with Šídák's multiple comparisons test, ns. Hoxb8 Cre^{+}, n=9 male n=4; Hoxb8 Cre^{-}, n=7, male n=5.

3.9. CASPR2 overexpression did not influence cold allodynia or spontaneous pain-like behaviour

Cold hypersensitivity is a neuropathic pain symptom (Descoeur et al., 2011). Our previous attempt at measuring cold allodynia after nerve injury using the dry ice assay was unsuccessful (**Appendix Figure 3**). Therefore, I examine the effects of CASPR2 overexpression in cold allodynia using the cold place preference assay (**Figure 9A**). After nerve injury, neuropathic mice spent less time in the cold 16°C chamber compared to the baseline (**Figure 9B**). There was no difference in the time

spent in the 16°C chamber between Hoxb8 Cre $^{+}$ and Cre $^{-}$ mice, suggesting that CASPR2 overexpression did not alleviate cold allodynia in nerve-injured mice.

I also used the conditioned place preference (CPP) assay to measure non-evoked, spontaneous pain in neuropathic mice. Spontaneous or ongoing pain is a common symptom reported by neuropathic pain patients (Colloca et al., 2017). In the CPP assay, a non-addictive analgesic is paired with a chamber in a two-chamber apparatus. Measurements of the change in the time spent in the treatment-associated chambers before and after conditioning quantify the animal's preference and indirectly assess the effectiveness of the applied treatment (**Figure 9B-i**). After conditioning, all mice exhibited an increase for the gabapentin-paired chamber (**Figure 9B-ii**), indicating that the conditioning paradigm was successful in establishing a preference for the drug-paired chamber. The assay variability was high, and there was no difference in the time spent in the drug-paired chamber between the Hoxb8 Cre^{+} and Cre^{-} mice.

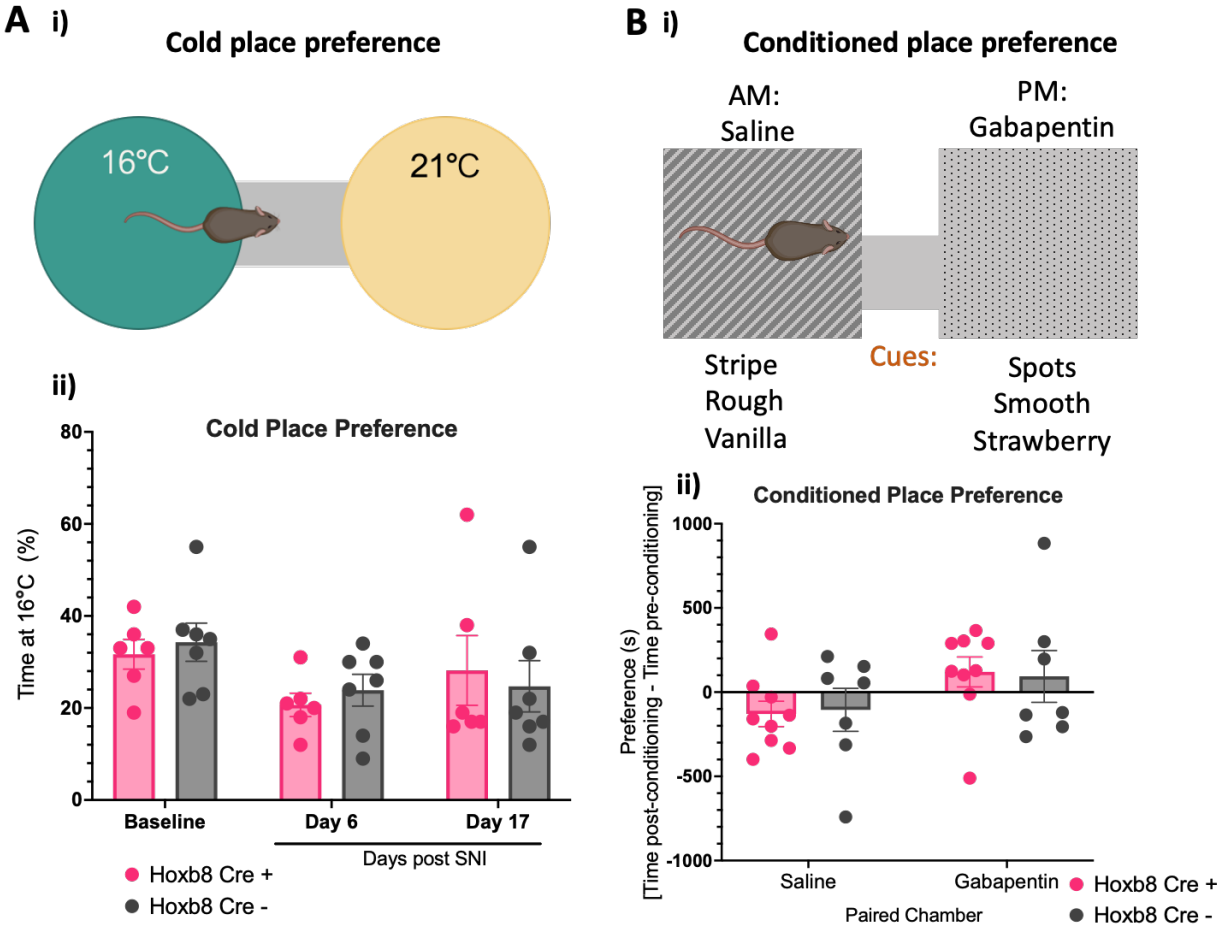


Figure 9. Measurement of cold hypersensitivity and spontaneous pain in Hoxb8 Cre: R26$^{LSL:hCNTNAP2(+/+)}$ mice after nerve injury

A) i) Schematic of the two-chamber cold place preference apparatus. The floor of one chamber is set at 16 °C and the other at room temperature. Mice are free to roam for 10 minutes during testing. The time spent in the cold chamber after nerve injury is shown in **ii)**. After nerve injury, all mice showed reduced time spent in the cold chamber. No difference was detected in time spent in the 16°C chamber between the Hoxb8 Cre + and Cre –mice. Two-way RM ANOVA with Šídák's multiple comparisons tests, ns. Hoxb8 Cre^{+}, n=9; Hoxb8 Cre^{-}, n=7. **B) i)** Diagram of the two-chamber apparatus for the conditioned place preference assay. Each chamber contains visual, tactile, and scent cues. Mice are conditioned by pairing the drug gabapentin with a particular chamber. Preference is calculated by subtracting the time spent in the drug- or vehicle-paired chamber post-conditioning from the pre-conditioning baseline times. A negative preference score indicates an aversion to the chamber, and a positive score indicates a preference for the chamber. Ii) Preference scores for saline and gabapentin-paired chambers in mice after nerve injury. **ii)** There was an overall reduction in time spent in the saline-paired chamber and an increased time spent in the gabapentin-paired chamber. However, there was no difference between the genotypes in the time spent in each chamber. Two-way RM ANOVA with Šídák's multiple comparisons test, ns. Hoxb8 Cre^{+}, n=9; Hoxb8 Cre^{-}, n=7

3.10. CASPR2 overexpression reduced capsaicin-induced calcium influx in medium DRG neurons

I noted the reduction in pain-related behaviours in response to capsaicin in three independent CASPR2-overexpressing transgenic mouse lines ($Na_v1.8$ Cre, Advilin CreERT2 and Hoxb8 Cre) and sought to further understand the effects of CASPR2 overexpression on neuronal excitability in response to different chemical stimuli. I tested cultured DRG neurons in calcium imaging assay and quantified their responses to capsaicin and ATP bath application (**Figure 10A**). Overall, there was no difference in the percentage of neurons that responded to each algogen (**Figure 10B**). To distinguish the responses from small and medium-sized neurons, I analysed these populations separately in **Figures 10B** and **10C.** There was a significant reduction in the amplitude in small Hoxb8 Cre^+ neurons in response to KCl, compared to control Hoxb8 Cre^- neurons (**Figure 10C**). Consistent with the in vivo capsaicin assay, I observed a significant reduction in calcium influx in medium Hoxb8 Cre^+ cells upon capsaicin application (**Figure 10D**).

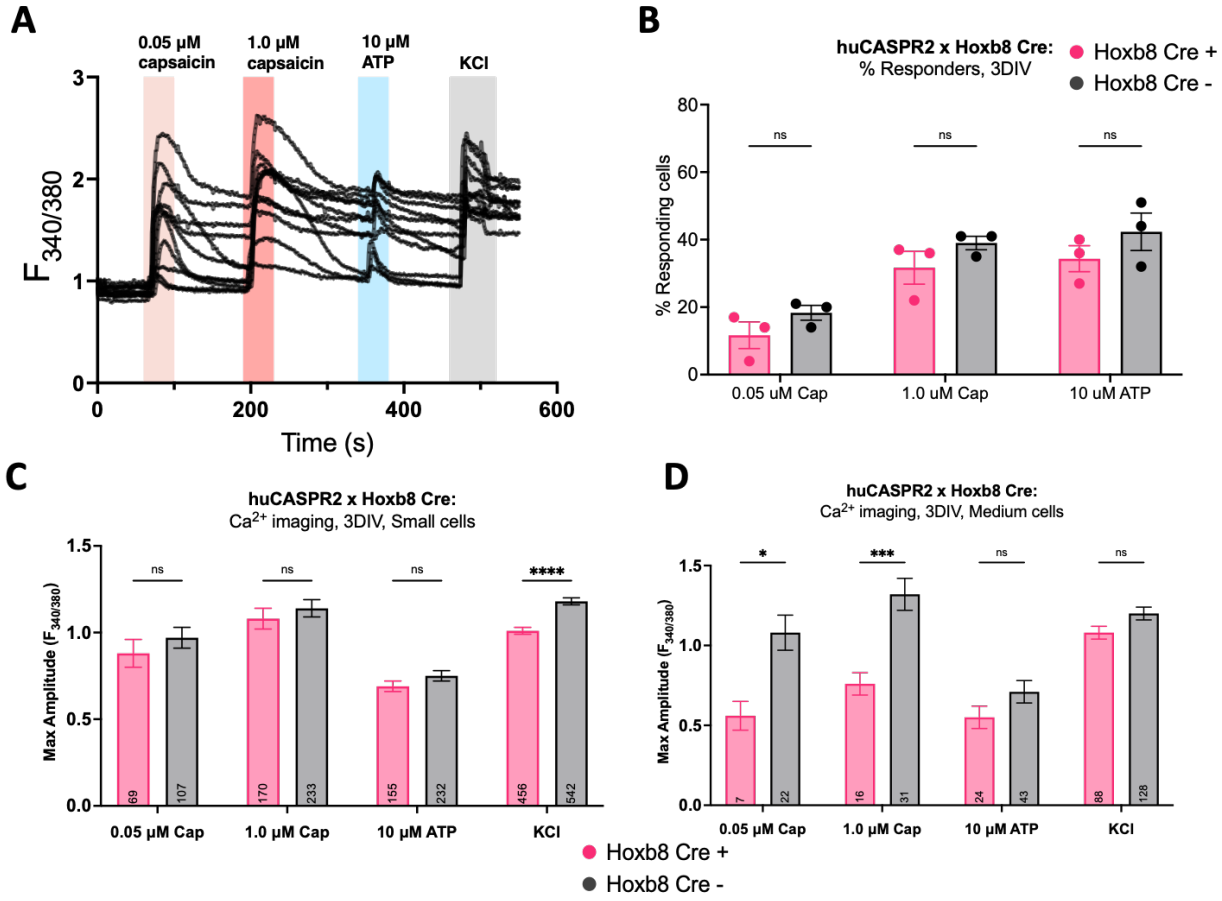


Figure 10. Calcium imaging characterisation of Hoxb8 Cre : R26$^{LSL:hCNTNAP2(+/+)}$ neurons
A) Example calcium imaging traces from DRG neurons showing the timing of chemical stimuli. Two concentrations (0.05 and 1.0 µM) of capsaicin were added for 30 seconds, followed by the application of 10 µM ATP and a final KCl pulse. A 120-second wash-out step was performed between each chemical stimulus. **B)** There was no difference between the Hoxb8Cre^{+} and Cre^{-} neurons ins the percentage of responders to each stimulus. Two-way ANOVA with Šídák's multiple comparisons test, ns, n=3 **C)** Comparison of the maximum amplitude in response to each stimulus in small neurons between Hoxb8 Cre^{+} and Cre^{-} DRG neurons (<25 µm in diameter). The response to KCl-induced depolarisation was significantly lower in the small Hoxb8 Cre^{+} neurons compared to small Hoxb8 Cre^{-} neurons. Two-way ANOVA with Šídák's multiple comparisons test, ****<0.0001, n= number of cells as indicated on the graphs from 3 animals per genotype **D)** There was a reduction in maximum amplitude in response to both capsaicin concentrations in medium-sized neurons from Hoxb8 Cre^{+} mice compared to those from Hoxb8 Cre^{-} control. Two-way ANOVA with Šídák's multiple comparisons test, * p<0.05, ** p<0.01, n= number of cells as indicated on the graphs from 3 animals per genotype. 3 to 4 coverslips were imaged per animal.

3.11. Adenoviral delivery of CASPR2 *in vivo*

To explore the feasibility of CASPR2 overexpression as a pain therapeutic, I used a more clinically relevant method of delivery of CASPR2 by AAVs. AAVs have been successfully used to transduce DRG neurons, and AAV gene therapies

have been approved by the FDA (Chan et al., 2017). To stay within the packaging limits of AAVs, I generated a truncated version of the huCASPR2 protein with an EGFP tag (**Figure 11A**) and placed it under the neuronal CAG promoter. A control AAV (AAV9-EGFP) was generated with the same promoter.

After injection of AAV9-huCASPR2-EGFP or AAV9-EGFP in pups, I quantified the protein expression in whole DRG lysates from adult mice and showed the expression of the CASPR2-EGFP (C2-EGFP) was significantly lower than EGFP (**Figure 11B**). I also looked at the percentage of DRG neurons transduced by AAVs using immunofluorescence and showed that AAV9-huCASPR2-EGFP transduced approximately 40% of DRG neurons compared to 60% of neurons by AAV9-EGFP (**Figure 11D**). The subpopulation analysis of expression in DRG neurons revealed that AAV9-EGFP transduced a significantly higher percentage of IB4-positive neurons (**Figure 11D**).

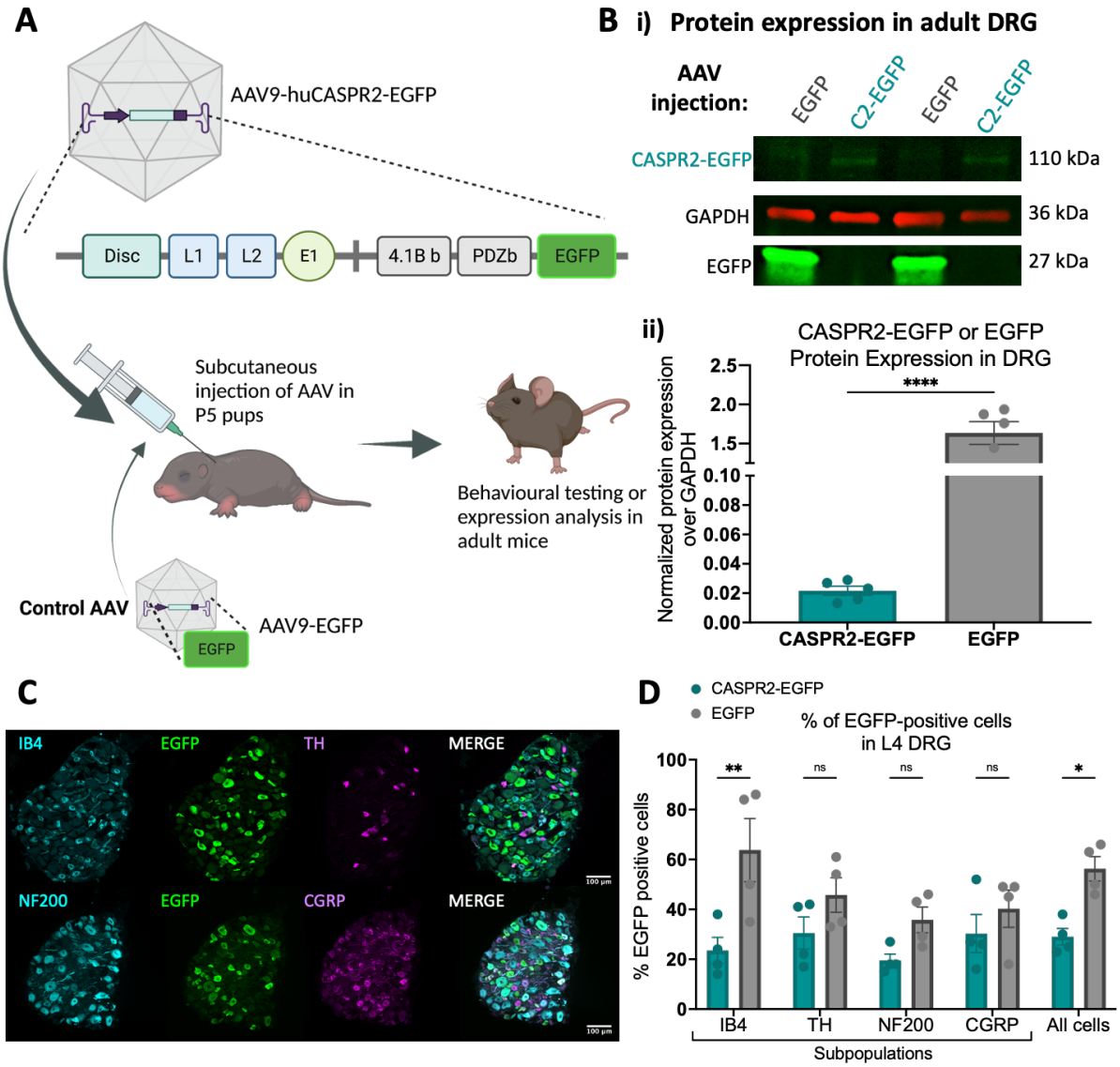


Figure 11. AAV delivery of CASPR2 in mice

A) Schematic of AAV9-huCASPR2-EGFP vector. A truncated version of huCASPR2 tagged with EGFP at the C-terminus was packaged in AAV9 and delivered via subcutaneous injection in P5 pups. A control AAV9 vector encoding EGFP was used as a negative control. Adult AAV-injected mice (8-12 weeks) were used for behavioural testing, and tissue was taken for transgene expression analysis. **B)** Expression of CASPR2-EGFP or EGFP in the adult mouse DRG **i)** Representative images from western blot using an anti-EGFP antibody to detect CASPR2-EGFP or EGFP in the DRG of mice injected with either virus. GAPDH (red, 26 kDa) was used as a loading control. C2-EGFP = CASPR2-EGFP **ii)** Quantification of CASPR2-EGFP or EGFP protein expression in DRG lysates. A high expression of EGFP (27 kDa) was detected, but the expression of CASPR2-EGFP was significantly lower. Unpaired two-tailed t-test, **** $p < 0.0001$, CASPR2-EGFP n=5, EGFP n=7. **C)** Representative images of AAV9-injected mouse DRGs stained with antibodies against EGFP, IB4, TH, NF200, and CGRP **D)** Subpopulation analysis of transgene expression in L4 DRGs of adult mice. The percentage of cells expressing EGFP was quantified for each subpopulation. AAV9-EGFP showed significantly higher transduction efficiency with a higher percentage of cells expressing EGFP in the DRG. There was also a higher percentage of IB4-positive neurons expressing EGFP in mice injected with AAV9-EGFP than those injected with AAV9-CASPR2-EGFP. Two-way ANOVA with Šídák's multiple comparisons test, * $p<0.05$, ** $p<0.01$, n=4.

3.12. CASPR2 Overexpression using AAV did not alter acute pain sensitivity

I assessed thermal and mechanical sensitivity using von Frey, Hargreaves test, and hot plate and did not observe any differences in mechanical or thermal sensitivity between AAV9-EGFP and AAV9-huCASPR2-EGFP-injected mice (**Figure 12A-C**). The open-field assay showed no difference in motor or exploratory behaviour (**Figure 12D**) between AAV9-huCASPR2-EGFP mice and control EGFP mice. Their responses to intraplantar capsaicin were similar- with no reduction in nocifensive behaviour duration at any time point. (**Figure 12E**)

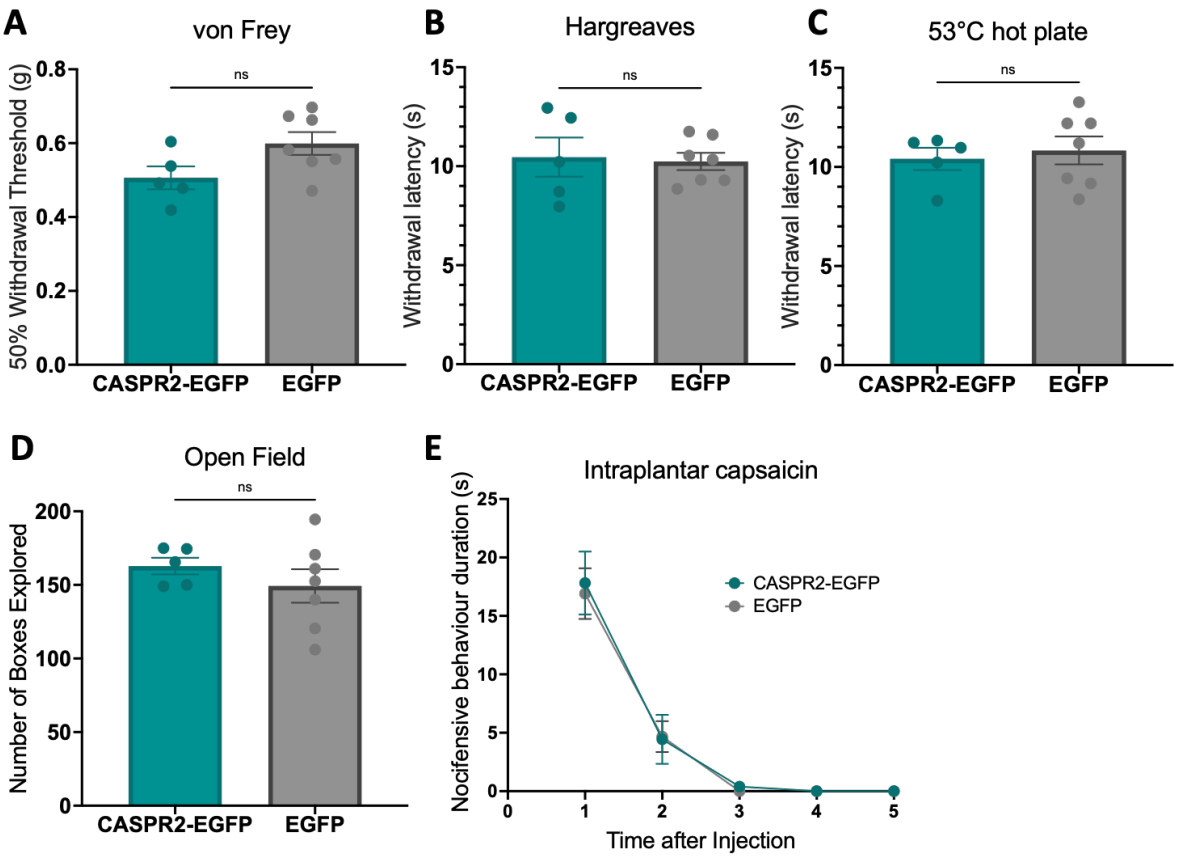


Figure 12. Behavioural testing in mice treated with AAV-CASPR2-EGFP or EGFP
A) Mechanical sensitivity assessed with von Frey hairs showed no difference between AAV9-CASPR2-EGFP or AAV-EGFP-injected mice. There was also no alteration in thermal sensitivity as measured by **B)** Hargreaves test and **C)** 53 °C hot plate. **D)** The number of boxes explored was not significantly different between CASPR2-overexpressing mice and control mice. **E)** There was no reduction in nocifensive behaviour in response to intraplantar capsaicin injection in AAV9-CASPR2-EGFP-injected mice compared to AAV9-EGFP control mice. Unpaired two-tailed t-test. ns. AAV9-CASPR2-EGFP n=5, AAV9-EGFP n=7

4. Discussion

Recent data has shown that the genetic ablation of CASPR2 in mice resulted in the loss of K_v1 membrane expression, hyperexcitability in sensory neurons, and neuropathic pain-like behaviour (Dawes et al., 2018). The downregulation of $K_v1.2$ and $K_v1.1$ in primary afferents after nerve injury has been well-described in literature (Calvo et al., 2016; Fan et al., 2014). Furthermore, in-house data has demonstrated the downregulation of CASPR2 mRNA after nerve injury. This suggests that increasing CASPR2 expression in neurons may reduce cell excitability and, consequently, reduce neuropathic pain after nerve injury (**Appendix Figure 1**). I used transgenic mouse lines that overexpress CASPR2 in nociceptors and DRG neurons and evaluated the therapeutic potential of modulating CASPR2 expression to regulate sensory neuron excitability and pain sensitivity.

I have confirmed that human CASPR2 is overexpressed in the two transgenic mouse lines described previously. The $Hoxb8^{Cre}$: $R26^{LSL:hCNTNAP2(+/+)}$ mice displayed robust overexpression CASPR2 protein in the sciatic nerve and DRG, but not in the brain, in line with the description of the Hoxb8 Cre mouse line (Witschi et al., 2010). The $Na_v1.8^{Cre}$: $R26^{LSL:hCNTNAP2(+/+)}$ mouse line showed comparatively lower protein overexpression of huCASPR2 in the sciatic nerve and only three-fold transcript overexpression in the DRG. It is important to note that the whole DRG will include $Na_v1.8$ negative neurons and non-neuronal cells, so the measured transcript and protein levels may be under-represented due to the unavoidable inclusion of irrelevant cell populations. Given the higher CASPR2 transcript expression in the DRG, it is also plausible that CASPR2 mRNA may be transported and translated along the axon. Using immunocytochemistry, I could stratify DRG

neurons by size and confirm surface overexpression of CASPR2 on small-diameter neurons, described in literature as mostly $Na_v1.8$-positive (Djouhri et al., 2003a). I also showed that huCASPR2 is overexpressed on the surface of DRG neurons of all sizes in the Hoxb8 Cre^+ mouse line.

The confirmed overexpression of CASPR2 is encouraging, but ultimately, the increased surface expression of K_v1 channels is the most critical factor in regulating cell excitability (Smith, 2020; Tsantoulas & McMahon, 2014). Due to the difficulty in obtaining consistent membrane staining of K_v1 channels in DRG neurons, I was not able to validate whether this higher surface expression of CASPR2 translated to increased K_v1 channel localisation at the surface of DRG neurons (**Appendix Figure 8**). Other ways of determining K_v1 channel expression include measurement of K_v1 channel currents in DRG neurons in the presence of known K_v channel blockers or using cell-surface biotinylation and western blot to detect the surface K_v1 channels. Both of these methods are laborious and time-consuming but would be instrumental in answering our question of K_v1 surface expression in the primary afferents of CASPR2-overexpressing mice.

Overexpression of huCASPR2 in the nociceptors or DRG and spinal cord neurons did not affect acute pain-related behaviours, as evidenced by the lack of significant difference in mechanical and thermal sensitivity between CASPR2-overexpressing and control mice. It is possible that the sensory tests utilised to detect mechanical and thermal thresholds were not able to disclose differences between the genotypes. However, pain-like behaviour was substantially reduced in response to the noxious chemical capsaicin in the CASPR2-overexpressing mice. This phenotype was observed across three CASPR2-overexpressing mouse lines, indicating that the overexpression of CASPR2 could reduce neuronal excitability

in TRPV1-expressing neurons. Indeed, the calcium imaging data from the Hoxb8 Cre^{+} DRG neurons showed a significant reduction in the amplitude of calcium transients in response to capsaicin in medium-sized cells compared to Hoxb8 Cre^{-} neurons. There was also a significant reduction in response to KCl-induced depolarisation in Hoxb8 Cre+ small cells. That being said, calcium imaging is only a proxy for measuring neuronal activity, as other factors could influence calcium influx that is not mediated through voltage-gated calcium channels. For example, the non-selective cation channel, TRPV1, could facilitate the direct entry of Ca^{2+} upon capsaicin stimulation. There may also be a release of calcium from internal stores through inositol triphosphate receptors (IP3R), a known interactor of CASPR2 (Argent et al., 2020). Using electrophysiology to measure cell excitability will allow us to gain insight into other electrophysiological properties, such as action potential duration, number, morphology, and frequency.

We therefore measured cell excitability parameters in the DRG neurons of $Na_v1.8^{Cre}$: $R26^{LSL:hCNTNAP2(+/+)}$ mice. Since the exogenous huCASPR2 is untagged, we focussed on the small and medium-sized cells, which are most likely $Na_v1.8$-positive (Djouhri et al., 2003a; Shields et al., 2012). No difference was observed in the threshold to action potential firing or repetitive firing, suggesting that CASPR2-overexpression in nociceptors did not alter cell excitability. This result is perhaps unsurprising, given the modest overexpression of CASPR2 and unconfirmed surface K_v1 channels in $Na_v1.8$ Cre^{+} DRG neurons. It is important to note that we did not inject currents exceeding the rheobase for medium-sized neurons to elicit repetitive firing in these experiments. Therefore, future experiments should be conducted using increased current injections that exceed the rheobase of medium-sized neurons (>2000 pA). More in-depth measures evaluating K^{+} currents in these neurons will help us understand if there is an increase in K_v1 channels on the

neuronal membrane driven by CASPR2 overexpression. Due to time constraints, I did not have the opportunity to measure cell excitability or perform any electrophysiological characterisations on the Hoxb8 Cre $^{+}$ mouse lines.

It is well-established that the mRNA or protein of K_v1 channels are downregulated after nerve injury, leading to altered cell excitability (Fan et al., 2014; Li et al., 2019; Tsantoulas & McMahon, 2014). Recent studies have shown that the upregulation of K_v1 channels in hyperactive neurons reduced excitability. In a rodent epilepsy model, the delivery of an engineered $K_v1.1$ gene reduced neuronal hyperexcitability, and the epigenetic restoration of $K_v1.2$ channels alleviated pain sensitivity after nerve injury (Qiu et al., 2022; Zhang et al., 2021). I utilised the spared nerve injury model to determine whether CASPR2 had any effects in alleviating pain-like behaviour after nerve injury, whether through direct upregulation of K_v1 channel expression or altered membrane trafficking of K_v1 channels. Using the spared sural nerve injury, I first evaluated the $Na_v1.8^{Cre}$: $R26^{LSL:hCNTNAP2(+/+)}$ and observed dramatic reductions in the paw withdrawal thresholds in the ipsilateral hindpaw, as expected. No difference was observed between the genotypes, indicating that CASPR2 overexpression in the nociceptors did not reduce mechanical pain sensitivity after injury at any time point. I looked at changes to transcript levels of key genes after injury by comparing the cDNA in ipsilateral and contralateral DRGs (**Appendix Figure 4**). As expected, the injury marker, ATF3, was significantly upregulated in the ipsilateral lumbar DRGs. Unexpectedly, I did not observe significant downregulation of K_v1 transcripts (*Kcna1, Kcna2*), CASPR2 (*Cntnap2*), or its partner protein, contactin-2 (*Cntn2*). This may have been due to experimenter error where the L3, L4, and L5 DRGs were pooled. The SNI impacts the L4 and L5 DRG; therefore, the inclusion of an uninjured DRG may have diluted the changes in transcript expression in the

injured DRGs. Additionally, whole DRGs were used, including Nav1.8 Cre- populations and other cell types, further reducing the signals observed.

I then evaluated whether the Hoxb8^{Cre} CASPR2-overexpressors had any differences in pain sensitivity after nerve injury, as I have seen much more robust overexpression in this mouse line. I used a modified spared nerve injury model- sparing the tibial nerve- and transecting the common peroneal and sural nerve. The sural nerve contains purely sensory fibres, whereas the tibial nerve contains both sensory and motor fibres; as such, sparing the tibial nerve should result in less motor deficit (Omori et al., 2009; Shields et al., 2003). The spared tibial nerve model also offers the advantage of testing in the middle of the plantar surface, as opposed to the less accessible lateral sural skin area (Decosterd & Woolf, 2000). I observed increased hypersensitivity to punctate and dynamic mechanical stimuli in the ipsilateral hindpaw, and as described in previous studies, the withdrawal thresholds to von Frey hairs were higher than those seen in the spared sural nerve injury model (Bourquin et al., 2006; Guan et al., 2015; He et al., 2022). While there was no reduction in mechanical pain sensitivity between the Hoxb8 Cre^{+} overexpressors and their control littermates, I noticed a trend of recovery on day 21. Separating the groups by sex revealed a significant increase in the paw withdrawal threshold and a non-significant reduction in nocifensive score to dynamic brush in male HoxB8 Cre^{+} male mice compared to Cre^{-} control male mice on day 21. It is important to note that there were fewer Hoxb8 Cre^{-} female mice than male mice (n=2 vs n= 5), so we were underpowered to detect sex differences after injury. Nevertheless, the reduction in pain mechanical pain hypersensitivity in male mice is encouraging, and it is worthwhile to pursue this in a larger cohort in the future.

I also performed free-choice assays to measure spontaneous or ongoing pain-like behaviour in neuropathic animals. The cold place preference assay showed a reduction in time spent at the colder chamber post-injury, though this change was non-significant for both Hoxb8 Cre CASPR2 overexpressors and control littermates. Perhaps a greater temperature difference is required to detect cold allodynia; as described by Caporoso *et al*., an optimised temperature pairing of 30 °C and 12.5 °C was capable of detecting and quantifying behavioural responses in nerve-injured animals (Caporoso et al., 2020). The tibial nerve contains the largest proportion of motor and sensory fibres, and the development of cold hypersensitivity may rely on the injury of a sufficient number of fibres that are not reached in the spared tibial nerve model. Nevertheless, our previous attempt using the dry ice assay after spared sural nerve injury in neuropathic animals did not reveal cold allodynia in neuropathic mice (**Appendix Figure 3**), contrary to what literature suggested (Brenner et al., 2012; Macdonald et al., 2021; Millecamps et al., 2022).

I utilised the conditioned place preference assay to determine whether CASPR2 overexpression affected spontaneous pain. I observed an overall increase for the gabapentin-paired chamber, regardless of mouse genotype, suggesting that the conditioning paradigm was successful in establishing a preference for the drug-paired chamber. However, I did not detect significant differences in the time spent in the gabapentin-paired chamber between the Hoxb8 Cre^{+} and Cre^{-} mice. The variability in the assay was high, and while I attempted to assign drug-paired chambers randomly, there was a slight inherent bias in baseline preference for the striped chamber. I also noted that the paradigm was only effective when the mice had the drug paired with the less-preferred chamber during the baseline testing. As shown in the graph in **Appendix Figure 9**, mice that received the same chamber

pairing (i.e. gabapentin paired with their baseline preferred chamber) did not exhibit an increase in the drug-paired chamber or a decrease in the saline-paired chamber. This calls the assay set-up in question- Should the less-preferred chamber be paired with the analgesic to maximise the window for detecting preference? Or should drug-paired chambers be randomly assigned and counter-balanced such that the group baselines are similar? Another confounding factor in the CPP assay is the choice of drug to induce preference. I used gabapentin to obviate intrathecal injections required for clonidine, a non-addictive analgesic (King et al., 2009). Gabapentin, on the other hand, is an addictive analgesic prone to misuse and dependence in humans and has been shown to induce drug-seeking behaviour in mice, albeit at a much higher dose than the one used in our study (300 mg/kg vs 30 mg/kg) (Althobaiti et al., 2020; Griggs et al., 2015). It has also been shown that neuropathic mice display anxiety- and depression-like symptoms and gabapentin reversed depression-like behaviours in nerve-injured mice (Fu et al., 2018; Norman et al., 2010). Therefore, we cannot exclude the confound of the anti-depression-like symptoms of gabapentin in conditioned place preference assays in nerve injury models.

The conditioned place preference assay can be a powerful tool to measure spontaneous and affective pain, but further optimisation and validation are needed to reduce the observed high variability. Other non-reflexive pain measures can also be applied to assess spontaneous and ongoing pain. The automated measurement of body pose and weight-bearing or gait analysis could be viable alternatives to detect pain-related behaviours in neuropathic mice (Berryman et al., 2009; Zhang et al., 2022). The measurement of cage-hanging, an elective, voluntary behaviour in mice has been demonstrated to be a promising translationally relevant outcome measure. Cage-hanging behaviour was significantly reduced in nerve-injured mice

and this reduction was sustained over longer timepoints, making it an ideal assay for nerve injury models. Cage-hanging can be performed without experimenter intervention and measured using automatic detection strategies (Zhang et al., 2021).

Adeno-associated virus gene therapy has emerged as a promising therapeutic modality, proven by the US FDA approval of two AAV-based gene therapies (Luxturna and Zolgesma) and the increasing number of clinical trials (Kuzmin et al., 2021). Furthermore, targeted delivery of AAV to peripheral sensory neurons has been tested as a treatment for pain (Iyer et al., 2014). I investigated the potential of AAV delivery of CASPR2 as a therapeutic for neuropathic pain. I utilised the AAV9 serotype for its tropism for peripheral sensory neurons and generated a truncated version of CASPR2 that fits within the packaging capacity of the AAV (Foust et al., 2009; Schuster et al., 2014). AAV vectors expressing CASPR2-EGFP (AAV9-CASPR2-EGFP) or control EGFP (AAV9-EGFP) under the mini neuronal CAG promoter were delivered by subcutaneous injection to neonatal mice. I then assessed transgene expression in adult mice and observed moderate transduction efficiencies of the AAV9 vectors, with approximately 40% and 60% of DRG neurons expressing CASPR2-EGFP or EGFP, respectively. There was a pronounced disparity in total protein levels between the two vectors; the amount of CASPR2-EGFP expressed was drastically lower than that of EGFP, as detected by western blot in whole DRG lysates. This might be caused by the lower number of AAV9-CASPR2-EGFP particles injected. An equal volume of viruses (10μL) was injected to maximise the number of injected particles, but the titre of AAV9-CASPR2-EGFP was approximately three-fold lower than that of AAV9- EGFP. Moreover, the CASPR2-EGFP transgene is nearly five times bigger than EGFP alone, potentially making it more energetically expensive to produce and causing

lower expression in transduced neurons. Given the extremely low expression of CASPR2-EGFP in the DRG, it is unsurprising that I did not observe changes to acute pain-related behaviours or reductions in pain-like behaviour in response to capsaicin. Thus, I did not carry out a nerve-injury model in the AAV9-injected mice.

Other factors may also influence the lack of efficacy observed in AAV9-CASPR2-EGFP mice. There could be epitopes that are occluded or lost due to the design of our truncated construct. Studies with domain-deletion experiments showed that CASPR2 associates with contactin-2 through the laminin-like G4 (L4) domain proximal to the membrane (Saint-Martin et al., 2019). However, our construct does not contain the domains necessary for the association of contactin-2 and theoretically would not partner with contactin-2 when expressed *in vivo*. It has been shown that the association of CASPR2 and contactin-2 is necessary for the clustering of VGKC at the JXP, so this delivery of truncated CASPR2 is perhaps unlikely to affect K_v1 channel expression (Horresh et al., 2008; Poliak et al., 2003). The intracellular domain of CASPR2 is relatively small (5 kDa) compared to the 26 kDa EGFP. Placing a large EGFP tag on CASPR2 may hinder the binding of CASPR2 to the adapter protein 4.1B. The interaction of CASPR2 and 4.1B is necessary for the accumulation of CASPR2 and K_v1 channels at the JXP, and the deletion of the 4.1B-binding domain of CASPR2 led to K_v1 spreading to paranodal regions (Horresh et al., 2008).

Looking forward, further optimisation of construct design, method of delivery, and capsid choice will hopefully aid in a more productive AAV-based therapeutic. In the case of CASPR2, selecting a truncated domain that can associate with its partner proteins would be crucial in ensuring the upregulation of K_v1

channels. For ease of detection of AAV transduction in tissues, a fluorescent protein can be paired with the untagged protein of interest separated by self-cleaving 2A peptides (Liu et al., 2017). The large EGFP tag can also be replaced with a smaller epitope tag, such as a HA or flag tag, reducing the likelihood of epitope hindrance of partner proteins.

For AAV therapeutics in general, further improvement of promoters will offer greater specificity of AAV transduction in cell populations of interest, potentially reducing the titre required for effective expression. As shown in a recent study by Qiu *et al*., using an AAV vector containing a c-Fos promoter specifically targeted hyperactive cells (Qiu et al., 2022). Intrathecal injection of AAV9 has yielded a high transduction efficiency in mouse DRG (Schuster et al., 2014) and clinical trials have shown no administration-related serious adverse events using the intrathecal route of injection (Kuzmin et al., 2021). Our use of a truncated construct stemmed from a need to fit within the packaging capacity of current capsids. Fortunately, significant advances have been made in optimising AAV capsids regarding payload capacity or tropism. Efforts using machine-guided approaches to generate synthetic AAV capsids or Cre-recombination-based AAV targeted evolution (CREATE) will propel the next generation of AAV capsids that circumvent the current limitations (Bryant et al., 2021; Kumar et al., 2020).

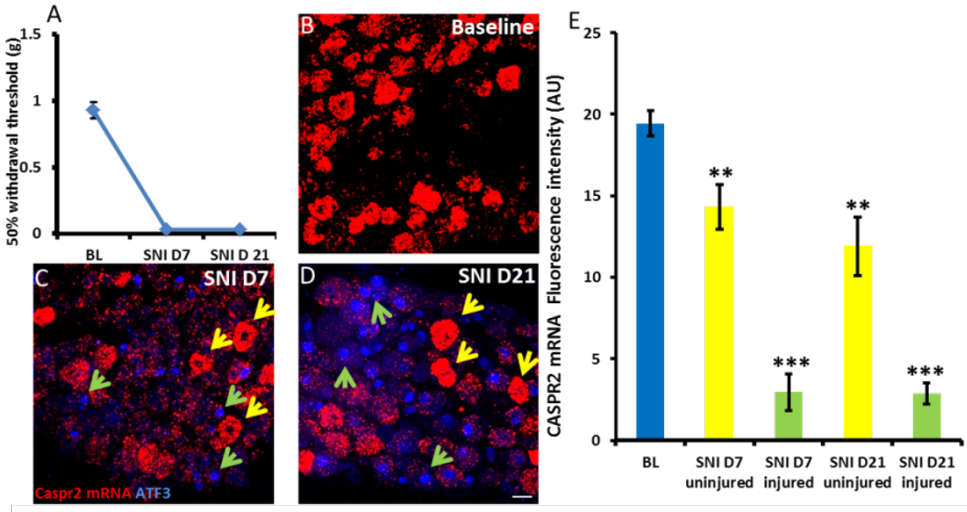
In summary, I have successfully generated transgenic mouse lines that overexpress full-length CASPR2 in the peripheral sensory neurons. Overexpression of CASPR2 did not affect acute sensory behaviours but reduced pain sensitivity to capsaicin. Additional experiments are required to determine whether CASPR2 overexpression is sufficient to increase the surface expression of K_v1 channels or other protein complexes that may reduce the sensitivity to

capsaicin. Further understanding of the mechanisms underlying CASPR2 modulation of K_v1 channels in the primary afferents will aid in designing next-generation, effective therapeutics that reduce neuronal hyperexcitability and ultimately alleviate neuropathic pain.

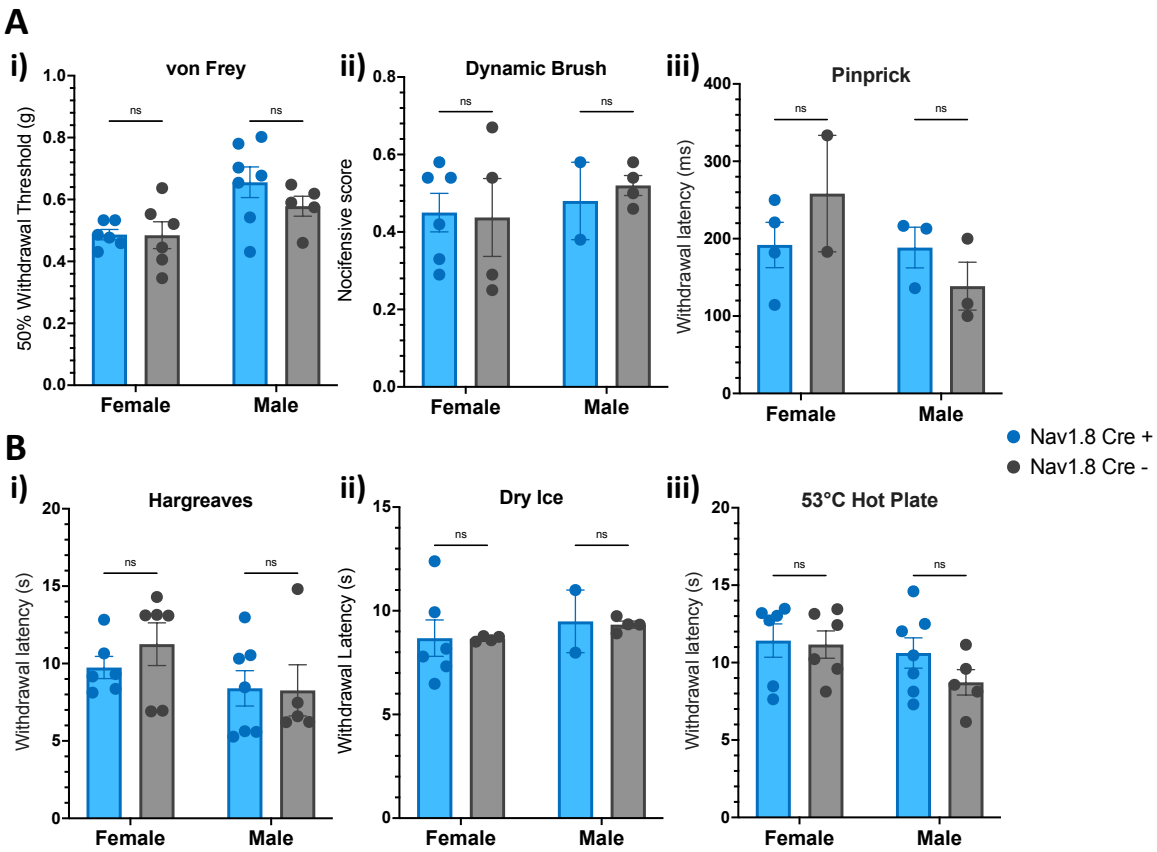
5. Acknowledgements

Rodent surgeries were performed by A/Prof J. Dawes or myself. The electrophysiology data from Figure 5 was generated by Dr A. Farah. The AAV9-CASPR2-EGFP and AAV9-EGFP vectors were designed by A/ Prof J. Dawes and Dr T. Trendafilova and generated by the Viral Vector Facility at the Neuroscience centre of Zurich. Appendix Figure 1 with the interesting colour scheme was by A/Prof J. Dawes. All figures were generated using BioRender or GraphPad Prism9.

6. Appendices

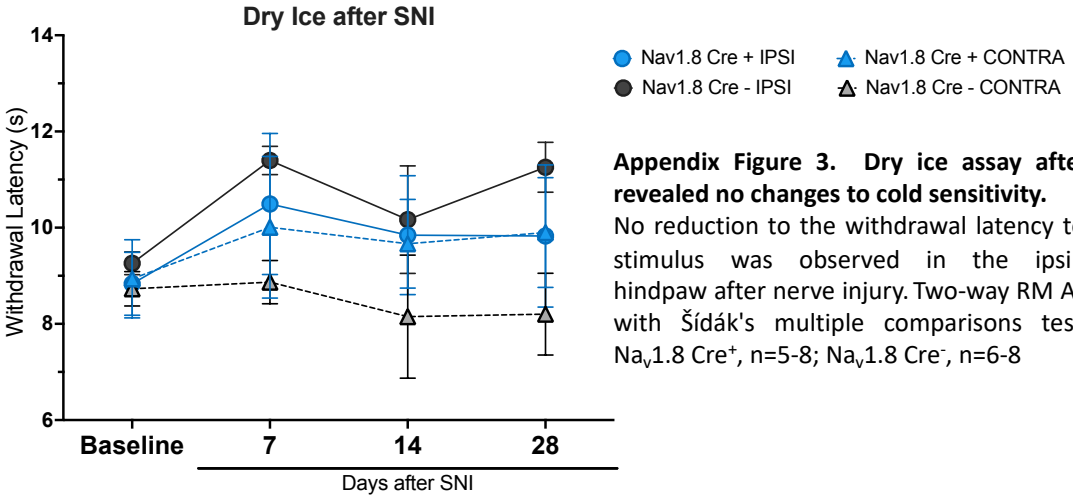


Appendix Figure 1. Down-regulation of Caspr2 in primary sensory neurons following nerve injury.
A) The spared nerve injury (SNI) model causes robust mechanical hypersensitivity in mice at 7 and 21 days post-injury (n=10). Representative images of ISH for Caspr2 mRNA expression (red) in mouse L4 DRG tissue section without injury **B)** or at 7 **C)** and 21 **D)** days post SNI. ATF3 (blue) marks injured neurons indicated by green arrows, yellow arrows indicate non-injured neurons. **E)** Quantification of Caspr2 mRNA expression shows a significant down-regulation at both 7 and 21 days post SNI; time points associated with neuropathic pain-like behaviour. Caspr2 mRNA down-regulation is most prominent in injured (green) neurons (n=5). One Way ANOVA *p<0.01, **p<0.001 vs baseline. Data shown as mean±SEM. Scale bar 25µm.

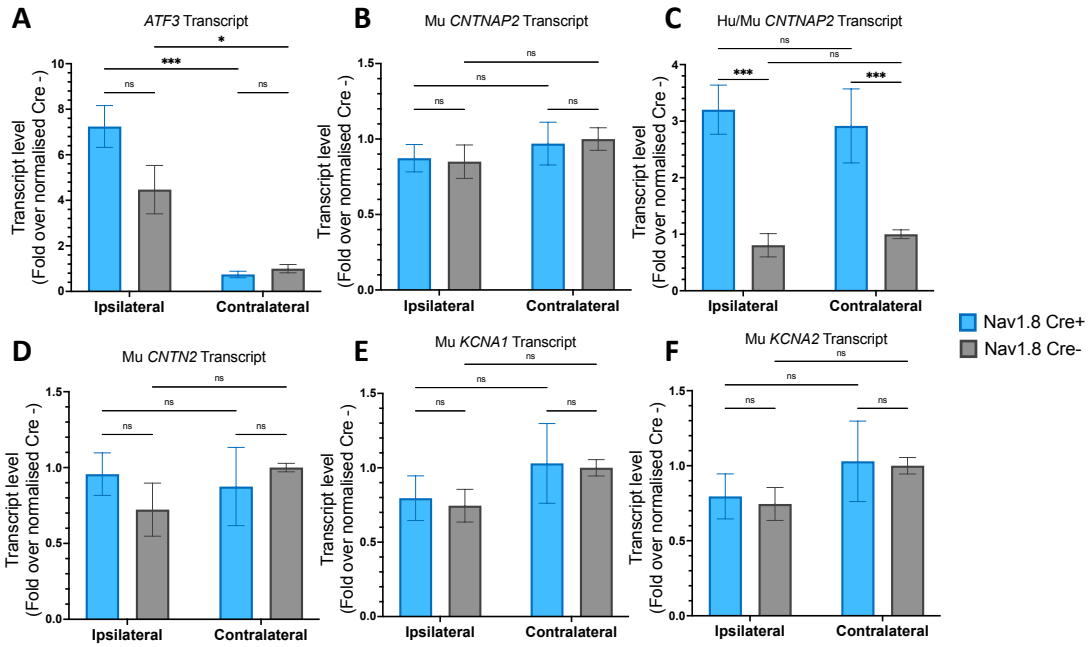


Appendix Figure 2. Baseline mechanical and thermal sensory testing in male and female Nav1.8Cre : R26$^{LSL:hCNTNAP2(+/+)}$ mice.

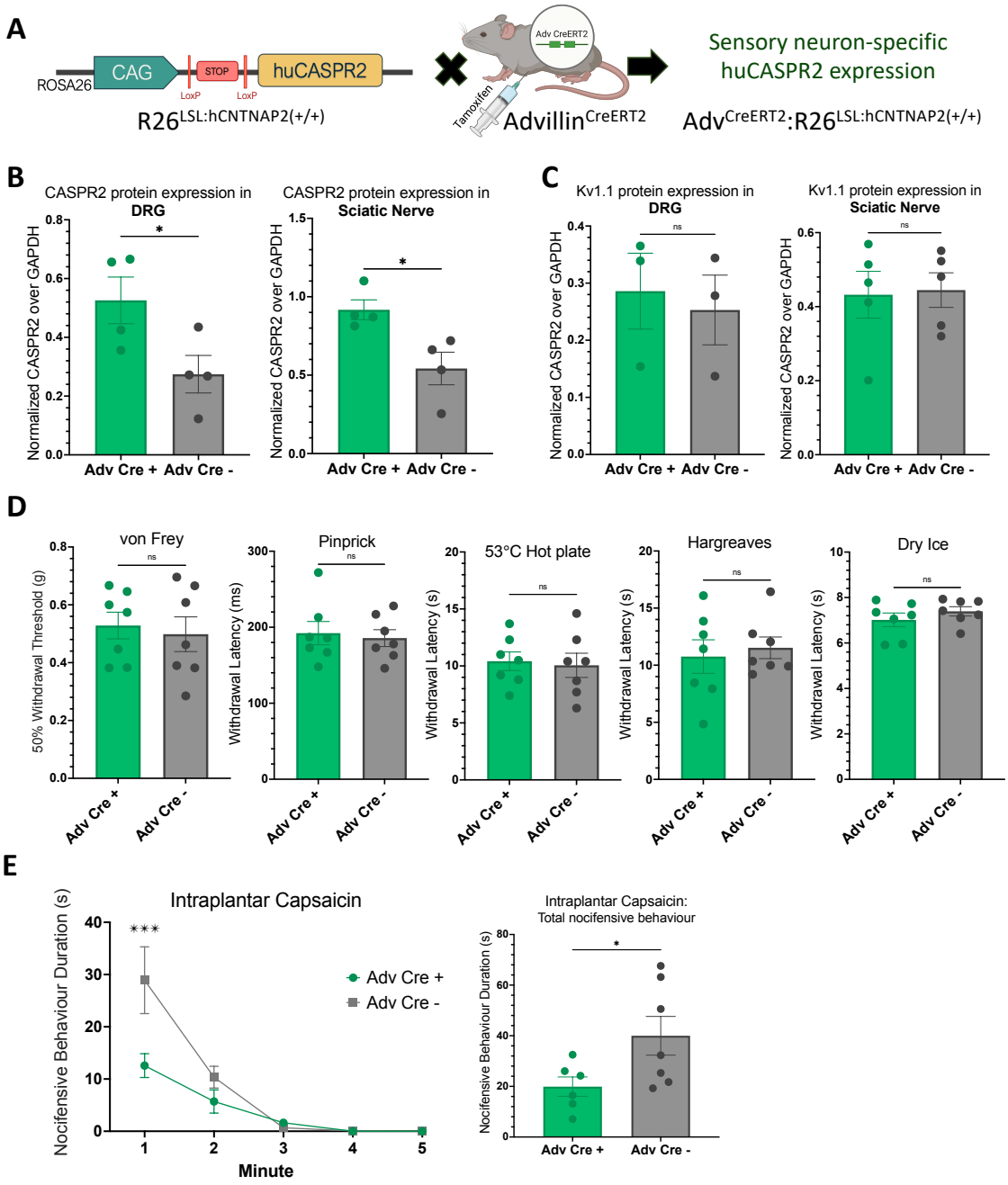
A) There were no significant differences in mechanical sensitivity detected between male and female $Na_v1.8$ Cre^+ and $Na_v1.8$ Cre^- control littermates measured by **i)** von Frey hairs **ii)** dynamic brush test iii) Pinprick **B)** There were no significant differences in thermal sensitivity between male and female $Na_v1.8$ Cre^+ and Nav1.8 Cre^- control littermates measured by **i)** Hargreaves test or **ii)** Dry ice assay, or **iii)** 53° hotplate,. Two-way ANOVA with Šídák's multiple comparisons test $Na_v1.8$ Cre^+ female n=4-6, male n=3-7; $Na_v1.8$ Cre^- female n=2-6, male n=3-5. ns.



Appendix Figure 3. Dry ice assay after SNI revealed no changes to cold sensitivity.
No reduction to the withdrawal latency to cold stimulus was observed in the ipsilateral hindpaw after nerve injury. Two-way RM ANOVA with Šídák's multiple comparisons test, ns, $Na_v1.8$ Cre^+, n=5-8; $Na_v1.8$ Cre^-, n=6-8

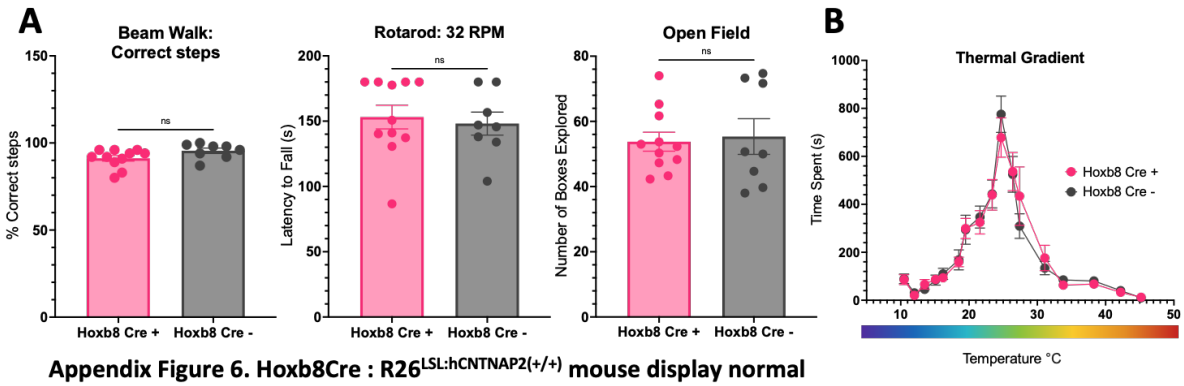


Appendix Figure 4. Transcript levels of voltage-gated potassium complex genes in pooled L3-L5 DRGs
Normalised transcript levels of RNA from pooled L3, L4, L5 DRGs in $Na_v1.8$ Cre^+ and $Na_v1.8$ Cre^- mice 28 days after $SNI_{(s)}$. **A)** Transcript of ATF3, a marker for neuronal injury, was upregulated in the ipsilateral DRG after injury **B)** No change in the murine *Cntnap2* transcript (encoding endogenous mouse CASPR2) in ipisilateral DRGs **C)** The overexpression of hu*Cntnap2* was sustained in the Nav1.8 Cre^+ mice after injury. No decrease of hu/mu *Cntnap2* was detected in the ipsilateral $Na_v1.8Cre^-$ DRG. **D)** No alterations in *Cntn2* (encodes mouse contactin-2, partner protein of CASPR2) transcript expression detected after injury. No changes in expression detected for **E)** *Kcna1* ($K_v1.1$) and **F)** *Kcna2* ($K_v1.2$) transcripts after injury between $Na_v1.8$ Cre^+ and $Na_v1.8$ Cre^- mice. Two-way RM ANOVA with Šídák's multiple comparisons test, *** $p<0.001$, ns. n=4



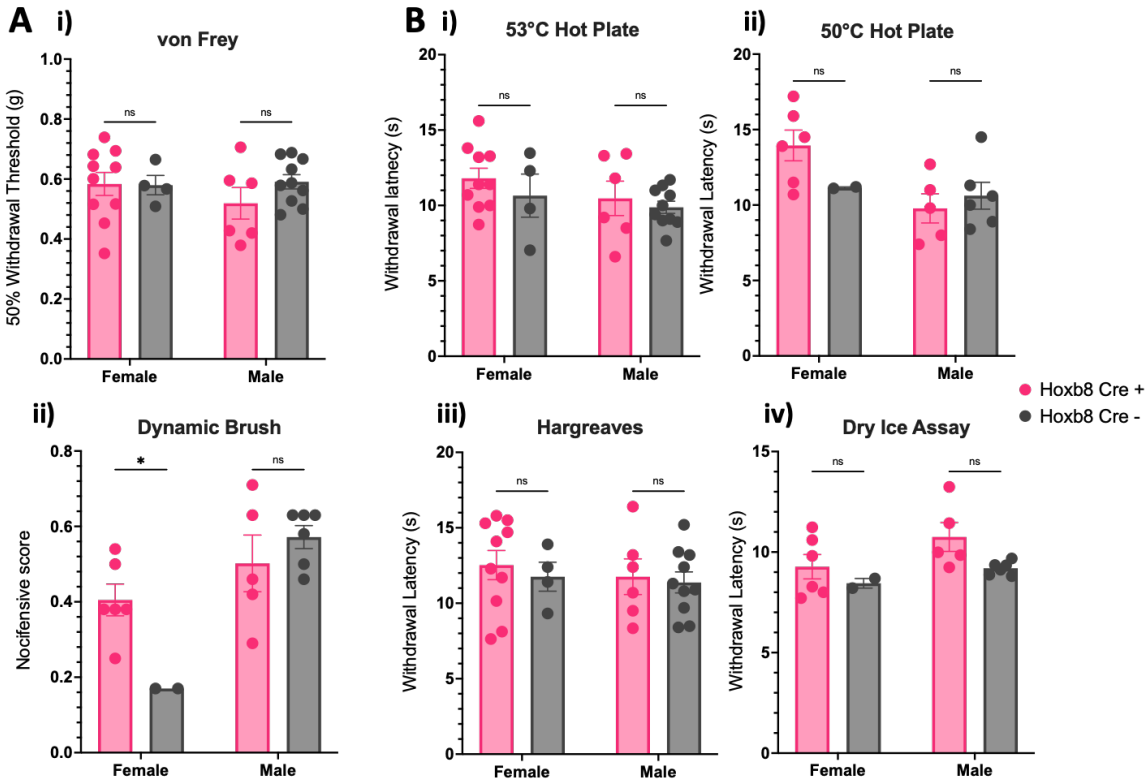
Appendix Figure 5. Expression and sensory testing of the Adv^{CreERT2}: R26$^{LSL:hCNTNAP2(+/+)}$ mouse line

A) The R26$^{LSL:hCNTNAP2(+/+)}$ mouse line was crossed with the tamoxifen-inducible Adv^{CreERT2}: R26$^{LSL:hCNTNAP2(+/+)}$ line to induce overexpression of CASPR2 in sensory neurons. This mouse line was not viable after tamoxifen-dosing, so a nerve injury model was not performed. Behaviour was performed 4 weeks after tamoxifen injection **B)** CASPR2 overexpression was detected in the DRG and sciatic nerve of Adv Cre + mice compared to control littermates **C)** Kv1.1 protein expression in whole DRG or sciatic nerve was not upregulated in the Adv Cre + mice. **D)** Baseline mechanical and thermal sensory testing did not differ disclose any differences between the Adv Cre + and Cre – mice. Unpaired two-tail t-test, ns. Adv Cre^{+} n=7, Na$_v$1.8 Cre^{-} n=7. E) Adv Cre $^{+}$ mice exhibited reduced nocifensive behaviour in the first minute after injection and in total nocifensive behaviour over 5 minutes, compared to Cre- littermates. Two-way RM ANOVA with Šídák's multiple comparisons test, ***p<0.001, Adv Cre^{+} n=6, Na$_v$1.8 Cre^{-} n=7.



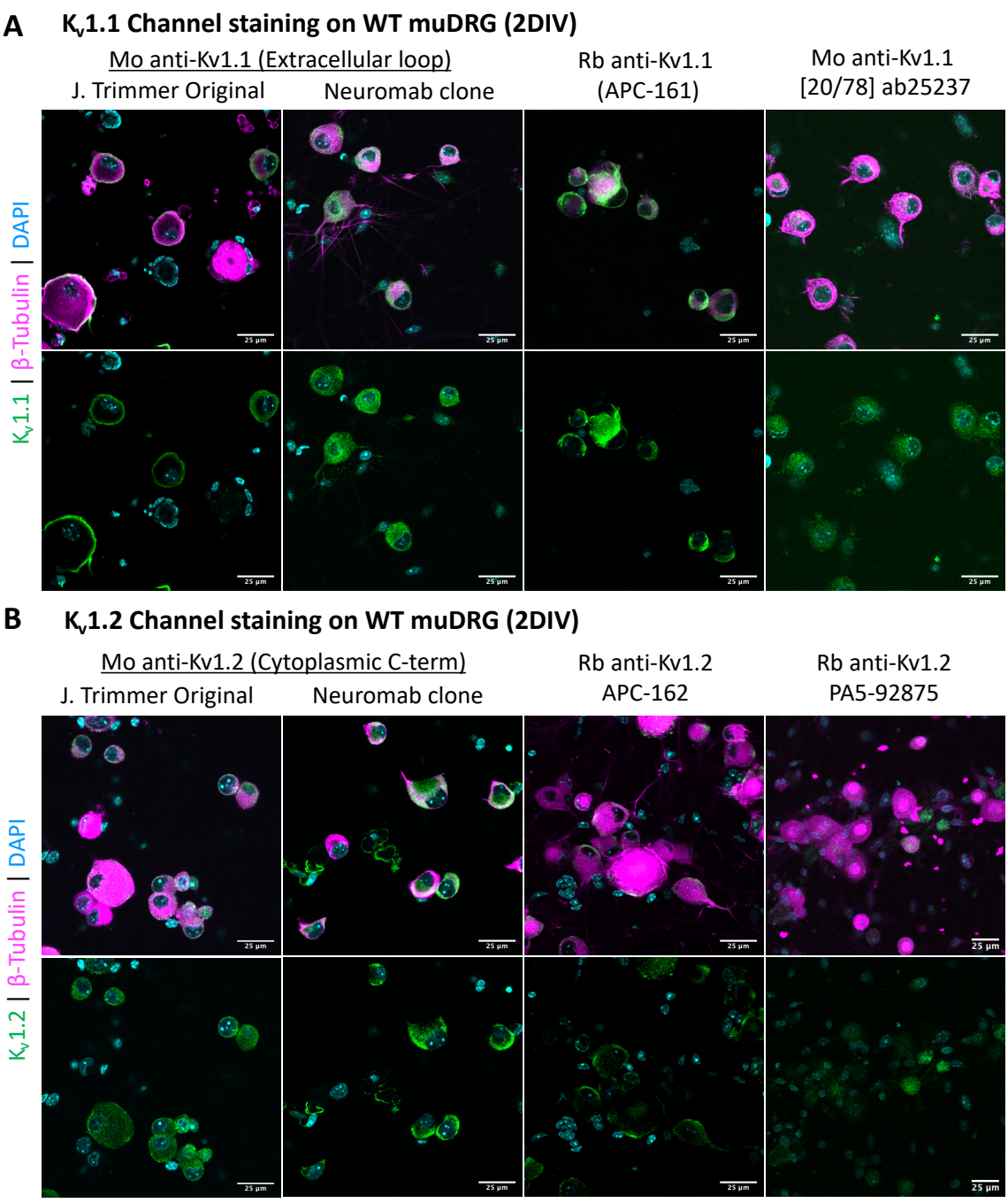
Appendix Figure 6. Hoxb8Cre : R26$^{LSL:hCNTNAP2(+/+)}$ mouse display normal motor activity and temperature preference,

A) Motor activity testing by beam walk, Rotarod, open field revealed no altered motor activity in CASPR2 overexpressing Hoxb8Cre+ mice compared to control littermates. Two-tailed unpaired t-test. $Na_v1.8$ Cre$^+$ **B)** Hoxb8 Cre$^+$ mice also exhibited the same thermal preference as the control mice. Hoxb8 Cre$^+$, *n*=11; Hoxb8 Cre$^-$, n=9, ns



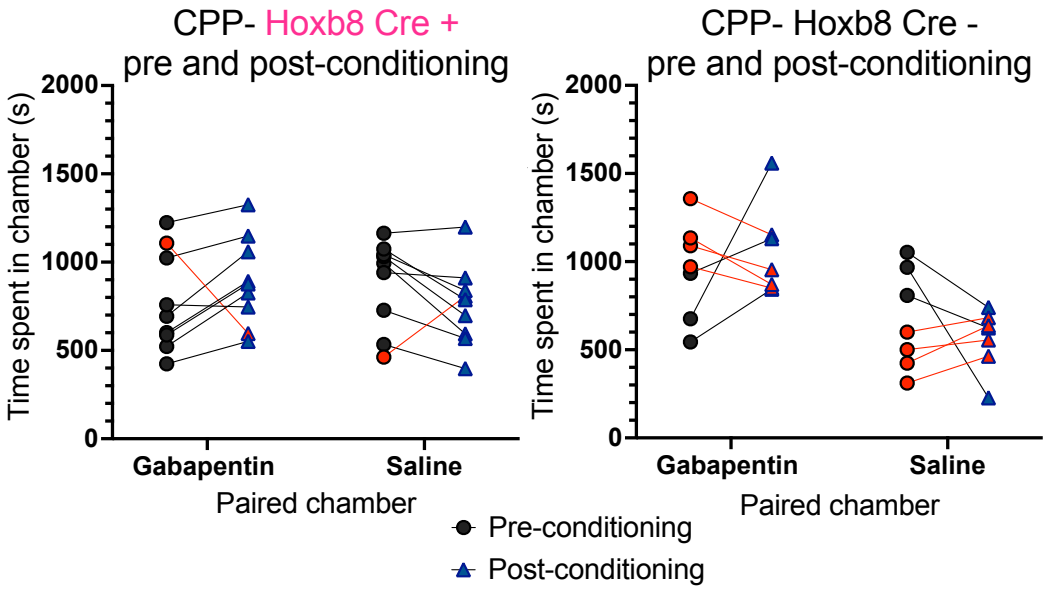
Appendix Figure 7. Baseline mechanical and thermal sensory testing in male and female Hoxb8Cre : R26$^{LSL:hCNTNAP2(+/+)}$ mice.

A) i) There were no significant differences in mechanical sensitivity detected between male and female Hoxb8 Cre$^+$ and Hoxb8 Cre$^-$ control littermates measured by von Frey hairs **ii)** Female Hoxb8 Cre- had a significantly lower nocifensive score in the dynamic brush test. **B)** There were no significant differences in thermal sensitivity between male and female Hoxb8 Cre$^+$ and Hoxb8 Cre$^-$ control littermates measured by **i)** 53°C or **ii)** 50°C hot plate, **iii)** Hargreaves test, or **iv)** dry ice assay. Two-way ANOVA with Šídák's multiple comparisons test HoxB8Cre$^+$ female n=6-10, male n=4-6; Hoxb8Cre$^-$ female n=2-4, male n=5-10. ns, *p<0.05

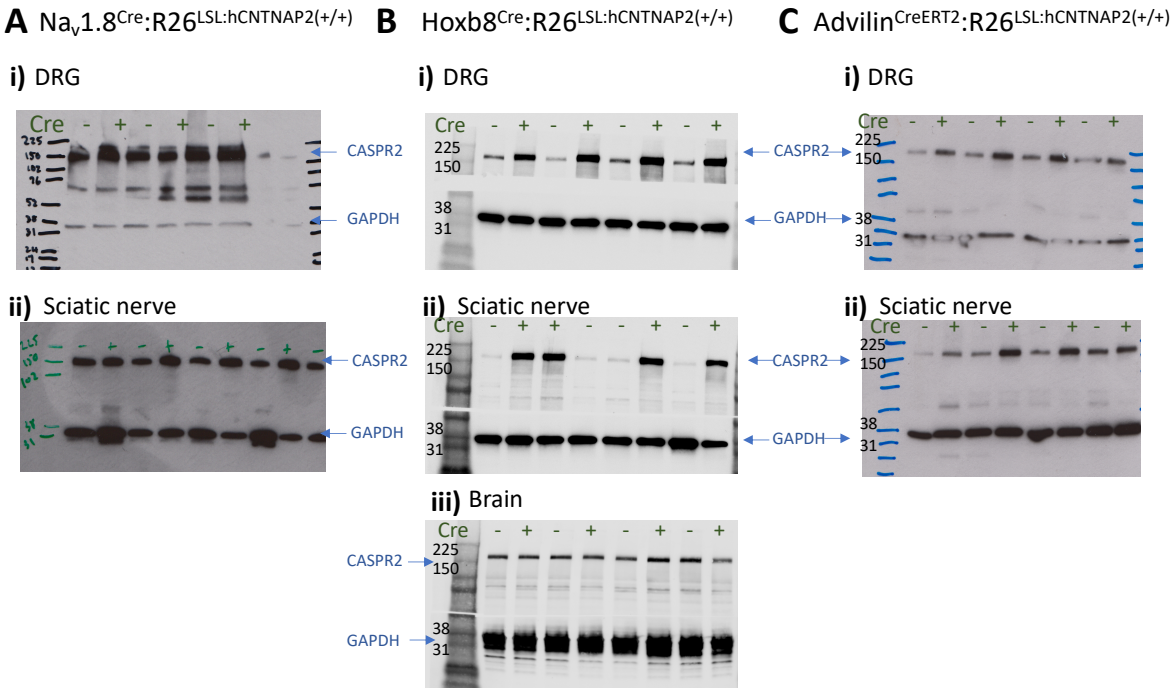


Appendix Figure 8. Staining of potassium channels is variable

Representative images of **A)** Kv1.1 and **B)** Kv1.2 staining of wild-type mouse DRGs at 2 days *in vitro.* None of the commercial clones recapitulated the membrane-staining of the original antibody from Dr J Trimmer. Similar results were obtained for DRG neurons at days 1 and 3 in culture. DAPI (cyan) and βIII-tubulin (magenta). Scale bar, 25 µm.



Appendix Figure 9. Assignment of drug-paired chamber affects induction of CPP.
Mice that received the same drug chamber pairing as their baseline preferred chamber (i.e. gabapentin paired with their baseline preferred chamber, in red) did not exhibit an increase in the drug-paired chamber, or a decrease in the saline-paired chamber .



Appendix Figure 10. Images of western blots detecting CASPR2 and GAPDH in tissues of A) Na$_v$1.8^{Cre}:R26$^{LSL:hCNTNAP2(+/+)}$ **B)** Hoxb8^{Cre}:R26$^{LSL:hCNTNAP2(+/+)}$ and **C)** Advilin^{CreERT2}:R26$^{LSL:hCNTNAP2(+/+)}$ mice. Molecular weights (KDa) are illustrated in the figure. CASPR2 and GAPDH bands are labelled in blue. Data from these blots are plotted in Figures 1, 6, and appendix figure 5.

Appendix Table1. List of antibodies used in this thesis

Antibody	Source	Catalog Number
Rabbit anti-4EBP1	Cell Signaling Technology	9644
Rabbit anti-Akt	Cell Signaling Technology	9272
Rabbit anti-pAkt (Ser473)	Cell Signaling Technology	4060
Mouse anti-beta actin	Abcam	ab8226
Rabbit anti-CASPR2	Alomone	APZ-005
Rabbit anti-CASPR2	Abcam	ab153856
Rabbit anti-CASPR2	Abcam	ab13705
Sheet anti-CGRP	Enzo Life Sciences	BML-CA1137
Chicken anti-EGFP	Abcam	ab13970
Rabbit anti-GAPDH	Abcam	ab16891
Isolectin B4 (IB4), biotin conjugate	Sigma-Aldrich	L2140
Mouse anti-Kv1.1 [K20/78]	Gift from J. Trimmer	N/A
Mouse anti-Kv1.1 [K20/78]	Neuromab / Antibodies Inc.	75-007
Rabbit anti-Kv1.1	Alomone	APC-161
Mouse anti-Kv1.1 [K20/78]	Abcam	ab25237
Mouse anti-Kv1.2 [K14/16]	Gift from J. Trimmer	N/A
Mouse anti-Kv1.2 [K14/16]	Neuromab / Antibodies Inc.	75-008
Rabbit anti-Kv1.2	Alomone	APC-162
Rabbit anti-p44/42 MAPK	Cell Signaling Technology	9102
Rabbit anti-p44/42 MAPK	Cell Signaling Technology	9101S
Mouse anti-NF200	Sigma-Aldrich	N0142
Chicken anti-NF200	Abcam	ab4680
Rabbit anti-NeuN	Abcam	ab177487
Mouse anti-beta III tubulin	Sigma-Aldrich	T8578
Rabbit anti-beta III tubulin, Alexa 555 conjugate	Abcam	ab202519
Rabbit anti-S6	Cell Signaling Technology	2217
Rabbit anti-pS6 (Ser235/236)	Cell Signaling Technology	4858
Sheep anti-Tyrosine Hydroxylase	Milipore	AB1542
Streptavidin Pacific Blue	Thermo Fisher Scientific	S11222
Goat anti-human IgG (H+L), Alexa 488	Thermo Fisher Scientific	A-11013
Goat anti-human IgG (H+L), Alexa 546	Thermo Fisher Scientific	A-21089
Goat ant-human IgG (Fc), Alexa 488	Thermo Fisher Scientific	A-55747
Goat anti-Chicken IgY (H+L) Alexa 488	Thermo Fisher Scientific	A-11039
Goat anti-Rabbit IgG (H+L), Alexa 488	Thermo Fisher Scientific	A-11008
Goat anti-Rabbit IgG (H+L), Alexa 546	Thermo Fisher Scientific	A-11010
Goat anti-Mouse IgG (H+L), Alexa 488	Thermo Fisher Scientific	A-11001
Goat anti-Mouse IgG (H+L), Alexa 546	Thermo Fisher Scientific	A-11030
Donkey anti-goat IgG, Alexa 488	Thermo Fisher Scientific	A-11055

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3

Patient Sera Screening on Rodent DRG Neurons

1. Introduction

Pain has been reported as a common clinical symptom in patients with LGI1 and CASPR2 autoantibodies. Multiple studies have reported that pain was experienced by over 50% of patients with CASPR2 or LGI1 antibodies, and in many of these patients, pain is the sole symptom (Binks et al., 2018; Gadoth et al., 2017; Ramanathan et al., 2021). As the most common clinical manifestations of CASPR2/LGI1 IgG-positive patients involve the CNS (e.g. encephalitis, seizures), most studies have focused on the binding and effects of patient sera on hippocampal neurons (Giannoccaro et al., 2019; Joubert et al., 2022; Lang et al., 2017; Saint-Martin et al., 2019). There are currently few studies characterising the binding of patient sera on peripheral sensory neurons; hence, the pathophysiology of autoantibody-mediated pain is still not well-understood (Cuhadar et al., 2019; Dawes et al., 2018; Goebel et al., 2021).

1.1. Neuropathic pain and autoantibodies

Autoantibodies toward CASPR2 and LGI-1 have been identified in patients with neuronal hyperexcitability disorders (Hart et al., 1997; Irani et al., 2010). Patients with anti-LGI1 or CASPR2 antibodies have a spectrum of neurological presentations, with differences in CNS and PNS involvement. Antibodies toward CASPR2 are commonly associated with Morvan's syndrome (MoS), neuromyotonia, and pain; while antibodies to LGI1 are more frequently associated with encephalitis and seizures (faciobrachial dystonic seizures) (Binks et al., 2018; Gadoth et al., 2017; Hart et al., 1997; Irani et al., 2010). Pain, often neuropathic, is a frequent clinical feature in patients with CASPR2 and LGI1 antibodies; it has been reported in 62% of patients with Morvan's syndrome (Irani et al., 2012). Additionally, a study with 316 patients with anti-VGKC antibodies reported that

50% of these patients experience pain. In the same study, subacute onset pain was reported by 21% of LGI1-IgG- positive and 46% of CASPR2-IgG- positive patients (Gadoth et al., 2017). Treatment of patients with immunotherapies showed improvements in their pain, suggesting that autoantibodies may be causative of pain (Klein et al., 2012; Lahoria et al., 2017). Furthermore, the passive transfer of neuropathic patient IgG in mice resulted in pain-related hypersensitivity, confirming the pathogenicity of autoantibodies *in vivo* (Dawes et al., 2018).

Given the expression of CASPR2 and LGI1 in the CNS and PNS- and their close associations with voltage-gated potassium channels- it is likely that autoantibodies toward these antigens may alter neuronal excitability (Poliak et al., 1999). Earlier studies demonstrated that the application of antibodies from neuromyotonia patients reduced K^+ currents in neuronal cell lines, which the authors suggested was due to increased degradation of VGKCs caused by bivalent binding of patient antibodies (Tomimitsu et al., 2004). In line with these findings, Dawes *et al*. showed that K_v1 channel expression was reduced on DRG neurons following treatment with pain patient plasma, supporting the notion that patient autoantibodies can directly target neuronal molecules that regulate cell excitability, therefore contributing to pain (Dawes et al., 2018).

The pathogenic potential of autoantibodies is bolstered by other studies investigating the role of antibodies in other pain conditions such as fibromyalgia syndrome (FMS) and complex regional pain syndrome (CRPS) (Cuhadar et al., 2019; Goebel et al., 2011, 2021). The passive transfer of IgG from patients with CRPS caused a reduction of rearing behaviour and motor impairment in mice (Goebel et al., 2011). In another study, the combination of paw incision and administration of CRPS patient IgG in mice induced post-surgical hypersensitivity to noxious mechanical and thermal stimulation. This painful hypersensitivity was caused by

the sensitisation of A and C nociceptors by autoantibodies (Cuhadar et al., 2019). More recently, Goebel *et al*. demonstrated that the passive transfer of IgG from fibromyalgia patients also induced pain hypersensitivity in mice which was also caused by the sensitisation of peripheral nociceptive afferents (Goebel et al., 2021). These studies add to the growing evidence of the significant involvement of autoantibodies in pain conditions. The additional characterisation of patient serum samples will help further our understanding of the mechanisms underlying the pathogenicity of autoantibodies.

1.2. Screening of patient antibodies

Screening of patient sera is typically done using live cell-based assays (CBAs) in which human embryonic kidney (HEK293) or Chinese hamster ovary (CHO) cells are transfected with antigens of interest. This assay offers high-throughput screening of patient sera and confirms their reactivity to specific antigens. The scoring of binding using the CBA is quick, as its usually done on epifluorescent microscopes using a semi-quantitative scale from 0 – 4 (van Coevorden-Hameete et al., 2016). However, heterologous expression of neuronal antigens does not completely recapitulate the native environment of proteins *in vivo*, as accessory proteins are not usually co-expressed. These partner proteins may modulate the structure and alter or mask epitopes associated with antigens. The CBA also requires the choice of isoforms when multiple isoforms or splice variants exist, whereas primary neuronal cultures express the relevant isoforms in the context of antibody binding (Miller et al., 2021; Waters et al., 2016).

Live neuronal cultures can be used to detect the binding of patient antibodies to extracellular antigens expressed on neurons (Miller et al., 2021; van Coevorden-Hameete et al., 2016; Waters et al., 2016). These cultures express the

relevant epitopes and isoforms and can distinguish the localisation of subcellular binding. For example, in cultured hippocampal neurons, developed synapses and neurites can be detected after 14 days in culture and binding to these regions can be assessed *in vitro* (Joubert et al., 2022; Pinatel et al., 2015; van Coevorden-Hameete et al., 2016).

However, binding assays using primary neuronal cultures are not without shortcomings. The expression of the neuronal antigen of interest may be low compared to heterologous expression systems. The lower antigen expression can reduce the assay sensitivity and require higher concentrations of antibodies for the detection of binding or the application of tertiary antibodies to amplify the signal. The protein expression levels may also differ depending on the length of culture, so careful optimisation of culture conditions is needed. Rodent neurons may also express proteins such as Fc gamma receptors that may mediate the binding of IgG without direct binding to the target antigen (Wang et al., 2019). Finally, as most laboratories utilise rodent neuronal cultures, there will be species differences between human and rodent versions of the proteins of interest. As such, there may be disparities in binding observed on rodent neurons and binding patients *in vivo*. Due to these drawbacks, extensive optimisation of culture conditions and patient serum dilutions are needed to maximise the detection window and minimise the non-specific binding to accurately capture the frequency and degree of patient sera binding.

1.3. Aims

I endeavour to further characterise sera from patients with LGI1 or CAPSR2 autoantibodies using rodent primary DRG neurons and iPSC-derived human sensory neurons (iPSNs). I compare the frequency of binding and titre of sera from

patients with or without neuropathic pain. I also investigate the frequency of binding of healthy control sera on rodent DRG neurons.

2. Methods

2.1. Animal Care

All procedures were carried out in accordance with UK home office regulations as well as the Animals Scientific Procedures Act 1986 at a licensed facility within the University of Oxford. Animals were group-housed in IVC cages on a 12-hour light-dark cycle in temperature and humidity-controlled rooms, with food and water available ad libitum. Study design, sample sizes, randomisation and blinding were determined and followed in accordance with the ARRIVE guidelines. Both male and female mice were used in this study.

The CASPR2 knock-out mice (Cntnap2 -/-) were a gift from Dr E. Peles and have previously been described (Poliak et al., 2003).

2.2. Cell Culture

2.2.1. Mouse Primary DRG Cell Culture

Adult mice were sacrificed in a CO_2 chamber. The spinal column was removed and bisected, and DRGs were taken at all levels and placed into Hanks' Balanced Salt Solution (HBSS, without Ca^{2+} and Mg^{2+}, Invitrogen). DRG were digested enzymatically at 37°C for 1.5 hrs in Collagenase II (4 mg/mL, Gibco) and Dispase II (4.7 mg/mL, Roche) diluted in HBSS. DRG were mechanically dissociated by gentle trituration with a fire-polished glass pipette and washed in HBSS. Dissociated cells were suspended in culture medium (Neurobasal medium with 2% B27 (v/v) and 1% glutaMAX (v/v), Gibco) supplemented with mouse NGF (50 ng/μl, Peprotech) and GDNF (10 ng/μL, Peprotech) and plated in laminin / poly-

D Lysine-coated 96-well plates (Greiner) or coverslips. Cultured cells were incubated at 37°C with 5% CO_2 until assay.

2.2.2. Rat Primary DRG Cell Culture

Rat DRG cell culture was done similarly to mouse DRG neuron culture, as described above. The only difference was the use of human NGF (500 ng/ul, Peprotech) instead of mouse NGF and GDNF in the culture medium.

2.2.3. HEK293 culture

Human embryonic kidney cells (HEK) were cultured in Dulbecco's modified Eagle's Medium (DMEM) with 10% foetal bovine serum (Sigma) at 37°C with 5% CO_2

2.3. Live Cell Staining

Patient sera were diluted in DRG culture medium (Neurobasal + B27 + GlutaMAX + mNGF, GDNF). 1/4th of the cultured cells' medium was removed and replaced with the diluted antibodies and incubated for one hour at 37°C. The subsequent steps were all completed at room temperature. The medium was removed, and cells were washed twice and then incubated with Alexa 488-goat anti-human (H+L) for an hour. The cells were washed twice, then fixed with 4% PFA and permeabilised with 0.3% Triton-X diluted in PBS. No blocking agents were used for this assay. The neuronal marker, Alexa 546-rabbit anti-βIII tubulin antibody (Abcam), was added and incubated with cells for one hour. After an hour of incubation, the cells were washed and mounted on slides with Vectashield mounting medium (Vector Labs). Slides were imaged using the Zeiss LSM 700 using 405nm, 488nm, and 546nm diode lasers. Images were processed using FIJI (ImageJ).

2.4. Myelinated iPS-derived neurons

Dr Alexander Davies (University of Oxford) generated and performed assays with myelinated iPS-derived neurons. Myelinating co-cultures of human iPSC-derived sensory neurons and rat Schwann cells were generated using a previously published method (Clark et al., 2017).

2.5. Patient Characterisation

All patient characterisations were performed by Dr S. Ramanathan (University of Oxford, University of Sidney).

Patients with antibodies against LGI1, CASPR2, or both targets were identified from the Oxford Autoimmune Neurology Group's clinical assessments, including 37 patients from previous studies. From 39 patients with pain, case notes (in all 39) and additional telephone interviews (23/39) retrospectively assessed clinical features, including patient-rated treatment responses (no response/worsening vs any improvement) and three validated questionnaires:

1. The Douleur Neuropathique 4 (DN4) was used to define neuropathic pain at disease nadir by a score ≥ 3 (without physical examination).
2. Patient-Reported Outcome Measurement Information System Pain Interference (PROMIS-PI; maximal score = 40) was used to quantify pain interference at nadir of pain, after immunotherapy, and at latest follow-up (median = 5 years, range = 1–17).
3. Five-level EuroQol 5-dimension quality of life assessment (EQ-5D) and EQ-5D visual analogue scale were used to evaluate functional domains and self-reported quality of life (QOL; 0 worst to 100 best health) at latest follow-up.

2.6. Statistical Analysis

Student's t-test was used to compare the mean of two groups. In experimental groups where multiple comparisons were made, one-way or two-way analysis of variance (ANOVA) tests with appropriate post-hoc tests were performed. Fisher exact test was used to compare discrete binary variables. All data is represented as mean ± the standard error of the mean (SEM) unless otherwise stated. Statistical significance for all experiments was placed at $p < 0.05$. Statistical significance is indicated as follows * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The statistical tests used are reported in the appropriate figure legend. All statistical tests were carried out with GraphPad Prism 9.

3. Results

3.1. Differential binding of LGI1 and CASPR2 patient IgG

Table 1 shows the number of patients identified with LGI1 or CASPR2 antibodies with or without pain. Patients were determined to have pain with DN4, PROMIS-PI and EQ-5D questionnaires as described in the Methods section by Dr S. Ramanathan. These patient sera have been confirmed for binding to LGI1 or CASPR2 using live CBAs (cut-off 1:20 for LGI1 and 1:100 for CASPR2, data not shown). Age- and gender-matched healthy controls (n=12) were included in subsequent binding assays.

Group	Pain	Number of Patients	Median Age of Onset (range)	Gender M (F)	% of Male
LGI1-IgG	No Pain	6	67 (53-84)	3 (3)	50%
	With Pain	8	68 (45-75)	7 (1)	87.5%
CASPR2-IgG	No Pain	8	64 (22-80)	6 (2)	75%
	With Pain	8	68 (56-75)	8 (0)	100%
Healthy Control	No Pain	12	64 (55-69)	7 (5)	58.3%

Table 1. LGI1 and CASPR2 IgG patient characteristics

The patient cohort characterised included patients with pain and LGI1 antibodies (n=8) or CASPR2 antibodies (n=8) and patients without pain but with characteristic central nervous system (CNS) manifestations with antibodies against LGI1 (n = 6), CASPR2 (n = 8).

To determine whether screening patient serum samples on rodent DRG neurons is feasible, I first tested select CASPR2 IgG patient sera on WT and CASPR2 Knockout (CASPR2 KO) cultured DRG neurons. **Figure 1** shows the serum binding of CASPR2 IgG patients with pain on WT DRG neurons. The binding of this serum was abolished in CASPR2 KO DRG neurons, confirming the sensitivity and specificity of the DRG neuron binding assay.

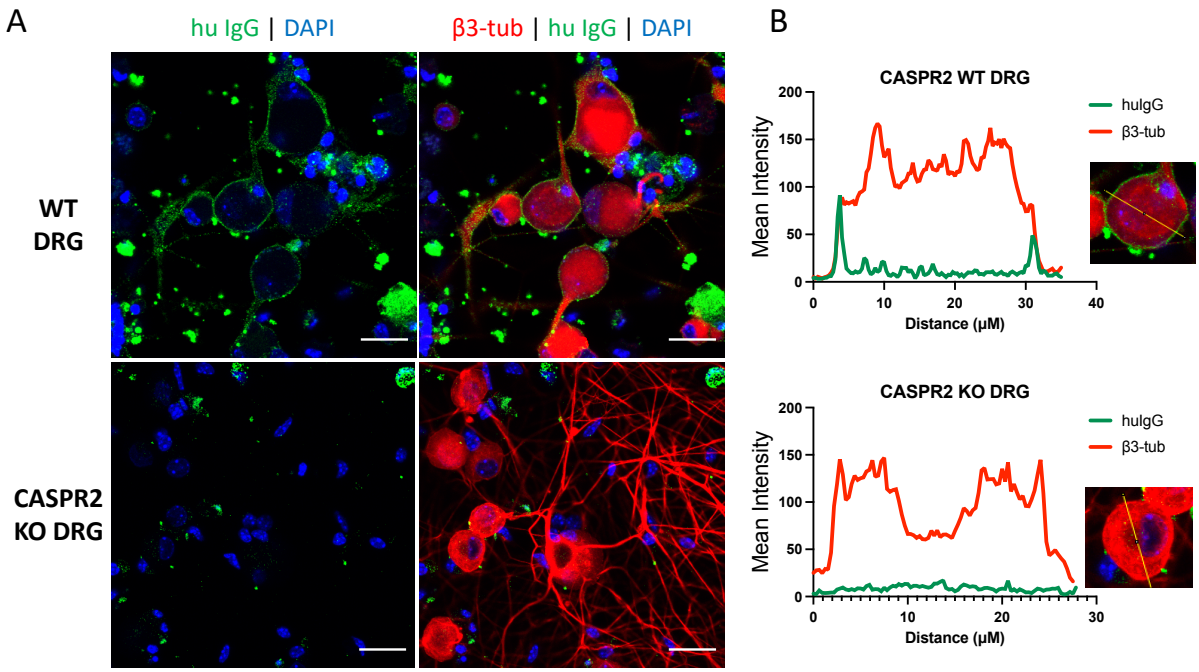

Figure 1. Binding of CASPR2 IgG patient serum on CASPR2 WT or CASPR2 KO mouse DRG

A) Representative images showing patient serum binding to WT mouse DRGs, but not to CASPR2 KO mouse DRGs. Cultures were stained with neuronal marker β3-tub (red) and DAPI (blue). Serum at 1:100 dilution on 2 DIV neurons. Scale bar = 25 μm **B)** Example profile plots of binding to WT DRG. Binding to live DRGs is localised to the membrane, which is demarcated by the β3-tubulin stain. No binding is detected on CASPR2 KO mouse DRGs.

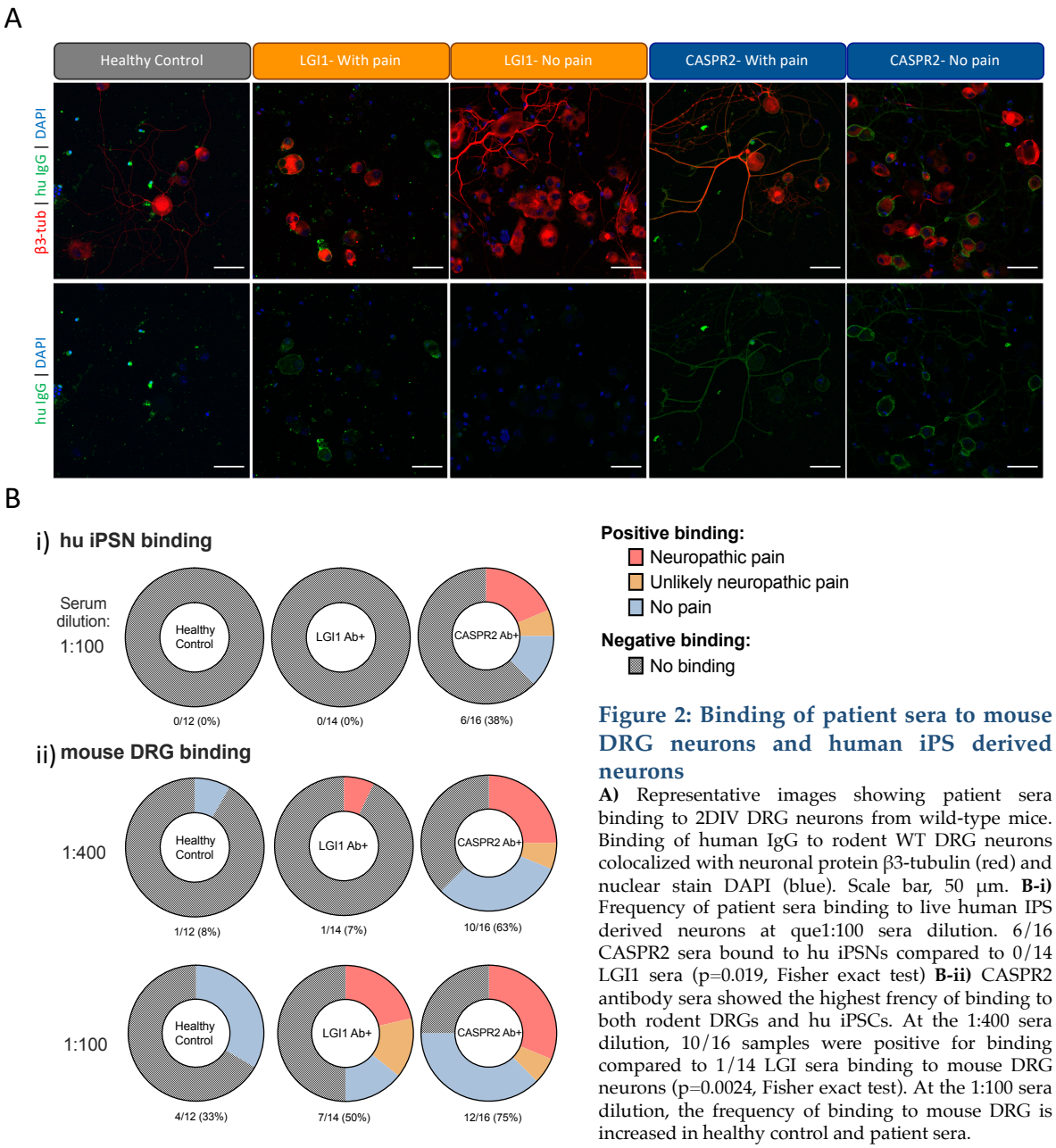

Figure 2: Binding of patient sera to mouse DRG neurons and human iPS derived neurons

A) Representative images showing patient sera binding to 2DIV DRG neurons from wild-type mice. Binding of human IgG to rodent WT DRG neurons colocalized with neuronal protein β3-tubulin (red) and nuclear stain DAPI (blue). Scale bar, 50 μm. **B-i)** Frequency of patient sera binding to live human IPS derived neurons at que1:100 sera dilution. 6/16 CASPR2 sera bound to hu iPSNs compared to 0/14 LGI1 sera (p=0.019, Fisher exact test) **B-ii)** CASPR2 antibody sera showed the highest frency of binding to both rodent DRGs and hu iPSCs. At the 1:400 sera dilution, 10/16 samples were positive for binding compared to 1/14 LGI sera binding to mouse DRG neurons (p=0.0024, Fisher exact test). At the 1:100 sera dilution, the frequency of binding to mouse DRG is increased in healthy control and patient sera.

After establishing the mouse DRG binding assay, I tested all patient sera at 1:100 and 1:400 dilution. Representative images of sera binding from each patient group are shown in **Figure 2A**. The binding of sera was determined by visually scoring the images by two experimenters blind to patient groups. **Figure 2B** shows the proportion of healthy control, LGI1-IgG, or CASPR2-IgG patient sera bound to iPSC-derived human sensory neurons (iPSNs) or mouse DRG neurons. Sera were tested at 1:100 dilution on hu iPSNs (**Figure 2B-i**) and 1:400 and 1:100 dilution on

mouse DRG neurons (**Figure 2B-ii**). CASPR2 IgG sera showed the highest binding frequency with 10/16 (63%) patient sera binding to mouse DRGs and 6/16 (38%) to hu iPSNs. 6/16 CASPR2 IgG bound to hu iPSNs in contrast with 0/14 of the LGI1 IgG (p=0.019, fisher exact test, **Figure 2B-i**). None of the healthy control sera bound to hu iPSNs. **Figure 2B-ii** shows CASPPR2-IgG sera, at the 1:400 dilution, exhibited higher frequency (10/16, 63%) of binding to mouse DRGs compared to LGI1 IgG (1/14, 7%) [p=0.0024, Fisher exact test). At the 1:100 dilution, an increased binding frequency on mouse DRGS was seen in healthy control and patient sera. 7/14 (50%) of LGI1-IgG sera showed binding at the lower dilution compared to the 1/14 (7%) at the 1:400 dilution (p=0.039, Fisher exact test). Though not significant, the frequency of healthy control binding to mouse DRGs also increased at the 1:100 dilution; 4/12 (33%) of the healthy control sera bound at the 1:100 dilution compared to only 1/12 (8%) at the 1:400 dilution (p=0.3168, Fisher exact test)

3.2. Increased titre of CASPR2 IgG patients with pain

To further characterise the patient sera that showed binding to mouse DRGs, we carried out sera titrations and determined the end-point titres of these samples on mouse DRG neurons, shown in **Figure 3**. **Figure 3A** shows that the titres of CASPR2 IgG patient sera with pain were significantly higher than those without pain (p=0.0265) and healthy controls (p=0.0121). In contrast, no significant difference was observed in end-point titres of LGI1 patient sera with or without pain (p= 0.6902) and healthy controls (p=0.9310), **Figure 3B**.

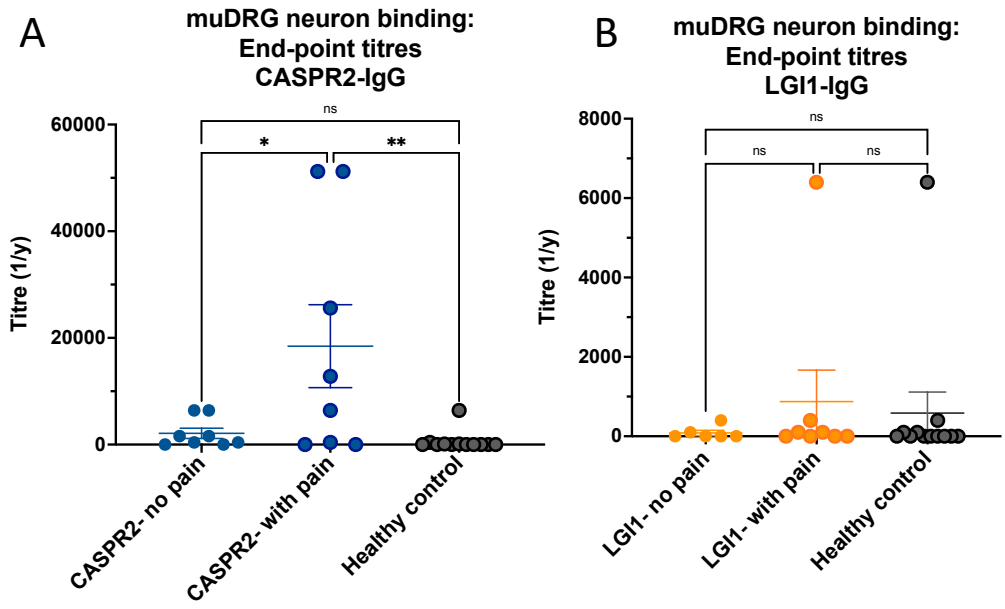

Figure 3. End-point dilutions of patient sera on mouse DRG neurons
End-point dilutions tested on live DRGs for both LGI1 and CASPR2 antibody sera ranged from 1:100 to 1:51,200. **A)** End-point titres of CASPR2 antibody sera showed significantly higher titres of CASPR2-IgG patients with pain compared to CASPR2-IgG without pain (p=0.0308) and healthy controls (p=0.0082). One way ANOVA followed by post-hoc Bonferroni test, *p<0.05. **B)** End-point titres of LGI1 antibody sera did not show significant differences between patients with and without pain. One way ANOVA, ns.

3.3. Healthy control binding on rodent DRG neurons

I noted the higher frequency of HC sera binding to mouse DRG neurons compared to hu iPSN (**Figure 2B**), which, though not significant, was higher when tested at the 1:100 dilution (p=0.3168, Fisher exact test). To better understand this elevated binding frequency of healthy control sera, I tested additional sera for binding to mouse DRGs (**Figure 4A**). Sera from 41 healthy controls ages 27-80 were collected from the Oxford Autoimmune Neurology group, Dr Andreas Goebel (University of Liverpool), and Dr Simon Rinaldi (University of Oxford). I included rat DRG neurons as an additional rodent neuronal line to determine whether the binding is specific to mouse DRG neurons. All healthy control sera were tested at a higher dilution at 1:100 to maximise the detection of binding. I observed that over half (22/41 -56%) of the healthy control sera was positive for binding to mouse

DRGs (**Figure 4B**). There was a non-significant reduction in the frequency (16/41-39%) of binding when tested on rat DRG neurons (p=0.2681, Fisher exact test). Representative images of sera binding are shown in **Figure 4C**. The binding pattern on rat and mouse DRGs are similar, with clear membrane-binding on neurons co-stained with β3-tubulin.

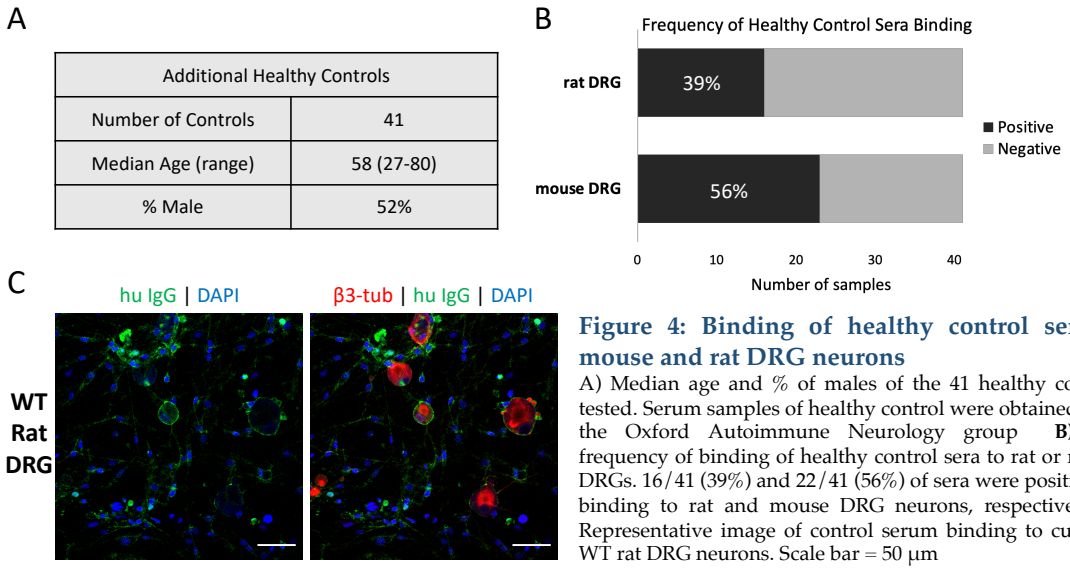

Figure 4: Binding of healthy control sera to mouse and rat DRG neurons
A) Median age and % of males of the 41 healthy controls tested. Serum samples of healthy control were obtained from the Oxford Autoimmune Neurology group **B)** The frequency of binding of healthy control sera to rat or mouse DRGs. 16/41 (39%) and 22/41 (56%) of sera were positive for binding to rat and mouse DRG neurons, respectively. C) Representative image of control serum binding to cultured WT rat DRG neurons. Scale bar = 50 μm

4. Discussion

In this study, I examined sera from LGI1 or CASPR2-IgG patients with or without pain in binding assays to human iPSNs and mouse DRGs. I observed differential binding of CASPR2 or LGI1 serum IgG to mouse DRGs and unmyelinated iPS-derived human sensory neurons. CASPR2 IgG bound to both human iPSNs and mouse DRGs, while LGI1-IgG displayed significantly reduced binding frequency in mouse DRG neurons and did not bind to human iPSNs. Additionally, I showed an increased titre of CASPR2 IgG in patients with pain compared to those without pain, suggestive of titres in differences in pain symptoms. I also investigated the frequency of healthy control sera binding and revealed a surprisingly high percentage of binding on rodent DRG neurons.

CASPR2 and LGI1 are part of the VGKC complex expressed in the CNS and PNS (Baudin et al., 2022; Poliak et al., 1999). There is a spectrum of clinical characteristics in patients and differences in treatment responsiveness seen in patients with CASPR2 or LGI1 autoantibodies (Gadoth et al., 2017; Ramanathan et al., 2021; Van Sonderen et al., 2016). Interestingly, I saw differences in patient sera binding to cultured sensory neurons. CASPR2-IgG patient sera bound to both hu iPSNs and mouse DRGs. In contrast, LGI1-IgG patient sera did not bind to hu iPSNs and showed reduced binding frequency to mouse DRGs. Similarly, Irani et al. showed that titres in CASPR2-IgG patients with Morvan's syndrome were higher than MoS patients with LGI1 autoantibodies (Irani et al., 2012). This difference in binding is suggestive of the pathophysiological difference in the clinical symptoms and treatment responsiveness. A higher percentage of patients with CASPR2 autoantibodies have been reported to have neuropathic pain (Gadoth et al., 2017). While it is possible that the higher binding of CASPR2-IgG to cultured neurons may contribute to the more sustained pain experienced by patients, it may be too simplistic to correlate the frequency of binding to the incidence of pain in patients. Several factors may influence our detection of antibody binding on neuronal cultures. Autoantibodies can mediate their effects in many ways that may not be detected as binding. As shown in autoantibodies against N-methyl-d-aspartate (NMDA) receptors (NMDARs) and Aquaporin-4 (AQP4), patient antibodies can bind to the target antigen and cause the internalisation of the antibody/antigen complex (Hinson et al., 2017; Masdeu et al., 2016; Takahashi et al., 2020).

I observed the lack of binding in LGI1 patient sera to iPSNs and mouse DRGs. This may be attributed to the fact that LGI1 is also secreted (Baudin et al., 2022). It is possible that the antibodies, at a low concentration, may have been

saturated with soluble LGI1 and did not bind to cell-surface LGI1. I saw an increase in the frequency of binding to mouse DRG neurons when the sera were tested at a higher concentration (50% at 1:100 dilution vs 7% at 1:400), suggesting that perhaps the concentration of the LGI1 patient sera was lower than other sera; therefore, a higher concentration is required for detection of binding on mouse DRG neurons. Alternatively, patient antibodies may target epitopes masked in live neuronal cultures. Ramberger *et al.* demonstrated that LGI1 antibodies that target the EPTP domain of LGI1 did not bind to hippocampal neurons in vitro; these antibodies also did not bind to HEK293 cells transfected with ADAM22/23 and pre-docked with soluble LGI-1, confirming that the EPTP epitope is masked by the association of partner proteins ADAM22/23. Some of these patients may have antibodies that exclusively target the same EPTP epitope domain. As shown in the same study by Ramberger et al., in which the binding of 2/7 patient CSFs was restricted to a single domain, patients could potentially have antibodies toward a sole domain (Ramberger et al., 2020). Further work on characterising the IgG concentration and epitopes of the LGI1 patient sera will help us understand what underlies the low frequency of binding of LGI1 sera to live neuronal cultures.

Additionally, as I only tested serum IgG in these assays, I may be missing the binding of antibodies in the CSF, which are produced intrathecally. These antibodies can mediate their effects in the CNS, as seen in the robust binding of LGI1 antibodies to hippocampal neurons and sections, corresponding to the array of CNS symptoms of these patients (Ramanathan et al., 2021; Ramberger et al., 2020).

Consistent with a previous study, I saw higher titres in CASPR2 IgG patient sera compared to LGI1 patient sera (Irani et al., 2012). Although this study's sample numbers are low, I observed significantly higher titres in CASPR2-IgG patients

with pain than those without pain. The increased binding to peripheral sensory neurons in patient samples with pain is intriguing and further supports the idea that patient autoantibodies may contribute to pain by binding to peripheral sensory neurons. However, the CASPR2 antibody concentration in the patient sera and the affinities of the antibodies may also affect the end-point titres. It may be helpful to determine the concentration of anti-CASPR2/LGI1 antibodies in patient serum samples. Purifying IgG from serum samples and using normalised IgG concentrations on DRG neurons will increase our confidence in this finding.

Screening additional healthy control sera on rodent DRG neurons revealed an unexpectedly high frequency of binding: Over 50% of the healthy control sera showed binding to mouse DRG neurons when tested at a 1:100 dilution. Literature suggests that antibodies against neuronal surfaces in healthy participants are low. A review from Lang and Prüss estimated that the prevalence of antibodies in healthy controls that bound to neuronal surface antigens was 0.23% (Lang & Prüss, 2017). However, their estimate was based on studies in which sera were screened for binding on single antigens using standard detection methods such as CBA or ELISA (i.e. against a single neuronal protein for each assay). The rodent neuronal cultures express a heterogenous collection of proteins in addition to neuronal antigens of interest. These primary neuronal cultures are particularly useful for presenting surface neuronal antigens in the native context but cannot discern specific binding to neuronal antigens or irrelevant binding to other non-pathogenic cell-surface proteins. Other studies utilising mouse DRG neuronal cultures showed the binding of healthy control IgG on the neuronal membranes. Although the percentage of healthy control samples bound to DRG neurons was not quantified or shown, it is clear that a large percentage of healthy control IgG binds to mouse DRG neurons (Goebel et al., 2021; Krock et al., 2022). In contrast to the relatively

high frequency of healthy control binding to rodent DRG neurons, there was no binding of healthy control IgG observed on hu iPSNs, indicating that the iPSN culture system may be a 'cleaner' assay to discern pathogenic IgG.

The binding seen in healthy control sera may be non-specific. Human serum can be 'sticky' due to the high quantities of polyclonal antibodies which may exhibit non-specific binding to cells (Waters et al., 2016). Thus, increased dilutions may be required to eliminate the non-specific binding. A blocking step or inclusion of a blocking reagent such as BSA or foetal bovine serum (FBS) at a low concentration may reduce the non-specific binding of serum samples. It is also possible that IgG may be binding to FC gamma receptors (FcγR) expressed on DRG neurons. The Fc region of immunoglobulins binds to FcγRs expressed on the cell surface, and this binding activates or inhibits cells depending on the type of FcγR bound. The activating FcγRI is expressed in rodent DRG neurons, and IgG has been described to bind to these receptors (Qu et al., 2011; Wang et al., 2019). The use of an FC receptor block will eliminate the binding of IgG to FcγRs and further increase the specificity of screening serum samples on rodent neuronal cultures. In these neuronal binding assays, I utilised a fluorescently labelled secondary antibody against human IgG (heavy and light chain), which detects all immunoglobulin classes (IgA, IgE, IgM, and IgD) due to the reactivity to the immunoglobulin light chains (Waters et al., 2016). The use of a secondary antibody against the Fc region of IgG would obviate the detection of other immunoglobulin classes.

Furthermore, as IgG concentrations in most patient or healthy control serum samples are unknown, titration of the serum samples is a useful method for assessing the degree of binding to neuronal antigens. The determination of an appropriate 'cut-off' for positive binding is subjective without normalising IgG concentration in serum. Finally, the determination of binding was done manually

by visual inspection of images. Although the experimenters were blind to the sample types, the scoring can be variable and subjective. Using a quantitative analysis will aid in the objective, consistent analysis of patient sera binding in neuronal cultures.

To summarise, I showed differential binding of sera from patients with LGI1 or CASPR2 antibodies on rodent and human iPSNs, suggesting pathophysiological differences that may influence differing clinical manifestations. Live neuronal cultures offer the advantage of presenting neuronal proteins in their native context and can be utilised as an additional diagnostic or research tool. However, further refinement and optimisation of these cultures are required to ensure the specificity and sensitivity required for patient sample screening. Comparing the binding and clinical manifestations of patient sera has revealed differences in LGI1 and CASPR2-IgG sera that can be further examined by taking a closer look at the mechanisms of patient antibodies. We investigate these mechanisms in the following chapter.

5. Acknowledgements

I performed all the DRG binding experiments in this chapter. Data from figures 2 and 3 have been published in a peer-reviewed journal. A/Prof J. Dawes and I visually scored the binding of patient sera to DRG neurons (Figure 2,3). The patient characterisations were done by Dr S. Ramanathan. The iPSN binding assays (Figure 3) were performed by Dr A. Davies.

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4

Mechanisms of CASPR2 Antibodies

1. Introduction

CASPR2 autoantibodies have been found in patients with syndromes associated with varying CNS and PNS involvement. Neuropathic pain is one of the common symptoms reported by CASPR2-IgG patients. The clinical heterogeneity displayed by patients with CASPR2 autoantibodies suggests that several autoantibody-mediated mechanisms may be responsible for the multi-faceted symptoms. The characteristics of CASPR2-IgG, such as IgG localisation (serum vs CSF), epitope, affinity, and concentration, may play a role in their pathogenic potential.

The differential binding of CASPR2-IgG from patients with neuropathic pain on mouse DRG neurons, described in the previous chapter, supports the notion that the binding properties of autoantibodies may influence the clinical manifestations in patients. Currently, there are a few studies investigating the mechanisms of patient CASPR2-IgG. Existing studies focus on the CNS symptoms and therefore utilise hippocampal neuron cultures or heterologous expression systems.

1.1. Known mechanisms of action of CASPR2 antibodies

Current studies investigating the mechanisms of CASPR2-autoantibodies have proposed that the blocking and inhibition of binding of CASPR2's partner protein, contactin-2, is the primary mechanism of action of CASPR2 autoantibodies (Patterson et al., 2018; Saint-Martin et al., 2019). The disruption of the CASPR2/contactin-2 complex is thought to decrease the clustering and expression of K_v1 channels on the cell surface, leading to neuron hyperexcitability (Patterson et al., 2018).

Using the solid-phase binding assay, Patterson *et al.* showed that CASPR2 associates with contactin-2 with nanomolar affinity, consistent with previous studies (Lu et al., 2016). The pre-incubation of CASPR2 patient sera with immobilised CASPR2 protein resulted in a concentration-dependent decrease of contactin-2 binding. The six patient samples tested in this study inhibited contactin-2 binding at varying degrees. Interestingly, two of the six patients had neuropathic pain symptoms (Patterson et al., 2018). Another study by Saint-Martin et al. using immunoprecipitation in transfected HEK cells demonstrated that the pre-incubation of patient IgG with HEK/CASPR2 cells slightly decreased the levels of contactin-2 that co-immunoprecipitated with CASPR2 (Saint-Martin et al., 2019).

The disruption of CASPR2/contactin-2 association was also shown in a recent study by Joubert *et al*., in which the infusion of patient CASPR2 IgG in mice reduced surface CASPR2 and a decrease in the colocalisation of CASPR2 and contactin-2 in the hippocampus. This confirms that patient CASPR2 autoantibodies can disrupt the interaction with contactin-2 *in vivo*. This study also observed decreased CASPR2 surface expression, indicating that CASPR2 IgG can internalise CASPR2. The surface level of $K_v1.1$ was also reduced in hippocampal neurons, indicating that the disruption of CASPR2/contactin-2 or CASPR2 internalisation caused this reduction of $K_v1.1$ expression and clustering *in vitro* (Joubert et al., 2022). In contrast, Saint-Martin et al. reported an unexpected increase in $K_v1.2$ expression in transfected HEK cells and hippocampal neurons treated with patient CASPR2 IgG without alterations in CASPR2 expression. They speculated that the patient IgG binding might restrict CASPR2 diffusion, hence promoting the formation of $K_v1.2$ clusters on the membrane (Saint-Martin et al., 2019).

CASPR2 autoantibodies in patients are predominantly of the IgG4 subclass, which undergoes Fab arm exchange *in vivo* and is functionally monovalent (Joubert

et al., 2016; Van Sonderen et al., 2016; Vidarsson et al., 2014). Consequently, the internalisation of CASPR2 autoantibodies has mainly been dismissed as a potential mechanism (Dalakas, 2022; Lacagnina et al., 2021; Patterson et al., 2018). Nonetheless, studies have shown that patient IgG can internalise in neurons. CASPR2 patient IgG was observed to be internalised in cultured hippocampal neurons and CASPR2-transfected HEKs. However, there were no reductions in the surface expression of CASPR2 (Giannoccaro et al., 2019). There might be compensatory or *de novo* synthesis of CASPR2 *in vitro* that offsets the reduction due to CASPR2-IgG internalisation. Further experiments using techniques such as fluorescence recovery after photobleaching (FRAP) will help address this issue.

The current studies that showed internalisation of CASPR2-IgG attributed the observed internalisation to the presence of IgG1 antibodies in the polyclonal IgG (Giannoccaro et al., 2019; Joubert et al., 2022). However, Fab fragments and an IgG4 antibody targeting LGI1 are internalised in hippocampal neurons (Ramberger et al., 2020). In patients with high autoantibody titres, it is possible that fab arm exchange could generate bivalent antibodies that target the same antigen (Jentzer et al., 2022; Ramberger et al., 2020). However, this possibility remains to be investigated for CASPR2 autoantibodies. Polyclonal patient sera are invaluable in identifying potential mechanisms. Nevertheless, as Ramberger *et al.* have shown, the characterisation of patient-derived monoclonal antibodies is particularly helpful in deconvoluting specific pathogenic mechanisms (Ramberger et al., 2020).

The findings from existing studies suggest that the antibody-mediated inhibition of the interaction of CASPR2 and contactin-2 can reduce K_v1 channel expression and thus alter neuronal excitability. The internalisation of the CASPR2 antibodies may also play a pathogenic role (Giannoccaro et al., 2019; Joubert et al., 2022; Patterson et al., 2018; Saint-Martin et al., 2019). These mechanisms have yet

to be established on peripheral sensory neurons. Understanding the pathogenic mechanisms of autoantibodies in the development of neuropathic pain will be beneficial in the pursuit of a targeted pain therapeutic.

1.2. Other pathways of pain

The generation of ectopic activity in primary afferents after peripheral nerve injury is thought to be a significant driver of neuropathic pain (Amir et al., 1999; Haroutounian et al., 2014). Altered expression of ion channels has been identified as a key contributor to increased neuronal excitability and ectopic firing in primary sensory neurons (Finnerup et al., 2021). Localised translation can occur in myelinated sensory fibres, producing rapid, maladaptive protein expression in the primary afferents, leading to neuronal hyperexcitability (Jiménez-Díaz et al., 2008).

Signalling pathways that regulate translation have been implicated in the development and maintenance of neuropathic pain through their effects on regulating mRNA translation (Yousuf et al., 2021). mRNA translation can be induced by the activation of cell-surface receptors, such as tyrosine receptor kinases (TRK), insulin-like receptors (IR), or epidermal growth factor receptors (EGFRs). Activation of these receptors triggers downstream pathways via the mammalian/mechanistic target of rapamycin complex 1 (mTOR1) and the extracellular-signal-regulated-kinases (ERK) signalling pathways that are illustrated in **Figure 1**. Upstream of mTOR1 are the PI3K and ERK signalling pathways which promote mTOR1 activity through suppression of TSC1.2. mTOR1 regulates mRNA translation by phosphorylation of two downstream targets, 4E-binding protein (4E-BP1) and p70S6 ribosomal kinase (p70S6K). The phosphorylation of 4E-BP1 by mTOR leads to the release of eIF4E protein, which leads to the formation of the eIF4F complex crucial for cap-dependent mRNA translation, while the phosphorylation p70S6K activates ribosomal protein S6. The

ERK pathway also promotes the formation of the eIF4F complex by phosphorylation of eIF4E (Khoutorsky & Price, 2018; Manning & Cantley, 2007; Porta et al., 2014).

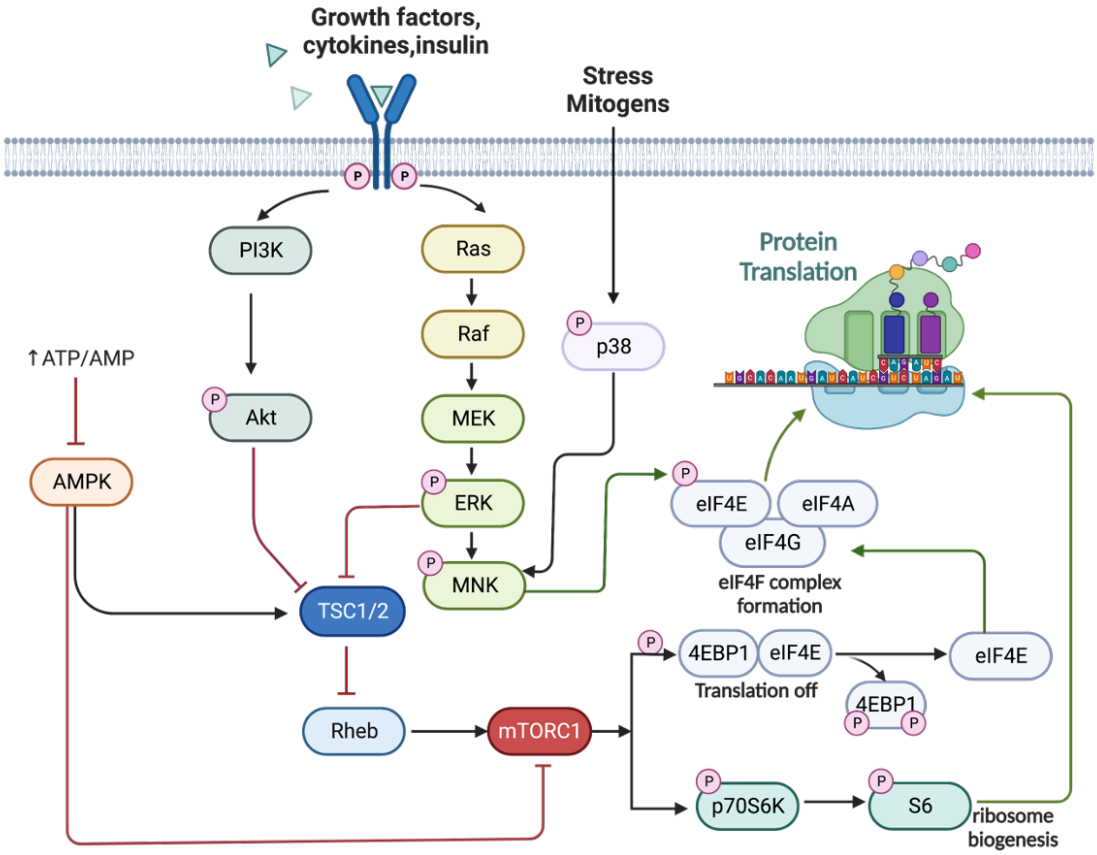


Figure 1. Simplified schematic of the mTOR signalling pathway that regulates translation
Cytokine or growth factor binding to the receptor activates the PI3K/AKT signalling pathway and triggers the phosphorylation of and activation of mTORC1. Activated mTORC1 phosphorylates p70S6K that is involved in ribosome synthesis. mTORC1 activation also relieves 4E-BP inhibition and thereby increases eIF4F complex formation, critical for the initiation of translation. The MAPK/ERK pathway converges with the PI3K/AKT pathway and also activates mTORC1. ERK/MNK also directly activates eIF4E in promoting the formation of the eIF4F complex.

Peripheral nerve injury in rats and mice resulted in the reorganisation of translation regulation signalling molecules and translation machinery in injured peripheral sensory neurons. These changes resulted in increases in eIF4F complex formation, which is required to initiate translation, and therefore augmented protein synthesis (Melemedjian et al., 2011). Activation of the serine/threonine

protein kinase, AKT, has been shown to contribute to pain-related hypersensitivity after nerve injury or capsaicin injection (Géranton et al., 2009; Xu et al., 2007). Furthermore, nerve injury by CCI in rats caused elevated levels of p-AKT, p-mTOR and P70S6K (Guo et al., 2017). The inhibition of the mTOR pathway attenuated mechanical and cold hypersensitivity after nerve injury (Obara et al., 2011). The findings from these studies support the idea that mRNA translation regulation pathways are vital influences in changes in nociceptor excitability.

The AKT-mTOR1 signalling pathway was described to be hyperactive in CASPR2 KO mice, which have been shown to exhibit increased pain-related hypersensitivity (Dawes et al., 2018; Xing et al., 2019). Xing *et al*. showed that phosphorylated AKT and the downstream ribosomal protein S6 were elevated in the DRG of CASPR2 KO mice. The repression of AKT by small molecular inhibitor LY294002 and the inhibition of mTOR by rapamycin reduced DRG neuron hyperexcitability and attenuated thermal and mechanical pain sensitivity in CASPR2 KO mice (Xing et al., 2020). Although the relationship between CAPSR2 deficiency and elevated AKT/mTOR activity is currently unclear, these studies suggest that modulation of the AKT/mTOR signalling pathway could reduce pain in patients with CASPR2 deficiency.

Studies have shown that mTOR signalling also contributes to the modulation of voltage-gated ion channel expression in neurons. Though these studies showed contradicting results in $K_v1.1$ expression when mTOR was activated (Nguyen & Anderson, 2018; Raab-graham et al., 2011). The physiological activation of mTOR was found to suppress the translation of $K_v1.1$ channels in the dendrites of hippocampal neurons, while the inhibition of mTOR by rapamycin increased the $K_v1.1$ protein and promoted $K_v1.1$ surface expression on dendrites (Raab-graham et al., 2011). On the contrary, deletion of PTEN, which

results in the downstream activation of the PI3K/AKT/mTOR pathway, resulted in the upregulation of $K_v1.1$ protein- but not $K_v1.1$ mRNA- in hippocampal neurons. The administration of rapamycin to PTEN KO mice reduced and normalised the levels of $K_v1.1$ expression in the hippocampus (Nguyen & Anderson, 2018). Nguyen & Anderson proposed that the differences in $K_v1.1$ protein expression resulting from mTOR activation may be due to local dendritic and axonal mechanisms, and mTOR functions may differ under physiological and pathological conditions (Nguyen & Anderson, 2018). While the exact mechanisms underlying mTOR regulation of $K_v1.1$ translation are currently unknown, these studies support the concept that mTOR dysregulation could contribute to neuronal hyperexcitability by modulating local translation of voltage-gated potassium channels.

The predominant subclass in CASPR2 patients, IgG4, is unable to activate complement or cellular immune responses (Van Sonderen et al., 2016; Vidarsson et al., 2014). it is therefore unlikely that CASPR2 IgG mediates its effects through complement-dependent cytotoxicity. Furthermore, the systemic administration of CASPR2-IgG caused a reduction of CASPR2 with no direct neuronal damage (Dawes et al., 2018). Hence, I focus on antibody internalisation and altered activation of signalling pathways as potential mechanisms of action of CASPR2 autoantibodies.

1.3. Aims

I extend the work from the previous chapter in an effort to understand how CASPR2-IgG may cause the development and maintenance of neuropathic pain. Here, I explore two putative mechanisms of action of CASPR2 autoantibodies on periphery sensory neurons that- to the best of our knowledge- have not yet been described.

2. Methods

2.1. Animal Care

All procedures were carried out in accordance with UK home office regulations as well as the Animals Scientific Procedures Act 1986 at a licensed facility within the University of Oxford. Animals were group-housed in IVC cages on a 12-hour light-dark cycle in temperature and humidity-controlled rooms, with food and water available *ad libitum*. Study design, sample sizes, randomisation, and blinding were determined and followed in accordance with the ARRIVE guidelines. Both male and female mice were used in this study.

The CASPR2 knock-out mice (Cntnap2 -/-) were a gift from Dr E. Peles and have previously been described (Poliak et al., 2003).

2.2. Cell Culture

2.2.1. Mouse Primary DRG Cell Culture

Adult mice were sacrificed in a CO_2 chamber. The spinal column was removed and bisected, and DRGs were taken at all levels and placed into Hanks' Balanced Salt Solution (HBSS, without Ca^{2+} and Mg^{2+}, Invitrogen). DRG were digested enzymatically at 37°C for 1.5 hrs in Collagenase II (4 mg/mL, Gibco) and Dispase II (4.7 mg/mL, Roche) diluted in HBSS. DRG were mechanically dissociated by gentle trituration with a fire-polished glass pipette and washed in HBSS. Dissociated cells were suspended in culture medium (Neurobasal medium with 2% B27 (v/v) and 1% glutaMAX (v/v), Gibco) supplemented with mouse NGF (50 ng/μl, Peprotech) and GDNF (10 ng/μL, Peprotech) and plated in laminin / poly-D Lysine-coated 96-well plates (Greiner) or coverslips. Cultured cells were incubated at 37°C with 5% CO_2 until assay.

2.2.2. Adenoviral transduction of cultured DRG neurons

Adenovirus vectors expressing CASPR2-EGFP or control vector EGFP were applied to cultured DRG neurons at one day in vitro. AAVs were diluted in complete DRG medium for a final concentration of 3.2 x 10^10 vector genomes / well. 5 μM of Cytosine β-D-arabinofuranoside (Ara-C, Sigma) was added to the cultures to limit the proliferation of non-neuronal cells. Cells were assayed after one week in culture for maximal transgene expression.

2.2.3. HEK293 culture & Transfection

Human embryonic kidney cells (HEK) were cultured in Dulbecco's modified Eagle's Medium (DMEM) with 10% foetal bovine serum (Sigma) at 37°C with 5% CO_2. Cells were transfected using JetPEI transfection reagent (Polyplus) according to the manufacturer's instructions. Dr J. Dawes and Dr T. Trendafilova generated CASPR2-EGFP constructs. The CASPR2-Flag construct was obtained from Origene, and the extracellular tag was introduced in-house.

2.3. IgG purification and labelling

2.3.1. Patient IgG purification

IgG was purified from patient plasma using protein G Sepharose beads (Sigma). Protein G beads were incubated with plasma for 4 hrs at 4°C with gentle rotation. Beads were washed 3 times with PBS, then eluted with 0.1 M glycine solution (pH 2.4) and neutralised with 1 M Tris (pH 8). Purified IgG was buffer-exchanged to PBS, and protein concentration was determined by spectrophotometry (A_{280}) using the NanoDrop2000 (Thermo Scientific).

2.3.2. Fluorescent labelling of IgG

Monoclonal antibodies or purified IgG were labelled with pHrodo™ Red, succinimidyl ester (SE) (Invitrogen) or Alexa Fluor488/555-SE (Invitrogen) by

covalent conjugation to the primary amines of antibodies, according to the manufacturer's instructions. The unbound dye was removed by desalting using BioGel P-30 medium (BioRad) in a spin column (Costar). Labelled antibodies were subsequently buffer-exchanged to PBS using Amicon Ultra-4 columns (Merck) or dialysis cassettes (Invitrogen). The degree of labelling and final protein concentration was determined using the Nanodrop One spectrophotometer (Invitrogen).

2.4. In vitro Assays

2.4.1. Live Cell Staining

Patient sera were diluted in DRG culture medium (Neurobasal + B27 + GlutaMAX + mNGF, GDNF). 1/4th of the cultured cells' medium was removed, replaced with diluted antibodies, and incubated for one hour at 37°C. The subsequent steps were all completed at room temperature. The medium was removed, and cells were washed twice and then incubated with Alexa 488-goat anti-human (H+L) for an hour. The cells were washed twice, then fixed with 4% PFA and permeabilised with 0.3% Triton-X diluted in PBS. The neuronal marker, Alexa 546-rabbit anti-βIII tubulin antibody (Abcam), was added and incubated with cells for one hour. After an hour of incubation, the cells were washed and mounted on slides with Vectashield mounting medium (Vector Labs). Slides were imaged using the Zeiss LSM 700 using 405nm, 488nm, and 546nm diode lasers. Images were processed using FIJI (ImageJ).

2.4.2. Analysis of IgG binding on cell membranes

Analysis of IgG binding to transfected HEK or muDRG neurons was performed using FIJI (Image J). Briefly, the cell membranes of transfected cells or neurons were

identified by thresholding or manually delineating the EGFP fluorescence or tubulin marker, respectively. The cells or neurons were then added as regions of interest (ROIs). The 'Enlarge' and 'Make band' tools were used to create a band surrounding the membrane, which should identify the extracellular membrane binding of IgGs. Then, the mean intensities of the identified bands were measured. To facilitate the analysis of large numbers of images, an ImageJ macro (unpublished) was written for the ease for data analysis. The mean intensity data was exported to Excel or GraphPad Prism9 for further analysis.

2.4.3. Internalisation Assay

DRG neurons or HEK cells were plated in 96-well black-wall clear bottom plates (Corning) for all internalisation assays. Fluorescently-conjugated antibodies were sterilised by filtration through a 0.22 μM spin column (Corning) before addition to cultured cells. Phenol red-free medium (Invitrogen) was used to reduce background fluorescence. Antibodies were added to cells and incubated in the Incucyte S3 imager (Sartorius) at 37°C for live cell imaging. Images were acquired using the 20X objective with phase, green (Ex/Em 441-491/503-544 nm), and red (567-607/622-704 nm) filters. Spectral overlap was adjusted based on background fluorescence. Data was analysed using the Incucyte software, or raw data was exported and analysed manually using FIJI/Image J (NIH).

2.4.4. Signalling pathway analysis in cultured DRG neurons

Cultured neurons were prepared as described above and plated in a 96-well plate. 2 DIV DRG neurons were treated with the following:

- Vehicle control (0.2 % DMSO) for 1 hour

- Inhibitors (100 μM resveratrol and 50 μM LY294002 dissolved in 0.2% DMSO) for 1 hour
- Activators (100 ng/μL GDNF and NGF for 50 minutes, then 100 μM ATP and 2 μM capsaicin for 5 minutes, and 50 mM KCl for 5 minutes)
- Purified pooled CASPR2 patient IgG (Patients JR002 and JR060, 100 μg/mL) for 1 hour
- Age-matched healthy control IgG (100 μg/mL) for 1 hour

After treatment, DRG neurons were washed with ice-cold TBS, lysed in 1X RIPA buffer supplemented with protease and phosphatase inhibitor (Invitrogen) and used for Western blotting.

2.5. Western Blot

DRGs were isolated and homogenised on ice in RIPA buffer (Sigma) with 1X protease and phosphatase inhibitors (Invitrogen). Insoluble cell debris was removed by centrifugation (12,000 RPM, 15 min). The protein concentration of the extract was quantified using the BCA assay (Pierce). Extracts were separated by SDS-PAGE (4-20% gel, Bio-Rad) and then transferred onto nitrocellulose membranes. Membranes were blocked with TBS-milk or EveryBlot blocking solution (Bio-rad) and incubated overnight with primary antibodies listed in the appendix. The blots were washed twice with TBS-Tween, then probed with HRP-labelled secondary antibodies. The blots were washed again and processed for chemiluminescence detection (ECL, Amersham). For fluorescent detection, Alexa 488, Alex 546, or Dylight 680-conjugated secondary antibodies were used at a 1:3,000 dilution. Immunoblots were imaged using the ChemiDoc imager (Bio-rad). Analysis was performed using the Image Studio software (Li-Cor).

3. Results

We endeavoured to characterise the mechanisms of autoantibodies on peripheral sensory neurons. Literature has suggested that patient CASPR2

antibodies internalise in hippocampal neurons (Giannoccaro et al., 2019; Joubert et al., 2022). However, these studies utilised polyclonal patient IgGs, which include non-CASPR2-specific antibodies that can internalise in neurons in a CASPR2-independent manner. Hence, we used monoclonal antibodies (mAb) from CASPR2-IgG patients to determine whether CASPR2 mAbs can internalise in DRG neurons in an antigen-specific manner. We also examined a larger sample of patient CASPR2-IgG and showed that they internalise in mouse DRG neurons in an antigen-specific fashion.

3.1. Patient-derived mAbs bind and internalise in CASPR2-expressing cells

I tested mAbs derived from two CASPR2-IgG patients with or without pain to understand whether antibody mechanisms are related to neuropathic pain symptoms. Both patients had encephalitis and seizures, but only patient JR060 experienced neuropathic pain.

Table 1 shows the details of antibodies derived from patients with or without neuropathic pain. These patient antibodies vary in epitope, affinity (data not shown), and degree of binding to HEK/huCASPR2 or hippocampal neurons. Antibodies E7, H1 and H2 bind to the N-terminal discoidin domain, while antibodies E8 and E9 bind to the more membrane-proximal Laminin G-like 3 domain.

Patient	Symptoms	% IgG4 in serum	Antibody	Epitope	Binds to			Internalisation	
					HEK/ huCASPR2	Hippocampal Neuron	DRG Neuron	HEK/ huCASPR2	DRG Neuron
JR002	Limbic encephalitis Seizures	100	**E7**	Discoidin domain	Yes	Yes	Yes	Yes	Yes
			E8	Laminin G-like Domain 3	Yes	Yes	Yes	Yes	ND
			E9		Yes	Yes	Yes	Yes	Yes
JR060	Limbic encephalitis Seizures Neuropathic pain	98	**H1**	Discoidin Domain	Weak	No	No	ND	ND
			H2		Yes	No	No	ND	ND

Table 1. Summary of patient monoclonal antibodies properties.

Monoclonal IgG4 antibodies (mAbs) were generated from CASPR2-IgG patients JR002 and JR060. All mAbs were confirmed to bind to HEK/huCASPR2. Only mAbs from patient JR002 exhibited binding to hippocampal or DRG neurons.

First, I confirmed the binding of the monoclonal antibodies to huCASPR2-transfected HEK cells. mAbs from patient JR002 (E7, E8, and E9) showed robust binding to HEK/huCASPR2. Comparatively, mAbs from patient JR060 exhibited lower binding (**Figure 2A & B**). I then tested mAbs E7, E8, and E9 in the internalisation assay. **Figure 2C** shows the schematic of the pHrodo internalisation assay. mAbs were conjugated to pH-sensitive dye, pHrodo Red (mAb-pHrodo), and added to transfected cells and imaged using the Incucyte imager. pHrodo does not fluoresce at neutral pH but at the lower pH in the endosome or lysosome after cell internalisation. The increase in pHrodo fluorescence can then be quantified as a measure of internalisation. **Figure 2D** shows an example image of the pHrodo fluorescence in HEK/CASPR2 cells after incubation with E9-pHrodo. **Figure 2E** shows the time course of Ab internalisation. E7, E8 and E9 all showed internalisation in huCASPR2-transfected HEKs at different rates and levels. I also titrated the pHrodo-mAbs and showed that the internalisation is concentration-dependent. mAbs E7 and E8 did not internalise at the lowest concentration, whereas mAb E9 internalised at all concentrations tested (**Figure 2F**).

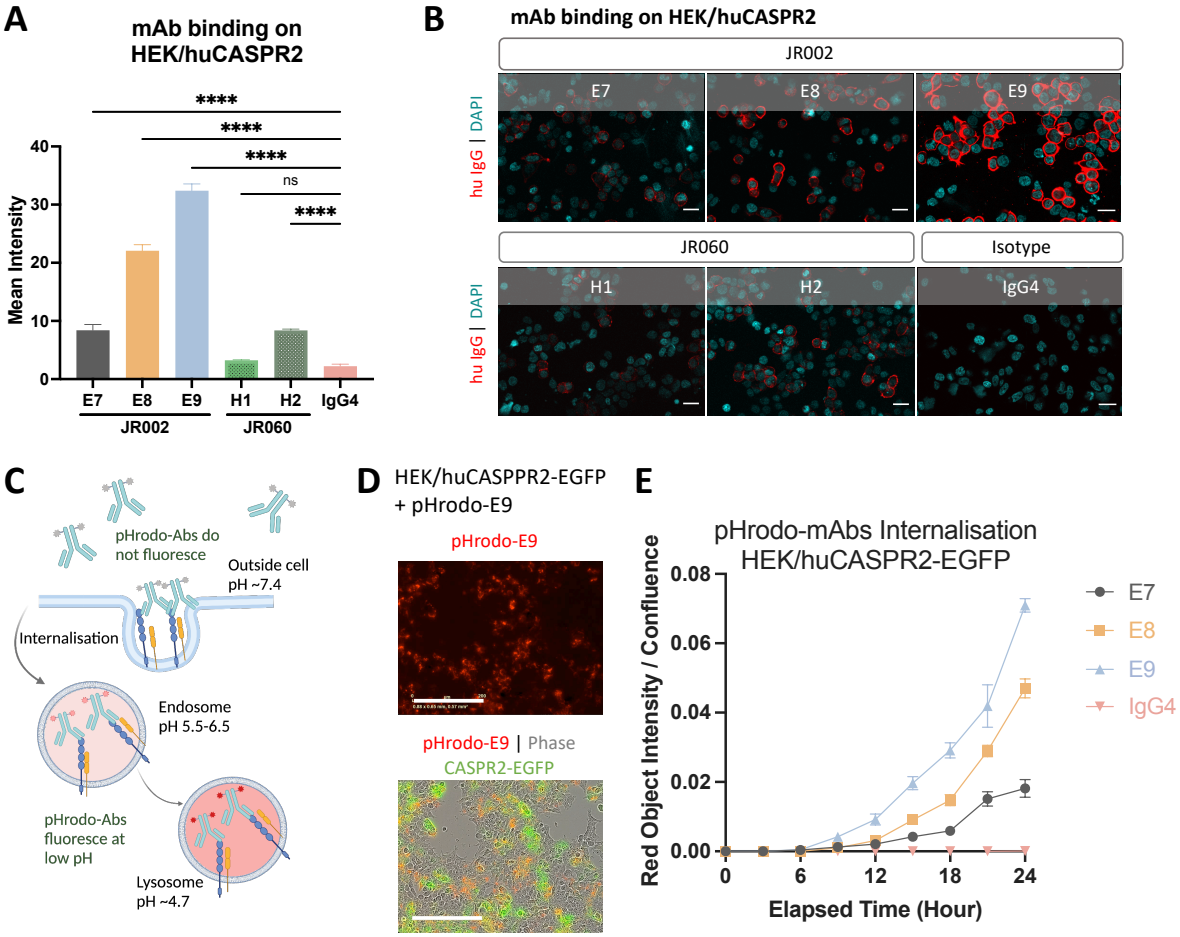


Figure 2. Patient monoclonal antibodies bind and internalise in huCASPR2- transfected HEKs

A) Quantification of monoclonal antibody binding on HEK/huCASPR2 cells. E7, E8, E9, and H2 showed significantly higher binding intensity on transfected HEKs than the IgG4 isotype control. One way ANOVA with Dunnett's multiple comparison test. n= cells from one transfection ****p<0.0001. **B)** Representative images of mAb binding (red) on CASPR2-transfected HEKs. H1 exhibited very low binding. Scale bar, 25 µm **C)** Schematic of the pHrodo internalisation assay. pHrodo-labelled antibodies do not fluoresce at neutral pH, but fluoresce brightly after internalisation in the endosome or lysosome. **D)** Top- Example image of E9-pHrodo fluorescence at the 21-hour timepoint in HEK/huCASPR2 cells; bottom- same image as above, but with phase and green fluorescence channel to detect transfected cells Scale bar, 200µm E) Time-course of pHrodo fluorescence increase in internalised pHrodo-mAbs in HEK/huCASPR2 cells. pHrodo-mAbs E7, E8, E9 showed internalisation. **F)** pHrodo-mAbs E7, E8, and E9 internalise in HEK/huCASPR2 cells in a concentration-dependent manner. Two-way ANOVA with Šídák's multiple comparisons test, ****p<0.0001, *p<0.05, ns, n=3 wells/condition.

To determine whether mAbs internalise in peripheral sensory neurons, I tested these mAbs in cultured WT DRG neurons for binding and internalisation.

I confirmed the binding of mAbs E7, E8, and E9 to DRG neurons, but unlike in HEK/huCASPR2 cells, mAbs H1 and H2 did not bind to DRG neurons. (**Figure 3A & B**). E7- and E9-pHrodo showed some internalisation in DRG neurons after a 24-hour incubation, with a 150 – 160% increase in pHrodo fluorescence in neurons, compared to the control IgG4-pHrodo (**Figure 3C &D**). Since DRG neurons express other neuronal antigens and Fc gamma receptors, I wanted to confirm that the internalisation is antigen-specific. I utilised the CASPR2 KO DRG neurons and transduced them with AAV-huCASPR2-EGFP or AAV-EGFP as a control. **Figure 3E** shows that the pHrodo fluorescence only increases in huCASPR2-expressing DRG neurons. The internalised E7-pHrodo is only observed in KO DRG neurons transduced with AAV-huCASPR2-EGFP and not in those transduced with AAV-EGFP, indicating that the internalisation of pHrodo mAbs is antigen-specific (**Figure 3F**).

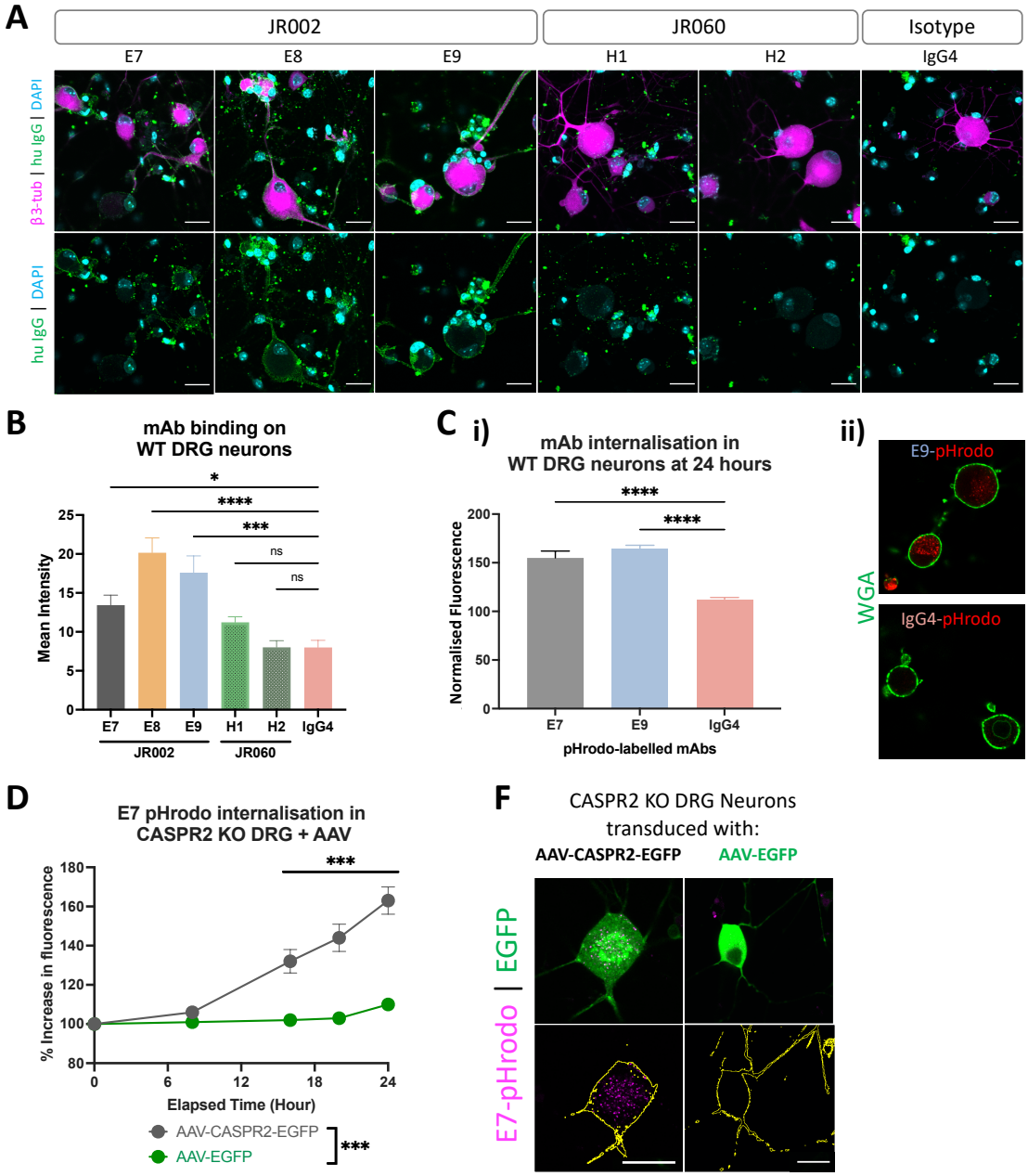


Figure 3. Patient monoclonal antibodies bind and internalisation in WT and transduced DRG neurons

A) Monoclonal antibodies from patient JR002 bound to wild-type muDRG neurons. No binding was observed for antibodies from patient JR060, or the IgG4 isotype control. **B)** Quantification of mAb binding on WT DRG neurons. E7, E8, and E9 showed binding to WT DRG neurons. One way ANOVA with Dunnett's multiple comparison test. n= cells from one culture ****p<0.0001, ***p<0.001, *p<0.05, ns. **C-i)** Quantification of the increase in pHrodo fluorescence in DRG neurons after 24-hour incubation with pHrodo-mAbs. % normalised to IgG isotype control. One way ANOVA with Dunnett's multiple comparison test. n= cells from one culture ****p<0.0001**. ii)** Example images of live WT DRG neurons treated with E9- (top) or IgG4-pHrodo (bottom) for 24 hours. Membrane stained with wheat germ agglutinin-488 (WGA). Scale bar, 25µm **D)** Time-course of pHrodo fluorescence in CASPR2 KO DRG neurons transduced with AAV-CASPR2-EGFP or AAV-EGFP control. E7-pHrodo internalised only in KO neurons transduced with AAV-CASPR2-EGFP. Two-way RM ANOVA with Šídák's multiple comparisons test, ***p<0.001, n=number of cells from one transduction. E) Example images of E7-pHrodo internalisation (magenta) in CASPR2 KO DRG neurons transduced with AAV-CASPR2-EGFP. No internalisation was observed in KO DRG neurons expressing EGFP control. Scale bar, 25µm

3.2. Testing a more extensive patient cohort

After determining that CASPR2 patient mAbs bind and internalise in an antigen-specific manner, I wanted to examine a bigger group of patients with or without neuropathic pain. As monoclonal antibodies are time-consuming to generate and might not capture all the antibody repertoire, I opted to utilise purified polyclonal patient IgGs instead. **Figure 4A** shows the patient samples that were tested in this study. These patient samples are a subset of the cohort described in Chapter 3. All the sera from the CASPR2-IgG group and the pooled healthy control sample were positive for binding to WT DRG neurons. I purified the IgG from patient sera and conjugated the polyclonal IgG to pHrodo for the internalisation assay at a normalised concentration (20 μg/mL). The CASPR2 KO DRG neurons were included as a negative control to determine whether the internalisation is CASPR2-specific. Most CASPR2-IgG samples showed internalisation in WT DRG neurons but not in KO DRG neurons at 24 hours post-incubation (**Figure 4B**). Sample JR35078 from the 'no pain' group showed similar levels of internalisation WT and KO DRG neurons, suggesting that the internalisation observed in this sample is likely not CASPR2-mediated. Interestingly, the samples with the highest level of internalisation (JR2028 and JR2042, yellow) also had the highest end-point titre (Ch 3 Fig 3). As expected, the healthy control IgGs did not internalise in WT or KO neurons. The images in **Figure 4C** show that the pHrodo fluorescence is mainly localised in neurons at 24 hours post-incubation.

A

Group	Pain	Samples
CASPR2-IgG	Neuropathic pain	3
	Unlikely neuropathic pain	2
	No pain	2
Pooled healthy control	No Pain	1
Healthy control	No pain	3

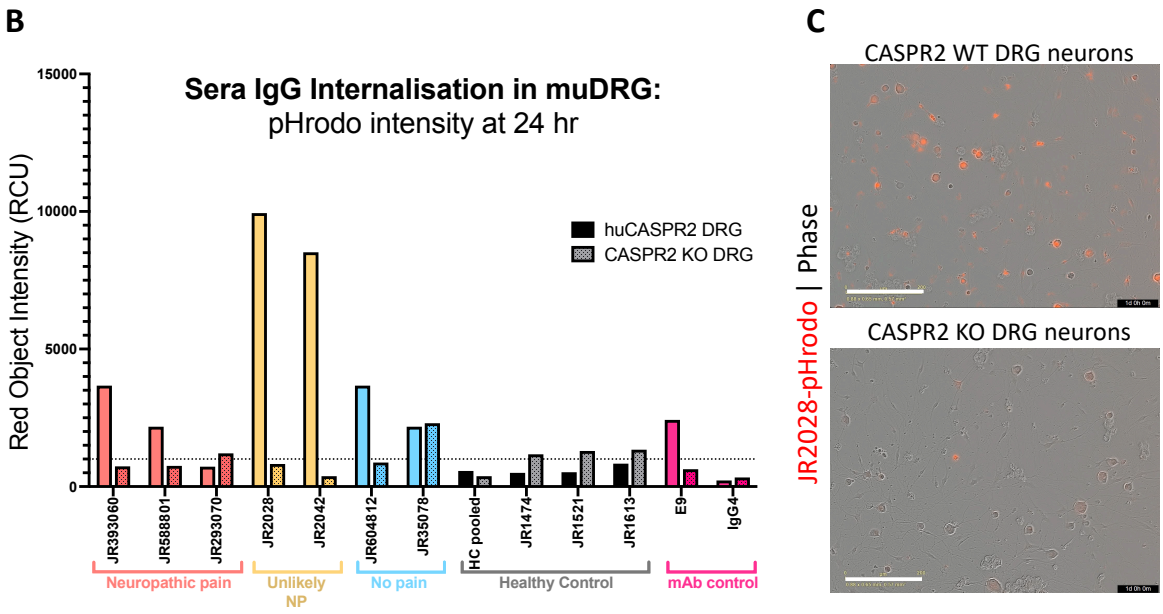


Figure 4. Purified patient IgG internalised in DRG neurons in an antigen-specific manner

A) CASPR2-IgG and healthy control sera samples tested for internalisation. Sera were from the same patient cohort described in Chapter 3. **B)** Quantification of pHrodo signal in CASPR2 WT or KO DRG neurons at 24 hours post-incubation. Most patient sera showed antigen-specific internalisation. Healthy control IgG and isotype control did not show any internalisation in WT or KO DRG neurons. **C)** Example images from the Incucyte live-cell imaging at 24 hours post-internalisation. Sample JR2028-pHrodo showed the highest level of internalisation in CASPR2 WT DRG neurons (top). Internalisation was abrogated in the CASPR2 KO DRG neurons (bottom). Scale bar, 200 μm

I also tested these patient IgG for internalisation in HEK/CASPR2-EGFP cells, as the expression of CASPR2 levels is higher in transfected HEKs than in DRG neurons. The internalisation of IgGs was imaged by live-cell confocal imaging to visualise the internalised antibodies. After a 24-hour incubation with transfected HEKs, the pHrodo Abs can be detected intracellularly and colocalised with CASPR2-EGFP (**Figure 5A**). Interestingly, the levels of internalisation of patient IgGs differed between transfected HEKs and WT DRG neurons. **Figure 5B** shows

that sample JR39070, from the neuropathic pain group, had the highest fluorescence in HEK/huCASPR2, while this sample did not internalise in WT DRG neurons. Conversely, sample JR393060 did not internalise in HEK/huCASPR2 but internalised in WT DRG neurons. None of the healthy control IgGs was internalised in HEK/huCASPR2 cells, while the control mAb E9 showed robust internalisation, as expected.

I wondered whether the internalisation of the IgG was mediated by clathrin/receptor-mediated endocytosis or by non-receptor-mediated methods, such as pinocytosis or phagocytosis. I utilised the dynamin inhibitor, Dyngo-4a, to halt all clathrin-mediated endocytosis *in vitro* and applied pHrodo-IgG samples to treated cells. Patient IgGs could internalise in the DMSO vehicle-treated HEK/huCASPR2 but not the Dyngo-4a pre-treated HEK/huCASPR2 (**Figure 5C**), suggesting that the observed internalisation is dynamin-dependent. **Figure 5D** shows the absence of pHrodo signal in Dyngo-4a-treated HEK cells upon incubation with patient-IgG; compared to the increase in pHrodo fluorescence in vehicle-treated HEK/huCASPR2 cells.

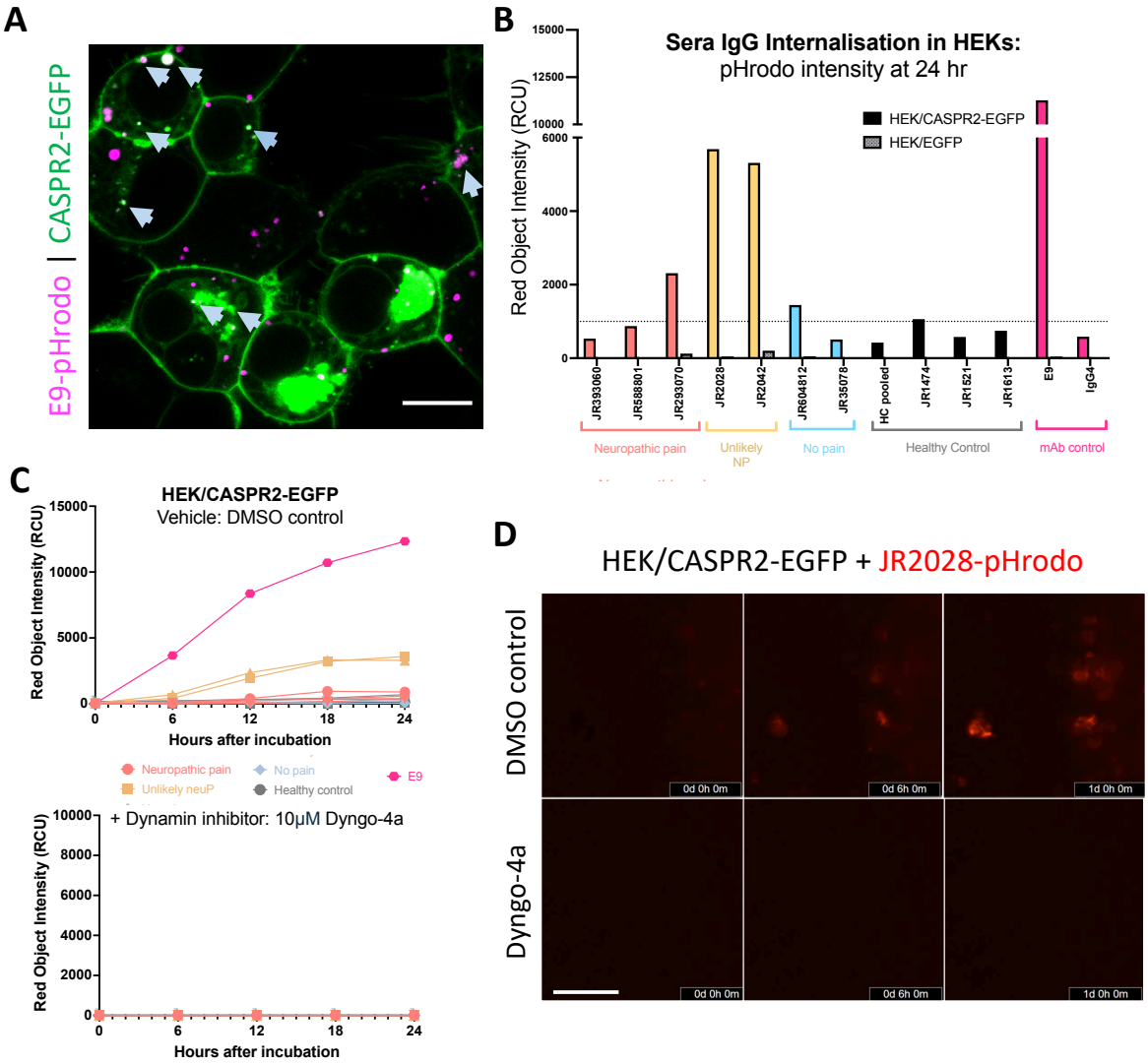


Figure 5. Purified patient IgG internalised in transfected HEKs in a dynamin-dependent manner.
A) Example image of HEK/huCASPR2-EGFP (green) treated with E9-pHrodo (magenta). At 24 hours, internalised antibodies can be detected inside the cells. Colocalisation of E9-pHrodo with CASPR2-EGFP protein can also be seen inside the cell (blue arrowheads). Scale bar, 10 μm **B)** Quantification of pHrodo signal in huCASPR2 or EGFP-transfected HEKs at 24 hours post-Ab incubation. Most patient sera showed internalisation in HEK/huCASPR2. Healthy control IgG and isotype control did not internalise. Control E9-pHrodo showed robust internalisation in HEK/huCASPR2. **C)** Time-course of Ab internalisation in transfected HEKs with (bottom) or without (top) pre-treatment with dynamin inhibitor, Dyngo-4a. Pre-treatment of transfected HEKs with Dyngo-4a attenuated the internalisation of patient IgG ad control mAbs, compared to vehicle-treated controls. D) Example time-course images of pHrodo internalisation in DMSO vehicle control or Dyngo-4a-treated cells. No pHrodo fluorescence was observed in cells treated with Dyngo-4a. Scale bar, 100 μm

3.3. Potential pathway modulation by CASPR2-Abs

Studies have shown that the genetic disruption of CASPR2 resulted in elevated AKT/mTOR signalling in the hippocampus and DRG of CASPR2 KO

mice (Xing et al., 2019, 2020). Pharmacological inhibition of this hyperactive signalling reduced DRG neuron hyperexcitability and attenuated pain-related hypersensitivity in CASPR2 KO mice (Xing et al., 2020; Zhang et al., 2021). As CASPR2-IgG from neuropathic pain patients have been shown to disrupt CASPR2 expression, I wondered whether this immune-mediated CASPR2 downregulation also induces changes in the downstream AKT/mTOR and MAPK signalling that could alter local mRNA translation and ultimately result in the observed increase in neuronal excitability (Dawes et al., 2018; Giannoccaro et al., 2019).

I evaluated the changes in the phosphorylation of proteins involved in the Akt-mTOR and MAPK pathways that are increased in nerve injury or inflammatory pain models. (Moy et al., 2017). **Figure 6A** shows the proteins examined in this study in green. AKT, which is upstream of mTOR in the signalling pathway; S6, which is downstream of mTOR and mediates ribosome biogenesis in translation; and ERK, which modulates MNK and the subsequent activation of eIF4E for cap-dependent translation. I first confirmed that AKT, S6 and ERK were activated in the CASPR2 KO mice by quantifying the phosphorylated protein levels in whole DRG lysates of CASPR2 KO mice (**Figure 6B**).

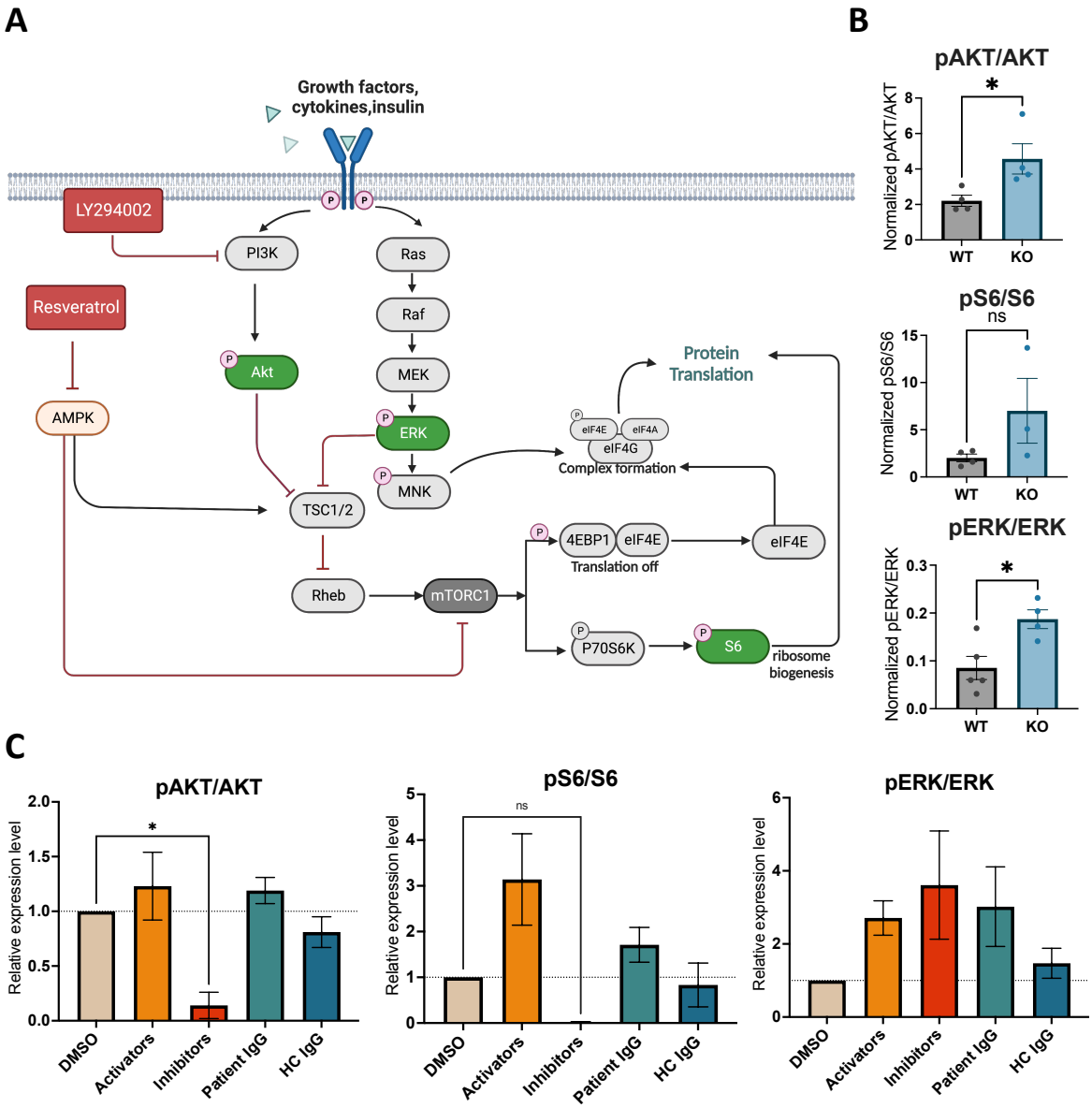


Figure 6. 6.The AKT/mTOR/MAPK pathway is hyperactive in CASPR2 KO DRG

A) Simplified schematic of the AKT/mTOR/MAPK pathway interactions. Growth factors or cytokines can trigger downstream pathways through binding and activation of the receptor tyrosine kinases. The PI3K/AKT pathway and MAPK pathway are activated through phosphorylation of proteins. Key pathway proteins investigated are in green. AMPK inhibitor, Resveratrol, and PI3K inhibitor LY294002, are highlighted in red. **B)** AKT/mTOR/MAPK pathway is hyperactive in CASPR2 KO DRG neurons. Quantification of normalised pAKT , pS6, and pERK levels in CASPR2 WT or KO DRG lysates showed a significant increase in pAKT and pERK activation in CASPR2 KO DRG than WT DRG. Unpaired two-tailed t-test, *p<0.05, n=3-4. **C)** Quantification of relative phospho-protein levels in cultured WT DRG neurons treated with DMSO, activators (growth factor, capsaicin, KCl), inhibitors (Reservatrol and LY294002), purified patient IgG (JR002+JR060), and healthy control IgG. Treatment of WT cultured DRG neurons with resveratrol and LY294002 inhibited the phosphorylation of AKT and S6. Activation and depolarisation of DRG neurons using a combination of growth factors, capsaicin, and KCl resulted in a trend of increase in S6 and ERK phosphorylation. Patient or healthy control IgG (HC IgG) did not alter phosphorylation. One way ANOVA with Dunnett's multiple comparison test, *p<0.05, ns, n=3 cultured WT mice DRGs.

As a pilot experiment to determine if there are any changes to pathway activation, I applied purified CASPR2 patient IgG to cultured WT DRG neurons and measured changes in the pathway proteins. As a proof-of-concept, I applied known inhibitors and activators to cultured WT DRG neurons for one hour and analysed levels of phosphorylated-AKT, S6 and ERK proteins by western blot. The combination of inhibitors, LY294002 and Resveratrol, which inhibit AKT phosphorylation and mTOR activation, reduced pAKT and pS6 levels in cultured DRG neurons compared to vehicle control (**Figure 6C**). I also used a combination of activators (NGF, GDNF, capsaicin, and KCl) to activate the AKT/mTOR pathway *in vitro* but did not observe any significant upregulation of phosphorylated proteins. The application of purified patient CASPR2-IgG to the cultured DRGs did not induce any changes in phosphorylation of AKT, S6 or ERK after one hour in culture (**Figure 6C**). While I could detect the inhibition of AKT and S6 phosphorylation in this assay, I could not distinguish any elevated phosphorylation of pathway proteins by adding growth factors. Since growth factors are necessary for the viability of DRG neurons *in vitro*, further optimisation of the assay and culture conditions are required to detect the upregulation of pathway proteins.

4. Discussion

In an effort to understand the pathogenic mechanisms underlying the common symptom of neuropathic pain CAPR2-patient antibodies, I characterised monoclonal antibodies generated from two CASPR2-IgG patients. Both patients had limbic encephalitis and seizures, but only one patient experienced neuropathic pain. The mAbs, E7, E8, and E9, from patient JR002 target the most common N-

terminal discoidin or the laminin-like G3 domain. mAbs H1 and H2 were derived from patient JR060 with neuropathic pain, and both target the discoidin domain.

I confirmed the binding of mAbs derived from both patients on CASPR2-transfected HEK cells but, surprisingly, did not observe the binding of mAbs H1 and H2 from the patient with neuropathic pain on cultured DRG neurons. The binding observed on peripheral sensory neurons correlated with that of cultured hippocampal neurons, indicating that there may be fundamental differences in epitope or receptor availability between heterologously-expressed and endogenously-expressed CASPR2 in neurons. The heterologous expression of CASPR2 on HEK cells provides a high level of CASPR2 surface expression without partner proteins blocking antibody epitopes. MAbs H1 and H2 may have low affinities for CASPR2 and need to bind bivalently, which requires a high level of CASPR2-expression.

Studies have shown that patient polyclonal CASPR2-IgG internalised in hippocampal neurons (Giannoccaro et al., 2019; Joubert et al., 2022). Using a live-cell imaging assay, I tested patient mAbs E7, E8, and E9 for internalisation and found that they could internalise in transfected HEKs and DRG neurons. The internalisation of mAbs in HEK/huCASPR2 cells was concentration-dependent, suggesting affinity differences between the mAbs. I also confirmed that the internalisation observed was antigen-specific using CASPR2 KO DRG neurons. I transduced CASPR2 KO DRGs with AAV-CASPR2-EGFP or control AAV-EGFP and demonstrated that the internalisation was abrogated in CASPR2 KO DRG neurons, suggesting that the internalisation is mediated through the binding of CASPR2.

However, it is possible that the mAbs may be internalised through the endogenous endocytosis of CASPR2 independent of antibody binding. mAbs can

bind to CASPR2 and be endocytosed with CASPR2, while non-binding mAbs would not be internalised in this manner. I used the dynamin inhibitor, Dyngo-4a and showed that the internalisation of mAbs in transfected HEKs was likely due to receptor-mediated endocytosis. Still, this method doesn't allow us to discern the physiological endocytosis from antibody-mediated endocytosis. Literature has shown the cell surface expression of CASPR2 is regulated through an endocytosis motif and that the selective endocytosis of CASPR2 is responsible for the polarised expression of CASPR2 in hippocampal neurons (Bel et al., 2009). In the DRG neuron, FcγR-blockers could distinguish antibody-mediated endocytosis by inhibiting the binding of IgG or IgG-immune complexes to FcγRs. Additionally, assays utilising cell-surface biotinylation of neurons and treatment with isotype controls and anti-CASPR2 antibodies may help us distinguish physiological vs antibody-mediated internalisation.

Measuring surface level CASPR2 after incubation with mAbs could help discern whether the internalisation observed is indeed mediated through antibody binding. Studies measuring the surface expression of CASPR2 on hippocampal neurons after patient-IgG incubation disclosed different results. Some reported no change or an increase in CASRP2 surface expression (Giannoccaro et al., 2019; Saint-Martin et al., 2019), while others observed a decrease in expression (Joubert et al., 2022). Furthermore, these studies utilised different patient IgGs that could alter CASPR2 expression through other mechanisms. Compensatory mechanisms or *de novo* synthesis of CASPR2 *in vitro* may add to the confound of varying CASPR2 surface expression, especially in longer timescales in internalisation assays. Experiments using techniques such as fluorescence recovery after photobleaching (FRAP) may help address this issue.

The small number of patient samples and monoclonal antibodies limits our ability to infer the relationship between antibody properties and their pathogenic potential in neuropathic pain patients. Monoclonal antibodies also do not recapitulate the entire repertoire of autoantibodies *in vivo*. Therefore, I examined a larger number of patient sera for internalisation in CASPR2-transfected HEKs and DRG neurons. I showed that most CASPR2 patient IgGs could internalise in DRG neurons and observed differences in internalisation between DRG neurons and HEK/CASPR2 cells. Notably, the mAb E9 exhibited a higher level of internalisation in HEK/CASPR2 cells than in DRG neurons. This difference is likely due to the epitope of the antibody; as E9 binds to the laminin-like G3 domain, its epitope could be hindered by the association of contactin-2 in DRG neurons. Thus, it is plausible that the different epitopes of patient IgG could contribute to the differential internalisation between cell types.

Due to the small sample sizes, I could not correlate IgG internalisation with the symptom of neuropathic pain in patients. Interestingly, the samples that showed the highest level of internalisation were from patients with likely non-neuropathic pain. However, more samples are required to validate this finding. Further insight into the downstream effects of internalisation could help us understand if the observed internalisation is pathogenic. For instance, measurement of changes in K_v1 channel expression after internalisation or assessing the neuronal excitability by calcium imaging or electrophysiology will broaden our understanding of the pathogenic mechanisms of antibody internalisation.

I also explored another potential antibody mechanism by evaluating changes in signalling pathways that regulate translation. Inspired by the study which showed that CASPR2 deficiency resulted in hyperactive AKT/mTOR

signalling, I studied whether the CASPR2 disruption by patient IgG could also activate the mTOR signalling pathway in the same manner (Xing et al., 2019, 2020). First, I confirmed that the DRG of CASPR2 KO mice showed elevated phosphorylation of AKT, S6 and ERK by western blot, as literature suggested (Xing et al., 2020; Zhang et al., 2021). However, the whole DRG lysates analysed include non-neuronal cells, such as satellite glial cells and immune cells; thus, the upregulation of these proteins cannot be exclusively attributed to neurons. Immunohistochemistry could be applied to confirm the elevated phosphorylation of pathway proteins. As shown by Pezet *et al*., the elevated phosphorylation of AKT in DRG neurons was detectable by immunohistochemistry (Pezet et al., 2005)

To reduce the potential confound of other cell types, I utilised cultured DRG neurons to examine the effects of patient IgG on pathway proteins. I applied purified patient IgG and healthy control IgG to DRG-cultured neurons and analysed phosphorylated AKT, S6 and ERK levels. Using known inhibitors of AKT (LY294002) and mTOR (Resveratrol), I could detect a significant reduction in the phosphorylation of the analysed pathway proteins. However, I could not see the upregulation of these proteins upon adding growth factors, capsaicin, and KCl to DRG neurons, indicating that the assay window is insufficient to detect changes in elevated pathway signalling. The mouse DRG cultures in this assay require the presence of growth factors, which could interfere with the activation of the pathway proteins examined. Pezet *et al*. showed that including a low concentration of growth factors and a two-day culture was optimal for providing a good assay window (Pezet et al., 2005). Further optimisation of the DRG culture conditions is needed to establish the conditions that give the lowest possible level of phosphorylated pathway proteins. I did not detect any changes in activation of the AKT/mTOR pathway in cultured DRG neurons treated with patient of healthy

control IgG. It is possible that the one-hour incubation with patient IgG was not sufficient to detect any downstream changes. I noted that the internalisation of CASPR2 by patient IgG was not detectable until 12 hours post-incubation.

The AKT/mTOR/ERK pathways are involved in numerous key cell regulatory processes, such as cell proliferation and survival (Manning & Cantley, 2007; Porta et al., 2014). Therefore, it is essential to confirm that the activation of the pathway by patient IgG leads to alterations in protein expression. As there is a wide heterogeneity of symptoms in CASPR2-IgG patients, more patient samples must be assayed for both internalisation and pathway activation before confirmation of these mechanisms as potentially pathogenic in patients.

While it may be simplistic to ascribe the mechanism of autoantibodies to neuropathic pain symptoms, the implication of elucidating the mechanisms of autoantibodies is paramount to the development of effective pain therapies. For example, the analgesic potential of blocking endocytosis was demonstrated by LX9211, an investigation drug for neuropathic pain that has shown efficacy in phase 2 of clinical trials. LX9211 is a small-molecule inhibitor of AP2-associated kinase 1 (AAK1) that is hypothesised to mediate its antinociceptive effects by inhibiting the endocytosis to $GABA_A$ receptor (Kostich et al., 2016). Research in targeting translation by modulating the upstream signalling pathway has resulted in the discovery of inhibitors that target dysregulated translation in pathological pain conditions (Khoutorsky & Price, 2018; Moy et al., 2017). MNK inhibitors have been described as a potential pain therapeutic as it is the only kinase for eIF4E phosphorylation, thus limiting possible off-target effects. It has been shown to reduce cold hypersensitivity in neuropathic pain models (Moy et al., 2017). Although the downstream mRNA targets for eIF4E phosphorylation are currently unknown, cell excitability studies suggest that eIF4E activation increases the

translation of mRNAs that influence the membrane trafficking of voltage-gated channels (Moy et al., 2017; Yousuf et al., 2021).

To summarise, I have shown that CASPR2 IgG samples from patients with or without pain internalise in DRG neurons in an antigen-specific manner. The wealth of literature on the effect of nerve injury in modulating signalling pathways regulating translation supports the more in-depth investigation of whether CASPR2-IgGs are involved in altering signalling pathways that leads to neuronal hyperexcitability. Understanding the steps between CASPR2-IgG binding to DRG neurons and the induction of pain *in vivo* will be instrumental in delivering targeted treatments for neuropathic pain. The synergistic use of binding and mechanistic assays will hopefully provide further insight into the role of autoantibodies in neuropathic pain.

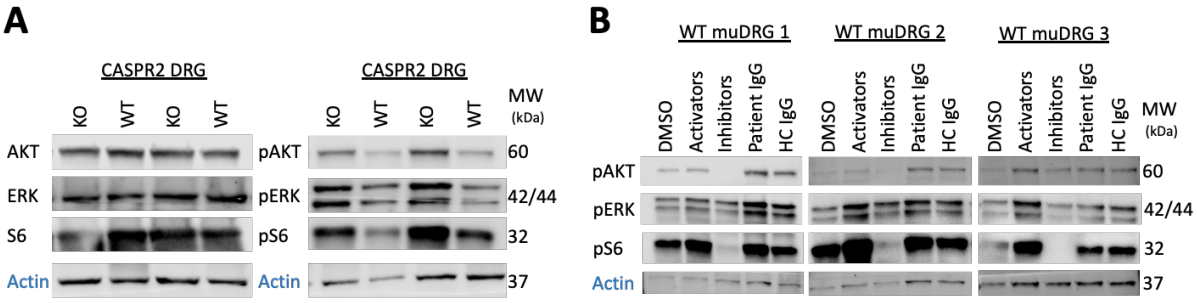
5. Acknowledgements

Prof Sarosh Irani and Dr Bo Sun provided clinical phenotype and binding data to hippocampal neurons in Table 1. Monoclonal antibodies were generated by Dr Bo Sun and the Oxford Autoimmune Neurology Group. Figures were made with BioRender and Graphpad Prism 9.

6. Appendices

Table 1. List of antibodies used.

Antibodies	Predicted Band Size (kDa)	Dilution	Host Species	Vendor and Catalogue number
CASPR2	152	1:1000 (in TBS-T + 1% milk)	Rabbit	Abcam Ab13705
Akt	60	1:1000 (in TBS-T + 5% BSA)	Rabbit	Cell Signaling Technology #9272
p-Akt (Ser473)	60	1:1000 (in TBS-T + 5% BSA)	Rabbit	Cell Signaling Technology #4060
S6	32	1:5000 (in TBS-T + 5% BSA)	Rabbit	Cell Signaling Technology #2217
p-s6 (Ser235/236)	32	1:1000 (in TBS-T + 5% BSA)	Rabbit	Cell Signaling Technology #4858
4EBP1	20	1:1000 (in TBS-T + 5% BSA)	Rabbit	Cell Signaling Technology #9644
β-actin	42	1:1000 (in TBS-T + 5% BSA)	Mouse	Abcam ab8226
p44/42 MAPK (Erk1/2)	42/44	1:1000 (in TBS-T + 5% BSA)	Rabbit	Cell Signaling Technology #9102
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	42/44	1:1000 (in TBS-T + 5% BSA)	Rabbit	Cell Signaling Technology #9101S



Appendix Figure 1. Western blot detection of pAKT, pS6, and pERK
A) Representative western blot images of whole DRG lysates from WT or CASPR2 KO mice. **B)** Western blot images of cultured DRG lysates from WT mice. Cultured neurons received treatments as described in the methods section.

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5

General Discussion

1. CASPR2 overexpression in pain

This work shows that CASPR2 overexpression in the sensory neurons of transgenic mouse lines did not significantly alter pain mechanical or thermal sensitivity in preclinical models, but reduced pain sensitivity to capsaicin stimuli. Without confirming K_v1 channel expression in CASPR2-overexpressing neurons, it is difficult to ascertain whether the absence of effect was due to insufficient CASPR2 overexpression or the lack of K_v1 channel surface upregulation. Future studies determining the surface expression of K_v1 channels in CASPR2-overexpressing neurons are therefore crucial in establishing the role of CASPR2 overexpression in regulating neuronal excitability. Encouraging evidence from the literature showed that the co-transfection of CASPR2 and $K_v1.2$ increased the surface localisation of Kv1.2 channels in a heterologous cell system, although this has yet to be confirmed in neurons (Saint-Martin et al., 2019). Beyond confirmation of surface expression, assessing neuronal excitability is also vital in understanding if CASPR2 overexpression can regulate neuronal hyperexcitability.

K_v1 channels play a crucial role in regulating cell excitability, and the disruption of these channels has been shown to cause neuronal hyperexcitability (Busserolles et al., 2016; Rasband et al., 2001). Accordingly, increasing the expression of K_v channels should decrease hyperexcitability. However, as Sutherland *et al*. have demonstrated in their study using $K_v1.1$-overexpressing mice- one can have too much of a good thing- at least in a physiological setting. The additional Kv1.1 transgene in naïve mice resulted in the downregulation of endogenous K_v1 channel expression, which resulted in hyperexcitability and increased network excitability (Sutherland et al., 1999). In this study, we utilised transgenic mouse lines that constitutively expressed CASPR2 in nociceptors or

sensory neurons. Could there be compensatory mechanisms of K_v1 channel expression from the constitutive overexpression of CASPR2 in these transgenic mouse lines, similar to what Sutherland et al. have observed? Given CASPR2's close association with K_v1 channels and their role in synapse formation, the consequence of disrupting coordinated CASPR2 gene expression during PNS development should be further examined.

The effects of CASPR2 overexpression on the potential disturbance of protein homeostasis must also be considered. $K_v\beta2$ is a known CASPR2-associated protein that is immunoprecipitated with CASPR2 in hippocampal and cortex lysates (Chen et al., 2015; Poliak et al., 1999). The K_v1 channels associate with accessory $K_v\beta$ subunits at a 1:1 stoichiometry (Long et al., 2005). The assembly of the $Kv\alpha$ and $K_v\beta$ influences subcellular targeting and increases K_v1 channel expression and cell surface stability (Shi et al., 1996). Could the introduction of exogenous CASPR2 disrupt the homeostasis of the K_v channel complex formation? Additional studies in the expression of associated proteins are required to determine if they are concomitantly upregulated on the cell surface.

Considering the possible disruption of endogenous K_v channel expression through constitutive expression and the most viable therapeutic approach for pain treatment, the optimal way of assessing CASPR2 overexpression is thus the delivery of the transgene after the nerve injury-induced downregulation of K_v1 channels. This approach would hopefully circumvent the potential disturbance of protein and channel homeostasis but restore membrane K_v channel expression to a physiologically relevant level and limit neuronal hyperexcitability after injury. Recent studies have shown the efficacy of restoring K_v channel expression in limiting neuronal hyperexcitability and reducing pain-related behaviour after nerve injury (Fan et al., 2014; Zhang et al., 2021).

The consistent phenotype of hyposensitivity to intraplantar capsaicin in all transgenic mouse lines was intriguing and perhaps hinted at a role of CASPR2 in the modulation of other ion channels. The overexpression of K_v1.2 in rats did not alter capsaicin-induced acute pain (Fan et al., 2014). In contrast, I showed that CASPR2 overexpression in nociceptors and sensory neurons was sufficient to decrease pain-like behaviour in mice. Consistent with the *in vivo* results, I noted a significant reduction in calcium influx in CASPR2-overexpressing neurons upon capsaicin application in the calcium imaging assay. The robust phenotype related to capsaicin points to the capsaicin-responsive TRPV1 channel as a plausible putative partner of CASPR2. TRPV1 is expressed in nociceptive neurons in the DRG and is activated by noxious heat and vanilloid molecules such as capsaicin (Caterina et al., 1997).

While there have not been any studies investigating the direct interactions between CASPR2 and TRPV1, the literature suggests that they share a few partner proteins. The K_v subunits $K_v\beta1$ and $K_v\beta2$, which have been shown to associate with CASPR2, have also been demonstrated to co-immunoprecipitate with TRPV1. $K_v\beta2$ associates with and enhances the surface expression of TRPV1, and electrophysiological studies in DRG neurons also showed that the $K_v\beta2$ subunit renders the TRPV1 channel more sensitive to capsaicin (Bavassano et al., 2013). Recent data showed that $K_v\beta1$ also enhances the stability of TRPV1 on the plasma membrane. Interestingly, this association also shifted the temperature threshold for TRPV1 thermal activation (Wang et al., 2020). Additionally, Whirlin, a calmodulin-dependent serine kinase (CASK)-interacting protein, has been identified as a putative TRPV1 partner. Whirlin contained a PDZ-binding domain and was shown to stabilise TRPV1 on the plasma membrane (Ciardo et al., 2016), and CASPR2 has been shown to bind to CASK through its intracellular PDZ-binding domain

(Horresh et al., 2008; Pinatel et al., 2017). Though there is currently no direct connection linking CASPR2 with TRPV1, exploring this potential relationship could further our understanding of how CASPR2 interactions in other protein complexes may regulate neuronal excitability.

2. The translational potential of this work

2.1. CASPR2 Overexpression: Implications in other diseases

There is a wealth of literature exploring the role of CASPR2 disruption in diseases with CNS involvement, such as limbic encephalitis and Morvan's syndrome (Irani et al., 2012; Joubert et al., 2016; Patterson et al., 2018; Van Sonderen et al., 2016). However, research on CNS- and PNS-involved diseases caused by CASPR2-IgG have been largely separated, which is understandable due to the diverse clinical manifestations that require thorough investigation. The role of K_v1 channels in regulating neuronal excitability is conserved across PNS and CNS neurons (Smith, 2020). Hence, treating the underlying disease aetiology of K_v1 channel expression could offer translational potential in diseases characterised by neuronal hyperexcitability. Notably, research in epilepsy, a brain disorder caused by abnormal firing of neuronal networks, has exemplified the effectiveness of increasing $K_v1.1$ expression in neurons in limiting neuronal hyperexcitability (Snowball et al., 2019; Snowball & Schorge, 2015). Furthermore, a recent study from Qiu *et al*. described a clever gene therapy strategy that selectively downregulates excitability in overactive neurons by using an immediate early gene promoter which induces the expression of $K_v1.1$ channels selectively in hyperactive neurons (Qiu et al., 2022). Combining cell type-specific promoters and novel-engineered

AAVs with higher tissue tropism increases the potential of reciprocally translating potassium gene therapy to attenuate neuronal hyperexcitability CNS and PNS.

Although the delivery of CASPR2 by AAV was ineffective due to low transgene levels in this study, the recent advances in engineered AAVs will hopefully facilitate the design of more efficient viral vectors. Machine-guided approaches to generate synthetic AAV capsids or Cre-recombination-based AAV targeted evolution (CREATE) have created capsids with greater payload capacity and tissue tropism (Bryant et al., 2021; Kumar et al., 2020). To treat neuropathic pain, a similar approach of promoter design by utilising the immediate early gene promoter, *c-fos*, to target delivery of AAV-CASPR2 to activated nociceptive neurons would increase the specificity of transgene delivery and avoid the potential disruption of cell surface protein homeostasis in non-injured cells. The diversity of cell types that can be targeted using different serotypes makes AAV delivery an attractive therapeutic modality.

CASPR2 overexpression may also have potential therapeutic potential in other diseases. Mutations in CASPR2 are strongly associated with autism spectrum disorders (ASDs), and CASPR2 KO mice have been shown to have behavioural features consistent with autism. Epilepsy and sensory processing abnormalities are frequently associated with ASD (Peñagarikano et al., 2011; Poot, 2015). The shed ectodomain of CASPR2 (CASPR2-ecto) regulates network synchrony in cortical neurons. A recent study has observed reduced levels of the CASPR2-ecto in the CSF of individuals with ASD (Martín-de-Saavedra et al., 2022). This observation revealed another avenue through which CASPR2 could suppress seizures by restricting excessive network activity and synchrony. The delivery of CASPR2 in a soluble form could be a potentially viable approach to modulate network excitability.

2.2. In-depth patient antibody screening

I showed that rodent neuronal cultures are a beneficial addition to the assay repertoire for patient sera screening. Though there is the caveat of species differences, rodent neurons present neuronal antigens in their native environment that better represents the *in vivo* autoantibody binding, which heterologous systems fail to capture (Ramanathan et al., 2021; Waters et al., 2016). Similarly, human iPS-derived sensory neuron cultures are invaluable in characterising patient autoantibodies. The use of myelinating co-cultures, in particular, provides the context for localising antibody binding in the nodal regions and can be further utilised in investigating antibody-mediated disruption of axoglial connections (Clark et al., 2017; Fehmi et al., 2021).

These neuronal assays can be extended to explore the putative mechanisms of autoantibodies. The internalisation assay is robust in detecting the internalisation of monoclonal or polyclonal antibodies in neurons or transfected cells and- when used in combination with antigen-specific knock-out cell lines- can establish if the neuronal antigen indeed mediates the antibody internalisation. I started to inspect signalling pathways that regulate translation in cultured DRG neurons and observed that genetic disruption of CASPR2 caused hyperactivation of the AKT/mTOR signalling pathway.

These neuronal assays can be applied in synergy to investigate other painful diseases that are considered to be autoimmune-mediated. Recent publications have demonstrated the presence of pronociceptive antibodies in patients with chronic primary pain conditions (CPP) (Goebel et al., 2022). Passive transfer experiments of IgG or IgM from patients with fibromyalgia syndrome, complex regional pain syndrome (CRPS), or non-inflammatory joint pain associated with rheumatoid arthritis have revealed causative roles of patient autoantibodies in these pain

syndromes (Goebel et al., 2011, 2021; Krock et al., 2022; Wigerblad et al., 2016). The target antigens and pathogenic mechanisms are unknown in many of these diseases. Thus, using neuronal assays would be advantageous in better understanding the cellular and molecular mechanisms underlying these diseases. In addition to neuronal binding assays, the sera from patients with CPP can be characterised for internalisation or activation of the aforementioned translation-related signalling pathways in an antigen-agnostic manner.

With the elucidation of the pathomechanisms of the patient autoantibodies, there are opportunities for more personalised therapies that target the precise mechanism for pathogenic antibodies. For instance, understanding the pathogenic mechanism of the Aquaporin-4 (AQP4) antibodies in patients with neuromyelitis optica (NMO) has led to the development of Aquaporumab. This humanised monoclonal antibody has been engineered to have no effector function and competitively displaces AQP4-IgG in patients, reducing AQP4-IgG-dependent cytotoxicity and NMO pathology (Tradtrantip et al., 2012). Since autoantibodies from patients have been shown to disrupt the interaction between contactin-2 and CASPR2, a similar approach could be applied to treating CASPR2-IgG-mediated diseases (Patterson et al., 2018; Saint-Martin et al., 2019).

Although the incidence of CASPR2 antibody disease is low, the notion of targeted therapies for autoimmune-mediated pain can be extended to other painful conditions. For example, the development of therapeutics that mitigate the downstream mechanisms, such as internalisation or activation of translation-regulating signalling pathways. Indeed, research on targeting the NGF signalling pathway for pain has led to the discovery of the NGF antagonist, Tanezumab (Mantyh et al., 2011; Patel et al., 2018). Currently, there are therapeutics in clinical or preclinical trials that could address the pathogenic mechanisms of

autoantibodies. For example, LX9211 is a small molecule kinase inhibitor which prevents clathrin-mediated endocytosis and has demonstrated efficacy in the reduction of neuropathic pain in phase 2 of clinical trials (Kostich et al., 2016). Although the exact mechanisms for LX9211 are still not fully understood (suggested inhibition of GABAa receptor internalisation), this drug is safe to use in patients and, where prior screening has suggested antibody internalisation, could be applied to specific autoantibody patient groups. Additionally, research in targeting translation by modulating the upstream signalling pathway has led to the discovery of MNK inhibitors that target dysregulated translation in pathological pain conditions (Khoutorsky & Price, 2018; Moy et al., 2017). The selective inhibition of pathogenic autoantibody mechanisms will hopefully address the shortcomings of current pain treatments for these patients.

3. General Conclusions

In conclusion, I have explored the potential of CASPR2 overexpression in regulating pain sensitivity by thoroughly characterising two transgenic mouse lines. The overexpression of CASPR2 in nociceptors and sensory neurons of mice did not significantly alter acute pain sensitivity but reduced capsaicin-induced acute pain. *In vitro* data corroborated with the hyposensitivity to capsaicin observed *in vivo*, suggesting that CASPR2 may regulate the excitability of specific neuronal populations. Future experiments are required to establish whether CASPR2 overexpression modulates K_v1 channel surface expression. Nonetheless, the widespread expression of CASPR2 in the CNS and PNS and its putative functions in protein modulation bolsters it as a worthwhile target of further investigation.

I showed differential binding of CASPR2 IgG in patients with and without neuropathic pain, demonstrating the value of rodent neuronal assays in

strengthening clinical-serological correlations. Our investigation of autoantibody mechanisms revealed that CASPR2-IgG could internalise in peripheral neurons in an antigen-specific fashion. Furthermore, the internalisation and analysis of signalling pathways can be applied to the study of other autoantibody-mediated painful diseases in an antigen-agnostic manner. Elucidating the pathogenic mechanisms of autoantibodies will hopefully promote the discovery and use of personalised, targeted therapies for neuropathic pain.

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