ELECTROPHYSIOLOGICAL CHARACTERISATION OF NEURONAL COMPONENTS OF COLD SENSITIVITY

Ryan Patel

A thesis submitted to University College London for the degree of Doctor of Philosophy

Department of Neuroscience, Physiology and Pharmacology
Gower Street | London | WC1E 6BT

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Declaration

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

Abstract

Aberrant cold sensitivity is apparent in several neuropathies of peripheral and central origin, and is poorly treated by currently available drugs. In an attempt to understand the mechanisms of cold evoked hyperalgesia and analgesia, these studies examined the dual pro- and anti-nociceptive roles of TRPM8, a cold temperature gated channel, and the role of calcium channels within cold sensitive pathways through a combination of *in vivo* electrophysiology, behavioural measures and gene ablation. Blocking TRPM8 with novel antagonists revealed lamina V/VI neuronal responses to innocuous and noxious cold stimulation were conserved in naïve rats. However, under neuropathic conditions inhibition of TRPM8 decreased neuronal responses to innocuous and noxious cold stimuli. This corresponded with an attenuation of behavioural hypersensitivity to innocuous cooling. Remarkably, systemically activating TRPM8 with a novel agonist resulted in identical neuronal and behavioural effects in neuropathic rats. Menthol is known to relieve various pain conditions as well as inducing hyperalgesia. Unlike in human subjects, menthol fails to induce central sensitisation in naïve rats, whereas in neuropathic rats topical menthol exerts some similar effects to the systemically dosed TRPM8 agonist. Gene ablation identifies a role of $\alpha_2\delta$-1, an auxiliary calcium channel subunit, in cold and mechanical sensory pathways, likely dependent on impaired trafficking of calcium channels. Furthermore, $\alpha_2\delta$-1 knockout mice exhibit a delay in the development of neuropathic like behaviours after injury. In neuropathic rats, systemic and spinal delivery of an activation state dependent Ca_v2 antagonist suppresses neuronal responses to mechanical stimuli but reveals no change in channel function within cold sensitive pathways. These findings expand the understanding of the neural basis of cold sensitivity and demonstrate TRPM8 is not essential to all forms of cold transduction in naïve rats, and that both inhibiting and activating TRPM8 have similar selective modality related inhibitory effects on cold transduction in neuropathic rats.

Publications

Some of the work presented in this thesis has been previously published:

Patel R, Rutten K, Valdor M, Schiene K, Wigge S, Schunk S, Damann N, Christoph T, Dickenson AH. Electrophysiological Characterisation of Activation State Dependent Ca_v2 Channel Antagonist TROX-1 in Spinal Nerve Injured Rats. *Neuroscience (2015) June; 297: 47-57*

Patel R, Gonçalves L, Newman R, Jiang FL, Goldby A, Reeve J, Hendrick A, Teall M, Hannah D, Almond S, Brice N, Dickenson AH. The Anti-Hyperalgesic Effects of a Novel TRPM8 Agonist in Neuropathic Rats: A Comparison with Topical Menthol. *Pain (2014) Oct; 155(10): 2097-2107*

Patel R, Gonçalves L, Newman R, Jiang FL, Goldby A, Reeve J, Hendrick A, Teall M, Hannah D, Almond S, Brice N, Dickenson AH. Novel TRPM8 antagonist attenuates cold hypersensitivity following peripheral nerve injury in rats. *J Pharm Exp Ther (2014) Apr; 349(1):47-55*

Patel R, Bauer CS, Nieto-Rostro M, Margas W, Ferron L, Chaggar K, Crews K, Ramirez-Rozo J, Bennett DLH, Schwartz A, Dickenson A, Dolphin AC. α2δ-1 gene deletion affects somatosensory neuron function and delays mechanical hypersensitivity in response to peripheral nerve damage. *J Neurosci (2013) 33(42):16412-16426*

Sikandar S, Patel R, Patel S, Sikander S, Bennett DL, Dickenson AH. Genes, molecules and patients—Emerging topics to guide clinical pain research. *Eur J Pharmacol. (2013) Sep 15;716(1-3):188-202*

Acknowledgments

This thesis is a lot like me; a bit short and straight to the point. In no particular order the following people deserve recognition.

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Before I started here, PhD students I knew repeatedly questioned whether it was worth it. Stubborn as always, I didn't listen. Maybe I was just lucky but it was all fine in the end. I rarely felt that work was a chore (apart from maybe the thesis writing at times) and luckily in 4 years nothing went majorly wrong. I have never felt more in my element than when I'm charging up and down the lab churning out experiments. I don't know if this work measures up to expectations, all I can say is I tried to make the most of it. The last few years feel like they have flown by, I hope this signals the start of something great rather than the end of it.

Abbreviations

-/-	Homozygous Knockout
+/+	Homozygous Wildtype
5-HT	5-hydroxytryptamine
ADP	Adenosine diphosphate
ACC	Anterior cingulated cortex
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
APs	Action potentials
ATF-3	Activating transcription factor 3
ATP	Adenosine triphosphate
AUC	Area Under the Curve
a.u.	Arbitrary Units
bc	Brachium conjunctivum
BDNF	Brain derived neurotrophic factor
BTX-A	Botulinum toxin A
CaMKII	Calcium/calmodulin dependent protein kinase
cAMP	Cyclic adenosine monophosphate
cc	Corpus callosum
Ce	Central nucleus of the amygdala
CI	Cold injury
CIN	Chemotherapy induced neuropathy
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin Gene Related Peptide
Contra	Contralateral
COX	Cyclooxygenase
CPSP	Central post stroke pain
DAG	Diacylglycerol
DRG	Dorsal root ganglia
DTA	Diptheria toxin A receptor

ERK	Extracellular signal regulated kinase
fMRI	Functonal magnetic resonance imaging
FRAP	Fluoride resistant acid phosphatase
GABA	γ-Aminobutyric acid
GAD	Glutamate decarboxylase
GFP	Green fluorescent protein
GFR	Glial cell derived neurotrophic factor receptor
GPI	Glycosylphosphatidylinositol
HCN	Hyperpolarisation and cyclic nucleotide gated channel
Hip	Hippocampus
HPC	Heat-pinch-cold
IASP	International Association of the study of Pain
I	Input
IB4	Isolectin B4
Ic	Internal capsule
IL	Interleukin
In	Insula cortex
$iPLA_2$	Calcium independent phospholipase A2
Ipsi	Ipsilateral
KCC2	Cl/K transporter 2
LC	Locus coeruleus
LTMR	Low threshold mechanoreceptor
MAPK	Mitogen activated protein kinase
MDvc	Medial dorsal nucleus of thalamus
mEPSC	Miniature excitatory post synaptic current
mIPSC	Miniature inhibitory post synaptic current
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NF	Neurofilament
NGF	Nerve growth factor

NK	Neurokinin
NKCC1	Na/Cl/K transporter 1
NMDA	N-methyl-D-aspartate
NNT	Number needed to treat
NO	Nitric oxide
NS	Nociceptive specific
SNL	Spinal Nerve Ligation
PAG	Periaqueductal grey
PB	Parabrachial
PD	Post-discharge
PG	Prostaglandins
PI3K	Phosphoinositde 3 kinase
PIP_2	Phosphatidylinositol 4,5 bisphosphate
Pirt	Phosphoinositide interacting regulator of TRP
PGB	Pregabalin
PHN	Post herpetic neuralgia
PK	Protein kinase
PLC	Phospholipase C
PNP	Polyneuropathy
Po	Posterior group of thalamic nuclei
PSNL	Partial Sciatic Nerve Ligation
PWT	Paw Withdrawal Threshold
Py	Pyramidal tract
R	Receptor
RM	Repeated measures
RVM	Rostral ventromedial medulla
SEM	Standard error of the mean
SNRI	Serotonin noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant

TNF	Tumour necrosis factor
TRP	Transient receptor potential
Trk	Tyrosine kinase receptor
V	Ventricle
VGCC	Voltage gated calcium channel
VMH	Ventral medial nucleus of hypothalamus
VMpo	Posterior portion of the ventral medial nucleus of the thalamus
VL	Ventral lateral nucleus of the thalamus
VPL/M/I	Ventral posterior nuclei of the thalamus
VWA	von Willebrand factor A
WDR	Wide Dynamic Range
WU	Wind-up

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1. Introduction

1.1 The burden of chronic pain

The ability of an organism to detect damage or potentially harmful stimuli is essential to survival. Pain exists as a complex sensory experience inextricably linked with emotion; both these aspects are reflected in the current International Association for the study of Pain (IASP) definition of pain. The term 'nociception' on the other hand refers to encoding and transduction of noxious stimuli before entering consciousness; substantial summation and modulation within the central nervous system occurs before this can be perceived as a painful experience. Pain in humans is highly subjective and difficult to quantify as the relationship between the intensity of the stimulus and perception is not always linear. It is clear that cognitive, emotional, expectative, attentive and genetic factors influence pain perception (Tracey & Mantyh, 2007).

Following tissue damage, adaptive pain processes promote survival by protecting against further injury and aiding the healing process. The consequences of failure to do so are particularly evident in those with congenital insensitivity to pain syndromes. Although this is a rare phenotype, these individuals often have bite injuries to the lips and tongue, susceptibility to burn injuries and multiple fractures leading to long-term disability (Cox *et al.*, 2006). Pain that outlasts the resolution of an injury becomes maladaptive and can be considered a disease in itself. Neuropathic syndromes are often characterised by a complex combination of positive and negative sensory phenomena including allodynia (perceiving non-noxious stimuli as pain), hyperalgesia (increased pain to normally painful stimuli), but also paroxysmal or persistent paraesthesias (abnormal sensations e.g. numbness, tingling) and dysaesthesias (abnormal and unpleasant sensations e.g. shocking, paradoxical burning). It should be noted, persistent pain is not an inevitable outcome of a nerve lesion, for example the rates of chronic pain following surgery vary from 10-50% depending on the nature of the procedure and this may be debilitating in 2-10% of cases (Kehlet *et al.*, 2006). In the UK, prevalence of diabetic neuropathy is estimated to occur in 26% of patients (Davies *et al.*, 2006b).

IASP defines neuropathic pain as the result of a lesion or disease to primary or secondary somatosensory neurones. Conditions are often subdivided into 4 broad categories: nociceptive (resulting from a transient injury), inflammatory (including rheumatoid or osteoarthritis) and neuropathic (including nerve injury, phantom limb, post herpetic neuralgia, diabetic neuropathy, central post stroke pain, multiple sclerosis). The last category is referred to as 'generalised' or 'dysfunctional' as no obvious pathology is apparent such as fibromyalgia. It could be argued that this classification system is fast becoming redundant as cancer related bone

pain and osteoarthritis for example could be considered to involve inflammatory, neuropathic and disease specific components (Falk & Dickenson, 2014; Thakur *et al.*, 2014). A move to a mechanism based classification may be more advantageous in determining appropriate treatment for patients (Baron, 2006).

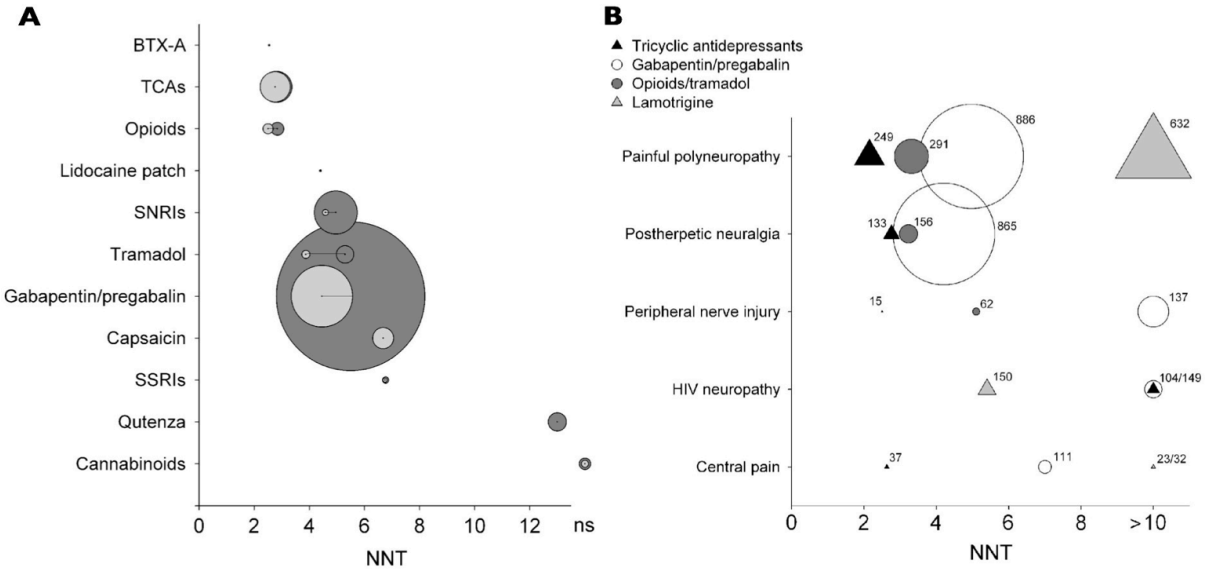


Figure 1.1 Meta-analysis of NNT values across different drug classes. (A) Combined NNT values for various drug classes across various neuropathies of differing aetiology. The figure illustrates the change from 2005 values in light grey to 2010 values in dark grey. **(B)** Combined NNT values for different drug classes against specific disease aetiologies. The symbol sizes indicate the relative number of patients who received active treatment drugs in trials for which dichotomous data were available. BTX-A - botulinum toxin type A, TCAs - tricyclic antidepressants, SNRIs - serotonin noradrenaline reuptake inhibitors, SSRIs - selective serotonin reuptake inhibitor. (Taken from Finnerup *et al.*, 2010).

Chronic pain occurs in 19% of the adult population in Europe, 59% of which have suffered for 2 to 15 years, and 40% report inadequate management of their symptoms (Breivik *et al.*, 2006). Recent estimates suggest pain with neuropathic characteristics has a prevalence of 7-10% (van Hecke *et al.*, 2014). Large numbers of neuropathic patients fail to achieve relief from currently available treatments. The number needed to treat (NNT) varies across drug classes (Fig. 1.1*A*) and between disease states within a drug class (Fig. 1.1*B*). At best only 1 in 2-3 patients achieve a >50% reduction of pain (Finnerup *et al.*, 2010). Quality of life is also severely affected as chronic pain conditions are commonly associated with co-morbidities such as depression, anxiety and sleep disorders (Nicholson & Verma, 2004). As well as having a devastating social impact, estimates of the economic cost of healthcare and lost working days in Europe *per annum* range from 3-10% of gross domestic product (€390-1300bn) (Breivik *et al.*, 2013). These studies highlight the urgent need for novel therapeutics and better utilisation of currently available treatments.

1.2 Clinical features of cold allodynia and hyperalgesia

Disturbances of cold sensitivity are evident in a range of neuropathic conditions of peripheral and central origin (Maier *et al.*, 2010). Across all neuropathies presentations of cold hypersensitivity is apparent in up to 20% of patients though the frequency of occurrence varies between conditions (Table 1.1). A decrease in the cold detection threshold is common to all neuropathies (cold hypoaesthesia), whereas increases in cold detection thresholds are rare. An increase in cold pain threshold, i.e. cold pain at warmer temperatures, is prevalent in conditions such as complex regional pain syndrome, peripheral nerve injury, trigeminal neuralgia and post-herpetic neuralgia. Many patients with these conditions also report paradoxical heat sensations though in polyneuropathy patients this appears to occur without any significant changes in cold thresholds.

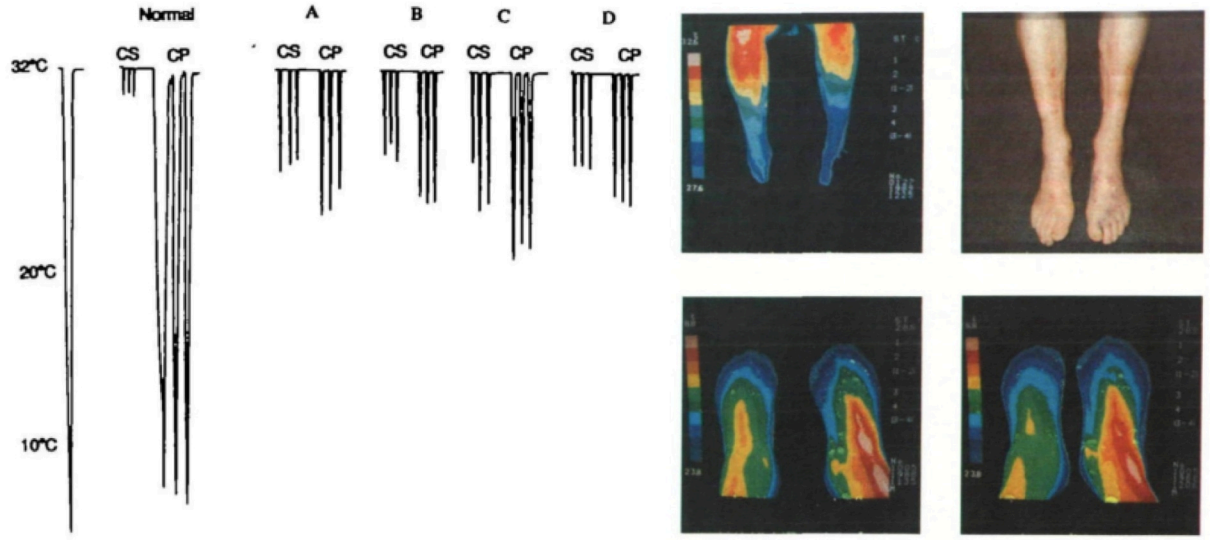


Figure 1.2 'Triple cold syndrome'. Quantitative thermal testing from different patients (A-D) reveals cold hypoaesthesia (CS - cold sensitivity) and cold hyperalgesia (CP - cold pain) compared to normal controls (left). Thermal imaging shows cooler skin in affected areas (top right) and no change in thermal output after 15 minutes warming at 45°C (bottom right). (Taken from Ochoa & Yarnitsky, 1994).

A combination of cold hypoaesthesia, cold hyperalgesia and hypothermic skin has been described as a 'triple cold syndrome' (Fig. 1.2). In a cohort of poly- and mono-neuropathy patients, approximately half exhibited cold skin and reduced blood flow on warming possibly symptomatic of a sympathetic denervation. Small myelinated axonopathy was apparent in three out of seven biopsies examined. Most described pain during cold exposure as having a paradoxical burning quality (Ochoa & Yarnitsky, 1994). In a separate study involving peripheral nerve injury patients, cold detection thresholds were comparable between the affected and un-affected side but also to normal control subjects. Cold pain thresholds in affected areas were on average 23-24°C compared

to normal thresholds of below 10°C (Morin & Bushnell, 1998; Jørum *et al.*, 2003). A significant sensitisation in un-affected areas was also apparent in these patients with cold pain thresholds around 17°C demonstrating a general sensitisation to cold temperatures (Jørum *et al.*, 2003). The presence of cold allodynia after nerve trauma does not appear to correlate with ongoing pain or mechanical allodynia (Kleggetveit & Jørum, 2010). In contrast postherniotomy patients with ongoing pain display greater cold hyopaesthesia compared to pain-free patients and experience cold pain at slightly warmer temperatures. This increase in cold pain threshold is also apparent on the un-operated side in these patients compared to those that are pain free (Aasvang *et al.*, 2010).

In addition to the conditions outlined in table 1.1, 30% of fibromyalgia syndrome patients report reduced tolerance to cold temperatures. Fibromyalgia patients exhibit abnormal temporal summation of pain including enhanced cold induced wind-up (Price *et al.*, 2002). The perceptual qualities of cold temperatures bears a marked resemblance to nerve injury patients in evoking paradoxical heat and dysaesthesia/paraesthesia (Berglund *et al.*, 2002). Paradoxical heat upon cooling is notable in post-stroke patients with thalamic lesions, supporting disruption in thalamocortical processing underlying these symptoms (Vestergaard *et al.*, 1995). Increased sensitivity to cold and warm temperatures is also apparent in adolescents with sickle cell anaemia. Although it is unclear whether this is due to peripheral or central sensitisation, the descriptors used to describe the pain include aching, burning, tingling, shooting and stabbing which are all commonly associated with neuropathic conditions (Brandow *et al.*, 2013).

Another critical area with great clinical need for novel therapeutic agents is for those undergoing chemotherapy. Platinum based agents such as oxaliplatin are widely used to treat solid tumours. Treatment is often hampered by the dose-limiting neurotoxic side effects. Acute symptoms include cold induced dysaesthesia and paraesthesia and are present in >90% of patients, though this typically resolves within a week (Argyriou *et al.*, 2013). Chronic oxaliplatin induced neuropathy persists between and after treatment for a large number of patients and is related to cumulative dose and duration of administration (Beijers *et al.*, 2014). The studies over the course of this thesis will aim to probe the neurobiology of normal and aberrant cold sensitivity with cooling compounds utilising behavioural and electrophysiological techniques.

Table 1.1 Summary of abnormal sensory functions in neuropathic patients. Data expressed as % of patients examined. (Taken from Maier *et al.*, 2010).

Gain of function	Poly-neuropathy *n*=343	Post-herpetic neuralgia *n*=72	Peripheral nerve injury *n*=154	Complex regional pain syndrome *n*=403	Trigeminal neuralgia *n*=92	Central pain *n*=51	Other *n*=121	All *n*=1236
Cold detection threshold	0.30	1.40	2.00	2.70	4.40	2.00	3.30	2.00
Warm detection threshold	0.90	1.40	0.70	2.50	1.10	3.90	1.70	1.60
Thermal sensory limen	0.6	4.2	1.3	2.7	3.3	3.9	4.1	2.30
Cold pain threshold	1.5	20.8	27.3	30.5	17.6	5.9	29.8	19.40
Heat pain threshold	7	20.8	24.7	40.1	25	9.8	23.1	23.80
Pressure pain threshold	5	38.5	51	66.3	9.2	16	36.3	36.40
Mechanical pain threshold	11.1	29.2	27	28.7	15.2	21.6	22.3	21.70
Mechanical pain sensitivity	8.5	36.1	29.6	46.6	25.8	23.5	30.8	29.20
Wind-up ratio	6.9	17.9	17.4	13.1	15.7	16.3	12.6	12.60
Mechanical detection threshold	0.3	5.6	9.2	9.5	-	-	3.3	5.00
Vibration detection threshold	-	2.6	2.6	1.5	4.4	-	4.2	1.90
Paradoxical heat sensation	37.3	15.3	14.9	9.4	-	26	11.6	18.40
Dynamic mechanical allodynia	12	48.6	17.5	24.1	14.6	17.7	17.5	19.70
Loss of function								
Cold detection threshold	40.2	62.5	48.7	32.5	38.1	49	41.3	40.40
Warm detection threshold	18.4	52.8	35.7	26.6	25	54.9	33.9	28.80
Thermal sensory limen	36.7	55.6	42.9	26.9	23.9	62.7	36.4	35.50
Cold pain threshold	-	5.6	14.3	5.2	6.6	9.8	10.7	5.80
Heat pain threshold	5	18.1	16.2	7.7	6.5	17.7	12.4	9.40
Pressure pain threshold	13.2	6.2	5.4	3.3	5.8	14	6.2	7.40
Mechanical pain threshold	21.9	26.4	25.7	10	12	23.5	19	17.80
Mechanical pain sensitivity	5	19.5	21.1	6.2	10.1	29.4	15.8	10.70
Wind-up ratio	0.4	1.5	2.3	2.7	3.4	-	5.4	2.20
Mechanical detection threshold	39.8	62.6	57.5	35.2	16.3	49	41.3	40.60
Vibration detection threshold	45.9	40.3	42.2	35.4	14.1	43.1	35.8	38.70

1.3 Theories of acute pain

The mechanisms by which primary afferents signal painful stimuli, including cold, have been the subject of much debate and are briefly outlined here. Specificity theory describes dedicated pathways for the transmission of non-noxious and noxious stimuli (Fig. 1.3*A*). Proponents of this theory advocate the existence of modality specific 'labelled lines' with segregated pathways conserved through the pain neuraxis (Craig, 2003; Cavanaugh *et al.*, 2009; Sun *et al.*, 2009; Knowlton *et al.*, 2013). Intensity theory is dependent on a lack of differentiation between low and high intensity fibres and that all fibres monotonically discharge to increasing intensity (Fig. 1.3*B*). This theory is discredited by the discovery of specialised nociceptors. Most natural stimuli are likely to activate a range of primary afferents. Pattern theory proposes somatic afferents display a range of responsiveness and relationships to intensity (Fig. 1.3*C*). Rather than labelled lines being independent, the spatiotemporal integration of peripheral inputs shapes specific somatic sensations. Pattern theory could explain thermal paradoxes such as the thermal grill illusion, which arises from the summation of warm and cool stimuli evoking sensations of burning, pain and unpleasantness (Green, 2004; Defrin *et al.*, 2008). According to gate control theory small and large diameter afferents converge on 'transmission neurones' of an 'action system' (Fig. 1.3*D*). A pre-synaptic gate in the dorsal horn is controlled by the balance between activity in A-fibres and C-fibres and is further subjected to descending control (Melzack & Wall, 1965). Although some of the details have been modified since first proposed, convergence and inhibitory control are recurring themes and are considered in the sections below.

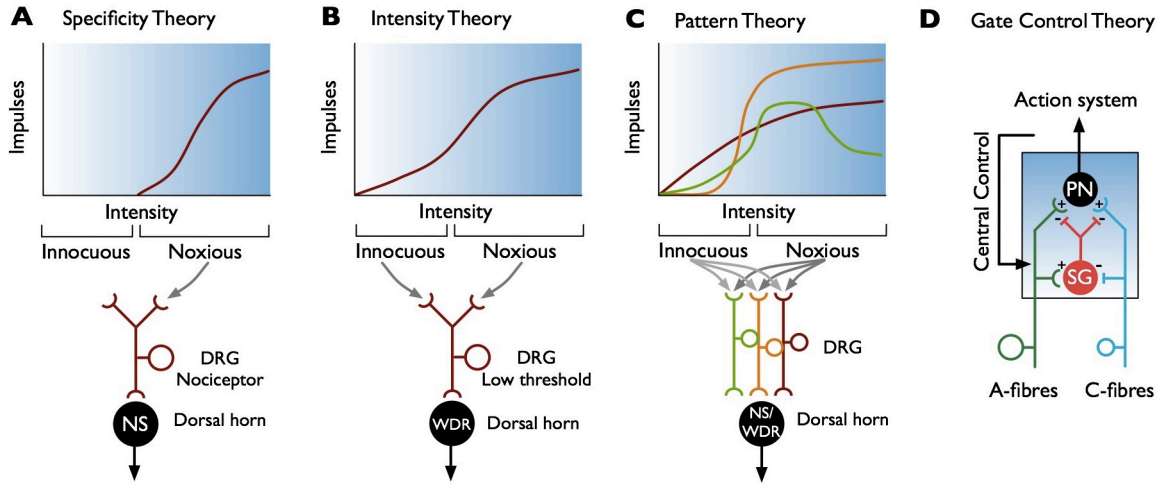


Figure 1.3 Theories of pain. Diagrams depicting theories of primary afferent signalling in pain. **(A)** Specificity theory, **(B)** intensity theory, **(C)** pattern theory and **(D)** gate control theory. NS – nociceptive specific, WDR – wide dynamic range, PN – projection neurone, SG – substantia gelatinosa neurone. (Modified from Perl, 2007).

1.4 Classification of peripheral sensory neurones

Peripheral sensory neurones innervating the skin, viscera and joints transduce and transmit non-noxious and noxious stimuli providing constant feedback of the external environment and alerting the organism to the existence or threat of injury. Peripheral neurones are pseudounipolar with the cell body residing within a dorsal root ganglion (DRG) projecting to the periphery and dorsal horn of the spinal cord. Sensory neurones have distinct termination patterns in the periphery, and the dorsal horn where synapses with second order neurones are formed (discussed in section 1.6), and are frequently sub-classified according to four main criteria: myelination/conduction velocity, modality, response characteristics and cytochemistry.

Aβ-fibres are heavily myelinated, have large calibre fibres (5-20 μm) and cell bodies (>40 μm), and conduct in the 30-70 m/s range. Aδ-fibres are lightly myelinated, have smaller diameter fibres (2-5 μm) and cell bodies (30-40 μm), and conduct between 12 and 30 m/s. C-fibres are unmyelinated, with thin axons (0.4-1.2 μm) and small cell bodies (<25 μm), and are slow conducting (<2 m/s) (Bourinet *et al.*, 2014). The responses of these afferents differ considerably. Aβ-fibres are responsive to low tactile stimuli and convey proprioceptive information, though a minor subset may also be nociceptive (Treede *et al.*, 1998). The two major classes of nociceptors are Aδ- and C-fibres responsible for 'first/sharp' pain and 'second/slow' pain respectively (Basbaum *et al.*, 2009). Aδ-fibres are sub-classified as type I or type II, the former responding to noxious mechanical and heat with slow increasing responses to prolonged stimulation and the latter exhibiting lower thresholds to heat, short latencies to intense stimuli followed by adapting responses and minimal mechanical sensitivity (Treede *et al.*, 1998). C-fibres are a heterogeneous population and most are polymodal. These can be subclassified as C-M (mechano), C-MH (mechano-heat), C-MC (mechano-cold) and C-MHC (mechano-heat-cold). Heat sensitive but mechanically insensitive C-fibres have also been described which additionally respond vigorously to chemical stimuli (e.g. histamine, mustard oil, capsaicin). These fibres develop mechanical sensitivity after injury and are often referred to as 'silent nociceptors' (Schmidt *et al.*, 1995). A proportion of Aδ- and C-fibres do not display characteristics of nociceptors and are classed as low threshold mechanoreceptors (LTMR). A- and C-LTMRs are diverse in terms of their biophysical properties and range from slow to rapidly adapting (reviewed by Abraira & Ginty, 2013). LTMRs in glabrous and hairy skin are associated with Pacinian corpuscles, Ruffini endings, Meissner corpuscles and Merkel's discs and transduce movement across the skin, stretch, hair deflection and vibration. C-LTMRs in particular are associated with pleasurable touch (Loken *et al.*, 2009).

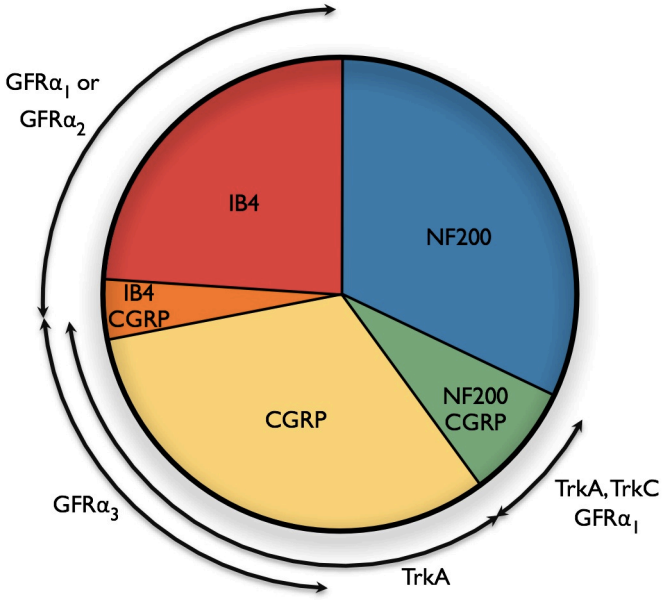


Figure 1.4 Summary of main neuronal populations residing within DRG. NF200+ neurones have myelinated axons. A subset express peptidergic transmitters and TRPV1 and are presumed to be nociceptive Aδ-fibres. C-fibres are broadly grouped according to the presence (CGRP+) or absence (IB4+) of peptides. CGRP+ neurones often co-express substance P, galanin, vasointestinal peptide, somatostatin, BDNF and TRPV1, IB4+ neurones co-express P2X3, TRPV1 and FRAP, NF200+ neurones express carbonic anhydrase and GM1 ganglioside. These populations can also be distinguished by expression of growth factor receptors (glial cell derived neurotrophic factor receptors – GFR, tyrosine receptor kinase - Trk). Note significant overlap between populations. (Modified from Priestley *et al.*, 2002).

Sensory neurones are further classified on the basis of neurochemistry (Fig. 1.4). All subtypes utilise glutamate to mediate fast synaptic transmission dependent on post-synaptic AMPA receptors; small diameter neurones can be further sub-divided as 'non-peptidergic' and 'peptidergic' neurones containing additional transmitters such as substance P, CGRP and somatostatin which mediate slow prolonged excitatory post synaptic potentials during high intensity stimulation (De Koninck & Henry, 1991; Snider & McMahon, 1998). The proportion of neurones expressing various markers varies between species, but in the rat 40% of all DRG neurones, 50% of C-fibres and 20% Aδ-fibres are classified as peptidergic. Most peptidergic neurones express tyrosine kinase receptor A (TrkA) and are dependent on nerve growth factor (NGF) for development and survival (Averill *et al.*, 1995). Non-peptidergic neurones contain fluoride resistant acid phosphatase (FRAP) and the lectin IB4 and constitute 30% of all DRG neurones (Silverman & Kruger, 1988b; a). Non-peptidergic neurones lack TrkA receptors but do express glial cell derived neurotrophic factor receptors ($GFR\alpha_{1\text{-}4}$) and receptor tyrosine kinase RET (Bennett *et al.*, 1998).

1.5 Properties of cold sensitive primary afferent fibres

Cold temperatures can elicit a range of sensations including pleasant and refreshing whereas further temperature decreases evoke aching, pricking and somewhat paradoxically, a sensation of burning. Defining the boundaries between innocuous and noxious cold is complex as cold pain thresholds can vary according to the rate of cooling; thresholds tend to be higher (i.e. sensation of cooling at warmer temperatures) in hairy skin compared to glabrous skin and with faster temperature ramps (Harrison & Davis, 1999). Spatial summation of cold pain is comparable between hairy and glabrous skin (Defrin *et al.*, 2011). Innocuous cold is commonly defined as temperatures between 30°C and 15°C, whereas noxious cold is generally perceived at temperatures below 15°C (Morin & Bushnell, 1998).

In primates, cold responsive fibres have been identified with receptive fields consisting of one or many cold spots and are thought to conduct in the Aδ- and C-fibre ranges. Low threshold 'cold' fibres often exhibit ongoing burst activity at skin temperature. Upon cooling from 35 to 20°C fibres exhibit high frequency discharge followed by slow adaptation (Dubner *et al.*, 1975; Kenshalo & Duclaux, 1977). High threshold cold fibres have also been described with thresholds of activation below 27°C and exhibit no ongoing activity and rapid adaptation (LaMotte & Thalhammer, 1982). The discharge rate in Aδ- and C-fibres responsive to noxious cold and mechanical stimuli increases monotonically with decreasing temperature down to -12°C and differ only in threshold for activation (higher in Aδ-fibres) (Simone & Kajander, 1996; 1997). Microneurography has been used to isolate C-fibres in human skin responding to innocuous and noxious cold stimulation. Response frequencies in high threshold polymodal C-fibres appear to be low in the 19-0°C temperature range and fire with a regular discharge rate rather than a burst pattern, whereas low threshold afferents respond vigorously to small decreases in temperature before adapting to steady state temperature (Campero *et al.*, 1996; Campero *et al.*, 2001; Campero *et al.*, 2009). The sensations evoked by different fibre types have largely been inferred through the use of pressure or ischaemic nerve blocks. Cool and pricking sensations are diminished following A-fibre block unmasking a sharp burning pain due to continuing activity in C-fibres (Wahren *et al.*, 1989; Yarnitsky & Ochoa, 1990; Davis, 1998).

1.5.1 Transduction of innocuous cold temperatures

1.5.1.1 Melastatin transient receptor potential channels

Transient receptor potential (TRP) channels are a superfamily of six transmembrane non-selective cation channels. Based on topology and sequence homology, TRP channels are separated into 7 subfamilies within two broad groups - group 1: TRPC, TRPV, TRPM, TRPA, TRPN. Group 2: TRPP and TRPML (Christensen & Corey, 2007) (Fig. 1.5). Often low homology exists between subfamilies and physiological roles of many channels remain undefined, but in general TRP channels act as multiple signal integrators and physiological sensors with diverse roles in thermosensation, mechanosensation, olfaction, hearing and vision. The melastatin TRP subfamily comprises 8 members in mammalian systems (Table. 1.2), and are characterised by a C-terminus TRP box and an absence of ankyrin repeats in the N-terminus (Venkatachalam & Montell, 2007).

Table 1.2 Properties, distribution and modulators of TRPM channels. (Modified from Venkatachalam & Montell, 2007).

Gene	Selectivity P_{Ca}:P_{Na}	Modulators	Highest expression
TRPM1	Non-selective	Zn^{2+} inhibited	Brain, melanosome, retina
TRPM2	~0.3	ADP-ribose, cADP-ribose, pyrimidine nucleotides, arachidonic acid, NAD, H_2O_2, Ca^{2+}	Brain, bone marrow, spleen, leukocytes
TRPM3	1.6	Osmotic cell swelling, store depletion (?), sphingolipids, heat >40°C	Kidney, brain, pituitary, DRG, retina
TRPM4	Monovalent cation selective	Ca^{2+}, voltage modulated, PIP_2	Prostate, colon, heart, kidney, testis
TRPM5	Monovalent cation selective	Ca^{2+}, voltage modulated, PIP_2, heat (15–35°C)	Intestine, liver, lung, taste cells
TRPM6	Divalent cation selective (Mg^{2+} and Ca^{2+})	Mg^{2+} inhibited	Kidney, small intestines
TRPM7	Divalent cation selective (Mg^{2+} and Ca^{2+})	Mg^{2+} inhibited, ATP, protons, phosphorylation, PIP_2	Kidney, heart, pituitary, bone, adipose
TRPM8	3.3	Cool (23–28°C), menthol, icilin, pH modulated, PIP_2	DRG, trigeminal ganglia, prostate, liver, brain

1.5.1.2 Structure and regulation of TRPM8

Two independent studies identified TRPM8 as a receptor for menthol, icilin and cold with a threshold below 25°C (McKemy *et al.*, 2002; Peier *et al.*, 2002). TRPM8 forms functional channels as homotetramers, as of yet hetero-oligomerisation has not been demonstrated (Stewart *et al.*, 2010). Temperature dependent gating is conferred by structures contained within the C-terminus as shown by switching temperature sensitivity using

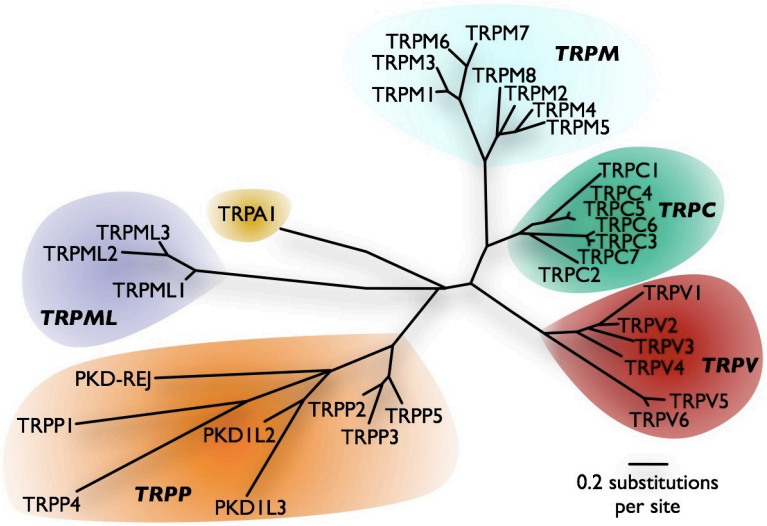


Figure 1.5 Phylogeny of mouse TRP channels. Scale bar represents 0.2 nucleotide substitutions per site. (Modified from Christensen & Corey, 2007).

chimeric recombination of TRPV1 and TRPM8 channels (Brauchi *et al.*, 2006). Two models have been proposed to explain thermodynamics of channels gating: a two-state and an allosteric model. Both support that cooling evoked opening of TRPM8 channels results in a leftward shift in the voltage dependence of activation (Brauchi *et al.*, 2004; Voets *et al.*, 2004). The allosteric model predicts cooling increases channel open probability in combination with voltage dependency converging onto a single gating mechanism (Brauchi *et al.*, 2004). Voltage changes alone are insufficient to gate TRPM8. Several sites have been identified as critical in the binding of menthol and icilin but also in determining cation selectivity and voltage gating (Fig. 1.6). Post-translational N-glycosylation of TRPM8 facilitates assembly and trafficking of channels, though N-glycosylation is not essential for this process (Erler *et al.*, 2006). Deletion of the N934 N-glycosylation site shifts the voltage dependency and decreases responses to cold and menthol (Pertusa *et al.*, 2012). The phospholipid PIP_2 acts as a general modulator of thermal responses in primary afferent fibres. PLC dependent degradation of PIP_2 following Ca^{2+} influx results in TRPM8 desensitisation (Daniels *et al.*, 2009). Other lipid modulators include products of calcium independent PLA_2 such as polyunsaturated fatty acids (PUFA) and lysophospholipids (LPL) (Andersson *et al.*, 2007) (Fig. 1.7). The two transmembrane domain protein Pirt interacts with PIP_2 and enhances both TRVP1 and TRMP8 activation (Kim *et al.*, 2008; Tang *et al.*, 2013). In addition Pirt knockout mice have reduced sensitivity to cold temperatures (Tang *et al.*, 2013). Several putative phosphorylation sites have been identified in TRPM8 (Fig. 1.6). Forskolin activation of PKA mediates TRPM8 desensitisation to menthol and icilin activation (De Petrocellis *et al.*, 2007). PKC dependent dephosphorylation of TRPM8 occurs through activation of phosphatases and functionally down-regulates TRPM8 channels (Fig. 1.7) (Premkumar *et al.*, 2005). Acidic pH

also reduces TRPM8 currents induced by icilin and cold temperatures, but not menthol, suggesting TRPM8 and TRPV1 might be differentially modulated by low pH during inflammation and that significant differences exist in the mechanism gating of TRPM8 by menthol and icilin (Andersson *et al.*, 2004). Short TRPM8 isoforms exerting dominant negative effects have been identified in the prostate but not DRG (Bidaux *et al.*, 2012).

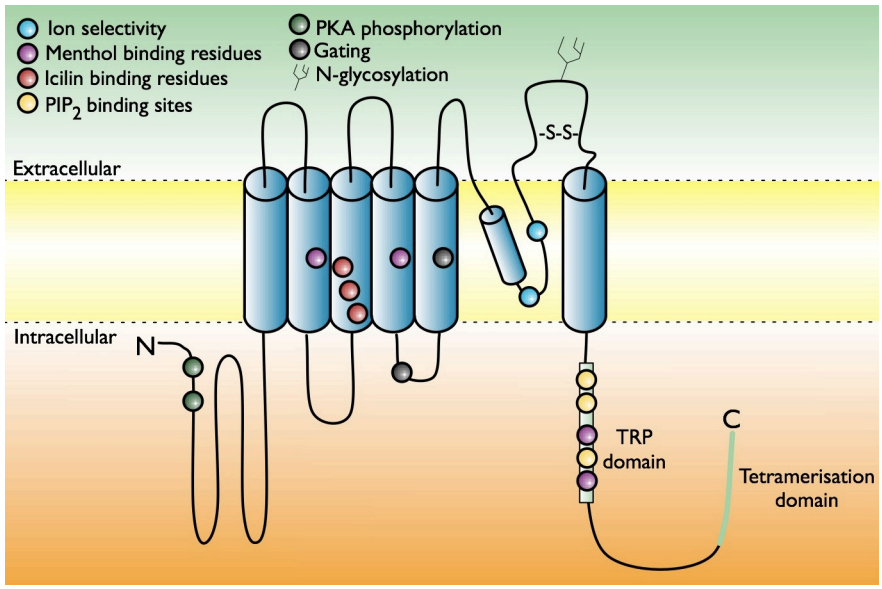


Figure 1.6 Representation of TRPM8 channel topology. Highlighted sites display approximate positions of residues implicated in ion selectivity (Q914, D920), menthol binding (Y745, R842, Y1005, L1009), icilin binding (N799, D802, G805), PIP_2 binding (K995, R998, R1008), PKA phosphorylation (S9, T17), voltage gating (R842, K856), N-glycosylation (N934), TRP domain (V993 to V1025) and tetramerisation domain (L1064 to K1104). (Modified from Latorre *et al.*, 2007 and Malkia *et al.*, 2011).

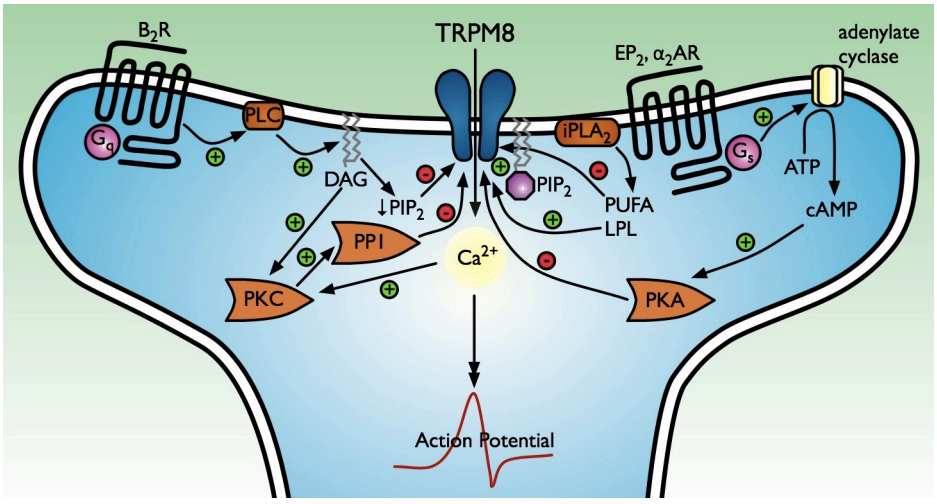


Figure 1.7 Modulation of TRPM8 by intracellular signalling pathways. Bradykinin mediates phospholipase C (PLC) and diacylglycerol (DAG) mediated depletion of PIP_2 and activation of PKC leading to channel down regulation. PIP_2 can also directly activate TRPM8 channels. Activation of prostaglandin (EP_2R) or adrenoreceptors (α_2R) increases cAMP activation of PKA, which mediates channel desensitisation. (Modified from Latorre *et al.*, 2011).

1.5.1.3 TRPM8 and cold sensitivity

Immunohistochemical analysis of mouse DRG neurones revealed expression of TRPM8 in a subset of small neurones (10-15%) with no co-expression with classical markers of nociceptors (Peier *et al.*, 2002). TRPM8 protein and mRNA has been detected in rat arterial myocytes implicating TRPM8 in the regulation of vasomotor responses to cooling (Johnson *et al.*, 2009). Distal cerebrospinal fluid-contacting neurones in the rat brain have also been demonstrated to be TRPM8 positive, though its function here is unresolved (Du *et al.*, 2009). *In vivo*, the peripheral and central projections of TRPM8 positive neurones have been identified with the insertion of green fluorescent protein (GFP) at the TRPM8 locus. In the periphery, TRPM8-GFP projections mark endings in the superficial layer of the epidermis including the mystacial pads but also the oral cavity and teeth (Takashima *et al.*, 2007; Dhaka *et al.*, 2008). TRPM8 positive neurones predominantly project to lamina I and IIo in the dorsal horn with no overlap with PKCγ+ interneurones in lamina IIi and III (Takashima *et al.*, 2007). Both these studies are largely consistent with previous observations that TRPM8 positive neurones are not typically CGRP, IB4 or NF150 positive (Peier *et al.*, 2002), though up to a third of TRPM8+ afferents may co-express CGRP (Takashima *et al.*, 2007). Between 19% and 38% of TRPM8+ trigeminal ganglion neurones and 12-24% of DRG neurones also co-express TRPV1 (Takashima *et al.*, 2007; Dhaka *et al.*, 2008). Innocuous and noxious cold sensitivity are retained following deafferentation of TRPV1+ fibres in adult mice (Cavanaugh *et al.*, 2009). In contrast TRPV1-DTA mice are insensitive to cold temperatures supporting TRPM8 neurones being derived from TRPV1 precursors during development (Mishra *et al.*, 2011).

The contribution of TRPM8 to cold transduction has been addressed through global knockouts and selective ablation of TRPM8+ afferents. TRPM8 -/- mice consistently display deficits in discriminating between warm and cool temperatures (Bautista *et al.*, 2007; Colburn *et al.*, 2007; Dhaka *et al.*, 2007). Noxious cold transduction appears partly dependent on TRPM8. 'Wet dog shakes' following intraperitoneal icilin are greatly reduced in TRPM8 -/- mice, presumed to represent a noxious stimulus (Bautista *et al.*, 2007; Dhaka *et al.*, 2007). An increased withdrawal latency to noxious cold temperatures in TRPM8 -/- mice has only been observed in one study (Dhaka *et al.*, 2007). In 2-plate preference tests, TRPM8 -/- mice continue to avoid noxious cold temperatures though with reduced efficiency (Bautista *et al.*, 2007). Although some cold sensitivity is retained following ablation of TRPM8 afferents, the severely impaired ability to detect innocuous and noxious cooling confirms TRPM8+ afferents as a significant and discrete population of cool fibres and cold nociceptors (Knowlton *et al.*, 2013).

1.5.2 Transduction of noxious cold temperatures

TRPA1 was identified as a TRP like channel expressed in peptidergic nociceptors expressing TRPV1 but no co-expression with TRPM8 (Story et al, 2003). TRPA1 acts as an integrator of multiple chemical irritants including cinnamaldehyde and bradykinin (Bandell *et al.*, 2004), mustard oil (Jordt *et al.*, 2004; Bautista *et al.*, 2006), formalin (McNamara *et al.*, 2007) and products of oxidative stress (Andersson *et al.*, 2008). Furthermore TRPA1 has also been implicated in mechanotransduction (Kwan *et al.*, 2006), and astrocyte function (Shigetomi *et al.*, 2013). Several groups have confirmed sensitivity of TRPA1 to temperatures below 17°C (Story *et al.*, 2003; Karashima *et al.*, 2009; Kremeyer *et al.*, 2010), but these findings have been equally disputed with others being unable to reproduce the data (Jordt *et al.*, 2004; Nagata *et al.*, 2005; Bautista *et al.*, 2006). TRPA1 activation by intracellular calcium could be an indirect mechanism of gating during cooling (Zurborg *et al.*, 2007). Knockout studies have failed to provide any further clarification. One line displayed no behavioural deficits to cold temperatures (Bautista *et al.*, 2006), whereas a second study only reported sex dependent differences (Kwan *et al.*, 2006). Genetic ablation of both TRPM8 and TRPA1 suggests the former and not the latter is required for aversion to noxious cold (Knowlton *et al.*, 2010). Significant species dependent differences in gating question whether TRPA1 is required for cold transduction in primates (Chen *et al.*, 2013). Individuals with a gain of function point mutation in TRPA1 exhibit no changes in baseline thermal and mechanical thresholds but often suffer episodes of intense pain triggered by fasting or cold temperatures (Kremeyer *et al.*, 2010). Alternatively TRPA1 may only have a pathological role in cold hyperalgesia. TRPA1 is up-regulated in TrkA expressing DRG neurones after an inflammatory insult or after nerve damage (Obata *et al.*, 2005) and is reversed by intrathecal TRPA1 anti-sense oligonucleotides (Obata *et al.*, 2005; Katsura *et al.*, 2006).

Cooling can cause numbness but the transmission of nociceptive inputs continues while innocuous stimuli are blocked. Populations of cold sensitive neurones can be distinguished on the basis of sodium channel expression. The tetrodotoxin resistant voltage gated sodium channel $Na_v1.8$ is expressed exclusively in a subset of small to medium diameter neurones, over 85% of which are classed as nociceptors and is localised to nerve endings (Djouhri *et al.*, 2003). $Na_v1.8$ knockout mice are almost completely unresponsive to noxious cold and have impaired responses to noxious mechanical stimuli (Zimmermann *et al.*, 2007; Abrahamsen *et al.*, 2008). In contrast, $Na_v1.7$ null mice retain noxious cold sensitivity but display deficits in detecting innocuous cool temperatures (Minett *et al.*, 2012; Minett *et al.*, 2014). Unlike other sodium channels the inactivation kinetics of $Na_v1.8$ are resistant to cold, identifying $Na_v1.8$ positive neurones as critical for the detection of noxious cold and noxious stimuli in cold environments (Zimmermann *et al.*, 2007).

1.5.3 Other molecular candidates for transduction of cold temperatures

A substantial number of rodent DRG neurones are excited by cooling but are insensitive to menthol (Babes *et al.*, 2004; Munns *et al.*, 2007). It is evident that other transducers of noxious cold exist, the identity of which remain elusive. A potential candidate is TRPC5, which is gated by cooling in the range of 37-25°C. TRPC5 is expressed in a subset of medium to small diameter neurones with partial overlap with TRPM8. Although TRPC5 knockout animals have no overall changes in thermal and mechanical thresholds; single unit recordings of peripheral afferents reveal that TRPM8 expressing afferents form a larger component of cold sensitivity (Zimmermann *et al.*, 2011).

Ion channel inhibition could be an alternative mechanism of cold transduction as inhibition of K^+ channels by cooling would reduce potassium efflux and increase neuronal excitability. Differences in K^+ currents identified between cold sensitive and cold insensitive trigeminal neurones suggest the presence of a 4-aminopyridine sensitive I_{KD} current in cold insensitive neurones acting as an 'excitatory brake' preventing excitation during cooling (Viana *et al.*, 2002). The 2-pore domain potassium channels TREK and TRAAK are expressed in sub-populations of TRPV1, TRPV2 and TRPM8 positive rat trigeminal neurones. Both channels have been implicated in modulating mechanical, heat and cold nociception (Maingret *et al.*, 1999; Maingret *et al.*, 2000; Alloui *et al.*, 2006). TREK1/TRAKK double knockout mice exhibit increased heat hyperalgesia, increased cold avoidance and cold hypersensitivity after nerve injury, suggesting TREK-1 and TRAAK dually modulate cold transmission (Noël *et al.*, 2009). More recently, TREK-2 has been identified in influencing mechanical, heat and cold detection thresholds without affecting noxious cold sensitivity (Pereira *et al.*, 2014). Molecular profiling of cold sensitive neurones also reveals the 2-pore potassium leak channel TASK-3 is highly enriched in TRPM8+ neurones and its ablation shifts the temperature threshold to warmer temperatures (Morenilla-Palao *et al.*, 2014). I_H currents are dependent on hyperpolarisation and cyclic nucleotide gated channels (HCN). HCN1 generated sub-threshold membrane oscillations are present in cold sensitive neurones and although dispensable for the transduction of cold stimuli, these oscillations are required to shape neuronal responses to cooling. This is particularly notable in corneal cold sensitive afferents where HCN antagonist ZD7288 alters bursting patterns during cooling (Orio *et al.*, 2012). In mice, pharmacological block or genetic ablation of HCN1 reduces sensitivity to noxious cold temperatures (Orio *et al.*, 2009). Membrane oscillation in cold sensitive afferents may also be dependent on the Na^+/K^+ ATPase (Schafer & Braun, 1990). The slow activating potassium M-current is generated by K_v7 channels. Pharmacological modulation with the antagonist XE991 and agonist retigabine identifies a role of the M-current in high threshold Aδ−fibres in determining membrane excitability

and inhibition facilitates dorsal horn neuronal responses to mechanical and noxious heat stimuli (Passmore *et al.*, 2012). In peripheral fibres, inhibition of M-currents sensitises high threshold TRPM8+ afferents to cold temperatures (Vetter *et al.*, 2013). The differential expression of ion channels in cold afferents could underlie the ability of a single transducer to confer multiple aspects of cold sensitivity.

1.6 Organisation of the dorsal horn

Primary afferent fibres enter the dorsal horn via dorsal root entry zones and terminate within specific regions known as Rexed laminae. Heavily myelinated Aβ-fibres conveying tactile stimuli project to the deeper laminae (III-VI). Myelinated Aδ-fibres innervate the most superficial laminae including projection neurones of lamina I, though some also terminate deeper. Most peptidergic C-fibres terminate in lamina I and the outer part of lamina II. In contrast, non-petidergic C-fibres target more ventral neurones within lamina II. Lamina I and V are most important in the processing of noxious stimuli and receive monosynaptic and polysynaptic inputs (Basbaum *et al.*, 2009). Laminae III/IV are composed of mechanoresponsive neurones receiving input from Aβ and Aδ fibres. Neurones in these regions poorly encode for intensity of stimulation and convey tactile and proprioceptive information. Retrograde labelling reveals lamina III/IV neurones project to the caudal ventrolateral medulla, parabrachial, thalamus and peraquiductal grey (Todd, 2002).

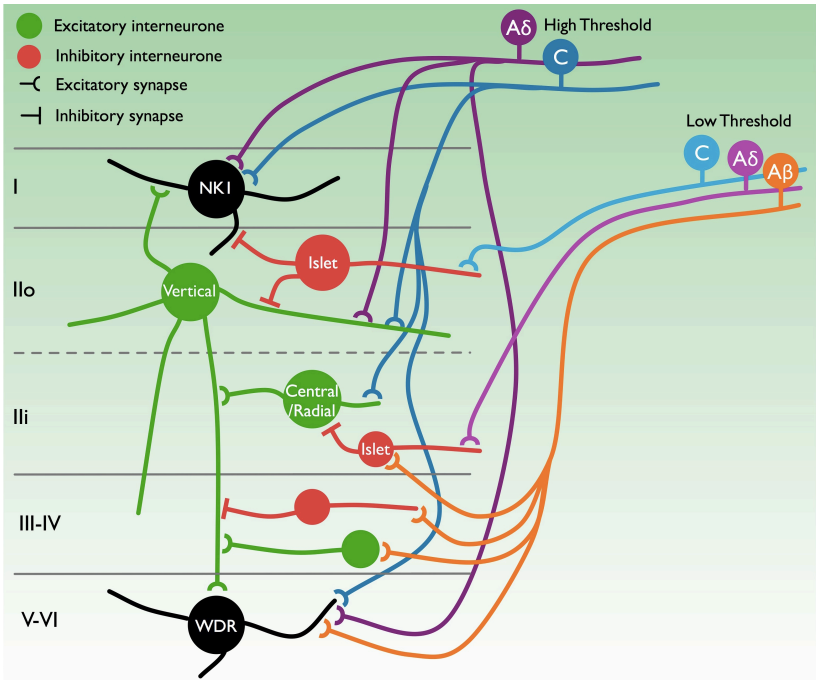


Figure 1.8 Organisation of dorsal horn circuitry. Projection neurones in the dorsal horn are subjected to substantial inhibitory and excitatory control. Nociceptive afferents can directly activate superficial NK1+ projection neurones and indirectly through activation of lamina II vertical and central interneurones. Low threshold afferents terminate deeper and can engage both excitatory and inhibitory interneurones. (Modified from Braz *et al.*, 2014).

1.6.1 Lamina I

Lamina I neurones are a major source of ascending output from the dorsal horn. The majority of these neurones (75-80%) respond linearly to increasing noxious stimuli and are known as nociceptive-specific (NS), whereas the remaining neurones are classed as wide dynamic range and are also moderately responsive to innocuous stimuli (Bester *et al.*, 2000; Craig *et al.*, 2001; Seagrove *et al.*, 2004). In general evoked responses appear to be smaller compared to lamina V neurones (Seagrove *et al.*, 2004). Electrophysiological characterisation identifies 3 main categories of neurones: nociceptive specific (NS), thermoreceptive specific (COOL or WARM) and polymodal nociceptive (HPC – heat, pinch, cold) (Han *et al.*, 1998). In rats, approximately one third of spinoparabrachial lamina I neurones are HPC with none responsive only to innocuous cooling (Bester *et al.*, 2000). In contrast, in cats 30-40% of spinothalamic lamina I neurones are estimated to be COOL; a similar proportion are also HPC and NS (Craig *et al.*, 2001).

1.6.2 Lamina II

Lamina II is predominantly comprised of interneurones and has no projections to higher centres. The complex interneuronal circuitry permits substantial modulation of incoming nociceptive activity (Fig. 1.8). Four morphologically distinct subtypes have been described: islet, central, radial and vertical (Grudt & Perl, 2002). Islet and central neurones arborise in the rostrocaudal plane and are contained within the lamina where the cell body lies. Ventrally directed dendrites of vertical neurones integrate inputs from low and high threshold afferents and local interneurone circuits. Immunohistochemical analysis reveals islet cells are largely GABAergic, occasionally co-express glycine and are inhibitory. In general radial and vertical cells are glutamatergic and considered excitatory. Both inhibitory and excitatory subsets of central interneurones have been described (Yasaka *et al.*, 2010).

High threshold Aδ- and C-fibres can directly activate NK1+ projection neurones in lamina I. Nociceptive afferents also activate lamina IIo vertical cells either directly or indirectly through central cells in lamina IIi, which in turn provides a feedforward excitatory drive to NK1+ neurones. Ventrally directed excitatory drive to deeper laminae, such as lamina V, runs in parallel (Braz *et al.*, 2014). Low threshold Aβ-fibres in contrast terminate deeper in the dorsal horn and engage excitatory and inhibitory circuits. Inhibitory interneurones are outnumbered by excitatory interneurones; the majority of inhibitory cells in lamina II are islet cells and are activated by low threshold C-fibres (Lu & Perl, 2003). A subset of excitatory interneurones in lamina II express PKCγ and are targeted by low threshold afferents (Polgár *et al.*, 1999; Neumann *et al.*, 2008). Mice lacking

PKCγ are resistant to developing neuropathy; an outcome accompanied by reduced post-synaptic up-regulation of NK1 receptors (Malmberg *et al.*, 1997). The glycine receptor inhibitor strychnine disinhibits superficial nociceptive neurones resulting in *de novo* responsiveness to light brushing, an effect which is blocked by PKCγ and NMDA antagonists. This study proposes a potential mechanism of dynamic mechanical allodynia by which dysfunction of tonically active gylcinergic interneurones allows low threshold mechanosensitive afferents to activate nociceptive circuits in the superficial laminae (Miraucourt *et al.*, 2007). Tonic inhibitory tone is also dependent on $GABA_A$ receptors; bicuculline facilitates superficial NS neuronal responses to punctate mechanical stimuli (Seagrove *et al.*, 2004). Behavioural signs of hypersensitivity are induced by pharmacological block of glycine or $GABA_A$ receptors (Yaksh, 1989).

1.6.3 Lamina V/VI

The monosynaptic and polysynaptic convergence of high and low threshold input into lamina V/VI neurones means that these neurones respond to both innocuous and noxious stimuli in a linear manner in relation to intensity. These neurones are also responsive to a range of modalities including mechanical, heat and chemical stimuli and are commonly referred to as wide dynamic range (WDR). Of particular importance to this thesis, most lamina V neurones exhibit intensity dependent responses in the innocuous to noxious cold temperature range (Khasabov *et al.*, 2001). Deep dorsal horn neurones are also distinguished by a short-term homosynaptic plasticity known as wind-up. The wind-up phenomenon was described as a frequency dependent progressive facilitation of neuronal responses to a constant stimulus and likely represents a temporal summation of inputs (Fig. 1.9) (Mendell & Wall, 1965). Whereas lamina I neurones exhibit minimal post-discharge and wind-up (Seagrove *et al.*, 2004), lamina V/VI neurones wind-up substantially to C-fibre strength stimuli optimally between frequencies of 0.5 to 2 Hz (Herrero *et al.*, 2000). The ability of deep dorsal horn WDR neurones to amplify pre-synaptic input is highly dependent on NMDA receptors (Dickenson & Sullivan, 1987). Wind-up is also reduced by pharmacological blockade of NK1 receptors and is absent in NK1 knockout mice (Budai & Larson, 1996; Suzuki *et al.*, 2003). At resting conditions or during low frequency firing, a Mg^{2+} ion 'plug' of NMDA receptors prevents opening of the pore. The co-release of substance P and CGRP in addition to glutamate contribute to the slow progressive depolarisation of second order neurones to allow release of the Mg^{2+} ion and increase permeability to Ca^{2+} (De Koninck & Henry, 1991).

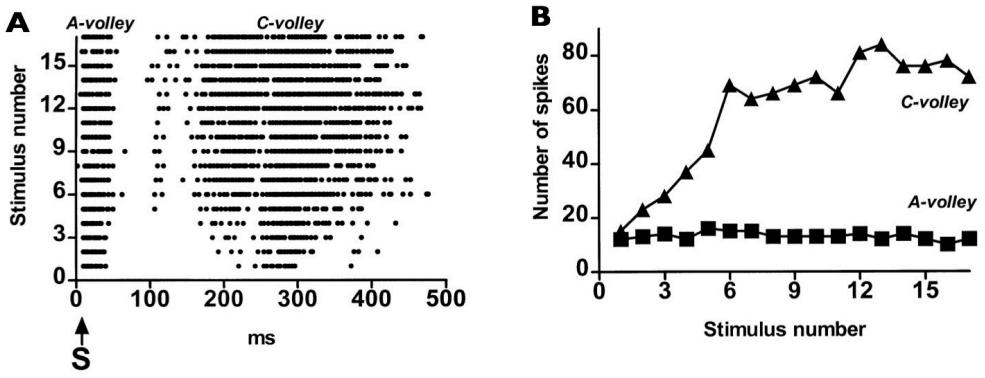


Figure 1.9 Wind-up of a deep dorsal horn neurone in an anaesthetised rat. The responses to 16 stimuli at 1 Hz are shown. **(A)** Each dot represents a single action potential with the position on the x-axis corresponding to the latency from stimulation (S). **(B)** Total number of action potentials from **(A)** separated according to latency and stimulus number. Note the increase in action potentials in the longer C-fibre latencies as opposed to shorter A-fibre latencies. (Taken from Herrero *et al*., 2000).

1.7 Ascending projection pathways

1.7.1 Spinothalamic tract

The majority of ascending activity from the dorsal horn emanates from lamina I and V projecting through the spinothalamic, spinoreticular, spinomesencephalic, spinoparabracial and spinotelencephalic tracts. Projection axons decussate at spinal level and convey pain related activity to higher centres associated with sensory/discriminative and affective components of pain (Fig. 1.10). Pathways engaging thalamic and cortical structures such as the somatosensory cortex, insula and anterior cingulated cortex are critical for the localisation and sensory discriminative aspects of pain, though the insula and anterior cingulated cortex are also important for the emotional and affective dimension of pain (Tracey & Mantyh, 2007). Spinothalamic tract neurones have been identified using anterograde and retrograde tracers. Although some species dependent differences exist, in primates half of all spinothalamic tract neurones originate from lamina I, a quarter from laminae IV-V and the remaining from laminae VII-VIII. In rats, a greater proportion of spinothalamic neurones originate from lamina V (Todd, 2002). Within the primate thalamus, spinothalamic tract neurones terminate within 6 distinct regions: the posterior portion of the ventral medial nucleus (VMpo), ventral posterior nuclei (VPL, VPM, VPI), ventral lateral nucleus (VL), central lateral nucleus, parafascicular nucleus and ventral caudal portion of the medial dorsal nucleus (MDvc). Lamina V spinothalamic tract neurones terminate within the VP nuclei and the VL. Neurones within these nuclei typically display WDR response profiles (Apkarian & Shi, 1994). In primates lamina I spinothalamic neurones heavily project to the VMpo, with weak input into the VP

nuclei and moderate projection to the MDvc. Anatomically the VMpo is arranged as a thalamic relay site with lamina I terminations topographically arranged in the rostrocaudal direction. Single unit recordings of cold sensitive neurones in the VMpo reveal ongoing activity that can be inhibited by warm temperatures and excited by cold. Nociceptive-specific neurones display graded responses to increasingly noxious stimuli (Craig *et al.*, 1994). VMpo neurones with similar properties have also been identified in humans undergoing stereotaxic thalamic procedures. Microstimulation of the VMpo evokes cooling sensations, which correlate with stimulus intensity. Upon application of a cooling ramp to the hand, neurones within this region have the ability to encode for intensity. Neuronal firing transiently increases during the cooling phase, decreases but stabilises during steady state cold temperatures and is inhibited during re-warming. The projection of the VMpo to the insula cortex suggests this pathway is an important spino-thalamo-cortical relay required for the perception of cold stimuli (Davis *et al.*, 1999).

1.7.2 Spinoparabrachial tract

NK1 receptors are expressed by approximately 80% of projection neurones in lamina I targeting areas such as the thalamus, periaqueductal grey, parabrachial and caudal ventrolateral medulla (Marshall *et al.*, 1996; Todd, 2002). Spinothalamic neurones are greatly outnumbered by those projecting to the parabrachial and caudal ventrolateral medulla (Todd, 2002; Spike *et al.*, 2003). Lamina I neurones negative or weakly expressing NK1 receptors are poorly characterised but express c-Fos after a noxious stimulus. A significant proportion express gephyrin, are under GABAergic inhibitory control and contribute to the spinoparabrachial pathway (Puskar *et al.*, 2001). Most parabrachial neurones selectively responsive to noxious cold stimulation reside within the central and external subnuclei of lateral parabrachial nucleus (Menendez *et al.*, 1996). These constituted a third of all neurones sampled, the remaining being only responsive to noxious heat and/or pinch. Thresholds for activation are typically between 25 and 10°C; neurones code linearly up to 0°C and more weakly between 0 and -10°C. Electrical stimulation of the receptive field confirms parabrachial neurones receive input consistent with Aδ- and C-fibre latencies. However, the early onset of neuronal activity upon cooling poorly correlates with the longer timescale for the perception of cold pain in psychophysical studies (Menendez *et al.*, 1996). The most prominent targets of parabrachial projections are the central nucleus of the amygdala and the hypothalamus but also the medulla, thalamus and periaqueductal grey (Moga *et al.*, 1990; Bernard *et al.*, 1993). Thus the parabrachial nucleus may be associated with affective and autonomic responses to noxious stimulation rather than sensory/discriminative components (Tracey & Mantyh, 2007).

1.8 Descending control of nociceptive transmission

The periaqueductal grey (PAG) is central to descending pain modulation and exerts dynamic control of the balance between descending inhibition and facilitation of nociceptive transmission in the dorsal horn (Fig. 1.10). The PAG is reciprocally connected with cortical structures, the amygdala, parabrachial, rostral ventromedial medulla (RVM) and pontine nuclei (Mantyh, 1983b; An *et al.*, 1998; Millan, 2002). Rostral projections from the PAG to thalamic and hypothalamic nuclei also implies an influence on ascending pathways (Mantyh, 1983a). Much heterogeneity exists within the subnuclei (dorsomedial, dorsolateral, lateral, and ventrolateral) on the basis of cytoarchitecture, neurochemistry and projection patterns (Millan, 2002). Seminal studies identified midbrain stimulation exerted powerful analgesic effects with selectivity of noxious inputs over non-noxious stimuli (Reynolds, 1969; Mayer *et al.*, 1971). Spinal neurones receiving predominantly A- or C-fibre input target distinct subnuclei with a larger proportion of C-fibre activated neurones targeting the ventrolateral nucleus (Parry *et al.*, 2008). Several studies support a differential control of A- and C- fibre evoked withdrawal reflexes (McMullan & Lumb, 2006b; a; Waters & Lumb, 2008). As with heat and mechanical stimuli, electrical stimulation of the ventrolateral PAG inhibits dorsal horn neuronal responses to noxious cold stimulation but not innocuous cold evoked responses (Leith *et al.*, 2010). Direct descending projections to the dorsal horn are sparse; the PAG largely exerts descending inhibition through the RVM but also through nuclei of the pontine tegmentum (Bajic *et al.*, 2001; Heinricher *et al.*, 2009). PAG neurones initiating descending inhibition are under tonic GABAergic control by inhibitory interneurones which mediate the effects of opioids in the midbrain (Depaulis *et al.*, 1987). Passive (e.g. quiescence, immobility) and active (e.g. confrontation, escape) coping strategies due to external stressors are dependent on ventrolateral and lateral/dorsolateral columns respectively. The integration of nociceptive activity and affective components from limbic structures within the PAG identifies pathways through which emotional state could modulate nociceptive activity in response to an external threat (Keay & Bandler, 2001; Heinricher *et al.*, 2009).

1.8.1 Serotonergic modulation of dorsal horn neuronal excitability

The rostral ventromedial medulla (RVM) includes the nucleus raphe magnus, nucleus raphe pallidus and gigantocellular reticular nucleus. Retrograde labelling reveals these nuclei receive substantial projections from the dorsal hypothalamic area, and dorsolateral and ventrolateral PAG but also the lateral preoptic area, central nucleus of the amygdala, parabrachial, and lateral and paraventricular hypothalamus (Hermann *et al.*, 1997). Electrical stimulation of the RVM supports an overall inhibitory influence on dorsal horn neuronal responses to

noxious stimulation (Basbaum *et al.*, 1976). However, it is clear parallel inhibitory and excitatory pathways originating from the RVM exist (Zhuo & Gebhart, 1992). Neurones within the RVM display distinct firing patterns in response to noxious somatic stimulation prior to a nocifensive reflex withdrawal. ON-cells increase firing prior to withdrawal and are considered to mediate descending facilitation, whereas tonically active OFF-cells abruptly cease firing, are necessary for the opioid analgesia and are considered to exert inhibitory influences. NEUTRAL cells continue to maintain firing upon stimulation and their role is largely unresolved (Fields *et al.*, 1983a; Fields *et al.*, 1983b; Heinricher *et al.*, 1989).

An extensive body of evidence supports the modulatory role of 5-HT in nociceptive pathways with the pro- and anti-nociceptive effects dependent on different receptor subtypes (Millan, 2002). Electrical stimulation of the RVM evokes the spinal release of 5-HT (Bourgoin *et al.*, 1980). Initially there was little compelling evidence that ON- and OFF-cells were serotonergic, however since then it is apparent that a significant proportion of serotonergic neurones in the raphe and reticular nuclei display characteristics of ON- and OFF-cells (Mason, 1997; Gao & Mason, 2000; Gau *et al.*, 2013). Most spinal serotonergic innervation is derived from the nucleus raphe magnus, though neurones within this region also co-localise with acetylcholine, GABA, gylcine, somatostatin, enkephalin and dynorphin all of which contribute to spinal modulation of nociceptive processing (Millan, 2002). Serotonergic afferents extensively innervate the spinal cord with 5-HT+ varicosities abundant throughout the dorsal horn laminae forming axo-somatic and dendritic contacts engaging projection neurones and interneuronal circuits. Axo-axo interactions with primary afferent fibre terminals are rare, though the pre-synaptic expression of 5-HT receptors suggests these are targeted through 'volume transmission' (Li *et al.*, 1997b). Intrathecal 5-HT increases behavioural thresholds to noxious stimuli identifying a net inhibitory role of 5-HT receptors in the spinal cord (Yaksh & Wilson, 1979). Lidocaine block of the RVM or depletion of endogenous spinal 5-HT however suppresses dorsal horn neuronal responses supportive of net facilitation in uninjured rats (Rahman *et al.*, 2006; Bee & Dickenson, 2007). The ablation of NK1+ projection neurones in the dorsal horn with a saporin-substance P conjugate suppresses deep dorsal horn neuronal excitability supporting the descending PAG-RVM pathway as the efferent arm of a spino-bulbo-spinal circuit acting as a positive feedback loop facilitating spinal neuronal responses during noxious stimulation. This in particular appears to be dependent on spinal 5-HT_3 receptors (Suzuki *et al.*, 2002b).

1.8.2 Noradrenergic modulation of dorsal horn neuronal excitability

Noradrenaline in descending axons within the dorsal horn is almost entirely derived from nuclei within the dorsolateral pontine tegmentum, in particular A6 (locus coeruleus) but also A5 and A7 (Kwiat & Basbaum, 1992). Micro-stimulation of these areas is known to be anti-nociceptive in naïve rats and can be reversed by intrathecal blockade of α_2-adrenoreceptors (Jones & Gebhart, 1986; Miller & Proudfit, 1990). Through an optogenetic approach, it is evident that locus coeruleus neurones are not a homogenous population and ventrally located neurones can exert pro-nociceptive effects compared to the anti-nociceptive role of dorsally located neurones (Hickey *et al.*, 2014). Given the reciprocal nature of projections from the locus coeruleus to multiple regions including the PAG and RVM, several putative pathways exist to form a dynamic descending modulatory feedback loop for the control of spinal neuronal excitability during prolonged noxious stimulation (Pertovaara, 2006).

Descending noradrenergic projections form minimal contact with the central terminals of primary afferent fibres (Hagihira *et al.*, 1990). Noradrenaline is predominantly located in non-synapse forming varicosities in the dorsal horn with few axo-somatic synapses. The inhibitory effects of noradrenaline may therefore be dependent on 'volume transmission' rather than through classical synapses (Rajaofetra *et al.*, 1992). Immunohistochemical analysis indicates α_{2A} receptors are located on primary afferent terminals and are co-expressed with substance P (Stone *et al.*, 1998), whereas α_{2C} is primarily associated with interneurones (Stone *et al.*, 1998; Olave & Maxwell, 2003). In general, activation of α_1 and α_2 receptors increases and decreases neuronal excitability respectively; both potentially contribute to the inhibitory effects of noradrenaline (Pertovaara, 2006). Several mechanisms could explain pre- and post-synaptic inhibitory effects including pontospinal projections directly terminating on spinal neurones inhibiting excitability by activating α_2 receptors (Sonohata *et al.*, 2004), activation of α_1 receptors on GABAergic and glycinergic inhibitory interneurones increasing inhibitory drive to projection neurones (Baba *et al.*, 2000), activation of α_{2A} receptors on primary afferent fibres preventing transmitter release (Kawasaki *et al.*, 2003), and activation of α_{2C} receptors on glutamatergic interneurones reducing excitatory drive to projection neurones (Olave & Maxwell, 2003).

Significant engagement of noradrenergic inhibitory pathways is apparent during extended periods of noxious nociceptive drive (Green *et al.*, 1998). In contrast there is little compelling evidence for the existence of a tonic noradrenergic control of spinal excitability. Knockouts of α_{2A}, $\alpha_{2B,}$ or α_{2C} receptors all have normal mechanical and heat thresholds and do not develop hypersensitivity any differently to wildtype mice suggesting no tonic

control of threshold levels of stimulation and that establishment of a neuropathic state is not dependent on these receptors (Malmberg *et al.*, 2001). Depletion of noradrenaline, through a saporin-dopamine β hydroxylase conjugate, or antagonism of spinal α-adrenoreceptors has no effect on acute nociception (Hylden *et al.*, 1991; Jasmin *et al.*, 2003). One study did demonstrate a small facilitation of dorsal horn neuronal responses to low threshold mechanical stimuli by spinal atipamezole (Rahman *et al.*, 2008). At best these data indicate minimal or no intrinsic inhibitory activity in naïve animals.

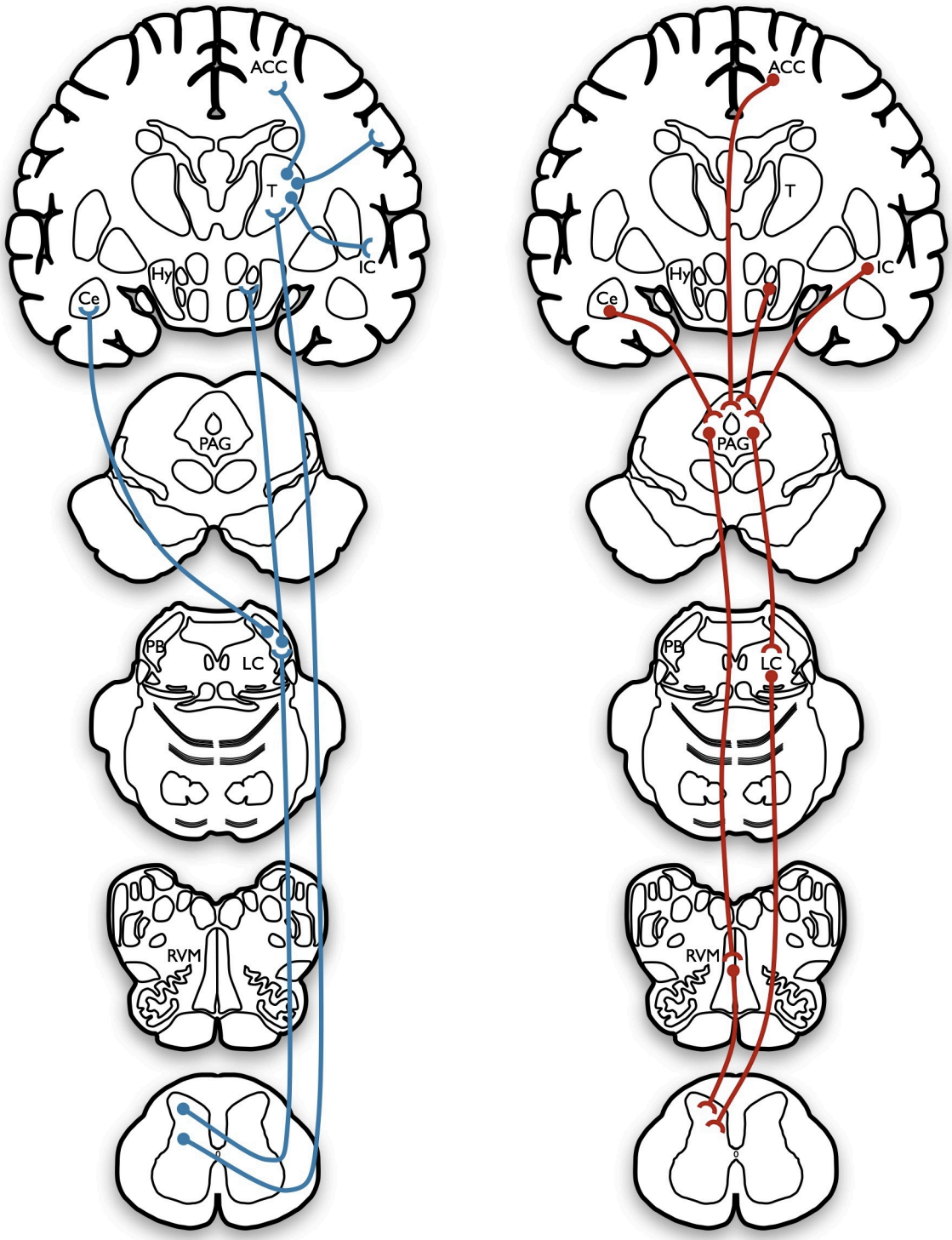


Fig 1.10 Main ascending and descending spinal pathways. Spinothalamic and spinoparabrachial pathways originating from the dorsal horn project to areas associated with discriminative/localisation and affective components of pain respectively (blue). Descending pathways originating from the amygdala, hypothalamus and periaqueductal grey activate descending controls through monoaminergic pontine and raphe nuclei (red). (ACC - anterior cingulated cortex; Ce - central nucleus of the amygdala; Hy - ventral medial nucleus of the hypothalamus; In - insula cortex; LC - locus coeruleus; PAG - periaqueductal grey; PB - parabrachial area; RVM - rostral ventromedial medulla; T - ventral posteriolateral nucleus and ventral posteriomedial nucleus of the thalamus). (Modified from Hunt & Mantyh, 2001 and Tracey & Mantyh, 2007).

1.9 Nerve trauma induced mechanisms of hyperalgesia

1.9.1 Peripheral and central inflammatory components

Following damage to a peripheral nerve, Wallerian degeneration occurs distal to the injury. Inflammation ensuing from an endoneurial infiltration of macrophages to clear damaged axons exposes remaining axons to inflammatory mediators leading to peripheral and central sensitisation. The presentation of neuropathic like behaviours is delayed in the *Wld*^{S} mouse and correlates with the delayed infiltration of macrophages (Sommer & Schafers, 1998). Infiltrating leukocytes and Schwann cells release a plethora of pro-inflammatory mediators including IL-1β, IL-6, nitric oxide (NO), prostaglandins (PG), NGF and TNFα (Fig. 1.11*A*). These factors may induce aberrant neuronal activity and sensitivity through several mechanisms including activation of their respective receptors, retrograde transport to DRG neurones, transcriptional changes and inducing sympathetic sprouting. The algogenic effects of NGF have been extensively examined. Cytokines such as IL-1β and TNFα promote the release of NGF from macrophages and Schwann cells. Upon binding to TrkA receptors, internalisation and retrograde transport to the soma of DRG neurones drives transcriptional changes including up-regulation of substance P, CGRP, P2X3, BDNF and $Na_v1.8$ leading to long term changes in nociceptor sensitivity (Fukuoka *et al.*, 2001; McMahon *et al.*, 2005; Basbaum *et al.*, 2009). NGF-TrkA complexes also activate signalling cascades including PLC, MAPK and PI3K leading to changes in expression and phosphorylation state of TRPV1 (Chuang *et al.*, 2001; Ji *et al.*, 2002).

Microglia are considered to be mediators of immunosurveillance in the central nervous system and share lineage with macrophages. A segmentally restricted hypertrophy of microglia is apparent following spinal nerve ligation, a process reliant on the activation of p38 MAPK pathways (Jin *et al.*, 2003). Inflammatory mediators are released in a self-propagating cycle and can influence nociceptive transmission by augmenting pre-synaptic transmitter release or increasing post-synaptic excitability (Fig. 1.11*B*). The initiating trigger for gliosis is unclear but may be dependent on excess release of glutamate, substance P and ATP during the ensuing afferent barrage following peripheral injury (McMahon *et al.*, 2005). Inhibiting microglial activity with minocycline would suggest that microgliosis is dispensable for the maintenance of a neuropathic state but shapes the development of neuropathic behaviours (Raghavendra *et al.*, 2003).

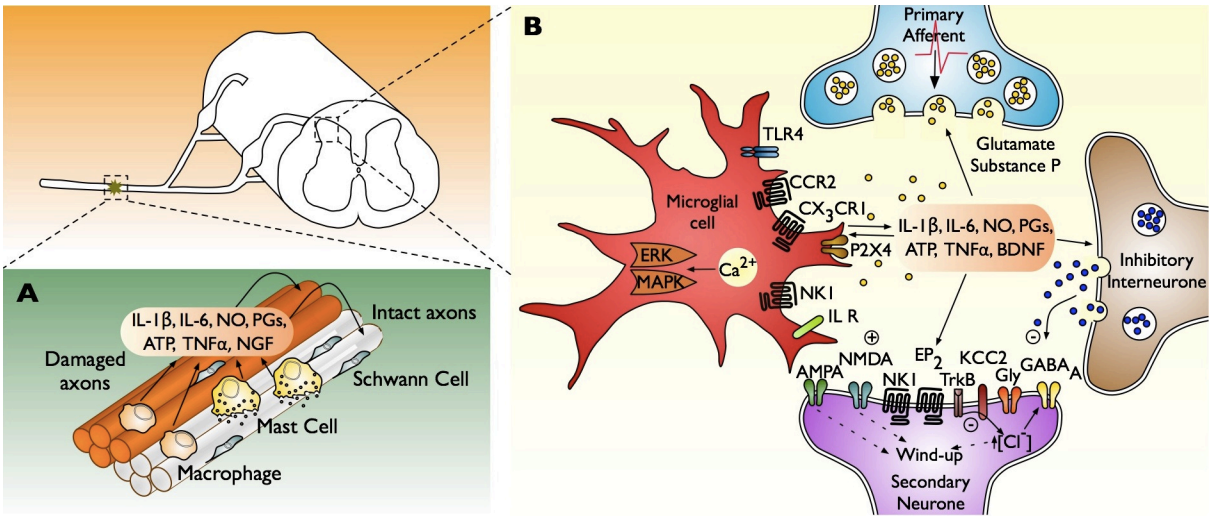


Figure 1.11 Peripheral and central inflammatory events occurring after nerve injury. (A) At peripheral sites of injury and along decaying axons, recruitment of macrophages, mast cells, and Schwann cells results in the release of pro-inflammatory mediators such as IL-β, TNFα, IL-6 and NGF. These factors induce activity in axons or can be retrogradely transported to the DRG where they induce transcriptional changes. **(B)** Within the dorsal horn activated microglia release numerous self-propagating cytokines, chemokines and other factors, which modulate nociceptive processing through pre-synaptic release of transmitters or post-synaptic excitability. (Modified from Marchand *et al.*, 2005 and Sikandar *et al.*, 2013a).

1.9.2 Peripheral afferent dysfunction

The term 'central sensitisation' was coined to describe an afferent activity dependent long-term change in spinal neuronal excitability increasing overall gain in somatosensory transduction. This manifests as an increase in receptive field size recruiting previously sub-threshold stimuli, enhanced temporal summation, reduction in response threshold and enhanced responsiveness to noxious stimuli (Woolf, 1983). This seminal study demonstrated sensitisation was not purely dependent on the periphery and identified potential substrates for allodynia, hyperalgesia and secondary hyperalgesia.

Spontaneous activity occurs following nerve damage presumably dependent on a dysregulation of ion channel function and/or expression, for example *de novo* expression of $Na_v1.3$ (Black *et al.*, 1999). With regards to a peripheral injury, how does aberrant peripheral activity evoke central changes? Injured afferents are an obvious candidate, however transection of the L5 spinal nerve appears to lead to ectopic discharge in A-fibres rather than C-fibres (Boucher *et al.*, 2000; Liu *et al.*, 2000). These occur several hours after injury and afferents typically display tonic or bursting firing patterns, which gradually decline with time. Irregular firing patterns dominate in the later stages of neuropathy (Sun *et al.*, 2005). This is somewhat surprising given that C-fibre strength stimuli are required to evoke central sensitisation. Two possibilities are that spontaneous activity develops in nociceptive Aβ-fibres or that a *de novo* synthesis of substance P/CGRP occurs resulting in a

'phenotypic switch' (Fig. 1.13*L*) (Neumann *et al.*, 1996; Treede *et al.*, 1998). Evidence for the latter is notable after inflammatory sensitisation but less so after spinal nerve ligation (Neumann *et al.*, 1996; Hughes *et al.*, 2007). Dorsal rhizotomy before or after L5 transection implies ectopic activity from the injured nerve is not essential for hyperalgesia to occur (Eschenfelder *et al.*, 2000; Li *et al.*, 2000). Following L5 ventral rhizotomy, Wallerian degeneration of myelinated fibres is sufficient to induce low frequency spontaneous firing in uninjured C-fibres and subsequent behavioural changes. In support of this, low frequency C-fibre strength electrical stimulation can induce behavioural signs of secondary hyperalgesia (Wu *et al.*, 2002).

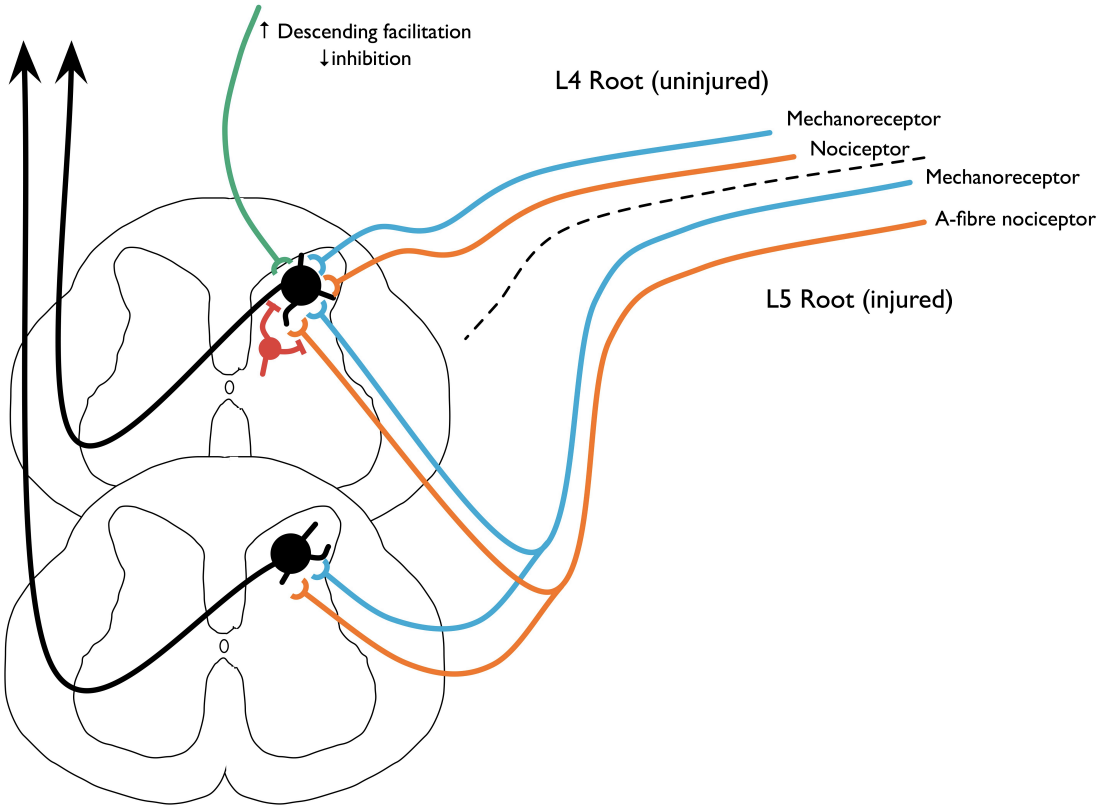


Figure 1.12 Induction of central sensitisation in SNL model. Up to a third of superficial dorsal horn neurones receive monosynaptic input from two to four segmental dorsal roots (Pinto *et al.*, 2008). Activity from injured myelinated L5 afferents induces spontaneous activity in intact L4 C-fibres. Enhanced activity leads to homosynaptic (synapses activated by conditioning stimulus) and heterosynaptic potentiation (recruiting adjacent synapses). Neuroimmune mechanisms in addition to concurrent increases in facilitation and decreases in inhibitory tone further augment neuronal excitability. (Modified from Campbell & Meyer, 2006)

Features of homosynaptic and heterosynaptic potentiation are evident clinically and in surrogate models of pain and are proposed to underlie Aβ- and Aδ- mechanisms of primary and secondary mechanical hyperalgesia (Campbell *et al.*, 1988; Magerl *et al.*, 1998). These processes depend on many mechanisms and in turn relates to long-term pre- and post-synaptic changes, alterations of interneuronal inhibitory control, changes in descending

modulation and neuroimmune mechanisms. Figure 1.12 proposes a model through which sensitisation of dorsal horn neurones occurs following spinal nerve ligation. Potential spinal mechanisms of hyperalgesia and allodynia are summarised in figure 1.13, some of the key mechanisms are discussed in the next sections.

1.9.3 Post-synaptic changes in dorsal horn excitability

Activity dependent sensitisation of spinal neurones is associated with early phosphorylative influences on ion channel function and longer term transcriptional processes. As observed with wind-up, ongoing afferent activity releases substance P and CGRP leading to slow depolarisations, release of Mg^{2+} ion block of NMDA receptors and Ca^{2+} influx (Latremoliere & Woolf, 2009). The pharmacological inhibition of NMDA receptors or genetic ablation of NR1 subunits is sufficient to prevent the establishment of central sensitisation (Woolf & Thompson, 1991; South *et al.*, 2003). Elevated intracellular Ca^{2+} activates several signalling pathways and is the key trigger for augmenting synaptic strength and initiating central sensitisation. Intracellular calcium can be derived from several sources including AMPA receptors, voltage gated calcium channels and release from intracellular stores coupled to metabotropic glutamate receptors (Latremoliere & Woolf, 2009).

Noxious stimulation can evoke PKC, PKA and CaMKII dependent phosphorylation of serine residues of AMPA receptor subunits leading to increased trafficking to synapses thereby increasing synaptic strength (Fang *et al.*, 2003; Galan *et al.*, 2004). Src phosphorylation of tyrosine residues in the NR2B subunit of NMDA receptors increases open times during channel activation and prevents endocytosis of channels (Yu *et al.*, 1997; Guo *et al.*, 2002). PKC can also increase the probability of channel activation by reducing the Mg^{2+} ion block (Chen & Huang, 1992). NO is synthesised by either inducible or neuronal NO synthase and can act as a diffusible messenger contributing to central sensitisation either directly or indirectly through disinhibitory mechanisms. This could involve activation of soluble guanylyl cyclase and cGMP cascades, ADP ribosylation or production of reactive oxygen species (Wu *et al.*, 1998; Ding & Weinberg, 2006; Schwartz *et al.*, 2008).

Both inflammation and nerve injury induces transcriptional changes through activation of PLC/PKC, PI3K and MAPK cascades. Inhibition of PI3K can prevent persistent pain states due to decreases in ERK, CaMKII and NR2B phosphorylation (Pezet *et al.*, 2008). ERK activation produces translational and post-translational effects on synaptic strength and neuronal excitability through phosphorylation of NR1 subunits of NMDA receptors, phosphorylation of potassium channels leading to a reduction of K^+ currents, and up-regulation of NK1R, c-Fos, TrkB and COX-2 (Hu *et al.*, 2006; Kohno *et al.*, 2008; Ji *et al.*, 2009). Behavioural signs of central sensitisation are also diminished in mice deficient in neuronal ERK (Karim *et al.*, 2006).

1.9.4 Alterations within spinal inhibitory circuits

A state of disinhibition could arise from a combination of reduced inhibitory transmitter synthesis, changes in release properties or a decrease in post-synaptic receptor expression and/or sensitivity (Fig. 1.13*D, F*). Opening of polysynaptic pathways from deeper laminae grants access of non-noxious mechanical afferents to pain pathways in the superficial laminae (Fig. 1.13*J* and Fig. 1.8). In the bone cancer model this may in part manifest as a change in the response properties of lamina I neurones. Under normal conditions the ratio of NS:WDR neurones is approximately 3:1. In contrast, under pathological conditions this shifts to a 1:1 relationship (Urch *et al.*, 2003). To date a similar change in the response profiles of superficial neurones has not been demonstrated in SNL rats.

There is conflicting evidence as to whether apoptosis of inhibitory interneurones occurs after peripheral nerve injury or whether this is a prerequisite for the development of neuropathy (Polgár *et al.*, 2003; Polgár *et al.*, 2004; Scholz *et al.*, 2005). After a partial nerve injury, *in vitro* electrophysiology reveals a reduction in the frequency of mIPSCs in lamina II neurones with no changes in the amplitude and is dependent on pre-synaptic GABA, but not glycine, release. This is accompanied by a reduction in the dorsal horn expression of the GABA synthesising enzyme GAD65 but not GAD67 (Moore *et al.*, 2002). In addition the density, dendritic morphology of GABAergic neurones and synaptic connections with excitatory neurones are all unaffected by peripheral nerve injury (Leitner *et al.*, 2013). Both these studies support a reduced excitatory drive to inhibitory interneurones underlying a diminution of inhibitory control (Moore *et al.*, 2002; Leitner *et al.*, 2013).

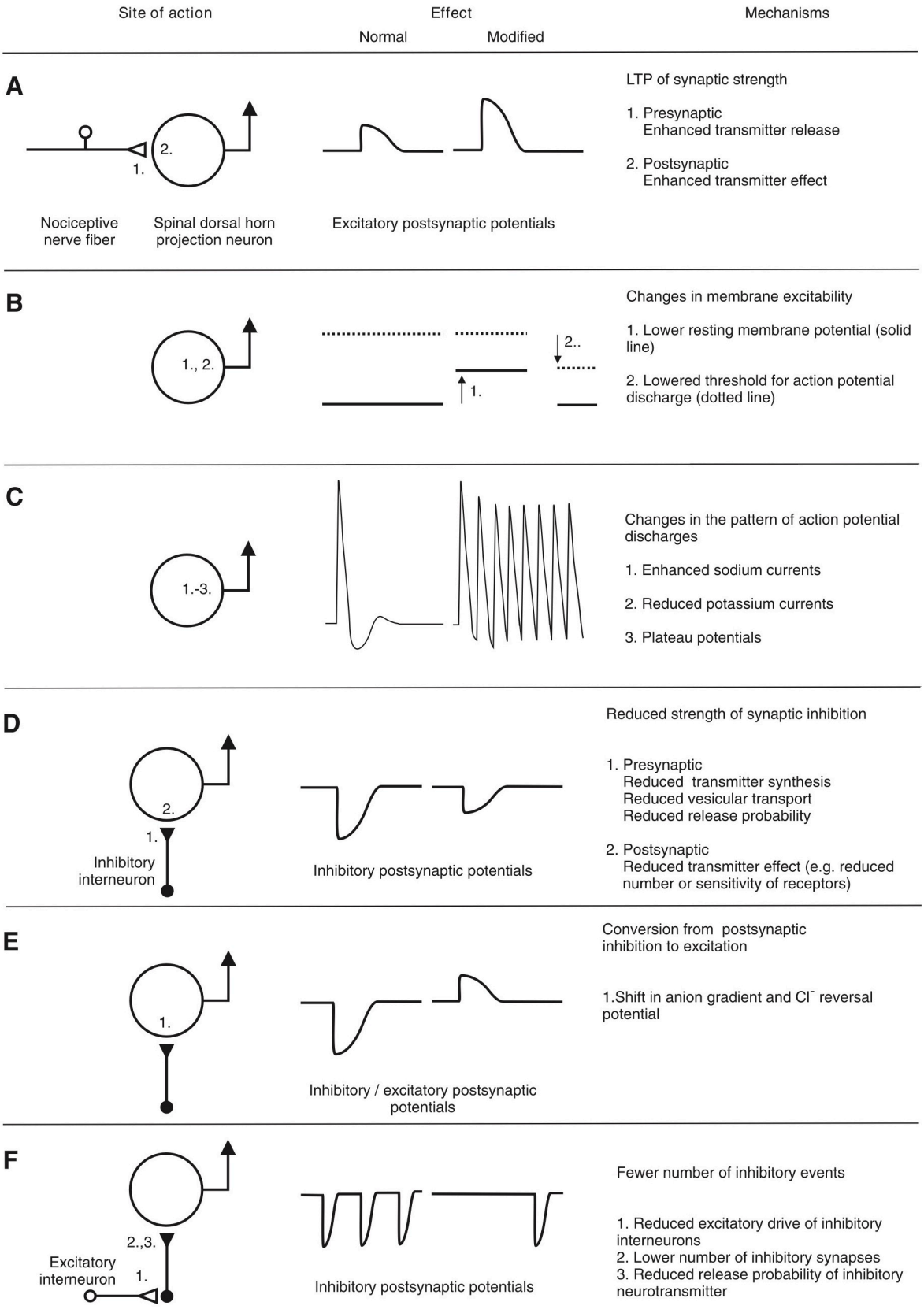
Another mechanism contributing to the reduction in spinal inhibition of lamina I neurones is the consequence of microglial derived BDNF on KCC2 transporters. Under normal conditions the Cl^-/K^+ transporter KCC2 and the $Na^+/Cl^-/K^+$ transporter NKCC1 maintain a net steady state Cl^- gradient so that activation of Cl^- permeable channels such as $GABA_A$ receptors results in hyperpolarisation of neurones. BDNF release results in downregulation of KCC2 shifting the anionic gradient diminishing GABAergic inhibition (Fig. 1.13*E*) (Coull *et al.*, 2005; Lu *et al.*, 2008). This can be replicated in naïve rats as pharmacological blockade of KCC2 converts inhibitory effects of GABA into excitatory effects on lamina I neurones and leads to mechanical and heat hypersensitivity (Coull *et al.*, 2003).

1.9.5 Plasticity in descending modulatory pathways

As discussed above, the RVM acts as a relay site for caudally projecting descending pathways. A balance between descending inhibition and facilitation supports a certain level of sensory gain without permitting excessive transmission of noxious stimuli. An increase in facilitation and/or a loss of inhibition could underlie sensitisation in pathological states (Fig. 1.13*H*). In inflammatory conditions, ON-cell activity dominates over OFF-cells and likely mediates the facilitatory effects of the RVM (Kincaid *et al.*, 2006). Growing evidence supports that supra-spinal loci actively maintain persistent pain and that mechanisms initiating and maintaining neuropathic pain are distinct. Reduced withdrawal thresholds to mechanical stimulation after SNL can be normalised by intra-RVM or intra-PAG injections of lidocaine whilst normal withdrawal thresholds are unaffected (Pertovaara *et al.*, 1996). Likewise, severance of descending projections through the dorsolateral funiculus also reverses behavioural hypersensitivity (Ossipov *et al.*, 2000). Plasticity within descending pathways is time dependent as inactivation of RVM neurones with lidocaine reverses behavioural hypersensitivity >6 days after injury but not at day 3. Lesioning of the dorsolateral funiculus does not delay the onset of behavioural hypersensitivity but prevents progression into chronicity (Burgess *et al.*, 2002). A similar effect is apparent following depletion of spinal 5-HT in SNL rats (Rahman *et al.*, 2006). Dermorphin-saporin ablation of μ-opioid receptor expressing neurones in the RVM, many of which are ON-cells, suppresses dorsal horn neuronal responses to noxious mechanical and heat stimuli and also prevents the emergence of neuropathic behaviours (Porreca *et al.*, 2001; Bee & Dickenson, 2008). ON- and OFF-cell responses are generally considered 'all or nothing' to noxious stimulation. In neuropathic conditions both groups obtain the capacity to encode intensity to low threshold mechanical stimuli in addition to prolonged firing or pause duration upon noxious stimulation (Carlson *et al.*, 2007). Correspondingly, in the dorsal horn, intra-RVM lidocaine additionally suppresses low threshold mechanical and heat stimuli in SNL rats compared to a selective inhibition of noxious stimuli evoked neuronal responses in sham rats (Bee & Dickenson, 2007). As well as sustaining evoked hypersensitivity, conditioned place preference tests reveal ongoing pain in SNL rats is dependent on descending influences and can be unmasked by intra-RVM lidocaine in 'pain free' injured animals (De Felice *et al.*, 2011).

The increased potency of α_2-adrenoreceptor agonists in neuropathy would support plasticity within descending noradrenergic pathways following an injury (Suzuki *et al.*, 2002a). This may be related to changes in α_2 receptor expression, alterations in engagement of G-proteins or BDNF induced sprouting of descending noradrenergic afferents (Stone *et al.*, 1999; Bantel *et al.*, 2005; Hayashida *et al.*, 2008). These mechanisms are hypothesised to be compensatory to counteract a loss of inhibitory drive. In the early stages following an injury

blocking spinal α_2 receptors exacerbates ipsilateral behavioural and neuronal hypersensitivity in nerve injured and osteoarthritic rats; no further sensitisation is induced by α_2 antagonists in the chronic stages (Rahman *et al.*, 2008; Burnham & Dickenson, 2013; Hughes *et al.*, 2013). Inhibition of spinal α_2 receptors can also unmask both hypersensitivity in animals with no symptoms following nerve ligation and contralateral hypersensitivity (Xu *et al.*, 1999; De Felice *et al.*, 2011; Hughes *et al.*, 2013). These studies suggest noradrenergic pathways act temporally and spatially to restrict spinal hypersensitivity and supports a loss of inhibitory drive. Others have postulated that noradrenergic neurones in the locus coeruleus are pro-nociceptive after injury (Brightwell & Taylor, 2009). The identification of pro-nociceptive neurones within this region provides a potential neural basis for these observations (Hickey *et al.*, 2014). Given the connectivity of the locus coeruleus with other mid-brain and brainstem nuclei, it is unclear as to whether the pro-nociceptive effects represent modulation of ascending or descending pathways.


Site of action
Effect
Normal
Modified
Mechanisms
A
2.
1.
LTP of synaptic strength
1. Presynaptic
Enhanced transmitter release
2. Postsynaptic
Enhanced transmitter effect
Nociceptive nerve fiber
Spinal dorsal horn projection neuron
Excitatory postsynaptic potentials
B
1., 2.
2..
1.
Changes in membrane excitability
1. Lower resting membrane potential (solid line)
2. Lowered threshold for action potential discharge (dotted line)
C
1.-3.
Changes in the pattern of action potential discharges
1. Enhanced sodium currents
2. Reduced potassium currents
3. Plateau potentials
D
2.
1.
Inhibitory interneuron
Inhibitory postsynaptic potentials
Reduced strength of synaptic inhibition
1. Presynaptic
Reduced transmitter synthesis
Reduced vesicular transport
Reduced release probability
2. Postsynaptic
Reduced transmitter effect (e.g. reduced number or sensitivity of receptors)
E
1.
Inhibitory / excitatory postsynaptic potentials
Conversion from postsynaptic inhibition to excitation
1.Shift in anion gradient and Cl- reversal potential
F
2.,3.
Excitatory interneuron
1.
Inhibitory postsynaptic potentials
Fewer number of inhibitory events
1. Reduced excitatory drive of inhibitory interneurons
2. Lower number of inhibitory synapses
3. Reduced release probability of inhibitory neurotransmitter

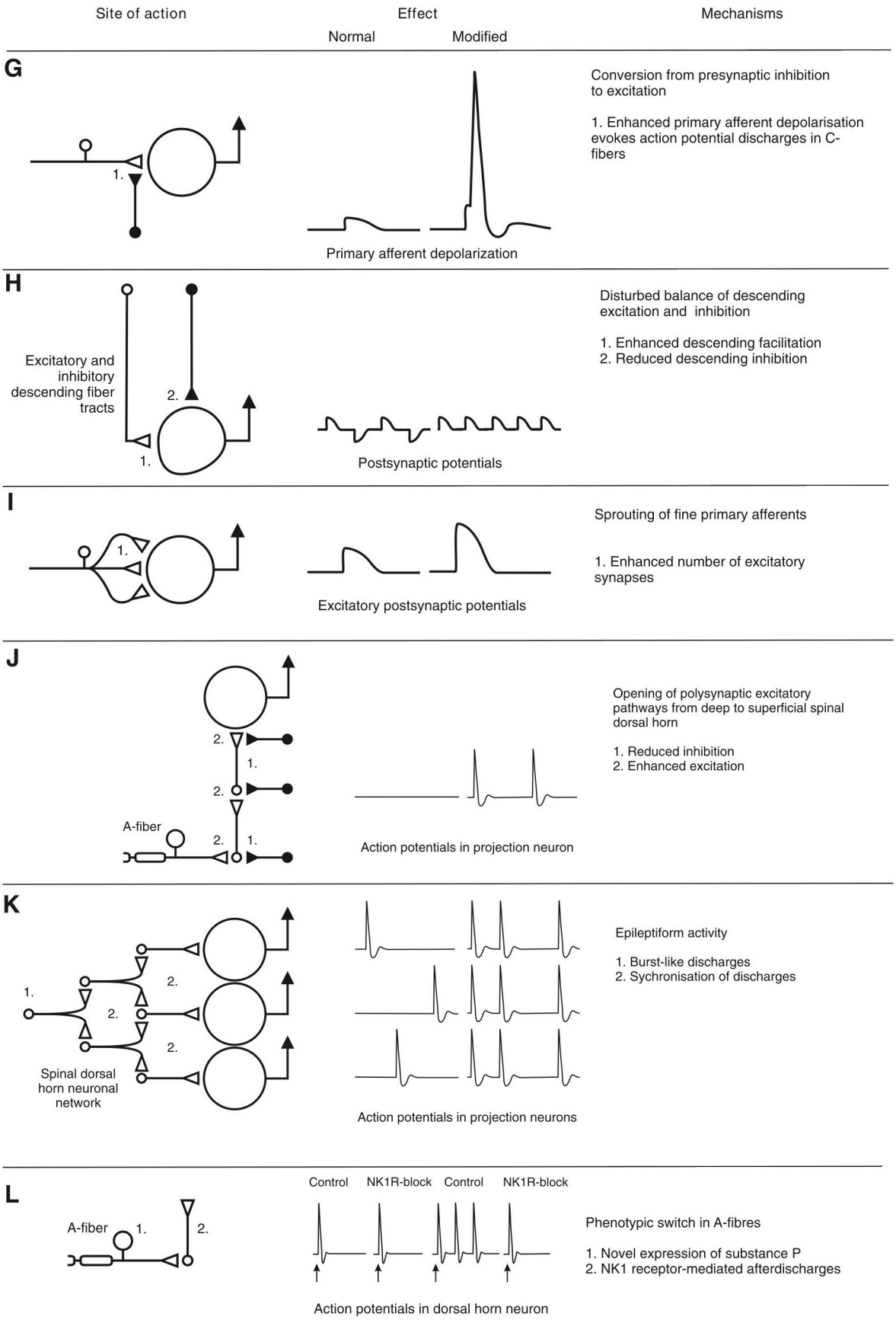


Figure 1.13 Spinal mechanisms of hyperalgesia. (Taken from Sandkuhler, 2009).

1.10 Aims of thesis

To date most studies of the effects of TRPM8 modulating compounds have focused on behavioural outcomes or peripheral neuronal recordings. These are limited in scope as behavioural tests are restricted to examining threshold responses whereas *in vivo* electrophysiology of spinal neuronal coding can extend these by allowing objective quantification of supra-threshold responses, which are more likely to relate to the high levels of pain described by patients. Furthermore, neurones in culture may also be phenotypically different to those *in vivo* and the physiological relevance is not always clear. Not all cold sensitive primary afferents are TRPM8+ and recordings from peripheral afferents *in vivo* fail to address the central integration and processing of all cold sensitive input and the relative contribution of TRPM8 to cold transduction. During the course of this thesis, the dual pro- and anti-nociceptive roles of TRPM8 will be examined in addition to the role of calcium channels in cold pathways in normal and neuropathic rats.

1. Initial studies will utilise *in vivo* electrophysiology to focus on the effect of TRPM8 blockade on spinal neuronal responses to innocuous and noxious cooling in uninjured conditions. These studies will be extended to examine the role of TRPM8 in cold hyperalgesia in the spinal nerve ligation model of neuropathy and ciguatoxin induced cold hyperalgesia.

2. Several studies in humans have suggested high concentration topical menthol induces hyperalgesia proposed to be analogous to the capsaicin induced hyperalgesia model. The second part of this study will explore the back-translation of this human surrogate model and the underlying neuronal mechanisms. Menthol is also analgesic in neuropathic patients; the effects of topical menthol and systemic administration of a novel TRPM8 agonist will be examined in spinal nerve ligated rats.

3. The $\alpha_2\delta$-1 subunit of calcium channels is the molecular target for pregabalin and gabapentin. However, the physiological role $\alpha_2\delta$-1 is less clear. This study will combine behavioural testing and *in vivo* electrophysiology in the $\alpha_2\delta$-1 knockout mouse to study the contribution of this subunit to cold, mechanical and heat sensitivity. The development of cold hypersensitivity after nerve ligation will also be examined.

4. Lastly, the effects of a state dependent Ca_v2 antagonist will be examined in SNL rats to determine whether a functional change of spinally expressed calcium channels in cold sensitive pathways occurs.

2. Material and Methods

2.1 *In vivo* electrophysiology recordings from dorsal horn neurones

2.1.1 Animals

Male Sprague Dawley rats (250-300g) and transgenic male $\alpha_2\delta$-1 knockout mice (10-12 weeks old) bred by the Biological Service Unit (University College London, UK) were used for behavioural and electrophysiological experiments. Knockout mice were originally generated through excision of exon 2 of the $\alpha_2\delta$-1 gene followed by electroporation of the construct into murine embryonic stem cells. Positive cell lines were injected into C57Bl/6 blastocysts and implanted into C57Bl/6x129J females (Fuller-Bicer *et al.*, 2009). Animals were group housed on a 12 h:12 h light-dark cycle; food and water were available *ad libitum*. Temperature (20-22°C) and humidity (65-75%) of holding and procedure rooms were closely regulated. All procedures described were approved by the UK Home Office and in accordance with the Animals (Scientific Procedures) Act 1986 and IASP ethics guidelines (Zimmermann, 1983).

2.1.2 Set-up (Rat)

In vivo electrophysiology was performed as previously described (Urch & Dickenson, 2003). Animals were anaesthetised in an induction box with 3.5% v/v isoflurane delivered in a 3:2 ratio of nitrous oxide and oxygen. Once areflexic, rats were transferred to a nose cone and the trachea exposed through blunt dissection of the surrounding muscle. A transverse incision was made into the trachea and a polyethylene cannula was inserted and secured with 3-0 silk threads. Isoflurane was delivered directly through the cannula for the remainder of the experiment. Rats were then placed in a stereotaxic frame with the head secured by ear bars. A rectal probe was inserted and body temperature was maintained at 37°C through feedback to a heat blanket. A laminectomy was performed under 2.5% v/v isoflurane. An incision was made into the skin along the vertebrae. The vertebral column was clamped following paraspinal incisions made approximately from T13 to L3. Muscle and vertebrae were removed to expose the L4 to L6 spinal segments. The dura was carefully removed with fine forceps. Two further paraspinal incisions were then made below the level of the laminectomy and a second clamp placed to straighten and stabilise the cord. Rats were maintained on 1.5% v/v isoflurane for the remainder of the experiment. Schedule 1 was performed at the end of the experiment through isoflurane overdose (5% v/v) and cervical dislocation.

2.1.3 Set-up (Mouse)

Mice were anesthetised with intraperitoneal 2.4 g/kg urethane (Sigma, UK) dissolved in 0.9% saline in 3 stages over an hour (approximately 4:1:1 of total dose at 20 minute intervals). Once areflexic, the trachea was exposed and a polyethylene cannula inserted. Mice were placed in a stereotaxic frame and the head secured with zygomatic bars. Body temperature was maintained at 37°C through a homeothermic blanket. An incision was made into the skin along the vertebrae. The vertebral column was clamped following paraspinal incisions made approximately from T12 to L5. Muscle and vertebrae were removed to expose the L3 to L5 spinal segments. Mineral oil was applied to the spinal cord to prevent dehydration. Schedule 1 was performed at the end of the experiment through cervical dislocation.

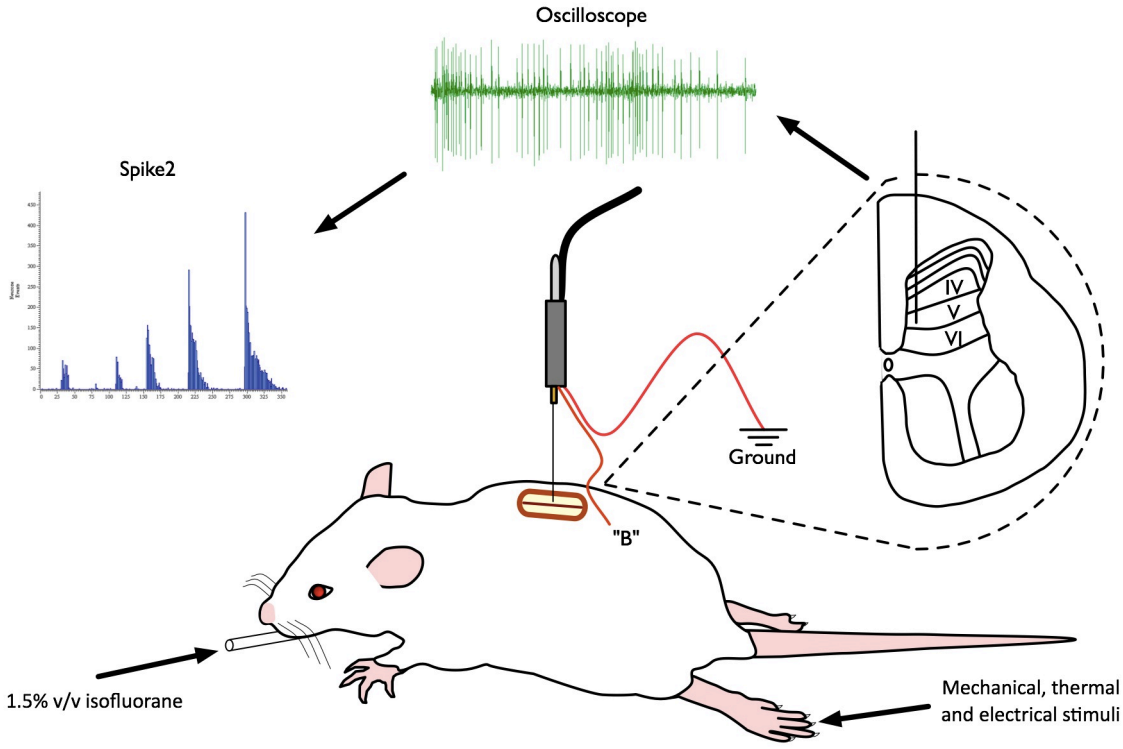


Figure 2.1 Overview of electrophysiology setup. Under anaesthesia, a laminectomy is performed to expose the lumbar spinal cord. An electrode is manually lowered through the dorsal horn; a second lead is attached to the skin of the rat. Action potentials generated by stimulating hind paw are visualised on an oscilloscope and recorded by Spike2.

2.1.4 Recording system

Recordings of single lamina V/VI neurones were obtained with an AC recording system (NeuroLog System, Digitimer, UK). A parylene coated tungsten microelectrode (125 µm diameter, 2 MΩ impedance, A-M systems Inc., USA), inserted into a headstage fixed to a 3-axis manipulator, was manually inserted into the spinal cord. The recording system was grounded via a lead attached to the stereotaxic frame. A second lead ("B") was

attached to the rat. The signal from the rat ("B") was subtracted from the signal from the electrode ("A") by the NeuroLog differential recording mode to reduce interference. Neurones were stimulated by tapping of the receptive field on the hind paw of the rat. Neuronal activity was amplified, filtered, and then displayed on an oscilloscope as well as being made audible via a speaker system. Quantification of neuronal activity was achieved with a CED 1401 interface coupled to Spike2 software (Cambridge Electronic Design, UK) (see Fig. 2.1 and 2.2). Once a single neurone had been isolated, based on a single amplitude series of action potentials, and a good signal to noise ratio (~4:1), a number of stimuli were then applied to the receptive field.

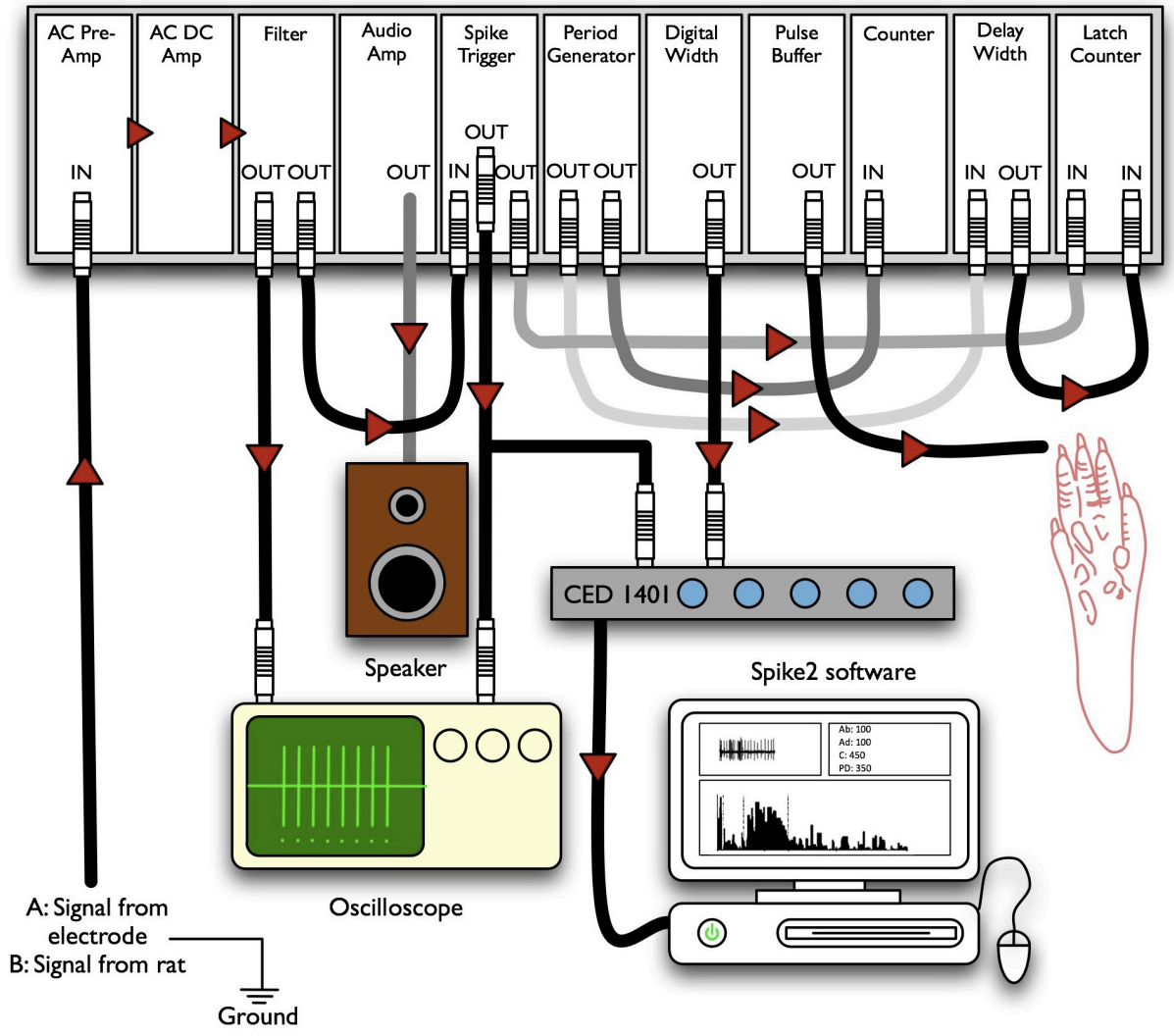


Figure 2.2 Overview of CED NeuroLog recording system. Input from the electrode (A), reference lead (B) and ground lead are fed into the head stage. The signal is amplified after interference from reference lead is subtracted, fed through low and high pass filters and outputted to a speaker and oscilloscope. The pulse buffer delivers electrical stimuli to the receptive field, the frequency and duration of which are controlled by the period generator, digital width, delay width and counter. The spike trigger determines the amplitude boundaries for counting events. The resulting evoked action potentials are captured by the CED 1401 and separated according to latency by Spike2 to construct a post stimulus time histogram. Modified from (Urch & Dickenson, 2003).

2.1.5 Quantifying stimulus evoked responses in rats

Deep dorsal horn neurones display graded firing to natural stimuli and are identified as WDR by confirming responses to dynamic brushing, noxious punctate mechanical and heat stimulation. Electrical stimulation of WDR neurones was delivered transcutaneously via needles inserted into the receptive field. A- and C-fibre thresholds were determined by delivering a single stimulus and observing the point at which action potentials were generated in the corresponding latencies. A train of 16 electrical stimuli (2 ms pulses, 0.5Hz) was applied at three times the threshold current for C-fibre activation. Responses evoked by Aβ- (0–20 ms), Aδ- (20–90 ms) and C-fibres (90–350 ms) were separated and quantified on the basis of latency. Neuronal responses occurring after the C-fibre latency band were classed as post-discharge (350-800 ms) (Fig. 2.4*A*). The input, a theoretical non-potentiated response, and the wind-up, the potentiated response, were calculated as Input = (action potentials evoked by first pulse) × total number of pulses (16), wind-up = (total action potentials after 16 train stimulus) − Input. The receptive field was also stimulated using a range of natural stimuli (dynamic brushing, von Frey filaments (Touch-Test, North Coast Medical, USA) – 2 g, 8 g, 15 g, 26 g and 60 g and heat - 35°C, 42°C, 45°C, 48°C) applied over a period of 10 s per stimulus and the evoked response quantified (Fig. 2.4*B*). The heat stimulus was applied with a constant water jet onto the centre of the receptive field. 100 μl acetone and ethyl chloride (Miller Medical Supplies, UK) were applied as an evaporative innocuous cooling and noxious cooling stimulus respectively (quantified over subsequent 10 s). 100 μl room temperature water was applied and evoked response subtracted to control for concomitant mechanical stimulation during application. Baseline values were calculated as an average of 3 consecutive stable readings (<10% variation). Receptive fields were determined prior to electrical stimulation using 8 g and 60 g von Frey filaments. An area was considered part of the receptive field if a response of >50 action potentials/10 s was obtained. A rest period of 30 s between applications was used to avoid sensitisation. Receptive field sizes are expressed as a percentage area of a standardised paw measured using ImageJ (NIH, Bethesda, MD) (Fig. 2.3).

Figure 2.3 Example of receptive field map. Receptive fields expressed as % area of total plantar surface.

2.1.6 Quantifying stimulus evoked responses in mice

Stimulus evoked responses in mice were quantified as described previously (see 2.15) with some modifications. Responses evoked by A- (0–50 ms) and C-fibres (50–250 ms) were separated and quantified on the basis of latency. Neuronal responses occurring after the C-fibre latency band were classed as post-discharge (250-800 ms). The receptive field was also stimulated using a range of natural stimuli (dynamic brushing, von Frey filaments (Touch-Test, North Coast Medical, USA) - 0.4 g, 1 g, 4 g, 8 g and 15 g and heat - 35°C, 42°C, 45°C and 48°C) applied over a period of 10 s per stimulus and the evoked response quantified. An acetone drop was applied as an innocuous cooling stimulus preceded by an equivalent volume of room temperature water. Data were captured and analysed by a Cambridge Electronic Design 1401 interface coupled to a Pentium computer with Spike2 software (Cambridge, UK) with post-stimulus time histogram and rate functions.

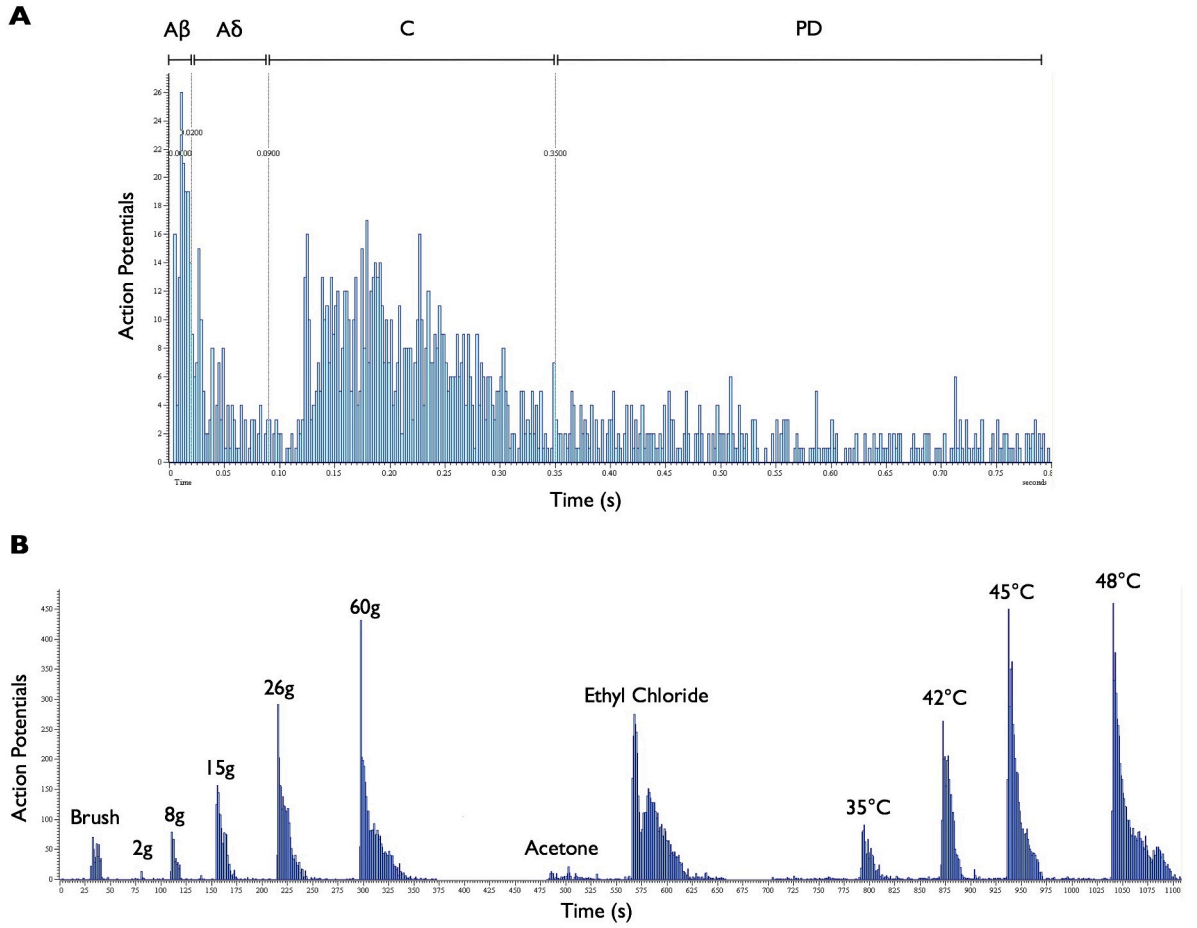


Figure 2.4 Example Spike2 traces of rat neuronal responses. (A) Post stimulus time histogram after train of 16 electrical stimuli. Action potentials are separated according to latency. **(B)** Evoked neuronal responses to mechanical and thermal stimuli.

2.2 Spinal nerve ligation (SNL) surgery

Surgery was performed as described by Kim and Chung, 1992. Rats (125-135 g) were initially anaesthetised with 3.5% v/v isoflurane, delivered in a 3:2 ratio of nitrous oxide and oxygen, in an induction box. Following shaving of the back and sterilisation of the skin with Betadine, rats were transferred to a nose cone and maintained under 2% v/v isoflurane anaesthesia. A heated mat was used to maintain core body temperature. An incision was made in the skin along the length of the vertebrae centred on the pelvis. A paraspinal incision was made and the left tail muscle excised. Part of the L6 transverse process was removed to expose the underlying nerves. The L5 and L6 spinal nerves were then isolated with a glass nerve hook (Ski-Ry Ltd, London, UK) and ligated with a non-absorbable 6-0 braided silk thread proximal to the formation of the sciatic nerve (see Fig. 2.5). The surrounding muscle was closed with absorbable 3-0 sutures. The skin was sutured with a subcuticular pattern with absorbable 3-0 sutures. Animals were allowed to recover in a heated recovery chamber before being returned to their cages.

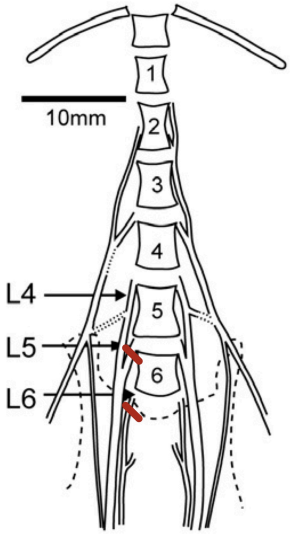


Figure 2.5 Spinal nerve ligation surgery. The left L5 and L6 spinal nerves (marked in red) were ligated distal to the DRG and proximal to the formation of sciatic nerve. The L4 spinal nerve was left uninjured. (Modified from Rigaud *et al*., 2008).

2.3 Assessment of stimulus evoked behaviours in rats

2.3.1 Mechanical sensitivity

Rats were allowed to acclimatise to the behaviour room for 1 hour prior to testing. Rats were then placed in isolation inside Perspex chambers on a wire mesh floor and allowed to acclimatise for a further 15 minutes. Mechanical sensitivity was assessed using von Frey filaments (Touch-Test, North Coast Medical, USA). Filaments were applied until they buckled for 5-6 s; lifting, flinching and shaking were considered positive responses. 50% withdrawal thresholds were determined using the up-down method described by (Chaplan *et al.*, 1994a) with von Frey filaments proving forces of 1.4 g, 2 g, 4 g, 6 g, 8 g, 10 g and 15 g. Testing began at 6 g then by the next weight up or down depending on a negative or positive response respectively. Following a change in direction of the response a further 4 filaments were applied increasing or decreasing in weight as required. 50% paw withdrawal thresholds (PWT) were calculated with the following formula: PWT = (10^(x + $k\delta$)/10,000), where x represents the log of the last von Frey tested, δ represents the mean difference between the von Frey filaments in log units (0.17) and k, a value dependent on the series of responses (Dixon et al., 1980). Rats were tested 4 at a time, order of testing was as follows: The starting filament was applied to the left paw of the first rat, then to the left paw of the second rat, then third and fourth. On returning to the first rat, the starting filament was then applied to the right paw, followed by the right paw of the second rat and so forth. Testing was continued, moving down or up to the next weight as necessary, until all required responses were obtained. For behavioural pharmacology, animals were randomly assigned to groups with all testing performed blind to treatment.

2.3.2 Cold sensitivity

Cold sensitivity was examined after the completion of von Frey testing. This was performed by applying a drop of acetone, using a modified 0.5 ml syringe with polythene tubing, to the left and right hind paws (same order of testing as described above). Acetone was applied 5 times, with at least 2 minutes recovery between applications. The number of withdrawals out of 5 was recorded. Licking, flinching and shaking were considered positive responses.

2.4 Partial sciatic nerve ligation (PSNL) surgery

Surgery was performed based on a method previously described (Seltzer *et al.*, 1990). Mice were initially anaesthetised with 3.5% v/v isoflurane, delivered in a 3:2 ratio of nitrous oxide and oxygen, in an induction box. Following shaving of the thigh and sterilisation of the skin with Betadine, mice were transferred to a nose cone and maintained under 1.5% v/v isoflurane anaesthesia. Under aseptic conditions the left sciatic nerve was exposed through incision of the biceps femoris above the trifurcation of the nerve. Approximately one third to a half of the nerve was ligated with a non-absorbable 7-0 braided silk thread (Fig. 2.6). The surrounding muscle was closed with absorbable 6-0 vicryl sutures and the skin with Michel clips. Sham surgery was performed in wildtype mice in an identical manner omitting the nerve ligation step. Animals were allowed to recover in a heated recovery chamber before being returned to their cages. All surgery, behaviour and pharmacology were performed blind to genotype and treatment.

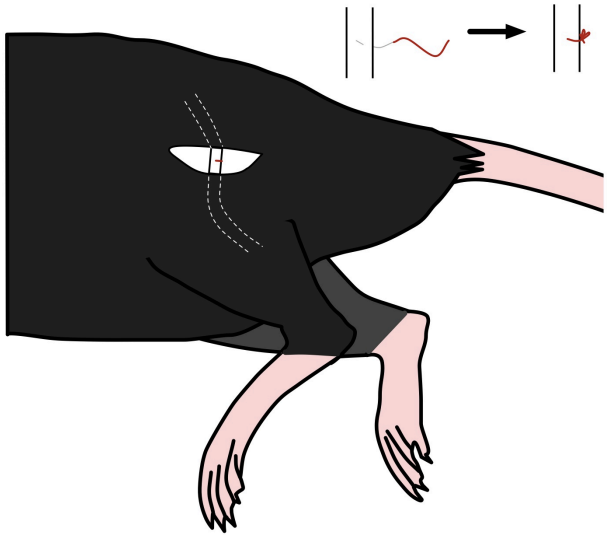


Figure 2.6 Partial sciatic nerve ligation surgery. The left sciatic nerve was partially ligated (in red) above the trifurcation of the nerve.

2.5 Assessment of stimulus evoked behaviours in mice

2.5.1 Mechanical sensitivity

Mice were allowed to acclimatise to the behaviour room for 1 hour prior to testing. Mice were then placed in isolation inside Perspex chambers on a wire mesh floor and allowed to acclimatise for a further hour. Mechanical sensitivity was assessed using von Frey filaments (Touch-Test, North Coast Medical, USA) providing forces of 0.07 g, 0.16 g and 0.4 g. Filaments were tested in ascending order and applied 10 times across left and

right hind paws. Filaments were applied until they buckled for 3-4 s; lifting, flinching and shaking were considered positive responses. Ambulation was considered ambiguous and the test was repeated. For behavioural pharmacology, only withdrawal frequency to 0.4 g von Frey was assessed.

2.5.2 Cold sensitivity

Cold sensitivity was tested by applying a drop of acetone, using a modified 0.5 ml syringe with polythene tubing, to the left and right hind paws. Acetone was applied 5 times, with at least 5 minutes recovery between applications, and the time spent licking, flinching and shaking measured over the next 45 s. An initial shake of the paw due to the mechanical nature of the stimulus was ignored. Ambulation was considered ambiguous and was discounted. Testing was performed in the following order: left paw of first mouse, followed by left of second mouse and so forth before returning and testing right paw of first mouse, then second mouse and so forth. Stimuli were applied in following order:

Acetone → 0.07 g → acetone → 0.16 g → acetone → 0.4 g → acetone → acetone

2.5.3 Heat sensitivity

Heat thresholds were determined using an infrared Nd:YAP laser with a wavelength of 1.34 μm (Electronical Engineering, Italy). A He–Ne laser illuminated the area to be stimulated with a 3 mm wide spot. The pulse time was 4 ms. Mice were lightly anaesthetised and maintained on 0.8% v/v isoflurane delivered in a 3:2 ratio of nitrous oxide and oxygen. Body temperature was regulated using a homeothermic blanket (Harvard Apparatus, Kent, UK). Reflexes were confirmed before each test by gently pinching between the toes. The laser was aligned across the footpads of the right hind paw before applying an increasing incremental heat stimulus starting at 1 J. If no response was observed, the stimulus was increased by 0.5 J. Mice were allowed 3 minutes to recover between tests. Once a positive withdrawal was observed, the intensity was reduced by 0.25 J to determine the approximate withdrawal threshold. Thresholds were confirmed by repeating the test on the left hind paw. The cut-off was set at 3.5 J to prevent tissue damage.

2.6 Statistical analysis

All statistical analyses were performed using SPSS v22 (IBM, NY, USA). Specific details of tests used are described in individual chapters.

3. Novel TRPM8 Antagonists Selectively Inhibit Innocuous and Noxious Cooling in Neuropathic Conditions

3.1 Introduction

As discussed previously, cold hypersensitivity and hyperalgesia are often symptoms of several neuropathic conditions including complex regional pain syndrome, trigeminal neuralgia, peripheral nerve injury (Maier *et al.*, 2010), but also chemotherapy-induced neuropathy where cancer treatment is often limited due to adverse effects and the resulting neuropathy is largely undertreated by currently available drugs (Pachman *et al.*, 2011). Neurological disturbances are also evident in ciguatera, a syndrome caused by consumption of ciguatoxin contaminated tropical fish. Symptoms include dysaesthesias in the extremities, pruritus, muscle pain and cold hyperalgesia (Lewis, 2006).

Much diversity exists in the sensations evoked by cold, however relatively little is known about transduction of cold temperatures at the molecular level or how this relates to the properties of cold perception. In rodents, cold sensitivity is predominantly conferred by TRPM8, which is gated by cold temperatures below 25 °C and cold mimetic compounds such as menthol (McKemy *et al.*, 2002; Peier *et al.*, 2002). TRPM8 is expressed in a subset of Aδ- and C-fibres; mice devoid of TRPM8 display deficits in aversion to innocuous cold temperatures (Bautista *et al.*, 2007; Colburn *et al.*, 2007; Dhaka *et al.*, 2007) and to a certain degree noxious cold temperatures (Knowlton *et al.*, 2010).

Numerous studies implicate TRPM8 in the development of cold hypersensitivity. Genetic ablation of TRPM8 abolishes cold evoked behaviours following peripheral inflammation or nerve injury (Colburn *et al.*, 2007) and in models of chemotherapy induced neuropathy (Descoeur *et al.*, 2011). Similar findings have also been observed following ablation of TRPM8+ afferents (Knowlton *et al.*, 2013). Pharmacological modulators of TRPM8 activity are often referred to as agonists and antagonists. Activation of TRPM8 by menthol is the result of shifting the voltage dependence of activation to negative potentials to increase channel opening at physiological temperatures, whereas antagonists shift voltage dependence towards more positive potentials (Mälkiä *et al.*, 2007). Studies using various TRPM8 antagonists are often complicated by actions at other TRP channels despite low homology between channels.

Several lines of evidence support the therapeutic potential of TRPM8 antagonists, which have been demonstrated to be efficacious in neuropathy and inflammatory models (Xing *et al.*, 2007; Knowlton *et al.*, 2011; Calvo *et al.*, 2012), and in chemotherapy induced neuropathy (Gauchan *et al.*, 2009), in addition to

inhibiting bladder reflexes (Lashinger *et al.*, 2008; Winchester *et al.*, 2014). TRPM8, like TRPV1, is necessary for thermoregulation with inhibition of peripheral, but not central, channels required for the hypothermic effects of TRPM8 antagonists (Almeida *et al.*, 2012). In the short term this would represent a significant and undesirable side effect, however the hypothermic effect appears to diminish with repeated dosing (Gavva *et al.*, 2012). The only human study to date reports a reduction in cold pain intensity during the cold pressor test in the absence of changes in core body temperature following TRPM8 blockade, though a surprising adverse effect was the unmasking of a predominantly peri-oral heat sensation in some subjects (Winchester *et al.*, 2014). This finding corresponds well with knockout studies implicating TRPM8 in both innocuous and noxious cold sensitivity and has raised the prospects that pharmacological modulation of TRPM8 could be beneficial in the treatment of aberrant cold sensitivity. These studies will focus on examining the TRPM8 dependence of cold hyperalgesia in peripheral sensitisation and nerve injury models.

Hypotheses and Aims:

Numerous lines of evidence implicate TRPM8 in innocuous and noxious cold sensitivity in rodents and humans. Despite this, the pharmacology of spinal neurones has not been characterised as extensively as peripheral neurones. The effects of 2 novel TRPM8 antagonists (synthesised by Takeda Cambridge Ltd (M8-An1 and M8-An2; see appendix 8.2)) on spinal neuronal responses in naïve rats will be examined to test the hypothesis that TRPM8 contributes to intensity dependent coding to cold temperatures. Subsequent studies will examine the role of TRPM8 in 2 models of cold hyperalgesia induced by spinal nerve injury and Pacific ciguatoxin-2.

3.2 Materials and Methods

3.2.1 Animals

Male Sprague Dawley rats (250-300 g), bred by the Biological Service Unit (UCL, London, UK), were used for behavioural and electrophysiological experiments.

3.2.2 *in vivo* electrophysiology

See section 2.12, 2.14 and 2.15. *In vivo* electrophysiology was performed between day 15 and 18 post SNL surgery and in age/weight matched naïve rats. Single WDR neurones were characterised for responses to repeated electrical stimulation followed by natural stimuli. For order of testing see figure 2.4*A, B*. A 1-minute rest period followed each stimulus. Following 3 stable rounds of testing (<10% variation; averaged to give baseline response) rats were dosed subcutaneously into the contralateral flank with vehicle (85% saline, 10% cremophor (Sigma, UK), 5% dimethylsulfoxide (Sigma, UK)), 30 mg/kg M8-An1, 30 mg/kg M8-An2 or 100 mg/kg M8-An2 in a volume of 1 ml/kg. Doses were chosen based on effects on core body temperature (Fig. 3.5), and preliminary pharmacokinetic data and efficacy in complete Freund's adjuvant induced hyperalgesia model (data not shown). Mechanical, thermal and electrical stimuli were next applied 30 minutes post dosing and then every 20 minutes up to 110 minutes post dosing. For Pacific ciguatoxin-2 studies (gift from R. Lewis, University of Queensland), in naïve rats 30 µl of 10 nM ciguatoxin-2 (vehicle of 0.1% bovine serum albumin (Sigma, UK), 0.05% methanol (Sigma, UK) and >99% saline) was injected into the receptive field. Concentration was chosen based on previously published findings (Zimmermann *et al.*, 2013). Rats were subsequently dosed subcutaneously with 30 mg/kg M8-An2. Tests were performed 20 and 40 minutes post dosing of each compound, peak change is plotted.

3.2.3 Spinal nerve ligation surgery

See section 2.2. SNL rats were used for either *in vivo* electrophysiology or behavioural testing and were euthanised at the end of the experiment.

3.2.4 Behavioural Assessments

See section 2.3.1 and 2.3.2. Ipsilateral and contralateral behavioural sensitivity to mechanical and cold stimuli was assessed in SNL rats on day 14 post surgery. Immediately after the conclusion of baseline testing, rats were dosed intraperitoneally with either vehicle, 30 mg/kg M8-An1 or 30 mg/kg M8-An2 in a volume of 1 ml/kg. Behavioural testing was repeated 30, 60 and 90 minutes post dosing. Rats were randomised into treatment groups and all behavioural testing was performed while blind to treatment.

3.2.5 Statistics

Mechanical and heat coding of neurones was compared with a 2 way repeated measures (RM) ANOVA followed by a Bonferroni *post hoc* test for paired comparisons. Cold, brush and electrically evoked neuronal responses were compared with a 1 way RM ANOVA followed by a Bonferroni *post hoc* test for paired comparisons. Sphericity was tested using Mauchly's test, the Greenhouse-Geisser correction was applied if violated. Behavioural time-courses were compared with a Friedman test followed by a Wilcoxon *post hoc*. Area under curve (AUC) values were calculated with the trapezoid rule and compared with a 1 way ANOVA and Bonferroni *post hoc*.

3.3 Results

3.3.1 Summary of baseline neuronal responses

In vivo electrophysiology was performed to examine the effect of TRPM8 inhibition on spinal neuronal responses to cold stimulation and its effect, if any, on other modalities under normal or neuropathic conditions. Table 3.1 summarises neuronal depths from the surface of the cord and pre-drug baseline responses to mechanical, thermal and electrical stimulation. Neurones were characterised from depths corresponding to lamina V/VI (Watson et al., 2009) and had receptive fields on the glabrous skin of the hind toes. Deep dorsal horn neurones displayed graded firing to natural stimuli and were identified as wide dynamic range by confirming responses to dynamic brushing, noxious punctate mechanical and heat stimulation.

Table 3.1 Baseline characterisations of deep dorsal horn WDR neurones from naïve and SNL rats. Range of depths recorded from in parentheses. Data represent mean ± SEM. (APs- action potentials)

	Naïve *n*=34	SNL *n*=21
Depth (μm)	725 ± 20.79 (550 - 950)	645 ± 34.32 (490 - 910)
A threshold (mA)	0.09 ± 0.04	0.07 ± 0.01
C threshold (mA)	0.35 ± 0.07	0.32 ± 0.05
Aβ-evoked (APs)	128 ± 6.86	124 ± 8.76
Aδ-evoked (APs)	229 ± 10.16	239 ± 20.56
C-evoked (APs)	521 ± 25.01	565 ± 36.86
Post-discharge (APs)	359 ± 24.11	392 ± 33.89
Brush (APs)	438 ± 23.76	480 ± 42.75
2g (APs)	34 ± 4.78	39 ± 6.38
8g (APs)	335 ± 24.60	363 ± 32.20
15g (APs)	639 ± 33.95	672 ± 39.43
26g (APs)	935 ± 35.00	1014 ± 39.93
60g (APs)	1290 ± 38.96	1350 ± 49.00
35°C (APs)	264 ± 27.52	353 ± 42.61
42°C (APs)	576 ± 40.08	561 ± 62.78
45°C (APs)	816 ± 50.11	822 ± 67.87
48°C (APs)	1225 ± 119.36	1269 ± 74.01
Acetone (APs)	68 ± 13.44	87 ± 23.01
Ethyl chloride (APs)	516 ± 46.98	625 ± 68.18

3.3.2 M8-An1 inhibits neuronal responses to noxious cooling in SNL rats

After isolating single dorsal horn neurones and following stable baseline recordings, naïve or SNL rats were dosed subcutaneously with 30 mg/kg M8-An1. Acetone and ethyl chloride were applied as evaporative cooling stimuli. From recordings of skin temperature, these solvents have been described previously to represent innocuous and noxious stimuli respectively as the latter induces a withdrawal reflex in normal animals (Leith *et al.*, 2010). Compared to baseline, 30 mg/kg M8-An1 did not significantly reduce the number of action potentials evoked by innocuous (1 way RM ANOVA $P>0.05$, $F_{1.56,15.6}=1.133$) or noxious cold (1 way RM ANOVA $P>0.05$, $F_{1.90,15.15}=2.690$) stimulation in naïve rats (Fig. 3.1*A*). In SNL rats, M8-An1 inhibited neuronal responses to noxious cold stimulation at 30 and 50 minutes post dosing (1 way RM ANOVA $P<0.01$, $F_{5,35}=4.597$, followed by Bonferroni *post hoc*) with no effect on acetone evoked responses (1 way RM ANOVA $P>0.05$, $F_{1.71,11.96}=1.723$) (Fig. 3.1*B*). Furthermore, M8-An1 did not alter neuronal responses to heat stimulation (2 way RM ANOVA $P>0.05$, Naïve: $F_{5,50}=1.152$, SNL: $F_{5,35}=0.688$) (Fig. 3.1*C, D*), punctate mechanical stimulation (2 way RM ANOVA $P>0.05$, Naïve: $F_{5,50}=0.994$, SNL: $F_{2.62,18.36}=1.664$) (Fig. 3.1*E, F*), or dynamic brushing of the receptive field (1 way RM ANOVA $P>0.05$, Naïve: $F_{5,50}=2.194$, SNL: $F_{2.46,17.26}=3.76$) (Fig. 3.1*G, H*) in either naïve or SNL rats. Following a sequence of supra-threshold electrical stimuli, the total number of action potentials attributed to Aβ- (Naïve: $F_{5,50}=1.287$, SNL: $F_{5,35}=1.530$), Aδ- (Naïve: $F_{5,50}=1.469$, SNL: $F_{5,35}=1.064$) and C-fibres (Naïve: $F_{5,50}=2.73$, SNL: $F_{5,35}=0.946$) did not significantly differ to baseline (1 way RM ANOVA $P>0.05$). Measures of neuronal excitability, input (Naïve: $F_{1.90,18.96}=1.498$, SNL: $F_{2.76,16.58}=2.408$), wind-up (Naïve: $F_{5,50}=1.043$, SNL: $F_{5,35}=1.879$) and post-discharge (Naïve: $F_{5,50}=1.668$, SNL: $F_{5,35}=0.430$) were also unaffected in both naïve and SNL rats (1 way RM ANOVA $P>0.05$) (Fig. 3.1*I, J*). Vehicle alone had no effect on neuronal responses for the duration of the testing period (Appendix 8.4).

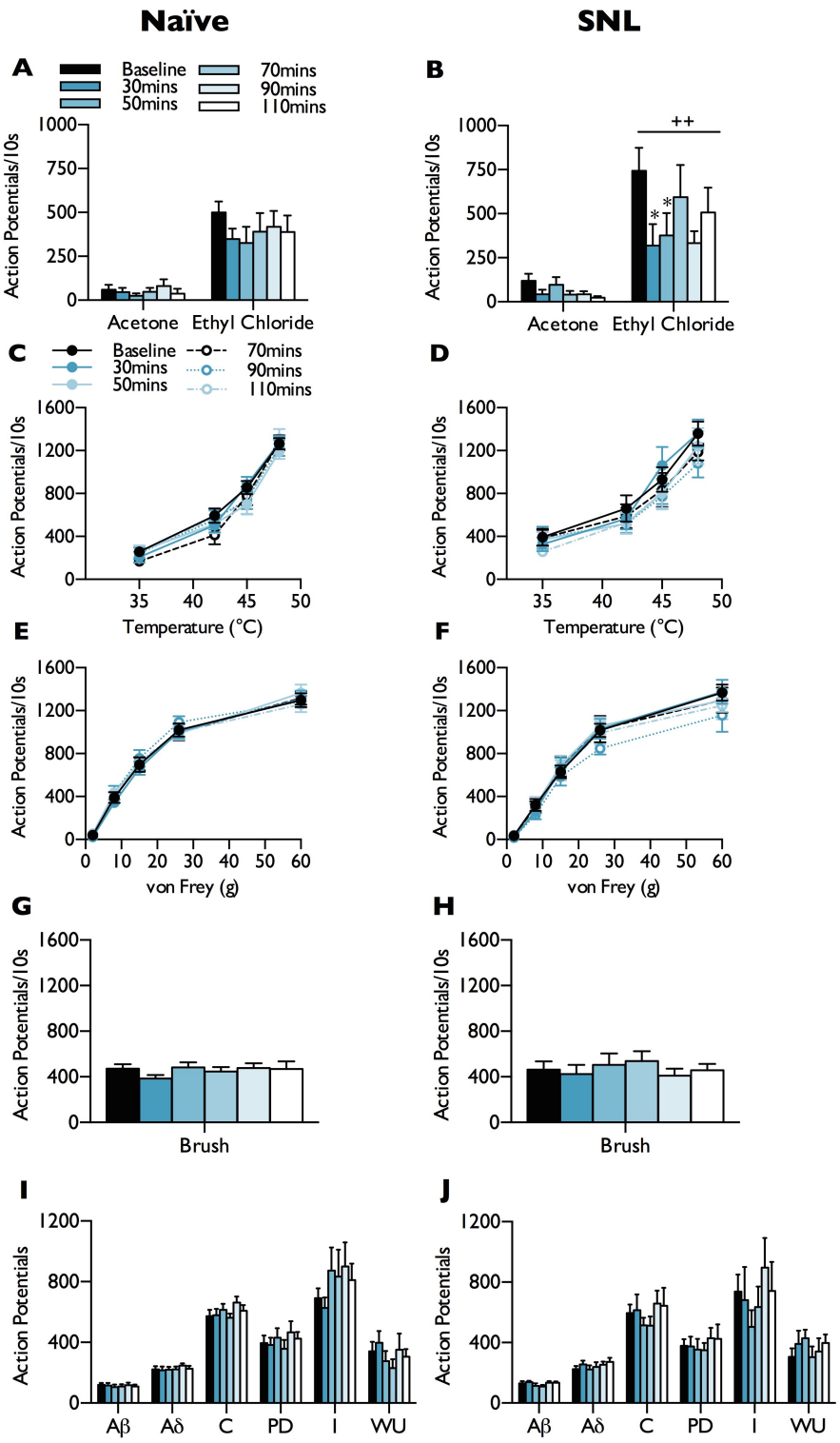


Figure 3.1 M8-An1 inhibits deep dorsal horn lamina V/VI neuronal responses to noxious cold stimulation in SNL rats but not naïve rats. Following stable baseline recordings, rats were dosed subcutaneously with 30 mg/kg M8-An1. After 30 minutes post dosing and then at 20 minute intervals, recordings were made of neuronal responses to cold **(A, B)**, heat **(C, D)**, punctate mechanical **(E, F)**, dynamic brushing **(G, H)** and electrical stimuli **(I, J)**. Left Panels - naïve (*n*=11), Right panels - SNL (*n*=9). *$P<0.05$. (+) represents Main Effect (1 Way RM ANOVA), asterisks denote difference from baseline (Bonferroni *post hoc*). Data represent mean ± SEM. (I – input, PD – post-discharge, WU – wind-up).

3.3.3 M8-An2 inhibits neuronal responses to innocuous and noxious cooling in SNL rats

Following stable baseline neuronal recordings, naïve rats were dosed subcutaneously with either 30 mg/kg or 100 mg/kg M8-An2. Neither dose of M8-An2 had any statistically significant effect on innocuous cold (1 way RM ANOVA $P>0.05$, 30 mg/kg: $F_{1.9,11.44}=0.815$, 100 mg/kg: $F_{5,30}=0.765$) or noxious cold (1 way RM ANOVA $P>0.05$, 30 mg/kg: $F_{5,30}=2.085$, 100 mg/kg: $F_{1.68.8.38}=2.05$) (Fig. 3.2*A, B*), heat (2 way RM ANOVA $P>0.05$, 30 mg/kg: $F_{5,30}=0.518$, 100 mg/kg: $F_{5,30}=1.785$) (Fig. 3.2*D, E*), punctate mechanical (2 way RM ANOVA $P>0.05$, 30 mg/kg: $F_{5,30}=0.377$, 100 mg/kg: $F_{5,30}=2.676$) (Fig. 3.2*G, H*) or dynamic brush (1 way RM ANOVA $P>0.05$, 30 mg/kg: $F_{5,30}=1.406$, 100 mg/kg: $F_{5,30}=1.272$) (Fig. 3.2*J, K*) evoked neuronal responses. Electrically evoked parameters, Aβ- (30 mg/kg: $F_{5,30}=2.088$, 100 mg/kg: $F_{5,30}=0.282$), Aδ- (30 mg/kg: $F_{5,30}=0.988$, 100 mg/kg: $F_{5,30}=1.264$) and C-fibre (30 mg/kg: $F_{5,30}=1.706$, 100 mg/kg: $F_{5,30}=2.272$) evoked activity and input (30 mg/kg: $F_{5,30}=0.563$, 100 mg/kg: $F_{5,30}=1.723$), wind-up (30 mg/kg: $F_{1.69,10.11}=0.493$, 100 mg/kg: $F_{5,30}=1.15$) and post-discharge (30 mg/kg: $F_{5,30}=0.432$, 100 mg/kg: $F_{5,30}=2.562$) were all unaffected by both doses (1 way RM ANOVA $P<0.05$) (Fig. 3.2*M, N*).

SNL rats were dosed subcutaneously with 30 mg/kg M8-An2. Compared to baseline, M8-An2 inhibited neuronal responses to noxious cold stimulation with significantly reduced neuronal responses at 30 and 50 minutes post dosing (1 way RM ANOVA $P<0.01$, $F_{5,55}=4.338$, followed by Bonferroni *post hoc* test) (Fig. 3.2*C*). In addition M8-An2 decreased overall neuronal responses to innocuous cooling with acetone (1 way RM ANOVA $P<0.05$, $F_{5,55}=2.52$) (Fig. 3.2*C*). As seen in naïve rats, M8-An2 did not alter neuronal responses to punctate mechanical stimulation (2 way RM ANOVA $P>0.05$, $F_{5,55}=1.782$) (Fig. 3.2*I*), heat stimulation (2 way RM ANOVA $P>0.05$, $F_{5,55}=0.778$) (Fig. 3.2*F*) or dynamic brushing of the receptive field (1 way RM ANOVA $P>0.05$, $F_{1.91,19.11}=2.154$) (Fig. 3.2*L*). Measures of neuronal excitability and total spinal neuronal events evoked by global stimulation of Aβ- ($F_{5,55}=0.544$), Aδ- ($F_{5,55}=1.059$) and C-fibres ($F_{1.96,22.96}=1.205$) were also unaffected in SNL rats indicating blocking TRMP8 does not alter electrical function of primary afferent fibres under normal or pathological conditions (1 way RM ANOVA $P>0.05$) (Fig. 3.2*O*).

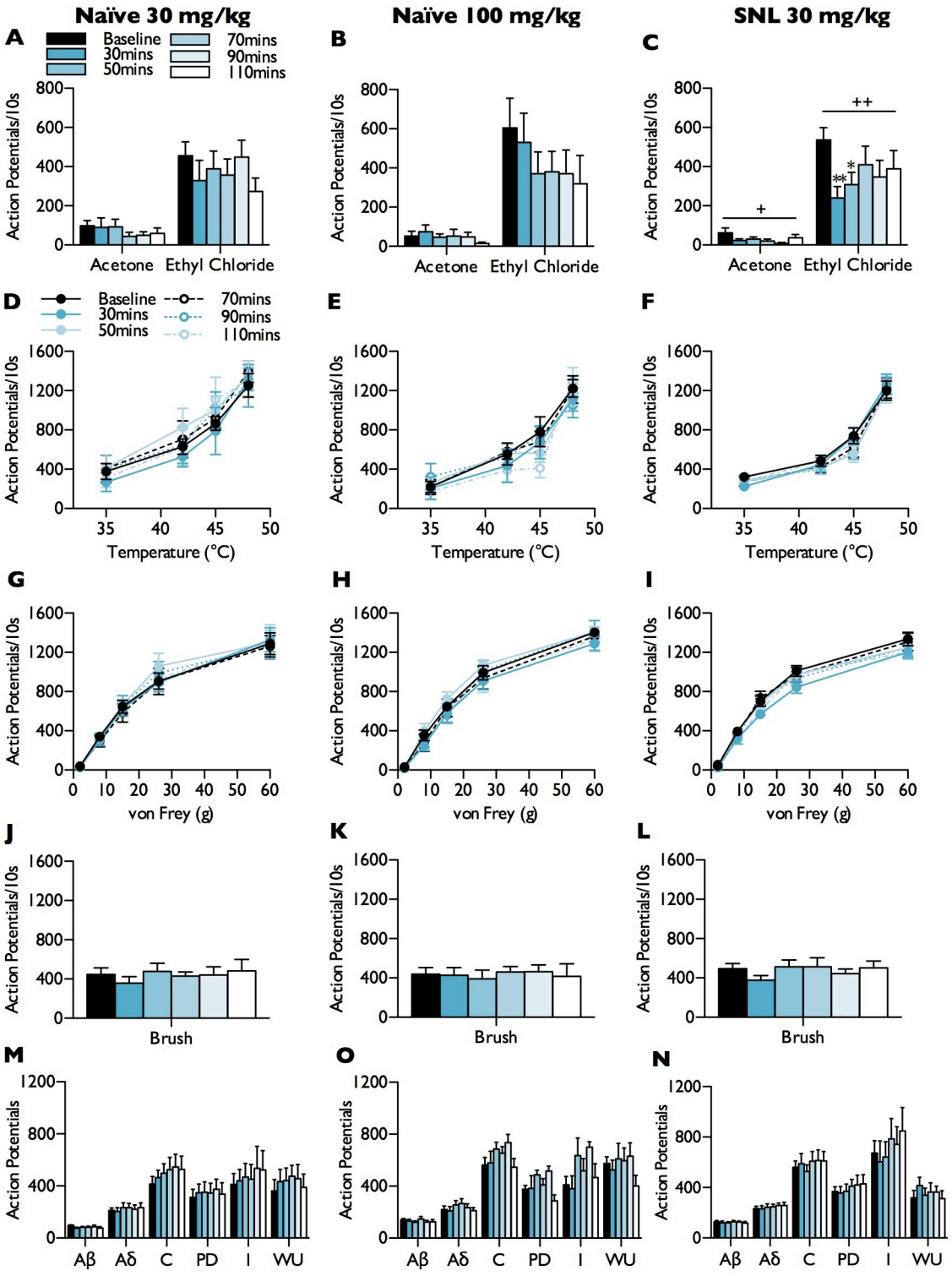


Figure 3.2 M8-An2 inhibits deep dorsal horn lamina V/VI neuronal responses to innocuous and noxious cold stimulation in SNL rats. Rats were dosed subcutaneously with 30 mg/kg M8-An2. After 30 minutes post dosing and then at 20 minute intervals, recordings were made of neuronal responses to cold **(A, B, C)**, heat **(D, E, F)**, punctate mechanical **(G, H, I)**, dynamic brushing **(J, K L)** and electrical stimuli **(M, N, O)**. Left Panels - naïve 30 mg/kg (*n*=7), Centre Panels – naïve 100 mg/kg (*n*=7), Right panels - SNL 30 mg/kg (*n*=12). **P*<0.05, ***P*<0.01. (+) represents Main Effect (1 Way RM ANOVA), asterisks denote difference from baseline (Bonferroni *post hoc*). Data represent mean ± SEM.

3.3.4 M8-An2 attenuates cold but not mechanical hypersensitivity in SNL rats

The ability of M8-An1 and M8-An2 to alleviate cold hypersensitivity was examined in SNL rats. Rats were dosed with either vehicle, 30 mg/kg M8-An1 or 30 mg/kg M8-An2 and tested for 90 minutes post dosing. M8-An2 reduced the behavioural response to cooling of the injured paw compared to pre-drug values (Friedman test $P<0.01$, followed by Wilcoxon's test and Bonferroni correction) (Fig. 3.3*A*), whereas M8-An1 and vehicle alone has no significant effect (Friedman test, $P>0.05$) (Fig. 3.3*A*). Contralateral responses to cooling were not affected by M8-An1 or M8-An2 (Friedman test, $P>0.05$) (Fig. 3.3*B*). AUC analysis between 30 and 90 minutes post dosing confirms behavioural signs of cold hypersensitivity are reduced by M8-An2 compared to vehicle treatment (1 way ANOVA $P<0.001$, $F_{5,28}=11.828$, followed by Bonferroni *post hoc* test) (Fig. 3.3*C*). Neither compound altered mechanically evoked responses on either the ipsilateral (Fig. 3.3*D*) or contralateral (Fig. 3.3*E*) paw compared to pre-drug values (Friedman test, $P>0.05$). AUC analysis between 30 and 90 minutes post dosing also confirms no overall effect of either drug or vehicle treatment on mechanical hypersensitivity (1 way ANOVA $P<0.001$, $F_{5,36}=34.569$, followed by Bonferroni *post hoc* test) (Fig. 3.3*F*). The behavioural observations correlated with electrophysiological recordings of neuronal responses to punctate mechanical stimulation and acetone induced cooling. AUC analysis was performed between 30 and 110 minutes post dosing to compare the effects of M8-An1 and M8-An2 on total neuronal events in uninjured and nerve ligated conditions. 30 mg/kg M8-An1 and M8-An2 did not affect mechanical coding of WDR neurones to below threshold and threshold stimuli in SNL or naïve rats (2 way ANOVA $P>0.05$, M8-An1: $F_{1,54}=3.132$, M8-An2: $F_{1,54}=0.732$) (Fig. 3.3*H, J*), whereas cooling evoked responses were significantly reduced by M8-An2 in SNL rats (unpaired Student's t-test, $P<0.05$) (Fig. 3.3*I*) but not by M8-An1 (unpaired Student's t-test, $P>0.05$) (Fig. 3.3*G*).

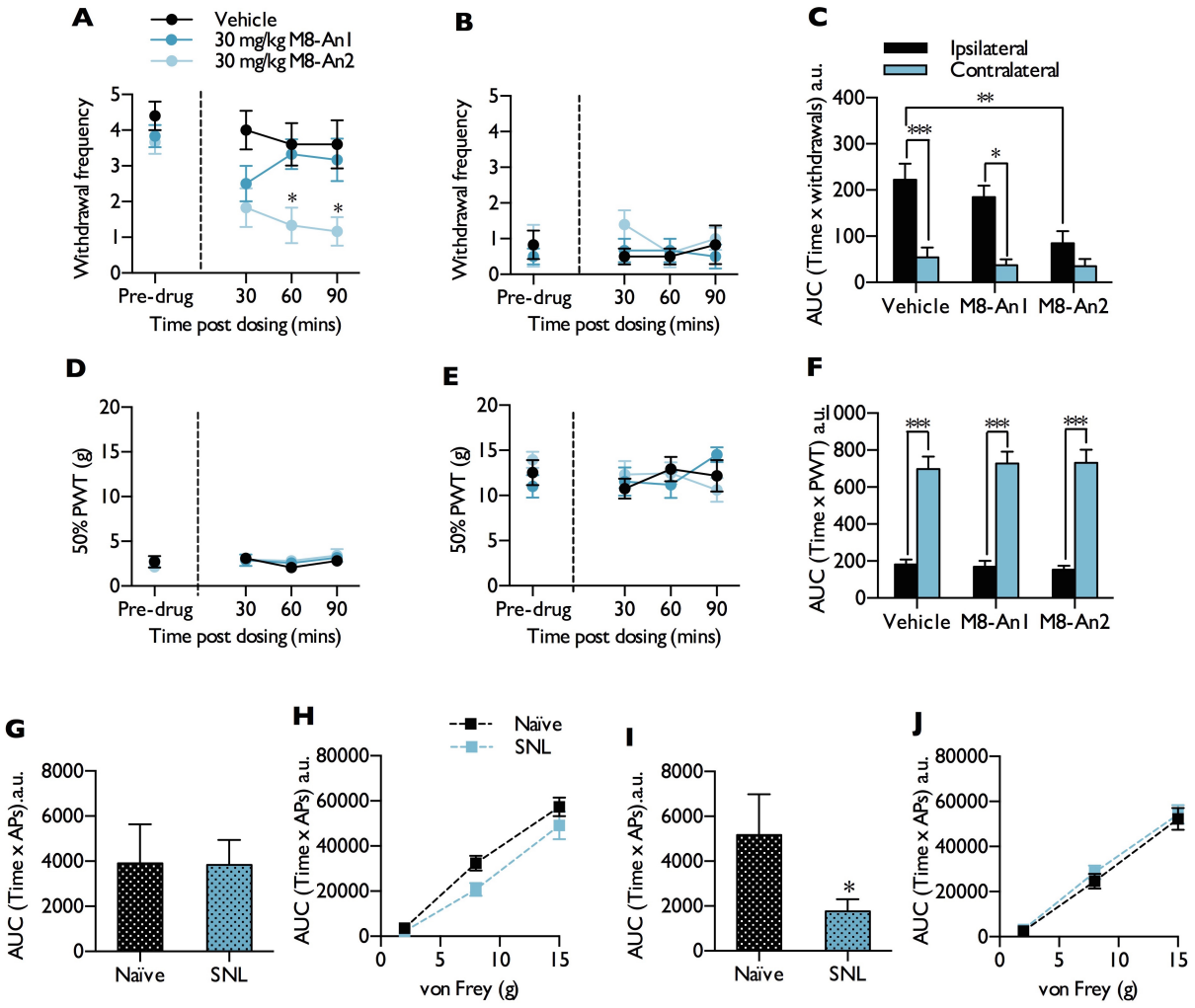


Figure 3.3 M8-An2 selectively reduces behavioural and neuronal responses to cooling following spinal nerve ligation. **(A)** Effect of 30 mg/kg M8-An1 (*n*=6), M8-An2 (*n*=6) and vehicle (*n*=5) on the behavioural response to acetone on the nerve injured ipsilateral side compared to pre-drug values. **(B)** Corresponding contralateral responses to innocuous cooling. **(C)** Overall effect of M8-An1 and M8-An2 compared to vehicle treatment on cold hypersensitivity. Effect of M8-An1, M8-An2 and vehicle on mechanical withdrawal thresholds on the ipsilateral side **(D)** and contralateral side **(E)** (*n*=7). **(F)** Overall effect of M8-An1 and M8-An2 compared to vehicle treatment on mechanical hypersensitivity. **(G)** Effect of M8-An1 on total neuronal responses between 30 and 110 minutes post dosing to mechanical stimulation in SNL (*n*=9) and naïve rats (*n*=11). **(H)** Effect of M8-An1 on total neuronal responses to acetone in SNL (*n*=9) and naïve rats (*n*=11). **(I)** Effect of M8-An2 on total neuronal responses to mechanical stimulation in SNL (*n*=12) and naïve rats (*n*=7). **(J)** Effect of M8-An2 on total neuronal responses to acetone in SNL (*n*=12) and naïve rats (*n*=7). Data represent mean ± SEM. **P*<0.05, ** *P*<0.01, ****P*<0.001. (a.u. - arbitrary units, AUC - area under curve, PWT - paw withdrawal threshold, APs - action potentials).

3.3.5 Ciguatoxin-2 induces cold hyperalgesia in naïve rats and is not reversed by TRPM8 antagonist M8-An2

The peripheral effects of Pacific ciguatoxin-2 were examined in a group of naïve rats. Following stable baseline responses, 10 nM ciguatoxin-2 was injected into the receptive field. Ciguatoxin-2 increased neuronal responses to innocuous and noxious cold stimulation (paired Student's T-test, $P<0.01$) (Fig. 3.4*A*). In a subset of rats, 30 mg/kg M8-An2 was dosed subcutaneously after the establishment of ciguatoxin induced cold hyperalgesia, and subsequently failed to reduce neuronal responses to cold stimulation (1 way RM ANOVA $P<0.05$, innocuous: $F_{2,10}=4.238$, noxious: $F_{2,10}=11.547$, followed by Bonferroni *post hoc* test) (Fig. 3.4*B*). In addition, ciguatoxin-2 potentiated neuronal responses to below threshold and threshold punctate mechanical stimulation but not to noxious stimuli (2 way RM ANOVA $P<0.01$, $F_{1,12}=17.486$) (Fig. 3.4*C*). No effect of the toxin was observed on brush (paired Student's T-test, $P>0.05$) (Fig. 3.4*D*), heat (2 way RM ANOVA $P>0.05$, $F_{1,12}=0.222$) (Fig. 3.4*E*) or electrically evoked neuronal responses (paired Student's T-test, $P>0.05$) (Fig. 3.4*F*).

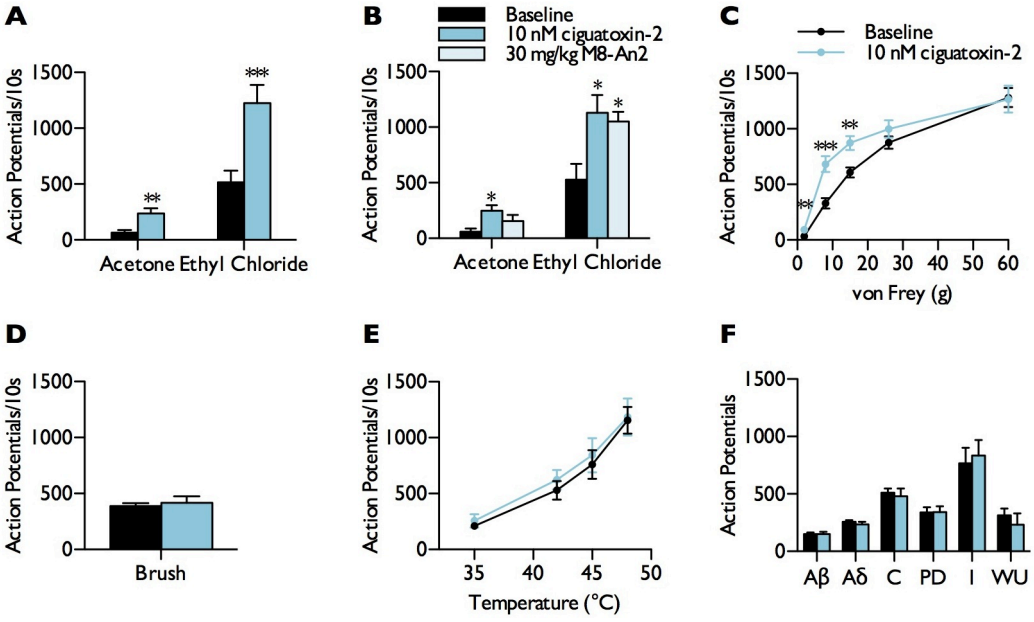


Figure 3.4 Ciguatoxin-2 induces cold and mechanical hypersensitivity. Naïve rats were injected with 10 nM ciguatoxin-2 into the receptive field. **(A)** Ciguatoxin-2 increases innocuous and noxious cold evoked neuronal responses ($n=9$) and **(B)** was not reversed by systemic administration of 30 mg/kg M8-An2 ($n=6$). **(C)** Ciguatoxin-2 potentiates neuronal responses to low threshold mechanical stimuli. No effect was observed on brush **(D)**, heat **(E)**, and electrically **(F)** evoked neuronal responses. Data represent mean ± SEM. Asterisks denote statistically significant difference from baseline. *$P<0.05$, ** $P<0.01$, ***$P<0.001$. (I – input, PD – post-discharge, WU – wind-up).

3.4 Discussion

The data presented here demonstrate that blocking peripheral TRPM8 channels only inhibits spinal neuronal firing to cold stimulation and attenuates cold evoked behaviours after a neuropathic insult. The effects of M8-An2 were greater than that of M8-An1, which only inhibited neuronal responses to noxious cold stimulation in SNL rats at the dose administered.

3.4.1 Role of TRPM8 in cold transduction in naïve rats

Rats display few withdrawal behaviours to innocuous cold stimulation in the absence of injury, which is mirrored by the low neuronal responses to cooling. Behavioural assays of cold sensitivity in normal animals, unlike heat tests, are often ambiguous, unreliable and display large variability complicating studies of cold sensitivity (McKemy, 2012). Several studies have demonstrated that TRPM8 is required for the aversion to innocuous cold in temperature preference assays (Bautista *et al.*, 2007; Colburn *et al.*, 2007; Dhaka *et al.*, 2007; Knowlton *et al.*, 2013). From a pharmacological perspective, it may prove difficult to discern between cold aversion and warm preference, as efficacious doses of TRPM8 antagonists are often associated with an undesired thermoregulatory effect. This is also apparent with M8-An2 which dose dependently decreases core body temperature (Fig. 3.5). *In vivo* electrophysiology was performed under homeothermic conditions to examine the contribution of TRPM8 to spinal neuronal responses to cold stimulation without the confounding impact of changes in body temperature.

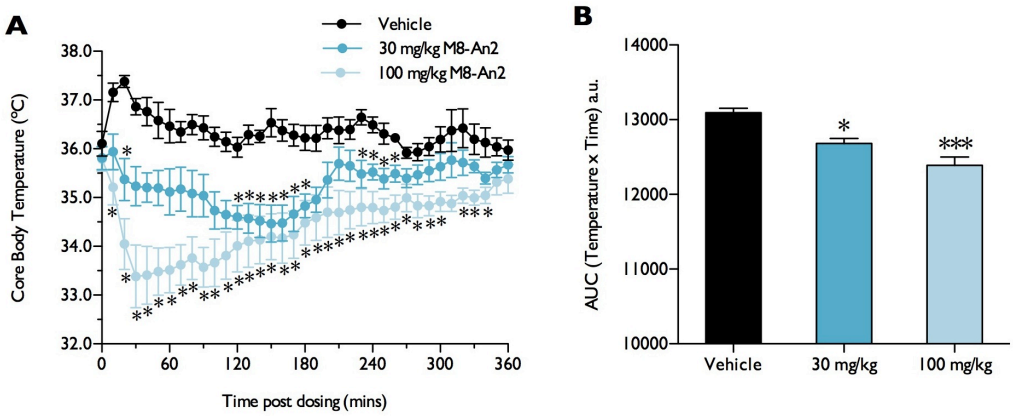


Figure 3.5 Decrease in core body temperature after M8-An2 treatment. (A) Rats were dosed intraperitoneally with vehicle (n=6), 30 mg/kg (n=7), or 100 mg/kg (n=7) M8-An2; core body temperature was monitored over the subsequent 6 hours. **(B)** AUC values for 6-hour period post dosing. Asterisks denote statistical difference compared with vehicle. *P<0.05, ***P<0.001. Data represent mean ± SEM. (a.u., arbitrary units; AUC, area under curve). (Modified from Patel *et al.*, 2014b).

In naïve rats, neither 30 mg/kg M8-An1 nor 100 mg/kg M8-An2 significantly reduced lamina V/VI WDR neuronal responses to innocuous or noxious cold stimulation. Despite the canonical view that TRPM8 is the primary transducer of cool temperatures, surprisingly innocuous cooling evoked responses were conserved at the highest dose examined. Additional cold sensitive ion channels may be expressed in TRPM8 positive peripheral terminals, which may include TRPC5 (Zimmermann *et al.*, 2011). The TRPM8 antagonist BCTC inhibits menthol evoked currents, but has minimal impact on cold evoked activity, in peripheral nerve endings in the cornea (Madrid *et al.*, 2006). A proportion of cold sensitive neurones are TRPM8 negative where the transduction mechanisms are less clear (Babes *et al.*, 2004; Munns *et al.*, 2007). Mechanical coding and heat evoked responses were unaffected by TRPM8 inhibition as were total spinal neuronal events evoked by global Aδ- or C-fibre activation following supra-threshold electrical stimulation of the receptive field, consistent with the restricted expression of TRPM8 in subsets of these fibres and the absence of effect on overall afferent excitability.

Although cross reactivity with other TRP channels was not comprehensively addressed, heat evoked responses of spinal neurones were unaffected suggesting no significant modulation of other thermo TRPs. M8-An2 does however activate TRPA1 (EC_{50} 2.6 μM) and inhibit TRPV1 (IC_{50} >3 μM) though at much higher concentrations than required for TRPM8 blockade (IC_{50} 10.9 nM). Mechanical coding was also unaffected suggesting little or no activity of M8-An1 or M8-An2 at putative mechanoreceptors TRPC3 and TRPC6 (Quick *et al.*, 2012). These data support previous observations that TRPM8 is not required for behavioural manifestations to heat or mechanical stimulation (Knowlton *et al.*, 2013).

3.4.2 Role of TRPM8 in cold hyperalgesia

Ideally a novel therapeutic would alleviate abnormal pain in affected areas without perturbing normal somatosensory function. Using behavioural assays in neuropathic rats, the systemic effect of a dose of M8-An2 that had small but significant effects on body temperature and no effect on neuronal responses to cold stimulation in naïve rats was examined. The impact of M8-An1 on core body temperature had not been examined. M8-An2 (30 mg/kg) reversed cold but not mechanical hypersensitivity in SNL rats without affecting behaviours in the uninjured contralateral paw. Furthermore, the same dose attenuated neuronal responses to noxious and innocuous cold stimulation in SNL but not naïve rats. As observed in naïve rats, M8-An2 had no impact on heat or mechanical coding of neurones in SNL rats. Measures of neuronal excitability, 'input' (the non-potentiated response), 'wind-up' (the potentiated response) and post-discharge also did not differ post

dosing of M8-An2 consistent with a peripheral inhibition rather than reduced spinal neuronal excitability. The effect of M8-An2 appears dependent on pathophysiological state, is confined to the damaged nerve territory and supports previous conclusions that TRPM8 contributes to cold hypersensitivity and hyperalgesia (Colburn *et al.*, 2007). The inability of M8-An1 to inhibit innocuous cooling evoked neuronal responses and behavioural responses could be due to pharmacokinetic differences between the two compounds tested. TRPM8 may have a more prominent role in transduction of noxious temperatures after neuropathy and therefore a higher dose of M8-An1 could have inhibited both innocuous and noxious cold transduction.

3.4.3 Mechanisms of cold hypersensitivity and hyperalgesia

In general, little is known about the mechanisms of cold hypersensitivity and the relative contributions of peripheral and central components. In reality this may be difficult to dissect as peripheral and central sensitisation exist in tandem. In healthy volunteers, A-fibre nerve block can unmask a C-fibre mediated burning pain following cooling leading to the hypothesis that a central disinhibition of primary afferent activity could underlie cold hyperalgesia (Ochoa & Yarnitsky, 1994). Disrupting the integrity of spinal circuits modifies gating of cold stimuli, for example ablation of CGRPα+ primary afferents results in a loss of tonic inhibition of cold sensitive spinal neurones (McCoy *et al.*, 2013). Spinal neuronal responses to cold are also subject to dynamic descending modulation via the PAG and RVM, which may sustain abnormal behaviours under pathological conditions (Rahman *et al.*, 2006; Taylor *et al.*, 2007; Leith *et al.*, 2010). Both ON- and OFF-cells in the RVM display abnormal responses to cold stimulation after nerve injury (increased and decreased response respectively) (Goncalves *et al.*, 2007). The role of NMDA receptors in mediating central sensitisation is well established (Woolf & Thompson, 1991). Correspondingly ketamine reduces spinal neuronal excitability after peripheral injury (Suzuki *et al.*, 2001), and also reduces pain intensity scores to cold stimulation in neuropathic subjects without affecting cold pain thresholds implicating a role for central sensitisation in cold hypersensitivity whereas NMDA independent mechanisms are likely involved in perceiving cold temperatures (Jørum *et al.*, 2003).

It has been noted previously that WDR neuronal responses to mechanical and heat stimulation do not differ between SNL, sham or naïve rats (Brignell *et al.*, 2008). This was also apparent in this study. At first this appears incongruous with increased behavioural sensitivity to below threshold mechanical and cold stimulation considering the experimental model induces substantial denervation of the lumbar spinal cord. The equivalent neuronal responses could be explained by an increase in neuronal excitability to compensate for a loss of

peripheral input. Also, an enlargement of receptive field size of spinal neurones will result in increased numbers excited by peripheral stimulation (Suzuki *et al.*, 2000). One exception to the profile of WDR neurones following SNL appears to be an increased response to noxious cold stimulation with ice, postulated to represent in part a peripheral mechanism of hypersensitivity (Brignell *et al.*, 2008). However, this is not apparent with the evaporative stimulus applied in this study (Table 3.1). A small trend towards increased neuronal responses to ethyl chloride in SNL animals was observed over the course of this thesis (Appendix 8.1). Although ice and ethyl chloride are both considered noxious, the discrepancy in the neuronal response could be explained by the differences in the rate of cooling or the peak temperature decrease. Cooling of the skin towards 0°C and below may excite increasing numbers of C-fibres (Simone & Kajander, 1996).

Most clinical and pre-clinical evidence supports a contribution of central mechanisms to cold hypersensitivity. Alternatives include a peripheral sensitisation of cold fibres, though the evidence for this is sparse (Serra *et al.*, 2009). Whether TRPM8 is sensitised following nerve injury is currently unclear. A nerve injury induced increase in menthol currents in DRG neurones and increased proportions of menthol responsive neurones has been proposed to represent a peripheral neuronal substrate of cold hypersensitivity (Xing *et al.*, 2007). However transcriptional changes of TRPM8 are not always apparent (Katsura *et al.*, 2006). Several cellular processes modify temperature sensitivity and gating of TRPM8 *in vitro* (outlined in section 1.5.1.2), which may also be altered after injury and potentiate channel activity in the absence of significant changes in expression levels. Neurotrophic factors such as artemin and NGF also sensitise cold evoked behaviours in a TRPM8 dependent manner (Lippoldt *et al.*, 2013). Many gene changes occur following injury which affect neuronal excitability (Wang *et al.*, 2002). Refinement of thresholds of cold sensitive neurones is determined by differential expression of background potassium channels in subsets of TRPM8+ fibres (Madrid *et al.*, 2009; Descoeur *et al.*, 2011). A loss of K^+ currents could confer cold sensitivity to previously unresponsive primary afferents (Viana *et al.*, 2002). The data presented here does not necessarily discount a peripheral component in addition to central neuroplastic changes.

The consequences of changes in ion channel function are more likely to be apparent in a model of peripheral sensitisation such as with ciguatoxin. The resultant hyperalgesia induced by peripheral ciguatoxin-2 demonstrates mechanisms of cold hyperalgesia not necessarily dependent on TRPM8 and may share overlapping mechanisms with nerve injured conditions. Subcutaneous injection of 0.1-10 nM ciguatoxin-1 in human subjects displays the somatosensory hallmarks of systemic ciguatera poisoning, namely pruritus at low concentrations and cold hyperalgesia at higher concentrations. As with neuropathic patients, innocuous cold

temperatures elicit sensations of intense stabbing, pricking and burning pain (Zimmermann *et al.*, 2013). Ciguatoxin-2 exists as a diastereomer of ciguatoxin-3 and has not been extensively characterised owing to the difficulty of purification (Lewis *et al.*, 1993). Ciguatoxin-2 increased neuronal responses to innocuous and noxious cold stimulation in the absence of any obvious changes in central neuronal excitability consistent with a peripheral mechanism of action. As previously observed, this was not reversed by blockade of TRPM8 channels (Vetter *et al.*, 2012). In mice, Pacific ciguatoxin-1 and ciguatoxin-3 induce spontaneous flinching and increased nocifensive behaviours in response to cold temperatures (Vetter *et al.*, 2012; Zimmermann *et al.*, 2013). *In vitro*, ciguatoxin-1 confers cold sensitivity to a subset of non-cold responsive neurones dependent on the expression of TRPA1 (Vetter *et al.*, 2012). An increase in membrane excitability in these neurones appears partly related to the inhibition of transient and delayed rectifier potassium currents by ciguatoxin-1, the former of which is sensitive to block by 4-aminopyridine and influences thresholds for cold sensitivity (Viana *et al.*, 2002; Birinyi-Strachan *et al.*, 2005). Consistent with this, behavioural hypersensitivity can be reversed by flupirtine, an activator of K_v7 channels. In contrast, varied results are obtained with sodium channel blockers, with behavioural hypersensitivity most effectively reversed with a frequency dependent antagonist and to a lesser extent with those stabilising the inactive channel state (Zimmermann *et al.*, 2013). *In vitro* studies of the effect of ciguatoxins on tetrodotoxin sensitive and resistant sodium channels reveal a voltage shift in activation and inactivation, and increased rate of recovery from inactivated states respectively (Strachan *et al.*, 1999; Yamaoka *et al.*, 2009). Genetic and pharmacological approaches both support that ciguatoxin induced cold hyperalgesia is partly dependent on $Na_v1.8$ and tetrodotoxin sensitive channels, one of which does not appear to be $Na_v1.7$ (Vetter *et al.*, 2012). Increased mechanical sensitivity is not observed clinically, however ciguatoxin-2 induced a sensitisation to low threshold mechanical stimuli. This disparity is likely due to the higher potency and abundance of ciguatoxin-1 in contaminated fish (Lewis, 2006). Whether this effect is dependent on tetrodotoxin sensitive or resistant channels would be an interesting future line of investigation.

3.4.4 Summary

In conclusion, these data suggest that cold evoked neuronal responses are conserved in naïve rats following TRPM8 inhibition supporting the existence of other cold transducers, and that peripheral and central sensitisation may underlie the contribution of TRPM8 to cold hypersensitivity and hyperalgesia following nerve injury. The identification of selective antagonists will in future provide useful tools to investigate further the role of TRPM8 in the transduction of cold temperatures.

4. The Anti-Hyperalgesic Effects of a Novel TRPM8 Agonist in Neuropathic Rats: A Comparison with Topical Menthol

4.1 Introduction

Cold temperatures can be refreshing and relieving but also evoke sensations of aching, burning and pricking. The cold mimetic compound menthol has been used to alleviate several painful conditions including musculoskeletal pain (Taylor *et al.*, 2012). A case study has suggested high concentration topical menthol reduces ongoing pain in a patient undergoing chemotherapy (Colvin *et al.*, 2008), but also cold and pinprick hyperalgesia after peripheral neuropathy in a small of group of patients (Namer *et al.*, 2008; Wasner *et al.*, 2008). High concentration menthol applied to uninjured areas has been reported to induce cold hyperalgesia, pinprick hyperalgesia and in most cases secondary hyperalgesia (Wasner *et al.*, 2004; Hatem *et al.*, 2006; Binder *et al.*, 2011).

The cold and menthol sensitive channel TRPM8 has a multifaceted role in cold sensitivity. Selective fibre blocks suggest Aδ-fibres are likely responsible for the cooling effects of menthol and cold temperatures whereas C-fibres are implicated in the paradoxical burning sensation (Wahren *et al.*, 1989; Wasner *et al.*, 2004). Both menthol and cooling induced analgesia are dependent on TRPM8 (Proudfoot *et al.*, 2006; Liu *et al.*, 2013), though a TRPM8 negative population of cold sensitive primary afferents are also important. Whilst cooling is analgesic in both phases of the formalin test in wildtype mice, cooling induced analgesia is absent in the first phase, but not in the second phase, following genetic ablation of TRPM8 (Dhaka *et al.*, 2007). Through genetic or pharmacological approaches, TRPM8 has been implicated in the development of cold hypersensitivity in several pre-clinical models (Colburn *et al.*, 2007; Descoeur *et al.*, 2011; Knowlton *et al.*, 2011). Paradoxically blocking or activating TRPM8 channels may both be beneficial in neuropathic conditions. This has parallels to the TRPV1 channel, a member of the same family, where capsaicin can produce both pain and pain relief (O'Neill *et al.*, 2012).

Clinical trials involving topical menthol are currently being undertaken for chronic pain conditions including osteoarthritis (clinical trials identifier: NCT01565070), muscle pain (NCT01542827) and chemotherapy induced neuropathy (NCT01855607). To date the investigation of whether a systemically available TRPM8 agonist would have more potent anti-hyperalgesic effects has not been examined.

Hypotheses and Aims:

The mechanisms of menthol-induced hyperalgesia are hypothesised to overlap with the capsaicin induced hyperalgesia model. Initial studies will examine the back-translation and neural mechanisms of the menthol induced hyperalgesia model in naïve rats. Paradoxically, hyperalgesic concentrations of menthol in normal human subjects can be analgesic in some neuropathic patients; the analgesic effects of menthol in a rodent model of neuropathy will also be examined and compared to patient data. Finally, the effects of a novel TRPM8 agonist, M8-Ag, synthesised by Takeda Cambridge Ltd (Appendix 8.2) will be tested in a nerve injury model of cold hypersensitivity through a systemic route of administration as this may have more potent effects compared to a topical agonist. M8-Ag displays >100 fold selectivity over TRPA1 and activates TRPM8 channels within the nanomolar range (Appendix 8.2).

4.2 Materials and Methods

4.2.1 Animals

Male Sprague Dawley rats (250-300g), bred by the Biological Service Unit (UCL, London, UK), were used for behavioural and electrophysiological experiments.

4.2.2 Spinal nerve ligation (SNL) surgery

See section 2.2. SNL rats were used for either *in vivo* electrophysiology or behavioural testing and were euthanised at the end of the experiment.

4.2.3 Behavioural testing

See section 2.3.1 and 2.3.2. Ipsilateral and contralateral behavioural sensitivity to mechanical and cold stimuli was assessed in SNL and sham rats on day 14 post surgery. For M8-Ag study, only rats with significant cold hypersensitivity were used for analysis, which was defined as 2 or more withdrawals out of 5 on the nerve injured side (4/18 rats were excluded). Rats were then dosed intraperitoneally with vehicle (85% normal saline, 10% cremophor (Sigma, UK) 5% dimethylsulfoxide (Sigma, UK)) or 30 mg/kg M8-Ag in dose volume of 2 ml/kg. Behavioural testing was repeated 30, 60 and 90 minutes post dosing. The experimenter was blind to the treatment during behavioural testing. For menthol study, approximately 150 μl 1%, 10% and 40% L-menthol (Alfa Aesar, Heysham, UK) (in absolute ethanol) were cumulatively applied to the entire plantar surface of the left hind paw. Behavioural testing was repeated 15 minutes after each application. Concentrations of menthol were chosen based on efficacy seen in neuropathic patients (Namer *et al.*, 2008; Fallon *et al.*, 2015).

4.2.4 *In vivo* electrophysiology

See section 2.12, 2.14 and 2.15. *In vivo* electrophysiology was performed between day 15 and 18 post SNL or sham surgery and in age/weight matched naïve rats. Single WDR neurones were characterised for responses to repeated electrical stimulation followed by natural stimuli. For order of testing see figure 2.4*A, B.* A 1-minute rest period followed each stimulus. After three consecutive stable baseline responses to natural stimuli (<10% variation, data were averaged to give control values), sham and SNL animals were injected subcutaneously into the contralateral flank with 10 or 30 mg/kg M8-Ag. Doses were chosen based on preliminary pharmacokinetic data (data not shown). Responses to electrical and natural stimuli were next measured 30 minutes post dosing

and then every 20 minutes up to 110 minutes. In separate groups of rats, 1%, 10% and 40% L-menthol (Alfa Aesar, Heysham, UK) (in absolute ethanol) were cumulatively applied to the receptive field of naïve and SNL rats (approximately 20-30 µl). Neuronal responses were tested 15 minutes later. One neurone was characterised per rat.

4.2.5 Statistics

For *in vivo* electrophysiology measures, statistical significance was tested using a 1 way or 2 way repeated measures (RM) ANOVA, followed by a Bonferroni *post hoc* test for paired comparisons. Sphericity was tested using Mauchly's test, the Greenhouse-Geisser correction was applied if violated. Behavioural time-courses and receptive field sizes were tested with the Friedman test, followed by a Wilcoxon *post hoc* and Bonferroni correction for paired comparisons. Area under curve (AUC) values were calculated using the trapezoid rule and compared with a 1 way ANOVA followed by a Bonferroni *post hoc* test.

4.3 Results

4.3.1 Summary of behavioural and neuronal responses in naïve, sham and SNL rats

Behavioural sensitivity to mechanical and cooling stimuli was examined 14 days post sham or spinal nerve ligation (SNL) surgery and in age matched naïve rats. SNL rats displayed guarding behaviour of the injured ipsilateral hind paw, which was absent on the uninjured contralateral side and in sham operated rats. SNL rats, but not sham or naïve, displayed significantly reduced withdrawal thresholds to punctate mechanical stimulation (Wilcoxon test, $P<0.05$) (Fig. 4.1*A*) and increased withdrawals to acetone induced innocuous cooling (Wilcoxon test, $P<0.05$) (Fig. 4.1*B*) compared to contralateral responses.

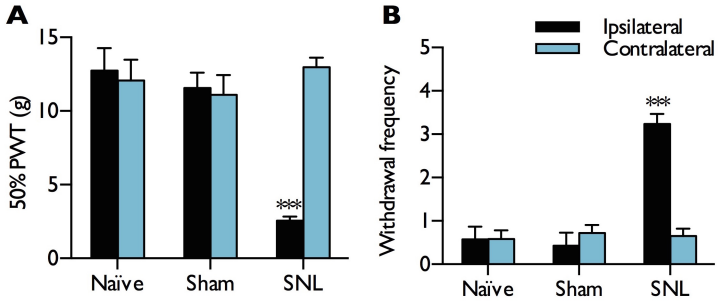


Figure 4.1 Behavioural hypersensitivity in SNL rats 14 days post surgery. (A) Unilateral ligation of L5 and L6 spinal nerves reduces mechanical withdrawal thresholds and **(B)** increases responsiveness to innocuous cooling in SNL rats. Sham rats exhibited similar behavioural sensitivity to naïve rats. Data represents mean ± SEM. *** $P<0.001$. Naïve $n=7$, Sham $n=7$, SNL $n=25$. PWT - Paw withdrawal threshold.

In vivo electrophysiology was performed to examine the effects of either menthol or M8-Ag on nociceptive processing by WDR spinal neurones in neuropathic and uninjured conditions. Neurones were characterised from depths relating to lamina V/VI in the deep dorsal horn (Watson *et al.*, 2009). Neurones were confirmed as WDR on the basis of responses to noxious heat, dynamic brushing and noxious punctate mechanical stimulation. Table 4.1 summarises neuronal depths from the surface of the cord recorded from and evoked baseline responses.

Table 4.1 Baseline characterisations of deep dorsal horn WDR neurones from naïve, sham and SNL rats. Range of neuronal depths recorded from in parentheses. Data represent mean ± SEM. (APs- action potentials)

	Naïve *n*=7	Sham *n*=7	SNL *n*=20
Depth (μm)	862 ± 68.72 (620 - 1080)	704 ± 66.22 (600 - 830)	693 ± 27.04 (530 - 870)
A threshold (mA)	0.03 ± 0.02	0.07 ± 0.02	0.09 ± 0.02
C threshold (mA)	0.29 ± 0.11	0.43 ± 0.16	0.38 ± 0.08
Aβ-evoked (APs)	102 ± 13.75	108 ± 6.94	106 ± 7.44
Aδ-evoked (APs)	178 ± 23.89	207 ± 21.94	231 ± 12.93
C-evoked (APs)	419 ± 45.45	484 ± 62.30	444 ± 30.01
Post-discharge (APs)	281 ± 47.22	312 ± 48.04	339 ± 28.44
Brush (APs)	411 ± 65.99	474 ± 51.06	411 ± 28.29
2g (APs)	22 ± 6.42	27 ± 8.85	28 ± 5.77
8g (APs)	305 ± 52.29	311 ± 31.91	332 ± 27.76
15g (APs)	554 ± 48.74	620 ± 66.70	598 ± 26.15
26g (APs)	855 ± 55.77	876 ± 63.47	896 ± 27.02
60g (APs)	1151 ± 77.44	1192 ± 47.53	1184 ± 33.28
35°C (APs)	172 ± 22.90	270 ± 61.58	210 ± 25.51
42°C (APs)	391 ± 32.25	570 ± 114.32	464 ± 72.28
45°C (APs)	562 ± 39.65	785 ± 109.61	617 ± 65.33
48°C (APs)	1041 ± 132.36	1151 ± 77.09	1074 ± 66.68
Acetone (APs)	75 ± 39.22	56 ± 36.17	78 ± 22.20
Ethyl chloride (APs)	411 ± 49.97	434 ± 53.32	557 ± 44.06

4.3.2 10% and 40% menthol alleviate cold hypersensitivity in spinal nerve ligated rats

Increasing concentrations of menthol were unilaterally and cumulatively applied to the left hind paw of SNL rats 14 days post surgery and weight/age matched naïve rats. Few rats displayed flinching/shaking behaviours post application of menthol (naïve 2/7, SNL 1/7). In naïve rats, no significant sensitisation (2/7 rats sensitised) to acetone induced innocuous cooling was observed following application of low and high concentrations of menthol (Friedman test, $P>0.05$) (Fig. 4.2*A*). In contrast, high concentrations of topical menthol reduced acetone-induced withdrawals in SNL rats (Friedman test, $P<0.05$, followed by Dunn's *post hoc* test) (Fig. 4.2*A*). Electrophysiological recordings of dorsal horn WDR neurones were made to examine spinal processing of threshold and supra-threshold stimuli. In naïve rats, menthol had no overall effect on neuronal responses to innocuous cooling (3/7 sensitised) or ethyl chloride induced noxious cooling (1 way RM ANOVA $P>0.05$, innocuous: $F_{3,18}=1.668$, noxious: $F_{3,18}=0.275$) (Fig. 4.2*B*). Only a weak trend towards reduced acetone evoked responses by 40% menthol was observed in SNL rats, with no effect on noxious cold responses (1 way RM ANOVA $P>0.05$, innocuous: $F_{3,15}=1.272$, noxious: $F_{3,15}=0.775$) (Fig. 4.2*C*). No ongoing neuronal activity was observed post menthol application in either SNL or naïve rats.

Menthol had minimal effects on mechanical sensitivity in naïve rats (Friedman test, *P*>0.05) (Fig. 4.2*D*), a feature that was mirrored by a lack of effect on mechanical coding of dorsal horn WDR neurones (2 way RM ANOVA *P*>0.05, $F_{3,15}$=0.318) (Fig. 4.2*E*). In SNL rats, menthol was unable significantly to attenuate mechanical hypersensitivity (Friedman test, *P*>0.05) (Fig. 4.2*D*), though 4/7 rats exhibited a small increase (~2-3 g) in withdrawal thresholds. Similarly neuronal responses to mechanical stimulation were also unaltered (2 way RM ANOVA *P*>0.05, $F_{3,15}$=0.517) (Fig. 4.2*F*).

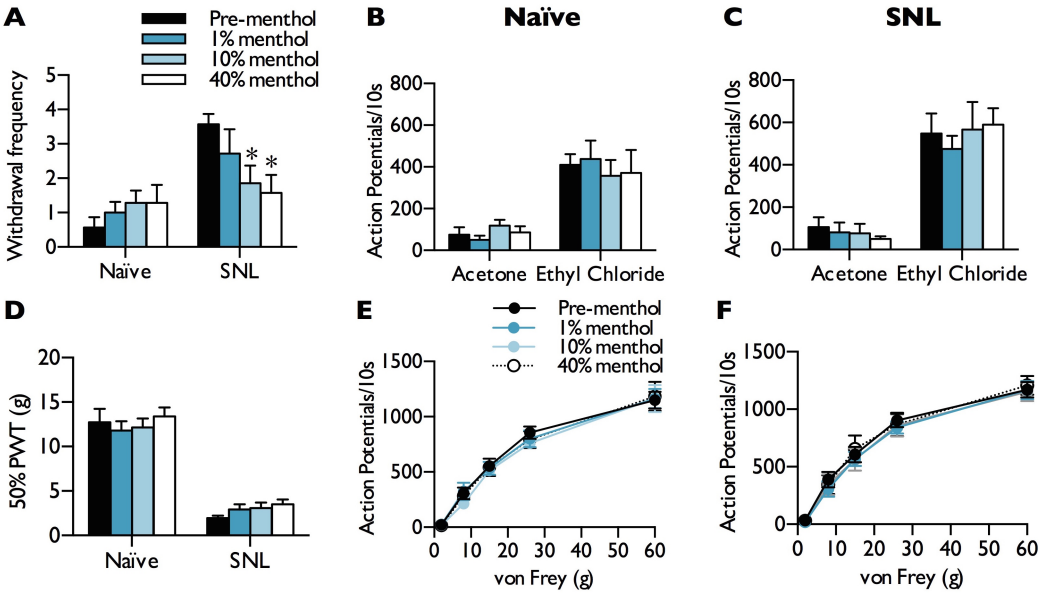


Figure 4.2 Behavioural and neuronal correlates of mechanical and cold sensitivity post menthol application in naïve and SNL rats. (A) Menthol alleviates cold hypersensitivity in SNL rats with no significant effect in naïve rats (*n*=7). Corresponding neuronal responses to innocuous and noxious cooling in **(B)** naïve rats (*n*=7) and **(C)** SNL rats (*n*=6). **(D)** Menthol does not significantly affect mechanical withdrawal thresholds in naïve or SNL rats. Neuronal responses to mechanical stimulation in **(E)** naïve and **(F)** SNL rats corroborates behavioural observations. Data represent mean ± SEM. **P*<0.05. (PWT – paw withdrawal threshold).

4.3.3 Menthol does not influence excitability of deep dorsal horn neurones in naïve and SNL rats

The effect of menthol on measures of neuronal excitability was also examined. High concentration menthol does not induce an expansion of receptive field sizes to innocuous (8 g) or noxious (60 g) punctate mechanical stimulation in naïve rats (Friedman test, *P*>0.05) (Fig. 4.3*A*) and were also unaltered in SNL rats (Friedman test, *P*>0.05) (Fig. 4.3*B*). In addition, menthol had no influence on neuronal responses to heat stimulation (2 way RM ANOVA *P*>0.05, Naïve: $F_{3,15}$=1.683, SNL: $F_{3,15}$=0.588) (Fig. 4.3*C, D*) or dynamic brushing (1 way RM ANOVA *P*>0.05, Naïve: $F_{1.14,6.83}$=1.163, SNL: $F_{3,15}$=0.814) (Fig. 4.3*E, F*) in naïve or SNL rats. Furthermore, menthol did not

sensitise Aδ- and C-fibre evoked responses or increase the wind-up and post-discharge of WDR neurones in naïve rats (1 way RM ANOVA $P>0.05$, Aδ: $F_{1.61,8.05}=0.805$, C: $F_{3,15}=0.674$, WU: $F_{3,15}=0.814$, PD: $F_{3,15}=0.071$) (Fig. 4.3*G*). After nerve injury, menthol was unable to reduce evoked activity attributed to primary afferent fibres or subsequent wind-up of spinal neurones (1 way RM ANOVA $P>0.05$, Aβ: $F_{3,15}=1.286$, Aδ: $F_{1.49,7.42}=1.668$, C: $F_{3,15}=0.334$, WU: $F_{3,15}=0.579$) (Fig. 4.3*H*).

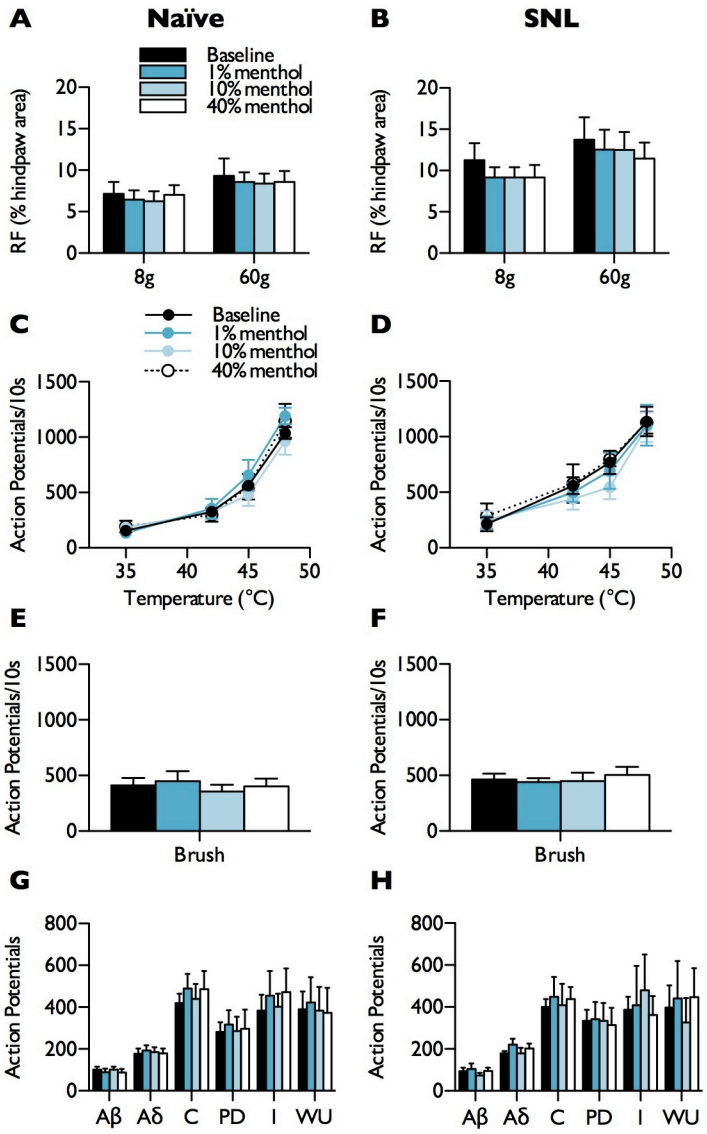


Figure 4.3 Neuronal responses to natural and electrical stimulation of the receptive field following menthol application in naïve and SNL rats. Following topical menthol application, no change in receptive field size was observed **(A, B)** or in heat **(C, D)**, dynamic brush **(E, F)** or electrically **(G, H)** evoked responses in naïve (left panels) or SNL rats (right panels). Data represent mean ± SEM, Naïve *n*=7, SNL *n*=6. (RF – receptive field, PD – post-discharge, WU – wind-up, I – input).

4.3.4 Neuronal responses to innocuous and noxious cooling attenuated by M8-Ag in spinal nerve ligated rats

In vivo electrophysiology was performed on SNL and sham rats between 15 and 18 days post surgery. After obtaining stable baseline neuronal recordings, rats were dosed subcutaneously with 10 or 30 mg/kg M8-Ag. In SNL rats, compared to baseline, 30 mg/kg M8-Ag inhibited neuronal responses to noxious cold stimulation with significantly reduced responses at 30, 70 and 110 minutes post dosing (1 way RM ANOVA $P<0.01$, $F_{2.64,13.19}=7.938$, followed by Bonferroni *post hoc* test) (Fig. 4.4*A*). In addition M8-Ag decreased overall neuronal responses to innocuous cooling with acetone (1 way RM ANOVA $P<0.05$, $F_{3,30}=4.383$) (Fig. 4.4*A*). These effects of M8-Ag were absent in sham-operated rats (1 way RM ANOVA $P>0.05$, innocuous: $F_{3,30}=0.197$, noxious: $F_{3,30}=0.116$) (Fig. 4.4*B*). Furthermore, 30 mg/kg M8-Ag did not alter neuronal responses to heat stimulation (2 way RM ANOVA $P>0.05$, sham: $F_{3,30}=1.284$, SNL: $F_{3,30}=0.781$) (Fig. 4.4*D, E*), punctate mechanical stimulation (2 way RM ANOVA $P>0.05$, sham: $F_{3,30}=1.976$, SNL: $F_{3,30}=0.969$) (Fig. 4.4*G, H*) or dynamic brushing of the receptive field (1 way RM ANOVA, $P>0.05$ sham: $F_{3,30}=0.668$, SNL: $F_{3,30}=0.823$) (Fig. 4.4*J, K*) in either sham or SNL rats. Neuronal events evoked by global stimulation of Aβ-, Aδ- and C-fibres were also unaffected in SNL and sham rats indicating activating TRMP8 does not increase overall afferent drive under normal (1 way RM ANOVA $P>0.05$, Aβ: $F_{5,30}=0.627$, Aδ: $F_{5,30}=2.536$, C: $F_{1.91,11.43}=2.572$) or pathological conditions (1 way RM ANOVA $P>0.05$, Aβ: $F_{5,30}=0.470$, Aδ: $F_{5,30}=0.720$, C: $F_{5,30}=1.381$) (Fig. 4.4*M, N*).

The effect of 10 mg/kg was also examined in SNL rats, however this lower dose had no effect on cold (1 way RM ANOVA $P>0.05$, innocuous: $F_{1.82,9.09}=0.667$, noxious: $F_{5,30}=2.275$) (Fig. 4.4*C*), heat (2 way RM ANOVA $P>0.05$, $F_{5,30}=0.801$) (Fig. 4.4*F*), mechanical (2 way RM ANOVA $P>0.05$, $F_{5,30}=0.652$) (Fig. 4.4*I*), brush (1 way RM ANOVA $P>0.05$, $F_{5,30}=2.205$) (Fig. 4.4*L*) or electrically (1 way RM ANOVA $P>0.05$, Aβ: $F_{5,30}=0.224$, Aδ: $F_{5,30}=0.462$, C: $F_{5,30}=0.760$, PD: $F_{5,30}=0.459$, I: $F_{5,30}=0.678$, WU: $F_{5,30}=1.658$) (Fig. 4.4*O*) evoked neuronal responses. Neuronal responses were also unaffected by vehicle alone (Appendix 8.3).

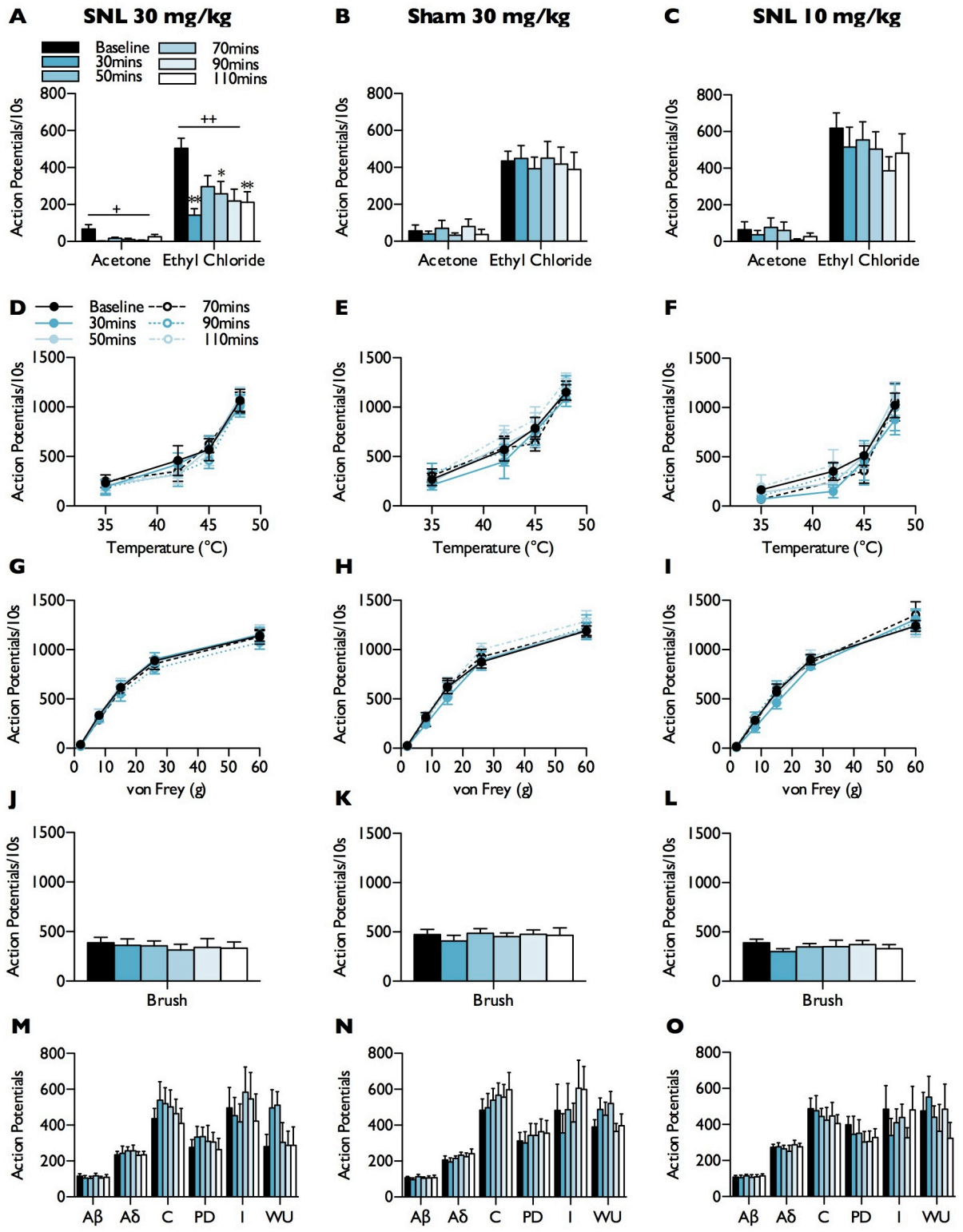


Figure 4.4 M8-Ag inhibits deep dorsal horn lamina V/VI neuronal responses to innocuous and noxious cold stimulation in spinal nerve ligated rats. After 30 minutes post dosing and then at 20 minute intervals, recordings were made of neuronal responses to cold **(A, B, C)**, heat **(D, E, F)**, punctate mechanical **(G, H, I)**, dynamic brush **(J, K, L)** and electrical stimuli **(M, N, O)**. (left panels – SNL 30 mg/kg, middle panels – sham 30 mg/kg, right panels – SNL 10 mg/kg). (+) represents statistically significant Main Effect (2 way RM ANOVA), asterisks denote significant difference from baseline (Bonferroni *post hoc*). **P*<0.05, ***P*<0.01, *n*=7. (PD – post-discharge, WU – wind-up, I – input).

4.3.5 M8-Ag reverses behavioural hypersensitivity to acetone induced cooling in SNL rats

The ability of M8-Ag to alleviate behavioural signs of cold hypersensitivity was examined in SNL rats 14 days after nerve ligation. Rats were dosed intraperitoneally with either 30 mg/kg M8-Ag or vehicle and tested up to 90 minutes post dosing. The majority of rats dosed with M8-Ag exhibited sporadic 'wet dog shakes' up to an hour post dosing (7/9). These behaviours were absent in vehicle treated rats. M8-Ag reduced the behavioural response to innocuous evaporative cooling of the injured paw compared to pre-drug responses (Friedman test $P<0.01$, followed by Wilcoxon *post hoc* and Bonferroni correction) (Fig. 4.5*A*), whereas the vehicle alone had no significant effect (Friedman test, $P>0.05$) (Fig. 4.5*A*). Contralateral responses to acetone induced cooling were minimal in comparison and were not affected by M8-Ag (Friedman test, $P>0.05$) (Fig. 4.5*B*). Overall behavioural signs of cold hypersensitivity are reduced by M8-Ag but not by vehicle treatment (1 way ANOVA $P<0.01$, $F_{3,24}=10.537$, followed by Bonferroni *post hoc*) (Fig. 4.5*C*). The behavioural observations correlate with electrophysiological recordings of neuronal responses to acetone. Comparing the effects of 30 mg/kg M8-Ag on total neuronal events in uninjured and nerve ligated rats between 30 and 110 minutes post dosing reveals cooling evoked responses were significantly reduced in SNL rats (unpaired Student's t-test, $P<0.05$) (Fig. 4.5*D*)

M8-Ag did not alter mechanically evoked withdrawals on either the ipsilateral (Fig. 4.5*E*) or contralateral (Fig. 4.5*F*) side compared to pre-drug values (Friedman test, $P>0.05$). AUC analysis also confirms no overall effect of either drug or vehicle treatment on mechanical sensitivity (1 way ANOVA $P<0.001$, $F_{3,32}=61.515$, followed by Bonferroni *post hoc*) (Fig. 4.5*G*). Correspondingly, M8-Ag did not affect mechanical coding of WDR neurones to below threshold and threshold stimuli in SNL or sham rats (2 way ANOVA $P>0.05$, $F_{1,36}=0.006$) (Fig. 4.5*H*).

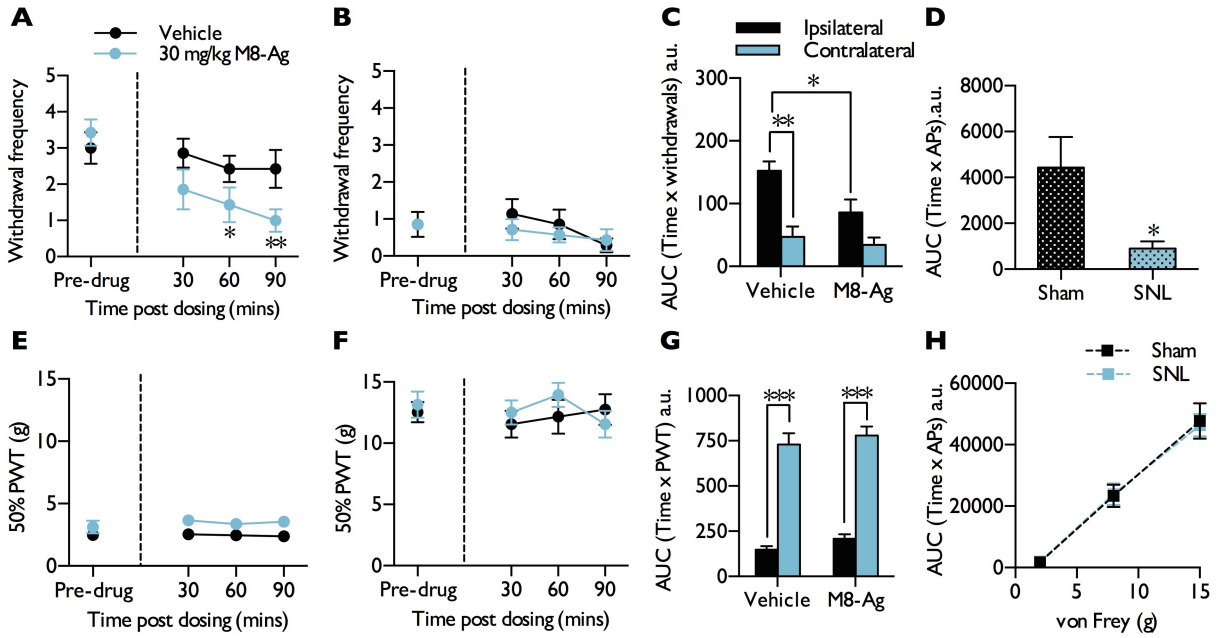


Figure 4.5 M8-Ag selectively reduces behavioural and neuronal responses to cooling following spinal nerve ligation. (A) 30 mg/kg M8-Ag reversed the behavioural response to acetone induced cooling on the nerve injured ipsilateral side compared to pre-drug values (*n*=7), whereas vehicle alone had no significant effect (*n*=7). **(B)** Contralateral responses to cooling were not affected by either treatment. **(C)** AUC analysis confirms a significant attenuation of cold hypersensitivity by M8-Ag compared to vehicle treatment. **(D)** A corresponding decrease in overall neuronal responses to acetone after dosing of M8-Ag was also observed in SNL rats (*n*=7) compared to sham rats (*n*=7). M8-Ag did not alter mechanical withdrawal thresholds of **(E)** the injured ipsilateral paw or **(F)** the uninjured contralateral side (*n*=9). **(G)** AUC analysis confirms that rats exhibited significant mechanical hypersensitivity, which was not affected by M8-Ag or vehicle treatment. **(H)** Neuronal responses to mechanical stimulation in SNL (*n*=7) and sham rats (*n*=7) were also unaffected by 30 mg/kg M8-Ag. Data represent mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001. (a.u. - arbitrary units, AUC - area under curve, PWT - paw withdrawal threshold, APs - action potentials).

4.4 Discussion

Several studies support menthol-induced hyperalgesia as a rapidly inducing surrogate model of cold hypersensitivity in human subjects (Wasner *et al.*, 2004; Hatem *et al.*, 2006; Namer *et al.*, 2008; Binder *et al.*, 2011). In addition, menthol also possesses analgesic and counterirritant properties (Wasner *et al.*, 2008; Olsen *et al.*, 2014). However, some discrepancies exist between these human studies, but also in animal models as menthol has been proposed to have complex pro- and anti-nociceptive effects in both uninjured and injured animals (Galeotti *et al.*, 2002; Proudfoot *et al.*, 2006; Brignell *et al.*, 2008; Caspani *et al.*, 2009; Gentry *et al.*, 2010; Klein *et al.*, 2010; Klein *et al.*, 2012; Gao *et al.*, 2013; Liu *et al.*, 2013). By combining behavioural measures and electrophysiological recordings of lamina V/VI neurones, the translational value of this model in uninjured and neuropathic rats was examined (summarised in Table 4.2 and Table 4.3). This study also investigates the effects of activating TRPM8, with a novel systemically available agonist, on behavioural and neuronal correlates of cold sensitivity in neuropathic animals. In chapter 3, antagonism of the TRPM8 receptor was shown to inhibit behavioural and spinal neuronal responses to innocuous and noxious cooling in SNL but not naïve rats. Interestingly, the TRPM8 agonist reported here displayed identical effects. This would imply TRPM8 is not essential for all forms of cold transduction in the absence of injury in rats and the effects of modulators of TRPM8 are dependent on changes induced by the pathophysiological state.

4.4.1 Menthol induced hyperalgesia as a translational model

Increasing concentrations of menthol applied topically to the hind paw of naïve rats rarely sensitised cooling evoked withdrawals and neuronal responses. Furthermore, menthol had no effect on noxious cold evoked neuronal responses. Previous studies have demonstrated that menthol sensitises peripheral afferents and trigeminal neurones to cold stimulation (Hensel & Zotterman, 1951; Schafer *et al.*, 1986; Zanotto *et al.*, 2007), and that TRPM8 antagonists can reverse menthol-induced activity in peripheral nerve endings (Madrid *et al.*, 2006). Menthol does not appear consistently to induce ongoing pain like behaviours in rats when applied to the paw mirrored by an absence of ongoing spinal neuronal activity. Menthol may however render cool temperatures more aversive in temperature preference assays (Klein *et al.*, 2010).

Menthol induced hyperalgesia was designed as a surrogate human model of cold hyperalgesia analogous to the capsaicin model and some parallels may exist between the two. Hyperexcitability of dorsal horn neurones is considered the neural basis of capsaicin-induced hyperalgesia where a prolonged stimulus results in ongoing Aδ- and C-fibre activity and subsequent wind-up, central sensitisation and secondary hyperalgesia (reviewed by

O'Neill *et al.*, 2012). Menthol does not appear to induce comparable changes in neuronal excitability in the rat, which in all likelihood reflects low overall afferent drive from TRPM8+ fibres. WDR neurones did not exhibit potentiated wind-up post menthol application; a similar lack of effect on a perceptual correlate of wind-up was observed in human subjects (Wasner *et al.*, 2004; Binder *et al.*, 2011). In addition, no expansion of receptive field sizes to below threshold (8 g) and noxious punctate mechanical (60 g) stimuli occurred. Total neuronal events evoked by electrical activation of Aδ- and C-fibres were also unaffected, though it is possible that the supra-threshold nature of the electrical stimulus masked the peripheral sensitisation of this small population of cold and menthol responsive primary afferents. All studies in humans have demonstrated an increase in the cold pain threshold (i.e. cold pain experienced at warmer temperatures) following topical menthol with varied effects on other modalities (Table 4.2). Binder *et al.*, reported incidences of secondary hyperalgesia and pin prick hyperalgesia as well as heat pain, though over longer time periods than other comparable studies. In a pilot study, no changes in evoked neuronal activity by 10% or 40% menthol up to 80 minutes post application were observed (data not shown).

Table 4.2 Comparison of effects of menthol in naïve rats and normal human subjects. + increased response frequently observed, +/- occasionally observed, - never/rarely observed, blank – not tested.

	Rat	Normal Human Subjects					
	Naïve	*n*=20	*n*=12	*n*=39	*n*=10	*n*=10	*n*=10
Increase in:		(Andersen *et al.*, 2015)	(Binder *et al.*, 2011)	(Hatem *et al.*, 2006)	(Wasner *et al.*, 2004)	(Namer *et al.*, 2008)	(Olsen *et al.*, 2014)
Dynamic mechanical	-	+	+/-	-	-	+/-	
Punctate mechanical	-	+	+	-	+		+
Cold	+/-	+	+	+	+	+	+
Heat	-	-	+	-	-	-	-
Wind-up	-	-	-		-		
Secondary hyperalgesia/ Receptive field	-	+	+		+/-		

4.4.2 Back translation of menthol analgesia in a rat model of neuropathy

The impact of topical menthol in neuropathic patients has been less extensively examined compared to normal subjects. Heterogeneity in the mechanisms of cold allodynia complicates direct comparisons with each other and with pre-clinical models. As a case in point, menthol appears to exacerbate wind-up like pain in amputees (Vase *et al.*, 2013), whereas menthol reversed wind-up in a case of chemotherapy induced neuropathy (Colvin *et al.*, 2008). The effects of topical menthol in a nerve injury model exhibiting cold hypersensitivity were examined (Table 4.3). The analgesic properties of menthol in SNL rats appeared restricted to alleviating cooling

hypersensitivity though with no effect on innocuous or noxious cold evoked neuronal responses. During behavioural characterisations, menthol was applied to the entire plantar surface, whereas with the electrophysiological characterisations, application was restricted to the receptive field of the neurone. Given the paucity of cutaneous TRPM8+ afferents (Takashima *et al.*, 2007), the spatial summation of menthol-evoked activity may be critical in mediating analgesia. Systemic M8-Ag alleviated cold hypersensitivity after SNL and reduced WDR neuronal responses to both innocuous and noxious cooling. The apparent lack of effect of menthol on noxious cold evoked neuronal activity might be due to the maximum concentration levels achievable through dermal diffusion. In neuropathic patients though, the effects of menthol on noxious cold temperatures is difficult to ascertain, as pain is reported at threshold temperatures and further decreases in temperature cannot be tolerated. These data could be consistent with a restoration of spinal gating of cold temperatures at threshold levels by menthol but not of noxious temperatures.

Table 4.3 Comparison of effects of menthol in neuropathic rats and neuropathic patients.

+ decreased response frequently observed, +/- occasionally observed, - never/rarely observed, blank – not tested. (CI – cold injury, PNP – polyneuropathy, PHN – post herpetic neuralgia, CPSP – central post stroke pain, CIN – chemotherapy induced neuropathy)

	Rat	Neuropathic Patients			
Decrease in:	SNL	PNP/PHN/CPSP *n*=8 (Wasner *et al.*, 2008)	CI *n*=12 (Namer *et al.*, 2008)	CIN *n*=51 (Fallon *et al.*, 2015)	PHN *n*=1 (Davies *et al.*, 2002)
Dynamic mechanical	-	-	-	+/-	-
Punctate mechanical	+/-	+/-	+/-	+/-	-
Cold	+	+	+/-	+	
Heat	-		- (increased)	+	
Wind-up	-			+/-	
Receptive field	-			+	
Ongoing pain				+	+

4.4.3 Mechanisms of TRPM8 mediated analgesia

The effects of TRPM8 ligands could result from different consequences of activation of TRPM8 at peripheral levels and this may be more apparent with topical application. Activation of cutaneous TRPV1 by capsaicin can be sensitising but also have a desensitising effect due to a combination of acute desensitisation, tachyphylaxis and withdrawal of epidermal nerve fibres (O'Neill *et al.*, 2012). The mechanism of desensitisation differs between chemical agonists of TRPM8 (Kuhn *et al.*, 2009). Systemic M8-Ag reversal of cold hypersensitivity and hyperalgesia could result from a depolarisation block of TRPM8+ afferents through Ca^{2+} dependent second

messenger pathways downregulating TRPM8 activity to prevent calcium excitotoxicity through prolonged activation of channels (Premkumar *et al.*, 2005; Daniels *et al.*, 2009).

There could also be central consequences, either as a result of peripheral TRPM8 targeting or actions on the receptor elsewhere – these could be more marked with systemic dosing. Following nerve damage, a disinhibition hypothesis has been proposed to underlie the paradoxical burning associated with small decreases in temperature (Ochoa & Yarnitsky, 1994). If sensitisation of Aδ- and C-fibres underlies the hyperalgesia associated with topical menthol, in an already sensitised state, rather than exacerbate cold hyperalgesia, activation of TRPM8 expressing afferents with menthol or M8-Ag may recruit inhibitory circuits within the dorsal horn restoring a loss of spinal inhibition (Wasner *et al.*, 2008). The paradox of a noxious stimulus inducing pain and analgesia according to context is also demonstrable with noxious cold temperatures applied to areas sensitised by capsaicin. The perception of relief could in part be determined by competing aversive and appetitive states converging on descending inhibitory pathways (Mohr *et al.*, 2008). fMRI analysis suggests the supraspinal integration of ascending spinal activity and engagement of descending inhibitory pathways through the PAG shapes relief or pain associated with topical menthol or cooling (Seifert & Maihofner, 2007; Mohr *et al.*, 2008; Lindstedt *et al.*, 2011). Although not directly related to the present study, patients with post-stroke pain often have abnormal cold sensitivity and this is thought to be due to damage to a cool-signaling lateral thalamic pathway that causes a disinhibition of a medial thalamic pathway promoting pain, resulting in the observed burning, cold, ongoing pain and cold allodynia (Greenspan *et al.*, 2004). It is possible that peripheral and then spinal mechanisms via TRPM8 could finally impact on this system.

In terms of central mechanisms, TRPM8+ afferents predominantly innervate the superficial dorsal horn though some afferents terminate in the deeper laminae (Takashima *et al.*, 2007; Dhaka *et al.*, 2008). Cold temperatures activate a distinct sub-population of spinothalamic and spinoparabrachical lamina I neurones described as 'cool' responsive or 'polymodal-nocicpetive' (Bester *et al.*, 2000; Andrew & Craig, 2002). These responses converge onto lamina V/VI neurones, which exhibit graded intensity dependent responses to decreasing temperatures (Khasabov *et al.*, 2001). Under normal conditions local glycinergic and GABAergic inhibitory interneurones tonically control inhibitory tone within the dorsal horn and excitability of projection neurones (Takazawa & MacDermott, 2010). A substantial cross inhibition exists within the dorsal horn whereby low and high threshold afferents can moderate central neuronal activity (Lu & Perl, 2003; McCoy *et al.*, 2013). Group III metabotropic glutamate receptors are highly expressed in the superficial laminae of the dorsal horn, as are group II receptors, which are primarily associated with interneurones in lamina IIi, in

proximity to small myelinated fibres, with a large overlap with GABAergic terminals (Li *et al.*, 1997a; Jia *et al.*, 1999; Azkue *et al.*, 2001). Both groups of receptors can regulate inhibitory tone within the dorsal horn and are subject to expressional changes following injury (Chen & Pan, 2005; Goudet *et al.*, 2008; Zhou *et al.*, 2011). This neuroplasticity is the likely determinant of the reversal of icilin-induced analgesia by group II and III antagonists LY341495 and UBP1112 in neuropathic mice (Proudfoot *et al.*, 2006). TRPM8+ afferents also synapse near GABAergic terminals in the superficial dorsal horn (Dhaka *et al.*, 2008). Menthol evokes excitatory postsynaptic potentials in GABAergic interneurones, presumed to activate cold afferents, subsequently gating transmission in projection neurones (Zheng *et al.*, 2010).

Activation of endogenous opioid signalling pathways by menthol has also been implicated in analgesia. Contradictory reports exist of the reversal of icilin and menthol analgesia by naloxone. The discord between these studies could be attributed to the systemic route of administration of menthol (Galeotti *et al.*, 2002; Liu *et al.*, 2013) as opposed to topically applied icilin (Proudfoot *et al.*, 2006), as centrally acting menthol could reduce neuronal excitability through TRPM8 independent partly opioid mechanisms (Pan *et al.*, 2012). Several molecular targets of menthol-mediated analgesia have been proposed including the cumulative inactivation of sodium channels (Gaudioso *et al.*, 2012), activation of $GABA_A$ currents (Zhang *et al.*, 2008), and inhibition of 5-HT_3 receptors (Ashoor *et al.*, 2013). The efficacy of menthol is lost in TRPM8 knockout mice suggesting these non-TRP mechanisms comprise minor components of anti-hyperalgesia by menthol (Liu *et al.*, 2013).

4.4.4 Summary

In summary, the data presented here suggest that some aspects of menthol-induced analgesia are comparable between rats and humans, whereas menthol does not appear to induce central sensitisation as appears to be the case in normal human subjects. Furthermore, M8-Ag attenuated cold behaviours and neuronal responses in neuropathic rats but not in the absence of injury. Thus, overall, an interplay between complex peripheral and central effects appear to underlie the bi-directional effects of TRPM8 ligands and changes in these functions appear to be driven by damage to peripheral nerves.

5. Reduced Cold and Mechanical Sensitivity in $\alpha_2\delta$-1 -/- Mice

5.1 Introduction

$\alpha_2\delta$-1 subunits are auxiliary components of voltage gated calcium channels (VGCCs) (discussed further in chapter 6). The main biophysical and pharmacological properties of VGCCs are determined by the pore forming α_1 subunit, whereas the $\alpha_2\delta$, β and γ components influence trafficking and activation kinetics (Arikkath & Campbell, 2003). Of particular interest, the $\alpha_2\delta$-1 subunit is the most extensively studied variant and is the key molecular target for the anti-hyperalgesic effects of gabapentin and pregabalin (Field *et al.*, 2006). Much insight has been gained into the cellular and synaptic functions of $\alpha_2\delta$, however relatively little is known about the normal physiological processes dependent on $\alpha_2\delta$-1 expression.

5.11 Topology of $\alpha_2\delta$ subunits

To date four mammalian genes have been cloned (CACNA2D1, CACNA2D2, CACNA2D3, CACNA2D4) encoding $\alpha_2\delta$-1, $\alpha_2\delta$-2, $\alpha_2\delta$-3 and $\alpha_2\delta$-4 respectively. The range of $\alpha_2\delta$ variants is expanded through alternate splicing in three regions described as A, B, and C. The tissue selective expression of splice variants may have implications for the functional impact on calcium currents (Kim *et al.*, 1992; Angelotti & Hofmann, 1996; Qin *et al.*, 2002; Lana *et al.*, 2014). All subunits are products of a single gene cleaved post-translation and joined by disulphide bridges (De Jongh *et al.*, 1990). Rather than having a trans-membrane domain as previously predicted from sequence analysis, $\alpha_2\delta$ subunits are associated with plasma membranes through a glycosylphosphatidylinositol (GPI) anchor on the δ domain (Davies *et al.*, 2010). Detailed structural analysis is limited, but all $\alpha_2\delta$ subunits contain a metal ion-dependent adhesion site motif and von Willebrand factor A (VWA) domain necessary for trafficking of α_1 subunits of calcium channels (Canti *et al.*, 2005).

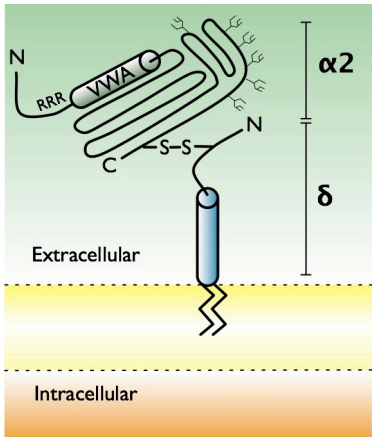


Figure 5.1 Topology of $\alpha_2\delta$ subunits. Disulphide linked α and δ subunits are anchored to the plasma membrane through a GPI anchor. Putative N-glycosylation sites have been identified on both domains. Approximate position of VWA domain shown in close proximity to gabapentin binding site in $\alpha_2\delta$-1 and $\alpha_2\delta$-2 (RRR). (Modified from Bauer *et al.*, 2010).

5.12 Distribution of $\alpha_2\delta$ subunits

In the cerebellum, $\alpha_2\delta$-2 subunits are concentrated within lipid raft microdomains (Davies *et al.*, 2006a). Lipid raft association has not been confirmed within the dorsal horn or in primary afferent fibres where $\alpha_2\delta$-1 has been shown to be anterogradely transported following nerve ligation (Bauer *et al.*, 2009). The localisation of $\alpha_2\delta$ to lipid rafts could have consequences for nociceptive transduction as depletion of cholesterol enhances peak calcium currents (Davies *et al.*, 2006a).

Within the spinal cord, $\alpha_2\delta$-1 is predominantly expressed pre-synaptically in the dorsal horn in the superficial laminae with moderate expression post-synaptically on deeper neurones (Li *et al.*, 2006; Bauer *et al.*, 2009). The pre-synaptic expression is most dense in a subset of small diameter DRG neurones, likely to be C-fibres, with moderate levels in a minor proportion of large diameter neurones (Taylor & Garrido, 2008). Within the 'pain matrix', moderate to strong expression has also been observed in the dorsal raphe, locus coeruleus and amygdala (Cole *et al.*, 2005; Taylor & Garrido, 2008). $\alpha_2\delta$-2 is also widely expressed throughout the CNS with a strong association with GABAergic interneurones in the thalamus, cerebellar Purkinje cells and hippocampus (Cole *et al.*, 2005). $\alpha_2\delta$-2 is also present in the amygdala, thalamus and periaqueductal grey (Cole *et al.*, 2005). In mice $\alpha_2\delta$-3 appears to be expressed exclusively in the brain, however in humans it is also present in the heart and skeletal muscle (Klugbauer *et al.*, 1999; Gong *et al.*, 2001). $\alpha_2\delta$-4 is largely found in the retina and endocrine organs with low levels in the CNS (Qin *et al.*, 2002; Wycisk *et al.*, 2006). The lack of availability of selective antibodies for $\alpha_2\delta$-2, $\alpha_2\delta$-3 and $\alpha_2\delta$-4, has hampered co-localisation studies.

Table 5.1 Summary of tissue distribution and function of $\alpha_2\delta$ subunits. (Modified from Arikkath & Campbell, 2003a and Dolphin, 2013).

Subunit	Distribution	Phenotype of knockout/mutation
$\alpha_2\delta$-1	Brain, spinal cord, heart, skeletal muscle (Gurnett *et al.*, 1997)	Knockout – Reduced myocardial contractibility, bladder dilatation (Fuller-Bicer *et al.*, 2009)
$\alpha_2\delta$-2	Lung, testis, brain, heart, pancreas, prostrate, skeletal muscle, spinal cord (Klugbauer *et al.*, 1999; Gao *et al.*, 2000)	ducky (du/du), du(2J) mutation – ataxia/seizures (Barclay *et al.*, 2001)
$\alpha_2\delta$-3	Brain, heart, skeletal muscle (Klugbauer *et al.*, 1999; Gong *et al.*, 2001)	Knockout – Impaired auditory processing, synaesthesia, impaired thermal nociception (Neely *et al.*, 2010; Pirone *et al.*, 2014)
$\alpha_2\delta$-4	Retina, pituitary, adrenal gland, colon (Qin *et al.*, 2002; Wycisk *et al.*, 2006)	Frame-shift mutation – retinal dystrophy (Wycisk *et al.*, 2006)

5.13 Effects on calcium channel kinetics

In vitro $\alpha_2\delta$ subunits indiscriminately associate with α_1 subunits of VGCCs (Table 5.2); the tissue selective expression of various calcium channel components likely determine the composition and biophysical properties of heteromeric complexes *in vivo* (Dolphin, 2013). In general, in heterologous expression systems, $\alpha_2\delta$ subunits increase maximum current density and accelerate activation and inactivation of calcium currents (Table 5.2 and Fig. 5.2). Splice variants of $\alpha_2\delta$-1 do not appear to exert significantly different effects on N-type current properties (Lana *et al.*, 2014). In comparison, various splice variants of $\alpha_2\delta$-2 can alter T-type calcium current density with minimal impact on activation kinetics (Hobom *et al.*, 2000). The influence of $\alpha_2\delta$ subunits alone on calcium channel kinetics is difficult to determine as expression systems have low endogenous levels of β subunits, which are required for membrane expression but also influence channel activation (Dolphin, 2013). In the absence of β subunits, $\alpha_2\delta$ subunits do not consistently exert influences on calcium channel properties (De Waard & Campbell, 1995; Klugbauer *et al.*, 1999; Hobom *et al.*, 2000) but can potentiate the effects of the β subunit when co-expressed (De Waard & Campbell, 1995). The increase in current density is dependent on $\alpha_2\delta$ subunits enhancing trafficking of α_1 to the cell membrane rather than directly influencing channel kinetics (Felix *et al.*, 1997). $\alpha_2\delta$-1 may also stabilise calcium channels at the cell surface as its absence promotes internalisation and degradation of channel complexes (Bernstein & Jones, 2007), though this is not observed in neuronal cultures (Cassidy *et al.*, 2014).

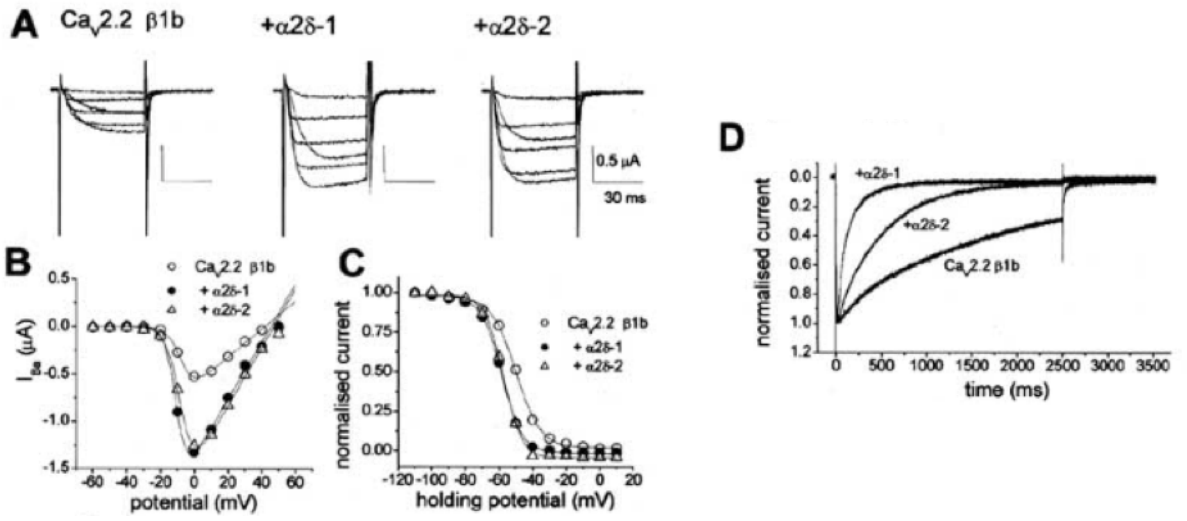


Figure 5.2 Effects of $\alpha_2\delta$-1 and $\alpha_2\delta$-2 on $Ca_v2.2$ (N-type)/β1b currents. (A) Example I_{Ba} in presence and absence of $\alpha_2\delta$ subunits. **(B)** Representative current-voltage relationships. **(C)** Representative steady-state inactivation curves. **(D)** Examples of currents normalised to show relative effects on inactivation kinetics. (Taken from Cantí *et al.*, 2003).

Table 5.2 Summary of $\alpha_2\delta$ effects on VGCC kinetics in expression systems.

$\alpha_2\delta$	α1	β	Effect on calcium currents	Reference
$\alpha_2\delta$-1	α_{1A} (P/Q-type)	None	No change in current amplitude or voltage dependence of activation or inactivation	(De Waard & Campbell, 1995)
	α_{1A} (P/Q-type)	β1a, β2a, β3, β4	Increased inactivation, potentiation of peak current increase by β subunit	(De Waard & Campbell, 1995)
	α_{1A} (P/Q-type)	β2a	Increased current amplitude, hyperpolarising shift in channel inactivation	(Hobom *et al.*, 2000)
	α_{1B} (N-type)	β3	Increased current amplitude, increase channel inactivation	(Gao *et al.*, 2000)
	α_{1C} (L-type)	β2a	Increased current amplitude, hyperpolarising shift in channel activation and inactivation	(Klugbauer *et al.*, 1999)
	α_{1E} (R-type)	β3	Increased current amplitude, hyperpolarising shift in channel inactivation	(Klugbauer *et al.*, 1999)
	α_{1G} (T-type)	None	No effect	(Hobom *et al.*, 2000)
$\alpha_2\delta$-2	α_{1A} (P/Q-type)	β2a	Increased current amplitude, hyperpolarising shift in channel inactivation, decrease time constant of activation	(Hobom *et al.*, 2000)
	α_{1B} (N-type)	β3	Increased current amplitude	(Gao *et al.*, 2000)
	α_{1C} (L type)	β3	Increased current amplitude	(Gao *et al.*, 2000)
	α_{1C} (L type)	β2a	Increased current amplitude, hyperpolarising shift in channel activation and inactivation	(Hobom *et al.*, 2000)
	α_{1E} (R-type)	β3	Increased current amplitude, hyperpolarising shift in channel activation and inactivation	(Hobom *et al.*, 2000)
	α_{1G} (T-type)	β3	Increased current amplitude	(Gao *et al.*, 2000)
	α_{1G} (T-type)	None	Increased current amplitude, hyperpolarising shift in channel inactivation, accelerates decay and steady state inactivation	(Hobom *et al.*, 2000)
$\alpha_2\delta$-3	α_{1C} (L-type)	None	No effect	(Klugbauer *et al.*, 1999)
	α_{1C} (L-type)	β2a	Increased current amplitude, hyperpolarising shift in channel activation and inactivation	(Klugbauer *et al.*, 1999)
	α_{1E} (R-type)	β3	Increased current amplitude, hyperpolarising shift in channel activation and inactivation	(Klugbauer *et al.*, 1999)
$\alpha_2\delta$-4	α_{1C} (L-type)	β3	Increased current amplitude	(Qin *et al.*, 2002)

5.14 $\alpha_2\delta$ subunits and synaptogenesis

$\alpha_2\delta$ subunits have been implicated in mediating synapse formation independent from their role with calcium channels. Through the WVA domain, $\alpha_2\delta$-1 interacts with the epidermal growth factor like domain of astrocyte-derived thrombospondins, which mediate synaptic adhesion and promote establishment of synapses

(Christopherson *et al.*, 2005; Eroglu *et al.*, 2009). RNA knockdown of $\alpha_2\delta$-1 inhibits synapse formation between cultured retinal ganglion cells, whereas *in vivo* the overexpression of $\alpha_2\delta$-1 increases the number of cortical excitatory synapses (Eroglu *et al.*, 2009). Lesioning of whiskers in neonatal mice induces synaptic remodelling within the barrel cortex (a somatotopic representation of the mystacial pads), a process that is sensitive to gabapentin inhibition, presumably by disrupting the interaction between $\alpha_2\delta$-1 and thrombospondins (Eroglu *et al.*, 2009). In Drosophila deficient in $\alpha_2\delta$-3 expression, embryos lack synaptic boutons at the neuromuscular junction despite normal navigation of motor neurones and formation of growth cones; cytoskeleton deficiencies are also apparent in addition to an absence of synaptic α_1 subunit expression and evoked transmission (Kurshan *et al.*, 2009). $\alpha_2\delta$-3 null mice on the other hand do not appear to present with any significant motor deficits (Neely *et al.*, 2010). RNA knockdown in Drosophila and gene ablation in mice results in impaired heat nociception. In mice this was attributed to impaired thalamocortical processing (Neely *et al.*, 2010). Surprisingly, in response to tactile and heat stimulation, $\alpha_2\delta$-3 knockout mice also display cross activation of the visual cortex, auditory cortex and olfactory tubercle. This would certainly be consistent with abnormalities in the formation of synaptic circuits within the brain.

5.15 Role of $\alpha_2\delta$-1 in experimental neuropathy

Transcriptional alterations that occur after nerve injury are considered to be an adaptive response to preserve neuronal function. These changes can contribute to neuronal hyperexcitability and spinal plasticity in neuropathic pain (Hokfelt *et al.*, 1994). Numerous studies have identified an up-regulation of $\alpha_2\delta$-1 in DRG neurones and the spinal cord after nerve injury; supra-spinal changes have not been examined in any great detail (Luo *et al.*, 2001; Costigan *et al.*, 2002; Wang *et al.*, 2002; Xiao *et al.*, 2002; Li *et al.*, 2004; Boroujerdi *et al.*, 2008; Bauer *et al.*, 2009). Spinal nerve ligation increases levels of $\alpha_2\delta$-1 in pre-synaptic terminals of primary afferent fibres in the dorsal horn in addition to an accumulation proximal to the ligation indicative of anterograde trafficking (Bauer *et al.*, 2009). Ectopic discharges have been hypothesised to be a key driving factor in pathology induced expressional changes of $\alpha_2\delta$-1 as lidocaine application to spinal nerves at the time of ligation reduces aberrant afferent activity, and delays up-regulation of $\alpha_2\delta$-1 and presentation of neuropathic behaviours (Boroujerdi *et al.*, 2008). Elevated DRG expression of $\alpha_2\delta$-1 is readily detectable after SNL peaking 7 days after injury and declining after several months, a feature that temporally correlates with the emergence and cessation of neuropathic like evoked behaviours (Luo *et al.*, 2001). A circadian fluctuation in DRG $\alpha_2\delta$-1 expression also appears to correlate with changes in behavioural hypersensitivity during light and dark phases

(Kusunose *et al.*, 2010). Established mechanical hypersensitivity is reversed by intrathecal antisense oligonucleotides and prevented by dorsal rhizotomy at the time of nerve ligation (Li *et al.*, 2004).

Some of the mechanisms by which increased $\alpha_2\delta$-1 expression facilitates excitatory transmission have been examined in transgenic mice over expressing $\alpha_2\delta$-1. In the absence of injury, these transgenic mice have mechanical withdrawal thresholds comparable to nerve ligated wildtype controls (Li *et al.*, 2006). DRG neurones from transgenic mice also exhibit a hyperpolarising shift in the voltage activation of VGCCs, increased peak conductance and an increased inactivation (Li *et al.*, 2006). Deep dorsal horn WDR neurones in transgenic mice exhibit responses to low threshold mechanical and heat stimulation of the receptive field greater than high threshold stimuli in wildtype mice. A pronounced and prolonged after firing characteristic of central neuronal hyperexcitability is also notable (Li *et al.*, 2006). Electrically evoked responses (input, wind-up, post-discharge) are unaltered in transgenic mice suggesting no change in intrinsic WDR excitability (Li *et al.*, 2006; Nguyen *et al.*, 2009). Over-expression of $\alpha_2\delta$-1 increases the frequency, but not the amplitude, of mEPSCs in the dorsal horn and is reversed by gabapentin, and inhibition of AMPA and NMDA receptors (Nguyen *et al.*, 2009; Zhou & Luo, 2014). These data support an increase in afferent excitability and subsequent spinal neuronal responses in mediating behavioural abnormalities in $\alpha_2\delta$-1 over-expressing mice. The increase in mechanical withdrawal thresholds in $\alpha_2\delta$-1 over-expressing mice following intrathecal ondansetron bears marked similarities to the inhibitory effect of ondansetron on mechanically evoked neuronal responses in SNL rats suggesting over-expression of $\alpha_2\delta$-1 in the absence of injury is sufficient to drive changes in descending serotonergic facilitations (Suzuki *et al.*, 2004; Chang *et al.*, 2013).

Hypotheses and Aims:

As $\alpha_2\delta$-1 is highly expressed in DRG neurones of all sizes, it is possible that $\alpha_2\delta$-1 -/- mice exhibit sensory deficits. Initial studies will determine whether sensitivity to cool temperatures but also mechanical and heat stimuli in $\alpha_2\delta$-1 -/- mice are altered and whether this corresponds with spinal neuronal responses. Given the correlation between the up-regulation of $\alpha_2\delta$-1 and the development of neuropathy, it could be expected that the absence of $\alpha_2\delta$-1 prevents or alters the course of cold and mechanical hypersensitivity after nerve injury. Finally, if $\alpha_2\delta$-1 -/- mice develop cold and mechanical hypersensitivity, the hypothesis that the analgesic effects of pregabalin are dependent on $\alpha_2\delta$-1 will be tested (Field *et al.*, 2006).

5.2 Materials and Methods

5.2.1 Animals

Male transgenic $\alpha_2\delta$-1 knockout (-/-) mice (Fuller-Bicer *et al.*, 2009) and wildtype (+/+) littermate controls aged between 10 and 12 weeks old, bred by the Biological Service Unit (UCL, London, UK), were used for behavioural and electrophysiological experiments. All behavioural and electrophysiological characterisations were performed while blind to the genotype and/or drug treatment.

5.2.2 Baseline behavioural testing

Mechanical, cooling and heat sensitivity were tested as described in sections 2.5.1, 2.5.2 and 2.5.3 respectively.

5.2.3 Partial sciatic nerve ligation

Nerve ligation and sham surgery was performed as described in section 2.4. Baseline behavioural testing was performed 5 days prior to surgery and on days 3, 7, 9, 11 and 14 days post surgery. Mechanical and cooling hypersensitivity were tested as described in sections 2.5.1 and 2.5.2 respectively. In a second group of animals, behavioural pharmacology was performed 14 days after injury. Mechanical hypersensitivity, using the 0.4 g von Frey filament, was tested before dosing and 30, 60, and 90 minutes after dosing. +/+ and -/- mice were dosed intraperitoneally with either vehicle (0.9% saline) or 10 mg/kg pregabalin (gift from Pfizer) in a volume of 5 ml/kg. Dose of pregabalin was chosen based on inhibition of spinal neuronal responses in SNL rats and minor motor deficits induced at this dose when administered systemically (Bee & Dickenson, 2008; Abbadie *et al.*, 2010).

5.2.4 *In vivo* electrophysiology

In vivo electrophysiology was performed as described in sections 2.1.3, 2.1.4 and 2.1.6. Wide dynamic range neurones were characterised from depths of 350-700 μm from the surface of the cord. Neuronal responses to mechanical, thermal and electrical stimulation were measured. Stimuli were applied in the following order: brush, 0.4 g, 1 g, 4 g, 8 g, 15 g, acetone, 42°C, 45°C, 48°C, electrical stimulation. A 1-minute rest period followed each stimulus. In total, 17 neurones were characterised from 14 -/- mice and 19 neurones were characterised from 15 +/+ mice.

5.2.5 Statistics

Baseline mechanical and heat withdrawals were compared with a Mann Whitney U test. Responses to cooling were compared with an unpaired Student's t-test. Mechanical and heat coding of WDR neurones were compared with a 2 way ANOVA, followed by Bonferroni *post hoc*. All other electrophysiological parameters were compared with an unpaired Student's t-test. Behavioural time-courses following PSNL were compared with the Kruskal-Wallis test, followed by Dunn's *post hoc* test for paired comparisons. AUC values were calculated using the trapezoid rule and compared with a 1 way ANOVA, followed by Bonferroni *post hoc* test. The effect of pregabalin in PSNL mice was compared to pre-drug responses with a Friedman test, followed by Wilcoxon *post hoc* and Bonferroni correction.

5.3 Results

5.3.1 Behavioural deficits in cold and mechanical, but not heat sensitivity, in $\alpha_2\delta$-1 -/- mice

$\alpha_2\delta$-1 -/- mice and +/+ littermate controls were initially examined for behavioural differences in cold, mechanical and heat sensitivity. $\alpha_2\delta$-1 -/- mice displayed significantly fewer nocifensive behaviours in response to acetone application to the hind paws as an evaporative cooling stimulus (unpaired Student's t-test with Welch's correction, $P<0.05$) (Fig. 5.3*A*). Mechanical sensitivity was tested using von Frey filaments, which were applied across the hind paws. Significantly fewer withdrawals were observed in $\alpha_2\delta$-1 -/- mice in response to 0.16 g and 0.4 g filaments but not 0.07 g, the lowest weight tested (Mann-Whitney U Test, $P<0.05$) (Fig. 5.3*B*). Nociceptive reflexes to heat stimulation were conducted in lightly anesthetised mice, using infrared laser stimulation, shown previously to stimulate Aδ- and C-fibres selectively (Sikandar et al., 2013). There was no significant difference in noxious heat withdrawal thresholds between $\alpha_2\delta$-1 +/+ and $\alpha_2\delta$-1 -/- mice (Mann-Whitney U Test, $P>0.05$) (Fig. 5.3*C*).

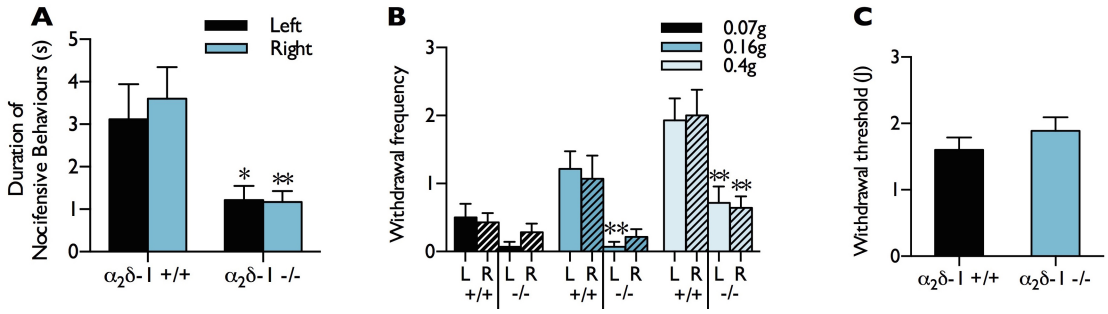


Figure 5.3 Reduced behavioural responses of $\alpha_2\delta$-1 -/- mice to cooling and mechanical stimuli. (A) Duration of nocifensive behaviours in response to acetone application (n=12). **(B)** Withdrawal frequency to punctate mechanical stimulation applied using von Frey filaments (L-left hind paw, R-right hind paw, n=14). **(C)** Withdrawal thresholds to infrared laser stimulation of hind paw (+/+ n=10, -/- n=9). Asterisks denote significant differences between respective paws between genotypes. No significant differences were observed between left and right responses within genotypes (Wilcoxon test, $P>0.05$). *$P<0.05$, **$P<0.01$. Data represent mean ± SEM.

5.3.2 Deficits in coding of dorsal horn lamina V/VI neurones in $\alpha_2\delta$-1 -/- mice

In vivo electrophysiology was performed to assess the impact of $\alpha_2\delta$-1 deletion on the spinal processing of a range of thermal and mechanical stimuli. All neurones characterised had receptive fields on the glabrous skin of the hind toes and were identified as WDR by confirming responses to light brushing, noxious punctate mechanical stimulation and heat stimulation. Neurones were characterised from similar depths, corresponding to deep dorsal horn laminae ($\alpha_2\delta$-1 +/+, 516 ± 26 μm; $\alpha_2\delta$-1 -/-, 542 ± 25 μm).

Deep dorsal horn neurones responded to mechanical and heat stimulation in an intensity-dependent manner. The reduced behavioural response to punctate mechanical stimulation in $\alpha_2\delta$-1 -/- mice (Fig. 5.3*B*) is corroborated by a deficit in mechanical coding of lamina V/VI neurones with significantly reduced neuronal responses to 1 g, 8 g and 15 g stimulation (2 way ANOVA $P<0.05$, $F_{1,170}=21.45$, followed by Bonferroni *post hoc*) (Fig. 5.4*A*). In addition, neuronal responses to dynamic brush stimulation were also attenuated (unpaired Student's t-test, $P<0.05$) (Fig. 5.4*B*). In contrast to the behavioural response to acetone-induced cooling (Fig. 5.3*A*), deep dorsal horn neuronal responses in $\alpha_2\delta$-1 -/- mice were not different from the $\alpha_2\delta$-1 +/+ controls (unpaired Student's t-test, $P>0.05$) (Fig. 5.4*C*). However, only 5/17 $\alpha_2\delta$-1 +/+ neurones and 5/19 $\alpha_2\delta$-1 -/- neurones were responsive to cooling. No significant difference in heat coding of lamina V/VI neurones was observed in $\alpha_2\delta$-1 -/- mice (2 way ANOVA $P>0.05$, $F_{1,170}=2.618$) (Fig. 5.4*D*), supporting earlier observations of unaltered heat withdrawal thresholds (Fig. 5.3*C*).

Electrical thresholds for activation of A- and C-fibres were indistinguishable between the genotypes (unpaired Student's t-test, $P>0.05$) (Fig. 5.4*E*). Following repeated supra-threshold electrical stimulation of the receptive field (16 stimuli, 2 ms pulse, 0.5 Hz), the cumulative total of neuronal events evoked by A- and C-fibre stimulation were not affected in $\alpha_2\delta$-1 -/- mice (unpaired Student's t-test, $P>0.05$) (Fig 5.4*F, G*). Lamina V/VI neurones from $\alpha_2\delta$-1 +/+ and $\alpha_2\delta$-1 -/- exhibited a similar rate of wind-up (Fig. 5.4*F*), in addition to a similar total wind-up (unpaired Student's t-test, $P>0.05$) (Fig. 5.4*G*). However, the 'input', the non-potentiated response, more indicative of pre-synaptic events, and the post-discharge, a property of spinal neurones and measure of hyperexcitability, were significantly decreased in $\alpha_2\delta$-1 -/- mice (unpaired Student's t-test, $P<0.05$) (Fig. 5.4*G*).

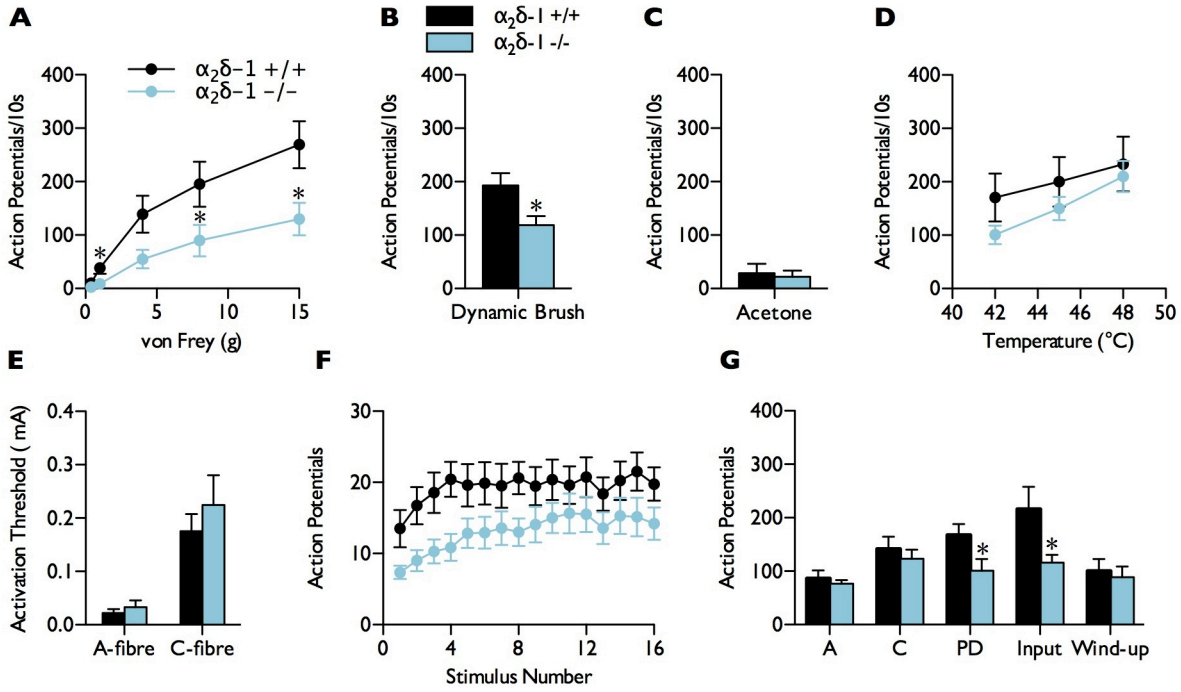


Figure 5.4 Reduced lamina V/VI neuronal responses to mechanical and electrical stimulation in α2δ-1 -/- mice. (A) Evoked neuronal responses to punctate mechanical stimulation, **(B)** dynamic brush, **(C)** acetone evoked cooling, and **(D)** heat stimulation. **(E)** Electrical thresholds for activation of A- and C-fibres. **(F)** Wind-up of deep dorsal horn neurones (16 stimuli, 0.5 Hz, 2 ms pulse), expressed as mean number of action potentials per stimulus number. **(G)** Total action potentials evoked in response to repeated electrical stimulation, separated according to latency. +/+ *n*=17, -/- *n*=19. **P*<0.05. Data represent mean ± SEM. (PD – post-discharge)

5.3.3 Altered mechanical hypersensitivity in α2δ-1 -/- mice following nerve injury

Baseline testing was performed 5 days prior to PSNL surgery, and then repeated on post-operative days 3, 7, 9, 11 and 14. Following surgery, mice groomed normally and maintained pre-surgery body weights. The $\alpha_2\delta$-1 +/+ mice developed rapid mechanical hypersensitivity on the injured side after PSNL, exhibiting increased withdrawal frequency compared to sham operated mice on day 14 to the 0.07 g von Frey (Fig. 5.5*A*), between days 7-11 for the 0.16 g von Frey (Fig. 5.5*B*), and from day 3 onwards for the 0.4 g (Fig. 5.5*C*) von Frey filament (Kruskal-Wallis *P*<0.05, Dunn's *post hoc* for paired comparisons). The ipsilateral withdrawal frequencies for $\alpha_2\delta$-1 -/- mice were reduced compared to $\alpha_2\delta$-1 +/+ mice at day 7 for the 0.07 g von Frey (Fig. 5.5*A*), and at days 3-9 for the 0.16 g (Fig. 5.5*B*) and 0.4 g (Fig. 5.5*C*) von Frey filaments (Kruskal-Wallis *P*<0.01, followed by Dunn's *post hoc*). On the contralateral side a small but non-significant sensitisation was observed in both genotypes (Friedman test, *P*>0.05) (Fig. 5.5*D, E, F*). The differences in the time-courses of hypersensitivity could be attributed to the difference in baseline sensitivity. $\alpha_2\delta$-1 -/- mice however appear to have a marked delay in the expression of neuropathic like behaviours as is illustrated by analysing total withdrawals to the 0.4 g von

Frey filament after the subtraction of baseline responses. $\alpha_2\delta$-1 +/+ exhibit a larger increase in withdrawals compared to $\alpha_2\delta$-1 -/- mice in the induction phase of the model (1 way ANOVA $P<0.001$, $F_{5,72}=10.334$, followed by Bonferroni *post hoc*) (Fig. 5.6*A*). $\alpha_2\delta$-1 -/- mice on the other hand do not exhibit significant neuropathic behaviours until the chronic phase (1 way ANOVA $P<0.001$, $F_{5,72}=7.646$, followed by Bonferroni *post hoc*) (Fig. 5.6*B*). Cold hypersensitivity following PSNL was an infrequent occurrence in both genotypes (5/13 $\alpha_2\delta$-1 -/-, 5/11 $\alpha_2\delta$-1 +/+). Overall $\alpha_2\delta$-1 +/+ mice did not exhibit significant cold sensitivity compared to sham operated animals and was not markedly different in $\alpha_2\delta$-1 -/- mice (Kruskal-Wallis $P>0.05$) (Fig. 5.5*G, H*).

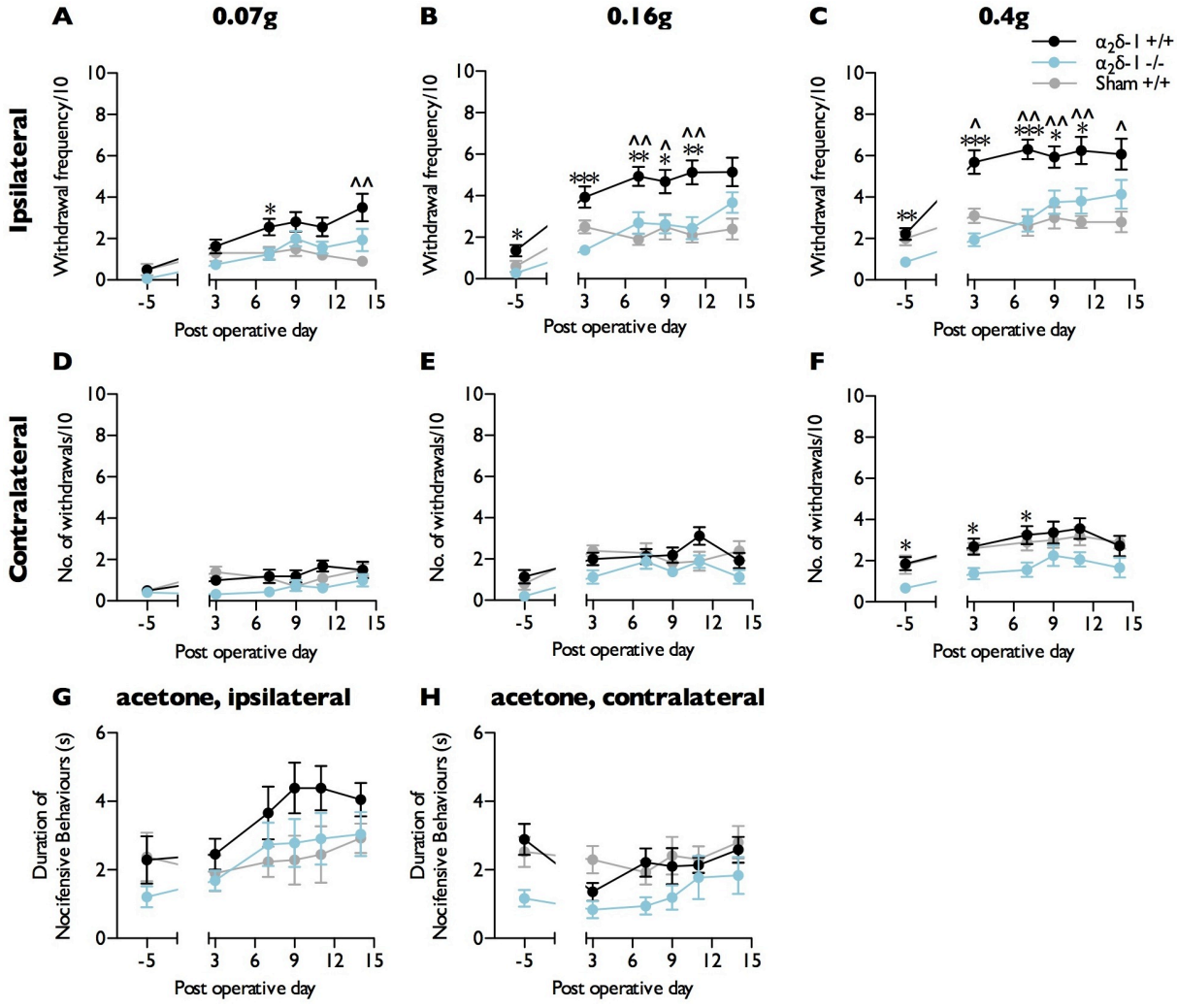


Figure 5.5 Time-course of mechanical and cold hypersensitivity after PSNL. Time-course of mechanical sensitivity of $\alpha_2\delta$-1 +/+ (n=16), $\alpha_2\delta$-1 -/- (n=16) and sham +/+ (n=10) mice to **(A)** 0.07 g, **(B)** 0.16 g, and **(C)** 0.4 g von Frey stimulation following nerve ligation. Corresponding contralateral responses to **(D)** 0.07 g, **(E)** 0.16 g, and **(F)** 0.4 g stimulation. **(G)** Time-course of cold hypersensitivity of $\alpha_2\delta$-1 +/+ (n=11), $\alpha_2\delta$-1 -/- (n=13) and sham +/+ (n=8) mice after injury. **(H)** Corresponding contralateral responses to cooling stimuli. Asterisks represent significant differences between $\alpha_2\delta$-1 +/+ and $\alpha_2\delta$-1 -/- groups. (^) represents significant differences between $\alpha_2\delta$-1 +/+ and sham +/+ groups. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Data represent mean ± SEM.

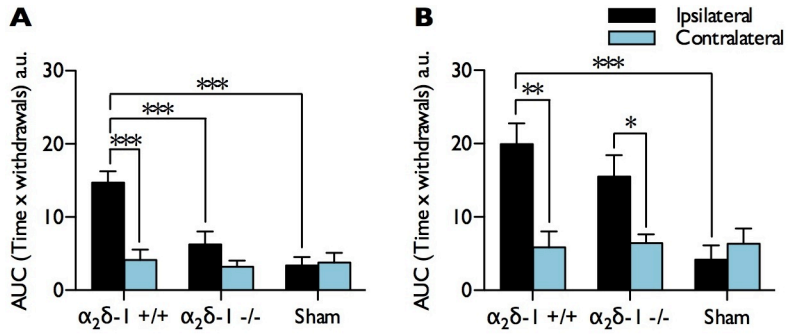


Figure 5.6 Delayed development of mechanical hypersensitivity after PSNL in $\alpha_2\delta$-1 -/- mice. (A) Change from baseline in withdrawal frequency to 0.4g von Frey filament during induction phase (day 3-7) of model, and **(B)** chronic phase (day 9-14) of model. **P*<0.05, ***P*<0.01, ****P*<0.001. +/+ *n*=16, -/- *n*=16, sham *n*=10. Data represent mean ± SEM. (a.u. – arbitrary units, AUC –area under curve).

5.3.4 Anti-hyperalgesic effect of pregabalin lost in $\alpha_2\delta$-1 -/- mice after nerve injury

As only a subset of animals exhibited cold hypersensitivity, the ability of pregabalin to attenuate mechanical hypersensitivity was examined in $\alpha_2\delta$-1 +/+ and $\alpha_2\delta$-1 -/- 14 days after PSNL. Mechanical hypersensitivity was tested prior to administration of either vehicle or 10 mg/kg pregabalin, and repeated 30, 60 and 90 min post-dosing. In $\alpha_2\delta$-1 +/+ mice, pregabalin reduced withdrawal frequency on the injured ipsilateral side compared to pre-drug values at 30 min post-dosing (Friedman test $P<0.01$, followed by Wilcoxon *post hoc*), and to the vehicle-treated group at 30 and 60 min post-dosing (Mann-Whitney U test, $P<0.05$) (Fig. 5.7*A*). Contralateral responses were not altered by vehicle or pregabalin treatment (Friedman test $P>0.05$) (Fig. 5.7*B*). In $\alpha_2\delta$-1 -/- mice, pregabalin did not affect mechanical hypersensitivity compared to pre-drug withdrawal frequencies (Friedman test $P>0.05$) or the vehicle-treated group (Mann-Whitney U test, $P>0.05$) (Fig. 5.7*C*). Contralateral responses were also unaffected by both treatments (Friedman test $P>0.05$) (Fig. 5.7*D*).

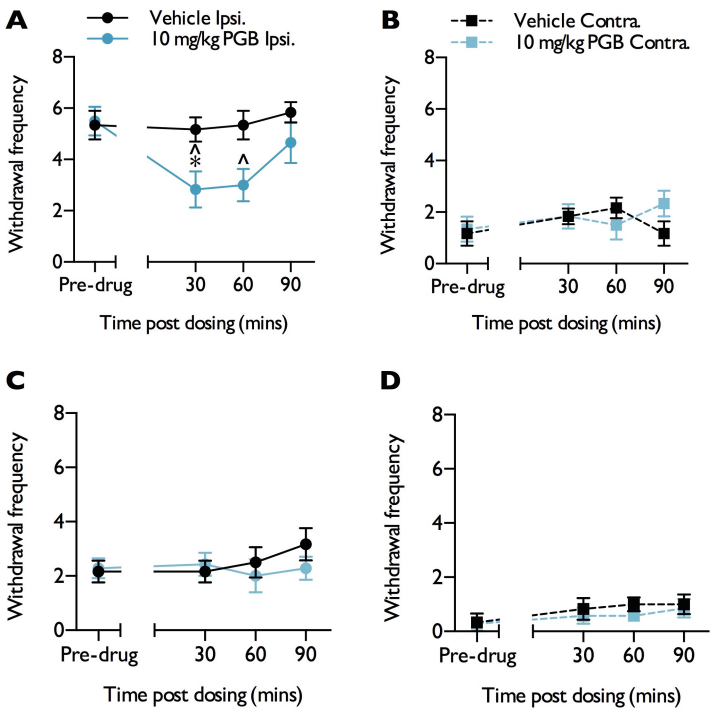


Figure 5.7 Effect of pregabalin absent in $\alpha_2\delta$-1 -/- mice following peripheral nerve injury. (A) 10 mg/kg pregabalin attenuates mechanical hypersensitivity in $\alpha_2\delta$-1 +/+ mice, whereas **(B)** contralateral sensitivity is unaffected (*n*=6). **(C)** Pregabalin does not modulate mechanical hypersensitivity in $\alpha_2\delta$-1 -/- mice, or **(D)** contralateral responses (vehicle *n*=6, pregabalin-treated *n*=7). Asterisks (*) denote statistically significant difference of response in pregabalin-treated mice, compared to pre-drug withdrawal frequency. (^) denotes statistically significant differences between ipsilateral vehicle-treated and ipsilateral pregabalin-treated responses. *P<0.05. Data represent mean ± SEM. (PGB-pregabalin, Ipsi.-ipsilateral, Contra.-contralateral).

5.4 Discussion

To date $\alpha_2\delta$-1 -/- mice have only been studied in terms of the impact on cardiovascular function. Knockout mice have reduced myocardial contractibility and decreased peak L-type calcium current amplitude in cardiac myocytes (Fuller-Bicer *et al.*, 2009). Although originally thought to be unviable, $\alpha_2\delta$-1 -/- mice appear to have no gross physical or behavioural abnormalities (personal observation and Fuller-Bicer *et al.*, 2009). Contrasting reports exist regarding whether $\alpha_2\delta$-1 is required for muscle development (Gach *et al.*, 2008; Garcia *et al.*, 2008), however no obvious muscle weakness was apparent in the -/- mice as they did not appear to have any deficits in mounting withdrawal reflexes. This would ideally have been examined with a grip strength test. Distension of the bladder was apparent in a minor proportion of $\alpha_2\delta$-1 -/- mice (<10%; personal observation), which could be attributed to a role of the $\alpha_2\delta$-1 subunit in the contractibility of smooth muscle cells (Bannister *et al.*, 2009).

5.4.1 The role of $\alpha_2\delta$-1 in baseline sensitivity to mechanical and cold stimuli

$\alpha_2\delta$-1 -/- mice show markedly reduced behavioural sensitivity to mechanical and cold stimuli, with no change in withdrawal threshold for noxious heat. $\alpha_2\delta$-1 is expressed in most excitable tissues, including peripheral and central neurones (Cole *et al.*, 2005; Taylor & Garrido, 2008). In a global knockout model, altered behavioural sensitivity could therefore be attributed to differences in supra-spinal circuits as well as spinal and somatosensory neurones. This data strongly supports a peripheral and spinal role for $\alpha_2\delta$-1 in modulating sensitivity to these stimuli. The behavioural profile was, in part, reflected by decreased punctate and dynamic mechanical responses of dorsal horn neurones while heat and cold responses were unaffected. The prominent mechanical phenotype is perhaps a little surprising given the low levels $\alpha_2\delta$-1 expression in Aβ-fibres, which likely dominate in mediating light touch in glabrous skin whereas low threshold Aδ− and C- mechanoreceptors are typically associated with hair follicles (Abraira & Ginty, 2013). TRPM8 appears to be the principal detector of cool temperatures in the mouse (Bautista *et al.*, 2007; Colburn *et al.*, 2007; Dhaka *et al.*, 2007). These neurones constitute a minor proportion of all peripheral primary afferents; synaptic transmission in this population may be particularly dependent on $\alpha_2\delta$-1. Although there was no significant difference between $\alpha_2\delta$-1 +/+ and $\alpha_2\delta$-1 -/- mice in the neuronal response to cooling, not all lamina V/VI neurones are excited by cool temperatures (Khasabov *et al.*, 2001). Only 5/17 neurones in $\alpha_2\delta$-1 +/+ mice and 5/19 neurones in $\alpha_2\delta$-1 -/-

mice responded to acetone induced cooling. The high level of anaesthesia required to perform such recordings may have suppressed transduction of low threshold stimuli.

The lower 'input' following electrical stimulation in $\alpha_2\delta$-1 -/- mice likely represents a decrease of synaptic transmitter release from primary afferents projecting to dorsal horn neurones, and the lack of change in wind-up also supports pre-synaptic alterations. However, there was no global reduction in primary afferent transmission via A- and C-fibres, in terms of evoked responses and thresholds, suggesting no major changes in conduction of afferent nerves. In light of the deficit in mechanical coding it might be expected that electrically evoked A-fibre events are reduced in -/- mice. The electrical stimulus however is more akin to supra threshold stimulation whereas natural stimulation examines threshold responses. A limitation with the mouse set-up is the inability to distinguish reliably between Aβ- and Aδ- evoked action potentials. Neurones from -/- mice also exhibited significantly reduced post-discharge. This could in part be explained by reduced primary afferent drive as the input was also decreased, though this does not preclude $\alpha_2\delta$-1 contributing to post-synaptic events. Moderate expression of $\alpha_2\delta$-1 has been observed post-synaptically in the deep dorsal horn (Li *et al.*, 2006), $\alpha_2\delta$-1 could influence calcium dependent plateau potentials, which appear to underlie the ability of a subset of deep dorsal horn neurones to mount significant trains of post-discharge following a noxious stimulus (Morisset & Nagy, 1999).

As discussed previously, in addition to its association with calcium channels, $\alpha_2\delta$-1 mediates thrombospondin dependent excitatory synapse formation *in vitro* and *in vivo* (Eroglu *et al.*, 2009). At the spinal level, $\alpha_2\delta$-1 is present predominantly in pre-synaptic terminals of primary afferents terminating in the superficial laminae (Bauer *et al.*, 2009), while lamina V/VI dorsal horn neurones directly or indirectly, through interneurones, receive input from Aβ-, Aδ- and C-fibres. Although synaptogenesis in $\alpha_2\delta$-1 -/- mice has not been directly addressed, the unaltered electrically evoked responses yet modality specific deficits argue against global synaptic dysregulation within the dorsal horn. Neurite outgrowth *in vitro* is unaffected by $\alpha_2\delta$-1 gene deletion (personal communication; A. Dolphin, UCL); impaired synapse formation is still a feasible possibility.

5.4.2 Properties of DRG neurones in $\alpha_2\delta$-1 -/- mice

Properties of DRG neurones have been examined to further investigate the modality selective effects of $\alpha_2\delta$-1 gene ablation. Loss of $\alpha_2\delta$-1 does not affect proportions of myelinated and unmyelinated DRG neurones (Appendix 8.4, Fig. 8.3*B*). Intraepidermal nerve fibre density is also normal in $\alpha_2\delta$-1 -/- mice (Appendix 8.4, Fig. 8.3*C, D*). *In vitro*, a greater proportion of $\alpha_2\delta$-1-/- DRG neurones exhibit responses to capsaicin treatment with

an increased peak calcium influx. In contrast, a reduced proportion of $\alpha_2\delta$-1 -/- neurones respond to menthol and could underlie reduced behavioural sensitivity to cold temperatures (Appendix 8.4, Fig. 8.4*B*). This appears unrelated to TRPM8 and TRPV1 mRNA levels in DRG neurones (Appendix 8.4, Fig. 8.5*A*). The difference in capsaicin sensitivity might reflect compensatory changes in translation to counteract a loss of transmission in nociceptors. Overall, no impact on behavioural or spinal neuronal responses to heat stimulation was observed. Calcium currents are reduced in $\alpha_2\delta$-1 -/- DRG neurones (Appendix 8.4, Fig. 8.6*A*), as are brain and spinal cord synaptosomal N-type calcium channel levels (Appendix 8.4, Fig. 8.2C, *D*), consistent with the role of $\alpha_2\delta$-1 in trafficking of calcium channels to active zones of synapses, and controlling calcium influx and subsequent transmitter release (Hoppa *et al.*, 2012).

5.4.3 The impact of $\alpha_2\delta$-1 gene ablation on cold and mechanical hypersensitivity after injury.

Increased sensitivity to innocuous cooling was not consistently observed in either genotype after PSNL. In those animals that did develop cold hypersensitivity, the time-course of development was not markedly different between $\alpha_2\delta$-1 +/+ and -/- mice with most behaviours presenting day 7-9 post injury. The time-course is in contrast to the rapid presentation of mechanical hypersensitivity, which was noticeable 3 days after injury supporting divergent mechanisms of cold and mechanical hypersensitivity in this model. The altered course of development of mechanical hypersensitivity after PSNL in $\alpha_2\delta$-1 -/- mice does not appear entirely attributable to a baseline difference in sensitivity. Nerve injury induces transcriptional changes in DRG neurones (Costigan *et al.*, 2002; Wang *et al.*, 2002; Xiao *et al.*, 2002), including $\alpha_2\delta$-1 (Luo *et al.*, 2001), demonstrated to be up-regulated in DRG neurones of all sizes (Bauer *et al.*, 2009). Immunohistochemical analysis of L3, L4 and L5 DRGs reveals similar ipsilateral expression of injury markers such ATF-3 and neuropeptide Y in wildtype and $\alpha_2\delta$-1 -/- mice 14 days after sciatic nerve ligation (Appendix 8.4, Fig. 8.8*A-C*). There was also no apparent compensatory up-regulation of $\alpha_2\delta$-2, $\alpha_2\delta$-3 or β3 subunits in DRG neurones (Appendix 8.4, Fig. 8.7*A-D*). The chronic phase of the model was defined in relation to the engagement of descending influences in the second week post injury (Burgess *et al.*, 2002). In $\alpha_2\delta$-1 -/- mice after nerve injury, aberrant input into the cord may be suppressed compared to wildtype mice in the early parts of the model. These findings suggest that $\alpha_2\delta$-1 expression is a limiting factor in transmitting abnormal peripheral activity to central neurones and is key in shaping the initiation of neuropathic pain but the absence of $\alpha_2\delta$-1 fails to prevent chronicity and is not essential in the maintenance of a neuropathic state.

5.4.4 Mechanism of pregabalin inhibition after neuropathic injury

Gabapentin and pregabalin display efficacy in various neuropathies of peripheral and central origin (Moore *et al.*, 2009; Moore *et al.*, 2011). The mechanism of action in neuropathy has been the subject of much debate. Originally designed as analogues of GABA, neither compound has any significant agonist-like effect on $GABA_A$ or $GABA_B$ receptors (Lanneau *et al.*, 2001; Jensen *et al.*, 2002). Gabapentin was discovered to bind to $\alpha_2\delta$ subunits (Gee *et al.*, 1996), with greater affinity for $\alpha_2\delta$-1 (K_d = 59 nM) compared to $\alpha_2\delta$-2 (K_d = 153 nM) and no affinity for $\alpha_2\delta$-3 (Marais *et al.*, 2001). Point mutation of arginine 217 in $\alpha_2\delta$-1 abolishes the anti-hyperalgesic effects of pregabalin in neuropathic mice (Field *et al.*, 2006). The data presented here supports this previous finding, as pregabalin had no effect on mechanical hypersensitivity in neuropathic $\alpha_2\delta$-1 -/- mice confirming $\alpha_2\delta$-1 is the sole molecular target for the analgesic actions of gabapentinoid drugs. Pregabalin had no effect on contralateral sensitivity to mechanical stimulation in wildtype mice; the pathophysiological state dependent effects of pregabalin and gabapentin implies other factors influence efficacy in neuropathic conditions.

Gabapentin and pregabalin are thought to inhibit transmitter release, though the molecular mechanism is still unclear. Calcium currents are not consistently reduced by acute gabapentin (Stefani *et al.*, 1998; Sutton *et al.*, 2002; Hendrich *et al.*, 2008), whereas chronically applied gabapentin can reduce P-type and N-type calcium currents (Hendrich *et al.*, 2008). Similarly in cultured tissue slices, gabapentin does not consistently inhibit transmitter release (Fink *et al.*, 2000; Fehrenbacher *et al.*, 2003; Brown & Randall, 2005; Quintero *et al.*, 2011).

Chronic systemic pregabalin treatment in SNL rats inhibits trafficking of $\alpha_2\delta$-1 to pre-synaptic membranes in the dorsal horn (Bauer *et al.*, 2009). Whether this would account for the acute effects of pregabalin dosing observed in this study, and previous studies (Hunter *et al.*, 1997; Field *et al.*, 2006; Miyazaki & Yamamoto, 2012), seems unlikely given the time scale of axonal trafficking. At the cell surface gabapentin does not disrupt the interaction between $\alpha_2\delta$-1 and α_{1B} subunits (Cassidy *et al.*, 2014). Gabapentin fails to inhibit the internalisation rate of $\alpha_2\delta$-2 but disrupts rab11 dependent recycling from endosomal compartments consequently reducing calcium currents (Tran-Van-Minh & Dolphin, 2010). It is tempting to speculate that at the spinal level, acute pregabalin and gabapentin treatment preferentially targets channel cycling pathways, the rate of which may be influenced by convergent factors governing pre-synaptic excitability. One candidate is PKC, as the up-regulation has been implicated in the development of neuropathy (Hua *et al.*, 1999). Gabapentin inhibits transmitter release only after PKC dependent phosphorylation within spinal circuits (Maneuf & McKnight, 2001; Fehrenbacher *et al.*, 2003). In neuropathy, permissible conditions for the inhibitory actions of

spinally delivered gabapentioids depend on interactions between $\alpha_2\delta$-1 and descending brainstem facilitations terminating on spinal 5-HT_3Rs. Targeted saporin conjugate disruption of a spino-bulbal-spinal loop comprising spinal NK1+ projection neurones, or μ-opioid receptor expressing neurones in the RVM negates gabapentin mediated inhibition of spinal neuronal excitability (Suzuki *et al.*, 2005; Bee & Dickenson, 2008). The depolarising effect of activating pre-synaptic 5-HT_3Rs could consequently alter the kinetics and/or cycling of calcium channels and create permissible conditions for gabapentin to inhibit $\alpha_2\delta$-1 (Suzuki *et al.*, 2005). In contrast the conditions for the inhibitory effects of pregabalin in visceral hyperalgesia are independent of μ-opioid receptor positive neurones of the RVM (Sikandar *et al.*, 2012), and likely reflects differential brainstem control of cutanueous and visceral stimulation (Sikandar & Dickenson, 2011). Central sensitisation and the subsequent engagement of descending influences, in some cases in the absence of pathology, is a key determinant of gabapentin analgesia. This is evident in humans and pre-clinical models. In a human surrogate model, acute gabapentin is anti-nociceptive after capsaicin-induced hyperalgesia where significant activity in brainstem nuclei is apparent (Gottrup *et al.*, 2004; Iannetti *et al.*, 2005). Pregabalin also suppresses spinal neuronal excitability in animal models of opioid-induced hyperalgesia, where $\alpha_2\delta$-1 at the transcriptional level does not appear to be up-regulated (Bannister *et al.*, 2011).

Both intrathecally and intracerebroventricularly delivered gabapentin are efficacious after peripheral nerve injury (Tanabe *et al.*, 2005; Hayashida *et al.*, 2007; Takeuchi *et al.*, 2007), the effect of the latter in part dependent on a disinhibition of locus coeruleus neurones promoting release of noradrenaline in the dorsal horn and is reversible by intrathecal α_2 receptor antagonists (Tanabe *et al.*, 2005; Takasu *et al.*, 2008). Thus it is conceivable that systemically delivered gabapentinoids exert their effects by modulating both noradrenergic and serotonergic pathways. Pregabalin also normalises elevated neuronal activity in the central nucleus of the amygdala in neuropathic states (Goncalves & Dickenson, 2012). These changes may occur secondary to reducing spinal neuronal activity and in turn influence the balance between inhibition and facilitation through the PAG, locus coeruleus and RVM (discussed in section 1.8). Pregabalin displays a small anxiolytic effect in generalised anxiety disorders (Feltner *et al.*, 2003), and could indirectly influence pain perception through these areas. Elevated expression of $\alpha_2\delta$-1 in the amygdala is associated with increased predator odour induced anxiety and can be reversed by pregabalin (Nasca *et al.*, 2013). Fear and anxiety responses were not examined in $\alpha_2\delta$-1 -/- mice, though this might be an interesting future avenue of investigation.

5.4.5 Summary

The data presented here for the first time demonstrate that $\alpha_2\delta$-1 -/- mice display significant sensory deficits and that $\alpha_2\delta$-1 regulates synaptic transmission in cold and mechanical pathways possibly through decreasing trafficking of N-type calcium channels. In addition, $\alpha_2\delta$-1 appears to influence the induction of neuropathy but not the maintenance of a chronic pain state. Finally, pregabalin efficacy is lost in $\alpha_2\delta$-1 -/- mice after neuropathy consistent with $\alpha_2\delta$-1 and not $\alpha_2\delta$-2 being essential in mediating the analgesic effects of gabapentinoid drugs.

6. State dependent Ca_v2 Channel Antagonist Attenuates Mechanical but not Cold Hypersensitivity

6.1 Introduction

Calcium influx through voltage gated calcium channels (VGCC) controls a multitude of physiological processes, including neurotransmitter/hormone release, membrane excitability, activation of second messenger pathways, gene transcription and plasticity. Ten pore forming α_1 subunits have been described, categorised as L-type (Ca_v1), N-, P/Q-, R-type (Ca_v2) and T-type (Ca_v3) (Catterall, 2000). The formation of heteromeric complexes with $\alpha_2\delta$, β and γ auxiliary subunits influences trafficking and biophysical properties of the α_1 subunit (Arikkath & Campbell, 2003).

6.1.1 Structure and properties of calcium channels

VGCCs were originally classified according to their biophysical properties. High voltage activated (L-, P/Q-, N-, R-type) or low voltage activated (T-type) channels were later divided into three families based on structure and functionality (Ertel *et al.*, 2000) (Table 6.1). Calcium channel complexes were initially purified from skeletal muscle and predicted to compose of a 190 kDa α_1, 170 kDa $\alpha_2\delta$, 54 kDa β and 30 kDa γ subunit (Takahashi *et al.*, 1987). Calcium channels in the brain do not typically appear to associate with γ subunits (Liu *et al.*, 1996). The α_1 pore forming component is organised into 4 repeated domains of 6 trans-membrane segments (Fig. 6.1). The β subunit is intracellular whereas the γ subunit has 4 trans-membrane segments (Takahashi *et al.*, 1987). The disulphide linked $\alpha_2\delta$ subunit was confirmed to be extracellularly attached by a GPI anchor (De Jongh *et al.*, 1990; Davies *et al.*, 2010). Splice variants exist for all α_1 subunits generating diversity in the functional properties of calcium channels (Lipscombe *et al.*, 2013). This is perhaps best illustrated through alternate splicing of α_{1A}, which gives rise to P- and Q- type currents, the former displaying slower inactivation kinetics during prolonged depolarisation and increased sensitivity to ω-agatoxin IVA (Bourinet *et al.*, 1999). L-type currents are differentiated by high voltage of activation, large single channel conductance and slow voltage dependent inactivation (Nowycky *et al.*, 1985). T-type currents activate transiently by weak depolarisations with low channel conductance whereas N-type channels display intermediate inactivation kinetics and voltage dependence for activation (Nowycky *et al.*, 1985).

The high voltage activated channels can be further distinguished by sensitivities to specific antagonists. L-type currents are sensitive to inhibition by dihydropyridines, P/Q channels are blocked by ω-agatoxin IVA

(Mintz *et al.*, 1992), N-type channels are blocked by ω-conotoxin GVIA (Reynolds *et al.*, 1986) and R-type channels are blocked by SNX-482 (Newcomb *et al.*, 1998). R(-)-efonipidine has been proposed to be a selective T-type antagonist of $Ca_v3.1$, efficacy against $Ca_v3.2$ and $Ca_v3.3$ was not examined (Furukawa *et al.*, 2004). TTA-A2 is a state dependent antagonist of Ca_v3 channels (Francois *et al.*, 2013).

Table 6.1 Summary of distribution and function of Ca^{2+} channel subtypes. (Taken from Catterall, 2000).

Ca^{2+} channel	Current type	Distribution	α_1	Specific blocker	Functions
$Ca_v1.1$	L	Skeletal muscle	α_{1S}	Dihydropyridines	Excitation-contraction coupling, calcium homeostasis, gene regulation
$Ca_v1.2$	L	Cardiac muscle, endocrine cells, neurones	α_{1C}	Dihydropyridines	Excitation-contraction coupling, hormone secretion, gene regulation
$Ca_v1.3$	L	Endocrine cells, neurones	α_{1D}	Dihydropyridines	Hormone secretion, gene regulation
$Ca_v1.4$	L	Retina	α_{1F}	Dihydropyridines	Tonic neurotransmitter release
$Ca_v2.1$	P/Q	Nerve terminals, dendrites	α_{1A}	ω-Agatoxin IVA	Neurotransmitter release, dendritic Ca^{2+} transients
$Ca_v2.2$	N	Nerve terminals, dendrites	α_{1B}	ω-Conotoxin GVIA	Neurotransmitter release, dendritic Ca^{2+} transients
$Ca_v2.3$	R	Cell bodies, nerve terminals, dendrites	α_{1E}	SNX-482	Ca^{2+} dependent action potentials, neurotransmitter release
$Ca_v3.1$	T	Cardiac muscle, skeletal muscle, neurones	α_{1G}	R(-)-efonipidine?	Repetitive firing
$Ca_v3.2$	T	Cardiac muscle, neurones	α_{1H}	None	Repetitive firing
$Ca_v3.3$	T	Neurones	α_{1I}	None	Repetitive firing

6.1.2 Modulation of calcium channels

Coupling of transmitter release to calcium influx may be limited by available 'slots' in active zones of synapses as increasing channel expression does not necessarily increase synaptic strength (Cao *et al.*, 2004). Calcium channel function is substantially regulated through multiple signalling pathways. In general β subunits traffic channels to the plasma membrane, increase current density and slows inactivation (De Waard & Campbell, 1995; Dolphin, 2003). These effects are larger than those exerted by $\alpha_2\delta$ subunits. γ subunits on the other hand appear to exert inhibitory influences on calcium currents (Arikkath & Campbell, 2003).

Activity of N-type channels is regulated by several G-protein coupled receptors including opioid receptors (Bourinet *et al.*, 1996). Single channel analysis suggests G-proteins delay latency to open (Patil *et al.*, 1996). At the cellular level, the $G_{\gamma\beta}$ subunit shifts the voltage dependence of activation of calcium currents to more positive potentials and decreases rate of activation (Herlitze *et al.*, 1996). α_1 and β subunits are phosphorylated by cAMP activated kinases and other kinases, which correlate with the regulation of channel conductance.

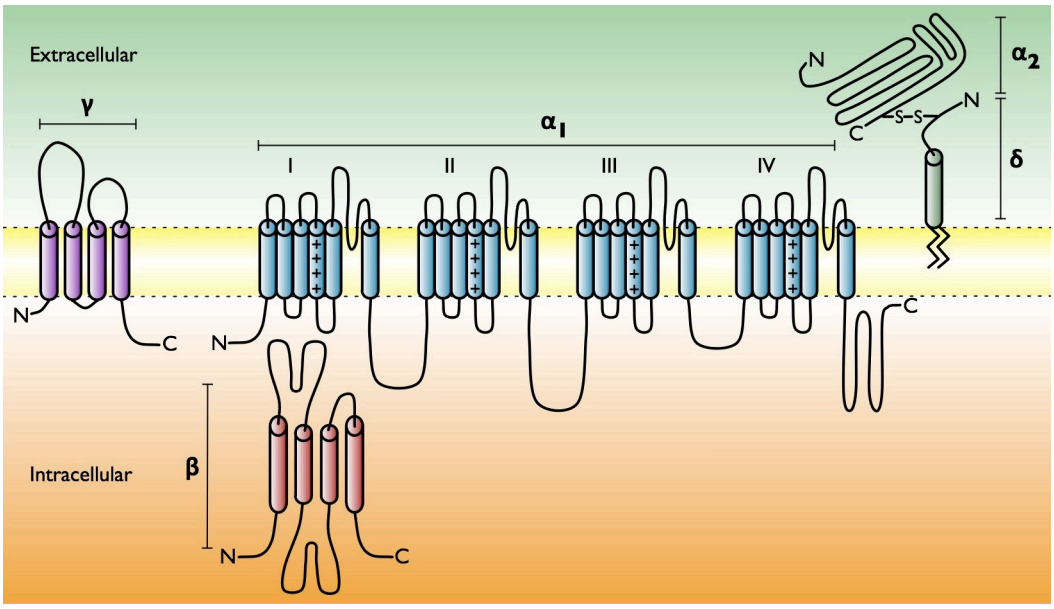


Figure 6.1 Stoichiometry and structure of calcium channels subunits. Predicated trans-membrane domains shown as cylinders. α_1 subunits are comprised of four domains each with 6 trans-membrane segments. Segment 4 contains positively charged residues (+) thought to contribute to voltage sensing. Loops between 5 and 6 form the pore of the channel. (Modified from Catterall, 2000).

6.1.3 Ca_v2 channels in nociceptive signalling

$Ca_v2.1$ (P/Q-type) and $Ca_v2.2$ (N-type) channels are responsible for initiating rapid synaptic transmission. In the dorsal horn, P/Q- and N-type channels are present in distinct populations of neurones with the N-type channel highly expressed in substance P positive presynaptic terminals of primary afferents projecting to the superficial laminae (Westenbroek *et al.*, 1998). Furthermore, P/Q channels, and to a lesser extent N-type channels, have been observed in the ventral horn on motor neurones. $Ca_v2.3$ (R-type) channels appear to be predominantly expressed in the soma of dorsal horn neurones (Westenbroek *et al.*, 1998). The Ca_v2 family of channels have been implicated in mediating the release of several transmitters including dopamine, glutamate and substance P (Luebke *et al.*, 1993; Turner *et al.*, 1993; Terashima *et al.*, 2013). *In vitro,* the release of CGRP, often co-expressed with substance P, in the dorsal horn is sensitive to inhibition by ω-conotoxin GVIA (Santicioli *et al.*,

1992). N-type calcium channels also mediate the release of noradrenaline from sympathetic neurones and are likely responsible for the potent hypotensive effect of parenteral ω-conotoxin GVIA (Clasbrummel *et al.*, 1989; Molderings *et al.*, 2000).

Genetic ablation reveals N-type but not R-type calcium channels are essential in basal heat nociception (Saegusa *et al.*, 2000; Hatakeyama *et al.*, 2001; Kim *et al.*, 2001; Saegusa *et al.*, 2001), however examining the role of P/Q channels in nociceptive pathways has been hampered by the severe neurological deficits and lethality of a global knockout (Jun *et al.*, 1999). Development of inflammatory and neuropathic pain is impaired in N-type knockout mice (Hatakeyama *et al.*, 2001; Saegusa *et al.*, 2001), whereas R-type knockout mice exhibit fewer phase 2 behaviours in response to formalin (Saegusa *et al.*, 2000). ω-conotoxin GVIA and MVIIA blockade of N-type channels is effective both acutely and in models of neuropathy (Chaplan *et al.*, 1994b; Bowersox *et al.*, 1996; Omote *et al.*, 1996), including the reversal of cold hypersensitivity (Lee *et al.*, 2010). Spinally delivered ω-agatoxin IVA has no effect on acute responses to mechanical and heat stimulation (Sluka, 1997; 1998), but P/Q channels may play a more prominent role in the establishment of a sensitised state and minor effects on the maintenance (Chaplan *et al.*, 1994b; Sluka, 1998; Matthews & Dickenson, 2001). SNX-482, an antagonist of R-type channels, exhibits complex pro-nociceptive and anti-nociceptive effects in the formalin test in contrast to the phenotype of knockout mice (Murakami *et al.*, 2004).

6.1.4 Activation state dependent calcium channel block

Neuropathic pain remains complex to treat with many patients failing to achieve adequate relief from frontline therapies (Finnerup *et al.*, 2005). The first in class drug Ziconotide (Prialt™) is a synthetic version of the $Ca_v2.2$ antagonist ω-conotoxin, derived from *Conus magus*, which is licensed for refractory chronic pain (McGivern, 2007). However, due to the narrow therapeutic window and considerable side effects associated with systemic dosing, Ziconotide is only administered intrathecally. Compounds such as TROX-1 have substantial advantages over peptide antagonists by being orally bioavailable and blood brain barrier penetrant. The calcium channel antagonist TROX-1 has been shown to inhibit P/Q-, N- and R-type VGCCs in a use dependent manner (Swensen *et al.*, 2012) (Fig. 6.2*B*). Calcium currents were inhibited to a greater degree under depolarised but not hyperpolarised conditions demonstrating a preferential inhibition of open/inactive channels (Abbadie *et al.*, 2010; Swensen *et al.*, 2012) (Fig. 6.2*A*). *In vivo*, TROX-1 attenuates mechanical hypersensitivity after inflammatory and neuropathic injury with no significant effect on acute nociception or motor function at anti-hyperalgesic doses (Abbadie *et al.*, 2010). In addition, the anti-hyperalgesic effect of TROX-1 is absent in $Ca_v2.2$

null mice demonstrating a predominant role of this particular channel in inflammatory heat hyperalgesia (Abbadie *et al.*, 2010) (Fig. 6.2*C*).

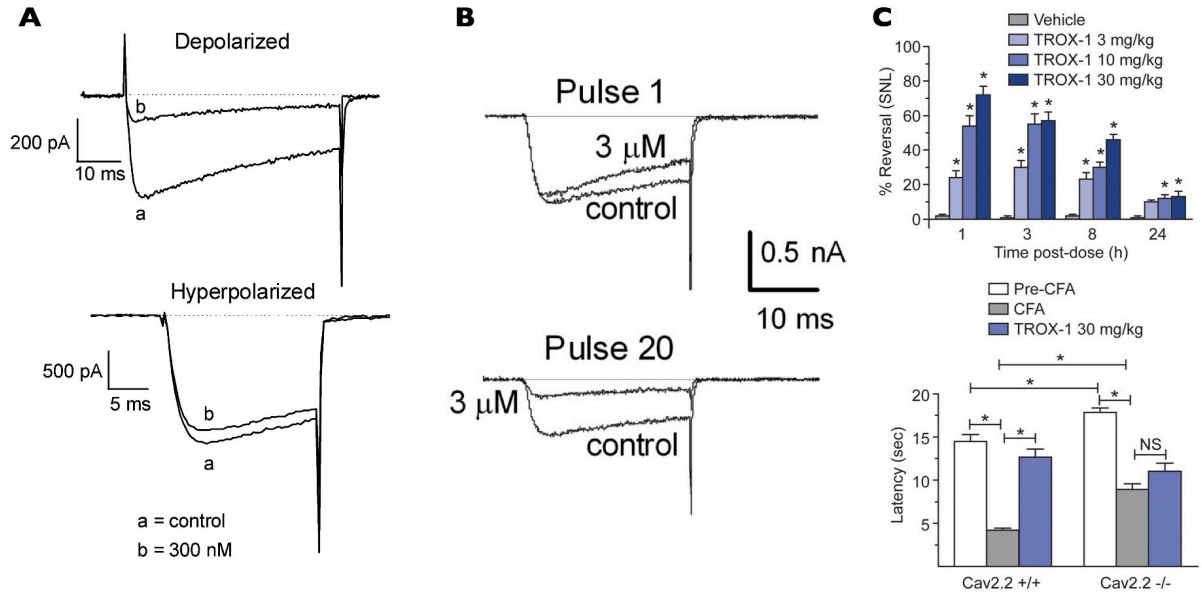


Figure 6.2 *In vitro* and behavioural effects of TROX-1. (A) Representative current traces illustrating the inhibition of $Ca_v2.2$ current by 300 nM TROX-1 using the depolarised and hyperpolarised conditions. **(B)** Representative $Ca_v2.2$ currents recorded voltage-clamped at -60 mV, and trains of 20 25-ms pulses to 20 mV were applied at a frequency of 2 Hz every 5 min. (From Swensen *et al.*, 2012). **(C)** Dose dependent reversal of mechanical hypersensitivity by TROX-1 in SNL rats (top) and reversal of heat hyperalgesia in $Ca_v2.2$ +/+ mice but not knockout (bottom). (From Abbadie *et al.*, 2010).

Hypotheses and Aims:

Given that in the absence of injury ω-conotoxin GVIA inhibits both innocuous and noxious mechanical and heat evoked spinal neuronal firing (Matthews & Dickenson, 2001), and that TROX-1 does not affect acute nociception (Abbadie *et al.*, 2010), these studies aim to examine the notion that in neuropathic rats this state dependent channel antagonist may selectively inhibit aberrant high frequency neuronal firing to cold, heat and mechanical stimuli. Whether any effects of TROX-1 are dependent on spinally expressed calcium channels will be also be investigated.

6.2 Materials and Methods

6.2.1 Animals

Male Sprague Dawley rats (250-300g), bred by the Biological Service Unit (UCL, London, UK), were used for behavioural and electrophysiological experiments.

6.2.2 *in vivo* electrophysiology

See section 2.12, 2.14 and 2.15. *In vivo* electrophysiology was performed between day 15 and 18 post SNL or sham surgery. After three consecutive stable baseline responses to natural stimuli (<10% variation, data were averaged to give control values), animals were injected subcutaneously into the contralateral flank with 20 mg/kg TROX-1 (gift from Grünenthal) in 85% normal saline, 10% cremophor (Sigma, UK) and 5% dimethylsulfoxide (Sigma, UK). Responses to electrical and natural stimuli were measured 10, 30 and 50 minutes post dosing. For spinal dosing, 0.1 μg, 1 μg and 10 μg of TROX-1 were cumulatively applied directly onto the cord in a volume of 50 μl. The vehicle for spinally applied drug consisted of >99% normal saline, <1% cremophor and <1% dimethylsulfoxide. Systemic and spinal drug doses were chosen based on efficacy in behavioural assays (Abbadie *et al.*, 2010). One neurone was characterised per rat.

6.2.3 Spinal nerve ligation surgery

See section 2.2.

6.2.4 Behavioural Assessments

See section 2.3.1 and 2.3.2. Ipsilateral and contralateral behavioural sensitivity to mechanical and cold stimuli was assessed in SNL and sham rats on day 14 post surgery.

6.2.5 Statistics

Mechanical and heat coding of neurones was compared with a 2 way repeated measures (RM) ANOVA followed by a Bonferroni *post hoc* test for paired comparisons. Cold, heat, brush and electrically evoked neuronal responses were compared with either a paired Student's T test or a 1 way RM ANOVA followed by a Bonferroni *post hoc* test for paired comparisons. Sphericity was tested using Mauchly's test, the Greenhouse-Geisser correction was applied if violated. Behavioural responses were compared with the Wilcoxon test.

6.3. Results

6.3.1 Summary of neuronal and behavioural responses in sham and SNL rats

Rats were examined 14 days post injury for signs of behavioural hypersensitivity following sham or SNL surgery. SNL rats displayed guarding behaviour of the injured ipsilateral hind paw, a feature that was absent on the uninjured contralateral side and in sham operated rats. SNL rats, but not sham, displayed significantly reduced withdrawal thresholds to punctate mechanical stimulation (Wilcoxon test, $P<0.05$) (Fig. 6.3*A*) and increased withdrawals to innocuous cooling (Wilcoxon test, $P<0.05$) (Fig. 6.3*B*).

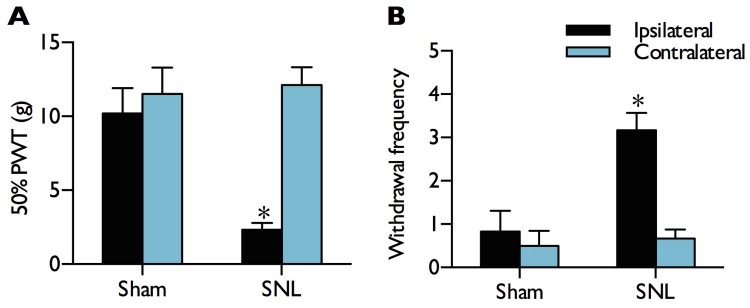


Figure 6.3 SNL rats exhibit behavioural hypersensitivity. (A) Unilateral ligation of L5 and L6 spinal nerves reduces mechanical withdrawal thresholds and **(B)** increases responsiveness to innocuous cooling in SNL rats. Data represents mean ± SEM. * $P<0.05$. Sham $n=11$, SNL $n=14$. PWT - Paw withdrawal threshold.

In vivo electrophysiology was subsequently performed to examine the effect of TROX-1 on mechanical and thermal coding of lamina V/VI spinal neurones under uninjured and neuropathic conditions in the chronic phase of the model. Table 6.2 summarises neuronal depths recorded from and evoked baseline responses. Neurones were characterised from depths relating to deep dorsal horn laminae (Watson *et al.*, 2009) and were selected on the basis of responses to noxious heat, dynamic brushing and noxious mechanical stimulation.

Table 6.2 Baseline characterisations of deep dorsal horn WDR neurones from sham and SNL rats. Range of neuronal depths recorded from in parentheses. Data represent mean ± SEM. (APs- action potentials).

	Sham *n*=11			SNL *n*=14		
Depth (μm)	773 (670	± -	32.88 910)	616 (490	± -	35.32 880)
A threshold (mA)	0.03	±	0.01	0.06	±	0.01
C threshold (mA)	0.63	±	0.17	0.47	±	0.13
Aβ-evoked (APs)	87	±	10.97	101	±	8.94
Aδ-evoked (APs)	199	±	25.61	203	±	15.82
C-evoked (APs)	435	±	30.65	499	±	37.25
Post-discharge (APs)	292	±	45.64	362	±	46.24
Brush (APs)	445	±	53.61	477	±	40.33
2g (APs)	28	±	4.31	29	±	4.77
8g (APs)	299	±	49.50	302	±	29.70
15g (APs)	575	±	52.39	627	±	37.02
26g (APs)	906	±	50.63	909	±	40.27
60g (APs)	1264	±	59.62	1203	±	64.02
35°C (APs)	203	±	32.84	232	±	28.63
42°C (APs)	461	±	61.18	412	±	43.97
45°C (APs)	676	±	71.89	595	±	74.33
48°C (APs)	1151	±	70.01	1068	±	46.29
Acetone (APs)	74	±	16.26	65	±	15.71
Ethyl chloride (APs)	570	±	118.25	680	±	100.73

6.3.2 Systemic TROX-1 inhibits neuronal responses to mechanical stimulation in spinal nerve ligated rats

Following isolation and characterisation of single WDR neurones, rats were dosed subcutaneously with 20 mg/kg TROX-1. In SNL and sham rats, TROX-1 had no effect on innocuous and noxious cold evoked neuronal responses (paired Student's t-test, $P>0.05$) (Fig. 6.4*A, B*). In SNL rats, TROX-1 suppressed mechanical coding of dorsal horn neurones with significantly reduced neuronal responses to low threshold and supra-threshold stimuli (2 Way RM ANOVA $P<0.01$, $F_{1,20}=22.78$, followed by Bonferroni *post hoc* test) (Fig. 6.4*E*). However, no effect was observed on mechanical coding in sham-operated rats (2 Way RM ANOVA $P>0.05$, $F_{1,16}=1.195$)(Fig. 6.2*F*). Neuronal responses to heat (2 Way RM ANOVA $P>0.05$, sham: $F_{1,12}=1.059$, SNL: $F_{1,15}=2.529$) (Fig. 6.4*C, D*) and dynamic brushing (paired Student's t-test, $P>0.05$) (Fig. 6.4*G, H*) were also unaffected in both SNL and sham rats. A train of electrical stimuli was applied to examine the effect of TROX-1 on pre- and post-synaptic measures of activity. The total number of evoked action potentials attributed to Aβ-, Aδ- and C-fibres did not significantly differ to baseline in SNL or sham rats. Indicators of neuronal excitability, input, wind-up and post-discharge were also unaffected (paired Student's t-test, $P>0.05$) (Fig. 6.4*I, J*).

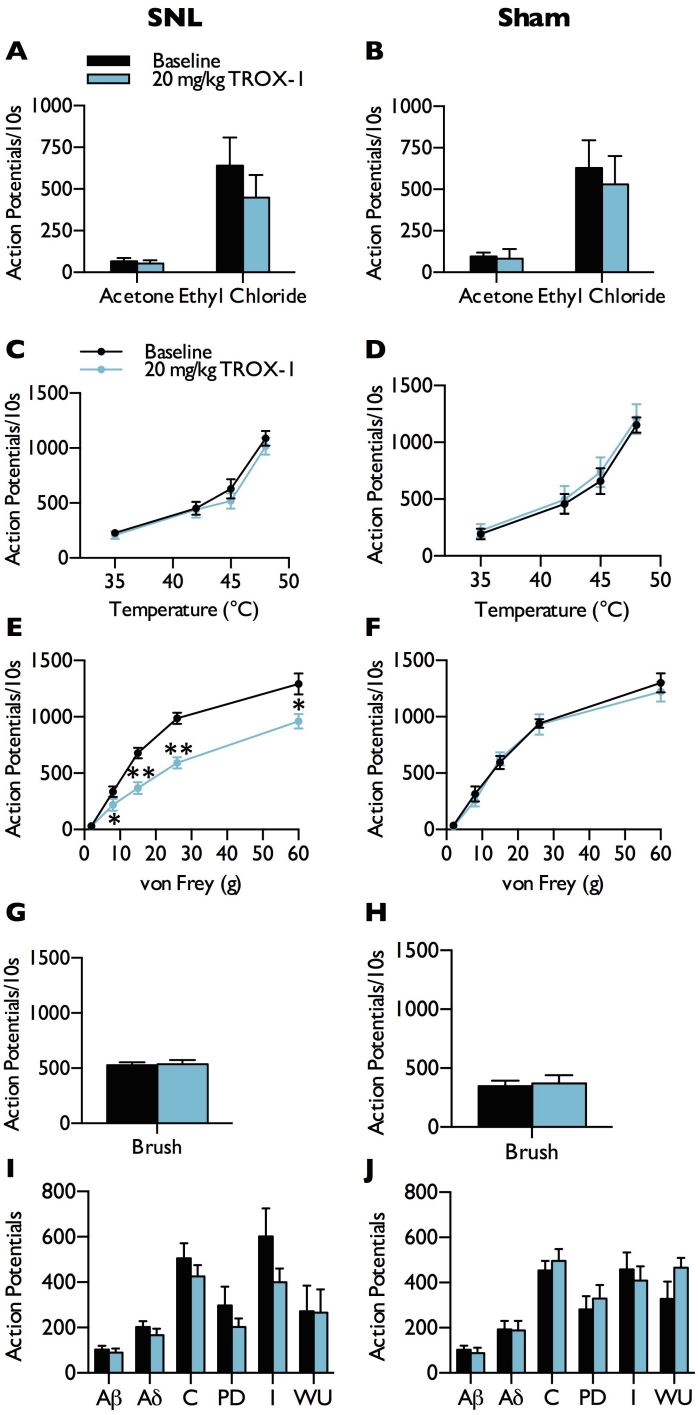


Figure 6.4 Systemic TROX-1 selectively inhibits mechanical coding of dorsal horn lamina V/VI neurones in spinal nerve ligated rats. SNL (*n*=6) or sham (*n*=5) rats were dosed subcutaneously with 20 mg/kg TROX-1. Neuronal responses to innocuous and noxious cooling **(A, B)**, heat **(C, D)**, punctate mechanical **(E, F)**, dynamic brushing **(G, H)**, and electrical stimuli **(I, J)** were recorded. Left panels – SNL, right panels – sham. Data displayed as maximum change from baseline. Data represents mean ± SEM. * $P<0.05$, ** $P<0.01$. (I – input, WU – wind-up, PD – post-discharge).

6.3.3 Spinal dosing replicates systemic effect of TROX-1 on mechanically evoked responses in spinal nerve ligated rats

The mechanism of TROX-1 was further investigated by examining the cumulative effects of 0.1 μg, 1 μg and 10 μg TROX-1 applied directly onto to the spinal cord. As previously observed through a systemic route of administration, TROX-1 did not alter evoked responses to innocuous cold (1 Way RM ANOVA $P>0.05$, sham: $F_{3,9}=0.579$, SNL: $F_{1.34,6.69}=1.594$) or noxious cold (1 Way RM ANOVA $P>0.05$, sham: $F_{3,9}=0.286$, SNL: $F_{3,18}=0.713$) (Fig. 6.5*A, B*), heat (2 Way RM ANOVA $P>0.05$, sham: $F_{3,15}=0.409$, SNL: $F_{3,9}=1.15$) (Fig. 6.5*C, D*) or dynamic brushing of the receptive field in either conditions (1 Way RM ANOVA $P>0.05$, sham: $F_{3,15}=0.714$, SNL: $F_{1.19,7.11}=0.03$) (Fig. 6.5*G, H*). The total number of evoked action potentials attributed to Aβ- (sham: $F_{1.04,5.22}=1.307$, SNL: $F_{2.22,13.31}=2.15$), Aδ- (sham: $F_{1.4,5.2}=2.574$, SNL: $F_{3,18}=0.335$) and C-fibres (sham: $F_{3,15}=2.667$, SNL: $F_{3,18}=1.75$) did not significantly differ to baseline in SNL or sham rats. Indicators of neuronal excitability, input (sham: $F_{3,15}=0.232$, SNL: $F_{1.49,8.96}=2.242$), wind-up (sham: $F_{3,15}=0.07$, SNL: $F_{1.29,7.74}=0.0556$) and post-discharge (sham: $F_{3,15}=1.126$, SNL: $F_{3,18}=3.155$) were also unaffected (1 Way RM ANOVA $P>0.05$) (Fig. 6.5*I, J*). Spinal TROX-1 dose dependently inhibited neuronal responses to punctate mechanical stimulation of the receptive field in SNL rats (2 way RM ANOVA $P<0.001$, $F_{3,18}=27.186$) (Fig. 6.5*E*) with no effect observed in sham rats (2 way RM ANOVA $P>0.05$, $F_{3,15}=1.161$) (Fig. 6.5*F*).

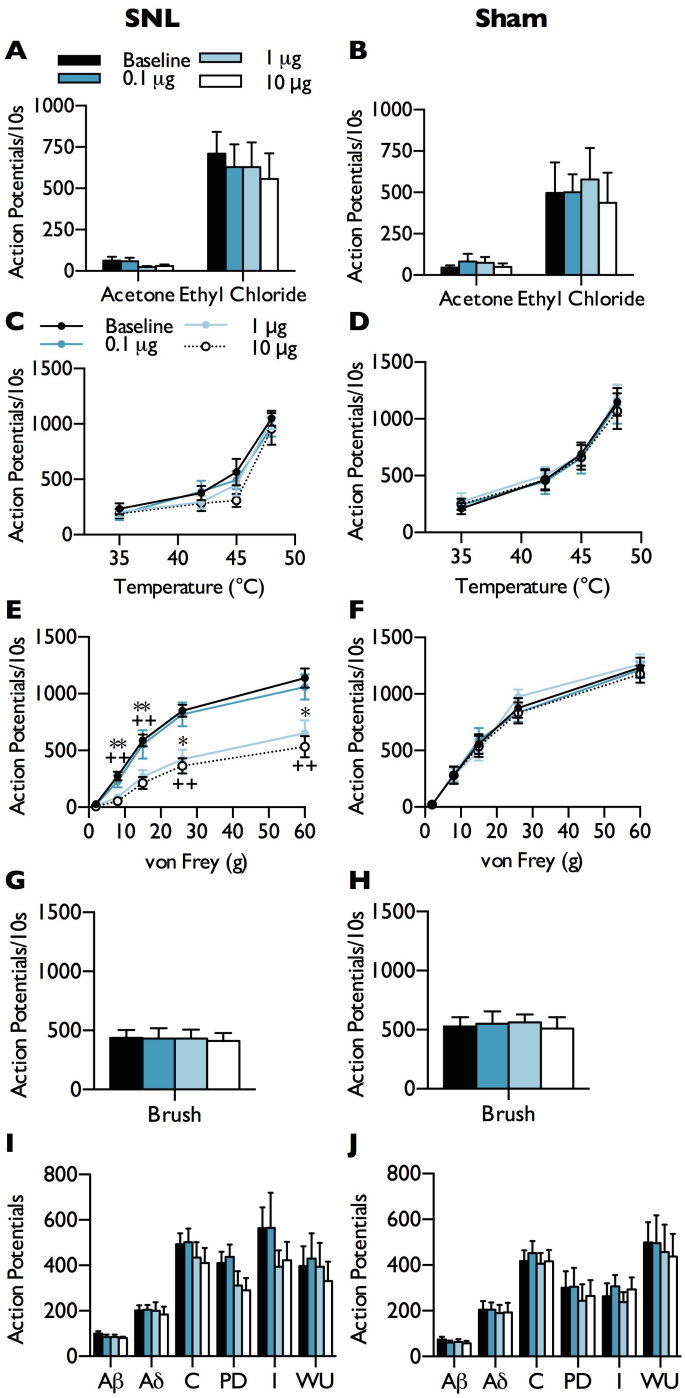


Figure 6.5 Spinal TROX-1 selectively inhibits mechanical coding of dorsal horn lamina V/VI neurones in spinal nerve ligated rats. Following stable baseline recordings (<10% variation), SNL (*n*=8) or sham (*n*=6) rats were dosed spinally with 0.1 µg, 1 µg and 10 µg TROX-1 in a volume of 50 µl. Neuronal responses to innocuous and noxious cooling **(A, B)**, heat **(C, D)**, punctate mechanical **(E, F)**, dynamic brushing **(G, H)**, and electrical stimuli **(I, J)** were recorded. Left panels – SNL, right panels – sham. Asterisks (*) denote significant differences between baseline and 1 µg. (+) denotes significant differences between baseline and 10 µg. Data displayed as maximum change from baseline. Data represents mean ± SEM. * *P*<0.05, ** *P*<0.01. (I – input, WU – wind-up, PD – post-discharge).

6.4 Discussion

This study extends previous observations of the effects of TROX-1 in nociceptive assays in providing a neuronal correlate of behavioural responses to threshold and supra-threshold stimulation in a model of neuropathic pain. Due to the ubiquitous expression of calcium channels, a blocker that inhibits open or inactive channels in neurones exhibiting high frequencies of firing could modulate abnormal nociceptive processing without affecting normal neuronal function (Winquist *et al.*, 2005). ω-conotoxin GVIA and MVIIA, unlike TROX-1, display no preference for activation state or frequency dependent inhibition (Feng *et al.*, 2003).

6.4.1 Comparison of TROX-1 to state independent inhibitors of Ca_v2 channels

The effects of antagonists of the N-type (ω-conotoxin GVIA), P/Q-type (ω-agatoxin IVA) and R-type (SNX-482) channels have been examined on spinal neuronal activity in nerve injured and uninjured conditions. All three antagonists inhibit neuronal responses to mechanical, heat and electrical stimuli in SNL rats to various degrees, though SNX-482 had minimal effects in sham rats (Matthews & Dickenson, 2001; Matthews *et al.*, 2007). The increased potency of ω-conotoxin GVIA and SNX-482, but not ω-agatoxin IVA, in SNL rats identifies significant neuroplastic changes that modulate N-type and R-type function and/or expression after injury (Matthews & Dickenson, 2001; Matthews *et al.*, 2007).

TROX-1 exhibits pathophysiological state dependent activity and selectively suppresses mechanical coding of WDR neurones in SNL rats. *In vitro*, TROX-1 increasingly inhibits calcium currents following repeated trains of depolarisations thereby demonstrating use-dependency (Swensen *et al.*, 2012). In contrast, TROX-1 had no impact on wind-up potentiation of dorsal horn neurones following repeated electrical stimulation *in vivo*. The nature of this stimulus is more akin to supra-threshold stimulation and involves synchronised activation of primary afferent terminals in the receptive field. The discord between the *in vitro* and *in vivo* scenarios could be explained by the differing stimulus parameters (*in vitro* - 20 x 25 ms pulse, 2 Hz. *In Vivo* – 16 x 2 ms pulse, 0.5 Hz). Wind-up occurs optimally between 0.5 and 2 Hz (Herrero *et al.*, 2000). In this respect, the stimulus frequencies were relatively comparable between the two studies, though it is unclear whether TROX-1 also exhibits frequency dependent inhibition. Additionally, the proportion of channels entering an inactivated state increases with prolonged stimulus duration (Freeze *et al.*, 2006). Mechanical stimuli applied for 10 s results in prolonged depolarisations at threshold levels of intensity and thus, natural stimulation may be more amenable to inhibition in comparison to a rapid electrical stimulus *in vivo*. Also, an important caveat to note is that expression systems do not fully recreate regulation of channel function.

Dynamic mechanical and cold allodynia are frequent features of several neuropathies (Maier *et al.*, 2010), however brushing and cooling evoked neuronal responses were not inhibited by TROX-1. Surprisingly, heat evoked responses were also unaffected by TROX-1 given the anti-nociceptive effects of TROX-1 in CFA induced heat hyperalgesia (Abbadie *et al.*, 2010). This perhaps reflects divergent mechanisms between inflammatory hyperalgesia and nerve injury models. A differential regulation of pre-synaptic N-type function may underlie the modality selective actions of TROX-1 in SNL rats.

6.4.2 Pathology dependent changes in calcium channel regulation

Functional diversity in N-type calcium channels is achieved through alternate splicing of the C-terminus. In particular the e37a and e37b variants are highly expressed in nociceptors and whereas the former is associated with mechanical and heat hypersensitivity, the latter only influences mechanical hypersensitivity (Altier *et al.*, 2007). The e37a variant, compared to e37b, exhibits increased channel open time and inactivates more slowly (Castiglioni *et al.*, 2006). Channels exhibiting these biophysical properties may be more favourable for inhibition by a state dependent antagonist. However, many splice variants are postulated to exist, some of which may be differentially expressed after injury, and could have modality selective consequences for sensory transduction. At the mRNA level, N-type channels do not appear up-regulated in DRG neurones following nerve ligation while some splice variants are down-regulated (Altier *et al.*, 2007). In contrast, an increase in the immunoreactivity of the pore forming α_{1B} subunit in the superficial laminae has been reported (Cizkova *et al.*, 2002). This is likely due to the up-regulation of the $\alpha_2\delta$-1 subunit that occurs after nerve injury (Luo *et al.*, 2001). Elevated levels of the $\alpha_2\delta$-1 subunit in the central terminals of primary afferent fibres enhances trafficking of channels to synapses in addition to determining kinetics of transmitter release (Hoppa *et al.*, 2012). Consistent with this role, synaptosomal levels of $Ca_v2.2$ are reduced in $\alpha_2\delta$-1 knockout mice (Appendix 8.4 Fig. 8.2). Although predominantly expressed in unmyelinated DRG neurones under normal conditions, the $\alpha_2\delta$-1 subunit is up-regulated in neurones of all types after injury (Bauer *et al.*, 2009). The increase in trafficking and inactivation rate of calcium channels by $\alpha_2\delta$-1 likely enhances pre-synaptic terminal excitability and facilitates excitatory transmission by supporting rapid transition between active and inactive states (Li *et al.*, 2006). Differential expression of $\alpha_2\delta$-1 splice variants also occurs following SNL, though these variants exert similar effects on the biophysical properties of Ca_v2 currents (Lana *et al.*, 2014). Although not consistently observed, expression of the β3 subunit also increases after nerve injury (Appendix 8.2 Fig. 8.7 and Li *et al.*,

2012), which could further influence the rate of channel activation and inactivation (Castellano *et al.*, 1993) resulting in enhanced calcium currents in small diameter DRG neurones (Li *et al.*, 2012).

As mentioned previously, calcium channels are subjected to substantial modulation by G-proteins (Herlitze *et al.*, 1996; Patil *et al.*, 1996). $GABA_B$ receptors, and to a lesser extent α_2 adrenoreceptors, localised in the dorsal horn are crucial in mediating tonic inhibitory influences on pre-synaptic terminals as well as influencing excitability of projection neurones and interneurones (Millan, 2002). Noradrenaline induced mobilisation of $G_{\beta\gamma}$ subunits has been demonstrated to inhibit transmitter release from neurones *in vitro* (Stephens & Mochida, 2005; Bucci *et al.*, 2011). Likewise, the inhibitory effect of $GABA_B$ receptors is also dependent on coupling to calcium channels (Takahashi *et al.*, 1998). G-protein coupled receptor moderation of primary afferent terminal excitability could occur through regulation of potassium currents consequently hyperpolarising pre-synaptic terminals (Yagi & Sumino, 1998). Descending serotonergic inhibitory pathways terminating on metabatropic 5-HTRs can directly, or indirectly through GABAergic interneurones, exert inhibitory influences on pre-synaptic calcium channel function and subsequent transmitter release (Brenchat *et al.*, 2010; Choi *et al.*, 2012). Numerous studies have identified deficits in inhibitory drive following a neuropathic injury (Moore *et al.*, 2002; Rahman *et al.*, 2008; Hughes *et al.*, 2013). Additionally, descending facilitatory pathways activating ionotropic 5-HT_3Rs will have further depolarising effects on pre-synaptic terminals. Spinally delivered ondansetron, a 5-HT_3R antagonist, inhibits mechanically evoked responses with minimal effects on heat evoked responses in SNL rats (Suzuki *et al.*, 2004). Spinalisation of SNL rats ablates mechanical hypersensitivity but not heat hypersensitivity indicating the latter is likely dependent on spinal disinhibition whereas the former requires intact spinal-supraspinal circuits (Ossipov *et al.*, 2000). One possibility is that a combination of neuroplastic changes in primary afferent excitability coupled with alterations in activity in descending pathways underlies the pathophysiological and modality selective actions of TROX-1 in SNL rats. This would be consistent with efficacy in the sodium iodoacetate induced osteoarthritis model (Abbadie *et al.*, 2010), which can be characterised by a neuropathic component resulting in hypersensitivity in areas of the dermatome outside the primary area of injury (Combe *et al.*, 2004). A time dependent loss of noradrenergic inhibition and an increased descending facilitatory drive has also been demonstrated in this model (Rahman *et al.*, 2009; Burnham & Dickenson, 2013).

6.4.3 Role of non-spinally expressed calcium channels

These data suggest spinally expressed calcium channels are critical for the anti-hyperalgesic effects of TROX-1. N-type calcium channels at the site of injury could contribute to afferent dysfunction, generation of ectopic discharges and hyperexcitability (Xiao & Bennett, 1995), though this does not appear consistent across models (Chaplan *et al.*, 1994b). The central nucleus of the amygdala has been implicated in the acquisition of conditioned fear and anxiety, and can influence aversive and evoked pain behaviours (Neugebauer *et al.*, 2004). The acquisition of conditioned aversive behaviour but not unconditioned behaviour appears in part dependent on the expression of N-type calcium channels in the amygdala and can be blocked by ω-conotoxin GVIA (Finn *et al.*, 2003). In addition, injection of ω-conotoxin GVIA into the central nucleus is pro-nociceptive in the early phase of the formalin test with no effect on the secondary phase supporting a role for the central nucleus in tonically inhibiting acute nociceptive drive (Finn *et al.*, 2003). Intra-RVM injections of ω-conotoxin MVIIA, but not ω-agatoxin IVA, alleviated mechanical hypersensitivity following SNL implicating N-type over P/Q type calcium channels in modulating activity in descending pathways (Urban *et al.*, 2005). However, the precise mechanism at this site remains unclear. These findings do not entirely preclude non-spinally mediated effects of TROX-1.

6.4.4 Summary

Systemic and spinally applied TROX-1 displays modality selective and pathology dependent activity in SNL rats. Phase II trials for CNV2197944, a state selective $Ca_v2.2$ antagonist, are currently being undertaken (Clinical Trials Identifier: NCT01848730). Meanwhile, phase II trials of a similar acting compound (Z160) have yielded poor results in post-herpetic neuralgia and lumbosacral radiculopathy (NCT01655849). Despite this, state dependent channel antagonists remain a promising area for selectively inhibiting aberrant neuronal activity. Further pre-clinical characterisations of the effects on nociceptive processing may help shape future trials and act as better predictors for success.

7. General discussion

7.1 Utilising models of cold hyperalgesia

The studies described here aimed to characterise components of cold sensitivity and characterise targets for the treatment of cold hyperalgesia. For the first time these data identify a role of $\alpha_2\delta$-1 in cold sensitive afferents. The findings presented also corroborate previous observations that cold hyperalgesia after nerve damage is TRPM8 dependent. In contrast, in naïve rats TRPM8 may be limited to encoding certain aspects of cold sensitivity such as subserving aversive components and driving warm seeking behaviours (Klein *et al.*, 2010; Almeida *et al.*, 2012). Unlike in human studies menthol fails to induce hyperalgesia in naïve rats highlighting significant species dependent differences in cold sensitivity. This may be related to the fact that testing in humans is performed on either the hands or forearm, however cold sensitivity in rodents is more prominent in trigeminal areas. Second order neurones in the trigeminal subnucleus caudalis respond vigorously to cooling and can be sensitised with low concentrations of menthol (<10%) in contrast to neurones innervating the hind paw (Zanotto *et al.*, 2007). With regards to analgesia, menthol had comparable effects in SNL rats and neuropathic patients supporting overlapping pathology dependent mechanisms. Surrogate models of pain have the potential to bridge the gap between pre-clinical testing and patients by screening treatments in healthy volunteers. Naturally a single model cannot express the full spectrum of symptoms/mechanisms experienced by patients such as sensory loss or long term transcriptional changes but a suitable model would ideally induce comparable and reversible symptomatology (Arendt-Nielsen & Yarnitsky, 2009). Perceptually A-fibre block, the thermal grill and menthol induced hyperalgesia model all evoke sensations of paradoxical burning and may have relevance to neuropathic patients.

There is little consensus over the mechanism of the thermal grill illusion. Leading theories include a warm temperature inhibition of lamina I COOL neurones leading to a disinhibition of HPC activity (Craig & Bushnell, 1994). Alternatively, increasing the temperature differential through either the warm or cold element can increase the intensity of the illusion supporting a theory of summation and convergence on thalamic pathways (Bouhassira *et al.*, 2005; Lindstedt *et al.*, 2011). Perception of unpleasantness during application of the thermal grill has been reported to be reduced in chronic pain patients of central and peripheral origin consistent with an underlying disruption of central integration of thermal stimuli (Morin *et al.*, 2002; Sumracki *et al.*, 2014). The inhibition of paradoxical pain intensity by ketamine, with no effects on noxious cold stimuli, supports the thermal grill illusion sharing some mechanisms with neuropathic patients (Kern *et al.*, 2008).

Areas of brain activation during menthol induced cold hyperalgesia and normal cold pain partially overlap and includes the anterior insula, anterior cingulated cortex and inferior frontal cortex. Activation of the dorsolateral prefrontal cortex and parabrachical were specific for menthol induced hyperalgesia (Seifert & Maihofner, 2007). Mechanisms of cold hyperalgesia differ between 'peripheral sensitisation' (menthol) and 'central disinhibition' (A-fibre block) models. fMRI analysis reveals menthol induced cold hyperalgesia preferentially activates a thermospecific lateral thalamic pathway whereas during a conduction block of A-fibres a stronger activation of the medial thalamus is apparent implying A-fibre block disinhibits activity in this ascending nociceptive channel (Binder *et al.*, 2007). Activity maps of these models frequently overlap with patients with abnormal cold sensitivity (Moisset & Bouhassira, 2007). That is not to say these processes are equivalent as changes in blood flow are merely surrogate markers of activity and do not permit inferences of inhibitory or excitatory transmission, but the insights gained from fMRI and the somatosensory profile of the menthol induced hyperalgesia model are suggestive of comparable underlying mechanisms with neuropathic patients and animal models of neuropathy (Fig. 7.1) (Binder *et al.*, 2011).

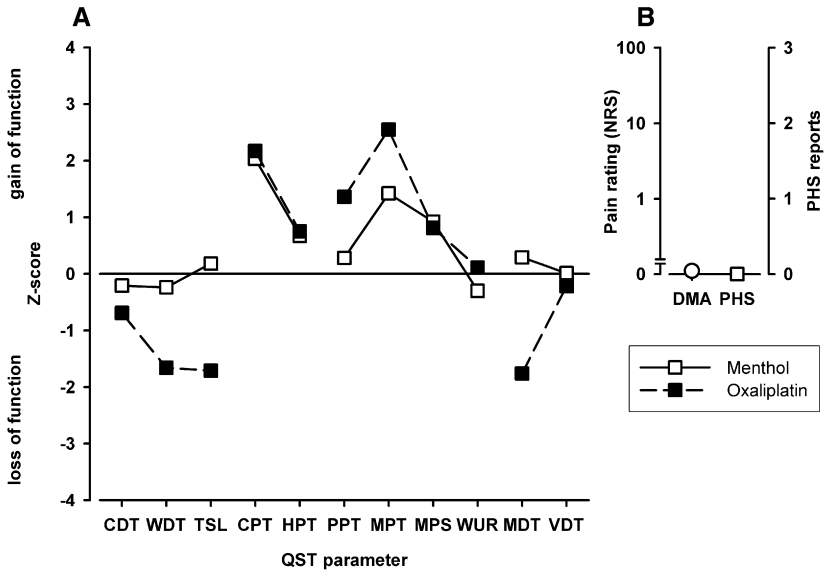


Figure 7.1 Somatosensory profiles of the menthol surrogate model and acute painful oxaliplatin neuropathy. Presented is the somatosensory (quantitative sensory testing) profile of the 12 volunteers and 9 patients with acute painful oxaliplatin neuropathy obtained on the dorsum of the right hand. Z-score: Volunteers: numbers of standard deviations between volunteer data after menthol application and normative group-specific mean value at baseline. Patients: numbers of standard deviations compared to normative age and sex matched data. CDT, cold detection threshold; WDT, warm detection threshold; TSL, thermal sensory limen; CPT, cold pain threshold; HPT, heat pain threshold; PPT, pressure pain threshold; MDT, mechanical detection threshold; MPT, mechanical pain threshold; VDT, vibration detection threshold; MPS, mechanical pain sensitivity; DMA, dynamic mechanical allodynia; WUR, wind-up; PHS, paradoxical heat sensation. NRS, numeric rating scale (pain intensity from 0–100, 0 indicating no pain, 100 worst pain). WUR, values give ratio series/single pain rating; PHS, the number of 3 observations. (Taken from Binder *et al.*, 2011).

Utilising *in vivo* electrophysiology in the SNL model may be useful for screening compounds for modulating cold hyperalgesia. State dependent channel antagonists are attractive targets as they are hypothesised to inhibit channels in neurones exhibiting high levels of activity. Both indirect and direct evidence implicates N-type calcium channels in cold transduction. In knockout mice reduced $\alpha_2\delta$-1 mediated trafficking of calcium channels, and the anti-nociceptive effects of ω-conotoxin in neuropathy models (Lee *et al.*, 2010), supports an N-type calcium channel dependence in cold sensitive afferents in normal and pathological conditions. However, here the state dependent Ca_v2 blocker TROX-1 had no effect on cold evoked neuronal responses but revealed an injury induced change in calcium channel function in mechanosensitive pathways.

High concentration topical menthol in humans consistently induces cold hyperalgesia and secondary pinprick hyperalgesia (Namer *et al.*, 2008; Binder *et al.*, 2011; Olsen *et al.*, 2014). It was not possible to induce similar symptoms in naïve rats making it difficult to infer underlying neural mechanisms, however some similarities may exist with the capsaicin induced hyperalgesia model. Intradermal capsaicin induces small areas of primary heat hyperalgesia and larger areas of secondary pinprick hyperalgesia and brush allodynia (LaMotte *et al.*, 1991). Whilst primary hyperalgesia is considered the effect of peripheral and central sensitisation, secondary effects are a central phenomenon. Superficial NS and deeper WDR dorsal horn neurones exhibit expanded receptive fields and become hyperexcitable to mechanical and heat stimuli following intradermal capsaicin, including a *de novo* response to innocuous brush stimulation in NS neurones (Simone *et al.*, 1991). Heterosynaptic mechanisms have been proposed to underlie Aβ- and Aδ-dependent secondary brush allodynia and pin prick hyperalgesia respectively, as low threshold mechanosensitive fibres are not involved in the conditioning C-fibre stimulus (Torebjork *et al.*, 1992; Magerl *et al.*, 1998; Ziegler *et al.*, 1999). Engagement of descending facilitatory influences could sustain a state of hyperexcitability after the cessation of peripheral drive (Zambreanu *et al.*, 2005). Menthol in contrast does not evoke dynamic mechanical allodynia or similar brainstem activity (Wasner *et al.*, 2004; Hatem *et al.*, 2006; Seifert & Maihofner, 2007; Namer *et al.*, 2008; Binder *et al.*, 2011). In the absence of a reproducible animal model, the role of descending controls in central sensitisation induced by menthol could be better clarified pharmacologically in human subjects as centrally acting drugs such as gabapentin or tapentadol may reduce signs of central sensitisation. A recently ended clinical trial has attempted to address this aspect (NCT01615510).

Most clinical trials rely heavily on average pain scores but a few report efficacy against cold hyperalgesia. In a small cohort of neuropathic patients with pain of central origin, gabapentin reduces pain intensity scores to noxious cold temperatures to levels comparable to uninjured areas (Attal *et al.*, 1998). Opioids in contrast have

moderate efficacy against cold pain thresholds in pain conditions of peripheral but not central origin (Eisenberg *et al.*, 2006). The serotonin-noradrenaline reuptake inhibitor duloxetine substantially reduces pain intensity scores to dynamic brushing and cooling in post stroke and spinal cord injury patients with no effect on pin prick and pressure pain (Vranken *et al.*, 2011). Tramadol but not pregabalin or ibuprofen increases cold pain thresholds in the menthol induced hyperalgesia model (Altis *et al.*, 2009). This study unfortunately did not assess other induced primary and secondary sensory changes associated with menthol. For example, gabapentin reduces the area of secondary sensitisation induced by capsaicin in addition to effects on the intensity of evoked stimuli (Dirks *et al.*, 2002; Gottrup *et al.*, 2004).

There are no reliable indicators as to whether a patient will respond to a particular treatment. Much heterogeneity exists between patients within a condition in terms positive and negative sensory abnormalities. Cluster analysis of neuropathic conditions identifies groups with distinct somatosensory profiles within a particular condition and between conditions, which may imply similar underlying mechanisms. Many have advocated identifying appropriate treatments through a mechanism based algorithm rather than simply grouping by aetiology (Baron *et al.*, 2009; Freeman *et al.*, 2014). In practice it would be difficult to determine an underlying mechanism from a particular symptom, but recent examples where this approach might successfully identify suitable patients includes studies demonstrating that diabetic neuropathy patients with inefficient diffuse noxious inhibitory controls were more likely to benefit from duloxetine and tapentadol presumably through enhancing descending inhibition (Yarnitsky *et al.*, 2012; Niesters *et al.*, 2014). It would be particularly interesting to see if patients across aetiologies exhibiting cold hyperalgesia were responsive to topical menthol and whether this predicted efficacy of a systemic TRPM8 agonist or antagonist.

7.2 Relation of lamina V coding to pain processing

Behavioural pharmacological studies are often the recipient of criticism given their partially subjective nature, susceptibility to environmental influences and whether a withdrawal reflex constitutes a valid measure of pain (Chesler *et al.*, 2002; Percie du Sert & Rice, 2014). In isolation, these studies can be informative but need to be interpreted with these limitations in mind. A reflex withdrawal equates to a threshold response involving spinal and bulbospinal circuits. In humans, pain is often rated on a visual analogue scale (0-10) and by definition threshold is rated one, hence from a clinical perspective a pharmacological agent reducing pain ratings from 7 to 4 would be of more significance to patients (Sikandar & Dickenson, 2013). Single unit recordings within the dorsal horn allow the objective assessment of responses to low and high threshold natural stimuli within the

context of intact spinal inhibitory and excitatory circuits and descending modulatory pathways. Several algesiometric tests have been designed to examine various aspects and co-morbidities of chronic pain, such as conditioned place preference and open field testing (Wallace *et al.*, 2008; King *et al.*, 2009). Convergent WDR neurones are aptly placed in the sensory pathway to examine mechanisms of central sensitisation, neuronal substrates of allodynia and hyperalgesia, and have successfully been utilised to examine the mechanisms of numerous clinically efficacious drugs including opioids, gabapentin, ω-conotoxin and tapentadol (Dickenson *et al.*, 1990; Matthews & Dickenson, 2001; Suzuki *et al.*, 2005; Bee *et al.*, 2011). Models of hyperalgesia can also be back translated as is evident with the effects of ciguatoxin in providing a neuronal correlate of the cold hyperalgesia observed clinically and in a surrogate model of ciguatera (Zimmermann *et al.*, 2013).

Electrophysiological studies in primates indicate WDR neurones are capable of encoding the intensity of noxious heat stimulation (Maixner *et al.*, 1986; Dubner *et al.*, 1989). These observations are supported by a recent parallel human and rat study which revealed that both lamina I NS and lamina V WDR neurones exhibit spatially summated and graded intensity dependent coding to laser evoked stimulation. Lamina V neurones however displayed a steeper stimulus-response curve and greater sensitivity to small differences in stimulus intensity, a feature that showed strong concordance with human pain scores to the same stimulus (Sikandar *et al.*, 2013b). Population coding by WDR neurones appears to discriminate between low and high threshold stimuli, as well as code stimulus intensity within the noxious range, likely to be dependent on numbers of neurones activated and frequency of firing (Coghill *et al.*, 1993; Quevedo & Coghill, 2009). As well as subserving aversive aspects of pain, NS neurones responding in tandem with WDR neurones may have the capacity to fine-tune intensity and localisation of stimuli (Price *et al.*, 2003).

Lamina I neurones unlike lamina V neurones exhibit minimal wind-up and post-discharge (Seagrove *et al.*, 2004). One of the criticisms often aimed at experimentally induced wind-up is that the synchronised supra-threshold nature of afferent stimulation bears little physiological relevance. A perceptual correlate can be induced in human subjects, and both neuronal and perceptual wind-up are inhibited by NMDA blockade (Dickenson & Sullivan, 1987; Price *et al.*, 1994). Although wind-up and central sensitisation are not considered equivalent processes, it undeniable that the two are closely linked (Woolf, 1996). Wind-up is a short-term process in comparison reflecting activity dependent increases in neuronal excitability and can lead to features shared with central sensitisation such as expansion of receptive field size, inducing wind-up at lower frequencies and overlapping pharmacological dependencies (e.g. reversal by NMDA antagonists) (Li *et al.*, 1999). Wind-up can initiate central sensitisation (though central sensitisation can be established without increased

wind-up), and abnormal temporal summation of pain is frequently present in neuropathic patients with signs characteristic of central sensitisation (Woolf, 1996; Woolf, 2011). Thus, wind-up could act as a read out of sensitisation state and pharmacological agents that inhibit wind-up may also reverse central sensitisation (Herrero *et al.*, 2000).

7.3 Drawbacks of search technique

When comparing baseline neuronal responses from naïve, SNL and sham operated rats, it is important to consider selection biases during interpretation. Neurones were chosen on the basis of exhibiting wind-up and evoked responses to low and high threshold mechanical and thermal stimuli. As a result neurones exhibiting high levels of response or spontaneous firing were often discarded as these typically failed to stabilise and in some cases depolarised or burst. Neurones with little or no cold sensitivity were also discarded, therefore it is likely that the samples of neurones characterised were not truly representative of the whole population to be able to draw reliable conclusions (Appendix 8.1). Previous studies quantifying spontaneous activity suggest firing rates of approximately 1 Hz and 4 Hz in a subset of neurones in sham and SNL rats respectively (Chapman *et al.*, 1998). Drug effects on spontaneous firing were not examined during these studies mainly owing to the difficulty in interpreting the source or the nature of ongoing activity.

An additional obstacle in knockout mice arises from potential phenotypic changes in neuronal excitability. Normal neurone selection criteria could be rendered obsolete if gene ablation substantially alters neuronal responses potentially resulting in discarding of neurones. If we consider a knockout paradigm that specifically attenuates transduction of low threshold stimuli, WDR neurones may now appear to have an NS phenotype. This was generally overcome by performing these studies while blinded to genotype to avoid selection bias and by characterising all polymodal neurones found within depths delineating lamina V and VI.

7.4 Confounding effects of anaesthesia

At physiologically relevant concentrations, isoflurane is claimed to exert a bimodal effect on TRPM8 function. After initially inducing calcium currents in HEK293 cells and DRG neurones, prolonged exposure results in inhibition of channel function. Both isoflurane suppression of respiratory drive and hypothermia are partially abolished in TRPM8 knockout mice (Vanden Abeele *et al.*, 2013). It could be argued that both TRPM8 antagonists failed to inhibit neuronal responses to cooling in naïve rats due to isoflurane effects on TRPM8 function. However, it should be noted the concentrations used in these *in vitro* studies might not accurately

represent the concentration at the level of cutaneous receptors *in vivo*. During the course of these studies, neuronal responses to cooling stimuli remained stable (minimum 3 tests over 1 hour) prior to drug administration in SNL, sham and naïve rats. Both TRPM8 antagonists tested inhibited neuronal responses to noxious cold stimulation confirming TRPM8 functionality, in SNL rats at least, during prolonged periods of anaesthesia. Additionally, behavioural responses in naïve rats following TRPM8 blockade were entirely consistent with neuronal recordings. It is possible that TRPM8 dependent mechanisms underlie some of the acute side effects during the induction of anaesthesia in humans (Vanden Abeele *et al.*, 2013). In rats there was little evidence that neuronal responses to cold stimulation were progressively suppressed by isoflurane during the timeframe experiments were conducted under.

For the knockout studies, neuronal recordings were performed under urethane anaesthesia as opposed to isoflurane used during the rat set-up. An injectable anaesthetic was chosen due to the difficulty of maintaining a constant level of anaesthesia over long periods of time with isoflurane in mice. Although the mechanism of action of urethane is not entirely clear, *in vitro* urethane inhibits NMDA and AMPA receptors and potentiates $GABA_A$ and glycine receptors (Hara & Harris, 2002). This would undoubtedly impact negatively on fast excitatory synaptic transmission and excitability of spinal neurones. Genotype dependent differences in tolerability to urethane cannot be ruled out given the cardiovascular phenotype of $\alpha_2\delta$-1 -/- mice and that high doses of urethane suppress cardiovascular function (Maggi *et al.*, 1984). The electrophysiological findings are largely consistent with behavioural observations suggesting overall this has not had any confounding effect.

7.5 Future studies

The studies described here largely focussed on TRPM8 as evidence from human and animal studies overwhelmingly support a role in multiple aspects of cold sensitivity. Numerous studies also implicate TRPA1, TRPC5, $Na_v1.8$, HCN1, K_v7, TASK-3 and TREK/TRAAK channels in transduction of cold temperatures. Small molecule inhibitors of some of these channels are available and could be delivered locally to the receptive field to examine the contribution to integrative dorsal horn neuronal responses to cold stimulation. The roles of these channels could also be examined under pathological conditions.

To date the coding properties of lamina I neurones to cold stimuli after SNL have not been examined. These studies could be complemented by examining the pharmacological basis of cold sensitivity in superficial neurones in addition to the WDR neurones examined here. This would pose some technical difficulties as the stability of lamina I neurones means they are not always conducive to long term pharmacological studies. In

light of the fact spinoparabrachical lamina I neurones in rats do not respond vigorously to cooling in the innocuous range and most lamina I neurones in the rat are medulla and parabrachial projecting, it is unclear as to whether a substantial spinothalamic COOL population exists in rodents (Bester *et al.*, 2000; Todd, 2002). Approximately half of spinothalamic HPC neurones exhibit graded responses to innocuous cooling in rats (Zhang *et al.*, 2006). Any thermospecific neurones could be identified more readily with an electrical searching stimulus, though a study in rats may be restricted to examining responses of polymodal neurones. Given the ability of descending controls to inhibit or facilitate spinal processing, and the clear roles of monoamines in these events, it would be interesting to investigate the roles of these central pathways, in normal and neuropathic conditions, in spinal mechanisms of cold transmission.

A drawback of the endpoints chosen during the course of this thesis is that ongoing pain could not be assessed. Behavioural studies could be extended to examine whether TRPM8 agonists (systemic or topical) or TROX-1 inhibit ongoing pain using conditioned place preference testing. Unfortunately, whether menthol alleviates ongoing pain in neuropathic patients is not always reported which at present makes it difficult to assess the predictive value of this test.

7.6 Closing remarks

Cold temperatures can evoke intense pain in many neuropathic patients. Acutely this can make a simple everyday task such as opening the fridge a painful experience. Cumulatively, repeated exposure to cool temperatures potentially subjects patients to several pain evoking events throughout the day, which may summate to produce ongoing pain (Bennett, 2012). Identifying novel agents to manage this symptom as an adjunct to other analgesics would be of great benefit to these patients. In some cases it may allow continuation of life saving chemotherapy in addition to managing the long-term pain associated with the resultant neuropathy. The studies outlined here support the use of TRPM8 agonists and antagonists for treating aberrant cold sensitivity. Further human studies are required to examine whether TRPM8 modulators are safe and tolerable in neuropathic patients.

8. Appendices

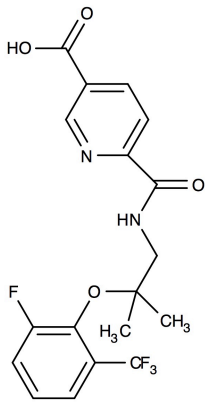
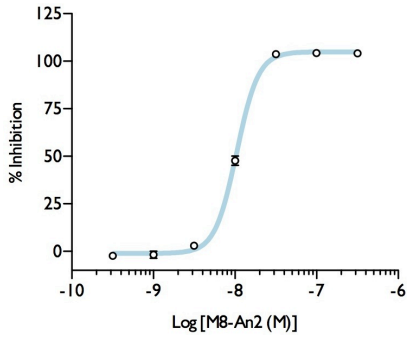
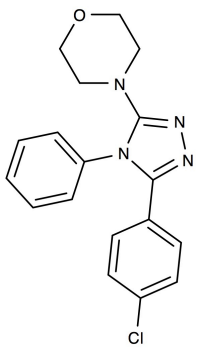
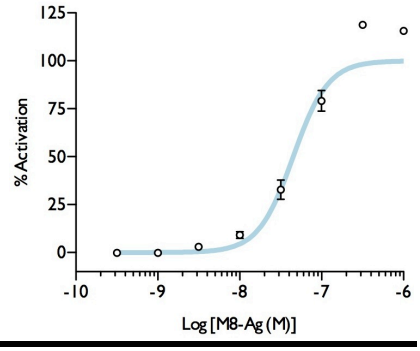
8.1 Collated baseline neuronal responses

Table 8.1 Comparison of baseline neuronal responses in naïve, sham and SNL rats.

	Naïve *n*=41	Sham *n*=18	SNL *n*=55	
Depth (μm)	750 ± 22.76 (620 - 1080)	741 ± 35.33 (600 - 830)	656 ± 18.79 (470 - 1030)	
A threshold (mA)	0.08 ± 0.03	0.05 ± 0.01	0.07 ± 0.01	1 way ANOVA P>0.05, $F_{2,111}$=0.486
C threshold (mA)	0.34 ± 0.06	0.55 ± 0.12	0.38 ± 0.05	1 way ANOVA P>0.05, $F_{2,111}$=1.674
Aβ-evoked (APs)	123 ± 6.27	95 ± 7.47	112 ± 4.96	1 way ANOVA P>0.05, $F_{2,111}$=2.249
Aδ-evoked (APs)	220 ± 9.72	202 ± 17.40	227 ± 9.98	1 way ANOVA P>0.05, $F_{2,111}$=0.836
C-evoked (APs)	504 ± 22.75	454 ± 30.06	504 ± 21.05	1 way ANOVA P>0.05, $F_{2,111}$=0.885
Post-discharge (APs)	346 ± 21.82	299 ± 32.73	365 ± 20.25	1 way ANOVA P>0.05, $F_{2,111}$=1.426
Brush (APs)	433 ± 22.36	456 ± 37.45	454 ± 21.74	1 way ANOVA P>0.05, $F_{2,111}$=0.249
2g (APs)	32 ± 4.13	28 ± 4.18	32 ± 3.45	2 way ANOVA P>0.05, $F_{2,555}$=1.179
8g (APs)	330 ± 22.07	304 ± 31.98	336 ± 17.61	
15g (APs)	624 ± 29.57	592 ± 40.32	633 ± 20.25	
26g (APs)	921 ± 30.66	894 ± 38.56	944 ± 21.78	
60g (APs)	1269 ± 35.64	1236 ± 40.75	1252 ± 29.02	
35°C (APs)	252 ± 24.51	229 ± 31.14	276 ± 22.73	2 way ANOVA P>0.05, $F_{2,555}$=1.518
42°C (APs)	551 ± 36.37	503 ± 57.50	491 ± 37.29	
45°C (APs)	782 ± 45.86	718 ± 60.62	697 ± 42.31	
48°C (APs)	1201 ± 82.60	1151 ± 50.77	1153 ± 41.20	
Acetone (APs)	69 ± 12.77	64 ± 19.36	78 ± 12.53	1 way ANOVA P>0.05, $F_{2,111}$=0.257
Ethyl chloride (APs)	498 ± 40.17	496 ± 60.93	614 ± 39.64	1 way ANOVA P>0.05, $F_{2,111}$=2.553

8.2 Pharmacological modulators of TRPM8

Table 8.2 Summary of TRPM8 modulating compounds used.

Compound	M_w	Structure	EC_{50}/IC_{50}	Dose response curve (Ca^{2+} influx assay)
M8-An1	359.8	Undisclosed	Undisclosed	Undisclosed
M8-An2 (Patel *et al.*, 2014b)	400		10.9 ± 2.6 nM	
M8-Ag (Patel *et al.*, 2014a)	340.8		44.97 ± 1.2 nM	

8.3 Effects of vehicle (85% saline, 10% cremophor and 5% dimethylsulfoxide) on WDR neuronal responses in naïve rats

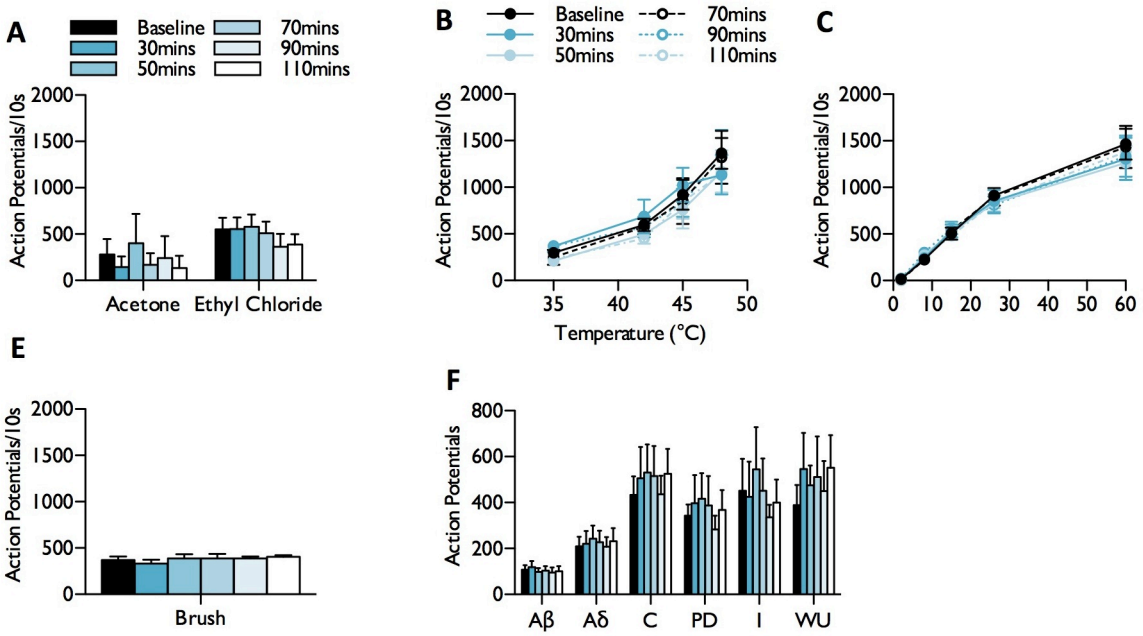


Figure 8.1. Neuronal responses to **(A)** cooling (1 way RM ANOVA $P>0.05$, innocuous: $F_{5,20}=1.466$, noxious: $F_{5,20}=2.466$), **(B)** heat (2 way RM ANOVA $P>0.05$, $F_{5,20}=1.126$), **(C)** mechanical (2 way RM ANOVA $P>0.05$, $F_{5,20}=0.456$), **(D)** brush (1 way RM ANOVA $P>0.05$, $F_{5,20}=0.578$) and **(E)** electrical stimulation (1 way RM ANOVA $P>0.05$, Aβ: $F_{5,20}=1.478$, Aδ: $F_{5,20}=0.792$, C: $F_{5,20}=1.263$, PD: $F_{5,20}=1.151$, I: $F_{5,20}=0.725$, WU: $F_{5,20}=0.642$) following subcutaneous injection of 1 ml/kg vehicle ($n=5$). Data represents mean ± SEM.

8.4 Figures from Patel *et al.*, 2013. *Journal of Neuroscience* 33(42):16412-16426

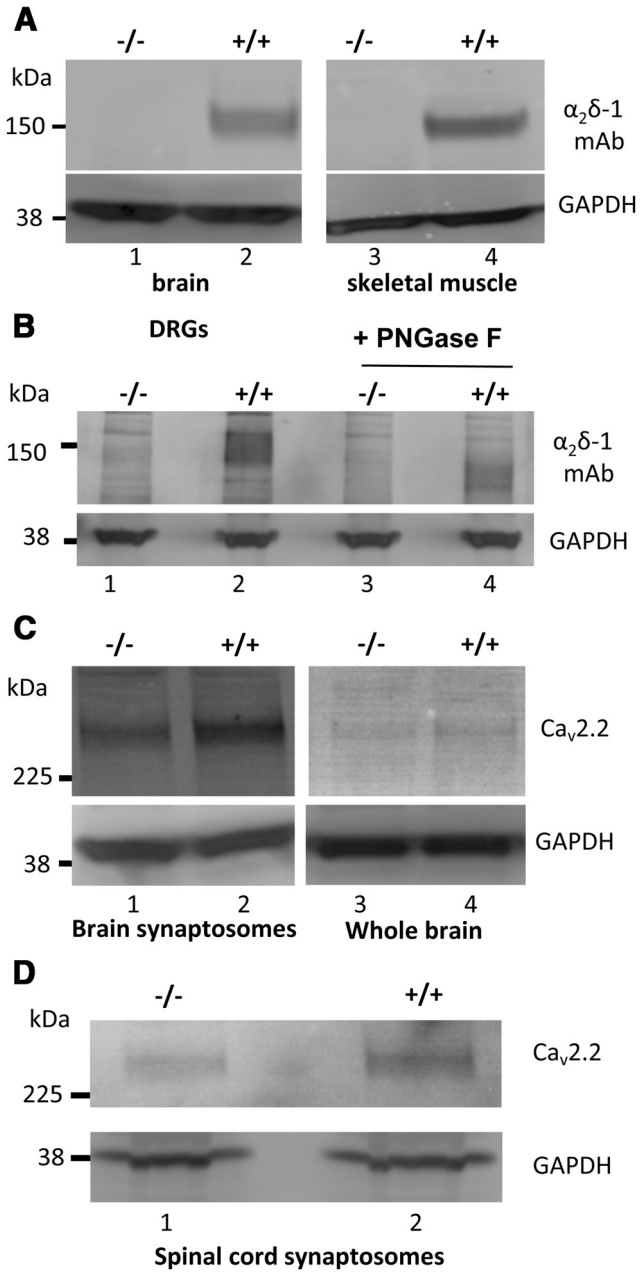


Figure 8.2 Immunoblotting for α2δ-1 and Cav2.2 in +/+ and −/− mice tissues. ***A***, Comparison of α2δ-1 levels in brain (left panel) and skeletal muscle (right panel) in α2δ-1$^{-/-}$ (lanes 1, 3) and α2δ-1$^{+/+}$ (lanes 2, 4). A total of 20 μg of protein was loaded/lane. No α2δ-1 is observed in α2δ-1$^{-/-}$ mice. Immunoblot for GAPDH (bottom panel) provides the loading control. ***B***, α2δ-1 levels in DRGs from α2δ-1$^{-/-}$ (lanes 1, 3) and α2δ-1$^{+/+}$ (lanes 2, 4) mice. Material (30 μg of protein/lane) in lanes 3 and 4 has been deglycosylated with PNGase F, showing the characteristic decrease in MW of α2δ-1 from ~150 kDa (lane 2) to ~105 kDa (lane 4). No corresponding bands are observed in α2δ-1$^{-/-}$ ganglia. GAPDH (bottom) provides a loading control. Data are representative of *n* = 3 experiments. ***C***, Cav2.2 levels in cerebral synaptosomes (left) and brain WCL (right) from α2δ-1$^{-/-}$ (lanes 1, 3) and α2δ-1$^{+/+}$ (lanes 2, 4) mice. GAPDH (bottom) provides a loading control for Cav2.2 quantification. Data are representative of *n* = 4 experiments. ***D***, Cav2.2 levels in spinal cord synaptosomes (top) from α2δ-1$^{-/-}$ (lane 1) and α2δ-1$^{+/+}$ (lane 2) mice. GAPDH (bottom) provides a loading control for Cav2.2 quantification. Data are representative of *n* = 4 experiments.

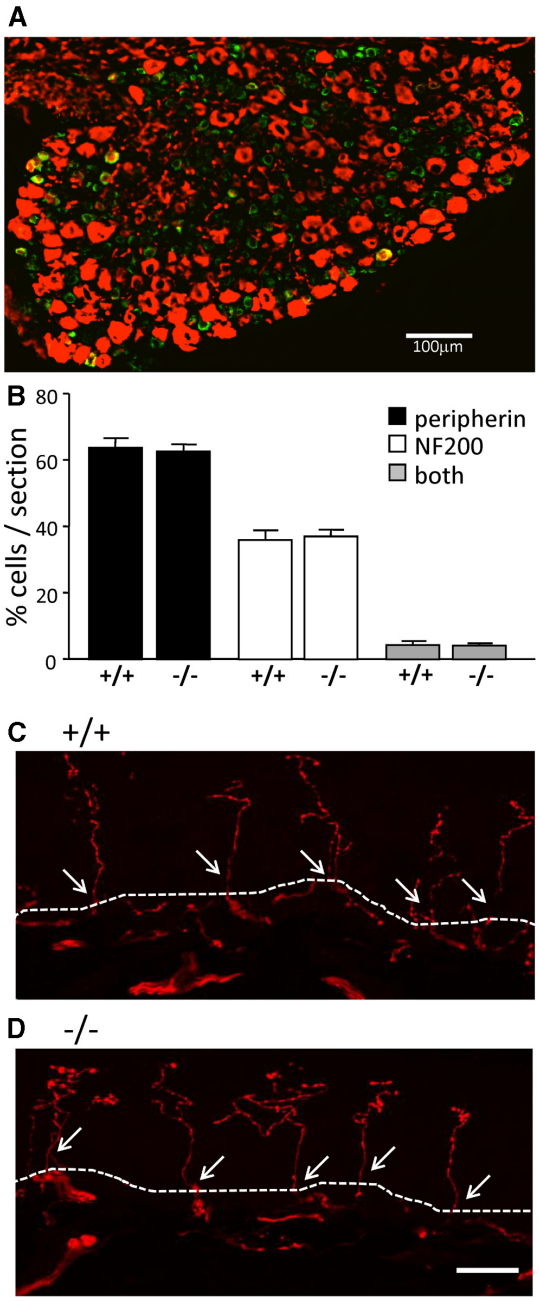


Figure 8.3 Sensory neuronal subpopulations and terminals in the skin are not different between $\alpha_2\delta\text{-}1^{+/+}$ and $\alpha_2\delta\text{-}1^{-/-}$ DRGs. ***A***, Representative double immunofluorescence staining for the neuronal intermediate filaments peripherin (green) and NF200 (red) in an L4 DRG section from an $\alpha_2\delta\text{-}1^{-/-}$ mouse. The majority of cell bodies are positive for peripherin or NF200, with a small number of cells showing staining for both intermediate filament types (yellow). ***B***, Quantification of peripherin (black bars), NF200 (white bars), and staining for both intermediate filament types (both, gray) in L4 DRGs from $\alpha_2\delta\text{-}1^{+/+}$ and $\alpha_2\delta\text{-}1^{-/-}$ mice, shown as percentage of cells stained per section. Data represent mean ± SEM. Statistical analysis was as follows: unpaired Student's *t* test comparing n = 14 +/+ with n = 13 −/− sections from four mice, respectively; p = 0.727 for peripherin, p = 0.073 for NF200, p = 0.70 for both. ***C***, ***D***, Images of PGP 9.5-positive axons crossing the dermal–epidermal boundary of glabrous skin in $\alpha_2\delta\text{-}1^{+/+}$ (***C***) and $\alpha_2\delta\text{-}1^{-/-}$ (***D***) mice. The dermal–epidermal junction is represented by the dotted line. Positive nerve profiles (stained with PGP 9.5 in red) crossing the dermal–epidermal junction are counted (arrows). Scale bar, 20 μm.

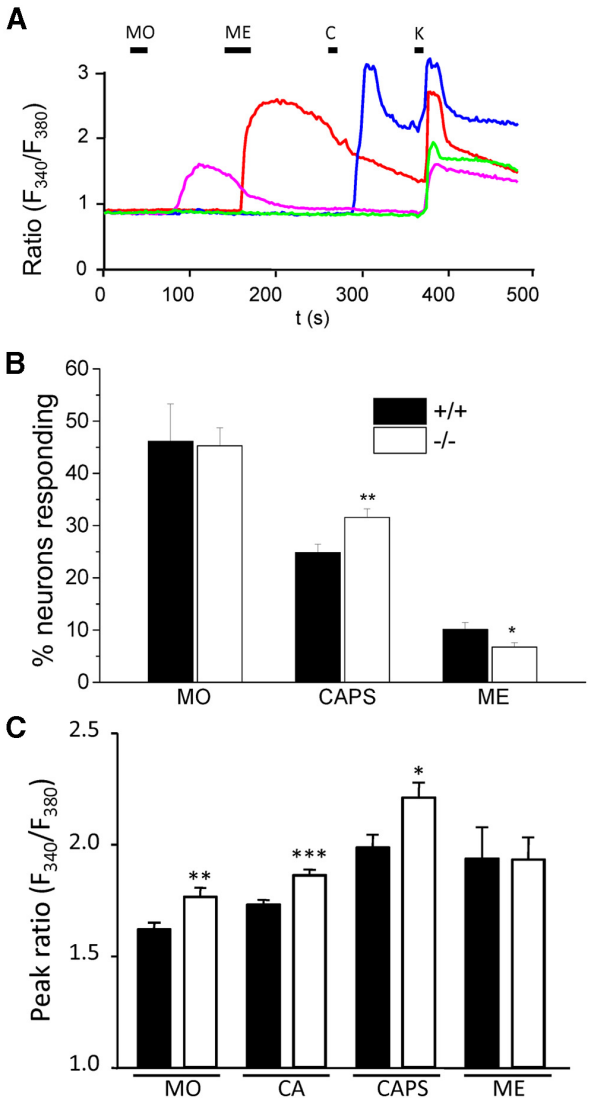


Figure 8.4 TRPA1, TRPV1, and TRPM8 responses in $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ DRG neurons. ***A***, Representative traces showing changes in cytoplasmic Ca^{2+} activity (fura-2 AM fluorescence ratio: ratio (F_{340}/F_{380})) in response to the TRPA1 agonist MO (100 μm, applied for 20 s), the TRPM8 agonist ME (500 μm, 30 s), the TRPV1 agonist CAPS (1 μm, 10 s), and 60 mm K^+ (K, 10 s) in $\alpha_2\delta$-1$^{+/+}$ neurons. ***B***, Quantification of percentage of $\alpha_2\delta$-1$^{+/+}$ (black bars) and $\alpha_2\delta$-1$^{-/-}$ (white bars) DRG neurons responding to MO (100 μm for 20 s, left), CAPS (1 μm for 5 or 10 s, middle), and ME (250 or 500 μm for 30 s, right). The number of responses to each agonist was normalized to number of responses to 60 mm K^+ per coverslip. Error bars indicate SEM. Statistical analysis was as follows: unpaired Student's *t* test comparing $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ responses: MO response, $p = 0.90$ ($n = 13$ $\alpha_2\delta$-1$^{+/+}$ and $n = 18$ $\alpha_2\delta$-1$^{-/-}$ coverslips); CAPS response, $p = 0.005$ ($n = 29$ $\alpha_2\delta$-1$^{+/+}$ and $n = 36$ $\alpha_2\delta$-1$^{-/-}$ coverslips); ME response, $p = 0.028$ ($n = 30$ $\alpha_2\delta$-1$^{+/+}$ and $n = 37$ $\alpha_2\delta$-1$^{-/-}$ coverslips). In total, $n = 982$ $\alpha_2\delta$-1$^{+/+}$ and $n = 1151$ $\alpha_2\delta$-1$^{-/-}$ cells were analyzed. ***C***, Peak fura-2 AM fluorescence ratio (peak ratio: (F_{340}/F_{380})) for MO, CA, CAPS, and ME responses. Error bars indicate SEM. Statistical analysis was as follows: unpaired Student's *t* test, comparing $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ neurons: MO peak response, $p < 0.003$ ($n = 290$ $\alpha_2\delta$-1$^{+/+}$, $n = 247$ $\alpha_2\delta$-1$^{-/-}$ neurons); CA peak response, $p = 0.0002$ ($n = 230$ $\alpha_2\delta$-1$^{+/+}$, $n = 325$ $\alpha_2\delta$-1$^{-/-}$ neurons); CAPS peak response, $p = 0.011$ ($n = 189$ $\alpha_2\delta$-1$^{+/+}$, $n = 191$ $\alpha_2\delta$-1$^{-/-}$ neurons); ME peak response (250 μm), $p = 0.98$ ($n = 55$ $\alpha_2\delta$-1$^{+/+}$, $n = 49$ $^{-/-}$ neurons). *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

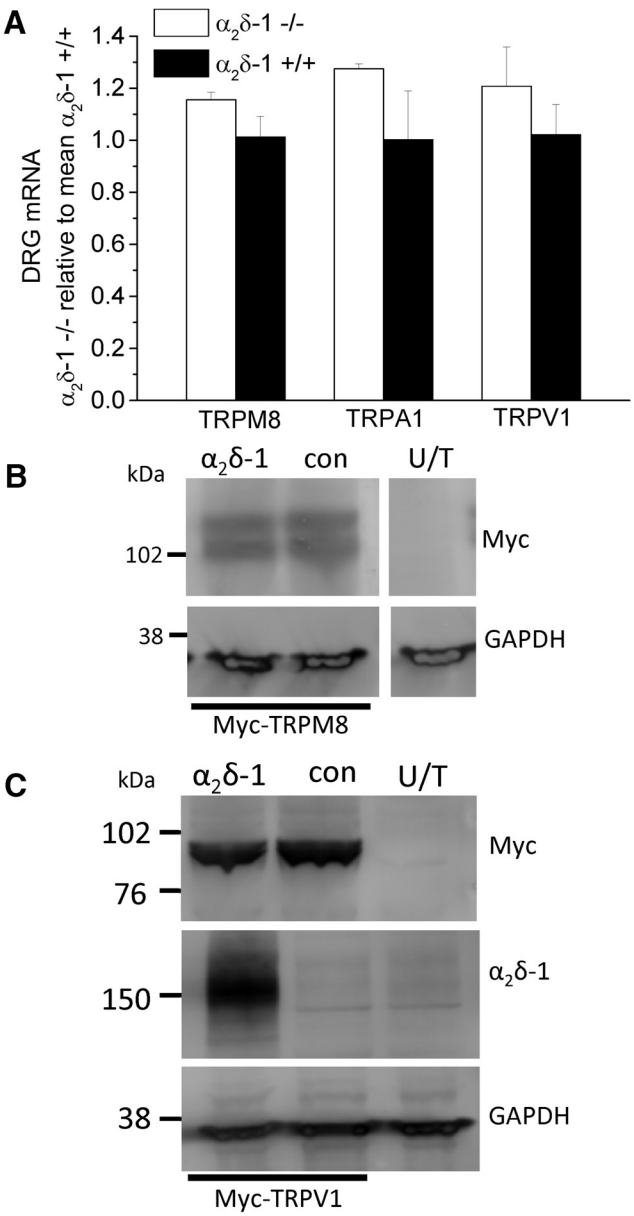


Figure 8.5 TRP channel mRNA and protein expression. ***A***, TRPM8, TRPA1, and TRPV1 mRNA levels in $\alpha_2\delta$-$1^{-/-}$ (white bar) and $\alpha_2\delta$-$1^{+/+}$ (black bar) DRGs (n = 3), normalized to the mean $\alpha_2\delta$-$1^{+/+}$ mRNA values. ***B***, ***C***, TRPM8 (***B***) and TRPV1 (***C***) expression, after transfection in tsA-201 cells, detected by their associated myc tag (top), was not affected by coexpression of $\alpha_2\delta$-1 (lane 1), compared with a control protein β1b (lane 2). No signal was observed in WCL from untransfected (U/T) tsA-201 cells (lane 3). Bottom, GAPDH loading controls. ***C***, $\alpha_2\delta$-1 expression is also shown (middle). All lanes are from the same gel. Data are representative of n = 2 experiments.

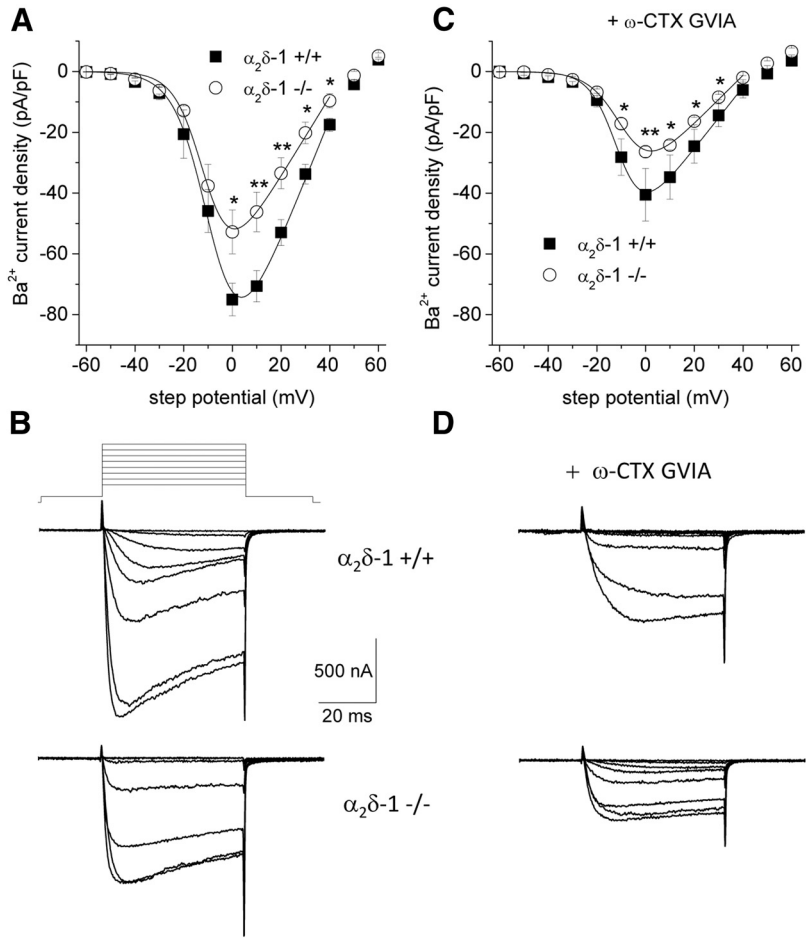


Figure 8.6 Calcium channel currents in $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ DRG neurons. ***A***, Calcium channel current density-voltage relationship for $\alpha_2\delta$-1$^{+/+}$ (closed squares, *n* = 19 cells, 4 mice) and $\alpha_2\delta$-1$^{-/-}$ (open circles, *n* = 29 cells, 5 mice) DRG neurons. Mean data were fit between −60 and 40 mV with a modified Boltzmann relationship (see Materials and Methods). For $\alpha_2\delta$-1$^{+/+}$, the $V_{50,act}$ was −10.2 mV and for $\alpha_2\delta$-1$^{-/-}$, the $V_{50,act}$ was −8.17 mV. ***B***, Representative current traces under the two conditions: $\alpha_2\delta$-1$^{+/+}$ (top) and $\alpha_2\delta$-1$^{-/-}$ (bottom). Top, Voltage protocol. Holding potential −90 mV, test potentials −60 to 10 mV in 10 mV steps. ***C***, Calcium channel current density-voltage relationship for $\alpha_2\delta$-1$^{+/+}$ (closed squares, *n* = 7 cells, 4 mice) and $\alpha_2\delta$-1$^{-/-}$ (open circles, *n* = 25 cells, 7 mice) for DRG neurons after preincubation for 15 min with ω-conotoxin GVIA (ω-CTX GVIA, 1 μm); otherwise as in ***A***. For $\alpha_2\delta$-1$^{+/+}$, the $V_{50,act}$ was −7.85 mV and for $\alpha_2\delta$-1$^{-/-}$, the $V_{50,act}$ was −10.3 mV. ***D***, Representative current traces under the two conditions, as in ***B***. Data were compared by two-way ANOVA, followed by the Tukey Honest Significant Differences test. ***A***, ***C***, The statistical significance of the difference in current density between $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ is indicated: **p* < 0.05, ***p* < 0.01. There was no effect of ω-conotoxin GVIA on the difference in current density between the mouse genotypes.

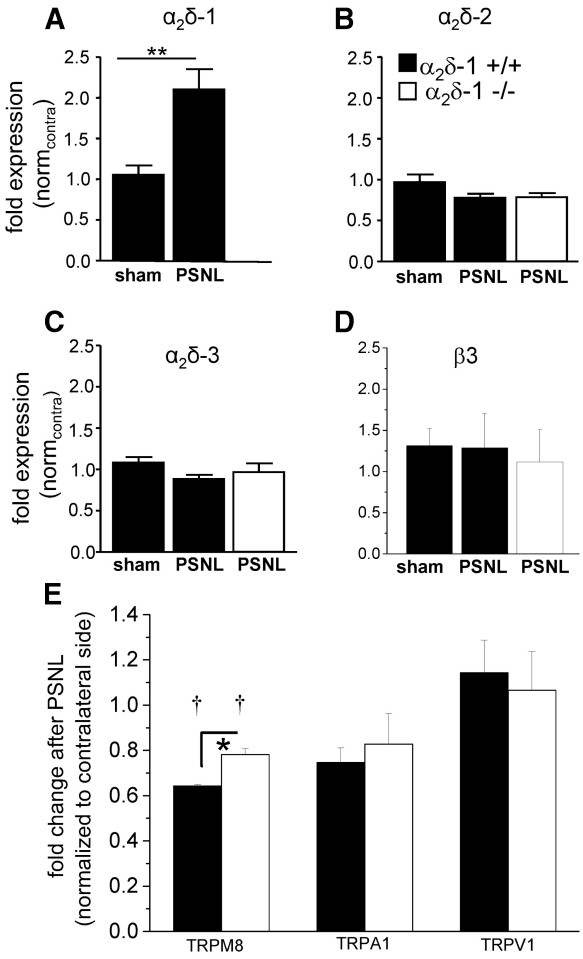


Figure 8.7 PSNL-mediated ipsilateral changes of mRNA of neuropathy markers in $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ DRGs. The legend applies to all parts of the figure. ***A***, Q-PCR results for $\alpha_2\delta$-1 mRNA levels (fold expression ($norm_{contra}$)) in pooled L3-L5 ipsilateral DRGs from sham-operated (n = 9) or PSNL-operated (n = 7) $\alpha_2\delta$-1$^{+/+}$ mice (black bars), 14 d after surgery. Data are normalized to the respective contralateral side. Error bars indicate SEM. Statistical analysis was performed using one-way ANOVA with p = 0.003 using Bonferroni's post test. No data are included for the $\alpha_2\delta$-1$^{-/-}$ DRGs because of the gene disruption. ***B***, Q-PCR results for $\alpha_2\delta$-2 mRNA levels (fold expression ($norm_{contra}$)) in pooled L3-L5 ipsilateral DRGs from sham-operated (n = 9) or PSNL-operated (n = 7) $\alpha_2\delta$-1$^{+/+}$ mice (black bars) or PSNL-operated $\alpha_2\delta$-1$^{-/-}$ mice (white bar, n = 7), 14 d after surgery. Data are normalized to the respective contralateral side. Error bars indicate SEM. Statistical analysis: one-way ANOVA with p = 0.11. ***C***, Q-PCR results for $\alpha_2\delta$-3 mRNA levels (fold expression ($norm_{contra}$)) in pooled L3-L5 ipsilateral DRGs from sham-operated (n = 9) or PSNL-operated (n = 7) $\alpha_2\delta$-1$^{+/+}$ mice (black bars) or PSNL-operated $\alpha_2\delta$-1$^{-/-}$ mice (white bar, n = 7), 14 d after surgery. Data are normalized to the respective contralateral side. Error bars indicate SEM. Statistical analysis: one-way ANOVA with p = 0.21. ***D***, Q-PCR results for β3 mRNA levels (fold expression ($norm_{contra}$)) in pooled L3-L5 ipsilateral DRGs from sham-operated (n = 9) or PSNL-operated (n = 7) $\alpha_2\delta$-1$^{+/+}$ mice (black bars) or PSNL-operated $\alpha_2\delta$-1$^{-/-}$ mice (white bar, n = 6), 14 d after surgery. Data are normalized to the respective contralateral side. Error bars indicate SEM. Statistical analysis: one-way ANOVA with Bonferroni's multiple-comparison test, $p > 0.05$ for all comparisons. ***E***, Q-PCR results for TRPM8, TRPA1, and TRPV1 mRNA levels (fold expression) in pooled L3-L5 ipsilateral DRGs from PSNL-operated $\alpha_2\delta$-1$^{+/+}$ mice (black bars n = 3) or PSNL-operated $\alpha_2\delta$-1$^{-/-}$ mice (white bars, n = 3), 14 d after surgery. Data are normalized to the respective contralateral side. Error bars indicate SEM. Statistical analysis shows that TRPM8 mRNA ratios for $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ DRGs are significantly less than a theoretical value of 1: †p = 0.0003 and p = 0.0142, respectively (one-sample t test). The difference between $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ TRPM8 ratios is also statistically significant.

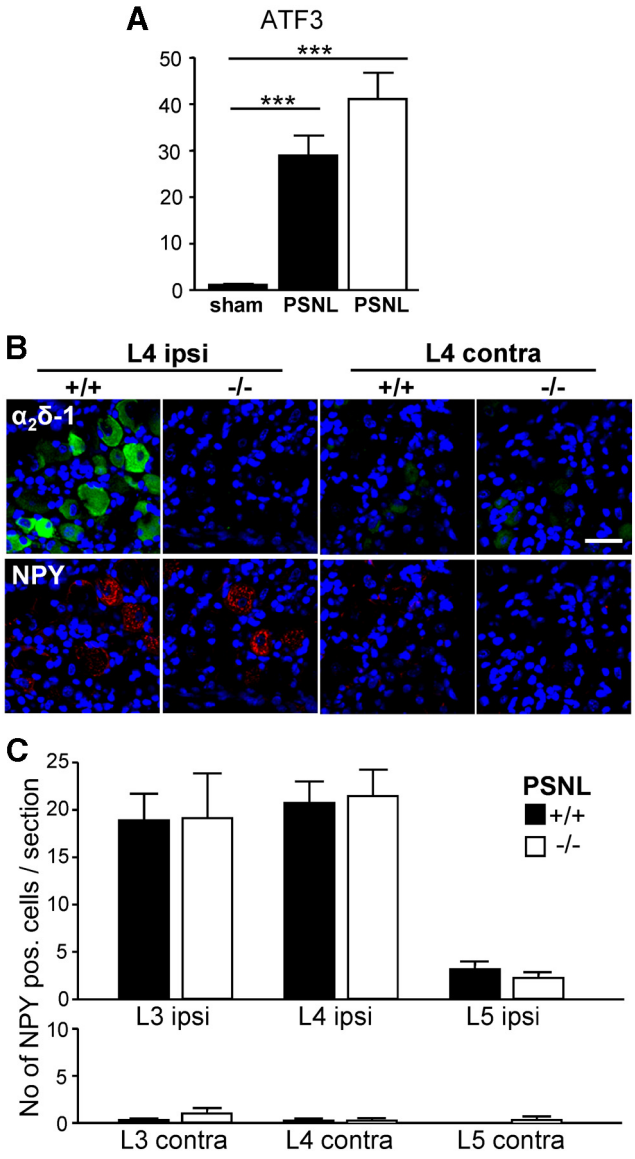


Figure 8.8 PSNL-mediated ipsilateral changes of mRNA and protein levels of injury markers in $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ DRGs. ***A***, Q-PCR results for ATF3 mRNA levels (fold expression) in pooled L3-L5 ipsilateral DRGs from sham-operated (n = 9) or PSNL-operated (n = 7) $\alpha_2\delta$-1$^{+/+}$ mice (black bars) or PSNL-operated $\alpha_2\delta$-1$^{-/-}$ mice (white bar, n = 7) 14 d after surgery. Data are normalized to the respective contralateral side. ***p < 0.001 (one-way ANOVA and Bonferroni's *post hoc* test). ATF3 mRNA levels in PSNL-operated $\alpha_2\delta$-1$^{+/+}$ were not significantly different from PSNL-operated $\alpha_2\delta$-1$^{-/-}$ mice. ***B***, Representative immunofluorescence images of ipsilateral (ipsi) and contralateral (contra) L4 DRG sections from $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ mice after PSNL. Double immunofluorescence staining for $\alpha_2\delta$-1 (green) and NPY (red); blue represents nuclear staining. Scale bar, 100 μm. ***C***, Quantification of NPY-positive cells per section (number of NPY-positive cells/section) in ipsilateral and contralateral $\alpha_2\delta$-1$^{+/+}$ (black bars) and $\alpha_2\delta$-1$^{-/-}$ (white bars) L3-L5 DRGs 14 d after PSNL. Error bars indicate SEM. Statistical analysis was as follows: unpaired Student's t test comparing number of NPY-positive cells. $\alpha_2\delta$-1$^{+/+}$ and $\alpha_2\delta$-1$^{-/-}$ DRG sections: ipsilateral L3, p = 0.97 (n = 18 $\alpha_2\delta$-1$^{+/+}$ sections, n = 19 $\alpha_2\delta$-1$^{-/-}$ sections); ipsilateral L4, p = 0.84 (n = 22 $\alpha_2\delta$-1$^{+/+}$ sections, n = 20 $\alpha_2\delta$-1$^{-/-}$ sections); ipsilateral L5, p = 0.37 (n = 13 $\alpha_2\delta$-1$^{+/+}$ sections, n = 13 $\alpha_2\delta$-1$^{-/-}$ sections); contra L3, p = 0.27 (n = 11 $\alpha_2\delta$-1$^{+/+}$ sections, n = 13 $\alpha_2\delta$-1$^{-/-}$ sections); contra L4 contra, p = 0.94 (n = 13 $\alpha_2\delta$-1$^{+/+}$ sections, n = 12 $\alpha_2\delta$-1$^{-/-}$ sections); and contra L5, p = 0.39 (n = 8 $\alpha_2\delta$-1$^{+/+}$ sections, n = 10 $\alpha_2\delta$-1$^{-/-}$ sections).

References

Aasvang, E.K., Brandsborg, B., Jensen, T.S. & Kehlet, H. (2010) Heterogeneous sensory processing in persistent postherniotomy pain. *Pain*, **150**, 237-242.

Abbadie, C., McManus, O.B., Sun, S.-Y., Bugianesi, R.M., Dai, G., Haedo, R.J., Herrington, J.B., Kaczorowski, G.J., Smith, M.M. & Swensen, A.M. (2010) Analgesic effects of a substituted N-triazole oxindole (TROX-1), a state-dependent, voltage-gated calcium channel 2 blocker. *Journal of Pharmacology and Experimental Therapeutics*, **334**, 545-555.

Abrahamsen, B., Zhao, J., Asante, C.O., Cendan, C.M., Marsh, S., Martinez-Barbera, J.P., Nassar, M.A., Dickenson, A.H. & Wood, J.N. (2008) The cell and molecular basis of mechanical, cold, and inflammatory pain. *Science*, **321**, 702-705.

Abraira, Victoria E. & Ginty, David D. (2013) The Sensory Neurons of Touch. *Neuron*, **79**, 618-639.

Alloui, A., Zimmermann, K., Mamet, J., Duprat, F., Noel, J., Chemin, J., Guy, N., Blondeau, N., Voilley, N. & Rubat-Coudert, C. (2006) TREK-1, a K+; channel involved in polymodal pain perception. *The EMBO journal*, **25**, 2368-2376.

Almeida, M.C., Hew-Butler, T., Soriano, R.N., Rao, S., Wang, W., Wang, J., Tamayo, N., Oliveira, D.L., Nucci, T.B. & Aryal, P. (2012) Pharmacological Blockade of the Cold Receptor TRPM8 Attenuates Autonomic and Behavioral Cold Defenses and Decreases Deep Body Temperature. *The Journal of Neuroscience*, **32**, 2086-2099.

Altier, C., Dale, C.S., Kisilevsky, A.E., Chapman, K., Castiglioni, A.J., Matthews, E.A., Evans, R.M., Dickenson, A.H., Lipscombe, D., Vergnolle, N. & Zamponi, G.W. (2007) Differential Role of N-Type Calcium Channel Splice Isoforms in Pain. *The Journal of Neuroscience*, **27**, 6363-6373.

Altis, K., Schmidtko, A., Angioni, C., Kuczka, K., Schmidt, H., Geisslinger, G., Lotsch, J. & Tegeder, I. (2009) Analgesic efficacy of tramadol, pregabalin and ibuprofen in menthol-evoked cold hyperalgesia. *Pain*, **147**, 116-121.

An, X., Bandler, R., Ongur, D. & Price, J.L. (1998) Prefrontal cortical projections to longitudinal columns in the midbrain periaqueductal gray in macaque monkeys. *The Journal of comparative neurology*, **401**, 455-479.

Andersen, H.H., Norgaard, J., Uchida, Y., Nikbakht, A., Arendt-Nielsen, L. & Gazerani, P. (2015) Cold and L-menthol-induced sensitization in healthy volunteers - a cold hypersensitivity analogue to the Heat/Capsaicin model. *Pain*.

Andersson, D.A., Chase, H.W. & Bevan, S. (2004) TRPM8 activation by menthol, icilin, and cold is differentially modulated by intracellular pH. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **24**, 5364-5369.

Andersson, D.A., Gentry, C., Moss, S. & Bevan, S. (2008) Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **28**, 2485-2494.

Andersson, D.A., Nash, M. & Bevan, S. (2007) Modulation of the cold-activated channel TRPM8 by lysophospholipids and polyunsaturated fatty acids. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **27**, 3347-3355.

Andrew, D. & Craig, A.D. (2002) Quantitative responses of spinothalamic lamina I neurones to graded mechanical stimulation in the cat. *J Physiol*, **545**, 913-931.

Angelotti, T. & Hofmann, F. (1996) Tissue-specific expression of splice variants of the mouse voltage-gated calcium channel alpha2/delta subunit. *FEBS letters*, **397**, 331-337.

Apkarian, A.V. & Shi, T. (1994) Squirrel monkey lateral thalamus. I. Somatic nociresponsive neurons and their relation to spinothalamic terminals. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **14**, 6779-6795.

Arendt-Nielsen, L. & Yarnitsky, D. (2009) Experimental and Clinical Applications of Quantitative Sensory Testing Applied to Skin, Muscles and Viscera. *The Journal of Pain*, **10**, 556-572.

Argyriou, A.A., Cavaletti, G., Briani, C., Velasco, R., Bruna, J., Campagnolo, M., Alberti, P., Bergamo, F., Cortinovis, D., Cazzaniga, M., Santos, C., Papadimitriou, K. & Kalofonos, H.P. (2013) Clinical pattern and associations of oxaliplatin acute neurotoxicity: a prospective study in 170 patients with colorectal cancer. *Cancer*, **119**, 438-444.

Arikkath, J. & Campbell, K.P. (2003) Auxiliary subunits: essential components of the voltage-gated calcium channel complex. *Curr Opin Neurobiol*, **13**, 298-307.

Ashoor, A., Nordman, J.C., Veltri, D., Yang, K.H., Shuba, Y., Al Kury, L., Sadek, B., Howarth, F.C., Shehu, A., Kabbani, N. & Oz, M. (2013) Menthol inhibits 5-HT3 receptor-mediated currents. *The Journal of pharmacology and experimental therapeutics*, **347**, 398-409.

Attal, N., Brasseur, L., Parker, F., Chauvin, M. & Bouhassira, D. (1998) Effects of gabapentin on the different components of peripheral and central neuropathic pain syndromes: a pilot study. *European neurology*, **40**, 191-200.

Averill, S., McMahon, S.B., Clary, D.O., Reichardt, L.F. & Priestley, J.V. (1995) Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *The European journal of neuroscience*, **7**, 1484-1494.

Azkue, J.J., Murga, M., Fernandez-Capetillo, O., Mateos, J.M., Elezgarai, I., Benitez, R., Osorio, A., Diez, J., Puente, N., Bilbao, A., Bidaurrazaga, A., Kuhn, R. & Grandes, P. (2001) Immunoreactivity for the group III metabotropic glutamate receptor subtype mGluR4a in the superficial laminae of the rat spinal dorsal horn. *The Journal of comparative neurology*, **430**, 448-457.

Baba, H., Shimoji, K. & Yoshimura, M. (2000) Norepinephrine facilitates inhibitory transmission in substantia gelatinosa of adult rat spinal cord (part 1): effects on axon terminals of GABAergic and glycinergic neurons. *Anesthesiology*, **92**, 473-484.

Babes, A., Zorzon, D. & Reid, G. (2004) Two populations of cold, sensitive neurons in rat dorsal root ganglia and their modulation by nerve growth factor. *European Journal of Neuroscience*, **20**, 2276-2282.

Bajic, D., Van Bockstaele, E.J. & Proudfit, H.K. (2001) Ultrastructural analysis of ventrolateral periaqueductal gray projections to the A7 catecholamine cell group. *Neuroscience*, **104**, 181-197.

Bandell, M., Story, G.M., Hwang, S.W., Viswanath, V., Eid, S.R., Petrus, M.J., Earley, T.J. & Patapoutian, A. (2004) Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron*, **41**, 849-857.

Bannister, J.P., Adebiyi, A., Zhao, G., Narayanan, D., Thomas, C.M., Feng, J.Y. & Jaggar, J.H. (2009) Smooth muscle cell alpha2delta-1 subunits are essential for vasoregulation by CaV1.2 channels. *Circulation research*, **105**, 948-955.

Bannister, K., Sikandar, S., Bauer, C.S., Dolphin, A.C., Porreca, F. & Dickenson, A.H. (2011) Pregabalin suppresses spinal neuronal hyperexcitability and visceral hypersensitivity in the absence of peripheral pathophysiology. *Anesthesiology*, **115**, 144-152.

Bantel, C., Eisenach, J.C., Duflo, F., Tobin, J.R. & Childers, S.R. (2005) Spinal nerve ligation increases alpha2-adrenergic receptor G-protein coupling in the spinal cord. *Brain Res*, **1038**, 76-82.

Barclay, J., Balaguero, N., Mione, M., Ackerman, S.L., Letts, V.A., Brodbeck, J., Canti, C., Meir, A., Page, K.M., Kusumi, K., Perez-Reyes, E., Lander, E.S., Frankel, W.N., Gardiner, R.M., Dolphin, A.C. & Rees, M. (2001) Ducky mouse phenotype of epilepsy and ataxia is associated with mutations in the Cacna2d2 gene and decreased calcium channel current in cerebellar Purkinje cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **21**, 6095-6104.

Baron, R. (2006) Mechanisms of disease: neuropathic pain--a clinical perspective. *Nature clinical practice. Neurology*, **2**, 95-106.

Baron, R., Tölle, T.R., Gockel, U., Brosz, M. & Freynhagen, R. (2009) A cross-sectional cohort survey in 2100 patients with painful diabetic neuropathy and postherpetic neuralgia: Differences in demographic data and sensory symptoms. *PAIN*, **146**, 34-40.

Basbaum, A.I., Bautista, D.M., Scherrer, G. & Julius, D. (2009) Cellular and molecular mechanisms of pain. *Cell*, **139**, 267-284.

Basbaum, A.I., Clanton, C.H. & Fields, H.L. (1976) Opiate and stimulus-produced analgesia: functional anatomy of a medullospinal pathway. *Proceedings of the National Academy of Sciences of the United States of America*, **73**, 4685-4688.

Bauer, C.S., Nieto-Rostro, M., Rahman, W., Tran-Van-Minh, A., Ferron, L., Douglas, L., Kadurin, I., Sri Ranjan, Y., Fernandez-Alacid, L., Millar, N.S., Dickenson, A.H., Lujan, R. & Dolphin, A.C. (2009) The increased trafficking of the calcium channel subunit alpha2delta-1 to presynaptic terminals in neuropathic pain is inhibited by the alpha2delta ligand pregabalin. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **29**, 4076-4088.

Bauer, C.S., Tran-Van-Minh, A., Kadurin, I. & Dolphin, A.C. (2010) A new look at calcium channel alpha2delta subunits. *Curr Opin Neurobiol*, **20**, 563-571.

Bautista, D.M., Jordt, S.-E., Nikai, T., Tsuruda, P.R., Read, A.J., Poblete, J., Yamoah, E.N., Basbaum, A.I. & Julius, D. (2006) TRPA1 Mediates the Inflammatory Actions of Environmental Irritants and Proalgesic Agents. *Cell*, **124**, 1269-1282.

Bautista, D.M., Siemens, J., Glazer, J.M., Tsuruda, P.R., Basbaum, A.I., Stucky, C.L., Jordt, S.-E. & Julius, D. (2007) The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature*, **448**, 204-208.

Bee, L.A., Bannister, K., Rahman, W. & Dickenson, A.H. (2011) Mu-opioid and noradrenergic alpha(2)-adrenoceptor contributions to the effects of tapentadol on spinal electrophysiological measures of nociception in nerve-injured rats. *Pain*, **152**, 131-139.

Bee, L.A. & Dickenson, A.H. (2007) Rostral ventromedial medulla control of spinal sensory processing in normal and pathophysiological states. *Neuroscience*, **147**, 786-793.

Bee, L.A. & Dickenson, A.H. (2008) Descending facilitation from the brainstem determines behavioural and neuronal hypersensitivity following nerve injury and efficacy of pregabalin. *Pain*, **140**, 209-223.

Beijers, A.J., Mols, F. & Vreugdenhil, G. (2014) A systematic review on chronic oxaliplatin-induced peripheral neuropathy and the relation with oxaliplatin administration. *Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer*, **22**, 1999-2007.

Bennett, D.L., Michael, G.J., Ramachandran, N., Munson, J.B., Averill, S., Yan, Q., McMahon, S.B. & Priestley, J.V. (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **18**, 3059-3072.

Bennett, G.J. (2012) What is spontaneous pain and who has it? *The journal of pain : official journal of the American Pain Society*, **13**, 921-929.

Berglund, B., Harju, E.L., Kosek, E. & Lindblom, U. (2002) Quantitative and qualitative perceptual analysis of cold dysesthesia and hyperalgesia in fibromyalgia. *Pain*, **96**, 177-187.

Bernard, J.F., Alden, M. & Besson, J.M. (1993) The organization of the efferent projections from the pontine parabrachial area to the amygdaloid complex: a Phaseolus vulgaris leucoagglutinin (PHA-L) study in the rat. *The Journal of comparative neurology*, **329**, 201-229.

Bernstein, G.M. & Jones, O.T. (2007) Kinetics of internalization and degradation of N-type voltage-gated calcium channels: role of the alpha2/delta subunit. *Cell Calcium*, **41**, 27-40.

Bester, H., Chapman, V., Besson, J.M. & Bernard, J.F. (2000) Physiological properties of the lamina I spinoparabrachial neurons in the rat. *J Neurophysiol*, **83**, 2239-2259.

Bidaux, G., Beck, B., Zholos, A., Gordienko, D., Lemonnier, L., Flourakis, M., Roudbaraki, M., Borowiec, A.S., Fernandez, J., Delcourt, P., Lepage, G., Shuba, Y., Skryma, R. & Prevarskaya, N. (2012) Regulation of activity of transient receptor potential melastatin 8 (TRPM8) channel by its short isoforms. *The Journal of biological chemistry*, **287**, 2948-2962.

Binder, A., Binder, A., Maag, R., Peller, M., Wasner, G., Siebner, H.R. & Baron, R. (2007) Differential coding of cold allodynia – A fmri study. *Clinical Neurophysiology*, **118**, e17.

Binder, A., Stengel, M., Klebe, O., Wasner, G. & Baron, R. (2011) Topical high-concentration (40%) menthol-somatosensory profile of a human surrogate pain model. *The journal of pain : official journal of the American Pain Society*, **12**, 764-773.

Birinyi-Strachan, L.C., Gunning, S.J., Lewis, R.J. & Nicholson, G.M. (2005) Block of voltage-gated potassium channels by Pacific ciguatoxin-1 contributes to increased neuronal excitability in rat sensory neurons. *Toxicology and applied pharmacology*, **204**, 175-186.

Black, J.A., Cummins, T.R., Plumpton, C., Chen, Y.H., Hormuzdiar, W., Clare, J.J. & Waxman, S.G. (1999) Upregulation of a silent sodium channel after peripheral, but not central, nerve injury in DRG neurons. *J Neurophysiol*, **82**, 2776-2785.

Boroujerdi, A., Kim, H.K., Lyu, Y.S., Kim, D.S., Figueroa, K.W., Chung, J.M. & Luo, Z.D. (2008) Injury discharges regulate calcium channel alpha-2-delta-1 subunit upregulation in the dorsal horn that contributes to initiation of neuropathic pain. *Pain*, **139**, 358-366.

Boucher, T.J., Okuse, K., Bennett, D.L., Munson, J.B., Wood, J.N. & McMahon, S.B. (2000) Potent analgesic effects of GDNF in neuropathic pain states. *Science*, **290**, 124-127.

Bouhassira, D., Kern, D., Rouaud, J., Pelle-Lancien, E. & Morain, F. (2005) Investigation of the paradoxical painful sensation ('illusion of pain') produced by a thermal grill. *Pain*, **114**, 160-167.

Bourgoin, S., Oliveras, J.L., Bruxelle, J., Hamon, M. & Besson, J.M. (1980) Electrical stimulation of the nucleus raphe magnus in the rat. Effects on 5-HT metabolism in the spinal cord. *Brain Research*, **194**, 377-389.

Bourinet, E., Altier, C., Hildebrand, M.E., Trang, T., Salter, M.W. & Zamponi, G.W. (2014) Calcium-permeable ion channels in pain signaling. *Physiological reviews*, **94**, 81-140.

Bourinet, E., Soong, T.W., Stea, A. & Snutch, T.P. (1996) Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 1486-1491.

Bourinet, E., Soong, T.W., Sutton, K., Slaymaker, S., Mathews, E., Monteil, A., Zamponi, G.W., Nargeot, J. & Snutch, T.P. (1999) Splicing of alpha 1A subunit gene generates phenotypic variants of P- and Q-type calcium channels. *Nat Neurosci*, **2**, 407-415.

Bowersox, S.S., Gadbois, T., Singh, T., Pettus, M., Wang, Y.X. & Luther, R.R. (1996) Selective N-type neuronal voltage-sensitive calcium channel blocker, SNX-111, produces spinal antinociception in rat models of acute, persistent and neuropathic pain. *The Journal of pharmacology and experimental therapeutics*, **279**, 1243-1249.

Brandow, A.M., Stucky, C.L., Hillery, C.A., Hoffmann, R.G. & Panepinto, J.A. (2013) Patients with sickle cell disease have increased sensitivity to cold and heat. *American journal of hematology*, **88**, 37-43.

Brauchi, S., Orio, P. & Latorre, R. (2004) Clues to understanding cold sensation: thermodynamics and electrophysiological analysis of the cold receptor TRPM8. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 15494-15499.

Brauchi, S., Orta, G., Salazar, M., Rosenmann, E. & Latorre, R. (2006) A hot-sensing cold receptor: C-terminal domain determines thermosensation in transient receptor potential channels. *The Journal of Neuroscience*, **26**, 4835-4840.

Braz, J., Solorzano, C., Wang, X. & Basbaum, A.I. (2014) Transmitting Pain and Itch Messages: A Contemporary View of the Spinal Cord Circuits that Generate Gate Control. *Neuron*, **82**, 522-536.

Breivik, H., Collett, B., Ventafridda, V., Cohen, R. & Gallacher, D. (2006) Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. *European journal of pain (London, England)*, **10**, 287-333.

Breivik, H., Eisenberg, E. & O'Brien, T. (2013) The individual and societal burden of chronic pain in Europe: the case for strategic prioritisation and action to improve knowledge and availability of appropriate care. *BMC public health*, **13**, 1229.

Brenchat, A., Nadal, X., Romero, L., Ovalle, S., Muro, A., Sánchez-Arroyos, R., Portillo-Salido, E., Pujol, M., Montero, A., Codony, X., Burgueño, J., Zamanillo, D., Hamon, M., Maldonado, R. & Vela, J.M. (2010) Pharmacological activation of 5-HT7 receptors reduces nerve injury-induced mechanical and thermal hypersensitivity. *PAIN*, **149**, 483-494.

Brightwell, J.J. & Taylor, B.K. (2009) Noradrenergic neurons in the locus coeruleus contribute to neuropathic pain. *Neuroscience*, **160**, 174-185.

Brignell, J.L., Chapman, V. & Kendall, D.A. (2008) Comparison of icilin-and cold-evoked responses of spinal neurones, and their modulation of mechanical activity, in a model of neuropathic pain. *Brain research*, **1215**, 87-96.

Brown, J.T. & Randall, A. (2005) Gabapentin fails to alter P/Q-type Ca2+ channel-mediated synaptic transmission in the hippocampus in vitro. *Synapse (New York, N.Y.)*, **55**, 262-269.

Bucci, G., Mochida, S. & Stephens, G.J. (2011) Inhibition of synaptic transmission and G protein modulation by synthetic CaV2. 2 Ca2+ channel peptides. *The Journal of Physiology*, **589**, 3085-3101.

Budai, D. & Larson, A.A. (1996) Role of substance P in the modulation of C-fiber-evoked responses of spinal dorsal horn neurons. *Brain Res*, **710**, 197-203.

Burgess, S.E., Gardell, L.R., Ossipov, M.H., Malan, T.P., Jr., Vanderah, T.W., Lai, J. & Porreca, F. (2002) Time-dependent descending facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **22**, 5129-5136.

Burnham, L.J. & Dickenson, A.H. (2013) The antinociceptive effect of milnacipran in the monosodium iodoacetate model of osteoarthritis pain and its relation to changes in descending inhibition. *The Journal of pharmacology and experimental therapeutics*, **344**, 696-707.

Calvo, R.R., Meegalla, S.K., Parks, D.J., Parsons, W.H., Ballentine, S.K., Lubin, M.L., Schneider, C., Colburn, R.W., Flores, C.M. & Player, M.R. (2012) Discovery of vinylcycloalkyl-substituted benzimidazole TRPM8 antagonists effective in the treatment of cold allodynia. *Bioorganic & Medicinal Chemistry Letters*, **22**, 1903-1907.

Campbell, J.N. & Meyer, R.A. (2006) Mechanisms of neuropathic pain. *Neuron*, **52**, 77-92.

Campbell, J.N., Raja, S.N., Meyer, R.A. & Mackinnon, S.E. (1988) Myelinated afferents signal the hyperalgesia associated with nerve injury. *Pain*, **32**, 89-94.

Campero, M., Baumann, T.K., Bostock, H. & Ochoa, J.L. (2009) Human cutaneous C fibres activated by cooling, heating and menthol. *J Physiol*, **587**, 5633-5652.

Campero, M., Serra, J., Bostock, H. & Ochoa, J.L. (2001) Slowly conducting afferents activated by innocuous low temperature in human skin. *The Journal of physiology*, **535**, 855-865.

Campero, M., Serra, J. & Ochoa, J.L. (1996) C-polymodal nociceptors activated by noxious low temperature in human skin. *The Journal of physiology*, **497**, 565-572.

Cantí, C., Davies, A. & Dolphin, A.C. (2003) Calcium Channel $\alpha 2\delta$ Subunits: Structure, Functions and Target Site for Drugs. *Current Neuropharmacology*, **1**, 209-217.

Canti, C., Nieto-Rostro, M., Foucault, I., Heblich, F., Wratten, J., Richards, M.W., Hendrich, J., Douglas, L., Page, K.M., Davies, A. & Dolphin, A.C. (2005) The metal-ion-dependent adhesion site in the Von Willebrand factor-A domain of alpha2delta subunits is key to trafficking voltage-gated Ca2+ channels. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 11230-11235.

Cao, Y.Q., Piedras-Renteria, E.S., Smith, G.B., Chen, G., Harata, N.C. & Tsien, R.W. (2004) Presynaptic Ca2+ channels compete for channel type-preferring slots in altered neurotransmission arising from Ca2+ channelopathy. *Neuron*, **43**, 387-400.

Carlson, J.D., Maire, J.J., Martenson, M.E. & Heinricher, M.M. (2007) Sensitization of pain-modulating neurons in the rostral ventromedial medulla after peripheral nerve injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **27**, 13222-13231.

Caspani, O., Zurborg, S., Labuz, D. & Heppenstall, P.A. (2009) The contribution of TRPM8 and TRPA1 channels to cold allodynia and neuropathic pain. *PLoS One*, **4**, e7383.

Cassidy, J.S., Ferron, L., Kadurin, I., Pratt, W.S. & Dolphin, A.C. (2014) Functional exofacially tagged N-type calcium channels elucidate the interaction with auxiliary $\alpha 2\delta$-1 subunits. *Proceedings of the National Academy of Sciences*.

Castellano, A., Wei, X., Birnbaumer, L. & Perez-Reyes, E. (1993) Cloning and expression of a third calcium channel beta subunit. *Journal of Biological Chemistry*, **268**, 3450-3455.

Castiglioni, A.J., Raingo, J. & Lipscombe, D. (2006) Alternative splicing in the C-terminus of CaV2.2 controls expression and gating of N-type calcium channels. *The Journal of Physiology*, **576**, 119-134.

Catterall, W.A. (2000) Structure and regulation of voltage-gated Ca2+ channels. *Annual review of cell and developmental biology*, **16**, 521-555.

Cavanaugh, D.J., Lee, H., Lo, L., Shields, S.D., Zylka, M.J., Basbaum, A.I. & Anderson, D.J. (2009) Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 9075-9080.

Chang, E.Y., Chen, X., Sandhu, A., Li, C.Y. & Luo, Z.D. (2013) Spinal 5-HT3 receptors facilitate behavioural hypersensitivity induced by elevated calcium channel alpha-2-delta-1 protein. *European journal of pain (London, England)*, **17**, 505-513.

Chaplan, S., Bach, F., Pogrel, J., Chung, J. & Yaksh, T. (1994a) Quantitative assessment of tactile allodynia in the rat paw. *Journal of neuroscience methods*, **53**, 55-63.

Chaplan, S.R., Pogrel, J.W. & Yaksh, T.L. (1994b) Role of voltage-dependent calcium channel subtypes in experimental tactile allodynia. *Journal of Pharmacology and Experimental Therapeutics*, **269**, 1117-1123.

Chapman, V., Suzuki, R. & Dickenson, A.H. (1998) Electrophysiological characterization of spinal neuronal response properties in anaesthetized rats after ligation of spinal nerves L5-L6. *J Physiol*, **507 (Pt 3)**, 881-894.

Chen, J., Kang, D., Xu, J., Lake, M., Hogan, J.O., Sun, C., Walter, K., Yao, B. & Kim, D. (2013) Species differences and molecular determinant of TRPA1 cold sensitivity. *Nat Commun*, **4**, 2501.

Chen, L. & Huang, L.Y. (1992) Protein kinase C reduces Mg2+ block of NMDA-receptor channels as a mechanism of modulation. *Nature*, **356**, 521-523.

Chen, S.R. & Pan, H.L. (2005) Distinct roles of group III metabotropic glutamate receptors in control of nociception and dorsal horn neurons in normal and nerve-injured Rats. *The Journal of pharmacology and experimental therapeutics*, **312**, 120-126.

Chesler, E.J., Wilson, S.G., Lariviere, W.R., Rodriguez-Zas, S.L. & Mogil, J.S. (2002) Identification and ranking of genetic and laboratory environment factors influencing a behavioral trait, thermal nociception, via computational analysis of a large data archive. *Neuroscience and biobehavioral reviews*, **26**, 907-923.

Choi, I.S., Cho, J.H., An, C.H., Jung, J.K., Hur, Y.K., Choi, J.K. & Jang, I.S. (2012) 5-HT1B receptors inhibit glutamate release from primary afferent terminals in rat medullary dorsal horn neurons. *British journal of pharmacology*, **167**, 356-367.

Christensen, A.P. & Corey, D.P. (2007) TRP channels in mechanosensation: direct or indirect activation? *Nature reviews. Neuroscience*, **8**, 510-521.

Christopherson, K.S., Ullian, E.M., Stokes, C.C., Mullowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P. & Barres, B.A. (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell*, **120**, 421-433.

Chuang, H.H., Prescott, E.D., Kong, H., Shields, S., Jordt, S.E., Basbaum, A.I., Chao, M.V. & Julius, D. (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature*, **411**, 957-962.

Cizkova, D., Marsala, J., Lukacova, N., Marsala, M., Jergova, S., Orendacova, J. & Yaksh, T.L. (2002) Localization of N-type Ca2+ channels in the rat spinal cord following chronic constrictive nerve injury. *Experimental brain research*, **147**, 456-463.

Clasbrummel, B., Osswald, H. & Illes, P. (1989) Inhibition of noradrenaline release by omega-conotoxin GVIA in the rat tail artery. *British journal of pharmacology*, **96**, 101-110.

Coghill, R.C., Mayer, D.J. & Price, D.D. (1993) The roles of spatial recruitment and discharge frequency in spinal cord coding of pain: a combined electrophysiological and imaging investigation. *Pain*, **53**, 295-309.

Colburn, R.W., Lubin, M.L., Stone, D.J., Jr., Wang, Y., Lawrence, D., D'Andrea, M.R., Brandt, M.R., Liu, Y., Flores, C.M. & Qin, N. (2007) Attenuated cold sensitivity in TRPM8 null mice. *Neuron*, **54**, 379-386.

Cole, R.L., Lechner, S.M., Williams, M.E., Prodanovich, P., Bleicher, L., Varney, M.A. & Gu, G. (2005) Differential distribution of voltage-gated calcium channel alpha-2 delta (alpha2delta) subunit mRNA-containing cells in the rat central nervous system and the dorsal root ganglia. *The Journal of comparative neurology*, **491**, 246-269.

Colvin, L.A., Johnson, P.R., Mitchell, R., Fleetwood-Walker, S.M. & Fallon, M. (2008) From bench to bedside: a case of rapid reversal of bortezomib-induced neuropathic pain by the TRPM8 activator, menthol. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **26**, 4519-4520.

Combe, R., Bramwell, S. & Field, M.J. (2004) The monosodium iodoacetate model of osteoarthritis: a model of chronic nociceptive pain in rats? *Neuroscience Letters*, **370**, 236-240.

Costigan, M., Befort, K., Karchewski, L., Griffin, R.S., D'Urso, D., Allchorne, A., Sitarski, J., Mannion, J.W., Pratt, R.E. & Woolf, C.J. (2002) Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC neuroscience*, **3**, 16.

Coull, J.A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., Gravel, C., Salter, M.W. & De Koninck, Y. (2005) BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature*, **438**, 1017-1021.

Coull, J.A., Boudreau, D., Bachand, K., Prescott, S.A., Nault, F., Sik, A., De Koninck, P. & De Koninck, Y. (2003) Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature*, **424**, 938-942.

Cox, J.J., Reimann, F., Nicholas, A.K., Thornton, G., Roberts, E., Springell, K., Karbani, G., Jafri, H., Mannan, J., Raashid, Y., Al-Gazali, L., Hamamy, H., Valente, E.M., Gorman, S., Williams, R., McHale, D.P., Wood, J.N., Gribble, F.M. & Woods, C.G. (2006) An SCN9A channelopathy causes congenital inability to experience pain. *Nature*, **444**, 894-898.

Craig, A. & Bushnell, M. (1994) The thermal grill illusion: unmasking the burn of cold pain. *Science*, **265**, 252-255.

Craig, A.D. (2003) Pain mechanisms: labeled lines versus convergence in central processing. *Annual review of neuroscience*, **26**, 1-30.

Craig, A.D., Bushnell, M.C., Zhang, E.T. & Blomqvist, A. (1994) A thalamic nucleus specific for pain and temperature sensation. *Nature*, **372**, 770-773.

Craig, A.D., Krout, K. & Andrew, D. (2001) Quantitative Response Characteristics of Thermoreceptive and Nociceptive Lamina I Spinothalamic Neurons in the Cat. *Journal of neurophysiology*, **86**, 1459-1480.

Daniels, R.L., Takashima, Y. & McKemy, D.D. (2009) Activity of the Neuronal Cold Sensor TRPM8 Is Regulated by Phospholipase C via the Phospholipid Phosphoinositol 4,5-Bisphosphate. *Journal of Biological Chemistry*, **284**, 1570-1582.

Davies, A., Douglas, L., Hendrich, J., Wratten, J., Tran Van Minh, A., Foucault, I., Koch, D., Pratt, W.S., Saibil, H.R. & Dolphin, A.C. (2006a) The calcium channel alpha2delta-2 subunit partitions with CaV2.1 into lipid rafts in cerebellum:

implications for localization and function. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **26**, 8748-8757.

Davies, A., Kadurin, I., Alvarez-Laviada, A., Douglas, L., Nieto-Rostro, M., Bauer, C.S., Pratt, W.S. & Dolphin, A.C. (2010) The alpha2delta subunits of voltage-gated calcium channels form GPI-anchored proteins, a posttranslational modification essential for function. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 1654-1659.

Davies, M., Brophy, S., Williams, R. & Taylor, A. (2006b) The prevalence, severity, and impact of painful diabetic peripheral neuropathy in type 2 diabetes. *Diabetes care*, **29**, 1518-1522.

Davies, S.J., Harding, L.M. & Baranowski, A.P. (2002) A novel treatment of postherpetic neuralgia using peppermint oil. *The Clinical journal of pain*, **18**, 200-202.

Davis, K.D. (1998) Cold-induced pain and prickle in the glabrous and hairy skin. *Pain*, **75**, 47-57.

Davis, K.D., Lozano, R.M., Manduch, M., Tasker, R.R., Kiss, Z.H. & Dostrovsky, J.O. (1999) Thalamic relay site for cold perception in humans. *J Neurophysiol*, **81**, 1970-1973.

De Felice, M., Sanoja, R., Wang, R., Vera-Portocarrero, L., Oyarzo, J., King, T., Ossipov, M.H., Vanderah, T.W., Lai, J., Dussor, G.O., Fields, H.L., Price, T.J. & Porreca, F. (2011) Engagement of descending inhibition from the rostral ventromedial medulla protects against chronic neuropathic pain. *Pain*, **152**, 2701-2709.

De Jongh, K.S., Warner, C. & Catterall, W.A. (1990) Subunits of purified calcium channels. Alpha 2 and delta are encoded by the same gene. *The Journal of biological chemistry*, **265**, 14738-14741.

De Koninck, Y. & Henry, J.L. (1991) Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons in vivo by noxious stimulation. *Proceedings of the National Academy of Sciences*, **88**, 11344-11348.

De Petrocellis, L., Starowicz, K., Moriello, A.S., Vivese, M., Orlando, P. & Di Marzo, V. (2007) Regulation of transient receptor potential channels of melastatin type 8 (TRPM8): effect of cAMP, cannabinoid CB(1) receptors and endovanilloids. *Experimental cell research*, **313**, 1911-1920.

De Waard, M. & Campbell, K.P. (1995) Subunit regulation of the neuronal alpha 1A Ca2+ channel expressed in Xenopus oocytes. *The Journal of Physiology*, **485**, 619-634.

Defrin, R., Benstein-Sheraizin, A., Bezalel, A., Mantzur, O. & Arendt-Nielsen, L. (2008) The spatial characteristics of the painful thermal grill illusion. *Pain*, **138**, 577-586.

Defrin, R., Sheraizin, A., Malichi, L. & Shachen, O. (2011) Spatial summation and spatial discrimination of cold pain: effect of spatial configuration and skin type. *Pain*, **152**, 2739-2745.

Depaulis, A., Morgan, M.M. & Liebeskind, J.C. (1987) GABAergic modulation of the analgesic effects of morphine microinjected in the ventral periaqueductal gray matter of the rat. *Brain Res*, **436**, 223-228.

Descoeur, J., Pereira, V., Pizzoccaro, A., Francois, A., Ling, B., Maffre, V., Couette, B., Busserolles, J., Courteix, C., Noel, J., Lazdunski, M., Eschalier, A., Authier, N. & Bourinet, E. (2011) Oxaliplatin-induced cold hypersensitivity is due to remodelling of ion channel expression in nociceptors. *EMBO Molecular Medicine*, **3**, 266-278.

Dhaka, A., Earley, T.J., Watson, J. & Patapoutian, A. (2008) Visualizing cold spots: TRPM8-expressing sensory neurons and their projections. *The Journal of Neuroscience*, **28**, 566-575.

Dhaka, A., Murray, A.N., Mathur, J., Earley, T.J., Petrus, M.J. & Patapoutian, A. (2007) TRPM8 is required for cold sensation in mice. *Neuron*, **54**, 371-378.

Dickenson, A.H. & Sullivan, A.F. (1987) Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. *Neuropharmacology*, **26**, 1235-1238.

Dickenson, A.H., Sullivan, A.F. & McQuay, H.J. (1990) Intrathecal etorphine, fentanyl and buprenorphine on spinal nociceptive neurones in the rat. *Pain*, **42**, 227-234.

Ding, J.D. & Weinberg, R.J. (2006) Localization of soluble guanylyl cyclase in the superficial dorsal horn. *Journal of Comparative Neurology*, **495**, 668-678.

Dirks, J., Petersen, K.L., Rowbotham, M.C. & Dahl, J.B. (2002) Gabapentin suppresses cutaneous hyperalgesia following heat-capsaicin sensitization. *Anesthesiology*, **97**, 102-107.

Djouhri, L., Fang, X., Okuse, K., Wood, J.N., Berry, C.M. & Lawson, S.N. (2003) The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons. *J Physiol*, **550**, 739-752.

Dolphin, A.C. (2003) G protein modulation of voltage-gated calcium channels. *Pharmacol Rev*, **55**, 607-627.

Dolphin, A.C. (2013) The alpha2delta subunits of voltage-gated calcium channels. *Biochimica et biophysica acta*, **1828**, 1541-1549.

Du, J., Yang, X., Zhang, L. & Zeng, Y.M. (2009) Expression of TRPM8 in the distal cerebrospinal fluid-contacting neurons in the brain mesencephalon of rats. *Cerebrospinal fluid research*, **6**, 3.

Dubner, R., Kenshalo Jr, D.R., Maixner, W., Bushnell, M.C. & Oliveras, J.L. (1989) The correlation of monkey medullary dorsal horn neuronal activity and the perceived intensity of noxious heat stimuli. *Journal of Neurophysiology*, **62**, 450-457.

Dubner, R., Sumino, R. & Wood, W.I. (1975) A peripheral "cold" fiber population responsive to innocuous and noxious thermal stimuli applied to monkey's face. *Journal of neurophysiology*, **38**, 1373-1389.

Eisenberg, E., McNicol, E.D. & Carr, D.B. (2006) Efficacy of mu-opioid agonists in the treatment of evoked neuropathic pain: Systematic review of randomized controlled trials. *European journal of pain (London, England)*, **10**, 667-676.

Erler, I., Al-Ansary, D.M.M., Wissenbach, U., Wagner, T.F.J., Flockerzi, V. & Niemeyer, B.A. (2006) Trafficking and Assembly of the Cold-sensitive TRPM8 Channel. *Journal of Biological Chemistry*, **281**, 38396-38404.

Eroglu, C., Allen, N.J., Susman, M.W., O'Rourke, N.A., Park, C.Y., Ozkan, E., Chakraborty, C., Mulinyawe, S.B., Annis, D.S., Huberman, A.D., Green, E.M., Lawler, J., Dolmetsch, R., Garcia, K.C., Smith, S.J., Luo, Z.D., Rosenthal, A., Mosher, D.F. & Barres, B.A. (2009) Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. *Cell*, **139**, 380-392.

Ertel, E.A., Campbell, K.P., Harpold, M.M., Hofmann, F., Mori, Y., Perez-Reyes, E., Schwartz, A., Snutch, T.P., Tanabe, T., Birnbaumer, L., Tsien, R.W. & Catterall, W.A. (2000) Nomenclature of voltage-gated calcium channels. *Neuron*, **25**, 533-535.

Eschenfelder, S., Habler, H.J. & Janig, W. (2000) Dorsal root section elicits signs of neuropathic pain rather than reversing them in rats with L5 spinal nerve injury. *Pain*, **87**, 213-219.

Falk, S. & Dickenson, A.H. (2014) Pain and nociception: mechanisms of cancer-induced bone pain. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **32**, 1647-1654.

Fallon, M.T., Storey, D.J., Krishan, A., Weir, C.J., Mitchell, R., Fleetwood-Walker, S.M., Scott, A.C. & Colvin, L.A. (2015) Cancer treatment-related neuropathic pain: proof of concept study with menthol-a TRPM8 agonist. *Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer*.

Fang, L., Wu, J., Lin, Q. & Willis, W.D. (2003) Protein kinases regulate the phosphorylation of the GluR1 subunit of AMPA receptors of spinal cord in rats following noxious stimulation. *Brain research. Molecular brain research*, **118**, 160-165.

Fehrenbacher, J.C., Taylor, C.P. & Vasko, M.R. (2003) Pregabalin and gabapentin reduce release of substance P and CGRP from rat spinal tissues only after inflammation or activation of protein kinase C. *Pain*, **105**, 133-141.

Felix, R., Gurnett, C.A., De Waard, M. & Campbell, K.P. (1997) Dissection of functional domains of the voltage-dependent Ca2+ channel alpha2delta subunit. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **17**, 6884-6891.

Feltner, D.E., Crockatt, J.G., Dubovsky, S.J., Cohn, C.K., Shrivastava, R.K., Targum, S.D., Liu-Dumaw, M., Carter, C.M. & Pande, A.C. (2003) A randomized, double-blind, placebo-controlled, fixed-dose, multicenter study of pregabalin in patients with generalized anxiety disorder. *Journal of clinical psychopharmacology*, **23**, 240-249.

Feng, Z.P., Doering, C.J., Winkfein, R.J., Beedle, A.M., Spafford, J.D. & Zamponi, G.W. (2003) Determinants of inhibition of transiently expressed voltage-gated calcium channels by omega-conotoxins GVIA and MVIIA. *The Journal of biological chemistry*, **278**, 20171-20178.

Field, M.J., Cox, P.J., Stott, E., Melrose, H., Offord, J., Su, T.-Z., Bramwell, S., Corradini, L., England, S. & Winks, J. (2006) Identification of the α2-δ-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. *Proceedings of the National Academy of Sciences*, **103**, 17537-17542.

Fields, H.L., Bry, J., Hentall, I. & Zorman, G. (1983a) The activity of neurons in the rostral medulla of the rat during withdrawal from noxious heat. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **3**, 2545-2552.

Fields, H.L., Vanegas, H., Hentall, I.D. & Zorman, G. (1983b) Evidence that disinhibition of brain stem neurones contributes to morphine analgesia. *Nature*, **306**, 684-686.

Fink, K., Meder, W., Dooley, D.J. & Gothert, M. (2000) Inhibition of neuronal Ca(2+) influx by gabapentin and subsequent reduction of neurotransmitter release from rat neocortical slices. *British journal of pharmacology*, **130**, 900-906.

Finn, D.P., Chapman, V., Jhaveri, M.D., Samanta, S., Manders, T., Bowden, J., Matthews, L., Marsden, C.A. & Beckett, S.R. (2003) The role of the central nucleus of the amygdala in nociception and aversion. *Neuroreport*, **14**, 981-984.

Finnerup, N.B., Otto, M., McQuay, H.J., Jensen, T.S. & Sindrup, S.H. (2005) Algorithm for neuropathic pain treatment: an evidence based proposal. *Pain*, **118**, 289-305.

Finnerup, N.B., Sindrup, S.H. & Jensen, T.S. (2010) The evidence for pharmacological treatment of neuropathic pain. *Pain*, **150**, 573-581.

Francois, A., Kerckhove, N., Meleine, M., Alloui, A., Barrere, C., Gelot, A., Uebele, V.N., Renger, J.J., Eschalier, A., Ardid, D. & Bourinet, E. (2013) State-dependent properties of a new T-type calcium channel blocker enhance Ca(V)3.2 selectivity and support analgesic effects. *Pain*, **154**, 283-293.

Freeman, R., Baron, R., Bouhassira, D., Cabrera, J. & Emir, B. (2014) Sensory profiles of patients with neuropathic pain based on the neuropathic pain symptoms and signs. *Pain*, **155**, 367-376.

Freeze, B.S., McNulty, M.M. & Hanck, D.A. (2006) State-dependent verapamil block of the cloned human CaV3. 1 T-type Ca2+ channel. *Molecular pharmacology*, **70**, 718-726.

Fukuoka, T., Kondo, E., Dai, Y., Hashimoto, N. & Noguchi, K. (2001) Brain-derived neurotrophic factor increases in the uninjured dorsal root ganglion neurons in selective spinal nerve ligation model. *Journal of Neuroscience*, **21**, 4891-4900.

Fuller-Bicer, G.A., Varadi, G., Koch, S.E., Ishii, M., Bodi, I., Kadeer, N., Muth, J.N., Mikala, G., Petrashevskaya, N.N., Jordan, M.A., Zhang, S.P., Qin, N., Flores, C.M., Isaacsohn, I., Varadi, M., Mori, Y., Jones, W.K. & Schwartz, A. (2009) Targeted disruption of the voltage-dependent calcium channel alpha2/delta-1-subunit. *American journal of physiology. Heart and circulatory physiology*, **297**, H117-124.

Furukawa, T., Miura, R., Honda, M., Kamiya, N., Mori, Y., Takeshita, S., Isshiki, T. & Nukada, T. (2004) Identification of R(-)-isomer of efonidipine as a selective blocker of T-type Ca2+ channels. *British journal of pharmacology*, **143**, 1050-1057.

Gach, M.P., Cherednichenko, G., Haarmann, C., Lopez, J.R., Beam, K.G., Pessah, I.N., Franzini-Armstrong, C. & Allen, P.D. (2008) Alpha2delta1 dihydropyridine receptor subunit is a critical element for excitation-coupled calcium entry but not for formation of tetrads in skeletal myotubes. *Biophys J*, **94**, 3023-3034.

Galan, A., Laird, J.M. & Cervero, F. (2004) In vivo recruitment by painful stimuli of AMPA receptor subunits to the plasma membrane of spinal cord neurons. *Pain*, **112**, 315-323.

Galeotti, N., Di Cesare Mannelli, L., Mazzanti, G., Bartolini, A. & Ghelardini, C. (2002) Menthol: a natural analgesic compound. *Neurosci Lett*, **322**, 145-148.

Gao, B., Sekido, Y., Maximov, A., Saad, M., Forgacs, E., Latif, F., Wei, M.H., Lerman, M., Lee, J.H., Perez-Reyes, E., Bezprozvanny, I. & Minna, J.D. (2000) Functional properties of a new voltage-dependent calcium channel alpha(2)delta auxiliary subunit gene (CACNA2D2). *The Journal of biological chemistry*, **275**, 12237-12242.

Gao, K. & Mason, P. (2000) Serotonergic raphe magnus cells that respond to noxious tail heat are not ON or OFF cells. *Journal of Neurophysiology*, **84**, 1719-1725.

Gao, T., Hao, J., Wiesenfeld-Hallin, Z. & Xu, X.-J. (2013) Activation of TRPM8 cold receptor triggers allodynia-like behavior in spinally injured rats. *Scandinavian Journal of Pain*, **4**, 33-37.

Garcia, K., Nabhani, T. & Garcia, J. (2008) The calcium channel alpha2/delta1 subunit is involved in extracellular signalling. *J Physiol*, **586**, 727-738.

Gau, R., Sevoz-Couche, C., Hamon, M. & Bernard, J.F. (2013) Noxious stimulation excites serotonergic neurons: a comparison between the lateral paragigantocellular reticular and the raphe magnus nuclei. *Pain*, **154**, 647-659.

Gauchan, P., Andoh, T., Kato, A. & Kuraishi, Y. (2009) Involvement of increased expression of transient receptor potential melastatin 8 in oxaliplatin-induced cold allodynia in mice. *Neurosci Lett*, **458**, 93-95.

Gaudioso, C., Hao, J., Martin-Eauclaire, M.F., Gabriac, M. & Delmas, P. (2012) Menthol pain relief through cumulative inactivation of voltage-gated sodium channels. *Pain*, **153**, 473-484.

Gavva, N.R., Davis, C., Lehto, S.G., Rao, S., Wang, W. & Zhu, D.X. (2012) Transient receptor potential melastatin 8 (TRPM8) channels are involved in body temperature regulation. *Mol Pain*, **8**, 36.

Gee, N.S., Brown, J.P., Dissanayake, V.U., Offord, J., Thurlow, R. & Woodruff, G.N. (1996) The novel anticonvulsant drug, gabapentin (Neurontin), binds to the alpha2delta subunit of a calcium channel. *The Journal of biological chemistry*, **271**, 5768-5776.

Gentry, C., Stoakley, N., Andersson, D.A. & Bevan, S. (2010) The roles of iPLA2, TRPM8 and TRPA1 in chemically induced cold hypersensitivity. *Mol Pain*, **6**, 4.

Goncalves, L., Almeida, A. & Pertovaara, A. (2007) Pronociceptive changes in response properties of rostroventromedial medullary neurons in a rat model of peripheral neuropathy. *The European journal of neuroscience*, **26**, 2188-2195.

Goncalves, L. & Dickenson, A.H. (2012) Asymmetric time-dependent activation of right central amygdala neurones in rats with peripheral neuropathy and pregabalin modulation. *The European journal of neuroscience*, **36**, 3204-3213.

Gong, H.C., Hang, J., Kohler, W., Li, L. & Su, T.Z. (2001) Tissue-specific expression and gabapentin-binding properties of calcium channel alpha2delta subunit subtypes. *The Journal of membrane biology*, **184**, 35-43.

Gottrup, H., Juhl, G., Kristensen, A.D., Lai, R., Chizh, B.A., Brown, J., Bach, F.W. & Jensen, T.S. (2004) Chronic oral gabapentin reduces elements of central sensitization in human experimental hyperalgesia. *Anesthesiology*, **101**, 1400-1408.

Goudet, C., Chapuy, E., Alloui, A., Acher, F., Pin, J.P. & Eschalier, A. (2008) Group III metabotropic glutamate receptors inhibit hyperalgesia in animal models of inflammation and neuropathic pain. *Pain*, **137**, 112-124.

Green, B.G. (2004) Temperature perception and nociception. *Journal of neurobiology*, **61**, 13-29.

Green, G.M., Lyons, L. & Dickenson, A.H. (1998) Alpha2-adrenoceptor antagonists enhance responses of dorsal horn neurones to formalin induced inflammation. *Eur J Pharmacol*, **347**, 201-204.

Greenspan, J.D., Ohara, S., Sarlani, E. & Lenz, F.A. (2004) Allodynia in patients with post-stroke central pain (CPSP) studied by statistical quantitative sensory testing within individuals. *Pain*, **109**, 357-366.

Grudt, T.J. & Perl, E.R. (2002) Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. *J Physiol*, **540**, 189-207.

Guo, W., Zou, S., Guan, Y., Ikeda, T., Tal, M., Dubner, R. & Ren, K. (2002) Tyrosine phosphorylation of the NR2B subunit of the NMDA receptor in the spinal cord during the development and maintenance of inflammatory hyperalgesia. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **22**, 6208-6217.

Gurnett, C.A., Felix, R. & Campbell, K.P. (1997) Extracellular interaction of the voltage-dependent Ca2+ channel alpha2delta and alpha1 subunits. *The Journal of biological chemistry*, **272**, 18508-18512.

Hagihira, S., Senba, E., Yoshida, S., Tohyama, M. & Yoshiya, I. (1990) Fine structure of noradrenergic terminals and their synapses in the rat spinal dorsal horn: an immunohistochemical study. *Brain Res*, **526**, 73-80.

Han, Z.S., Zhang, E.T. & Craig, A.D. (1998) Nociceptive and thermoreceptive lamina I neurons are anatomically distinct. *Nat Neurosci*, **1**, 218-225.

Hara, K. & Harris, R.A. (2002) The anesthetic mechanism of urethane: the effects on neurotransmitter-gated ion channels. *Anesthesia and analgesia*, **94**, 313-318, table of contents.

Harrison, J.L. & Davis, K.D. (1999) Cold-evoked pain varies with skin type and cooling rate: a psychophysical study in humans. *Pain*, **83**, 123-135.

Hatakeyama, S., Wakamori, M., Ino, M., Miyamoto, N., Takahashi, E., Yoshinaga, T., Sawada, K., Imoto, K., Tanaka, I., Yoshizawa, T., Nishizawa, Y., Mori, Y., Niidome, T. & Shoji, S. (2001) Differential nociceptive responses in mice lacking the alpha(1B) subunit of N-type Ca(2+) channels. *Neuroreport*, **12**, 2423-2427.

Hatem, S., Attal, N., Willer, J.C. & Bouhassira, D. (2006) Psychophysical study of the effects of topical application of menthol in healthy volunteers. *Pain*, **122**, 190-196.

Hayashida, K., Clayton, B.A., Johnson, J.E. & Eisenach, J.C. (2008) Brain derived nerve growth factor induces spinal noradrenergic fiber sprouting and enhances clonidine analgesia following nerve injury in rats. *Pain*, **136**, 348-355.

Hayashida, K., DeGoes, S., Curry, R. & Eisenach, J.C. (2007) Gabapentin activates spinal noradrenergic activity in rats and humans and reduces hypersensitivity after surgery. *Anesthesiology*, **106**, 557-562.

Heinricher, M.M., Barbaro, N.M. & Fields, H.L. (1989) Putative nociceptive modulating neurons in the rostral ventromedial medulla of the rat: firing of on- and off-cells is related to nociceptive responsiveness. *Somatosensory & motor research*, **6**, 427-439.

Heinricher, M.M., Tavares, I., Leith, J.L. & Lumb, B.M. (2009) Descending control of nociception: Specificity, recruitment and plasticity. *Brain research reviews*, **60**, 214-225.

Hendrich, J., Van Minh, A.T., Heblich, F., Nieto-Rostro, M., Watschinger, K., Striessnig, J., Wratten, J., Davies, A. & Dolphin, A.C. (2008) Pharmacological disruption of calcium channel trafficking by the alpha2delta ligand gabapentin. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 3628-3633.

Hensel, H. & Zotterman, Y. (1951) The effect of menthol on the thermoreceptors. *Acta Physiol Scand*, **24**, 27-34.

Herlitze, S., Garcia, D.E., Mackie, K., Hille, B., Scheuer, T. & Catterall, W.A. (1996) Modulation of Ca2+ channels by G-protein beta gamma subunits. *Nature*, **380**, 258-262.

Hermann, D.M., Luppi, P.H., Peyron, C., Hinckel, P. & Jouvet, M. (1997) Afferent projections to the rat nuclei raphe magnus, raphe pallidus and reticularis gigantocellularis pars alpha demonstrated by iontophoretic application of choleratoxin (subunit b). *Journal of chemical neuroanatomy*, **13**, 1-21.

Herrero, J.F., Laird, J.M. & Lopez-Garcia, J.A. (2000) Wind-up of spinal cord neurones and pain sensation: much ado about something? *Prog Neurobiol*, **61**, 169-203.

Hickey, L., Li, Y., Fyson, S.J., Watson, T.C., Perrins, R., Hewinson, J., Teschemacher, A.G., Furue, H., Lumb, B.M. & Pickering, A.E. (2014) Optoactivation of locus ceruleus neurons evokes bidirectional changes in thermal nociception in rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **34**, 4148-4160.

Ho Kim, S. & Mo Chung, J. (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain*, **50**, 355-363.

Hobom, M., Dai, S., Marais, E., Lacinova, L., Hofmann, F. & Klugbauer, N. (2000) Neuronal distribution and functional characterization of the calcium channel alpha2delta-2 subunit. *The European journal of neuroscience*, **12**, 1217-1226.

Hokfelt, T., Zhang, X. & Wiesenfeld-Hallin, Z. (1994) Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends in neurosciences*, **17**, 22-30.

Hoppa, M.B., Lana, B., Margas, W., Dolphin, A.C. & Ryan, T.A. (2012) $\alpha 2\,\delta$ expression sets presynaptic calcium channel abundance and release probability. *Nature*, **486**, 122-125.

Hu, H.-J., Carrasquillo, Y., Karim, F., Jung, W.E., Nerbonne, J.M., Schwarz, T.L. & Gereau Iv, R.W. (2006) The Kv4.2 Potassium Channel Subunit Is Required for Pain Plasticity. *Neuron*, **50**, 89-100.

Hua, X.Y., Chen, P. & Yaksh, T.L. (1999) Inhibition of spinal protein kinase C reduces nerve injury-induced tactile allodynia in neuropathic rats. *Neurosci Lett*, **276**, 99-102.

Hughes, D.I., Scott, D.T., Riddell, J.S. & Todd, A.J. (2007) Upregulation of substance P in low-threshold myelinated afferents is not required for tactile allodynia in the chronic constriction injury and spinal nerve ligation models. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **27**, 2035-2044.

Hughes, S.W., Hickey, L., Hulse, R.P., Lumb, B.M. & Pickering, A.E. (2013) Endogenous analgesic action of the pontospinal noradrenergic system spatially restricts and temporally delays the progression of neuropathic pain following tibial nerve injury. *Pain*, **154**, 1680-1690.

Hunt, S.P. & Mantyh, P.W. (2001) The molecular dynamics of pain control. *Nature reviews. Neuroscience*, **2**, 83-91.

Hunter, J.C., Gogas, K.R., Hedley, L.R., Jacobson, L.O., Kassotakis, L., Thompson, J. & Fontana, D.J. (1997) The effect of novel anti-epileptic drugs in rat experimental models of acute and chronic pain. *Eur J Pharmacol*, **324**, 153-160.

Hylden, J.L., Thomas, D.A., Iadarola, M.J., Nahin, R.L. & Dubner, R. (1991) Spinal opioid analgesic effects are enhanced in a model of unilateral inflammation/hyperalgesia: possible involvement of noradrenergic mechanisms. *Eur J Pharmacol*, **194**, 135-143.

Iannetti, G.D., Zambreanu, L., Wise, R.G., Buchanan, T.J., Huggins, J.P., Smart, T.S., Vennart, W. & Tracey, I. (2005) Pharmacological modulation of pain-related brain activity during normal and central sensitization states in humans. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 18195-18200.

Jasmin, L., Boudah, A. & Ohara, P.T. (2003) Long-term effects of decreased noradrenergic central nervous system innervation on pain behavior and opioid antinociception. *The Journal of comparative neurology*, **460**, 38-55.

Jensen, A.A., Mosbacher, J., Elg, S., Lingenhoehl, K., Lohmann, T., Johansen, T.N., Abrahamsen, B., Mattsson, J.P., Lehmann, A. & Bettler, B. (2002) The anticonvulsant gabapentin (Neurontin) does not act through γ -aminobutyric acid-B receptors. *Molecular pharmacology*, **61**, 1377-1384.

Ji, R.-R., Gereau Iv, R.W., Malcangio, M. & Strichartz, G.R. (2009) MAP kinase and pain. *Brain research reviews*, **60**, 135-148.

Ji, R.R., Samad, T.A., Jin, S.X., Schmoll, R. & Woolf, C.J. (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron*, **36**, 57-68.

Jia, H., Rustioni, A. & Valtschanoff, J.G. (1999) Metabotropic glutamate receptors in superficial laminae of the rat dorsal horn. *The Journal of comparative neurology*, **410**, 627-642.

Jin, S.X., Zhuang, Z.Y., Woolf, C.J. & Ji, R.R. (2003) p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **23**, 4017-4022.

Johnson, C.D., Melanaphy, D., Purse, A., Stokesberry, S.A., Dickson, P. & Zholos, A.V. (2009) Transient receptor potential melastatin 8 channel involvement in the regulation of vascular tone. *American Journal of Physiology - Heart and Circulatory Physiology*, **296**, H1868-H1877.

Jones, S.L. & Gebhart, G.F. (1986) Characterization of coeruleospinal inhibition of the nociceptive tail-flick reflex in the rat: mediation by spinal alpha 2-adrenoceptors. *Brain Res*, **364**, 315-330.

Jordt, S.E., Bautista, D.M., Chuang, H.H., McKemy, D.D., Zygmunt, P.M., Hogestatt, E.D., Meng, I.D. & Julius, D. (2004) Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature*, **427**, 260-265.

Jørum, E., Warncke, T. & Stubhaug, A. (2003) Cold allodynia and hyperalgesia in neuropathic pain: the effect of N-methyl-D-aspartate (NMDA) receptor antagonist ketamine: a double-blind, cross-over comparison with alfentanil and placebo. *Pain*, **101**, 229-235.

Jun, K., Piedras-Renteria, E.S., Smith, S.M., Wheeler, D.B., Lee, S.B., Lee, T.G., Chin, H., Adams, M.E., Scheller, R.H., Tsien, R.W. & Shin, H.S. (1999) Ablation of P/Q-type Ca(2+) channel currents, altered synaptic transmission, and progressive ataxia in mice lacking the alpha(1A)-subunit. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 15245-15250.

Karashima, Y., Talavera, K., Everaerts, W., Janssens, A., Kwan, K.Y., Vennekens, R., Nilius, B. & Voets, T. (2009) TRPA1 acts as a cold sensor in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 1273-1278.

Karim, F., Hu, H.J., Adwanikar, H., Kaplan, D. & Gereau, R.W.t. (2006) Impaired inflammatory pain and thermal hyperalgesia in mice expressing neuron-specific dominant negative mitogen activated protein kinase kinase (MEK). *Mol Pain*, **2**, 2.

Katsura, H., Obata, K., Mizushima, T., Yamanaka, H., Kobayashi, K., Dai, Y., Fukuoka, T., Tokunaga, A., Sakagami, M. & Noguchi, K. (2006) Antisense knock down of TRPA1, but not TRPM8, alleviates cold hyperalgesia after spinal nerve ligation in rats. *Exp Neurol*, **200**, 112-123.

Kawasaki, Y., Kumamoto, E., Furue, H. & Yoshimura, M. (2003) Alpha 2 adrenoceptor-mediated presynaptic inhibition of primary afferent glutamatergic transmission in rat substantia gelatinosa neurons. *Anesthesiology*, **98**, 682-689.

Keay, K.A. & Bandler, R. (2001) Parallel circuits mediating distinct emotional coping reactions to different types of stress. *Neuroscience and biobehavioral reviews*, **25**, 669-678.

Kehlet, H., Jensen, T.S. & Woolf, C.J. (2006) Persistent postsurgical pain: risk factors and prevention. *Lancet*, **367**, 1618-1625.

Kenshalo, D.R. & Duclaux, R. (1977) Response characteristics of cutaneous cold receptors in the monkey. *Journal of neurophysiology*, **40**, 319-332.

Kern, D., Pelle-Lancien, E., Luce, V. & Bouhassira, D. (2008) Pharmacological dissection of the paradoxical pain induced by a thermal grill. *Pain*, **135**, 291-299.

Khasabov, S.G., Cain, D.M., Thong, D., Mantyh, P.W. & Simone, D.A. (2001) Enhanced responses of spinal dorsal horn neurons to heat and cold stimuli following mild freeze injury to the skin. *J Neurophysiol*, **86**, 986-996.

Kim, A.Y., Tang, Z., Liu, Q., Patel, K.N., Maag, D., Geng, Y. & Dong, X. (2008) Pirt, a phosphoinositide-binding protein, functions as a regulatory subunit of TRPV1. *Cell*, **133**, 475-485.

Kim, C., Jun, K., Lee, T., Kim, S.S., McEnery, M.W., Chin, H., Kim, H.L., Park, J.M., Kim, D.K., Jung, S.J., Kim, J. & Shin, H.S. (2001) Altered nociceptive response in mice deficient in the alpha(1B) subunit of the voltage-dependent calcium channel. *Molecular and cellular neurosciences*, **18**, 235-245.

Kim, H.L., Kim, H., Lee, P., King, R.G. & Chin, H. (1992) Rat brain expresses an alternatively spliced form of the dihydropyridine-sensitive L-type calcium channel alpha 2 subunit. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 3251-3255.

Kincaid, W., Neubert, M.J., Xu, M., Kim, C.J. & Heinricher, M.M. (2006) Role for medullary pain facilitating neurons in secondary thermal hyperalgesia. *J Neurophysiol*, **95**, 33-41.

King, T., Vera-Portocarrero, L., Gutierrez, T., Vanderah, T.W., Dussor, G., Lai, J., Fields, H.L. & Porreca, F. (2009) Unmasking the tonic-aversive state in neuropathic pain. *Nat Neurosci*, **12**, 1364-1366.

Kleggetveit, I.P. & Jørum, E. (2010) Large and small fiber dysfunction in peripheral nerve injuries with or without spontaneous pain. *The journal of pain : official journal of the American Pain Society*, **11**, 1305-1310.

Klein, A.H., Sawyer, C.M., Carstens, M.I., Tsagareli, M.G., Tsiklauri, N. & Carstens, E. (2010) Topical application of L-menthol induces heat analgesia, mechanical allodynia, and a biphasic effect on cold sensitivity in rats. *Behavioural brain research*, **212**, 179-186.

Klein, A.H., Sawyer, C.M., Takechi, K., Davoodi, A., Ivanov, M.A., Carstens, M.I. & Carstens, E. (2012) Topical hindpaw application of L-menthol decreases responsiveness to heat with biphasic effects on cold sensitivity of rat lumbar dorsal horn neurons. *Neuroscience*, **219**, 234-242.

Klugbauer, N., Lacinova, L., Marais, E., Hobom, M. & Hofmann, F. (1999) Molecular diversity of the calcium channel alpha2delta subunit. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **19**, 684-691.

Knowlton, W.M., Bifolck-Fisher, A., Bautista, D.M. & McKemy, D.D. (2010) TRPM8, but not TRPA1, is required for neural and behavioral responses to acute noxious cold temperatures and cold-mimetics in vivo. *Pain*, **150**, 340-350.

Knowlton, W.M., Daniels, R.L., Palkar, R., McCoy, D.D. & McKemy, D.D. (2011) Pharmacological blockade of TRPM8 ion channels alters cold and cold pain responses in mice. *PLoS One*, **6**, e25894.

Knowlton, W.M., Palkar, R., Lippoldt, E.K., McCoy, D.D., Baluch, F., Chen, J. & McKemy, D.D. (2013) A Sensory-Labeled Line for Cold: TRPM8-Expressing Sensory Neurons Define the Cellular Basis for Cold, Cold Pain, and Cooling-Mediated Analgesia. *The Journal of Neuroscience*, **33**, 2837-2848.

Kohno, T., Wang, H., Amaya, F., Brenner, G.J., Cheng, J.K., Ji, R.R. & Woolf, C.J. (2008) Bradykinin enhances AMPA and NMDA receptor activity in spinal cord dorsal horn neurons by activating multiple kinases to produce pain hypersensitivity. *Journal of Neuroscience*, **28**, 4533-4540.

Kremeyer, B., Lopera, F., Cox, J.J., Momin, A., Rugiero, F., Marsh, S., Woods, C.G., Jones, N.G., Paterson, K.J. & Fricker, F.R. (2010) A gain-of-function mutation in TRPA1 causes familial episodic pain syndrome. *Neuron*, **66**, 671-680.

Kuhn, F.J., Kuhn, C. & Luckhoff, A. (2009) Inhibition of TRPM8 by icilin distinct from desensitization induced by menthol and menthol derivatives. *The Journal of biological chemistry*, **284**, 4102-4111.

Kurshan, P.T., Oztan, A. & Schwarz, T.L. (2009) Presynaptic alpha2delta-3 is required for synaptic morphogenesis independent of its Ca2+-channel functions. *Nat Neurosci*, **12**, 1415-1423.

Kusunose, N., Koyanagi, S., Hamamura, K., Matsunaga, N., Yoshida, M., Uchida, T., Tsuda, M., Inoue, K. & Ohdo, S. (2010) Molecular basis for the dosing time-dependency of anti-allodynic effects of gabapentin in a mouse model of neuropathic pain. *Mol Pain*, **6**, 83.

Kwan, K.Y., Allchorne, A.J., Vollrath, M.A., Christensen, A.P., Zhang, D.-S., Woolf, C.J. & Corey, D.P. (2006) TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron*, **50**, 277-289.

Kwiat, G.C. & Basbaum, A.I. (1992) The origin of brainstem noradrenergic and serotonergic projections to the spinal cord dorsal horn in the rat. *Somatosensory & motor research*, **9**, 157-173.

LaMotte, R.H., Shain, C.N., Simone, D.A. & Tsai, E.F. (1991) Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms. *J Neurophysiol*, **66**, 190-211.

LaMotte, R.H. & Thalhammer, J.G. (1982) Response properties of high-threshold cutaneous cold receptors in the primate. *Brain Research*, **244**, 279-287.

Lana, B., Schlick, B., Martin, S., Pratt, W.S., Page, K.M., Goncalves, L., Rahman, W., Dickenson, A.H., Bauer, C.S. & Dolphin, A.C. (2014) Differential upregulation in DRG neurons of an alpha2delta-1 splice variant with a lower affinity for gabapentin after peripheral sensory nerve injury. *Pain*, **155**, 522-533.

Lanneau, C., Green, A., Hirst, W.D., Wise, A., Brown, J.T., Donnier, E., J Charles, K., Wood, M., Davies, C.H. & Pangalos, M.N. (2001) Gabapentin is not a GABA B receptor agonist. *Neuropharmacology*, **41**, 965-975.

Lashinger, E.S., Steiginga, M.S., Hieble, J.P., Leon, L.A., Gardner, S.D., Nagilla, R., Davenport, E.A., Hoffman, B.E., Laping, N.J. & Su, X. (2008) AMTB, a TRPM8 channel blocker: evidence in rats for activity in overactive bladder and painful bladder syndrome. *American Journal of Physiology-Renal Physiology*, **295**, F803-F810.

Latorre, R., Brauchi, S., Madrid, R. & Orio, P. (2011) A cool channel in cold transduction. *Physiology (Bethesda, Md.)*, **26**, 273-285.

Latorre, R., Brauchi, S., Orta, G., Zaelzer, C. & Vargas, G. (2007) ThermoTRP channels as modular proteins with allosteric gating. *Cell Calcium*, **42**, 427-438.

Latremoliere, A. & Woolf, C.J. (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *The journal of pain : official journal of the American Pain Society*, **10**, 895-926.

Lee, S., Kim, Y., Back, S.K., Choi, H.W., Lee, J.Y., Jung, H.H., Ryu, J.H., Suh, H.W., Na, H.S., Kim, H.J., Rhim, H. & Kim, J.I. (2010) Analgesic effect of highly reversible omega-conotoxin FVIA on N type Ca2+ channels. *Mol Pain*, **6**, 97.

Leith, J.L., Koutsikou, S., Lumb, B.M. & Apps, R. (2010) Spinal processing of noxious and innocuous cold information: differential modulation by the periaqueductal gray. *The Journal of Neuroscience*, **30**, 4933-4942.

Leitner, J., Westerholz, S., Heinke, B., Forsthuber, L., Wunderbaldinger, G., Jager, T., Gruber-Schoffnegger, D., Braun, K. & Sandkuhler, J. (2013) Impaired excitatory drive to spinal GABAergic neurons of neuropathic mice. *PLoS One*, **8**, e73370.

Lewis, R.J. (2006) Ciguatera: Australian perspectives on a global problem. *Toxicon : official journal of the International Society on Toxinology*, **48**, 799-809.

Lewis, R.J., Norton, R.S., Brereton, I.M. & Eccles, C.D. (1993) Ciguatoxin-2 is a diastereomer of ciguatoxin-3. *Toxicon : official journal of the International Society on Toxinology*, **31**, 637-643.

Li, C.Y., Song, Y.H., Higuera, E.S. & Luo, Z.D. (2004) Spinal dorsal horn calcium channel alpha2delta-1 subunit upregulation contributes to peripheral nerve injury-induced tactile allodynia. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **24**, 8494-8499.

Li, C.Y., Zhang, X.L., Matthews, E.A., Li, K.W., Kurwa, A., Boroujerdi, A., Gross, J., Gold, M.S., Dickenson, A.H., Feng, G. & Luo, Z.D. (2006) Calcium channel alpha2delta1 subunit mediates spinal hyperexcitability in pain modulation. *Pain*, **125**, 20-34.

Li, H., Ohishi, H., Kinoshita, A., Shigemoto, R., Nomura, S. & Mizuno, N. (1997a) Localization of a metabotropic glutamate receptor, mGluR7, in axon terminals of presumed nociceptive, primary afferent fibers in the superficial layers of the spinal dorsal horn: an electron microscope study in the rat. *Neurosci Lett*, **223**, 153-156.

Li, J., Simone, D.A. & Larson, A.A. (1999) Windup leads to characteristics of central sensitization. *Pain*, **79**, 75-82.

Li, J.L., Kaneko, T., Nomura, S., Li, Y.Q. & Mizuno, N. (1997b) Association of serotonin-like immunoreactive axons with nociceptive projection neurons in the caudal spinal trigeminal nucleus of the rat. *The Journal of comparative neurology*, **384**, 127-141.

Li, L., Cao, X.-H., Chen, S.-R., Han, H.-D., Lopez-Berestein, G., Sood, A.K. & Pan, H.-L. (2012) Up-regulation of Cavβ3 subunit in primary sensory neurons increases voltage-activated Ca2+ channel activity and nociceptive input in neuropathic pain. *Journal of Biological Chemistry*, **287**, 6002-6013.

Li, Y., Dorsi, M.J., Meyer, R.A. & Belzberg, A.J. (2000) Mechanical hyperalgesia after an L5 spinal nerve lesion in the rat is not dependent on input from injured nerve fibers. *Pain*, **85**, 493-502.

Lindstedt, F., Johansson, B., Martinsen, S., Kosek, E., Fransson, P. & Ingvar, M. (2011) Evidence for thalamic involvement in the thermal grill illusion: an FMRI study. *PLoS One*, **6**, e27075.

Lippoldt, E.K., Elmes, R.R., McCoy, D.D., Knowlton, W.M. & McKemy, D.D. (2013) Artemin, a Glial Cell Line-Derived Neurotrophic Factor Family Member, Induces TRPM8-Dependent Cold Pain. *The Journal of Neuroscience*, **33**, 12543-12552.

Lipscombe, D., Andrade, A. & Allen, S.E. (2013) Alternative splicing: functional diversity among voltage-gated calcium channels and behavioral consequences. *Biochimica et biophysica acta*, **1828**, 1522-1529.

Liu, B., Fan, L., Balakrishna, S., Sui, A., Morris, J.B. & Jordt, S.E. (2013) TRPM8 is the principal mediator of menthol-induced analgesia of acute and inflammatory pain. *Pain*, **154**, 2169-2177.

Liu, C.N., Wall, P.D., Ben-Dor, E., Michaelis, M., Amir, R. & Devor, M. (2000) Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. *Pain*, **85**, 503-521.

Liu, H., De Waard, M., Scott, V.E., Gurnett, C.A., Lennon, V.A. & Campbell, K.P. (1996) Identification of three subunits of the high affinity omega-conotoxin MVIIC-sensitive Ca2+ channel. *The Journal of biological chemistry*, **271**, 13804-13810.

Loken, L.S., Wessberg, J., Morrison, I., McGlone, F. & Olausson, H. (2009) Coding of pleasant touch by unmyelinated afferents in humans. *Nat Neurosci*, **12**, 547-548.

Lu, Y. & Perl, E.R. (2003) A specific inhibitory pathway between substantia gelatinosa neurons receiving direct C-fiber input. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **23**, 8752-8758.

Lu, Y., Zheng, J., Xiong, L., Zimmermann, M. & Yang, J. (2008) Spinal cord injury-induced attenuation of GABAergic inhibition in spinal dorsal horn circuits is associated with down-regulation of the chloride transporter KCC2 in rat. *J Physiol*, **586**, 5701-5715.

Luebke, J.I., Dunlap, K. & Turner, T.J. (1993) Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. *Neuron*, **11**, 895-902.

Luo, Z.D., Chaplan, S.R., Higuera, E.S., Sorkin, L.S., Stauderman, K.A., Williams, M.E. & Yaksh, T.L. (2001) Upregulation of dorsal root ganglion $\alpha 2 \delta$ calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. *The Journal of Neuroscience*, **21**, 1868-1875.

Madrid, R., de la Pena, E., Donovan-Rodriguez, T., Belmonte, C. & Viana, F. (2009) Variable threshold of trigeminal cold-thermosensitive neurons is determined by a balance between TRPM8 and Kv1 potassium channels. *The Journal of Neuroscience*, **29**, 3120-3131.

Madrid, R., Donovan-Rodriguez, T., Meseguer, V., Acosta, M.C., Belmonte, C. & Viana, F. (2006) Contribution of TRPM8 channels to cold transduction in primary sensory neurons and peripheral nerve terminals. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **26**, 12512-12525.

Magerl, W., Wilk, S.H. & Treede, R.D. (1998) Secondary hyperalgesia and perceptual wind-up following intradermal injection of capsaicin in humans. *Pain*, **74**, 257-268.

Maggi, C.A., Manzini, S., Parlani, M. & Meli, A. (1984) An analysis of the effects of urethane on cardiovascular responsiveness to catecholamines in terms of its interference with Ca++ mobilization from both intra and extracellular pools. *Experientia*, **40**, 52-59.

Maier, C., Baron, R., Tolle, T.R., Binder, A., Birbaumer, N., Birklein, F., Gierthmuhlen, J., Flor, H., Geber, C., Huge, V., Krumova, E.K., Landwehrmeyer, G.B., Magerl, W., Maihofner, C., Richter, H., Rolke, R., Scherens, A., Schwarz, A., Sommer, C., Tronnier, V., Uceyler, N., Valet, M., Wasner, G. & Treede, R.D. (2010) Quantitative sensory testing in the German Research Network on Neuropathic Pain (DFNS): Somatosensory abnormalities in 1236 patients with different neuropathic pain syndromes. *Pain*, **150**, 439-450.

Maingret, F., Fosset, M., Lesage, F., Lazdunski, M. & Honore, E. (1999) TRAAK is a mammalian neuronal mechano-gated K+ channel. *Journal of Biological Chemistry*, **274**, 1381-1387.

Maingret, F., Lauritzen, I., Patel, A.J., Heurteaux, C., Reyes, R., Lesage, F., Lazdunski, M. & Honore, E. (2000) TREK-1 is a heat-activated background K+ channel. *The EMBO journal*, **19**, 2483-2491.

Maixner, W., Dubner, R., Bushnell, M.C., Kenshalo Jr, D.R. & Oliveras, J.-L. (1986) Wide-dynamic-range dorsal horn neurons participate in the encoding process by which monkeys perceive the intensity of noxious heat stimuli. *Brain Research*, **374**, 385-388.

Mälkiä, A., Madrid, R., Meseguer, V., de la Pena, E., Valero, M., Belmonte, C. & Viana, F. (2007) Bidirectional shifts of TRPM8 channel gating by temperature and chemical agents modulate the cold sensitivity of mammalian thermoreceptors. *The Journal of physiology*, **581**, 155-174.

Mälkiä, A., Morenilla-Palao, C. & Viana, F. (2011) The emerging pharmacology of TRPM8 channels: hidden therapeutic potential underneath a cold surface. *Curr Pharm Biotechnol*, **12**, 54-67.

Malmberg, A.B., Chen, C., Tonegawa, S. & Basbaum, A.I. (1997) Preserved acute pain and reduced neuropathic pain in mice lacking PKCgamma. *Science*, **278**, 279-283.

Malmberg, A.B., Hedley, L.R., Jasper, J.R., Hunter, J.C. & Basbaum, A.I. (2001) Contribution of alpha(2) receptor subtypes to nerve injury-induced pain and its regulation by dexmedetomidine. *British journal of pharmacology*, **132**, 1827-1836.

Maneuf, Y.P. & McKnight, A.T. (2001) Block by gabapentin of the facilitation of glutamate release from rat trigeminal nucleus following activation of protein kinase C or adenylyl cyclase. *British journal of pharmacology*, **134**, 237-240.

Mantyh, P.W. (1983a) Connections of midbrain periaqueductal gray in the monkey. I. Ascending efferent projections. *J Neurophysiol*, **49**, 567-581.

Mantyh, P.W. (1983b) Connections of midbrain periaqueductal gray in the monkey. II. Descending efferent projections. *J Neurophysiol*, **49**, 582-594.

Marais, E., Klugbauer, N. & Hofmann, F. (2001) Calcium channel alpha(2)delta subunits-structure and Gabapentin binding. *Mol Pharmacol*, **59**, 1243-1248.

Marchand, F., Perretti, M. & McMahon, S.B. (2005) Role of the immune system in chronic pain. *Nature reviews. Neuroscience*, **6**, 521-532.

Marshall, G.E., Shehab, S.A., Spike, R.C. & Todd, A.J. (1996) Neurokinin-1 receptors on lumbar spinothalamic neurons in the rat. *Neuroscience*, **72**, 255-263.

Mason, P. (1997) Physiological identification of pontomedullary serotonergic neurons in the rat. *J Neurophysiol*, **77**, 1087-1098.

Matthews, E.A., Bee, L.A., Stephens, G.J. & Dickenson, A.H. (2007) The Cav2.3 calcium channel antagonist SNX-482 reduces dorsal horn neuronal responses in a rat model of chronic neuropathic pain. *The European journal of neuroscience*, **25**, 3561-3569.

Matthews, E.A. & Dickenson, A.H. (2001) Effects of spinally delivered N- and P-type voltage-dependent calcium channel antagonists on dorsal horn neuronal responses in a rat model of neuropathy. *Pain*, **92**, 235-246.

Mayer, D.J., Wolfle, T.L., Akil, H., Carder, B. & Liebeskind, J.C. (1971) Analgesia from electrical stimulation in the brainstem of the rat. *Science*, **174**, 1351-1354.

McCoy, E.S., Taylor-Blake, B., Street, S.E., Pribisko, A.L., Zheng, J. & Zylka, M.J. (2013) Peptidergic CGRPα Primary Sensory Neurons Encode Heat and Itch and Tonically Suppress Sensitivity to Cold. *Neuron*, **78**, 138-151.

McGivern, J.G. (2007) Ziconotide: a review of its pharmacology and use in the treatment of pain. *Neuropsychiatric disease and treatment*, **3**, 69-85.

McKemy, D.D. (2012) The Molecular and Cellular Basis of Cold Sensation. *ACS Chemical Neuroscience*, **4**, 238-247.

McKemy, D.D., Neuhausser, W.M. & Julius, D. (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature*, **416**, 52-58.

McMahon, S.B., Cafferty, W.B.J. & Marchand, F. (2005) Immune and glial cell factors as pain mediators and modulators. *Experimental Neurology*, **192**, 444-462.

McMullan, S. & Lumb, B.M. (2006a) Midbrain control of spinal nociception discriminates between responses evoked by myelinated and unmyelinated heat nociceptors in the rat. *Pain*, **124**, 59-68.

McMullan, S. & Lumb, B.M. (2006b) Spinal dorsal horn neuronal responses to myelinated versus unmyelinated heat nociceptors and their modulation by activation of the periaqueductal grey in the rat. *J Physiol*, **576**, 547-556.

McNamara, C.R., Mandel-Brehm, J., Bautista, D.M., Siemens, J., Deranian, K.L., Zhao, M., Hayward, N.J., Chong, J.A., Julius, D. & Moran, M.M. (2007) TRPA1 mediates formalin-induced pain. *Proceedings of the National Academy of Sciences*, **104**, 13525.

Melzack, R. & Wall, P.D. (1965) Pain mechanisms: a new theory. *Science*, **150**, 971-979.

Mendell, L.M. & Wall, P.D. (1965) RESPONSES OF SINGLE DORSAL CORD CELLS TO PERIPHERAL CUTANEOUS UNMYELINATED FIBRES. *Nature*, **206**, 97-99.

Menendez, L., Bester, H., Besson, J.M. & Bernard, J.F. (1996) Parabrachial area: electrophysiological evidence for an involvement in cold nociception. *J Neurophysiol*, **75**, 2099-2116.

Millan, M.J. (2002) Descending control of pain. *Progress in neurobiology*, **66**, 355-474.

Miller, J.F. & Proudfit, H.K. (1990) Antagonism of stimulation-produced antinociception from ventrolateral pontine sites by intrathecal administration of alpha-adrenergic antagonists and naloxone. *Brain Res*, **530**, 20-34.

Minett, M.S., Eijkelkamp, N. & Wood, J.N. (2014) Significant Determinants of Mouse Pain Behaviour. *PLoS One*, **9**, e104458.

Minett, M.S., Nassar, M.A., Clark, A.K., Passmore, G., Dickenson, A.H., Wang, F., Malcangio, M. & Wood, J.N. (2012) Distinct Nav1.7-dependent pain sensations require different sets of sensory and sympathetic neurons. *Nat Commun*, **3**, 791.

Mintz, I.M., Venema, V.J., Swiderek, K.M., Lee, T.D., Bean, B.P. & Adams, M.E. (1992) P-type calcium channels blocked by the spider toxin omega-Aga-IVA. *Nature*, **355**, 827-829.

Miraucourt, L.S., Dallel, R. & Voisin, D.L. (2007) Glycine inhibitory dysfunction turns touch into pain through PKCgamma interneurons. *PLoS One*, **2**, e1116.

Mishra, S.K., Tisel, S.M., Orestes, P., Bhangoo, S.K. & Hoon, M.A. (2011) TRPV1-lineage neurons are required for thermal sensation. *The EMBO journal*, **30**, 582-593.

Miyazaki, R. & Yamamoto, T. (2012) The efficacy of morphine, pregabalin, gabapentin, and duloxetine on mechanical allodynia is different from that on neuroma pain in the rat neuropathic pain model. *Anesthesia and analgesia*, **115**, 182-188.

Moga, M.M., Herbert, H., Hurley, K.M., Yasui, Y., Gray, T.S. & Saper, C.B. (1990) Organization of cortical, basal forebrain, and hypothalamic afferents to the parabrachial nucleus in the rat. *The Journal of comparative neurology*, **295**, 624-661.

Mohr, C., Leyendecker, S., Mangels, I., Machner, B., Sander, T. & Helmchen, C. (2008) Central representation of cold-evoked pain relief in capsaicin induced pain: an event-related fMRI study. *Pain*, **139**, 416-430.

Moisset, X. & Bouhassira, D. (2007) Brain imaging of neuropathic pain. *NeuroImage*, **37**, S80-S88.

Molderings, G., Likungu, J. & Göthert, M. (2000) N-type calcium channels control sympathetic neurotransmission in human heart atrium. *Circulation*, **101**, 403-407.

Moore, K.A., Kohno, T., Karchewski, L.A., Scholz, J., Baba, H. & Woolf, C.J. (2002) Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **22**, 6724-6731.

Moore, R.A., Straube, S., Wiffen, P.J., Derry, S. & McQuay, H.J. (2009) Pregabalin for acute and chronic pain in adults. *The Cochrane database of systematic reviews*, Cd007076.

Moore, R.A., Wiffen, P.J., Derry, S. & McQuay, H.J. (2011) Gabapentin for chronic neuropathic pain and fibromyalgia in adults. *The Cochrane database of systematic reviews*, Cd007938.

Morenilla-Palao, C., Luis, E., Fernandez-Pena, C., Quintero, E., Weaver, J.L., Bayliss, D.A. & Viana, F. (2014) Ion Channel Profile of TRPM8 Cold Receptors Reveals a Role of TASK-3 Potassium Channels in Thermosensation. *Cell reports*, **8**, 1571-1582.

Morin, C. & Bushnell, M. (1998) Temporal and qualitative properties of cold pain and heat pain: a psychophysical study. *Pain*, **74**, 67-73.

Morin, C., Bushnell, M.C., Luskin, M.B. & Craig, A.D. (2002) Disruption of thermal perception in a multiple sclerosis patient with central pain. *The Clinical journal of pain*, **18**, 191-195.

Morisset, V. & Nagy, F. (1999) Ionic basis for plateau potentials in deep dorsal horn neurons of the rat spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **19**, 7309-7316.

Munns, C., AlQatari, M. & Koltzenburg, M. (2007) Many cold sensitive peripheral neurons of the mouse do not express TRPM8 or TRPA1. *Cell Calcium*, **41**, 331-342.

Murakami, M., Nakagawasai, O., Suzuki, T., Mobarakeh, II, Sakurada, Y., Murata, A., Yamadera, F., Miyoshi, I., Yanai, K., Tan-No, K., Sasano, H., Tadano, T. & Iijima, T. (2004) Antinociceptive effect of different types of calcium channel inhibitors and the distribution of various calcium channel alpha 1 subunits in the dorsal horn of spinal cord in mice. *Brain Res*, **1024**, 122-129.

Nagata, K., Duggan, A., Kumar, G. & Garc√≠a-A√±overos, J. (2005) Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing. *The Journal of Neuroscience*, **25**, 4052-4061.

Namer, B., Kleggetveit, I.P., Handwerker, H., Schmelz, M. & Jorum, E. (2008) Role of TRPM8 and TRPA1 for cold allodynia in patients with cold injury. *Pain*, **139**, 63-72.

Nasca, C., Orlando, R., Marchiafava, M., Boldrini, P., Battaglia, G., Scaccianoce, S., Matrisciano, F., Pittaluga, A. & Nicoletti, F. (2013) Exposure to predator odor and resulting anxiety enhances the expression of the alpha2 delta subunit of voltage-sensitive calcium channels in the amygdala. *Journal of neurochemistry*, **125**, 649-656.

Neely, G.G., Hess, A., Costigan, M., Keene, A.C., Goulas, S., Langeslag, M., Griffin, R.S., Belfer, I., Dai, F., Smith, S.B., Diatchenko, L., Gupta, V., Xia, C.P., Amann, S., Kreitz, S., Heindl-Erdmann, C., Wolz, S., Ly, C.V., Arora, S., Sarangi, R., Dan, D., Novatchkova, M., Rosenzweig, M., Gibson, D.G., Truong, D., Schramek, D., Zoranovic, T., Cronin, S.J., Angjeli, B., Brune, K., Dietzl, G., Maixner, W., Meixner, A., Thomas, W., Pospisilik, J.A., Alenius, M., Kress, M., Subramaniam, S., Garrity, P.A., Bellen, H.J., Woolf, C.J. & Penninger, J.M. (2010) A genome-wide Drosophila screen for heat nociception identifies alpha2delta3 as an evolutionarily conserved pain gene. *Cell*, **143**, 628-638.

Neugebauer, V., Li, W., Bird, G.C. & Han, J.S. (2004) The amygdala and persistent pain. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry*, **10**, 221-234.

Neumann, S., Braz, J.M., Skinner, K., Llewellyn-Smith, I.J. & Basbaum, A.I. (2008) Innocuous, not noxious, input activates PKCgamma interneurons of the spinal dorsal horn via myelinated afferent fibers. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **28**, 7936-7944.

Neumann, S., Doubell, T.P., Leslie, T. & Woolf, C.J. (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. *Nature*, **384**, 360-364.

Newcomb, R., Szoke, B., Palma, A., Wang, G., Chen, X., Hopkins, W., Cong, R., Miller, J., Urge, L., Tarczy-Hornoch, K., Loo, J.A., Dooley, D.J., Nadasdi, L., Tsien, R.W., Lemos, J. & Miljanich, G. (1998) Selective peptide antagonist of the class E calcium channel from the venom of the tarantula Hysterocrates gigas. *Biochemistry*, **37**, 15353-15362.

Nguyen, D., Deng, P., Matthews, E.A., Kim, D.S., Feng, G., Dickenson, A.H., Xu, Z.C. & Luo, Z.D. (2009) Enhanced pre-synaptic glutamate release in deep-dorsal horn contributes to calcium channel alpha-2-delta-1 protein-mediated spinal sensitization and behavioral hypersensitivity. *Mol Pain*, **5**, 6.

Nicholson, B. & Verma, S. (2004) Comorbidities in chronic neuropathic pain. *Pain medicine (Malden, Mass.)*, **5 Suppl 1**, S9-s27.

Niesters, M., Proto, P.L., Aarts, L., Sarton, E.Y., Drewes, A.M. & Dahan, A. (2014) Tapentadol potentiates descending pain inhibition in chronic pain patients with diabetic polyneuropathy. *British journal of anaesthesia*, **113**, 148-156.

Noël, J., Zimmermann, K., Busserolles, J., Deval, E., Alloui, A., Diochot, S., Guy, N., Borsotto, M., Reeh, P. & Eschalier, A. (2009) The mechano-activated K+; channels TRAAK and TREK-1 control both warm and cold perception. *The EMBO journal*, **28**, 1308-1318.

Nowycky, M.C., Fox, A.P. & Tsien, R.W. (1985) Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature*, **316**, 440-443.

O'Neill, J., Brock, C., Olesen, A.E., Andresen, T., Nilsson, M. & Dickenson, A.H. (2012) Unravelling the Mystery of Capsaicin: A Tool to Understand and Treat Pain. *Pharmacological Reviews*, **64**, 939-971.

Obata, K., Katsura, H., Mizushima, T., Yamanaka, H., Kobayashi, K., Dai, Y., Fukuoka, T., Tokunaga, A., Tominaga, M. & Noguchi, K. (2005) TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. *Journal of Clinical Investigation*, **115**, 2393.

Ochoa, J.L. & Yarnitsky, D. (1994) The triple cold syndrome: Cold hyperalgesia, cold hypoaesthesia and cold skin in peripheral nerve disease. *Brain*, **117**, 185-197.

Olave, M.J. & Maxwell, D.J. (2003) Neurokinin-1 projection cells in the rat dorsal horn receive synaptic contacts from axons that possess alpha2C-adrenergic receptors. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **23**, 6837-6846.

Olsen, R.V., Andersen, H.H., Moller, H.G., Eskelund, P.W. & Arendt-Nielsen, L. (2014) Somatosensory and vasomotor manifestations of individual and combined stimulation of TRPM8 and TRPA1 using topical L-menthol and trans-cinnamaldehyde in healthy volunteers. *European journal of pain (London, England)*.

Omote, K., Kawamata, M., Satoh, O., Iwasaki, H. & Namiki, A. (1996) Spinal antinociceptive action of an N-Type voltage-dependent calcium channel blocker and the synergistic interaction with morphine. *Anesthesiology*, **84**, 636-643.

Orio, P., Madrid, R., de la Pena, E., Parra, A., Meseguer, V., Bayliss, D.A., Belmonte, C. & Viana, F. (2009) Characteristics and physiological role of hyperpolarization activated currents in mouse cold thermoreceptors. *J Physiol*, **587**, 1961-1976.

Orio, P., Parra, A., Madrid, R., Gonzalez, O., Belmonte, C. & Viana, F. (2012) Role of Ih in the firing pattern of mammalian cold thermoreceptor endings. *J Neurophysiol*, **108**, 3009-3023.

Ossipov, M.H., Hong Sun, T., Malan, P., Jr., Lai, J. & Porreca, F. (2000) Mediation of spinal nerve injury induced tactile allodynia by descending facilitatory pathways in the dorsolateral funiculus in rats. *Neurosci Lett*, **290**, 129-132.

Pachman, D.R., Barton, D.L., Watson, J.C. & Loprinzi, C.L. (2011) Chemotherapy-Induced Peripheral Neuropathy: Prevention and Treatment. *Clin Pharmacol Ther*, **90**, 377-387.

Pan, R., Tian, Y., Gao, R., Li, H., Zhao, X., Barrett, J.E. & Hu, H. (2012) Central mechanisms of menthol-induced analgesia. *The Journal of pharmacology and experimental therapeutics*, **343**, 661-672.

Parry, D.M., Macmillan, F.M., Koutsikou, S., McMullan, S. & Lumb, B.M. (2008) Separation of A- versus C-nociceptive inputs into spinal-brainstem circuits. *Neuroscience*, **152**, 1076-1085.

Passmore, G.M., Reilly, J.M., Thakur, M., Keasberry, V.N., Marsh, S.J., Dickenson, A.H. & Brown, D.A. (2012) Functional significance of M-type potassium channels in nociceptive cutaneous sensory endings. *Frontiers in molecular neuroscience*, **5**, 63.

Patel, R., Bauer, C.S., Nieto-Rostro, M., Margas, W., Ferron, L., Chaggar, K., Crews, K., Ramirez, J.D., Bennett, D.L.H., Schwartz, A., Dickenson, A.H. & Dolphin, A.C. (2013) $\alpha 2\delta$-1 Gene Deletion Affects Somatosensory Neuron Function and Delays Mechanical Hypersensitivity in Response to Peripheral Nerve Damage. *The Journal of Neuroscience*, **33**, 16412-16426.

Patel, R., Goncalves, L., Leveridge, M., Mack, S.R., Hendrick, A., Brice, N.L. & Dickenson, A.H. (2014a) Anti-hyperalgesic effects of a novel TRPM8 agonist in neuropathic rats: A comparison with topical menthol. *Pain*, **155**, 2097-2107.

Patel, R., Goncalves, L., Newman, R., Jiang, F.L., Goldby, A., Reeve, J., Hendrick, A., Teall, M., Hannah, D., Almond, S., Brice, N. & Dickenson, A.H. (2014b) Novel TRPM8 Antagonist Attenuates Cold Hypersensitivity after Peripheral Nerve Injury in Rats. *The Journal of pharmacology and experimental therapeutics*, **349**, 47-55.

Patil, P.G., de Leon, M., Reed, R.R., Dubel, S., Snutch, T.P. & Yue, D.T. (1996) Elementary events underlying voltage-dependent G-protein inhibition of N-type calcium channels. *Biophysical Journal*, **71**, 2509-2521.

Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P. & Bevan, S. (2002) A TRP channel that senses cold stimuli and menthol. *Cell*, **108**, 705-715.

Percie du Sert, N. & Rice, A.S. (2014) Improving the translation of analgesic drugs to the clinic: animal models of neuropathic pain. *British journal of pharmacology*, **171**, 2951-2963.

Pereira, V., Busserolles, J., Christin, M., Devilliers, M., Poupon, L., Legha, W., Alloui, A., Aissouni, Y., Bourinet, E., Lesage, F., Eschalier, A., Lazdunski, M. & Noël, J. (2014) Role of the TREK2 potassium channel in cold and warm thermosensation and in pain perception. *PAIN®*.

Perl, E.R. (2007) Ideas about pain, a historical view. *Nature reviews. Neuroscience*, **8**, 71-80.

Pertovaara, A. (2006) Noradrenergic pain modulation. *Prog Neurobiol*, **80**, 53-83.

Pertovaara, A., Wei, H. & Hamalainen, M.M. (1996) Lidocaine in the rostroventromedial medulla and the periaqueductal gray attenuates allodynia in neuropathic rats. *Neurosci Lett*, **218**, 127-130.

Pertusa, M., Madrid, R., Morenilla-Palao, C., Belmonte, C. & Viana, F. (2012) The N-glycosylation of TRPM8 channels modulates the temperature sensitivity of cold-thermoreceptor neurons. *Journal of Biological Chemistry*.

Pezet, S., Marchand, F., D'Mello, R., Grist, J., Clark, A.K., Malcangio, M., Dickenson, A.H., Williams, R.J. & McMahon, S.B. (2008) Phosphatidylinositol 3-kinase is a key mediator of central sensitization in painful inflammatory conditions. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **28**, 4261-4270.

Pinto, V., Szûcs, P., Derkach, V.A. & Safronov, B.V. (2008) Monosynaptic convergence of C- and A δ -afferent fibres from different segmental dorsal roots on to single substantia gelatinosa neurones in the rat spinal cord. *The Journal of Physiology*, **586**, 4165-4177.

Pirone, A., Kurt, S., Zuccotti, A., Ruttiger, L., Pilz, P., Brown, D.H., Franz, C., Schweizer, M., Rust, M.B., Rubsamen, R., Friauf, E., Knipper, M. & Engel, J. (2014) alpha2delta3 is essential for normal structure and function of auditory nerve synapses and is a novel candidate for auditory processing disorders. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **34**, 434-445.

Polgár, E., Fowler, J.H., McGill, M.M. & Todd, A.J. (1999) The types of neuron which contain protein kinase C gamma in rat spinal cord. *Brain Res*, **833**, 71-80.

Polgár, E., Gray, S., Riddell, J.S. & Todd, A.J. (2004) Lack of evidence for significant neuronal loss in laminae I–III of the spinal dorsal horn of the rat in the chronic constriction injury model. *Pain*, **111**, 144-150.

Polgár, E., Hughes, D.I., Riddell, J.S., Maxwell, D.J., Puskar, Z. & Todd, A.J. (2003) Selective loss of spinal GABAergic or glycinergic neurons is not necessary for development of thermal hyperalgesia in the chronic constriction injury model of neuropathic pain. *Pain*, **104**, 229-239.

Porreca, F., Burgess, S.E., Gardell, L.R., Vanderah, T.W., Malan, T.P., Jr., Ossipov, M.H., Lappi, D.A. & Lai, J. (2001) Inhibition of neuropathic pain by selective ablation of brainstem medullary cells expressing the mu-opioid receptor. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **21**, 5281-5288.

Premkumar, L.S., Raisinghani, M., Pingle, S.C., Long, C. & Pimentel, F. (2005) Downregulation of transient receptor potential melastatin 8 by protein kinase C-mediated dephosphorylation. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **25**, 11322-11329.

Price, D.D., Greenspan, J.D. & Dubner, R. (2003) Neurons involved in the exteroceptive function of pain. *Pain*, **106**, 215-219.

Price, D.D., Mao, J., Frenk, H. & Mayer, D.J. (1994) The N-methyl-D-aspartate receptor antagonist dextromethorphan selectively reduces temporal summation of second pain in man. *Pain*, **59**, 165-174.

Price, D.D., Staud, R., Robinson, M.E., Mauderli, A.P., Cannon, R. & Vierck, C.J. (2002) Enhanced temporal summation of second pain and its central modulation in fibromyalgia patients. *Pain*, **99**, 49-59.

Priestley, J.V., Michael, G.J., Averill, S., Liu, M. & Willmott, N. (2002) Regulation of nociceptive neurons by nerve growth factor and glial cell line derived neurotrophic factor. *Canadian journal of physiology and pharmacology*, **80**, 495-505.

Proudfoot, C.J., Garry, E.M., Cottrell, D.F., Rosie, R., Anderson, H., Robertson, D.C., Fleetwood-Walker, S.M. & Mitchell, R. (2006) Analgesia mediated by the TRPM8 cold receptor in chronic neuropathic pain. *Current biology : CB*, **16**, 1591-1605.

Puskar, Z., Polgar, E. & Todd, A.J. (2001) A population of large lamina I projection neurons with selective inhibitory input in rat spinal cord. *Neuroscience*, **102**, 167-176.

Qin, N., Yagel, S., Momplaisir, M.L., Codd, E.E. & D'Andrea, M.R. (2002) Molecular cloning and characterization of the human voltage-gated calcium channel alpha(2)delta-4 subunit. *Mol Pharmacol*, **62**, 485-496.

Quevedo, A.S. & Coghill, R.C. (2009) Filling-in, spatial summation, and radiation of pain: evidence for a neural population code in the nociceptive system. *J Neurophysiol*, **102**, 3544-3553.

Quick, K., Zhao, J., Eijkelkamp, N., Linley, J.E., Rugiero, F., Cox, J.J., Raouf, R., Gringhuis, M., Sexton, J.E. & Abramowitz, J. (2012) TRPC3 and TRPC6 are essential for normal mechanotransduction in subsets of sensory neurons and cochlear hair cells. *Open biology*, **2**.

Quintero, J.E., Dooley, D.J., Pomerleau, F., Huettl, P. & Gerhardt, G.A. (2011) Amperometric measurement of glutamate release modulation by gabapentin and pregabalin in rat neocortical slices: role of voltage-sensitive Ca2+ alpha2delta-1 subunit. *The Journal of pharmacology and experimental therapeutics*, **338**, 240-245.

Raghavendra, V., Tanga, F. & DeLeo, J.A. (2003) Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *The Journal of pharmacology and experimental therapeutics*, **306**, 624-630.

Rahman, W., Bauer, C.S., Bannister, K., Vonsy, J.L., Dolphin, A.C. & Dickenson, A.H. (2009) Descending serotonergic facilitation and the antinociceptive effects of pregabalin in a rat model of osteoarthritic pain. *Mol Pain*, **5**, 45.

Rahman, W., D'Mello, R. & Dickenson, A.H. (2008) Peripheral Nerve Injury–Induced Changes in Spinal α 2-Adrenoceptor–Mediated Modulation of Mechanically Evoked Dorsal Horn Neuronal Responses. *The Journal of Pain*, **9**, 350-359.

Rahman, W., Suzuki, R., Webber, M., Hunt, S.P. & Dickenson, A.H. (2006) Depletion of endogenous spinal 5-HT attenuates the behavioural hypersensitivity to mechanical and cooling stimuli induced by spinal nerve ligation. *Pain*, **123**, 264-274.

Rajaofetra, N., Ridet, J.L., Poulat, P., Marlier, L., Sandillon, F., Geffard, M. & Privat, A. (1992) Immunocytochemical mapping of noradrenergic projections to the rat spinal cord with an antiserum against noradrenaline. *Journal of neurocytology*, **21**, 481-494.

Reynolds, D.V. (1969) Surgery in the rat during electrical analgesia induced by focal brain stimulation. *Science*, **164**, 444-445.

Reynolds, I.J., Wagner, J.A., Snyder, S.H., Thayer, S.A., Olivera, B.M. & Miller, R.J. (1986) Brain voltage-sensitive calcium channel subtypes differentiated by omega-conotoxin fraction GVIA. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 8804-8807.

Rigaud, M., Gemes, G., Barabas, M.E., Chernoff, D.I., Abram, S.E., Stucky, C.L. & Hogan, Q.H. (2008) Species and strain differences in rodent sciatic nerve anatomy: implications for studies of neuropathic pain. *Pain*, **136**, 188-201.

Saegusa, H., Kurihara, T., Zong, S., Kazuno, A., Matsuda, Y., Nonaka, T., Han, W., Toriyama, H. & Tanabe, T. (2001) Suppression of inflammatory and neuropathic pain symptoms in mice lacking the N-type Ca2+ channel. *The EMBO journal*, **20**, 2349-2356.

Saegusa, H., Kurihara, T., Zong, S., Minowa, O., Kazuno, A., Han, W., Matsuda, Y., Yamanaka, H., Osanai, M., Noda, T. & Tanabe, T. (2000) Altered pain responses in mice lacking alpha 1E subunit of the voltage-dependent Ca2+ channel. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 6132-6137.

Sandkuhler, J. (2009) Models and mechanisms of hyperalgesia and allodynia. *Physiological reviews*, **89**, 707-758.

Santicioli, P., Del Bianco, E., Tramontana, M., Geppetti, P. & Maggi, C.A. (1992) Release of calcitonin gene-related peptide like-immunoreactivity induced by electrical field stimulation from rat spinal afferents is mediated by conotoxin-sensitive calcium channels. *Neurosci Lett*, **136**, 161-164.

Schafer, K. & Braun, H.A. (1990) Modulation of periodic cold receptor activity by ouabain. *Pflugers Archiv : European journal of physiology*, **417**, 91-99.

Schafer, K., Braun, H.A. & Isenberg, C. (1986) Effect of menthol on cold receptor activity. Analysis of receptor processes. *The Journal of general physiology*, **88**, 757-776.

Schmidt, R., Schmelz, M., Forster, C., Ringkamp, M., Torebjork, E. & Handwerker, H. (1995) Novel classes of responsive and unresponsive C nociceptors in human skin. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **15**, 333-341.

Scholz, J., Broom, D.C., Youn, D.H., Mills, C.D., Kohno, T., Suter, M.R., Moore, K.A., Decosterd, I., Coggeshall, R.E. & Woolf, C.J. (2005) Blocking caspase activity prevents transsynaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **25**, 7317-7323.

Schwartz, E.S., Lee, I., Chung, K. & Chung, J.M. (2008) Oxidative stress in the spinal cord is an important contributor in capsaicin-induced mechanical secondary hyperalgesia in mice. *PAIN*, **138**, 514-524.

Seagrove, L.C., Suzuki, R. & Dickenson, A.H. (2004) Electrophysiological characterisations of rat lamina I dorsal horn neurones and the involvement of excitatory amino acid receptors. *Pain*, **108**, 76-87.

Seifert, F. & Maihofner, C. (2007) Representation of cold allodynia in the human brain--a functional MRI study. *NeuroImage*, **35**, 1168-1180.

Seltzer, Z., Dubner, R. & Shir, Y. (1990) A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain*, **43**, 205-218.

Serra, J., Sola, R., Quiles, C., Casanova-Molla, J., Pascual, V., Bostock, H. & Valls-Sole, J. (2009) C-nociceptors sensitized to cold in a patient with small-fiber neuropathy and cold allodynia. *Pain*, **147**, 46-53.

Shigetomi, E., Jackson-Weaver, O., Huckstepp, R.T., O'Dell, T.J. & Khakh, B.S. (2013) TRPA1 channels are regulators of astrocyte basal calcium levels and long-term potentiation via constitutive D-serine release. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **33**, 10143-10153.

Sikandar, S., Bannister, K. & Dickenson, A.H. (2012) Brainstem facilitations and descending serotonergic controls contribute to visceral nociception but not pregabalin analgesia in rats. *Neurosci Lett*, **519**, 31-36.

Sikandar, S. & Dickenson, A.H. (2011) Pregabalin modulation of spinal and brainstem visceral nociceptive processing. *Pain*, **152**, 2312-2322.

Sikandar, S. & Dickenson, A.H. (2013) II. No need for translation when the same language is spoken. *British journal of anaesthesia*, **111**, 3-6.

Sikandar, S., Patel, R., Patel, S., Sikander, S., Bennett, D.L. & Dickenson, A.H. (2013a) Genes, molecules and patients--emerging topics to guide clinical pain research. *Eur J Pharmacol*, **716**, 188-202.

Sikandar, S., Ronga, I., Iannetti, G.D. & Dickenson, A.H. (2013b) Neural coding of nociceptive stimuli-from rat spinal neurones to human perception. *Pain*, **154**, 1263-1273.

Silverman, J.D. & Kruger, L. (1988a) Acid phosphatase as a selective marker for a class of small sensory ganglion cells in several mammals: spinal cord distribution, histochemical properties, and relation to fluoride-resistant acid phosphatase (FRAP) of rodents. *Somatosensory research*, **5**, 219-246.

Silverman, J.D. & Kruger, L. (1988b) Lectin and neuropeptide labeling of separate populations of dorsal root ganglion neurons and associated "nociceptor" thin axons in rat testis and cornea whole-mount preparations. *Somatosensory research*, **5**, 259-267.

Simone, D.A. & Kajander, K.C. (1996) Excitation of rat cutaneous nociceptors by noxious cold. *Neuroscience Letters*, **213**, 53-56.

Simone, D.A. & Kajander, K.C. (1997) Responses of Cutaneous A-Fiber Nociceptors to Noxious Cold. *Journal of neurophysiology*, **77**, 2049-2060.

Simone, D.A., Sorkin, L.S., Oh, U., Chung, J.M., Owens, C., LaMotte, R.H. & Willis, W.D. (1991) Neurogenic hyperalgesia: central neural correlates in responses of spinothalamic tract neurons. *J Neurophysiol*, **66**, 228-246.

Sluka, K.A. (1997) Blockade of calcium channels can prevent the onset of secondary hyperalgesia and allodynia induced by intradermal injection of capsaicin in rats. *Pain*, **71**, 157-164.

Sluka, K.A. (1998) Blockade of N- and P/Q-type calcium channels reduces the secondary heat hyperalgesia induced by acute inflammation. *The Journal of pharmacology and experimental therapeutics*, **287**, 232-237.

Snider, W.D. & McMahon, S.B. (1998) Tackling pain at the source: new ideas about nociceptors. *Neuron*, **20**, 629-632.

Sommer, C. & Schafers, M. (1998) Painful mononeuropathy in C57BL/Wld mice with delayed wallerian degeneration: differential effects of cytokine production and nerve regeneration on thermal and mechanical hypersensitivity. *Brain Res*, **784**, 154-162.

Sonohata, M., Furue, H., Katafuchi, T., Yasaka, T., Doi, A., Kumamoto, E. & Yoshimura, M. (2004) Actions of noradrenaline on substantia gelatinosa neurones in the rat spinal cord revealed by in vivo patch recording. *J Physiol*, **555**, 515-526.

South, S.M., Kohno, T., Kaspar, B.K., Hegarty, D., Vissel, B., Drake, C.T., Ohata, M., Jenab, S., Sailer, A.W., Malkmus, S., Masuyama, T., Horner, P., Bogulavsky, J., Gage, F.H., Yaksh, T.L., Woolf, C.J., Heinemann, S.F. & Inturrisi, C.E. (2003) A conditional deletion of the NR1 subunit of the NMDA receptor in adult spinal cord dorsal horn reduces NMDA currents and injury-induced pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **23**, 5031-5040.

Spike, R.C., Puskar, Z., Andrew, D. & Todd, A.J. (2003) A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. *The European journal of neuroscience*, **18**, 2433-2448.

Stefani, A., Spadoni, F. & Bernardi, G. (1998) Gabapentin inhibits calcium currents in isolated rat brain neurons. *Neuropharmacology*, **37**, 83-91.

Stephens, G.J. & Mochida, S. (2005) G protein $\beta\ \gamma$ subunits mediate presynaptic inhibition of transmitter release from rat superior cervical ganglion neurones in culture. *The Journal of physiology*, **563**, 765-776.

Stewart, A.P., Egressy, K., Lim, A. & Edwardson, J.M. (2010) AFM imaging reveals the tetrameric structure of the TRPM8 channel. *Biochemical and biophysical research communications*, **394**, 383-386.

Stone, L.S., Broberger, C., Vulchanova, L., Wilcox, G.L., Hokfelt, T., Riedl, M.S. & Elde, R. (1998) Differential distribution of alpha2A and alpha2C adrenergic receptor immunoreactivity in the rat spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **18**, 5928-5937.

Stone, L.S., Vulchanova, L., Riedl, M.S., Wang, J., Williams, F.G., Wilcox, G.L. & Elde, R. (1999) Effects of peripheral nerve injury on alpha-2A and alpha-2C adrenergic receptor immunoreactivity in the rat spinal cord. *Neuroscience*, **93**, 1399-1407.

Story, G.M., Peier, A.M., Reeve, A.J., Eid, S.R., Mosbacher, J., Hricik, T.R., Earley, T.J., Hergarden, A.C., Andersson, D.A. & Hwang, S.W. (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*, **112**, 819-829.

Strachan, L.C., Lewis, R.J. & Nicholson, G.M. (1999) Differential actions of pacific ciguatoxin-1 on sodium channel subtypes in mammalian sensory neurons. *The Journal of pharmacology and experimental therapeutics*, **288**, 379-388.

Sumracki, N.M., Buisman-Pijlman, F.T., Hutchinson, M.R., Gentgall, M. & Rolan, P. (2014) Reduced response to the thermal grill illusion in chronic pain patients. *Pain medicine (Malden, Mass.)*, **15**, 647-660.

Sun, Q., Tu, H., Xing, G.G., Han, J.S. & Wan, Y. (2005) Ectopic discharges from injured nerve fibers are highly correlated with tactile allodynia only in early, but not late, stage in rats with spinal nerve ligation. *Exp Neurol*, **191**, 128-136.

Sun, Y.G., Zhao, Z.Q., Meng, X.L., Yin, J., Liu, X.Y. & Chen, Z.F. (2009) Cellular basis of itch sensation. *Science*, **325**, 1531-1534.

Sutton, K.G., Martin, D.J., Pinnock, R.D., Lee, K. & Scott, R.H. (2002) Gabapentin inhibits high-threshold calcium channel currents in cultured rat dorsal root ganglion neurones. *British journal of pharmacology*, **135**, 257-265.

Suzuki, R., Green, G.M., Millan, M.J. & Dickenson, A.H. (2002a) Electrophysiologic characterization of the antinociceptive actions of S18616, a novel and potent alpha 2-adrenoceptor agonist, after acute and persistent pain states. *The journal of pain : official journal of the American Pain Society*, **3**, 234-243.

Suzuki, R., Hunt, S.P. & Dickenson, A.H. (2003) The coding of noxious mechanical and thermal stimuli of deep dorsal horn neurones is attenuated in NK1 knockout mice. *Neuropharmacology*, **45**, 1093-1100.

Suzuki, R., Kontinen, V.K., Matthews, E., Williams, E. & Dickenson, A.H. (2000) Enlargement of the Receptive Field Size to Low Intensity Mechanical Stimulation in the Rat Spinal Nerve Ligation Model of Neuropathy. *Experimental neurology*, **163**, 408-413.

Suzuki, R., Matthews, E.A. & Dickenson, A.H. (2001) Comparison of the effects of MK-801, ketamine and memantine on responses of spinal dorsal horn neurones in a rat model of mononeuropathy. *Pain*, **91**, 101-109.

Suzuki, R., Morcuende, S., Webber, M., Hunt, S.P. & Dickenson, A.H. (2002b) Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. *Nat Neurosci*, **5**, 1319-1326.

Suzuki, R., Rahman, W., Hunt, S.P. & Dickenson, A.H. (2004) Descending facilitatory control of mechanically evoked responses is enhanced in deep dorsal horn neurones following peripheral nerve injury. *Brain research*, **1019**, 68-76.

Suzuki, R., Rahman, W., Rygh, L.J., Webber, M., Hunt, S.P. & Dickenson, A.H. (2005) Spinal-supraspinal serotonergic circuits regulating neuropathic pain and its treatment with gabapentin. *Pain*, **117**, 292-303.

Swensen, A.M., Herrington, J., Bugianesi, R.M., Dai, G., Haedo, R.J., Ratliff, K.S., Smith, M.M., Warren, V.A., Arneric, S.P. & Eduljee, C. (2012) Characterization of the Substituted N-Triazole Oxindole TROX-1, a Small-Molecule, State-Dependent Inhibitor of Cav2 Calcium Channels. *Molecular pharmacology*, **81**, 488-497.

Takahashi, M., Seagar, M.J., Jones, J.F., Reber, B.F. & Catterall, W.A. (1987) Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 5478-5482.

Takahashi, T., Kajikawa, Y. & Tsujimoto, T. (1998) G-Protein-coupled modulation of presynaptic calcium currents and transmitter release by a GABAB receptor. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **18**, 3138-3146.

Takashima, Y., Daniels, R.L., Knowlton, W., Teng, J., Liman, E.R. & McKemy, D.D. (2007) Diversity in the neural circuitry of cold sensing revealed by genetic axonal labeling of transient receptor potential melastatin 8 neurons. *The Journal of Neuroscience*, **27**, 14147-14157.

Takasu, K., Ono, H. & Tanabe, M. (2008) Gabapentin produces PKA-dependent pre-synaptic inhibition of GABAergic synaptic transmission in LC neurons following partial nerve injury in mice. *Journal of neurochemistry*, **105**, 933-942.

Takazawa, T. & MacDermott, A.B. (2010) Glycinergic and GABAergic tonic inhibition fine tune inhibitory control in regionally distinct subpopulations of dorsal horn neurons. *J Physiol*, **588**, 2571-2587.

Takeuchi, Y., Takasu, K., Ono, H. & Tanabe, M. (2007) Pregabalin, S-(+)-3-isobutylgaba, activates the descending noradrenergic system to alleviate neuropathic pain in the mouse partial sciatic nerve ligation model. *Neuropharmacology*, **53**, 842-853.

Tanabe, M., Takasu, K., Kasuya, N., Shimizu, S., Honda, M. & Ono, H. (2005) Role of descending noradrenergic system and spinal alpha2-adrenergic receptors in the effects of gabapentin on thermal and mechanical nociception after partial nerve injury in the mouse. *British journal of pharmacology*, **144**, 703-714.

Tang, Z., Kim, A., Masuch, T., Park, K., Weng, H., Wetzel, C. & Dong, X. (2013) Pirt functions as an endogenous regulator of TRPM8. *Nat Commun*, **4**, 2179.

Taylor, B.K., Abhyankar, S.S., Vo, N.-T.T., Kriedt, C.L., Churi, S.B. & Urban, J.H. (2007) Neuropeptide Y acts at Y1 receptors in the rostral ventral medulla to inhibit neuropathic pain. *Pain*, **131**, 83-95.

Taylor, C.P. & Garrido, R. (2008) Immunostaining of rat brain, spinal cord, sensory neurons and skeletal muscle for calcium channel alpha2-delta (alpha2-delta) type 1 protein. *Neuroscience*, **155**, 510-521.

Taylor, R., Jr., Gan, T.J., Raffa, R.B., Gharibo, C., Pappagallo, M., Sinclair, N.R., Fleischer, C. & Tabor, A. (2012) A randomized, double-blind comparison shows the addition of oxygenated glycerol triesters to topical mentholated cream for the treatment of acute musculoskeletal pain demonstrates incremental benefit over time. *Pain practice : the official journal of World Institute of Pain*, **12**, 610-619.

Terashima, T., Xu, Q., Yamaguchi, S. & Yaksh, T.L. (2013) Intrathecal P/Q- and R-type calcium channel blockade of spinal substance P release and c-Fos expression. *Neuropharmacology*, **75c**, 1-8.

Thakur, M., Dickenson, A.H. & Baron, R. (2014) Osteoarthritis pain: nociceptive or neuropathic? *Nature reviews. Rheumatology*, **10**, 374-380.

Todd, A.J. (2002) Anatomy of primary afferents and projection neurones in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor. *Experimental physiology*, **87**, 245-249.

Torebjork, H.E., Lundberg, L.E. & LaMotte, R.H. (1992) Central changes in processing of mechanoreceptive input in capsaicin-induced secondary hyperalgesia in humans. *J Physiol*, **448**, 765-780.

Tracey, I. & Mantyh, P.W. (2007) The Cerebral Signature for Pain Perception and Its Modulation. *Neuron*, **55**, 377-391.

Tran-Van-Minh, A. & Dolphin, A.C. (2010) The alpha2delta ligand gabapentin inhibits the Rab11-dependent recycling of the calcium channel subunit alpha2delta-2. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **30**, 12856-12867.

Treede, R.D., Meyer, R.A. & Campbell, J.N. (1998) Myelinated mechanically insensitive afferents from monkey hairy skin: heat-response properties. *J Neurophysiol*, **80**, 1082-1093.

Turner, T.J., Adams, M.E. & Dunlap, K. (1993) Multiple Ca2+ channel types coexist to regulate synaptosomal neurotransmitter release. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 9518-9522.

Urban, M.O., Ren, K., Sablad, M. & Park, K.T. (2005) Medullary N-type and P/Q-type calcium channels contribute to neuropathy-induced allodynia. *Neuroreport*, **16**, 563-566.

Urch, C. & Dickenson, A. (2003) In vivo single unit extracellular recordings from spinal cord neurones of rats. *Brain research protocols*, **12**, 26-34.

Urch, C.E., Donovan-Rodriguez, T. & Dickenson, A.H. (2003) Alterations in dorsal horn neurones in a rat model of cancer-induced bone pain. *Pain*, **106**, 347-356.

van Hecke, O., Austin, S.K., Khan, R.A., Smith, B.H. & Torrance, N. (2014) Neuropathic pain in the general population: a systematic review of epidemiological studies. *Pain*, **155**, 654-662.

Vanden Abeele, F., Kondratskyi, A., Dubois, C., Shapovalov, G., Gkika, D., Busserolles, J., Shuba, Y., Skryma, R. & Prevarskaya, N. (2013) Complex modulation of the cold receptor TRPM8 by volatile anaesthetics and its role in complications of general anaesthesia. *Journal of cell science*, **126**, 4479-4489.

Vase, L., Svensson, P., Nikolajsen, L., Arendt-Nielsen, L. & Jensen, T.S. (2013) The effects of menthol on cold allodynia and wind-up-like pain in upper limb amputees with different levels of phantom limb pain. *Neurosci Lett*, **534**, 52-57.

Venkatachalam, K. & Montell, C. (2007) TRP channels. *Annual review of biochemistry*, **76**, 387-417.

Vestergaard, K., Nielsen, J., Andersen, G., Ingeman-Nielsen, M., Arendt-Nielsen, L. & Jensen, T.S. (1995) Sensory abnormalities in consecutive, unselected patients with central post-stroke pain. *Pain*, **61**, 177-186.

Vetter, I., Hein, A., Sattler, S., Hessler, S., Touska, F., Bressan, E., Parra, A., Hager, U., Leffler, A., Boukalova, S., Nissen, M., Lewis, R.J., Belmonte, C., Alzheimer, C., Huth, T., Vlachova, V., Reeh, P.W. & Zimmermann, K. (2013) Amplified cold transduction in native nociceptors by M-channel inhibition. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **33**, 16627-16641.

Vetter, I., Touska, F., Hess, A., Hinsbey, R., Sattler, S., Lampert, A., Sergejeva, M., Sharov, A., Collins, L.S., Eberhardt, M., Engel, M., Cabot, P.J., Wood, J.N., Vlachova, V., Reeh, P.W., Lewis, R.J. & Zimmermann, K. (2012) Ciguatoxins activate specific cold pain pathways to elicit burning pain from cooling. *The EMBO journal*, **31**, 3795-3808.

Viana, F., de la Pena, E. & Belmonte, C. (2002) Specificity of cold thermotransduction is determined by differential ionic channel expression. *Nature neuroscience*, **5**, 254-260.

Voets, T., Droogmans, G., Wissenbach, U., Janssens, A., Flockerzi, V. & Nilius, B. (2004) The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature*, **430**, 748-754.

Vranken, J.H., Hollmann, M.W., van der Vegt, M.H., Kruis, M.R., Heesen, M., Vos, K., Pijl, A.J. & Dijkgraaf, M.G. (2011) Duloxetine in patients with central neuropathic pain caused by spinal cord injury or stroke: a randomized, double-blind, placebo-controlled trial. *Pain*, **152**, 267-273.

Wahren, L.K., Torebjork, E. & Jorum, E. (1989) Central suppression of cold-induced C fibre pain by myelinated fibre input. *Pain*, **38**, 313-319.

Wallace, V.C., Segerdahl, A.R., Blackbeard, J., Pheby, T. & Rice, A.S. (2008) Anxiety-like behaviour is attenuated by gabapentin, morphine and diazepam in a rodent model of HIV anti-retroviral-associated neuropathic pain. *Neurosci Lett*, **448**, 153-156.

Wang, H., Sun, H., Della Penna, K., Benz, R., Xu, J., Gerhold, D., Holder, D. & Koblan, K. (2002) Chronic neuropathic pain is accompanied by global changes in gene expression and shares pathobiology with neurodegenerative diseases. *Neuroscience*, **114**, 529-546.

Wasner, G., Naleschinski, D., Binder, A., Schattschneider, J., McLachlan, E.M. & Baron, R. (2008) The effect of menthol on cold allodynia in patients with neuropathic pain. *Pain medicine (Malden, Mass.)*, **9**, 354-358.

Wasner, G., Schattschneider, J., Binder, A. & Baron, R. (2004) Topical menthol--a human model for cold pain by activation and sensitization of C nociceptors. *Brain*, **127**, 1159-1171.

Waters, A.J. & Lumb, B.M. (2008) Descending control of spinal nociception from the periaqueductal grey distinguishes between neurons with and without C-fibre inputs. *Pain*, **134**, 32-40.

Watson, C., Paxinos, G., Kayalioglu, G. & Heise, C. (2009) Chapter 15 - Atlas of the Rat Spinal Cord. In Charles, W., George, P., Gulgun, K. (eds) *The Spinal Cord*. Academic Press, San Diego, pp. 238-306.

Westenbroek, R.E., Hoskins, L. & Catterall, W.A. (1998) Localization of Ca2+ channel subtypes on rat spinal motor neurons, interneurons, and nerve terminals. *The Journal of neuroscience*, **18**, 6319-6330.

Winchester, W., Gore, K., Glatt, S., Petit, W., Gardiner, J.C., Conlon, K., Postlethwaite, M., Saintot, P.P., Roberts, S., Gosset, J.R., Matsuura, T., Andrews, M.D., Glossop, P.A., Palmer, M.J., Clear, N., Collins, S., Beaumont, K. & Reynolds, D.S. (2014) Inhibition of TRPM8 channels reduces pain in the cold pressor test in humans. *The Journal of pharmacology and experimental therapeutics*.

Winquist, R.J., Pan, J.Q. & Gribkoff, V.K. (2005) Use-dependent blockade of Cav 2.2 voltage-gated calcium channels for neuropathic pain. *Biochemical pharmacology*, **70**, 489-499.

Woolf, C.J. (1983) Evidence for a central component of post-injury pain hypersensitivity. *Nature*, **306**, 686-688.

Woolf, C.J. (1996) Windup and central sensitization are not equivalent. *Pain*, **66**, 105-108.

Woolf, C.J. (2011) Central sensitization: Implications for the diagnosis and treatment of pain. *PAIN*, **152**, S2-S15.

Woolf, C.J. & Thompson, S.W. (1991) The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. *Pain*, **44**, 293-299.

Wu, G., Ringkamp, M., Murinson, B.B., Pogatzki, E.M., Hartke, T.V., Weerahandi, H.M., Campbell, J.N., Griffin, J.W. & Meyer, R.A. (2002) Degeneration of myelinated efferent fibers induces spontaneous activity in uninjured C-fiber afferents. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **22**, 7746-7753.

Wu, J., Lin, Q., McAdoo, D.J. & Willis, W.D. (1998) Nitric oxide contributes to central sensitization following intradermal injection of capsaicin. *Neuroreport*, **9**, 589-592.

Wycisk, K.A., Zeitz, C., Feil, S., Wittmer, M., Forster, U., Neidhardt, J., Wissinger, B., Zrenner, E., Wilke, R., Kohl, S. & Berger, W. (2006) Mutation in the auxiliary calcium-channel subunit CACNA2D4 causes autosomal recessive cone dystrophy. *American journal of human genetics*, **79**, 973-977.

Xiao, H.S., Huang, Q.H., Zhang, F.X., Bao, L., Lu, Y.J., Guo, C., Yang, L., Huang, W.J., Fu, G., Xu, S.H., Cheng, X.P., Yan, Q., Zhu, Z.D., Zhang, X., Chen, Z., Han, Z.G. & Zhang, X. (2002) Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 8360-8365.

Xiao, W. & Bennett, G. (1995) Synthetic omega-conopeptides applied to the site of nerve injury suppress neuropathic pains in rats. *Journal of Pharmacology and Experimental Therapeutics*, **274**, 666-672.

Xing, H., Chen, M., Ling, J., Tan, W. & Gu, J.G. (2007) TRPM8 mechanism of cold allodynia after chronic nerve injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **27**, 13680-13690.

Xu, M., Kontinen, V.K. & Kalso, E. (1999) Endogenous noradrenergic tone controls symptoms of allodynia in the spinal nerve ligation model of neuropathic pain. *Eur J Pharmacol*, **366**, 41-45.

Yagi, J. & Sumino, R. (1998) Inhibition of a hyperpolarization-activated current by clonidine in rat dorsal root ganglion neurons. *J Neurophysiol*, **80**, 1094-1104.

Yaksh, T.L. (1989) Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: effects of modulatory receptor systems and excitatory amino acid antagonists. *Pain*, **37**, 111-123.

Yaksh, T.L. & Wilson, P.R. (1979) Spinal serotonin terminal system mediates antinociception. *The Journal of pharmacology and experimental therapeutics*, **208**, 446-453.

Yamaoka, K., Inoue, M., Miyazaki, K., Hirama, M., Kondo, C., Kinoshita, E., Miyoshi, H. & Seyama, I. (2009) Synthetic ciguatoxins selectively activate Nav1.8-derived chimeric sodium channels expressed in HEK293 cells. *The Journal of biological chemistry*, **284**, 7597-7605.

Yarnitsky, D., Granot, M., Nahman-Averbuch, H., Khamaisi, M. & Granovsky, Y. (2012) Conditioned pain modulation predicts duloxetine efficacy in painful diabetic neuropathy. *Pain*, **153**, 1193-1198.

Yarnitsky, D. & Ochoa, J.L. (1990) Release of cold-induced burning pain by block of cold-specific afferent input. *Brain*, **113**, 893-902.

Yasaka, T., Tiong, S.Y., Hughes, D.I., Riddell, J.S. & Todd, A.J. (2010) Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. *Pain*, **151**, 475-488.

Yu, X.-M., Askalan, R., Keil, G.J. & Salter, M.W. (1997) NMDA Channel Regulation by Channel-Associated Protein Tyrosine Kinase Src. *Science*, **275**, 674-678.

Zambreanu, L., Wise, R.G., Brooks, J.C., Iannetti, G.D. & Tracey, I. (2005) A role for the brainstem in central sensitisation in humans. Evidence from functional magnetic resonance imaging. *Pain*, **114**, 397-407.

Zanotto, K.L., Merrill, A.W., Carstens, M.I. & Carstens, E. (2007) Neurons in superficial trigeminal subnucleus caudalis responsive to oral cooling, menthol, and other irritant stimuli. *J Neurophysiol*, **97**, 966-978.

Zhang, X., Davidson, S. & Giesler, G.J., Jr. (2006) Thermally identified subgroups of marginal zone neurons project to distinct regions of the ventral posterior lateral nucleus in rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **26**, 5215-5223.

Zhang, X.B., Jiang, P., Gong, N., Hu, X.L., Fei, D., Xiong, Z.Q., Xu, L. & Xu, T.L. (2008) A-type GABA receptor as a central target of TRPM8 agonist menthol. *PLoS One*, **3**, e3386.

Zheng, J., Lu, Y. & Perl, E.R. (2010) Inhibitory neurones of the spinal substantia gelatinosa mediate interaction of signals from primary afferents. *J Physiol*, **588**, 2065-2075.

Zhou, C. & Luo, Z.D. (2014) Electrophysiological characterization of spinal neuron sensitization by elevated calcium channel alpha-2-delta-1 subunit protein. *European journal of pain (London, England)*, **18**, 649-658.

Zhou, H.Y., Chen, S.R., Chen, H. & Pan, H.L. (2011) Functional plasticity of group II metabotropic glutamate receptors in regulating spinal excitatory and inhibitory synaptic input in neuropathic pain. *The Journal of pharmacology and experimental therapeutics*, **336**, 254-264.

Zhuo, M. & Gebhart, G.F. (1992) Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. *J Neurophysiol*, **67**, 1599-1614.

Ziegler, E.A., Magerl, W., Meyer, R.A. & Treede, R.D. (1999) Secondary hyperalgesia to punctate mechanical stimuli. Central sensitization to A-fibre nociceptor input. *Brain*, **122 (Pt 12)**, 2245-2257.

Zimmermann, K., Deuis, J.R., Inserra, M.C., Collins, L.S., Namer, B., Cabot, P.J., Reeh, P.W., Lewis, R.J. & Vetter, I. (2013) Analgesic treatment of ciguatoxin-induced cold allodynia. *Pain*, **154**, 1999-2006.

Zimmermann, K., Leffler, A., Babes, A., Cendan, C.M., Carr, R.W., Kobayashi, J., Nau, C., Wood, J.N. & Reeh, P.W. (2007) Sensory neuron sodium channel Nav1. 8 is essential for pain at low temperatures. *Nature*, **447**, 856-859.

Zimmermann, K., Lennerz, J.K., Hein, A., Link, A.S., Kaczmarek, J.S., Delling, M., Uysal, S., Pfeifer, J.D., Riccio, A. & Clapham, D.E. (2011) Transient receptor potential cation channel, subfamily C, member 5 (TRPC5) is a cold-transducer in the peripheral nervous system. *Proceedings of the National Academy of Sciences*, **108**, 18114-18119.

Zimmermann, M. (1983) Ethical guidelines for investigations of experimental pain in conscious animals. *Pain*, **16**, 109-110.

Zurborg, S., Yurgionas, B., Jira, J.A., Caspani, O. & Heppenstall, P.A. (2007) Direct activation of the ion channel TRPA1 by Ca2+. *Nature neuroscience*, **10**, 277-279.