CONTRIBUTION OF AXONAL TRANSPORT DISRUPTION TO THE DEVELOPMENT OF NEUROPATHIC PAIN

IEVA SATKEVICIUTE

PhD 2018

Abstract

Axonal transport disruption is a major part of the mechanism of neuropathic pain. Following traumatic nerve injury there is complete cessation of axonal transport. When intact axons are inflamed, axonal transport is also disrupted. The main aim of the present study was to determine the role of axonal transport disruption along intact axons in the mechanisms of neuropathic pain. Low doses of vinblastine were applied to the rat sciatic nerve in order to disrupt axonal transport in the absence of axonal degeneration or inflammation. Comparisons were made to a model of local nerve inflammation (neuritis). An examination of evoked neuropathic pain behaviours revealed reversible signs of mechanical and cold allodynia following both vinblastine treatment and neuritis. Burrowing behaviour also showed signs of reduction in both models. *In vivo* electrophysiological recordings were performed to look for peripheral ongoing activity, which is reputed to drive central mechanisms that lead to neuropathic pain behaviours. Whereas ongoing activity developed in 25% of C-fibre neurons in the neuritis model, ongoing activity did not increase following vinblastine treatment. An electrophysiological examination of the spinal cord following neuritis revealed an increase in wind-up response in wide dynamic range neurons within deep laminae. Following vinblastine treatment, there was a reduction in mechanical responses of such neurons. Both vinblastine treatment and neuritis caused an increase in activity-dependent c-fos expression within superficial laminae, which indicates a central neuropathic pain mechanism in both models. Following vinblastine treatment, substance P expression was decreased in the dorsal horn, suggestive of altered nociceptor transmission into the spinal cord. Taken together, these findings infer a role for axonal transport disruption along intact axons in the development of neuropathic pain. The lack of increased ongoing activity

following vinblastine treatment suggests that central mechanisms may be maintained by an alternative signal. Finally, vinblastine treated animals do not show any adverse effects, and therefore in terms of the 3Rs, this model provides an alternative less severe model to assess the mechanisms of neuropathic pain.

Table of contents

List of tables

List of figures

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ASIC	acid sensing ion channels
AUC	area under the curve
CCI	chronic constriction injury
CFA	complete Freund's adjuvant
CIPN	chemotherapy-induced peripheral neuropathy
CREB	cyclic adenosine monophosphate response element-binding protein
CRPS1	complex regional pain syndrome type 1
DAPI	4',6-diamino-2-phenylindole
DRG	dorsal root ganglion
GABA	γ-aminobutyric acid
HCN	hyperpolarisation-activated cyclic nucleotide-gated
HIV	human immunodeficiency virus
IASP	International Association for the Study of Pain
IL	interleukin
IQR	interquartile range
K_v	voltage-gated potassium channels
LTMR	low threshold mechanoreceptor
mRNA	messenger ribonucleic acid
Na_v	voltage-gated sodium channel

NC3Rs	the national centre for the replacement, refinement and reduction of animals in research
NGF	nerve growth factor
NICE	The National Institute for Health and Clinical Excellence
NK-1R	neurokinin 1 receptor
NMDA	N-Methyl-D-aspartic acid
NSAP	non-specific arm pain
PBS	phosphate-buffered saline
PSL	partial sciatic nerve ligation
SD	standard deviation
SEM	standard error of the mean
SNI	spared nerve injury
SNL	spinal nerve ligation
SNRI	serotonin-noradrenaline reuptake inhibitor
TCA	tricyclic antidepressant
TNF-α	tumour necrosis factor alpha
TRPV1	transient receptor potential cation channel subfamily V member 1
TRPM8	transient receptor potential cation channel subfamily M member 8
TRPA1	transient receptor potential cation channel subfamily A member 1
WAD	whiplash-associated disorder
WDR	wide dynamic range

Acknowledgements

I would like to express my gratitude to my supervisor Dr. Andrew Dilley for allowing me to be a part of his research group and for his guidance, help and teaching during the project.

I would also like to thank Dr. Kim Pulman for teaching me surgical techniques.

Special thanks to George Goodwin for his help and cooperation in the lab, office and conferences throughout the three years.

Thanks to everyone in the BSMS medical research building for philosophical and scientific debates, tea and cakes.

A very special gratitude goes out to all down at the national centre for the replacement, refinement and reduction of animals in research (NC3Rs) for providing the funding for the work.

An exceptional acknowledgement goes to my family, who provided me with support and encouragement throughout every step of my life.

Finally, thanks to my boyfriend Mohsen Faraji and dear friends Tessa Brianne Frank and Dineswari R Ganesan for believing in me and for help in achieving my goals.

Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed: 

Dated: 08/10/2018

CHAPTER 1:

Introduction

1.1 Neuropathic pain

Pain, "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (IASP), has a biologically important protective function. The complexity of pain, and diverse symptomology, means that it is difficult to classify. Although there are various classification systems, it is generally accepted that pain can be nociceptive, inflammatory and neuropathic. Pain can also be divided into 1) acute pain, which is sharp and short-lived (< 12 weeks), and 2) chronic pain, which is pain lasting for more than 12 weeks. Neuropathic pain, which has a negative impact on up to 10% of the general population (van Hecke *et al.*, 2014), is currently defined as "pain caused by a lesion or disease of the somatosensory system" (IASP) (Jensen *et al.*, 2011).

1.1.1 Signs and symptoms

To understand the impact of neuropathic pain on the wellbeing of an individual and to therefore appreciate the problems faced by pain researchers, it is important to be aware of the common signs and symptoms. Generally, neuropathic pain symptoms often have no definite cause and can be broadly grouped into those that are spontaneous and those that are evoked. Patients may experience paraesthesias and/or dysesthesias (Table 1.1), which include deep aching, burning, itching or electric shock-like sensations (Boureau *et al.*, 1990, Backonja and Stacey, 2004, Bennett, 2001, Baron *et al.*, 2017, Rasmussen *et al.*, 2004). Additionally, they may report allodynia, when a normally unpainful stimulus becomes painful, or hyperalgesia, when a normally painful stimulus leads to exaggerated painful response (Jensen and Finnerup, 2014). Although patients can avoid stimuli that evoke hyperalgesic responses, allodynia can be prompted by the simplest every day's tasks such as

dressing oneself. Pain can be also experienced during limb movements that mechanically stimulate nerve trunks. For example, clinical tests that stretch peripheral nerves or apply digital pressure over nerve trunks can cause an exacerbation of pain. Interestingly, for patients, neuropathic pain does not only associate with painful sensations (i.e. positive symptoms) but it can also cause negative symptoms such as partial or full loss of sensation or a reduction in painful response to noxious stimulus. Moreover, neuropathic pain usually results in changes in mood, development of anxiety, depression and sleep disorders that have a negative impact on the general pain experience (Meyer-Rosberg *et al.*, 2001).

Terms	IASP definitions
Allodynia	Pain due to a stimulus that does not normally provoke pain.
Dysesthesia	An unpleasant abnormal sensation, whether spontaneous or evoked.
Hyperalgesia	Increased pain from a stimulus that normally provokes pain.
Hyperesthesia	Increased sensitivity to stimulation, excluding the special senses.
Hypoalgesia	Diminished pain in response to a normally painful stimulus.
Hypoesthesia	Decreased sensitivity to stimulation, excluding the special senses.
Paraesthesia	An abnormal sensation, whether spontaneous or evoked.

Table 1.1 IASP definitions of neuropathic pain signs and symptoms.

1.1.2 Current treatments

The complexity of patient symptoms, and the mechanisms that lead to these, have resulted in limited treatment success with less than half of patients who receive medical treatment reporting improved pain state (Dworkin *et al.*, 2003, Finnerup *et al.*, 2005). Patients with neuropathic pain require multidisciplinary management including physiotherapy, psychological therapy and occasionally palliative care (NICE). In cases that are refractory to pharmacotherapy or those with significant side effects, neuropathic pain may be managed by neurostimulation therapies, such as spinal cord stimulation (Verrills *et al.*, 2016). Currently, the classes of drugs that are used to treat patients include antidepressants, psychotropic drugs, anticonvulsants, opioids and antiarrhythmics (Table 1.2). The National Institute for Health and Clinical Excellence (NICE) guidelines recommend prescribing oral amitriptyline, duloxetine, gabapentin, pregabalin, imipramine or nortriptyline as a first-line treatment. If the first-line treatment is unsuccessful, patients are usually offered treatment with another drug (mentioned above) instead or in combination with the original drug. In a case of second-line treatment failure, a patient can be prescribed oral tramadol instead or in combination with the second-line treatment. Additionally, a topical lidocaine patch can be offered. In cases where pain is localized and oral treatment is not possible, 0.075% capsaicin cream is suggested.

Drug	Class	Known mechanism of action
Amitriptyline	Antidepressant: TCA	Monoamine reuptake inhibitor
Duloxetine	Antidepressant: SNRI	Serotonin-noradrenaline reuptake inhibitor
Gabapentin	Anticonvulsant	Interacts with presynaptic voltage-gated calcium channels (binds to the $\alpha_{2\delta}$ subunit)
Pregabalin	Anticonvulsant: *indicated for fibromyalgia and neuropathic pain specifically*	Interacts with presynaptic voltage-gated calcium channels (binds to the $\alpha_{2\delta}$ subunit)
Imipramine	Antidepressant: TCA	Monoamine reuptake inhibitor
Nortriptyline	Antidepressant: TCA	Monoamine reuptake inhibitor
Tramadol	Opiate analgesic	μ-opioid-receptor agonist, monoamine reuptake inhibitor
Lidocaine patch	Antiarrhythmic	Blocks peripheral sodium channels and in turn ectopic discharges.
Capsaicin cream	Topical analgesic	TRPV1 agonist; depolarizes cell membrane, initially stimulates skin nerve fibres (causing pain) then blocks them (causing pain relief).

TCA, tricyclic antidepressant; SNRI, serotonin-noradrenaline reuptake inhibitor; TRPV1, transient receptor potential cation channel subfamily V member 1.

Table 1.2 The mechanisms of action of drugs recommended for the treatment of neuropathic pain (NICE).

1.1.3 Causes

As previously stated, neuropathic pain is defined as a consequence of a lesion or a disease of the somatosensory nervous system. There is no single cause for neuropathic pain. The aetiology is often complex, and for many patients it can be difficult to identify any preceding event that has led to the initiation of symptoms. It is associated with a multitude of different possible causes, which include surgery, disease (diabetes, kidney or liver disease), infection (herpes zoster virus, human immunodeficiency virus), tumours, chemicals (chemotherapy agents, antiretroviral treatment), toxins or hereditary diseases (e.g. paroxysmal extreme pain disorder). Commonly patients that experience neuropathic pain symptoms have a partial nerve injury (e.g. nerve compression, sever or increased traction) that can lead to both pain as well as loss of sensory innervation, which underlies negative symptoms. In neuropathic pain syndromes, inflammation undoubtedly plays a role and may contribute to the symptomology discussed in section 1.1.1. Significant examples of inflammation-driven neuropathic pain include painful peripheral neuropathies such as herpetic neuralgia, HIV neuropathy and Guillain Barré syndrome. These conditions are frequently preceded by a viral or bacterial infection and are mainly characterised by axonal degeneration and/or demyelination (Watson *et al.*, 1991, Martin *et al.*, 2003, Polydefkis *et al.*, 2002, Asbury *et al.*, 1969, Pritchard and Hughes, 2004, Jones *et al.*, 2005, Wallace *et al.*, 2007). There are many other factors that are considered to increase the risk of developing neuropathic pain. Gender undoubtedly plays a role, since neuropathic pain is more prevalent in women compared to men. A study has shown that 8% of women suffer from neuropathic pain compared to 5.7% of men (Bouhassira *et al.*, 2008). Furthermore, age is also considered a risk factor, with neuropathic pain being more prevalent in those at least

50 years old (Dieleman *et al.*, 2008, Bouhassira *et al*., 2008). More recently, genetic risk factors have been also identified. For example, mutations in genes coding for ion channels (e.g.$Na_v1.7$) are linked with rare neuropathic pain conditions, such as erythromelalgia (Veluchamy *et al.*, 2018, reviewed in Zorina-Lichtenwalter *et al.*, 2018).

1.1.4 Neuropathic pain in the absence of a traumatic nerve injury

Of particular interest to this study are patients with neuropathic pain who do not have a clear gross neuropathology on routine clinical testing and thus are difficult to diagnose. Pain in these patients is often diffuse and does not follow specific dermatomes. Unlike the previous inflammatory conditions, the symptoms in these patients cannot usually be attributed to a viral or bacterial infection. This group forms a large number of the patients seen in clinics and they are often diagnosed with a number of different syndromes, which are reviewed below.

Non-specific arm pain

Non-specific arm pain (NSAP), also known as a repetitive strain injury or cumulative trauma disorder, is associated with repetitive activity such as office work (Macfarlane *et al.*, 2000, Fogleman and Brogmus, 1995). Together with common neuropathic pain symptoms, patients also present with tenderness of deep muscles, pain with arm movements, muscle weakness and difficulty in coordination of fine finger movement (Dilley and Greening, 2012). Current evidence indicates involvement of peripheral nerves and inflammation, since changes in the function of unmyelinated and myelinated nerve fibres (Greening *et al.*, 2003) as well as increased serum levels of C-reactive protein and inflammatory mediators (i.e. IL-1β, TNF-α, and IL-6) have been reported in patients (Carp *et al.*, 2007). Additionally,

signal intensity changes during magnetic resonance imaging and raised vibration thresholds in these patients are also indicative of neuropathology of the peripheral nervous system (Dilley *et al.*, 2011, Greening and Lynn, 1998).

Whiplash-associated disorder

Following a whiplash injury, approximately half of patients suffer from persistent pain, known as whiplash-associated disorder (WAD). The main cause of whiplash is a hyperextension injury following a rear impact (Lovell and Galasko, 2002), such as during automobile collisions, sports activities, falls and slips. Among common neuropathic pain symptoms, patients also present with neck pain and stiffness, headaches and pain in the shoulders and arms (Sterling *et al.*, 2003, Sterling and Kenardy, 2006). Inflammation is thought to play an important role in WAD, since it is indicated following positron emission tomography scans (Linnman *et al.*, 2011), magnetic resonance imaging scans (Greening *et al.*, 2018) and upregulation in inflammatory cytokines, such as IL-6, has been reported (Gerdle *et al.*, 2008).

Complex regional pain syndrome type 1

Complex regional pain syndrome type 1 (CRPS1), also known as a reflex sympathetic dystrophy, is a neuropathic pain disorder affecting one or more distal extremities. In most cases it is triggered by tissue injuries (e.g. post-surgical conditions, strains, contusions and fractures) and manifests with disproportionally severe neuropathic pain signs and symptoms. Additionally, patients experience motor dysfunction, swelling and changes in skin colour (white, red, blue) as well as temperature of the affected extremity (Galer *et al.*, 2000). Increased levels of cytokines (e.g. IL-6, IL-1β, TNF-α), activated macrophages, activated monocytes and peptides (e.g. calcitonin gene-related protein, substance P, bradykinin), have all

been reported (Huygen *et al.*, 2002, Heijmans-Antonissen *et al.*, 2006, Alexander *et al.*, 2005, Uceyler *et al.*, 2007, Schinkel *et al.*, 2006, Birklein *et al.*, 2001, Blair *et al.*, 1998, Birklein *et al.*, 2014).

Fibromyalgia

Fibromyalgia is a condition of widespread pain that affects no less than seven areas of the body (Wolfe *et al.*, 1995, Wolfe *et al.*, 2011). It is suggested that abnormalities within the central nervous system and peripheral nervous system play a role (Staud *et al.*, 2008, Uceyler *et al.*, 2013). Altered expression of neurochemicals, including substance P, nerve growth factor and glutamate (Russell, 1998, Harris *et al.*, 2009, Russell *et al.*, 1992), as well as changes in small nerve fibre properties, such as reduced intraepidermal innervation, regeneration of unmyelinated fibres and changes in cold and warm detection thresholds, have been reported (Uceyler *et al*., 2013). Evidence is also present for nerve inflammation (Dilley *et al*., 2011).

1.1.5 Chemotherapy-induced peripheral neuropathy

Of particular interest to this study is chemotherapy-induced peripheral neuropathy (CIPN), which, as the name suggests, can affect patients following chemotherapy treatment. CIPN can occur following treatment with a number of different chemotherapy agents, which includes vinca alkaloids such as vinblastine (reviewed in Miltenburg and Boogerd, 2014). Vinca alkaloids are currently used for the treatment of haematologic and lymphatic malignancies as well as breast, ovarian, testicular, brain and non-small cell lung tumours and sarcomas and are known as microtubule destabilizing agents. CIPN often manifests with symmetric neuropathic pain symptoms, where numbness of distal extremities, tingling or burning sensations are frequent features. Loss of sensation in the feet commonly impairs the gait of

patients, while that in the hands leads to difficulties in fine hand movements (Boyette-Davis *et al.*, 2013, Verstappen *et al.*, 2005, Postma *et al.*, 1993). In addition, treatment with vinca alkaloids can impair the function of motor neurons, leaving patients with distal muscle weakness and cramps as well as lessened motor reflexes (Haim *et al.*, 1994). If autonomic nervous system is also affected, orthostatic hypotension, bladder or bowel disorders, ocular palsies as well as heart rate abnormalities may accompany sensory symptoms (Hirvonen *et al.*, 1989, Hancock and Naysmith, 1975). Various pathophysiological changes have been indicated in CIPN patients and include altered function of ion channels (e.g. sodium channels) subsequently leading to changes in neuronal excitability (Park *et al.*, 2009a, Park *et al.*, 2009b). Evidence is also present for mitochondria dysfunction, whereby swollen, vacuolated mitochondria are observed in sensory neurons (Sahenk *et al.*, 1994, Fazio *et al.*, 1999, reviewed in Flatters *et al.*, 2017). In the areas of skin with most severe symptoms, a loss of small and large diameter peripheral sensory nerve fibres as well as Meissner's corpuscles is present (Kroigard *et al.*, 2014, Sahenk *et al*., 1994, Boyette-Davis *et al*., 2013).

1.2 Introduction to primary sensory neurons: fibre types

Many of the signs and symptoms of neuropathic pain can be attributed to alterations in the electrophysiological properties of primary sensory neurons. Based on anatomy and function, this group of neurons can be categorized into different classes and subclasses. In this section, an overview of the different primary sensory nerve fibre neurons has been provided (Table 1.3; note that neurons with B-fibres are not discussed here).

1.2.1 Aβ-fibre neurons

In the rat, heavily myelinated Aβ-fibre neurons have a diameter ranging between 1.5-12.5 μm. Such neurons have fast conduction velocities (14-30 m/s) and short duration of action potentials (0.6 - 2.9 ms) (Lynn and Carpenter, 1982, Harper and Lawson, 1985, Vejsada *et al.*, 1985). These low threshold nerve fibres are the most common and detect innocuous stimuli applied to the skin (Bai *et al.*, 2015). However, a small population of Aβ-fibre neurons in mammals such as rodents, cats and primates are nociceptive and respond to high threshold stimuli (Lynn and Carpenter, 1982, Fang *et al.*, 2002, reviewed in Djouhri and Lawson, 2004). Aβ-fibre neurons, based on their responses to continuous mechanical stimulation, are subdivided into two groups (reviewed in Zimmerman *et al.*, 2014):

1) Slowly adapting Aβ-fibre neurons

This group of low threshold mechanoreceptors (LTMRs) is characterized by slow adaptation (Perl, 1968). They tend to have static or phasic firing patterns and are important in perceiving information about displacement of the skin. Studies in mammals have identified two types of slowly adapting Aβ-LTMRs. One group includes those with irregular firing pattern and nerve terminals associated with Merkel cells in the glabrous skin (Iggo and Muir, 1969, Iggo, 1977, Woodbury and Koerber, 2007). In mice, these nerve fibres have also been demonstrated to associate with follicles of straight long hair, known as guard hair (Li *et al.*, 2011). Another group of slowly adapting Aβ-LTMRs includes those with regular firing pattern in response to skin stretching or pressure and nerve terminals associated with Ruffini endings in both hairy and glabrous skin (Iggo, 1977).

2) Rapidly adapting Aβ-fibre neurons

This group of Aβ-LTMRs is characterized by rapid adaptation. In other words, they only fire in response to application or removal of the stimulus and detect changes in intensity of the stimulus and movement of the skin (e.g. vibration, walking). These sensory nerve fibres are mainly found in glabrous skin and are associated with Meissner's corpuscles and Pacinian corpuscles, which are located within the dermis. Studies in humans and primates revealed that Meissner's corpuscles have small receptive fields and respond to mechanical stimuli and low frequency vibrations (Cauna and Ross, 1960, Lindblom, 1965, Talbot *et al.*, 1968, Cauna, 1956). In contrast, Pacinian corpuscles have been shown to have large receptive fields and respond to mechanical stimuli and high frequencies (150-400 Hz) in the cat (Hubbard, 1958, Lynn, 1971, Gray and Matthews, 1951). Some of these nerve fibres also innervate follicles and are activated by hair movement (Sinclair, 1981). For example, in mice rapidly adapting Aβ-LTMRs wrap around the base of guard hair and medium length Awl/auchene hair (Li *et al*., 2011).

1.2.2 Muscle spindles and Golgi tendon organs

Muscle spindles and Golgi tendon organs are large diameter myelinated nerve fibres that provide proprioceptive feedback to the central nervous system and are involved in reflex responses. They fire in response to muscle movement and have a role in prevention of muscle injury (Houk and Simon, 1967, Matthews, 1933, Hunt and Kuffler, 1951). Muscle spindles are composed of Aα- (Group Ia) and Aβ-fibre neurons (Group II) with phasic and static firing patterns respectively. In contrary, Golgi tendon organs are composed of Aα-fibres (Group Ib) only. While muscle spindles respond to muscle lengthening and prevent muscle overstretching, Golgi

tendon organs respond to changes in muscle tension and prevent high tension build-up in muscle tendons, which is a potential injury source (Houk and Simon, 1967).

1.2.3 Aδ-fibre neurons

Medium diameter Aδ-fibre neurons are thinly myelinated. In the rat, their conduction velocities range between 2.2-8 m/s and they have short duration of action potentials (0.5-1.7 ms) (Harper and Lawson, 1985). Studies in the rat have shown that Aδ-fibre neurons have free nerve endings and the majority respond to noxious heat, chemical and mechanical stimuli. This population of nociceptors mediates the first pain response (i.e. acute/sharp pain). Based on their receptive characteristics, they are further divided into two types: 1) Type I nerve fibres are high-threshold mechanoreceptors, which respond to chemical and mechanical stimuli and have a very high threshold to heat stimuli (> 50^{o}C) (Lynn and Carpenter, 1982, Lynn and Shakhanbeh, 1988, Nagy and Rang, 1999); 2) Type II nerve fibres are high threshold mechanoreceptors that have a relatively low heat threshold (~43^{o}C) (Nagy and Rang, 1999). Aδ-fibre neurons that are responsive to noxious cold (Simone and Kajander, 1997) or convey itch sensation (Ringkamp *et al.*, 2011) have been also identified in the rat and human/primate respectively. In contrast, a population of Aδ-fibre neurons that carry innocuous information and act as rapidly adapting mechanoreceptors that innervate hair follicles, including zigzag hairs (also known as down or D-hairs) (Li *et al*., 2011), and respond to hair movement (Adriaensen *et al.*, 1983, Brown and Iggo, 1967) has been reported.

1.2.4 C-fibre neurons

Thin unmyelinated C-fibre neurons represent most (~80%) of the cutaneous nerve fibres in the rat (Lynn, 1984). They have a diameter ranging between 0.1-0.83 μm,

slow conduction velocities (< 1.4 m/s) and broad action potentials (0.6-7.4 ms), and form free nerve endings (Harper and Lawson, 1985, Vejsada *et al*., 1985). The majority of C-fibre neurons are considered to be polymodal and respond to noxious thermal, mechanical and chemical stimuli (Lynn and Carpenter, 1982, Dray, 1992b, Dray, 1992a, Fang *et al.*, 2005). These nociceptors mediate the second pain response (i.e. dull/ burning/aching) and can be divided into two groups depending on their expression profile of neuropeptides and receptors: 1) Peptidergic C-fibre neurons express substance P, calcitonin gene-related peptide and tropomyosin receptor kinase, a receptor for nerve growth factor. This population of neurons represents around 40-45% of dorsal root ganglion (DRG) population in adult rodents (Molliver *et al.*, 1997, Snider and McMahon, 1998); 2) Non-peptidergic C-fibre neurons express c-Ret neurotrophin receptor, which is activated by glial-derived neurotrophic factor, neurturin and artemin. Additionally, a proportion of these neurons express isolectin B4 as well G-protein-coupled and purinergic receptors (Dong *et al.*, 2001). Non-peptidergic C-fibre neurons are found to represent around 30% of DRG neurons in adult rodents (Snider and McMahon, 1998). Although polymodal C-fibre neurons are considered to represent the majority of nociceptive non-myelinated neurons in rodents, newly emerging techniques, such as in vivo GCaMP optical imaging suggests that in healthy mice >85% of C-fibre neurons are modality specific (Emery *et al.*, 2016).

A subgroup of C-fibre neurons, known as silent nociceptors, responds to mechanical and heat stimuli only when they are sensitized (Schmidt *et al.*, 1995, Emery *et al*., 2016). Moreover, distinctive types of nociceptive neurons (both C- and Aδ-fibre neurons) that covey itch sensation have also been identified (Davidson and Giesler, 2010, Usoskin *et al.*, 2015, Green and Dong, 2016, Ringkamp *et al*., 2011).

Further studies in rodents, primates and humans have identified a population of C-fibre neurons, which are LTMRs (also known as C-tactile afferents). C-LTMRs respond to innocuous mechanical stimulation, such as light stroking of the skin (Leem *et al.*, 1993, Iggo, 1969, Olausson *et al.*, 2008, Loken *et al.*, 2009, reviewed in Liljencrantz and Olausson, 2014) as well as innocuous cooling (Seal *et al.*, 2009, Li *et al.*, 2011). These nerve fibres are thought to be responsible for a pleasant sensation associated with a gentle touch.

Fibre type	Myelination state	Diameter (μm; rat)	CV (m/s; rat)	Subtype	Stimuli	Sensation
Aα/β	Myelinated	> 1.5	> 14	Muscle spindle; Golgi tendon organ	Mechanical	Proprioception
Aβ	Myelinated	1.5-12.5	14-30	High threshold mechanoreceptor ***(Noxious)***	Mechanical; Thermal	Temperature, Pain
				Low threshold SA mechanoreceptor (Merkel cell; Ruffini ending)	Movement of hair, touch to the skin, vibration	Touch
				Low threshold RA mechanoreceptor (Meissner corpuscle; Pacinian corpuscle; neuron innervating hair follicles)	Movement of hair (guard and Awl/auchene hair), touch to the skin vibration	Touch
Aδ	Thinly myelinated	0.1-1.5	2.2-8	Type I ***(Noxious)***	Mechanical, high heat	Pain
				Type II ***(Noxious)***	Heat, high mechanical	Temperature, pain

Fibre	Myelination state	Diameter (µm; rat)	CV (m/s; rat)	Subtype	Stimuli	Sensation
Aδ	Thinly myelinated	0.1-1.5	2.2-8	Cold sensitive ***(Noxious)***	Cold	Temperature, pain
				Mechanoreceptor ***(Noxious)***	Pressure	Pain
				Low threshold mechanoreceptor	Awl/auchene and zigzag hair movement	Touch
C	Non-myelinated	0.1- 0.83	< 1.4	High threshold mechanoreceptor; Mechano-heat and -cold receptor ***(Noxious)***	Noxious thermal, mechanical and chemical stimuli	Pain, heat, cold
				Mechanoreceptor ***(Noxious)***	Pressure	Pain
				Mechano-insensitive ***(Noxious)***	Histamine application to the skin	Itch
				Low threshold mechanoreceptor	Stroking of skin; Awl/auchene and zigzag hair movement	Pleasant touch sensation
				Low threshold cooling	Cooling	Cool

CV, conduction velocity; RA, rapidly adapting; SA, slowly adapting. Note that diameter and CV parameters are those of the rat species.

Table 1.3 Classification of primary sensory neurons based on rodent, cat, primate and human nerve fibre characteristics.

1.2.5 Deep afferents innervating muscles, joints and tendons

A population of Aδ- and C-fibre neurons, known as Group IV afferents, innervate deeper structures, such as muscles and joints (Iggo, 1961, Mense *et al.*, 1985, Mense and Stahnke, 1983). These neurons respond to pressure changes that are associated with muscle contractions. Nociceptive fibres found in deep tissues express a variety of neuropeptides and receptors, including substance P, calcitonin gene-related protein and somatostatin (Molander *et al.*, 1987).

1.3 Ion channels

Ion channels are a major component of excitable cells, such as neurons. In addition to their role of transmitting action potentials, ion channels also act as transducers, converting tactile, thermal or chemical stimuli into an electrical potential. Notable examples of such channels are detailed in this section.

1.3.1 Propagation of action potentials

Voltage-gated sodium channels (Na_v)

Underlying the propagation of an action potential are voltage-gated sodium channels. These ion channels are heteromeric and are composed of α and β subunits (reviewed in Catterall, 2000). Their main role is the initiation and transmission of action potentials and regulation of excitability by enabling a rapid influx of sodium ions into neuronal cells. Since mutations in genes coding for $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ have been linked with several pain conditions in humans, they have been of specific interest in the pain field. In normal conditions, $Na_v1.7$ and $Na_v1.9$ channels are expressed in small diameter nociceptive DRG neurons (Sangameswaran *et al.*, 1997, Djouhri *et al.*, 2003, Dib-Hajj *et al.*, 1998, Dib-Hajj *et al.*, 1999, Fang *et al*., 2002), while $Na_v1.8$ channels are expressed in small/medium nociceptive DRG neurons and

their terminals as well as large diameter neurons (Djouhri *et al*., 2003, Fjell *et al.*, 2000, Akopian *et al.*, 1996, Belkouch *et al.*, 2014). The three channels are indicated to play a role in pain mechanisms, since, in pathological conditions, their upregulation in DRGs (Black *et al.*, 2004, Belkouch *et al*., 2014, Black *et al.*, 1997, Tanaka *et al.*, 1998), redistribution at injury/inflammatory sites (Belkouch *et al*., 2014, Gold *et al.*, 2003, Novakovic *et al.*, 1998) and involvement in pain behaviours (Amaya *et al.*, 2006, Lai *et al.*, 2002, Dong *et al.*, 2007, Joshi *et al.*, 2006, Bulaj *et al.*, 2006, Gaida *et al.*, 2005, Jarvis *et al.*, 2007, Payne *et al.*, 2015) have all been reported. Different subtypes of voltage-gated sodium channels and their association with human disorders are reviewed in table 1.4.

Voltage-gated potassium channels (K_v)

The role of the voltage-gated potassium channels is to enable repolarization of the cell membrane. By allowing efflux of potassium ions into neuronal cells, such channels also regulate firing frequency and duration of action potentials. Sensory neurons express a variety of K_v channels, which can be homomeric and heteromeric and are formed of α and β subunits (reviewed in Gutman *et al.*, 2005). Their expression patterns are summarized in table 1.5. Following nerve injury, expression of these channels is mainly reduced leading to membrane hyperexcitability and in turn neuropathic pain behaviours (Clark and Tempel, 1998, Chi and Nicol, 2007, Tsantoulas *et al.*, 2014, Ishikawa *et al.*, 1999, Chien *et al.*, 2007, Zheng *et al.*, 2013, Passmore *et al.*, 2003, Rose *et al.*, 2011, Hao *et al.*, 2013). This is also observed in patients with autoantibodies against K_v channels who develop thermal hyperalgesia (Klein *et al.*, 2012).

Na_v type	Location	Associated disorders	References
Na_v1.1	CNS neurons	Epilepsy	(Catterall, 2012)
	DRG neurons	Familial hemiplegic migraine	(Dichgans *et al.*, 2005)
Na_v1.2	CNS neurons	Epilepsy	(Catterall, 2012)
	DRG neurons		
Na_v1.3	CNS	N/A	N/A
	Immature DRG neurons (can be upregulated under specific conditions in adult neurons)		
Na_v1.4	Skeletal muscle	N/A	N/A
Na_v1.5	Mostly in cardiac muscle	N/A	N/A
	Observed in DRG neurons in small amounts		
Na_v1.6	CNS neurons	Epileptic disorders	(Veeramah *et al.*, 2012)
	DRG sensory neurons		

Na_v type	Location	Associated disorders	References
Na_v1.7	DRG sensory neurons	Insensitivity to pain	(Goldberg *et al.*, 2007, Cox *et al.*, 2006)
		Familial erythromelalgia	(Yang *et al.*, 2004, Dib-Hajj *et al.*, 2005, Cummins *et al.*, 2007, Sheets *et al.*, 2007)
		Paroxysmal extreme pain disorder	(Fertleman *et al.*, 2006)
Na_v1.8	DRG sensory neurons	Associated with painful small-fibre peripheral neuropathy	(Faber *et al.*, 2012)
Na_v1.9	DRG sensory neurons	Familial episodic pain	(Zhang *et al.*, 2013)
		Painful peripheral neuropathy	(Han *et al.*, 2015, Huang *et al.*, 2014)
		Insensitivity to pain	(Leipold *et al.*, 2013)

CNS, central nervous system; DRG, dorsal root ganglion.

Table 1.4 Subtypes of Na_v channels and associated disorders observed in humans (reviewed in Cummins *et al.*, 2007).

K_v type	K_v subtype	Location	References
K_v1	K_v1.1; K_v1.2	Large diameter neurons	(Rasband *et al.*, 2001, Hao *et al*., 2013)
	K_v1.4	Small diameter neurons	(Rasband *et al*., 2001)
K_v2	K_v2.1; K_v2.2	Large and small diameter neurons	(Tsantoulas *et al*., 2014)
K_v3	K_v3.4	Small diameter neurons	(Chien *et al*., 2007)
K_v4	K_v4.3	Cell bodies of non-peptidergic small diameter neurons	(Chien *et al*., 2007)
	K_v4.2	Very low levels	(Phuket and Covarrubias, 2009)
K_v7	K_v7.2; K_v7.3; K_v7.5	Large and small diameter neurons	(Passmore *et al*., 2003, King and Scherer, 2012)
	K_v7.2	Nodes of Ranvier in large diameter neurons	(Devaux *et al*., 2004)
K_v9	K_v9.1	Large diameter neurons	(Tsantoulas, 2012)

Table 1.5 Subtypes of K_v channels and their expression patterns in rodents.

Hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels

A group of ion channels known as HCN channels play a significant role in modulating the firing frequency of action potentials. These channels are activated by hyperpolarization of the membrane (Pape, 1996; reviewed in Wahl-Schott and Biel, 2009), and their activity can be enhanced following an increase in intracellular cyclic adenosine monophosphate levels (e.g. following inflammation) (Luthi and McCormick, 1999a, Luthi and McCormick, 1999b, Wainger *et al.*, 2001). There are four members of the HCN family. HCN1 channels are widely expressed in DRGs and large diameter neurons as well as a small proportion of small diameter cold sensing neurons (Chaplan *et al.*, 2003, Jiang *et al.*, 2008, Tu *et al.*, 2004). Following nerve injury, expression of these channels is reduced in DRGs and accumulation at the injury site is observed (Chaplan *et al*., 2003, Jiang *et al*., 2008). HCN2 channels can be found on the membrane of large sensory neurons as well as in the cytoplasm of small diameter DRG neurons (Jiang *et al*., 2008, Wainger *et al*., 2001, Tu *et al*., 2004). Both HCN1 and HCN2 channels have a potential role in the maintenance of neuropathic pain behaviours (Schnorr *et al.*, 2014, Chaplan *et al*., 2003, Jiang *et al*., 2008, Momin *et al.*, 2008, Emery *et al.*, 2011, Young *et al.*, 2014). In contrary, HCN3 and HCN4 channels can be found in DRG neurons only at low levels (Chaplan *et al*., 2003, Tu *et al*., 2004).

1.3.2 Transducers: TRP channels

A major family of ion channels that act as transducers are the transient receptor potential (TRP) channels. TRP channels are homo- or hetero-tetrameric non-selective cation channels permeable to sodium, calcium and magnesium with each tetramer formed of six transmembrane domains (reviewed in Pedersen *et al.*, 2005). TRP channels are expressed in a variety of mammalian tissues, including primary

sensory neuron terminals where they are activated by sensory stimuli. Herein, TRP channel subtypes TRPV1, TRPM8 and TRPA1 are briefly reviewed.

TRPV1

TRPV1 channels are activated by capsaicin, other vanilloids and noxious heat (threshold of 43^{o}C) and are found in C- and some Aδ- sensory nerve fibres (30-50% of all somatosensory neurons in rodents) (Kirschstein *et al.*, 1999, Nagy and Rang, 1999, Kobayashi *et al.*, 2005). This group of TRP channels play a role in inflammatory pain transmission including heat hyperalgesia as well as cold allodynia (Bolcskei *et al.*, 2005, McGaraughty *et al.*, 2006, Honore *et al.*, 2005, Cobos *et al.*, 2018).

TRPM8

TRPM8 channels are activated by cooling agents, such as menthol or thermal cold stimuli (threshold of ~26^{o}C), and are found in around 15% of sensory nerve fibres (mostly C-fibre neurons), with up to 30% of them expressing TRPV1 channels (Bautista *et al.*, 2007, Colburn *et al.*, 2007). Modulation of TRM8 channel activity can result in either analgesic or painful responses (Premkumar *et al.*, 2005, Pan *et al.*, 2012, Knowlton *et al.*, 2013, Descoeur *et al.*, 2011).

TRPA1

TRPA1 channels mediate chemonociception by detecting various chemicals that can cause both acute and inflammatory pain and are activated by air pollutants (e.g. formalin), oxidative/nitrative stress by-products as well as inflammatory mediators (Andersson *et al.*, 2008, Bandell *et al.*, 2004, McNamara *et al.*, 2007, Bautista *et al.*, 2006). Moreover, these channels play a role in cold detection, since they can be activated at temperatures below 17˚C (Story *et al.*, 2003). TRPA1 channels are

mostly located in peptidergic C-fibre neurons, which also express TRPV1 channels, and are thought to amplify TRPV1 responses (Salas *et al.*, 2009). Supporting a role of TRPA1 channels in neuropathic pain, a recent study reported that TRPA1 activation by chemotherapeutic agents (i.e. vinca alkaloids) may underlie enhanced neuronal excitability in CINP (Boiko *et al.*, 2017).

1.3.3 Transducers: ASIC channels

Acid sensing ion channels (ASIC) are involved in neuronal responses to changes in extracellular pH, which follows tissue injury, inflammation or ischemia. Some recent studies also suggest that ASIC channels also play a role mechanosensation, such as sensation of light touch (Price *et al.*, 2000). These voltage insensitive, ligand-gated ion channels are mainly activated by protons (acidosis), although several studies have reported activation by non-proton ligands (Yu *et al.*, 2010, Li *et al.*, 2010), and are mainly permeable to sodium ions (Waldmann *et al.*, 1997). Three homomeric or heteromeric subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 or ASIC4) form a functional channel in mammals (Deval *et al.*, 2010, Grunder and Pusch, 2015, Wemmie *et al.*, 2013). Generally, ASIC subunits are expressed in both peripheral and central nervous system, with ASIC1b and ASIC3 being predominantly found in the peripheral (i.e. DRGs and peripheral terminals). Activation of ASIC1, ASIC2 and ASIC3 channels leads to development of neuropathic pain behaviours (Bohlen *et al.*, 2011, Yu *et al*., 2010, Diochot *et al.*, 2016). Further supporting the role for ASIC channels in pain mechanisms, inhibition of ASIC 1 homomers as well as ASIC1 and ASIC2 heterodimers in both peripheral and central nervous system leads to reduced inflammatory pain (Diochot *et al.*, 2012). While activation of ASIC1, ASIC2 and ASIC3 channels is directly linked with pain modulation, association of ASIC4 with other ASIC channels plays a role in regulating their function (Donier *et al.*, 2008).

1.3.4 Mechanoreceptors: Piezo channels

A recently discovered group of channels that act as mechanical transducers are Piezo channels (Syeda *et al.*, 2016) that are involved in responses to light touch, proprioception, and vascular blood flow. Large Piezo proteins are predicted to have more than 14 transmembrane domains and form a pore, which when opened allows influx of positively charged ions (e.g. calcium ions) into the cell (Coste *et al.*, 2012, Coste *et al.*, 2010). Two channel isoforms, Piezo 1 and Piezo 2, are found in vertebrate organisms and their expression is essential for survival (Ranade *et al.*, 2014a, Ranade *et al.*, 2014b). While, Piezo 1 is mainly expressed in non-sensory tissues (e.g. kidneys, lungs, bladder, etc.), Piezo 2 is expressed in sensory tissues, such as dorsal root ganglion, Merkel cells and sensory neurons (reviewed in Wu *et al.*, 2017). Piezo 2 is responsible for light but not harsh touch sensation (Woo *et al.*, 2014, Maksimovic *et al.*, 2014) as well as proprioception (Woo *et al.*, 2015, Florez-Paz *et al.*, 2016). Among other abnormalities, mutations in Piezo 2 are linked with defects in proprioception and sensory ataxia (Haliloglu *et al.*, 2017).

1.4 Axonal transport

Ion channel components that are required for neurons to function are transported by a process of axonal transport from the cell bodies towards the peripheral terminals. Such process can be disrupted along primary sensory neurons following a traumatic nerve injury, nerve inflammation or its exposure to chemical agents, which are the common methods used to induce animal models of neuropathic pain. Importantly, disruption of such process can occur along intact axons, which may underlie some of the symptomology in patients without an obvious nerve injury. Undoubtedly, axonal transport disruption plays a role in neuropathic pain mechanisms and therefore is a major focus of this study.

Axonal transport is also responsible for the removal of damaged or misfolded proteins as well as transmission of chemical signals from the periphery to the cell body. The role of axonal transport is to maintain long-distance signalling throughout the lifetime of the neuron ensuring its survival (Figure 1.1). Axonal transport of macromolecules depends on microtubules (Kreutzberg, 1969), the main component of the cytoskeleton of the cell. Microtubules are composed of α- and β-tubulin heterodimers, with the slow growing minus end facing the cell body and the fast growing plus end facing the axon tips (Burton and Paige, 1981). Stabilized by microtubule-associated proteins (e.g. tau), microtubules form tracks along the axon that motor proteins travel along carrying various cargoes. This process involves fast movements, pauses and directional changes affecting the speed of the cargo transported (Lasek *et al.*, 1984, Roy *et al.*, 2000, Wang *et al.*, 2000).

Axonal transport is mediated by motor proteins and occurs in both retrograde and anterograde directions (Allen *et al.*, 1982, Brady *et al.*, 1982). That is, from the cell body to the tip of the axon and vice versa respectively. Kinesins are the main motor proteins in the anterograde direction (Vale *et al.*, 1985, Hirokawa *et al.*, 1991). Once cargoes are attached to the kinesin tail and adenosine triphosphate is hydrolysed, motor domains start 'walking' along the microtubule towards the plus end, transporting newly synthesized proteins, lipids and mitochondria to the distal part of the axon. Dyneins are the main motor proteins in the retrograde direction (Paschal *et al.*, 1987, Hirokawa *et al.*, 1990) and like kinesins use their motor domains and adenosine triphosphate to 'walk' along the microtubule towards the minus end. Retrograde transport maintains cellular homeostasis by clearing misfolded and aggregated proteins from the axon (Komatsu *et al.*, 2006), as well as ensures neuronal viability by transporting distal trophic signals to the nerve body where they

can alter gene expression (reviewed in Chowdary *et al.*, 2012). Cargo-bound motors are continuously regulated by scaffolding and adaptor proteins, which avoids the accumulation or depletion of transported cellular components. The importance of axonal transport is reflected by impairments in microtubule mechanisms that induce or contribute to various neurodegenerative diseases, such as Alzheimer's, Huntington's and Parkinson's disease (reviewed in Perlson *et al.*, 2010).

1.4.1 Fast axonal transport

Fast axonal transport moves membrane-bound organelles at a speed of around 1 μm/sec (Kaether *et al.*, 2000). Fast anterograde axonal transport ensures rapid delivery of newly synthesized axonal proteins (Kaether *et al.*, 2000) as well as synaptic components to the distal end of the axon (Yonekawa *et al.*, 1998), while fast retrograde axonal transport is vital for transporting trophic signals (Heerssen *et al.*, 2004) and autophagosomes (Maday *et al.*, 2012) to the cell body. Mitochondrion are moved bidirectionally along the axon by fast axonal transport and positioned at the sites with energy needs (reviewed in Saxton and Hollenbeck, 2012). Following nerve injury, fast retrograde axonal transport is responsible for a rapid delivery of transcription factors to the nucleus (Hanz *et al.*, 2003, Perry *et al.*, 2012).

1.4.2 Slow axonal transport

Slow axonal transport moves cytoplasmic and cytoskeletal proteins at a speed of 0.2-10 mm/day (Lasek *et al.*, 1984). Slow anterograde axonal transport is responsible for transporting neurofilaments (Wang and Brown, 2001), actin (Flynn *et al.*, 2009), tubulin (Wang and Brown, 2002) as well as cytosolic proteins (reviewed in Roy, 2014).

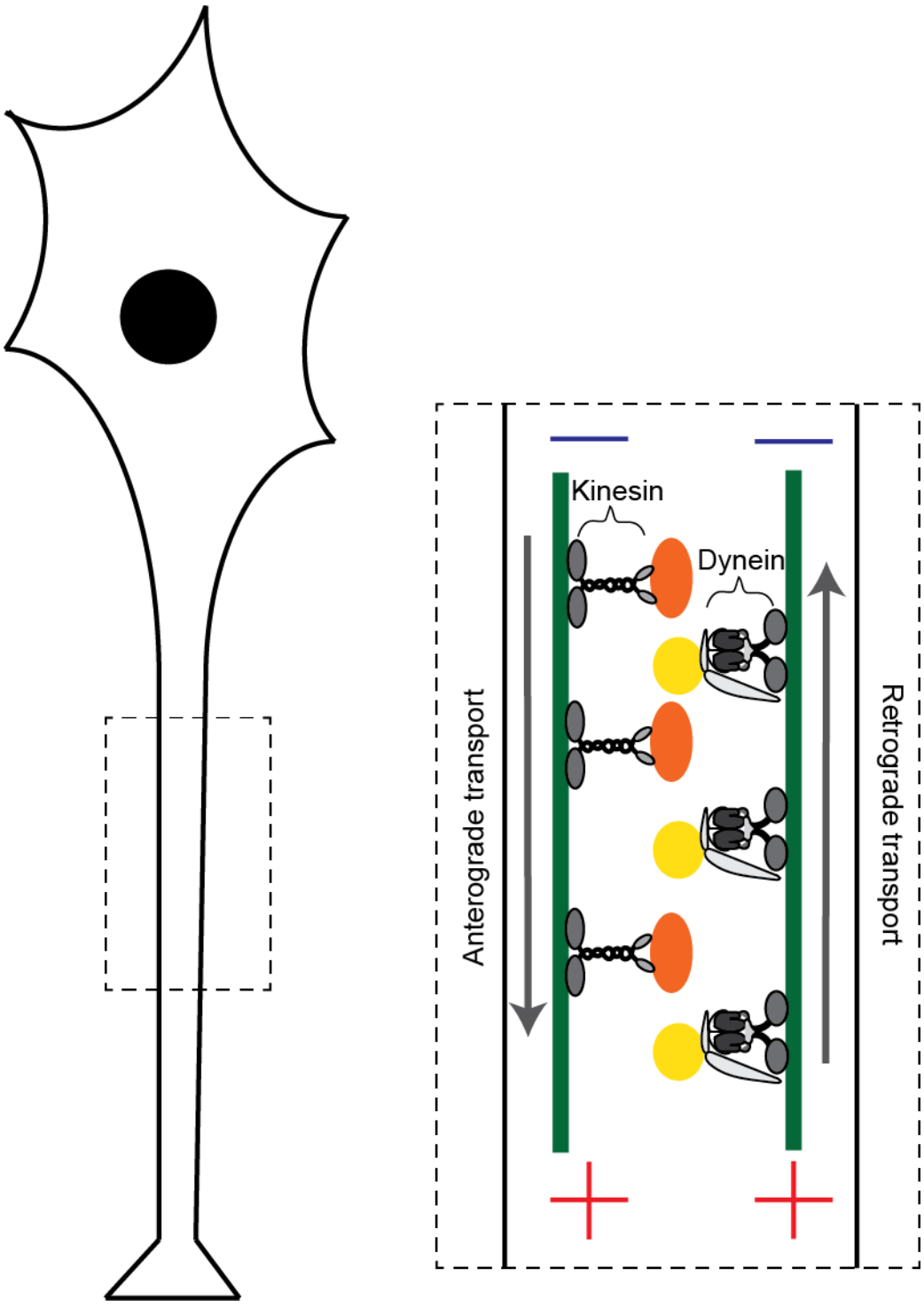


Figure 1.1 Axonal transport mechanisms. Motor proteins kinesin and dynein drive the movement of cargoes along polarized microtubules. Kinesin-driven fast and slow anterograde axonal transport towards periphery enables supply of newly synthesized proteins and synaptic components to the axon terminals. Dynein-driven fast retrograde axonal transport towards the cell body enables survival signalling and degradative trafficking.

1.5 Introduction to the dorsal horn

The dorsal horn is the primary entry point for primary afferent axons to the spinal cord enabling transmission of pain, itch and touch (Braz *et al.*, 2014, Abraira and Ginty, 2013, Todd, 2017). Based on the size of neurons and their density, the grey matter of the spinal cord can be divided into ten parallel laminae (Rexed, 1952, Molander *et al.*, 1984) with laminae I-V located within the dorsal horn (Figure1.2). Primary afferent axons enter the spinal cord via dorsal roots and terminate within these laminae to form synapses with dorsal horn neurons that based on their function can be divided into interneurons, which have axons that remain within the spinal cord, and projection neurons with their axons projecting to the brain. Most projection neurons involved in pain and itch pathways ascend the spinothalamic tract and project to several brain areas, which include the thalamus, periaqueductal grey matter, nucleus of the solitary tract, lateral parabrachial area, as well as caudal ventral medulla (Al-Khater and Todd, 2009, Spike *et al.*, 2003, Todd *et al.*, 2000, Al-Khater *et al.*, 2008, Polgár *et al.*, 2010)**.**

1.5.1 Dorsal horn circuitry

Nociceptive Aδ- and C-fibre neurons terminate within the laminae I-II region (Light and Perl, 1979), which is also known as the superficial dorsal horn. Interestingly, termination patterns of peptidergic and non-peptidergic C-fibres are slightly different: while peptidergic axons terminate in both lamina I and the outer part of lamina II, non-peptidergic axons synapse in the central area of lamina II (Lorenzo *et al.*, 2008). Generally, the inner part of lamina II receives inputs from non-nociceptive Aδ- and C-fibre LTMRs (Abraira and Ginty, 2013, Abraira *et al.*, 2017, Seal *et al.*, 2009). Afferent nerve fibres that terminate within the superficial laminae

form synapses with a diverse population of interneurons, which can be broadly grouped into two categories based on their function: excitatory and inhibitory interneurons. Excitatory (glutamatergic) interneurons enable the transmission of somatosensory signals between different laminae (Yasaka *et al.*, 2014, Grudt and Perl, 2002), while inhibitory (GABAergic/glycinergic) interneurons inhibit this activity by acting on either excitatory interneurons or projection cells.

Based on their morphology, interneurons in lamina II can be classified into islet, central, vertical and radial cells (Grudt and Perl, 2002). Studies in rodents have demonstrated that islet, vertical, radial and a large proportion of central interneurons are innervated by C-fibres, whereas Aδ-fibre neurons innervate only vertical and radial cells (Grudt and Perl, 2002, Lu and Perl, 2005). Interneurons in lamina II can also be classified based on their neurochemical properties (reviewed in Todd, 2017; Table 1.6). Based on neurochemical properties, four distinct non-overlapping populations of inhibitory interneurons have been recently identified in the superficial dorsal horn of the rat (Polgar *et al.*, 2013, Petitjean *et al.*, 2015), which include those that express either galanin, neuropeptide Y, neuronal nitric oxide synthase or parvalbumin (Tiong *et al.*, 2011). Specifically, parvalbumin and neuropeptide Y expressing interneurons have been demonstrated to form inhibitory synapses with excitatory neurons of the more superficial laminae (Lu *et al.*, 2013, Polgar *et al.*, 2011, Polgar *et al.*, 1999). Several other studies have demonstrated distinct laminar distribution of excitatory interneurons expressing substance P, gastrin-releasing peptide, neurotensin and neurokinin B, which represents separate neuronal populations in mice (Gutierrez-Mecinas *et al.*, 2016, Gutierrez-Mecinas *et al.*, 2017).

Afferent nerve fibres that terminate in lamina I also form synapses with projection neurons. These cells are mainly glutamatergic and can also be found in laminae III-V

in a rat. Similar to interneurons, projection neurons can be classified based on their morphology and neurochemical properties. Based on morphology (shape of cell body and branching pattern of dendrites) projection neurons of lamina I can be divided into fusiform, pyramidal and multipolar cells (Spike *et al*., 2003, Zhang *et al.*, 1996, Han *et al.*, 1998, Lima and Coimbra, 1986). Furthermore, projection neurons can be classified based on expression patterns of the neurokinin-1 receptor (NK-1R). More than half of these neurons in lamina I and around one-third of those in laminae III-IV express NK-1R (Nakaya *et al.*, 1994, Todd *et al.*, 1998, Marshall *et al.*, 1996, Ding *et al.*, 1995, Naim *et al.*, 1997, Al Ghamdi *et al.*, 2009). Such receptor is the main receptor for substance P, which is released by innervating peptidergic primary sensory neurons (Todd *et al.*, 2002) and local interneurons (Gutierrez-Mecinas *et al*., 2017, Ljungdahl *et al.*, 1978). In addition to direct activation by afferent nerve fibres, lamina I NK-1R positive projection neurons receive peripheral sensory inputs via polysynaptic connections mediated by lamina II interneurons (Lu and Perl, 2005). Projection neurons that are NK-1R negative, such as giant projection neurons, are also present in lamina I. The activity of giant projection neurons is regulated by inhibitory as well as excitatory interneurons (Puskar *et al.*, 2001, Todd *et al.*, 2003, Polgar *et al.*, 2008) without an apparent input from the primary afferent neurons. The remaining NK-1R negative projection neurons have been demonstrated to receive inputs from mainly Aδ-fibre afferents (Baseer *et al.*, 2014).

The area extending from lamina II to V forms the LTMR recipient zone, which receives inputs form LTMRs (Abraira and Ginty, 2013, Abraira *et al*., 2017, Seal *et al*., 2009). Within this zone, 11 subtypes of interneurons (7 excitatory and 4 inhibitory) have been recently identified in mice by Abraira and colleagues (2017).

These interneurons receive inputs not only from Aβ, Aδ- or C-fibre LTMRs but also from other spinal cord interneurons and corticospinal projection neurons. Their formed connections mainly remain within the deep dorsal horn. Another population of deep dorsal horn neurons, tyrosine kinase Ret expressing interneurons, was identified in neonatal mice (Cui *et al.*, 2016). In addition to direct excitatory inputs from Aβ-fibre neurons and in some cases C-fibre afferents, Ret positive interneurons receive polysynaptic and direct inhibitory inputs from primary sensory nerve fibres and other Ret positive interneurons. Interestingly, this group of neuronal cells not only inhibits activity of excitatory interneurons but also forms presynaptic inhibitory synapses onto primary afferent terminals.

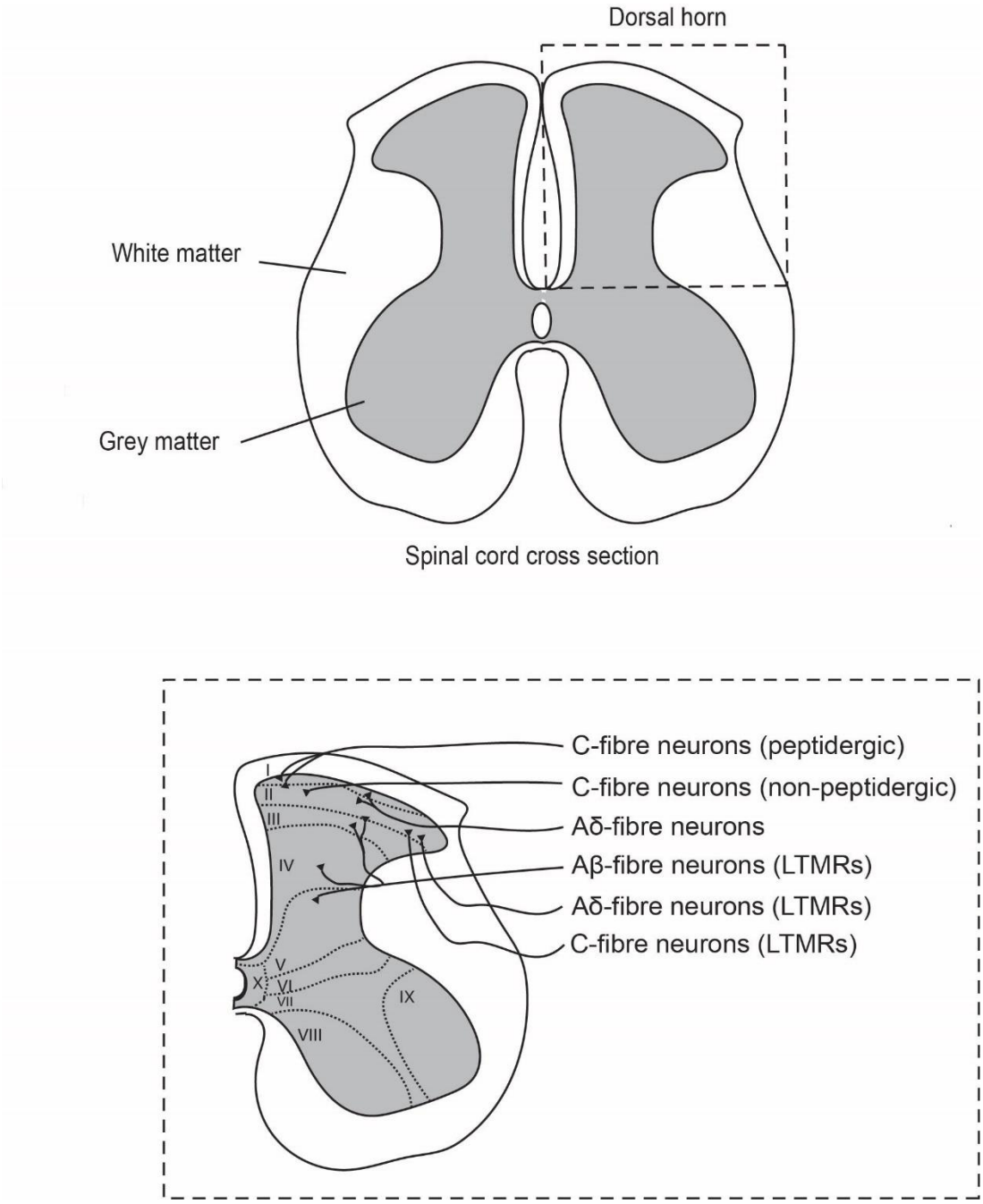


Figure 1.2 Laminar organization of the dorsal horn based on the Rexed system (adapted from Todd, 2010 and Abraira and Ginty, 2013). A transverse section of the L5 spinal cord is represented. Dashed lines represent boundaries of different laminae. Direct afferent inputs are represented. LTMRs, low threshold mechanoreceptors.

	Type of interneuron	
	Inhibitory	***Excitatory***
Neuropeptides	Neuropeptide Y	Somatostatin
	Galanin	Neurotensin
	Enkephalin	Neurokinin B
	Dynorphin	Substance P
		Enkephalin
		Dynorphin
Receptors	Sst2A	NK-1
	NK3	NK-3
		MOR-1
		Neuropeptide Y Y1

NK-1, neurokinin-1; MOR-1, μ-opioid receptor 1; sst2A, somatostatin receptor subtype.

Table 1.6 Neurochemical markers present in interneurons in laminae I-III of the dorsal horn (reviewed in Todd, 2010, Todd and Spike, 1993).

1.5.2 Wide dynamic range neurons

Wide dynamic range (WDR) neurons are polysynaptic interneurons and projection cells (Willis and Coggeshall, 1991) that receive multiple inputs from Aβ-, Aδ- and C- fibre neurons (Mendell, 1966, Wagman and Price, 1969) as well as internal organs, muscles and joints. WDR neurons are located in both superficial and deep layers of the dorsal horn, with a greater population found in the latter (i.e. lamina V region). Due to their responsiveness to innocuous and noxious stimuli (Wagman and Price, 1969, Mendell, 1966, Willis and Coggeshall, 1991) and importantly wind-up (Mendell, 1966, Mendell and Wall, 1965), which is a frequency-dependent progressive increase in dorsal horn responses to repetitive electrical stimulation of C-fibres, WDR neurons have received a specific interest within pain field. Such increase in post-synaptic responses is short-lived and requires low frequency stimulation. Although the mechanism that underlies wind-up is not entirely clear, it shares similarities with that of central sensitization (for comparison of wind-up and central sensitisation please refer to Section 1.7.2; Figure 1.4). Consistent with such processes, increases in wind-up have been reported in models of chronic hind paw and joint inflammation (Traub, 1997, Herrero and Cervero, 1996, Stanfa *et al.*, 1992). Wind-up is a result of glutamate, substance P and calcitonin gene-related protein release from nociceptive nerve fibres (Sivilotti *et al.*, 1993) following repetitive noxious stimulation. This leads to a cumulative membrane depolarization, which activates N-Methyl-D-aspartic acid (NMDA) receptors by removing the Mg^{++} block (Davies and Lodge, 1987, Dickenson and Sullivan, 1987, Fossat *et al.*, 2007). Consequently, an influx of calcium ions causes an increase in responses to nociceptive stimuli in a nonlinear fashion (reviewed in Latremoliere and Woolf, 2009).

1.6 Animal models of neuropathic pain

Due to the complexity of neuropathic pain, research into the underlying mechanisms requires the use of experimental animal models. Although various neuropathic pain syndromes have been modelled in animals, those requiring a direct nerve injury (i.e. nerve ligation, cut or constriction) are the most commonly used. In such models, physical injury of the peripheral nerve leads to inflammation as well as axonal transport disruption. As such, axonal transport is likely to play a significant role in neuropathic pain mechanisms. Axonal transport can also be impaired along intact axons following neuritis or exposure to chemical agents, such as vinca alkaloids, which can avoid inflammatory processes. In this section, animal models induced by traumatic nerve injury, neuritis and vinca alkaloids are discussed. For each model, a description of the histology, pattern of pain behaviours and principal electrophysiological changes is given (Figure 1.3; Tables 1.7 and 1.8).

1.6.1 Traumatic nerve injury

Traumatic peripheral nerve injuries are commonly induced by nerve ligation, severance or constriction and frequently result in axonal transport disruption, Wallerian degeneration and endoneural inflammation. The most commonly used models that involve traumatic nerve injury include:

Neuroma

Neuroma is induced by tightly ligating the sciatic nerve at the level of the mid-thigh and cutting the nerve distal to the ligature (complete axotomy) or by crushing the nerve with a pair of forceps (Devor *et al.*, 1979, Wall *et al.*, 1979).

Histology

Following surgery, Wallerian degeneration occurs distal to the neuroma and at the injury site in both A- and C-fibre neurons (Devor *et al*., 1979). Proximal to the suture, a bulbous neuroma forms and contains a tangled mass of regenerating small diameter axonal sprouts (Wall *et al*., 1979, Fried and Devor, 1988, Devor and Wall, 1976). Additionally, a decrease in nerve fibre diameter has been reported proximal to the neuroma (Cragg and Thomas, 1961). At around one-week post-surgery, there is an increase in macrophage infiltration followed by expression of inflammatory mediators (Il-6, TNF-a) and neurotrophins (brain derived neurotrophic factor and nerve growth factor (NGF)) in the DRG (Kwon *et al*., 2013).

Behaviour

Following complete axotomy, autotomy of the affected limb is very common 3-9 weeks post-surgery (around 82% of rats) (Wall *et al*., 1979). Following a nerve crush, levels of autotomy are much lower (Devor *et al*., 1979), and mechanical and cold allodynia develop. Mechanical and cold allodynia are observed at three and 14 weeks post-surgery respectively and persist for approximately 52 weeks (Bester *et al*., 2000).

Electrophysiology

Ongoing activity develops in A-fibre neurons at around three days post-surgery and persists for seven weeks (Scadding, 1981, Devor, 1983, Blumberg and Janig, 1984), with around 17% of myelinated fibres firing in a rhythmic pattern (Devor and Govrin-Lippmann, 1983, Devor, 1983). Around 6% of C-fibre neurons are ongoing at 2-3 weeks post-axotomy (Welk *et al*., 1990). Mechanical sensitivity develops in around 40-66% of A-fibre neurons with ongoing activity (Chen and Devor, 1998,

Wall and Devor, 1983). In C-fibre neurons, only 13% of axons become mechanically sensitive, which peaks at three weeks (Welk *et al*., 1990). A slowing of conduction velocities proximal to the injury site is present at around one month and persists for at least 5 months (Cragg and Thomas, 1961, Devor and Govrin-Lippmann, 1986). Furthermore, chemosensitivity has been reported at the neuroma site in both A- and C- fibre neurons (Welk *et al*., 1990, Devor and Govrin-Lippmann, 1983).

Within the superficial dorsal horn, the majority of dorsal horn neurons have no receptive fields, while neurons with receptive fields show altered responses to mechanical stimulation (irregular and bursting patterns) (Hylden *et al.*, 1987).

Chronic Constriction Injury (CCI)

The CCI model was originally reported by Bennet and Xie in 1988 and was used to study pain in compressive neuropathies. Four 4-0 chromic gut sutures are loosely placed around the sciatic nerve proximal to the sciatic nerve trifurcation (mid-thigh level), leading to nerve constriction (Bennett and Xie, 1988).

Histology

CCI leads to a reduction in blood flow through the nerve's vasculature, causing swelling and strangulation of the nerve, Wallerian degeneration as well as nerve inflammation (Maves *et al.*, 1993, Bennett and Xie, 1988, Coggeshall *et al.*, 1993). Constriction of the nerve by ligature reduces the nerve diameter by 25-75%. This leads to a significant decrease in the number of large-diameter myelinated and small-diameter unmyelinated axons where a preferential loss of large fibres is observed (i.e. Aβ-fibre neurons) (Kajander and Bennett, 1992). An increase in mRNA of pro-inflammatory cytokines in the sciatic nerve of the rat is present at weeks 1 and 2 post-surgery, which include IL-1β, IL-6 (week 1) and TNF (week 2). Additionally,

elevation in anti-inflammatory cytokine IL-10 is reported at week 6 post-surgery (Okamoto *et al.*, 2001).

Behaviour

Animals that undergo CCI develop neuropathic pain behaviours as early as one day post-surgery, which peak at around 1-2 weeks post-surgery and last for up to two months. These include mechanical and cold allodynia, heat and mechanical hyperalgesia as well as chemogenic pain (Bennett and Xie, 1988, Maves *et al*., 1993, Kim *et al.*, 1997, Coggeshall *et al.*, 1993, Dowdall *et al.*, 2005). Commonly, mild to moderate autotomy, gate and posture changes, overgrown claws, guarding, licking and spontaneous lifting of ipsilateral hind paw as well as reduced rate of body weight gain are reported, which are consistent with spontaneous pain (Bennett and Xie, 1988).

Electrophysiology

Increased levels of ongoing activity have been reported in both Aβ- and Aδ-nerve fibre neurons as early as one day post-surgery (35% and 15% respectively), which corresponds to the time of onset of neuropathic pain behaviours. Such activity occurs in ongoing (tonic), bursting as well as irregular patterns and originates from the site of injury (Tal and Eliav, 1996, Kajander and Bennett, 1992). Increased levels of ongoing activity in C-fibre neurons develop at later stages (several weeks post-operative) (Xie and Xiao, 1990, Chen and Devor, 1998). Both types of axons (those with and without increased levels of ongoing activity), as well as neuronal sprouts, develop axonal mechanical sensitivity (Tal and Eliav, 1996, Chen and Devor, 1998). Additionally, the number of A-fibre neurons conducting though the treatment site is substantially reduced (55-85%), and the remaining myelinated axons are slowed

(Kajander and Bennett, 1992, Gabay and Tal, 2004). In contrast, far fewer C-fibre neurons are lost (9-32%).

Following CCI, dorsal horn neurons show abnormal characteristics, which include increased levels of ongoing activity, increased responses to mechanical stimulation of the injury site, absence of peripheral fields, reduced responsiveness to low intensity mechanical stimulation of the receptive field and prolonged after-discharge (Laird and Bennett, 1993, Sotgiu and Biella, 2000).

Partial Sciatic Nerve Ligation (PSL)

The PSL model was developed by Seltzer and colleagues (1990). In this model, dorsal 30-50% of the sciatic nerve thickness is tightly ligated using 8-0 silk sutures (Seltzer *et al.*, 1990).

Histology

Wallerian degeneration and demyelination are the features of the PSL model (Yagasaki *et al.*, 2013). At one week post-surgery, myelinated fibre density is reduced and myelin loss is present. At three weeks, the processes of remyelination and regeneration of small myelinated fibre neurons occurs. In mice, neutrophils and macrophages infiltrate the sciatic nerve following nerve injury (Liou *et al.*, 2011). Additionally, mRNA of pro-inflammatory cytokines such as IL-1β and TNF is rapidly upregulated (1-3 days post-surgery) in the distal stump of the sciatic nerve (Nadeau *et al.*, 2011).

Behaviour

Stimulus evoked pain behaviours, which include cold and mechanical allodynia as well as heat and mechanical hyperalgesia, develop at around one hour post-surgery and last for at least seven months (Seltzer *et al*., 1990, Kim *et al*., 1997). Animals

develop spontaneous pain behaviours a few hours after the surgery, which include altered gait and posture, licking, gentle biting of the skin and pulling on the nails of the affected limb (Seltzer *et al*., 1990). Some of these behavioural changes are present at mirror image sites on the contralateral limb and last for several months.

Electrophysiology

Spontaneous ongoing activity with a phasic firing pattern is a feature of this model and occurs in Aβ-, Aδ- and C-fibre neurons (Pan *et al.*, 1999, Chen *et al.*, 2002).

Extracellular recordings from the spinal cord showed increased receptive field size of spinal neurons (Takaishi *et al.*, 1996, Behbehani and Dollberg-Stolik, 1994).

Spinal Nerve Ligation (SNL)

The SNL model developed by Kim and Chung (1992) involves ligating the L5 and/or L6 spinal nerves with a 6-0 silk thread, which leaves the saphenous nerve (L3 root) and the L4 component of the sciatic nerve undamaged (Wu *et al.*, 2001, Djouhri *et al.*, 2006, Sapunar *et al.*, 2005).

Histology

One of the main features of the SNL model is the comingling of intact L4 afferents with injured L5 axons within the sciatic nerve. It is mainly axons of A-fibre neurons that undergo Wallerian degeneration (Vestergaard *et al.*, 1997, Sapunar *et al*., 2005, Roytta *et al.*, 1999). An influx of inflammatory cells (e.g. activated macrophages) into neurons leads to endoneural inflammation (Hu and McLachlan, 2002, Roytta *et al.*, 1999). Moreover, axonal sprouting has been reported distal to the ligation site, in the dorsal roots and in the sciatic nerve (low number of sprouts) (Roytta *et al.*, 1999).

Behaviour

SNL induces evoked pain behaviours that begin one day post-surgery, peak at around one week post-surgery and persist for several months (Kim *et al*., 1997, Choi *et al.*, 1994, Kim and Chung, 1992). These pain behaviours include mechanical and cold allodynia as well as heat and mechanical hyperalgesia. Additionally, deformities of the foot and associated limping are present. Spontaneous pain behaviours develop immediately after surgery and last for up to six weeks, which include overgrowth of nails, licking and guarding of the affected paw as well as pulling on the nails (Kim and Chung, 1992).

Electrophysiology

Transection of the L5 spinal nerve causes an increase in ongoing activity in 33% of A-fibre neurons 1-3 days post-surgery. By around day 21 post-surgery, the levels of ongoing activity decrease to 14% (Liu *et al.*, 2000a, Liu *et al.*, 2000b). Almost half of uninjured C-fibre neurons in the uninjured L4 spinal nerve develop ongoing activity within the first day of the lesion and persist for at least a week (Djouhri *et al.*, 2006, Wu *et al.*, 2001, Djouhri *et al.*, 2012, Boucher *et al.*, 2000). Intact L4 C-fibre neuros also display activity-dependent conduction velocity slowing (Shim *et al.*, 2007). Axonal mechanical sensitivity develops at the injury site (Liu *et al.*, 2000a).

In the spinal cord, increased ongoing activity levels are observed in WDR neurons between 1-2 weeks post-surgery. Responses to brush are increased during the first week post-surgery but decreased the following week. Wind-up in these animals is not changed (Chapman *et al.*, 1998, Suzuki *et al.*, 2001).

1.6.4 Spared Nerve Injury (SNI)

The SNI model involves transection of the tibial and common peroneal branches of the sciatic nerve distal to the sciatic trifurcation but sparing the sural branch (Decosterd and Woolf, 2000).

Histology

The comingling of healthy neurons from the sural nerve with injured neurons from the tibial and peroneal nerves is present in the DRG (Decosterd and Woolf, 2000). Nerve fibre sprouting occurs, where uninjured nerve fibres sprout into denervated skin territories (Decosterd and Woolf, 2000, Gong *et al.*, 2017, McKelvey *et al.*, 2015, Vega-Avelaira *et al.*, 2012, Cobos *et al.*, 2018).

Behaviour

Mechanical and cold allodynia, mechanical hyperalgesia as well as signs of heat hyperalgesia occur within the sural innervation area by 24 hours post-surgery and continue for more than six months (Decosterd and Woolf, 2000, Howard *et al.*, 2005, Cobos *et al.*, 2018).

Electrophysiology

Primary afferent fibres (Aδ- and C-fibre neurons) from SNI mice become mechanically sensitive and fire around 22% and 24% more action potentials in response to mechanical stimulation respectively when compared to control animals (Smith *et al.*, 2013). In rats, ongoing activity from A-fibre neurons is indicated (Suter *et al.*, 2009).

In the spinal cord, WDR neurons develop ongoing activity and are more responsive to cold stimulation of the receptive field one-week post-surgery (McKelvey *et al.*, 2015).

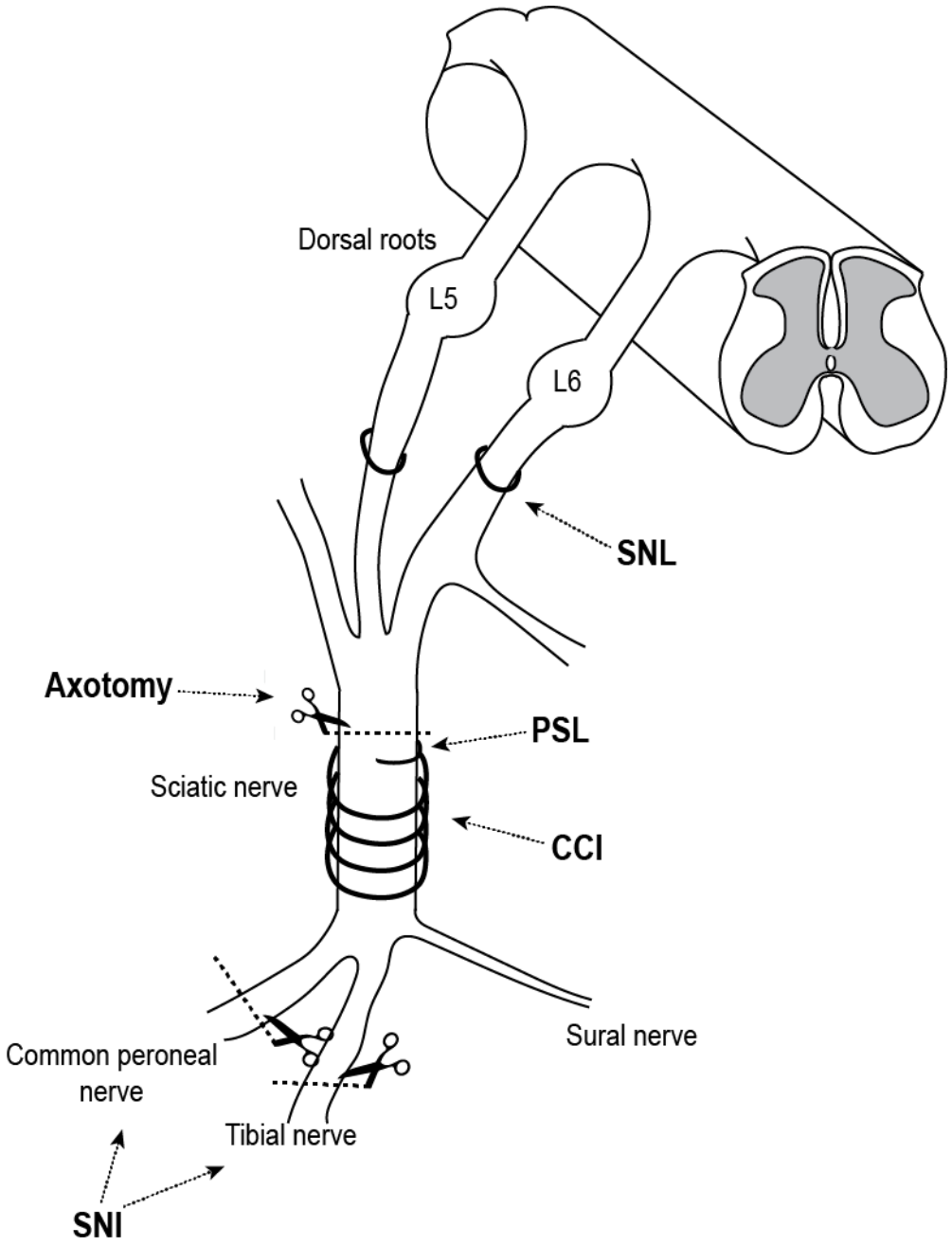


Figure 1.3 Schematic representation of the common traumatic nerve injury models used in neuropathic pain research (adapted from Suter *et al.*, 2011). Spinal nerve ligation (SNL) consists of tight L5 and/or L6 spinal nerve ligation; partial sciatic nerve ligation (PSL) is induced by a ligation of a part of the sciatic nerve; chronic constriction injury (CCI) consists of loose ligation of the sciatic nerve using chromic gut sutures; spared nerve injury (SNI) includes transection of the tibial and the peroneal branches; axotomy (neuroma) is induced by cutting or crushing the sciatic nerve.

1.6.2 Neuritis

Animal models that involve a traumatic nerve injury do not represent clinical cases where such nerve damage is not apparent during routine clinical examination (e.g. non-specific arm pain). In these patients, nerve inflammation in the absence of axonal degeneration may play a significant role. To examine the potential mechanisms in such patients, neuritis model may prove to be beneficial (Dilley *et al*., 2011, Eliav *et al.*, 1999).

Neuritis induced by complete Freund's adjuvant

The neuritis model is induced by applying an immune stimulant composed of inactivated and dried mycobacteria, complete Freund's adjuvant (CFA), to the sciatic nerve. This is performed by saturating oxidised cellulose in CFA and wrapping it loosely around the exposed sciatic nerve in the mid-thigh level (Eliav *et al*., 1999, Dilley *et al.*, 2005, Bove *et al.*, 2003).

Histology

Immune cells, which include macrophages, T-lymphocytes and granulocytes, infiltrate the epineurium within two days post-surgery, with concentrations peaking at around four weeks post-surgery (Eliav *et al*., 1999, Bove *et al*., 2003, Bove *et al.*, 2009). Additionally, raised TNF-α and chemokine (C-C motif) ligand 2 mRNA are present at the treatment site on day 1 post-surgery (Pulman *et al.*, 2013). By the first week, perineurium shows signs of oedema, which is followed by thickening and the presence of more dense fibrous tissue at later stages (Bove *et al*., 2009). None of the immune cells penetrate the endoneurium. The epineural inflammation is sustained for up to 12 weeks post-surgery with a minimal structural damage to the axons or

glia (Eliav *et al*., 1999, Bove, 2009). Additionally, anterograde axonal transport disruption is a feature of this model (Dilley *et al.*, 2013).

Behaviour

The time course of neuropathic pain behaviours is short-lived (Bennett, 1999). Mechanical and cold allodynia as well as mechanical and heat hyperalgesia of the ipsilateral hind paw develop between 2-3 days and peak between 3-5 days post-surgery (Eliav *et al*., 1999, Bennett, 2000, Bove *et al*., 2003, Pulman *et al*., 2013, Bennett, 1999). Recovery is observed 1-2 weeks post-surgery.

Electrophysiology

Nerve trunk inflammation induces ongoing activity and axonal mechanical sensitivity in functionally intact A- and C-fibre neurons, which peaks at day 4-5 post-surgery (Bove *et al*., 2003, Dilley *et al*., 2005, Bove and Dilley, 2010, Richards *et al.*, 2011). Axonal mechanical sensitivity develops at the site of inflammation mainly in nociceptive C- and Aδ-nerve fibres that innervate deep structures (Bove *et al*., 2003, Dilley and Bove, 2008b, Eliav *et al.*, 2001). These mechanically sensitive axons respond to both direct pressure and stretch (Dilley *et al*., 2005). In the most sensitive nerve fibres, 3% stretch is sufficient to trigger neuronal firing (Dilley *et al*., 2005). Axonal mechanical sensitivity peaks during the first week and recovers at two months post-surgery (Dilley and Bove, 2008a, Bove, 2009). Neuritis also causes a decrease in the conduction velocity of C-fibre neurons along the L5 dorsal root by week one, which peaks at four weeks and returns to the normal range by two months. Around 80% of axons treated with CFA develop axonal chemical sensitivity to inflammatory chemicals 6-7 days post-surgery (Govea *et al.*, 2017).

Neuritis induced by zymosan

A neuritis model can also be induced using an immune activator, zymosan, which is prepared from yeast cell wall. A strip of sterile Gelfoam is loosely wrapped around the sciatic nerve and a peri-sciatic catheter is implanted. Several days later, the Gelfoam is injected with zymosan through the catheter (Chacur *et al.*, 2001, Twining *et al.*, 2004, Gazda *et al.*, 2001).

Histology

In this model, mild to moderate subepineural oedema as well as minor Schwan cell activation occurs. Immune cells (e.g. activated macrophages and neutrophils) invade the Gelfoam and epineurium, which release IL-1β, TNF-α and reactive oxygen species (Gazda *et al.*, 2001). Raised levels of other cytokines (e.g. IL-6, IL-1) have also been reported in the sciatic nerve (Twining *et al*., 2004).

Behaviour

Dose-dependent mechanical allodynia develops three hours following zymosan administration and is still present at 24 hours (Gazda *et al*., 2001, Chacur *et al*., 2001). No changes in response to thermal stimulation are present (Gazda *et al*., 2001).

1.6.3 Vinblastine-induced axonal transport disruption

Experimentally, application of chemotherapy agents, such as vinblastine, to the sciatic nerve of the rat allows examination of the direct effects chemotherapy agents may have on neuronal tissues. Vinblastine is commonly used for the treatment of neoplastic diseases, which include lymphoblastic leukaemia, Hodgkin's and non-Hodgkin's lymphoma (Duflos *et al.*, 2002). Local application of vinblastine in low doses (e.g. 0.1 mM) for a short duration (e.g. 15 min) can disrupt axonal transport

along a peripheral nerve without causing inflammation or axonal degeneration (Fitzgerald *et al.*, 1984, Katoh *et al.*, 1992, Dilley and Bove, 2008a, Zhuo *et al.*, 1995, Kashiba *et al.*, 1992). This approach can be used to examine the role of axonal transport disruption along intact axons in neuropathic pain.

Histology

Following the local application of low doses of vinblastine (i.e. 0.1-0.15 mM), axons remain intact and there are no signs of Wallerian degeneration, axonal demyelination or nerve inflammation (Fitzgerald *et al.*, 1984, Kashiba *et al.*, 1992). At a dose of 0.15 mM, the number of myelinated and unmyelinated axons is decreased by 20%, which has been linked with increased endoneurial fluid (Fitzgerald *et al.*, 1984, Kashiba *et al.*, 1992). An examination of protein accumulation proximal to the treatment site (e.g. fluoride-resistant acid phosphatase, acetylcholinesterase and kinesin) has confirmed the disruption of anterograde axonal transport by day two post-surgery, with signs of recovery at day six (Dilley *et al.*, 2013, Fitzgerald *et al.*, 1984). Following the application of higher doses of vinblastine ($\geq$ 0.25 mM), significant demyelination and degeneration of both myelinated and unmyelinated nerve fibres have been reported (Fitzgerald *et al.*, 1984, Kashiba *et al.*, 1992). Additionally, abnormal Schwann cells, axonal debris and macrophages are present.

Behaviour

At low dose (0.1 mM), neuropathic pain behaviours occur within 24 hours post-surgery. Mechanical allodynia peaks at around day four, and recovery is observed from day seven post-surgery. By day 11, almost full recovery is evident (Dilley *et al.*, 2013). Heat and mechanical hyperalgesia does not develop (Dilley *et al.*, 2013,

Fitzgerald *et al*., 1984). At higher doses (0.25mM, 0.5 mM), animals develop insensitivity to mechanical and heat hyperalgesia (Fitzgerald *et al*., 1984).

Electrophysiology

At low dose (0.1 mM), vinblastine does not cause an increase in ongoing activity from A- or C-fibre neurons at 4-7 days post-surgery (Dilley *et al.,* 2013, Govea *et al*., 2017). There is slowing in conduction velocities in C-fibre neurons proximal to the treatment site (Dilley *et al*., 2013, Fitzgerald *et al*., 1984) as well as in both A- and C- fibres through the treatment site (Dilley and Bove, 2008a). A rapid development of axonal mechanical sensitivity has been reported. Around 33.3% of C-fibre neurons develop axonal mechanical sensitivity peaking on days 4-5 post-surgery (Dilley and Bove, 2008a). Additionally, around 38% of axons develop axonal chemical sensitivity to inflammatory chemicals 3-4 days post-surgery (Govea *et al*., 2017). Local vinblastine application proximal to a nerve injury has been used to determine the role of axonal transport in the development of axonal mechanical sensitivity (Dilley and Bove, 2008a), ongoing activity (Devor and Govrin-Lippmann, 1983) and heat hypersensitivity (Yamamoto and Yaksh, 1993).

1.6.4 Chemotherapy-induced peripheral neuropathy

As mentioned in the previous section (section 1.6.3), vinca alkaloids disrupt axonal transport and can be used to examine such mechanisms along intact peripheral nerves. Typically, vinca alkaloids are systemically injected into animals when modelling CIPN. Currently, one of the most commonly used vinca alkaloids for such purposes is vincristine (Siau and Bennett, 2006, Schappacher *et al.*, 2017, Aley *et al.*, 1996, Authier *et al.*, 2003). Below, vincristine-induced peripheral neuropathy animal model is reviewed.

Histology

Vincristine treatment leads to alterations in axonal microtubule cytoskeleton and impaired anterograde axonal transport (Tanner *et al.*, 1998a, Topp *et al.*, 2000). As a result, swelling of non-myelinated and myelinated nerve fibres is common in vincristine-induced peripheral neuropathy (VIPN) models (Tanner *et al*., 1998a, Topp *et al*., 2000, Authier *et al*., 2003, Boehmerle *et al.*, 2014). VIPN is associated with axonal degeneration of the intraepidermal nerve fibres (Siau *et al.*, 2006, Schappacher *et al*., 2017, axonal degeneration mechanisms reviewed by Fukuda *et al.*, 2017) as well as fragmentation of mitochondria (Berbusse *et al.*, 2016). Furthermore, increase in active Langerhans cells in the skin is indicative of inflammatory processes.

Behaviour

Although VIPN induction protocols vary, vincristine treatment is associated with a rapid development of cold and mechanical allodynia as well as heat and mechanical hyperalgesia in adult rats (Aley *et al*., 1996, Authier *et al*., 2003, Hu *et al.*, 2017). The time course of such behaviours fluctuates depending on the model used, but they tend to reverse 2-4 weeks following discontinuation of treatment in adult animals.

Electrophysiology

An increase in the levels of ongoing activity have been reported in both A- and C-fibre neurons, with 10% and 36% firing spontaneously respectively at the peak of neuropathic pain behaviours (Xiao and Bennett, 2008). Such ongoing activity occurs in continuous or bursting irregular firing patterns. Both non-myelinated and myelinated nerve fibres are slowed (Xiao and Bennett, 2008, Authier *et al*., 2003, Tanner *et al.*, 1998b).

In the spinal cord, WDR neurons display increased levels of ongoing activity and respond to mechanical stimulation of the receptive filed with augmented afterdischarge (Weng *et al.*, 2003). Additionally, an increase in wind-up response is present when nerve is stimulated at 0.1 Hz frequency (Weng *et al*., 2003). At higher stimulation frequencies (i.e. 0.3 and 1.0 Hz), WDR cells demonstrate wind-down following the first eight electrical stimuli.

	Neuropathic pain behaviours (present at days/weeks PO)				
	Mechanical allodynia	**Cold allodynia**	**Mechanical hyperalgesia**	**Heat hyperalgesia**	**References**
Neuroma	3-55 weeks	14-66 weeks	N/A	N/A	(Bester *et al*., 2000, Devor *et al*., 1979, Wall *et al*., 1979)
CCI	1-8 weeks	1-8 weeks	1-8 weeks	1-8 weeks	(Bennett and Xie, 1988, Maves *et al*., 1993, Kim *et al*., 1997, Coggeshall *et al*., 1993, Dowdall *et al*., 2005)
PSL	1->28 weeks	1->28 weeks	1->28 weeks	1->28 weeks	(Seltzer *et al*., 1990, Kim *et al*., 1997)
SNL	1->8 weeks	1->8 weeks	1->8 weeks	1->8 weeks	(Kim *et al*., 1997, Choi *et al*., 1994, Kim and Chung, 1992)
SNI	1->24 weeks	1->24 weeks	1- >24 weeks	1- >24 weeks	(Decosterd and Woolf, 2000, Howard *et al*., 2005, Cobos *et al*., 2018)
Neuritis (CFA)	1-7 days	2-7 days	1-7 days	1-7 days	(Eliav *et al*., 1999, Bennett, 2000, Bove *et al*., 2003, Pulman *et al*., 2013, Bennett, 1999)
Vinblastine	1-11 days	N/A	Absent	Absent	(Dilley *et al*., 2013, Fitzgerald *et al*., 1984)
VIPN	2-4 weeks (post-treatment)	2-4 weeks (post-treatment)	2-4 weeks (post-treatment)	2-4 weeks (post-treatment)	(Aley *et al*., 1996, Authier *et al*., 2003, Hu *et al*., 2017)

PO, post-operative; CCI, chronic constriction injury; PSL, partial sciatic nerve ligation; SNL, spinal nerve ligation; SNI, spared nerve injury; CFA, complete Freund's adjuvant; VIPN, vincristine-induced peripheral neuropathy; N/A, no reported studies

Table 1.7. A summary table of neuropathic pain behaviours in rat models of neuropathic pain.

	Electrophysiology of peripheral nerves			
	↑OA	**AMS**	**CV**	**References**
Neuroma	A- and C-fibre neurons	A- and C-fibre neurons	Slowed (*nerve fibre type not indicated*)	(Devor and Govrin-Lippmann, 1983, Devor, 1983, Scadding, 1981, Blumberg and Janig, 1984, Welk *et al*., 1990, Chen and Devor, 1998, Wall and Devor, 1983, Cragg and Thomas, 1961, Devor and Govrin-Lippmann, 1986)
CCI	A- and C-fibre neurons	A- and C-fibre neurons	Aβ-fibre neurons slowed	(Tal and Eliav, 1996, Kajander and Bennett, 1992, Xie and Xiao, 1990, Chen and Devor, 1998, Gabay and Tal, 2004)
PSL	A- and C-fibre neurons	N/A	N/A	(Pan *et al*., 1999, Chen *et al*., 2002)
SNL	Injured A-fibre neurons; Intact C-fibre neurons (L4)	Present at the treatment site	Activity-dependent slowing in intact C-fibre neurons (L4)	(Liu *et al*., 2000a, Liu *et al*., 2000b, Djouhri *et al*., 2006, Wu *et al*., 2001, Djouhri *et al*., 2012, Boucher *et al*., 2000, Shim *et al*., 2007)
SNI	A-fibre neurons	A- and C-fibre neurons*	Unchanged*	(Smith *et al*., 2013, Suter *et al*., 2009)
Neuritis (CFA)	Aδ- and C-fibre neurons	Aδ- and C-fibre neurons	C-fibre neurons slowed	(Richards *et al*., 2011, Bove *et al*., 2003, Bove and Dilley, 2010, Dilley and Bove, 2008a)
Vinblastine	Absent	C-fibre neurons	A- and C-fibre neurons slowed	(Dilley *et al*., 2013, Govea *et al*., 2017, Fitzgerald *et al*., 1984, Dilley and Bove, 2008a)
VIPN	A- and C-fibre neurons	N/A	A- and C-fibre neurons slowed	(Xiao and Bennett, 2008, Authier *et al*., 2003, Tanner *et al*., 1998b)

↑, increase; PO, post-operative; OA, ongoing activity; AMS, axonal mechanical sensitivity; CV, conduction velocity; CCI, chronic constriction injury; PSL, partial sciatic nerve ligation; SNL, spinal nerve ligation; SNI, spared nerve injury; CFA, complete Freund's adjuvant; VIPN, vincristine-induced peripheral neuropathy; N/A, no reported studies. * in mice

Table 1.8. A summary table of peripheral electrophysiological changes in rat models of neuropathic pain.

1.7 Current understanding of the mechanisms of neuropathic pain

Neuropathic pain is a result of a complex interplay between peripheral nervous system, central nervous system and immune system (reviewed in Ji *et al.*, 2016, Latremoliere and Woolf, 2009, Campbell and Meyer, 2006). Historically, the multifactorial nature of neuropathic pain has led to multiple challenges when trying to identify and understand the underlying neuropathic pain mechanisms. Fortunately, the development of animal models together with continuously progressing research techniques facilitates deeper understanding of such pathophysiology. In this section, current peripheral and central mechanisms of neuropathic pain are reviewed.

1.7.1 Peripheral mechanisms

Following a traumatic nerve injury, axonal transport is ceased at the injury site, which leads to degeneration of distal tissues, and immediate immune response is initiated. In attempt to re-innervate denervated tissues, axonal sprouts develop from the proximal stump of the injured nerve, which forms a neuroma (Devor and Wall, 1976). At this site, ion channels (e.g. Na_v channels) accumulate, which results in a 'hot spot' of excitability characterized by ongoing activity, axonal mechanical sensitivity as well as chemical sensitivity (Scadding, 1981, Matzner and Devor, 1994, Michaelis *et al.*, 1995, Tal and Eliav, 1996, Chen and Devor, 1998, Djouhri *et al.*, 2006, Wu *et al.*, 2001). Such alterations in neuronal excitability are associated with the symptoms of spontaneous pain (i.e. ongoing activity) as well as movement-evoked radiating pain (i.e. axonal mechanical sensitivity) commonly reported by patients (Greening *et al.*, 2005). In addition to its role in spontaneous pain, ongoing activity is considered to drive central mechanisms (Section 1.7.2) that are implicated

in the development and maintenance of neuropathic pain behaviours (Campbell and Meyer, 2006, Woolf, 2011, Xie *et al.*, 2005, Ji *et al.*, 2003). Although following a traumatic nerve injury ongoing activity firstly develops in myelinated axons, it is ongoing activity from C-fibre neurons that is considered to drive central changes (Ji and Woolf, 2001).

Ongoing activity not only develops in injured axons, but also in neighbouring intact afferents, specifically C-fibre neurons (Shim *et al.*, 2005, Wu et al., 2001, Djouhri *et al*., 2006). The role of intact afferents (those that are non-ligated, non-constricted, non-severed and through conducting) in neuropathic pain mechanisms is further supported by the neuritis model (Dilley *et al*., 2013, Bove *et al*., 2003). When nerves are inflamed, axonal transport is locally disrupted (Dilley and Bove, 2008a, Dilley *et al*., 2013). Such disruption may lead to ion channel accumulation and a formation of a 'hot spot' of excitability along otherwise uninjured axons, which leads to the development of ongoing activity and mechanical sensitivity (Dilley *et al*., 2013, Dilley and Bove, 2008a, Bove *et al*., 2003, Govea *et al*., 2017, Eliav *et al*., 2001, Dilley *et al*., 2005, Dilley and Bove, 2008b). Generally, sensitization of afferent nerve fibres highly depends on a decrease in the threshold for sodium channel activation (Devor, 2006) and altered expression/excitatory properties of other ion channels (refer to Section 1.3). In contrary, modulation of ion channel expression or function (e.g. a slowing of sodium channel kinetics or a decrease in channel density) may underlie conduction velocity slowing, which occurs not only along injured axons (Gallego and Adrover, 1990) but is also observed along intact nociceptors (Dilley *et al*., 2005, Shim *et al*., 2007, Dilley and Bove, 2008b).

In addition to changes in neuronal excitability, several studies have suggested a role for a phenotypic switch of nerve fibres, whereby Aβ-fibre neurons start to express

and release neuropeptides such as brain-derived neurotrophic factor or substance P (Fukuoka *et al.*, 2012, Noguchi *et al.*, 1995). In normal conditions, these are expressed by nociceptive neurons. Phenotypic switch theory is in agreement with an increase in ongoing activity from large myelinated fibres following nerve injury (Boucher *et al*., 2000), which could potentially lead to central sensitization, since such fibres now act as nociceptors.

Peripheral nerve damage leads to an immediate cascade of immune responses. Inflammatory mediators, such as substance P and calcitonin gene-related protein, are released from nociceptive terminals leading to increased vascular permeability and formation of oedema. As a result, endogenous factors released by non-neuronal cells (e.g. bradykinin, NGF, cytokines, chemokines, proteins and lipids) accumulate and sensitise nociceptors (reviewed in McMahon *et al.*, 2005, Julius and Basbaum, 2001, Ji *et al*., 2016). One of the earliest non-neuronal cells to respond to nerve injury are mast cells, which release neuronal mediators, such as histamine and TNF-α, at the lesion site (Olsson, 1967). These mediators act directly on nerve fibres, which enhance their activity, and also recruit more immune cells (e.g. neutrophils and macrophages) (Zuo *et al*., 2003). Similarly, recruited cells release cytokines (e.g. TNF-α, IL-1β) that contribute to neuropathic pain behaviours via modulation of ion channel activity (DeFrancesco-Lisowitz *et al*., 2015, Schuh *et al*., 2014, Zelenka *et al*., 2005). Peripheral nerve injury not only initiates the innate immune response, but also activates cells of the adaptive immune system (reviewed in Austin and Moalem-Taylor, 2010). An example of such activation is infiltration of T-cells into the injured peripheral nerves (Moalem *et al.*, 2004) and the soma of dorsal root ganglion neurons (Vicuna *et al.*, 2015).

Another type of immune cells that play a role in neuropathic pain mechanisms is glial cells. In the periphery these include Schwann and satellite cells (reviewed in Ji *et al.*, 2013). For example, Schwann cells stimulate immune cell infiltration into injured nerves and release pro-inflammatory mediators, while satellite glial cells produce cytokines at the soma of dorsal root ganglion neurons (Zhang *et al.*, 2007). In fact, a recent study by Lim and colleagues (2017) has demonstrated that peripheral nerve injury induced satellite cell activation leads to production of colony stimulating factor 1 in the dorsal root ganglion of the mouse, subsequently activating microglia in the spinal cord.

In agreement with previous reports, a role for immune system in peripheral mechanisms of neuropathic pain has been elegantly demonstrated in mice (Cobos *et al*., 2018). Following a peripheral nerve injury, animals lacking T-cells or macrophages did not develop mechanical allodynia. Such effect was independent of TRPV1-expressing nociceptors, which is indicative of large-diameter primary afferent fibre involvement in the underlying mechanisms (Xu *et al.*, 2015), whereby they may be sensitized by the immune system.

1.7.2 Central mechanisms

As mentioned in a previous section (Section 1.7.1), peripheral nerve injury leads to the hyperexcitability of primary sensory neurons as well as immune cell activation. Such processes are considered to drive alterations within the central nervous system that underlie neuropathic pain. The principal spinal cord mechanism is central sensitisation, which is defined as an increase in the excitability of dorsal horn neurons (Woolf, 2011, Woolf, 2014).

Intracellular changes

The underlying mechanisms of increased dorsal horn excitability involve a process similar to that of long term potentiation (Figure 1.4) (Randic *et al.*, 1993, Woolf, 2011). Repetitive nociceptive peripheral input activates NMDA (Woolf and Thompson, 1991) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Gu *et al.*, 1996). The resulting temporal summation of postsynaptic depolarization can relieve the Mg^{++} block of NMDA channels and in turn allow influx of calcium ions. Such increase in intracellular calcium levels promotes activation of calcium dependent kinases that phosphorylate receptors and ion channels (Esteban *et al.*, 2003, Chen and Huang, 1992) as well as activate signalling pathways (e.g. extracellular-signal-regulated kinase pathway) (Kawasaki *et al.*, 2004, Hu *et al.*, 2007). In turn, the distribution and function of ion channels is affected so that cell excitability is enhanced. This means that low level C-fibre neuron input, which is not sufficient to activate dorsal horn neurons in normal conditions, can now cause their depolarization as a result of an increase in synaptic efficacy.

Similar mechanisms that lead to intracellular calcium increase are also implied in the wind-up response of WDR neurons (section 1.5.2) (Woolf, 1996). Although wind-up has many similarities with central sensitisation, such as expanded receptive fields and increased responsiveness to C-fibre stimulation, the two phenomena are not the same. While central sensitization persists after the conditioning stimuli, wind-up only manifests during such stimulation. Furthermore, central sensitization represents a form of heterosynaptic potentiation leading to amplification of responses of non-conditioned nerve fibres (Latremoliere and Woolf, 2009). This is remarkably different from wind-up, which is an example of homosynaptic potentiation, whereby

amplified responses are observed only in conditioned nerve fibres. Therefore, it is important to highlight that central sensitisation is distinct from wind-up and the latter is instead thought to represent a short-lasting activity-dependent synaptic plasticity (Woolf, 1996, Li *et al.*, 1999, Ji *et al.*, 2003). Wind-up can precede central sensitisation but is not required for the latter process to occur (Woolf, 1996).

Alterations within neuronal circuits

In addition to intracellular changes, neuronal circuits within the dorsal horn are also affected (Figure 1.5). Early studies into neuronal connections within the dorsal horn indicated formation of additional Aβ-fibre sprouts that synapse onto neurons in the superficial laminae (Woolf *et al.*, 1992, Lekan *et al.*, 1996, Shortland *et al.*, 1997). Historically, such theory received a lot of interest. However, this mechanism has been refuted (Hughes *et al.*, 2003), since the presence of such arborisations in the superficial lamina of healthy adult mice has been demonstrated (Woodbury *et al.*, 2008). A theory of Aβ-fibre sprouting is currently outweighed by the evidence for Aβ-fibre gating of nociceptive circuits, which was first proposed by the Gate Control Theory of Pain (Melzack and Wall, 1965). This theory was revisited in recent years and suggests that projection cells receive inputs not only from nociceptors but also LTMRs, which in normal conditions are gated via feedforward activation of inhibitory interneurons (Duan *et al.*, 2014, Christensen *et al.*, 2016, Cheng *et al.*, 2017, reviewed in Duan *et al.*, 2018). There are several inhibitory interneurons identified, mainly in laminae II and III, which gate the polysynaptic Aβ-fibre pathways. These include cells expressing parvalbumin, receptor tyrosine kinase, dynorphin as well as glycinergic interneurons (Foster *et al.*, 2015, Petitjean *et al.*, 2015, Cui *et al.*, 2016, Duan *et al.*, 2014). In neuropathic pain states, disinhibition of such circuits occurs via a variety of different mechanisms allowing Aβ-fibre

mediated activation of nociceptive inputs (Takazawa and MacDermott, 2010, Petitjean *et al*., 2015). Disinhibition can occur as a result of: a decrease in the synthesis and release of inhibitory neurotransmitters (Moore *et al.*, 2002, Scholz *et al.*, 2005, Ibuki *et al.*, 1997); desensitization of GABA and glycine receptors (Lin *et al.*, 1996); reduction in the number of inhibitory synapses on to excitatory cells (Petitjean *et al*., 2015); lowered expression of the potassium-chloride exporter KCC2, which leads to an increase in intracellular Cl^- concentration leading to impaired GABAergic/glycinergic transmission (Zhang *et al.*, 2008). A loss of inhibitory interneurons within the dorsal horn has also been previously suggested (Scholz *et al*., 2005) however it is still debatable whether physical neuronal loss actually occurs.

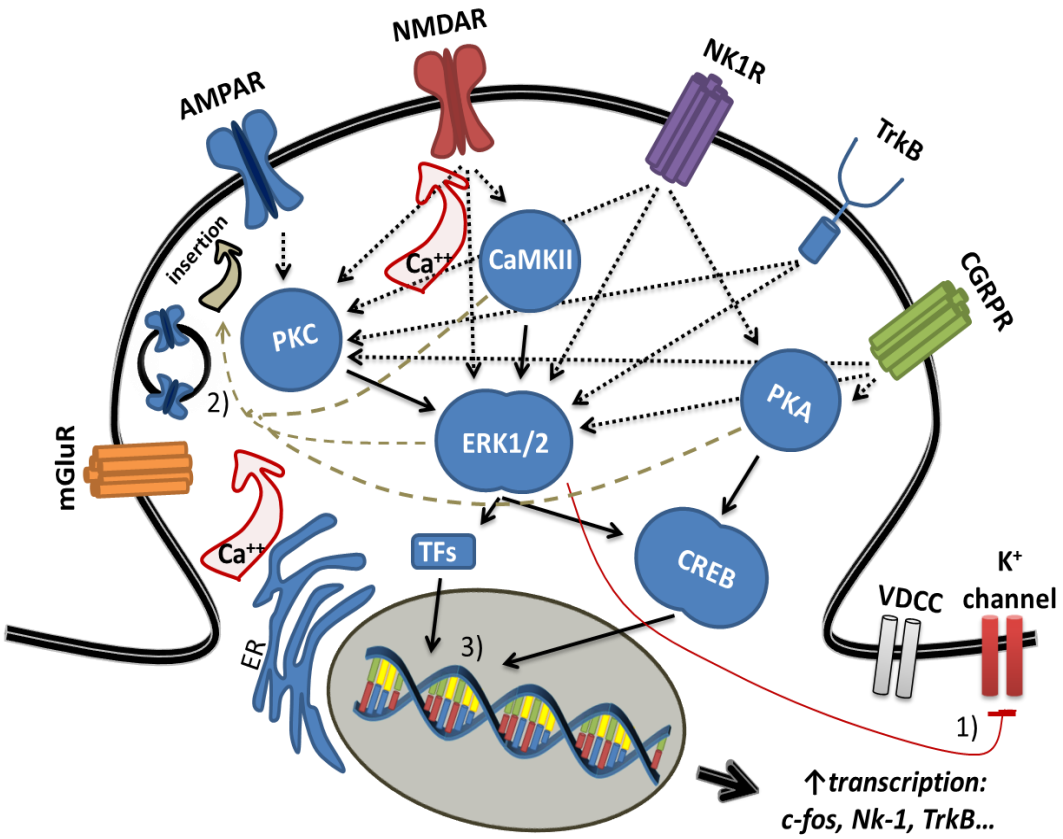


Figure 1.4 Key intracellular mechanisms that play a role in central sensitization (adapted from Latremoliere and Woolf, 2009).

Immune responses

The key non-neuronal spinal cord cells that are responsible for the pathogenesis of neuropathic pain are microglia, astrocytes and oligodendrocytes. Microglia cells are activated following both peripheral nerve injury and inflammation (reviewed in Inoue and Tsuda, 2018). Once activated, they release pro-inflammatory mediators (e.g. TNF-α, IL-1β, IL-18, brain-derived neurotrophic factor) that can enhance nociceptive transmission via effects on neuronal circuits. For example, an increase in brain-derived neurotrophic factor levels leads to inactivation of inhibitory GABAergic neurons (Coull *et al.*, 2005, Coull *et al.*, 2003), while TNF-α or IL-1β can enhance excitatory synaptic transmission (Kawasaki *et al.*, 2008). Such

Figure 1.4 Key intracellular mechanisms that play a role in central sensitization (adapted from Latremoliere and Woolf, 2009). When nociceptive neurons fire, neurotransmitters such as glutamate, substance P, CGRP and BDNF are released from pre-synaptic terminals to act on postsynaptic receptors. Following continuous nociceptive input the consequent membrane depolarization relieves magnesium block from NMDARs. These receptors are then activated by glutamate allowing influx of calcium ions. Calcium is also released from endoplasmic reticulum (ER) following activation of mGluRs, which are coupled to ER calcium channels. An increase in intracellular calcium leads to activation of various kinases and intracellular signalling pathways (i.e. PKC; PKA; CaMKII; ERK; indicated by black dotted arrows). As a result, phosphorylation-induced changes occur in the threshold and activation kinetics of NMDAR and AMPAR contributing to increased synaptic efficacy. Other changes include: 1) ERK-reduced potassium currents and increased membrane excitability; 2) PKA, CaMKII and ERK-induced recruitment of AMPAR to the membrane (indicated by brown dashed arrow); and 3) CREB and TF initiated expression of various genes needed for synapse strengthening. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDNF, brain derived neurotrophic factor; CaMKII, calcium/calmodulin-dependent protein kinase type II; CGRP, calcitonin gene related peptide; CREB, cyclic adenosine monophosphate response element-binding protein ERK, extracellular signal-regulated kinases; NMDAR, N-Methyl-D-aspartic acid receptor; PKA, protein kinase A; PKC, protein kinase C; VDKK, voltage-dependent calcium channels.

modulation of dorsal horn excitability has been demonstrated to be sex dependent, whereby the severity of mechanical allodynia can be modulated by manipulating the activity of microglia in male but not female mice (Sorge *et al.*, 2015, Sorge *et al.*, 2011). It has been suggested that in female mice adaptive immune system is responsible for neuropathic pain (Sorge *et al.*, 2015, Sorge *et al.*, 2011). Such argument is in agreement with higher expression of T-cells in the periphery of female mice (Scotland *et al.*, 2011) as well as T-cell infiltration into the female rat's dorsal horn following nerve injury (Costigan *et al.*, 2009). Similarly to glial cells, astrocytes enhance neuropathic pain transmission within the spinal cord (Sant'Anna *et al.*, 2016). Following peripheral nerve injury, these cells start to proliferate (Tsuda *et al.*, 2011) and express pro-inflammatory cytokines (e.g. IL-1β) as well as chemokines (reviewed in Ji *et al.*, 2014). A study by Chen and colleagues (2014) has demonstrated that levels of connexin 43, which in normal concentrations facilitates gap junction intracellular communication, are elevated in injured mice. Such increases in concentration of connexin 43 were shown to maintain neuropathic pain behaviours via the release of glutamate, adenosine triphosphate and chemokines. Although microglia and astrocytes have an established role in neuropathic pain, the role for oligodendrocytes is just emerging. For example, it was recently demonstrated that following peripheral nerve injury in mice, oligodendrocytes overexpress IL-33 leading to an increase in pro-inflammatory cytokines (i.e. TNF-α and IL-1β) and development of mechanical hyperalgesia (Zarpelon *et al.*, 2016).

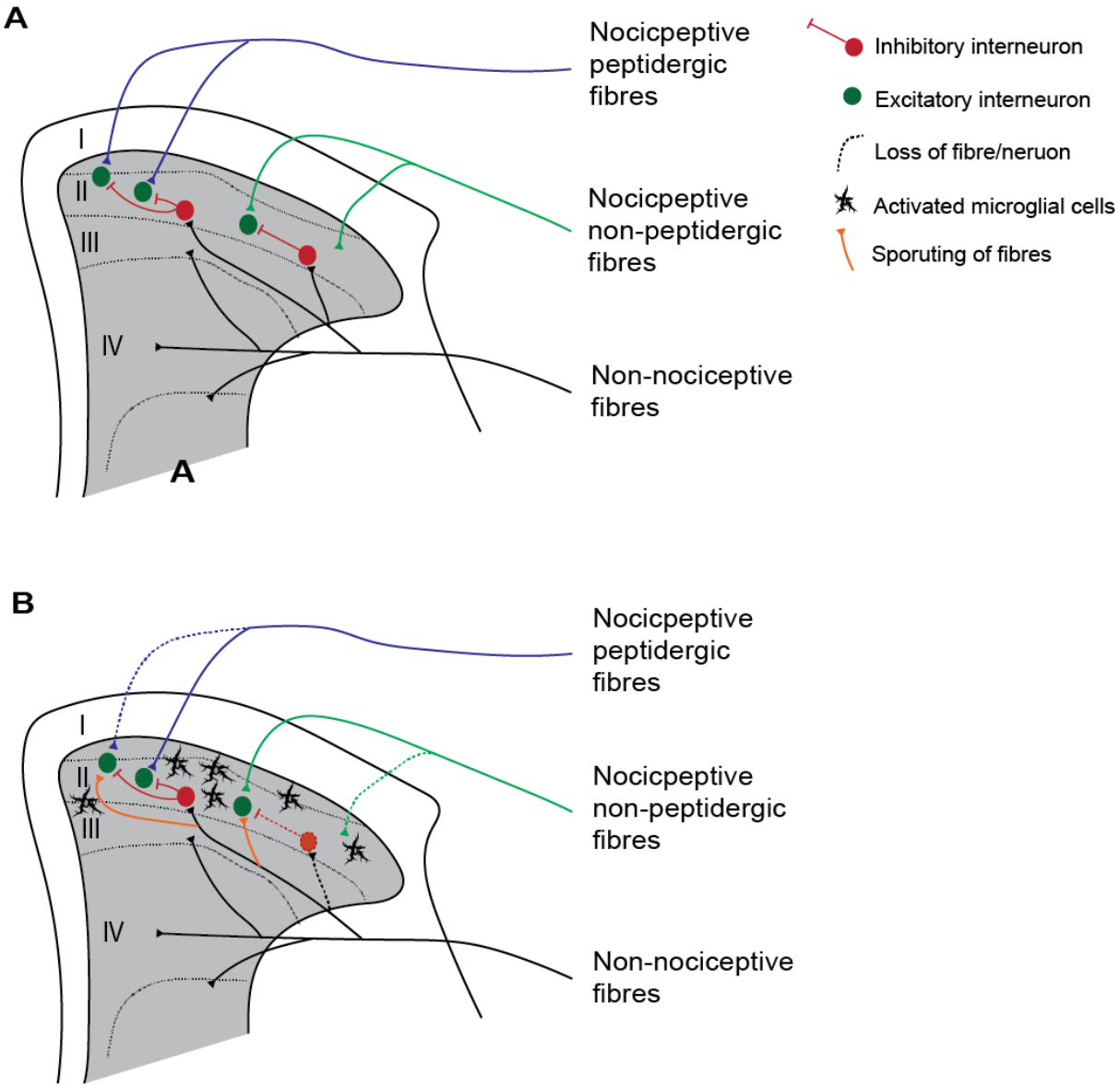


Figure 1.5 Changes within the dorsal horn in neuropathic pain conditions (adapted from Latremoliere and Woolf, 2009). A schematic representation of the dorsal horn: A) In normal conditions, nociceptive peptidergic and non-peptidergic fibres terminate in laminae I-II and contact excitatory interneurons. Non-nociceptive fibres terminate in laminae II-V and contact inhibitory interneurons. B) In neuropathic pain conditions, a number of alterations occur within the dorsal horn that lead to central sensitization. These include increased synaptic efficacy between primary sensory afferents and dorsal horn neurons, altered gating mechanisms, which enable Aβ-fibre driven activation of superficial dorsal horn neurons, and activation of microglial cells. Historically, it has been also suggested that non-nociceptive large diameter fibres develop sprouts to the superficial laminae and that there is a loss of inhibitory interneurons. Such theories remain controversial.

1.8 The 3Rs and neuropathic pain research

Due to the complex nature of neuropathic pain, research in the context of the whole nervous system is essential, and the use of live animals is hard to avoid. The most commonly used animal models in the pain field are rodent traumatic nerve injury models (Bennett and Xie, 1988, Seltzer *et al*., 1990, Kim and Chung, 1992, Decosterd and Woolf, 2000). Induction of these models requires surgery, which in some cases can be extensive and involve removal of substantial muscle or bone (Kim and Chung, 1992). In addition, the sciatic nerve, sciatic nerve branches or spinal nerves are often crushed, ligated or cut, which leads to significant axonal degeneration and demyelination. Animals undergoing such surgeries develop not only neuropathic pain behaviours, but also display signs of adverse effects, which are long lasting and barely reversible. These include muscle weakness, paralysis, altered gait (Bennett and Xie, 1988, Seltzer *et al*., 1990, Kim and Chung, 1992) and autotomy (Bennett and Xie, 1988, Zeltser *et al.*, 2000). In addition, porphyrin staining (Bennett and Xie, 1988), weight loss (Bennett and Xie, 1988), and changes in grooming activities (Bennett and Xie, 1988, Kim and Chung, 1992, Seltzer *et al*., 1990) are commonly observed. Although evoked pain is less frequent in patients, and the most common feature is spontaneous pain (Maier *et al.*, 2010, Backonja and Stacey, 2004), the majority of neuropathic pain studies that use rodents focus on measuring withdrawal reflexes to sensory stimuli (Mogil and Crager, 2004) and not complex ethologically relevant behaviours.

Translation from animal models to patients is relatively poor in the pain field and in part reflects a lack of understanding of the underlying mechanisms (Percie du Sert and Rice, 2014, Mao, 2012). Traumatic nerve injuries used to induce neuropathic pain models in rodents are not commonly observed in the clinic, and intact sensory

neurons may play an important role (Dilley and Greening, 2012). Therefore, there is a need for better refined and less severe animal models as well as measure outcomes that are clinically relevant.

1.9 Aims of the project

Axonal transport disruption following a traumatic nerve injury or chemotherapy treatment is unavoidable. Additionally, evidence suggests that local axonal transport disruption along intact, through conducting, inflamed axons may contribute to the development of neuropathic pain in those patients who do not have signs of a nerve injury on clinical examination. This has led to the main aim of this project, which is to investigate the role of axonal transport disruption along intact axons in both peripheral and central mechanisms of neuropathic pain. To achieve this, the following hypotheses are proposed:

1) It is hypothesised that local axonal transport disruption in rats will lead to the development of short-lived neuropathic pain behaviours, similarly to the neuritis model.
    - It has already been reported that vinblastine-induced axonal transport disruption in rats causes the development of short-lived mechanical allodynia, which is comparable to that observed following neuritis. However, currently there is negligible evidence for the presence of other neuropathic pain behaviours in this model. Therefore, to gain a better idea of the extent of neuropathic pain behaviours, the development of both stimulus-evoked and ethologically relevant behaviours will be assessed following vinblastine-induced axonal transport disruption and compared to the neuritis model.

2) It is hypothesised that inflammation is necessary for the development of ongoing activity, and that it is not a feature of vinblastine-induced axonal transport disruption.
    - It has been shown that neuritis leads to an increase in ongoing activity of C-fibre neurons at the peak of pain behaviours (days 4-5 post-surgery). However, a similar increase seems to be absent following vinblastine treatment. Currently, it is not known whether ongoing activity is elevated at other time points post-vinblastine treatment. Therefore, as part of this study, ongoing activity from A- and C-fibre neurons will be assessed from 3-4 hours to 15 days following vinblastine-induced axonal transport disruption and compared to neuritis.
3) It is hypothesised that local axonal transport disruption and neuritis will lead to an increase in ongoing activity and wind-up in deep WDR neurons, as well as an augmented responsiveness to mechanical stimulation.
    - The role of WDR neurons in neuropathic pain is not yet entirely clear. Previous studies have shown changes in the excitability of these neurons following nerve injury and chronic hind paw inflammation, which are consistent with the development of central sensitisation. Therefore, to determine whether similar signs of increased excitability occur following vinblastine treatment and neuritis, recordings will be made from WDR neurons in deep laminae at the peak of neuropathic pain behaviours (i.e. 3-6 days post-surgery). Ongoing activity levels, wind-up responses and responses to a range of mechanical stimuli will be assessed as measures of altered excitability.

4) It is hypothesised that local axonal transport disruption as well as neuritis will lead to increased activity of superficial dorsal horn neurons.
    - Following activation of nociceptive nerve fibres, dorsal horn neurons within superficial laminae express c-fos. In models of neuropathic pain, this expression is further increased, and as such c-fos is considered an indirect measure of sensitisation. Therefore, to determine whether there are signs of increased excitability within the superficial dorsal horn in vinblastine treated and neuritis animals, c-fos immunolabelling will be performed following electrical stimulation of the sciatic nerve at an intensity that activates nociceptors.
5) It is hypothesised that following local axonal transport disruption, and neuritis, peptidergic nociceptor transmission will be altered.
    - Following activation of primary peptidergic nerve fibres, there is a release substance P from their central terminals, and this release is altered in models of nerve injury and chronic hind paw inflammation. Therefore, to assess whether nociceptor transmission within the dorsal horn is also altered following vinblastine treatment and neuritis, substance P immunolabelling of the superficial dorsal horn will be performed at the peak of neuropathic pain behaviours (i.e. 4-5 days post-surgery).

CHAPTER 2:

Materials and Methods

Experiments were carried out in a strict accordance with the UK Animals (Scientific Procedures) Act (1986) and Home Office guidelines. Adult male Sprague Dawley rats (n = 213; 175-500g; Charles River Laboratories, Kent, UK) were used in this study, which avoids the possible quagmire associated with the oestrus cycle in female rodents. Rats were housed in groups of 2-4 in cages (50 x 35 x 26 cm) with sawdust, soft nesting material, soft wood blocks and plastic tubes. They were given *ad libitum* access to food and water.

2.1 Surgery

Surgeries were performed under aseptic conditions using isoflurane anaesthesia. Anaesthesia was induced with 3.5% isoflurane in 3 l O_2 and maintained at 2% isoflurane in 2 l O_2, and the animal was kept on a heat mat throughout the surgery. During this project, preliminary studies were carried out to examine the use of ultrasound to accurately deliver substances around the sciatic nerve without the need for surgery (Appendix 1). However, due to variability that occurred with agent delivery, a surgical approach was utilized throughout the study.

2.1.1 Vinblastine surgery

Vinblastine was applied to the sciatic nerve of adult Sprague Dawley rats (n = 56) as previously described (Dilley and Bove, 2008a) (Figure 2.1). Under general anaesthesia, the left thigh was shaved and cleaned with 70% ethanol, and the exposed skin was disinfected with Videne antiseptic aqueous solution (Williams Medical Supplies, Rhymney, UK). The area was draped with sterile drapes, and a small incision through the skin was made posterior to the femur. By blunt dissection, the biceps femoris and the vastus lateralis muscles were separated exposing the sciatic nerve. An 8 mm length was cleared from its connective tissue, which allowed

a short length of sterile parafilm (6 x 20 mm; Sigma Aldrich, Dorset, UK) to be positioned under the nerve using curved forceps to prevent leakage of vinblastine onto the surrounding tissue. The sciatic nerve was loosely wrapped with a strip of sterile Gelfoam (5 x 2 x 10 mm; SpongostanTM; Ferrosan, Denmark) saturated in approximately 150 µl of 0.1 mM vinblastine (Sigma Aldrich, Dorset, UK; diluted in 0.9% w/v sterile saline). Both the Gelfoam and parafilm were carefully removed after 15 minutes. The nerve was thoroughly rinsed with sterile saline, and the muscle and skin were closed using 4-0 monofilament sutures (Vicryl; Ethicon, West London, United Kingdom). The wound was cleaned with sterile saline and sprayed with antiseptic betadine topical spray (Williams Medical Supplies, Rhymney, UK). The animal was allowed to recover on a heat mat and was transferred to a clean cage. An additional group of animals (n = 39) underwent an identical surgery, except that the Gelfoam was saturated in 0.09% w/v sterile saline.

Animals were monitored immediately after the surgery, at four hours and daily for adverse effects. Appearance of the nest, grooming behaviours and interactions with cage-mates were inspected. Additionally, animals were placed on a flat surface to observe their gait and to look for signs of side effects, such as paralysis, foot guarding or indicators of stress (porphyrin staining around the eyes and nose, piloerection, weight loss).

2.1.2 Neuritis surgery

Neuritis was induced as previously described (n = 57; Figure 2.1) (Dilley and Bove, 2008b). Under general anaesthesia, the left thigh was shaved and cleaned with 70% ethanol. The exposed skin was disinfected with Videne antiseptic aqueous solution (Williams Medical Supplies, Rhymney, UK), and the area was draped with sterile drapes. A small incision through the skin was made posterior to the femur. The

biceps femoris and vastus lateralis muscles were then separated by blunt dissection, exposing the sciatic nerve. An 8 mm length was cleared from the surrounding connective tissue. A sterile strip of Gelfoam (5 x 2 x 10 mm) saturated in approximately 150 µl of 50% CFA (diluted in 0.9% w/v sterile saline) was loosely wrapped around the nerve using curved forceps (Figure 2.1). The muscle and skin were closed using 4-0 monofilament sutures, and the wound was cleaned with sterile saline and sprayed with antiseptic betadine topical spray (Williams Medical Supplies, Rhymney, UK). The animal was allowed to recover on a heat mat and was transferred to a clean cage. Monitoring of adverse effects and signs of stress was performed as described above (see section 2.1.1).

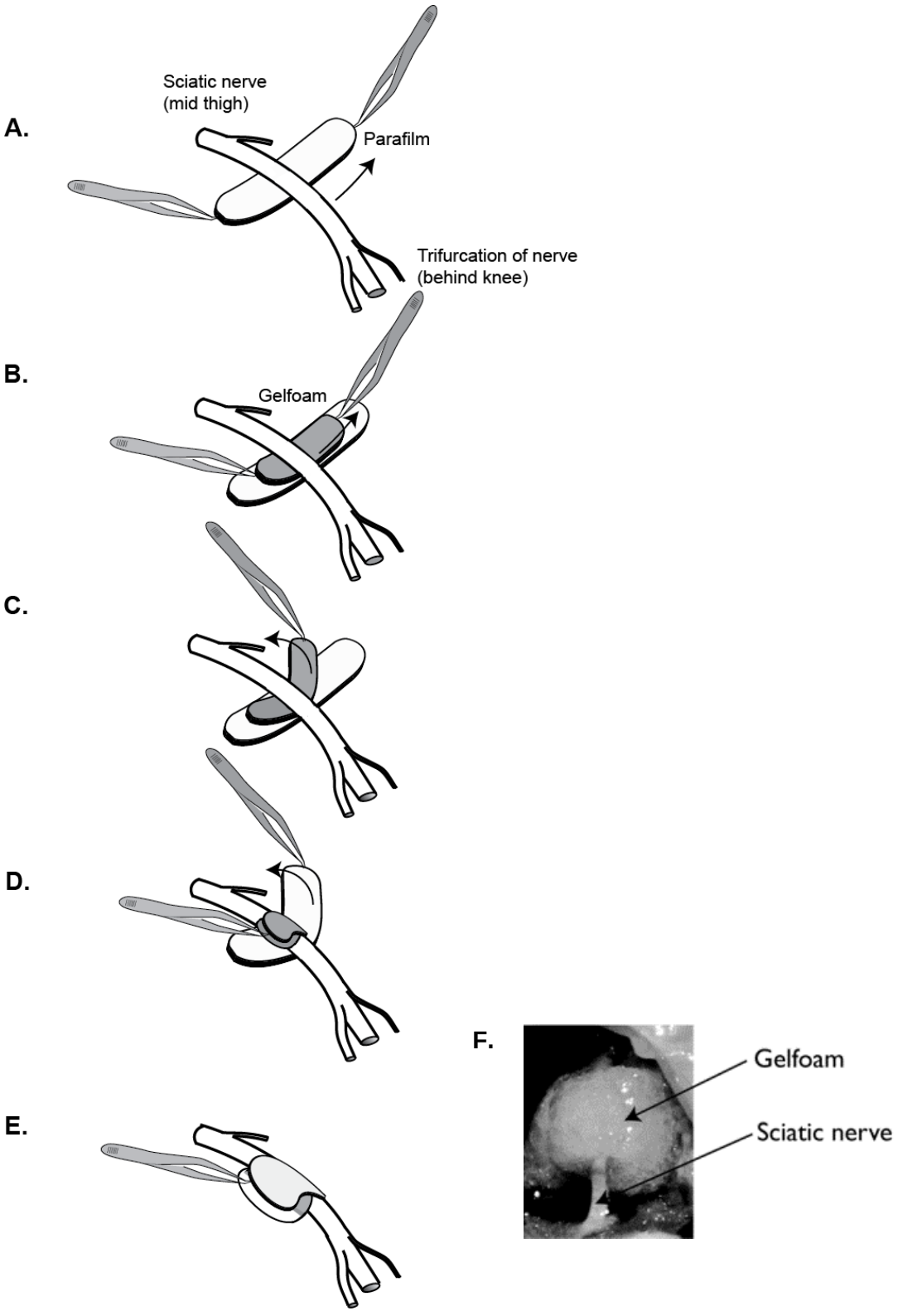


Figure 2.1 Surgery techniques.

2.1.3 Intraperitoneal injections

Unanesthetised animals were injected i.p. with 150 µl of 0.1 ml 0.1 mM vinblastine (n = 6) or 0.9%w/v saline (n = 6) using a 25G sterile hypodermic needle, which was equivalent to the volume of each agent applied to the Gelfoam during the surgery (described above).

Figure 2.1 Surgery techniques. A. A strip of sterile parafilm is placed under the sciatic nerve that is cleared of its connective tissue; B-D. Sterile Gelfoam saturated in 0.1 mM vinblastine or 0.9% w/v sterile saline is placed above parafilm and is loosely wrapped around the sciatic nerve; E. Parafilm is wrapped around the Gelfoam. Both the Gelfoam and parafilm are removed after 15 minutes and the nerve is rinsed with sterile saline. The wound is then closed, and the animal is allowed to recover. F. Image of the neuritis surgery site (A. Dilley). Neuritis surgery is performed by carefully wrapping sterile Gelfoam saturated in compete Freund's adjuvant around the nerve. The wound is then closed, and the animal is allowed to recover.

2.2 Behavioural testing

Behavioural testing was always performed at the same time on each day during the light period of the circadian cycle.

2.2.1 Stimulus-evoked measures

Mechanical and cold allodynia as well as mechanical and heat hyperalgesia were tested in animals. Testing of allodynia involved the application of stimuli to the plantar surface of the foot that were not considered to be noxious in non-lesioned animals (i.e. von Frey monofilaments and acetone), whereas testing of hyperalgesia involved the application of stimuli that could be perceived as painful (i.e. noxious heat and punctate stimuli). Each animal (n = 36) was habituated to the test apparatus (i.e. acrylic glass enclosures raised on a metal perforated floor and those raised on clear glass flooring) for one hour on three consecutive days before the start of the testing period. On the day of testing, animals were acclimatized for 15 minutes to allow exploration and major grooming activities to cease. For each behaviour both hind paws were tested with a five minute rest period between sides. The side to be tested first was randomized, except for heat hyperalgesia, where the ipsilateral side was initially examined, since in previous studies the second foot to be tested tended to respond faster than the first (Pulman *et al*., 2013). Six animals were examined in each treatment group. Cold allodynia, heat hyperalgesia and mechanical hyperalgesia were tested on the same animals with a 60 minute rest period between each test. Following i.p. injection of vinblastine or saline, the same animals were tested for mechanical and cold allodynia. Baseline values were obtained on three separate days prior to surgery or injection. All behaviours were tested up to three weeks post-

surgery or post-i.p. injection (see Figure 2.2 for timelines of experimental procedures).

2.2.1.1 Mechanical allodynia

Mechanical allodynia was assessed using calibrated von Frey monofilaments of increasing stiffness (0.4, 0.7, 1.2, 2, 4, 6, 9, 15 g; Ugo Basile, Varese, Italy) on day 1, 4, 5, 6, 7, 8 and 11 post-surgery. Animals that were injected i.p. with either vinblastine or saline were examined on day 1, 2, 4, 9, 14 and 21 post-injection. Animals were placed into individual acrylic glass enclosures raised on a metal perforated floor. Von Frey monofilaments were applied perpendicular to the plantar surface of the glabrous foot and held for five seconds. A positive response was recorded if the paw was sharply withdrawn, held or licked. Paw movements due to postural changes were not considered to be positive responses. The 50% paw withdrawal threshold was determined using the up-down method (Chaplan *et al.*, 1994, Dixon, 1980). Testing commenced with the 2.0 g monofilament. If no response was initiated, von Frey monofilaments of increasing stiffness were presented. A negative response followed by the first positive response was considered the response threshold. At the response threshold, hairs of decreasing stiffness were applied to the point where there was no positive response. At this point, von Frey monofilaments of increasing stiffness were applied until the next positive response was recorded. This pattern was repeated moving up and down von Frey monofilaments until a maximum of four stimuli had been applied after the response threshold. If all responses were only negative or positive, values of 15.0 g or 0.4 g were assigned respectively. The following formula was used to determine the 50% paw withdrawal threshold: 50% g threshold = $(10^{[Xf+k\delta]})/10{,}000$; where Xf = value of the final von Frey monofilament used (in log units); k = tabular value for

the pattern of positive/negative responses (Chaplan *et al*., 1994); δ = mean difference in log units between stimuli (here, 0.224). There was a one minute interval between each von Frey monofilament application.

2.2.1.2 Cold allodynia

Cold allodynia was tested on day 1, 2, 3, 4, 8, 14 and 21 post-surgery using the acetone test as previously described (Choi *et al*., 1994). Regarding animals that were injected i.p. with either vinblastine or saline, cold allodynia was examined on day 1, 2, 4, 9, 14 and 21 post-injection. An acetone bubble (approx. 10 µl) was formed at the end of a polyethylene tube that was connected to a syringe. With the animals in individual acrylic glass enclosures, the bubble was carefully applied to the plantar surface of the foot through the perforated floor. It was important to ensure that the tubing did not touch the skin. A brisk foot withdrawal was considered a positive response. Acetone was applied five times to each paw at 2.5 minute intervals. The percent paw withdrawal frequency was calculated.

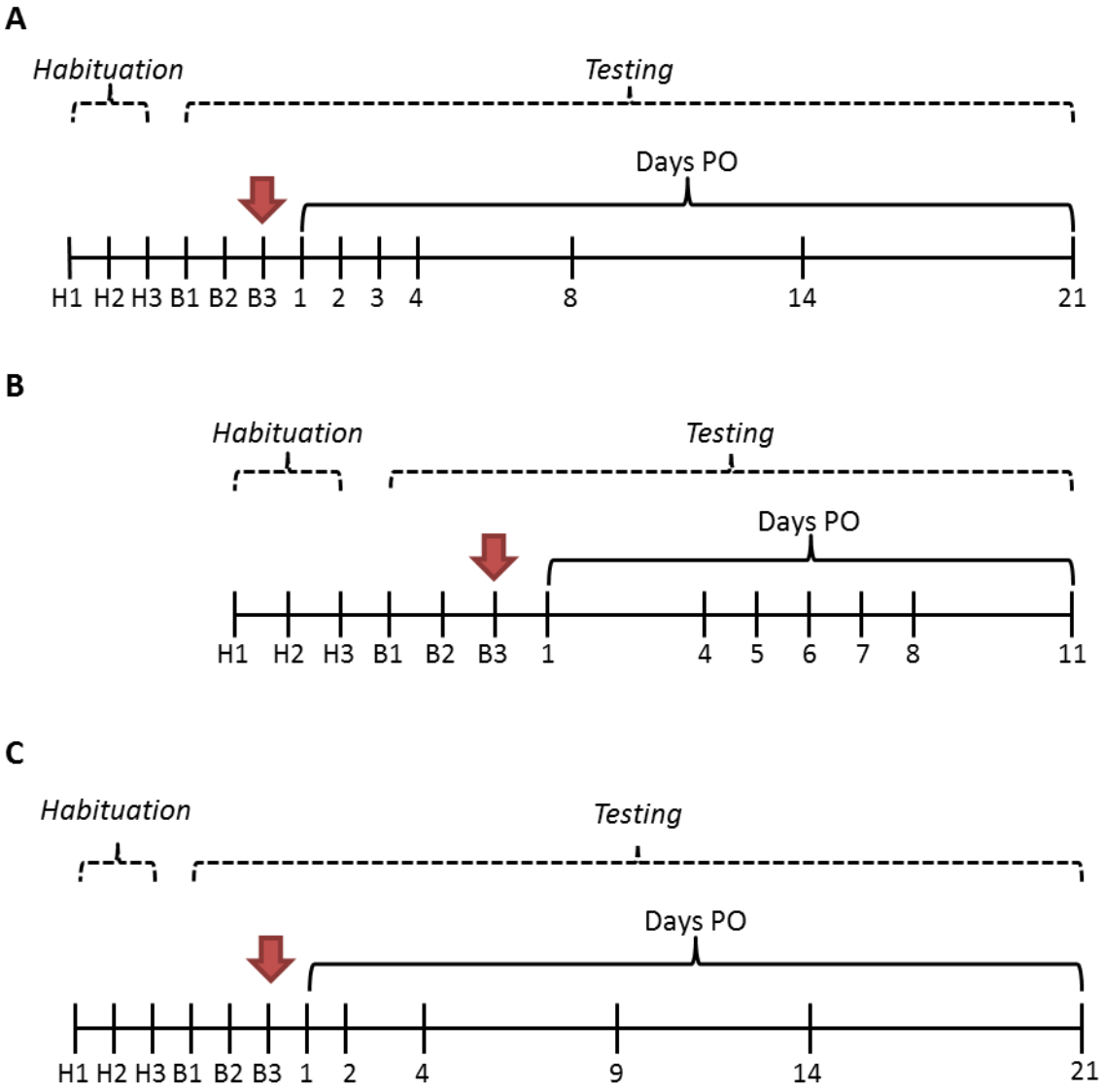


Figure 2.2 Experimental timelines of behavioural testing procedures: **(**A) cold allodynia, mechanical and heat hyperalgesia following standard surgery procedure; (B) mechanical allodynia following standard surgery procedure; (C) mechanical and cold allodynia following i.p. injections of substances (saline or vinblastine). Please note that the red arrow indicates the day of surgery (or injection) following B3 testing. H, habituation; B, baseline testing; PO, post-operative (post-i.p. injection).

2.2.1.3 Mechanical hyperalgesia

Mechanical hyperalgesia was assessed using a modified pin-prick test (Tal and Bennett, 1994). With the animals in the acrylic glass enclosures, an insect pin that was attached to a 4.0 g von Frey monofilament was applied to the mid-plantar surface of the foot through the perforated floor. The pin was applied against the skin with an intensity that was sufficient to indent but not penetrate the skin. The stimulus was repeated only once to each foot at an interval of five minutes. The duration of paw withdrawal was measured. A normal response consisted of a relatively small amplitude brisk reflex that was arbitrarily assigned 0.5 sec. A measurement cut-off of 15 seconds was applied to long-duration withdrawals.

2.2.1.4 Heat hyperalgesia

Heat hyperalgesia was tested using the Hargreaves method (Hargreaves *et al.*, 1988). Animals were placed into acrylic glass enclosures that were raised on a clear glass flooring (Ugo Basile, Italy). A calibrated movable radiant heat source below the floor was positioned directly under the glabrous skin of the mid-plantar hind paw. Onset of the stimulus activated a timer that automatically stopped when the animal responded. A sharp paw withdrawal with or without licking was considered to be a positive response. If the animal moved off the heat source, 10 minutes were allowed before retesting. Additionally, if the animal urinated, the surface of the enclosure was cleaned and dried, and 10 minutes were allowed before testing. The intensity of the heat source was set to produce latencies of approximately 10-15 seconds. In order to avoid the sensitization effect, only one test was performed on each side at an interval of 10 minutes (Pulman *et al*., 2013).

2.2.2 Burrowing behaviour assessment

Burrowing behaviour was assessed as previously described (Deacon, 2009). A burrow constructed of a non-transparent (brown) plastic tube (320 mm long by 100 mm diameter), raised at one end (60 mm) and closed at the other, was positioned in each animal cage (26 x 35 x 50 cm; Figure 2.3). The burrow was filled with 2 Kg of 10 mm gravel (Wickes, United Kingdom). Animals (n = 18) were habituated to the testing apparatus for two hours on four consecutive days prior to testing. During the first day of habituation, animals were placed into the test cage in pairs, allowing them to explore the new environment together. On the following three habituation days, animals were habituated individually. During testing, animals were allowed to burrow individually in the test cage for two hours. The amount of gravel remaining in the burrow was weighed and used as a measure of burrowing. Three pre-surgery baseline values were obtained. Testing was performed on day 1, 4 and 5 post-surgery (see Figure 2.4 for timelines of experimental procedures). One animal failed to burrow during the three pre-surgery test days and was therefore removed from the study.

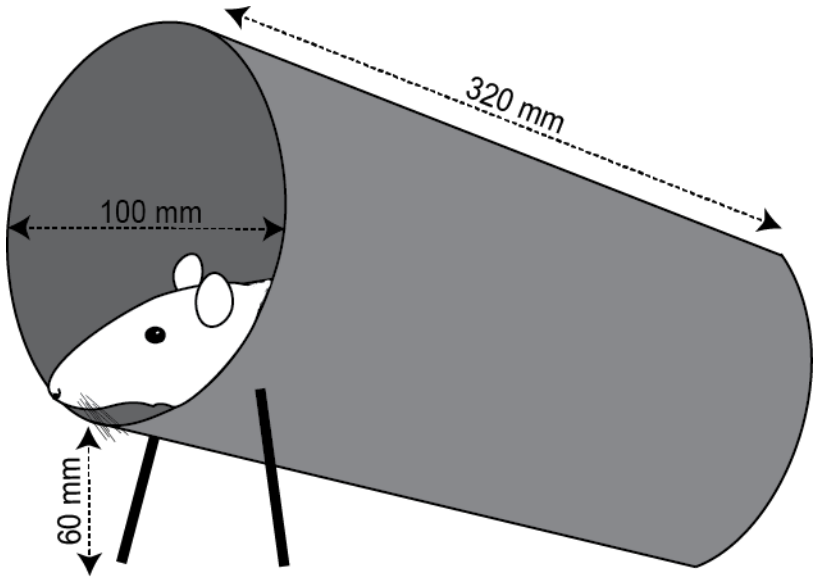


Figure 2.3 Schematic diagram of a burrow used for the assessment of burrowing behaviour in rats. A burrow was constructed of a non-transparent (brown) plastic tube with one closed end and filled with 2 Kg of gravel.

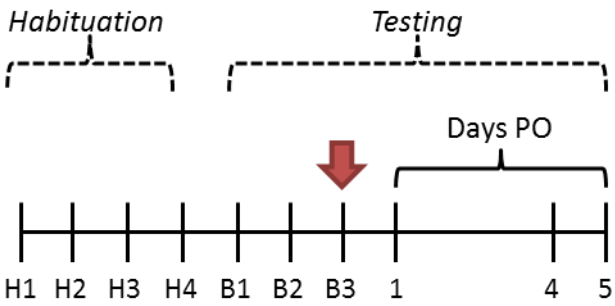


Figure 2.4 Experimental timeline of burrowing behaviour testing. Please note that the red arrow indicates the day of surgery following B3 testing. H, habituation; B, baseline testing; PO, post-operative.

2.3 Single-unit extracellular electrophysiological recordings

Single-unit extracellular electrophysiological recordings were made from A- and C-fibre axons in the L5 dorsal root as previously described (Dilley *et al*., 2013). The L5 dorsal root innervates much of the plantar surface of the foot (Dilley *et al*., 2013) that was examined during behavioural testing (i.e. 94% of neurons in the L5 dorsal root pass through the sciatic nerve in the thigh). Experiments were carried out at two hours to 15 days following vinblastine treatment (n = 14), neuritis (n = 13) and saline treatment (n = 9). A group of untreated animals (n = 3) was also examined using the same protocol. Animals were deeply anesthetized (1.5g/kg 25%w/v urethane i.p.) and smaller doses of anaesthetic were administered as necessary in order to maintain the absence of pinch withdrawal and corneal reflexes. The body temperature was maintained at 37°C using a rectal thermistor probe attached to a feedback controlled heat mat (Harvard Apparatus, Kent, UK). A mid-sagittal skin incision was made in the lumbar region of the back, and a laminectomy was performed from the L2 to L5 vertebrae. The body of the rat was secured by hip pins and the skin flaps were sutured to a metal ring to form a pool. The dura mater was cut to expose the caudal end of the spinal cord and cauda equina, and the pool was filled with mineral oil warmed to 37°C to prevent drying. The ipsilateral L5 dorsal root was identified and cut just before it enters the spinal cord and loosely placed onto a black glass platform (9 x 5 mm). Individual fine filaments were teased from the cut end of the nerve with finely sharpened forceps and carefully positioned on to the recording electrodes that were made from fine gold wires. Receptive fields were not searched, since noxious mechanical stimulation that is necessary to activate the terminals of nociceptors can cause ongoing activity (Bove and Dilley, 2010, Richards *et al*., 2011).

In the mid-thigh, the sciatic nerve was exposed and cleared from its connective tissue. In a case of a neuritis animal, the sciatic nerve was carefully cleared from the Gelfoam soaked in CFA. The surrounding skin flaps were stitched to a metal ring to form a second mineral oil pool. A small strip of plastic (6 x 25 mm) was placed under the cleared sciatic nerve in order to prevent stimulating electrodes from touching the surrounding tissues. Platinum bipolar stimulating electrodes were positioned under the nerve immediately distal to the treatment site or equivalent nerve segment (Figure 2.5). A-fibre neurons were identified by electrical stimulation (square wave pulses; 0.05 ms duration, 3-10 V amplitude) at this site using an isolated-voltage stimulator (Digitimer, Hertfordshire, United Kingdom). During stimulation, the limb was held in place by a noose around the foot. Note that muscular contractions of the foot during electrical stimulation at A-fibre strength were only small. The long conduction distance (range: 61-88 mm) allowed identification of individual A-fibre neurons.

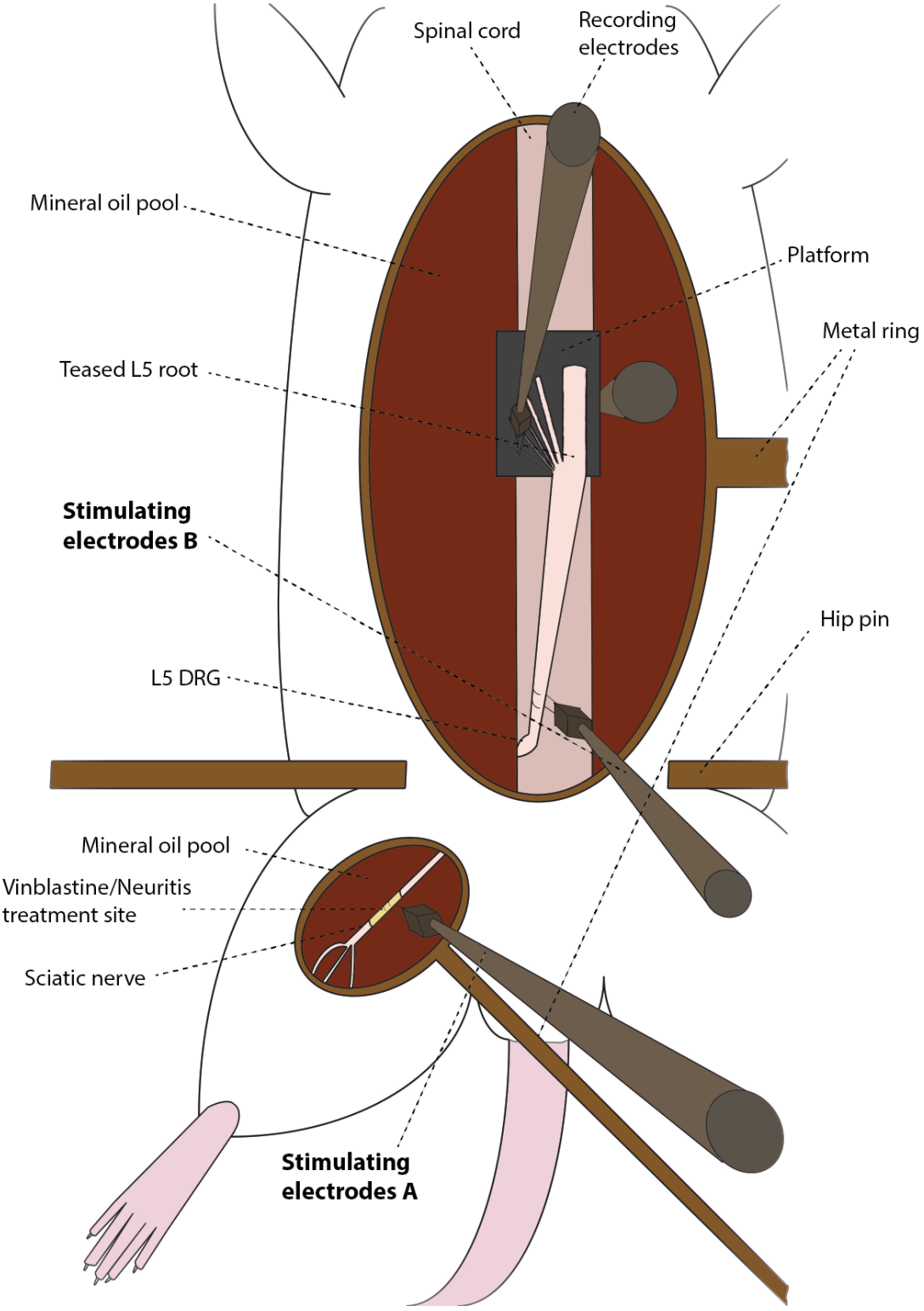


Figure 2.5 Schematic diagram of the experimental set up for performing single-unit extracellular electrophysiological recordings.

Bipolar stimulating electrodes were also positioned under the L5 dorsal root (Figure 2.5). C-fibre neurons were identified by electrical stimulation (square wave pulses; 0.5 ms duration, 30-50 V amplitude) at this site using an isolated-voltage stimulator (Digitimer, Hertfordshire, United Kingdom), which avoided electrically evoked leg movements that are observed in response to sciatic nerve stimulation in the C-fibre range. The longer conduction latencies of C-fibre neurons allowed identification of individual waveforms in response to dorsal root stimulation.

Figure 2.5 Schematic diagram of the experimental set up for performing single-unit extracellular electrophysiological recordings. The body of a rat is secured with hip pins and the skin flaps are sutured to a metal ring forming a pool that is filled up with mineral oil. Laminectomy is performed, and the L5 dorsal root is cut just before it enters the spinal cord. The cut end is placed on a glass platform. The sciatic nerve is exposed (Gelfoam is removed in neuritis animals) and a mineral oil pool is formed as described above. Fine filaments are teased from the cut end of the root and placed over gold bipolar recording electrodes. For sciatic nerve stimulation, platinum bipolar stimulating electrodes (A) are placed under the sciatic nerve. For dorsal root stimulation, platinum bipolar stimulating electrodes (B) are placed under the dorsal root.

Action potentials were amplified (1-2 K), band-pass filtered (10-5000 Hz) and monitored with an oscilloscope. Neuronal activity was digitized and recorded with Spike 2 software (Cambridge Electronic Designs, Cambridge, UK) for off-line analysis. Only fine filaments with clearly identifiable waveforms (1-7 neurons) were examined. To assess for the presence of ongoing activity, neurons were recorded for three minutes. Neurons with less than one action potential per minute were excluded from the data analysis (Bove and Dilley, 2010). Muscle spindles were also excluded, which were characterized by moving the limb. Neurons that altered their firing pattern in response to muscle stretch were considered muscle spindles. Neurons with ongoing activity were identified as either A- or C-fibre neurons by spike shape. C-fibre neurons typically had a longer duration and were often biphasic compared with A-fibre neurons, which were monophasic (Dilley *et al*., 2005). At the end of each experiment, the sciatic nerve and L5 dorsal root were removed and the conduction distance was measured. Conduction velocities of each neuron were calculated (conduction velocity = conduction distance / latency of the unit).

2.4 Spinal cord recordings

Spinal cord recordings from the L5 spinal segment were made as previously described (Urch and Dickenson, 2003, Dickenson and Sullivan, 1990, Chapman *et al*., 1998) following vinblastine treatment (n = 9), neuritis (n = 11), saline treatment (n = 9) and a group of untreated animals (n = 13). Experiments were carried out between post-operative days 3-6. Animals were deeply anesthetized (1.5g/kg 25%w/v urethane i.p.) and smaller doses of anaesthetic were administered as necessary in order to maintain absence of pinch withdrawal and corneal reflexes. The body temperature was maintained at 37°C using a rectal thermistor probe attached to a feedback controlled heat mat (Harvard Apparatus, Kent, UK). A mid-sagittal skin

incision was made in the lumbar region of the back and a short laminectomy was performed at vertebrae levels L1-L3 to expose the spinal cord at L4-L5 spinal segments (i.e. lumbosacral enlargement). In addition, at the end of experiment the L5 dorsal root was identified and traced back to the spinal cord to confirm the location. The body of the rat was secured by hip pins, and the skin flaps were sutured to a metal ring to form a pool. The dura mater was cut to expose the caudal end of the spinal cord and cauda equina, and the pool was filled with mineral oil warmed to 37°C to prevent drying. The spinous processes were clamped rostral and caudal to the exposed spinal cord. A reference electrode was attached to the metal O-ring with a crocodile clip. An insulated tungsten electrode (76 mm, 2.0 MOhm, 1 µm tip, 0.127 mm shaft diameter; World Precision Instruments, Inc., USA) was lowered using a micromanipulator onto the pia surface of the ipsilateral side of the spinal cord immediately lateral to the posterior spinal artery (Figure 2.6).

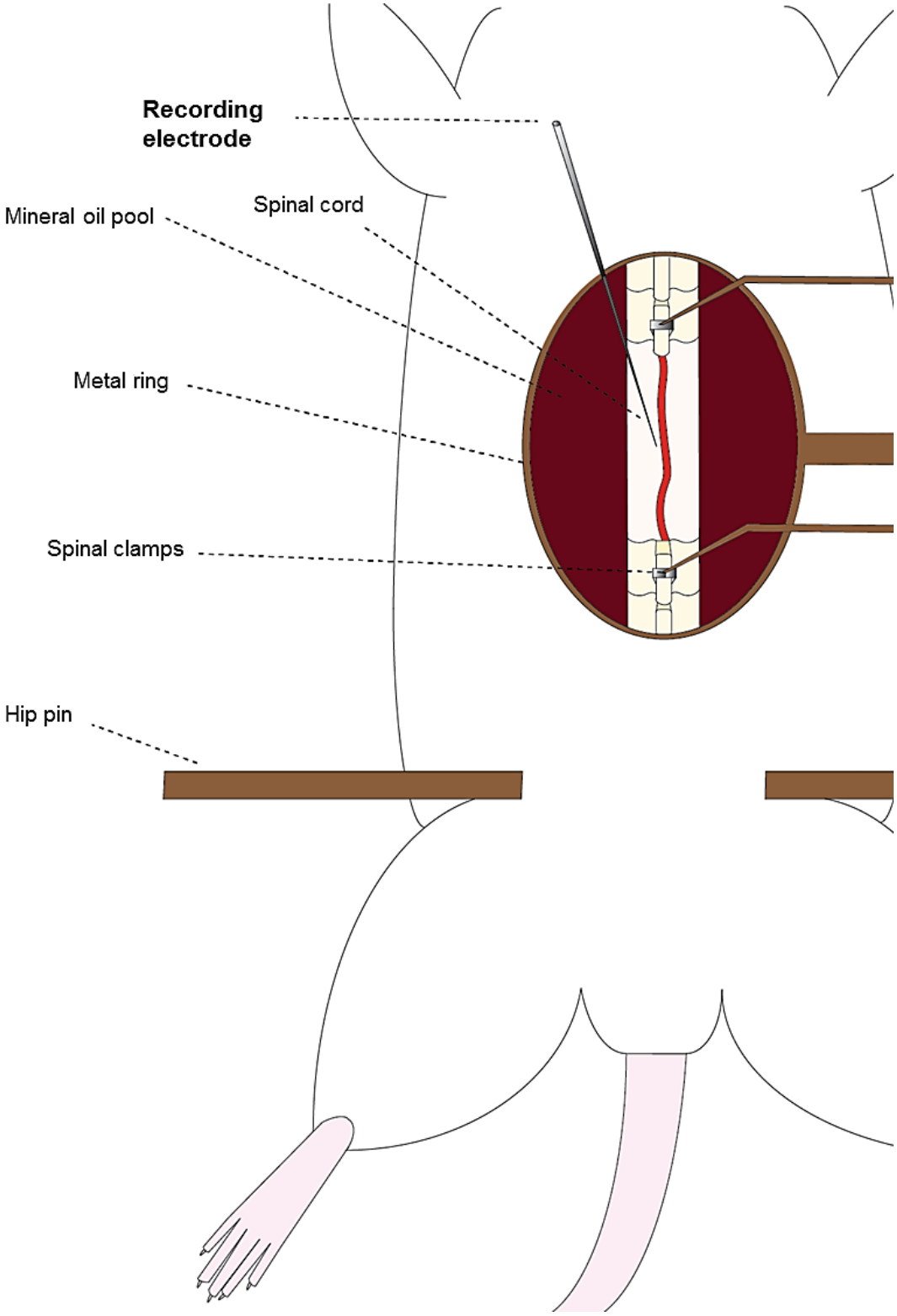


Figure 2.6 Schematic diagram of the experimental set up for performing spinal cord electrophysiological recordings.

The electrode was lowered into the spinal cord in 10-50 μm steps, while the plantar surface of the foot was being gently stroked/tapped until a response was observed (depth range 200-900 μm from the surface of the dorsal horn). Then the size and centre of the receptive field was identified using a fine von Frey hair (0.25g), and the unit was recorded for three minutes to assess ongoing activity levels. Neurons that fired more than once per minute were considered to be ongoing. These neurons were included in further analysis of ongoing activity levels and rates. The neuron was then characterized by assessing its responses to a range of mechanical stimuli applied to the receptive field (Figure 2.7), since such stimulation represents a range of innocuous as well as noxious stimuli. Initially, a painter's brush was used to lightly brush the centre of the receptive field representing innocuous stimulation. This was followed by the application of ascending series of von Frey monofilaments (1, 4, 8, 15, 26, 60 g), after which curved forceps were used to squeeze the receptive field. Each stimulus was applied for 10 seconds with 10 second intervals. If a clear graded response to the applied stimuli was observed, assessment of wind-up commenced.

Two needle electrodes were inserted into the receptive field (transcutaneously), and the threshold required to initiate a C-fibre-evoked response was identified (three consecutive single pulses separated by a minimum of 20 s; 0.75-5 V) using an

Figure 2.6 Schematic diagram of the experimental set up for performing spinal cord electrophysiological recordings. The body of a rat is secured with hip pins and the skin flaps are sutured to a metal ring forming a pool that is filled up with mineral oil. Laminectomy is performed and the spine is fixed with spinal clamps. An electrode is inserted into the ipsilateral side of the spinal cord immediately lateral to the posterior spinal artery. The electrode is lowered into the spinal cord in 10-50 μm steps, and neurons are identified by their responses to natural stimuli. To assess wind-up, transcutaneous electrical stimulation of the receptive field in the hind foot is used.

isolated-voltage stimulator (Digitimer, Hertfordshire, United Kingdom). To assess wind-up, 16 consecutive stimuli (0.5 Hz) at three times C-fibre threshold were applied to the receptive field (2 ms wide pulses, 0.5 Hz). The responses evoked by Aβ-, Aδ- and C- fibre neurons were separated and analysed based on their latencies. In the preliminary studies, clustering of waveforms with conduction velocities of 0-20 ms, 20-90 ms, 90-350 ms and 350-800 ms could be observed and is consistent with responses to Aβ-, Aδ- and C-fibre activation and after-discharge respectively in agreement with the recent studies (Patel *et al.*, 2015a, Patel *et al.*, 2014, Patel *et al.*, 2015b)(Figure 5.5). Wind-up was calculated as the sum of action potentials between 90 and 800 ms (C-fibre responses and after-discharge) evoked by the first 16 consecutive stimuli minus the input. Input was calculated from the sum of the C-fibre-evoked responses after the first stimulus multiplied by 16. WDR neurons were grouped based on the mean depth from the dorsal surface of the spinal cord (those < 550 μm and those ≥ 550 μm from the dorsal surface of the spinal cord). At the end of the experiment, approximate conduction distance was measured by dissecting out the sciatic nerve and L5 dorsal root.

Action potentials were amplified (1 K), band-pass filtered (10-5000 Hz) and monitored with an oscilloscope. Neuronal activity was digitized and recorded with Spike 2 software (Cambridge Electronic Designs, Cambridge, UK) for off-line analysis. Exclusion criteria were as follows: 1) units that developed ongoing activity, which did not stop for 15 minutes after the insertion of stimulating electrodes, (n = 8), were excluded due to mechanical damage to the peripheral terminals; 2) units that disappeared during the recording (n = 21) were excluded due to possible shift in the electrode position; 3) units with no identifiable C-fibre-evoked response (n = 39) were excluded, due to the lack of C-fibre input; 4) units that did not show graded

responses to increasing von Frey stimuli (n = 7) or those that did not respond to touch (n = 3) were excluded due to the lack of WDR properties; 6) recordings with a defined breathing artefact (n = 7 units) were also excluded due to unstable recording baseline, which disabled off-line analysis. Number of neurons recorder per animal ranged from 1 to 7. To avoid injury-induced receptive field sensitization, only neurons with non-overlapping receptive fields were examined in the same animals.

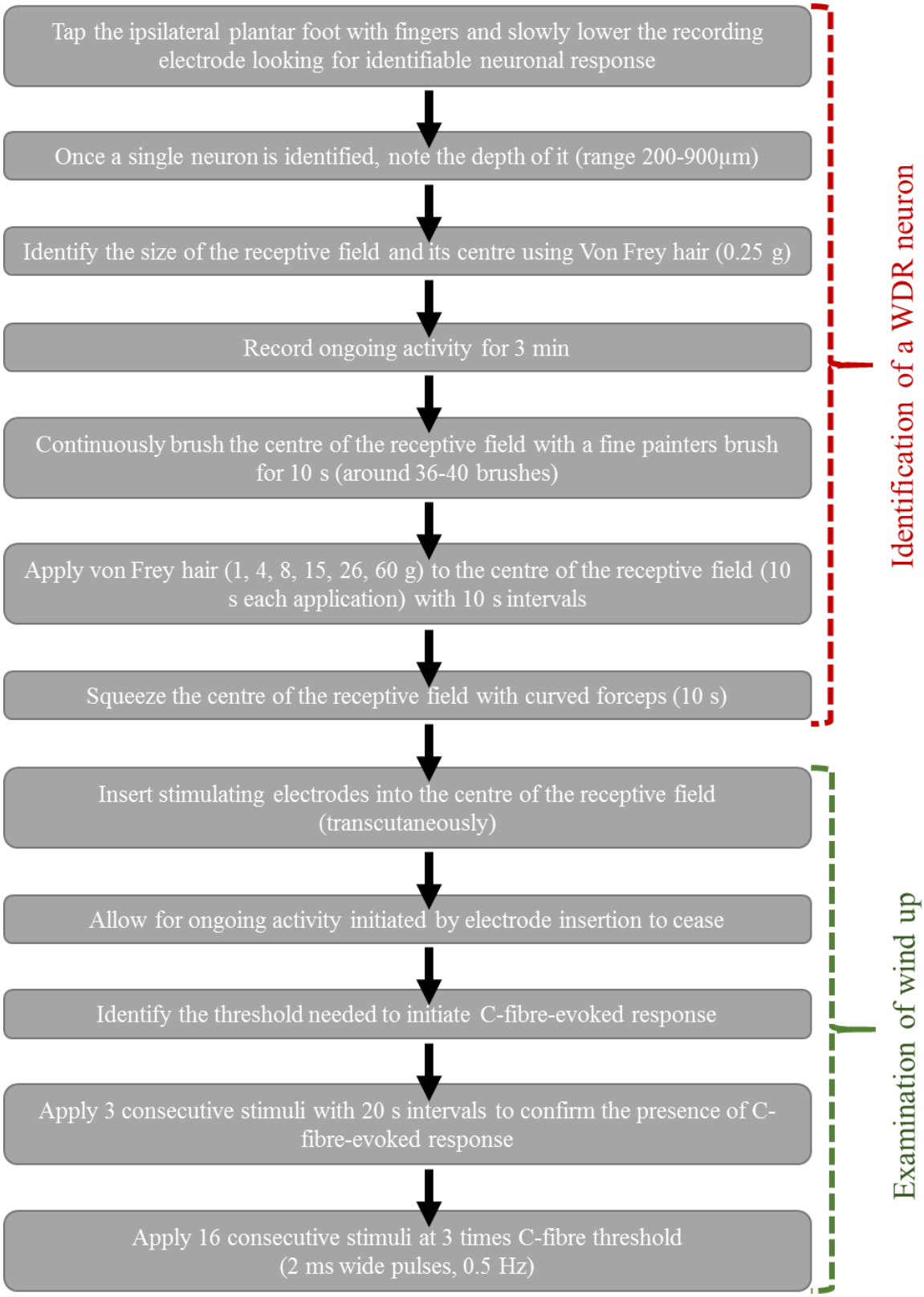


Figure 2.7 Flow chart of a typical spinal cord electrophysiological recording. Please note the identification of a wide dynamic range (WDR) neuron and examination of wind-up parts.

2.5 Imaging of the spinal cord

The expression of c-fos and substance P in the spinal cord was examined by immunohistochemistry. All animals were examined on day 4-5 post-surgery (i.e. at the peak of mechanical allodynia). Moreover, several L5 spinal cord sections were stained with the Nissl stain in order to determine the depth of superficial and deep dorsal horn laminae.

2.5.1 Electrical stimulation-induced c-fos expression in the dorsal horn

For the examination of activity-dependent c-fos expression, a group of vinblastine-treated (n = 12), neuritis (n = 12) and untreated (n = 12) animals were anaesthetized with pentobarbital (50mg/kg, i.p.), and the ipsilateral sciatic nerve was either electrically stimulated for 5 min at A- (3V, 0.05 ms, 5 Hz; n = 4 per group) or C-fibre strength (30V, 0.5 ms, 5 Hz; n = 4 per group; protocol modified from Molander *et al.*, 1994), or it received no electrical stimulation (n = 4 per group). After one hour, animals were deeply anesthetized and transcardially perfused with ice-cold 0.1 M phosphate-buffered saline (PBS) followed by ice-cold 4% paraformaldehyde (in 0.1 M PBS, pH 7.4). L5 spinal cord segments were then removed from all animals. The segment of the spinal cord to be extracted was chosen by tracking back the L5 dorsal root to its spinal cord entry point. Then the spinal cord was cut approximately 2-3 mm away from the spinal cord entry point at both sides and a spinal cord segment was extracted (around 4-6 mm in length). Spinal cords were snap frozen in isopentane on dry ice, post-fixed in 4% paraformaldehyde for 60 minutes and then transferred to 30% sucrose (in 0.1 M PBS) overnight at 4^{o}C for tissue cryoprotection. Transverse sections were cut at 20 µm using a cryostat (Leica Microsystems,

Wetzlar, Germany). The first 5 spinal cord sections were discarded in order to prevent imaging damaged tissue as well as tissue from the subsequent lamina. Three sections from approximately the middle of the L5 segment of spinal cord from vinblastine-treated, neuritis and untreated animals that underwent similar electrical stimulation were thaw-mounted on the same gelatin-coated slides. Sections from the same animal were mounted on two slides each. Only those mounted spinal cord sections that were complete (i.e. not folded or torn) were included for analysis.

Sections were blocked in 3% normal goat serum (Vector Laboratories, Burlingame, USA) in 0.2% Triton X-100 for one hour at room temperature and incubated overnight at 4^{o}C with c-fos polyclonal rabbit (1:1000; sc-52; Santa Cruz Biotechnology, Dallas, Texas, USA) primary antibody. The sections were then incubated for one hour at room temperature with Alexa Fluor 488 goat anti-rabbit (1:200; Thermo Fisher Scientific, Paisley, UK) conjugated secondary antibody. Sections were also labelled with DAPI (4',6-diamino-2-phenylindole; 1:2500; Thermo Fisher Scientific, Paisley, UK) and coverslipped using glycerol/PBS mounting medium (Citiflour, London, UK). All antibodies were diluted in 3% normal goat serum and 0.2% Triton X-100. Between each step, slides were washed in PBS (3 x 5 min). For double labelling experiments, c-fos anti-rabbit (1:1000) and NeuN monoclonal mouse (1:100; MAB377; Millipore Corporation, Bedford, MA, USA) primary antibodies were incubated together, followed by Alexa Fluor 488 goat anti-rabbit (1:200; Thermo Fisher Scientific, Paisley, UK) and Alexa Fluor 546 goat anti-mouse (1:200; Thermo Fisher Scientific, Paisley, UK) conjugated secondary antibodies using the same protocols.

Slides were viewed under a fluorescence microscope (Leica Microsystems) at 488 and 350 nm excitation and photographed at 100x at each wavelength using the same

settings. In addition, double labelled sections were viewed under a confocal microscope (Leica Microsystems SP8) at 488 and 546 nm excitation and photographed at 200x at each wavelength. Non-specific staining with secondary antibody was not found in the absence of primary antibody in any case.

2.5.1.1 Repeated electrical stimulation of the sciatic nerve

As part of the examination of activity depend c-fos expression in the dorsal horn, an additional series of experiments were conducted to check whether repeated electrical stimulation of the sciatic nerve at C-fibre strength blocks conduction in A-fibre neurons (n = 3 animals). In these experiments, the left sciatic nerve was stimulated for five minutes at 30 V (0.5 ms duration, 5 Hz frequency), and compound amplitudes as well as area under the curve in response to supramaximal electrical stimulation (2x maximal) of the sciatic nerve in the A-fibre range (0.05 ms duration, 3-10 V amplitude) were assessed before and immediately after stimulation.

2.5.2 Substance P expression in the dorsal horn

Animals from vinblastine-treated (n = 3), neuritis (n = 3) and saline-treated (n = 3) groups were transcardially perfused with ice-cold 0.1 M PBS followed by ice-cold 4% paraformaldehyde (in 0.1 M PBS, pH 7.4). L5 spinal cord segments were then removed from all animals. The segment of the spinal cord to be extracted was chosen by tracking back the L5 dorsal root to its spinal cord entry point. Then the spinal cord was cut approximately 2-3 mm away from the spinal cord entry point at both sides and a spinal cord segment was extracted (around 4-6 mm in length). Spinal cords were snap frozen in isopentane on dry ice, post-fixed in 4% paraformaldehyde for 60 minutes and then transferred to 30% sucrose (in 0.1 M PBS) overnight at 4^{o}C for tissue cryoprotection. Transverse sections were cut at 20 µm using a cryostat

(Leica Microsystems, Wetzlar, Germany). The first 5 spinal cord sections were discarded in order to prevent imaging damaged tissue as well as tissue from the subsequent lamina. Three sections from approximately the middle of the L5 segment of spinal cord from vinblastine-treated, neuritis and saline-treated animals were thaw-mounted on the same gelatin-coated slides. Sections from the same animal were mounted on two slides each. Only those mounted spinal cord sections that were complete (i.e. not folded or torn) were included for analysis.

Sections were blocked in 3% normal goat serum (Vector Laboratories, Burlingame, USA) in 0.2% Triton X-100 for one hour at room temperature and incubated overnight at 4^{o}C with monoclonal mouse (1:1000; ab14184; Abcam, Cambridge, UK) primary antibody. The sections were then incubated for one hour at room temperature with Alexa Fluor 546 goat anti-mouse (1:500; Thermo Fisher Scientific, Paisley, UK) conjugated secondary antibody. Sections were also labelled with DAPI (Thermo Fisher Scientific, Paisley, UK) and coverslipped using glycerol/PBS mounting medium (Citiflour, London, UK). All antibodies were diluted in 3% normal goat serum and 0.2% Triton X-100. Between each step, slides were washed in PBS (3 x 5 min).

Slides were viewed under a fluorescence microscope (Leica Microsystems) at 546 and 350 nm excitation and photographed at 100x at each wavelength using the same settings. Non-specific staining with secondary antibody was not found in the absence of primary antibody in any case.

2.5.3 Nissl staining of the L5 dorsal horn

Transverse L5 spinal cord sections of an untreated A-fibre strength stimulated animal (which was initially used for the c-fos study) were cut at 20 µm using a

cryostat (Leica Microsystems, Wetzlar, Germany). Approximately ten sections were thaw-mounted on gelatin-coated glass slides. Slides were washed in 0.01mM PBS (2 x 5 min), rinsed in double-distilled water (1 min) and stained with cresyl violate acetate (Sigma Aldrich, Dorset, UK) for 20 minutes. After a rinse with double-distilled water (2 x 5 min), three different concentrations of ethanol were used to dehydrate the slides (3 min 90% ethanol; 3 min 95% ethanol; 2 x 3 min 100% ethanol). Then, they were rinsed in 100 % histoclear (National diagnostics, Fisher Scientific, Leicestershire, UK; 3 x 3 min) and coverslipped with histomount mounting medium (National diagnostics, Fisher Scientific, Leicestershire, UK).

Slides were viewed under a fluorescent microscope (Leica Microsystems) and photographed at 100x and 200x using the same settings. Minor contrast and brightness adjustments as well as production of image collages were made with Adobe Photoshop CC2015 (Adobe Systems) for figure production.

2.6 Data analysis

Shapiro-Wilk tests were used to test all data for normality. Statistical analysis was carried out using SPSS Statistics V24 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism V7.03 (GraphPad Software Inc., San Diego, California) software. All data were considered statistically significant when p values were below 0.05 (i.e. there was a 95% probability that the effect was not due to chance).

Behavioural data

Stimulus-evoked behavioural data are expressed as mean ± standard error of the mean (SEM). Comparisons between groups at each time point were performed using two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc tests. Burrowing behaviour data is expressed as median (interquartile range (IQR)) and the

comparisons between groups at each time point were made using Kruskal-Wallis tests.

Electrophysiology data

Conduction velocities of A- and C-fibre neurons and rates of ongoing activity from C-fibre neurons were compared between time points and to saline treatment using Kruskal-Wallis tests followed by Dunn's post hoc tests. Due to the small number of A-fibre neurons with ongoing activity, rates could not be statistically compared. The conduction velocity and ongoing activity rate data are presented as median (IQR). Comparisons between the proportions of neurons with ongoing activity were made using Fisher's exact tests.

Regarding the spinal cord data, data are presented as mean ± SEM, except for the rates of ongoing activity that are expressed as median (IQR). Comparisons between groups were performed using one-way (brushing receptive field, pinching receptive field, electrical stimulation of receptive field and wind-up responses) or two way ANOVAs (von Frey monofilament responses) followed by Bonferroni post hoc tests. Proportion of ongoing activity was compared using Fisher Exact tests, and rates were compared using Kruskal Wallis tests. Animal weights were compared using Student's t-tests.

Immunohistochemistry data

For analysis of c-fos immunolabelling, images of the dorsal horn were converted to greyscale using Image J software (U. S. National Institutes of Health, Bethesda, Maryland, USA). A region of interest was drawn around the superficial dorsal horn as follows: a line was drawn from medial to lateral following the posterior surface of the dorsal horn. At the lateral margin, the line was extended anteriorly around the

curve of the dorsal horn, and a straight line was drawn parallel to the posterior surface, medially, to reach the dorsal column. This line was then extended to the posterior surface and the area of the region of interest was measured (Figure 2.8). The threshold was set so that individual cells could be identified. From the resulting binary image, the number of positively labelled cells within the marked area was determined using the 'analyze particles' function within Image J. To allow for variations in the size of the region of interest, the number of positive cells per 0.1 mm^2 was calculated for each section, and estimates from multiple sections of the same dorsal horn (n = 3 sections from each animal) were averaged. C-fos data are presented as mean ± SEM. The numbers of positive cells per 0.1 mm^2 were compared between treatment groups and sides using two-way ANOVAs followed by Bonferroni's post hoc tests.

Following experiments on whether repeated electrical stimulation of the sciatic nerve at C-fibre strength blocks conduction in A-fibre neurons, the differences between compound amplitude and area under the curve (AUC) pre- and post-electrical stimulation were compare using pared Student's t-tests. Such data is expressed as mean (standard deviation (SD)).

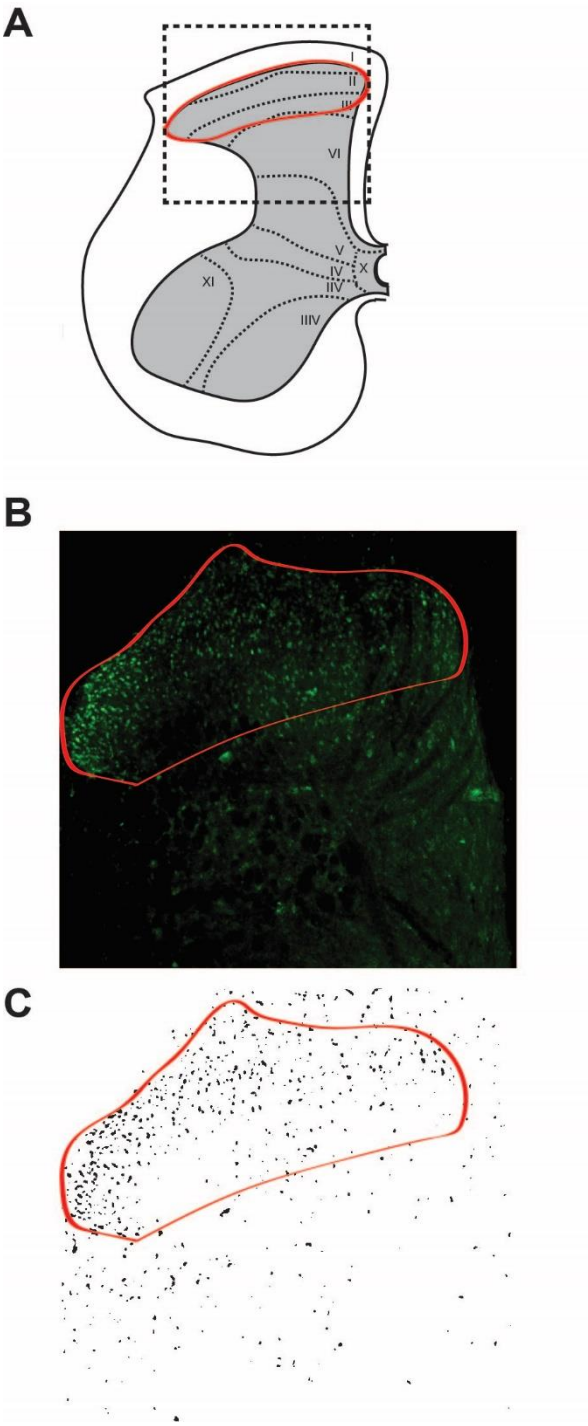


Figure 2.8 ImageJ analysis of c-fos immunolabelling in the superficial dorsal horn. (A) A schematic diagram indicating the marked superficial area (mainly laminae I, II and III). (B) The superficial area is marked and measured on the greyscale (16-bit) image. (C) The image after thresholding and removal of noise and outliers is used to count cell number within the marked dorsal horn area ('analyze particles' function). Note some labelling in the posterior column–medial lemniscus pathway, lateral spinal nucleus and deeper laminae.

For analysis of substance P labelling, images of the dorsal horn were converted to greyscale using Image J software (Figure 2.9). For each image, a greyscale value that was two standard deviations above the mean was determined from an area of approximately 140 x 75 pixels at the base of the dorsal horn. This value was used for thresholding the image. A region of interest was drawn around the superficial dorsal horn as follows: a line was drawn from medial to lateral following the posterior surface of the dorsal horn. At the lateral margin, the line was extended anteriorly around the curve of the dorsal horn, and a straight line was drawn parallel to the posterior surface, medially, to reach the dorsal column. This line was then extended to the posterior surface (Figure 2.9). The area of positive labelling (i.e. above the threshold) within the superficial laminae was measured. A ratio of the difference in the size of the area expressing substance P on the ipsilateral compared to contralateral side was calculated to look for difference between sides. A value of < 1 indicated the area with positive labelling was less on the ipsilateral compared to contralateral side; a value > 1 indicated the area with positive labelling was greater on the ipsilateral compared to contralateral side. Approximately 3 sections per animal were averaged. Substance P data are presented as mean ratio [ipsilateral area/contralateral area] ± SEM. Mean area ratios were compared using a one-way ANOVA followed by a Bonferroni post hoc test.

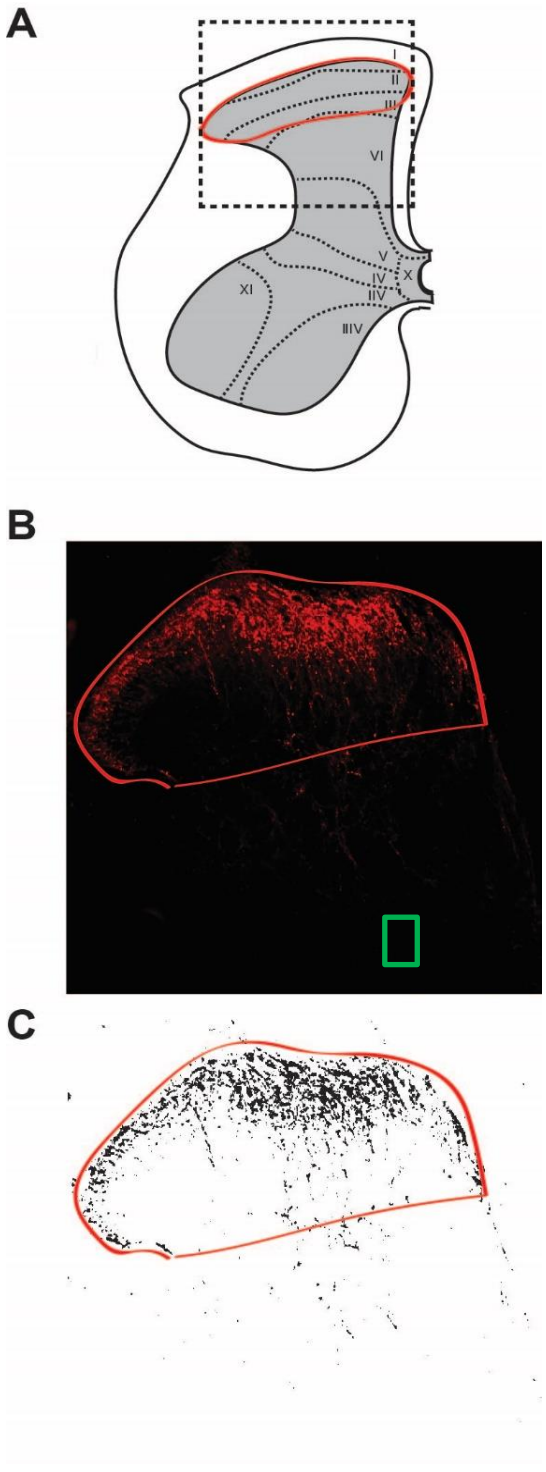


Figure 2.9 ImageJ analysis of substance P immunolabelling in the superficial dorsal horn. (A) A schematic diagram indicating the marked superficial area (mainly laminae I, II and III). (B) On the greyscale (16-bit) image, area is marked at the base of the dorsal horn and a greyscale value that is two standard deviations above the mean is determined for thresholding of the image (see green square). The area of the superficial dorsal horn is also marked. (C) The image after thresholding is used to measure the area of positive labelling within the marked dorsal horn area.

2.7 Avoiding bias

Every effort was made to minimise bias. During the surgery and for behavioural testing, the experimenter was blinded to vinblastine and saline treatments. Blinding to CFA could not be performed, since it forms an emulsion with saline, which is cloudy in appearance. However, following surgery, all animals were coded (by marking the tail) to avoid identification of group. Decoding was carried out after behavioural testing. Due to the nature of electrophysiological experiments, it was not possible for the experimenter to be blinded to the tested group. However, analysis of the data was partially automated to avoid bias. Spike 2 software was used to identify and count the spikes. Similarly, for the analysis of immunohistochemical data Image J software was used. The 'analyze particles' function was used to count the number of c-fos positive cells, while a threshold value of two standard deviations above the mean was determined from an area (around 140 x 75 pixels) at the base of the dorsal horn before measuring the area positively labelled for substance P (above the threshold).

CHAPTER 3:

Development of neuropathic pain behaviours

3.1 Introduction

Reflex paw withdrawal in response to various stimuli applied to the hind paw is commonly assessed in animal models of neuropathic pain. For example, following traumatic nerve injuries (e.g. CCI, SNL, PSL, SNI), mechanical and cold allodynia as well as mechanical and heat hyperalgesia develop (Bennett and Xie, 1988, Maves *et al*., 1993, Seltzer *et al*., 1990, Kim and Chung, 1992, Decosterd and Woolf, 2000). These behaviours persist for a long period of time and show no signs of recovery. Changes in evoked reflex responses are also observed following neuritis, although the behaviours are short-lived, with full recovery times ranging from one day to one week post-surgery (Chacur *et al*., 2001, Gazda *et al*., 2001, Eliav *et al*., 1999, Bove *et al*., 2003). The ability to test such behaviours enables the investigation of the mechanisms that underlie most common symptoms observed in neuropathic pain patients (see section 1.1.1). In contrary, mimicking clinical features of neuropathic pain in rodents by performing reflex-withdrawal-based tests does not reflect the global impact of pain. In patients, neuropathic pain commonly has a pronounced negative effect on their wellbeing, which can present as lack of motivation to perform daily tasks. In laboratory animals, ethologically relevant measures of pain, such as burrowing behaviour, enable evaluation of such processes. For example, a reduction in the latter behaviour is observed after physical nerve injury or chronic inflammation of the paw in the rat (Andrews *et al.*, 2012, Rutten *et al.*, 2014, Muralidharan *et al.*, 2016) and is indicative of reduced wellbeing and possibly pain. Behavioural investigations following low-dose vinblastine-induced axonal transport disruption are limited. The development of short-lived mechanical allodynia has been reported (Dilley *et al*., 2013), which is comparable to that observed following neuritis. However, previous studies indicate that heat and mechanical hyperalgesia is

not a feature of this model (Dilley *et al*., 2013, Fitzgerald *et al*., 1984). Ethologically relevant pain behaviours, such as burrowing, have not been assessed following neuritis or vinblastine. Here, it is hypothesised that local axonal transport disruption in rats will lead to the development of short-lived neuropathic pain behaviours, which include mechanical and cold allodynia, similarly to the neuritis model, but not heat or mechanical hyperalgesia. It is also proposed that a reduction in burrowing behaviour will be present following neuritis and vinblastine treatment.

Aims

- To investigate whether stimulus-evoked neuropathic pain behaviours, including mechanical and cold allodynia as well as mechanical and heat hyperalgesia, develop in the rat as a result of vinblastine-induced axonal transport disruption and to make comparisons with those behaviours induced by neuritis.
- To investigate the effect of local axonal transport disruption and neuritis on rat's burrowing behaviour.

3.2 Results

3.2.1 Stimulus-evoked neuropathic pain behaviours

Mechanical allodynia

The mean baseline 50% paw withdrawal threshold was 14.1 ± 0.79 g (n = 6), 15.0 g (n = 6), and 14.48 ± 0.74 g (n = 6) in vinblastine-treated, neuritis and saline-treated groups respectively. On the ipsilateral side, there was a significant interaction between treatment groups and von Frey threshold at each time point ($p < 0.01$, two-way ANOVA; Figure 3.1A). Signs of mechanical allodynia were clearly observed on

the ipsilateral plantar foot as early as day one post-surgery in the vinblastine-treated group. For both treatments, the 50% withdrawal threshold reached a maximum reduction on days 5-6 post-surgery (mean = 5.68 ± 1.92 g following vinblastine treatment on day five post-surgery and mean = 8.25 ± 1.49 g following neuritis on day six post-surgery; $p < 0.01$ compared to saline treatment, Bonferroni post hoc test). The mean percent decrease was 59.7 ± 13.6 % in the vinblastine-treated group on day five post-surgery and 45.01 ± 9.9 % in the neuritis group on day six post-surgery. A reversal was observed by day 11 post-surgery in both groups (Figure 3.1A). On the contralateral side, there was no significant interaction ($p = 0.28$). However, significant main effect for group was present ($p < 0.001$, two-way ANOVA) with signs of mechanical allodynia following vinblastine treatment (Figure 3.1A). In this group, the mean baseline 50% withdrawal threshold on the contralateral side was 12.51 ± 1.3 g, which reduced to 8.1 ± 2.5 g on day five post-surgery.

Following systemic i.p. administration of vinblastine (100 µl, 0.1 M) or saline (100 µl), there was no significant interaction between treatment groups and von Frey threshold ($p > 0.18$), or main effect for group ($p > 0.23$; two-way ANOVA) on both ipsilateral and contralateral sides. The mean baseline 50% paw withdrawal threshold on the left side was 14.94 ± 0.06 g ($n = 6$) and 15 g ($n = 6$) in vinblastine- and saline-treated groups respectively (Figure 3.2A). On day four of testing, the 50% withdrawal threshold was 15 g in both vinblastine- and saline-treated groups (Figure 3.2A). No signs of mechanical allodynia were present on the contralateral side (Figure 3.2A).

Cold allodynia

The mean baseline percent paw withdrawal frequency was 5.56 ± 3.18% (n = 6), 1.11 ± 1.11% (n = 6) and 6.67 ± 3.44% (n = 6) in vinblastine-treated, neuritis and saline-treated groups respectively. On the ipsilateral side, there was a significant interaction between treatment groups and paw withdrawal frequency at each time point ($p < 0.01$, two-way ANOVA; Figure 3.1B). Following vinblastine treatment, an increase in paw withdrawal frequency was observed as early as day 1 postoperative. A maximum paw withdrawal frequency was observed on day 3-4 in both neuritis and vinblastine-treated groups ($p < 0.05$ compared to saline treatment, Bonferroni post hoc test) and the mean values in the vinblastine-treated and neuritis groups were 36.67 ± 9.55% and 53.33 ± 9.89% respectively. Cold allodynia reversed by day 14 post-surgery in the vinblastine-treated group and by day 21 post-surgery in the neuritis group. On the contralateral side, there was also a significant interaction ($p < 0.05$, two-way ANOVA) with signs of cold hypersensitivity in the neuritis group (Figure 3.1B). In this group, the mean percent paw withdrawal frequency on the contralateral side increased to 30 ± 10 % on day 14 post-surgery.

Following systemic i.p. administration of vinblastine (100 µl, 0.1 M) or saline (100 µl), there was no significant interaction between treatment groups and paw withdrawal frequency ($p > 0.05$), or main effect for group ($p > 0.38$; two-way ANOVA) on both ipsilateral and contralateral sides. The mean baseline percent withdrawal frequency on the left side was 2.2 ± 1.41% (n = 12) and 5.6 ± 2.68% (n = 6) in vinblastine- and saline-treated groups respectively (Figure 3.2B). On day four post-surgery, the mean percent withdrawal frequency was 10 ± 6.83% in vinblastine and 6.7 ± 6.67% in saline-treated groups. No signs of cold allodynia were present on the contralateral side (Figure 3.2B).

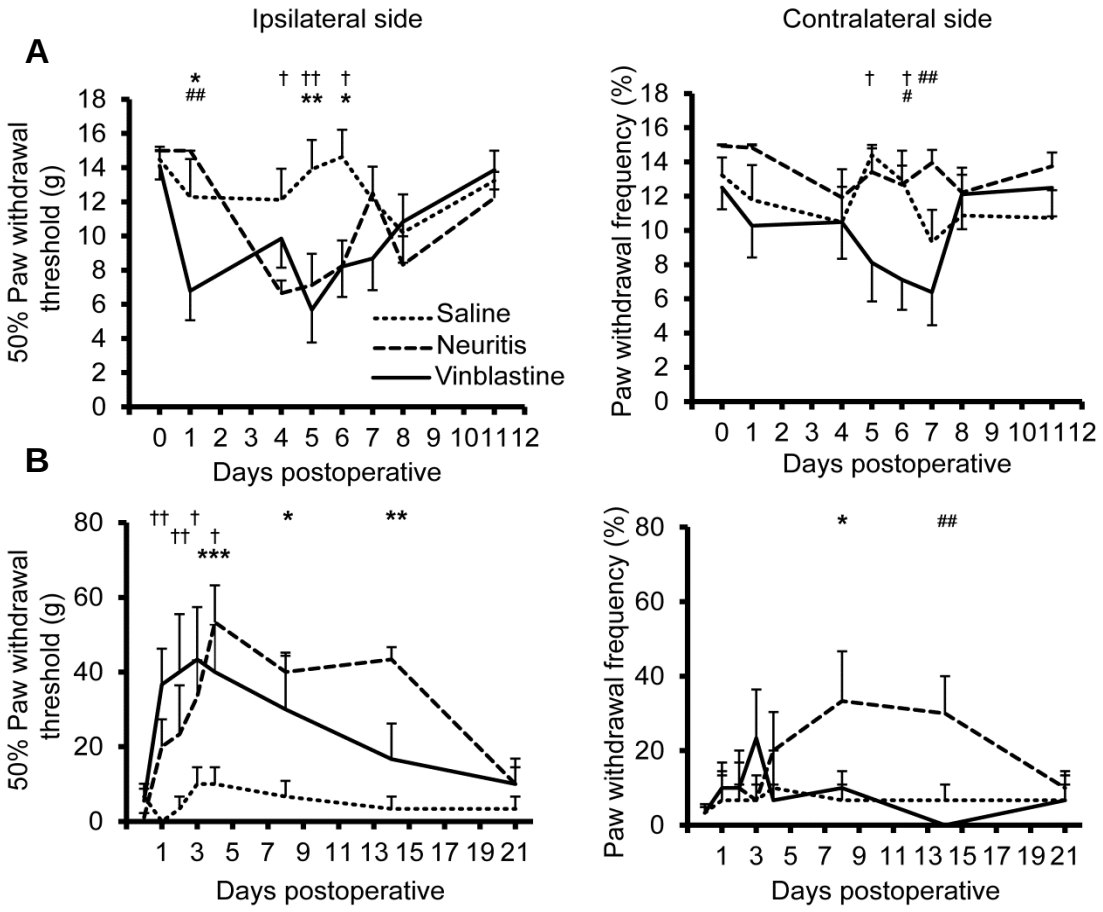


Figure 3.1 Development of mechanical (A) and cold (B) allodynia. Signs of evoked pain behaviours were examined in vinblastine-treated, neuritis and saline-treated animals. Ipsilateral (left) and contralateral (right) data are shown for each test. Each data point represents the mean of six animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing neuritis to saline-treated group; † $p<0.05$, †† $p<0.01$ comparing vinblastine-treated to saline-treated group; # $p<0.05$, ## $p<0.01$ comparing neuritis to vinblastine-treated group (two-way ANOVA followed by Bonferroni's post hoc tests at each time point). Error bars = SEM.

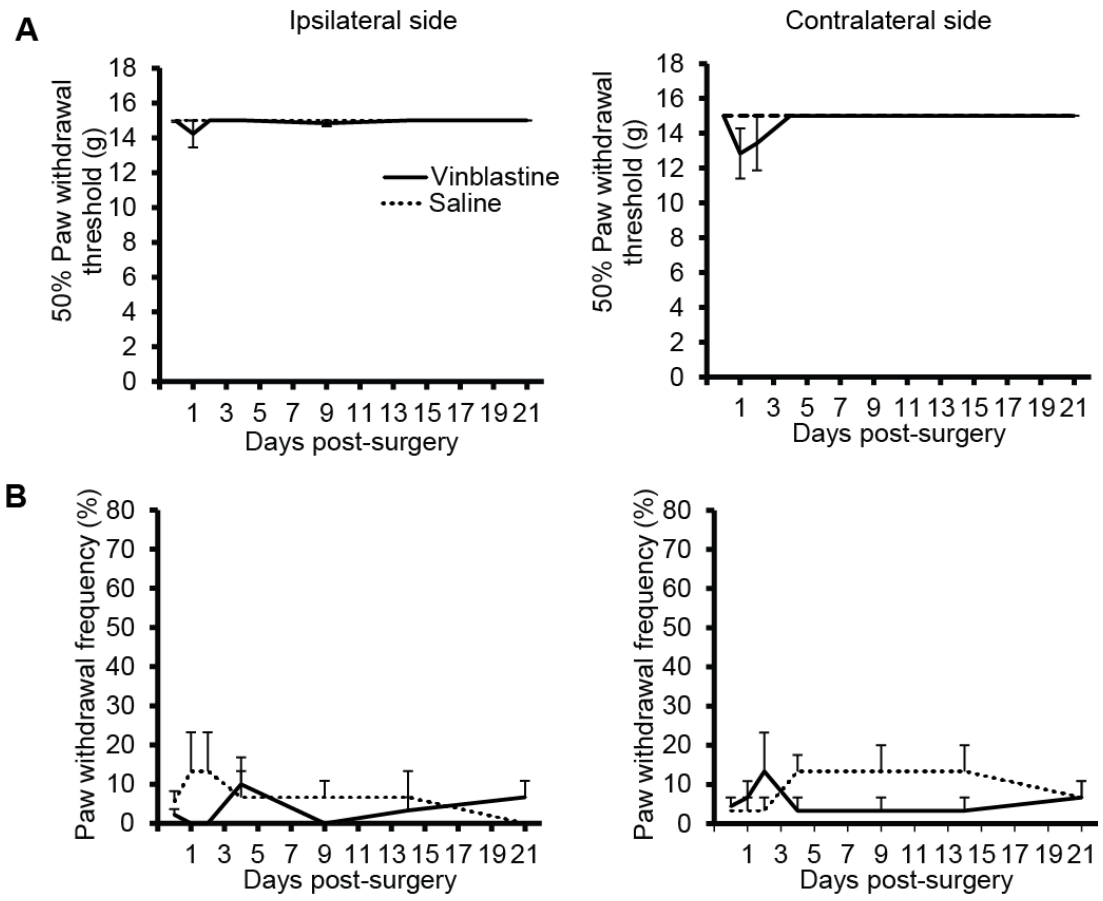


Figure 3.2 Development of neuropathic pain behaviours following systemic i.p. administration of vinblastine (100 µl, 0.1 M) or saline (100 µl). Signs of (A) mechanical allodynia (von Frey) and (B) cold allodynia were examined in vinblastine- and saline saline-treated animals. Ipsilateral (left) and contralateral (right) data are shown for each test. Each data point represents the mean of six animals.

Heat hyperalgesia

The mean baseline withdrawal latency was 17.7 ± 0.49 s (n = 6), 17.9 ± 0.49 s (n = 6) and 17.4 ± 1.26 s (n = 6) in vinblastine-treated, neuritis and saline-treated groups respectively. On the ipsilateral side, there was no significant interaction between treatment groups and withdrawal latency ($p = 0.07$ two-way ANOVA; Figure 3.3A). However, signs of heat hyperalgesia were present in both vinblastine-treated and neuritis groups on day two post-surgery (mean = 10.8 ± 1.69 s and 8.7 ± 1.28 s following vinblastine treatment and neuritis respectively). The effect was short-lived, with signs of a reversal by day three post-surgery. On the contralateral side, there was also no significant interaction between treatment groups and withdrawal latency ($p = 0.41$ two-way ANOVA; Figure 3.3A).

Mechanical hyperalgesia

The mean baseline withdrawal duration was 0.5 s (n = 6), 0.6 ± 0.08 s (n = 6) and 0.5 s (n = 6) in vinblastine-treated, neuritis and saline-treated groups respectively. On the ipsilateral side, there was a significant interaction between treatment groups and withdrawal duration ($p < 0.05$, two-way ANOVA; Figure 3.3B). Signs of mechanical hyperalgesia were present following neuritis and reached a maximum withdrawal duration on day four post-surgery (mean = 3.8 ± 2.3 s; $p < 0.05$ compared to saline and vinblastine treatments, Bonferroni post hoc test). Vinblastine treatment also induced signs of mechanical hyperalgesia (mean = 3.55 ± 2.4 s g on day eight post-surgery; $p < 0.05$ compared to saline treatment, Bonferroni post hoc test). On the contralateral side, there was no significant interaction ($p = 0.08$; two-way ANOVA; Figure 3.3B).

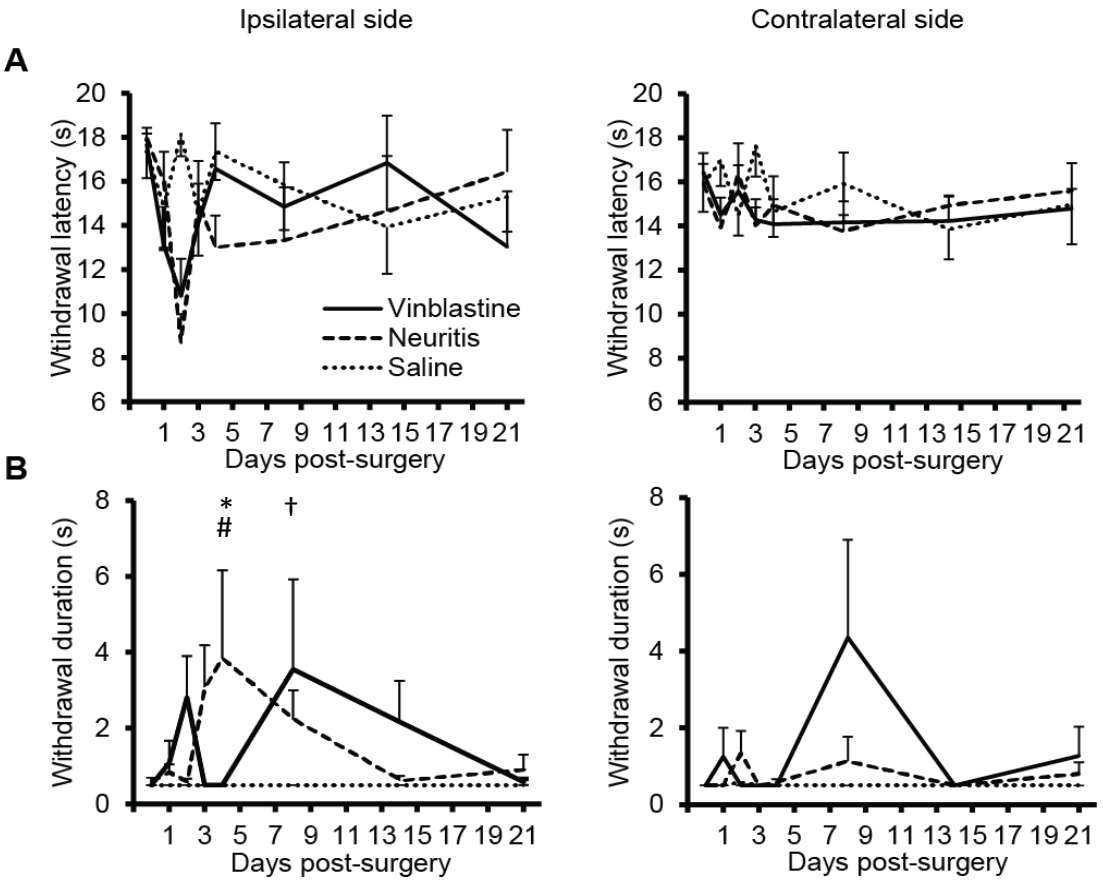


Figure 3.3 Development of heat (A) and mechanical (B) hyperalgesia. Signs of evoked pain behaviours were examined in vinblastine-treated, neuritis and saline-treated animals. Ipsilateral (left) and contralateral (right) data are shown for each test. Each data point represents the mean of 6 animals. * $p < 0.05$ comparing neuritis to saline-treated group; † $p<0.05$ comparing vinblastine-treated to saline-treated group; # $p<0.05$ comparing neuritis to vinblastine-treated group (two-way ANOVA followed by Bonferroni's post hoc tests at each time point). Error bars = SEM.

3.2.2 Ethologically relevant outcome measures: Burrowing behaviour

The results for burrowing are summarized in figure 3.4. The median baseline gravel displacement values were 98.17 g (IQR = 520.42; n = 6), 325.67 g (IQR = 719.67; n = 6) and 281.67 g (IQR = 453; n = 5) in vinblastine-treated, neuritis and saline-treated groups respectively. On day one post-surgery, a sharp decrease in burrowing behaviour was observed in neuritis and vinblastine-treated animals with 0 g and 4.5 g (IQR = 111) of gravel removed respectively, while saline-treated animals removed 64 g (IQR = 1461) of gravel. Although not significant, there was an increase in burrowing behaviour in the saline-treated group on day five post-surgery (mean gravel displaced on day five = 1263 g (IQR = 79); p = 0.16 Kruskal-Wallis compared with vinblastine treatment and neuritis).

.

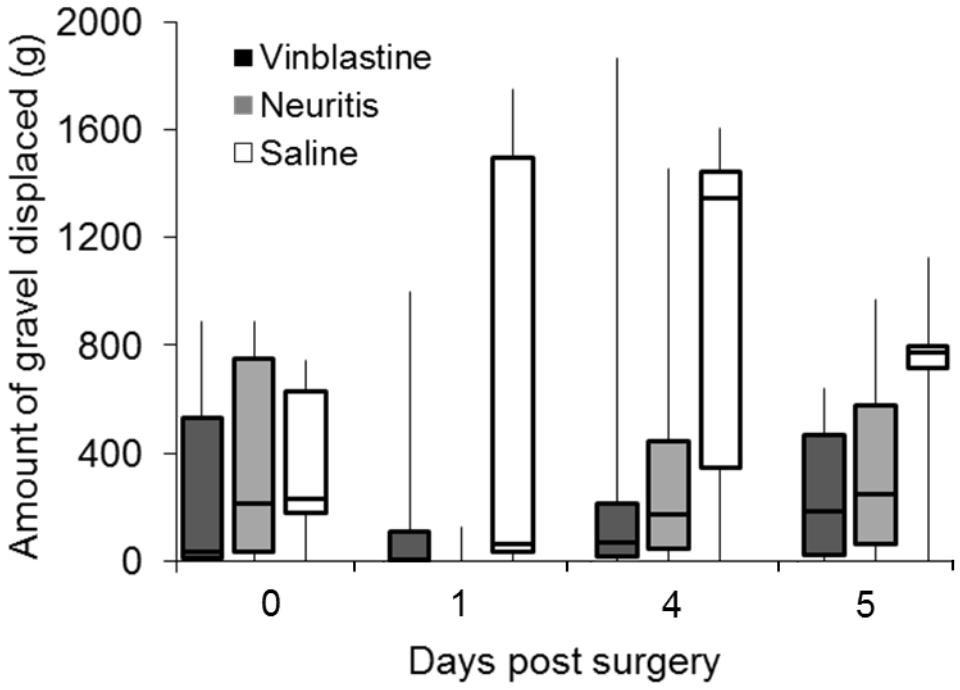


Figure 3.4 Box-plot diagram showing the amount of gravel removed by rats in vinblastine-treated, neuritis and saline-treated groups. The median values of removed gravel together with the range and outliers are represented (n = 6 in each group, except for the saline-treated group, where n = 5).

3.3 Summary of findings

- Signs of mechanical allodynia developed on the ipsilateral side following both vinblastine treatment and neuritis, peaking on days 5-6 post-surgery ($p < 0.01$ compared to saline treatment) and reversed within one week.
- Signs of mechanical allodynia developed on the contralateral side following vinblastine treatment on days 5 and 7 post-surgery ($p < 0.01$ compared to saline treatment).
- Signs of cold allodynia developed on the ipsilateral side following both vinblastine treatment and neuritis, peaking on days 3-4 post-surgery ($p < 0.01$ compared to saline treatment) and reversed by day 14 and 21 post-surgery in the vinblastine-treated and neuritis groups respectively.
- Signs of cold allodynia developed on the contralateral side following neuritis on day 14 post-surgery ($p < 0.01$ compared to saline treatment).
- No signs of mechanical or cold allodynia were present following i.p. injection of vinblastine or saline.
- Signs of mechanical hyperalgesia developed on the ipsilateral side following neuritis and vinblastine treatment on day four and eight post-surgery respectively ($p < 0.05$ compared to saline treatment).
- No signs of heat hyperalgesia developed on the contralateral side following neuritis or vinblastine treatment.
- Although not significant, the amount of gravel removed in the vinblastine-treated and neuritis groups was reduced on days 4-5 post surgery.

CHAPTER 4:

Electrophysiology of peripheral nerves

4.1 Introduction

Ongoing activity from axons of primary sensory neurons is reputed to drive central mechanisms that underlie neuropathic pain behaviours (Campbell and Meyer, 2006, Woolf, 2011, Xie *et al*., 2005, Ji *et al*., 2003). In neuropathic pain models that require a significant injury (e.g. constriction, ligation, crush) ongoing activity occurs in both myelinated and unmyelinated nerve fibres within a day and persists for several weeks post-surgery (Kajander *et al.*, 1992, Amir and Devor, 1993, Tal and Eliav, 1996, Xie and Xiao, 1990, Liu *et al*., 2000a, Liu *et al*., 2000b, Djouhri *et al*., 2006). Such activity originates from the peripheral terminals, injury site (Tal and Eliav, 1996) or DRGs (Wall and Devor, 1983) in the absence of peripheral sensory stimulus. In contrast, following neuritis, ongoing activity occurs within the first week in the absence of axonal degeneration, demyelination or neuroma formation (Bove *et al*., 2003, Bove and Dilley, 2010). Although axonal transport is disrupted following neuritis, in the absence of inflammation, axonal transport disruption (induced by vinblastine) does not cause an increase in ongoing activity at the peak of neuropathic pain behaviours (Dilley *et al*., 2013). Interestingly, in both models the conduction velocities of C-fibre neurons are slowed (Dilley *et al*., 2013, Fitzgerald *et al*., 1984, Dilley and Bove, 2008b, Dilley *et al*., 2005). Similarly, a recent study reported activity-dependent slowing of C-fibre conduction velocities following repetitive electrical stimulation in paw inflammation model (Dickie *et al.*, 2017).

In this study, it is hypothesised that inflammation is necessary for the development of ongoing activity and that it is not a feature of vinblastine-induced axonal transport disruption. Currently there have been no systematic studies on ongoing activity or conduction velocities during neuritis or vinblastine-induced axonal transport

disruption, in particular, whether ongoing activity occurs at different time points following vinblastine-treatment.

Aims

- To examine ongoing activity levels of peripheral nerves from early (3-4 hrs) to late (15 days) time points following vinblastine treatment and to make comparisons to that of neuritis.
- To examine changes in conduction velocities from early (3-4 hrs) to late (15 days) time points following vinblastine treatment and neuritis.

4.2 Results

4.2.1 Conduction velocities

The conduction velocity data is summarized in figure 4.1 and table 4.1. Recordings were made from a total of 1976 neurons in the L5 dorsal root following vinblastine and saline treatments, neuritis and in untreated animals. Figure 4.1A represents histograms of the conduction velocities of the untreated group and of the treatment groups on day 4-5 post-surgery (i.e. at the peak of mechanical allodynia). Two distinct populations of neurons could be identified: those that corresponded to Aα/β-(myelinated; > 10 m/s; n = 909) and C/slow Aδ-fibre neurons (unmyelinated/thinly myelinated; 0.2-2.1 m/s; n = 1067) (Waddell *et al.*, 1989). Neurons with conduction velocities between 2.2 and 10 m/s were absent in these electrophysiological experiments.

In the untreated group (n = 76 neurons), the median conduction velocity of the C/slow Aδ-fibre neurons was 0.63 m/s (IQR = 0.33; Figure 4.1B; Table 4.1). Following vinblastine treatment (n = 387 neurons), saline treatment (n = 179

neurons) and neuritis (n = 425 neurons), there was a significant slowing of conduction at each time point when compared to the untreated group ($p < 0.0001$, Kruskal-Wallis test; $p < 0.001$, Dunn's post hoc tests; Figure 4.1B; Table 4.1). The most pronounced slowing was seen in the neuritis group at 3-4 hours (median = 0.34 m/s, IQR = 0.21) and at 12-15 days post-surgery (median = 0.37 m/s, IQR = 0.19). moreover, differences were observed between the neuritis and saline-treated groups ($p < 0.0001$, Kruskal-Wallis test; $p < 0.001$, Dunn's post hoc test; Figure 4.1B; Table 4.1) as well as neuritis and vinblastine-treated groups ($p < 0.0001$, Kruskal-Wallis test; $p < 0.0001$, Dunn's post hoc test; Figure 4.1B; Table 4.1) at 3-4 hours post-surgery, with conduction velocities in the neuritis group being significantly slower.

In the untreated group (n = 78 neurons), the median conduction velocity of the Aα/β-fibre neurons was 33.01 m/s (IQR = 12.78; Figure 4.1C). Following vinblastine (n = 334 neurons) and saline treatment (n = 160 neurons), the conduction velocities of Aα/β-fibre neurons were comparable to the untreated group at the time points examined ($p > 0.03$, Kruskal-Wallis test; Figure 4.1C; Table 4.1). In the neuritis group (n = 337 neurons), conduction velocities were significantly reduced at 3-4 hours (median = 26.06 m/s, IQR = 8.55) and 1-2 (median = 28.85 m/s, IQR = 8.19) days post-surgery (Figure 4.1C; Table 4.1). Moreover, differences were observed between the neuritis and saline-treated groups ($p < 0.0001$, Kruskal-Wallis test; $P < 0.0001$, Dunn's post hoc test; Figure 4.1C; Table 4.1) as well as neuritis and vinblastine-treated groups ($p < 0.0001$, Kruskal-Wallis test; $P < 0.0001$, Dunn's post hoc test; Figure 4.1C; Table 4.1) at 3-4 hours post-surgery, with conduction velocities in the neuritis group being significantly slower.

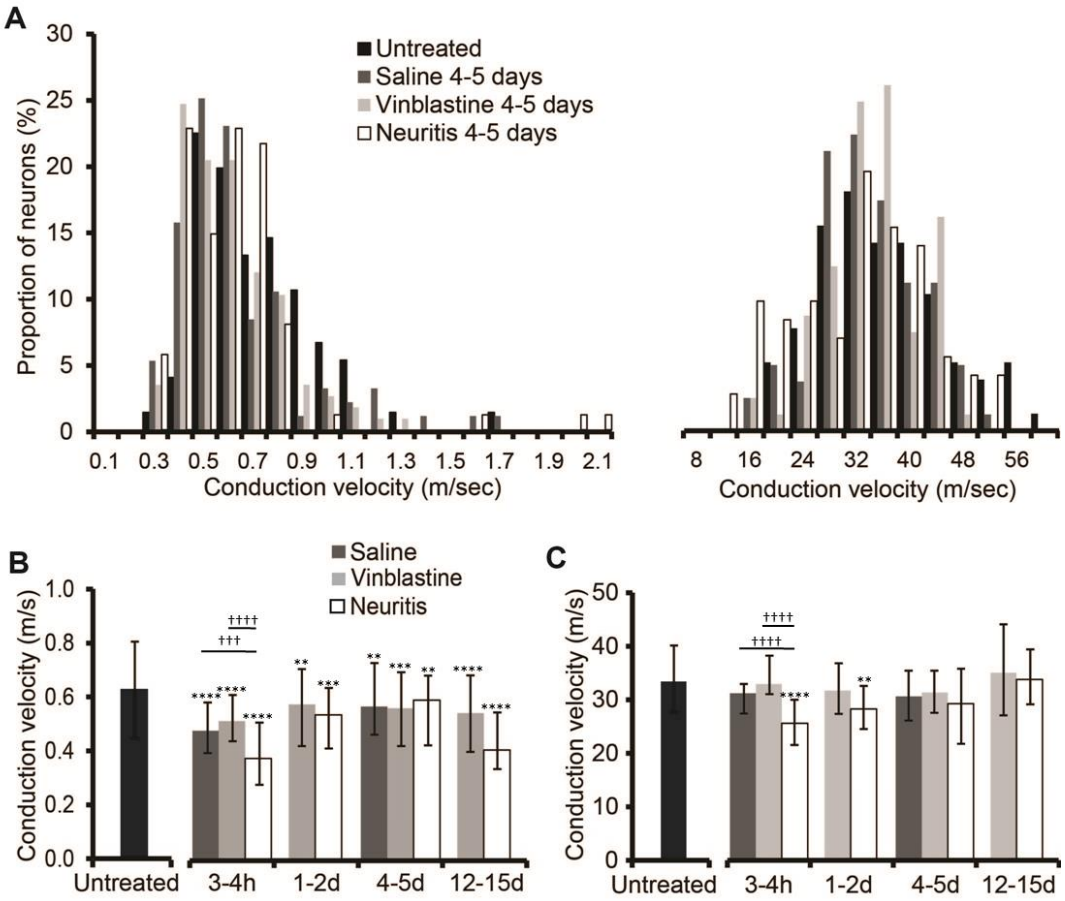


Figure 4.1 Conduction velocities of C/slow Aδ- and Aα/β-fibre neurons. (A) A histogram of the conduction velocities in the untreated group (n = 154 neurons) and on day 4-5 post-surgery following neuritis (n = 160 neurons), vinblastine (n = 199 neurons) and saline (n = 177 neurons) treatments. C/slow Aδ-fibre neurons were characterized by electrical stimulation of the L5 dorsal root, whereas Aα/β-fibre neurons were characterized by electrical stimulation of the sciatic nerve. The median conduction velocities for C/slow Aδ-fibre (B) and Aα/β-fibre (C) neurons are shown at different time points post-surgery. Saline-treated animals were only examined at 3-4 hours post-surgery and on days 4-5. **p < 0.01; ***p < 0.001; ****p < 0.0001 compared with untreated. †††p < 0.001; ††††p < 0.0001 compared with saline treatment (Kruskal-Wallis test followed by Dunn's post hoc test). Error bars are interquartile range (IQR).

Treatment	Time PS	Min value	1st QR	Median	3rd QR	Max value	n
			C/slow Aδ -fibre neurons				
Untreated	0	0.29	0.48	0.63	0.81	1.64	76
Vinblastine	3-4hr	0.31	0.42	0.47	0.57	0.90	74
	1-2d	0.27	0.40	0.52	0.66	1.52	100
	4-5d	0.26	0.39	0.51	0.65	1.22	118
	12-15d	0.24	0.38	0.49	0.63	1.18	95
Neuritis	3-4hr	0.20	0.26	0.34	0.47	1.41	101
	1-2d	0.21	0.40	0.50	0.61	1.07	131
	4-5d	0.25	0.39	0.54	0.63	2.05	88
	12-15d	0.23	0.31	0.37	0.51	1.44	105
Saline	3-4hr	0.27	0.37	0.44	0.54	1.92	83
	4-5d	0.24	0.43	0.52	0.68	1.61	96
			Aα/β-fibre neurons				
Untreated	0	16.89	27.50	33.01	40.28	58.67	78
Vinblastine	3-4hr	13.68	31.54	33.64	38.79	51.35	75
	1-2d	10.03	27.80	32.38	37.50	48.21	88
	4-5d	13.02	28.02	32.03	35.96	44.89	81
	12-15d	13.93	27.08	35.78	44.89	60.77	90
Neuritis	3-4hr	9.91	21.94	26.06	30.49	50.00	81
	1-2d	12.23	25.30	28.85	33.48	52.67	108
	4-5d	10.00	21.96	29.90	36.61	51.90	72
	12-15d	9.67	28.99	34.52	38.71	47.28	76
Saline	3-4hr	13.04	28.24	31.86	33.93	48.65	79
	4-5d	13.65	26.67	31.25	36.36	50.00	81

PS, post-surgery; QR, quartile; hr, hour; d, day.

Table 4.1 Conduction velocities of C/slow Aδ- and Aα/β- fibre neurons. Note that n represents the number of units.

4.2.2 Ongoing activity

The ongoing activity data is summarized in figures 4.2 (C/slow Aδ-fibre neurons) and 4.3 (Aα/β-fibre neurons) and table 4.2. Ongoing activity developed in 5.26% of neurons in the untreated group with a median firing rate of 0.6 Hz. In the vinblastine-treated group, the proportion of C/slow Aδ-fibre neurons with ongoing activity ranged from 6.00% to 10.81% at the time points examined (3-4 hrs, 1-2, 4-5, 12-15 days). In this group, ongoing activity levels were not significantly different from the untreated group ($p > 0.24$, comparing individual time points to untreated) or following saline treatment ($p > 0.12$ at 3-4 hours and 4-5 days postoperative, Fisher's exact tests). The median rate of ongoing activity in vinblastine treated neurons ranged from 0.08 to 0.35 Hz, which was comparable between time points ($p = 0.21$, Kruskal-Wallis test; Table 4.2). Different from vinblastine treatment, in the neuritis group, a significant increase in the proportion of ongoing units was present on days 4-5 post-surgery with 23.86% (21/88) of neurons firing spontaneously ($p < 0.05$ Fisher's exact test, compared to untreated and saline-treated). Their median firing rates ranged from 0.11 to 1.27 Hz with a significant increase on days 1-2 post-surgery compared to 3-4 hours and 12-15 days postoperative (Table 4.2; $p < 0.05$, Kruskal-Wallis test; $p < 0.05$, Dunn's post hoc tests). The firing patterns of C-fibre neurons tested were all irregular (Figure 4.2B).

Ongoing activity was present in only 1.27% (1/79) of Aα/β-fibre neurons in the untreated group with an irregular slow (0.02 Hz) firing pattern (Figure 4.3; Table 4.2). In the vinblastine treated group, the proportion of Aα/β-fibre neurons with ongoing activity ranged from 1.12% to 5.26% at the time points examined, which was not significantly different from the untreated group ($p > 0.20$ comparing individual time points to untreated) or from saline treatment ($p > 0.68$ at 3-4 hours

and 4-5 days postoperative, Fisher's exact tests). Similarly, in the neuritis group, ongoing activity was present in 1.31% to 6.48% of Aα/β-fibre neurons, which was also not significantly different from the untreated group ($p > 0.14$ comparing individual time points to untreated) or from saline treatment ($p > 0.44$ at 3-4 hours and 4-5 days postoperative, Fisher's exact tests). Median rates of ongoing activity in all Aα/β-fibre neurons tested ranged from 0.02 to 0.24 Hz (Table 4.2), and the firing patterns were irregular (Figure 4.3B).

		C/slow Aδ-fibre neurons			Aα/β-fibre neurons		
Treatment	**Time PS**	**Median**	**IQR**	**N**	**Median**	**IQR**	**N**
Untreated	0	0.60	1.03	4	0.02	0.00	1
Vinblastine	3-4hr	0.35	0.62	8	0.14	0.28	4
	1-2d	0.17	0.28	6	0.11	0.00	1
	4-5d	0.26	0.48	12	0.04	0.08	4
	12-15d	0.08	0.54	9	0.24	0.03	2
Neuritis	3-4hr	0.11	0.19	10	0.04	0.01	2
	1-2d	1.27*	1.31	15	0.09	0.15	7
	4-5d	0.55†#	1.30	22	0.11	0.09	2
	12-15d	0.10	0.13	5	0.02	0.00	1
Saline	3-4hr	0.14	0.10	7	0.03	0.15	4
	4-5d	0.47	0.56	4	0.21	0.20	2

* $p < 0.05$ compared to 3-4 hours and 12-15 days postoperative ; † $p < 0.05$ compared to 3-4 hours postoperative (Kruskal-Wallis test followed by Dunn's post hoc tests). # Based on 20 neurons (Rate could not accurately be determined for one neuron). PO postoperative, hr = hour, d = day.

Table 4.2 Rates (Hz) of ongoing C/slow Aδ- and Aα/β- fibre neurons.

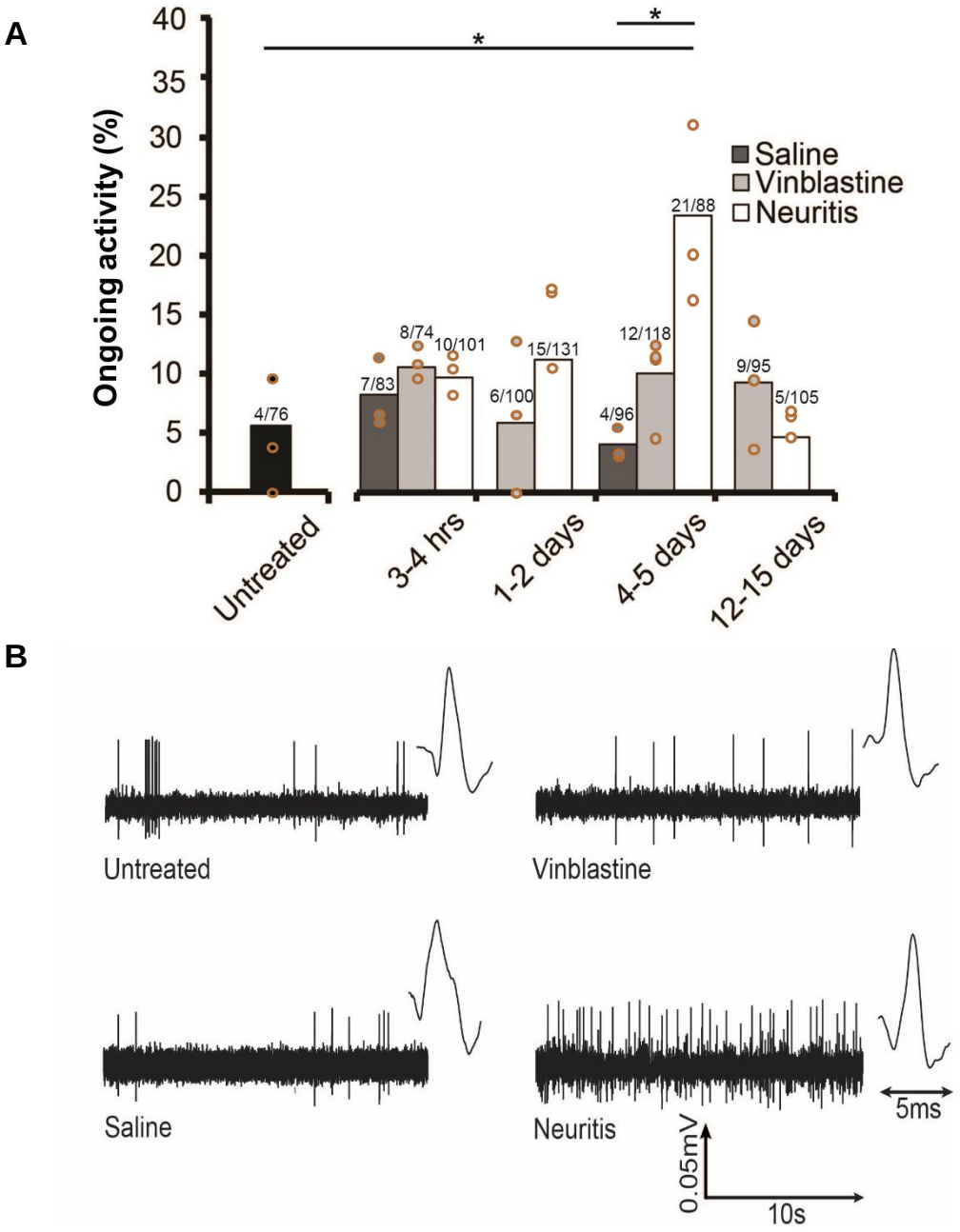


Figure 4.2 Development of ongoing activity in C/slow Aδ-fibre neurons. (A) The percentage of ongoing activity is shown for the untreated group and the neuritis, vinblastine and saline-treated groups at different time points post-surgery. Note the significant increase in ongoing activity in the neuritis group at day 4-5 post-surgery. Numbers of ongoing neurons are shown. Ongoing activity levels in each animal are indicated by orange circles (n = 3-4 animals in each group [day 1-2 vinblastine: 2 animals with 0% ongoing activity]; 16-53 fibres per rat). (B) Typical patterns of ongoing activity are also shown for each group. Inserts: expanded waveforms. *** $p < 0.001$ (Fisher's exact test)

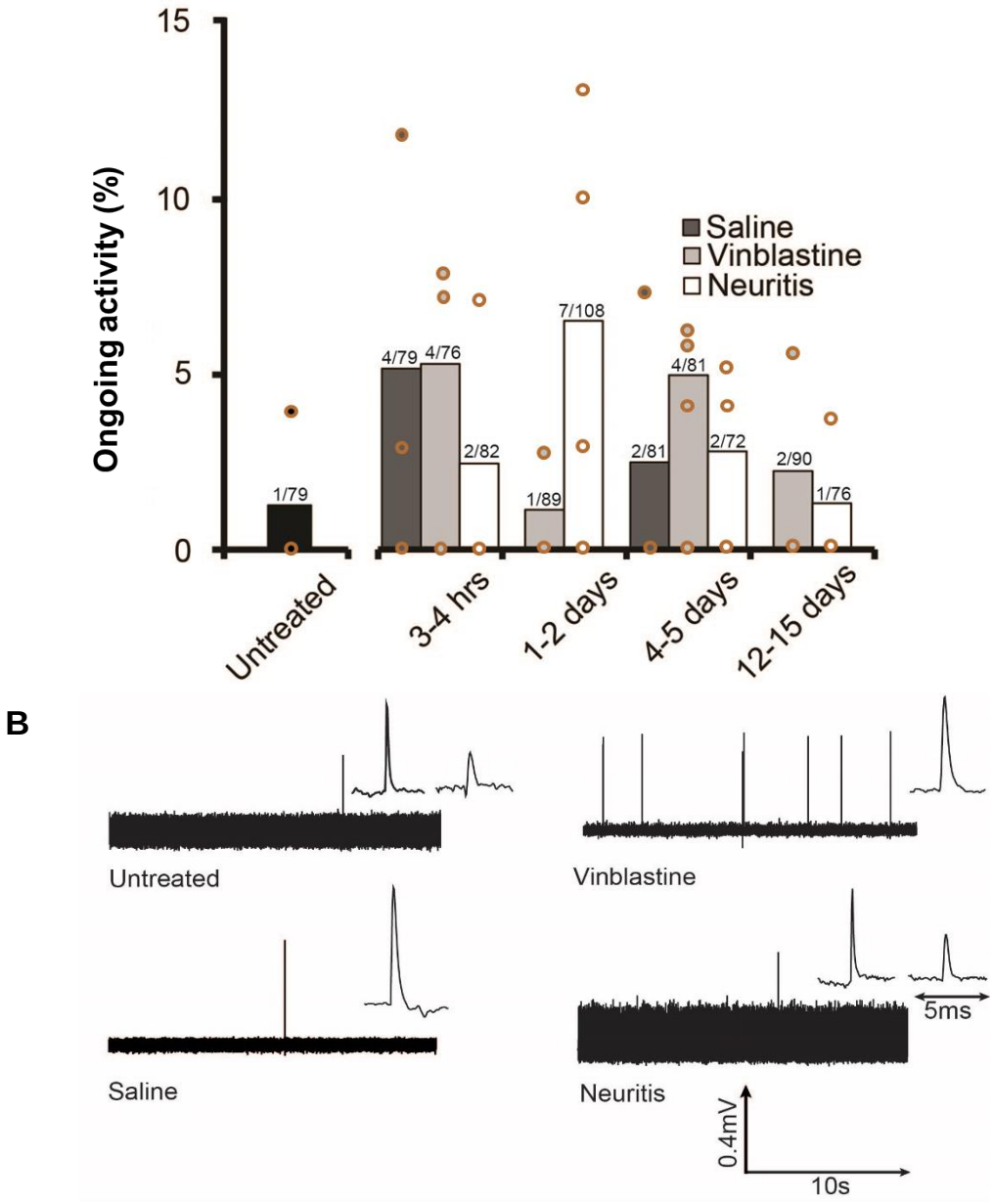


Figure 4.3 Development of ongoing activity in Aα/β-fibre neurons. (A) The percentage of ongoing activity is shown for the untreated group and the neuritis, vinblastine and saline-treated groups at different time points post-surgery. Numbers of ongoing neurons are shown. Ongoing activity levels in each animal are indicated by orange circles (n = 3-4 animals in each group [day 1-2 vinblastine: 3 animals with 0% ongoing activity; day 12-15 vinblastine: 2 animals with 0% ongoing activity; 3-4 hours neuritis: 2 animals with 0% ongoing activity; day 12-15 neuritis: 2 animals with 0% ongoing activity; day 4-5 saline: 2 animals with 0% ongoing activity; untreated: 2 animals with 0% ongoing activity]; 7-37 fibres per rat). (B) Typical patterns of ongoing activity are also shown for each group. Ongoing spindles are also present in the untreated and neuritis traces (smaller units). Inserts: expanded waveforms.

4.3 Summary of findings

- Conduction velocity slowing was present in C/slow Aδ-fibre neurons following vinblastine and saline treatments and neuritis at 3-4 hours, 1-2, 4-5 and 12-15 days post-surgery ($p < 0.05$ compared to pre-surgery).
- Conduction velocity slowing was present in Aα/β-fibre neurons at 3-4 hours and 1-2 days post-surgery following neuritis only ($p < 0.05$ compared to untreated).
- C/slow Aδ-fibre ongoing activity levels peaked on day 4-5 following neuritis with 23.86% of neurons firing spontaneously ($p < 0.05$ compared to untreated).
- C/slow Aδ-fibre ongoing activity levels following vinblastine (10.81%) and saline (8.43%) treatments were comparable to those in the untreated group (5.26%; $p > 0.19$).
- A significant increase in C/slow Aδ-fibre firing rates was observed only on day 1-2 following neuritis ($p < 0.05$ compared to 3-4 hours and 12-15 days postoperative).
- Aα/β-fibre ongoing activity levels following vinblastine (< 5.26%) and saline (< 5.12%) treatments as well as neuritis (< 6.48%) were comparable to that of untreated group (< 7%) at 3-4 hours, 1-2, 4-5 and 12-15 days post-surgery ($p > 0.1$).

CHAPTER 5:

Electrophysiology of the spinal cord

5.1 Introduction

Electrophysiological properties of spinal cord neurons following peripheral nerve injury and chronic hind paw inflammation have been extensively studied in order to identify central mechanisms underlying neuropathic pain behaviours. The common changes in dorsal horn neurons include increased ongoing activity levels, size of receptive fields, responses to peripheral stimulation and after-discharge (Laird and Bennett, 1993, Chapman *et al*., 1998, Behbehani and Dollberg-Stolik, 1994, Takaishi *et al*., 1996, Hylden *et al.*, 1989, Sotgiu and Biella, 2000, Suzuki *et al*., 2001), which are in agreement with central involvement. Examination of a subpopulation of dorsal horn neurons known as WDR neurons has revealed increases in excitability to repetitive noxious input, wind-up, following chronic inflammation (Traub, 1997, Herrero and Cervero, 1996, Stanfa *et al*., 1992), but not following a traumatic nerve injury (Chapman *et al*., 1998). Although wind-up, a short form of synaptic plasticity in WDR neurons, is not the same as central sensitization, they share common mechanisms (Li *et al*., 1999, Woolf, 1996) that have led to a high interest in this population of dorsal horn neurons among the scientific community. Unfortunately, information on electrophysiological properties of WDR dorsal horn neurons following neuritis or vinblastine-induced axonal transport disruption is absent. Since both neuritis and vinblastine-treatment cause the development of neuropathic pain behaviours (e.g. mechanical allodynia) and since much of the evidence supports a central mechanism for such behaviours, in this study it is hypothesised that vinblastine treatment as well as neuritis will lead to alterations in properties of deep WDR neurons.

Aims

- To examine ongoing activity levels of WDR neurons following vinblastine treatment and neuritis.
- To examine if there are any changes in responses of WDR neurons to mechanical stimulation of the receptive field following vinblastine treatment and neuritis.
- To examine if neuronal depth within the dorsal horn determines the response of the receptive field to mechanical stimulation.
- To examine wind-up responses of WDR neurons following vinblastine treatment and neuritis.

5.2 Results

5.2.1 Characterisation of a WDR neuron

Recordings were made from a total of 70 dorsal horn WDR neurons (L5 segment) that responded to both light innocuous touch (brushing) and noxious pinch (untreated group, n = 19; saline group, n = 18; neuritis group, n = 20; vinblastine group, n = 13). All cells had receptive fields on the ipsilateral hindpaw. Most of the neurons recorded were deep dorsal horn neurons located in approximately the lamina V region (mean depth from the surface of the spinal cord = 551.43 ± 142.69 μm; range: 200 – 900 μm; Figure 5.1).

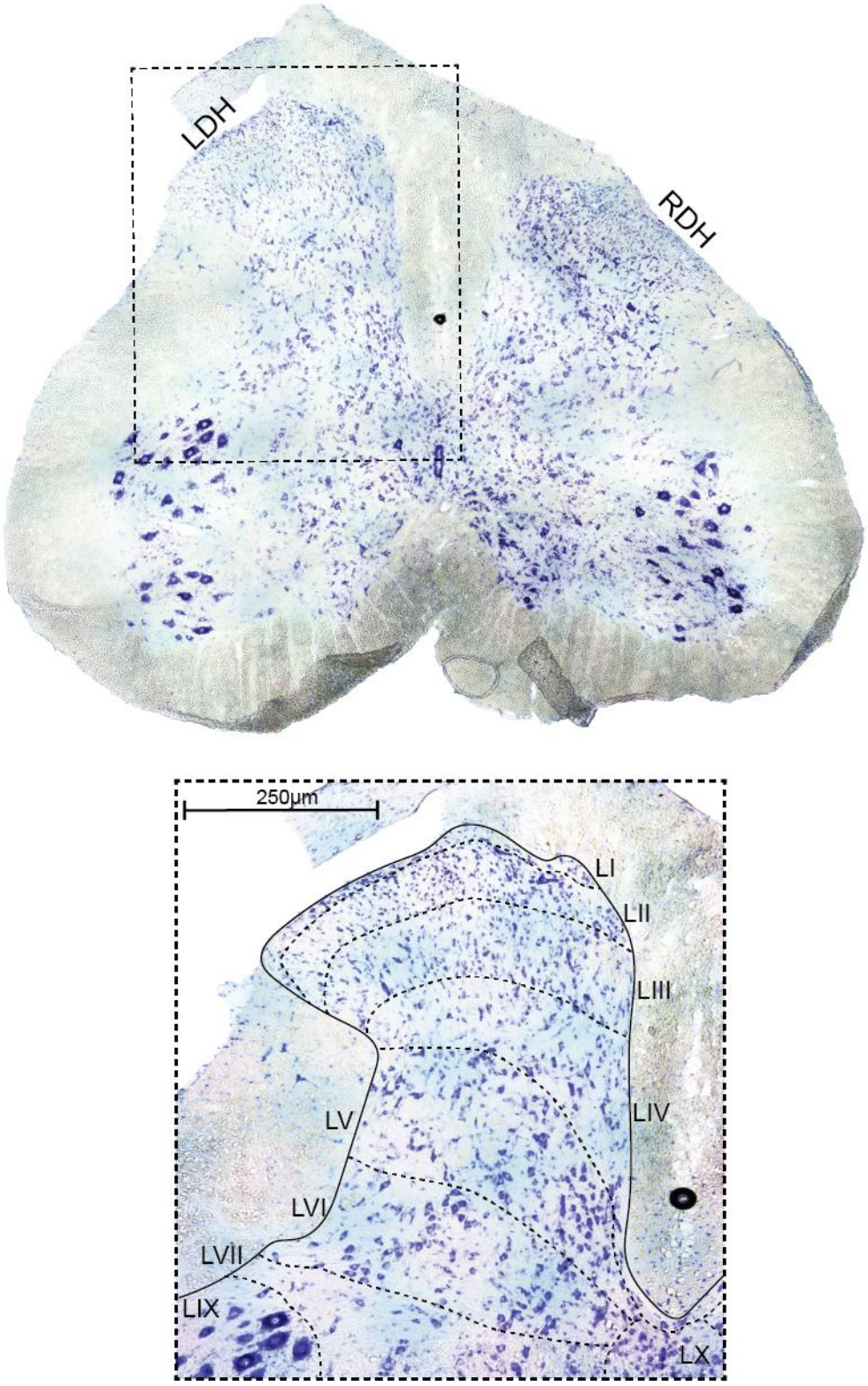


Figure 5.1 A transverse section of the rat L5 spinal cord stained with a Nissl stain.

5.2.2 WDR neuronal ongoing activity levels

Following isolation of a WDR neuron, background ongoing activity was recorded for three minutes. The proportion of neurons with ongoing activity was similar between the four groups with > 75.0% of neurons firing spontaneously (i.e. > 0.017 Hz; $p > 0.18$, Fisher's exact tests; Table 5.1). There were no differences in ongoing activity rates between the treatments ($p = 0.32$ Kruskal-Wallis test). The median rates of ongoing activity were slow, ranging from 0.06-0.24 Hz. The firing pattern was irregular (Figure 5.2).

Figure 5.1 A transverse section of the rat L5 spinal cord stained with a Nissl stain. The top image collage was produced using 100X magnification photographs. The insert represents the left dorsal horn imaged at 200X. Here, the Rexed laminar boundaries are marked by the dashed lines (based on Kayalioglu and Heise, 2009). Nissl staining confirmed that the majority of WDR neurons were located in the deep dorsal horn with the mean depth of 551.43 ± 142.69 μm from the surface of the spinal cord (approximately the lamina V region). LDH, left dorsal horn; RDH, right dorsal horn.

Treatment	Number of WDR neurons	%OA	Rate, Median (IQR)
Untreated	19	89.5	0.12 (0.13)
Saline	18	94.4	0.13 (0.19)
Neuritis	20	75.0	0.06 (0.20)
Vinblastine	13	76.9	0.24 (0.08)

WDR, wide dynamic range; OA, ongoing activity;
IQR, interquartile range

Table 5.1 Levels (%) and rates (Hz) of ongoing wide dynamic range neurons.

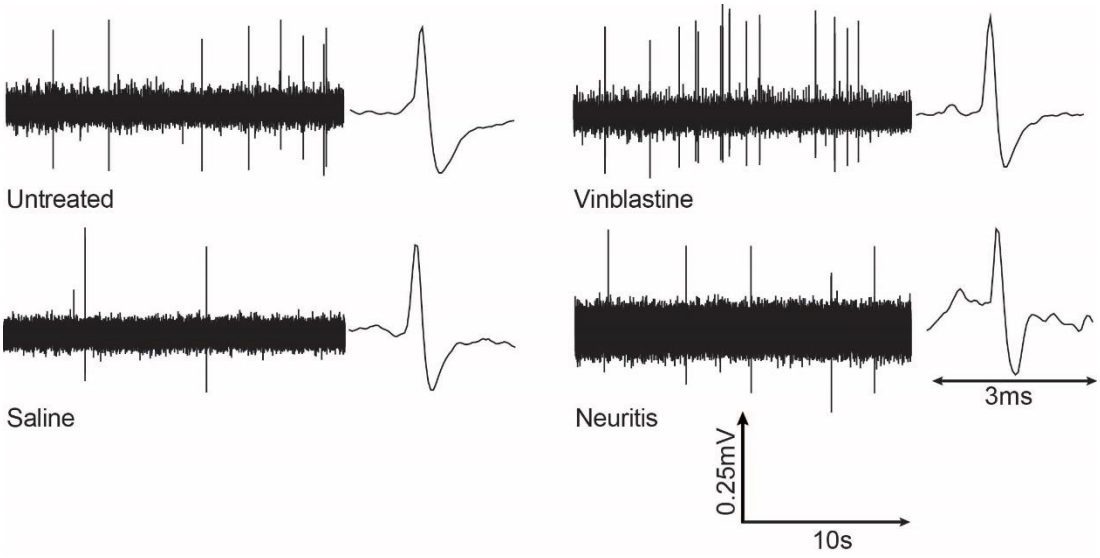


Figure 5.2 Ongoing activity levels in wide dynamic range neurons. Example traces of ongoing activity levels in the untreated, saline- and vinblastine-treated and neuritis groups. Inserts: expanded waveforms.

5.2.3 Responses to natural stimuli

For all WDR neurons, action potentials were evoked in response to both innocuous and noxious mechanical stimulation of the ipsilateral distal hind paw (Figure 5.3). The number of action potentials evoked by brushing the centre of each receptive field was comparable between groups ($p = 0.47$, one-way ANOVA). A significant interaction was present between the treatment group and von Frey monofilaments of increasing stiffness ($p < 0.05$ two-way ANOVA). In the untreated, saline-treated and neuritis groups, WDR neurons responded to von Frey monofilaments in a progressive manner, with higher number of spikes evoked by increasing force. In the vinblastine-treated group, the response plateaued with stiffer von Frey monofilaments (26-60 g). In fact, the number of action potentials evoked by 60 g von Frey monofilament was significantly lower in the vinblastine-treated group when compared to the untreated ($p < 0.001$) and neuritis groups ($p < 0.05$, Bonferroni's post hoc tests). The number of evoked average number of spikes in response to 10 s pinching was comparable between the four treatment groups ($p = 0.14$ one-way ANOVA).

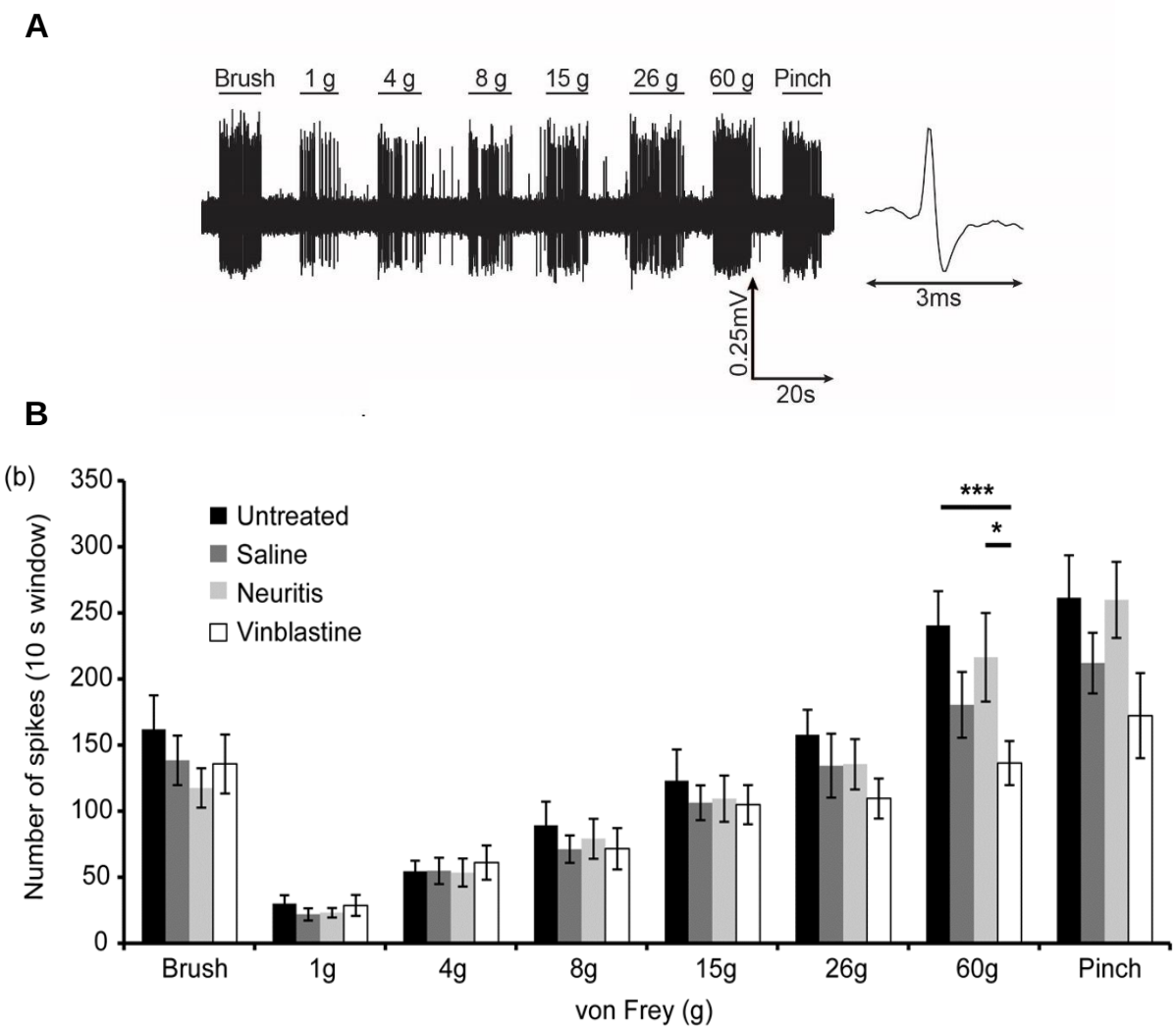


Figure 5.3 Responses of wide dynamic range neurons to mechanical stimulation of the peripheral receptive field (10 seconds from the stimulus onset). (A) A typical wide dynamic range neuron response (from a saline-treated animal) to lightly brushing the receptive field, applying von Frey hairs of ascending stiffness and pinching the skin with curved forceps. Insert: expanded waveform. (B) The mean number of action potentials elicited by brushing, von Frey monofilaments and pinching the skin in untreated, saline-treated, neuritis and vinblastine-treated animals (n = 19, 18, 20 and 13 wide dynamic range neurons in the untreated, saline-treated, neuritis and vinblastine treated group respectively). Note the decreased response to stiffer von Frey monofilaments in the vinblastine-treated group. * $p < 0.05$, *** $p < 0.001$ Two-way ANOVA followed by Bonferroni's post hoc test. Error bars = SEM.

5.2.4 Neuronal depth analysis

For analysis of neuronal depth on the response to mechanical stimulation of the receptive fields, WDR neurons were grouped into neurons < 550 μm (range: 200-550 μm) and neurons ≥ 550 μm (range: 550-900 μm; Figure 5.4) from the dorsal surface of the spinal cord. The cut off value was chosen based on the mean depth of all WDR neurons from the dorsal surface of the spinal cord (mean = 551.43 ± 142.69 μm).

In the untreated and saline-treated groups, there was no significant interaction between responses evoked by each von Frey monofilament and neuronal depth ($p > 0.06$) or main effect for depth ($p > 0.39$, two-way ANOVA). In both neuritis and vinblastine-treated groups, WDR neurons ≥ 550 μm from the dorsal surface of the spinal cord were less responsive to thinner von Frey monofilaments (1-15 g) than those < 550 μm from the dorsal surface of the spinal cord. In the neuritis group, there was no significant interaction between neuronal depth and von Frey monofilament stiffness ($p = 0.66$) or main effect for depth ($p = 0.13$, two-way ANOVA). In contrast, following vinblastine treatment, a significant interaction between neuronal depth and von Frey monofilament stiffness was present ($p < 0.001$, two-way ANOVA). In this group, WDR neurons < 550 μm from the dorsal surface of the spinal cord reached a plateau in responses to 8-60g von Frey monofilaments (Figure 5.4 interactions). Such reduction in responses was not observed with WDR neurons ≥ 550 μm from the dorsal surface of the spinal cord. In order to rule out the effects of animal size on neuronal depth, animal weights were compared between experiments that examined WDR neurons < 500 μm and ≥ 550 μm from the dorsal surface of the spinal cord. No significant differences were found between animal weights in the saline-treated, neuritis or vinblastine-treated groups. There was however a significant

difference observed in the untreated group (mean weight; neurons < 500 μm: 292 ± 18 g; neurons ≥ 550 μm: 373 ± 20 g; $p < 0.05$ Student's t-test).

No differences in ongoing activity rates were observed between WDR neurons located < 500 μm and ≥ 550 μm from the dorsal surface of the spinal cord in each treatment group ($p > 0.15$ Mann-Whitney U test) or between treatment groups in populations of WDR neurons located < 500 μm and ≥ 550 μm from the dorsal surface of the spinal cord ($p > 0.195$ Kruskal-Wallis test).

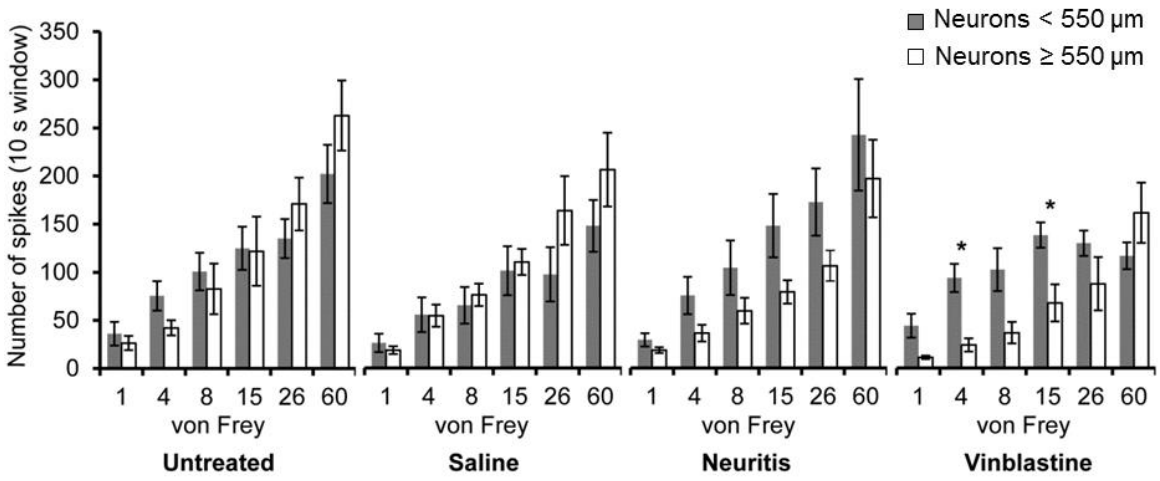


Figure 5.4 Mechanical responses of wide dynamic range neurons based on neuronal depth within the dorsal horn. Neurons were grouped into those with depths < 550 µm from the dorsal surface of the spinal cord (7, 8, 9 and 7 WDR neurons in the untreated, saline-treated, neuritis and vinblastine-treated groups respectively) and those with depths ≥ 550 µm from the dorsal surface (12, 10, 11 and 6 WDR neurons in the untreated, saline-treated, neuritis and vinblastine-treated groups respectively). *$p < 0.05$ Two-way ANOVA followed by Bonferroni's post hoc tests.

5.2.5 Wind-up

Forty-two of the characterized WDR neurons showed signs of wind-up in response to repeated electrical stimulation (x16) of the receptive field (untreated group, n = 10; saline group, n = 11; neuritis group, n = 10; vinblastine group, n = 10; e.g. Figure 5.5). The total number of Aβ-, Aδ- and C-fibre evoked responses, input (i.e. number of action potentials evoked by first pulse x total number of pulses) and after-discharge of the neurons that showed signs of wind-up is summarised table 5.2. There were no significant differences between groups in the number of action potentials evoked by Aβ-fibre neurons ($p = 0.47$, one-way ANOVA). Although not quite significant, there was a notable reduction in the number of action potentials evoked by Aδ- and C-fibre neurons in the vinblastine-treated group compared to neuritis ($p < 0.05$, one-way ANOVA; $p = 0.06$ and 0.07 respectively, Bonferroni post-hoc tests). Input and after discharge were comparable between groups ($p = 0.17$ and 0.11 respectively, one-way ANOVA).

The untreated, saline- and vinblastine-treated groups showed similar wind-up responses (mean wind-up (action potentials): untreated, 50.70 ± 9.07; saline, 56.73 ± 13.89; vinblastine, 43.70 ± 10.05; Figure 5.6). In contrast, there was a significant increase in wind-up response following neuritis with a mean number of 115.80 ± 21.67 spikes ($p < 0.05$ one-way ANOVA; $p < 0.05$ compared to untreated and saline-treated groups and $p < 0.01$ compared to vinblastine-treated group, Bonferroni post hoc test; Figure 5.7).

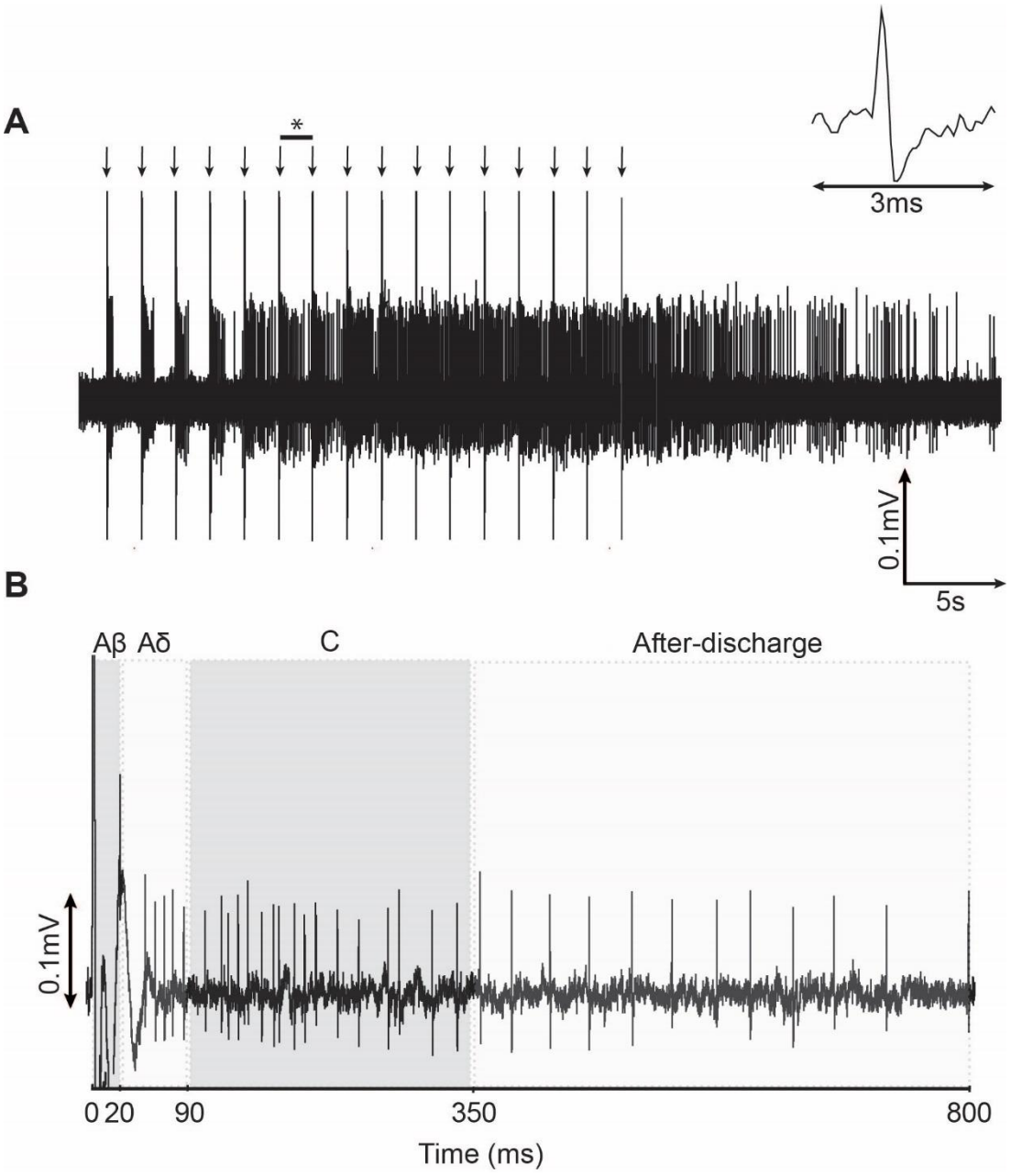


Figure 5.5 A typical wind-up response. (A) A typical wind-up response from a neuritis animal. The centre of the receptive field was electrically stimulated 16 times at 3x C-fibre threshold (arrows). Insert: expanded waveform; (B) Expanded section of trace (* on part A), showing action potentials evoked by electrical stimulation. Responses were analysed based on the latency of each spike. Please note that shading represents clusters of Aβ- (0-20 ms), Aδ- (20-90 ms), C-fibre (90-350 ms) responses as well as after-discharge (350-800 ms).

	Treatment group			
	Untreated	**Saline**	**Neuritis**	**Vinblastine**
Aβ-fibre	7.90±3.57	16.64±8.09	12.30±6.47	4.60±2.00
Aδ-fibre	29.10±6.92	46.09±11.84	50.00±8.88	15.60±5.87
C-fibre	209.90±27.68	158.36±20.52	229.70±35.27	129.40±21.18
Input	233.60±31.21	162.91±31.47	224.00±46.86	132.80±33.23
After-discharge	74.40±12.85	61.27±16.98	110.10±24.47	47.10±15.31

Table 5.2 A comparison of the mean total electrically-evoked responses of wide dynamic range neurons in the untreated, saline, neuritis and vinblastine treatment groups. Data is expressed as mean total number of spikes per group ± SEM. The responses evoked by Aβ-, Aδ- and C- fibre neurons are separated and analysed based on their latencies (0-20 ms, 20-90 ms and 90-350 ms respectively). Input is calculated as the sum of the C-fibre-evoked responses after the first stimulus multiplied by 16. After-discharge represents responses at latencies between 350 and 800 ms (note that wind-up can be calculated from these values by subtracting the input from the sum of action potentials between 90 and 800 ms (C-fibre responses and after-discharge) that are evoked by the first 16 consecutive stimuli).

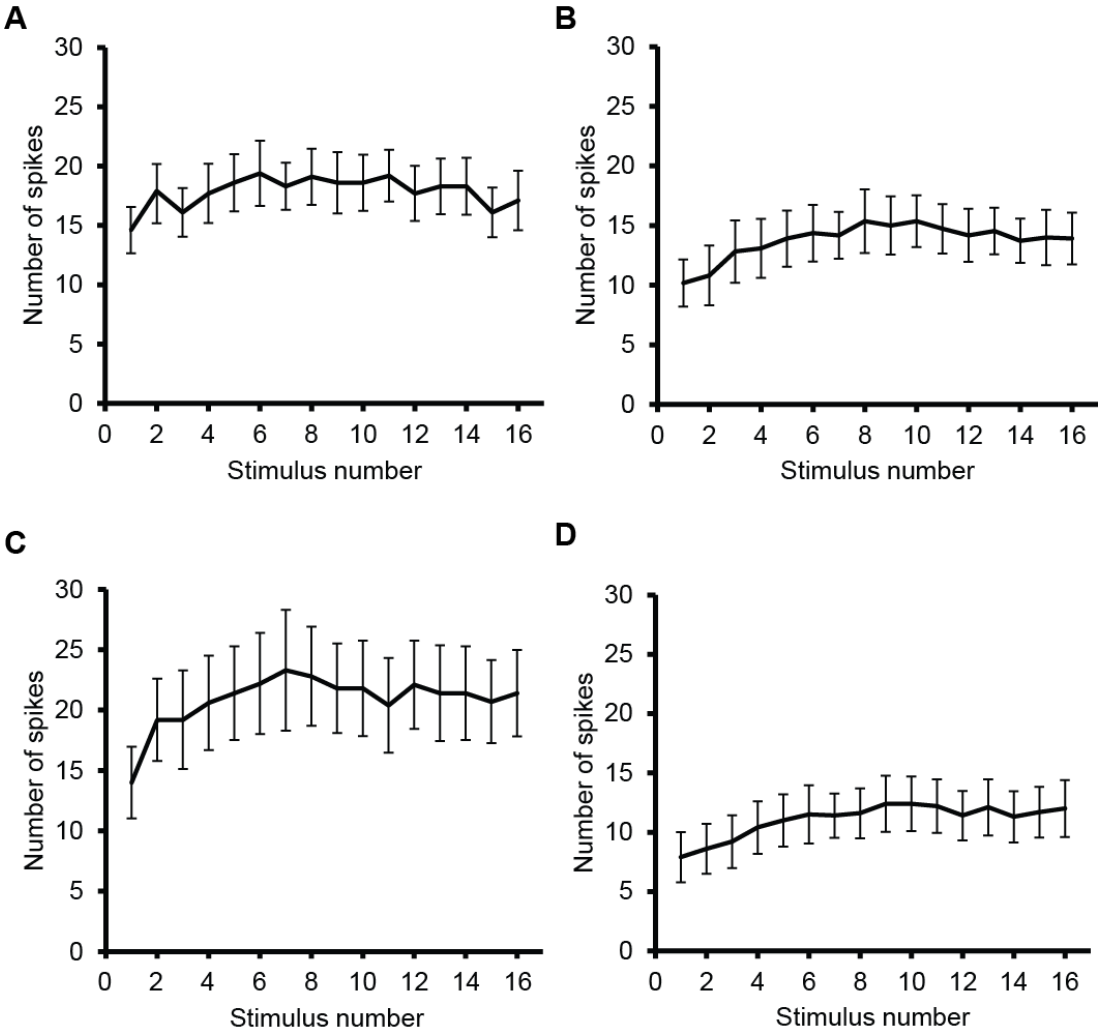


Figure 5.6 The mean total number of spikes in wide dynamic range neurons in (A) untreated, (B) saline-treated, (C) neuritis and (D) vinblastine-treated groups evoked per stimulus of the electrical train used to induce wind-up. An increase in the mean number of spikes evoked by each stimulus (up until the 8th stimulus, after which the number of spikes tends to plateau) can be observed in all four groups. However, it is more pronounced in the neuritis group. Error bars = SEM.

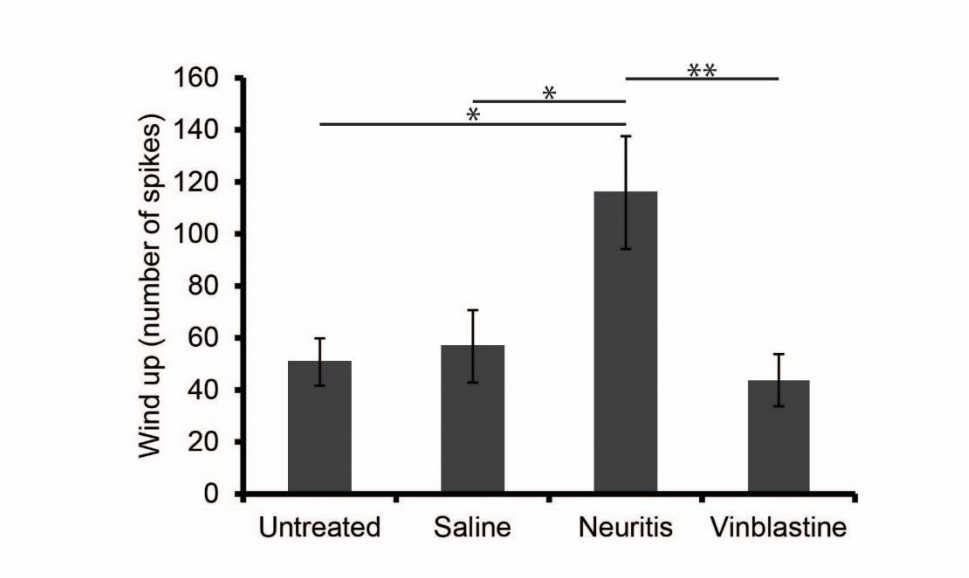


Figure 5.7 Comparison of the mean wind-up responses of wide dynamic range neurons in untreated, saline-treated, neuritis and vinblastine-treated groups. Wind-up was calculated as the sum of action potentials between 90 and 800 ms (C-fibre responses) produced by 16 consecutive stimuli minus C-fibre responses evoked by the first stimulus multiplied by the total number of stimuli (16; input). Number of neurons: 10 in untreated group (from 6 animals), 11 in saline-treated group (from 7 animals), 10 in neuritis group (from 5 animals) and 10 in vinblastine-treated group (from 6 animals). *$p < 0.05$ and **$p < 0.01$ (one-way ANOVA followed by a Bonferroni post-hoc test). Error bars = SEM.

5.3 Summary of findings

- Similar levels of WDR neuronal ongoing activity were found between the untreated, saline-treated, vinblastine-treated and neuritis groups (> 75.0% of neurons firing spontaneously; $p = 0.32$).
- Progressive responses to von Frey monofilaments of increasing stiffness were present in untreated, saline-treated and neuritis groups ($p < 0.05$).
- In the vinblastine-treated group, the response plateaued with stiffer von Frey monofilaments with the number of action potentials evoked by 60 g von Frey monofilament being less than that in the untreated ($p < 0.001$) and neuritis groups ($p < 0.05$).
- WDR neurons located ≥ 550 μm from the dorsal surface of the spinal cord were less responsive to thinner von Frey monofilaments (1-15 g) than those WDR neurons located < 550 μm from the dorsal surface of the dorsal horn following both vinblastine treatment and neuritis.
- WDR neurons located < 550 μm from the dorsal surface of the dorsal horn reached a plateau in responses to 8-60g von Frey monofilaments.
- Wind-up response was increased in the neuritis group only ($p < 0.05$ compared to untreated and saline-treated groups and $p < 0.01$ compared to vinblastine-treated group).

CHAPTER 6:

Immunohistochemistry of the spinal cord

6.1 Introduction

C-fos, the nuclear protein of the proto-oncogene *c-fos*, is rapidly expressed in the laminae I, II, V and VI of dorsal horn after noxious stimulation (e.g. electrical stimulation) (Hunt *et al.*, 1987, Coggeshall, 2005, Gao and Ji, 2009, Molander *et al*., 1994). C-fos expression is induced following an increase in cellular calcium, and may be dependent on activation of cyclic adenosine monophosphate response element-binding protein (Morgan and Curran, 1986, Latremoliere and Woolf, 2009). Together with c-jun, c-fos forms a heterodimer that is capable of binding to deoxyribonucleic acid, leading to the transcription of various genes, such as those coding for neuropeptides and dynorphin. In turn, the newly synthesised proteins may act on excitatory circuits to cause neuronal hyperexcitability (Ji *et al*., 2003). Although the exact role of c-fos in pain perception is not well understood, since c-fos plays a role in signalling pathways involved in modulation of spinal nociceptive mechanisms (Hunt *et al*., 1987, Kajander *et al.*, 1996), it is considered to be a marker of injury-induced neuronal activation, as well as an indirect indicator of central sensitization. Under normal conditions, basal c-fos expression in the spinal cord is minimal, and innocuous stimulation does not induce its expression. In contrast, following nerve injury, c-Fos expression in the superficial dorsal horn neurons is increased (Molander *et al*., 1994, Molander *et al.*, 1998, Catheline *et al.*, 1999). Currently, there are no studies that examined c-fos expression in the superficial dorsal horn, which is the area of termination for nociceptors, following neuritis or vinblastine-treatment. In this study, these neurons were not examined electrophysiologically. Therefore, c-fos expression in the superficial dorsal horn in response to electrical stimulation of the sciatic nerve was assessed. It was hypothesised that since neuropathic pain behaviours develop in both models, they

both will show an increase in c-fos expression in the superficial laminae, indicative of increased neuronal excitability. It is also hypothesised that such increase will be more pronounced following neuritis due to the presence of ongoing activity from primary sensory neurons.

Substance P is an excitatory neurotransmitter (von Euler and Gaddum, 1931) found in peripheral and central terminals of nociceptive afferent fibre neurons (Nilsson *et al.*, 1974, Ljungdahl *et al*., 1978, McCarthy and Lawson, 1989, Otsuka and Konishi, 1976, White, 1997). It is transported in storage vesicles from the cell body towards the terminals by axonal transport (Brimijoin *et al.*, 1980) and accumulates in the central terminals that are located in the superficial layer of the dorsal horn (Ljungdahl *et al*., 1978). Once released, it binds to NK-1Rs expressed by projection cells or interneurons, which play a role in nociception (Todd *et al*., 1998, Al Ghamdi *et al*., 2009). The role for substance P in neuropathic pain mechanisms is reflected by changes in its spinal cord levels following nerve injury (Barbut *et al.*, 1981, Fitzgerald *et al.*, 1985, Garrison *et al.*, 1993, Villar *et al.*, 1991) or chronic hindpaw inflammation (Galeazza *et al.*, 1995, Abbadie *et al.*, 1996). As both neuritis and vinblastine-treatment disrupt axonal transport and lead to the development of neuropathic pain behaviours, here it is hypothesised that substance P levels will be altered in the superficial dorsal horn as an indication of altered peptidergic nociceptor transmission.

Aims

- To investigate activity-dependent c-fos expression in the superficial dorsal horn following vinblastine-treatment and neuritis.

- To examine substance P expression in the superficial dorsal horn following vinblastine-treatment and neuritis.

6.2 Results

6.2.1 Electrical stimulation-induced c-fos expression

C-fos expression in the superficial dorsal horn is summarized in figure 6.1. C-fos immunofluorescence was present within the superficial dorsal horn in all groups (depth of staining: < 300 µm from the dorsal surface of spinal cord; Figure 6.1 A, n = 4 animals in each group). Double-labelling with neuronal marker NeuN confirmed that the majority of c-fos labelled cells were neurons (Figure 6.1 B).

In the unstimulated groups, there was no significant interaction between treatment and side for the numbers of c-fos positive cells ($p = 0.08$) or main effect ($p > 0.23$, two-way ANOVA; Figure 6.1 C). Repeated electrical stimulation of the sciatic nerve at C- and A-fibre strength led to an increase in c-fos expression in all treatment groups. In the groups stimulated at C-fibre strength, there was no significant interaction between treatment and side ($p = 0.27$). However, there were significant main effects for treatment ($p < 0.05$) and side ($p < 0.01$, two-way ANOVA), with increased c-Fos expression on the ipsilateral side in the neuritis and vinblastine-treated groups compared to the untreated group ($p < 0.05$, Bonferroni's post hoc tests) and contralateral sides ($p < 0.05$, Bonferroni's post hoc tests). In the groups stimulated at A-fibre strength, there was no significant interaction between treatment and side ($p = 0.33$) or main effect ($p > 0.06$, two-way ANOVA).

To evaluate whether repeated electrical stimulation had any effects on conduction of A-fibre neurons, nine compound action potentials in the A-fibre neuron conduction velocity range (mean range 14.48-51.43 m/s; n = 3 animals) were recorded before

and after five minutes of C-fibre electrical stimulation. There was no significant difference between the peak of the compound amplitudes before (mean = 0.26 mV, SD = 0.2) and after stimulation (mean = 0.27 mV, SD = 0.2; p = 0.47 paired Student's t-test). Similarly, no significant difference was present between area under the curve before (mean = 0.56, SD = 0.14) and after stimulation (mean = 0.59, SD = 0.14; p = 0.34 paired Student's t-test). The mean percent change in amplitude and area under the curve was 7.6% and 6.04% respectively, indicating that repeated electrical stimulation did not block A-fibre neurons (Figure 6.2).

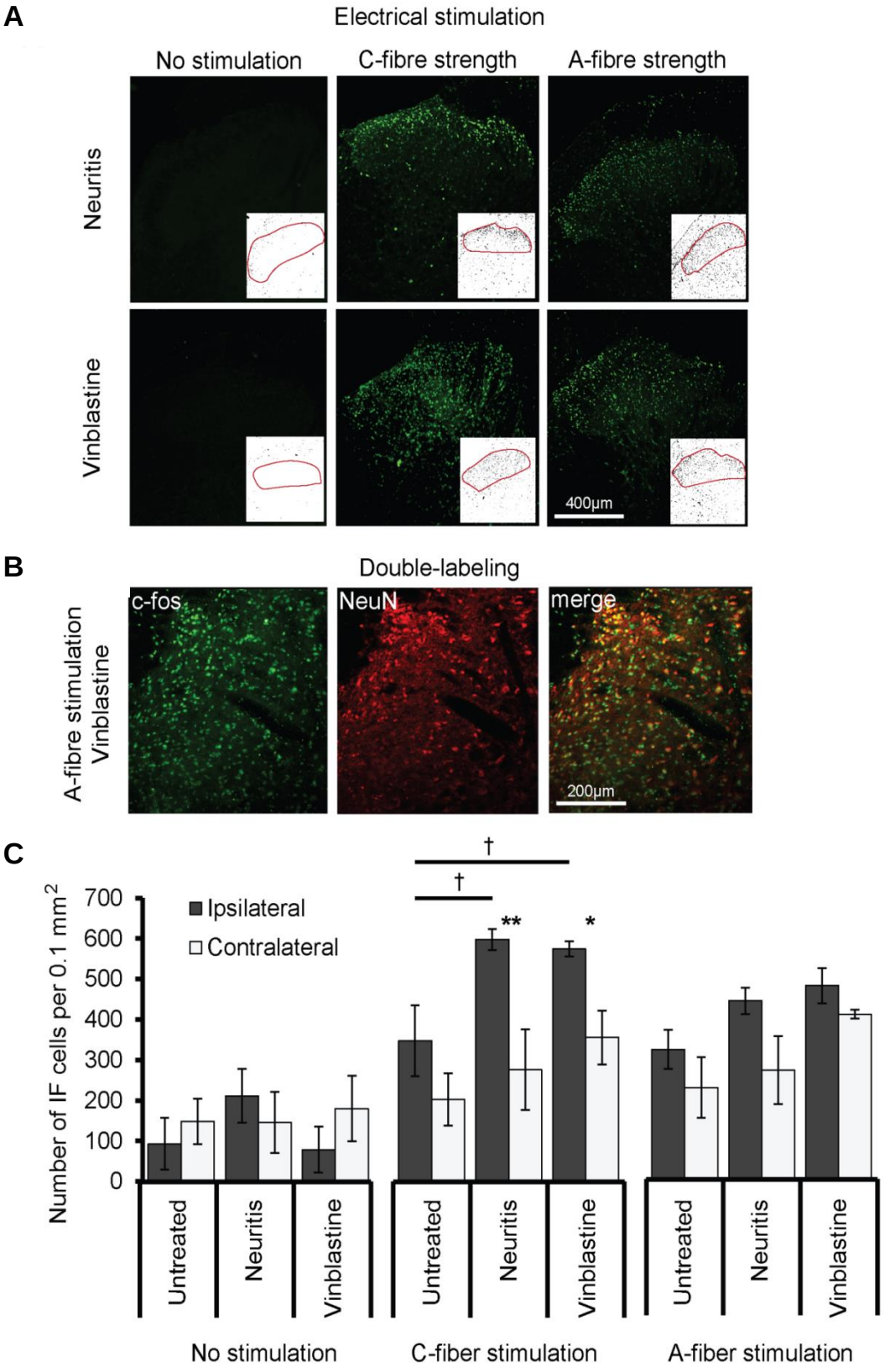


Figure 6.1 Electrical stimulation-induced c-fos expression in the L5 dorsal horn.

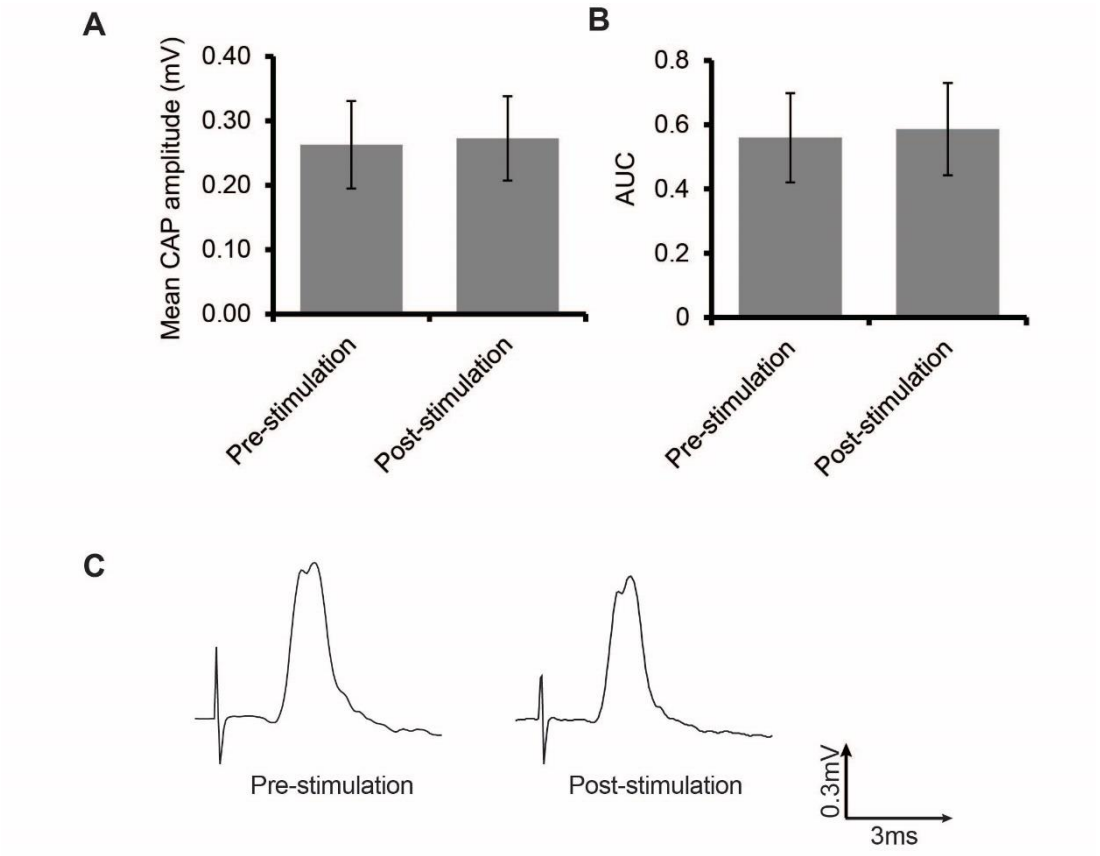


Figure 6.2 (A) Peak compound amplitude (CAP; mV) and (B) area under the curve (AUC) of A-fibre neurons before and after repeated five minute stimulation at C-fibre strength. (C) Examples of A-fibre compound action potential pre- and post-stimulation.

6.2.2 Substance P expression

Substance P labelling was present in the superficial lamina of the dorsal horn in the neuritis, vinblastine- and saline-treated groups (n = 3 animals in each group; Figure 6.3A). The size of the area with positive substance P labelling was comparable between ipsilateral and contralateral sides in the untreated (mean ratio [ipsilateral area/contralateral area] = 1.05 ± 0.10) and neuritis groups (mean ratio = 0.97 ± 0.07). Following vinblastine treatment there was a significant decrease in the size of the area positive for substance P on the ipsilateral compared to contralateral side (mean ratio = 0.71 ± 0.09; $p < 0.05$ one-way ANOVA; $p < 0.05$ Bonferroni post hoc test; Figure 6.3 B).

Figure 6.1 Electrical stimulation-induced c-fos expression in the L5 dorsal horn. (A) Example images of c-fos expression (green staining) in the ipsilateral dorsal horn following vinblastine treatment and neuritis. Prior to removal of the tissue, animals received either 5 min repeated stimulation of the sciatic nerve at C- (middle) or A-fibre strength (right), or no stimulation (left). The majority of c-fos positive cells were located in the superficial laminae. Note inserts that represent respective image thresholding used for ImageJ analysis. (B) Co-localization of c-fos (green) with NeuN (red). (C) Mean number of c-fos positive cells per 0.1 mm^2 in each group (n = 4 animals in each group; 1-8 sections per animal). † $p < 0.05$ comparing between treatments; * $p < 0.05$, ** $p < 0.01$ comparing between sides (two-way ANOVA followed by Bonferroni's post hoc tests). IF, Immunofluorescent. Error bars = SEM.

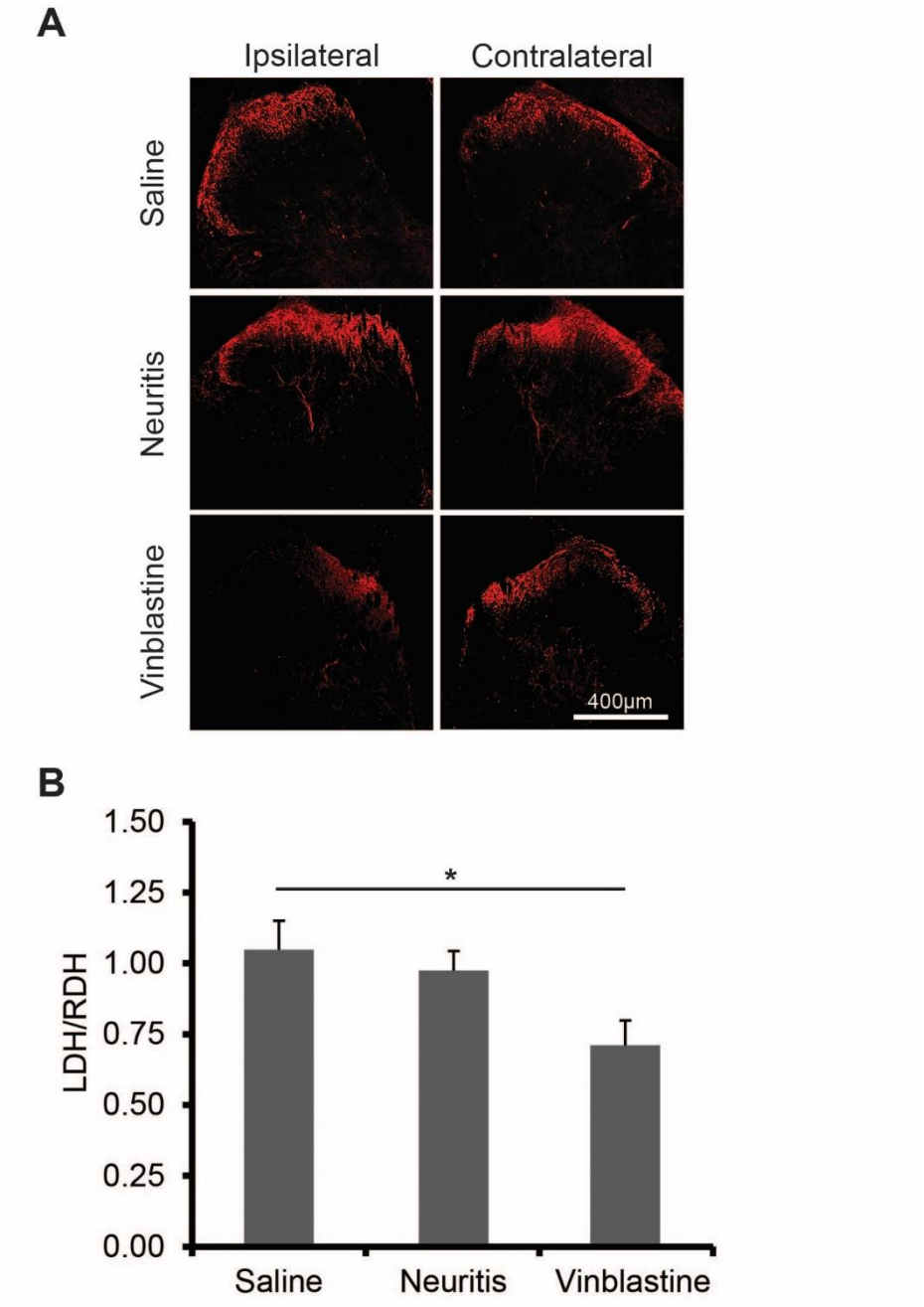


Figure 6.3 Substance P expression in the dorsal horn. (A) Typical example images of substance P immunolabelling in the L5 dorsal horn in the saline-treated, neuritis and vinblastine-treated groups. The majority of substance P expression is observed within the superficial lamina (red staining). (B) Mean ratios of the dorsal horn area with positive substance P labelling. Calculation was made by dividing the ipsilateral area by the contralateral area (n = 3 animals in each group; 2-5 sections per animal). *$p<0.05$ (one-way ANOVA followed by a Bonferroni post-hoc test). Error bars = SEM.

6.3 Summary of findings

- In the absence of electrical stimulation, no significant difference in the numbers of c-fos positive cells was found between untreated, vinblastine-treated and neuritis groups ($p > 0.23$).
- Repeated electrical stimulation of the sciatic nerve at C- fibre strength led to increased c-fos expression on the ipsilateral side in the neuritis and vinblastine-treated groups compared to the untreated group ($p < 0.05$) and contralateral sides ($p < 0.05$).
- Following repeated electrical stimulation of the sciatic nerve at A-fibre strength, the number of c-fos positive cells was comparable between untreated, vinblastine-treated and neuritis groups ($p > 0.06$).
- A significant decrease in the area with positive substance P immunolabelling was present in the ipsilateral superficial dorsal horn following vinblastine treatment ($p < 0.05$ compared to saline treatment).
- In saline-treated and neuritis groups, there were no differences in the area with positive substance P immunolabelling between the ipsilateral and contralateral superficial dorsal horns.

CHAPTER 7:

Discussion

7.1 Rationale and overview

Widely used animal models in neuropathic pain research are induced by nerve ligation, crush, or compression (Bennett and Xie, 1988, Seltzer *et al*., 1990, Kim and Chung, 1992, Decosterd and Woolf, 2000). However, such nerve injuries are uncommon in clinical practice (Percie du Sert and Rice, 2014, Rice *et al.*, 2008) and many patients present without an obvious nerve injury upon clinical examination. It has been suggested that in such cases, healthy-appearing axons might be inflamed (Djouhri *et al*., 2012, Bove, 2009, Bove *et al*., 2003, Dilley *et al*., 2005, Eliav *et al*., 1999). Consistent with this, inducing a local neuritis in rats leads to the development of pain behaviours that are typically neuropathic (Bove *et al*., 2003, Pulman *et al*., 2013). Since nerve inflammation results in axonal transport disruption along intact through conducting nerve fibres (Dilley *et al*., 2013), it has been hypothesised that such process may play a significant role in the underlying mechanisms.

To examine the role of axonal transport disruption in neuropathic pain, the chemotherapeutic agent vinblastine was locally applied to the rat sciatic nerve. Stimulus-evoked pain behaviours as well as burrowing activity were assessed in this model to determine the extent of neuropathic pain behaviours that are associated with axonal transport disruption. Single-unit extracellular recordings were also performed from the L5 dorsal root to look for ongoing activity in peripheral nerves, since such activity is reputed to drive central mechanisms that lead to neuropathic pain behaviours. Within the dorsal horn, intracellular recordings were performed from deep WDR neurons to look for signs of increased dorsal horn excitability. In the superficial laminae, c-fos was examined as an indicator of altered neuronal function since these neurons were not examined electrophysiologically, while

substance P levels were assessed as a measure of altered nociceptor transmission into the spinal cord. Throughout the study, comparisons were made to the neuritis model.

Vinblastine treatment led to the development of short-lived mechanical and cold allodynia as well as signs of reduced burrowing behaviour in the absence of an increase in A- or C-fibre ongoing activity. Similarly, wind-up in deep dorsal horn WDR neurons was not altered. Despite the development of mechanical allodynia, vinblastine-induced axonal transport disruption led to a reduction in WDR neuronal responses to mechanical stimulation of the receptive field. Within the superficial dorsal horn, there was an upregulation in c-fos expression together with reduced substance P immunolabelling. Although similar neuropathic pain behaviours and an increase in c-fos expression were observed following neuritis, C-fibre ongoing activity levels and wind-up in WDR neurons were increased in this model.

The development of transient neuropathic pain behaviours following vinblastine treatment and neuritis contrasts from traumatic nerve injury models where such behaviours persist (Bennett and Xie, 1988, Kim and Chung, 1992, Decosterd and Woolf, 2000, Seltzer *et al*., 1990). While an increase in peripheral ongoing activity is a significant feature of the latter models (Kajander *et al*., 1992, Amir and Devor, 1993, Tal and Eliav, 1996, Xie and Xiao, 1990, Liu *et al*., 2000a, Liu *et al*., 2000b, Djouhri *et a*l., 2006, Xiao and Bennett, 2008), it is absent following vinblastine-induced axonal transport disruption. Within the dorsal horn, the absence of an increase in deep WDR neuron excitability differs from inflammatory neuropathic pain models (Traub, 1997, Herrero and Cervero, 1996, Stanfa *et al*., 1992), including neuritis. However, the upregulation of activity-dependent c-fos expression in the superficial laminae following both vinblastine treatment and neuritis is consistent with findings from traumatic nerve injury models (Molander *et al*., 1994, Molander

et al., 1998, Catheline *et al*., 1999). Finally, a reduction in substance P levels as a result of vinblastine-induced axonal transport disruption is consistent with that following a traumatic nerve injury (Villar *et al*., 1991, Fitzgerald *et al*., 1985).

In summary, a relatively minor nerve insult, such as local vinblastine-induced axonal transport disruption or neuritis, can lead to the development of neuropathic pain behaviours. Such findings are consistent with patients who have symptoms of neuropathic pain but have no evidence of a nerve injury during routine clinical examination. Although neuritis disrupts axonal transport, the lack of an increase in peripheral ongoing activity following vinblastine treatment suggests that inflammation is required for its development. Furthermore, these observations suggest that ongoing activity from peripheral sensory axons is not critical for the development of neuropathic pain behaviours and are indicative of an alternative peripheral signal. Mechanistic differences between both models are further outlined by the lack of increased excitability in deep WDR dorsal horn neurons following vinblastine treatment when compared to neuritis. Finally, evidence for an increase in c-fos expression in superficial dorsal horn neurons is consistent with a central neuropathic pain mechanism.

7.2 Animal models used in the study

Clinical trial data from a decade ago revealed that only 9% of trials included patients who presented with a traumatic peripheral nerve injury (Rice *et al*., 2008). Contrary to this, neuropathic pain research remains mainly focused on traumatic nerve injury models, where peripheral nerves are cut, ligated, or constricted (e.g. spinal nerve ligation or chronic constriction injury models) (Berge, 2011). As such, the use of traumatic nerve injury models in preclinical research does not reflect the clinical

picture (Baron *et al.*, 2010). In turn, the translation of scientific data into the development of effective new medications has been very poor (Kissin, 2010, Mogil, 2009). Recently, preclinical rodent models that more closely reflect the pathophysiology of human conditions have been developed. Several examples of these disease-specific models include chemotherapy-induced neuropathy (Hu *et al*., 2017), diabetic neuropathy (D'Almeida *et al.*, 1999), HIV-associated peripheral neuropathy (Cao *et al.*, 2012), varicella zoster infection (Garry *et al.*, 2005) and anti-retroviral therapy models (Huang *et al.*, 2013). The use of such models in neuropathic pain research has provided novel therapeutic targets, such as oxidative stress, mitochondria dysfunction (Flatters *et al*., 2017) and loss of distal innervation (Boyette-Davis *et al.*, 2011), which has led to the initiation of new clinical trials (e.g. NCT01906008).

Currently, there is evidence to suggest that nerve inflammation is an important part of the neuropathology of patients who present with neuropathic pain but have no signs of nerve injury upon routine clinical examination (Greening *et al*., 2018, Dilley *et al*., 2011, Linnman *et al*., 2011). It has been demonstrated that local neuritis leads to axonal transport disruption (Dilley *et al*., 2013) and therefore, transient treatment of peripheral nerves with vinblastine may provide a novel model for examining the role of such processes in these patients. In comparison to traumatic nerve injury models (Bennett and Xie, 1988, Seltzer *et al*., 1990, Kim and Chung, 1992, Decosterd and Woolf, 2000) that feature a complete cessation of axonal transport, vinblastine treatment provides a less severe approach to examining the role of axonal transport disruption in neuropathic pain. Importantly, treating nerves with vinblastine avoids gross surgery and allows the exploration of axonal transport disruption along intact axons.

Although vinblastine-induced axonal transport disruption and neuritis models are relevant to patients without a traumatic nerve injury or history of infectious disease, it must be taken into consideration that there are obvious species differences between humans and rodents. This means that data presented in this study is only predictive of the potential mechanisms that may underlie neuropathic pain in patients. It must be noted that the short duration (i.e. several weeks) of neuropathic pain behaviours in both models contrasts clinical cases where symptoms persist for many years (Daniel *et al.*, 2008). Furthermore, in the present study, male rats were chosen to avoid the possible quagmire associated with the oestrus cycle in female rats. However, in humans, neuropathic pain is more common in women (Bouhassira *et al*., 2008, reviewed in Palmeira *et al.*, 2011). In agreement, differences in the severity of neuropathic pain behaviours and mechanisms have been observed between male and female rodents (Tall and Crisp, 2004, Tall *et al.*, 2001, Sorge *et al*., 2015, Lopes *et al.*, 2017).

7.3 Local axonal transport disruption leads to short-lived neuropathic pain behaviours

The extent of neuropathic pain behaviours that are associated with localised axonal transport disruption have not been explored in detail. Therefore, this study examined both stimulus-evoked and ethologically relevant behavioural measures to determine whether such minor injury contributes to behavioural changes that are frequently associated with traumatic nerve injury models. While pain cannot be directly measured in animals, it can be inferred from the assessment of stimulus-evoked pain behaviours, which is considered the 'gold standard' within the pain field. In the present study, behavioural tests to assess mechanical and cold allodynia as well as

mechanical and heat hyperalgesia were performed. Since these are common tests, they enable easy comparison of our obtained results to previously studied animal models. In addition to these measures, burrowing activity, which is considered an ethologically relevant behaviour in rodents (Deacon, 2006), was also assessed. The main advantages of burrowing assessment are that it is objective and requires minimal input from the investigator. Furthermore, it takes into account the effects of spontaneous pain and provides a measure of the general effect of pain on the whole organism, including the brain. A reduction in burrowing behaviour indicates reduced wellbeing of the rats, which might be comparable to a reduction in activities of daily living and general wellbeing in patients with neuropathic pain (Andrews *et al*., 2012, Andrews *et al.*, 2011).

Several limitations associated with the techniques used in this study must be considered. Firstly, cutaneous hypersensitivity to applied stimuli only reflects one of the many features of neuropathic pain (Rice *et al*., 2008, Maier *et al*., 2010). Secondly, while neuropathic pain has a significant effect on the general wellbeing of patients (Meyer-Rosberg *et al*., 2001), stimulus-evoked pain measures are reflexive and do not take higher pain centres into account. With regards to burrowing behaviour assessment, inter-rat variability is a significant issue, which other studies have tackled by using a selective training period prior to testing (Muralidharan *et al*., 2016, Wodarski *et al.*, 2016). For example, a previous cross-centre study reported that animals with poor burrowing performance were paired with those who were considered ‘good burrowers’ in order to initiate burrowing (Wodarski *et al*., 2016). In the present study, animal pairing based on burrowing ability was not carried out. Additionally, there is a lack of understanding as to whether reduced burrowing is a result of lower motivational status inflicted by painful sensations (either limb

movement-evoked or spontaneous) or due to the individual characteristics of the rat (e.g. easily loses interest).

Although it has been previously reported that transient mechanical allodynia develops following vinblastine treatment (Dilley *et al*., 2013), to our knowledge, this is the first study to demonstrate that cold allodynia is also a feature of this model. The absence of neuropathic pain behaviours following systemic vinblastine injection suggests that the observed behavioural effects following transient vinblastine application to the sciatic nerve are likely caused by local axonal transport disruption. A similar pattern of neuropathic pain behaviours was also observed in the neuritis model, which was consistent with previously published data (Dilley *et al*., 2013, Eliav *et al*., 1999, Bove *et al*., 2003, Pulman *et al*., 2013). However, the onset of these behaviours was slightly later than that following vinblastine treatment. In line with the current findings, stimulus-evoked pain behaviours are frequently reported following a traumatic nerve injury (Bennett and Xie, 1988, Kim and Chung, 1992, Decosterd and Woolf, 2000, Seltzer *et al*., 1990) as well as chemotherapy treatment (Aley *et al*., 1996, Authier *et al*., 2003, Hu *et al*., 2017). However, both vinblastine-induced axonal transport disruption and neuritis contrast significantly from traumatic nerve injury models with regard to the severity of evoked pain behaviours. In the latter models, pain behaviours tend to persist for no less than two months, together with additional adverse effects, such as motor dysfunction, autotomy and signs of stress (e.g. porphyrin staining, weight loss and changes in grooming activities) (Bennett and Xie, 1988, Kim and Chung, 1992, Decosterd and Woolf, 2000, Seltzer *et al*., 1990). Contrastingly, following vinblastine treatment and neuritis, there were no obvious signs of such adverse effects and neuropathic pain behaviours reversed within several weeks. Despite the transient development of allodynia, there were no

significant signs of heat hyperalgesia in vinblastine-induced axonal transport and neuritis models, which is in agreement with previous observations following vinblastine treatment (Dilley *et al*., 2013, Fitzgerald *et al*., 1984), but not neuritis (Dilley *et al*., 2013, Eliav *et al*., 1999, Pulman *et al*., 2013). Though previous reports have found that mechanical hyperalgesia develops following neuritis (Eliav *et al*., 1999), we were unable to come to the same conclusion; due to the considerable variability of responses to noxious mechanical stimuli observed in this study, the development of mechanical hyperalgesia following both neuritis and vinblastine remains uncertain.

In addition to the ipsilateral development of neuropathic pain behaviours, brief signs of contralateral mechanical and cold allodynia were also observed following vinblastine treatment and neuritis respectively, which is consistent with less pronounced and shorter-lived contralateral neuropathic pain behaviours in traumatic nerve injury (Seltzer *et al*., 1990, Kim and Chung, 1992, Tabo *et al.*, 1999, Arguis *et al.*, 2008, Takaishi *et al*., 1996) and chronic hind limb inflammation (Radhakrishnan *et al.*, 2003, Gao *et al.*, 2010) models.

With regard to the ethologically relevant behaviours, our finding of reduced burrowing behaviour in both models is consistent with the findings from traumatic nerve injury and chronic inflammation models (Andrews *et al*., 2012, Rutten *et al*., 2014, Muralidharan *et al*., 2016). However, it contrasts from a recent study where no such changes were found following bortezomib treatment (Duggett and Flatters, 2017), which leads to altered axonal transport (Staff *et al.*, 2013), similar to vinblastine treatment.

Similarities in the pattern of neuropathic pain behaviours following vinblastine treatment and neuritis may indicate common mechanisms. Since inflammation is not

present following vinblastine treatment, the common mechanistic feature is likely to be axonal transport disruption. As such, the subtle difference in the onset of mechanical and cold allodynia between vinblastine treatment and neuritis is consistent with earlier axonal transport disruption following vinblastine treatment (Dilley *et al*., 2013). In both models, neuropathic pain behaviours were less severe than those observed following a traumatic nerve injury. This, in fact, may reflect the differences in the extent of tissue damage. Wallerian degeneration and demyelination are features of traumatic nerve injury models (Roytta *et al*., 1999, Fried and Devor, 1988, Coggeshall *et al*., 1993) and therefore, the lack of full recovery of neuropathic pain behaviours is likely due to incomplete axonal regeneration and the slow process of re-myelination. Following vinblastine treatment (Fitzgerald *et al*., 1984, Kashiba *et al*., 1992) and neuritis (Eliav *et al*., 1999), such gross neuropathology is absent, enabling recovery of neuropathic pain behaviours within a few weeks. This is also in agreement with the reversal of axonal transport disruption, which is observed at day six following vinblastine treatment (Dilley *et al*., 2013). In fact, the transient nature of neuropathic pain behaviours observed in this study is comparable to models of chemotherapy-induced neuropathy, where neuropathic pain behaviours return to baseline values several weeks following discontinuation of the treatment (Aley *et al*., 1996, Authier *et al*., 2003, Hu *et al*., 2017). Together, these findings suggest that the processes which disrupt axonal transport must persist to maintain the symptoms. For example, in patients, this process could be the ongoing inflammation of tissue surrounding the nerve. The observed development of allodynia in the contralateral limb is suggestive of central involvement and may be driven by the activation of commissural interneurons within the dorsal horn. These interneurons have axons which extend from one dorsal horn to the other, enabling chemical and/or electrical

signals to reach neurons located on the contralateral side (reviewed in Koltzenburg *et al.*, 1999).

The reduced burrowing behaviour observed following vinblastine treatment and neuritis is indicative of reduced animal wellbeing. Furthermore, it may reflect impaired learning processes. In healthy adult male Sprague Dawley rats, the range of gravel removed from the burrow is reported to be between 1400-1700 g (one hour period) (Muralidharan *et al*., 2016). In the present study, a similar amount of gravel (~1200g) was displaced on day five following saline treatment only. In agreement, previously reported studies indicate that burrowing performance in rodents improves with practice (Deacon, 2006), however, this did not manifest in vinblastine-treated or neuritis animals. The observed difference between the results of this study and the reported unchanged burrowing behaviour following bortezomib treatment (Duggett and Flatters, 2017) suggests that different animal models may have distinct effects on this ethologically relevant behaviour.

7.4 Local axonal transport disruption does not increase peripheral ongoing activity

It is widely accepted that peripheral ongoing activity is necessary to drive central mechanisms that lead to neuropathic pain behaviours (Campbell and Meyer, 2006, Gracely *et al.*, 1992, Woolf, 2011, Xie *et al.*, 2005). Therefore, as part of this study, ongoing activity levels were assessed following vinblastine-treatment and comparisons were made to the neuritis model. *In vivo* single-unit extracellular recordings were performed from the L5 dorsal root to measure the levels of such activity in individual neurons. Such techniques are highly advantageous over tissue culture, since it allows examination of intact neurons without removing them from

their natural environment (e.g. presence of blood supply and immune system). It also enables recordings from the same population of neurons that are tested during behavioural studies (i.e. neurons that innervate the plantar surface of the hind paw). An additional advantage of single-unit electrophysiological recordings is that the properties of specific nerve fibre types can easily be explored, which include conduction velocity, receptive field characteristics and levels of ongoing activity, and origin of such activity (i.e. peripheral terminals, nerve trunk, or DRG). Nevertheless, several limitations are associated with such recording techniques. The method is technically challenging and therefore, significant tissue injury during the experimental set up could cause ongoing activity. Tissue longevity is another associated limitation, since the cut dorsal root, which is extremely fine (6-10 μm), can dry out during the recording.

The lack of an increase in ongoing activity levels at any of the examined time points following vinblastine treatment is consistent with a previous investigation of ongoing activity levels in A- and C-fibre neurons at the peak of mechanical allodynia (Dilley *et al*., 2013). Because recordings were made from the L5 dorsal root, ongoing activity from the peripheral terminals, treatment site and the DRG were ruled out. Such observations contrast from the reported increase in ongoing activity levels in both intact (Djouhri *et al*., 2006, Djouhri *et al*., 2012, Wu *et al*., 2001) and cut/ligated axons following a traumatic nerve injury (Tal and Eliav, 1996, Kajander *et al*., 1992, Chen and Devor, 1998, Pan *et al*., 1999, Matzner and Devor, 1994). Similarly, elevated levels of peripheral ongoing activity are featured in chemotherapy-induced peripheral neuropathy models (Xiao and Bennett, 2008), which again differs from the vinblastine-induced axonal transport disruption model. While behavioural changes were comparable between vinblastine-treated and

neuritis groups, there was a striking difference between the levels of ongoing activity in C/slow Aδ-fibre neurons, which is indicative of mechanistic differences between the models. Consistent with previous reports, ongoing activity levels were increased in nociceptive neurons at the peak of mechanical and cold allodynia following neuritis (Bove *et al*., 2003, Dilley *et al*., 2005). The observed electrophysiological changes were rapid, with increased firing rates on days 1-2 post-surgery. In contrast, no such increase was present in Aα/β-fibre neurons. The development of ongoing activity in C/slow Aδ-fibre neurons following neuritis is partially consistent with previous studies on traumatic nerve injury models, where ongoing activity in Aβ-fibre neurons is the predominant feature (Boucher *et al*., 2000, Kajander *et al*., 1992, Liu *et al*., 2000b, Xiao and Bennett, 2007). Such findings are also in line with reports of increased afferent ongoing activity in other inflammatory models (Xiao and Bennett, 2007, Kelly *et al*., 2012) and are suggestive of a role for inflammation. An observed slowing of C/slow Aδ-fibre conduction velocities in the L5 dorsal root following both vinblastine treatment and neuritis is in agreement with previous studies, where a reduction in conduction velocity was reported during the first week post-surgery (Dilley *et al*., 2013, Dilley and Bove, 2008b, Dilley *et al*., 2005). Moreover, this is comparable to conduction velocity slowing along intact nociceptive nerve fibres following spinal nerve ligation (Shim *et al*., 2007). In contrast, conduction velocity slowing along Aα/β-fibre neurons was absent following vinblastine treatment, and such alterations were observed only a few hours after neuritis surgery, which differs from traumatic nerve injury models (Kajander and Bennett, 1992, Gabay and Tal, 2004).

While vinblastine treatment does not evoke a significant increase in ongoing activity in nociceptor axons, the presence of such activity following neuritis is consistent

with the ‘peripheral generator’ theorem, whereby peripheral ongoing activity drives spinal changes responsible for neuropathic pain symptoms. These observations are suggestive of mechanistic differences between both models. Vinblastine is in fact anti-inflammatory by virtue of its anti-mitotic properties (Norris *et al.*, 1977), and the lack of an increase in ongoing activity in A- or C-fibre neurons following vinblastine-induced axonal transport disruption is consistent with the necessity for inflammation to activate such aberrant activity. While increase in peripheral ongoing activity may underlie the symptoms of spontaneous pain, which are frequently reported in the clinic (e.g. Bove *et al.*, 2005), the lack of such activity following vinblastine treatment suggests that spontaneous pain may not be a feature of this model.

We hypothesised that following neuritis, axonal transport disruption leads to an accumulation of anterogradely transported channels that are otherwise destined for the peripheral terminals. Examples of such channels may include HCN, Nav1.8 and TRP channels (Emery *et al*., 2011, Richards and Dilley, 2015, Belkouch *et al*., 2014, Jung *et al.*, 2008). Since ongoing activity is not increased following vinblastine treatment, it is likely that following neuritis, the accumulated channels are sensitised by the inflammatory response. Consistent with this hypothesis, a recent study has demonstrated that the application of an inflammatory soup to the site of axonal transport disruption following vinblastine treatment causes nociceptive neurons to become ongoing (Govea *et al*., 2017). As such, the present data also supports that if undamaged peripheral nociceptors exhibit ongoing activity, there must be a source of inflammation. The observed lack of altered activity in Aα/β-fibre neurons may in fact reflect the minor nature of nerve insult following vinblastine treatment and neuritis.

In both models, conduction velocity slowing in nociceptive nerve fibres is consistent with the widespread effects of a relatively minor nerve injury. However, the cause of such changes remains unclear. It may involve the generation of a chemical or electrical retrograde signal from the treatment site, leading to altered kinetics or expression of ion channels along proximal nerve segments (Gould *et al.*, 2000, Black *et al*., 2004, Tanaka *et al*., 1998). Alternatively, changes in axon morphology, such as reduced diameter, could also lead to a reduction in conduction velocities, which has previously been observed in patients with diabetic neuropathy (Malik *et al.*, 2001).

7.5 Local axonal transport disruption induces spinal cord changes

To determine whether there were signs of increased excitability in the spinal cord that may underlie neuropathic pain behaviours following vinblastine treatment and neuritis, *in vivo* single-cell spinal cord recordings were employed. The main focus was on WDR neurons, since they display a frequency-dependent increase in excitability to repetitive noxious input, known as wind-up, which shows similarities to central sensitization (Li *et al*., 1999). As such, changes in wind-up may indicate an increase in dorsal horn excitability, which may underlie neuropathic pain development. In addition, ongoing activity and mechanical thresholds of WDR neurons were also examined, since alterations in these properties could also indicate excitability changes. Similarly to L5 dorsal root recordings, single-cell spinal cord recordings allow the examination of intact neurons *in situ*. Furthermore, since neuronal circuits remain intact, the identification of the receptive field can be used to confirm that recordings are made from a population of neurons that correspond to the

area of the hind paw tested during behavioural studies. The main limitations associated with single-cell spinal cord recordings include a technically challenging set up and the inability to distinguish which neuron in the spinal cord circuitry (i.e. excitatory vs. inhibitory, interneuron vs. projection neuron) recordings are made from. Additionally, in this study, experiments were conducted on non-decerebrate animals; therefore, activity of recorded WDR neurons may have been influenced by descending inputs.

None of the neurons recorded during electrophysiological experiments were in the superficial laminae, which receives nociceptive input and therefore, an alternative approach was used to examine these neurons. To look for signs of increased excitability within the superficial dorsal horn, c-fos expression following electrical stimulation of the sciatic nerve was examined immunohistochemically. C-fos is a recognized marker of injury-induced neuronal activation (Hunt *et al.*, 1987), although its precise role in pain pathways is not well understood. Additionally, c-fos is an indirect indicator of central sensitization, since its expression is driven by the activation of CREB, which mediates transcriptional changes that are associated with such mechanisms (reviewed in Latremoliere and Woolf, 2009). As part of this study, substance P levels were also assessed in the superficial laminae of the dorsal horn as an indicator of increased spinal activation by nociceptive neurons. Immunohistochemistry is a relatively simple technique with well-defined protocols, which allows easy visualisation of specific antigens in a large number of samples. Such technique also facilitates a direct comparison between samples from different models, since different tissues can be mounted on the same slide. Although a popular technique, immunohistochemistry is also associated with several drawbacks. These

include non-specific labelling, a large window for human error due to a multi-step process and difficulties with accurately quantifying the protein of interest.

The lack of an increase in wind-up following vinblastine treatment is in line with findings in models of neuropathic pain that involve cutting or constricting nerves (Chapman *et al*., 1998, Suzuki *et al*., 2001, Xu *et al.*, 1995, Laird and Bennett, 1993, Patel *et al*., 2015b). In contrast, an increase in wind-up following neuritis is consistent with changes in chronic inflammation models (e.g. injection of CFA or carrageenan into the hind paw or joints) (Traub, 1997, Herrero and Cervero, 1996, Stanfa *et al*., 1992). Unchanged ongoing activity levels from tested WDR neurons following vinblastine treatment and neuritis contrasts from findings following traumatic nerve injury (Chapman *et al*., 1998, Laird and Bennett, 1993), chronic hind paw inflammation (Kitagawa *et al.*, 2005) and vincristine-induced peripheral neuropathy (Weng *et al*., 2003). It was expected that at the peak of mechanical allodynia, responses to direct mechanical stimulation of the receptive field would be increased following vinblastine treatment and neuritis. However, there was no such change. In fact, there was a significant decrease in the responsiveness of WDR neurons to noxious cutaneous mechanical stimulation (i.e. 60 g von Frey monofilament) following vinblastine treatment. Interestingly, the present data revealed differences in mechanical responses between those WDR neurons at depths of $\geq$ 550 μm from the surface of the dorsal horn compared to those < 550 μm (deeper neurons were less responsive to mechanical stimuli), a finding that has not been previously reported.

The increase in C-fibre stimulated c-fos expression in the superficial dorsal horn following both vinblastine treatment and neuritis was comparable to findings in

models of traumatic nerve injury (Catheline *et al*., 1999, Molander *et al*., 1994, Molander *et al*., 1998, Ro *et al.*, 2004). Similarly, the increase in c-fos expression observed following A-fibre stimulation, though not significant, is in line with reports using models of traumatic nerve injury (Bester *et al*., 2000, Kosai *et al.*, 2001, Molander *et al*., 1994) and vincristine-induced neuropathy (Thibault *et al.*, 2013). Furthermore, the decrease in the superficial dorsal horn area positively labelled for substance P following vinblastine treatment is consistent with traumatic nerve injury (Fitzgerald *et al*., 1985, Garrison *et al*., 1993, Villar *et al*., 1991, Barbut *et al*., 1981) and inflammatory models (Marlier *et al.*, 1991, Galeazza *et al*., 1995). Following neuritis, no alterations in substance P labelling were found. However, findings in other inflammatory models are varied and report an initial increase in substance P labelling followed by a decrease (Marlier *et al*., 1991, Honore *et al.*, 2000).

While the increase in wind-up response from deep WDR neurons following neuritis suggests that this neuronal population is sensitized, the absence of such change following vinblastine treatment questions the importance of wind-up in the development/maintenance of neuropathic pain, especially since the pattern of pain behaviours was similar between both models. This is further supported by the observed differences in wind-up response between traumatic nerve injury (Chapman *et al*., 1998, Suzuki *et al.*, 2001, Xu *et al*., 1995, Laird and Bennett, 1993) and inflammatory (Traub, 1997, Herrero and Cervero, 1996, Stanfa *et al*., 1992) models. These discrepancies may reflect differences in the inflammatory response between models, as well as the pattern of peripheral ongoing activity. For example, while ongoing activity from nociceptors (i.e. C/slow Aδ-fibre neurons) is present in neuritis (Bove and Dilley, 2010, Richards and Dilley, 2015, Bove *et al*., 2003) and chronic inflammatory models (Djouhri *et al*., 2006, Kocher *et al.*, 1987, Kelly *et al*.,

2012, Xiao and Bennett, 2007), following traumatic nerve injury, such ongoing activity is rarely observed (Boucher *et al*., 2000, Liu *et al*., 2000b) and when present, rates are extremely low (Wu *et al*., 2001, Djouhri *et al*., 2006). Therefore, it is plausible that activity from peripheral nociceptor axons may be responsible for the neuronal changes necessary to increase wind-up response. In agreement, vincristine-induced peripheral neuropathy, which is associated with significant ongoing activity in C-fibre neurons (Xiao and Bennett, 2008), also leads to signs of increased wind-up (Weng *et al*., 2003). Therefore, ongoing activity from nociceptive neurons may indirectly contribute to the increase in wind-up despite the fact that their input to the deep dorsal horn is mediated via polysynaptic pathways (reviewed in Herrero *et al*., 2000). In contrast, ongoing activity in WDR neurons is reported to be directly driven by injury-induced activity from primary sensory neurons (Pitcher and Henry, 2008). As such, unaltered ongoing activity levels from deep WDR neurons following vinblastine treatment and neuritis may reflect the absence of injury-induced discharge from Aβ-fibre neurons (Bove et al., 2003, Bove and Dilley, 2010, Dilley *et al*., 2013), which mainly synapse in the region of lamina V where the examined WDR neurons are located (Todd, 2010).

The observed reduction in responsiveness of deep WDR neurons to noxious mechanical stimuli following vinblastine treatment suggests altered neuronal connectivity, or inhibition, within the dorsal horn, which may dampen the nociceptive input. For example, activation of descending inhibitory pathways that act on dorsal horn neurons has been demonstrated following chronic inflammation of the hind paw (Green *et al.*, 1998). A similar central mechanism might also underlie the reduction in the responses to innocuous mechanical stimulation of the receptive fields of WDR neurons at depths $\geq$ 550 μm compared to those $<$ 550 μm.

Furthermore, it suggests that there may be sub-populations of WDR neurons that respond differently to the treatment. Since the mechanistic features of such changes are still unclear, a peripheral mechanism, such as a decrease in the mechanical sensitivity of the nociceptor peripheral terminals, cannot be ruled out. Mechanically sensitive ion channel components are transported to the peripheral terminals by fast axonal transport (Koschorke *et al.,* 1994) and therefore, if transport is impeded, it may impact the mechanical sensitivity of nociceptors. In the neuritis model, axonal transport disruption occurs at a later time point compared to vinblastine treatment (Dilley *et al.,* 2013), which might explain why a similar decrease in noxious mechanical sensitivity was not observed. The decrease in mechanical responses from deep WDR neurons following vinblastine treatment despite the development of mechanical allodynia suggests that these neurons may not be part of the mechanism leading to neuropathic pain behaviours in this model. Other mechanisms, such as increased activity of the superficial dorsal horn neurons, may play an important role.

Upregulation of c-fos expression may indirectly indicate central sensitization. Therefore, it is likely that neurons expressing c-fos as a result of electrical stimulation were sensitized following vinblastine treatment and neuritis. Since repeated C-fibre stimulation did not block A-fibre neurons, potential contribution of A-fibre nociceptors to the increase in c-fos expression when stimulating at C-fibre strength cannot be ruled out. Furthermore, the observed increase in A-fibre induced c-fos expression is most likely due to the activation of Aβ- and Aδ-fibre nociceptors.

The decrease in substance P levels suggests that nociceptor transmission is altered. It is possible that such alteration may be caused by the repeated activation of nociceptors, which may cause the depletion of neuropeptides from central terminals (Klein *et al.,* 1990). Although ongoing activity is lacking following vinblastine

treatment, the development of axonal mechanical sensitivity has been reported, similar to neuritis (Dilley and Bove, 2008a, Dilley *et al*., 2013, Bove *et al*., 2003, Dilley and Bove, 2008b). As such, mechanically sensitive nociceptive axons could be repetitively activated at the treatment site during limb movements (Dilley *et al*., 2005, Boyd *et al.*, 2005). Alternatively, the disruption of axonal transport following vinblastine treatment may be sufficient to affect the retrograde transport of trophic factors, such as NGF, from the peripheral terminals to the cell bodies. Since NGF is considered to modulate substance P production (Nielsch *et al.*, 1987, Fitzgerald *et al*., 1985, Goedert *et al.*, 1981), a decrease in NGF may impact substance P levels in the central terminals of primary sensory neurons. The lack of a similar loss of substance P following neuritis might reflect the slower time course of axonal transport disruption.

7.6 Conclusions

The main aim of the present study was to determine if axonal transport disruption along otherwise healthy axons is a potential cause of painful symptoms in patients who have signs and symptoms of neuropathic pain, but no overt nerve damage. Specifically, it was aimed to determine the contribution of peripheral and central neuronal excitability as well as the role of nerve inflammation in the development of neuropathic pain behaviours following axonal transport disruption. It was hypothesized that localised axonal transport disruption will lead to the development of neuropathic pain behaviours and that such behavioural changes will occur in the absence of increased peripheral ongoing activity. It was also hypothesized that axonal transport disruption will cause an increase in neuronal excitability within the dorsal horn as well as alterations in peptidergic nociceptive transmission. This study demonstrated that localised axonal transport disruption leads to the development of

transient neuropathic pain behaviours in the absence of increased peripheral ongoing activity. Consistent with the development of such behaviours, aberrant axonal transport caused changes within the superficial dorsal horn that are typically associated with central sensitization, but surprisingly, deep WDR neurons showed signs of decreased excitability. In contrast, following neuritis, ongoing activity from nociceptors was increased and there were signs of increased excitability in deep WDR neurons, which is more in line with our current understanding of neuropathic pain mechanisms.

Localised axonal transport disruption along intact axons drives transient cutaneous hypersensitivities associated with both tactile and thermal stimuli (Section 7.3). These findings further emphasise the importance of through-conducting axons in the mechanisms of neuropathic pain, which may be relevant to those patients who lack signs of a traumatic nerve injury upon clinical examination. In these patients, axonal transport disruption may be driven by nerve inflammation (i.e. neuritis) (Dilley *et al*., 2013). Supporting this proposal, magnetic resonance imaging studies in these patients have identified increases in T2-weighted signal intensity along nerve trunks that are consistent with neuritis (Dilley *et al*., 2011, Greening *et al*., 2018). This is further supported by the persistent painful symptoms in a rat model of repetitive motion disorders, where neuritis is a prominent feature (Barbe *et al*. 2013). The potential mechanisms underlying neuropathic pain as a result of nerve inflammation and subsequently axonal transport disruption are discussed below and are summarised in Figure 7.1.

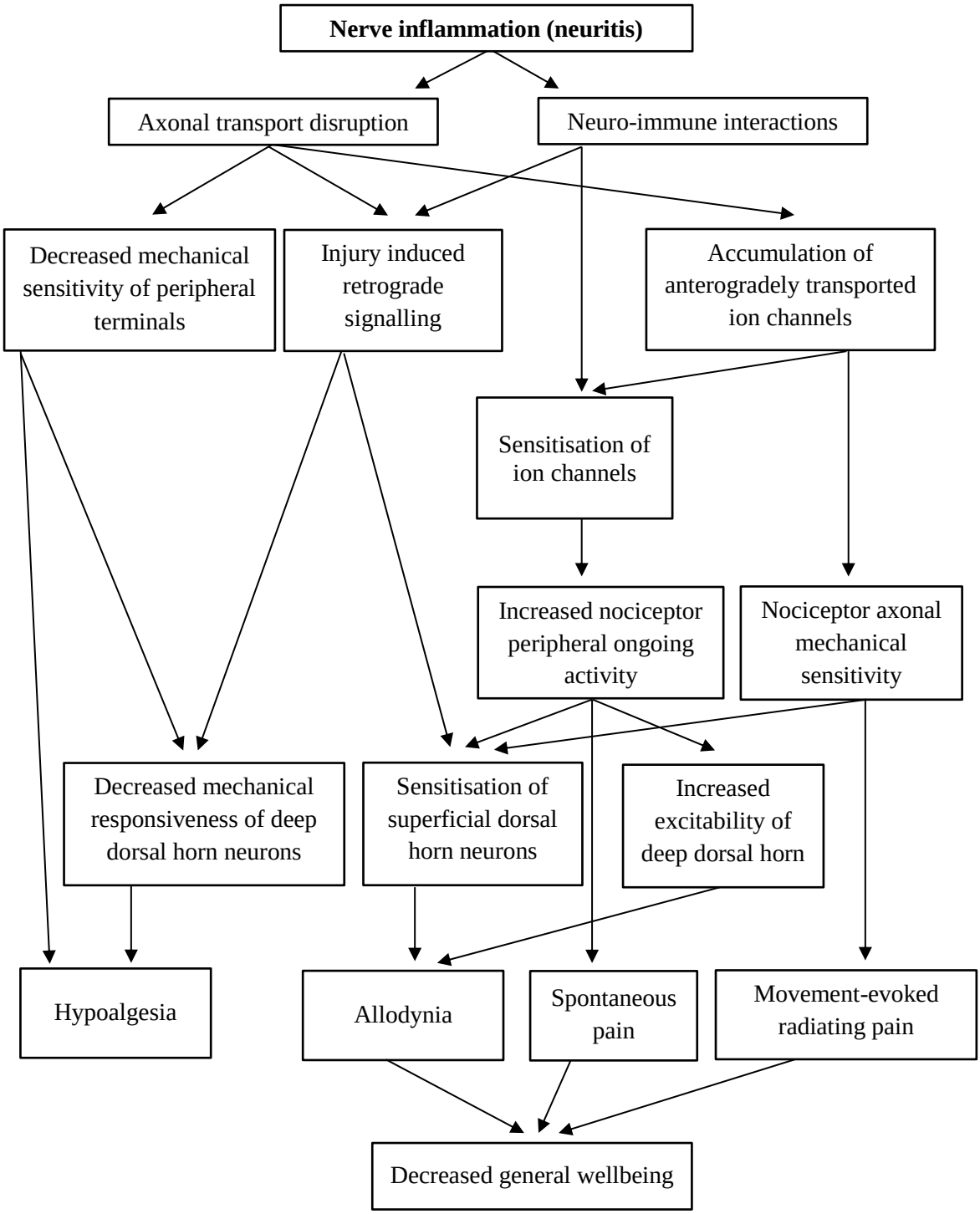


Figure 7.1 Potential mechanisms underlying the development of neuropathic pain behaviours following nerve inflammation and subsequently axonal transport disruption.

Localised axonal transport disruption leads to the accumulation of anterogradely transported ion channels, which drives changes in axonal excitability (Section 7.4). Interestingly, this accumulation itself is not sufficient to drive an increase in ongoing activity levels, indicating that an alternative mechanism may underlie changes in central neuropathic pain mechanisms responsible for allodynia. For example, at the site of axonal transport disruption, proteins may be produced and retrogradely transported to the cell bodies, where phenotypic/transcriptional changes are initiated. In agreement with a retrograde chemical signal, an increase in activating

Figure 7.1 Potential mechanisms underlying the development of neuropathic pain behaviours following localised axonal transport disruption and nerve inflammation. In brief, local nerve inflammation (neuritis) leads to axonal transport disruption. Axonal transport disruption in isolation leads to the accumulation of ion channels at the treatment site, which become mechanically sensitive. The development of axonal mechanical sensitivity may in fact underlie movement-evoked radiating pain and may also lead to sensitisation of superficial dorsal horn neurons. At the treatment site, proteins are produced that are retrogradely transported to the cell bodies ("injury signal"), where phenotypic/transcriptional changes are initiated and may also partially mediate excitability of the superficial dorsal horn. Such excitatory changes underlie the development of stimulus-evoked neuropathic pain behaviours (i.e. allodynia). A decrease in deep dorsal horn responses to mechanical stimulation observed following local axonal transport disruption may be due to the lack of newly synthesized ion channels, or their components, reaching peripheral terminals as a result of aberrant anterograde transport. Alternatively, altered neuronal connectivity, or inhibition, within the dorsal horn may play a role. The observed reduction in mechanical sensitivity of deep dorsal horn neurons may be partially responsible for hypoalgesia observed in patients. In the presence of local inflammation, inflammatory mediators sensitise ion channels that have accumulated at the treatment site and lead to the development of ongoing activity in nociceptive nerve fibres. Such increase in ongoing activity may underlie spontaneous pain and may drive excitability of the superficial and deep dorsal horn. Together, spontaneous pain, allodynia, and movement-evoked radiating pain are responsible for reduced general well-being, which in the rat is reflected by reduced burrowing behaviour.

transcription factor-3 in the nuclei of primary sensory neurons has been reported following neuritis (Dilley *et al.*, 2005). As previously discussed (Section 7.5), localised axonal transport disruption is also likely to cause a reduction in the transport of trophic factors, such as NGF, through the treatment site. As such, trophic factors required for survival of adult sensory neurons as well as their phenotype maintenance (Verge *et al.*, 1996) may not be sufficiently transported to the cell bodies, thus affecting the pattern of neurotransmitter release at the central terminals. Therefore, instead of peripheral ongoing activity, alterations in retrograde chemical signalling may lead to sensitisation of the dorsal horn. As proposed in Section 7.4, increased peripheral ongoing activity is likely dependent on neuro-immune interactions, which leads to sensitisation of accumulated ion channels. Such activity undoubtedly contributes to central neuropathic pain mechanisms following neuritis and may underlie spontaneous pain frequently reported by patients. Although localised axonal transport disruption alone is not sufficient to drive an increase in peripheral ongoing activity, it does lead to the development of axonal mechanical sensitivity (discussed Section 7.5), which may be responsible for movement-evoked pain and may also contribute to sensitisation of the dorsal horn. Such findings may be directly applicable to patients with persistent radiating pain, as this could be due to focal inflammation anywhere along the clinically implicated peripheral nerve.

Functional changes observed in dorsal horn neurons following localised axonal transport disruption and neuritis are consistent with central neuropathic pain mechanisms. These changes are driven by a peripheral signal, such as axonal mechanical sensitivity, chemical retrograde signal, or in the presence of inflammation, peripheral ongoing activity. Importantly, the likely sensitisation of the superficial dorsal horn, which was indicated by an increase in c-fos

immunolabelling, provides evidence that an increase in dorsal horn neuronal excitability can be driven by localised axonal transport disruption alone. Based on the 'peripheral generator' theorem (Campbell and Meyer, 2006, Gracely *et al*., 1992, Woolf, 2011, Xie *et al*., 2005), the transient development of neuropathic pain behaviours and increased c-fos expression in superficial laminae following axonal transport disruption seems incongruous with the lack of increased peripheral ongoing activity. However, the decrease in substance P levels in the dorsal horn suggests that nociceptor transmission is altered in the absence of inflammation. Such observations further support the role for a retrograde chemical signal in the initiation/maintenance of central neuropathic pain mechanisms.

The presence of inflammation initiates distinct changes within the deep dorsal horn. Specifically, increased excitability of deep WDR dorsal horn neurons is likely dependent on inflammation-induced increases in peripheral ongoing activity from nociceptors (Section 7.5). It is unclear whether deep WDR neurons are responsible for the development of allodynia, however, their involvement in such mechanisms following neuritis cannot be ruled out. In contrast, the reduction in mechanical responses of deep WDR following localised axonal transport disruption suggests that these neurons may not be implicated in mechanical allodynia in the absence of inflammation. In fact, such reduction in mechanical responses suggests that a central mechanism may underlie the decrease in cutaneous sensitivity observed in some patients. Alternatively, this could be driven by decreased mechanical sensitivity of the nociceptor peripheral terminals.

Taken together, localised axonal transport disruption along intact axons can induce short-lived neuropathic pain behaviours, similar to neuritis. Despite similarities in

the evoked behaviours, distinct mechanistic differences exist between the neuritis and vinblastine models. The lack of increased peripheral ongoing activity following localised axonal transport disruption contrasts from the widely accepted 'peripheral generator' theorem, in which peripheral ongoing activity is thought to drive central neuropathic pain mechanisms. Importantly, localized axonal transport disruption is sufficient to induce excitatory changes within the superficial dorsal horn, which is consistent with central sensitization. Such findings indicate that dorsal horn plasticity can be initiated via alternative peripheral pathways. In fact, the development of ongoing activity is likely to be inflammation-dependent and undoubtedly contributes to the increased excitability of the superficial and deep dorsal horn following neuritis.

Our findings confirm that axonal degeneration is not required for pain development and that a relatively minor insult to the nerve is sufficient to lead to the peripheral and central alterations that are implicated in neuropathic pain. These findings are consistent with observations of neuropathic pain patients with no signs of frank nerve injury and include those with non-specific arm and lower back pain, fibromyalgia, complex regional pain syndrome 1 and whiplash-associated disorder. Our findings provide further evidence for the role of inflammation in such patients and suggest that aberrant axonal transport may underlie some of the symptoms.

Consistent with our findings, several studies have suggested a role for the disruption of axonal transport of vital cellular components in chemotherapy-induced peripheral neuropathy. Specifically, vinca alkaloids bind to β-tubulin and interfere with tubulin assembly, thus inhibiting its polymerization (Gigant *et al.*, 2005, Lobert *et al.*, 1996) and impeding anterograde and retrograde axonal transport (LaPointe *et al.*, 2013). In

agreement with such disruption, it has been demonstrated that there is a reduction in the levels of axonal mRNA in the distal nerves following paclitaxel treatment (Bobylev *et al.*, 2015), which also disrupts axonal transport. Based on the current data, it is likely that axonal transport disruption in sensory neurons may be at least partially responsible for the development of painful symptoms in patients undergoing vinca alkaloid treatment. Therefore, the development of therapies that aim to repair dysfunctional axonal transport might be of specific importance to patients with vinca alkaloid-induced peripheral neuropathy.

Future directions

In the present study, we demonstrated that a chemical signal may drive central changes and the development of painful behaviours following vinblastine treatment. Therefore, extensive investigation of retrograde axonal transport both distal and proximal to the treatment site would be beneficial. Investigations could be carried out using retrograde axonal transport tracing techniques (e.g. fluoro-gold) and by looking at the accumulation of proteins, such as ion channels, within the proximity of the treatment site. In addition, since decreased mechanosensitivity of the peripheral terminals may play a role following vinblastine-induced axonal transport disruption, there is a possibility that a small number of nociceptive nerve fibres are dying back in the dermis (Siau *et al.*, 2006). To examine this hypothesis, receptive fields should be characterised and an assessment of PGP 9.5 expression, a marker of intraepidermal nerve fibres, should be performed.

The role of the dorsal horn in the underlying mechanisms of neuropathic pain requires further investigation. Other spinal cord activation markers could be examined, such as extracellular-signal-regulated kinase, which may be a better

marker of central sensitisation than c-fos (Ji *et al.*, 1999). In addition, the physiological properties of WDR neurons should be assessed at earlier and later time points. In particular, studies should focus on WDR neurons within the superficial laminae. Additionally, the role of ongoing activity in the development of increased excitability in the dorsal horn in the neuritis model requires further investigation. In these experiments, conduction along small-diameter neurons could be blocked (e.g. using resiniferatoxin) prior to dorsal horn neuronal assessment. A role for microglia in the spinal cord following neuritis and vinblastine treatment has not been previously addressed. Activation of microglia is linked to the maintenance of pain behaviours. A microglia marker (e.g. OX-42) or microglial activation marker (e.g. phosphorylated p38) could be used for such a study (Jin *et al.*, 2003, Hains and Waxman, 2006).

The current study revealed a negative effect of vinblastine and neuritis treatments on the general well-being of the animals as assessed by burrowing activity. In order to further examine the extent of pain effects, additional ethologically relevant behavioural tests could be used. These include: 1) the grimace scale, a method of assessing the presence of pain based on facial expressions (Langford *et al.*, 2010, Sotocinal *et al.*, 2011); 2) thigmotaxis, a predator avoidance measure (Wallace *et al.*, 2007, Hasnie *et al.*, 2007); and 3) ultrasonic vocalizations, which are produced by rodents in stressful situations in order to communicate with members of their social group (Calvino *et al.*, 1996, Han *et al.*, 2005).

The findings of the current study, together with the future studies proposed here, could reveal a novel mechanism of neuropathic pain driven by axonal transport disruption along intact axons. Understanding such a mechanism would be of significant clinical importance, as it could provide us with greater knowledge about the pathophysiology of neuropathic pain symptoms in patients who present without a traumatic nerve injury and who represent a majority of clinical cases. Such cases are among the hardest to treat and clinical outcomes are not satisfactory for the patients, since current treatments are aimed at masking painful symptoms rather than targeting their aetiology. This research would additionally advance our understanding of the mechanisms underlying vinca alkaloid-induced peripheral neuropathy. The study presented here marks just the beginning of an exciting and promising research topic. Future research on axonal transport disruption may initiate new clinical trials, leading to the development of novel therapies for the treatment of neuropathic pain, which currently represents an unmet need in the pain field.

References

Abbadie, C., Brown, J. L., Mantyh, P. W. and Basbaum, A. I. (1996) 'Spinal cord substance P receptor immunoreactivity increases in both inflammatory and nerve injury models of persistent pain', *Neuroscience,* 70(1), pp. 201-9.

Abraira, V. E. and Ginty, D. D. (2013) 'The sensory neurons of touch', *Neuron,* 79(4), pp. 618-39.

Abraira, V. E., Kuehn, E. D., Chirila, A. M., Springel, M. W., Toliver, A. A., Zimmerman, A. L., *et al.* (2017) 'The Cellular and Synaptic Architecture of the Mechanosensory Dorsal Horn', *Cell,* 168(1-2), pp. 295-310 e19.

Adriaensen, H., Gybels, J., Handwerker, H. O. and Van Hees, J. (1983) 'Response properties of thin myelinated (A-delta) fibers in human skin nerves', *J Neurophysiol,* 49(1), pp. 111-22.

Akopian, A. N., Sivilotti, L. and Wood, J. N. (1996) 'A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons', *Nature,* 379(6562), pp. 257-262.

Al-Khater, K. M., Kerr, R. and Todd, A. J. (2008) 'A quantitative study of spinothalamic neurons in laminae I, III, and IV in lumbar and cervical segments of the rat spinal cord', *J Comp Neurol,* 511(1), pp. 1-18.

Al-Khater, K. M. and Todd, A. J. (2009) 'Collateral projections of neurons in laminae I, III, and IV of rat spinal cord to thalamus, periaqueductal gray matter, and lateral parabrachial area', *J Comp Neurol,* 515(6), pp. 629-46.

Al Ghamdi, K. S., Polgar, E. and Todd, A. J. (2009) 'Soma size distinguishes projection neurons from neurokinin 1 receptor-expressing interneurons in lamina I of the rat lumbar spinal dorsal horn', *Neuroscience,* 164(4), pp. 1794-804.

Alexander, G. M., van Rijn, M. A., van Hilten, J. J., Perreault, M. J. and Schwartzman, R. J. (2005) 'Changes in cerebrospinal fluid levels of pro-inflammatory cytokines in CRPS', *Pain,* 116(3), pp. 213-9.

Aley, K. O., Reichling, D. B. and Levine, J. D. (1996) 'Vincristine hyperalgesia in the rat: a model of painful vincristine neuropathy in humans', *Neuroscience,* 73(1), pp. 259-65.

Allen, R. D., Metuzals, J., Tasaki, I., Brady, S. T. and Gilbert, S. P. (1982) 'Fast axonal transport in squid giant axon', *Science,* 218(4577), pp. 1127-9.

Amaya, F., Wang, H., Costigan, M., Allchorne, A. J., Hatcher, J. P., Egerton, J., *et al.* (2006) 'The voltage-gated sodium channel Na(v)1.9 is an effector of peripheral inflammatory pain hypersensitivity', *J Neurosci,* 26(50), pp. 12852-60.

Amir, R. and Devor, M. (1993) 'Ongoing activity in neuroma afferents bearing retrograde sprouts', *Brain Research,* 630(1-2), pp. 283-288.

Andersson, D. A., Gentry, C., Moss, S. and Bevan, S. (2008) 'Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress', *J Neurosci,* 28(10), pp. 2485-94.

Andrews, N., Harper, S., Issop, Y. and Rice, A. S. (2011) 'Novel, nonreflex tests detect analgesic action in rodents at clinically relevant concentrations', *Ann N Y Acad Sci,* 1245, pp. 11-3.

Andrews, N., Legg, E., Lisak, D., Issop, Y., Richardson, D., Harper, S., *et al.* (2012) 'Spontaneous burrowing behaviour in the rat is reduced by peripheral nerve injury or inflammation associated pain', *Eur J Pain,* 16(4), pp. 485-95.

Arguis, M. J., Perez, J., Martinez, G., Ubre, M. and Gomar, C. (2008) 'Contralateral neuropathic pain following a surgical model of unilateral nerve injury in rats', *Reg Anesth Pain Med,* 33(3), pp. 211-6.

Asbury, A. K., Arnason, B. G. and Adams, R. D. (1969) 'The inflammatory lesion in idiopathic polyneuritis. Its role in pathogenesis', *Medicine (Baltimore),* 48(3), pp. 173-215.

Attal, N., Jazat, F., Kayser, V. and Guilbaud, G. (1990) 'Further evidence for 'pain-related' behaviours in a model of unilateral peripheral mononeuropathy', *Pain,* 41(2), pp. 235-51.

Authier, N., Gillet, J. P., Fialip, J., Eschalier, A. and Coudore, F. (2003) 'A new animal model of vincristine-induced nociceptive peripheral neuropathy', *Neurotoxicology*, 24(6), pp. 797-805.

Backonja, M. M. and Stacey, B. (2004) 'Neuropathic pain symptoms relative to overall pain rating', *J Pain*, 5(9), pp. 491-7.

Bai, L., Lehnert, B. P., Liu, J., Neubarth, N. L., Dickendesher, T. L., Nwe, P. H., *et al.* (2015) 'Genetic Identification of an Expansive Mechanoreceptor Sensitive to Skin Stroking', *Cell*, 163(7), pp. 1783-1795.

Bandell, M., Story, G. M., Hwang, S. W., Viswanath, V., Eid, S. R., Petrus, M. J., *et al.* (2004) 'Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin', *Neuron.*, 41(6), pp. 849-857.

Barbut, D., Polak, J. M. and Wall, P. D. (1981) 'Substance P in spinal cord dorsal horn decreases following peripheral nerve injury', *Brain Research.*, 205(2), pp. 289-298.

Baron, R., Binder, A. and Wasner, G. (2010) 'Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment', *Lancet Neurol*, 9(8), pp. 807-19.

Baron, R., Maier, C., Attal, N., Binder, A., Bouhassira, D., Cruccu, G., *et al.* (2017) 'Peripheral neuropathic pain: a mechanism-related organizing principle based on sensory profiles', *Pain*, 158(2), pp. 261-272.

Baseer, N., Al-Baloushi, A. S., Watanabe, M., Shehab, S. A. S. and Todd, A. J. (2014) 'Selective innervation of NK1 receptor–lacking lamina I spinoparabrachial neurons by presumed nonpeptidergic Aδ nociceptors in the rat', *Pain*, 155(11), pp. 2291-2300.

Bautista, D. M., Jordt, S. E., Nikai, T., Tsuruda, P. R., Read, A. J., Poblete, J., *et al.* (2006) 'TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents', *Cell*, 124(6), pp. 1269-82.

Bautista, D. M., Siemens, J., Glazer, J. M., Tsuruda, P. R., Basbaum, A. I., Stucky, C. L., *et al.* (2007) 'The menthol receptor TRPM8 is the principal detector of environmental cold', *Nature,* 448(7150), pp. 204-8.

Behbehani, M. M. and Dollberg-Stolik, O. (1994) 'Partial sciatic nerve ligation results in an enlargement of the receptive field and enhancement of the response of dorsal horn neurons to noxious stimulation by an adenosine agonist', *Pain,* 58(3), pp. 421-8.

Belkouch, M., Dansereau, M. A., Tetreault, P., Biet, M., Beaudet, N., Dumaine, R., *et al.* (2014) 'Functional up-regulation of Nav1.8 sodium channel in Abeta afferent fibers subjected to chronic peripheral inflammation', *J Neuroinflammation,* 11, pp. 45.

Bennett, G. J. (1999) 'Does a neuroimmune interaction contribute to the genesis of painful peripheral neuropathies?', *Proceedings of the National Academy of Sciences of the United States of America,* 96(14), pp. 7737-7738.

Bennett, G. J. (2000) 'A neuroimmune interaction in painful peripheral neuropathy', *Clin.J.Pain,* 16(3 Suppl), pp. S139-S143.

Bennett, G. J. and Xie, Y. K. (1988) 'A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man', *Pain,* 33(1), pp. 87-107.

Bennett, M. (2001) 'The LANSS Pain Scale: the Leeds assessment of neuropathic symptoms and signs', *Pain,* 92(1-2), pp. 147-57.

Berbusse, G. W., Woods, L. C., Vohra, B. P. and Naylor, K. (2016) 'Mitochondrial Dynamics Decrease Prior to Axon Degeneration Induced by Vincristine and are Partially Rescued by Overexpressed cytNmnat1', *Front Cell Neurosci,* 10, pp. 179.

Berge, O.-G. (2011) 'Predictive validity of behavioural animal models for chronic pain', *British Journal of Pharmacology,* 164(4), pp. 1195-1206.

Bester, H., Beggs, S. and Woolf, C. J. (2000) 'Changes in tactile stimuli-induced behavior and c-Fos expression in the superficial dorsal horn and in parabrachial nuclei after sciatic nerve crush', *J Comp Neurol,* 428(1), pp. 45-61.

Birklein, F., Drummond, P. D., Li, W., Schlereth, T., Albrecht, N., Finch, P. M., *et al.* (2014) 'Activation of cutaneous immune responses in complex regional pain syndrome', *J Pain*, 15(5), pp. 485-95.

Birklein, F., Schmelz, M., Schifter, S. and Weber, M. (2001) 'The important role of neuropeptides in complex regional pain syndrome', *Neurology*, 57(12), pp. 2179-84.

Black, J. A., Langworthy, K., Hinson, A. W., Dib-Hajj, S. D. and Waxman, S. G. (1997) 'NGF has opposing effects on Na+ channel III and SNS gene expression in spinal sensory neurons', *NeuroReport*, 8(9-10), pp. 2331-5.

Black, J. A., Liu, S., Tanaka, M., Cummins, T. R. and Waxman, S. G. (2004) 'Changes in the expression of tetrodotoxin-sensitive sodium channels within dorsal root ganglia neurons in inflammatory pain', *Pain*, 108(3), pp. 237-47.

Blair, S. J., Chinthagada, M., Hoppenstehdt, D., Kijowski, R. and Fareed, J. (1998) 'Role of neuropeptides in pathogenesis of reflex sympathetic dystrophy', *Acta Orthop Belg*, 64(4), pp. 448-51.

Blumberg, H. and Janig, W. (1984) 'Discharge pattern of afferent fibers from a neuroma', *Pain*, 20, pp. 335-353.

Bobylev, I., Joshi, A. R., Barham, M., Ritter, C., Neiss, W. F., Hoke, A., *et al.* (2015) 'Paclitaxel inhibits mRNA transport in axons', *Neurobiol Dis*, 82, pp. 321-331.

Boehmerle, W., Huehnchen, P., Peruzzaro, S., Balkaya, M. and Endres, M. (2014) 'Electrophysiological, behavioral and histological characterization of paclitaxel, cisplatin, vincristine and bortezomib-induced neuropathy in C57Bl/6 mice', *Sci Rep*, 4, pp. 6370.

Bohlen, C. J., Chesler, A. T., Sharif-Naeini, R., Medzihradszky, K. F., Zhou, S., King, D., *et al.* (2011) 'A heteromeric Texas coral snake toxin targets acid-sensing ion channels to produce pain', *Nature*, 479(7373), pp. 410-4.

Boiko, N., Medrano, G., Montano, E., Jiang, N., Williams, C. R., Madungwe, N. B., *et al.* (2017) 'TrpA1 activation in peripheral sensory neurons underlies the ionic basis

of pain hypersensitivity in response to vinca alkaloids', *PLoS ONE,* 12(10), pp. e0186888.

Bolcskei, K., Helyes, Z., Szabo, A., Sandor, K., Elekes, K., Nemeth, J., *et al.* (2005) 'Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice', *Pain,* 117(3), pp. 368-76.

Boucher, T. J., Okuse, K., Bennett, D. L. H., Munson, J. B., Wood, J. N. and McMahon, S. B. (2000) 'Potent analgesic effects of GDNF in neuropathic pain states', *Science,* 290(5489), pp. 124-127.

Bouhassira, D., Lantéri-Minet, M., Attal, N., Laurent, B. and Touboul, C. (2008) 'Prevalence of chronic pain with neuropathic characteristics in the general population', *Pain,* 136(3), pp. 380-7.

Boureau, F., Doubrere, J. F. and Luu, M. (1990) 'Study of verbal description in neuropathic pain', *Pain,* 42(2), pp. 145-152.

Bove, G. M. (2009) 'Focal nerve inflammation induces neuronal signs consistent with symptoms of early complex regional pain syndromes', *Exp Neurol*.

Bove, G. M. and Dilley, A. (2010) 'The conundrum of sensitization when recording from nociceptors', *J Neurosci Methods,* 188(2), pp. 213-8.

Bove, G. M., Ransil, B. J., Lin, H. C. and Leem, J. G. (2003) 'Inflammation induces ectopic mechanical sensitivity in axons of nociceptors innervating deep tissues', *J.Neurophysiol.,* 90(3), pp. 1949-1955.

Bove, G. M., Weissner, W. and Barbe, M. F. (2009) 'Long lasting recruitment of immune cells and altered epi-perineurial thickness in focal nerve inflammation induced by complete Freund's adjuvant.', *J Neuroimmunol.,* 213, pp. 26-30.

Bove, G. M., Zaheen, A. and Bajwa, Z. H. (2005) 'Subjective nature of lower limb radicular pain', *J Manipulative Physiol Ther,* 28(1), pp. 12-4.

Boyd, B. S., Puttlitz, C., Gan, J. and Topp, K. S. (2005) 'Strain and excursion in the rat sciatic nerve during a modified straight leg raise are altered after traumatic nerve injury', *J Orthop Res,* 23(4), pp. 764-70.

Boyette-Davis, J., Xin, W., Zhang, H. and Dougherty, P. M. (2011) 'Intraepidermal nerve fiber loss corresponds to the development of taxol-induced hyperalgesia and can be prevented by treatment with minocycline', *Pain,* 152(2), pp. 308-13.

Boyette-Davis, J. A., Cata, J. P., Driver, L. C., Novy, D. M., Bruel, B. M., Mooring, D. L., *et al.* (2013) 'Persistent chemoneuropathy in patients receiving the plant alkaloids paclitaxel and vincristine', *Cancer Chemother Pharmacol,* 71(3), pp. 619-26.

Brady, S. T., Lasek, R. J. and Allen, R. D. (1982) 'Fast axonal transport in extruded axoplasm from squid giant axon', *Science,* 218(4577), pp. 1129-31.

Braz, J., Solorzano, C., Wang, X. and Basbaum, A. I. (2014) 'Transmitting pain and itch messages: a contemporary view of the spinal cord circuits that generate gate control', *Neuron,* 82(3), pp. 522-36.

Brimijoin, S., Lundberg, J. M., Brodin, E., Hokfelt, T. and Nilsson, G. (1980) 'Axonal transport of substance P in the vagus and sciatic nerves of the guinea pig', *Brain Res,* 191(2), pp. 443-57.

Brown, A. G. and Iggo, A. (1967) 'A quantitative study of cutaneous receptors and afferent fibres in the cat and rabbit', *J Physiol,* 193(3), pp. 707-33.

Bulaj, G., Zhang, M. M., Green, B. R., Fiedler, B., Layer, R. T., Wei, S., *et al.* (2006) 'Synthetic muO-conotoxin MrVIB blocks TTX-resistant sodium channel NaV1.8 and has a long-lasting analgesic activity', *Biochemistry,* 45(23), pp. 7404-14.

Burton, P. R. and Paige, J. L. (1981) 'Polarity of axoplasmic microtubules in the olfactory nerve of the frog', *Proc Natl Acad Sci U S A,* 78(5), pp. 3269-73.

Calvino, B., Besson, J. M., Boehrer, A. and Depaulis, A. (1996) 'Ultrasonic vocalization (22-28 kHz) in a model of chronic pain, the arthritic rat: effects of analgesic drugs', *Neuroreport,* 7(2), pp. 581-4.

Campbell, J. N. and Meyer, R. A. (2006) 'Mechanisms of neuropathic pain', *Neuron,* 52(1), pp. 77-92.

Campoy, L., Martin-Flores, M., Looney, A. L., Erb, H. N., Ludders, J. W., Stewart, J. E., *et al.* (2008) 'Distribution of a lidocaine-methylene blue solution staining in brachial plexus, lumbar plexus and sciatic nerve blocks in the dog', *Vet Anaesth Analg*, 35(4), pp. 348-54.

Cao, L., Butler, M. B., Tan, L., Draleau, K. S. and Koh, W. Y. (2012) 'Murine immunodeficiency virus-induced peripheral neuropathy and the associated cytokine responses', *J Immunol*, 189(7), pp. 3724-33.

Carp, S. J., Barbe, M. F., Winter, K. A., Amin, M. and Barr, A. E. (2007) 'Inflammatory biomarkers increase with severity of upper-extremity overuse disorders', *Clin Sci (Lond)*, 112(5), pp. 305-14.

Catheline, G., LeGuen, S., Honore, P. and Besson, J. M. (1999) 'Are there long-term changes in the basal or evoked Fos expression in the dorsal horn of the spinal cord of the mononeuropathic rat?', *Pain*, 80(1-2), pp. 347-357.

Catterall, W. A. (2000) 'From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels', *Neuron*, 26(1), pp. 13-25.

Catterall, W. A. (2012) 'Sodium Channel Mutations and Epilepsy', in Noebels, J.L., Avoli, M., Rogawski, M.A., Olsen, R.W. & Delgado-Escueta, A.V. (eds.) *Jasper's Basic Mechanisms of the Epilepsies.* 4th ed. Bethesda (MD).

Cauna, N. (1956) 'Nerve supply and nerve endings in Meissner's corpuscles', *Am J Anat*, 99(2), pp. 315-50.

Cauna, N. and Ross, L. L. (1960) 'The fine structure of Meissner's touch corpuscles of human fingers', *J Biophys Biochem Cytol*, 8, pp. 467-82.

Chacur, M., Milligan, E. D., Gazda, L. S., Armstrong, C., Wang, H. C., Tracey, K. J., *et al.* (2001) 'A new model of sciatic inflammatory neuritis (SIN): induction of unilateral and bilateral mechanical allodynia following acute unilateral peri-sciatic immune activation in rats', *Pain*, 94(3), pp. 231-244.

Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M. and Yaksh, T. L. (1994) 'Quantitative assessment of tactile allodynia in the rat paw', *Journal of Neuroscience Methods,* 53(1), pp. 55-63.

Chaplan, S. R., Guo, H. Q., Lee, D. H., Luo, L., Liu, C., Kuei, C., *et al.* (2003) 'Neuronal hyperpolarization-activated pacemaker channels drive neuropathic pain', *J.Neurosci.,* 23(4), pp. 1169-1178.

Chapman, V., Suzuki, R. and 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), pp. 881-94.

Chen, G., Park, C. K., Xie, R. G., Berta, T., Nedergaard, M. and Ji, R. R. (2014) 'Connexin-43 induces chemokine release from spinal cord astrocytes to maintain late-phase neuropathic pain in mice', *Brain,* 137(Pt 8), pp. 2193-209.

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

Chen, S. R., Wozniak, K. M., Slusher, B. S. and Pan, H. L. (2002) 'Effect of 2-(phosphono-methyl)-pentanedioic acid on allodynia and afferent ectopic discharges in a rat model of neuropathic pain', *J Pharmacol Exp Ther,* 300(2), pp. 662-7.

Chen, Y. and Devor, M. (1998) 'Ectopic mechanosensitivity in injured sensory axons arises from the site of spontaneous electrogenesis', *Eur J Pain,* 2(2), pp. 165-178.

Cheng, L., Duan, B., Huang, T., Zhang, Y., Chen, Y., Britz, O., *et al.* (2017) 'Identification of spinal circuits involved in touch-evoked dynamic mechanical pain', *Nat Neurosci,* 20(6), pp. 804-814.

Chi, X. X. and Nicol, G. D. (2007) 'Manipulation of the potassium channel Kv1.1 and its effect on neuronal excitability in rat sensory neurons', *J Neurophysiol,* 98(5), pp. 2683-92.

Chien, L. Y., Cheng, J. K., Chu, D., Cheng, C. F. and Tsaur, M. L. (2007) 'Reduced expression of A-type potassium channels in primary sensory neurons induces mechanical hypersensitivity', *J Neurosci,* 27(37), pp. 9855-65.

Choi, Y., Yoon, Y. W., Na, H. S., Kim, S. H. and Chung, J. M. (1994) 'Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain', *Pain*, 59(3), pp. 369-376.

Chowdary, P. D., Che, D. L. and Cui, B. (2012) 'Neurotrophin signaling via long-distance axonal transport', *Annu Rev Phys Chem*, 63, pp. 571-94.

Christensen, A. J., Iyer, S. M., Francois, A., Vyas, S., Ramakrishnan, C., Vesuna, S., *et al.* (2016) 'In Vivo Interrogation of Spinal Mechanosensory Circuits', *Cell Rep*, 17(6), pp. 1699-1710.

Clark, J. D. and Tempel, B. L. (1998) 'Hyperalgesia in mice lacking the Kv1.1 potassium channel gene', *Neurosci Lett*, 251(2), pp. 121-4.

Cobos, E. J., Nickerson, C. A., Gao, F., Chandran, V., Bravo-Caparros, I., Gonzalez-Cano, R., *et al.* (2018) 'Mechanistic Differences in Neuropathic Pain Modalities Revealed by Correlating Behavior with Global Expression Profiling', *Cell Rep*, 22(5), pp. 1301-1312.

Coggeshall, R. E. (2005) 'Fos, nociception and the dorsal horn', *Prog Neurobiol*, 77(5), pp. 299-352.

Coggeshall, R. E., Dougherty, P. M., Pover, C. M. and Carlton, S. M. (1993) 'Is large myelinated fiber loss associated with hyperalgesia in a model of experimental peripheral neuropathy in the rat?', *Pain*, 52, pp. 233-242.

Colburn, R. W., Lubin, M. L., Stone, D. J., Jr., Wang, Y., Lawrence, D., D'Andrea, M. R., *et al.* (2007) 'Attenuated cold sensitivity in TRPM8 null mice', *Neuron*, 54(3), pp. 379-86.

Coste, B., Mathur, J., Schmidt, M., Earley, T. J., Ranade, S., Petrus, M. J., *et al.* (2010) 'Piezo1 and Piezo2 are essential components of distinct mechanically-activated cation channels', *Science (New York, N.Y.)*, 330(6000), pp. 55-60.

Coste, B., Xiao, B., Santos, J. S., Syeda, R., Grandl, J., Spencer, K. S., *et al.* (2012) 'Piezo proteins are pore-forming subunits of mechanically activated channels', *Nature*, 483(7388), pp. 176-81.

Costigan, M., Moss, A., Latremoliere, A., Johnston, C., Verma-Gandhu, M., Herbert, T. A., *et al.* (2009) 'T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity', *J Neurosci,* 29(46), pp. 14415-22.

Coull, J. A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., *et al.* (2005) 'BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain', *Nature,* 438(7070), pp. 1017-21.

Coull, J. A., Boudreau, D., Bachand, K., Prescott, S. A., Nault, F., Sik, A., *et al.* (2003) 'Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain', *Nature,* 424(6951), pp. 938-42.

Cox, J. J., Reimann, F., Nicholas, A. K., Thornton, G., Roberts, E., Springell, K., *et al.* (2006) 'An SCN9A channelopathy causes congenital inability to experience pain', *Nature,* 444(7121), pp. 894-8.

Cragg, B. G. and Thomas, P. K. (1961) 'Changes in conduction velocity and fibre size proximal to peripheral nerve lesions', *J Physiol,* 157, pp. 315-27.

Cui, L., Miao, X., Liang, L., Abdus-Saboor, I., Olson, W., Fleming, M. S., *et al.* (2016) 'Identification of Early RET+ Deep Dorsal Spinal Cord Interneurons in Gating Pain', *Neuron,* 91(5), pp. 1137-1153.

Cummins, T. R., Sheets, P. L. and Waxman, S. G. (2007) 'The roles of sodium channels in nociception: Implications for mechanisms of pain', *Pain,* 131(3), pp. 243-57.

D'Almeida, J. A., de Castro-Costa, C. M., Frota, C. H., Severo, J. F., Rocha, T. D. and Nogueira, T. F. (1999) 'Behavioral changes of Wistar rats with experimentally-induced painful diabetic neuropathy', *Arq Neuropsiquiatr,* 57(3B), pp. 746-52.

Daniel, H. C., Narewska, J., Serpell, M., Hoggart, B., Johnson, R. and Rice, A. S. (2008) 'Comparison of psychological and physical function in neuropathic pain and nociceptive pain: implications for cognitive behavioral pain management programs', *Eur J Pain,* 12(6), pp. 731-41.

Davidson, S. and Giesler, G. J. (2010) 'The multiple pathways for itch and their interactions with pain', *Trends Neurosci,* 33(12), pp. 550-8.

Davies, S. N. and Lodge, D. (1987) 'Evidence for involvement of N-methylaspartate receptors in 'wind-up' of class 2 neurones in the dorsal horn of the rat', *Brain Res,* 424(2), pp. 402-6.

Deacon, R. M. (2006) 'Burrowing in rodents: a sensitive method for detecting behavioral dysfunction', *Nat Protoc,* 1(1), pp. 118-21.

Deacon, R. M. (2009) 'Burrowing: a sensitive behavioural assay, tested in five species of laboratory rodents', *Behav Brain Res,* 200(1), pp. 128-33.

Decosterd, I. and Woolf, C. J. (2000) 'Spared nerve injury: an animal model of persistent peripheral neuropathic pain', *Pain,* 87(2), pp. 149-158.

Descoeur, J., Pereira, V., Pizzoccaro, A., Francois, A., Ling, B., Maffre, V., *et al.* (2011) 'Oxaliplatin-induced cold hypersensitivity is due to remodelling of ion channel expression in nociceptors', *EMBO Mol Med,* 3(5), pp. 266-78.

Deval, E., Gasull, X., Noel, J., Salinas, M., Baron, A., Diochot, S., *et al.* (2010) 'Acid-sensing ion channels (ASICs): pharmacology and implication in pain', *Pharmacol Ther,* 128(3), pp. 549-58.

Devaux, J. J., Kleopa, K. A., Cooper, E. C. and Scherer, S. S. (2004) 'KCNQ2 is a nodal K+ channel', *J Neurosci,* 24(5), pp. 1236-44.

Devor, M. (1983) 'Nerve pathophysiology and mechanisms of pain in causalgia', *J Auton Nerv Syst,* 7(3-4), pp. 371-84.

Devor, M. (2006) 'Sodium channels and mechanisms of neuropathic pain', *J Pain,* 7(1 Suppl 1), pp. S3-S12.

Devor, M. and Govrin-Lippmann, R. (1983) 'Axoplasmic transport block reduces ectopic impulse generation in injured peripheral nerves', *Pain,* 16(1), pp. 73-85.

Devor, M. and Govrin-Lippmann, R. (1986) 'Retrograde slowing of conduction in sensory axons central to a sciatic nerve neuroma', *Exp Neurol,* 92(3), pp. 522-32.

Devor, M., Schonfeld, D., Seltzer, Z. and Wall, P. D. (1979) 'Two modes of cutaneous reinnervation following peripheral nerve injury', *J Comp Neurol,* 185(1), pp. 211-220.

Devor, M. and Wall, P. D. (1976) 'Type of sensory nerve fibre sprouting to form a neuroma', *Nature,* 262, pp. 705-708.

Dib-Hajj, S. D., Rush, A. M., Cummins, T. R., Hisama, F. M., Novella, S., Tyrrell, L., *et al.* (2005) 'Gain-of-function mutation in Nav1.7 in familial erythromelalgia induces bursting of sensory neurons', *Brain,* 128(Pt 8), pp. 1847-54.

Dib-Hajj, S. D., Tyrrell, L., Black, J. A. and Waxman, S. G. (1998) 'NaN, a novel voltage-gated Na channel, is expressed preferentially in peripheral sensory neurons and down-regulated after axotomy', *Proc.Natl.Acad.Sci.USA,* 95(15), pp. 8963-8968.

Dib-Hajj, S. D., Tyrrell, L., Cummins, T. R., Black, J. A., Wood, P. M. and Waxman, S. G. (1999) 'Two tetrodotoxin-resistant sodium channels in human dorsal root ganglion neurons', *FEBS Lett.,* 462(1-2), pp. 117-120.

Dichgans, M., Freilinger, T., Eckstein, G., Babini, E., Lorenz-Depiereux, B., Biskup, S., *et al.* (2005) 'Mutation in the neuronal voltage-gated sodium channel SCN1A in familial hemiplegic migraine', *Lancet,* 366(9483), pp. 371-7.

Dickenson, A. H. and 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(8), pp. 1235-8.

Dickenson, A. H. and Sullivan, A. F. (1990) 'Differential effects of excitatory amino acid antagonists on dorsal horn nociceptive neurones in the rat', *Brain Research,* 506(1), pp. 31-39.

Dickie, A. C., McCormick, B., Lukito, V., Wilson, K. L. and Torsney, C. (2017) 'Inflammatory Pain Reduces C Fiber Activity-Dependent Slowing in a Sex-Dependent Manner, Amplifying Nociceptive Input to the Spinal Cord', *The Journal of Neuroscience,* 37(27), pp. 6488-6502.

Dieleman, J. P., Kerklaan, J., Huygen, F. J., Bouma, P. A. and Sturkenboom, M. C. (2008) 'Incidence rates and treatment of neuropathic pain conditions in the general population', *Pain,* 137(3), pp. 681-8.

Dilley, A. and Bove, G. M. (2008a) 'Disruption of axoplasmic transport induces mechanical sensitivity in intact rat C-fibre nociceptor axons', *J Physiol,* 586(2), pp. 593-604.

Dilley, A. and Bove, G. M. (2008b) 'Resolution of inflammation-induced axonal mechanical sensitivity and conduction slowing in C-fiber nociceptors', *J Pain,* 9(2), pp. 185-92.

Dilley, A. and Greening, J. (2012) 'Non-specific arm pain', in McMahon, S.B., Koltzenburg, M., Tracey, I. & Turk, D.C. (eds.) *Wall & Melzack's Textbook of Pain.* 6 ed. Philadelphia: Elsevier Saunders, pp. 694-702.

Dilley, A., Greening, J., Walker-Bone, K. and Good, C. (2011) 'Magnetic resonance imaging signal hyperintensity of neural tissues in diffuse chronic pain syndromes: a pilot study', *Muscle Nerve,* 44(6), pp. 981-4.

Dilley, A., Lynn, B. and Pang, S. J. (2005) 'Pressure and stretch mechanosensitivity of peripheral nerve fibres following local inflammation of the nerve trunk', *Pain,* 117(3), pp. 462-472.

Dilley, A., Richards, N., Pulman, K. G. and Bove, G. M. (2013) 'Disruption of fast axonal transport in the rat induces behavioral changes consistent with neuropathic pain', *J Pain,* 14(11), pp. 1437-49.

Ding, Y. Q., Takada, M., Shigemoto, R. and Mizumo, N. (1995) 'Spinoparabrachial tract neurons showing substance P receptor-like immunoreactivity in the lumbar spinal cord of the rat', *Brain Res,* 674(2), pp. 336-40.

Diochot, S., Alloui, A., Rodrigues, P., Dauvois, M., Friend, V., Aissouni, Y., *et al.* (2016) 'Analgesic effects of mambalgin peptide inhibitors of acid-sensing ion channels in inflammatory and neuropathic pain', *Pain,* 157(3), pp. 552-9.

Diochot, S., Baron, A., Salinas, M., Douguet, D., Scarzello, S., Dabert-Gay, A. S., *et al.* (2012) 'Black mamba venom peptides target acid-sensing ion channels to abolish pain', *Nature,* 490(7421), pp. 552-5.

Dixon, W. J. (1980) 'Efficient analysis of experimental observations', *Annu.Rev.Pharmacol.Toxicol.,* 20, pp. 441-462.

Djouhri, L., Fang, X., Koutsikou, S. and Lawson, S. N. (2012) 'Partial nerve injury induces electrophysiological changes in conducting (uninjured) nociceptive and nonnociceptive DRG neurons: Possible relationships to aspects of peripheral neuropathic pain and paresthesias', *Pain,* 153(9), pp. 1824-36.

Djouhri, L., Fang, X., Okuse, K., Wood, J. N., Berry, C. M. and 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', *Journal of Physiology.,* 550(Pt:3), pp. 3-52.

Djouhri, L., Koutsikou, S., Fang, X., McMullan, S. and Lawson, S. N. (2006) 'Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C-fiber nociceptors', *J Neurosci,* 26(4), pp. 1281-1292.

Djouhri, L. and Lawson, S. N. (2004) 'Abeta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals', *Brain Res Brain Res Rev,* 46(2), pp. 131-45.

Dong, X., Han, S., Zylka, M. J., Simon, M. I. and Anderson, D. J. (2001) 'A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons', *Cell.,* 106(5), pp. 619-632.

Dong, X. W., Goregoaker, S., Engler, H., Zhou, X., Mark, L., Crona, J., *et al.* (2007) 'Small interfering RNA-mediated selective knockdown of Na(V)1.8 tetrodotoxin-resistant sodium channel reverses mechanical allodynia in neuropathic rats', *Neuroscience,* 146(2), pp. 812-21.

Donier, E., Rugiero, F., Jacob, C. and Wood, J. N. (2008) 'Regulation of ASIC activity by ASIC4--new insights into ASIC channel function revealed by a yeast two-hybrid assay', *Eur J Neurosci,* 28(1), pp. 74-86.

Dowdall, T., Robinson, I. and Meert, T. F. (2005) 'Comparison of five different rat models of peripheral nerve injury', *Pharmacol Biochem Behav,* 80(1), pp. 93-108.

Dray, A. (1992a) 'Mechanism action of capsaicin-like molecules on sensory neurons', *Life Sci.,* 51, pp. 1759-1765.

Dray, A. (1992b) 'Neuropharmacological mechanisms of capsaicin and related substances', *Biochem.Pharmacol.,* 44, pp. 611-615.

Duan, B., Cheng, L., Bourane, S., Britz, O., Padilla, C., Garcia-Campmany, L., *et al.* (2014) 'Identification of spinal circuits transmitting and gating mechanical pain', *Cell,* 159(6), pp. 1417-1432.

Duan, B., Cheng, L. and Ma, Q. (2018) 'Spinal Circuits Transmitting Mechanical Pain and Itch', *Neuroscience Bulletin,* 34(1), pp. 186-193.

Duflos, A., Kruczynski, A. and Barret, J. M. (2002) 'Novel aspects of natural and modified vinca alkaloids', *Curr.Med.Chem.Anticancer Agents,* 2(1), pp. 55-70.

Duggett, N. A. and Flatters, S. J. L. (2017) 'Characterization of a rat model of bortezomib-induced painful neuropathy', *Br J Pharmacol,* 174(24), pp. 4812-4825.

Dworkin, R. H., Backonja, M., Rowbotham, M. C., Allen, R. R., Argoff, C. R., Bennett, G. J., *et al.* (2003) 'Advances in neuropathic pain: diagnosis, mechanisms, and treatment recommendations', *Arch.Neurol.,* 60(11), pp. 1524-1534.

Echeverry, D. F., Gil, F., Laredo, F., Ayala, M. D., Belda, E., Soler, M., *et al.* (2010) 'Ultrasound-guided block of the sciatic and femoral nerves in dogs: a descriptive study', *Vet J,* 186(2), pp. 210-5.

Eliav, E., Benoliel, R. and Tal, M. (2001) 'Inflammation with no axonal damage of the rat saphenous nerve trunk induces ectopic discharge and mechanosensitivity in myelinated axons', *Neurosci.Lett.,* 311(1), pp. 49-52.

Eliav, E., Herzberg, U., Ruda, M. A. and Bennett, G. J. (1999) 'Neuropathic pain from an experimental neuritis of the rat sciatic nerve', *Pain,* 83(2), pp. 169-182.

Emery, E. C., Luiz, A. P., Sikandar, S., Magnusdottir, R., Dong, X. and Wood, J. N. (2016) 'In vivo characterization of distinct modality-specific subsets of somatosensory neurons using GCaMP', *Sci Adv*, 2(11), pp. e1600990.

Emery, E. C., Young, G. T., Berrocoso, E. M., Chen, L. and McNaughton, P. A. (2011) 'HCN2 ion channels play a central role in inflammatory and neuropathic pain', *Science*, 333(6048), pp. 1462-6.

Esteban, J. A., Shi, S. H., Wilson, C., Nuriya, M., Huganir, R. L. and Malinow, R. (2003) 'PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity', *Nat Neurosci*, 6(2), pp. 136-43.

Faber, C. G., Lauria, G., Merkies, I. S., Cheng, X., Han, C., Ahn, H. S., *et al.* (2012) 'Gain-of-function Nav1.8 mutations in painful neuropathy', *Proc Natl Acad Sci U S A*, 109(47), pp. 19444-9.

Fang, X., Djouhri, L., Black, J. A., Dib-Hajj, S. D., Waxman, S. G. and Lawson, S. N. (2002) 'The presence and role of the tetrodotoxin-resistant sodium channel Na(v)1.9 (NaN) in nociceptive primary afferent neurons', *J.Neurosci.*, 22(17), pp. 7425-7433.

Fang, X., Djouhri, L., McMullan, S., Berry, C., Okuse, K., Waxman, S. G., *et al.* (2005) 'trkA is expressed in nociceptive neurons and influences electrophysiological properties via Nav1.8 expression in rapidly conducting nociceptors', *J Neurosci*, 25(19), pp. 4868-4878.

Fazio, R., Quattrini, A., Bolognesi, A., Bordogna, G., Villa, E., Previtali, S., *et al.* (1999) 'Docetaxel neuropathy: a distal axonopathy', *Acta Neuropathol*, 98(6), pp. 651-3.

Fertleman, C. R., Baker, M. D., Parker, K. A., Moffatt, S., Elmslie, F. V., Abrahamsen, B., *et al.* (2006) 'SCN9A mutations in paroxysmal extreme pain disorder: allelic variants underlie distinct channel defects and phenotypes', *Neuron*, 52(5), pp. 767-74.

Finnerup, N. B., Otto, M., McQuay, H. J., Jensen, T. S. and Sindrup, S. H. (2005) 'Algorithm for neuropathic pain treatment: an evidence based proposal', *Pain,* 118(3), pp. 289-305.

Fitzgerald, M., Wall, P. D., Goedert, M. and Emson, P. C. (1985) 'Nerve growth factor counteracts the neurophysiological and neurochemical effects of chronic sciatic nerve section', *Brain Res*, 332(1), pp. 131-41.

Fitzgerald, M., Woolf, C. J., Gibson, S. J. and Mallaburn, P. S. (1984) 'Alterations in the structure, function, and chemistry of C fibers following local application of vinblastine to the sciatic nerve of the rat', *J.Neurosci.,* 4(2), pp. 430-441.

Fjell, J., Hjelmstrom, P., Hormuzdiar, W., Milenkovic, M., Aglieco, F., Tyrrell, L., *et al.* (2000) 'Localization of the tetrodotoxin-resistant sodium channel NaN in nociceptors', *Neuroreport,* 11(1), pp. 199-202.

Flatters, S. J. L., Dougherty, P. M. and Colvin, L. A. (2017) 'Clinical and preclinical perspectives on Chemotherapy-Induced Peripheral Neuropathy (CIPN): a narrative review', *Br J Anaesth,* 119(4), pp. 737-749.

Florez-Paz, D., Bali, K. K., Kuner, R. and Gomis, A. (2016) 'A critical role for Piezo2 channels in the mechanotransduction of mouse proprioceptive neurons', *Sci Rep,* 6, pp. 25923.

Flynn, K. C., Pak, C. W., Shaw, A. E., Bradke, F. and Bamburg, J. R. (2009) 'Growth cone-like waves transport actin and promote axonogenesis and neurite branching', *Dev Neurobiol*, 69(12), pp. 761-79.

Fogleman, M. and Brogmus, G. (1995) 'Computer mouse use and cumulative trauma disorders of the upper extremities', *Ergonomics*, 38(12), pp. 2465-75.

Fossat, P., Sibon, I., Le Masson, G., Landry, M. and Nagy, F. (2007) 'L-type calcium channels and NMDA receptors: a determinant duo for short-term nociceptive plasticity', *Eur J Neurosci,* 25(1), pp. 127-35.

Foster, E., Wildner, H., Tudeau, L., Haueter, S., Ralvenius, W. T., Jegen, M., *et al.* (2015) 'Targeted ablation, silencing, and activation establish glycinergic dorsal horn

neurons as key components of a spinal gate for pain and itch', *Neuron,* 85(6), pp. 1289-304.

Fried, K. and Devor, M. (1988) 'End-structure of afferent axons injured in the peripheral and central nervous system', *Somatosens.Mot.Res.,* 6, pp. 79-99.

Fukuda, Y., Li, Y. and Segal, R. A. (2017) 'A Mechanistic Understanding of Axon Degeneration in Chemotherapy-Induced Peripheral Neuropathy', *Front Neurosci,* 11, pp. 481.

Fukuoka, T., Yamanaka, H., Kobayashi, K., Okubo, M., Miyoshi, K., Dai, Y., *et al.* (2012) 'Re-evaluation of the phenotypic changes in L4 dorsal root ganglion neurons after L5 spinal nerve ligation', *Pain,* 153(1), pp. 68-79.

Gabay, E. and Tal, M. (2004) 'Pain behavior and nerve electrophysiology in the CCI model of neuropathic pain', *Pain,* 110(1-2), pp. 354-360.

Gaida, W., Klinder, K., Arndt, K. and Weiser, T. (2005) 'Ambroxol, a Nav1.8-preferring Na(+) channel blocker, effectively suppresses pain symptoms in animal models of chronic, neuropathic and inflammatory pain', *Neuropharmacology,* 49(8), pp. 1220-7.

Galeazza, M. T., Garry, M. G., Yost, H. J., Strait, K. A., Hargreaves, K. M. and Seybold, V. S. (1995) 'Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation', *Neuroscience,* 66(2), pp. 443-58.

Galer, B. S., Henderson, J., Perander, J. and Jensen, M. P. (2000) 'Course of symptoms and quality of life measurement in complex regional pain syndrome: A pilot survey', *Brit.J.Anaesth.,* 20(4), pp. 286-292.

Gallego, R. and Adrover, E. (1990) 'Axotomy decreases conduction velocity of unmyelinated sensory fibres', *Brain Res,* 523(2), pp. 298-300.

Gao, Y. J. and Ji, R. R. (2009) 'c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury?', *Open Pain J,* 2, pp. 11-17.

Gao, Y. J., Xu, Z. Z., Liu, Y. C., Wen, Y. R., Decosterd, I. and Ji, R. R. (2010) 'The c-Jun N-terminal kinase 1 (JNK1) in spinal astrocytes is required for the maintenance of bilateral mechanical allodynia under a persistent inflammatory pain condition', *Pain,* 148(2), pp. 309-19.

Garrison, C. J., Dougherty, P. M. and Carlton, S. M. (1993) 'Quantitative analysis of substance P and calcitonin gene-related peptide immunohistochemical staining in the dorsal horn of neuropathic MK-801-treated rats', *Brain Res,* 607(1-2), pp. 205-14.

Garry, E. M., Delaney, A., Anderson, H. A., Sirinathsinghji, E. C., Clapp, R. H., Martin, W. J., *et al.* (2005) 'Varicella zoster virus induces neuropathic changes in rat dorsal root ganglia and behavioral reflex sensitisation that is attenuated by gabapentin or sodium channel blocking drugs', *Pain,* 118(1-2), pp. 97-111.

Gazda, L. S., Milligan, E. D., Hansen, M. K., Twining, C. M., Poulos, N. M., Chacur, M., *et al.* (2001) 'Sciatic inflammatory neuritis (SIN): behavioral allodynia is paralleled by peri-sciatic proinflammatory cytokine and superoxide production', *Journal of the Peripheral Nervous System,* 6(3), pp. 111-129.

Gerdle, B., Lemming, D., Kristiansen, J., Larsson, B., Peolsson, M. and Rosendal, L. (2008) 'Biochemical alterations in the trapezius muscle of patients with chronic whiplash associated disorders (WAD)--a microdialysis study', *Eur J Pain,* 12(1), pp. 82-93.

Gerner, P., Binshtok, A. M., Wang, C. F., Hevelone, N. D., Bean, B. P., Woolf, C. J., *et al.* (2008) 'Capsaicin combined with local anesthetics preferentially prolongs sensory/nociceptive block in rat sciatic nerve', *Anesthesiology,* 109(5), pp. 872-8.

Gigant, B., Wang, C., Ravelli, R. B., Roussi, F., Steinmetz, M. O., Curmi, P. A., *et al.* (2005) 'Structural basis for the regulation of tubulin by vinblastine', *Nature,* 435(7041), pp. 519-22.

Goedert, M., Stoeckel, K. and Otten, U. (1981) 'Biological importance of the retrograde axonal transport of nerve growth factor in sensory neurons', *Proc Natl Acad Sci U S A,* 78(9), pp. 5895-8.

Gold, M. S., Weinreich, D., Kim, C. S., Wang, R. Z., Treanor, J., Porreca, F., *et al.* (2003) 'Redistribution of Na(V)1.8 in uninjured axons enables neuropathic pain', *Journal of Neuroscience*, 23(1), pp. 158-166.

Goldberg, Y. P., MacFarlane, J., MacDonald, M. L., Thompson, J., Dube, M. P., Mattice, M., *et al.* (2007) 'Loss-of-function mutations in the Nav1.7 gene underlie congenital indifference to pain in multiple human populations', *Clin Genet*, 71(4), pp. 311-9.

Gong, X., Chen, Y., Fu, B., Jiang, J. and Zhang, M. (2017) 'Infant nerve injury induces delayed microglial polarization to the M1 phenotype, and exercise reduces delayed neuropathic pain by modulating microglial activity', *Neuroscience*, 349, pp. 76-86.

Gould, H. J., III, Gould, T. N., England, J. D., Paul, D., Liu, Z. P. and Levinson, S. R. (2000) 'A possible role for nerve growth factor in the augmentation of sodium channels in models of chronic pain', *Brain Res.*, 854(1-2), pp. 19-29.

Govea, R. M., Barbe, M. F. and Bove, G. M. (2017) 'Group IV nociceptors develop axonal chemical sensitivity during neuritis and following treatment of the sciatic nerve with vinblastine', *J Neurophysiol*, pp. jn 00395 2017.

Gracely, R. H., Lynch, S. A. and Bennett, G. J. (1992) 'Painful neuropathy: altered central processing maintained dynamically by peripheral input', *Pain*, 51, pp. 175-194.

Gray, J. A. and Matthews, P. B. (1951) 'Response of Pacinian corpuscles in the cat's toe', *J Physiol*, 113(4), pp. 475-82.

Green, D. and Dong, X. (2016) 'The cell biology of acute itch', *The Journal of Cell Biology*, 213(2), pp. 155-161.

Green, G. M., Lyons, L. and Dickenson, A. H. (1998) 'Alpha2-adrenoceptor antagonists enhance responses of dorsal horn neurones to formalin induced inflammation', *Eur J Pharmacol*, 347(2-3), pp. 201-4.

Greening, J., Anantharaman, K., Young, R. and Dilley, A. (2018) 'Evidence for Increased MRI Signal Intensity and Morphological Changes in the Brachial Plexus and Median Nerves of Patients With Chronic Arm and Neck Pain Following Whiplash Injury', *J Orthop Sports Phys Ther*, pp. 1-35.

Greening, J., Dilley, A. and Lynn, B. (2005) 'In vivo study of nerve movement and mechanosensitivity of the median nerve in whiplash and non-specific arm pain patients', *Pain,* 115(3), pp. 248-253.

Greening, J. and Lynn, B. (1998) 'Vibration sense in the upper limb in patients with repetitive strain injury and a group of at-risk office workers', *Int.Arch.Occup.Environ.Health,* 71(1), pp. 29-34.

Greening, J., Lynn, B. and Leary, R. (2003) 'Sensory and autonomic function in the hands of patients with non-specific arm pain (NSAP) and asymptomatic office workers', *Pain,* 104(1-2), pp. 275-281.

Grudt, T. J. and Perl, E. R. (2002) 'Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn', *J Physiol,* 540(Pt 1), pp. 189-207.

Grunder, S. and Pusch, M. (2015) 'Biophysical properties of acid-sensing ion channels (ASICs)', *Neuropharmacology,* 94, pp. 9-18.

Gu, J. G., Albuquerque, C., Lee, C. J. and MacDermott, A. B. (1996) 'Synaptic strengthening through activation of Ca2+-permeable AMPA receptors', *Nature,* 381(6585), pp. 793-6.

Gutierrez-Mecinas, M., Bell, A. M., Marin, A., Taylor, R., Boyle, K. A., Furuta, T., *et al.* (2017) 'Preprotachykinin A is expressed by a distinct population of excitatory neurons in the mouse superficial spinal dorsal horn including cells that respond to noxious and pruritic stimuli', *Pain,* 158(3), pp. 440-456.

Gutierrez-Mecinas, M., Furuta, T., Watanabe, M. and Todd, A. J. (2016) 'A quantitative study of neurochemically defined excitatory interneuron populations in laminae I-III of the mouse spinal cord', *Mol Pain,* 12.

Gutman, G. A., Chandy, K. G., Grissmer, S., Lazdunski, M., McKinnon, D., Pardo, L. A., *et al.* (2005) 'International Union of Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels', *Pharmacol Rev*, 57(4), pp. 473-508.

Haim, N., Epelbaum, R., Ben-Shahar, M., Yarnitsky, D., Simri, W. and Robinson, E. (1994) 'Full dose vincristine (without 2-mg dose limit) in the treatment of lymphomas', *Cancer*, 73(10), pp. 2515-9.

Hains, B. C. and Waxman, S. G. (2006) 'Activated Microglia Contribute to the Maintenance of Chronic Pain after Spinal Cord Injury', *Journal of Neuroscience*, 26(16), pp. 4308-4317.

Haliloglu, G., Becker, K., Temucin, C., Talim, B., Kucuksahin, N., Pergande, M., *et al.* (2017) 'Recessive PIEZO2 stop mutation causes distal arthrogryposis with distal muscle weakness, scoliosis and proprioception defects', *J Hum Genet*, 62(4), pp. 497-501.

Han, C., Yang, Y., de Greef, B. T., Hoeijmakers, J. G., Gerrits, M. M., Verhamme, C., *et al.* (2015) 'The Domain II S4-S5 Linker in Nav1.9: A Missense Mutation Enhances Activation, Impairs Fast Inactivation, and Produces Human Painful Neuropathy', *Neuromolecular Med*, 17(2), pp. 158-69.

Han, J. S., Bird, G. C., Li, W., Jones, J. and Neugebauer, V. (2005) 'Computerized analysis of audible and ultrasonic vocalizations of rats as a standardized measure of pain-related behavior', *J Neurosci Methods*, 141(2), pp. 261-9.

Han, Z. S., Zhang, E. T. and Craig, A. D. (1998) 'Nociceptive and thermoreceptive lamina I neurons are anatomically distinct', *Nat Neurosci*, 1(3), pp. 218-25.

Hancock, B. W. and Naysmith, A. (1975) 'Vincristine-induced autonomic neuropathy', *Br Med J*, 3(5977), pp. 207.

Hanz, S., Perlson, E., Willis, D., Zheng, J. Q., Massarwa, R., Huerta, J., *et al.* (2003) 'Axoplasmic importins enable retrograde injury signaling in lesioned nerve', *Neuron*, 40, pp. 1095-1104.

Hao, J., Padilla, F., Dandonneau, M., Lavebratt, C., Lesage, F., Noel, J., *et al.* (2013) 'Kv1.1 channels act as mechanical brake in the senses of touch and pain', *Neuron,* 77(5), pp. 899-914.

Hargreaves, K., Dubner, R., Brown, F., Flores, C. and Joris, J. (1988) 'A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia', *Pain,* 32(1), pp. 77-88.

Harper, A. A. and Lawson, S. N. (1985) 'Electrical properties of rat dorsal root ganglion neurones with different peripheral nerve conduction velocities', *J.Physiol.Lond.,* 359, pp. 47-63.

Harris, R. E., Sundgren, P. C., Craig, A. D., Kirshenbaum, E., Sen, A., Napadow, V., *et al.* (2009) 'Elevated insular glutamate in fibromyalgia is associated with experimental pain', *Arthritis Rheum,* 60(10), pp. 3146-52.

Hasnie, F. S., Breuer, J., Parker, S., Wallace, V., Blackbeard, J., Lever, I., *et al.* (2007) 'Further characterization of a rat model of varicella zoster virus-associated pain: Relationship between mechanical hypersensitivity and anxiety-related behavior, and the influence of analgesic drugs', *Neuroscience,* 144(4), pp. 1495-508.

Heerssen, H. M., Pazyra, M. F. and Segal, R. A. (2004) 'Dynein motors transport activated Trks to promote survival of target-dependent neurons', *Nat Neurosci,* 7(6), pp. 596-604.

Heijmans-Antonissen, C., Wesseldijk, F., Munnikes, R. J., Huygen, F. J., van der Meijden, P., Hop, W. C., *et al.* (2006) 'Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1', *Mediators Inflamm,* 2006(1), pp. 28398.

Henriques-Coelho, T., Gonzaga, S., Endo, M., Zoltick, P. W., Davey, M., Leite-Moreira, A. F., *et al.* (2007) 'Targeted gene transfer to fetal rat lung interstitium by ultrasound-guided intrapulmonary injection', *Mol Ther,* 15(2), pp. 340-7.

Herrero, J. F. and Cervero, F. (1996) 'Changes in nociceptive reflex facilitation during carrageenan-induced arthritis', *Brain Res,* 717(1-2), pp. 62-8.

Herrero, J. F., Laird, J. M. and Lopez-Garcia, J. A. (2000) 'Wind-up of spinal cord neurones and pain sensation: much ado about something?', *Prog Neurobiol*, 61(2), pp. 169-203.

Hirokawa, N., Sato-Yoshitake, R., Kobayashi, N., Pfister, K. K., Bloom, G. S. and Brady, S. T. (1991) 'Kinesin associates with anterogradely transported membranous organelles in vivo', *J Cell Biol*, 114(2), pp. 295-302.

Hirokawa, N., Sato-Yoshitake, R., Yoshida, T. and Kawashima, T. (1990) 'Brain dynein (MAP1C) localizes on both anterogradely and retrogradely transported membranous organelles in vivo', *J Cell Biol*, 111(3), pp. 1027-37.

Hirvonen, H. E., Salmi, T. T., Heinonen, E., Antila, K. J. and Valimaki, I. A. (1989) 'Vincristine treatment of acute lymphoblastic leukemia induces transient autonomic cardioneuropathy', *Cancer*, 64(4), pp. 801-5.

Honore, P., Rogers, S. D., Schwei, M. J., Salak-Johnson, J. L., Luger, N. M., Sabino, M. C., *et al.* (2000) 'Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons', *Neuroscience*, 98(3), pp. 585-98.

Honore, P., Wismer, C. T., Mikusa, J., Zhu, C. Z., Zhong, C., Gauvin, D. M., *et al.* (2005) 'A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea], a novel transient receptor potential type V1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats', *J Pharmacol Exp Ther*, 314(1), pp. 410-21.

Houk, J. and Simon, W. (1967) 'Responses of Golgi tendon organs to forces applied to muscle tendon', *J Neurophysiol*, 30(6), pp. 1466-81.

Howard, R. F., Walker, S. M., Mota, P. M. and Fitzgerald, M. (2005) 'The ontogeny of neuropathic pain: postnatal onset of mechanical allodynia in rat spared nerve injury (SNI) and chronic constriction injury (CCI) models', *Pain*, 115(3), pp. 382-9.

Hu, C., Zhao, Y. T., Zhang, G. and Xu, M. F. (2017) 'Antinociceptive effects of fucoidan in rat models of vincristine-induced neuropathic pain', *Mol Med Rep*, 15(2), pp. 975-980.

Hu, D., Hu, R. and Berde, C. B. (1997) 'Neurologic evaluation of infant and adult rats before and after sciatic nerve blockade', *Anesthesiology,* 86(4), pp. 957-65.

Hu, H. J., Alter, B. J., Carrasquillo, Y., Qiu, C. S. and Gereau, R. W. T. (2007) 'Metabotropic glutamate receptor 5 modulates nociceptive plasticity via extracellular signal-regulated kinase-Kv4.2 signaling in spinal cord dorsal horn neurons', *J Neurosci,* 27(48), pp. 13181-91.

Hu, P. and McLachlan, E. M. (2002) 'Macrophage and lymphocyte invasion of dorsal root ganglia after peripheral nerve lesions in the rat', *Neuroscience,* 112(1), pp. 23-38.

Huang, J., Han, C., Estacion, M., Vasylyev, D., Hoeijmakers, J. G., Gerrits, M. M., *et al.* (2014) 'Gain-of-function mutations in sodium channel Na(v)1.9 in painful neuropathy', *Brain,* 137(Pt 6), pp. 1627-42.

Huang, W., Calvo, M., Karu, K., Olausen, H. R., Bathgate, G., Okuse, K., *et al.* (2013) 'A clinically relevant rodent model of the HIV antiretroviral drug stavudine induced painful peripheral neuropathy', *Pain,* 154(4), pp. 560-75.

Hubbard, S. J. (1958) 'A study of rapid mechanical events in a mechanoreceptor', *J Physiol,* 141(2), pp. 198-218.

Hughes, D. I., Scott, D. T., Todd, A. J. and Riddell, J. S. (2003) 'Lack of evidence for sprouting of Abeta afferents into the superficial laminas of the spinal cord dorsal horn after nerve section', *J Neurosci,* 23(29), pp. 9491-9.

Hunt, C. C. and Kuffler, S. W. (1951) 'Stretch receptor discharges during muscle contraction', *J Physiol,* 113(2-3), pp. 298-315.

Hunt, S. P., Pini, A. and Evan, G. (1987) 'Induction of c-fos-like protein in spinal cord neurons following sensory stimulation', *Nature,* 328, pp. 632-634.

Huygen, F. J., De Bruijn, A. G., De Bruin, M. T., Groeneweg, J. G., Klein, J. and Zijlstra, F. J. (2002) 'Evidence for local inflammation in complex regional pain syndrome type 1', *Mediators Inflamm,* 11(1), pp. 47-51.

Hylden, J. L., Nahin, R. L. and Dubner, R. (1987) 'Altered responses of nociceptive cat lamina I spinal dorsal horn neurons after chronic sciatic neuroma formation', *Brain Res,* 411(2), pp. 341-50.

Hylden, J. L. K., Nahin, R. L., Traub, R. J. and Dubner, R. (1989) 'Expansion of receptivve fields of spinal lamina 1 projection neurones in rats with unilateral adjuvant-induced inflammation: the contribution of dorsal horn mechanisms', *Pain,* 37, pp. 229-243.

Ibuki, T., Hama, A. T., Wang, X. T., Pappas, G. D. and Sagen, J. (1997) 'Loss of GABA-immunoreactivity in the spinal dorsal horn of rats with peripheral nerve injury and promotion of recovery by adrenal medullary grafts', *Neuroscience,* 76(3), pp. 845-58.

Iggo, A. (1961) 'Non-myelinated afferent fibres from mammalian skeletal muscle.', *Journal of Physiology,* 155, pp. 52-53P.

Iggo, A. (1969) 'Cutaneous thermoreceptors in primates and sub-primates', *J Physiol,* 200(2), pp. 403-30.

Iggo, A. (1977) 'Cutaneous and subcutaneous sense organs', *Br Med Bull,* 33(2), pp. 97-102.

Iggo, A. and Muir, A. R. (1969) 'The structure and function of a slowly adapting touch corpuscle in hairy skin', *J Physiol,* 200(3), pp. 763-96.

Inoue, K. and Tsuda, M. (2018) 'Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential', *Nat Rev Neurosci,* 19(3), pp. 138-152.

Ishikawa, K., Tanaka, M., Black, J. A. and Waxman, S. G. (1999) 'Changes in expression of voltage-gated potassium channels in dorsal root ganglion neurons following axotomy', *Muscle Nerve,* 22(4), pp. 502-507.

Jarvis, M. F., Honore, P., Shieh, C. C., Chapman, M., Joshi, S., Zhang, X. F., *et al.* (2007) 'A-803467, a potent and selective Nav1.8 sodium channel blocker, attenuates

neuropathic and inflammatory pain in the rat', *Proc Natl Acad Sci U S A,* 104(20), pp. 8520-5.

Jensen, T. S., Baron, R., Haanpaa, M., Kalso, E., Loeser, J. D., Rice, A. S., *et al.* (2011) 'A new definition of neuropathic pain', *Pain,* 152(10), pp. 2204-5.

Jensen, T. S. and Finnerup, N. B. (2014) 'Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms', *Lancet Neurol,* 13(9), pp. 924-35.

Ji, R.-R., Chamessian, A. and Zhang, Y.-Q. (2016) 'Pain Regulation by Non-neuronal Cells and Inflammation', *Science (New York, N.Y.),* 354(6312), pp. 572-577.

Ji, R.-R., Xu, Z.-Z. and Gao, Y.-J. (2014) 'Emerging targets in neuroinflammation-driven chronic pain', *Nature reviews. Drug discovery,* 13(7), pp. 533-548.

Ji, R. R., Baba, H., Brenner, G. J. and Woolf, C. J. (1999) 'Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity', *Nat Neurosci,* 2(12), pp. 1114-9.

Ji, R. R., Berta, T. and Nedergaard, M. (2013) 'Glia and pain: is chronic pain a gliopathy?', *Pain,* 154 Suppl 1, pp. S10-28.

Ji, R. R., Kohno, T., Moore, K. A. and Woolf, C. J. (2003) 'Central sensitization and LTP: do pain and memory share similar mechanisms?', *Trends Neurosci,* 26(12), pp. 696-705.

Ji, R. R. and Woolf, C. J. (2001) 'Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain', *Neurobiol Dis,* 8(1), pp. 1-10.

Jiang, Y. Q., Xing, G. G., Wang, S. L., Tu, H. Y., Chi, Y. N., Li, J., *et al.* (2008) 'Axonal accumulation of hyperpolarization-activated cyclic nucleotide-gated cation channels contributes to mechanical allodynia after peripheral nerve injury in rat', *Pain,* 137(3), pp. 495-506.

Jin, S. X., Zhuang, Z. Y., Woolf, C. J. and 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', *Journal of Neuroscience,* 23(10), pp. 4017-4022.

Jones, G., Zhu, Y., Silva, C., Tsutsui, S., Pardo, C. A., Keppler, O. T., *et al.* (2005) 'Peripheral nerve-derived HIV-1 is predominantly CCR5-dependent and causes neuronal degeneration and neuroinflammation', *Virology,* 334(2), pp. 178-93.

Joshi, S. K., Mikusa, J. P., Hernandez, G., Baker, S., Shieh, C. C., Neelands, T., *et al.* (2006) 'Involvement of the TTX-resistant sodium channel Nav 1.8 in inflammatory and neuropathic, but not post-operative, pain states', *Pain,* 123(1-2), pp. 75-82.

Julius, D. and Basbaum, A. I. (2001) 'Molecular mechanisms of nociception', *Nature,* 413(6852), pp. 203-210.

Jung, H., Toth, P. T., White, F. A. and Miller, R. J. (2008) 'Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root ganglia neurons', *J Neurochem,* 104(1), pp. 254-63.

Kaether, C., Skehel, P. and Dotti, C. G. (2000) 'Axonal membrane proteins are transported in distinct carriers: a two-color video microscopy study in cultured hippocampal neurons', *Mol Biol Cell,* 11(4), pp. 1213-24.

Kajander, K. C. and Bennett, G. J. (1992) 'Onset of a painful peripheral neuropathy in rat: a partial and differential deafferentation and spontaneous discharge in A beta and A delta primary afferent neurons', *J.Neurophysiol.,* 68(3), pp. 734-744.

Kajander, K. C., Madsen, A. M., Iadarola, M. J., Draisci, G. and Wakisaka, S. (1996) 'Fos-like immunoreactivity increases in the lumbar spinal cord following a chronic constriction injury to the sciatic nerve of rat', *Neurosci Lett,* 206(1), pp. 9-12.

Kajander, K. C., Wakisaka, S. and Bennett, G. J. (1992) 'Spontaneous discharge originates in the dorsal root ganglion at the onset of a painful peripheral neuropathy in the rat', *Neurosci.Lett.,* 138(2), pp. 225-228.

Kashiba, H., Senba, E., Kawai, Y., Ueda, Y. and Tohyama, M. (1992) 'Axonal blockade induces the expression of vasoactive intestinal polypeptide and galanin in rat dorsal root ganglion neurons', *Brain Res.*, 577(1), pp. 19-28.

Katoh, K., Tohyama, M., Noguchi, K. and Senba, E. (1992) 'Axonal flow blockade induces alpha-CGRP mRNA expression in rat motoneurons', *Brain Res.*, 599(1), pp. 153-157.

Kawasaki, Y., Kohno, T., Zhuang, Z. Y., Brenner, G. J., Wang, H., Van Der, M. C., *et al.* (2004) 'Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization', *J Neurosci*, 24(38), pp. 8310-8321.

Kawasaki, Y., Zhang, L., Cheng, J. K. and Ji, R. R. (2008) 'Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord', *J Neurosci*, 28(20), pp. 5189-94.

Kayalioglu, G. and Heise, C. (2009) 'Atlas of the Rat Spinal Cord', in Watson, C. & Paxinos, G. (eds.) *The Spinal Cord.* 1 ed. China: Elsevier Academic Press, pp. 64-93.

Kelly, S., Dunham, J. P., Murray, F., Read, S., Donaldson, L. F. and Lawson, S. N. (2012) 'Spontaneous firing in C-fibers and increased mechanical sensitivity in A-fibers of knee joint-associated mechanoreceptive primary afferent neurones during MIA-induced osteoarthritis in the rat', *Osteoarthritis Cartilage*, 20(4), pp. 305-13.

Kim, K. J., Yoon, Y. W. and Chung, J. M. (1997) 'Comparison of three rodent neuropathic pain models', *Experimental Brain Research*, 113, pp. 200-206.

Kim, S. H. and Chung, J. M. (1992) 'An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat', *Pain*, 50, pp. 355-363.

King, C. H. and Scherer, S. S. (2012) 'Kv7.5 is the primary Kv7 subunit expressed in C-fibers', *J Comp Neurol*, 520(9), pp. 1940-50.

Kirschstein, T., Greffrath, W., Busselberg, D. and Treede, R. D. (1999) 'Inhibition of rapid heat responses in nociceptive primary sensory neurons of rats by vanilloid receptor antagonists', *Journal of Neurophysiology,* 82(6), pp. 2853-2860.

Kissin, I. (2010) 'The development of new analgesics over the past 50 years: a lack of real breakthrough drugs', *Anesth Analg,* 110(3), pp. 780-9.

Kitagawa, J., Kanda, K., Sugiura, M., Tsuboi, Y., Ogawa, A., Shimizu, K., *et al.* (2005) 'Effect of chronic inflammation on dorsal horn nociceptive neurons in aged rats.', *Journal of Neurophysiology,* 93(6), pp. 3594-3604.

Klein, C. J., Lennon, V. A., Aston, P. A., McKeon, A. and Pittock, S. J. (2012) 'Chronic pain as a manifestation of potassium channel-complex autoimmunity', *Neurology,* 79(11), pp. 1136-44.

Klein, C. M., Coggeshall, R. E., Carlton, S. M., Westlund, K. N. and Sorkin, L. S. (1990) 'Changes in calcitonin gene-related peptide immunoreactivity in the rat dorsal horn following electrical stimulation of the sciatic nerve', *Neurosci Lett,* 115(2-3), pp. 149-54.

Knowlton, W. M., Palkar, R., Lippoldt, E. K., McCoy, D. D., Baluch, F., Chen, J., *et al.* (2013) 'A sensory-labeled line for cold: TRPM8-expressing sensory neurons define the cellular basis for cold, cold pain, and cooling-mediated analgesia', *J Neurosci,* 33(7), pp. 2837-48.

Kobayashi, K., Fukuoka, T., Obata, K., Yamanaka, H., Dai, Y., Tokunaga, A., *et al.* (2005) 'Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with adelta/c-fibers and colocalization with trk receptors', *J Comp Neurol,* 493(4), pp. 596-606.

Kocher, L., Anton, F., Reeh, P. W. and Handwerker, H. O. (1987) 'The effect of carrageenan-induced inflammation on the sensitivity of unmyelinated skin nociceptors in the rat', *Pain,* 29, pp. 363-373.

Kohane, D. S., Yieh, J., Lu, N. T., Langer, R., Strichartz, G. R. and Berde, C. B. (1998) 'A re-examination of tetrodotoxin for prolonged duration local anesthesia', *Anesthesiology,* 89(1), pp. 119-31.

Koltzenburg, M., Wall, P. D. and McMahon, S. B. (1999) 'Does the right side know what the left is doing?', *Trends Neurosci,* 22(3), pp. 122-127.

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., *et al.* (2006) 'Loss of autophagy in the central nervous system causes neurodegeneration in mice', *Nature,* 441(7095), pp. 880-4.

Kosai, K., Tateyama, S., Ikeda, T., Uno, T., Nishimori, T. and Takasaki, M. (2001) 'MK-801 reduces non-noxious stimulus-evoked Fos-like immunoreactivity in the spinal cord of rats with chronic constriction nerve injury', *Brain Res,* 910(1-2), pp. 12-8.

Koschorke, G. M., Meyer, R. A. and Campbell, J. N. (1994) 'Cellular components necessary for mechanoelectrical transduction are conveyed to primary afferent terminals by fast axonal transport', *Brain Res.,* 641(1), pp. 99-104.

Kreutzberg, G. W. (1969) 'Neuronal dynamics and axonal flow. IV. Blockage of intra-axonal enzyme transport by colchicine', *Proc Natl Acad Sci U S A,* 62(3), pp. 722-8.

Kroigard, T., Schroder, H. D., Qvortrup, C., Eckhoff, L., Pfeiffer, P., Gaist, D., *et al.* (2014) 'Characterization and diagnostic evaluation of chronic polyneuropathies induced by oxaliplatin and docetaxel comparing skin biopsy to quantitative sensory testing and nerve conduction studies', *Eur J Neurol,* 21(4), pp. 623-9.

Kwon, M. J., Kim, J., Shin, H., Jeong, S. R., Kang, Y. M., Choi, J. Y., *et al.* (2013) 'Contribution of macrophages to enhanced regenerative capacity of dorsal root ganglia sensory neurons by conditioning injury', *J Neurosci,* 33(38), pp. 15095-108.

Lai, J., Gold, M. S., Kim, C. S., Bian, D., Ossipov, M. H., Hunter, J. C., *et al.* (2002) 'Inhibition of neuropathic pain by decreased expression of the tetrodotoxin-resistant sodium channel, NaV1.8', *Pain,* 95(1-2), pp. 143-152.

Laird, J. M. and Bennett, G. J. (1993) 'An electrophysiological study of dorsal horn neurons in the spinal cord of rats with an experimental peripheral neuropathy', *J Neurophysiol.,* 69(6), pp. 2072-2085.

Langford, D. J., Bailey, A. L., Chanda, M. L., Clarke, S. E., Drummond, T. E., Echols, S., *et al.* (2010) 'Coding of facial expressions of pain in the laboratory mouse', *Nat Methods,* 7(6), pp. 447-9.

LaPointe, N. E., Morfini, G., Brady, S. T., Feinstein, S. C., Wilson, L. and Jordan, M. A. (2013) 'Effects of eribulin, vincristine, paclitaxel and ixabepilone on fast axonal transport and kinesin-1 driven microtubule gliding: implications for chemotherapy-induced peripheral neuropathy', *Neurotoxicology,* 37, pp. 231-9.

Lasek, R. J., Garner, J. A. and Brady, S. T. (1984) 'Axonal transport of the cytoplasmic matrix', *J Cell Biol,* 99(1 Pt 2), pp. 212s-221s.

Latremoliere, A. and Woolf, C. J. (2009) 'Central sensitization: a generator of pain hypersensitivity by central neural plasticity', *J Pain,* 10(9), pp. 895-926.

Leem, J. W., Willis, W. D. and Chung, J. M. (1993) 'Cutaneous sensory receptors in the rat foot', *J Neurophysiol,* 69(5), pp. 1684-99.

Leipold, E., Liebmann, L., Korenke, G. C., Heinrich, T., Giesselmann, S., Baets, J., *et al.* (2013) 'A de novo gain-of-function mutation in SCN11A causes loss of pain perception', *Nat Genet,* 45(11), pp. 1399-404.

Lekan, H. A., Carlton, S. M. and Coggeshall, R. E. (1996) 'Sprouting of A beta fibers into lamina II of the rat dorsal horn in peripheral neuropathy', *Neuroscience Letters,* 208(3), pp. 147-150.

Li, J., Simone, D. A. and Larson, A. A. (1999) 'Windup leads to characteristics of central sensitization', *Pain,* 79(1), pp. 75-82.

Li, L., Rutlin, M., Abraira, V. E., Cassidy, C., Kus, L., Gong, S., *et al.* (2011) 'The functional organization of cutaneous low-threshold mechanosensory neurons', *Cell,* 147(7), pp. 1615-27.

Li, W. G., Yu, Y., Zhang, Z. D., Cao, H. and Xu, T. L. (2010) 'ASIC3 channels integrate agmatine and multiple inflammatory signals through the nonproton ligand sensing domain', *Mol Pain,* 6, pp. 88.

Light, A. R. and Perl, E. R. (1979) 'Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers', *J.Comp.Neurol.,* 186, pp. 133-150.

Liljencrantz, J. and Olausson, H. (2014) 'Tactile C fibers and their contributions to pleasant sensations and to tactile allodynia', *Front Behav Neurosci,* 8, pp. 37.

Lim, H., Lee, H., Noh, K. and Lee, S. J. (2017) 'IKK/NF-kappaB-dependent satellite glia activation induces spinal cord microglia activation and neuropathic pain after nerve injury', *Pain,* 158(9), pp. 1666-1677.

Lima, D. and Coimbra, A. (1986) 'A Golgi study of the neuronal population of the marginal zone (lamina I) of the rat spinal cord', *J Comp Neurol,* 244(1), pp. 53-71.

Lin, Q., Peng, Y. B. and Willis, W. D. (1996) 'Inhibition of primate spinothalamic tract neurons by spinal glycine and GABA is reduced during central sensitization', *J Neurophysiol,* 76(2), pp. 1005-14.

Lindblom, U. (1965) 'Properties of touch receptors in distal glabrous skin of the monkey', *J Neurophysiol,* 28(5), pp. 966-85.

Linnman, C., Appel, L., Fredrikson, M., Gordh, T., Soderlund, A., Langstrom, B., *et al.* (2011) 'Elevated [11C]-D-deprenyl uptake in chronic Whiplash Associated Disorder suggests persistent musculoskeletal inflammation', *PLoS One,* 6(4), pp. e19182.

Liou, J. T., Liu, F. C., Mao, C. C., Lai, Y. S. and Day, Y. J. (2011) 'Inflammation confers dual effects on nociceptive processing in chronic neuropathic pain model', *Anesthesiology,* 114(3), pp. 660-72.

Liu, C. N., Wall, P. D., BenDor, E., Michaelis, M., Amir, R. and Devor, M. (2000a) 'Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury', *Pain,* 85(3), pp. 503-521.

Liu, X., Eschenfelder, S., Blenk, K. H., Janig, W. and Habler, H. (2000b) 'Spontaneous activity of axotomized afferent neurons after L5 spinal nerve injury in rats', *Pain,* 84(2-3), pp. 309-318.

Ljungdahl, A., Hokfelt, T. and Nilsson, G. (1978) 'Distribution of substance P-like immunoreactivity in the central nervous system of the rat--I. Cell bodies and nerve terminals', *Neuroscience,* 3(10), pp. 861-943.

Lobert, S., Vulevic, B. and Correia, J. J. (1996) 'Interaction of vinca alkaloids with tubulin: a comparison of vinblastine, vincristine, and vinorelbine', *Biochemistry,* 35(21), pp. 6806-14.

Loken, L. S., Wessberg, J., Morrison, I., McGlone, F. and Olausson, H. (2009) 'Coding of pleasant touch by unmyelinated afferents in humans', *Nat Neurosci,* 12(5), pp. 547-8.

Lopes, D. M., Malek, N., Edye, M., Jager, S. B., McMurray, S., McMahon, S. B., *et al.* (2017) 'Sex differences in peripheral not central immune responses to pain-inducing injury', *Sci Rep,* 7(1), pp. 16460.

Lorenzo, L. E., Ramien, M., St Louis, M., De Koninck, Y. and Ribeiro-da-Silva, A. (2008) 'Postnatal changes in the Rexed lamination and markers of nociceptive afferents in the superficial dorsal horn of the rat', *J Comp Neurol,* 508(4), pp. 592-604.

Lovell, M. E. and Galasko, C. S. (2002) 'Whiplash disorders--a review', *Injury,* 33(2), pp. 97-101.

Lu, Y., Dong, H., Gao, Y., Gong, Y., Ren, Y., Gu, N., *et al.* (2013) 'A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia', *J Clin Invest,* 123(9), pp. 4050-62.

Lu, Y. and Perl, E. R. (2005) 'Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (laminae I and II)', *J Neurosci,* 25(15), pp. 3900-7.

Luthi, A. and McCormick, D. A. (1999a) 'Ca(2+)-mediated up-regulation of Ih in the thalamus. How cell-intrinsic ionic currents may shape network activity', *Ann N Y Acad Sci,* 868, pp. 765-9.

Luthi, A. and McCormick, D. A. (1999b) 'Modulation of a pacemaker current through Ca(2+)-induced stimulation of cAMP production', *Nat Neurosci,* 2(7), pp. 634-41.

Lynn, B. (1971) 'The form and distribution of the receptive fields of Pacinian corpuscles found in and around the cat's large foot pad', *J Physiol,* 217(3), pp. 755-71.

Lynn, B. (1984) 'Effect of neonatal treatment with capsaicin on the numbers and properties of cutaneous afferent units from the hairy skin of the rat', *Brain Res,* 322(2), pp. 255-60.

Lynn, B. and Carpenter, S. E. (1982) 'Primary afferent units from the hairy skin of the rat hind limb', *Brain Res,* 238(1), pp. 29-43.

Lynn, B. and Shakhanbeh, J. (1988) 'Properties of A delta high threshold mechanoreceptors in the rat hairy and glabrous skin and their response to heat', *Neurosci Lett,* 85(1), pp. 71-6.

Macfarlane, G. J., Hunt, I. M. and Silman, A. J. (2000) 'Role of mechanical and psychosocial factors in the onset of forearm pain: prospective population based study', *BMJ,* 321(7262), pp. 676-9.

Maday, S., Wallace, K. E. and Holzbaur, E. L. (2012) 'Autophagosomes initiate distally and mature during transport toward the cell soma in primary neurons', *J Cell Biol,* 196(4), pp. 407-17.

Maier, C., Baron, R., Tolle, T. R., Binder, A., Birbaumer, N., Birklein, F., *et al.* (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(3), pp. 439-50.

Maksimovic, S., Nakatani, M., Baba, Y., Nelson, A. M., Marshall, K. L., Wellnitz, S. A., *et al.* (2014) 'Epidermal Merkel cells are mechanosensory cells that tune mammalian touch receptors', *Nature,* 509(7502), pp. 617-21.

Malik, R. A., Veves, A., Walker, D., Siddique, I., Lye, R. H., Schady, W., *et al.* (2001) 'Sural nerve fibre pathology in diabetic patients with mild neuropathy: relationship to pain, quantitative sensory testing and peripheral nerve electrophysiology', *Acta Neuropathol,* 101(4), pp. 367-74.

Marlier, L., Poulat, P., Rajaofetra, N. and Privat, A. (1991) 'Modifications of serotonin-, substance P- and calcitonin gene-related peptide-like immunoreactivities in the dorsal horn of the spinal cord of arthritic rats: a quantitative immunocytochemical study', *Exp Brain Res,* 85(3), pp. 482-90.

Marshall, G. E., Shehab, S. A., Spike, R. C. and Todd, A. J. (1996) 'Neurokinin-1 receptors on lumbar spinothalamic neurons in the rat', *Neuroscience,* 72(1), pp. 255-63.

Martin, C., Solders, G., Sonnerborg, A. and Hansson, P. (2003) 'Painful and non-painful neuropathy in HIV-infected patients: an analysis of somatosensory nerve function', *Eur J Pain,* 7(1), pp. 23-31.

Matthews, B. H. C. (1933) 'Nerve endings in mammalian muscle', *J Physiol,* 78(1), pp. 1-53.

Matzner, O. and Devor, M. (1994) 'Hyperexcitability at sites of nerve injury depends on voltage-sensitive Na+ channels', *Journal of Neurophysiology,* 72(1), pp. 349-359.

Maves, T. J., Pechman, P. S., Gebhart, G. F. and Meller, S. T. (1993) 'Possible chemical contribution from chromic gut sutures produces disorders of pain sensation like those seen in man', *Pain,* 54, pp. 57-69.

McCarthy, P. W. and Lawson, S. N. (1989) 'Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity', *Neuroscience,* 28, pp. 745-753.

McGaraughty, S., Chu, K. L., Faltynek, C. R. and Jarvis, M. F. (2006) 'Systemic and site-specific effects of A-425619, a selective TRPV1 receptor antagonist, on wide dynamic range neurons in CFA-treated and uninjured rats', *J Neurophysiol,* 95(1), pp. 18-25.

McKelvey, R., Berta, T., Old, E., Ji, R. R. and Fitzgerald, M. (2015) 'Neuropathic pain is constitutively suppressed in early life by anti-inflammatory neuroimmune regulation', *J Neurosci,* 35(2), pp. 457-66.

McMahon, S. B., Cafferty, W. B. and Marchand, F. (2005) 'Immune and glial cell factors as pain mediators and modulators', *Exp.Neurol.*, 192(2), pp. 444-462.

McNamara, C. R., Mandel-Brehm, J., Bautista, D. M., Siemens, J., Deranian, K. L., Zhao, M., *et al.* (2007) 'TRPA1 mediates formalin-induced pain', *Proc Natl Acad Sci U S A,* 104(33), pp. 13525-30.

Melzack, R. and Wall, P. D. (1965) 'Pain mechanisms: a new theory', *Science,* 150(3699), pp. 971-9.

Mendell, L. M. (1966) 'Physiological properties of unmyelinated fiber projection to the spinal cord', *Exp Neurol,* 16, pp. 316-332.

Mendell, L. M. and Wall, P. D. (1965) 'Responses of Single Dorsal Cord Cells to Peripheral Cutaneous Unmyelinated Fibres', *Nature,* 206, pp. 97-9.

Mense, S., Craig, A. D., Lehmann-Willenbrock, E. and Meyer, H. (1985) 'Neurobiology of small-diameter afferent fibers from deep tissues', *Development, Organization, and Processing in Somatosensory Pathways*: Alan R. Liss, Inc., pp. 299-308.

Mense, S. and Stahnke, M. (1983) 'Responses in muscle afferent fibres of slow conduction velocity to contractions and ischaemia in the cat', *J.Physiol.Lond.*, 342, pp. 383-397.

Meyer-Rosberg, K., Burckhardt, C. S., Huizar, K., Kvarnstrom, A., Nordfors, L. O. and Kristofferson, A. (2001) 'A comparison of the SF-36 and Nottingham Health Profile in patients with chronic neuropathic pain', *Eur J Pain,* 5(4), pp. 391-403.

Michaelis, M., Blenk, K. H., Janig, W. and Vogel, C. (1995) 'Development of spontaneous activity and mechanosensitivity in axotomized afferent nerve fibers during the first hours after nerve transection in rats', *J.Neurophysiol.*, 74(3), pp. 1020-1027.

Miltenburg, N. C. and Boogerd, W. (2014) 'Chemotherapy-induced neuropathy: A comprehensive survey', *Cancer Treat Rev*, 40(7), pp. 872-82.

Moalem, G., Xu, K. and Yu, L. (2004) 'T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats', *Neuroscience*, 129(3), pp. 767-77.

Mogil, J. S. (2009) 'Animal models of pain: progress and challenges', *Nat Rev Neurosci*, 10(4), pp. 283-94.

Mogil, J. S. and Crager, S. E. (2004) 'What should we be measuring in behavioral studies of chronic pain in animals?', *Pain*, 112(1-2), pp. 12-15.

Molander, C., Hongpaisan, J. and Persson, J. K. (1994) 'Distribution of c-fos expressing dorsal horn neurons after electrical stimulation of low threshold sensory fibers in the chronically injured sciatic nerve', *Brain Res*, 644(1), pp. 74-82.

Molander, C., Hongpaisan, J. and Shortland, P. (1998) 'Somatotopic redistribution of c-fos expressing neurons in the superficial dorsal horn after peripheral nerve injury', *Neuroscience*, 84(1), pp. 241-53.

Molander, C., Xu, Q. and Grant, G. (1984) 'The cytoarchitectonic organization of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord', *J.Comp.Neurol.*, 230, pp. 133-141.

Molander, C., Ygge, J. and Dalsgaard, C. J. (1987) 'Substance P-, somatostatin- and calcitonin gene-related peptide-like immunoreactivity and fluoride resistant acid phosphatase-activity in relation to retrogradely labeled cutaneous, muscular and visceral primary sensory neurons in the rat', *Neurosci Lett*, 74(1), pp. 37-42.

Molliver, D. C., Wright, D. E., Leitner, M. L., Parsadanian, A. S., Doster, K., Wen, D., *et al.* (1997) 'IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life', *Neuron*, 19(4), pp. 849-861.

Momin, A., Cadiou, H., Mason, A. and McNaughton, P. A. (2008) 'Role of the hyperpolarization-activated current Ih in somatosensory neurons', *J Physiol*, 586(Pt 24), pp. 5911-29.

Moore, K. A., Kohno, T., Karchewski, L. A., Scholz, J., Baba, H. and Woolf, C. J. (2002) 'Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord', *Journal of Neuroscience,* 22(15), pp. 6724-6731.

Morgan, J. I. and Curran, T. (1986) 'Role of ion flux in the control of c-fos expression', *Nature,* 322(6079), pp. 552-5.

Muralidharan, A., Kuo, A., Jacob, M., Lourdesamy, J. S., Carvalho, L. M. S. P. D., Nicholson, J. R., *et al.* (2016) 'Comparison of Burrowing and Stimuli-Evoked Pain Behaviors as End-Points in Rat Models of Inflammatory Pain and Peripheral Neuropathic Pain', *Frontiers in Behavioral Neuroscience,* 10, pp. 88.

Murray, J. M., Derbyshire, S. and Shields, M. O. (2010) 'Lower limb blocks', *Anaesthesia,* 65 Suppl 1, pp. 57-66.

Nadeau, S., Filali, M., Zhang, J., Kerr, B. J., Rivest, S., Soulet, D., *et al.* (2011) 'Functional recovery after peripheral nerve injury is dependent on the pro-inflammatory cytokines IL-1beta and TNF: implications for neuropathic pain', *J Neurosci,* 31(35), pp. 12533-42.

Nagy, I. and Rang, H. (1999) 'Noxious heat activates all capsaicin-sensitive and also a sub-population of capsaicin-insensitive dorsal root ganglion neurons', *Neuroscience.,* 88(4), pp. 995-997.

Naim, M., Spike, R. C., Watt, C., Shehab, S. A. and Todd, A. J. (1997) 'Cells in laminae III and IV of the rat spinal cord that possess the neurokinin-1 receptor and have dorsally directed dendrites receive a major synaptic input from tachykinin-containing primary afferents', *J Neurosci,* 17(14), pp. 5536-48.

Nakaya, Y., Kaneko, T., Shigemoto, R., Nakanishi, S. and Mizuno, N. (1994) 'Immunohistochemical localization of substance P receptor in the central nervous system of the adult rat', *J Comp Neurol,* 347(2), pp. 249-74.

Nielsch, U., Bisby, M. A. and Keen, P. (1987) 'Effect of cutting or crushing the rat sciatic nerve on synthesis of substance P by isolated L5 dorsal root ganglia', *Neuropeptides,* 10(2), pp. 137-45.

Nilsson, G., Hokfelt, T. and Pernow, B. (1974) 'Distribution of substance P-like immuno-reactivity in the rat central nervous system as revealed by immunohistochemistry', *Med Biol,* 52(6), pp. 424-7.

Noguchi, K., Kawai, Y., Fukuoka, T., Senba, E. and Miki, K. (1995) 'Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons', *Journal of Neuroscience,* 15(11), pp. 7633-7643.

Norris, D. A., Weston, W. L. and Sams, W. M., Jr. (1977) 'The effect of immunosuppressive and anti-inflammatory drugs on monocyte function in vitro', *J Lab Clin Med,* 90(3), pp. 569-80.

Novakovic, S. D., Tzoumaka, E., McGivern, J. G., Haraguchi, M., Sangameswaran, L., Gogas, K. R., *et al.* (1998) 'Distribution of the Tetrodotoxin-Resistant Sodium Channel PN3 in Rat Sensory Neurons in Normal and Neuropathic conditions', *J Neurosci,* 18(6), pp. 2174-2187.

Okamoto, K., Martin, D. P., Schmelzer, J. D., Mitsui, Y. and Low, P. A. (2001) 'Pro- and anti-inflammatory cytokine gene expression in rat sciatic nerve chronic constriction injury model of neuropathic pain', *Exp Neurol,* 169(2), pp. 386-91.

Olausson, H., Cole, J., Rylander, K., McGlone, F., Lamarre, Y., Wallin, B. G., *et al.* (2008) 'Functional role of unmyelinated tactile afferents in human hairy skin: sympathetic response and perceptual localization', *Exp Brain Res,* 184(1), pp. 135-40.

Otsuka, M. and Konishi, S. (1976) 'Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat', *Nature,* 264(5581), pp. 83-4.

Palmeira, C. C., Ashmawi, H. A. and Posso Ide, P. (2011) 'Sex and pain perception and analgesia', *Rev Bras Anestesiol,* 61(6), pp. 814-28.

Pan, H. L., Eisenach, J. C. and Chen, S. R. (1999) 'Gabapentin suppresses ectopic nerve discharges and reverses allodynia in neuropathic rats', *J Pharmacol.Exp.Ther.,* 288(3), pp. 1026-1030.

Pan, R., Tian, Y., Gao, R., Li, H., Zhao, X., Barrett, J. E., *et al.* (2012) 'Central mechanisms of menthol-induced analgesia', *J Pharmacol Exp Ther,* 343(3), pp. 661-72.

Pape, H. C. (1996) 'Queer current and pacemaker: the hyperpolarization-activated cation current in neurons', *Annu Rev Physiol,* 58, pp. 299-327.

Park, S. B., Goldstein, D., Lin, C. S., Krishnan, A. V., Friedlander, M. L. and Kiernan, M. C. (2009a) 'Acute abnormalities of sensory nerve function associated with oxaliplatin-induced neurotoxicity', *J Clin Oncol,* 27(8), pp. 1243-9.

Park, S. B., Lin, C. S., Krishnan, A. V., Goldstein, D., Friedlander, M. L. and Kiernan, M. C. (2009b) 'Oxaliplatin-induced neurotoxicity: changes in axonal excitability precede development of neuropathy', *Brain,* 132(Pt 10), pp. 2712-23.

Paschal, B. M., Shpetner, H. S. and Vallee, R. B. (1987) 'MAP 1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties', *J Cell Biol,* 105(3), pp. 1273-82.

Passmore, G. M., Selyanko, A. A., Mistry, M., Al Qatari, M., Marsh, S. J., Matthews, E. A., *et al.* (2003) 'KCNQ/M currents in sensory neurons: significance for pain therapy', *Journal of Neuroscience,* 23(18), pp. 7227-7236.

Patel, R., Brice, N. L., Lewis, R. J. and Dickenson, A. H. (2015a) 'Ionic mechanisms of spinal neuronal cold hypersensitivity in ciguatera', *The European Journal of Neuroscience,* 42(11), pp. 3004-3011.

Patel, R., Goncalves, L., Newman, R., Jiang, F. L., Goldby, A., Reeve, J., *et al.* (2014) 'Novel TRPM8 antagonist attenuates cold hypersensitivity after peripheral nerve injury in rats', *J Pharmacol Exp Ther,* 349(1), pp. 47-55.

Patel, R., Rutten, K., Valdor, M., Schiene, K., Wigge, S., Schunk, S., *et al.* (2015b) 'Electrophysiological characterization of activation state-dependent Ca(v)2 channel antagonist TROX-1 in spinal nerve injured rats', *Neuroscience,* 297, pp. 47-57.

Payne, C. E., Brown, A. R., Theile, J. W., Loucif, A. J., Alexandrou, A. J., Fuller, M. D., *et al.* (2015) 'A novel selective and orally bioavailable Nav 1.8 channel blocker,

PF-01247324, attenuates nociception and sensory neuron excitability', *Br J Pharmacol,* 172(10), pp. 2654-70.

Pedersen, S. F., Owsianik, G. and Nilius, B. (2005) 'TRP channels: an overview', *Cell Calcium,* 38(3-4), pp. 233-52.

Percie du Sert, N. and Rice, A. S. (2014) 'Improving the translation of analgesic drugs to the clinic: animal models of neuropathic pain', *Br J Pharmacol,* 171(12), pp. 2951-63.

Perl, E. R. (1968) 'Myelinated afferent fibres innervating the primate skin and their response to noxious stimuli', *J.Physiol.Lond.,* 197, pp. 593-615.

Perlson, E., Maday, S., Fu, M. M., Moughamian, A. J. and Holzbaur, E. L. (2010) 'Retrograde axonal transport: pathways to cell death?', *Trends Neurosci,* 33(7), pp. 335-44.

Perry, R. B., Doron-Mandel, E., Iavnilovitch, E., Rishal, I., Dagan, S. Y., Tsoory, M., *et al.* (2012) 'Subcellular knockout of importin beta1 perturbs axonal retrograde signaling', *Neuron,* 75(2), pp. 294-305.

Petitjean, H., Pawlowski, S. A., Fraine, S. L., Sharif, B., Hamad, D., Fatima, T., *et al.* (2015) 'Dorsal Horn Parvalbumin Neurons Are Gate-Keepers of Touch-Evoked Pain after Nerve Injury', *Cell Rep,* 13(6), pp. 1246-1257.

Phuket, T. R. and Covarrubias, M. (2009) 'Kv4 Channels Underlie the Subthreshold-Operating A-type K-current in Nociceptive Dorsal Root Ganglion Neurons', *Front Mol Neurosci,* 2, pp. 3.

Pitcher, G. M. and Henry, J. L. (2008) 'Governing role of primary afferent drive in increased excitation of spinal nociceptive neurons in a model of sciatic neuropathy', *Exp Neurol,* 214(2), pp. 219-28.

Polgar, E., Al-Khater, K. M., Shehab, S., Watanabe, M. and Todd, A. J. (2008) 'Large projection neurons in lamina I of the rat spinal cord that lack the neurokinin 1 receptor are densely innervated by VGLUT2-containing axons and possess GluR4-containing AMPA receptors', *J Neurosci,* 28(49), pp. 13150-60.

Polgar, E., Sardella, T. C., Tiong, S. Y., Locke, S., Watanabe, M. and Todd, A. J. (2013) 'Functional differences between neurochemically defined populations of inhibitory interneurons in the rat spinal dorsal horn', *Pain,* 154(12), pp. 2606-15.

Polgar, E., Sardella, T. C., Watanabe, M. and Todd, A. J. (2011) 'Quantitative study of NPY-expressing GABAergic neurons and axons in rat spinal dorsal horn', *J Comp Neurol,* 519(6), pp. 1007-23.

Polgar, E., Shehab, S. A., Watt, C. and Todd, A. J. (1999) 'GABAergic neurons that contain neuropeptide Y selectively target cells with the neurokinin 1 receptor in laminae III and IV of the rat spinal cord', *J Neurosci,* 19(7), pp. 2637-46.

Polgár, E., Wright, L. L. and Todd, A. J. (2010) 'A quantitative study of brainstem projections from lamina I neurons in the cervical and lumbar enlargement of the rat', *Brain Res,* 1308(5), pp. 58-67.

Polydefkis, M., Yiannoutsos, C. T., Cohen, B. A., Hollander, H., Schifitto, G., Clifford, D. B., *et al.* (2002) 'Reduced intraepidermal nerve fiber density in HIV-associated sensory neuropathy', *Neurology,* 58(1), pp. 115-9.

Postma, T. J., Benard, B. A., Huijgens, P. C., Ossenkoppele, G. J. and Heimans, J. J. (1993) 'Long-term effects of vincristine on the peripheral nervous system', *J Neurooncol,* 15(1), pp. 23-7.

Premkumar, L. S., Raisinghani, M., Pingle, S. C., Long, C. and Pimentel, F. (2005) 'Downregulation of transient receptor potential melastatin 8 by protein kinase C-mediated dephosphorylation', *J Neurosci,* 25(49), pp. 11322-9.

Prendiville, T. W., Ma, Q., Lin, Z., Zhou, P., He, A. and Pu, W. T. (2014) 'Ultrasound-guided transthoracic intramyocardial injection in mice', *J Vis Exp,* (90), pp. e51566.

Price, M. P., Lewin, G. R., McIlwrath, S. L., Cheng, C., Xie, J., Heppenstall, P. A., *et al.* (2000) 'The mammalian sodium channel BNC1 is required for normal touch sensation', *Nature,* 407(6807), pp. 1007-11.

Pritchard, J. and Hughes, R. A. (2004) 'Guillain-Barre syndrome', *Lancet,* 363(9427), pp. 2186-8.

Pulman, K. G., Smith, M., Mengozzi, M., Ghezzi, P. and Dilley, A. (2013) 'The erythropoietin-derived peptide ARA290 reverses mechanical allodynia in the neuritis model', *Neuroscience,* 233, pp. 174-83.

Puskar, Z., Polgar, E. and Todd, A. J. (2001) 'A population of large lamina I projection neurons with selective inhibitory input in rat spinal cord', *Neuroscience,* 102(1), pp. 167-76.

Radhakrishnan, R., Moore, S. A. and Sluka, K. A. (2003) 'Unilateral carrageenan injection into muscle or joint induces chronic bilateral hyperalgesia in rats', *Pain,* 104(3), pp. 567-77.

Ranade, S. S., Qiu, Z., Woo, S. H., Hur, S. S., Murthy, S. E., Cahalan, S. M., *et al.* (2014a) 'Piezo1, a mechanically activated ion channel, is required for vascular development in mice', *Proc Natl Acad Sci U S A,* 111(28), pp. 10347-52.

Ranade, S. S., Woo, S.-H., Dubin, A. E., Moshourab, R. A., Wetzel, C., Petrus, M., *et al.* (2014b) 'Piezo2 is the major transducer of mechanical forces for touch sensation in mice', *Nature,* 516(7529), pp. 121-125.

Randic, M., Jiang, M. C. and Cerne, R. (1993) 'Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord', *J Neurosci,* 13(12), pp. 5228-41.

Rasband, M. N., Park, E. W., Vanderah, T. W., Lai, J., Porreca, F. and Trimmer, J. S. (2001) 'Distinct potassium channels on pain-sensing neurons', *Proceedings of the National Academy of Sciences of the United States of America.,* 98(23), pp. 13373-13378.

Rasmussen, P. V., Sindrup, S. H., Jensen, T. S. and Bach, F. W. (2004) 'Symptoms and signs in patients with suspected neuropathic pain', *Pain,* 110(1-2), pp. 461-9.

Raykova, K., Jones, M. V., Huang, H., Hoffman, P. F. and Levy, M. (2015) 'Minimally-invasive Technique for Injection into Rat Optic Nerve', *Journal of Visualized Experiments : JoVE*, (99), pp. 52249.

Rexed, B. (1952) 'The cytoarchitectonic organization of the spinal cord in the cat', *J Comp Neurol*, 96(3), pp. 414-95.

Rice, A. S., Cimino-Brown, D., Eisenach, J. C., Kontinen, V. K., Lacroix-Fralish, M. L., Machin, I., *et al.* (2008) 'Animal models and the prediction of efficacy in clinical trials of analgesic drugs: a critical appraisal and call for uniform reporting standards', *Pain*, 139(2), pp. 243-7.

Richards, N., Batty, T. and Dilley, A. (2011) 'CCL2 has similar excitatory effects to TNF-alpha in a subgroup of inflamed C-fiber axons', *J Neurophysiol*, 106(6), pp. 2838-48.

Richards, N. and Dilley, A. (2015) 'Contribution of hyperpolarization-activated channels to heat hypersensitivity and ongoing activity in the neuritis model', *Neuroscience*, 284, pp. 87-98.

Ringkamp, M., Schepers, R. J., Shimada, S. G., Johanek, L. M., Hartke, T. V., Borzan, J., *et al.* (2011) 'A role for nociceptive, myelinated nerve fibers in itch sensation', *J Neurosci*, 31(42), pp. 14841-9.

Ro, L. S., Li, H. Y., Huang, K. F. and Chen, S. T. (2004) 'Territorial and extra-territorial distribution of Fos protein in the lumbar spinal dorsal horn neurons in rats with chronic constriction nerve injuries', *Brain Res*, 1004(1-2), pp. 177-87.

Rose, K., Ooi, L., Dalle, C., Robertson, B., Wood, I. C. and Gamper, N. (2011) 'Transcriptional repression of the M channel subunit Kv7.2 in chronic nerve injury', *Pain*, 152(4), pp. 742-54.

Roy, S. (2014) 'Seeing the Unseen – the Hidden world of Slow Axonal Transport', *Neuroscientist*, 20(1), pp. 71-81.

Roy, S., Coffee, P., Smith, G., Liem, R. K., Brady, S. T. and Black, M. M. (2000) 'Neurofilaments are transported rapidly but intermittently in axons: implications for slow axonal transport', *J Neurosci,* 20(18), pp. 6849-61.

Roytta, M., Wei, H. and Pertovaara, A. (1999) 'Spinal nerve ligation-induced neuropathy in the rat: sensory disorders and correlation between histology of the peripheral nerves', *Pain,* 80(1-2), pp. 161-170.

Russell, I. J. (1998) 'Neurochemical pathogenesis of fibromyalgia', *Z Rheumatol,* 57 Suppl 2, pp. 63-6.

Russell, I. J., Vaeroy, H., Javors, M. and Nyberg, F. (1992) 'Cerebrospinal fluid biogenic amine metabolites in fibromyalgia/fibrositis syndrome and rheumatoid arthritis', *Arthritis Rheum,* 35(5), pp. 550-6.

Rutten, K., Robens, A., Read, S. J. and Christoph, T. (2014) 'Pharmacological validation of a refined burrowing paradigm for prediction of analgesic efficacy in a rat model of sub-chronic knee joint inflammation', *Eur J Pain,* 18(2), pp. 213-22.

Sahenk, Z., Barohn, R., New, P. and Mendell, J. R. (1994) 'Taxol neuropathy. Electrodiagnostic and sural nerve biopsy findings', *Arch Neurol,* 51(7), pp. 726-9.

Salas, M. M., Hargreaves, K. M. and Akopian, A. N. (2009) 'TRPA1-mediated responses in trigeminal sensory neurons: interaction between TRPA1 and TRPV1', *Eur J Neurosci,* 29(8), pp. 1568-78.

Sangameswaran, L., Fish, L. M., Koch, B. D., Rabert, D. K., Delgado, S. G., Ilnicka, M., *et al.* (1997) 'A novel tetrodotoxin-sensitive, voltage-gated sodium channel expressed in rat and human dorsal root ganglia', *Journal of Biological Chemistry,* 272(23), pp. 14805-14809.

Sant'Anna, M. B., Kusuda, R., Bozzo, T. A., Bassi, G. S., Alves-Filho, J. C., Cunha, F. Q., *et al.* (2016) 'Medial plantar nerve ligation as a novel model of neuropathic pain in mice: pharmacological and molecular characterization', *Sci Rep,* 6, pp. 26955.

Sapunar, D., Ljubkovic, M., Lirk, P., McCallum, J. B. and Hogan, Q. H. (2005) 'Distinct Membrane Effects of Spinal Nerve Ligation on Injured and Adjacent Dorsal Root Ganglion Neurons in Rats', *Anesthesiology*, 103(2), pp. 360-376.

Saxton, W. M. and Hollenbeck, P. J. (2012) 'The axonal transport of mitochondria', *J Cell Sci*, 125(Pt 9), pp. 2095-104.

Scadding, J. W. (1981) 'Development of ongoing activity, mechanosensitivity, and adrenaline sensitivity in severed peripheral nerve axons', *Exp.Neurol.*, 73(2), pp. 345-364.

Schappacher, K. A., Styczynski, L. and Baccei, M. L. (2017) 'Early life vincristine exposure evokes mechanical pain hypersensitivity in the developing rat', *Pain*, 158(9), pp. 1647-1655.

Schinkel, C., Gaertner, A., Zaspel, J., Zedler, S., Faist, E. and Schuermann, M. (2006) 'Inflammatory mediators are altered in the acute phase of posttraumatic complex regional pain syndrome', *Clin J Pain*, 22(3), pp. 235-9.

Schmidt, R., Schmelz, M., Forster, C., Ringkamp, M., Torebjork, E. and Handwerker, H. (1995) 'Novel classes of responsive and unresponsive C nociceptors in human skin', *Journal of Neuroscience*, 15, pp. 333-341.

Schnorr, S., Eberhardt, M., Kistner, K., Rajab, H., Kasser, J., Hess, A., *et al.* (2014) 'HCN2 channels account for mechanical (but not heat) hyperalgesia during long-standing inflammation', *Pain*, 155(6), pp. 1079-90.

Scholz, J., Broom, D. C., Youn, D. H., Mills, C. D., Kohno, T., Suter, M. R., *et al.* (2005) 'Blocking caspase activity prevents transsynaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury', *J Neurosci*, 25(32), pp. 7317-7323.

Scotland, R. S., Stables, M. J., Madalli, S., Watson, P. and Gilroy, D. W. (2011) 'Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice', *Blood*, 118(22), pp. 5918-27.

Seal, R. P., Wang, X., Guan, Y., Raja, S. N., Woodbury, C. J., Basbaum, A. I., *et al.* (2009) 'Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors', *Nature,* 462(7273), pp. 651-5.

Seltzer, Z., Dubner, R. and Shir, Y. (1990) 'A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury', *Pain,* 43(2), pp. 205-218.

Sheets, P. L., Jackson, J. O., 2nd, Waxman, S. G., Dib-Hajj, S. D. and Cummins, T. R. (2007) 'A Nav1.7 channel mutation associated with hereditary erythromelalgia contributes to neuronal hyperexcitability and displays reduced lidocaine sensitivity', *J Physiol,* 581(Pt 3), pp. 1019-31.

Shim, B., Kim, D. W., Kim, B. H., Nam, T. S., Leem, J. W. and Chung, J. M. (2005) 'Mechanical and heat sensitization of cutaneous nociceptors in rats with experimental peripheral neuropathy', *Neuroscience,* 132(1), pp. 193-201.

Shim, B., Ringkamp, M., Lambrinos, G. L., Hartke, T. V., Griffin, J. W. and Meyer, R. A. (2007) 'Activity-dependent slowing of conduction velocity in uninjured L4 C fibers increases after an L5 spinal nerve injury in the rat', *Pain,* 128(1-2), pp. 40-51.

Shortland, P., Kinman, E. and Molander, C. (1997) 'Sprouting of A-fibre primary afferents into lamina II in two rat models of neuropathic pain', *Eur J Pain,* 1(3), pp. 215-27.

Siau, C. and Bennett, G. J. (2006) 'Dysregulation of cellular calcium homeostasis in chemotherapy-evoked painful peripheral neuropathy', *Anesth Analg,* 102(5), pp. 1485-90.

Siau, C., Xiao, W. and Bennett, G. J. (2006) 'Paclitaxel- and vincristine-evoked painful peripheral neuropathies: loss of epidermal innervation and activation of Langerhans cells', *Exp Neurol,* 201(2), pp. 507-14.

Simone, D. A. and Kajander, K. C. (1997) 'Responses of cutaneous A-fiber nociceptors to noxious cold', *J Neurophysiol,* 77(4), pp. 2049-60.

Sinclair, D. (1981) 'Cutaneous sensation 1980', *Anaesth Intensive Care,* 9(2), pp. 163-73.

Sivilotti, L. G., Thompson, S. W. and Woolf, C. J. (1993) 'Rate of rise of the cumulative depolarization evoked by repetitive stimulation of small-caliber afferents is a predictor of action potential windup in rat spinal neurons in vitro', *J Neurophysiol,* 69(5), pp. 1621-31.

Smith, A. K., O'Hara, C. L. and Stucky, C. L. (2013) 'Mechanical sensitization of cutaneous sensory fibers in the spared nerve injury mouse model', *Mol Pain,* 9, pp. 61.

Snider, W. D. and McMahon, S. B. (1998) 'Tackling pain at the source: new ideas about nociceptors', *Neuron,* 20(4), pp. 629-632.

Sorge, R. E., LaCroix-Fralish, M. L., Tuttle, A. H., Sotocinal, S. G., Austin, J. S., Ritchie, J., *et al.* (2011) 'Spinal cord Toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice', *J Neurosci,* 31(43), pp. 15450-4.

Sorge, R. E., Mapplebeck, J. C., Rosen, S., Beggs, S., Taves, S., Alexander, J. K., *et al.* (2015) 'Different immune cells mediate mechanical pain hypersensitivity in male and female mice', *Nat Neurosci,* 18(8), pp. 1081-3.

Sotgiu, M. L. and Biella, G. (2000) 'Contribution of central sensitization to the pain-related abnormal activity in neuropathic rats', *Somatosens.Motor Res.,* 17(1), pp. 32-38.

Sotocinal, S. G., Sorge, R. E., Zaloum, A., Tuttle, A. H., Martin, L. J., Wieskopf, J. S., *et al.* (2011) 'The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions', *Mol Pain,* 7, pp. 55.

Spike, R. C., Puskar, Z., Andrew, D. and Todd, A. J. (2003) 'A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord', *Eur J Neurosci,* 18(9), pp. 2433-48.

Staff, N. P., Podratz, J. L., Grassner, L., Bader, M., Paz, J., Knight, A. M., *et al.* (2013) 'Bortezomib alters microtubule polymerization and axonal transport in rat dorsal root ganglion neurons', *Neurotoxicology,* 39, pp. 124-31.

Stanfa, L. C., Sullivan, A. F. and Dickenson, A. H. (1992) 'Alterations in neuronal excitability and the potency of spinal mu, delta and kappa opioids after carrageenan-induced inflammation', *Pain,* 50(3), pp. 345-54.

Staud, R., Bovee, C. E., Robinson, M. E. and Price, D. D. (2008) 'Cutaneous C-Fiber Pain Abnormalities of Fibromyalgia Patients are Specifically Related to Temporal Summation', *Pain,* 139(2), pp. 315-323.

Sterling, M., Jull, G., Vicenzino, B. and Kenardy, J. (2003) 'Sensory hypersensitivity occurs soon after whiplash injury and is associated with poor recovery', *Pain,* 104(3), pp. 509-17.

Sterling, M. and Kenardy, J. (2006) 'The relationship between sensory and sympathetic nervous system changes and posttraumatic stress reaction following whiplash injury--a prospective study', *J Psychosom Res,* 60(4), pp. 387-93.

Story, G. M., Peier, A. M., Reeve, A. J., Eid, S. R., Mosbacher, J., Hricik, T. R., *et al.* (2003) 'ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures', *Cell,* 112(6), pp. 819-29.

Suter, M. R., Berta, T., Gao, Y. J., Decosterd, I. and Ji, R. R. (2009) 'Large A-fiber activity is required for microglial proliferation and p38 MAPK activation in the spinal cord: different effects of resiniferatoxin and bupivacaine on spinal microglial changes after spared nerve injury', *Mol Pain,* 5, pp. 53.

Suter, M. R., Siegenthaler, A., Decosterd, I. and Ji, R. R. (2011) 'Perioperative Nerve Blockade: Clues from the Bench', *Anesthesiology Research and Practice,* 2011, pp. 1-12.

Suzuki, R., Matthews, E. A. and 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(1-2), pp. 101-9.

Syeda, R., Florendo, M. N., Cox, C. D., Kefauver, J. M., Santos, J. S., Martinac, B., *et al.* (2016) 'Piezo1 Channels Are Inherently Mechanosensitive', *Cell Rep,* 17(7), pp. 1739-1746.

Tabo, E., Jinks, S. L., Eisele, J. H. and Carstens, E. (1999) 'Behavioral manifestations of neuropathic pain and mechanical allodynia, and changes in spinal dorsal horn neurons, following L4-L6 dorsal root constriction in rats', *Pain,* 80(3), pp. 503-520.

Takaishi, K., Eisele, J. H., Jr. and Carstens, E. (1996) 'Behavioral and electrophysiological assessment of hyperalgesia and changes in dorsal horn responses following partial sciatic nerve ligation in rats', *Pain,* 66(2-3), pp. 297-306.

Takazawa, T. and MacDermott, A. B. (2010) 'Glycinergic and GABAergic tonic inhibition fine tune inhibitory control in regionally distinct subpopulations of dorsal horn neurons', *J Physiol,* 588(Pt 14), pp. 2571-87.

Tal, M. and Bennett, G. J. (1994) 'Extra-territorial pain in rats with a peripheral mononeuropathy: mechano-hyperalgesia and mechano-allodynia in the territory of an uninjured nerve', *Pain,* 57, pp. 375-382.

Tal, M. and Eliav, E. (1996) 'Abnormal discharge originates at the site of nerve injury in experimental constriction neuropathy (CCI) in the rat', *Pain,* 64(3), pp. 511-518.

Talbot, W. H., Darian-Smith, I., Kornhuber, H. H. and Mountcastle, V. B. (1968) 'The sense of flutter-vibration: comparison of the human capacity with response patterns of mechanoreceptive afferents from the monkey hand', *J Neurophysiol,* 31(2), pp. 301-34.

Tall, J. M. and Crisp, T. (2004) 'Effects of gender and gonadal hormones on nociceptive responses to intraplantar carrageenan in the rat', *Neurosci Lett,* 354(3), pp. 239-41.

Tall, J. M., Stuesse, S. L., Cruce, W. L. and Crisp, T. (2001) 'Gender and the behavioral manifestations of neuropathic pain', *Pharmacol Biochem Behav,* 68(1), pp. 99-104.

Tanaka, M., Cummins, T. R., Ishikawa, K., Dib-Hajj, S. D., Black, J. A. and Waxman, S. G. (1998) 'SNS Na+ channel expression increases in dorsal root ganglion neurons in the carrageenan inflammatory pain model', *Neuroreport,* 9(6), pp. 967-972.

Tanner, K. D., Levine, J. D. and Topp, K. S. (1998a) 'Microtubule disorientation and axonal swelling in unmyelinated sensory axons during vincristine-induced painful neuropathy in rat', *J Comp Neurol,* 395(4), pp. 481-92.

Tanner, K. D., Reichling, D. B. and Levine, J. D. (1998b) 'Nociceptor hyper-responsiveness during vincristine-induced painful peripheral neuropathy in the rat', *J Neurosci,* 18(16), pp. 6480-91.

Thibault, K., Rivals, I., M'Dahoma, S., Dubacq, S., Pezet, S. and Calvino, B. (2013) 'Structural and molecular alterations of primary afferent fibres in the spinal dorsal horn in vincristine-induced neuropathy in rat', *J Mol Neurosci,* 51(3), pp. 880-92.

Tiong, S. Y., Polgar, E., van Kralingen, J. C., Watanabe, M. and Todd, A. J. (2011) 'Galanin-immunoreactivity identifies a distinct population of inhibitory interneurons in laminae I-III of the rat spinal cord', *Mol Pain,* 7, pp. 36.

Todd, A. J. (2010) 'Neuronal circuitry for pain processing in the dorsal horn', *Nat Rev Neurosci,* 11(12), pp. 823-36.

Todd, A. J. (2017) 'Identifying functional populations among the interneurons in laminae I-III of the spinal dorsal horn', *Mol Pain,* 13, pp. 1744806917693003.

Todd, A. J., Hughes, D. I., Polgar, E., Nagy, G. G., Mackie, M., Ottersen, O. P., *et al.* (2003) 'The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn', *Eur J Neurosci,* 17(1), pp. 13-27.

Todd, A. J., McGill, M. M. and Shehab, S. A. (2000) 'Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem', *Eur J Neurosci,* 12(2), pp. 689-700.

Todd, A. J., Puskar, Z., Spike, R. C., Hughes, C., Watt, C. and Forrest, L. (2002) 'Projection neurons in lamina I of rat spinal cord with the neurokinin 1 receptor are selectively innervated by substance p-containing afferents and respond to noxious stimulation', *J Neurosci,* 22(10), pp. 4103-13.

Todd, A. J. and Spike, R. C. (1993) 'The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn', *Prog Neurobiol,* 41(5), pp. 609-45.

Todd, A. J., Spike, R. C. and Polgar, E. (1998) 'A quantitative study of neurons which express neurokinin-1 or somatostatin sst2a receptor in rat spinal dorsal horn', *Neuroscience,* 85(2), pp. 459-73.

Topp, K. S., Tanner, K. D. and Levine, J. D. (2000) 'Damage to the cytoskeleton of large diameter sensory neurons and myelinated axons in vincristine-induced painful peripheral neuropathy in the rat', *J.Comp Neurol.,* 424(4), pp. 563-576.

Traub, R. J. (1997) 'Spinal modulation of the induction of central sensitization', *Brain Res,* 778(1), pp. 34-42.

Tsantoulas, C. (2012) 'Sensory Neuron Downregulation of the Kv9.1 Potassium Channel Subunit Mediates Neuropathic Pain following Nerve Injury', 32(48), pp. 17502-13.

Tsantoulas, C., Zhu, L., Yip, P., Grist, J., Michael, G. J. and McMahon, S. B. (2014) 'Kv2 dysfunction after peripheral axotomy enhances sensory neuron responsiveness to sustained input', *Exp Neurol,* 251, pp. 115-26.

Tsuda, M., Kohro, Y., Yano, T., Tsujikawa, T., Kitano, J., Tozaki-Saitoh, H., *et al.* (2011) 'JAK-STAT3 pathway regulates spinal astrocyte proliferation and neuropathic pain maintenance in rats', *Brain,* 134(Pt 4), pp. 1127-39.

Tu, H., Deng, L., Sun, Q., Yao, L., Han, J. S. and Wan, Y. (2004) 'Hyperpolarization-activated, cyclic nucleotide-gated cation channels: roles in the differential electrophysiological properties of rat primary afferent neurons', *J Neurosci Res,* 76(5), pp. 713-22.

Tumati, V., Mathur, S., Song, K., Hsieh, J. T., Zhao, D., Takahashi, M., *et al.* (2013) 'Development of a locally advanced orthotopic prostate tumor model in rats for assessment of combined modality therapy', *Int J Oncol*, 42(5), pp. 1613-9.

Twining, C. M., Sloane, E. M., Milligan, E. D., Chacur, M., Martin, D., Poole, S., *et al.* (2004) 'Peri-sciatic proinflammatory cytokines, reactive oxygen species, and complement induce mirror-image neuropathic pain in rats', *Pain*, 110(1-2), pp. 299-309.

Uceyler, N., Eberle, T., Rolke, R., Birklein, F. and Sommer, C. (2007) 'Differential expression patterns of cytokines in complex regional pain syndrome', *Pain*, 132(1-2), pp. 195-205.

Uceyler, N., Zeller, D., Kahn, A. K., Kewenig, S., Kittel-Schneider, S., Schmid, A., *et al.* (2013) 'Small fibre pathology in patients with fibromyalgia syndrome', *Brain*, 136(Pt 6), pp. 1857-67.

Urch, C. E. and Dickenson, A. H. (2003) 'In vivo single unit extracellular recordings from spinal cord neurones of rats', *Brain Res Brain Res Protoc*, 12(1), pp. 26-34.

Usoskin, D., Furlan, A., Islam, S., Abdo, H., Lonnerberg, P., Lou, D., *et al.* (2015) 'Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing', *Nat Neurosci*, 18(1), pp. 145-53.

Vale, R. D., Reese, T. S. and Sheetz, M. P. (1985) 'Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility', *Cell*, 42(1), pp. 39-50.

van Hecke, O., Austin, S. K., Khan, R. A., Smith, B. H. and Torrance, N. (2014) 'Neuropathic pain in the general population: a systematic review of epidemiological studies', *Pain*, 155(4), pp. 654-62.

Veeramah, K. R., O'Brien, J. E., Meisler, M. H., Cheng, X., Dib-Hajj, S. D., Waxman, S. G., *et al.* (2012) 'De novo pathogenic SCN8A mutation identified by whole-genome sequencing of a family quartet affected by infantile epileptic encephalopathy and SUDEP', *Am J Hum Genet*, 90(3), pp. 502-10.

Vega-Avelaira, D., McKelvey, R., Hathway, G. and Fitzgerald, M. (2012) 'The emergence of adolescent onset pain hypersensitivity following neonatal nerve injury', *Molecular Pain*, 8, pp. 30-30.

Vejsada, R., Paleček, J., Hník, P. and Soukup, T. (1985) 'Postnatal development of conduction velocity and fibre size in the rat tibial nerve', *International Journal of Developmental Neuroscience*, 3(5), pp. 583-589.

Veluchamy, A., Hebert, H. L., Meng, W., Palmer, C. N. A. and Smith, B. H. (2018) 'Systematic review and meta-analysis of genetic risk factors for neuropathic pain', *Pain*, 159(5), pp. 825-848.

Verge, V. M., Gratto, K. A., Karchewski, L. A. and Richardson, P. M. (1996) 'Neurotrophins and nerve injury in the adult', *Philos Trans R Soc Lond B Biol Sci*, 351(1338), pp. 423-30.

Verrills, P., Sinclair, C. and Barnard, A. (2016) 'A review of spinal cord stimulation systems for chronic pain', *J Pain Res*, 9, pp. 481-92.

Verstappen, C. C., Koeppen, S., Heimans, J. J., Huijgens, P. C., Scheulen, M. E., Strumberg, D., *et al.* (2005) 'Dose-related vincristine-induced peripheral neuropathy with unexpected off-therapy worsening', *Neurology*, 64(6), pp. 1076-7.

Vestergaard, S., Tandrup, T. and Jakobsen, J. (1997) 'Effect of permanent axotomy on number and volume of dorsal root ganglion cell bodies', *J.Comp.Neurol.*, 388(2), pp. 307-312.

Vicuna, L., Strochlic, D. E., Latremoliere, A., Bali, K. K., Simonetti, M., Husainie, D., *et al.* (2015) 'The serine protease inhibitor SerpinA3N attenuates neuropathic pain by inhibiting T cell-derived leukocyte elastase', *Nat Med*, 21(5), pp. 518-23.

Villar, M. J., Wiesenfeld-Hallin, Z., Xu, X. J., Theodorsson, E., Emson, P. C. and Hokfelt, T. (1991) 'Further studies on galanin-, substance P-, and CGRP-like immunoreactivities in primary sensory neurons and spinal cord: effects of dorsal rhizotomies and sciatic nerve lesions', *Exp Neurol*, 112(1), pp. 29-39.

von Euler, U. S. and Gaddum, J. H. (1931) 'An unidentified depressor substance in certain tissue extracts', *J Physiol,* 72(1), pp. 74-87.

Vos, B. P., Strassman, A. M. and Maciewicz, R. J. (1994) 'Behavioral evidence of trigeminal neuropathic pain following chronic constriction injury to the rat's infraorbital nerve', *Journal of Neuroscience,* 14(5 Pt 1), pp. 2708-2723.

Waddell, P. J., Lawson, S. N. and McCarthy, P. W. (1989) 'Conduction velocity changes along the processes of rat primary sensory neurons', *Neuroscience,* 30(3), pp. 577-84.

Wagman, I. H. and Price, D. D. (1969) 'Responses of dorsal horn cells of M. mulatta to cutaneous and sural nerve A and C fiber stimuli', *J Neurophysiol,* 32(6), pp. 803-17.

Wahl-Schott, C. and Biel, M. (2009) 'HCN channels: structure, cellular regulation and physiological function', *Cell Mol Life Sci,* 66(3), pp. 470-94.

Wainger, B. J., DeGennaro, M., Santoro, B., Siegelbaum, S. A. and Tibbs, G. R. (2001) 'Molecular mechanism of cAMP modulation of HCN pacemaker channels', *Nature,* 411(6839), pp. 805-10.

Waldmann, R., Champigny, G., Bassilana, F., Heurteaux, C. and Lazdunski, M. (1997) 'A proton-gated cation channel involved in acid-sensing', *Nature,* 386(6621), pp. 173-7.

Walker, S. M., Tochiki, K. K. and Fitzgerald, M. (2009) 'Hindpaw incision in early life increases the hyperalgesic response to repeat surgical injury: critical period and dependence on initial afferent activity', *Pain,* 147(1-3), pp. 99-106.

Wall, P. D. and Devor, M. (1983) 'Sensory afferent impulses originate from dorsal root ganglia as well as from the periphery in normal and nerve injured rats', *Pain,* 17, pp. 321-339.

Wall, P. D., Devor, M., Inbal, R., Scadding, J. W., Schonfeld, D., Seltzer, Z., *et al.* (1979) 'Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa', *Pain,* 7(2), pp. 103-11.

Wallace, V. C. J., Blackbeard, J., Pheby, T., Segerdahl, A. R., Davies, M., Hasnie, F., *et al.* (2007) 'Pharmacological, behavioural and mechanistic analysis of HIV-1 gp120 induced painful neuropathy', *Pain,* 133(1-3), pp. 47-63.

Wang, L. and Brown, A. (2001) 'Rapid Intermittent Movement of Axonal Neurofilaments Observed by Fluorescence Photobleaching', *Mol Biol Cell,* 12(10), pp. 3257-67.

Wang, L. and Brown, A. (2002) 'Rapid movement of microtubules in axons', *Current Biology,* 12(17), pp. 1496-1501.

Wang, L., Ho, C. L., Sun, D., Liem, R. K. and Brown, A. (2000) 'Rapid movement of axonal neurofilaments interrupted by prolonged pauses', *Nat Cell Biol,* 2(3), pp. 137-41.

Watson, C. P., Deck, J. H., Morshead, C., Van der Kooy, D. and Evans, R. J. (1991) 'Post-herpetic neuralgia: further post-mortem studies of cases with and without pain', *Pain,* 44(2), pp. 105-17.

Welk, E., Leah, J. D. and Zimmermann, M. (1990) 'Characteristics of A- and C-fibers ending in a sensory nerve neuroma in the rat', *J Neurophysiol.,* 63(4), pp. 759-766.

Wemmie, J. A., Taugher, R. J. and Kreple, C. J. (2013) 'Acid-sensing ion channels in pain and disease', *Nature reviews. Neuroscience,* 14(7), pp. 461-471.

Weng, H. R., Cordella, J. V. and Dougherty, P. M. (2003) 'Changes in sensory processing in the spinal dorsal horn accompany vincristine-induced hyperalgesia and allodynia', *Pain,* 103(1-2), pp. 131-8.

White, D. M. (1997) 'Release of substance P from peripheral sensory nerve terminals', *J Peripher Nerv Syst,* 2(3), pp. 191-201.

Willis, W. D. and Coggeshall, R. E. (1991) *Sensory Mechanisms of the Spinal Cord.* New York: Plenum Press.

Wodarski, R., Delaney, A., Ultenius, C., Morland, R., Andrews, N., Baastrup, C., *et al.* (2016) 'Cross-centre replication of suppressed burrowing behaviour as an

ethologically relevant pain outcome measure in the rat: a prospective multicentre study', *Pain*, 157(10), pp. 2350-2365.

Wolfe, F., Clauw, D. J., Fitzcharles, M. A., Goldenberg, D. L., Hauser, W., Katz, R. S., *et al.* (2011) 'Fibromyalgia criteria and severity scales for clinical and epidemiological studies: a modification of the ACR Preliminary Diagnostic Criteria for Fibromyalgia', *J Rheumatol*, 38(6), pp. 1113-22.

Wolfe, F., Ross, K., Anderson, J. and Russell, I. J. (1995) 'Aspects of fibromyalgia in the general population: sex, pain threshold, and fibromyalgia symptoms', *J Rheumatol*, 22(1), pp. 151-6.

Woo, S. H., Lukacs, V., de Nooij, J. C., Zaytseva, D., Criddle, C. R., Francisco, A., *et al.* (2015) 'Piezo2 is the principal mechanotransduction channel for proprioception', *Nat Neurosci*, 18(12), pp. 1756-62.

Woo, S. H., Ranade, S., Weyer, A. D., Dubin, A. E., Baba, Y., Qiu, Z., *et al.* (2014) 'Piezo2 is required for Merkel-cell mechanotransduction', *Nature*, 509(7502), pp. 622-6.

Woodbury, C. J. and Koerber, H. R. (2007) 'Central and peripheral anatomy of slowly adapting type I low-threshold mechanoreceptors innervating trunk skin of neonatal mice', *J Comp Neurol*, 505(5), pp. 547-61.

Woodbury, C. J., Kullmann, F. A., McIlwrath, S. L. and Koerber, H. R. (2008) 'Identity of Myelinated Cutaneous Sensory Neurons Projecting to Nocireceptive Laminae Following Nerve Injury in Adult Mice', *The Journal of comparative neurology*, 508(3), pp. 500-509.

Woolf, C. J. (1996) 'Windup and central sensitization are not equivalent', *Pain*, 66(2-3), pp. 105-108.

Woolf, C. J. (2011) 'Central sensitization: implications for the diagnosis and treatment of pain', *Pain*, 152(3 Suppl), pp. S2-15.

Woolf, C. J. (2014) 'What to call the amplification of nociceptive signals in the central nervous system that contribute to widespread pain?', *Pain,* 155(10), pp. 1911-2.

Woolf, C. J., Shortland, P. and Coggeshall, R. E. (1992) 'Peripheral nerve injury triggers central sprouting of myelinated afferents', *Nature,* 355, pp. 75-78.

Woolf, C. J. and 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(3), pp. 293-9.

Wu, G., Ringkamp, M., Hartke, T. V., Murinson, B. B., Campbell, J. N., Griffin, J. W., *et al.* (2001) 'Early onset of spontaneous activity in uninjured C-fiber nociceptors after injury to neighboring nerve fibers', *J.Neurosci.,* 21(8), pp. RC140.

Wu, J., Lewis, A. and Grandl, J. (2017) 'Touch, Tension, and Transduction – the Function and Regulation of Piezo Ion Channels', *Trends in biochemical sciences,* 42(1), pp. 57-71.

Xiao, W. H. and Bennett, G. J. (2007) 'Persistent low-frequency spontaneous discharge in A-fiber and C-fiber primary afferent neurons during an inflammatory pain condition', *Anesthesiology,* 107(5), pp. 813-21.

Xiao, W. H. and Bennett, G. J. (2008) 'Chemotherapy-evoked neuropathic pain: Abnormal spontaneous discharge in A-fiber and C-fiber primary afferent neurons and its suppression by acetyl-L-carnitine', *Pain,* 135(3), pp. 262-70.

Xie, W., Strong, J. A., Meij, J. T., Zhang, J. M. and Yu, L. (2005) 'Neuropathic pain: early spontaneous afferent activity is the trigger', *Pain,* 116(3), pp. 243-256.

Xie, Y. K. and Xiao, W. H. (1990) 'Electrophysiological evidence for hyperalgesia in the peripheral neuropathy', *Sci China B,* 33(6), pp. 663-72.

Xu, X. J., Zhang, X., Hokfelt, T. and Wiesenfeld-Hallin, Z. (1995) 'Plasticity in spinal nociception after peripheral nerve section: reduced effectiveness of the

NMDA receptor antagonist MK-801 in blocking wind-up and central sensitization of the flexor reflex', *Brain Res*, 670(2), pp. 342-6.

Xu, Z. Z., Kim, Y. H., Bang, S., Zhang, Y., Berta, T., Wang, F., *et al.* (2015) 'Inhibition of mechanical allodynia in neuropathic pain by TLR5-mediated A-fiber blockade', *Nat Med,* 21(11), pp. 1326-31.

Yagasaki, Y., Hayashi, M., Tamura, N. and Kawakami, Y. (2013) 'Gamma Knife Irradiation of Injured Sciatic Nerve Induces Histological and Behavioral Improvement in the Rat Neuropathic Pain Model', *PLoS ONE,* 8(4), pp. e61010.

Yamamoto, T. and Yaksh, T. L. (1993) 'Effects of colchicine applied to the peripheral nerve on the thermal hyperalgesia evoked with chronic nerve constriction', *Pain,* 55(2), pp. 227-233.

Yang, Y., Wang, Y., Li, S., Xu, Z., Li, H., Ma, L., *et al.* (2004) 'Mutations in SCN9A, encoding a sodium channel alpha subunit, in patients with primary erythermalgia', *J Med Genet,* 41(3), pp. 171-4.

Yasaka, T., Tiong, S. Y., Polgar, E., Watanabe, M., Kumamoto, E., Riddell, J. S., *et al.* (2014) 'A putative relay circuit providing low-threshold mechanoreceptive input to lamina I projection neurons via vertical cells in lamina II of the rat dorsal horn', *Mol Pain,* 10, pp. 3.

Yonekawa, Y., Harada, A., Okada, Y., Funakoshi, T., Kanai, Y., Takei, Y., *et al.* (1998) 'Defect in synaptic vesicle precursor transport and neuronal cell death in KIF1A motor protein-deficient mice', *J Cell Biol,* 141(2), pp. 431-41.

Young, G. T., Emery, E. C., Mooney, E. R., Tsantoulas, C. and McNaughton, P. A. (2014) 'Inflammatory and neuropathic pain are rapidly suppressed by peripheral block of hyperpolarisation-activated cyclic nucleotide-gated ion channels', *Pain,* 155(9), pp. 1708-19.

Yu, Y., Chen, Z., Li, W. G., Cao, H., Feng, E. G., Yu, F., *et al.* (2010) 'A nonproton ligand sensor in the acid-sensing ion channel', *Neuron,* 68(1), pp. 61-72.

Zarpelon, A. C., Rodrigues, F. C., Lopes, A. H., Souza, G. R., Carvalho, T. T., Pinto, L. G., *et al.* (2016) 'Spinal cord oligodendrocyte-derived alarmin IL-33 mediates neuropathic pain', *FASEB J*, 30(1), pp. 54-65.

Zeltser, R., Beilin, B., Zaslansky, R. and Seltzer, Z. (2000) 'Comparison of autotomy behavior induced in rats by various clinically-used neurectomy methods', *Pain*, 89(1), pp. 19-24.

Zhang, E. T., Han, Z. S. and Craig, A. D. (1996) 'Morphological classes of spinothalamic lamina I neurons in the cat', *J Comp Neurol*, 367(4), pp. 537-49.

Zhang, W., Liu, L. Y. and Xu, T. L. (2008) 'Reduced potassium-chloride co-transporter expression in spinal cord dorsal horn neurons contributes to inflammatory pain hypersensitivity in rats', *Neuroscience*, 152(2), pp. 502-10.

Zhang, X., Chen, Y., Wang, C. and Huang, L. Y. (2007) 'Neuronal somatic ATP release triggers neuron-satellite glial cell communication in dorsal root ganglia', *Proc Natl Acad Sci U S A*, 104(23), pp. 9864-9.

Zhang, X. Y., Wen, J., Yang, W., Wang, C., Gao, L., Zheng, L. H., *et al.* (2013) 'Gain-of-function mutations in SCN11A cause familial episodic pain', *Am J Hum Genet*, 93(5), pp. 957-66.

Zheng, Q., Fang, D., Liu, M., Cai, J., Wan, Y., Han, J. S., *et al.* (2013) 'Suppression of KCNQ/M (Kv7) potassium channels in dorsal root ganglion neurons contributes to the development of bone cancer pain in a rat model', *Pain*, 154(3), pp. 434-48.

Zhuo, H., Lewin, A. C., Phillips, E. T., Sinclair, C. M. and Helke, C. J. (1995) 'Inhibition of axoplasmic transport in the rat vagus nerve alters the numbers of neuropeptide and tyrosine hydroxylase messenger RNA-containing and immunoreactive visceral afferent neurons of the nodose ganglion', *Neuroscience*, 66(1), pp. 175-187.

Zimmerman, A., Bai, L. and Ginty, D. D. (2014) 'The gentle touch receptors of mammalian skin', *Science*, 346(6212), pp. 950-4.

Zorina-Lichtenwalter, K., Parisien, M. and Diatchenko, L. (2018) 'Genetic studies of human neuropathic pain conditions: a review', *Pain*, 159(3), pp. 583-594.

Publications

1) Satkeviciute I, Goodwin G, Bove GM, & Dilley A (2018). Time course of ongoing activity during neuritis and following axonal transport disruption. J Neurophysiol 119: 1993-2000.

Abstract

Local nerve inflammation (neuritis) leads to ongoing activity and axonal mechanical sensitivity (AMS) along intact nociceptor axons and disrupts axonal transport. This phenomenon forms the most feasible cause of radiating pain, such as sciatica. We have previously shown that axonal transport disruption without inflammation or degeneration also leads to AMS but does not cause ongoing activity at the time point when AMS occurs, despite causing cutaneous hypersensitivity. However, there have been no systematic studies of ongoing activity during neuritis or noninflammatory axonal transport disruption. In this study, we present the time course of ongoing activity from primary sensory neurons following neuritis and vinblastine-induced axonal transport disruption. Whereas 24% of C/slow Aδ-fiber neurons had ongoing activity during neuritis, few (<10%) A- and C-fiber neurons showed ongoing activity 1-15 days following vinblastine treatment. In contrast, AMS increased transiently at the vinblastine treatment site, peaking on days 4-5 (28% of C/slow Aδ-fiber neurons) and resolved by day 15. Conduction velocities were slowed in all groups. In summary, the disruption of axonal transport without inflammation does not lead to ongoing activity in sensory neurons, including nociceptors, but does cause a rapid and transient development of AMS. Because it is proposed that AMS underlies mechanically induced radiating pain, and a transient disruption of axonal transport (as previously reported) leads to transient AMS, it follows that processes that disrupt axonal transport, such as neuritis, must persist to maintain AMS and the associated symptoms. New & Noteworthy: Many patients with radiating pain lack signs of nerve injury on clinical examination but may have neuritis, which disrupts axonal transport. We have shown that axonal transport disruption does not induce ongoing activity in primary sensory neurons but does cause transient axonal mechanical sensitivity. The present data complete a profile of key axonal sensitivities following axonal transport disruption. Collectively, this profile supports that an active peripheral process is necessary for maintained axonal sensitivities.

2) Satkeviciute I, Dilley A. [EXPRESS] Neuritis and vinblastine-induced axonal transport disruption lead to signs of altered dorsal horn excitability. Mol Pain 2018:1744806918799581

Abstract

Background: Many patients with neuropathic pain present without signs of nerve injury on routine clinical examination. Some of these patients may have inflamed peripheral nerves (neuritis). In this study, we have examined whether neuritis causes changes within the dorsal horn that may contribute to a central pain mechanism. Comparisons have been made to a model of axonal transport disruption induced using vinblastine, since neuritis disrupts such processes.

Results: At the peak of cutaneous hypersensitivities, recordings from deep wide dynamic range (WDR) neurons revealed increases in wind-up following neuritis but not vinblastine treatment. Ongoing activity from these neurons was unchanged. Vinblastine treatment caused a reduction in the responses of WDR neurons to noxious mechanical stimulation of the receptive field. The response of neurons to innocuous mechanical stimulation was also reduced in WDR neurons that were at a depth ≥550 μm following vinblastine treatment. An examination of the superficial dorsal horn revealed an increase in c-Fos-positive neurons in both groups following electrical stimulation of the sciatic nerve. The area of dorsal horn expressing substance P was also decreased following vinblastine treatment.

Conclusion: These findings indicate that a minor nerve insult, such as neuritis, can lead to changes within the dorsal horn that are consistent with a central neuropathic pain mechanism.

Appendix:

Ultrasound-guided injections

Introduction

The majority of rat and mouse models of neuropathic pain are surgically induced, which is necessary to expose peripheral nerves that are then cut or ligated. This leads to extensive axonal degeneration. The most commonly used surgical models are the spinal nerve ligation (Kim and Chung, 1992), spared nerve injury (Decosterd and Woolf, 2000) and chronic constriction injury models (Bennett and Xie, 1988). The surgery required to induce these models can be extensive. For example, induction of the spinal nerve ligation model involves removing part of the back muscles and vertebrae. These models have significant adverse effects on the welfare of the animals. In addition to pain, animals may suffer weakness, paralysis and weight loss (Kim and Chung, 1992, Decosterd and Woolf, 2000). Foot deformities frequently occur and animals can develop abnormal postures and gait (Kim and Chung, 1992, Attal *et al.*, 1990). Grooming behaviour can also be affected (Vos *et al.*, 1994), and animals can show signs of autotomy (Zeltser *et al*., 2000). Postoperative pain is a significant issue in nerve injury models, and many of the adverse effects associated with extensive tissue damage are not reversible. As such, there is a need for techniques that could be used to induce animal models of neuropathic pain without the need for extensive tissue damage. Potentially, chemicals could be delivered around peripheral nerves using direct injections.

Direct injections of local anaesthetics are routinely applied around the sciatic nerve of an animal without physical exposure. During such experiments, laboratory animals are commonly under inhalation aesthesia when a drug (~ 100 – 300 µl) is injected using a 23-30 gauge needle. Injections can be performed at the sciatic notch of the hind limb (Gerner *et al.*, 2008, Kohane *et al.*, 1998, Walker *et al.*, 2009) or at a point between the greater trochanter and the knee just posterior to the femur (Hu *et*

al., 1997). The effectiveness of nerve block is commonly assessed by ipsilateral motor block and loss of withdrawal reflex response. However, if substances that do not have such effect on neurons were to be delivered, the effectiveness of delivery could not be easily assessed. In such cases, the ability to visualise agent delivery may be useful. One method that can visually confirm agent delivery to the nerve is injection of an agent in combination with a dye (e.g. methyl blue). As such, the nerve tissue exposed to the delivered agent changes colour confirming successful delivery (Raykova *et al.*, 2015). The downfall of such procedure is the need of an open surgery and use of different equipment (e.g. microscopes). Delivery of an agent-dye mixture to the nerve can also be confirmed following a gross nerve dissection (Campoy *et al.*, 2008). However, this requires a terminal procedure and is not indicative of a successful agent delivery at the time of initial procedure. Currently, ultrasound-guided injections are widely used by anaesthesiologists to induce nerve block (regional anaesthesia) in humans (reviewed in Murray *et al.*, 2010). Although such injections of substances around the nerve have not been reported in rodents, this technique has been successfully used for other experimental procedures such as cancer induction (Tumati *et al.*, 2013), intramyocardial delivery of substances (Prendiville *et al.*, 2014) and transfer of genes *in utero* (Henriques-Coelho *et al.*, 2007). Furthermore, ultrasound-guided sciatic nerve block in dogs has been successfully performed (Echeverry *et al.*, 2010). In this study, medical ultrasound has been used as a tool to guide injection of CFA and vinblastine around rat nerves as a method for inducing models of neuropathic pain. In line with the 3Rs, this approach provides a refined alternative to surgery and will avoid the need to cut nerves.

Aims

- To investigate whether ultrasound-guided injections of vinblastine or CFA around the sciatic nerve induce mechanical and cold allodynia in rats.

Methods

In a group of animals (n = 19), ultrasound-guided injections of vinblastine (5.5 mM, 11 mM, 100 mM, 120 mM, 150 mM; n = 9), CFA (n = 7) and 0.9% w/v saline (n = 2) were made around the left sciatic nerve (Appendix Figure 1). Under general isoflurane anaesthesia, the sciatic nerve in the thigh was imaged using a SonoSite M-Turbo ultrasound unit (SonoSite, Lonodn, UK) with a 15 MHz linear array transducer. When a clear view of the sciatic nerve was achieved under ultrasound guidance, a hypodermic needle (19-30G; 19G: CFA; 23G: vinblastine; 25-30G: initial studies on vinblastine) fitted with a 1 ml syringe was inserted into the thigh between the myofascial planes of the hamstring muscles. Once the tip could be visualized approaching the nerve trunk, a 150 µl bolus of agent (vinblastine or CFA) was slowly injected around the nerve. The animal was allowed to recover on a heat mat and was transferred to a clean cage. Monitoring of adverse effects and signs of stress was performed as described earlier (see section 2.1.1).

Animals were tested for signs of neuropathic pain behaviours including mechanical and cold allodynia. For detailed information regarding the methods used to test such behaviours please refer to section 2.2. For experimental timeline see Appendix Figure 2. Briefly, baseline behavioural values were obtained on three separate days prior to ultrasound-guided injection and further behavioural testing was performed for up to six days post-ultrasound-guided injection. Note that animals were not tested at exactly same days post-injection. This varied between experiments. In six animals

(three in each vinblastine and CFA groups), the delivery of the agent to the nerve failed. Such animals were removed from analysis.

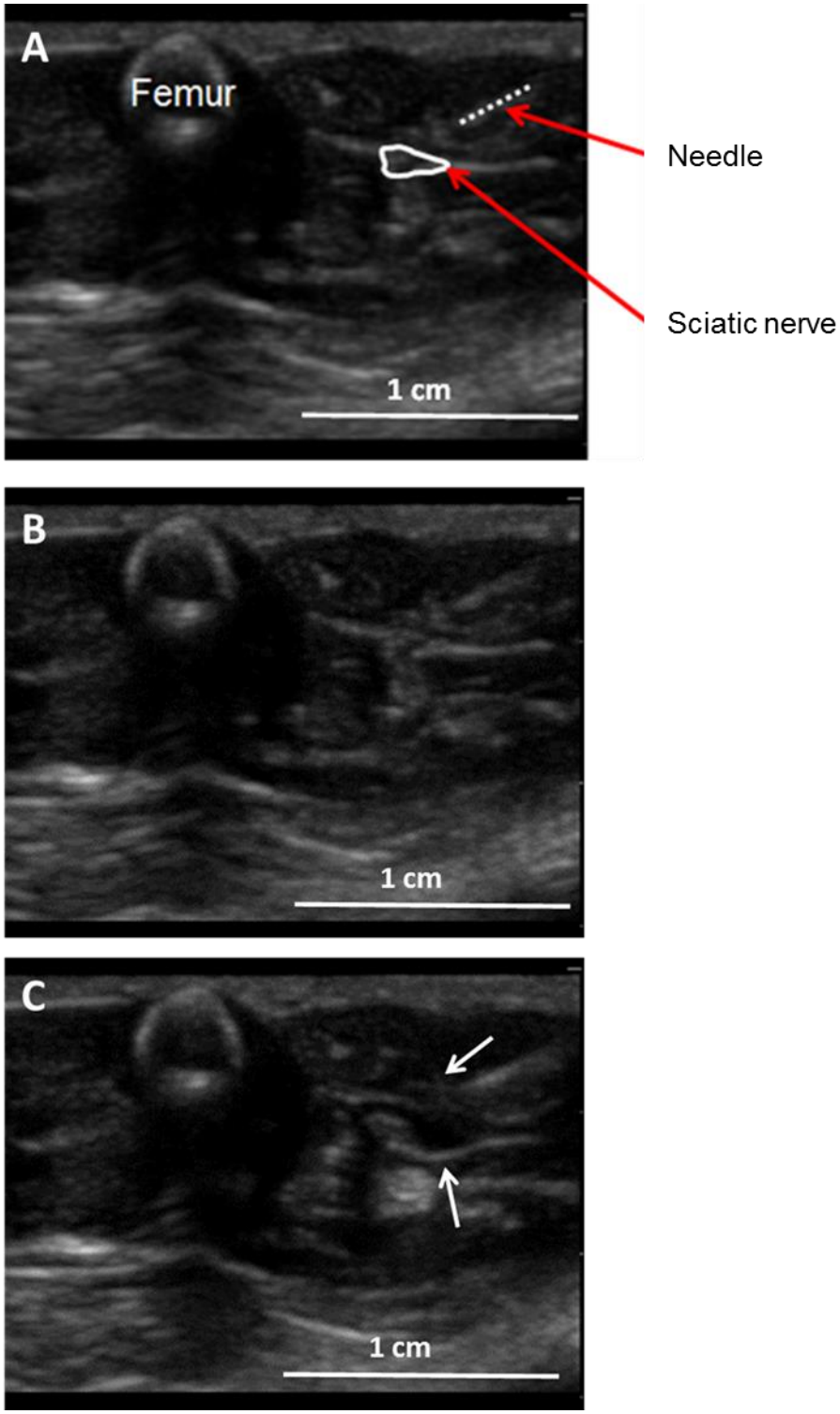
Results

Mechanical allodynia

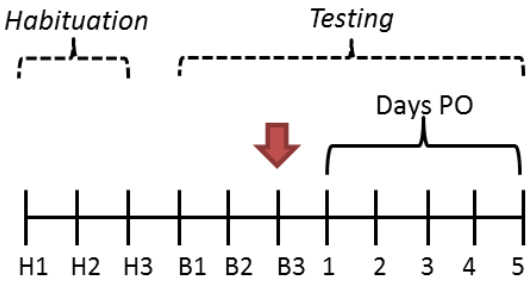
The mean baseline 50% paw withdrawal threshold was 14.55 ± 0.45 g in the vinblastine-treated group (n = 4), which remained unchanged following ultrasound-guided injection (Appendix Figure 3A). In the neuritis group, the mean baseline 50% paw withdrawal threshold was 15 g (n = 5), which reached a maximum reduction on day one post-injection (mean 8.88 ± 1.15 g; Appendix Figure 3B). In case of saline-treated animal, the baseline value was 15 g (n = 1) and no signs of mechanical allodynia were observed (Appendix Figure 3C).

Cold allodynia

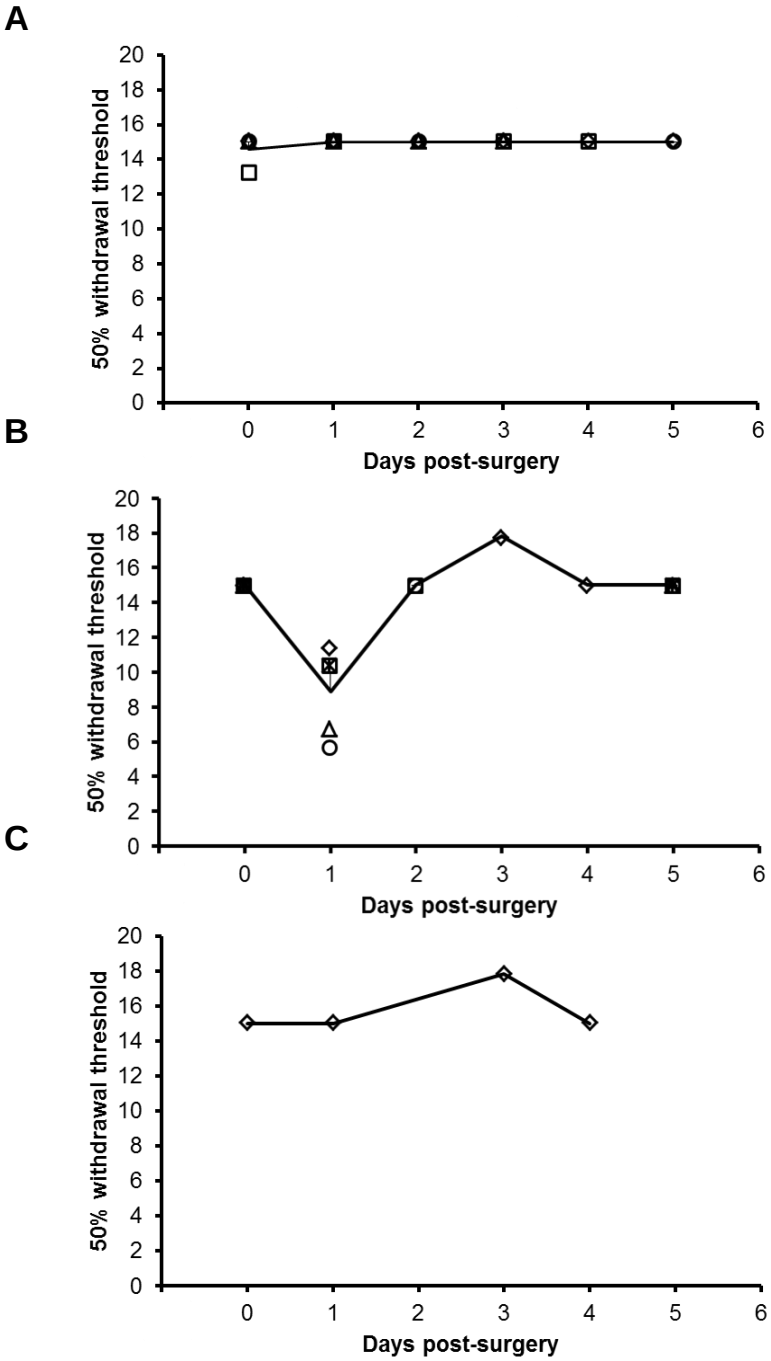
The mean baseline percent paw withdrawal frequency was 1.67 ± 1.67% in the vinblastine-treated group (n = 6), with the peak of behaviours observed on day two post-injection (mean 33.33 ± 17.64%; Appendix Figure 4A). The mean baseline percent withdrawal was 4 ± 4% in the neuritis group (n = 5), with no obvious changes present following ultrasound-guided injection (Appendix Figure 4B). In case of saline-treated animals, the mean baseline value was 3.33 ± 3.33 % (n = 2). On day 3 post-injection, the mean percent paw withdrawal frequency was 28.28 ± 20% (Appendix Figure 4C).



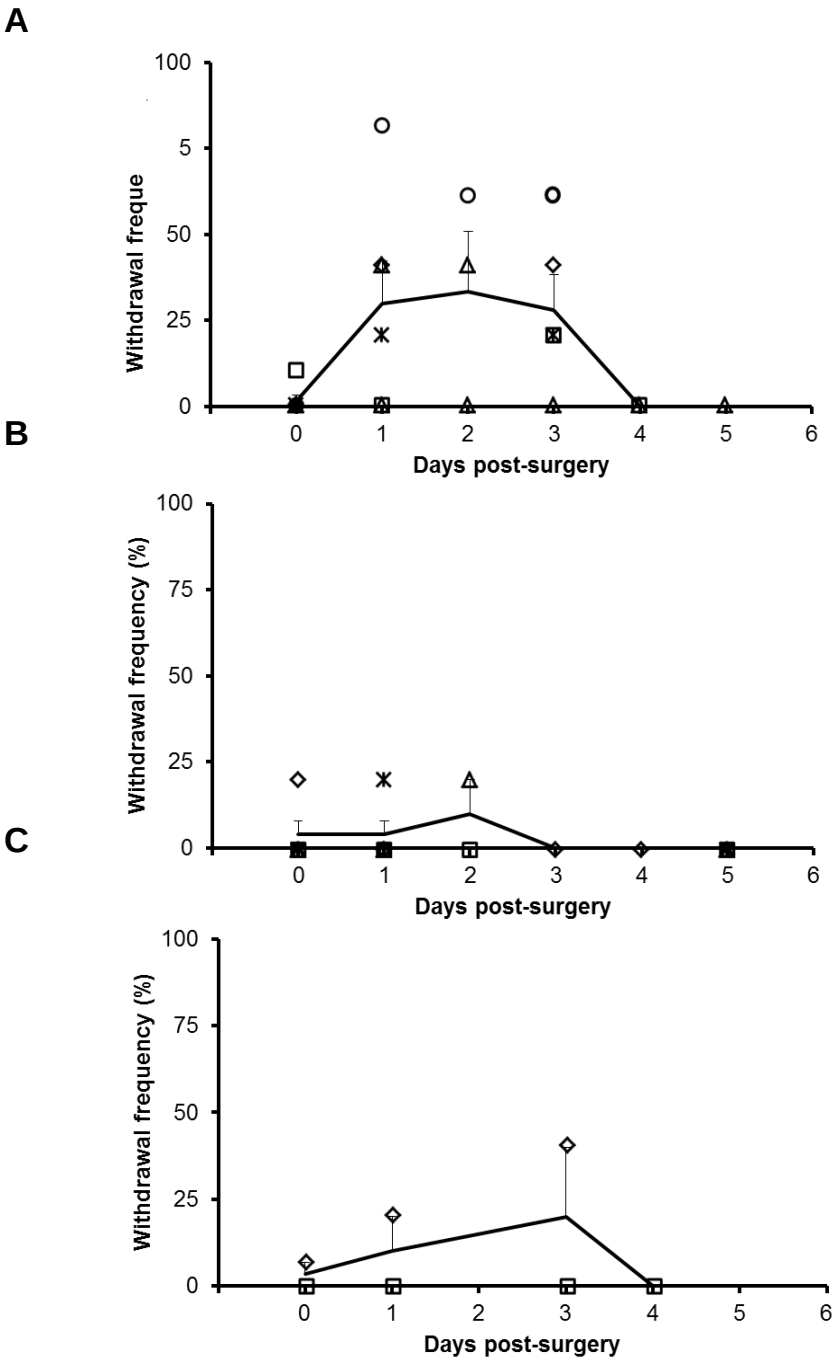
Appendix Figure 1 High-frequency ultrasound-guided injections of vinblastine, CFA or saline around the sciatic nerve of the hind limb. A) A schematic representation of the injection. The femur is used as a landmark to trace the sciatic nerve; B) Prior to injection: the nerve is being approached by the needle; C) After injection: a pool of liquid can be seen around the sciatic nerve (indicated by white arrows).



Appendix Figure 2 Timeline of behavioural testing procedures: mechanical and cold allodynia following ultrasound-guided injections of substances (vinblastine, CFA or saline) around the sciatic nerve. Please note that the red arrow indicates the day of injection following B3 testing. H, habituation; B, baseline testing; PO, post-operative (post-i.p. injection).



Appendix Figure 3 Ipsilateral development of mechanical allodynia following ultrasound-guided injections of (A) vinblastine, (B) CFA or (C) saline. Raw data for each animal is represented by a different symbol. The mean values for each treatment group are represented by solid black lines. Total number of animals was 4, 5 and 1 in vinblastine-treated, neuritis and saline treated groups respectively. Note that the mean number of animals varies at each time point, since animals were tested on different days post-injection. Error bars = SEM.



Appendix Figure 4 Ipsilateral development of cold allodynia following ultrasound-guided injections of (A) vinblastine, (B) CFA or (C) saline. Raw data for each animal is represented by a different symbol. The mean values for each treatment group are represented by solid black lines. Total number of animals was 6, 5 and 2 in vinblastine-treated, neuritis and saline treated groups respectively. Note that the mean number of animals varies at each time point, since animals were tested on different days post-injection. Error bars = SEM.

Summary of findings

- Ultrasound-guided injection of vinblastine or CFA around the sciatic nerve induced signs of neuropathic pain.
- A substantial variability between animals was observed post substance injection in all treatment groups, especially when testing for cold allodynia.