

**Temporal expression of neurochemical
markers in primary sensory neurones in
traumatic and non-traumatic models of
neuropathic pain**

**Thesis presented for the degree of Philosophy in Imperial
College London**

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I certify that the work and content presented in this thesis is my own and that where applicable I have acknowledged all material and sources including research articles and books used in its preparation.

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Abstract

Introduction: Expression of neurochemical markers in dorsal root ganglion (DRG) has been shown to underlie nerve injury induced neuropathic pain and are usually examined once post injury in rodent models. Here I hypothesize that neurochemical markers are expressed differentially at various time points post nerve injury and that expression of these markers differ between traumatic and non-traumatic models of neuropathic pain, thus reflecting variations in pathophysiology.

In two different rat models of neuropathic pain, namely, the tibial nerve transection (TNT) and the d4T (HIV antiretroviral drug) associated toxic neuropathy; immunohistochemical labelling was used to determine the expression of various neurochemical markers across time. Double labelling with peripherin or NF-200 (labels small and large neurons respectively) and a cell size analysis were conducted to validate cellular phenotype. Pain behaviour was measured *in vivo* using mechanical and thermal hypersensitivity assays.

To confirm tibial nerve distribution in the DRGs, Fluorogold retrograde labelling was performed. I also explored the effect of the omega-3 polyunsaturated fatty acid docosahexonoic acid (DHA) on TNT associated mechanical hypersensitivity and inflammation, as previous studies have shown DHA to be both anti-inflammatory and neuroprotective in animal models of spinal cord injury and stroke, and therefore could be relevant in peripheral nerve injury.

Results:

% expression in TNT injured rats ($\pm$ standard error of mean)					
Markers	Day1	Day 7	Day 14	Day 28	Day 43
<u>Activating transcription factor -3</u>					
Peripherin	11.9 $\pm$ 5.6	19.0 $\pm$ 4.9	18.9 $\pm$ 6.6	15.2 $\pm$ 1.4	25.4 $\pm$ 8.3
NF-200	71.1 $\pm$ 7.2	25.7 $\pm$ 7.7	30.4 $\pm$ 8.7	18.7 $\pm$ 6.0	11.5 $\pm$ 4.2
<u>Gap associated protein-43</u>					
Peripherin	3.8 $\pm$ 1.4	7.2 $\pm$ 0.7	15.7 $\pm$ 1.8	11.6 $\pm$ 2.5	10.0 $\pm$ 6.4
NF-200	3.2 $\pm$ 2.1	0.3 $\pm$ 0.3	15.9 $\pm$ 4.8	10.0 $\pm$ 1.9	8.3 $\pm$ 1.0
<u>Neuropeptide Y</u>					
Peripherin	0	2.1 $\pm$ 0.6	3.4 $\pm$ 1.4	6.5 $\pm$ 0.8	5.0 $\pm$ 0.8
NF-200	0	51.3 $\pm$ 4.8	51.7 $\pm$ 8.3	27.3 $\pm$ 3.4	14.5 $\pm$ 2.9
<u>Galanin</u>					
Peripherin	0	15.7 $\pm$ 2.7	19.3 $\pm$ 4.9	5.7 $\pm$ 1.3	7.7 $\pm$ 1.7
NF-200	0.7 $\pm$ 0.7	11.8 $\pm$ 1.2	25.8 $\pm$ 4.2	5.8 $\pm$ 1.6	7.8 $\pm$ 2.0

From day 7, TNT rats developed mechanical hypersensitivity ($\downarrow$ 62% from baseline at peak, day 28). Rats injected with d4T exhibited bilateral hind paw mechanical hypersensitivity ($\downarrow$ ~47% from baseline at peak, day 22), but failed to exhibit any neurochemical changes in the DRG such as that observed in TNT injury. Seven days following Fluorogold injection into the tibial nerve of naive rats, labelling was identified in the L4 (38.5%) and L5 (27.5%) DRGs. Since DHA did not alter TNT induced mechanical hypersensitivity, key neurochemical markers were not evaluated. Investigations examined the potential of DHA to modulate the DRG macrophage response, and revealed no differences compared to saline controls at day 28 post TNT injury.

Conclusion: The results of the study demonstrate completely different expression patterns of neurochemical markers following TNT injury and treatment with d4T, and highlight the mechanistic difference between nerve trauma and antiretroviral associated neuropathic pain.

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Abbreviations

5-HT	5-hydroxytryptamine (Serotonin)
β-APP	β-amyloid precursor protein
ACE	Angiotensin converting enzyme
AA	Arachidonic acid
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
ALA	α linolenic acid
AMPA	2-amino-3-(5-methyl-3-oxo-1,2 oxazol-4yl) propanoic acid
ANOVA	Analysis of variance
AP-1	Activating protein-1 (AP-1)
ATF-3	Activating transcription factor-3
ATN	Antiretroviral toxic neuropathy
ATP	Adenosine triphosphate
AZT	Zidovudine
B7-1 (CD80)	Co-stimulatory molecule B7-1
B7-2 (CD86)	Co-stimulatory molecule B7-2
BDNF	Brain derived neurotrophic factor
BL	Baseline
CAMK	Calcium-calmodulin kinase
βCTB	Cholera toxin beta subunit
CB	Cannabinoids
CCI	Chronic constriction injury
CCK	Cholecystokinin
CCL2	Chemokine ligand 2
CCR2	Chemokine receptor 2
CD11b/18	Cluster of differentiation 11b/18
CD68	Cluster of differentiation 68
CGRP	Calcitonin gene related peptide
CNS	Central Nervous System

COX-2	Cyclooxygenase-2
CR-3	Complement receptor-3
CREB	ATF/cAMP responsive element-binding
CRPS	Complex regional pain syndrome
CSNT	Complete sciatic nerve transection
CX3CL1	CX3C chemokine ligand 1 (fractalkine)
CX3CR1	CX3C chemokine receptor 1, also known as the fractalkine receptor or G-protein coupled receptor 13 (GPR13)
CXCR	chemokine receptor
d4T	Stavudine
DAPI	4',6-diamidino-2-phenylindole
ddC	Zalcitabine
ddI	Didanosine
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPN	Diabetic poly neuropathy
DRG	Dorsal root ganglion
ED-1	Macrophage, monocyte, microglia marker (also known as CD-68)
EPA	Eicosapentaenoic acid
ERK	Extracellular signal regulated mitogen activated protein kinase
Fat-1	Fatty acid desaturase
FEPS	Familial episodic pain syndrome
FG	Fluorogold
FK-506	Tacrolimus (calcineurin inhibitor)
GABA	γ -aminobutyric acid
GAD	Glutamic acid decarboxylase
GalR	Galanin Receptor
GAP-43	Growth associated protein 43
GDNF	Glial derived neurotrophic factor

GFAP	Glial fibrillary acidic protein
Gp120	Glycoprotein 120
GPCRs	Gene protein coupled receptors
GRNP	German research network on neuropathic pain
HCN	hyperpolarization activated cyclic nucleotide modulated channels
HIV	Human immunodeficiency virus
HRP	Horse raddish peroxidase
Iba1	Ionizing calcium-binding adapter molecule 1
i.p.	Intraperitoneal
i.v.	Intravenous
IASP	International Association for the Study of Pain
IB4	Isolectin <i>Bandeiraea simplicifolia</i>
IBS	Irritable bowel syndrome
IENF	Intra-epidermal nerve fibre
IFNγ	Interferon gamma
IL	Interleukin
JNK	c-Jun N-terminal kinase
K_{2p}	Two pore potassium ion channels
K_{Ca}	Calcium activating potassium channels
KCC	Potassium/chloride co-transporter
KCNQ	K _M channel
K_{ir}	Inward rectifying potassium ion channel
LC	Locus coeruleus
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCA-O	Middle cerebral artery occlusion
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOR	μ opioid receptor

mRNA	Messenger Ribonucleic acid
mtRNA	Mitochondrial Ribonucleic acid
NeuN	Neuronal specific nuclear marker
NF-200	Neurofilament 200
NGF	Nerve growth factor
NKCC1	Sodium-potassium-chloride co-transporter 1
NMDA	N-Methyl-D-aspartic acid
NPD1	Neuroprotectin D1
NPY	Neuropeptide Y
NRTIs	Nucleoside analog reverse transcriptase inhibitors
NRTIs	Nucleoside analog reverse transcriptase inhibitors
NSAIDs	Non steroidal anti inflammatory drugs
NT-3	Neurotrophin-3
OCT	Optimum cryostat temperature
P2Y12	Purinergic receptor P2Y, G-protein coupled, 12
PAG	Periaqueductal grey
PB	Parabrachial area
PBN	phenyl- <i>N-tert</i> -butylnitron
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGE	Prostaglandin
PHN	Postherpetic neuralgia
PLA2	Phospholipase A2
PNS	Peripheral nervous system
PPAR	Peroxisome proliferator-activated receptors
PSNL	Partial sciatic nerve ligation
PSNT	Partial sciatic nerve transection
PUFAs	Poly unsaturated fatty acids
PWL	Paw withdrawal latency

PWT	Paw withdrawal threshold
Quin-2	Fluorescent calcium indicator
Reg-2	Regenerating gene protein-2 (also known as pancreatitis associated protein 1 (PAP1))
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RVM	Rostral ventromedial medulla
RXR	Retinoic-x-receptor
SCI	Spinal cord injury
SCNA	Synuclein, alpha
SEM	Standard error of the mean
SK	Small conductance potassium ion channel
SNI	Spared nerve injury
SNL	Spinal nerve ligation
SNT	Spinal nerve transection
SOD-2	Superoxide dismutase-2
SPRR1A	Small Proline Rich Protein 1A
Tc	T cytotoxic
TDF	Tenofovir
Th	T helper
TLR3	Toll like receptor 3
TMB-8	8-(N, N-diethylamino)-octyl-3, 4, 5-trimethoxybenzoate
TNFR1	Tumor necrosis factor α receptor 1
TNFα	Tumor necrosis factor α
TNT	Tibial nerve transection
tPA	Tissue plasminogen activator
TRAAK	Two pore-forming potassium ion channel
TREK-1	Two pore-forming potassium ion channel also known as KCNQ2
TrkA	Tyrosine kinase receptor for NGF
TRPV1	Transient receptor potential vanilloid 1

TTX	Tetrodotoxin
TTXr	Tetrodotoxin resistant
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UK	United Kingdom
VGCC	Voltage gated calcium ion channel
VGKC	Voltage gated potassium ion channels
VGSC	Voltage gated sodium channel
VIP	Vasoactive intestinal peptide
VZV	Varicella zoster virus
WDR	Wide dynamic range
WIN 55,212-2	(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo

Hypothesis

Peripheral nerve damage initiates neurochemical changes in the dorsal root ganglion, and the development pain-like behaviours, which can be modulated via the administration of ω -3 polyunsaturated fatty acids.

Chapter 1

1 General Introduction

1.1 Pain

Pain is defined by the International Association for the Study of Pain (IASP) as ‘**An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage**’ (Merskey and Bogduk, 1994). Pain is an intrinsic warning system that allows the body to withdraw and be protected from harm or trauma, and also helps deter future similar situations. Pain is also a multidimensional experience that is highly subjective and essential for the maintenance and preservation of bodily tissues. In addition to the physiological effects of pain, there are also psychological implications such as anxiety, stress, anticipation and empathy to pain (Brooks and Tracey, 2005).

1.1.1 Nociception

The concept of a distinct class of peripheral nerve pain signal transduction mechanism was first proposed by Sir Charles Sherrington (Sherrington, 1906). The consensus now is that the transmission of pain (nociception) involves activation of specialized sensory receptors called the nociceptors (Brooks and Tracey, 2005). There are two types of nociceptive nerve fibres; the A δ fibres and the nociceptive C-fibres. C-fibers are considered polymodal since they react to various stimuli such as hypoxia and hypoglycemia (Craig, 2003), and some C-fibers are exquisitely sensitive to non-noxious light touch (Vallbo et al., 1999). C-fibres can be further subdivided into the peptidergic and non-peptidergic populations (Snider and McMahon, 1998). The A δ fibre is thinly myelinated, and has medium conduction velocities (6-25 m s⁻¹) whereas the nociceptive C-fibres are non-myelinated and have slow conduction velocities (1 m s⁻¹). The A δ and nociceptive C-fibres normally mediate 'first' and 'second' pain respectively, namely the rapid, acute, sharp pain and the delayed, more diffuse, dull type of pain (Julius and Basbaum, 2001). When peripheral A δ and nociceptive C-fibres are activated by noxious stimuli they generate action potentials that travel to their cell bodies, i.e. dorsal root ganglion (DRG) neurons, and then to the spinal cord and ultimately synapse at the superficial dorsal horn. These messages are eventually sent to the midbrain, limbic, and cortical areas of the brain where pain sensation is registered (Snider and McMahon, 1998).

1.1.2 Gate control theory of pain

The gate control theory of pain was first proposed in 1965 (Melzack and Wall, 1965). In this paper the authors summarized two schools of thought regarding the perception and central control of pain, before then explaining the gate control theory. The first theory suggests that pain is a specific modality like vision, or hearing, which requires a specialized set of receptors. The second is the pattern theory, which suggests that nerve impulse patterns for pain are produced via intense stimulation of non-specific receptors. While both theories are plausible they do not attempt to explain other factors such as the physiological influence on pain behaviour. In the newly proposed gate control theory the authors proposed that both small and large diameter neurons transmit information from the periphery to 'inhibitory' and 'transmission' cells in the spinal cord. When this input surpasses a certain threshold (e.g. noxious stimulation of skin), pain is then transmitted to the spinal cord and the gate is 'open'. During noxious stimulation, the activation of large diameter fibres also activates inhibitory cells in the substantia gelatinosa which acts to shut the 'gate' thus reducing transmission of sensory information. Activation of small fibres during noxious stimulation reduces activation of the inhibitory cells thus causing the gate to 'open'. In addition, a connection from the periphery straight to central control was also described, which by-passes the inhibitory and transmission cells, which can then go on to influence the mechanistics of the 'gate'. The advantage of the new concept of the gate control was that it could account for the hyperalgesia, spontaneous pain, and long delays after stimulation characteristic of pathological pain syndromes which could not be easily explained in the earlier two theories. The gate control theory allowed a benchmark for further studies to investigate and test the mechanisms of neuropathic pain, however the gate control theory is now considered to be an incomplete hypothesis since the theory does not incorporate long term changes in the central nervous system (CNS), plasticity of neurons, and inflammatory factors which influence nociceptor sensitivity (Loeser and Melzack, 1999).

1.2 Pain classifications

Pain can be categorized according to whether the pain is acute or chronic in nature; both have different approaches in terms of their mechanisms, management, and treatment.

1.2.1 Acute pain

Pain that is characterized by a rapid onset of symptoms is termed acute pain, and persists for a short period of time, ranging from a few days to weeks. Acute pain may range from mild to severe depending on the nature of the tissue injury and the emotional state; some of the common causes are surgery, disease, childbirth, broken bones, burns, and cuts. The acute inflammation caused by tissue injury acts to sensitize nociceptors, which leads to an increase in discharge rate and a lowered threshold for stimulus activation (Carr and Goudas, 1999). Acute pain usually remits when the injured tissue has healed and may not require medical intervention (Loeser and Melzack, 1999). To aid the healing process and reduce symptoms, acute pain can be managed by the effective use of opioids, non steroidal anti-inflammatory drugs (NSAIDS), paracetamol, and local anaesthetics (Carr and Goudas, 1999).

1.2.2 Chronic pain

Chronic pain can be divided into two main broad subtypes; (1) inflammatory pain e.g. rheumatoid osteoarthritis, and (2) neuropathic pain e.g. diabetic polyneuropathy (DPN) (Baron, 2006).

1.3 Inflammatory pain

Tissue injury results in the local release of numerous chemicals (such as substance P and bradykinin) that mediate or facilitate inflammatory processes which leads to the sensitisation of primary afferent nociceptive terminals. This sensitisation initiates an increase in nociceptive activity that is ultimately responsible for the primary hyperalgesia. Inflammatory processes in neurons along with corresponding ectopic nerve activity may represent a source for central sensitisation which may contribute to chronic persistent pain. Chronic pain may encompass features such as **allodynia** which is pain caused by a stimulus that does not normally provoke pain (mechanical or thermal generated) or **hyperalgesia**; an increased pain sensation in response to a normally painful stimulus; symptoms that both feature in neuropathic pain.

1.4 Neuropathic pain

Neuropathic pain has always been prevalent throughout documented history, but it was not until the IASP was founded in 1973 that attention was focused on the causes and treatment of neuropathic pain. The presence of neuropathic pain whether it is peripherally or centrally mediated continues to be a significant health burden which reduces the quality of life for the individual, and has wide scale impacts on healthcare resources. IASP originally defined neuropathic pain as ‘pain arising from a primary lesion or dysfunction of the nervous system’ (Merskey and Bogduk, 1994). While this current definition has been useful in diagnosing neuropathic pain, it lacks the specificity that would differentiate it from other types of pain where there is a ‘dysfunction’ in sensory processing, which may not be neuropathic in origin such as musculoskeletal and inflammatory pain. A revised definition of neuropathic pain has been proposed as ‘pain arising as a direct consequence of a lesion or disease affecting the somatosensory system’ (Treede et al., 2008). ISAP have now adopted the new version omitting the word ‘direct’ (Jensen et al., 2011). This new definition replaces the word ‘dysfunction’ with disease as it is somewhat misleading and could be interpreted to include the normal plasticity that occurs within the nociceptive system. The word ‘disease’ implies that there is a known pathology such as postherpetic neuralgia (PHN) or diabetes, while the word ‘lesion’ refers to macro or microscopically identifiable damage. A lesion or disease affecting the nervous system may cause other types of pain that should be differentiated from neuropathic, such as pain associated with spasticity and rigidity that is initiated by nociceptive afferents from muscles. Consequently, the replacement of the wording ‘nervous system’ with ‘somatosensory system’ is anatomically precise and recognizes that in neuropathic pain it is somatosensory processing that is perturbed beyond the normal plasticity of undamaged neurons. The intrinsic ‘nociceptive pain system’ is important because it forms a natural human defense mechanism and serves a useful biological function which is the avoidance of potentially harmful situations. However neuropathic pain serves no useful biological purpose and is regarded as a symptom of a disease process. Neuropathic pain may also represent abhorrent attempts of the nervous system to regenerate after an insult.

1.4.1 Neuropathic pain disorders

There is no international consensus to the characterization of neuropathic pain disorders; however the traditional approach is via the anatomical location of nerve injury and aetiology (see Table 1.1) (Asbury and Fields, 1984). Unlike the CNS, the PNS is not protected by bone and skull and therefore it is more exposed to the risks of mechanical, thermal, or chemical injury. Since cutaneous tissues are the most exposed organ innervated by the PNS these tissues are often the subject of nerve damage. Neuropathic pain may be idiopathic in nature; however the most widespread causes arise from a number of different aetiologies including: diabetes, herpes zoster, human immunodeficiency virus (HIV) infection, amputation, surgery, and certain cancer chemotherapy agents (Jensen et al., 2001). The lesion or disease affecting the somatosensory system may result in damage to peripheral nerves in cutaneous regions of the body, the DRG or the dorsal root neurons, as well as the CNS. Central related pain is distinct to that of peripheral origin, and can be differentiated by injury to the spinal cord or brain (Finnerup, 2008).

Peripheral	Central
Postherpetic neuralgia	Stroke
Diabetic polyneuropathy	Multiple sclerosis
Trigeminal neuralgia	Spinal cord injury
HIV related (including antiretroviral associated)	HIV related (including antiretroviral associated)
Phantom limb	Cancer and chemotherapy agents
Radiculopathies	Parkinson's disease
Cancer and chemotherapy agents	Epilepsy
Complex regional pain syndrome (type 2)	
Nerve trauma injury	
Alcoholic polyneuropathy	

Table 1.1 List of different examples of neuropathic pain conditions according to disease/trauma and anatomical site. Table adapted from Dworkin et al (2003).

The specific mechanisms that elicit neuropathic pain are not completely clear and at present is the subject of ongoing research. Although classifying neuropathic pain in terms of the aetiology is useful in terms of identifying the root cause of the pain, the diagnosis does not attempt to explain the underlying mechanisms which may give a functional framework for the clinical management of the pain. The pain that manifests in response to a specific lesion or disease in patients may operate through different mechanisms while different aetiologies may share common pain mechanisms. Additionally, more than one mechanism may be operating within a single patient; this may lead to different symptoms, further complicating treatment. A different approach has been proposed that classifies neuropathic pain according to the presumed mechanisms (Baron, 2006; Sindrup and Jensen, 1999; Woolf and Mannion, 1999). This focus on the actual mechanisms is important because, for example, HIV antiretroviral associated neuropathy has a clear mitochondrial toxicity brought on by NRTI treatments (Bacellar et al., 1994), and this toxicity could be specifically targeted. Conversely, mitochondrial toxicity does not appear to have a major role in traumatic neuropathies and may involve dysregulation of proteins/ion channels that is associated with ectopic activity of peripheral nerves (Lai et al., 2004). By identifying and targeting the nerve damage mechanisms specifically with therapeutic interventions, there is a possibility to limit the effects of nerve damage which could develop into neuropathic pain, thus leading to better treatment outcomes (Finnerup et al., 2010b; Finnerup et al., 2010a).

Diagnosis of neuropathic pain

There is no standard diagnostic procedure for neuropathic pain and diagnosis is based upon clinical judgment that involves two steps both of which are essential: (1) demonstration of lesion or disease and (2) demonstration that the pain is neuropathic. This can involve physical and neurological examinations, questionnaires, magnetic resonance imaging, and electrophysiological studies. Nerve and skin biopsies may also be taken to examine abnormal nerve fibres. In most cases the pain can be explained via a demonstrable lesion, previous traumatic injury or disease process e.g. herpes zoster, diabetes. However when no lesion can be demonstrated the array of diagnostic tools mentioned cannot always exclude the likelihood that the pain is of neuropathic origin.

1.4.2 Clinical aspects of neuropathic pain

Clinically, neuropathic pain is characterized by the presence of spontaneous (stimulus independent) or stimulus-evoked type pain (stimulus dependent) (Dworkin, 2002).

1.4.2.1 Stimulus independent

Spontaneous pain is present in the absence of any stimulation and it can be either continuous or intermittent (Jensen et al., 2001). The pain may demonstrate different qualities, for example the pain can be described as aching, stabbing, and burning in nature. Spontaneous pain can present shooting, electric shock-like paroxysms lasting a few seconds. Often patients will experience continuous pain which may vary in its intensity. Shooting type pains often occur in instances where there is nerve compression e.g. slipped disc. Spontaneous activity in small (nociceptive) unmyelinated C-fibres is thought to be responsible for mediating burning type pains and the sensitisation of dorsal horn neurons (Jensen et al., 2001). Equally, spontaneous activity in the large (non nociceptive) myelinated fibres is related to abnormal stimulus-independent sensations such as paraesthesiae (which are not unpleasant) and, after central sensitisation, to dysaesthesiae (unpleasant) (Woolf and Mannion, 1999).

1.4.2.2 Stimulus dependent pain

The stimulus evoked type pain is a common feature of neuropathic pain and can be initiated via mechanical, thermal, and chemical stimulation (Jensen et al., 2001). Several types of evoked pain may co-exist within a patient, in others only one may be present. The evoked pains are usually brief, lasting for the duration of the stimulation, but at times they can persist in the aftermath of stimulation lasting from a few minutes to hours or even days (Jensen et al., 2001). Such evoked stimulation produces allodynia (pain caused by innocuous stimuli) and hyperalgesia (increased pain response to a normally noxious stimulus). Allodynia is mediated via A β fibres, and human studies have demonstrated that the blockade of A β fibres can abolish allodynia (Torebjork et al., 1992). Hyperalgesia can be evoked via pinprick (e.g. von Frey hair) and is mediated via sensitised A δ fibres or C-fibres

(Ziegler et al., 1999). It is important to recognise other phenomena such as hypoalgesia (sensory loss) which can reside in areas of pain, representing a paradoxical type of pain. Hypoalgesia appears to affect all sensory modalities, and has been observed with respect to thermal in PHN patients (Besson et al., 2005; Rowbotham and Fields, 1996). This response may reflect a loss of afferent fibres in affected areas with the addition of sensitized peripheral neurons. A loss of spinothalamic function has been hypothesized since they propagate information regarding temperature, touch, and pain (Jensen and Lenz, 1995). Although some patients will use descriptors such as burning, shooting, and electric to describe their pain, Rasmussen and colleagues (2004) reported some difficulties in using these descriptors to differentiate between pain of neuropathic origin and non-neuropathic pain, given that these words can be used to describe non neuropathic types of pain (Rasmussen et al., 2004).

1.4.3 Heterogeneity of neuropathic pain symptoms

The past few years has seen an increasing realization of the heterogeneity of neuropathic pain symptoms, even in patients with the same aetiology (Baron et al., 2009; Maier et al., 2010). Screening tools including quantitative sensory testing (QST) and pain questionnaires have been used to evaluate symptoms of pain that maybe neuropathic in origin (Haanpaa et al., 2011).

The use of QST has allowed the quantification of sensory function in patients that either have or might be at risk of developing neuropathic pain, and is useful in identifying abnormalities of both small and large fibre function (Cruccu et al., 2010). QST typically involves measuring the detection thresholds to a range of stimuli including; mechanical, pressure, vibration, coolness, warmth, cold, and heat (Rolke et al., 2006b; Rolke et al., 2006a). Various QST protocols exist and therefore a standard protocol has been proposed by the German research network on neuropathic pain (GRNP) that can provide an assessment of a patient within a 30 minute time frame (Rolke et al., 2006a). A previous study using the GRNP-QST protocol demonstrated notable differences in the somatosensory profiles of patients with PHN, complex regional pain syndrome (CRPS; type I and II), and trigeminal neuralgia (Maier et al., 2010). For example, thermal and

mechanical hyperalgesias were most frequent in complex regional pain syndrome patients and peripheral nerve injury patients, and allodynia in postherpetic neuralgia patients.

The use of pain questionnaires, which broadly focuses on sensory descriptors of pain, provides useful information to the clinician regarding quality and intensity of pain which can aid diagnosis or the monitoring of pain. In a previous study, using the painDETECT questionnaire, 498 patients with PHN and 1623 patients with PDN were analyzed, and the results revealed that patients broadly fitted into 5 symptomatic characteristic subgroups (Baron et al., 2009). This again demonstrates the diverse range of symptoms that can exist within one neuropathic pain aetiology and it may be more appropriate to treat patients according to specific symptoms rather on the basis of aetiology.

Taken together, the clinical data from the QST and questionnaire studies suggest that a diverse range of mechanisms is likely to be operating for the manifestation of a particular symptom within any given aetiology. It is important to recognise such phenomena in preclinical studies which can be addressed by investigating a variety of peripheral nerve injury models to account for the diverse mechanisms.

1.4.4 Peripheral mechanisms of neuropathic pain

To date, the understanding of neuropathic pain mechanisms has derived from basic science research involving both traditional *in vivo* and *in vitro* as well as novel cell-molecular approaches. In addition to pre-clinical research, there has been a steady focus on experimental human pain models which have provided further insight into human pain pathology. Following peripheral nerve injury there are biochemical changes at the site of injury as well as the affected nerve which may set to drive the symptoms of neuropathic pain.

1.4.4.1 Dysregulation of ion channels and ectopic activity of neurons

Ectopic activity from injured primary afferents neurons in the peripheral nervous system is related to the dysregulation of the synthesis and function of sodium ion channels. Voltage gated sodium channels (VGSCs) play a fundamental role in regulating neuronal excitability, which is important to the physiology of neurons. There are different isoforms of VGSC (named Na_v1.1 to Na_v1.9) and they all differ in terms of their kinetic and pharmacological properties and some have distinct distribution patterns (Wood et al., 2004). The Na_v1.8 and Na_v1.9 ion channels are all exclusively expressed in small unmyelinated C-fibres. Other channels such as Na_v1.4 and Na_v1.5 are located in the muscle and heart respectively and are important in determining the functional specialization of those tissues. Following peripheral nerve injury the Na_v1.3 channel (which is expressed at low levels in the adult DRG and at high levels during neuronal development) is upregulated in the DRG (Lai et al., 2004). This upregulation is associated with ectopic activity which correlates with the development of neuropathic pain like behaviour in rodents (Lai et al., 2004). Additionally, Na_v1.3 is upregulated in the dorsal horn of animals with experimental spinal cord injury (SCI) and anti sense knockdown of Na_v1.3 attenuates dorsal horn excitability and pain like behaviours (Hains et al., 2003), although this result is in regards to the CNS, it provides evidence for Na_v1.3 role in pain. Despite this compelling evidence, the global knockout of Na_v1.3 in mice still results in pain related behaviours (Nassar et al., 2006). Furthermore, the use of conditional knockouts via cre-recombinase systems to exercise out the Na_v1.3 gene which can bypass any genetic possible compensatory mechanisms additionally resulted in the development of pain related behaviours. Other channels such as the Na_v1.8 and Na_v1.9 isoforms are down-regulated in injured DRG neurons following axotomy in both humans and animal pain models, while uninjured afferent expression levels are unchanged (Decosterd et al., 2002; Dib-Hajj et al., 1998). A direct involvement of Na_v1.8 in neuropathic pain was observed in a study where a knockdown of Na_v1.8 in the spinal nerve ligation model (SNL) of nerve injury reversed both the mechanical and thermal hypersensitivity associated with this injury (Gold et al., 2003; Lai et al., 2002). Since Na_v1.8 is down-regulated in the DRG, it is unlikely to contribute to the phenotype described. Interestingly an upregulation of Na_v1.8 is observed at sites along uninjured nerve afferents (Novakovic et al., 1998); this upregulation may

result in the development neuropathic pain-like behaviours. This dynamic shift in expression suggests that uninjured or spared neurons play a role in neuropathic pain; furthermore, redistribution of Na_v1.8 channels in injured sciatic nerve neurons has been described in the chronic constriction injury (CCI) model (Novakovic et al., 1998) and neuropathic pain patients (Coward et al., 2000).

Ectopic activity can be reduced by sodium channel blockers such as lidocaine and tetrodotoxin (TTX) (Matzner and Devor, 1994). Clinical trials have shown the lidocaine (5% patch) to be effective in the treatment of PHN and is considered a first-line treatment for neuropathic pain states due to its tolerability and safety (Markman and Dworkin, 2006). This provides direct evidence of sodium ion channel involvement in neuropathic pain. Other human data linking VGSC to pain derives from patients with erythromelalgia and paroxysmal extreme pain disorder. These hereditary disorders are caused by 'gain in function' missense mutation in the SCNA gene that encodes the Na_v1.7 VGSC (Yang et al., 2004). Electrophysiological recordings have indicated that the mutation lowers the threshold for channel activation and slows down the rate of deactivation thus leading to increased excitability and ectopic activity in neurons (Cummins et al., 2004; Dib-Hajj et al., 2005). Differing mutations in the SCNA can result the inability to experience pain, which is linked to a loss of channel function (Cox et al., 2006). The discovery of this has raised the possibility of Na_v1.7 VGSC can become an attractive drug target if selectively targeted.

Voltage gated potassium ion channels (VGKC) play a role in modulating excitability in neurons by acting as a break and repolarising neurons to restore baseline membrane potentials. The major potassium currents arising in neurons are regulated by the VGKC and the calcium activating potassium channels (K_{Ca}) (Wickenden, 2002). Other potassium currents are regulated by the inward rectifying (K_{ir}) and two pore ion channels (K_{2p}) (Wickenden, 2002). Experimental peripheral nerve injury leads to the reduction in VGKCs in the DRG such as K_v1.4 (which is expressed on small size cells), thus suggesting a potential mechanism for ectopic activity in injured neurons (Rasband et al., 2001). One VGKC that can regulate the excitability of neurons is the K_M channel (KCNQ). The K_M channel operates at low thresholds in the CNS and is responsible for the inhibitory M current in DRG neurons (Marrion, 1997). Retigabine, a drug that can facilitate the M

current, can reverse the associated mechanical hypersensitivity in experimental nerve injury models (Blackburn-Munro and Jensen, 2003). VGKCs may be a potential target that can be exploited for the treatment of neuropathic pain. The small conductance potassium ion channel (SK) may play a role in modulating sensory transmission from the periphery to the spinal cord. *In vivo* electrophysiological recordings reveal that neuronal responses to nociceptive stimulation increase after application of a selective SK channel blocker, thus demonstrating that SK channels can modulate afferent input (Bahia et al., 2005). Furthermore, these SK channels are present on peripherin immunoreactive neurons which represent the C-fibre (nociceptive) class of sensory neurons, which suggest the SK channels may be involved in processing nociceptive input.

Permeable to both potassium and sodium ions are the hyperpolarization activated cyclic nucleotide modulated channels (HCN). These ion channels are also known as pacemaker channels and are critical to the production of rhythmic activity in cardiac cells (Biel et al., 2009). However, the HCN channels are also located on primary afferent neurons, and therefore likely to play a role in regulating resting membrane potentials in sensory neurons. SNL injury can increase pacemaker currents in large diameter DRG neurons which results in pacemaker driven spontaneous action potentials in the ligated nerve. Blockade of the HCN channels can reverse hypersensitivity produced by light touch and decreases the firing frequency of ectopic discharges originating in A β and A δ , in a dose dependent manner (Chaplan et al., 2003). This result suggests that HCN channels may be, at least in part, an underlying factor in ectopic discharge in nerve injury.

A specific role of voltage gated calcium ion channels (VGCC) has been recognised in neuropathic pain. There are several different types of VGCC found within the body, all distinct via their electrophysiology or response to particular toxins/channel blockers. L-type channels are key determinants of membrane excitability whereas N and P/Q channels are involved with neurotransmitter release. Pharmacological evidence suggests that the N-type VGCC plays a role in neuropathic pain since intrathecal delivery of N-type antagonists reduces the mechanical hypersensitivity associated with SNL injury (Calcutt and Chaplan, 1997; Chaplan et al., 1994). The L-type Ca²⁺ channels may also play a role in neuropathic pain given that its knockdown in the dorsal horn reverses the mechanical

hypersensitivity (Fossat et al., 2010). The auxiliary $\alpha_2\delta$ subunit of the calcium ion channel could act to maintain excitability of neurons. Following spinal nerve injury the $\alpha_2\delta$ subunit is upregulated in the DRG and sciatic nerve axons this also correlates with neuropathic pain like behaviours (Bauer et al., 2010; Luo et al., 2001). In addition, transgenic animals which over express the $\alpha_2\delta 1$ subunit in neuronal tissues demonstrate exaggerated dorsal horn neuronal responses to both mechanical and thermal stimulation in the hind paws in both uninjured and nerve injured neurons (Li et al., 2006). Gabapentin, which is used in the treatment of clinical neuropathic pain, binds to the $\alpha_2\delta$ of the VGCC and this is associated with reduced Ca^{2+} currents in excitable neurons and ectopic discharges (Pan et al., 1999; Stefani et al., 1998). The exact mechanism of pregabalin induced analgesia is not fully understood, however a recent study has suggested that the *in vivo* application of pregabalin is associated with impaired trafficking of $\alpha_2\delta$ subunit on presynaptic terminals, which results in reduced neurotransmitter release and neuronal hyperexcitability (Bauer et al., 2010).

Transient receptor potential (TRP) receptors are ligand gated, non-selective cation channels involved in the biological transduction of thermal mediated nociceptor responses (Veronesi and Oortgiesen, 2006). The TRP channels allow the influx of Na^+ and Ca^{2+} ions resulting in depolarization of neurons, which may lead to the generation of action potentials. The TRPV1 channel was the first recognized pain transducer and was originally characterized by its activation by capsaicin; the pungent chemical found in chilli peppers (Immke and Gavva, 2006). The TRPV1 channel is present on both A δ and C-fibres. It was found that TRPV1 channels could be activated by temperatures above 43°C, low pH conditions, and pharmacologically via anandamide (Ramsey et al., 2006). Other heat sensing channels include TRPV2 which activates at temperatures >50°C (Tominaga and Caterina, 2004) and TRPM3 which exhibits robust responses to heat *in vitro* (Vriens et al., 2011). Conversely, the TRPA1 channel responds to noxious cold temperatures (<17°C) and other substances such as mustard, cinnamon, and ginger oils. The TRPV1 receptor is of immense interest given their role in nociception, and their activation can result in burning-like pain sensations; a frequently reported symptom of neuropathic pain. The exact role of TRPV1 channels in nerve injury is not well established however changes in TRPV1 expression have been reported in nerve injury models. TRPV1 messenger ribonucleic acid (mRNA)

and protein is down-regulated in the spinal cord and somata of damaged sensory neurons following sciatic nerve axotomy (Ma et al., 2005; Michael and Priestley, 1999). A further study had demonstrated in addition to the down-regulation of TRPV1 in the damaged sensory neurons, an upregulation of TRPV1 in the undamaged population (determined by tract tracing) – this may be crucial to the development or maintenance of neuropathic pain (Hudson et al., 2001). In addition, an increase of TRPV1 is observed in A β fibres which indicates a likely phenotypic switch since TRPV1 is normally expressed on A δ and C-fibres (Immke and Gavva, 2006). Evidence from transgenic studies reveal that mice with a knockout in the gene coding the TRPV1 channel exhibit normal responses to noxious mechanical and thermal stimuli, however in the context of inflammation this response is impaired (Caterina et al., 2000). This indicates a role of TRPV1 in mediating mechanical and thermal hypersensitivity in conditions of tissue injury.

The TRPA1 channel has also been shown to be dysregulated following nerve injury, given the increased expression in small diameter cells (Obata et al., 2003). It may be that this dysregulation is linked to the development of cold hypersensitivity that is evident in neuropathic pain models. Indeed a mutation of the gene coding for TRPA1 is responsible for a heritable episodic human pain syndrome termed familial episodic pain syndrome (FEPS), that can be triggered when exposed to cold (Kremeyer et al., 2010).

1.4.4.2 Sensitization of nociceptors by inflammatory mediators

Injury to a peripheral nerve is usually associated with neuroinflammation with the release of proinflammatory cytokines and chemokines from different cell types. Such phenomena can occur in Wallerian degeneration whereby the axon becomes separated from the cell soma and degenerates distal to the injury (Gaudet et al., 2011). The release of proinflammatory mediators can act to sensitize neurons by lowering action potential thresholds, which is associated with ectopic discharges, thus leading to an increase in neuronal excitability (McMahon et al., 2005). Such proinflammatory mediators include tumor necrosis factor α (TNF α), interleukin (IL) 1 β , IL-6, adenosine triphosphate (ATP), and bradykinin (Ji and Strichartz, 2004). Proinflammatory mediators can be released from damaged axons or Schwann cells, Langerhan cells, infiltrating leukocytes and

macrophages, and are likely to contribute to allodynia and hyperalgesia observed in human neuropathic pain conditions. Intraplantar or intrathecal injections of both TNF α and IL-1 β cytokines are associated with pain-like behaviour in rodents (Cunha et al., 1992; Safieh-Garabedian et al., 1995; Youn et al., 2008); and similarly the use of neutralizing antibodies can attenuate neuropathic pain related behaviours (Schafers et al., 2001; Sommer et al., 1999). The administration of TNF α to sensory neurons *in vivo* is known to affect the firing properties of injured and uninjured DRG neurons (Schafers et al., 2003b); this may occur through an interaction with VGSCs and VGCCs (Wilkinson et al., 1996). A consequence of IL-1 β is the genesis of signaling cascade that leads to the release of pronociceptive molecules (e.g. nitric oxide; certain prostaglandins such as PGE₂) from immune cells or Schwann cells. The cytokines IL-6 and leukemia inhibitory factor (LIF) can elicit the release of neuropeptides (e.g. galanin and SP) and BDNF, all of which can modulate nociceptive processing (McMahon et al., 2005). Further evidence for a role of interleukins stem from transgenic studies whereby a knockout of IL-6 markedly delays the onset of thermal induced hypersensitivity in mice following nerve injury (Ramer et al., 1998). Recently, there has been increasing interest in the role of CCL2, which has been linked to the pathogenesis of pain in the PNS (Sun et al., 2006). CCL2 is upregulated in the cell soma of DRG neurons after trauma nerve injury (White et al., 2005b) and is transported and released in an activity dependent manner in the central terminals of these fibres in the dorsal horn of the spinal cord (Thacker et al., 2009).

Aside from the direct action of cytokines on nociceptors, there are other circumstances that can result in the activation of nociceptors. Firstly, the chemokine fractalkine (CX3CL1) is normally expressed in sensory neurons in the DRG, and its receptor (CX3CR1) is located in satellite cells (as well as microglia in the cord) (Verge et al., 2004). However in response to neuronal activity, fractalkine can be cleaved by matrix metalloproteinase (MMPs), which can act on its receptors where its activation have been shown to be involved in the initiation of neuropathic pain-like behaviour in rats (Milligan et al., 2004). Secondly, the viral protein gp120, which is a cell surface protein found on HIV, when applied to the sciatic nerve can interact with CCR5 and CXCR4 expressed on sensory neurons (and Schwann cells), which initiates a macrophage infiltration at application site and has been associated with mechanical hypersensitivity in rats (Wallace et al., 2007a). Upregulation of

satellite cell marker GFAP is additionally observed in the DRG which may also release proinflammatory cytokines (Wallace et al., 2007a).

In addition to cytokines and chemokines, peptides have a role in regulating nociceptive responses. Bradykinin, a small peptide of the kinin family which regulates contraction of blood vessels can additionally mediate acute and chronic inflammatory responses (White et al., 2005a). Traumatic peripheral nerve injury initiates the release of bradykinin from non neuronal cells, which binds to the two bradykinin receptors (BK₁ and BK₂), therefore leading to the activation of the secondary messenger protein kinase C which promotes nociceptor sensitivity (Julius and Basbaum, 2001; White et al., 2005a) most likely through the TRPV1 receptor (Vellani et al., 2004). The B1 receptor appears to be of relevance since this receptor is significantly upregulated on sensory neurons following tissue injury (Fox et al., 2003) and is located in small diameter neurons in the DRG (Ma, 2001; Wotherspoon and Winter, 2000). B1 receptor activation additionally evokes the release of proinflammatory cytokines including PGE₂ and PGI₂, reactive oxygen species (ROS), and nitric oxide from endothelial cells (Walker et al., 1995). Pharmacological studies have revealed that the intraplantar administration of bradykinin B1 agonists does not affect paw withdrawal thresholds (PWT) to mechanical stimuli (Fox et al., 2003), however it can argument nociceptive behaviours following traumatic nerve injury through similarly administration routes (Werner et al., 2007). Antagonists of B1 receptor when administered systemically (i.v) to rats can reduce the mechanical and thermal hypersensitivity associated with traumatic peripheral nerve injury (Ferreira et al., 2005; Levy and Zochodne, 2000; Yamaguchi-Sase et al., 2003). Furthermore, ablation of the gene for the B1 receptor results in a significant reduction in early stages of mechanical and thermal hypersensitivity (Ferreira et al., 2005).

Neuropeptides such as calcitonin gene related peptide (CGRP) and Substance P are involved in what is defined as neurogenic inflammation – the inflammation arising from neurons which is characterized by plasma extravasation and vasodilation in the affected region. Sensitization of unmyelinated nociceptive afferents has been hypothesised to play a pivotal role in neuropathic pain conditions such as CRPS (Holzer, 1998). Studies in patients have demonstrated an increase in systemic CGRP, Substance P, and bradykinin in

the acute stages of CRPS (Birklein et al., 2001). Moreover, neurogenic inflammation was elicited by directly activating the skin by transcutaneous electrical stimulation via intradermal microdialysis capillaries. Animal experiments involving neurogenic inflammation have observed similar findings in regards to increases in neuropeptides, and pharmacological blockaded of the neurokinin-1 (NK-1) receptor results in a reversal of plasma extravasation in rodents (Holzer, 1998; Xu et al., 1992). The neurogenic inflammation was associated with alteration in thermal coding and decreases in mechanical withdrawal thresholds.

Angiotensin-converting enzyme (ACE) is a key kinase involved in the degradation of substance P and bradykinin. A clinical study have indicated an association between the the use of ACE inhibitors and the risk of developing CRPS (de Mos et al., 2009), which suggests a role for substance P and bradykinin in the underlying pathogenic process.

1.4.4.3 Apoptosis in the DRG

Apoptosis is a distinct form of cell death that can be triggered by a variety of physiological and pathological stimuli (Earnshaw et al., 1999). Nerve damage and pain related behaviours demonstrated in nerve injury models are accompanied by apoptotic signaling cascades in sensory neurons of the DRG (Joseph and Levine, 2004; Sekiguchi et al., 2009). Activated caspase-3 (the critical executioner of cell death) is induced in the L5 DRG following crush injury to the ipsilateral L5 spinal nerve (Sekiguchi et al., 2009). Recent findings by Schaeffer et al., 2010, using the terminal deoxynucleotidyl transferase nick end labelling (TUNEL) technique as a marker of apoptosis, demonstrated immunolabeling of satellite cells in the DRG of CCI animals 30 days after injury. Previous investigations using traumatic, ddC (Zalcitabine) antiretroviral, and vincristine-induced models of nerve damage have demonstrated that the administration of caspases inhibitors can attenuate pain-related behaviours, and therefore caspases signaling pathways may contribute towards neuropathic pain in humans (Joseph and Levine, 2004; Scholz et al., 2005; Sekiguchi et al., 2009). Apoptotic pathways may be activated via ROS, ceramide, and nitric oxide (Benn and Woolf, 2004) or stimulation of death receptors such as tumor necrosis factor receptor 1 (TNFR1) which can act via the c-Jun N-terminal kinase (JNK) pathway to activate effector

capsases (Earnshaw et al., 1999). Activation of the TNFR1 occurs by binding with TNF α which is released by immune cells such as macrophages in the aftermath of nerve injury (Scholz and Woolf, 2007) and is found in the DRG (Schafers et al., 2003a) where it co-localises with caspases-3 (Kim et al., 2011). This suggests TNF α could potentially play a role in initiating apoptosis. Apoptotic signaling may be of particular relevance in ‘die back’ neuropathies where the terminal of sensory neurons are diminished while the cell body remains, and has the potential to regenerate a new terminal. Although it is assumed that once the apoptosis process passes critical checkpoints, cell death ensues, evidence from a recent study using a variety of cells including brain cells indicates that late stage apoptosis can be reversed (Tang et al., 2012).

1.4.4.4 Altered gene expression and functional genomics

When peripheral sensory neurons undergo damage such as nerve transection, transcriptional changes lead to alterations in neuronal physiology which may influence the metabolic state, capacity to regenerate and neuronal plasticity, all of which bear relevance in neuropathic pain conditions. Since the pathophysiology of neuropathic pain is complex and may involve several mechanisms, a more rational approach has been considered taking into context changes in gene expression. Recent advances in molecular biology have led to the development of deoxyribonucleic acid (DNA) microarray based technologies, which have the capacity to measure changes in gene expression by analyzing the entire genome (Reilly et al., 2004). This technique has led to the identification of many potential targets which maybe relevant to the development or maintenance and symptoms of neuropathic pain. There are now several published studies documenting altered expression of neuropeptides, ion channels, neuroinflammatory markers and transcription factors, in the DRGs of traumatic models of nerve injury (Costigan et al., 2002;Valder et al., 2003;Wang et al., 2002). In an attempt to identify dysregulated genes in a model of peripheral nerve damage representing a clinically relevant aetiology, DRG gene expression in the gp120 + ddC (zalcitabine) was investigated and compared to the spinal nerve transection (SNT) model (Maratou et al., 2009). These models were associated with distinct sets of gene expression changes, with 5140 dysregulated genes in the SNT model and 656 in the gp120 + ddC model compared to sham control, interestingly, only 39 genes were found to be

commonly dysregulated (in the same direction) between the two models, some of which had not been previously implicated in mechanical hypersensitivity. Several of the genes identified such as pancreatitis associated protein-1/regenerating gene protein-2 (PaP1/Reg2) and neuropeptide Y (NPY) have previously been reported to be implicated in the onset of nerve injury related paw hypersensitivity in rodents (Maratou et al., 2009). However, other genes which have been previously unidentified such as VGF which is significantly upregulated in the g120 + ddC and SNT model has not been examined fully, and consequently warrants further investigation. Microarray technology therefore provides a basis to which novel targets can be identified and then specifically investigated.

1.4.4.5 Summary

In summary, research over the past 20 years has resulted in an increased understanding of neuropathic pain mechanisms. The notion that there is a single underlying hypothesis for the development and perhaps maintenance of neuropathic pain is not likely given the array of literature encompassing many potential targets and mechanisms (Bridges et al., 2001b). In addition, psychological and cognitive factors are increasingly recognized in their ability to profoundly influence the overall pain experience, which adds another level of complexity (Daniel et al., 2008; Nicholson and Verma, 2004). Research is still ongoing and advances in technology such as next generation sequencing technologies may afford the possibility of identifying novel targets which may lead to the development of new treatments.

Apoptosis in DRG ($\uparrow$ caspase-3)

Dysregulation of ion channels in DRG:

Receptors: $\downarrow$ Na_v1.8, Na_v1.9, Kv1.4, TRPV1 (damaged neurons) P2X3 (damaged neurons)

$\uparrow$ Ca_vα2δ1, Na_v1.3, TRPV1 (undamaged neurons), P2X3 (undamaged neurons),

Peptides: $\uparrow$ Galanin, NPY, PaP1 (Reg2), Substance P, VGF

$\downarrow$ BDNF

$\uparrow$ Statelite cell proliferation and macrophage infiltration

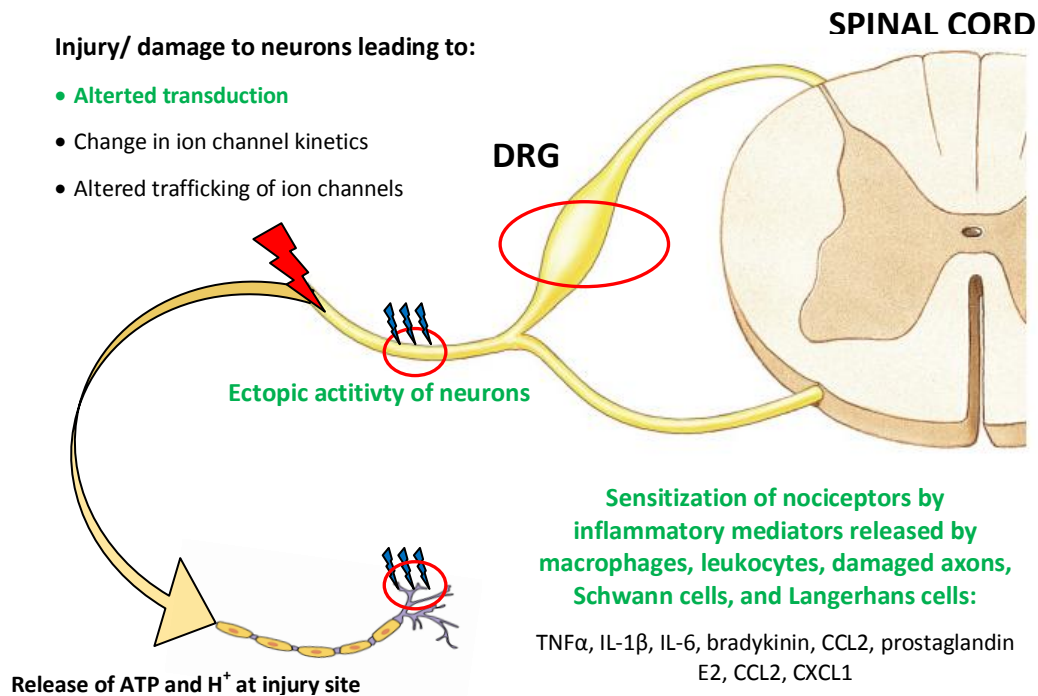


Figure 1.1 Diagram to summarise the various peripheral mechanisms of neuropathic pain following partial nerve injury. Such mechanisms involve dysregulation of ion channels and ectopic activity of neurons, sensitization of nociceptors by inflammatory mediators, apoptosis in the DRG, and altered gene expression.

1.4.5 Central mechanisms of neuropathic pain

1.4.5.1 Central sensitization – wind up

Central sensitization involves changes to the plasticity of neurons at the spinal level, whereby afferent inputs such as those arising from low threshold mechanical neurons which do not signal pain then begin to do so (D'Mello and Dickenson, 2008). Additionally, responsiveness to normally painful stimuli are now interpreted as more painful. Peripheral sensitization on the other hand is usually restricted to the site of tissue injury and results in the lowered threshold to a particular stimulus. The phenomenon of central sensitization was first demonstrated in a study where thermal cutaneous nerve injury produced long lasting changes to the receptive field properties of flexor motor neurons, which was used as a measure of functional plasticity in the spinal cord (Woolf, 1983). Since this time, the neuronal substrate for the activation of central sensitization has largely focused on glutamate. Glutamate is the major excitatory neurotransmitter of the nervous system and therefore essential to pain signaling. The vast majority of primary afferents synapsing at the dorsal horn utilize glutamate as a neurotransmitter, which then acts on a number of post synaptic receptors including the metabotropic glutamate receptors, and the ionotropic 2-amino-3-(5-methyl-3-oxo-1,2 oxazol-4yl) propanoic acid (AMPA) & N-Methyl-D-aspartic acid (NMDA) receptors (Bleakman et al., 2006). The release of glutamate and the activation of the AMPA receptors are usually in response to noxious and non-noxious stimuli, which represents the constitutive baseline response of dorsal horn neurons (D'Mello and Dickenson, 2008). However in the context of nerve injury where repetitive high frequency stimulation of C-fibres occurs, there is an increased release of glutamate from synapses in the spinal cord which acts on the NMDA receptor (Kawamata and Omote, 1996), leading to a non-proportional amplification in the response of dorsal horn neurons, also known as wind up (Dickenson and Sullivan, 1987). This high frequency stimulation as well as phosphorylation of the NMDA receptor is necessary for the removal of the Mg^{2+} block at the NMDA receptor and the opening of the receptor pore. This results in the influx of Ca^{2+} and the activation of downstream cellular kinases calcium-calmodulin (CAMKII) and extracellular signal regulated mitogen activated protein kinase (ERK) (Ji et al., 1999; Matsumura et al., 2010), both of which can promote the plasticity of neurons.

Wind up can be ablated through the use of NMDA antagonists such as memantine, MK-801, and the clinically used ketamine; all of which have been shown to be antinociceptive in SNL induced peripheral nerve injury (Suzuki et al., 2001). Similar antinociceptive responses can be observed with the more specific NR2B subunit NMDA antagonist (Boyce et al., 1999). Taken together, the evidence supports the role of NMDA contributing towards neuropathic pain.

1.4.5.2 Loss of inhibitory systems in spinal cord

The neurotransmitter γ -aminobutyric acid (GABA) is primarily responsible for some of the key inhibitory processes that occur within the CNS. GABA acts through the fast acting ionotropic GABA_A receptor and the slow acting metabotropic GABA_B receptor to initiate hyperpolarization in the mature nervous system. Evidence of GABA role in pain processing derives from pharmacological studies, which have demonstrated reduced mechanical hypersensitivity in SNL injured rats following intrathecal administration of GABA_A and GABA_B receptor agonists (Hwang and Yaksh, 1997). Furthermore, partial nerve injury results in decreases in the dorsal horn levels of GABA and its synthesizing enzyme glutamic acid decarboxylase (GAD) 65 kDa ipsilateral to the injury (Moore et al., 2002). GABA-mediated inhibitory currents and extracellular concentrations are additionally decreased. Interestingly, apoptosis can also be observed in the dorsal horn in nerve injured animals as demonstrated by TUNEL labelling, which may be attributed to excitotoxicity caused by excessive nerve discharge (Moore et al., 2002; Sugimoto et al., 1990). This apoptosis may reflect a selective degeneration of GABAergic interneurons which may give rise to the reduced GABA and GAD65 concentration that occurs following nerve injury.

Peripheral nerve injury results in the loss of opioids receptors in the DRG (Zhang et al., 1998) and spinal cord (Besse et al., 1991; Besse et al., 1992b) which most likely reflects the loss of primary afferents. These opioids receptors are localised predominately on pre synaptic terminals of primary afferents in the superficial lamina of the dorsal horn, and there is greater loss of mu opioid receptors than the delta isoform following peripheral nerve injury (Besse et al., 1992a). In addition to opioid receptor loss, there is a concurrent

increase in spinal mRNA cholecystokinin (CCK, an endogenous antagonist of the opioid receptor) and its receptor (CCK_B) (Kovelowski et al., 2000). This happening may act to lessen the potency of systemic or intrathecal delivered opioids in neuropathic pain and may explain the reduced effectiveness of opioids over time (Rashid et al., 2004).

1.4.5.3 Anatomical reorganization

A particular feature of neuropathic pain in humans is the development of allodynia, which is thought to mediate from A β fibres; however the mechanism by which this happens is unclear. One proposed mechanism is anatomical reorganization within the spinal cord. Evidence taken from labelling studies suggest that following peripheral nerve injury A β fibres sprout from their normal termination sites in the deeper lamina of the cord up to the superficial dorsal horn, which is normally innervated by C-fibres (Woolf et al., 1992). This reorganization could lead to situation whereby innocuous stimulation is now coded as nociceptive. The A β fibre sprouting is prevented by treatment with NGF or glial derived neurotrophic factor (GDNF), thereby indicating that trophic support during nerve injury is integral to nerve plasticity, however the degree to which this relates to pain-like behaviour is less clear (Bennett et al., 1996a). Interestingly, there are some studies that have raised concerns regarding whether A β fibre sprouting is a true phenomenon or is indeed artefact. There is evidence to suggest that uptake of the label cholera toxin β subunit (CTB) is altered following nerve injury, such that the label is taken up by C-fibres rather than sprouting A β fibres (Hughes et al., 2003; Tong et al., 1999). In addition, optimal sprouting does not occur until 2 weeks after injury, which would suggest other mechanisms are in place regarding hyper-reflexive responses (Woolf et al., 1992). Despite conflicting information in the literature, electrophysiological evidence supports the sprouting theory since stimulation of A β fibres after nerve injury can activate neurons in the superficial lamina II of the spinal cord (Okamoto et al., 2001). It has become clear that further studies are warranted in order to clarify contradictory reports in the literature regarding A β fibre sprouting and the extent to which this may contribute to hind paw hypersensitivity following peripheral nerve injury.

1.4.5.4 Descending modulation of primary afferents in the spinal cord

Supraspinal modulation of the spinal cord is thought to play an important role in neuropathic pain (D'Mello and Dickenson, 2008). This descending modulation encompasses both facilitatory and inhibitory controls (Zhuo and Gebhart, 1997); the balance of the two ultimately determines the excitability of neurons in the spinal cord. The descending projection pathways originate directly or indirectly from several brain stem nuclei which include the rostral ventromedial medulla (RVM) - which represents one of the main output nuclei for descending control, the parabrachial area (PB), and periaqueductal grey (PAG) (Millan, 2002). Within the RVM those facilitatory cells have been defined as 'ON cells' and the inhibitory cells, 'OFF cells' (Fields et al., 1995). Following peripheral nerve injury, there is enhanced descending excitatory drive from the RVM to the spinal cord, which has been described to contribute to the rather than induction of neuropathic pain-like behaviours in rodents (Burgess et al., 2002; Vera-Portocarrero et al., 2006) and persistent pain in humans (Gebhart, 2004). This excitatory drive may represent a method by which the CNS attempts to counteract the loss of sensory input caused by damaged neurons. Investigations have indicated that activation of spinal descending serotonin pathways play a pivotal role since the pharmacological blockade of 5-hydroxytryptamine-3 (5-HT₃) receptors using ondansetron suppresses spinal responses to punctate mechanical stimuli after SNL (Suzuki et al., 2004). In addition, depletion of serotonin in the spinal cord attenuates behavioural hypersensitivity after spinal nerve ligation injury (Rahman et al., 2006). Nociceptive information arrives on superficial regions of the dorsal horn where they terminate on NK-1 (substance p receptor) neurons (Todd, 2002).

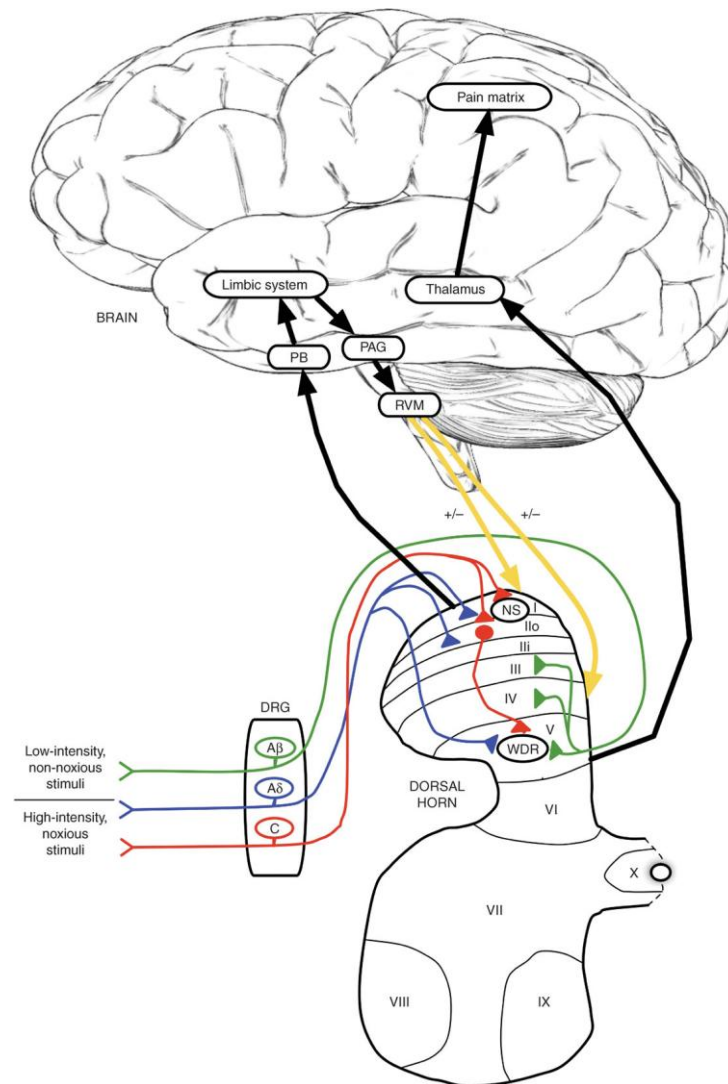


Figure 1.2 Pain pathways from periphery to brain. Primary afferent fibres transmit impulses from the periphery, through the dorsal root ganglion (DRG) and into the dorsal horn of the spinal cord. Nociceptive specific (NS) cells are mainly found in the superficial dorsal horn (laminae I–II), whereas most wide dynamic ranges (WDRs) are located deeper (lamina V). Projection neurones from lamina I innervate areas such as the parabrachial area (PB) and periaqueductal grey (PAG) and such pathways. From here descending pathways (yellow arrows) from brainstem nuclei such as the rostral ventromedial medulla (RVM) are activated and modulate spinal processing. Lamina V neurones mainly project to the thalamus (spinothalamic tract), and from here the various cortical regions. Figure adapted from D’Mello and Dickenson, 2008.

This nociceptive information is then conveyed to higher centers within the brain - such as the thalamus, where sensations are perceived thus representing the sensory-discriminative aspect of pain. NK-1R expressing neurons represent a small part of the neuronal population in superficial lamina, however they represent approximately 77% of the nociceptive cells that project supraspinally (Cheunsuang and Morris, 2000). A large proportion of these NK-1 cells make connections with the RVM, PAG, PB, thalamus, and hypothalamus (Braz et al., 2005). The ablation of NK-1 positive neurons using substance p-saporin results in reduced excitability of deep dorsal horn wide dynamic range (WDR) neurons which suggests that the descending connections are mainly facilitatory (Suzuki et al., 2002). Additionally, this ablation can reduce the receptor field sizes in the hind paw and their ability to accurately code both mechanical and thermal stimuli (in an animal model of peripheral neuropathy) compared to saporin controls (Suzuki et al., 2005). Further connections are made from the PB to the amygdala with similarly descending projections. Consequently, the amygdala has the ability to modulate the PB and accounts for the affective aspect of pain. Other transmitters in addition to serotonin have been implicated in descending facilitation – the peptide CCK when microinjected into the RVM of naive rats produces a robust mechanical hypersensitivity (Kovelowski et al., 2000), that is associated with the activation of OFF cells, and additionally prevents the antinociceptive actions of systemic morphine (Heinricher et al., 2001). Over 80% of RVM cells co-express the mu opioid receptor (MOR) and CCK_B receptor, and ablation of these cells using CCK-saporin or dermorphin-saporin respectively does not alter basal sensory thresholds but abolishes the tactile hypersensitivity induced by microinjection of CCK into the RVM (Zhang et al., 2009). Finally, the neuropathic pain drug pregabalin has been proposed to require exacerbated descending facilitations as seen in nerve injury to exert its mechanistic action (Bee and Dickenson, 2008), most likely by restoring the normal responses to nociceptive inputs.

Descending noradrenergic pathways can exert inhibitory influences onto the spinal cord, acting primarily via spinal α_2 -adrenoceptors. These pathways originate from regions such as the locus coeruleus (LC) and RVM. This descending inhibition is observed following peripheral nerve injury. It is possible that this is a mechanism to restore increased excitatory drive in the spinal cord. In fact clonidine – a partial α_2 -adrenoceptor agonist is

used clinically for the alleviation of some neuropathic pain states, and is effective in cancer pain (Eisenach et al., 1996). Upregulation of α_2 -adrenoceptor in the cord may explain the added effect of α_2 -adrenoceptor agonist in reducing pain-like behaviour following peripheral nerve injury. The release of noradrenaline at the spinal cord level could additionally provide inhibition by activating inhibitory interneurons (e.g. GABA or glycine) in the superficial lamina. Other neurotransmitters may account for the descending inhibition as spinal projections of RVM contain GABA, glycine, serotonin somatostatin, and enkephalins (Millan, 2002). The cannabinoid system may also have a role to play; the cannabinoid receptor 1 (CB₁) is particularly abundant in the PAG, the dorsolateral funiculus, and the superficial lamina of the dorsal horn (Farquhar-Smith et al., 2000; Herkenham, 1991). Microinjection of CB₁ agonists into the PAG or RVM of naïve rats results in antinociceptive in regards to the plantar test (Lichtman et al., 1996; Maione et al., 2006), however pronociceptive effects have been observed with low doses (Maione et al., 2006). These paradoxical effects have been associated enhanced or reduced activity of RVM OFF cells (Maione et al., 2006), suggesting that these actions occur via stimulation or inhibition of excitatory PAG projection neurons. The endocannabinoids additionally appear important in manipulating descending pain pathways (Petrosino et al., 2007), and CB₁ agonists are being perused clinically as novel analgesics, though they are yet to be fully evaluated and there are concerns regarding their psychoactive associations and potential for abuse (Rice, 2008).

1.4.5.5 Immune cell mediated sensitivity of the spinal cord

It was previously thought that only neurons were involved in regulating pain responses, but there is an increasing body of evidence from animal studies indicating the role of glial cells in maintaining both acute and chronic pain states (Watkins et al., 2001b). Several types of glial cells exist in the CNS, namely, microglia, astrocytes, and oligodendrocytes which differ in their associative functions but collectively act to support neuronal function and maintain homeostasis. Glia which outnumbers neurons 10 to 1 in the CNS, provides an additional important function which is to provide protection from pathogens such as viruses and bacteria that can cause their activation. However peripheral nerve injury can result in the activation of glia and cascade of immune responses in the spinal cord, of which,

microglia and astrocytes have been particularly implicated (Tsuda et al., 2005). There is also evidence to suggest immune cells other than astrocytes and microglia such as T cells are involved in neuropathic pain mechanisms (Zenonos and Kim, 2010).

Microglia

Microglia constitutes 5-10% of the glial population in the adult CNS, and are considered to be of macrophage lineage (Gehrmann et al., 1995). Microglia under normal conditions are not actively implicated in nociceptive processing and often considered to be in a 'resting' state, but they are constantly surveying the environment for potential foreign antigens. In the context of peripheral nerve injury, spinal microglia aggregate in affected regions of the spinal cord where they become activated and interact with sensory neurons. When activated, microglia experience morphological changes where they become hypertrophic and adopt an amoeboid shape in contrast to their normal dendritic shape (Kreutzberg, 1996). Several proinflammatory cytokines are synthesized and released including IL- β , TNF α , PGE $_2$, cytotoxic mediators such as ROS, proteases, and excitatory amino acids, which act to sensitise nociceptors and are associated with pain hypersensitivity (Ji and Strichartz, 2004). An important driver in the production of proinflammatory cytokines in microglia following nerve injury is the activation of p38 mitogen activated protein kinase (MAPK) and the resulting signaling cascade. Inhibitors of p38 can reverse mechanical hypersensitivity in a range of nerve injury models (Ji and Woolf, 2001). Ectopic dischargers from primary afferents following nerve injury can result in the release of neurotransmitters such as ATP, substance P, and glutamate in the dorsal horn of the spinal cord. The receptors for these neurotransmitters are expressed on microglia; therefore they are likely to activate p38 as well as other MAPKs such as ERK (Ji and Strichartz, 2004). The activation of microglia has been suggested to be associated with large A fibre activity since the blockade of TRPV1 fibres (putative C-fibres) using resiniferatoxin in the sciatic nerve could not prevent peripheral nerve injury induced microglial activation (Suter et al., 2009). The pharmacological blockade of spinal P2X4 receptors (receptor for ATP) results in attenuation of tactile mechanical hypersensitivity following nerve injury (Tsuda et al., 2003). There is also involvement of the chemokine CCL2 and its CCR2 (which is expressed on microglia) since intrathecal CCL2 in naive animals leads to activation of

microglia and the induction of neuropathic pain-like behaviours (Dansereau et al., 2008;Thacker et al., 2009), correspondingly a knockdown of the CCL2 (Abbadie et al., 2003) or the use of CCL2 antagonists (Dansereau et al., 2008) leads to the reduction of neuropathic pain-related behaviours. Minocycline, an antibiotic that can inhibit the activation of microglia can prevent the initiation of mechanical hypersensitivity following SNT and gp120-associated nerve damage (Ledeboer et al., 2005;Raghavendra et al., 2003). Although no effects were observed on establish nerve damage induced mechanical hypersensitivity despite the attenuation in inflammatory mediators in the L5 spinal cord, thus indicating that microgliosis is important for the initiation rather than maintenance of neuropathic pain. Coull and collages (2005) investigations proposed a further mechanism where microglia activation indirectly alters GABA_A currents so that its activation causes depolarization rather than hyperpolarisation and hence the sensitization of spinal neurons. The release of BDNF from activated microglia leads to the reduction in the potassium-chloride co-transporter (KCC) transporter in lamina I of the spinal cord, thus leading to an increased intracellular chloride concentration. This shift in GABA_A currents can be mimicked by administration of BDNF (Coull et al., 2005). Furthermore, inhibiting the release of BDNF from microglia via treatment with interfering RNA prevents the alteration in chloride currents, and the associated behavioural hypersensitivity (Coull et al., 2005). Additionally, the eventual depolarization by GABA_A currents may lead to the removal of the voltage dependent Mg²⁺ block of the NMDA receptor, which is required for the receptor activation.

Several immunophenotypical changes occur to microglia following nerve injury whereby microglia upregulates cell surface markers. These include major histocompatibility complex (MHC) class 1 & II (Streit et al., 1989), complement receptor-3 (CR-3 or CD11b/18) (Graeber et al., 1988), co-stimulatory molecules B7-1 & B7-2 (Williams et al., 1994), TLR3 & TLR4 (Obata et al., 2008;Tanga et al., 2005), and CXCR1 (Lindia et al., 2005). The upregulation of cell surface markers can further activate microglia and enable them to interact with other immune cells such as T cells (Hickey et al., 1991). The identification of activated microglia is often performed using the antibody OX42 which labels CD11b and the morphological status regarding their amoeboid shape can be observed (Graeber et al., 1994). The purinergic receptor, P2Y₁₂, which binds adenosine

diphosphate (ADP) has been reported to be upregulated (Tozaki-Saitoh et al., 2008), but the consequences of its expression is yet to be elucidated. Finally, the cannabinoid (CB)₂ receptor which demonstrates limited expression in healthy CNS tissue, (Graeber and Christie, 2012) is upregulated in microglia. Moreover, the activation of the CB₂ receptor has been shown to attenuate hypersensitivity associated with inflammation and nerve damage (Ibrahim et al., 2003;Quartilho et al., 2003) and therefore this activation may be related to reducing microglial activity or the inflammatory burden on neurons in the spinal cord. Spinal microgliosis appears to be a consequence of traumatic rather than non-traumatic nerve injury. Previous studies have demonstrated via immunohistochemical analysis or flow cytometry, increased numbers of cells expressing CD11b (microglial marker) in SNT or CCI models and to a lesser extent in the HIV drug induced neuropathy models but not the varicella zoster virus (VZV) or cancer chemotherapy drug induced models (Blackbeard et al., 2012;Zheng et al., 2011a).

Astrocytes

The astrocytes are the most abundant glial cells, representing 40-50% of the total glial population in the CNS and are in close proximity to neurons (Aldskogius and Kozlova, 1998). Although both microglia and astrocytes are activated following peripheral nerve injury, the microglial response precedes that of astrocytes. An increase in microglial markers occurs within a few hours of nerve injury whereas the increase in the astrocyte marker – glial fibrillary acidic protein (GFAP) is not detected until at least 4 days (DeLeo et al., 2004). In addition, ERK activation shifts from microglia to astrocytes 2-3 weeks after nerve injury therefore astrocytes may play a role in the maintenance of neuropathic pain (Zhuang et al., 2005). Astrocytes are thought to be activated in response to neurotransmitters/neuromodulators (e.g. glutamate, ATP, TNF α) following nerve injury resulting in the activation of JNK and ERK signaling pathways (Dominguez et al., 2008;Tsuda et al., 2011). There is an increasing list of signaling molecules/events released from astrocytes that can contribute directly or indirectly to the sensitization of nociceptors. Astrocytes release proinflammatory cytokines (e.g. IL-1 β and TNF α); chemokines (e.g. CCL2) (Gao and Ji, 2010); and proteases, such as tissue-type plasminogen activator (tPA) and matrix metalloproteinase (MMP)-2 and 9, which are involved in the cleavage and release of signalling molecules (Kozai et al., 2007). The glutamate transporter-1 is

abundantly expressed on astrocytes (Beart and O'Shea, 2007), and aids in the clearance of glutamate released from synapses (Huang and Bergles, 2004). However in nerve injured animals, following an initial upregulation, this transporter is down-regulated (Sung et al., 2003). This may lead to elevated concentrations of glutamate at the synapse which can act on NMDA receptors, thus modulating neuronal plasticity. The use of pharmacological agents to depress activation of astrocytes can reduce tactile mechanical hypersensitivity associated with nerve injury (Sweitzer et al., 2001).

T cells

T lymphocytes (or T-cells) are key constituents of the immune system and collectively act to defend the body against pathogens or foreign antigens that can cause harm or disease. There are two main types of effector T cells which have different functional roles, namely, T helper (Th) and T cytotoxic (Tc) (Parkin and Cohen, 2001). Previous studies have demonstrated Th cell infiltration in the dorsal horn of the spinal following SNI and is associated with the induction of neuropathic pain-like hypersensitivity (Cao and Deleo, 2008) with interferon (IFN) γ and its receptor - IFN γ R1, being a key mechanism (Costigan et al., 2002). This infiltration peaked at 14 days post initial injury, a time point which is often the peak of behavioural hypersensitivity. It is not entirely clear how T-cells are able to infiltrate the dorsal horn. T-cells can enter the CNS through the blood brain barrier by leukocyte extravasation and this process may be substantially elevated in nerve injury (White et al., 2007). Other studies support IFN γ in regulating nociception; the intrathecal administration of IFN γ elicits behavioural hypersensitivity in naive animals (Vikman et al., 2005; Xu et al., 1994), which is likely to act on the spinal IFN γ R1, where it has been shown to sensitise dorsal horn neurons (Vikman et al., 2005; Vikman et al., 2003). A knockout of the IFN γ R reduces the mechanical hypersensitivity following SNI, and expression of both ligand and receptor increases after peripheral nerve injury (Costigan et al., 2009). A more recent study has shown that the intrathecal administration of triptolide, a T cell inhibitor, attenuates CCI-induced mechanical hypersensitivity in rats (Hu et al., 2012). Taken together these studies further support the role of T cells in mediating neuropathic pain.

1.4.6 Models of peripheral nerve injury

Neuropathic pain is a complex condition which involves several different underlying etiologies, and may elicit a diverse range of symptoms and associated co-morbidities. There are both ethical and legal reasons for why humans cannot be subjected to explicit experimentation and therefore animal models have become an alternative and useful approach to investigate the potential mechanisms and treatments for neuropathic pain. By using a living animal the interactions between different organ systems, tissues, and cells can be investigated and therefore a better understanding of the pathological process can be attained.

Over the past 30 years there has been a focus and development on producing animal models of nerve injury that are intended to be a basis upon which mechanisms of neuropathic pain can be understood. These animal models have been largely based on peripheral nerve injuries usually via traumatic means and the subsequent development of hypersensitivity to mechanical stimuli. These animal models have proved useful in advancing understanding of pain mechanisms. However there are a number of shortcomings in animal models of nerve injury that should be addressed. Firstly, the major outcome measure in animal models of peripheral neuropathy is response to evoked hind limb withdrawal in response to mechanical stimulation. While the mechanical hypersensitivity that develops following nerve injury is a reliable outcome measure, it can only be described as ‘pain-like’ behaviour because animals cannot verbally communicate, therefore the notion that the animal is in pain cannot be confirmed. It is worth mentioning that hyperalgesia and allodynia are terms used to describe types of pain in humans but are often used to describe the same phenomena in rodent models of peripheral nerve injury. The use of such terminology is misguided since there are no tools to actually measure pain in rodents per se, therefore the word ‘hypersensitivity’ is more appropriate. Secondly, while evoked pain is common in neuropathic pain patients, the overwhelming symptom is spontaneous and/or lancinating pain. Spontaneous hypersensitivity is not routinely measured in nerve injury animal models (Mogil and Crager, 2004). Although some attempts have been made to measure ‘spontaneous pain’ in rodents, through the use of grimace scales (Sotocinal et al., 2011), animals cannot verbally convey the sensation of

spontaneous pain; therefore this assumption cannot be made. Thirdly, there are issues on how clinically relevant nerve injury models are and how the research is translated to benefit humans. Certain types of neuropathic pain maybe more prevalent in females than in males e.g. trigeminal neuralgia (Sadosky et al., 2008); additionally certain types of neuropathic pain such as PHN demonstrate increase prevalence with age, however studies typically use young male rodents. There are practical, ethical, and methodical reasons why young male rodents are commonly used, for example, using younger rodents makes experimental research cost effective. Additionally, the preference of male rodents is justified on the basis that female rats have fluctuations in ovarian hormones that could alter experimental outcomes, and could lead to misinterpretation of data. It is to be noted that not all humans who obtain nerve injuries develop neuropathic pain; it is only a subset of patients. However animal models of traumatic nerve injury for ethical reasons have been especially designed to consistently demonstrate a common primary outcome measure of nerve injury models; namely mechanical hypersensitivity, in order to minimize the total number of animals used in experiments. Neuropathic pain is chronic in nature, however nerve injury animal models are usually studied for short periods of time; typically between 2-4 weeks post injury. It is currently unclear the extent to which the chronicity of animal models extrapolates to the clinical situation. There also seems to be a uniform approach in regards to the species and strains of animals used to study, with a large focus on rodents, however rodent models may not always prove the best model in investigating aspects of neuropathic pain and perhaps a more integrated approach is warranted. Nevertheless, improving or refining a number of factors in the design of nerve injury animal models may improve their predictive validity when testing the efficacy of new compounds in clinical trials.

1.4.6.1 Models of traumatic peripheral nerve injury

The first animal models of peripheral nerve injury involved axotomy of the sciatic nerve which resulted in the complete absence of the flexion reflex and the self-mutilation (autotomy) of the denervated hind paw (Wall et al., 1979). This model was thought to mimic the symptoms of ‘phantom pain’ often experienced in patients with amputation. The self-mutilation was often observed and interpreted as a response to pain – the authors indicating that the neuroma formed at the site of nerve transection is the source of afferent discharge and could be perceived as pain. However it is argued whether the autotomy is actually a pain-related behaviour and not a result of excessive grooming in the absence of sensory feedback (Blumenkopf and Lipman, 1991;Coderre et al., 1986;Rodin and Kruger, 1984). It was later found that the infusion of lidocaine on the transected sciatic nerve could reverse autotomy, indicating that this behaviour was a response to ‘pain’ rather than an attempt to remove a denervated part of the paw. Destruction of the DRG via suicide transport of ricin failed to reverse the autotomy behaviour; with this conflicting observation it is still unclear what the self-mutilation infers (Blumenkopf and Lipman, 1991). Since the hind paw is completely denervated evoked mechanical hypersensitivity cannot be measured. Partial nerve injury models are now used in favor of the older neuroma models to avoid complications in the interpretation of autotomy behaviour associated with such injury. The first peripheral nerve injury models described were the CCI model (Bennett and Xie, 1988), the partial sciatic nerve ligation model (PSNL) (Seltzer et al., 1990), and the SNL (Kim and Chung, 1992). The advantage of the partial nerve injury models over the neuroma model is that not all of the axons in the sciatic nerve are damaged and therefore responses to mechanical and thermal stimuli in the hind paw can be measured. In addition, autotomy behaviour is rarely observed in the partial nerve injury models. Autotomy is an undesirable outcome measure of nerve injury associated behaviour, and there are also ethical issues regarding this behaviour as an experimental endpoint in preclinical studies.

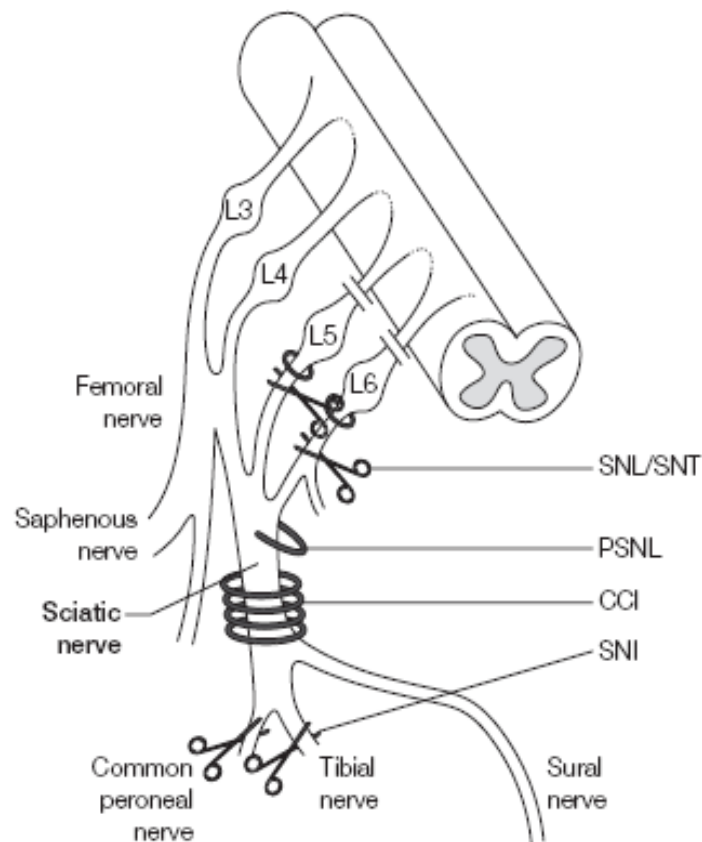


Figure 1.3 Illustration of the different types of peripheral nerve injury models used. Injury to the spinal nerve involves the SNL (spinal nerve ligation) or the SNT (spinal nerve transection) models. The sciatic nerve trifurcates into the common, tibial, and sural nerve branches. TNT (tibial nerve transection) involves ligation and transection of the tibial nerve which is the biggest of the three terminal sciatic nerve branches. SNI (sparing nerve injury) involves ligation and transection of the common and tibial nerves, leaving the sural nerve intact. CCI (chronic constriction injury) involves 4 separate loose ligations of the sciatic nerve. PSNL (partial sciatic nerve injury) involves a 30-50% ligature of the sciatic nerve trunk. Image taken from Wallace and Rice (2008).

Bennett and Xie (1988) developed the CCI model of peripheral neuropathy that attempted to reflect the clinical condition of causalgia or complex regional pain syndrome in patients. The CCI model involves 4 loose ligations of the sciatic nerve (1-2mm apart) at the mid-thigh level with chromic gut sutures. Gait and posture in these animals are altered; animals tend to adopt a protective guard-like position, excessive licking, and limping which suggest elements of spontaneous pain. Hypersensitivity to mechanical, thermal, and cold stimuli is observed following CCI (Bennett and Xie, 1988). The ligation of the sciatic nerve leads to swelling in the area surrounding the constriction, with degeneration and loss of the larger diameter A β and A δ fibres with the smaller diameter C-fibre population being less affected. The PSNL model involves ligation of approximately 30-50% of the trunk of the sciatic nerve at the mid thigh level and is associated with both mechanical and thermal hypersensitivity in the hind paw that lasts several months (Seltzer et al., 1990). The disadvantages of the PSNL and CCI models are that there are intra and inter operator differences in the size and tightness of the ligation between each animal, and therefore the degree of nerve injury is not the same – this can translate to behavioural differences as well as variation in the expression of neurochemical markers. In addition, chromic gut sutures, when used to produce the CCI, leads to rats exhibiting more time with the hindpaw in an abnormal position compared to CCI conducted with plain gut, or polyglactin (Vicryl) sutures, suggestive of greater paw hypersensitivity. This may also suggest that chromic gut sutures may have more than just a constrictive effect on the sciatic nerve compared to the use of plain gut, or polyglactin sutures, therefore inducing variability amongst the CCI model (Kajander et al., 1996). The SNL model represents the most frequent and commonly used model and involves the unilateral tight ligation of the L5 or both L5 and L6 spinal nerves. The nature of the injury means that the damage can be isolated to a particular DRG sparing adjacent DRGs from actual injury – this allows investigations into both the injured and uninjured afferents following nerve injury. For this reason, the SNL model is unique because it provides a standardization of nerve injury which in comparison is difficult to achieve with the previous PSNL or CCI models. The SNL model results in hypersensitivity that lasts for a period of at least 4 months (Kim and Chung, 1992). Moreover, the SNL model is also associated with what has been interpreted as ‘spontaneous pain-like behavior’ similar to that observed in the PSNL and CCI models (Kim et al., 1997). One disadvantage however is the SNL surgical procedure is more complex than the PSNL or

CCI models and requires more extensive surgical exposure which allows the possibility of unintentional collateral damage. Nonetheless, the high reproducibility and consistency of the mechanical hypersensitivity makes it a popular model. A modification of the SNL has been developed which involves the subsequent transection of the spinal nerves following ligation (spinal nerve transection (SNT) model) (Bridges et al., 2001a). The consequences of this adaptation are identical to that of the SNL animals however this step alleviates any concerns regarding the rigidity of the ligation (Decosterd and Woolf, 2000). The ligation of the spinal nerve is intended to be so tight that it induces axotomy and therefore 'transection' of the spinal nerve. However, it can be argued the extent to which axotomy occurs, therefore completing a true transection would provide confirmation of the axotomy. A modification of the SNT has been described that has been proposed as a model of 'spontaneous pain' – this involves the ligation of the L4 spinal nerve (Djouhri et al., 2006). The spontaneous activity of intact C-fibre DRG neurons and foot lifting are linked to the neuroinflammation of the L4 spinal nerve.

Spared nerve injury

Recently, the ligation or transection of the sciatic nerve branches has been described as a model of partial nerve injury. The sciatic nerve forms a trifurcation and branches off into the; tibial, common peroneal, and sural nerves. One model that has been described is the spared nerve injury model (SNI) which involves ligation of the common peroneal and tibial nerves thus leaving the sural nerve intact, which results in a robust mechanical and cold hypersensitivity (Decosterd and Woolf, 2000). The SNI model differs from the earlier partial sciatic nerve injuries models in the sense that there is separation of injured and uninjured axons in the sciatic nerve, and it permits sensory testing in distinct areas of the hind paw associated with axonal injury, as well as those adjacent areas where innervation is intact. Behavioural changes are observed in the ipsilateral sural and to a lesser extent in the saphenous territories. Ligation and transection of one or a combination of the sciatic nerve branches has been associated with the development of hypersensitivity in the hind paw of rats (Lee et al., 2000). Out of all the possible combinations, the tibial and sural nerve transection, and the tibial nerve transection alone produced the most robust mechanical hypersensitivity which is still present at 10 weeks post injury (Lee et al., 2000).

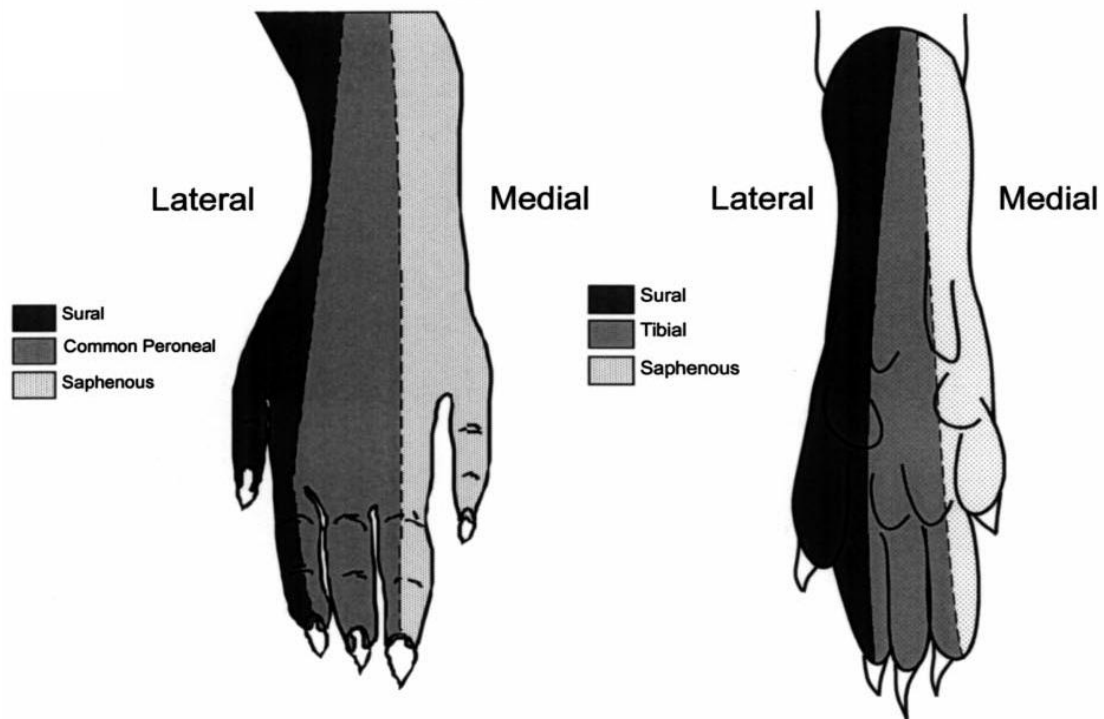


Figure 1.4 Illustration of the different zones of the dorsal and plantar surfaces of the rat paw innervated by the sciatic nerve terminal branches and saphenous nerve. The lateral glabrous part of the paw is innervated by the sural nerve. The mid-plantar region is innervated by the tibial nerve and the medial part is innervated by the saphenous nerve. (adapted from Decostered and Woolf, 2000).

Tibial nerve transection

Since robust mechanical hypersensitivity can be achieved through the ligation and transection of the tibial nerve alone, it has led to the use of TNT injury as a model to study neuropathic pain mechanisms (Andrews et al., 2011; Lee et al., 2000; Unal-Cevik and Oaklander, 2011). Like the SNL model, the nerve injury is isolated to one nerve region which means the distribution of injured and uninjured populations can be standardised and is less variable between animals. It is to be noted that the ease of preparation and reproducibility is a factor in the development of peripheral nerve injury models. TNT surgery offers a less complex and time consuming procedure that is in contrast to the SNL which involves removal of the L6 spinal transverse process, increasing the likelihood of collateral damage that could prolong recovery. Following TNT injury the mid-plantar region of the paw becomes denervated and is reportedly non-responsive towards applied mechanical stimuli for up to 63 days (Unal-Cevik and Oaklander, 2011). The mechanical hypersensitivity that develops is evident in the sural nerve territory (lateral region) of the paw (Lee et al., 2000; Unal-Cevik and Oaklander, 2011). Although the mid-plantar region of the paw is non-responsive towards applied mechanical stimuli, there is evidence to suggest the contrary. Mechanical hypersensitivity has been reported 2-3 weeks following injury lasting for at least 2 months (Andrews et al., 2011; Hofmann et al., 2003). In addition, a response to pin prick is observed in some animals 21 days following injury (Unal-Cevik and Oaklander, 2011). This suggests that sensory function in the denervated skin may gradually recover, though the reason for this is not clear. One aspect of the TNT model is the development of robust cold hypersensitivity. This element of the model could provide a suitable platform to investigate cold related neuropathic pain mechanisms.

1.4.6.2 Non-traumatic models of peripheral nerve injury

While physical trauma and nerve compression (e.g. radiculopathies) to peripheral nerves can trigger neuropathic pain, the most widespread causes arise from a number of different aetiologies including; diabetes, herpes zoster, human immunodeficiency virus (HIV) infection and its therapy, and certain cancer chemotherapy agents (Jensen et al., 2001). Traumatic animal models of nerve damage are frequently used preclinically to understand the mechanisms of neuropathic pain and test new potential drugs, however the majority of patients that undergo clinical trials are those with PHN or DPNs (Finnerup et al., 2010a). Traumatic models of peripheral nerve injury are used for their high intra and inter user reproducibility and reliability (in terms of the development of mechanical hypersensitivity), however the relevance to other neuropathic pain states such as PHN or DPN is understated since there may be different underlying mechanisms. Several groups have now developed, characterized, and refined clinically relevant rat models of peripheral neuropathy that are specific to several human neuropathic pain conditions. These include the VZV associated neuropathy (Fleetwood-Walker et al., 1999;Garry et al., 2005;Hasnie et al., 2007), HIV-1 gp120 induced neuropathy (Herzberg and Sagen, 2001;Wallace et al., 2007a;Wallace et al., 2007b) and drug-related neuropathy models (Flatters and Bennett, 2004;Joseph et al., 2004;Wallace et al., 2007a;Wallace et al., 2007b). These animal models may be useful in elucidating the mechanisms behind those neuropathic pain states.

VZV-associated neuropathic pain

PHN is the most frequent complication following an outbreak of herpes zoster (shingles) and risk factors are associated with those who have a decline in cell mediated immunity, especially the elderly (Gilden et al., 2003). PHN is the pain that arises after resolution of the herpes zoster rash lesions, usually occurring in 1-3 dermatomes (specific region of the skin innervated by a particular DRG) (Gilden et al., 2003). During infection free VZV virions are able to infect sensory neuronal terminals and are retrogradely transported to the cells bodies of sensory neurones in the DRG where they persist, usually, for the life time of the host (Cohrs et al., 2000). In humans the virus persists in a latent state (Kennedy et al., 2001) whereby the host is asymptomatic. However, under conditions of depressed cell-

mediated immunity (e.g. HIV disease) the virus may ‘re-activate’ to cause an acutely painful herpes zoster rash in affected dermatomes. It was previously thought that VZV could not infect sensory neurons of other species because VZV is largely species specific *in vitro* and *in vivo* with humans as its preferential hosts. Initial attempts to create a model of Varicella or VZV-related neuropathy by experimental inoculation (using VZV infected cells) only led to seroconversion with no overt clinical symptoms (Matsunaga et al., 1982; Myers et al., 1980; Myers et al., 1985). In 1990 Sadzot-Delvaux and colleagues reported for the first time an *in vivo* rat model, the presence of VZV viral proteins and nucleic acid in the Lamina 5 (L5) DRG of VZV infected rats at multiple intervals during a 9 month period (Sadzot-Delvaux et al., 1990). Fleetwood-Walker and colleagues (1999) then went on to infect with VZV (Dumas strain). Rats infected with VZV developed mechanical and thermal hypersensitivity within 14 days of subcutaneous inoculation of the glabrous paw of the hind limb (Fleetwood-Walker et al., 1999; Garry et al., 2005). The pain-related behaviour in VZV infected rats was replicated in an identical study (Garry et al., 2005). Moreover, in these animals there was evidence of immunoreactivity against viral proteins in the L4-L6 DRGs. Western blot techniques has demonstrated the presence of activating transcription factor-3 (ATF-3) nerve injury marker and the neuropeptides NPY & galanin (Garry et al., 2005), which are all expressed in traumatic nerve trauma models (Ma and Bisby, 1997; Ma and Bisby, 1998; Tsujino et al., 2000). The mechanical hypersensitivity developed after VZV inoculation has been reported to persist for up to 90 days (Dalziel et al., 2004) and is sensitive to clinically used analgesics such as gabapentin and amitriptyline (Hasnie et al., 2007). In a more recent model where the Dumas strain was used, neither thermal nor cold hypersensitivity were observed (Hasnie et al., 2007). Although a lower VZV viral “dose” was used, this model improves on the earlier model (Fleetwood-Walker et al., 1999) and draws parallels to the clinical situation where there can be degree of thermal sensory loss in PHN patients (Besson et al., 2005; Rowbotham and Fields, 1996). In addition, the refined model also exhibits anxiety-like behaviours which was shown to correlate with the pain-related behaviour (Hasnie et al., 2007) (see **Section 1.4.7.1** Assessment of anxiety-like behaviours). The rat VZV model is proving useful for the identification of potential drug targets; two novel histamine H₃ receptor antagonists have shown efficacy in reversing VZV-associated mechanical hypersensitivity of the hind limb when administered orally (Medhurst et al., 2008). The VZV model epitomizes features of

neuropathic pain and has been replicated in several laboratories. However, the VZV model has some limitations and emphasis should be placed on the fact that this is not a model of PHN, nor a model of herpes zoster, but simply a model whereby 'pain' behaviours are exhibited in rodents whose DRG are infected by VZV. Aspects of the model draws some parallels with the clinical situations of herpes zoster and PHN (Kinchington and Goins, 2011). The reproducibility of the model depends on the viral virulence which may vary between strains, in addition there are posed risks in the handling of virus that is a known pathogen to humans. Inoculation of cell associated VZV into the hind limb of rats infects sensory neurons; however there is no truly active infection. No varicella-like disease has been observed with this rodent model. An ideal model of PHN would constitute a resolving period of the primary infection (chickenpox), followed by a reactivation period of herpes zoster and the development of neuropathic pain-related behaviour after resolution of the rash. However this is not attainable in the rat model due to the nature of VZV being species specific to humans.

HIV disease associated neuropathic pain models

A common complication of HIV disease is the development of peripheral neuropathies, which can affect up to 50% of patients (Verma S et al., 2007). These neuropathies may develop as a direct consequence of HIV infection (HIV-related neuropathy), or as a result of highly active antiretroviral therapy agents (antiretroviral toxic neuropathy (ATN)). Both types of neuropathies can lead to the development of neuropathic pain symptoms which are characterised by a progressive 'die back' degeneration of small fibres, usually affecting those with the longest axons - typically the hands and feet. Although the clinical presentations of these two are very similar, they have distinct pathological aetiologies.

Gp-120 associated neuropathy

HIV-related neuropathies are not thought to occur via direct infection of sensory neurons (Lipton, 1991;Michaels et al., 1988), but rather via an interaction of the HIV envelope glycoprotein 120 (gp120) with neuronally-expressed chemokine receptors such as CCR5 and CXCR4 (Kaul and Lipton, 1999;Oh et al., 2001). Macrophages also play a significant

role in the gp120 associated sensory neuropathy (Kamerman et al., 2012). Immunohistochemical analysis has revealed the expression of the CXCR4 and CCR4 receptors on DRG neurons that also expressed substance P and TRPV1 indicating that they are small diameter neurons of a nociceptive phenotype (Oh et al., 2001). The toxic effects of soluble gp120 can be observed on cultured DRG neurons where they induce apoptosis through caspase-3 dependent mechanisms (Keswani et al., 2003b; Melli et al., 2006) and axonal injury resulting in a reduction of neurite length. Furthermore, gp120 when applied to DRG neurons results in excitation and the release of substance P (Oh et al., 2001). In line with *in vitro* findings, a number of studies have now reported toxic effects that is associated with the development of mechanical hypersensitivity following either intrathecal or perineural administration of gp120 in rats (Bhangoo et al., 2007; Herzberg and Sagen, 2001; Milligan et al., 2000; Wallace et al., 2007a) and mice (Minami et al., 2003). Thermal hypersensitivity has additionally been observed (Herzberg and Sagen, 2001; Milligan et al., 2000), and may be related to the higher doses of gp120 protein used in those studies, or the spinal administration as performed in Milligan et al (2000). The intrathecal administration may be considered a more robust approach since the spinal cord is usually acknowledged as an immunological privileged area. The mechanical hypersensitivity that develops is linked with the infiltration of microglia in the spinal cord and macrophages in the sciatic nerve and DRG (Wallace et al., 2007a). These immune cells can increase the production of cytokines such as TNF α , nitric oxide, eicosanoids and other proinflammatory mediators (Holguin et al., 2004) which when released can act to sensitize neurons. Inflammatory responses such as these are known to contribute towards neuropathic pain mechanisms, and the microglial response in this model is sensitive to treatment with microglial inhibitor minocycline (Ledeboer et al., 2005). Several studies have reported that inflammatory mediators released by HIV-infected macrophages may contribute indirectly to the neurotoxicity (Hahn et al., 2008; Jones et al., 2005; Zhang et al., 2003). Hahn et al., (2008) demonstrated that supernatant taken from cultured human monocytic cells infected with HIV can inhibit neurite growth in cultured human foetal DRGs. Experiments using recombinant HIV-1 clones derived from virus extracted from peripheral nerves of HIV-infected individuals has been shown to increase the expression of IL-1 β and TNF- α mRNA in cultured rat DRG, with the most likely source being macrophages (Jones et al., 2005).

The presence of ATF-3 and caspase-3 in the DRG of gp120 sciatic-nerve treated animals suggests nerve injury and apoptotic processes, with small diameter cells particularly affected (Wallace et al., 2007a). This correlates with the decreased intra epidermal nerve fibre density which mirrors the 'dying back' of small fibres that is observed clinically (Martin et al., 2003). An increase in the expression of the NPY, galanin, GFAP (satellite cell marker) are additionally observed, these findings are features associated with traumatic nerve injury models (Ma and Bisby, 1997; Ma and Bisby, 1998; Wynick et al., 2001). Collectively, this *in vivo* evidence suggests a likely role of gp120 facilitating neuropathic pain in humans. In addition to the sensory changes, the gp120 model also develops correlates of anxiety like behavior when placed in the open field, reflecting the emotional burdens of neuropathic pain (Wallace et al., 2007a). An important requisition of animal models is the demonstration of predictive (pharmacological) validity. Analgesics such as morphine and gabapentin which are effective for treating neuropathic pain in the clinic both demonstrate efficacy in reversing mechanical hypersensitivity in the gp120 model (Wallace et al., 2007a). Although, it is to be noted that the effectiveness of morphine and gabapentin reducing mechanical hypersensitivity associated with peripheral nerve injury (including trauma) is a phenomenon well documented in the literature (Abdi et al., 1998; Backonja et al., 1995; De Vry et al., 2004; Lee et al., 1994). Nevertheless, the use of amitriptyline (which was not effective in reducing mechanical hypersensitivity associated with gp120 exposure to the sciatic nerve) (Wallace et al., 2007a) further validates the use of this model, given that this drug has been shown not to be effective for treatment in HIV related neuropathy (Kiebert et al., 1998; Shlay et al., 1998). The CB₁ agonist WIN55,212-2 was equally efficacious, which may have positive ramifications for therapy. Recently, there has been an increasing amount of literature proposing an alternative hypothesis for gp-120 related toxicity, involving an interaction with Schwann cells and their release of TNF α (Keswani et al., 2003b). Although this is data gathered from *in vitro* studies, it is a plausible *in vivo* hypothesis given gp-120 can induce TNF α release in the spinal cord of gp-120 treated animals (Ledeboer et al., 2005). Since the HIV infected individuals are subjected to the toxic antiretroviral treatments a model of this has been described to address this scenario. The combination of both gp120 + ddC to rats produces a mechanical hypersensitivity that is more robust than either treatment alone which indicates a synergistic action (Wallace et al., 2007b). This hypersensitivity has been shown to be

related to a further increase in microglia infiltration into the spinal cord. An alternative approach has additionally been described whereby the oral administration of didanosine (ddI) to transgenic mice expressing gp120 under a GFAP promoter results in mechanical hypersensitivity (Keswani et al., 2006). These findings indicate that the gp120 model may be useful for investigating the mechanism of HIV related pain in humans.

Antiretroviral associated neuropathy

The treatment of HIV infection involves the use of nucleoside analog reversetranscriptase inhibitors (NRTIs) which are known to be toxic to humans, and patients are likely to develop a painful neuropathy at the beginning of treatment. Risk factors have been shown to be associated with increasing age, height, and TNF α genotype (Affandi et al., 2008). A model of NRTI induced peripheral neuropathy has been described in many research laboratories whereby rats injected with NRTIs (e.g d4T, ddC, ddI) develop a dose-dependent bilateral behavioral mechanical hypersensitivity (Bhangoo et al., 2007; Dorsey et al., 2009; Joseph et al., 2004; Wallace et al., 2007b). Additional studies have demonstrated the development of thermal hypersensitivity (Joseph et al., 2004). These behaviours are robust and occur within 3 days of a single injection and can last up to 45 days before it resolves to pre-injury levels (Wallace et al., 2007b). The induction of mechanical or thermal hypersensitivity has been shown to occur through a variety of administration routes including intravenous and intraperitoneal at doses ranging from 10-50mg/kg. Drugs are best when prepared fresh in saline on the day of injection. The arrival of the ATN model has allowed an opportunity to study potential mechanisms of the disease process. Biochemical and anatomical studies have revealed that an eminent feature of ATN is the associated mitochondrial toxicity caused by persistent exposure to treatment (Zhu et al., 2007); however the exact mechanisms are not entirely clear. NRTIs inhibit mitochondrial DNA polymerase gamma - the enzyme responsible for the replication of mtDNA. Affected animals also demonstrate myelin splitting and intramyelin oedema in the sciatic nerve following chronic oral administration (20 weeks) (Schmued et al., 1996). Furthermore, injected animals demonstrate a loss of intraepidermal nerve fibre (IENF) density (Wallace et al., 2007b), but do not experience some of the features observed in traumatic injury such as microgliosis in the spinal cord (Zheng et al., 2011a). The analgesics morphine,

gabapentin, and the CB₁ agonist WIN55,212-2 demonstrated efficacy in reversing mechanical hypersensitivity associated with the NRTI peripheral neuropathy model, while amitriptyline was not effective (Wallace et al., 2007b). Further experiments using the NRTI peripheral neuropathy model have demonstrated the presence of anxiety-like behaviours in the open field (Wallace et al., 2007b), a co-morbidity frequently observed in neuropathic pain patients. Co-morbid conditions may also contribute towards the painful neuropathy - alcohol abuse, which alone also produces a recognized neuropathy, is one of the most important co-morbid risk factors for peripheral neuropathy in patients with HIV/ acquired immunodeficiency syndrome (AIDS). A model has been described that combines both alcohol consumption and NRTI treatment, which results in profound mechanical hypersensitivity (Ferrari and Levine, 2010), and could be used to study aspects of this clinical scenario.

Diabetic model

Although patients with diabetic related neuropathic pain can improve symptoms by appropriate management of circulating blood glucose, neuropathic pain can still persist in those patients. A model of diabetic neuropathy has been developed; this involves the systemic administration of streptozotocin, which selectively ablates the beta islets of langerhans cells in pancreas. This treatment leads to pathological changes in peripheral nerves which are associated with the development of both bilateral mechanical and thermal hypersensitivity (Courteix et al., 1993). The nature of the sensory changes is complex since both tactile and thermal changes are observed at various time points post injury. This model has nonetheless been frequently used in the testing of new pharmaceuticals such as gabapentin (Field et al., 1999). A recognizable feature of the model is the associative ill health of the animals which not only raises ethical questions but it can be argued the extent to which it influences behavioural sensory thresholds (Fox et al., 1999).

Trigeminal neuralgia

Trigeminal neuralgia is a disease characterized by paroxysmal and sharp lancinating attacks of pain in the facial area innervated by the trigeminal nerve (Shakur et al., 2011). The pain can be brought about via light touch and is often initiated by innocuous tasks such as chewing and talking. A model has now been described in the literature that involves CCI injury to the infra-orbital branch of the trigeminal nerve (Idanpaan-Heikkila and Guilbaud, 1999; Martin and Avendano, 2009). This injury results in the development of mechanical and thermal hypersensitivity in the vibrissal pad (whisker area). This model has acquired wide acceptance for investigating mechanisms of trigeminal neuralgia. The fan-like geometry of the multi-fascicled infra-orbital nerve results in different degrees of constriction on different fascicles; this could be a possible shortcoming of the model however that is natural consequence of the injury given the anatomy of the nerve. The ease of performing sensory threshold testing on the hind paw makes the sciatic nerve associated injury models a more appropriate choice in neuropathic pain research studies.

Summary

In summary, although the past 30 years has seen developments in neuropathic pain models there still many concerns that need to be addressed. Currently, with the different treatment options available for neuropathic pain, only one third of patients achieve pain relief of 50% or more, usually at the consequence of undesirable side effects (Dworkin et al., 2007; Finnerup et al., 2010b; Hempenstall et al., 2005; Phillips et al., 2010). These side effects may reduce patient compliance and thus further decrease quality of life for the patient. While it is of comfort that some patients achieve sufficient pain relief, the majority of patients will not receive adequate pain relief. This may be regarded as a therapeutic failure, which in part, may be explained by flaws in the approaches used to assess new therapies preclinically. Many studies investigating neuropathic pain mechanisms or examining potential therapies rely on the use of clinically non-relevant animal models of neuropathic pain (Mogil, 2009; Rice et al., 2008). These models involve direct injury to a peripheral nerve e.g. PSNL, CCI or SNL. While physical trauma to peripheral nerves can trigger neuropathic pain, the most widespread causes include other conditions such as

diabetes, herpes zoster, and HIV infection (Jensen et al., 2001) (see **Section 1.4.1**). There is an appreciation that animal models need to be refined in order to reflect the clinical scenario and thus improve their predictive accuracy for clinical trials (Mogil, 2009). In humans, only a few develop pain as a consequence of nerve injury, while in animal models, most will develop mechanical hypersensitivity that is analogous to pain in humans. The intricacies of this have not yet been addressed in animal models. Researches may need to analyse the ‘global picture’ of pain encompassing the emotional burdens such as anxiety or depression, therefore providing more outcome measures which will reduce the reliance on evoked sensory testing that only answers to one facet of a multi-dimensional condition. The risk factors of certain aetiologies of neuropathic pain, such as PHN are associated with the elderly; however this factor is rarely of concern in studies that use young rodents (Legg et al., 2009). The strain of the rodent, supplier, sex, and testing procedures are also important factors to consider. Experimental bias is acknowledge to negatively influence experimental outcome measures and therefore observer blinding and experimental bias should not be understated (Rice et al., 2008). Other more subtle factors are related to husbandry, dietary, and handling of animals all of which can affect experimental outcomes (Deacon, 2006). In light of the preceding discussion, the use of animal models has furthered understanding of neuropathic pain mechanisms, but it is clear that many improvements to the way animal models are designed and utilized are needed to overcome this ‘therapeutic failure’. These improvements may be time consuming, labour intensive or expensive, but ultimately may be necessary to address the obvious challenges that face neuropathic pain research and the discovery of new treatments.

1.4.7 Co-morbidities of neuropathic pain

Patients that suffer from neuropathic pain syndromes exhibit co-morbidities such as insomnia, stress, anxiety, and depression, that can affect quality of life, and are of a direct consequence of living with chronic pain (Daniel et al., 2008; Meyer-Rosberg et al., 2001; Nicholson and Verma, 2004). In certain neuropathic pain syndromes such as CRPS, comorbidities such as migraine, asthma, and osteoporosis have all been associated with the CRPS, thus presenting further clinical burdens. It is possible that the medical history of the patient prior to the onset of CRPS might provide an insight into its risk factors and potential underlying disease mechanisms (de Mos et al., 2008).

One factor that could explain an individual's poor response to treatment is the inability to appropriately address these comorbidities related to pain which may exacerbate the patient's entire experience of pain. For that reason, there has been a continuing drive to make animal models of neuropathic pain more 'clinically relevant' by assessing co-morbidities such as anxiety and depression in order to make them more predictive for efficacy in clinical trials. Although some progress has been made, there are obvious challenges since such co-morbidities are heterogeneous in origin, with symptoms at the psychological, behavioural, and physiological level.

1.4.7.1 Assessment of anxiety like behaviours

Behavioural tests such as the open field paradigm are classically used as a measure of anxiety-like behaviour in rodents (Cryan and Holmes, 2005; Suaudeau et al., 2000; Vaeroy et al., 2005) and are minimally invasive. This test takes advantage of ethologically relevant behaviour that consists of aversion to exposed well-lit areas, and the tendency to explore new environments. In the context of the open field, thigmotaxis is a wall hugging behaviour which is thought to reflect predator avoidance strategies (Prut and Belzung, 2003), and is sensitive to anxiolytics (Treit and Fundytus, 1988), therefore it demonstrates predictive (pharmacological) validity. A potential confounding factor of the open field paradigm is that it is based on the ambulation, and in nerve trauma injured models, full motor function may be of concern. In order to rule out any motor dysfunction the total

distance travelled inside the arena is often measured - if identical to sham or naïve control animals, this can rule out the likelihood of motor deficits. Previous studies using the open field have demonstrated anxiety-like behaviours in animal models of traumatic (Hasnie et al., 2007;Roeska et al., 2008;Seminowicz et al., 2009;Wallace et al., 2007c), HIV-related neuropathy (Wallace et al., 2007a;Wallace et al., 2007c;Wallace et al., 2007b), and VZV induced neuropathy (Hasnie et al., 2007). Other well characterized paradigms exist for analyzing anxiety-like behaviour such as the elevated plus maze (EPM) and the light dark box (Walf and Frye, 2007). The EPM consists of two exposed open arms and two closed arms whereas the light-dark box consists of a brightly lit chamber separated by an accessible dark chamber. It is likely that the various paradigms differ in their sensitivity to demonstrating anxiety-like behaviours. It is important to recognize that protocols regarding lighting and external factors such as noise can vary between laboratories; these are factors that are particularly sensitive to the outcome of these paradigms. Male rats should be considered for these sensitive assays since fluctuations in ovarian hormones in female rats could lead to behavioural changes that may be related to emotionality or anxiety (Palanza, 2001). Reductions in anxiety-like behaviour in female rats have been reported during the phases of proestrous and oestrus, when compared to the other stages metaestrous and diestrous (Mora et al., 1996). Finally, the genetic background of the rat could influence the reflex and behavioural parameters as seen in nerve injury models (Lopez-Rubalcava and Lucki, 2000;Palanza, 2001;Shepard and Myers, 2008).

1.4.7.2 Assessment of depressive-like behaviors

The forced swim test (FST) and the tail suspension test (TST) are two of the most widely used tools for assessing antidepressant activity preclinically (Cryan et al., 2005;Cryan and Holmes, 2005). Both tests have been pharmacologically validated based on their responses to clinically utilized antidepressants i.e. the persistence of escape-directed behaviors for longer time periods. The FST (also known as behavioural despair or learned helplessness) was initially developed by Porsolt and colleagues (1977). It involves two swim sessions in a glass cylinder: an initial 5 minute pre-test followed 24 hours later by a 15 minute actual test session. In the first session (pre-conditioning) - the animal is able to discover the test, explore the water surface and dive. The first session is where the animal realizes that it

cannot escape. In the second session (test session) - the rat is familiarised to the FST, and will not try to dive (Petit-Demouliere et al., 2005). The depth of the water ensures rats will not try to support themselves by touching the bottom of the cylinder with their paws (Detke et al., 1995). Behavioural despair is judged when the rodent is immobile, and both hind legs are not moving, and no additional activity is observed other than that required to keep its head above water (Porsolt et al., 1977). The FST has been used preclinically to observe the effect of drugs in nerve injured rats (Gonçalves et al., 2008;Hu et al., 2009;Hu et al., 2007). Like the FST, the TST is based on immobility when placed in an inescapable situation; mice are hung upside down by the tail until they cease to struggle (Cryan et al., 2005). The TST however is not an appropriate test for rats given that they are unable to support their own body weight when suspended.

Since anxiety and depression can co-exist within an individual neuropathic pain patient, one study have attempted to co-analyse this phenomena (Gonçalves et al., 2008). Caution should be exerted when interpreting data, because experiments have shown that there is an order effect when the FST is executed before the EPM test (Andreatini and Bacellar, 1999), with animals showing more anxiety-like behaviour in the EPM (after the initial FST), even after a one week interval. Other groups have reported a decrease in exploratory behaviour in rodents following previous maze experience (Bertoglio and Carobrez, 2000;Holmes and Rodgers, 1998). It is therefore important to establish the ideal sequence and timing of the emotional behaviour tests, so that the behaviours displayed in one test does not go on to then influence the observed behaviours in another test.

1.5 Neurochemical markers in the DRG

The DRG contains cell bodies of peripheral sensory neurons which synapse onto second order CNS neurons in the dorsal horn of the spinal cord. These cell bodies in the DRG vary in size, with the smaller sized cells representing the C-fibre & A δ populations, and larger sized cells representing the A β population. Over the past 30 years a wide range of cell markers have been developed based on the expression of neurofilaments and/or receptors in order to classify the different sensory neurons and study their respective roles in sensory processes (see Table 1.2).

Markers	Cell size diameter	Information
Peripherin	Small	Peripherin is a type III intermediate filament protein that is expressed in many small diameter peripheral and some CNS neurons
Transient receptor potential vanilloid 1 (TRPV1)	Small	TRPV1 receptors are members of the TRP/vanilloid receptor ion channels and are expressed on C-fibres and involved in the process of thermal nociception
Isolectin Bandeiraea simplicifolia (IB4)	Small-medium	IB4 is a plant lectin that binds to a subgroup of small diameter sensory neurons. IB4 labelled DRG cells represent the non-peptidergic population of sensory neurons and relies on GDNF in postnatal development
Calcitonin gene related peptide (CGRP)	Small-medium	CGRP is a member of the calcitonin family of peptides and is abundant within the nervous system where its activity has been linked to the transmission of nociception. CGRP expression neurons relies on NGF for postnatal development
Neurofilament 200 (NF-200)	Large	NF-200 is a neurofilament protein is particularly abundant in the soma of large diameter neurons where it is slowly transported within axons towards the synaptic terminals.

Table 1.2 List of different markers used to identify distinct populations of DRG cells

The neurofilaments are one of the five major groups of intermediate filaments (distinct class of heterogeneous protein subunits, 10nm diameter) found in the PNS and CNS, which are composed of three major proteins of molecular weights 68kD, 160kD, and 200kD (Perry et al., 1991). Once synthesized in the neuronal cell body, they are slowly transported within axons towards the synaptic terminals. Large myelinated neurons in the DRG can be labelled with an antibody that detects phosphorylated neurofilament-200 (NF-200, also known as N52) (Lawson and Waddell, 1991), whereas the smaller sized unmyelinated neurons express the type III intermediate filament protein, peripherin (Yu et al., 2008). Peripherin is expressed within the cell of many peripheral and some CNS neurons in the adult rat (Barclay et al., 2007) and co-localises with TRPV1, the receptor responsible for transducing sensations of noxious heat and pain (Veronesi and Oortgiesen, 2006). Small-diameter sensory neurons can be further divided into two main subtypes based on their neurochemical biomarkers and their response to growth factors (Snider and McMahon, 1998). These are the Isolectin *Bandeiraea simplicifolia* (IB4) binding, P2X containing neurons (non-peptidergic) (Bennett et al., 1996b), and the Substance P/ CGRP containing peptidergic neurons. The IB4 positive neurons rely on GDNF for development whereas the IB4-negative (peptidergic) population depend on NGF for survival during postnatal development (Molliver et al., 1997). This differential response to growth factors suggests that these two populations of cells may play different roles in processing of nociceptive inputs. IB4 positive neurons have a longer action potential duration and higher densities of TTX resistant sodium ion channel currents than the peptidergic population of sensory neurons, therefore both populations of these small diameter sensory neurons have distinct electrophysiological functions (Lawson and Waddell, 1991). CGRP is a member of the calcitonin family of peptides and expressed in the CNS as well as the PNS and its activity has been linked to the transmission of nociception, particularly migraine (Benemei et al., 2009). CGRP co-localises with the TrkA receptor for which NGF is the ligand (Bennett et al., 1996b). While it may be common practice to classify nociceptive cells as either peptidergic or non-peptidergic, it is important to note that there is a degree of overlap with some cells co-expressing both CGRP and IB4 (Bergman et al., 1999). Other populations of DRG neurons may exist to which are yet to be defined electrophysiologically or by cellular markers.

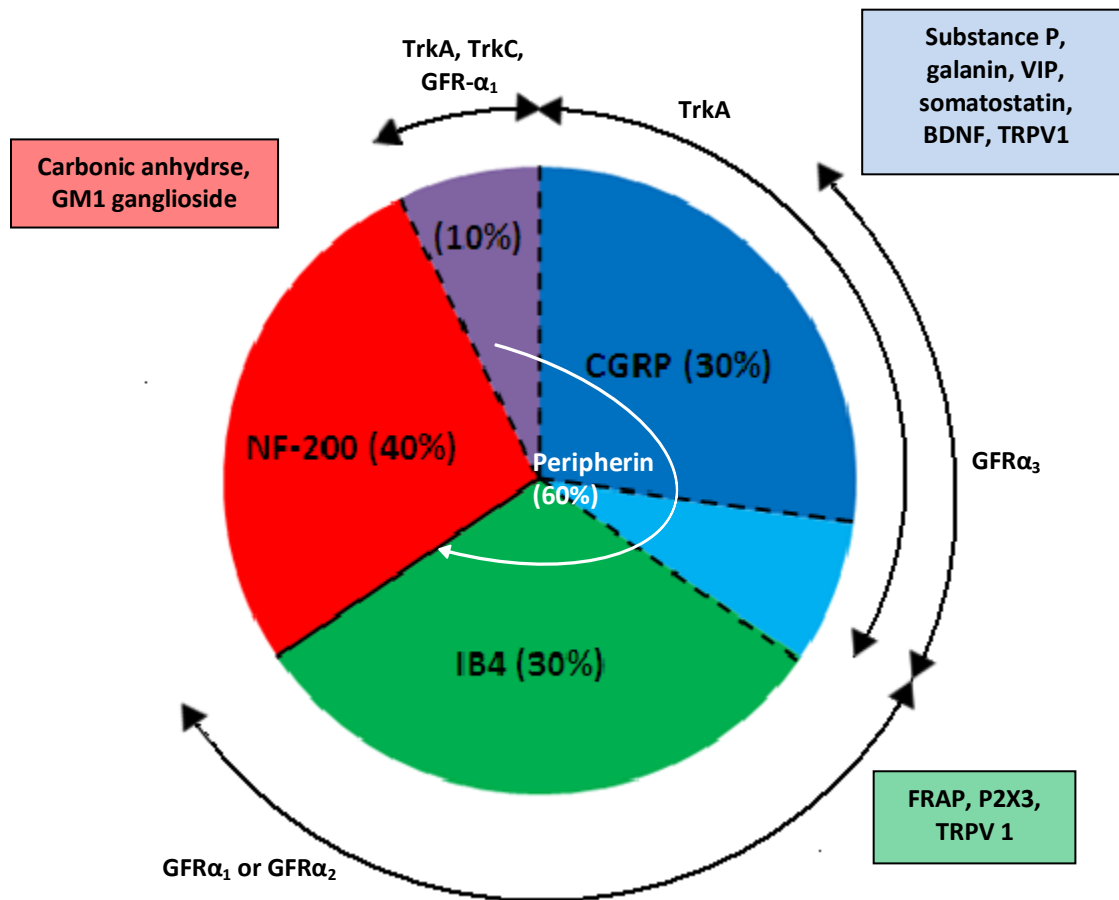


Figure 1.5 Schematic summary of the different neuronal cell populations in the DRG based on the markers expressed. Nociceptive unmyelinated neurons comprise of small-medium diameter cells that express CGRP and IB4. There is an overlap between DRG cells expressing CGRP and IB4. Non-nociceptive myelinated cells can be identified by NF200 and represent ~40% of DRG cells or even up to ~50% (Goldstein et al., 1991). Nociceptive unmyelinated cells can be identified by peripherin and represent ~50-60% of DRG cells. There is also an overlap between NF-200 and CGRP cells and ~6% of peripherin/NF-200 cells (Goldstein et al., 1991). Arrows around the edge of the pie chart represent receptor components for neurotrophic factors expressed by different populations of cells; trkA for NGF-responsive; GFR α_1 , GFR α_2 , GFR α_3 for GDNF-responsive; trkC for neurotrophin-3 (NT-3) responsive cells. The boxes represent the peptides that are additionally expressed in the relevant subpopulations. Image edited from McMahon & Koltzenburg; Wall and Melzack's textbook of pain, 5th Edition.

1.6 Dysregulated proteins in the DRG

The DRG is a frequently analysed tissue in neuropathic pain research, since it is a key junction where peripheral sensory information travels. It was once assumed that the DRG was not involved in nociceptive signal generation or processing and its role limited to metabolic demands of neurons, however evidence suggested a more direct role. Spontaneous discharge of DRG neurons may occur, which may be perpetuated by peripheral nerve injury and there is evidence of cross excitation of cell soma (Amir and Devor, 1996). Electrophysiological and immunohistochemical evidence exists for the expression of the receptors for NPY, galanin, and SP, and their activation by their respective ligands can modulate membrane excitability after micropipette application (Abdulla et al., 2001; Abdulla and Smith, 1999; Kerekes et al., 2003). The release of substance P from the DRG following potassium/chloride (KCL) stimulation further supports this notion (Huang and Neher, 1996). Peripheral nerve injury results in a variety of changes which involves alterations in gene expression, protein expression, and post-translational protein modification which can alter neuronal excitability (Lacroix-Fralish et al., 2011; Woolf and Salter, 2000; Zimmermann, 2001). The proceeding section will expand on the neurochemicals that have exhibited robust changes in their expression following peripheral nerve injury, namely; ATF-3, the neuropeptides NPY & galanin, and GAP-43.

1.6.1 Activating transcription factor- 3 (ATF-3)

Activating transcription factor- 3 (ATF-3) is a member of the ATF/cAMP responsive element-binding (CREB) family of transcription factors and is an immediate early gene protein (Hai et al., 1989; Ziff, 1990). ATF-3 regulates transcription by binding to DNA sites as a homodimer or heterodimer with jun proteins (Chen et al., 1994; Hai and Curran, 1991). The biological effect of ATF-3 *in vivo* is less clear than that of the neuropeptides (i.e. NPY and galanin), but a previous study has shown that ATF-3 deficient mice injected with a sublethal dose of murine cytomegalovirus possess significantly reduced titres and pathology in their livers 3 days after injection, compared to wild type (Rosenberger et al., 2008). A previous *in vitro* study has shown that ATF-3 expression in macrophages represses IL-6, IL-12, and other cytokine genes downstream of toll-like receptor 4 (TLR4)

(Litvak et al., 2009). Taken together, this evidence suggests ATF-3 reduces the inflammatory response and is therefore involved in the regulation of the immune system. Other studies have demonstrated ATF-3 to be involved in tumour suppression, (Thompson et al., 2009), however a more detailed understanding of how ATF-3 regulates oncogenic signalling pathways is required. The actual mechanisms regulating ATF-3 expression are not clear, but ATF-3 expression can be induced experimentally in preparations such as the mechanically injured liver and ischaemic heart, accordingly it is likely to play a role in stressed tissues (Chen et al., 1996).

A study by Tsujino and colleagues (2000) identified ATF-3 as a novel marker of the DRG cell body response to peripheral nerve trauma. In naïve animals mRNA for ATF-3 was not expressed, however following spinal nerve axotomy, ATF-3 was dramatically induced (within 24 hours) in virtually all ipsilateral L5 DRG neurons. Thereafter the expression remained until its downregulation at 10 weeks where it was then detected at low levels. ATF-3 expression in the spinal cord was modest and limited to the ventral horn where motor axons terminate, and not the dorsal horn which receives sensory input, thus indicating differential regulation of gene expression. Other studies using microarray technologies have demonstrated limited ATF-3 expression following dorsal root injury compared to spinal root injury indicating that ATF-3 is more associated with nerve injury to the PNS rather than CNS (Stam et al., 2007). The upregulation of ATF-3 observed by Tsujino and colleagues (2000) was present in the formalin nerve injury model, but not the CFA model where there is only inflammation and no tissue injury per se. Moreover, stimulation of neurons at C-fibre strength did not induce ATF-3 expression in the DRGs. Other experiments have shown that mustard oil, capsaicin, and methanol, and again formalin can all induce ATF-3 expression in the DRG, and this occurs in distinct subpopulations of sensory neurons (Braz and Basbaum, 2010). Taken together this information further suggests that nerve damage is the basis of ATF-3 expression rather than increased nociceptive transmission or inflammation (Tsujino et al., 2000). The subsequent labelling of the ATF-3 protein via immunohistochemistry revealed that ATF-3 co-localized with c-jun (Tsujino et al., 2000), which is a well characterized immediate early gene induced in neurons following axotomy (Herdegen et al., 1991). ATF-3 has since been suggested as a reliable marker for nerve injury in human tissue following dorsal root

avulsions (Linda et al., 2011). Additional studies have demonstrated that ATF-3 expression is not just limited to neurons but also to Schwann cells, epineurium, and glia following sciatic nerve axotomy and is associated with Wallerian degeneration (Hunt et al., 2004; Isacson et al., 2005; Saito and Dahlin, 2008). When the nerve is reattached following transection, therefore allowing the regeneration of axons, ATF-3 expression reduces, which suggests that ATF-3 may be an indicator of disturbed axonal processes (Hunt et al., 2004; Saito and Dahlin, 2008). Indeed, reduced ATF-3 expression has also been observed following reinnervation of muscle after crush lesion, thus allowing normal retrograde transport of growth factors (Kataoka et al., 2007). Previous studies have demonstrated that both NGF and GDNF can influence ATF-3 expression. The intrathecal administration of trophic factors NGF or GDNF attenuates ATF-3 expression in the DRG following axotomy (Averill et al., 2004). However, even in the absence of nerve repair ATF-3 expression in Schwann cells in the distal section of the sciatic nerve decreases over time suggesting that regenerating axons are not entirely responsible for the downregulation (Kataoka et al., 2007). *In vitro* studies have demonstrated that the over expression of ATF-3 (using a viral vector) in cultured DRG neurons taken from naïve animals results in increased neurite outgrowth (Seijffers et al., 2006). This observation has been further supported in transgenic studies where mice constitutively expressing ATF-3 experience enhanced peripheral nerve regeneration in DRG neurons comparable to that of a preconditioning nerve injury (Seijffers et al., 2007). Co-expression studies have showed that a variety of neurochemical makers associated with traumatic nerve injury are additionally expressed in ATF-3 positive cells such as galanin, NPY, NOS, and reg-2 (Averill et al., 2002; Shortland et al., 2006). ATF-3 has been shown to be induced in a number of other non-trauma neuropathic pain models such as the VZV model using western blot techniques (Garry et al., 2005) and HIV-1 gp120 induced neuropathy model (Wallace et al., 2007a), but not the antiretroviral related peripheral neuropathy model (Wallace et al., 2007b). The physiological significance of ATF-3 expression is unknown. The amount of ATF-3 expression has been shown to be proportional to the degree of thermal behavioural hypersensitivity exhibited, but not mechanical (Obata et al., 2003). However the transient-dynamic expression of ATF-3 following peripheral nerve injury does not correlate with the chronic pain-related behaviours observed in the animal models. It has been demonstrated that L5 SNL induces ATF-3 expression in the supposedly uninjured L4 DRG and these changes are associated

with upregulation of neuropeptides (Shortland et al., 2006) and downregulation of TTXr sodium ion channels Na_v1.8, and Na_v1.9 (Fukuoka et al., 2012). These expression patterns are similar to what is frequently observed in the directly injured L5 DRG following L5 SNL. However it can be argued that these findings are related to collateral damage incurred during surgical manipulations which is heavily influenced by the surgical operator. Even in sham operated animals, limited expression of ATF-3 can occur in the DRG. In any case, the very nature of surgery results in unavoidable damage, but can be minimized by careful attention, in order to carefully reflect the consequences of the intended injury (and subsequent ATF-3 expression).

1.6.2 Neuropeptide Y (NPY)

Neuropeptide Y (NPY) is an abundant and widespread neurotransmitter within the nervous system and is involved in regulating physiological events such as appetite, vasoconstriction, and pain (Intondi et al., 2008; Ji et al., 1994b). The 36 amino acid peptide was originally isolated in the porcine hypothalamus where it was found to be particularly abundant in the paraventricular and arcuate nucleus of the hypothalamus (Gibson et al., 1984; Tatemoto et al., 1982). Since its discovery, numerous studies have denoted NPY to be a potent stimulator of food intake that can decrease energy expenditure and promote weight gain (White, 1993). Later studies have now implicated NPY in the regulation of pain responses. NPY and its receptors are located in regions of the spinal cord that are associated with nociceptive transmission such as the superficial lamina of the dorsal horn (Gibson et al., 1984; Ma and Bisby, 1998). The intrathecal administration of NPY attenuates behavioural responses to noxious heat in normal animals (Hua et al., 1991) and inflammatory model of pain (Taiwo and Taylor, 2002), and mechanical and cold hypersensitivity in the SNI peripheral nerve injury model (Intondi et al., 2008). Equally the administration of NPY Y1 and Y2 receptor antagonists can reverse the NPY mediated antinociceptive effects, but only when co-administered since the effect of either antagonist is limited when administer individually (Intondi et al., 2008). Conversely, pronociceptive effects have been observed in the flexor reflex model of naive animals but this is likely attributed to the low doses used in the respective studies (White, 1997; Xu et al., 1999). After nerve injury the Y1 receptor, normally present on small DRG neurons, is down-

regulated, and the Y2 receptor is robustly up-regulated on large neurons (Zhang et al., 1997). The knockout of the NPY Y1 receptor induces a hypersensitive state to mechanical stimuli (Naveilhan et al., 2001; Shi et al., 2006). Sciatic nerve axotomy in these Y1 receptor knockouts protects mice from the associated loss of DRG neurons that is observed in wild type animals with similar injury (Shi et al., 2006). NPY is virtually undetectable in healthy rat DRG neurons (Gibson et al., 1984), or present at very low levels (Zhang et al., 1993b), but is present in superficial dorsal horn neurons. Following nerve injury NPY is dramatically upregulated in L4/L5 DRG and axonal fibres in superficial and deeper spinal lamina. This upregulation occurs primarily in large diameter DRG neurons, but some small cells are affected. NPY co-localises with other neuropeptides that have been associated with nerve injury such as vasoactive intestinal peptide (VIP) & galanin (Landry et al., 2000; Ma and Bisby, 1998), reg-2 (Averill et al., 2002), and ATF-3 (Shortland et al., 2006). The histological, transgenic, pharmacological, and behavioural studies together support the notion that NPY plays a role in regulating nociceptive transmission as well as nerve injury, and thus neuropathic pain.

1.6.3 Galanin

Galanin is a 29 (rat)/ 30 (human) amino-acid neuropeptide first identified in the porcine intestine (Tatemoto et al., 1983) and is widely distributed throughout the PNS and CNS. Galanin is thought to regulate numerous physiological actions in the adult nervous system such as energy homeostasis, and can rapidly stimulate food intake following acute injection into the hypothalamic paraventricular, lateral and ventromedial nuclei, and the central nucleus of the amygdala (Gundlach, 2002). In the gastrointestinal tract, where galanin is abundant, it inhibits gastric acid secretion and the release of numerous pancreatic peptides, including insulin, amylase, glucagon, and somatostatin (Lang et al., 2007). Galanin also regulates osmotic functions, and the intracerebroventricular administration of galanin has been shown to decrease free water intake in rats (Brewer et al., 2005). Other putative actions includes regulation of arousal/sleep, reproduction, anxiety (induces anxiolytic effects), and cognition where the ventral hippocampal administration of galanin has negative effects on learning and memory tests (Lang et al., 2007). The actions of galanin are mediated and regulated via interaction with three G-protein coupled receptors

(GPCRs), GalR₁₋₃ (Mitsukawa et al., 2010). The anatomical location of galanin and its receptors in DRG and the superficial dorsal horn is of interest and suggests that galanin may also participate in the regulation of nociceptive transmission and thus pain (Hokfelt et al., 1987; Michener et al., 1990). Galanin is constitutively expressed in healthy animals (<10% in the DRG) and exhibits diffuse granule-like cytoplasmic staining. There is evidence from electron and confocal microscopy that galanin expression in the DRG is even greater than what has been observed under light microscopy (>90%) but this technique has not got sufficient power to detect these differences (Zhang et al., 1993a). Following peripheral nerve injury galanin mRNA and protein is robustly upregulated (Landry et al., 2003; Ma and Bisby, 1997; Villar et al., 1989; Wang et al., 2002). This upregulation occurs early (4 days) following partial nerve injury and peaks at 10-14 days with medium-large diameter cells being proportionally affected in L4/L5 DRGs (Ma and Bisby, 1997). Complete transection of the sciatic nerve (axotomy) shifts the distribution so that small diameter cells increase expression of galanin. The proportion of galanin immunoreactivity in DRG neurons are significantly higher in partial nerve injuries compared to complete nerve transection 14 days after nerve injury, which suggests galanin expression also occurs in uninjured fibres (Ma and Bisby, 1997). This is corroborated by another study that has demonstrated approximately 50% of galanin labelled cells in the L5 DRG co-express the nerve injury marker ATF-3 14 days after injury indicating that those cells are damaged, but also intact neurons are equally affected (Averill et al., 2002). Expression of galanin also increases in the deeper lamina of the dorsal horn, where large fibres terminate. Early pharmacological experiments revealed that intrathecal administration of galanin in rats at high or low doses could facilitate or inhibit respectively the flexor reflex in response to noxious stimulation (Wiesenfeld-Hallin et al., 1988). Low dose galanin (10 ng) caused facilitation of the flexor reflex. Low dose galanin (0.1 and 1 nmol) has additionally been shown to facilitate decreases in mechanical PWTs in the rat (Kuraishi et al., 1991). The antinociceptive response is mediated by the activation of the galanin receptor-1 (Gal₁R) which has been suggested to inhibit the release of substance P (Hua et al., 2005), and glutamate in the spinal cord (Hua et al., 2004). The Gal₁R mediated hyperpolarisations of sensory neurons may account for the reported synergistic effects with opiates (Hua et al., 2004). Other experiments have shown that the administration of galanin antagonist and antisense nucleotides can reduce galanin expression in the DRG and this is

associated with a higher rate of autotomy after axotomy, a facet that is argued to be an outcome measure of neuropathic pain related behaviour (Ji et al., 1994a; Verge et al., 1993). The physiological stimuli that results in the release of galanin in the spinal cord of healthy animals is yet to be identified, however it is not released in response to noxious, thermal, or mechanical stimulation. The location of galanin in small GABA and enkephalin putative inhibitory interneurons of the spinal lamina II (Simmons et al., 1995; Zhang et al., 1993c) in conjunction with data corroborated from various studies suggest that galanin main actions are mainly antinociceptive. The knockout of galanin in mice impairs the ability of peripheral nerves to regenerate following sciatic nerve crush and is associated with long-term sensorimotor functional deficits and therefore galanin may play a role in nerve regeneration (Holmes et al., 2000). These effects however are not thought to be mediated through the Gal₁R since animals with a knock out in this receptor have no reduction in regenerative capacity following sciatic nerve crush (Blakeman et al., 2003). Rather the Gal₂R is thought to be involved given that these events are observed in the Gal₂R knockouts (Hobson et al., 2006). Furthermore, mice carrying a loss-of-function mutation of galanin exhibited a 13% reduction in the total number of adult DRG neurons (Holmes et al., 2000). These deficits were associated with a 2.8-fold increase in the number of apoptotic cells at postnatal day 3 (Holmes et al., 2000). Galanin expression may also act as a neuroprotective agent in peripheral nerve injury based on the preceding evidence and such neuroprotection has been demonstrated in the CNS, in the hippocampus of galanin overexpressing transgenic animals after kainate injection (Elliott-Hunt et al., 2004). This highlights some of the potential of galanin, and the reported effects of galanin in the literature could be explored fully for its therapeutic potential. Galanin has been shown to reduce action potentials in the locus coeruleus (LC) and the dorsal raphe nucleus by causing hyperpolarisations most likely through activation of potassium channels, thus reducing the release of serotonin and noradrenaline (Hokfelt et al., 1998; Seutin et al., 1989; Sevcik et al., 1993), indicating galanin is involved in the modulation of neurotransmitter release

1.6.4 Growth associated protein-43 (GAP-43)

Nerve growth or axonal elongation coincides with the expression of growth associated proteins (GAPs) in the cell bodies of corresponding neurons. The interest in GAPs led to the discovery of GAP-43, a membrane-associated phosphoprotein that is significantly expressed during axonal growth and synapse formation (Meiri et al., 1986). The precise biological actions of GAP-43 remains to be determined, however GAP-43 is considered to be an early marker of post-mitotic neurons and is highly expressed in the developing nervous system and exhibits significantly lower expression in the adult nervous system (Meiri et al., 1986). GAP-43 appears to be involved in transducing intra- and extracellular signals to regulate cytoskeletal organization in nerve endings (Benowitz and Routtenberg, 1997). Later studies have also demonstrated GAP-43 to be present in non-neuronal cells such as non-myelin forming Schwann cells (Curtis et al., 1992). Over expression of GAP-43 in mice leads to neuronal sprouting and conversely, the down-regulation of GAP-43 is associated with the regression of neurons *in vivo* (Aigner et al., 1995; Moya et al., 1988). In the mature ganglia GAP-43 is present in few small-diameter cells in DRG, and is undetectable in large diameter cells (Reynolds et al., 1991; Sommerville et al., 1991). GAP-43 exhibits cytoplasmic staining but also labels axons. Following traumatic peripheral nerve injury, damaged neurons have the capacity to regenerate and this has been associated with the increased expression of GAP-43 mRNA or protein in the terminals of peripheral afferents, DRG, and the superficial lamina of the dorsal horn (Nahin et al., 1994; Schreyer and Skene, 1991; Schreyer and Skene, 1993; Woolf et al., 1990). This increased expression occurs early after injury (within at least 3 days) with the immunoreactivity pattern initially predominant in small-diameter neurons, and peaks at approximately 21 days, before becoming undetectable at 9 weeks following crush injury or 36 weeks following sciatic nerve cut (Woolf et al., 1990). There is evidence for differential regulation since at later time points; medium-large diameter cells have been shown to express GAP-43 mRNA at >4-10 days (Wiese et al., 1992), and protein >14 days post injury (Sommerville et al., 1991). Increased GAP-43 expression is not thought to be directly related to the onset of mechanical hypersensitivity in nerve trauma (indicative of neuropathic pain like behaviour) (Jaken et al., 2011). Given the role of GAP-43 in the

nerve regeneration and association with nerve injury, it represents a suitable candidate for study when investigating neuropathic pain mechanisms.

1.7 Distribution of tibial nerve neurons within the DRG

To date it is unclear with regards to the literature the distribution of tibial nerve neurons found within the DRG. It is worth recognizing the relative distributions of tibial axons to the lower lumbar/sacral DRGs since this will reveal the proportion of damaged/undamaged neurons in affected DRGs. This is important because varying degrees of damage may reflect in the expression profiles of biochemical markers e.g. neuropeptides and ATF-3. Retrograde tracers are frequently used in experimental studies of the PNS to determine the number and origin of neurons projecting to a specific area (Zelev et al., 2010). Early tracing studies used Horseradish peroxidase (HRP) which is taken up by axons and into the cell soma, and has previously been used to describe tibial nerve distribution in the DRG (Swett et al., 1991). Recent years has seen the introduction of newer retrograde tracers that are superior to HRP. Fluorogold (FG) and cholera toxin β subunit (CTB) have become one of most frequently used tracers for retrograde labelling studies (Kobbert et al., 2000). One advantage of FG over the earlier tracer HRP is that it is not taken up by undamaged axons of passage (Schmued and Fallon, 1986). FG has a wide range of survival times ranging from one day to over a month whereas HRP has a short window in which tissue as to be harvested which is usually between 3-5 days (Kobbert et al., 2000). FG is a hydrophilic tracer and therefore is evenly distributed throughout the cytoplasm and survival times range from a day to several months, although 7-10 days are the typical time frames for study (Zelev et al., 2010). FG is taken up by virtually all neurons (Schmued and Fallon, 1986) however there is evidence to suggest that CTB has a differential distribution following nerve injury (Tong et al., 1999). As a result FG will be the more suitable choice for retrograde experiments involving traumatic nerve injury.

1.8 Macrophage associated neuroinflammation

Macrophages are immune cells that are produced by the differentiation of monocytes that originate from the bone marrow. In the PNS, the macrophages reside in the endoneurium accounting for up to 9% of the cell population, and are often in close proximity to blood vessels (Mueller et al., 2001). Within the DRG, macrophages are present around neurons where they lie outside the basal lamina of satellite cells. The main role of macrophages is to phagocytose cellular debris and pathogens in order to stimulate other immune cells to respond. This is enabled by the presence of MHC class II protein expressed on the cell surface, which makes macrophages special antigen presenting cells in the immune system (Griffin et al., 1993). Macrophages can be identified immunohistochemically in neuronal tissue (e.g. sciatic nerve or DRG) by the presence of cell surface markers e.g. CD68 (also known as ED-1) or CD11b (recognized by the OX-42 antibody) (Scholz and Woolf, 2007). A high density of macrophages in the DRG is suggestive of an active immune response (Bisby and Chen, 1990).

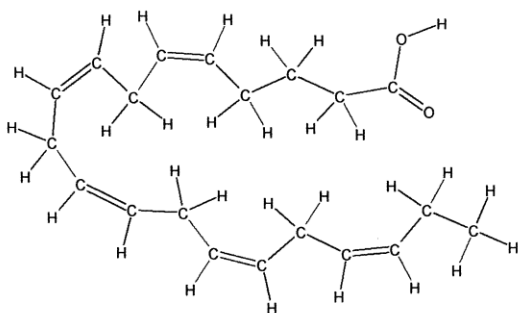
Following traumatic peripheral nerve injury, Wallerian degeneration takes place; distal axons degenerate, Schwann cells differentiate, and macrophages become activated (Stoll et al., 1989), where they co-ordinate an immune response releasing proinflammatory mediators such as cytokines, interleukins, interferons, prostanooids, growth factors, and nitric oxide (Scholz and Woolf, 2007). A macrophage infiltration has also been described in the DRG of ddC (HIV-antiretroviral drug) treated rats (non-traumatic neuropathy model) (Wallace et al., 2007b). Fundamentally, the infiltration of macrophages has also been reported in human nerve biopsies and the severity of the response is related to the degree of neuropathic pain (Bennett et al., 2008). This suggests that macrophage infiltration may be involved in the initiation (or maintenance) of neuropathic pain – experiments have shown that mice exhibiting slowed Wallerian degeneration (Myers et al., 1996; Ramer et al., 1997) or depleted macrophages (Liu et al., 2000a) have reduced hind paw hypersensitivity after traumatic peripheral nerve injury. The recruitment of macrophages and subsequent release of proinflammatory mediators after peripheral nerve injury in rodents can interact and activate their respective receptors on sensory neurons, directly altering transduction

properties of nociceptive afferents, leading to increased responsiveness (sensitization) (Calvo et al., 2012b).

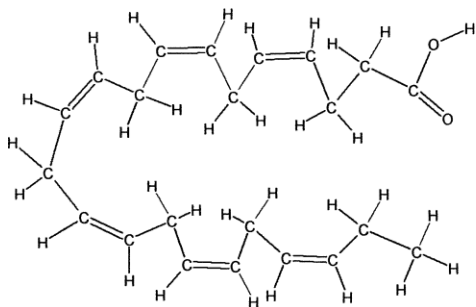
The macrophage response to nerve damage is important for the regeneration of injured sensory neurons (Bisby and Chen, 1990), and the suppression of the inflammatory response via toxic mediated macrophage destruction (Barrette et al., 2008) or combined ablation of IL-1 β and TNF α (Nadeau et al., 2011) impairs axon regeneration in mice. The therapeutical challenge is to modulate the inflammatory response to reduce persistent pain without impairing the important repair process.

1.9 Modulation of neuroinflammation via omega-3 poly unsaturated fatty acids

Since macrophages play a role in neuropathic pain pathophysiology (Watkins et al., 2001a), one approach to counteract this phenomena may involve ω -3 long chain poly unsaturated fatty acids (PUFAs). There are different types of PUFAs but the most frequently described in the literature are the ω -3 and ω -6 fatty acids (Holub and Holub, 2004). The ω -6 PUFAs such as arachidonic acid (AA) are considered to be proinflammatory whereas ω -3 PUFAs such as eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) have anti-inflammatory properties (see Figure 1.6 for structure). A large body of evidence now exists documenting the health benefits of ω -3 in the prevention and management of various inflammatory conditions including chronic inflammatory joint pain (Goldberg and Katz, 2007), inflammatory bowel syndrome (IBS), chronic spinal pain, multiple sclerosis, and coronary artery disease (Oomen et al., 2000). Much of these benefits are likely attributed to DHA, a major constituent of fish oil (Dyall and Michael-Titus, 2008). Several studies, both *in vitro* and *in vivo*, have demonstrated the neuroprotective effects of DHA (Belayev et al., 2009; Menard et al., 2009; Ward et al., 2010). This neuroprotective effect may be related to a potential modulation of macrophages and the immune response to nerve injury. Data from *in vitro* experiments supports a dose dependent inhibition of IFN γ /lipopolysaccharide (LPS) induced macrophage activation when exposed to DHA (Dustin et al., 1990; Lu et al., 2010; Ward et al., 2010). This inhibition is associated with a reduction of eicosanoids such as leukotrienes and prostaglandins (Lokesh et al., 1988), both of which contribute towards the sensitization of neurons (Ji and Strichartz, 2004). Of particular interest is a series of studies demonstrating that dietary or systemic administration of DHA following SCI in rats can reduce the associative spinal inflammatory response, and improve both the morphological and functional outcomes of nerve injury (Huang et al., 2007; King et al., 2006; Ward et al., 2010). Although DHA has been shown to be beneficial in SCI, few studies have investigated whether DHA or other ω -3 PUFAs are relevant in peripheral neuropathic pain syndromes.



EPA, Eicosapentaenoic Acid, 20:5 ω 3 (20:5n-3) (contains 20 carbon atoms and 5 double bonds/unsaturation sites)



DHA, Docosahexaenoic Acid, 22:6 ω 3 (22:6n-3) (contains 22 carbon atoms and 6 double bonds/unsaturation sites)

Figure 1.6 The structure of ω -3 PUFAs: Eicosapentaenoic (EPA) and Docosahexaenoic acid (DHA). The first of the double bonds is adjacent to the third carbon atom (when counting from the methyl carbon end of the molecule) hence the term omega-3. Figure adapted from Holub and Holub (2004).

No preclinical study has specifically investigated the modulation of the macrophage response via ω -3 PUFAs following peripheral nerve injury. Although there is no direct clinical evidence for the role of omega-3 PUFAs in neuropathic pain syndromes, they form an essential part of the diet, and their anti inflammatory properties can possibly be exploited to form a safe, cost-effective, and efficient approach in the management of neuropathic pain.

1.10 Aim of thesis

Peripheral nerve injury results in a variety of changes which involves alterations in gene expression and protein expression in sensory neurons (Elliott-Hunt et al., 2004;Lacroix-Fralish et al., 2011;Maratou et al., 2009;Woolf and Salter, 2000;Zimmermann, 2001). These neurobiological events often occur in the DRG, and therefore this tissue represents a topical point of study in neuropathic pain research. Understanding these biochemical changes may reveal possible mechanisms regarding some features of neuropathic pain such as spontaneous pain and hyperalgesia. Biochemical changes include the expression of neuronal injury marker ATF-3 (Tsujino et al., 2000), upregulation of neuropeptides, particularly NPY (Ma and Bisby, 1998), galanin (Hokfelt et al., 1987;Ma and Bisby, 1997), and GAP-43 (Sommerville et al., 1991;Woolf et al., 1990). These responses have even been proposed as generic biomarkers of neuropathic pain, and in the case of galanin suggested as a drug development target (Hulse et al., 2012). Although the expressions of these markers have been investigated in various nerve trauma models, the results of these studies are difficult to compare due to different analysis methodologies, variability of nerve injury which may reflect in reported expression patterns, and differences in species and strains of animals used. Moreover, these neurochemical markers have not been comprehensively investigated in non-traumatic nerve injury models such as the d4T-induced neuropathy model where nerve damage and pain related behaviours are also evident. Lastly, the temporal expression profiles encompassing both the acute and chronic stages of nerve injury as well as phenotypic cell effects have not been comprehensively documented.

One way to try to counteract the long term maladaptive changes is to provide neuroprotection. The ω -3 PUFAs have been described in the literature as a potent neuroprotector and their benefits are already acknowledged in other disease areas such as the cardiovascular and joint pain field. There are some indications that ω -3 PUFAs are beneficial following spinal nerve injury in animal models (Huang et al., 2007). However, ω -3 PUFAs have not been comprehensively investigated in the context of peripheral neuropathic pain.

The TNT model of nerve injury has been chosen for investigation because TNT surgery offers a less complex and time consuming procedure that avoids potential collateral damage that could prolong recovery.

The d4T model of nerve injury was chosen for investigation because d4T is widely used in resource limited settings due to its low cost and widespread availability and is associated with sensory neuropathy and pain and therefore understanding the mechanisms of this sensory neuropathy is still relevant (Cherry et al., 2012; Gilks et al., 2006; Makinson et al., 2008; Renaud-Thery et al., 2007). Previously, the ddC and ddI models have been examined (Bhangoo et al., 2007; Joseph et al., 2004; Wallace et al., 2007b), but the d4T model to date is yet to be characterised in rats.

Aims of the thesis:

- ❖ To provide temporal profile expressions of commonly dysregulated neurochemical markers (i.e. ATF-3, GAP-43, NPY, and galanin) in traumatic and non-traumatic nerve injury using the TNT and d4T induced models of peripheral nerve injury respectively.
- ❖ To compare and contrast these changes in both models.
- ❖ Characterize, compare, and contrast evoked sensory parameters (limb withdrawal responses) in both models.
- ❖ Investigate whether DHA (ω -3 PUFA) can reverse mechanical and thermal hypersensitivity associated with TNT injury and alter the expression of neurochemical markers in the DRG.

1.11 Hypothesis

Peripheral nerve damage initiates neurochemical changes in the dorsal root ganglion, and the development pain-like behaviours, which can be modulated via the administration of ω -3 polyunsaturated fatty acids.

Specific investigations:

- ❖ To investigate the expression of specific neurochemical markers indicative of pathological process in two distinct models of peripheral nerve injury: (1) traumatic nerve injury and (2) non-traumatic HIV-antiretroviral drug induced nerve injury at both the acute (24 hours) and chronic stages of nerve injury. Further investigations will reveal how this relates to changes in outcome measures of neuropathic pain-like behaviours (i.e mechanical hypersensitivity). A key part of the study will seek to make comparisons between the two models described. This will highlight the mechanistic differences between the two nerve injury models that are likely reflective of the mechanistic differences that occur between neuropathic pain patients with different aetiologies. This can support the idea of targeted treatment strategies based on mechanisms, which could be more effective.

- ❖ Determine whether chronic administration of DHA (an ω -3 PUFA) can modulate neuropathic pain-like behaviours and provide neuroprotection following traumatic peripheral nerve injury. This will be conducted with particular attention to the mechanism, i.e. the expression of neurochemical markers in DRG (referred to in **Section 1.6**). Assessment of simple reflex withdrawal behaviours in response to punctate mechanical stimuli will be the outcome measure of behavioural like pain.

Chapter 2

2 General methods

2.1 Animal maintenance

Adult male Wistar rats weighing 200-250g were used for all experiments (Charles River, UK), and were housed in individually ventilated cages (2-4 animals per cage) in a temperature controlled environment. Animals were provided with food and water *ad libitum* and maintained on a 12:12 hr light–dark cycle. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and the International Association for the Study of Pain (IASP) guidelines.

Project licence number: 70/7162

Personal licence number: 70/21129

2.2 Randomisation and blinding

All behavioural studies and immunohistochemical analysis procedures were conducted using randomisation and blinding to reduce allocation and observer bias, respectively. Experiments were conducted in accordance to Macleod Good Clinical Practice: Preventing Introduction of Bias at the Bench (Macleod et al., 2009).

Randomisation was performed by using a computerized random sequence generator (www.random.org) in which the range of numbers required are entered into specific fields on a computer program (animal's cage number) and the computer generates a list of the numbers in a randomized fashion.

Peripheral nerve injured animals (tibial nerve transection (TNT) and stavudine (d4T) treated animals, see **Section 2.9** for sample size analysis) were assigned per cage in a randomized fashion (as described above) and the investigator performing the sensory assessments analysis was not aware of each treatment group until the cessation of study i.e. when the animals were culled. Assessment of the immunohistochemical analysis was conducted by 're-blinding' via the masking of animal and cage identification's on slide labels and assigning a number to each slide. Before analysis, slide labels of nerve injured

and sham/control animals were randomised as previously described using the random sequence generator.

2.3 Allocation concealment

The allocation of DHA/saline to the animals was conferred by selecting pre labelled identification's out of a hat by and therefore treatments were not assigned per cage. Upon initial administration of DHA the treatments were coded (by an independent investigator) as either 'A' or 'B' and maintained the same code so throughout the study. The visual of the solution were indistinguishable and therefore there was no likely potential for bias. The 'blinding' of the surgical procedure was not necessary in this case, since both experimental groups received identical nerve trauma injuries (TNT surgery).

2.4 Inclusion/exclusion criteria

In order to maintain the consistency of the studies, a specific set of inclusion/exclusion criteria were established to retrospectively include/exclude data from analysis:

Inclusion criteria

- Confirmation of tibial nerve transection upon postmortem

Exclusion criteria

- <30% reduction in mechanical PWT from baseline
- Significant weight loss (>20% of pre surgery body weight),
- Autotomy of more than 1 digit
- Infected wound
- Excessive motor defect or paralysis

2.5 Reporting of animals excluded from analysis

All randomized animals (both overall and by treatment group) were accounted for in the data presented. A flow chart in the beginning of the each results section illustrates the total numbers of animals used and the total number of exclusions.

2.6 Fluorogold injections

Following confirmation of adequate anaesthesia, the tibial nerve was exposed in naive animals (n=2) in a similar manner required to that of TNT surgery. Fluorogold (FG; 3µl, 4% solution in 0.9% saline) was injected into the tibial nerve using a 10µl Hamilton syringe with a 30G needle. Following injection, haemostasis was confirmed and the wound was sutured at both the muscle and skin level with 4/0 silk suture. Animals received appropriate post operative analgesia (as indicated in **Section 2.7.1**).

2.7 Induction of peripheral nerve injury

2.7.1 TNT surgery

TNT/sham surgery was performed under general anaesthesia with isoflurane in O₂ (2-3%, 1.5L/min), using an aseptic technique (Lee et al., 2000). Following confirmation of adequate anaesthesia (absence of front paw reflex), a skin incision was made over and in line with the femur in the left hind limb. Connective tissue was cleared under the skin via blunt dissection and an incision made along the fascial plane between the gluteus superficialis and biceps femoris muscles to expose the trifurcation of the sciatic nerve. The tibial nerve was identified in terms of its anatomical position and 7/0 silk suture was used to ligate the nerve 2-3 mm distal to the tibial nerve junction from the sciatic nerve. A subsequent ligature was made approximately 5mm further distally on the tibial nerve proximal to the subsequent branching of the nerve (see Figure 2.1). On completion of the tight ligations the tibial nerve was transected between the two ligatures and the nerve gently placed back into its correct anatomical position. Haemostasis was confirmed and the wound was sutured at both the muscle and skin level with 4/0 silk suture. Sterile cotton bud swabs with sterile saline were used to clean blood away from the surgical procedure. Sham surgery was performed by exposing the trifurcation of the sciatic nerve as described above, but ligation and transection of the nerve were not performed. Care and attention was taken not to touch/damage the nerve itself. Transection of the tibial nerve was confirmed postmortem. All animals received postoperative analgesia consisting of a single s.c. infiltration of Bupivacaine (Astra) (0.25% 0.05ml) around the wound site. Carprofen (20%, 0.1ml/200g body weight) was administered i.p. 2-4 hours post-operatively. Animals were

monitored closely post-surgery for any potential complications such as infection, insufficient closure of wound, or excessive motor defects (i.e. complete motor loss in one limb so that locomotion only possible by ‘dragging’ that limb, see Table 2.1 for motor impairment scale).

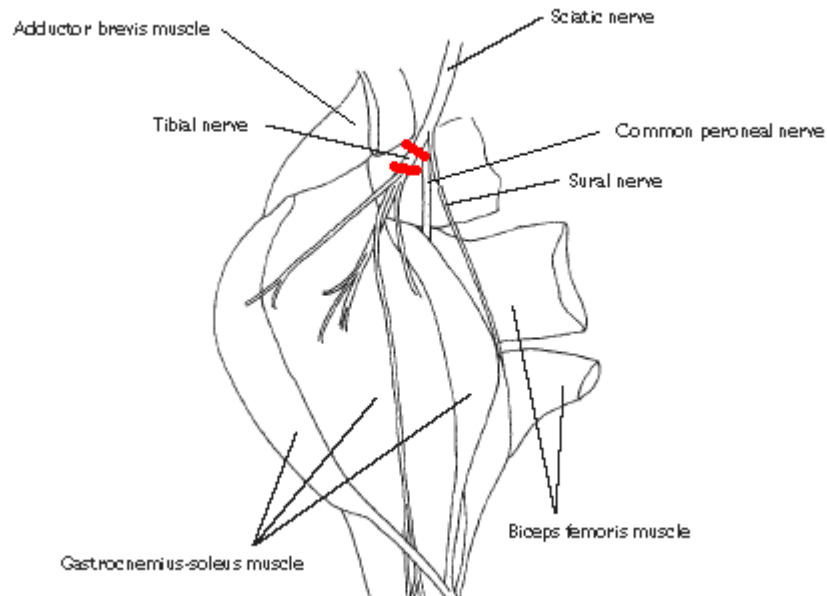


Figure 2.1 Schematic view of sciatic nerve trifurcation. The tibial nerve branch of the sciatic nerve is the final, and thickest, nerve portion to appear from the sciatic nerve. Ligation areas are illustrated in this diagram with two thick red lines, and transection between these ligations occurs on this branch before the subsequent trifurcation of the tibial nerve (Illustration adapted from Lee et al., 2000).

Motor impairment scale	
1	Normal motor function
2	Weight bearing on all four paws. Normal locomotor rhythm but some eccentric placement of paws. Speed of locomotion within normal range.
3	Temporary dragging of up to two paws (occurring within 2 days of operation and lasting up to 1 week) with impaired locomotor function for that period (but animals still able to rapidly reach food and water). After 1 week, motor impairments as in 1 above.
4	Temporary motor impairment of up to two paws (starting within 2 days and persisting for up to 1 week) but animals still able to rapidly reach food and water. After 1 week, persistent motor impairment of paws resulting in incorrect limb placement, delayed and/or incomplete swing phase of walking, but animals still able to move, albeit at less than half the normal speed to reach food or water.
5	Permanent motor impairment of one paw. Locomotion only possible by 'dragging' that limb.

Table 2.1 The motor impairment scale used to determine the degree of motor deficit after recovery from TNT surgery. Each animal was assessed independently and the gait examined inside and outside cage of their cage on a raised platform. Assessment was approximately 1 minute per animal. The scale was extracted from Seltzer et al., 1990.

2.7.2 d4T administration

Injections were performed under brief general anaesthesia using isoflurane in O₂ (2-3%, 1.5L/min). Following confirmation of adequate anaesthesia (absence of front paw reflex), animals received 0.5ml of d4T (i.v. 50 mg/kg in saline; gift from Pfizer) or saline via a tail vein. Fresh solutions were prepared as required using 0.9% sterile saline. d4T/saline injections were repeated 4 days after initial injection. Once haemostasis was confirmed in the tail vein, animals were returned to their cages. The repeated administration regime was chosen based on a pilot study from a parallel d4T project within the group which demonstrated that this regime was optimum.

2.8 Behavioural reflex testing

2.8.1 Reflex withdrawal to mechanical stimuli

Reflex responses to static punctate mechanical stimuli were assessed using a hand held electronic von Frey device (Moller et al., 1998) at baseline and at a time point post-surgery or initial d4T injection shortly before cessation of study. At the time of testing the animal was placed into a Plexiglas clear box (23 x 18 x 14cm) with a 0.8cm diameter mesh floor, and allowed to acclimatise to the testing environment until exploratory behaviour had ceased (approximately 10-15 minutes). A calibrated force transducer (0.5mm² diameter tip) was applied at a rate of 8-15 grams/sec to the mid-plantar surface of each hind paw in turn until an active limb withdrawal response was observed. A minimum of 1 minute was allowed before sampling the same hind paw. Readings were discarded when the animal did not actively withdraw its hind limb. A mean paw withdrawal threshold (PWT) was then calculated after 5 applications. At the end of each experiment the animal was placed back into its cage and the Plexiglas box cleaned thoroughly with disinfectant.

2.8.2 Reflex withdrawal to thermal stimuli

Behavioural reflex responses to thermal stimuli were assessed using a Hargreaves device at baseline and at a time point post surgery shortly before cessation of study (Bridges et al.,

2001a;Hargreaves et al., 1988). At the time of testing the animal was placed into a clear Plexiglas box to acclimatize for 15 minutes. The Hargreaves device was then calibrated: the infrared intensity level was set to 70; and the heat source was set to a maximum exposure time of 21.4 seconds (this was to ensure that there was no permanent tissue damage to the animal). Upon testing, the Hargreaves device was placed directly below the mid-plantar region of the hind paw and switched on. The timer automatically starts on heat emission and automatically stops on withdrawal of the hind paw. A minimum of 1 minute was allowed before sampling the same hind paw. A mean paw withdrawal latency (PWL) from the radiant heat source was then calculated after 3 applications for each hind paw. At the end of each experiment the animal was placed back into its cage and the Plexiglas box cleaned thoroughly with disinfectant.

2.9 Experimental study groups

A sample size analysis was performed based on previous data using the SNT and ddC model:

Sample size calculation for TNT rats based on previous data using SNT rats (Hasnie et al., 2007):

- ❖ The minimum detectable difference in the mean PWTs in SNT rats was 25g and the standard deviation was 8g. Using SigmaStat v3.5, a sample size of 4 animals was calculated per group at the desired power of 0.8 and alpha 0.05.

Sample size calculation for d4T treated rats based on previous data using ddC treated rats (Wallace et al., 2008):

- ❖ The minimum detectable difference in the mean PWTs in ddC animals was 16g and the standard deviation was 5g. Using SigmaStat v3.5, a sample size of 4 animals was calculated per group at the desired power of 0.8 and alpha 0.05.

The time points examined using the TNT and d4T treated rats differ due to the nature of the hypersensitivity in the respective models:

- ❖ TNT/sham animals were sacrificed at days 1, 7, 14, 28 and 43 post surgeries, shortly after recording of behavioural responses (**Chapter 3**). These time points represent the early (day 1 and 7), peak (day 14), and chronic stages of nerve injury (day 28 and 43) (Lee et al., 2000).
- ❖ d4T/saline treated animals were sacrificed at day 7, 22, and day 43 following initial injection, shortly after recording of behavioural responses (**Chapter 4**). Day 7 represents an early time point post injury (3 days post second d4T injection) that has not previously been examined. Day 22 represents a time at which peak behavioural hypersensitivity is generally observed towards mechanical stimuli in this model (Wallace et al., 2008). Day 43 represents a time at which hypersensitivity is still evident, but past the peak (Wallace et al., 2007b).

2.10 DHA preparation

Pure docosahexaenoic acid (DHA) solution (25 μ l, Sigma, UK) was opened and handled in a bubble sealed nitrogen gas atmosphere and 276 μ l of 100% ethanol was added to the pure DHA to make a 1M solution. Following this step, 5 μ l of pure DHA was aliquoted into 1ml light-protective glass screw top vials, using a pipette gun and glass pipette tips. The nitrogen gas atmosphere was required to protect the DHA from being oxidized, which is possible at normal atmosphere conditions. DHA is also light sensitive and therefore was handled at low light levels and required the use of light-protective vials. Pure DHA aliquots (5 μ l) were subsequently stored at -80°C.

Immediately before animals were prepared for DHA administration, a vial of DHA was taken from the -80°C freezer and placed in a bubble sealed nitrogen gas atmosphere. Animals were planned to have DHA administration at 2.5mls/kg. The 5 μ l of DHA was diluted to form a working concentration of 200 μ M by using serial dilutions to achieve a dilution equivalent to 25ml. The cap of the vial was unscrewed and 1ml of sterile saline

was added to stock DHA solution, and this solution was then extracted via an electronic pipette gun and aliquoted into a 10ml glass vial, where a further 4mls of sterile saline was added (total 5mls). Following this step, 1ml of the DHA-saline solution in glass vial was extracted via an electronic pipette gun and aliquoted into another 10ml glass vial, where a further 4 ml of sterile saline was added, thus producing a final 5ml solution of 500 nmol/kg of DHA-saline. The final ethanol concentration was negligible (8.0 pmol/ml).

2.11 DHA administration

Following TNT surgery animals were kept anesthetized in preparation for the administration of treatments. Animals received an i.v. tail vein injection of either saline (sterile, 0.9%) or DHA (500 nmol/2.5mls/kg; Sigma, UK) 30 minutes after transection of the tibial nerve. Animals were then placed back into their respective cages following recovery from anesthetic. Animals continued treatment of either saline or DHA every 3 days following surgery until day 28 when animals were sacrificed. Mechanical and thermal behaviour assessment was conducted every 7 days post surgery. The treatment groups were coded as either 'A' or 'B' and were administered in the order originally received upon initial administration.

2.12 Tissue preparation

All animals were sacrificed with an intraperitoneal injection of pentobarbital (300 mg/kg Animal Care Ltd) and transcardially perfused with 200ml of heparinised 0.9% saline followed by 300ml of 4% paraformaldehyde (PFA) solution (pH 7.4) in 0.1 phosphate buffer. DRG tissue from lumbar segments L4 and L5 were harvested and subsequently post-fixed in 4% PFA for 2-4 hours before being submerged in a 30% sucrose solution (in phosphate buffered saline (PBS)) for 72 hours. The L4 and L5 DRG was identified via position relative to the iliac crests and rib cage (Rigaud et al., 2008). Following incubation with 30% sucrose solution the DRGs were then embedded in optimum cryostat temperature (OCT) compound (VWR; Poole, UK) under liquid nitrogen or dry ice. Embedded tissue was stored at -80°C. Serial cryostat sections (10µm) were taken and tissue was thaw-mounted on superfrost slides (VWR) before storage in a -20°C freezer.

2.13 DRG Immunohistochemistry

All fluorescence immunohistochemistry experiments were conducted at room temperature (25°C). DRG L5 sections were rinsed in 0.1M PBS solution. Slides were then pre-incubated for 1 hour in 0.1M PBS containing 0.3% triton X-100 and 10% normal donkey or goat serum where appropriate, in order to block non-specific binding. In this thesis, the following primary antibodies were used (also see Table 2.2); anti-rabbit polyclonal activating transcription factor-3 (1:200, 1:200, Santa Cruz Biotechnology, CA); anti-rabbit polyclonal galanin (1:4000, Peninsula Laboratories, CA); anti-rabbit polyclonal neuropeptide Y (1:1000, Peninsula Laboratories, CA); anti-rabbit polyclonal growth associated protein-43 (1:1000, Chemicon); and anti-mouse ED-1 (1:500, Serotec). Primary antisera consisted of 0.1M PBS and 0.3% triton X-100. Double labelling experiments with previously mentioned antibodies were performed using anti-mouse peripherin (1:100, Santa Cruz); anti-mouse peripherin (1:1000, Abcam); or anti-mouse Neurofilament 200 (1:1000, Sigma) primary antisera to label small diameter and large diameter cells respectively. Following incubation of primary antisera, slides were rinsed with 0.1 PBS and incubated using the following secondary anti-sera where appropriate: Cy3-goat anti-rabbit (1:300, Jacksons Laboratories), FITC-donkey anti-mouse (1:200, Jacksons Laboratories), Alexa flour 568 goat anti-mouse (1:400, Invitrogen), Alexa flour 488 goat anti-rabbit (1:400, Invitrogen). Secondary antisera consisted of 0.1M PBS and 0.3% triton X-100. For the double labelling experiments, two sets of primary antisera were applied and developed sequentially to avoid any possible cross immunoreactivity. After 2 hour incubation with secondary reagents, sections were washed in 0.1M PBS and incubated with 2µg/ml of Hoechst 33342 for 2 minutes, thoroughly rinsed with distilled water, before being coverslipped with PBS/glycerol (1:8).

Controls:

Negative controls were carried out for each primary antibody used. This involved using an identical protocol as above but with omission of primary antisera on slides.

Antibody	Host	Dilution	Supplier
Primary's			
ATF-3	Rabbit	1:200	Santa Cruz
GAP-43	Rabbit	1:1000	Chemicon
NPY	Rabbit	1:1000	Peninsula Laboratories
Galanin	Rabbit	1:4000	Peninsula Laboratories
NeuN	Mouse	1:1000	Chemicon
Peripherin	Mouse	1:100	Santa Cruz
Peripherin	Mouse	1:1000	Abcam
NF-200	Mouse	1:1000	Sigma
Secondary's			
Alexa flour 488 (green)	goat (anti-rabbit)	1:400	Invitrogen
Alexa flour 568 (red)	goat (anti-mouse)	1:400	Invitrogen

Table 2.2 Summary of primary and secondary antibodies used in thesis

2.14 Image analysis

Fluorescent images were visualised using a Leica fluorescence microscope (DMR model 020-575-719, Leica, Milton Keynes, UK), equipped with the appropriate filter blocks, namely FITC (adsorption 492, emission 520; Leica, part # L5 513840), TRITC (absorption 550nm, emission 570nm; Leica, part # A4 513839) and UV (adsorption 350nm, emission 450nm; Leica, part # N2.1 513812) and captured at consistent exposure times using a Hamamatsu CCD camera (model 3CCD c5810, Japan). Images were analysed using Leica QWin v.3.0 software (Leica, Milton Keynes, UK). All images were taken at x10 magnification from 3-4 serial sections (100-150µm apart). Monochromatic grey scale images were loaded onto QWin software and the detection threshold level for immunolabelled cells was determined for each section taking into account background immunoreactivity which was determined by selecting an area of the DRG where

immunolabeling was not present. An 'intensity versus number of pixels' graph was constructed and a binary overlay was constructed to identify immunoreactive cells (see Figure 2.2). Positive immunolabeling was conferred when there was a clear demarcation point between background (large number of pixels with low intensity) and the immunopositive reaction (relatively small number of pixels with high intensity).

The number of peripherin/NF-200 neurons that co-labelled with investigative markers (i.e. ATF-3, GAP-43, NPY, and galanin) and the total number of peripherin/NF-200 cells in the ipsilateral L5 DRG were calculated and expressed as a percentage at each time point studied and plotted in a line graph. The cell size area (μm^2) for each peripherin/NF-200 immunoreactive cell was subsequently calculated based on the number of pixels (at x10 magnification, 1 pixel = $1.42\mu\text{m}$) using the measure feature function in the QWin software. The cell size analysis data was exported into an Excel (Microsoft) spreadsheet and each peripherin/ NF-200 and immunoreactive neuron along with the co-labeled investigative marker (i.e. ATF-3, GAP-43, NPY, and galanin) were then categorized according to cell size area in groups of increasing $100\mu\text{m}^2$ increments. The percentage cell size distribution of investigative markers was then plotted on bar graphs for both peripherin and NF-200 populations of sensory DRG neurons. Parallel color images were taken with nuclear marker Hoechst 33342 and the immunolabeling cross checked during analysis so that only those neurons that demonstrated a nucleus were included in the analysis. To quantify macrophage infiltration in the DRG (ED-1 immunohistochemistry), the mean percentage area of ED-1 immunoreactivity per $250,000\mu\text{m}^2$ ($500\mu\text{m} \times 500\mu\text{m}$) was calculated.

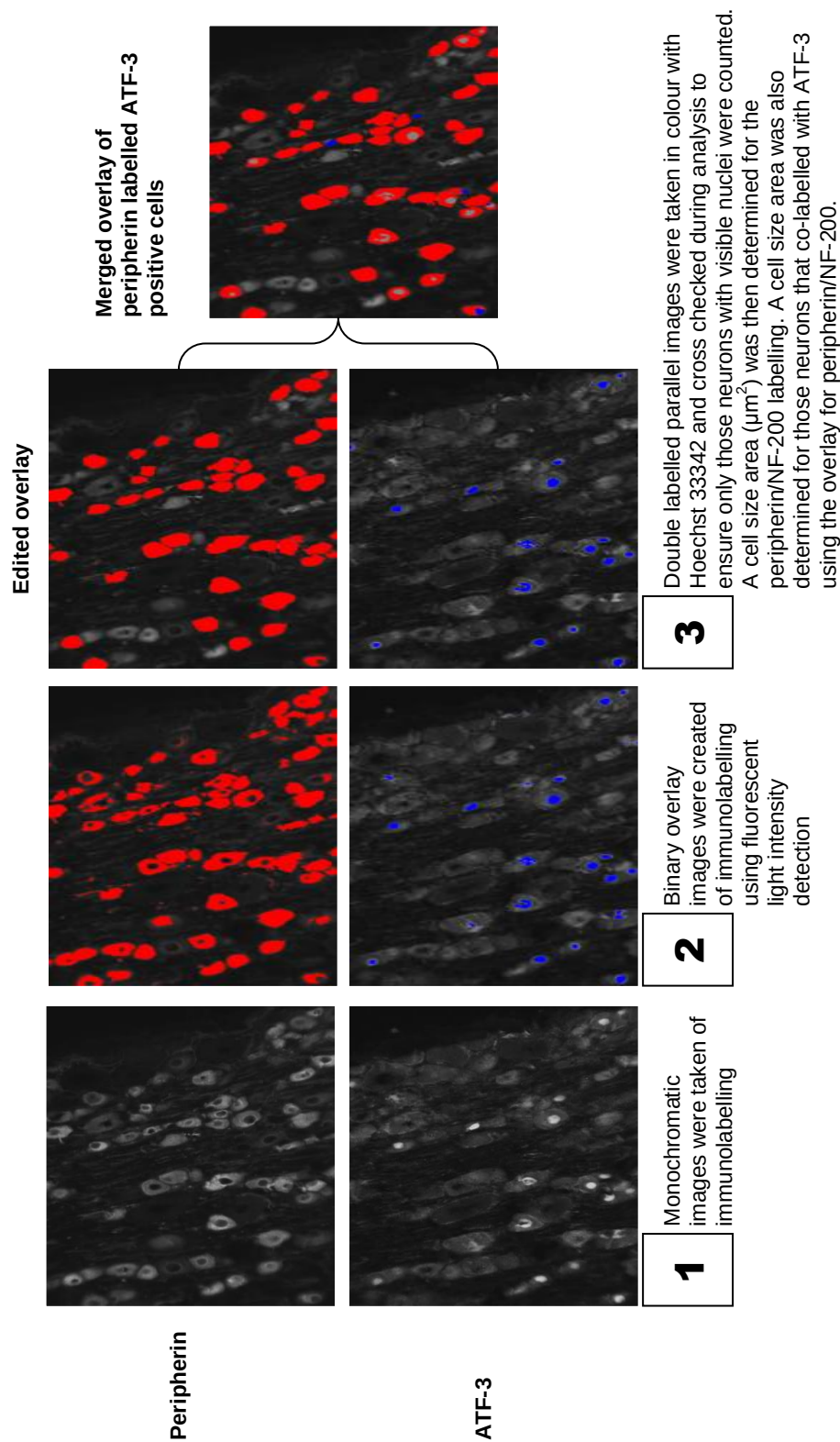


Figure 2.2 Semi-automated analysis of DRG immunohistochemistry using QWin software. An example using ATF-3 and peripherin immunohistochemistry in the L5 DRG of a TNT operated rat 14 days post surgery. Grey scale images were taken in the same plane in the respective filters for peripherin and ATF-3 immunoreactive cells and uploaded onto QWin. Once a binary mask had been completed of the immunolabelling, the cell size area was determined and those cells that were double labelled were clearly evident.

2.15 Statistical analysis

Statistical analysis for behavioural data was performed using Sigmastat (version 3.5, Systat Software, Inc). All data is represented as mean $\pm$ standard error of the mean (sem).

- ❖ To compare mean behavioural response at baseline and at each time point, a Student's paired t test was performed.
- ❖ To compare mean ipsilateral (or left hind limb) behavioural responses between nerve injured and control animals; a Student's unpaired t test was performed.
- ❖ A one way analysis of variance (ANOVA) was performed to identify specific trends in the temporal profile expressions of investigative markers in both peripherin and NF-200 DRG populations.
- ❖ A two way ANOVA followed by Holm-Sidak post hoc test was used to analyse treatment effects (nerve injury vs. controls) and cell size effects (NF-200 vrs peripherin)
- ❖ A one way ANOVA followed by a Dunnett's post hoc test was performed to identify significant differences at each time point compared to baseline values in DHA/saline treated animals.
- ❖ A one way ANOVA followed by a Tukey post hoc test was used for pairwise comparisons between ipsilateral and contralateral paws at each time point in DHA/saline treated animals.
- ❖ Where equal variance test failed a Kruskal-Wallis one way ANOVA on ranks was performed followed by a Tukey test.
- ❖ Significance level for data was taken at $p < 0.05$ unless indicated otherwise.

Chapter 3

3 Evaluation of DRG neurochemical markers in an animal model of tibial nerve transection induced peripheral nerve trauma injury

3.1 Introduction

Neuropathic pain may arise due to a disease or injury to the peripheral nervous system (PNS) and a number of peripheral nerve trauma animal models have been developed to in an attempt to understand the resulting pathophysiology (Treede et al., 2008). Both peripheral and central mechanisms contribute towards the development of neuropathic pain, although in most cases initial injury occurs to the PNS (Dworkin et al., 2003). Of consequence, much research has focused on addressing peripheral mechanisms of neuropathic pain. All sensory information from the periphery travels to the cell soma which resides in the dorsal root ganglion (DRG), which subsequently relays information to the spinal cord. Following experimental peripheral nerve injury there are a number of neurochemical changes that occur within associated DRG cells which may be related to the induction or maintenance of neuropathic pain. These changes include the dysregulation of neuropeptides, ion channels, and release of inflammatory mediators. The use of global gene expression in the DRG via microarray technologies and/or quantitative polymerase chain reactions (qPCR) has become a useful strategy in identifying genes that maybe involved in neuropathic pain processing.

Significant preclinical effort however is required to translate an observation based on a change in gene expression into a clear demonstration of a key role for that gene in the manifestation of neuropathic pain (Kinloch and Cox, 2005). Although gene microarrays, qPCR, and even Western blotting techniques are useful in demonstrating the presence of a dysregulated protein in the DRG, they are all limited by the inability to demonstrate the actual cellular localization of a particular protein. A more informative technique which can identify cellular localization of proteins in cells is immunohistochemistry and is a well utilized technique. Furthermore there is the possibility of double/triple labelling which can maximize study efficiently. Amongst the proteins that are dysregulated in nerve injury models, activating transcription factor-3 (ATF-3), Neuropeptide Y (NPY), and galanin represent some of the most frequently studied upregulated proteins (Hokfelt et al., 1987;Ma and Bisby, 1998;Tsujino et al., 2000;Woolf et al., 1990). Although these markers are commonly investigated in nerve injury studies, there are some reasons that would require the revaluation of their expression profiles. Expression profiles in the DRG are usually

examined at a single time point post injury, i.e. the peak of behavioural hypersensitivity towards mechanical von Frey stimuli, which is usually 14 days following initial nerve injury. The temporal expression profiles in the acute and chronic stages of nerve injury are often not investigated. Single time point studies are informative but they are limited in their approach since they only provide a snapshot and do not encompass the dynamic changes that can occur over time. In addition, different models of traumatic nerve injury encompass varying degrees of nerve injury which may reflect in the expression patterns, therefore making it difficult to draw conclusions when making comparisons between studies. The methods used to analyse and quantify immunolabeling has varied between studies as many laboratories employ different immunohistochemical image analysis protocols, some of which involve manual counting, others a semi-automated approach using imaging software. Furthermore, an observer judgement of the immunolabeling is an inherent step in the analysis and therefore there is potential for observer bias. This can be alleviated via adequate blinding and randomisation, but this prerequisite is seldom declared and may influence reported outcomes. Therefore, there may be different reported expression patterns in some studies even where the study time point and model used are matched. Characterizing the temporal expression profiles of these markers will allow for direct comparisons to be made between time points since variables such as nerve injury, image analysis, and strain of animals are all standardized. In addition the temporal expression patterns can be correlated with the outcome measures of neuropathic pain-like behaviours which may provide further insight to their role in neuropathic pain. Although the behavioural profiles of the tibial nerve transection (TNT) injured animals has been previously reported, no study to date has provided immunohistochemical characterization of dysregulated proteins associated with nerve injury in this model.

No study to date has described the distribution of tibial nerve neurons in the DRG using the more modern FG tracer and therefore this project attempted to address this issue. This study would reveal the proportion of damage exhibited by the the respective DRGs and give an indication of the proportion of damage acquired as a result of TNT injury.

3.1.1 Aims of study

There are a number of aims in the following study:

- 1) Characterize behavioural responses towards mechanical and thermal stimulus following TNT surgery in both the acute and chronic stages injury and compare to sham rats.
- 2) Provide a temporal profile expression of ATF-3, NPY, galanin, and growth associated protein (GAP-43) in the ipsilateral L5 DRG following TNT injury using immunohistochemical techniques and characterize the cellular phenotype of cells with profiles via double labelling using markers of small (peripherin) and large diameter (NF-200) neurons.
- 3) Perform a cell size analysis of investigative markers to validate immunohistochemical/phenotype data.
- 4) Correlate mechanical behavioural hypersensitivity with expression of investigative markers.
- 5) Determine in naïve animals the distribution of tibial nerve terminals in the L3-S1 DRGs using the retrograde tracer FG in order to gain insight into the proportion of nerve damage.

3.2 Results

Animals were sacrificed at days 1, 7, 24, 28, and 43 post TNT/sham surgery shortly after recording of mechanical and thermal behavioural responses. Animals exhibited no obvious signs of motor impairment; this was assessed by observing gait when animals were freely moving within the cage. A total of 44 animals were used (Figure 3.1).

Total animals used in Study:

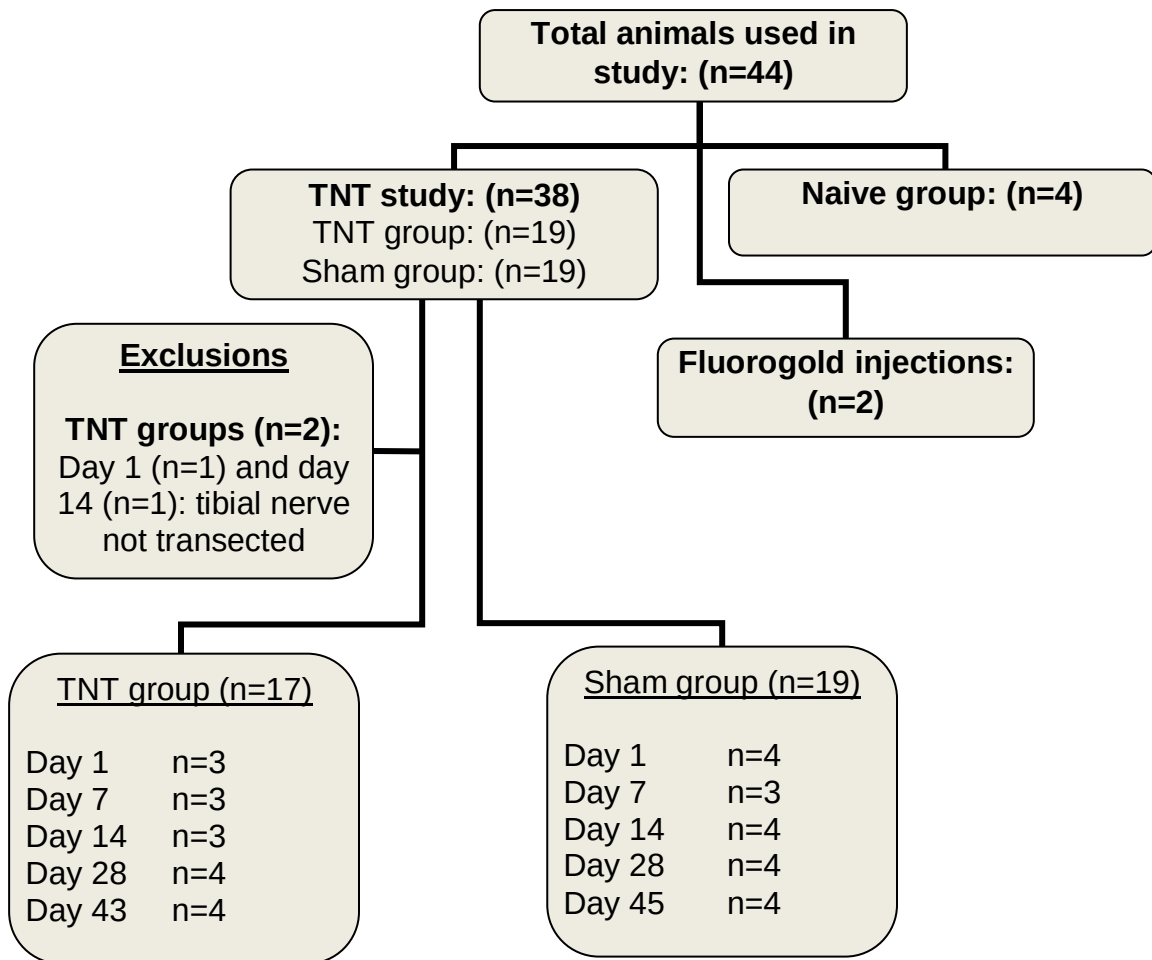


Figure 3.1 Animals were divided into the following groups: Naïve (n=4), Fluorogold injected (n=2), TNT surgery (n=17), and sham surgery (n=19) and further sub divided according the time of sacrifice (1, 7, 14, 28, and 43 days post TNT/sham surgery). All animals were checked post mortem to identify transection of tibial nerve with aid of a dissecting microscope. Two animals were excluded from the analysis due to incorrect nerve injury. Naïve animals were used as additional controls.

3.3 Distribution of tibial neurons in the L3-S1 DRG

In order to determine the extent of damage in the DRG produced by TNT injury, FG was injected into the tibial nerve of naïve animals (n=2) which were culled after 7 days. Immunohistochemistry for neuronal specific nuclear marker (NeuN) was conducted to identify nucleated neurons (since I could not use DAPI/Hoechst because FG excites at similar wavelength). The total number of NeuN and FG labelled neurons were counted for L3-S1 DRGs and the percentage co-expression from the total number of colabelled NeuN neurons was calculated. The results reveal that FG was taken up in only L4 and L5 and not in L3, L6, and S1 DRGs (see Table 3.1). There are large values for the standard error in both experimental animals; however the percentage co-expression was consistently greater in the L4 compared to the L5 DRG. The immunohistochemical images and cell size analysis suggests that FG labelling was present in mainly small size DRG cells but also some large size cells (Figure 3.2).

Table 3.1
Fluorogold/NeuN labelling percentages across DRG L3-S1
Data presented as mean $\pm$ SEM
(n=number of nucleated neuronal profiles)

DRG	Rat 1 Fluorogold/NeuN	Rat 2 Fluorogold/NeuN	Mean (Rat 1 & Rat 2) Fluorogold/NeuN
L3	0% (n=153)	0% (n=263)	0%
L4	21.3% (n=89)	55.6% (n=171)	38.5 $\pm$ 17.2 %
L5	19% (n=122)	35.5% (n=180)	27.5 $\pm$ 8.3 %
L6	0% (n=141)	0% (n=200)	0%
S1	0% (n=107)	0% (n=172)	0%

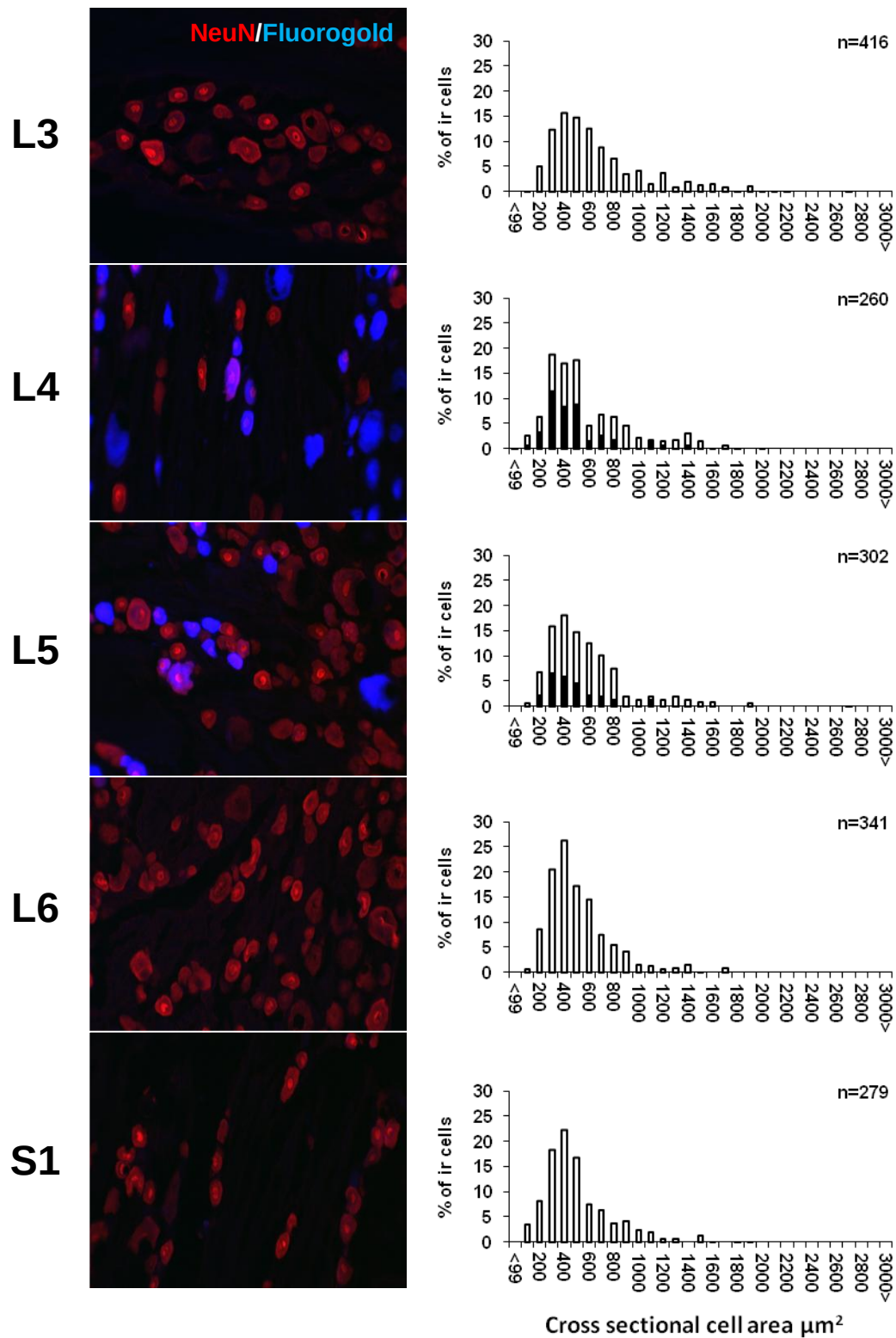


Figure 3.2 Representative images of immunoreactivity and cell size analysis of NeuN/Fluorogold labelled neurons in the L3-S1 DRGs of naïve animals (n=2). Magnification x20, Scale bars 50 μm .

3.3.1 Assessment of mechanical behaviours

From at least 7 days following TNT injury, animals exhibited significant mechanical hypersensitivity in the ipsilateral but not the contralateral mid-plantar region of the hind paw with respect to baseline values (Figure 3.3B-E). No significant hypersensitivity was observed in sham-operated animals at the various time points throughout the study. However at days 28 post TNT surgery in the contralateral paw, animals demonstrated a significant increase in paw withdrawal thresholds (PWTs). A significant increase in PWTs was also observed in the ipsilateral paw of sham animals at 43 days post surgery. The ipsilateral PWTs of animals at baseline and at day 1 post TNT injury were 39.1 ± 2.0 g and 30.1 ± 2.8 g respectively (Figure 3.3A), thus representing a mean percentage decrease of 22.0 ± 11.4 , but no significant change. Ipsilateral PWTs at baseline and at day 7 post TNT injury were 47.1 ± 3.0 g and 25.5 ± 1.0 g respectively (Figure 3.3B), representing a mean percentage decrease of 45.1 ± 6.1 ($p < 0.05$). The ipsilateral PWTs of animals sacrificed at 14 days decreased to 25.3 ± 1.0 g from a baseline value of 40.4 ± 0.6 g, representing a mean percentage decrease of 37.3 ± 3.4 ($p < 0.01$). This was a slightly smaller decrease than responses observed in the TNT day 7 group. Behavioural mechanical hypersensitivity reached a peak at 28 days post TNT injury with ipsilateral PWTs decreasing to 18.3 ± 2.0 g compared to baseline values of 48.3 ± 5.6 g (Figure 3.3D), representing a mean percentage decrease of 61.6 ± 2.9 ($p < 0.05$). In the longest experimental time of 43 days post TNT surgery, ipsilateral PWTs decreased to 27.3 ± 2.5 g from a baseline of 44.3 ± 3.8 g (Figure 3.3E), representing a mean percentage decrease of 37.6 ± 5.8 g. There were significant differences in the mean percentage decrease from baseline when comparing the ipsilateral paws of TNT animals and their sham counterparts at days 7, 14, 28, and 43 post surgery ($p < 0.05$, Figure 3.5A). Sham animals at 43 days post surgery demonstrated 46.5 ± 8.0 % increase in the ipsilateral PWT from baseline.

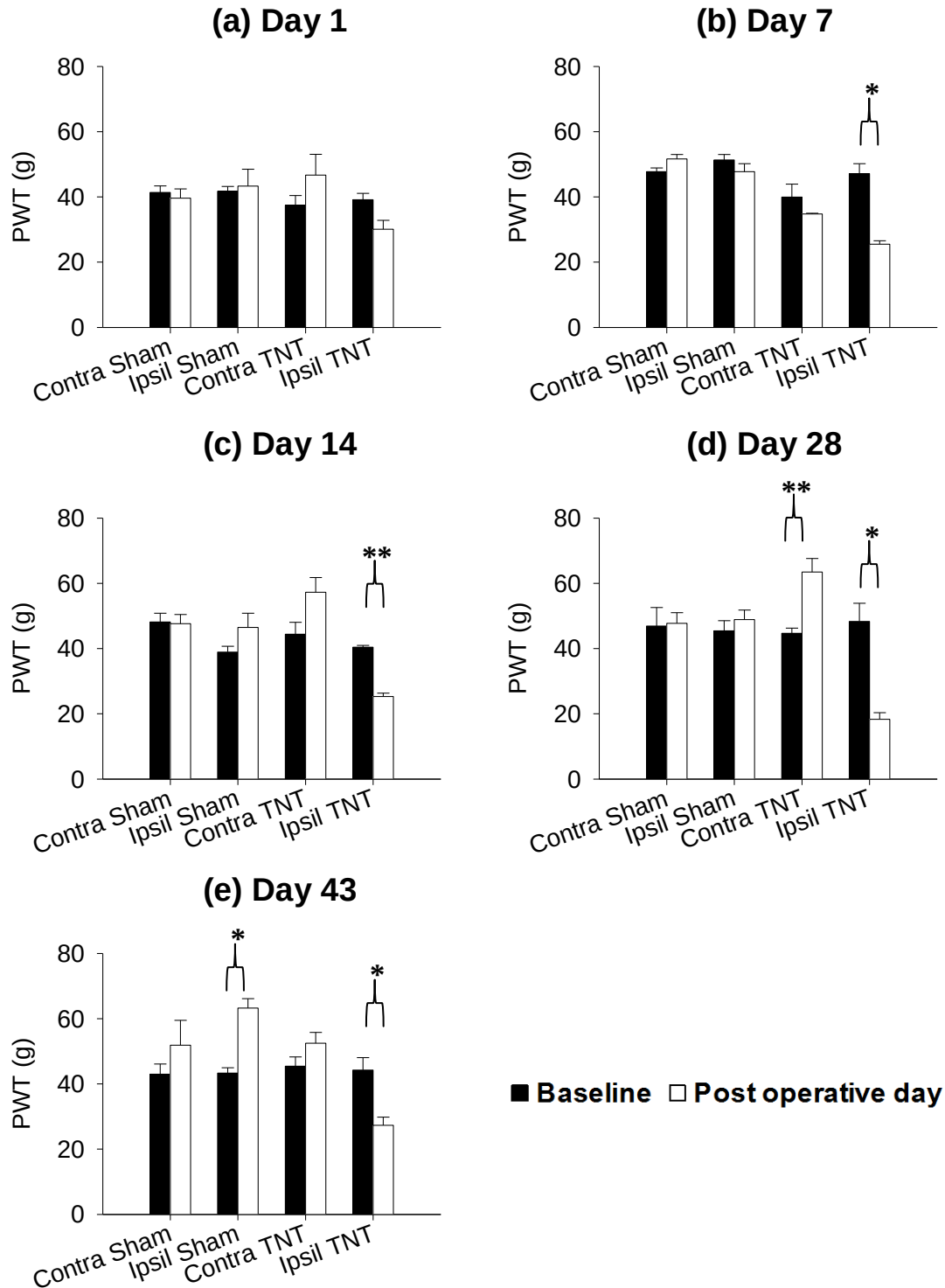


Figure 3.3 Mean paw withdrawal thresholds (PWT) to punctate mechanical stimuli measured using an electronic von Frey device at various time points post TNT/sham surgery in different groups of rats. Data presented as mean $\pm$ SEM. ** $P < 0.01$ and * $P < 0.05$ vs. respective baseline value using a Student's paired t test.

3.3.2 Assessment of thermal behaviours

Thermal hypersensitivity in the ipsilateral paws of TNT animals at day 1,7,14, and 43 post surgery was not evident, however significant responses were observed at day 28 post TNT injury. Ipsilateral PWLs at baseline and at day 1 post TNT injury were 13.5 ± 0.8 s and 14.3 ± 0.5 s respectively (Figure 3.4A), representing a mean percentage increase of 6.1 ± 2.7 s. The ipsilateral PWL at baseline and at day 7 post TNT injury were 9.0 ± 0.1 s and 6.8 ± 0.8 s respectively (Figure 3.4B), representing noticeable but not significant mean percentage decrease of 23.5 ± 9.4 . Similarly at day 14 there was a decrease in thermal PWL that did not reach significance, with recorded PWLs of 8.3 ± 1.5 s from baseline PWLs of 10.7 ± 2.1 s (mean percentage decrease of 16.5 ± 24.9 , Figure 3.4C). Significant thermal hypersensitivity in the ipsilateral paw was only evident in TNT animals at post operative day 28 with a mean PWL of 6.3 ± 0.5 s when compared to the respective mean baseline value of 8.9 ± 0.6 s ($p < 0.05$, Figure 3.4D), representing a mean percentage decrease of 29.0 ± 3.9 . At post operative day 43, there were no significant changes in PWL from baseline, 9.7 ± 0.8 s and 10.0 ± 1.1 s respectively (mean percentage decrease of 0.7 ± 10.3 , Figure 3.4E). No thermal hypersensitivity was observed in the contralateral paws of all study animals. Similarly, no thermal hypersensitivity was evident in sham animals at all time points within the study. There was however a significant increase in thermal PWL in the contralateral paws of sham animals at post operative day 14 from preoperative values (14.2 ± 0.3 s and 11.6 ± 0.5 s respectively, $p < 0.05$, Figure 3.4C). On comparison of the ipsilateral mean percentage decrease from baseline between TNT and sham operated animals, significant differences were only observed in the 28 days post surgery group ($p < 0.05$, Figure 3.5).

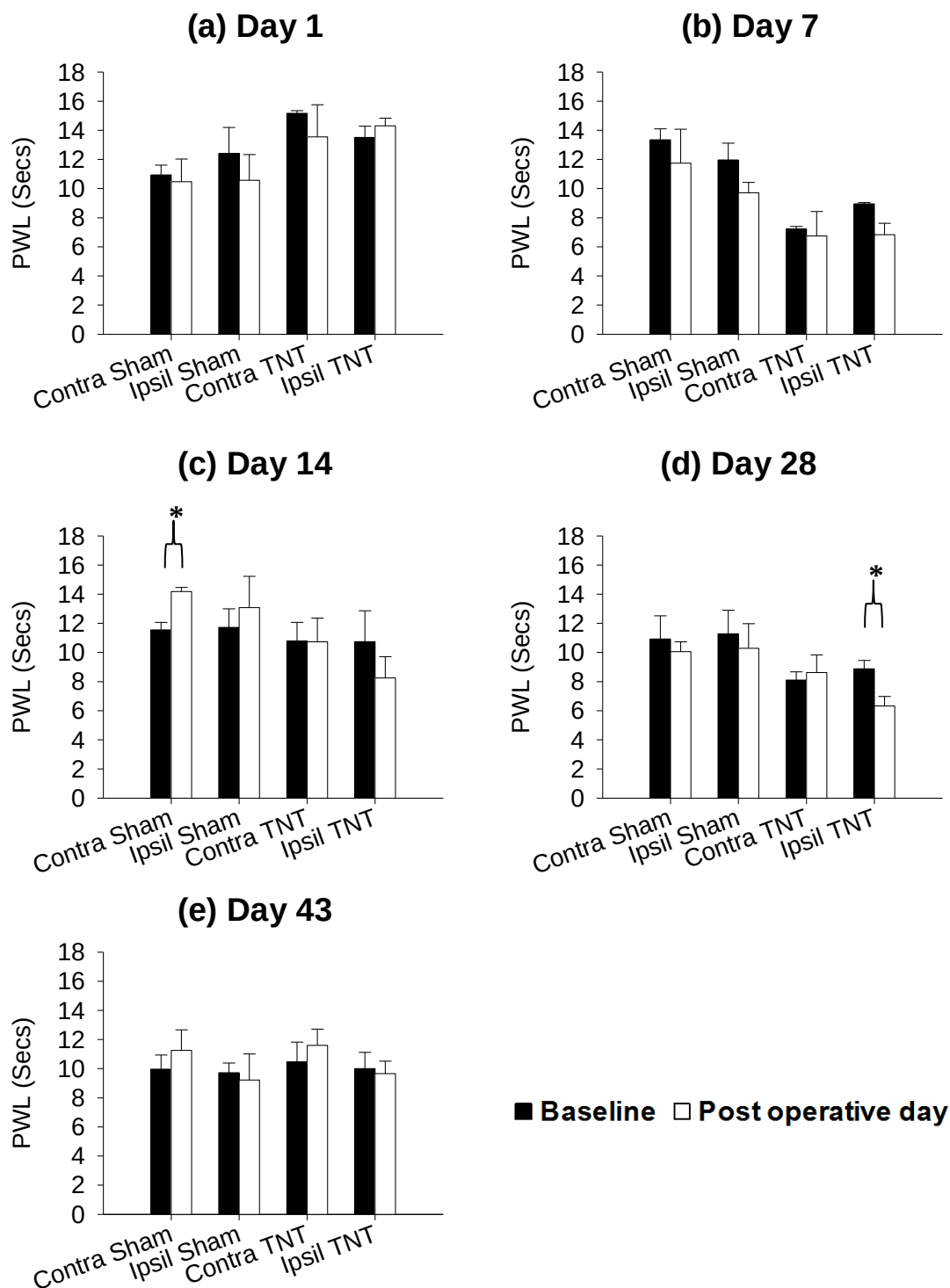


Figure 3.4 Mean paw withdrawal latencies (PWL) to thermal stimuli measured using a Hargreaves' device at various time point post TNT/sham surgery in different groups of rats. Data presented as mean $\pm$ SEM. * $P < 0.05$ vs. respective baseline value using a Student's paired t test.

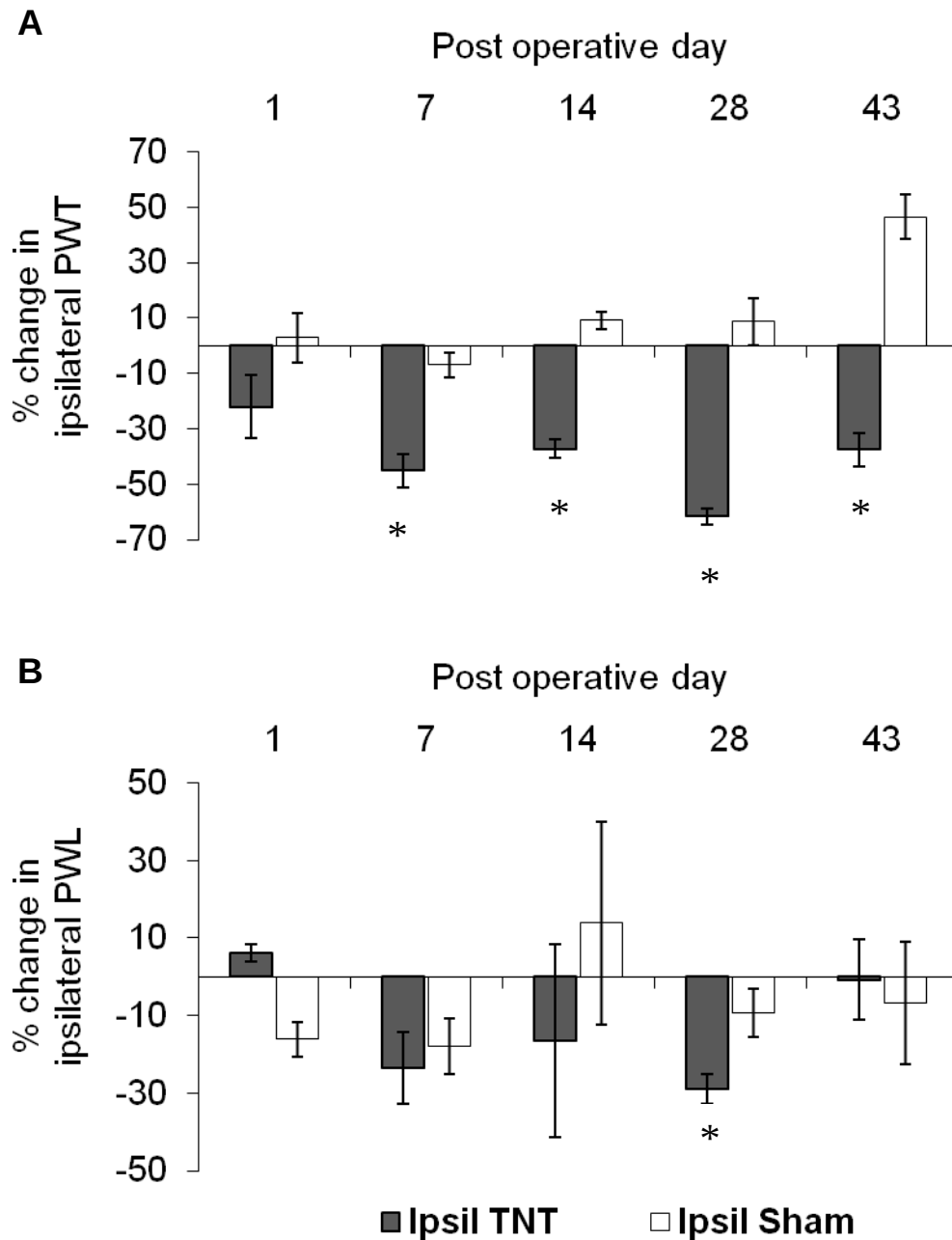


Figure 3.5 Mean percentage change from baseline at various time points post TNT injury in response to (A) punctate mechanical stimulation (using electronic von Frey device) and (B) Thermal Hargreaves device. * $P < 0.05$ vs. respective baseline value using a Student's paired t test. (PWT, paw withdrawal threshold; PWL, paw withdrawal latency). Sham animals at 43 days post surgery demonstrated a 46.5 ± 8.0 % increase in the ipsilateral PWT from baseline; an unusual finding.

3.3.3 Temporal profile expression of ATF-3 in ipsilateral L5 DRG

As previously reported, ATF-3 has been described as a marker of neuronal injury, and is not normally expressed or is present in low levels in uninjured animals (Tsujino et al., 2000). Consistent with this, ATF-3 immunoreactivity was not evident in DRGs harvested from naïve rats (see Table 3.2 for percentage immunoreactivity/mean cell size analysis and Figure 3.9 for immunohistochemistry images).

TNT injury was associated with significant ATF-3 immunoreactivity in both NF-200 and peripherin cell populations compared to sham operated rats except at 1 day post injury in peripherin characterised cells, and 43 days post injury in NF-200 characterised cells (Figure 3.6). A significant temporal profile effect was observed in the NF-200 characterised population in TNT injured rats, which was particularly evident one day after nerve injury but not in the peripherin characterised population. Other populations of DRG cells (e.g. IB4 or CGRP) could not be simultaneously examined because labeling with an additional fourth distinct colour was not possible due to the design of the experiment (all three filters were already designated).

One day post TNT injury ATF-3 immunoreactivity was robustly upregulated with 71.1 ± 7.2 % immunoreactivity observed in NF-200 characterised cells and was significantly different to values obtained from respective sham operated rats. ATF-3 immunoreactivity in peripherin characterised cells at this time point was 11.9 ± 5.6 %, a value that was not significantly different to that observed in respective sham operated rats, but was significantly different compared to that observed in NF-200 characterised cells. ATF-3 immunoreactivity was nuclear and present in neuronal cells only (Figure 3.7). Labeling was limited in sham animals (Figure 3.8). By 7 days post TNT injury ATF-3 immunoreactivity reduced to 25.7 ± 7.7 % in NF-200 characterised cells but increased slightly to 19.0 ± 5.0 % in peripherin characterised cells. There were no significant differences in the percentage of ATF-3 immunoreactivity between these two characterised cell populations, however both values were significantly different compared to that observed in respective sham operated rats. At 14 days post TNT injury, ATF-3 immunoreactivity was similar to that observed in day 7 rats, with 30.4 ± 8.6 % in NF-200 characterised cells and was not significantly different compared to the 18.9 ± 6.6 % observed in the peripherin characterised population. Percentage ATF-3 immunoreactivity at

this time point in both characterised cell populations was significantly different compared to that observed in respective sham operated rats. By 28 days post TNT injury, ATF-3 immunoreactivity was observed in 18.7 ± 6.0 % of NF-200 characterised cells and was not significantly different compared to the 15.2 ± 1.4 % observed in peripherin characterised cells, however both values were significantly different to those obtained from respective sham operated rats. At 43 days post injury, ATF-3 immunoreactivity was still evident, with 11.5 ± 4.2 % in NF-200 characterised cells, but was not significantly different compared to the 25.4 ± 8.3 % observed in peripherin characterised cells. Both values at this time point were significantly different to those obtained from respective sham operated rats.

A cell size analysis reinforced immunohistochemical observations regarding ATF-3 immunoreactivity following TNT injury in large diameter ($\geq 900 \mu\text{m}^2$) and small diameter cells ($\leq 500 \mu\text{m}^2$) at all time points investigated. The distribution was homogeneous in both neuronal populations up until day 14 (Figure 3.10 and Figure 3.11), but at day 28 post TNT injury and thereafter, ATF-3 immunoreactivity in large size NF-200 immunoreactive cells demonstrated a skewed distribution to the left (Figure 3.11) which coincides with the decreasing mean cell size area in this population (see Table 3.3 for mean cell size areas of ATF-3 immunoreactive cells across time in peripherin/NF-200 cells).

ATF-3

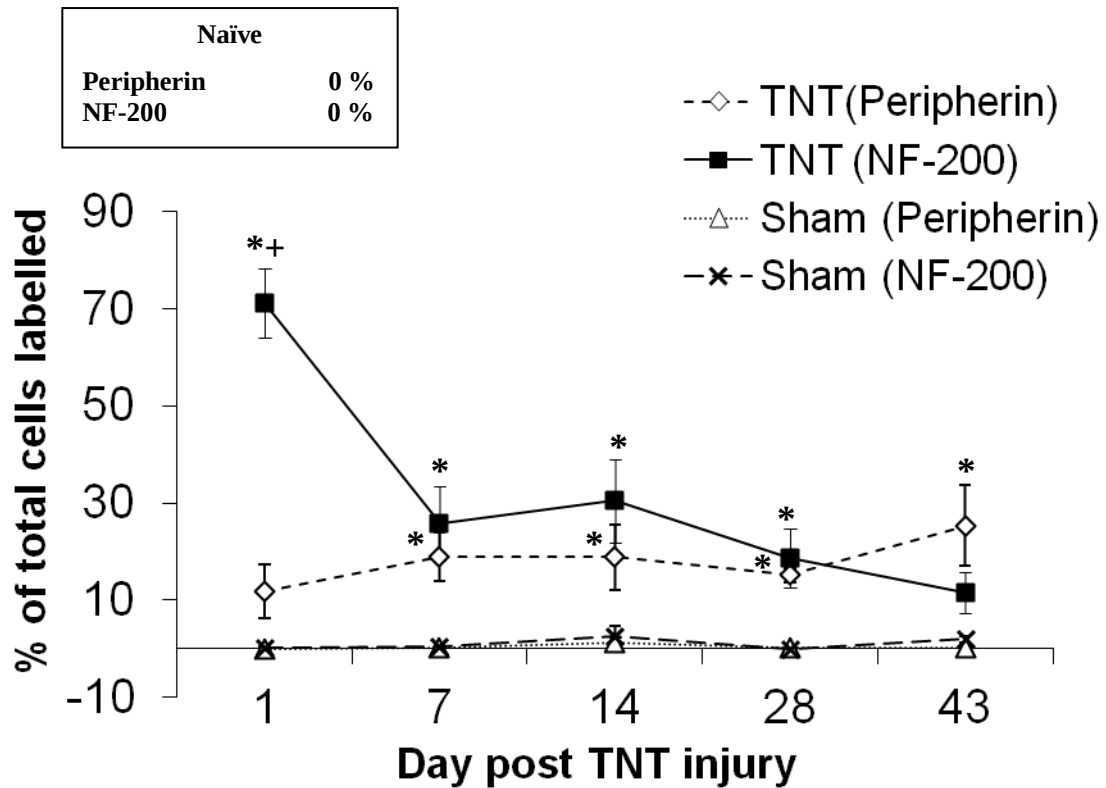


Figure 3.6 Mean percentage of total peripherin and NF-200 immunoreactive cells expressing ATF-3 in TNT, sham, and naïve animals (ipsilateral L5 DRG n=3-4 animals per group). Data presented as mean $\pm$ SEM. Statistical test at each time point was conducted using a two way ANOVA followed by Holm-Sidak post hoc analysis: * $P < 0.05$ vs. respective sham; + $P < 0.05$ vs. respective TNT (NF-200) immunoreactive cells. In TNT injured rats, ATF-3 immunoreactivity in NF-200 immunoreactive cells demonstrated a significant temporal profile expression, while ATF-3 immunoreactivity in peripherin immunoreactive cells did not exhibit a significant temporal profile.

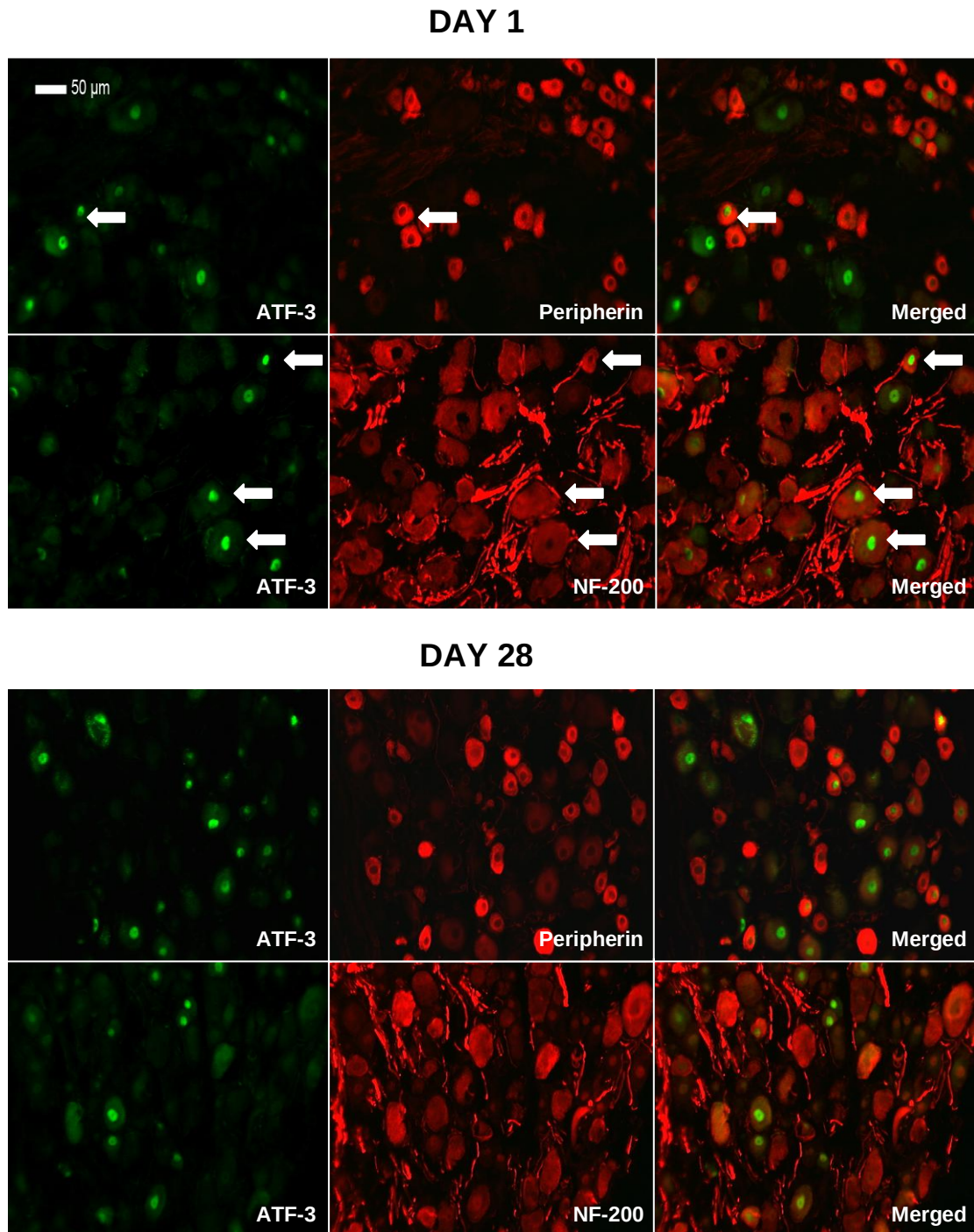


Figure 3.7 Representative images of ATF-3 immunoreactivity in peripherin (small diameter) and NF-200 (large diameter) neuronal sub-populations at 1 and 28 days following TNT injury (Ipsilateral L5 DRG), magnification x20. Arrows indicate co-localisation of immunolabeling.

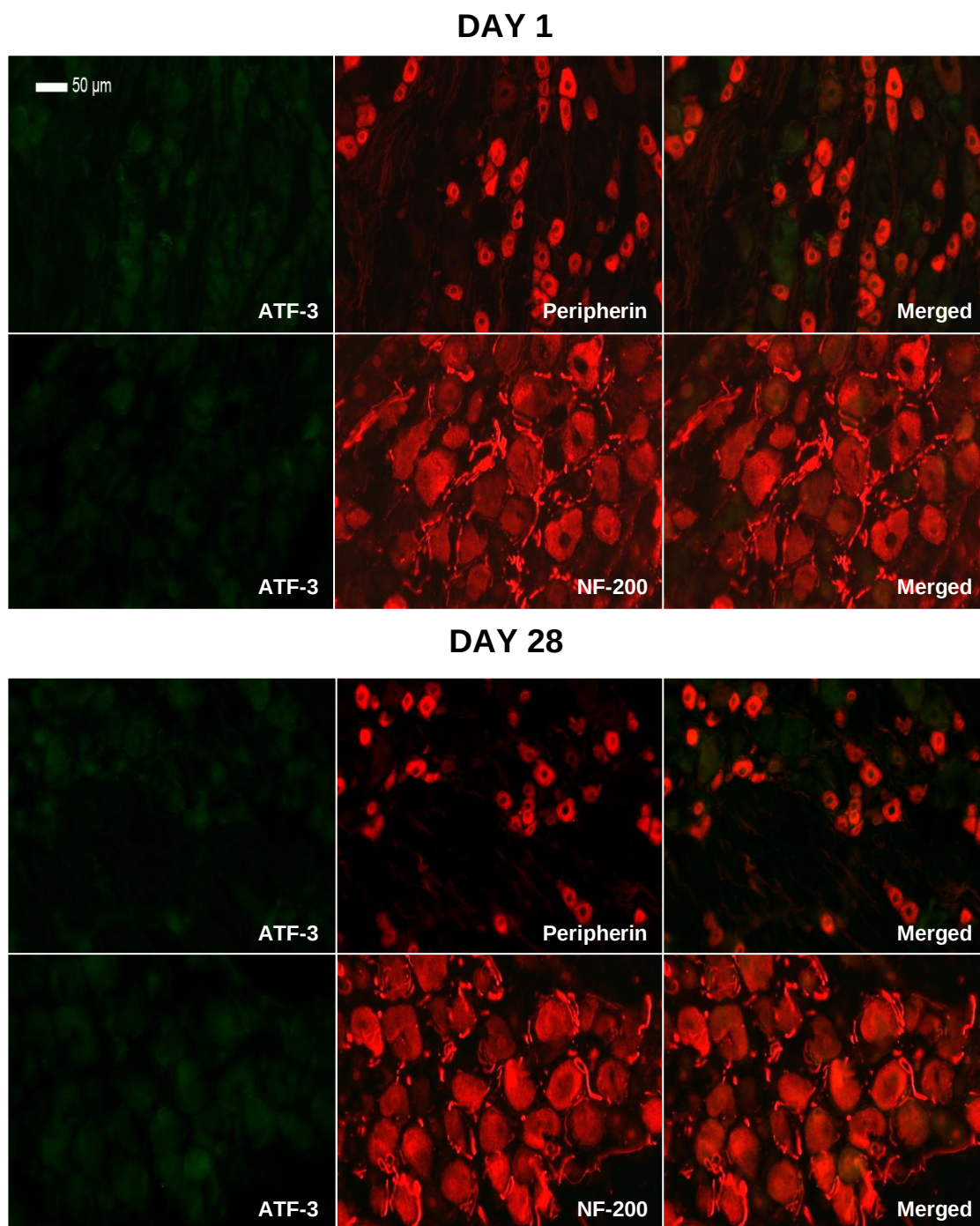


Figure 3.8 Representative images of ATF-3 immunoreactivity in peripherin (small diameter) and NF-200 (large diameter) neuronal sub-populations at 1 and 28 days following sham surgery (Ipsilateral L5 DRG). Magnification x20, Scale bar 50μm.

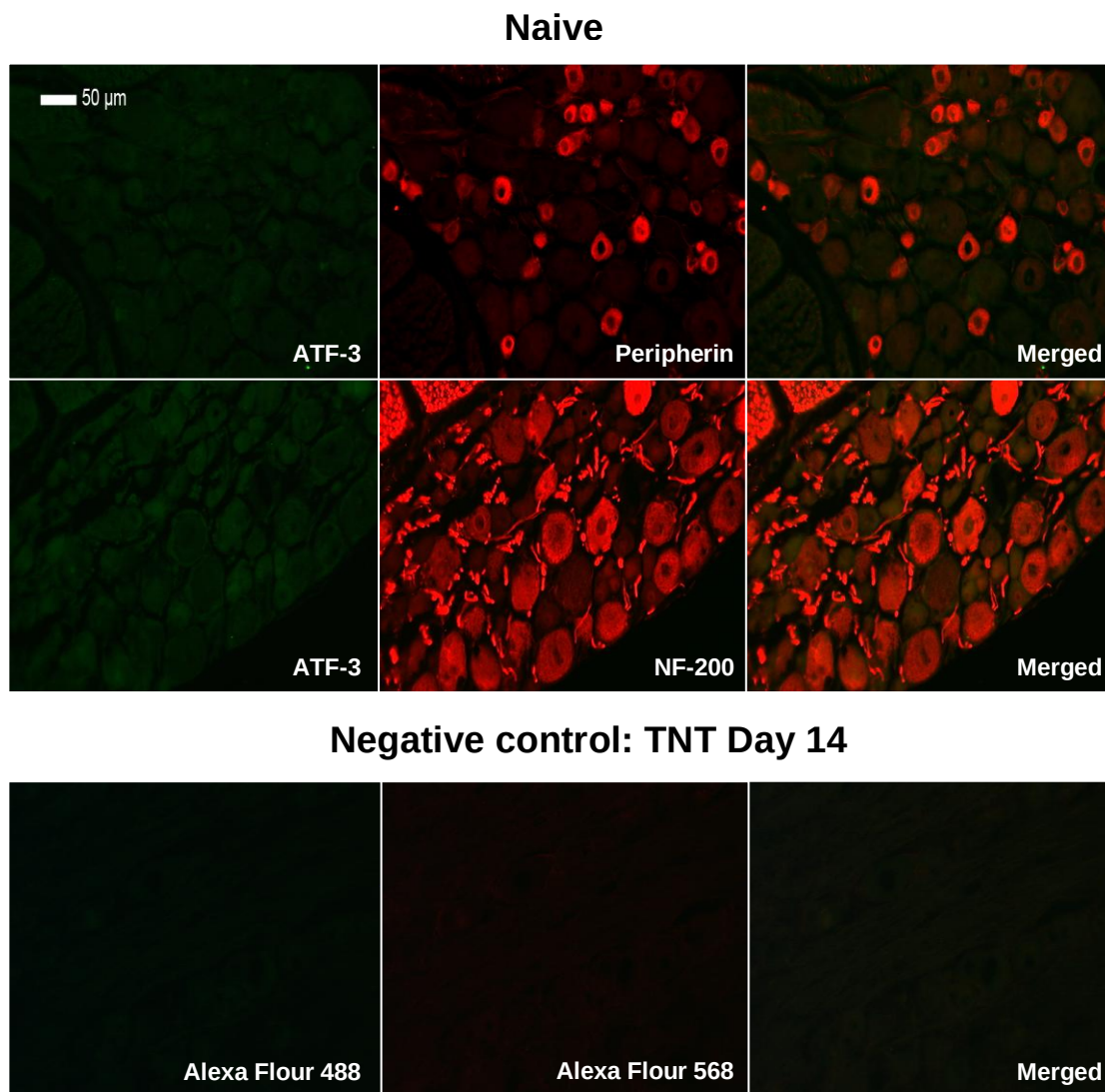


Figure 3.9 Representative images of ATF-3 immunohistochemistry in naive L5 DRG. Double labelling was conducted to identify ATF-3 immunoreactivity in both peripherin and NF-200 sub-populations of DRG cells; magnification x20. Bottom montages are representative images of negative controls (omission of primary antisera) taken from TNT ipsilateral L5 DRGs at post operative day 14.

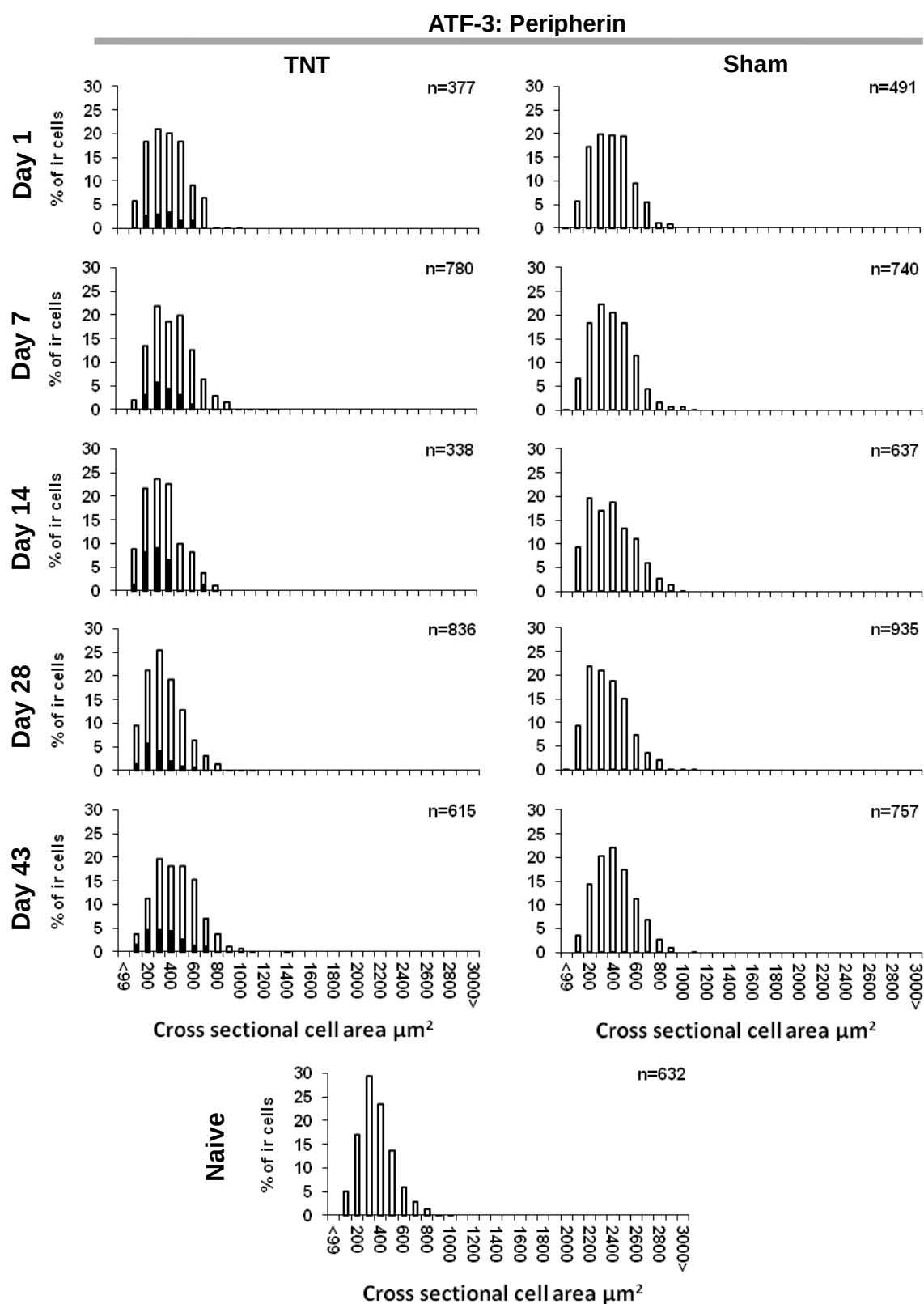


Figure 3.10 Percentage cell size distribution of ATF-3 immunoreactivity in peripherin immunoreactive cells (ipsilateral L5 DRG) at various time points following TNT/sham surgery and in naive animals (n = total number of nucleated peripherin immunoreactive cells).

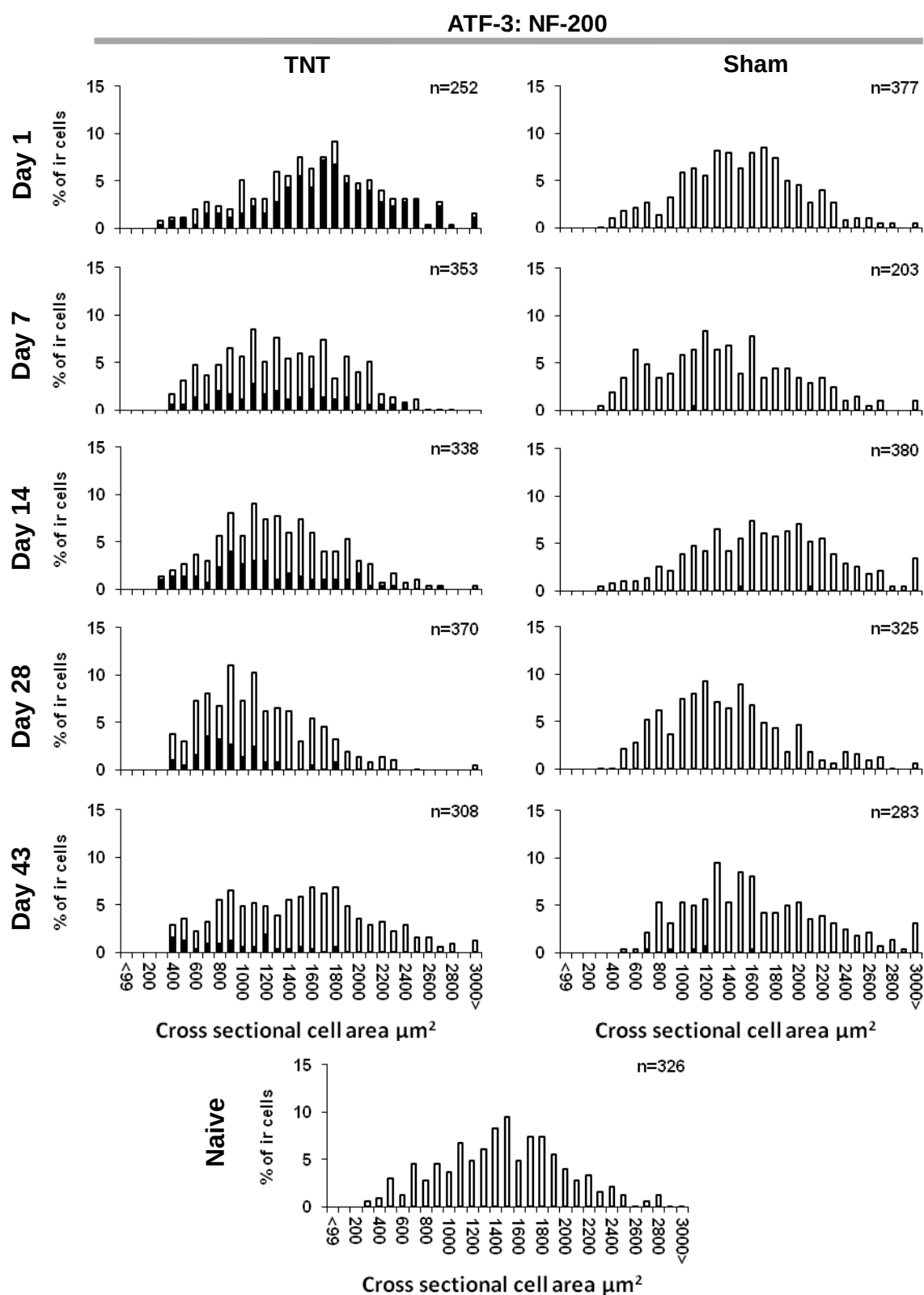


Figure 3.11 Percentage cell size distribution of ATF-3 immunoreactivity in NF-200 immunoreactive cells (ipsilateral L5 DRG) at various time points following TNT/sham surgery and in naive animals (n= total number of nucleated NF-200 immunoreactive cells).

3.3.4 Dynamic expression of GAP-43 in ipsilateral L5 DRG cells

GAP-43 immunoreactivity in naïve ganglia was relatively low (<2%) and only observed in peripherin immunoreactive cells (see Table 3.2 for mean percentage immunoreactivity/mean cell size analysis and Figure 3.13 for immunoreactivity images).

TNT injury was associated with significant GAP-43 immunoreactivity in both NF-200 and peripherin characterised cell populations at different time points post injury compared to sham operated rats (Figure 3.12). Significant temporal profile effects were evident in the NF-200 but not the peripherin characterised cell populations. GAP-43 immunoreactivity was cytoplasmic in nature, and was observed in both NF-200 and peripherin DRG cell populations, and additionally observed in axons (Figure 3.14 and Figure 3.15).

At one day post TNT injury, GAP-43 immunoreactivity was not significantly differently compared to sham operated rats. However at 7 days post TNT injury, GAP-43 immunoreactivity had increased in the peripherin characterised population to 7.2 ± 0.7 % and was significantly different to values obtained from respective sham operated rats. GAP-43 immunoreactivity in NF-200 characterised cells at this time point was 0.3 ± 0.3 %, a value that was not significantly different to that observed in respective sham operated rats, but was significantly different compared to that observed in peripherin characterised cells. By 14 days post TNT injury, GAP-43 immunoreactivity had peaked in both characterised DRG cell populations examined, with 15.9 ± 4.8 % immunoreactivity in NF-200 characterised cells and 15.7 ± 1.8 immunoreactivity in peripherin characterised cells. There were no significant differences in the percentage of GAP-43 immunoreactivity between these two characterised cell populations, however both values were significantly different compared to that observed in respective sham operated rats. At 28 days post TNT injury, GAP-43 immunoreactivity had reduced to 10.0 ± 1.9 % in NF-200 characterised cells and was not significantly different to the 11.6 ± 2.5 % observed in peripherin characterised cells, however both values were significantly different to those obtained from respective sham operated rats. At 43 days post TNT injury, GAP-43 immunoreactivity further reduced in both NF-200 and peripherin characterised cell populations and was not significantly different compared to those values obtained from respective sham operated rats. GAP-43 immunoreactivity was observed in 8.3 ± 1.0 % of NF-200 cells and was not

significantly different compared to the $10.0 \pm 6.4\%$ observed in the peripherin characterised cells.

A cell size distribution analysis of GAP-43 expression in TNT animals revealed a homogenous distribution in peripherin characterised cells at all time points (Figure 3.16); mean cell size area ($\leq 500 \mu\text{m}^2$, Table 3.3). However a skewed distribution to the left was observed in large diameter NF-200 characterised cells (Figure 3.17); mean cell size area ($\geq 600 \mu\text{m}^2$). See Table 3.3 for mean cell size areas of GAP-43 immunoreactive cells across time in peripherin/NF-200 cells.

GAP-43

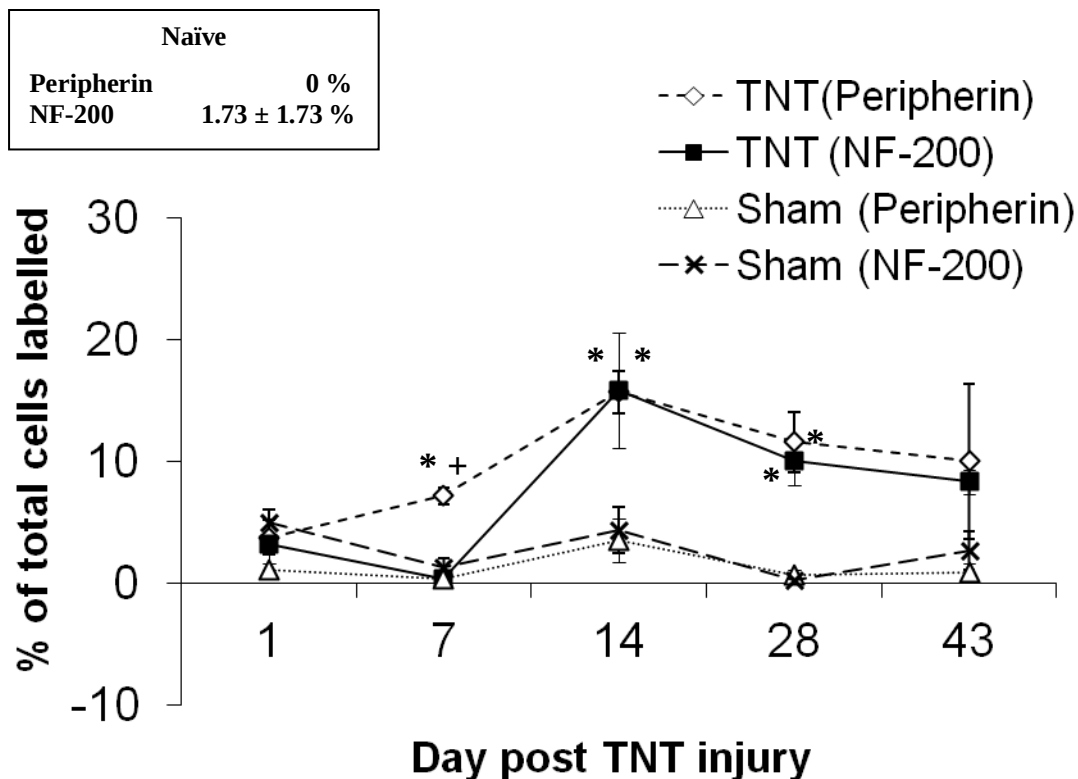


Figure 3.12 Mean percentage of total peripherin and NF-200 immunoreactive cells expressing GAP-43 in TNT, sham, and naïve animals (ipsilateral L5 DRG n=3-4 animals per group). Data presented as mean $\pm$ SEM. Statistical test at each time point was conducted using a two way ANOVA followed by Holm-Sidak post hoc analysis: * $P < 0.05$ vs. respective sham; + $P < 0.05$ vs. respective TNT (NF-200) immunoreactive cells. In TNT injured rats, GAP-43 expression in NF-200 immunoreactive cells demonstrated a significant temporal profile expression, while GAP-43 expression in peripherin immunoreactive cells did not exhibit a significant temporal profile.

Naive

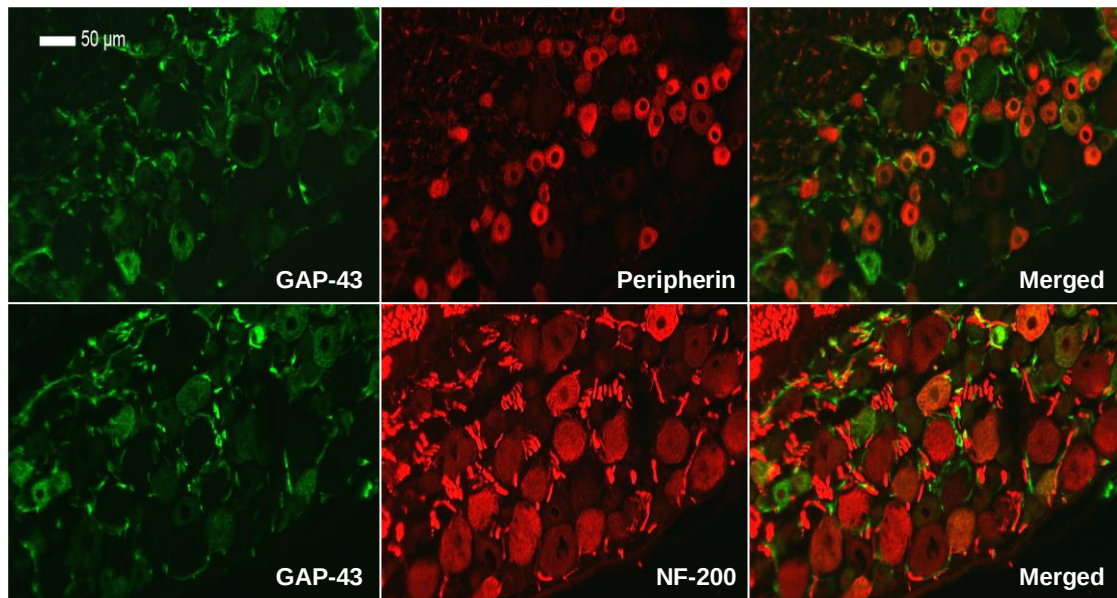


Figure 3.13 Representative images of GAP-43 immunohistochemistry in naive L5 DRG. Double labelling was conducted to identify GAP-43 immunoreactivity in both peripherin and NF-200 sub-populations of DRG cells; magnification x20.

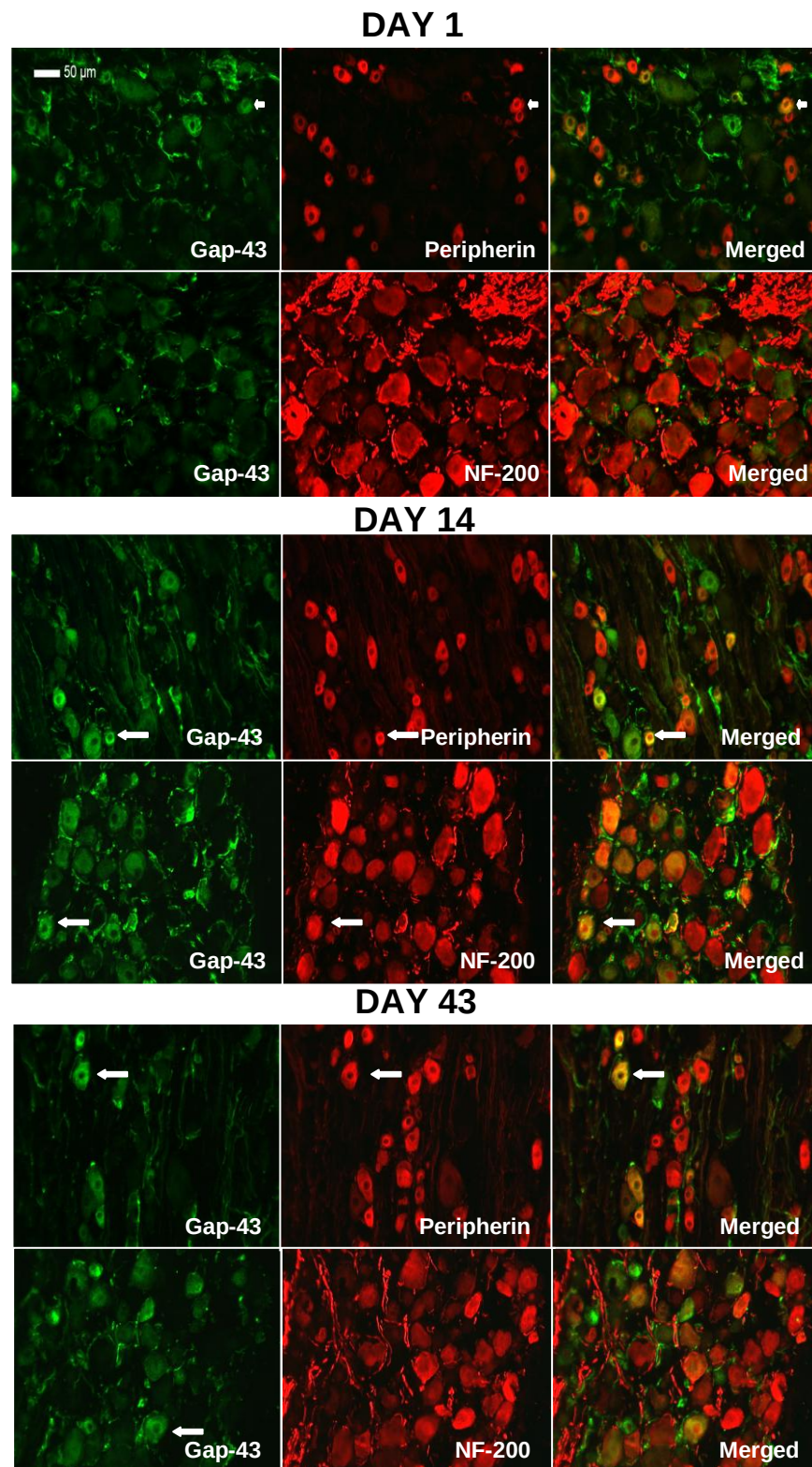


Figure 3.14 Representative images of GAP-43 immunoreactivity at 1,14, and 43 days post TNT injury (ipsilateral L5 DRG) in peripherin and NF-200 neuronal sub-populations, magnification x20. Arrows indicate co-localisation of immunolabeling.

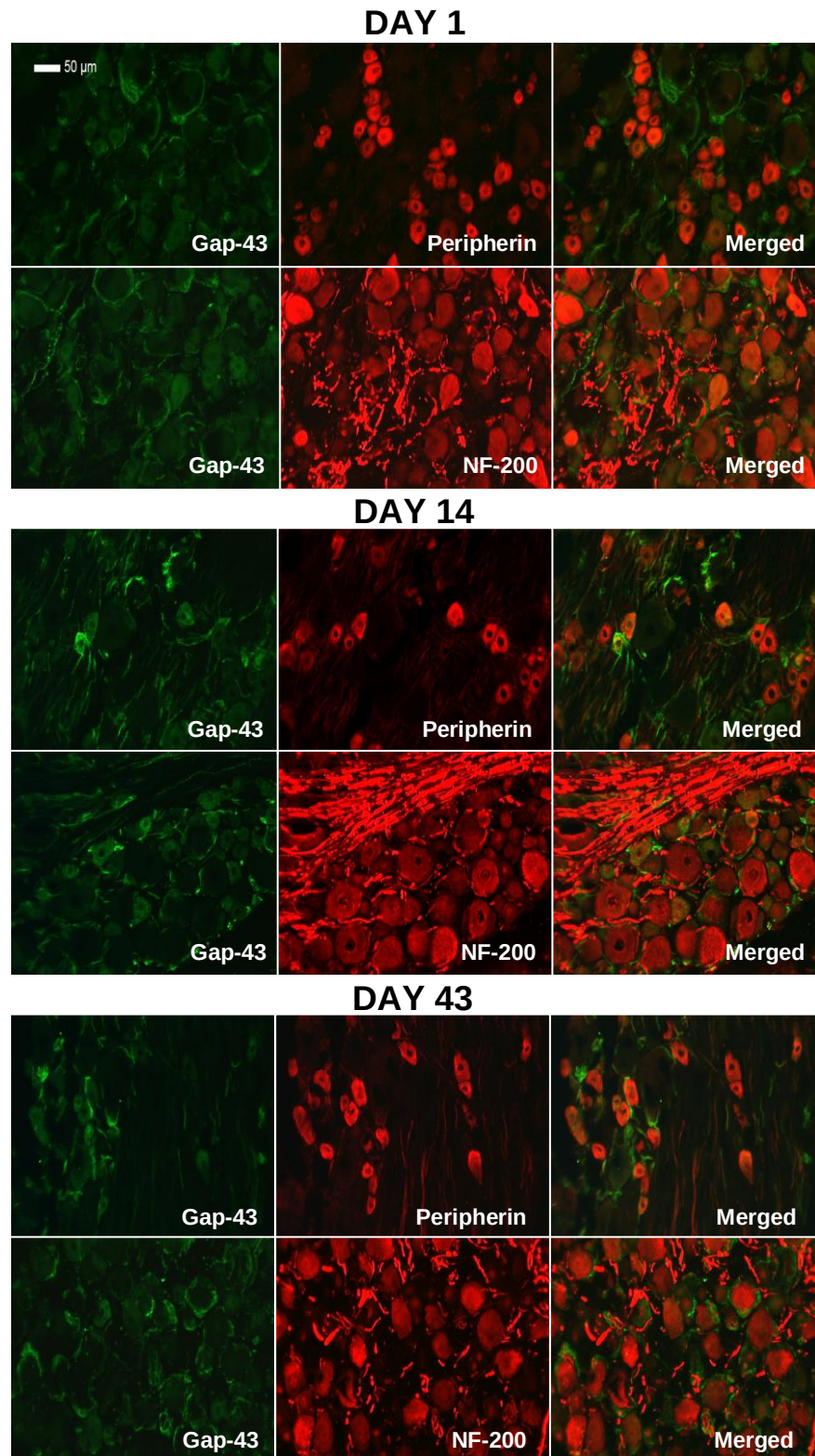


Figure 3.15 Representative images of GAP-43 immunoreactivity at 1, 14, and 43 days post sham surgery (ipsilateral L5 DRG) in peripherin and NF-200 neuronal sub-populations, magnification x20.

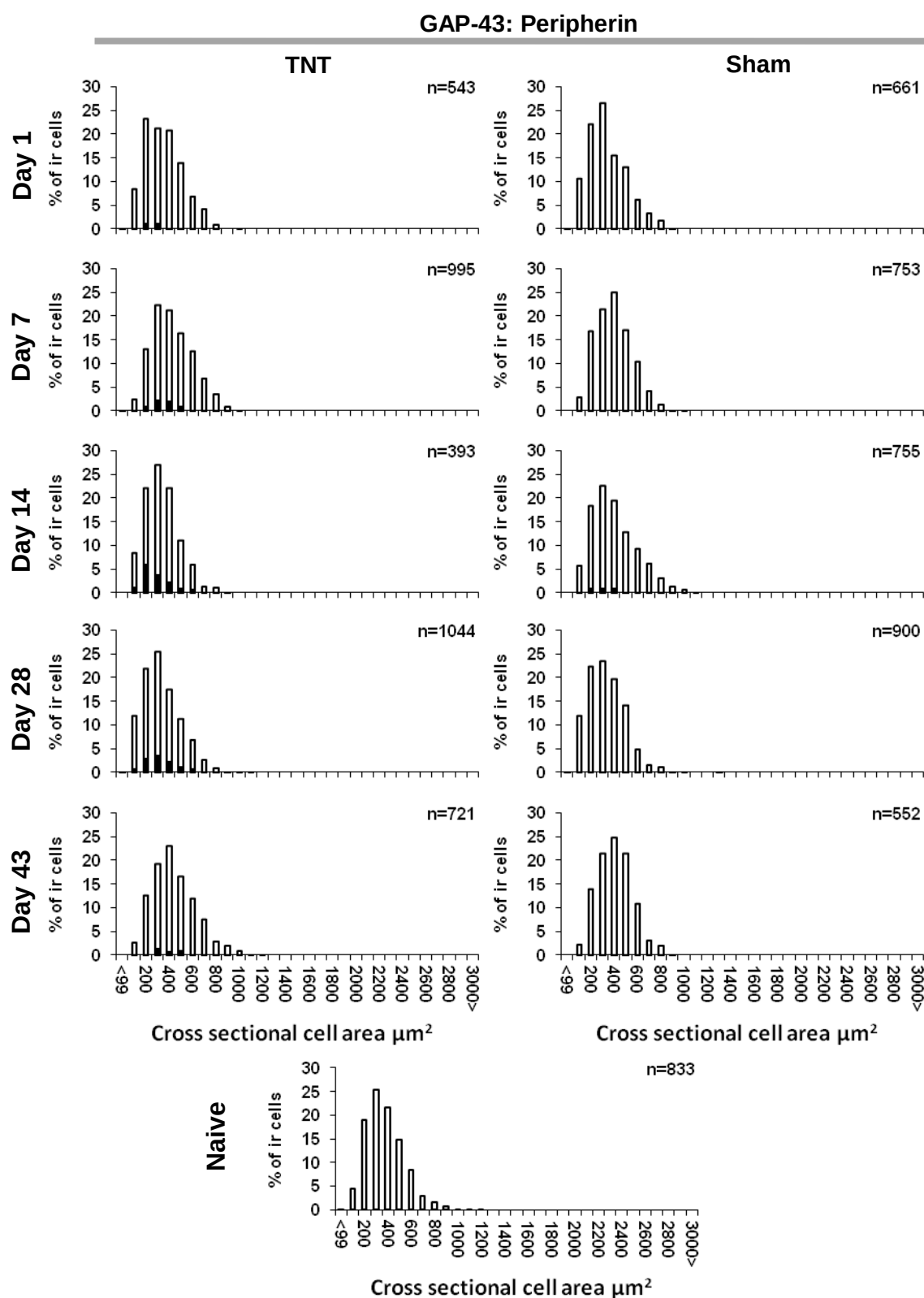


Figure 3.16 Percentage cell size distribution of GAP-43 immunoreactivity in peripherin immunoreactive cells (ipsilateral L5 DRG) at various time points following TNT/sham surgery and in naive animals (n= total number of nucleated peripherin immunoreactive cells).

Gap-43: NF-200

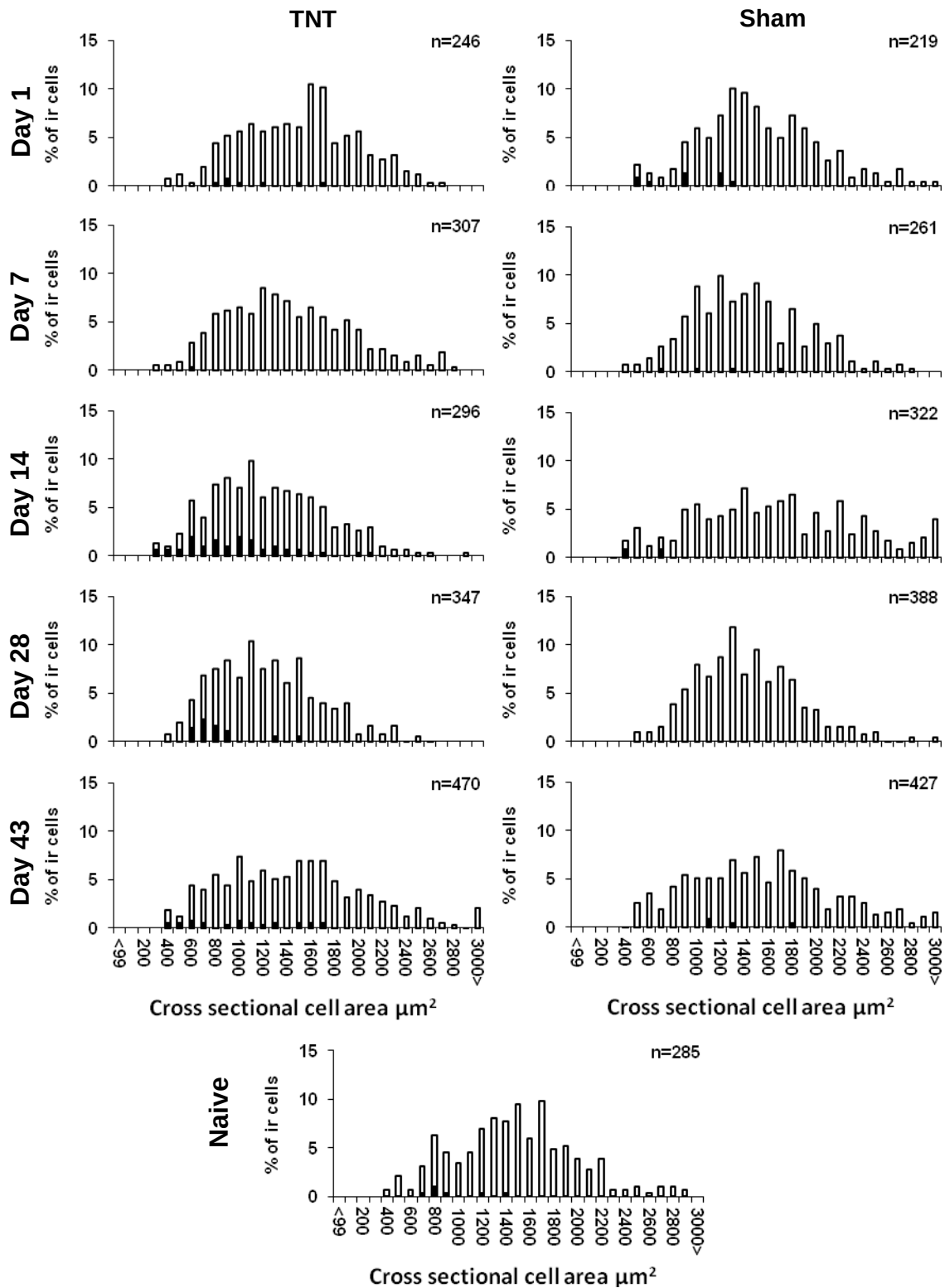


Figure 3.17 Percentage cell size distribution of GAP-43 immunoreactivity in NF-200 immunoreactive cells (ipsilateral L5 DRG) at various time points following TNT/sham surgery and in naive animals (n= total number of nucleated NF-200 immunoreactive cells).

3.3.5 NPY exhibits dynamic temporal expression in ipsilateral L5 DRG cells

NPY immunoreactivity in naïve ganglia was only observed only in few NF-200 immunoreactive cells (<0.3 %, Table 3.2) and not in the peripherin characterised population.

Following TNT injury, NPY exhibited significant temporal profile expressions in both NF-200 and peripherin characterised DRG cells (Figure 3.18, $p < 0.05$, one way ANOVA). NPY immunoreactivity remained relatively low in peripherin characterised cells across time (<6.5%), compared to immunoreactivity in NF-200 cells, which was more robust (peak of ~51% at 14 days survival). NPY immunoreactivity was cytoplasmic in nature (see Figure 3.20 and Figure 3.21), and some immunoreactivity was additionally observed in axons (Figure 3.22).

At one day post TNT injury, NPY immunoreactivity was not observed in injured ganglia. However, at 7 days post injury NPY immunoreactivity was robustly upregulated, with 51.3 ± 4.8 % observed in NF-200 characterised cells and was significantly different to values obtained from respective sham operated rats. NPY immunoreactivity in peripherin characterised cells at this time point was 2.1 ± 0.6 %, a value that was not significantly different to that observed in respective sham operated rats, but was significantly different compared to that observed in NF-200 characterised cells. By 14 days post injury, NPY immunoreactivity was comparable to that observed at 7 days post injury. NPY immunoreactivity was 51.7 ± 8.3 % in NF-200 characterised cells and significantly different to values obtained from respective sham operated rats. NPY immunoreactivity in peripherin characterised cells at this time point was 3.4 ± 1.4 %, a value that was not significantly different to that observed in respective sham operated rats, but was significantly different compared to that observed in NF-200 characterised cells. At 28 days post injury, NPY immunoreactivity had reduced to 27.3 ± 3.4 % in NF-200 characterised cells, while immunoreactivity in peripherin characterised cells peaked at 6.5 ± 0.8 %, with significant differences present between these two cell populations and their respective sham counterparts. NPY immunoreactivity further reduced at 43 days post injury in NF-200 characterised cells to 14.5 ± 2.9 %, while immunoreactivity in peripherin characterised cells stabilized at 5.0 ± 0.8 % – significant differences were present between these two cell populations and their respective sham counterparts.

A cell size distribution analysis supported immunohistochemical observations regarding NPY immunoreactivity following TNT injury in NF-200 large diameter ($\geq 900 \mu\text{m}^2$) and peripherin immunoreactive small diameter cells ($\leq 600 \mu\text{m}^2$) at all time points investigated (see Table 3.3 for mean cell size areas of NPY immunoreactive cells across time in peripherin/NF-200 cells, and Figure 3.23 and Figure 3.24 for cell size distribution analysis).

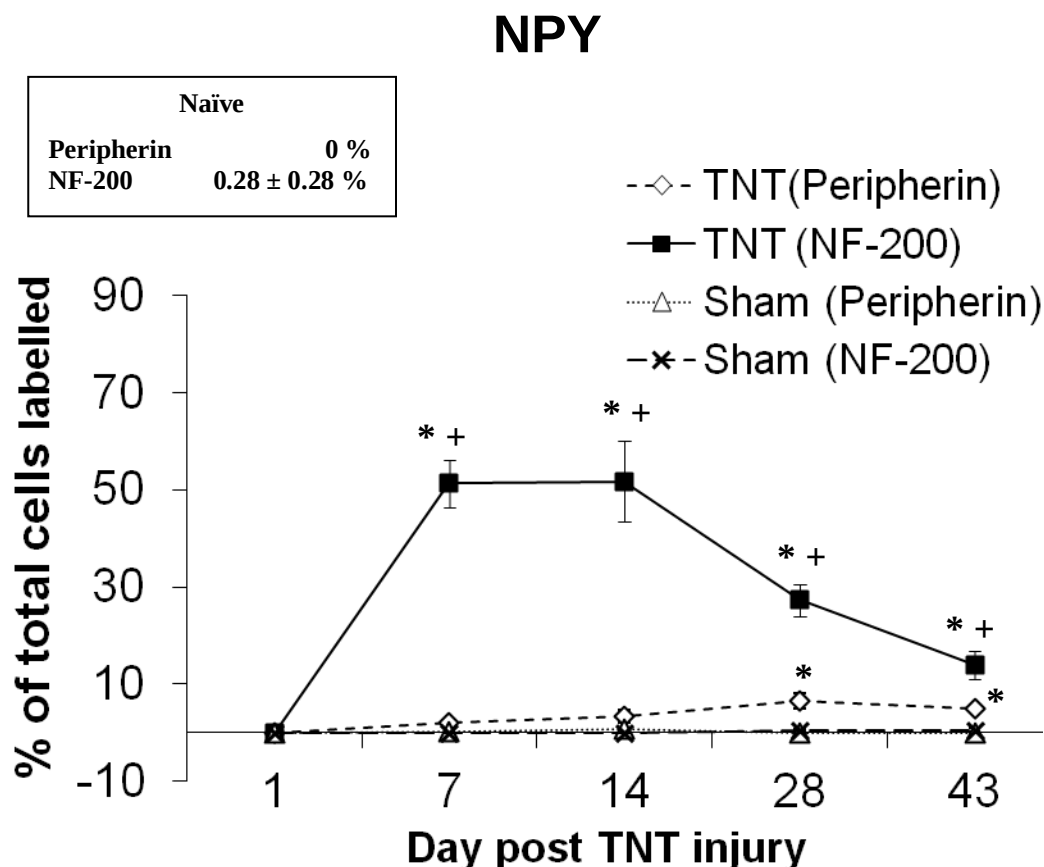


Figure 3.18 Mean percentage of total peripherin and NF-200 immunoreactive cells expressing NPY in TNT, sham, and naïve animals (ipsilateral L5 DRG n=3-4 animals per group). Data presented as mean $\pm$ SEM. Statistical test at each time point was conducted using a two way ANOVA followed by Holm-Sidak post hoc analysis: * $P < 0.05$ vs. respective sham; $^+P < 0.05$ vs. Respective TNT (peripherin) immunoreactive cells. In TNT injured rats, NPY immunoreactivity in both NF-200 and peripherin immunoreactive cells demonstrated significant temporal profile expressions.

Naive

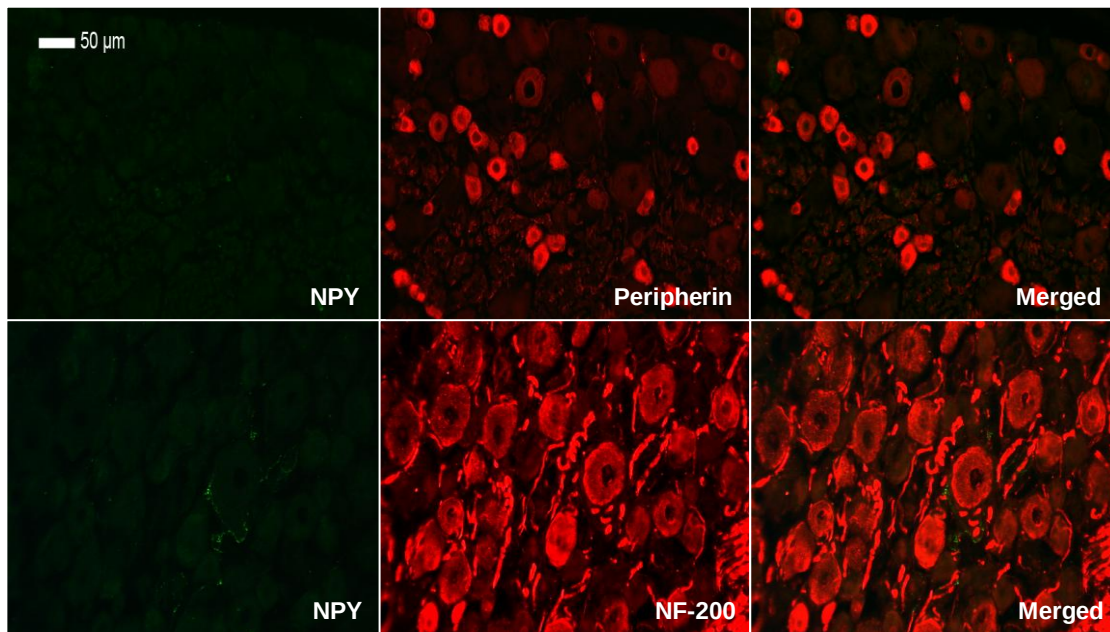


Figure 3.19 Representative images of NPY immunohistochemistry in naive L5 DRG. Double labelling was conducted to identify NPY immunoreactivity in both peripherin and NF-200 sub-populations of DRG cells; magnification x20.

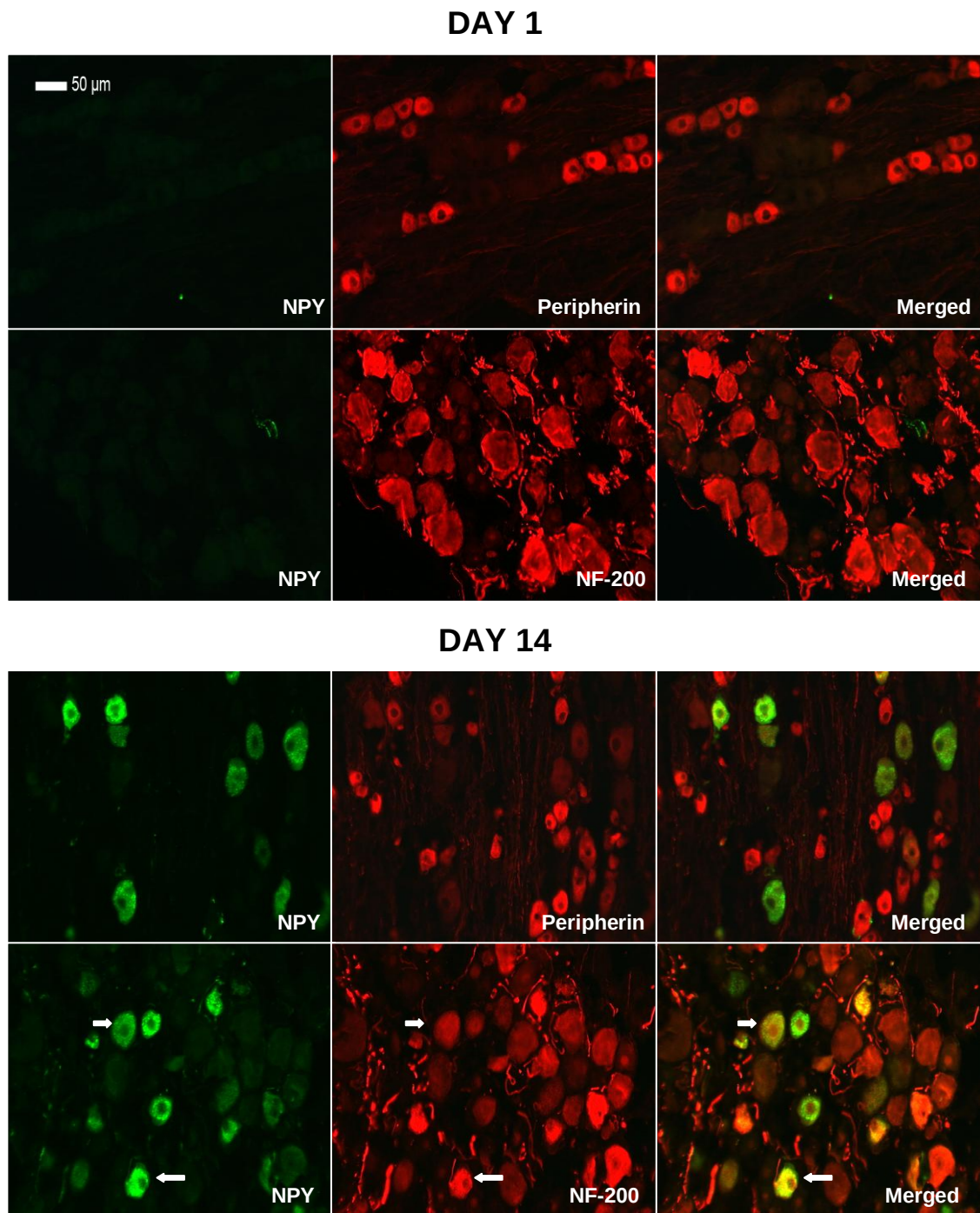


Figure 3.20 Representative images of NPY immunoreactivity in peripherin (small diameter) and NF-200 (large diameter) neuronal sub-populations at 1 and 14 days following TNT injury (ipsilateral L5 DRG), magnification x20. Arrows indicate co-localisation of immunolabeling.

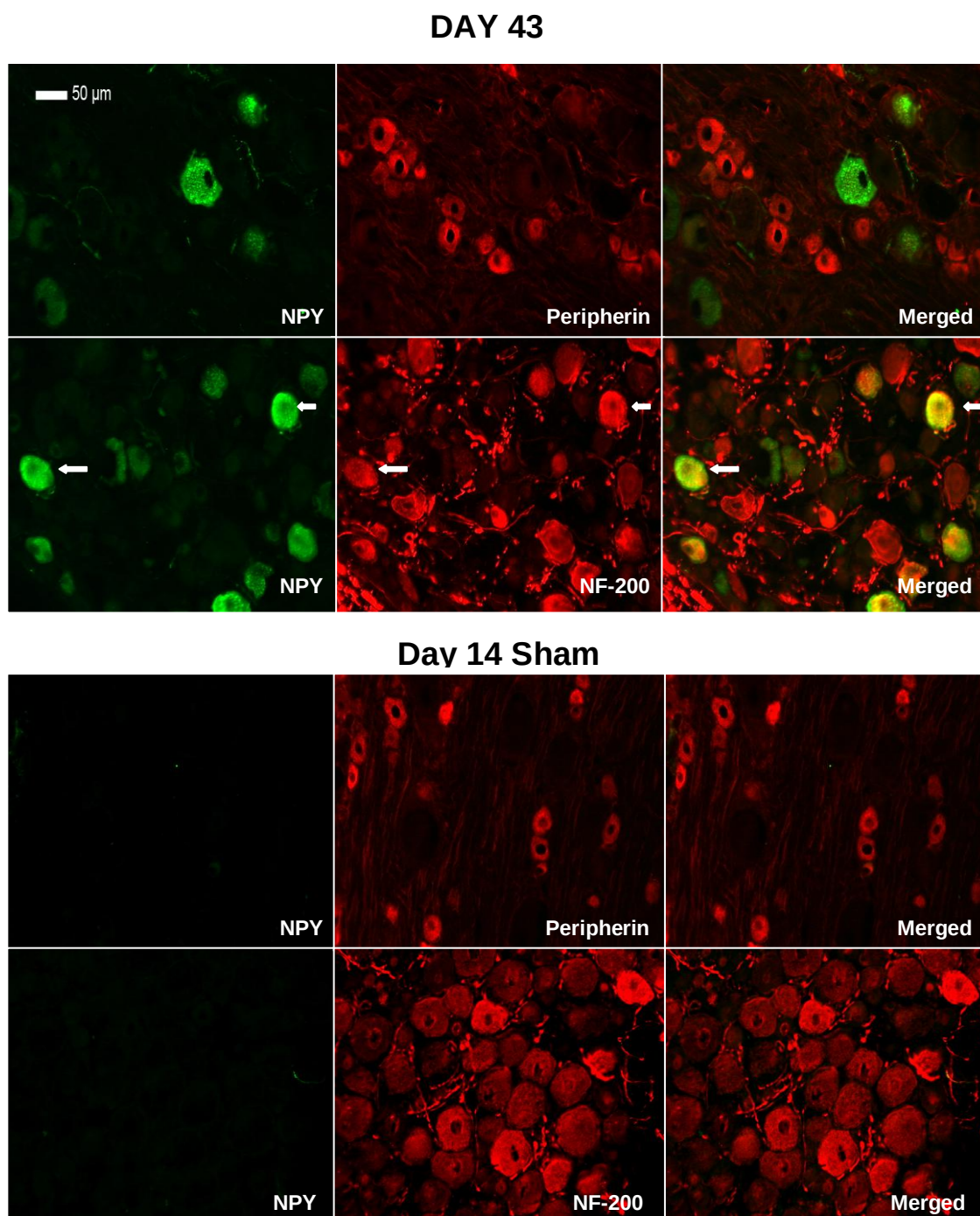


Figure 3.21 Representative images of NPY immunoreactivity in peripherin (small diameter) and NF-200 (large diameter) neuronal sub-populations at 43 days post TNT injury & 14 days after sham surgery (ipsilateral L5 DRG), magnification x20. Arrows indicate co-localisation of immunolabeling.

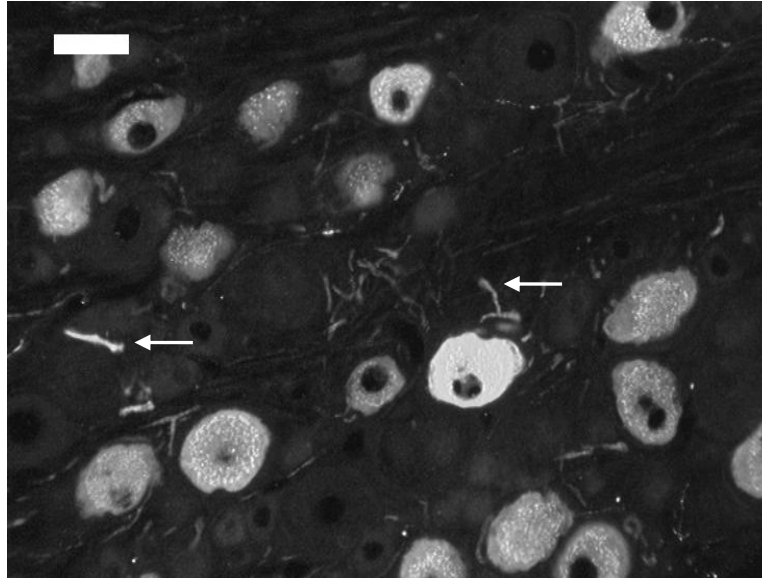


Figure 3.22 Representative image of NPY immunoreactivity in the mid-section of the L5 DRG at 14 days post TNT injury using rabbit polyclonal antisera. NPY is induced in the cytoplasm of mainly large diameter cells but is also induced in axons as indicated by the red arrows. Neuronal magnification x20, scale bars 50 μ m.

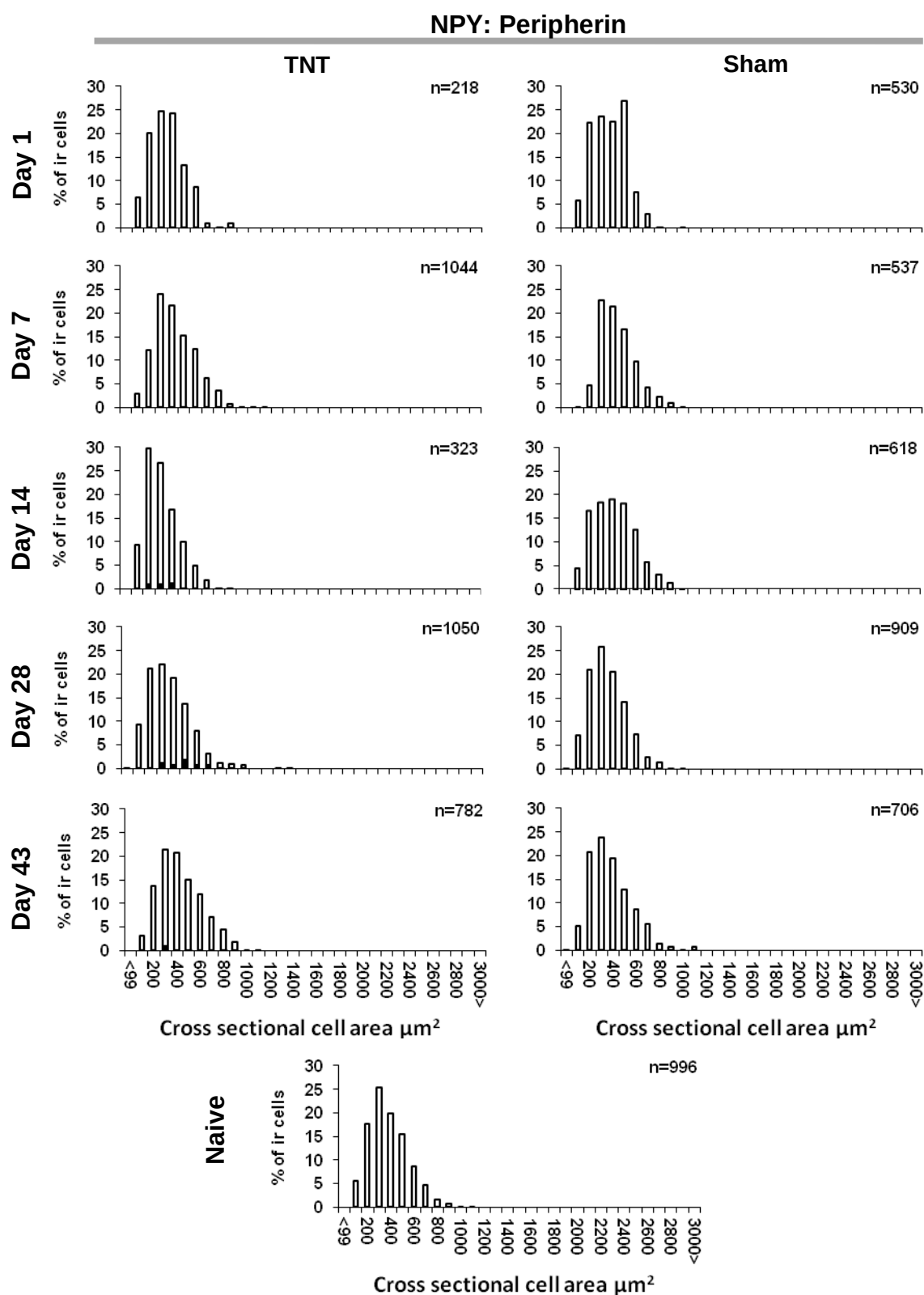


Figure 3.23 Percentage cell size distribution of NPY immunoreactivity in peripherin immunoreactive cells (ipsilateral L5 DRG) at various time points following TNT/sham surgery and in naive animals (n = total number of nucleated peripherin immunoreactive cells).

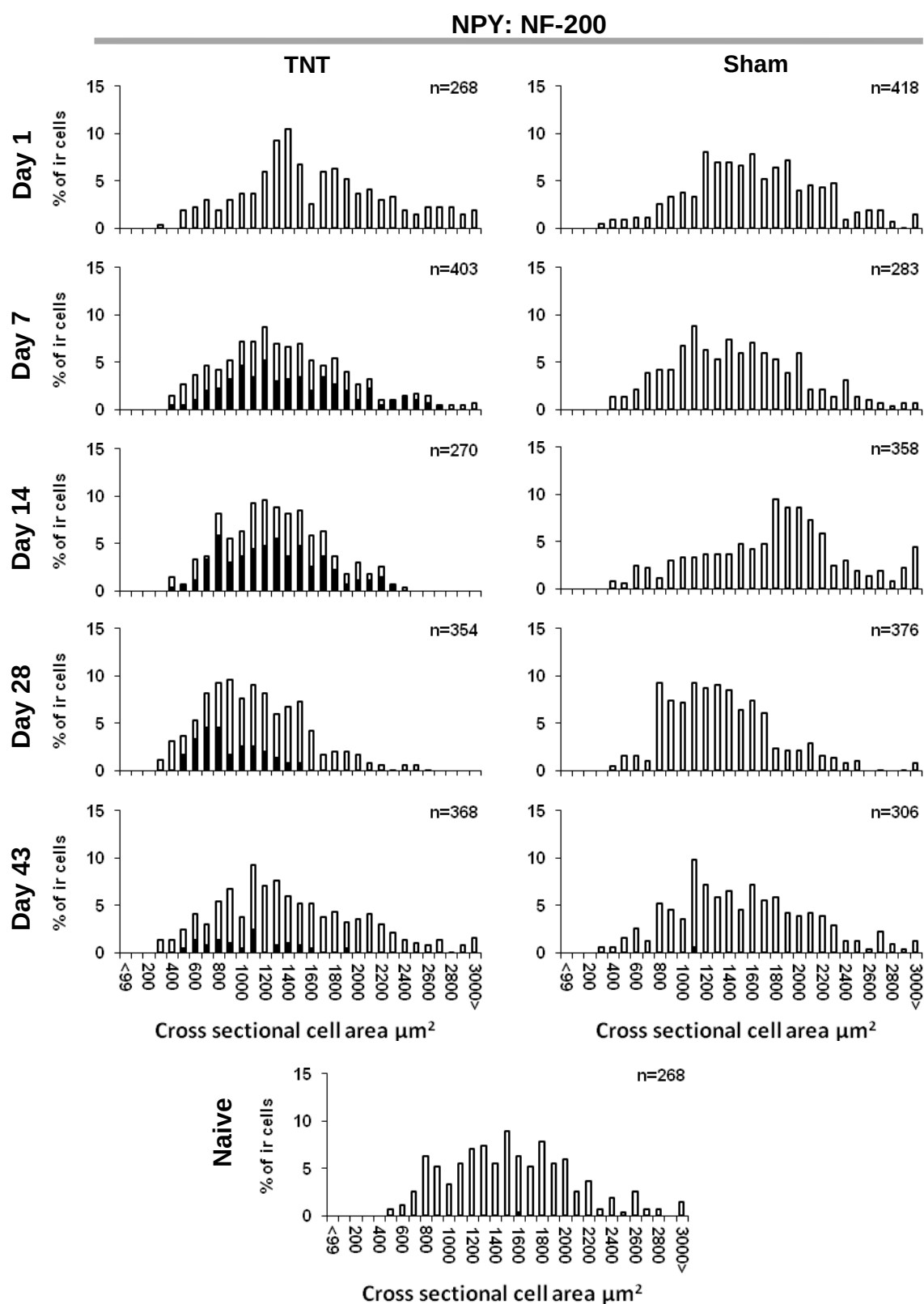


Figure 3.24 Percentage cell size distribution of NPY immunoreactivity in NF-200 immunoreactive cells (ipsilateral L5 DRG) at various time points following TNT/sham surgery and in naive animals (n= total number of nucleated NF-200 immunoreactive cells).

3.3.6 Dynamic expression of galanin in ipsilateral L5 DRG cells

Galanin immunoreactivity in naïve ganglia was relatively low with 0.56 ± 0.56 % of galanin immunoreactivity in peripherin immunoreactive cells and no immunoreactivity present in NF-200 immunoreactive cells (see Table 3.2 for mean percentage immunoreactivity/mean cell size analysis and Figure 3.26 for immunohistochemistry images).

Following TNT injury, galanin exhibited dynamic expression, upregulating at similar levels in both peripherin and NF-200 characterised cells at 7 and 14 days, before down-regulating at 28 and 43 days post injury (Figure 3.25). These patterns were significant in both cell populations ($p < 0.05$, one way ANOVA). Galanin exhibited granular cytoplasmic staining (Figure 3.27), not evident in sham controls (Figure 3.28).

At one day post TNT injury, galanin immunoreactivity was $<2\%$ in peripherin and NF-200 cells examined and no different to respective sham operated rats. However at 7 days post injury, galanin immunoreactivity increased to 11.8 ± 1.2 % in NF-200 characterised cells and 15.7 ± 2.7 % in peripherin characterised cells. There were no significant differences in the percentage of galanin immunoreactivity between these two characterised cell populations, however both values were significantly different compared to that observed in respective operated sham rats. At 14 days post TNT injury, galanin immunoreactivity had peaked in both characterised DRG cell populations examined, with 25.8 ± 4.2 % immunoreactivity in NF-200 characterised cells and 19.3 ± 4.8 immunoreactivity in peripherin characterised cells. By 28 days post TNT injury, galanin immunoreactivity had reduced to 5.8 ± 1.60 in NF-200 characterised cells and was not significantly different to the $5.7 \pm 1.3\%$ observed in peripherin characterised cells, however both values were significantly different to those obtained from respective sham operated rats. Percentage galanin immunoreactivity appeared to stabilize at 43 days post TNT injury with 7.8 ± 2.0 % immunoreactivity in NF-200 characterised cells which was not significantly different compared to that observed in respective sham operated rats. Galanin immunoreactivity at this time point was 7.7 ± 1.7 % in peripherin characterised cells and was significantly different compared to that observed in NF-200 characterised cells and to that observed in respective sham operated rats.

A cell size analysis supported immunohistochemical observations regarding galanin immunoreactivity in NF-200 large diameter ($\geq 900 \mu\text{m}^2$) and peripherin immunoreactive small diameter cells ($\leq 600 \mu\text{m}^2$) at all time points following TNT injury (see Table 3.3 for mean cell size areas of galanin immunoreactive cells across time in peripherin/NF-200 cells. The distribution appeared to be homogenous (Figure 3.29 and Figure 3.30).

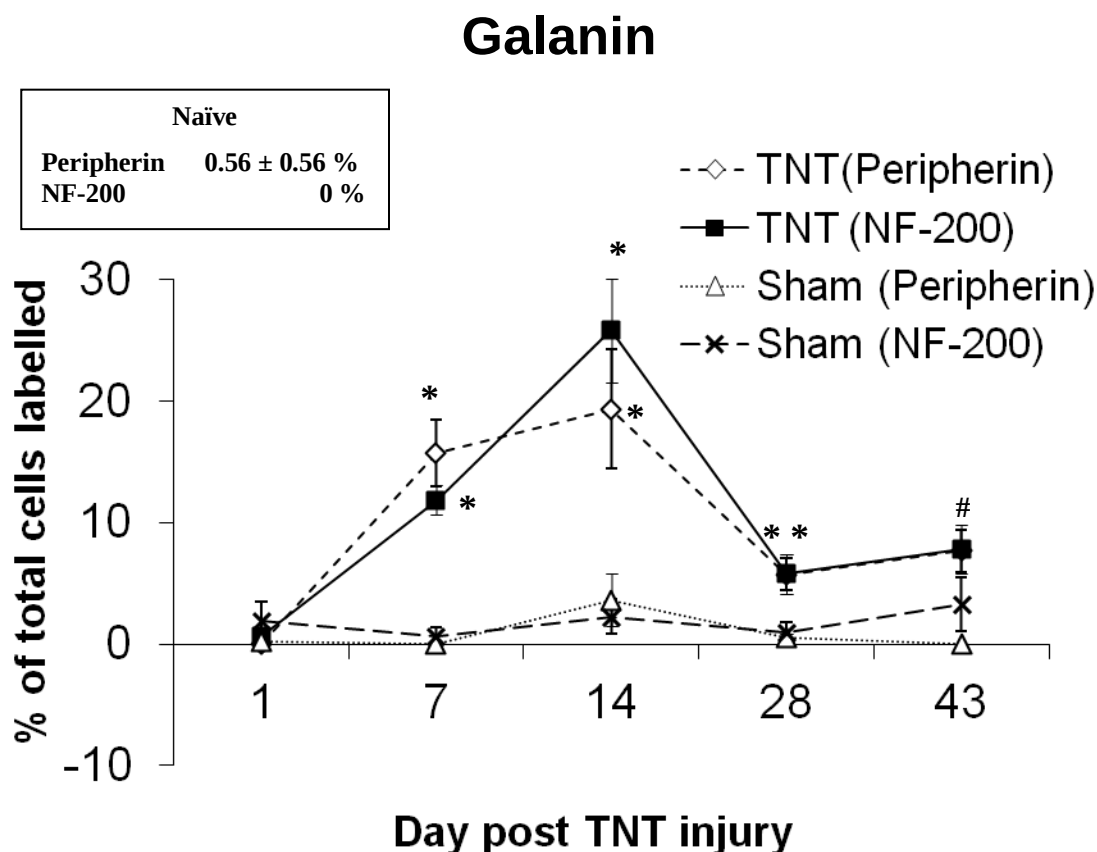


Figure 3.25 Mean percentage of total peripherin and NF-200 immunoreactive cells expressing galanin in TNT, sham, and naïve animals (ipsilateral L5 DRG n=3-4 animals per group). Data presented as mean $\pm$ SEM. Statistical test at each time point was conducted using a two way ANOVA followed by Holm-Sidak post hoc analysis: * $P < 0.05$ vs. respective sham, # $P < 0.05$ TNT (peripherin) vs. Sham (peripherin). In TNT injured rats, galanin immunoreactivity in both NF-200 and peripherin immunoreactive cells demonstrated significant temporal profile expressions.

Naive

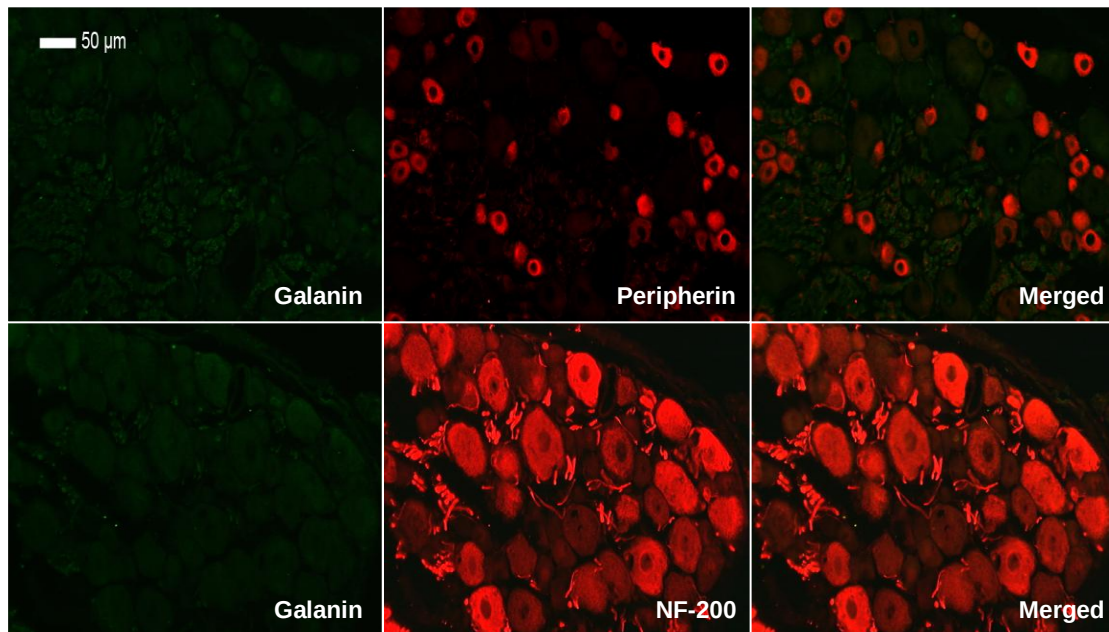


Figure 3.26 Representative images of galanin immunohistochemistry in naive L5 DRG. Double labelling was conducted to identify galanin immunoreactivity in both peripherin and NF-200 sub-populations of DRG cells; magnification x20.

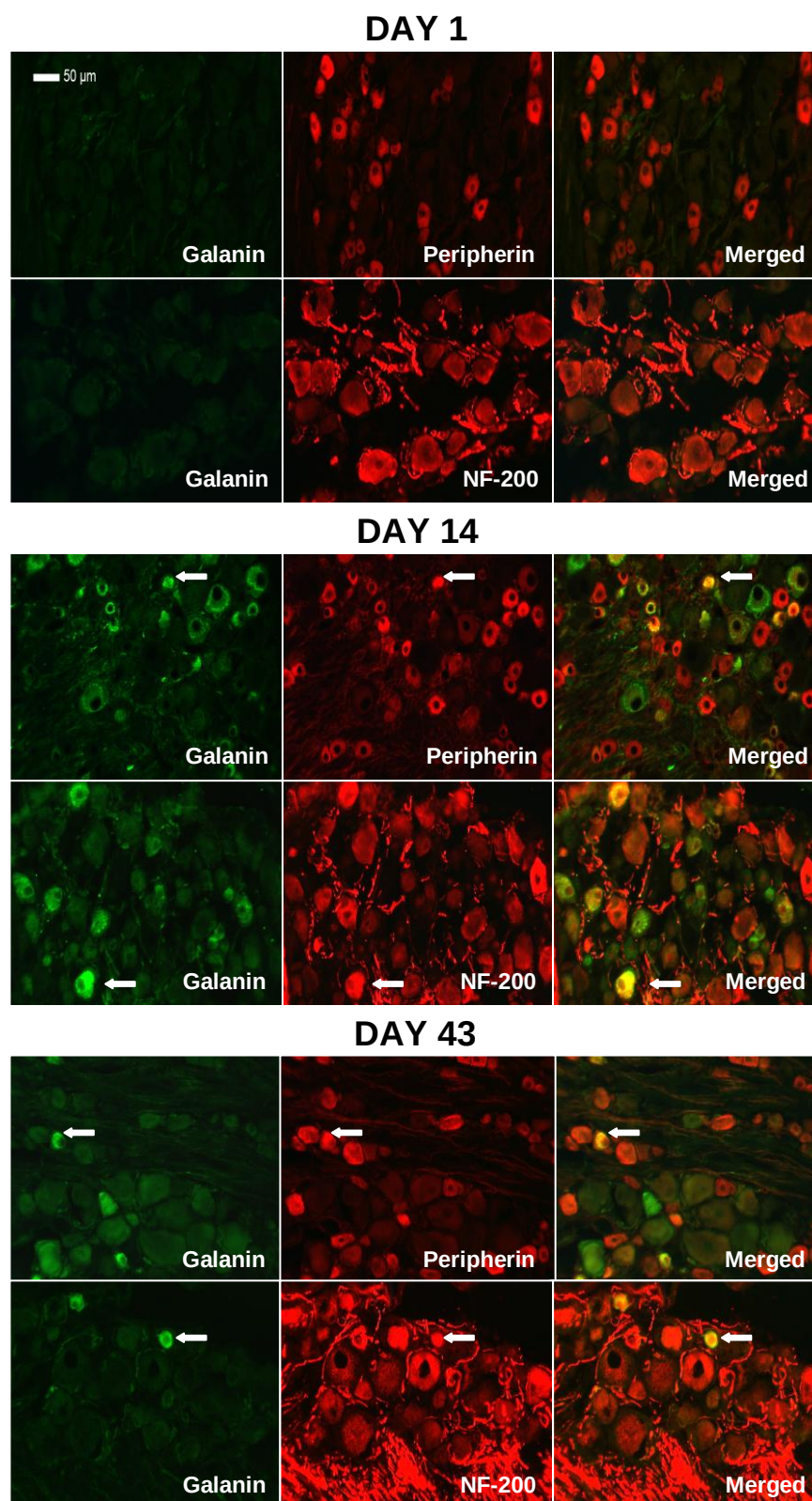


Figure 3.27 Representative images of galanin immunoreactivity at 1, 14, and 43 days post TNT injury (ipsilateral L5 DRG) in peripherin and NF-200 neuronal sub-populations, magnification x20. Arrows indicate co-localisation of immunolabeling.

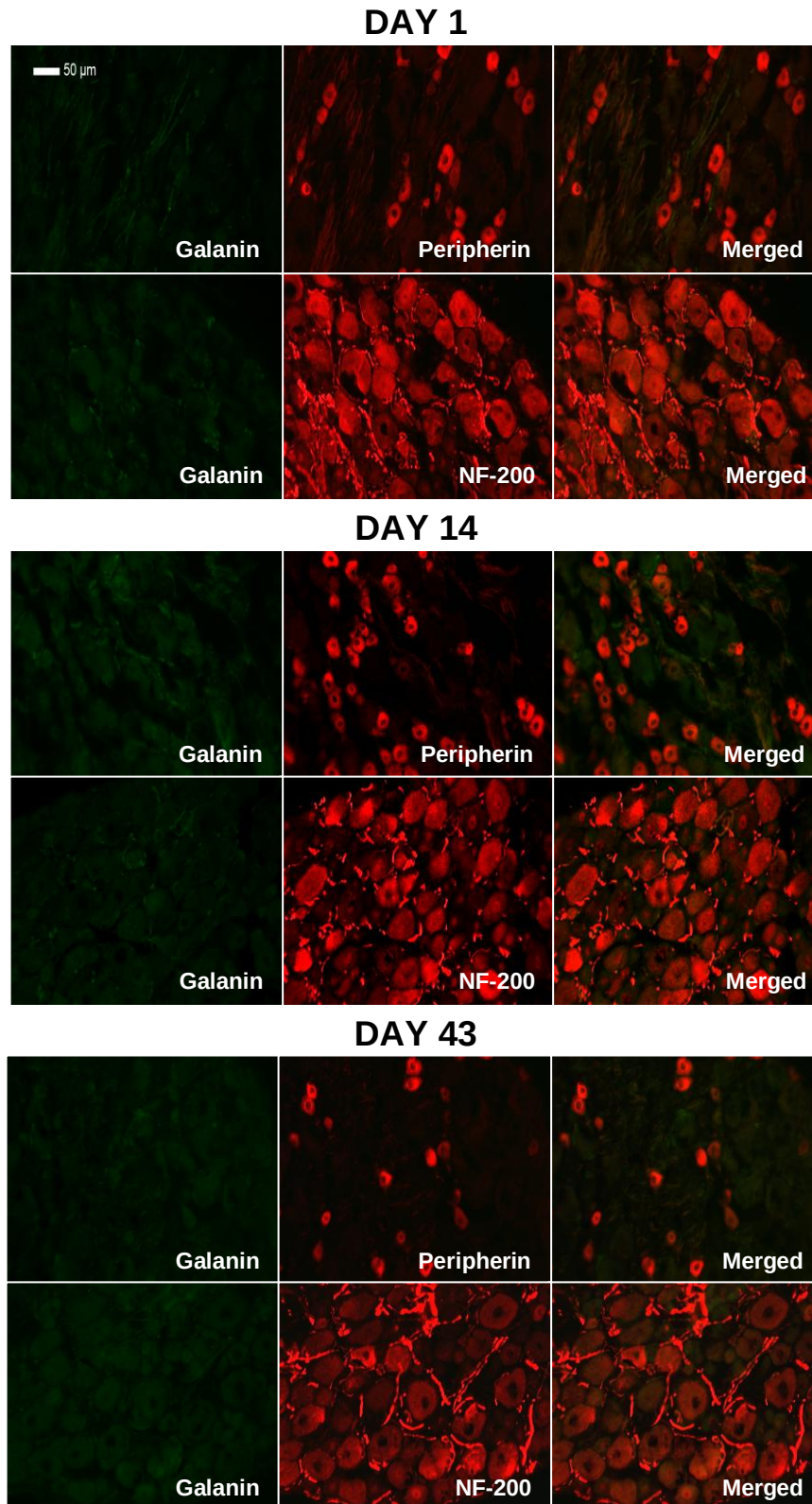


Figure 3.28 Representative images of galanin immunoreactivity at 1, 14, and 43 days post sham surgery (ipsilateral L5 DRG) in peripherin and NF-200 neuronal sub-populations, magnification x20

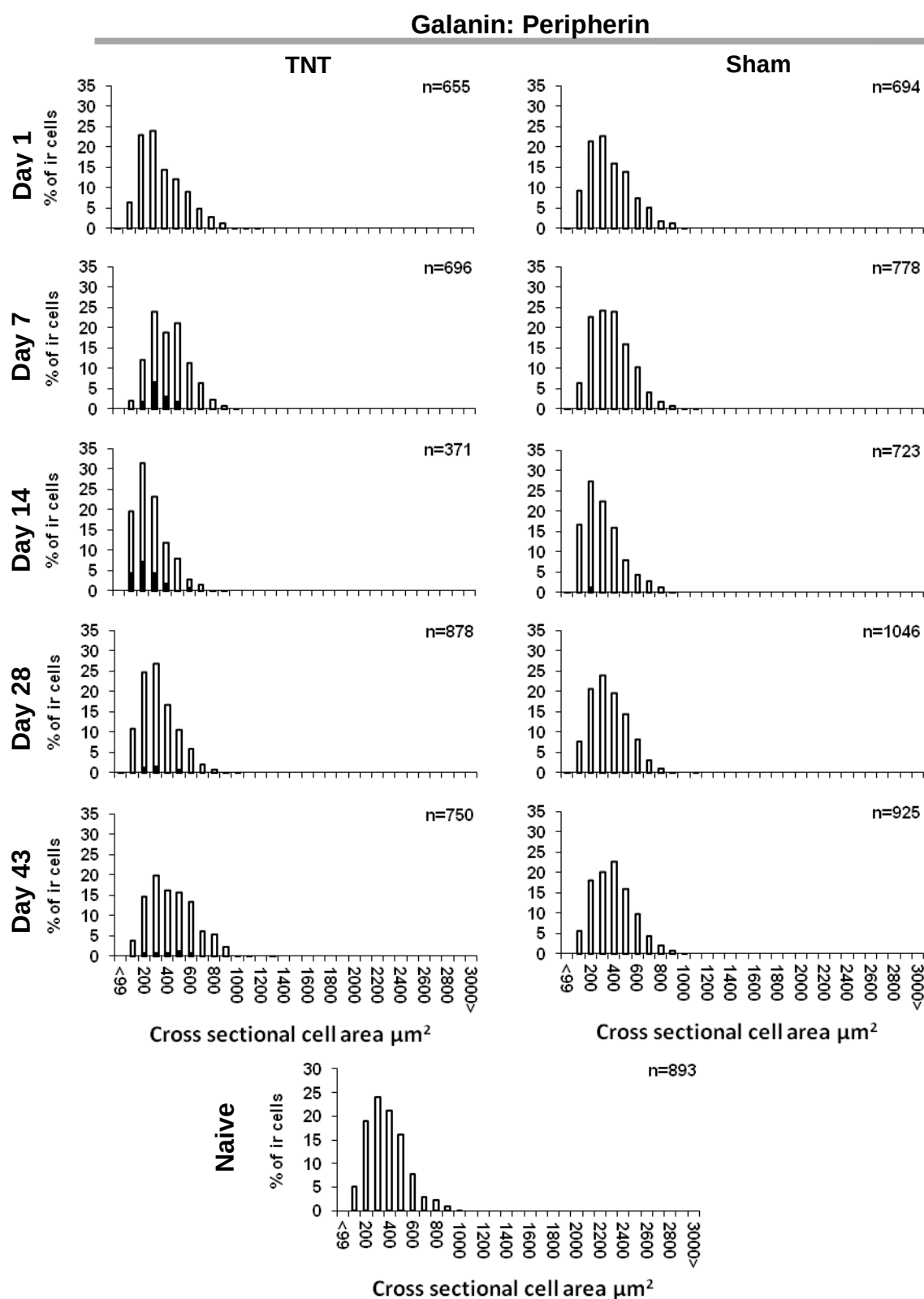


Figure 3.29 Percentage cell size distribution of galanin immunoreactivity in peripherin immunoreactive cells (ipsilateral L5 DRG) at various time points following TNT/sham surgery and in naive animals (n = total number of nucleated peripherin immunoreactive cells).

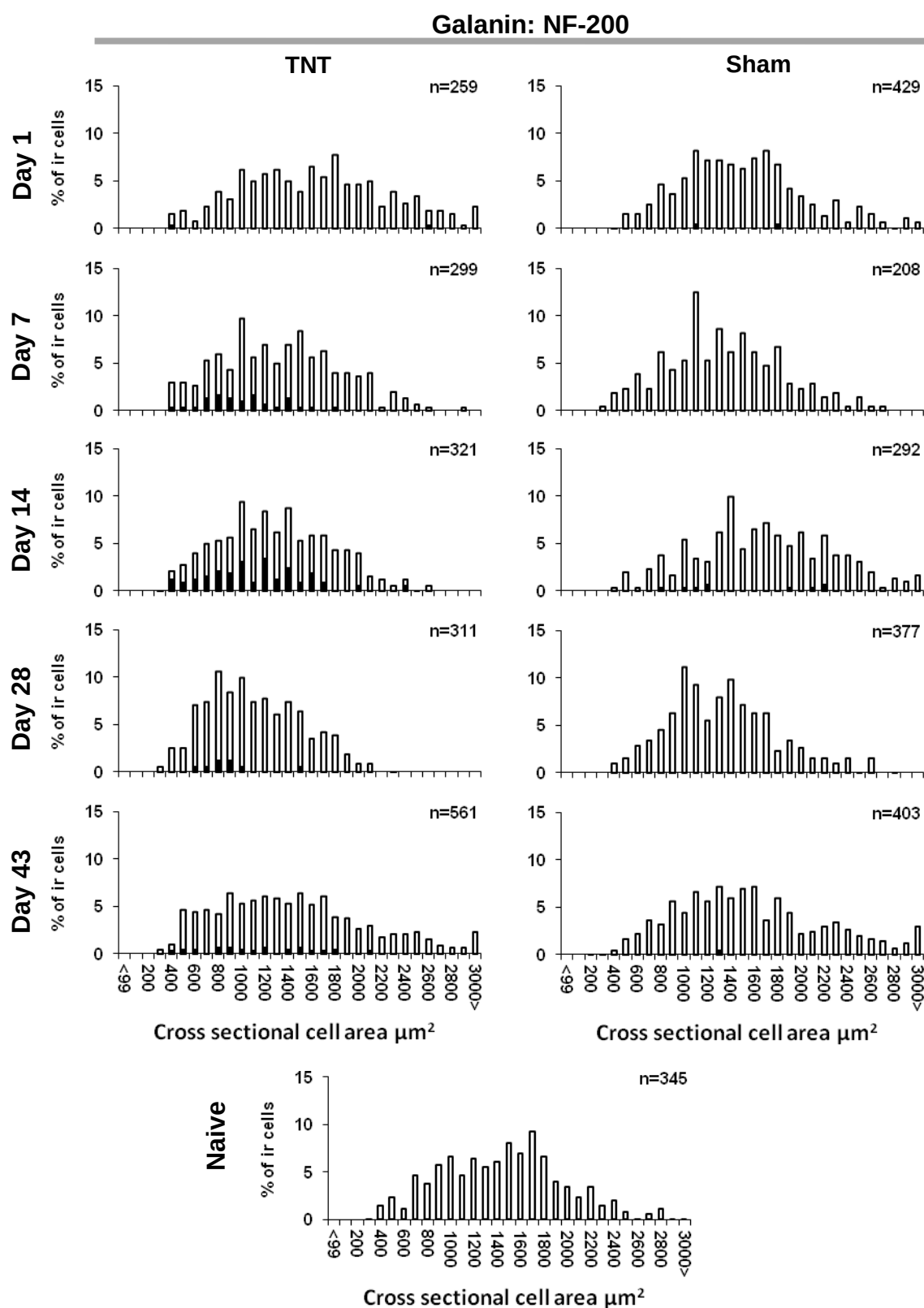


Figure 3.30 Percentage cell size distribution of galanin immunoreactivity in NF-200 immunoreactive cells (ipsilateral L5 DRG) at various time points following TNT/sham surgery and in naive animals (n= total number of nucleated NF-200 immunoreactive cells).

Naïve L5 DRG		
Markers	Mean % immunoreactivity	Mean cell area μm^2
<u>ATF-3</u>		
Peripherin	0	n/a (0/632)
NF-200	0	n/a (0/326)
<u>GAP-43</u>		
Peripherin	0	n/a (0/833)
NF-200	1.73 ± 1.73	997.5 ± 100.5 (7/285)
<u>NPY</u>		
Peripherin	0	n/a (0/996)
NF-200	0.28 ± 0.28	1609.1 (1/268)
<u>Galanin</u>		
Peripherin	0.56 ± 0.56	477.2 ± 33.0 (6/893)
NF-200	0	n/a (0/345)

Table 3.2 Mean percentage immunoreactivity and mean cell area (μm^2) of ATF-3, GAP-43, NPY, and galanin in NF-200 and peripherin characterised cell populations in naïve L5 DRG. Data presented as mean $\pm$ SEM). The numbers of ATF-3, GAP-43, NPY, and galanin immunoreactivity cell profiles out of the total number of peripherin/NF-200 DRG immunoreactive cells are indicated in brackets (n/a = not applicable as no data was available).

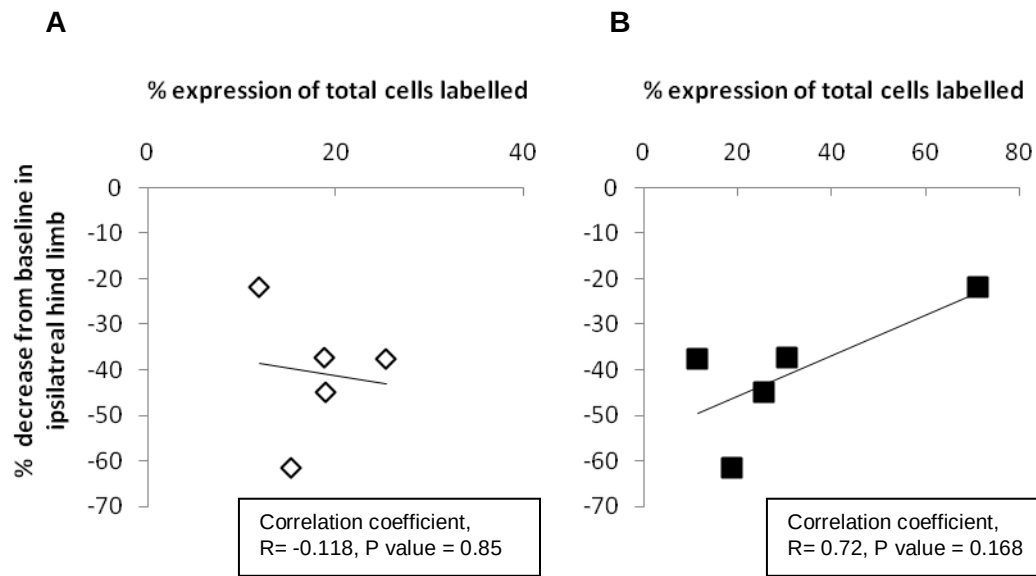
	TNT/sham operated animals [mean cell area $\mu\text{m}^2 \pm$ standard error of mean]				
Markers	Day1	Day 7	Day 14	Day 28	Day 43
<u>ATF-3</u> TNT					
Peripherin	429.1 $\pm$ 21.2 (50 of 337)	433.1 $\pm$ 13.8 (154 of 780)	366.7 $\pm$ 39.9 (94 of 338)	350.5 $\pm$ 12.5 (131 of 836)	416.3 $\pm$ 15.4 (132 of 615)
NF-200	1773.5 $\pm$ 42.4 (181 of 252)	1389.8 $\pm$ 53.9 (93 of 353)	1210.5 $\pm$ 51.8 (95 of 298)	997.0 $\pm$ 43.1 (76 of 370)	996.5 $\pm$ 66.5 (37 of 308)
Sham					
Peripherin	n/a (0 of 491)	253.1 $\pm$ 115.9 (2 of 696)	307.1 $\pm$ 39.9 (8 of 637)	425.5 $\pm$ 14.1 (2 of 935)	320.4 $\pm$ 99.0 (2 of 757)
NF-200	2032.8 (1 of 377)	1141.3 (1 of 203)	1391.1 $\pm$ 186.9 (10 of 380)	n/a (0 of 325)	1174.5 $\pm$ 125.8 (6 of 283)
<u>GAP-43</u> TNT					
Peripherin	442.0 $\pm$ 7.2 (23 of 543)	427.3 $\pm$ 16.2 (73 of 995)	336.9 $\pm$ 16.2 (60 of 393)	401.9 $\pm$ 15.7 (126 of 1044)	469.7 $\pm$ 30.8 (34 of 721)
NF-200	1195.4 $\pm$ 135.3 (7 of 246)	609.0 (1 of 307)	1024.3 $\pm$ 59.6 (47 of 296)	970.6 $\pm$ 59.2 (33 of 347)	1127.0 $\pm$ 76.5 (39 of 470)
Sham					
Peripherin	500.1 $\pm$ 63.6 (9 of 661)	550.5 $\pm$ 108.9 (2 of 753)	380.9 $\pm$ 37.6 (27 of 755)	471.8 $\pm$ 50.3 (6 of 900)	457.1 $\pm$ 50.7 (4 of 552)
NF-200	982.2 $\pm$ 96.0 (10 of 219)	1218.4 $\pm$ 213.2 (4 of 261)	1005.2 $\pm$ 148.9 (13 of 322)	740.0 (1 of 288)	1385.7 $\pm$ 101.3 (15 of 427)
<u>NPY</u> TNT					
Peripherin	n/a (0 of 218)	574.6 $\pm$ 75.9 (20 of 1044)	388.6 $\pm$ 31.9 (11 of 323)	564.1 $\pm$ 24.2 (72 of 1050)	541 $\pm$ 40.4 (34 of 782)
NF-200	n/a (0 of 268)	1488.3 $\pm$ 38.0 (208 of 403)	1323.1 $\pm$ 34.5 (149 of 270)	971.0 $\pm$ 35.9 (96 of 354)	1154.2 $\pm$ 60 (49 of 368)
Sham					
Peripherin	n/a (0 of 471)	137.1 (1 of 649)	378.3 $\pm$ 63.4 (6 of 618)	n/a (0 of 909)	n/a (0 of 706)
NF-200	n/a (0 of 419)	n/a (0 of 283)	1417.7 $\pm$ 282.9 (3 of 358)	1594.0 $\pm$ 376.1 (2 of 376)	1144.3 $\pm$ 27.8 (2 of 306)
<u>Galanin</u> TNT					
Peripherin	n/a (0 of 655)	400.2 $\pm$ 12.9 (110 of 696)	319.8 $\pm$ 18.2 (76 of 371)	410.1 $\pm$ 28.8 (51 of 878)	538.4 $\pm$ 33.1 (48 of 750)
NF-200	1564.4 $\pm$ 1071.1 (2 of 259)	1064.8 $\pm$ 55.2 (34 of 299)	1190.6 $\pm$ 48.7 (83 of 321)	981.5 $\pm$ 63.5 (20 of 311)	1292.1 $\pm$ 78.3 (47 of 561)
Sham					
Peripherin	419.4 (1 of 694)	n/a (0 of 696)	364.7 $\pm$ 29.1 (29 of 723)	501.3 $\pm$ 102.7 (5 of 1046)	n/a (0 of 925)
NF-200	1608.4 $\pm$ 256.8 (8 of 429)	n/a (0 of 208)	1568.1 $\pm$ 187.0 (9 of 292)	1359.1 $\pm$ 174.4 (4 of 377)	1282.1 $\pm$ 117.9 (11 of 403)

Table 3.3 Mean cell area (μm^2) of ATF-3, GAP-43, NPY, and galanin immunoreactivity in NF-200 and peripherin characterised cell populations in ipsilateral L5 DRG of TNT/sham animals at 1, 7, 14, 28, and 43 days post surgery. Data are presented as means $\pm$ SEM. The numbers of ATF-3, GAP-43, NPY, and galanin immunoreactivity cell profiles out of the total number of peripherin/NF-200 DRG immunoreactive cells are indicated in brackets (n/a = not applicable as no data was available).

3.3.7 No correlation of investigative markers with mechanical hypersensitivity

In order to determine if there were a relationship between the temporal expression of investigative markers in both cell populations (peripherin and NF-200) and the degree of behavioural mechanical hypersensitivity, a Pearson's correlation co-efficient was calculated. The results of the study indicate that there is no correlation between TNT induced mechanical hypersensitivity and the expression of ATF-3, GAP-43, NPY, and galanin (see Figure 3.31 and Figure 3.32). However there appears to be a trend towards an association in both peripherin and NF-200 cells with regards to GAP-43, NPY, and galanin and with ATF-3 in peripherin characterized cells.

ATF-3



GAP-43

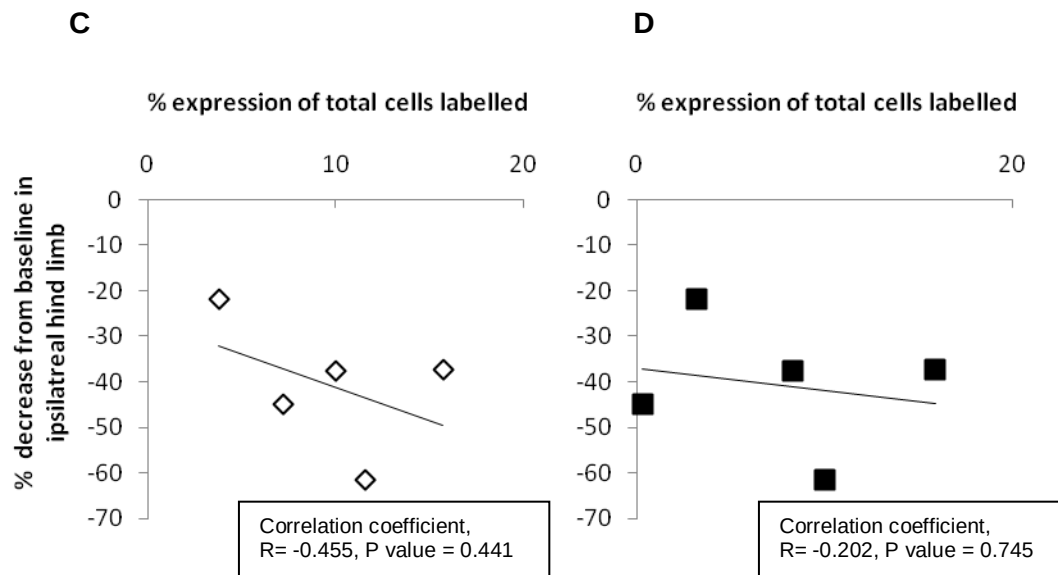
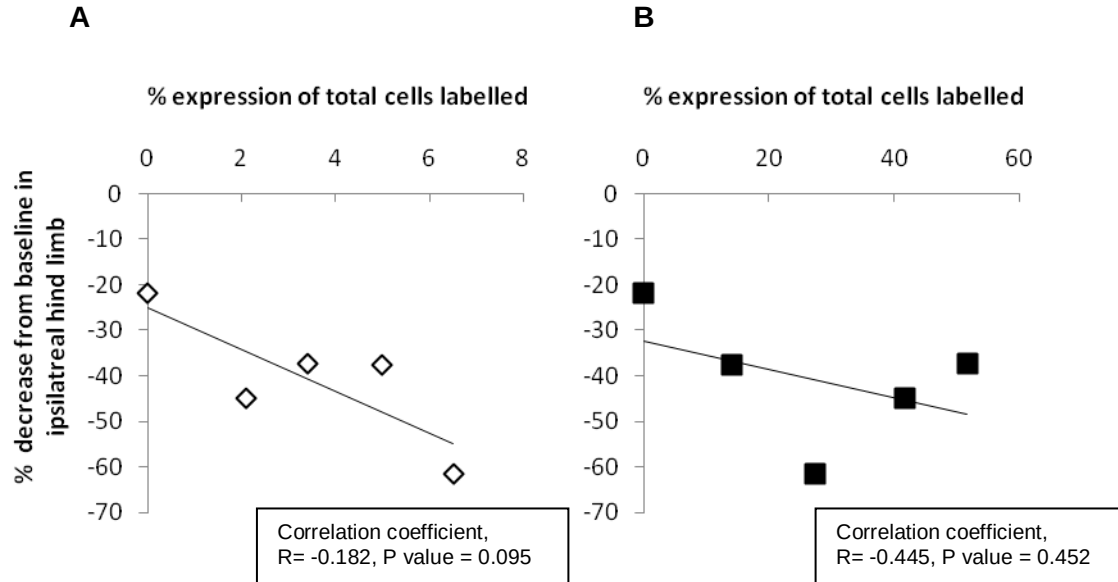


Figure 3.31 Relationship between TNT-induced mechanical hypersensitivity and mean percentage immunoreactivity of (A) ATF-3, peripherin immunoreactive cells; (B) ATF-3, NF-200 immunoreactive cells (C) GAP-43, peripherin immunoreactive cells (D) GAP-43, NF-200 immunoreactive cells. Key: (■) NF-200 and (◇) Peripherin immunoreactive cells. (Ipsilateral L5 DRG n=3-4 animals per group). Data presented as mean $\pm$ SEM.

NPY



Galanin

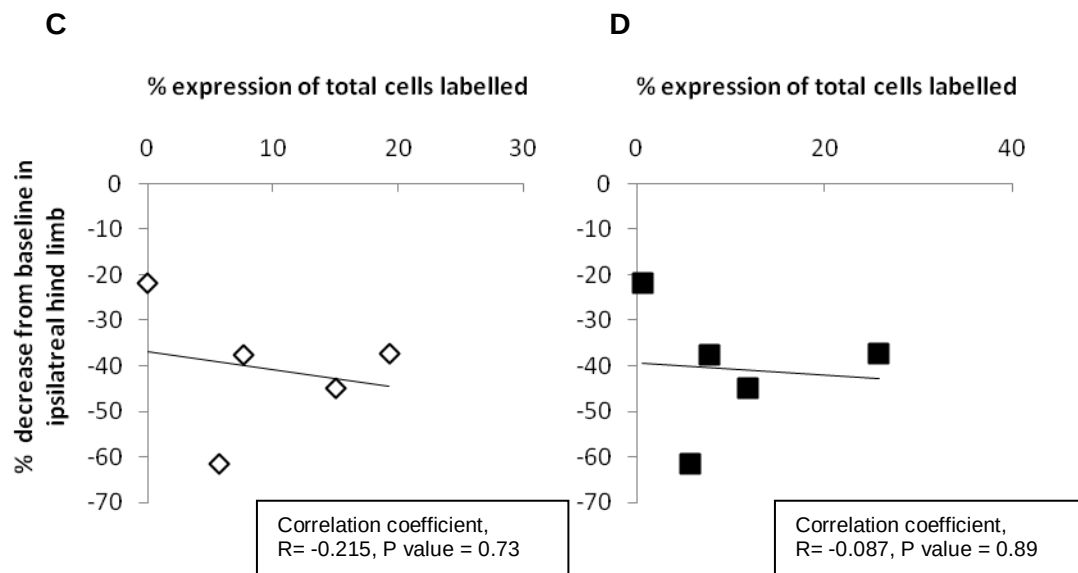


Figure 3.32 Relationship between TNT-induced mechanical hypersensitivity and mean percentage immunoreactivity of (A) NPY, peripherin immunoreactive cells; (B) NPY, NF-200 immunoreactive cells (C) Galanin, peripherin immunoreactive cells (D) Galanin, NF-200 immunoreactive cells. Key: (■) NF-200 and (◇) Peripherin immunoreactive cells. (Ipsilateral L5 DRG n=3-4 animals per group). Data presented as mean $\pm$ SEM.

3.4 Discussion

The aim of the investigation was to characterize the behavioural responses to mechanical and thermal stimuli following TNT injury and to provide temporal profile expressions of neurochemical markers in the DRG that have been associated with traumatic nerve injury using immunohistochemical techniques. The other aim of this investigation was to determine the distribution of tibial nerve neurons in the DRG via FG retrograde tracing.

3.4.1 Distribution of tibial nerve axons in L3-S1 DRGs

At the present time, the tibial nerve distribution between different lumbar DRGs is not known. Seven days following FG injections into naive animals, labelling was present in the L4 and L5 DRGs suggesting that these ganglia receive the majority of tibial nerve axons. The mean percentage FG/NeuN co-labelling in the L4 DRG was higher than that observed in the L5 DRG. This result reflects similar observations by Swett et al., 1991 (using retrograde tracer horseradish peroxidase) which reported 99% of labelling in the L4 and L5 DRGs (with higher proportions in L4 DRG [68%] compared to L5 DRG [31%]) following injection in to the tibial nerve. Further experiments regarding the TNT model focused on investigating neurochemical changes in the L5 DRG since the L5 DRG is bigger than the L4 DRG and more sections could be made which was important given the number of sections required to complete the study. In addition, the results could be compared to other studies involving mixed neuronal injury (i.e. PSNL and CCI) and also the SNT or spinal nerve ligation (SNL) model where the L5 is commonly assessed. The variability between the two animals examined limits the strengths of the study which could be improved with a larger sample size. I propose two main reasons that could explain the variability. Firstly, the injected FG may have dispersed to the spinal cord – such tissue samples were not examined in analysis. Secondly, the fact that some FG labelled cells did not co-label NeuN meant that these cells were not included in the analysis and thus likely underestimated the percentage expression. Nonetheless, this tract tracing study has been an informative approach to estimate DRG neuronal damage that occurs following TNT injury.

3.4.2 TNT associated mechanical hypersensitivity

Behavioural mechanical hypersensitivity in the mid-plantar paw was assessed using an electronic von Frey device. In the acute stages of TNT injury (day 1 post surgery), mechanical hypersensitivity was not evident. However, at 7 days following TNT injury, and at subsequent time points (day 14, 28, and 43), animals demonstrated a robust mechanical hypersensitivity that was not apparent in sham animals. Once established, the mechanical hypersensitivity remained persistent. These results are in concordant with previous findings which depict mechanical hypersensitivity following TNT surgery (Andrews et al., 2011; Lee et al., 2000; Unal-Cevik and Oaklander, 2011). Although it is to be noted that there are some differences regarding the site of sensory testing. In the present study the mid-plantar region of the paw (which receives tibial nerve innervation) was used to measure behavioural responses towards a mechanical stimulus, a method that mirrors that of Andrews et al., 2011. However other studies have used the lateral part of the paw which is innervated by the sural nerve. This lateral part of the paw is reported to be the initial hypersensitive part since it remains surgically intact (Lee et al., 2000; Unal-Cevik and Oaklander, 2011). The advantage of the assessing the mid-plantar part of the paw is that it is more accessible than the lateral part of the paw for testing and the results are comparable to other studies that mostly assess the mid-plantar paw. The disadvantage of assessing the mid-plantar paw, with respect to the TNT model, is that the paw maybe hyposensitive, whereas the lateral paw maybe hypersensitive with an appropriate response – the lateral paw is less accessible than the mid-plantar paw and more patience and time is required during testing to reflect this. Despite the differences at the site of stimulation, the latency to the onset of mechanical hypersensitivity in the current study is comparable to what has been described in other studies that has been reported to be as early as 3 (Unal-Cevik and Oaklander, 2011) and 4 days (Lee et al., 2000) post injury. The lack of behavioural hypersensitivity in the acute stages of nerve injury (day 1 post TNT injury) may be related to the lack of sensory input that is likely to occur following the transection of the tibial nerve. Alternatively, this occurrence maybe explained by delayed neurochemical changes in the DRG that might underlie behavioural hypersensitivity. At days 28 post TNT surgery in the contralateral paw, animals demonstrated a 22.8 ± 18.8 % increase in PWTs. Sham animals at 43 days post surgery demonstrated a 46.5 ± 8.0 % increase in the ipsilateral PWT from baseline. These results may be explained by an increase in the weight of the animals which subsequently results

in increases in PWTs over time, a phenomenon that seems to occur at longer time points. This is further discussed in **Section 6.6.1** of the general discussion (assessment of mechanical hypersensitivity) in context of study limitations.

3.4.3 TNT injury induces transient thermal hypersensitivity

In this study, thermal PWLs were assessed using a Hargreaves device. Following TNT injury thermal hypersensitivity was only apparent in the day 28 group (29% decrease from baseline). Several models of peripheral nerve trauma injury (e.g. partial sciatic nerve ligation (PSNL); chronic constriction injury (CCI), and spinal nerve transection(SNT)) are known to exhibit thermal hypersensitivity of similar magnitudes and therefore this observation falls in line with previous findings (Bennett and Xie, 1988;Kim and Chung, 1992;Seltzer et al., 1990). Although, in one particular study, there were no reported changes in PWL following spared nerve injury (SNI) or sciatic nerve crush, but an increase in duration of withdrawal was observed (Decosterd and Woolf, 2000). The results of the current study are the first to describe thermal hypersensitivity in the TNT model. However this hypersensitivity was not consistent across time points. TNT animals at 7 and 14 days post injury demonstrated a decreasing but not significant trend in PWLs. There appears to be large standard deviation values that reflect the variations amongst PWL readings. The reason for this is not clear but may relate to alterations in thermal coding as a consequence of nerve injury. Thermal hyperalgesia is not thought to be a common feature of neuropathic pain in humans and this inconsistency may reflect this notion (Koltzenburg, 1998). Data from animal studies suggests inflammation may play a role and has been shown to be critical for the development of thermal hypersensitivity in the CCI model (Clatworthy et al., 1995). Moreover the administration of IL-10, an anti-inflammatory cytokine at the site of injury can reduce the thermal hypersensitivity associated with CCI (Wagner et al., 1998) and adds further weight to this argument.

3.4.4 Temporal profile expression of ATF-3

ATF-3 was examined because it is a widely used marker of damaged DRG neurons following peripheral nerve injury. In this study, ATF-3 expression was not observed in the L5 DRG of naive animals, which is in agreement with previous observations (Tsujino

et al., 2000). After TNT injury, ATF-3 was robustly upregulated in ipsilateral L5 DRG in both peripherin and NF-200 cells. The percentage immunoreactivity in peripherin/NF-200 diameter cells remained similar and constant across time (10-25%), except at the acute stage of nerve injury (day 1), where percentage immunoreactivity was evidently greater in NF-200 cells (71%). The robust upregulation of ATF-3 has been observed in various types of peripheral nerve injury (Averill et al., 2004;Averill et al., 2002;Djoughri et al., 2006;Fukuoka et al., 2012;Obata et al., 2003;Peters et al., 2005;Shortland et al., 2006;Tsujino et al., 2000;Wright et al., 2004). The method used to quantify ATF-3 expression (i.e. double labelling to determine phenotype) differs to that of previous studies. The majority of studies calculate the percentage expression of the total nucleated neuronal DRG population. The percentage expression of ATF-3 varies amongst the use of different trauma models. For example, Shortland et al., 2006 using the SNT model demonstrated greater expression levels than that observed in the present study (L4/L5 DRG 1 and 14 days post injury, >98%). Obata et al., 2003 using the CCI model noted 9-39% ATF-3 expression in L4/L5 DRG 14 days post injury. Other studies involving complete axotomy of the sciatic nerve have demonstrated differing levels of expression: Averill et al., 2002 (L5 DRG, within 30 days of injury, 58-73%), Averill et al., 2004 (L4 DRG,14 days post injury, 82%) and Linda et al., 2011 (L4-L6 DRG, 1-21 days of injury, 45-60%). The reason for the variability between studies is not entirely clear, but I propose three plausible arguments. Firstly, the nature of the nerve injury varies between studies and more severe injuries may increase ATF-3 expression. Secondly, there may be differences in the level of surgical expertise; less experienced operators are more likely to incur unnecessary collateral damage that may influence ATF-3 expression. Lastly, the potential differences in response to nerve injury amongst various rat strains could be an influential factor. The results of the study indicate that the large diameter neurons are primarily responsible for the down-regulation that is commonly observed following ATF-3 induction, a reflection on the differing degrees of injury sustained between small and large diameter neurons. In certain types of nerve trauma injury, large myelinated fibres incur massive microscopic damage and degeneration with less severe changes evident in smaller unmyelinated fibres (Basbaum et al., 1991;Munger et al., 1992). Though such changes have been observed at 10-14 days post injury, similar process could occur in the in the early part of TNT injury, leading to increased ATF-3 expression (indication of nerve damage). The subsequent down-regulation of ATF-3 observed at 7 days post TNT injury could be due to a loss of some large diameter neurons, a

phenomena that has been observed in certain neuropathic states (Coggeshall et al., 1993). This finding is suggestive of a partial recovery. Double labelling experiments, in conjunction with the cell size distribution analysis reinforced the evidence regarding the down-regulation of ATF-3 in large cells. Despite this down-regulation, ATF-3 expression is still evident in the chronic stages of nerve injury which ties in with the persistent mechanical hypersensitivity observed 43 days post TNT injury. The ATF-3 expression at this stage may be suggestive of other processes. For example, ATF-3 has been linked to regeneration of peripheral sensory neurons (Hunt et al., 2004; Saito and Dahlin, 2008). When the sciatic nerve is transected and reattached, allowing the regeneration of axons, ATF-3 expression down-regulates 30 days post surgery (Hunt et al., 2004). Transgenic studies have revealed that the over expression of ATF-3 in non-injured adult mice is associated with an increased rate of peripheral nerve regeneration comparable to nerve injured animals, although not through GAP-43 mechanisms but through the expression of another growth associated protein named small proline rich protein 1A (SPRR1A) (Seijffers et al., 2007). ATF-3 may even bind with the closely related c-jun proteins (Chen et al., 1994; Hai and Curran, 1991) since they have been shown to co-localize in the injured DRG (Tsujino et al., 2000) and therefore this could precipitate regeneration. The co-transfection of ATF-3 and c-Jun into neuronal cell lines has been shown to produce greater neurite outgrowth than transfection with either genes alone (Pearson et al., 2003) and therefore a plausible hypothesis. The expression of ATF-3 in the DRG could also indicate neuroprotective strategies. The over expression of ATF-3 to sympathetic neurons via a viral vector has been shown to reduce cell death associated with growth factor deprivation, and additionally enhanced neurite outgrowth by a specific pathway involving c-jun protein (Nakagomi et al., 2003).

3.4.5 Temporal profile expression of GAP-43

Following traumatic peripheral nerve injury, damaged neurons have the capacity to regenerate and this has been associated with an increased expression of GAP-43 in the L4/L5 DRG (Woolf et al., 1990). GAP-43 has also been identified in the growth cones of regenerating axons in culture (Meiri et al., 1988; Meyer et al., 1994) and *in vivo* (Hall et al., 1992) which can influence neuron survival *in vivo* (Aigner et al., 1995). The results of this study show that GAP-43 was expressed at low levels in naïve L5 DRG tissue (0%, Peripherin; <2% NF-200). Previous studies have shown higher expression levels in naïve

animals: ~35% lightly labelled and <10% heavily labelled in L4/L5 ganglia, (Schreyer and Skene, 1991); and 50% of GAP-43 expression in large diameter cells (Sommerville et al., 1991). However, observations by Schreyer and Skene, 1991 included lightly labelled cells which were omitted in the current study due to the ambiguous immunoreactivity. This could additionally explain discrepancies regarding observations by Sommerville et al., 1991. Following TNT injury, GAP-43 was upregulated in both peripherin and NF-200 cells in ipsilateral L5 DRG; temporal profile expressions were similar amongst both cell populations. These observations were reinforced by the cell size distribution analysis. The mean size of GAP-43 immunoreactive cells in peripherin and NF-200 cells throughout the study was $<500\mu\text{m}^2$ and $>1200\mu\text{m}^2$ respectively. Past research findings have shown GAP-43 expression in predominantly small-medium diameter neurons during the early stages (4 days) of nerve injury (Woolf et al., 1990) which correlates with the observations of this study at 7 days post TNT injury. The temporal expression pattern also corroborates with data from in situ hybridization studies showing GAP-43 mRNA upregulation in initially small cells before a subsequent increase in large diameter cells after unilateral ligation of the sciatic nerve (Wiese et al., 1992). Peak GAP-43 mRNA expression in Wiese et al., 1992 was observed at 10 days in both small and large cells which are comparable to the peak expression time of 14 days observed in the current study. Although it is to be noted that large cells did not demonstrate similar intensities of hybridisation signal when compared to smaller cells. Previous studies have shown higher percentages of GAP-43 expression following nerve injury. For example, Schreyer and Skene, 1991 demonstrated ~45% of L4/L5 DRG cells expressing high intensity labelling of GAP-43, 7 and 14 days following nerve crush injury (compared to ~15% observed in both cell populations in TNT animals). Sommerville *et al* (1991) examined the expression of GAP-43 in RT 97 labelled cells (large diameter cells), in the ipsilateral L5 DRG at various time points following sciatic nerve section injury. In the acute stages of nerve injury (4 days), GAP-43 expression was reported to be 8% in large diameter cells, a figure that is greater than the 3.2 % observed at in the current study (day 1 post TNT). Moreover at 14 days post sciatic nerve transection, 29% of the GAP-43 labelled cells were found to be in large diameter cells, a figure once more greater than the 15.9% observed in NF-200 immunoreactive cells at identical time points in the present study. The comparatively modest GAP-43 expression observed (compared to literature findings) may be explained by the differing nature of

the nerve injury, since sciatic nerve transection arguably results in greater proportions of axotomized axons than TNT injury.

3.4.6 Temporal profile expression of NPY

The results of the study demonstrated that in naïve animals, NPY is not expressed in L5 DRG. After TNT injury, NPY was robustly upregulated in ipsilateral L5 DRG, exhibiting a dynamic temporal profile expression which was predominant in NF-200 characterised cells (~30-51%). An increase in NPY immunoreactivity was additionally observed in the smaller peripherin immunoreactive cells amongst the various time points (1-6.5%) and was confirmed via a cell size distribution analysis. These results are consistent with previous findings regarding NPY expression following nerve injury (Ma and Bisby, 1997; Ohara et al., 1994; Wakisaka et al., 1991; Wakisaka et al., 1992), although the method of analysis used to quantify NPY expression differs amongst the various studies. For example, Ma and Bisby (1997) calculated the total percentage expression in ipsilateral L4/L5 ganglia, comparing three different types of sciatic nerve injury; complete sciatic nerve transection (CSNT), partial sciatic nerve transection (PSNT), and CCI injury. NPY expression was found to be similar amongst the nerve injury types (~7-10%). In a model of complete median nerve transection where C6 and C7 DRGs were examined, NPY expression remained ~70% and ~20% amongst medium and large diameter fibres respectively across various time points within a 20 week time period (Tsai et al., 2007). Finally, in experiments conducted by Brumovsky et al., 2004, the number of NPY immunoreactive profiles per 100,000 micrometers method was used to quantify expression in L4 and L5 DRG following single ligature CCI.

While there have been contradictory reports regarding the effects of NPY, much of the evidence points towards NPY eliciting anti-nociceptive actions (Hua et al., 1991; Intondi et al., 2008; Taiwo and Taylor, 2002), through the NPY Y1 & Y2 receptors (Intondi et al., 2008; Naveilhan et al., 2001; Shi et al., 2006). After peripheral nerve injury the Y1 receptor, normally present on small DRG neurons, is down regulated, and the Y2 receptor is robustly up-regulated on large diameter neurons (Zhang et al., 1997). A proposed mechanism of neuropathic pain relates to ectopic discharges originating from large diameter fibres or phenotypic switching so that they begin to convey nociceptive

signals (Devor, 2009). The increased NPY observed in large diameter cells, upon release, may represent an attempt to reduce this ectopic activity by acting on the Y1 receptors, thus restoring normal nociceptive processing. It has been argued that NPY enhances the effect of noradrenaline in peripheral nerve injury, increasing the discharge rate of damaged neurons (Benoliel et al., 2001); a facet of sympathetically maintained pain. While this may be a phenomenon observed in some traumatic models (e.g. PSNL and CCI) (Shir and Seltzer, 1991), it is unlikely in the TNT model which has been described as a sympathetically independent model of peripheral nerve injury (Lee et al., 2000).

3.4.7 Temporal profile expression of galanin

Galanin expression in naïve L5 DRG was low (<1%) in both peripherin and NF-200 cells, a figure comparable the 2-3% expression observed by Hokfelt et al (1987) but less than that observed by Ma and Bisby, 1997 in L5 DRG (~12%) and Shortland et al 2006 in L4 DRG (10%). After TNT injury, galanin was upregulated in peripherin and NF-200 cells in ipsilateral L5 DRG, with expression peaking at 14 days in both cell populations (~20%) before dramatically reducing at 28 and 43 days post injury (<10%). The temporal expression profiles remained similar between both cell populations. A cell size distribution analysis indicated a uniform distribution of galanin immunoreactivity in peripherin immunoreactive cells across all time points studied. Although in NF-200 immunoreactive cells (most notably at day 7 and 14 post TNT injury), the distribution was slightly skewed to the left indicating that these were most likely medium-diameter cells. The mean size of galanin immunoreactive cells in peripherin and NF-200 cells throughout the study was $<600\mu\text{m}^2$ and $>900\mu\text{m}^2$ respectively. The upregulation of galanin has been observed at various time points in several models of peripheral nerve injury, mainly in small diameter cells (Averill et al., 2002;Fukuoka et al., 1998;Hokfelt et al., 1987;Hokfelt et al., 1994;Holmes et al., 2003;Ma and Bisby, 1997;Shi et al., 1999;Shortland et al., 2006;Villar et al., 1989). In the acute stage of nerve injury (one day post TNT injury), like NPY, galanin expression is no different to sham operated or naive control rats in peripherin/NF-200 cell populations (<2%). This percentage expression of galanin, one day after nerve trauma differs markedly from those reported by Villar et al 1989 (~25% after sciatic nerve crush), Averill et al., 2002 (22.5% after CSNT), and Shortland et al 2006 (17% after L5 SNT). One possible explanation for this is the

differing nature of the nerve injury. SNT surgery involves the removal of L6 transverse process (hemi-laminectomy) and extensive manipulation of muscle which increases the likelihood of collateral damage that could enhance nerve injury, thus increasing galanin expression. SNT is also more robust in terms of the nerve injury since the nerve damage is more proximal to the DRGs compared to the TNT model where the nerve injury is more distal. This may additionally explain the lower galanin expression observed at 14 days post TNT injury in the current study (20-25%) compared to that observed by Shortland et al 2006 (77% after L5 SNT); Ma and Bisby, 1997 (~50-60% after CSNT, PSNT and CCI); Hokfelt et al., 1994 (40-50% after CSNT); and Shi et al., 1999 (20-40% after PSNL and CCI). The sensitivity of the immunohistochemical techniques between studies also cannot be ruled out when explaining variability amongst studies. Despite this disagreement, my results are in concord with other studies that have reported the peak of galanin expression to be at 14 days post nerve injury (Averill et al., 2002). Although the expression of galanin down-regulates 14 days post TNT injury, expression is still maintained at chronic stages of nerve injury (43 days post TNT surgery) when pain related behaviours are still evident. These findings differ to that reported by Villar et al 1989, in which a return of galanin to near normal expression levels in L4/L5 ganglia was observed 45 days following sciatic nerve transection. Again, the nature of the nerve injury differs here. Nevertheless, it is conceivable that the galanin protein is expressed at 43 days post TNT injury and beyond, given the mRNA for galanin is still abundant at 60 days after traumatic peripheral nerve injury (Villar et al., 1989).

3.4.8 Mechanisms underlying neuropeptide upregulation

Following peripheral nerve injury many neuropeptides in the DRG become dysregulated (Maratou et al., 2009; Valder et al., 2003; Wang et al., 2002). A well known regulatory factor of DRG neuropeptide expression is nerve growth factor (NGF) (Pezet and McMahon, 2006). The decreased expression of substance P (SP) and calcitonin gene related peptide (CGRP) and the increased expression of galanin, NPY and other neuropeptides (e.g. Vasoactive intestinal peptide (VIP) and cholecystokinin (CCK)) in DRG cells following axotomy are attenuated by the administration of exogenous NGF (Verge et al., 1995). The evidence from the literature supports a negative regulation of NPY and galanin with NGF. It is possible that following TNT injury, the resulting decrease in NGF release deprives peripheral axons of tropic support, causing an

increased synthesis of NPY and galanin in the DRG. Galanin could be more affected here given that the TrkA - the receptor for NGF, is present predominately on small diameter DRG cells (Verge et al., 1992). Further evidence regarding the role of NGF is supported by experiments demonstrating an increase in CCI-induced NPY expression following the administration of antisera against NGF (Obata et al., 2004). Other growth factors have been implicated such as brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), Neurotrophin-3 (NT-3), and leukemia inhibitory factor (LIF) (see Figure 3.33). Under normal circumstances, BDNF is constitutively expressed in 11-38% of DRG neurons, mainly in small TrkA-positive neurons (Michael et al., 1999). After a peripheral nerve transaction, BDNF mRNA is upregulated mainly in medium-large diameter neurons expressing the TrkB and TrkC receptors (NGF-insensitive) (Fukuoka et al., 2001). BDNF can stimulate the promoter for NPY (Minthorby, 1994; Wernersson et al., 1998; Williams et al., 1998) which may result in increased NPY protein production. GDNF, like NGF, is critical for the maturation of sensory neurons in development and may play a role in the regulation of NPY and galanin following nerve injury. Intrathecal administration of GDNF (or NGF) via an osmotic pump to nerve injured rats (L5/L6 SNT) can prevent the upregulation of NPY and galanin (and ATF-3) in the DRG and additionally prevent the development of pain related behaviours (Wang et al., 2003a). The predominant expression of NPY in large diameter neurons observed in the current study may be explained by a loss of neuronal trophic factor 3 (NT-3), which can suppress NPY expression (Ohara et al., 1995).

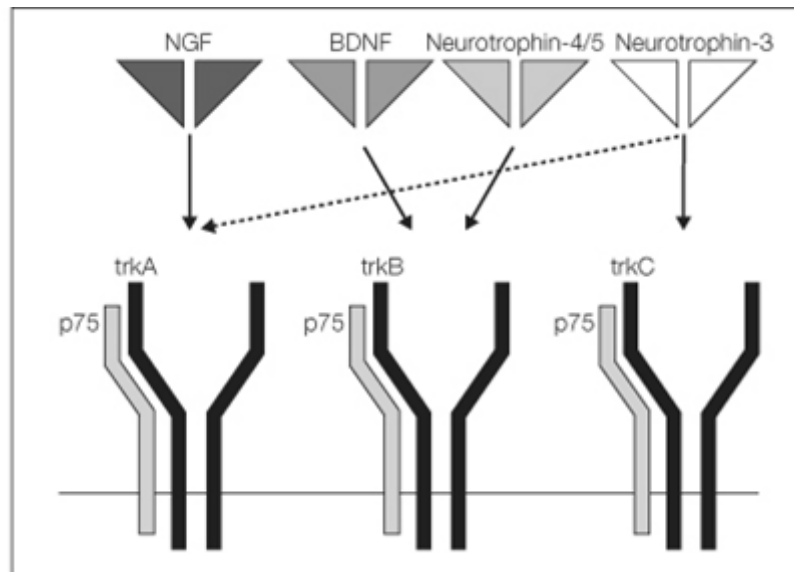


Figure 3.33 The neurotrophin interactions of the Trk receptors. Nerve growth factor (NGF) is the preferred ligand for trkA, neurotrophin-4/5 and brain-derived nerve factor (BDNF) are the preferred ligands at the trkB receptor, and neurotrophin-3 (NT-3) is the preferred ligand at the trkC receptor, although it will bind to all the trk receptors (albeit with different potencies). All the neurotrophins bind the p75 receptor with equal affinity. NGF negatively regulates NPY, galanin, and ATF-3 expression and therefore is a proposed main mechanism regarding their dysregulation. Image taken from Wall and Melzacks Textbook of Pain 5th Edition.

After peripheral nerve injury, Wallerian degeneration takes place and there is a reduction in the release of NT-3 from the endoneurium (Ji et al., 1994a). This reduction may result in reduced activation of trkC (the receptor for NT-3) which is expressed predominately on large diameter neurons (Hendry et al., 1992). Indeed the exogenous administration of NT-3 through nerve grafting in sciatic nerve transected rats has been shown to attenuate the upregulation of NPY (Sterne et al., 1998). The reduced presence of NT-3 at the site of injury will additionally affect adjacent non-axotomised neurons, which could lead to upregulation of NPY in non-injured neurons. In regard to galanin, evidence from previous studies has demonstrated that galanin upregulation is impaired in mice lacking the gene for LIF (Rao et al., 1993) and IL-6 (Murphy et al., 1999). This suggests that LIF and/or IL-6 is required for galanin to be expressed and is likely that this is the case in the TNT injured animals since LIF and IL-6 are upregulated in Schwann cells following traumatic nerve injury (Murphy et al., 1993; Murphy et al., 1999; Sun and Zigmond, 1996). Both intact and injured sensory neurons express the receptors for LIF β and gp130 (Scott et al., 2000). The presence of LIF and IL-6 can also elicit the release of galanin, therefore its upregulation could have potential consequences of modulating nociceptive processing (McMahon et al., 2005). NGF can negatively regulate the expression of LIF (Corness et al., 1998). It is likely that a reduction in NGF expression following TNT injury could initiate an increase in LIF synthesis, thus leading towards increased galanin production in the cell soma. The down-regulation of both NPY and galanin at day 43 post TNT injury suggests there is a partial reversal of nerve injury and is supported by the down-regulation of ATF-3 this time point. Regenerated axons may restore the normal expression levels of neurotrophic factors thus leading to a normal expression of trophic factors, and a reduction of NPY and galanin expression. However the reduction of neuropeptide expression over time did not result in a reduction of TNT induced mechanical hypersensitivity, which indicates that other factors must be involved in maintaining chronic neuropathic pain responses.

3.4.9 ATF-3, GAP-43, NPY, and galanin do not correlate with mechanical hypersensitivity

Data from this study indicates a lack of correlation between the temporal profile expression of investigative markers (i.e. ATF-3, GAP-43, NPY and galanin) in both peripherin and NF-200 DRG cells and the degree of mechanical hypersensitivity exhibited after TNT injury. Although there was a noticeable trend towards an association in both peripherin and NF-200 cells with regards to GAP-43, NPY, and galanin immunoreactivity and also with ATF-3 expression in peripherin characterized cells. A significant association may have been a possibility through the use of increased experimental group sizes. The nervous system is extremely plastic and pain as well as being highly sensory/discriminative, also involves both affective-motivational and cortical processing, so perhaps such a correlation was oversimplified. The observed lack of correlation regarding ATF-3 is consistent with previous observations using the SNL model (Djouhri et al., 2006; Shortland et al., 2006) and CCI model (Obata et al., 2003). Of interest, Obata et al., 2003 examined the relationship between axotomized neurons and the incidence of pain-related behavior by dividing rats into three groups according to the percentage of ATF-3 expression (group 1 <12.5%, group 2 12.5-25%, group 3 >25%). Mechanical and thermal hypersensitivity were observed in group 2 and 3 animals (ATF-3 expression >12.5%). Interestingly, no pain-related behaviour was observed in animals exhibiting ATF-3 expression <12.5%. The expression of GAP-43 may be related to the interruption of axonal transport as a consequence of nerve injury. The inhibition of axonal transport via capsaicin or vinblastine is enough to induce GAP-43 expression to similar levels observed following nerve crush injury (Woolf et al., 1990). The lack of correlation regarding NPY and galanin are supported by previous studies (Ma and Bisby, 1997). In another study of a differing nature, Benoliel et al (2000) examined the expression of NPY in the trigeminal ganglia at 14 days post infraorbital CCI injury and indicated no correlation between expression and the degree of mechanical hypersensitivity in the vibrissal pads. Shi et al 1999 reported an inverse relationship between galanin expression and the degree of mechanical hypersensitivity across various models (PSNL, CSNT, and CCI.) i.e. low galanin expression → low PWT. Moreover, galanin expression was always found to be greater in the nerve injured animals which did not display mechanical hypersensitivity (<10% with PSNL, ~20% with CCI and ~35%

greater with total axotomy). This finding conflicts with both personal observations and that of other research groups and warrant further investigation.

3.4.10 Conclusion

To conclude, in this chapter I have characterized the temporal profile expressions of markers commonly dysregulated after nerve injury, namely, ATF-3, GAP-43, NPY, and galanin. In addition, I have correlated the expression profiles with the associated mechanical hypersensitivity. To summarize, in the acute stages of nerve injury, large diameter fibres were more proportionally affected (i.e. high percentage of cells immunoreactive for ATF-3 - an indicator of nerve damage), which is a novel finding. The neuropeptides NPY and galanin were robustly upregulated once neuropathic pain-like behaviours were established, both demonstrating a dynamic expression, with NPY particularly abundant in large diameter NF-200 immunoreactive cells. The upregulation of GAP-43 in injured ganglia provides evidence of nerve regeneration. There were no correlations between mechanical hypersensitivity and the markers investigated suggesting their expression is not critical towards the magnitude of neuropathic pain states.

Chapter 4

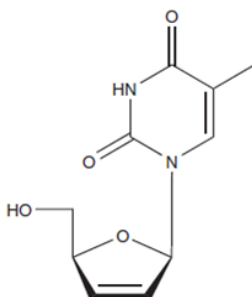
4 Evaluation of DRG neurochemical markers in an animal model of antiretroviral drug d4T-induced peripheral toxic neuropathy

4.1 Introduction

The introduction of highly active antiretroviral therapy (HAART) for the treatment of human immunodeficiency virus (HIV) infection/acquired immunodeficiency syndrome has resulted in improved patient clinical health and increased survival time (Beck et al., 1999; Brinkman et al., 1998; Verma S et al., 2007). The drugs belonging to the group nucleoside reverse transcriptase inhibitors (NRTIs) form an integral component of HAART, which acts to suppress HIV replication, therefore reducing the viral load and the deterioration of the immune system (Shafer and Vuitton, 1999). The development of peripheral neuropathies is a known consequence of HIV disease, however the prevalence has increased in association with the widespread use of NRTIs, particularly zalcitabine (ddC), stavudine (d4T) and didanosine (ddI) (13% in 1993 vs 42% in 2006) (Smyth et al., 2007), and is now recognized as the disorder antiretroviral toxic neuropathy (ATN) (Bacellar et al., 1994). These treatments can cause a progressive ‘die back’ degeneration of small diameter unmyelinated sensory neurons, that is associated with a reduced IENF density and significant pain in patients (Pardo et al., 2001). The degeneration of sensory nerve fibers is usually bilateral, symmetrical, and distal in nature affecting those with the longest axons - typically in the hands and feet. The symptoms experienced by patients include numbness, burning pain, and paraesthesiae, with motor dysfunction being relatively rare, but reduced or absent deep tendon reflexes can be observed (Verma et al., 2004). The pain that results from antiretroviral therapies has a detrimental effect on the patient’s quality of life and is often a motivating factor for withdrawal of treatment (Pandya et al., 2005). The use of ddC, one of the most neurotoxic NTRIs, has been phased out of use in both developed and developing countries, and been replaced by other less toxic NRTIs such as zidovudine (AZT) or tenofovir (TDF). The use of d4T has also been indicated to be phased out of use by the WHO¹, and although less toxic drugs are available, d4T is still widely used in resource limited settings due to its low cost and widespread availability (Cherry et al., 2012; Gilks et al., 2006; Makinson et al., 2008; Renaud-Thery et al., 2007). There are currently 34 million people worldwide living with HIV, and much of the social-economic burdens of HIV-related sensory neuropathies are in Sub-Saharan Africa¹. From the 34 million people worldwide living with HIV, approximately 6.6 million individuals are receiving antiretroviral therapy, and 5.5 million

of those are in Sub-Saharan Africa¹, therefore this region are most at risk from developing painful neuropathies as a consequence of continued exposure to treatment. Previous research has already indicated that the incidence of d4T-induced neuropathy is related to both the dose and length of use (Simpson and Tagliati, 1995). Although symptoms can improve upon cessation of treatment (Petersen et al., 1995), this improvement is often preceded by a period of augmented symptoms that may last several weeks (Simpson and Tagliati, 1995), therefore treatment strategies remain challenging. Although the prevalence of HIV related sensory neuropathy has increased since 1993 (pre HAART era) amongst the use of d4T and other NRTIs such as ddI and ddC, the prevalence has remained unchanged from 2001 (44%) to 2006 (42%) despite the general decreased use of NRTIs (Smyth et al., 2007). There may be various factors involved to explain this outcome. This finding could be indicative of the direct neurotoxicity associated with HIV disease, or could indicate an inability of peripheral nerves to regenerate and recover from the effects of previous exposure to neurotoxic NRTIs, and therefore the neuropathic symptoms persist.

d4T chemistry



d4T is a synthetic pyrimidine nucleoside analogue and is a potent inhibitor of HIV replication *in vitro* in human cells (Guimaraes et al., 2010). When administered to a subject, d4T enters cells by non-facilitated diffusion where it is converted into its active triphosphate form. d4T competes for the binding site of HIV reverse transcriptase enzyme with the normal cellular triphosphates needed for DNA synthesis therefore mimicking their action. This disrupts HIV- reverse transcriptase activity, resulting in the termination of viral DNA replication.

¹ World health organization: Progress report 2011: Global HIV/AIDS response (http://www.who.int/hiv/pub/progress_report2011/summary_en.pdf accessed 17/2/2012)

4.1.1 Effects of antiretroviral toxic neuropathy

The exact pathogenesis of painful ATN to date remains unclear and is the topic of many investigations. The elucidation of specific mechanisms regarding ATN in human subjects is confounded by the concurrent viral (HIV) interaction with peripheral sensory neurons, and their supporting cells, which has also been identified to precipitate small fibre peripheral neuropathy in itself. The use of preclinical *in vitro* and *in vivo* studies has therefore provided a platform to which direct mechanisms can be investigated independently of HIV influence.

Effects of NRTIs *in vitro*

When primary DRG cultures are exposed to NRTIs they elicit a dose dependant toxicity which is characterized by neuronal cell death, decreased total number of neuritis per neuron, and reduced neurite length (Cui et al., 1997; Keswani et al., 2003a). Reduced soma size has additionally been observed in feline DRG cultures (Zhu et al., 2007). The NRTI induced cell death is reported to involve both apoptotic and necrotic types of cell death. Evidence of necrotic cell death derives from the increased size of dead cells and the lack of TUNEL labelling in ddC treated DRG cultures, a common method for identifying apoptosis (Keswani et al., 2003a). Exposure of ddI to neonatal DRG cultures can induce a c-Jun N-terminal kinase dependent apoptosis (Bodner et al., 2004). This NRTI toxicity is associated with a reduced rate of glycolysis and a decreased cellular content of mitochondrial DNA upon continuous exposure (Chen and Cheng, 1989; Cui et al., 1997), which can be reversed following NRTI removal (Chen and Cheng, 1989). These results are suggestive of a mitochondrial toxicity mechanism which has become a recognised avenue of investigation. Antiretroviral drugs can alter mitochondrial DNA (mtDNA) content by inhibiting DNA polymerase γ ; the enzyme responsible for the replication of mtDNA (Brinkman et al., 1999). Studies using various cell lines (e.g. T-lymphoblastic cells, human myocytes, isolated rat mitochondria from brain, liver, and skeletal muscle) have demonstrated differing levels of potency regarding DNA polymerase inhibition with some minor discrepancies depending on the cell line used and NRTI concentration. The NRTI ddC, which is being phased out of use, demonstrates the most potency (Chen et al., 1991), followed by d4T and ddI which are still widely used (Kakuda, 2000). Other studies have reported ultrastructural abnormalities following

NRTI treatments in DRG culture such as loss of cristae in mitochondria, and the clustering of microtubules in neurons (Liu et al., 2008). NRTI toxicity has been associated with a reduced expression of mRNA for brain derived neurotrophic factor (BDNF) and its receptor, TrkB (Zhu et al., 2007). BDNF is an important modulator of peripheral neuronal function, released by neurons, providing support and encouraging growth and differentiation of new neurons and synapses (Pezet and McMahon, 2006). The sustained neuronal toxicity induced by NRTIs maybe a due to a lack of trophic support as a direct consequence of their inability to produce and respond to growth factors such as BDNF (Zhu et al., 2007).

Effects of NRTIs *in vivo*

In the earliest animal model of ATN, rabbits were exposed to chronic administration of oral ddC which resulted in reduced conduction velocities and amplitudes in the sural nerve compared to controls (Anderson et al., 1992). In parallel to the observations made *in vitro*, these animals exhibited evidence of neuronal damage which included myelin splitting, intramyelinic oedema, demyelination, and axonal loss. Similar observations have been made regarding chronic exposures in rats (Schmued et al., 1996). This chronic exposure of ddC also elicits a reduction in intra-epidermal nerve fibre (IENF) density (Wallace et al., 2007b) which corresponds to the clinical situation of small fibre loss of distal axons and associated pain (Pardo et al., 2001; Polomano et al., 2001). Decreases in the conduction velocities (Chen and Levine, 2007) and morphological changes - including mitochondrial damage (Van Steenwinckel et al., 2008), have been observed within a week of a single dose of ddC in rats. This indicates that acute exposures are sufficient to elicit a neuropathy, which reflects the clinical scenario. However it is to be noted that some reports indicate that this acute exposure is not associated with the induction of demyelination and axonal degeneration (Bhangoo et al., 2007; Van et al., 2008) or decreases in paw IENF density in the paws that is evident upon chronic exposures (Siau et al., 2006).

Despite an array of studies demonstrating *in vivo* models of ATN, other researchers have been unable to replicate the model, and have highlighted potential difficulties that may be related to species differences or varied drug treatment regimes. Zhu and colleagues (2007) failed to induce nerve fibre loss, electrophysiological changes, or reductions in

IENF density in cats administered oral ddI daily for 6 weeks. Furthermore, no evidence of neuropathy was reported in monkeys administered ddI on a daily basis for 1 year (Kaul et al., 1999) or rabbits for administered ddI for a 24 week period (Warner et al., 1995). It is not clear why this is the case. Nonetheless it seems that rat models demonstrate more consistency regarding peripheral neuropathies upon exposure to NRTIs. While biochemical studies in animals have revealed some cellular effects of NRTIs that underlies ATN, including, most importantly, mitochondrial toxicity (Cherry and Wesselingh, 2003; Dalakas et al., 2001; Kakuda, 2000; Keilbaugh et al., 1997) the mechanisms underlying the pain experienced by these patients on these medications is yet to be fully elucidated. Several laboratories have now developed and reproduced models of ATN following injections with NRTIs (e.g. d4T, ddC, ddI) that develop a dose-dependent indices of pain-related behaviour (Bhangoo et al., 2007; Dorsey et al., 2009; Joseph et al., 2004; Wallace et al., 2007b; Zheng et al., 2011a). These behaviours are also associated with reductions in IENF density (Wallace et al., 2007b). The nature of the pain related behaviours may be related to the expression of particular markers known to be dysregulated following traumatic nerve injury that may alter neuronal excitability (Lacroix-Fralish et al., 2011; Woolf and Salter, 2000; Zimmermann, 2001). These include markers of cell injury/stress e.g. activating transcription factor-3 (ATF-3), neuropeptides e.g. galanin and neuropeptide (NPY), Vgf, regenerating gene protein-2 (Reg-2), and vasoactive intestinal peptide (VIP). Further evidence for their involvement derives from the closely related model of gp120 and ddC combined toxicity, in which such proteins are upregulated (Maratou et al., 2009; Wallace et al., 2007b).

Why d4T?

Since the use of d4T is still prevalent in resource limited settings and has been associated with nerve damage and significant pain in humans, it is worth investigating the mechanisms associated with d4T use in order to reduce the clinical burden associated with HIV disease. Neurochemical marker dysregulation is a major consequence of nerve trauma and is associated with pain-related mechanical hypersensitivity. However, these neurochemical markers have not been comprehensively investigated in non-traumatic nerve injury models such as the d4T-induced neuropathy model where nerve damage and pain-related behaviours are also evident.

4.1.2 Aims of the study

There are a number of aims in the following study:

- 6) Characterize behavioural responses towards mechanical stimuli following treatment with d4T at acute and chronic time points
- 7) Provide a temporal profile expression of ATF-3, NPY, galanin, and GAP-43 in the L5 DRG using immunohistochemical techniques and characterize the phenotype of those immunoreactive neurons via double labelling using markers for peripherin and NF-200.
- 8) Perform a cell size analysis of investigative markers as a quality control for immunohistochemical data
- 9) Provide a comparison with the effects of traumatic nerve injury as described in **Chapter 3.**

4.2 Results

Animals were sacrificed at days 7, 21, and 43 post initial d4T injections, shortly after recording of mechanical behavioural responses, and immunohistochemistry for ATF-3, GAP-43, NPY, and galanin was performed in the left L5 DRG of d4T/saline treated animals. Double labelling with each investigative marker was conducted using antisera for peripherin and NF-200, as well as a cell size analysis. Following injections, animals exhibited no obvious signs of motor impairment; this was assessed by observing gait when animals were freely moving within the cage. Phenotypic changes were only evident upon formal behavioural testing. A total of 28 animals were used in this study (Figure 4.1).

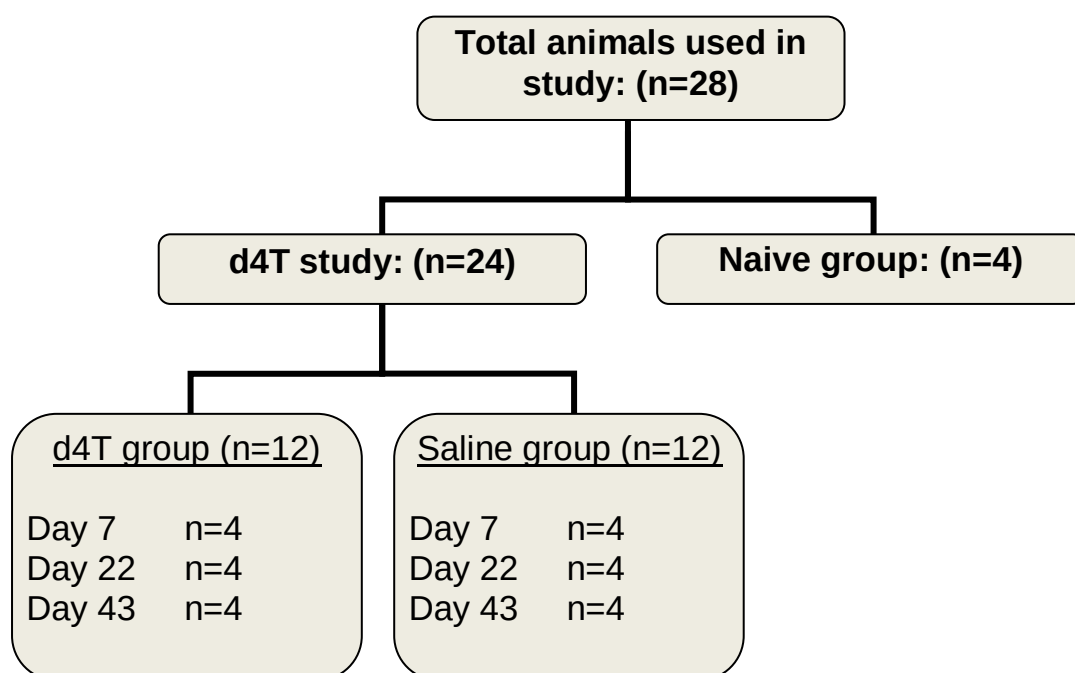


Figure 4.1 A breakdown of the numbers of animals used in the study. Animals were divided into two groups: d4T groups (n=12) and saline groups (n=12) and further subdivided according to the time of sacrifice (7, 22, and 43 days post injection). Naïve animals were used as additional controls

4.2.1 d4T induces bilateral mechanical hypersensitivity

At 7, 22, and 43 days post initial d4T injection, rats exhibited significant bilateral mechanical hypersensitivity in the mid-plantar region of the hind paws with respect to baseline values (Figure 4.2A-C). The PWTs in the left hind limb of animals at baseline and at day 7 post initial d4T injections were 47.5 ± 1.2 g and 32.7 ± 0.7 g respectively, representing a mean percentage decrease of 30.9 ± 3.1 (* $p < 0.01$). This was similar to the right hind limb which was 45.1 ± 0.8 g and 31.0 ± 0.4 g at baseline and at day 7 post initial d4T injection respectively with a mean percentage decrease of 31.2 ± 2.0 (* $p < 0.01$). Behavioural mechanical hypersensitivity reached a peak in both hind limbs at 22 days post initial d4T injection. The PWTs in the left hind limb of animals at baseline and at day 22 post initial d4T injections were 47.9 ± 1.1 g and 24.7 ± 0.5 g respectively, representing a mean percentage decrease of 48.3 ± 1.4 (* $p < 0.01$). The PWTs in the right limb at baseline and at day 22 post initial d4T injections were 45.8 ± 1.6 g and 24.3 ± 1.6 g respectively (mean percentage decrease from baseline of 46.7 ± 3.5 , * $p < 0.01$). Behavioural mechanical hypersensitivity at day 43 then reduced to values below that observed at day 7 and day 22 post d4T injection. The PWTs in the left hind limb at baseline and at day 43 post initial d4T injection were 46.9 ± 0.5 g and 38.5 ± 0.7 g respectively (mean percentage decrease from baseline of 17.9 ± 1.2 , * $p < 0.05$). Similarly, the reductions in the PWTs in the right hand limb were identical to that of the left with the baseline and day 43 values 45.1 ± 1.7 g and 37.8 ± 2.1 g respectively (mean percentage decrease from baseline of 16.0 ± 3.6 g, * $p < 0.01$). Significant hypersensitivity in saline treated animals was only observed at day 22 in the right hind limb (mean percentage decrease from baseline, 5.9 ± 1.8 , * $p < 0.05$). There were significant differences in the mean percentage decrease from baseline when comparing the left or right paws of d4T animals and their saline counterparts at all study groups (day 7, ** $p < 0.01$; day 22, ** $p < 0.01$; and day 43, * $p < 0.05$ post initial d4T injection, Figure 4.3).

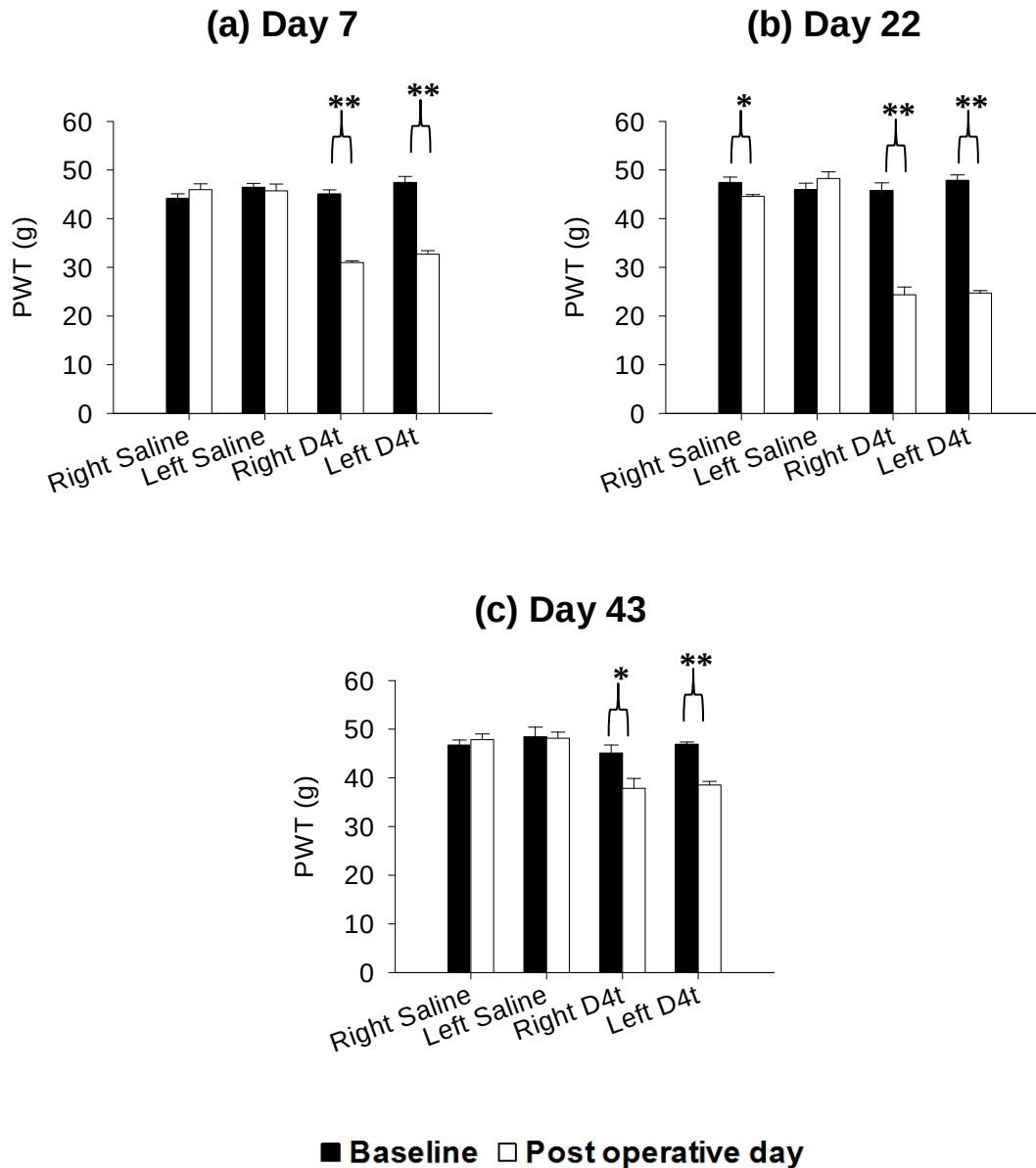


Figure 4.2 Mean paw withdrawal thresholds (PWT) to punctate mechanical stimuli measured using an electronic von Frey device at the various time point post d4T/saline injections in left and right hind limbs. Data presented as mean $\pm$ SEM. ** $P < 0.01$ and * $P < 0.05$ vs. respective baseline value using a Student's paired t test. An unusual significant hypersensitivity in saline treated animals was observed at day 22 in the right hind limb.

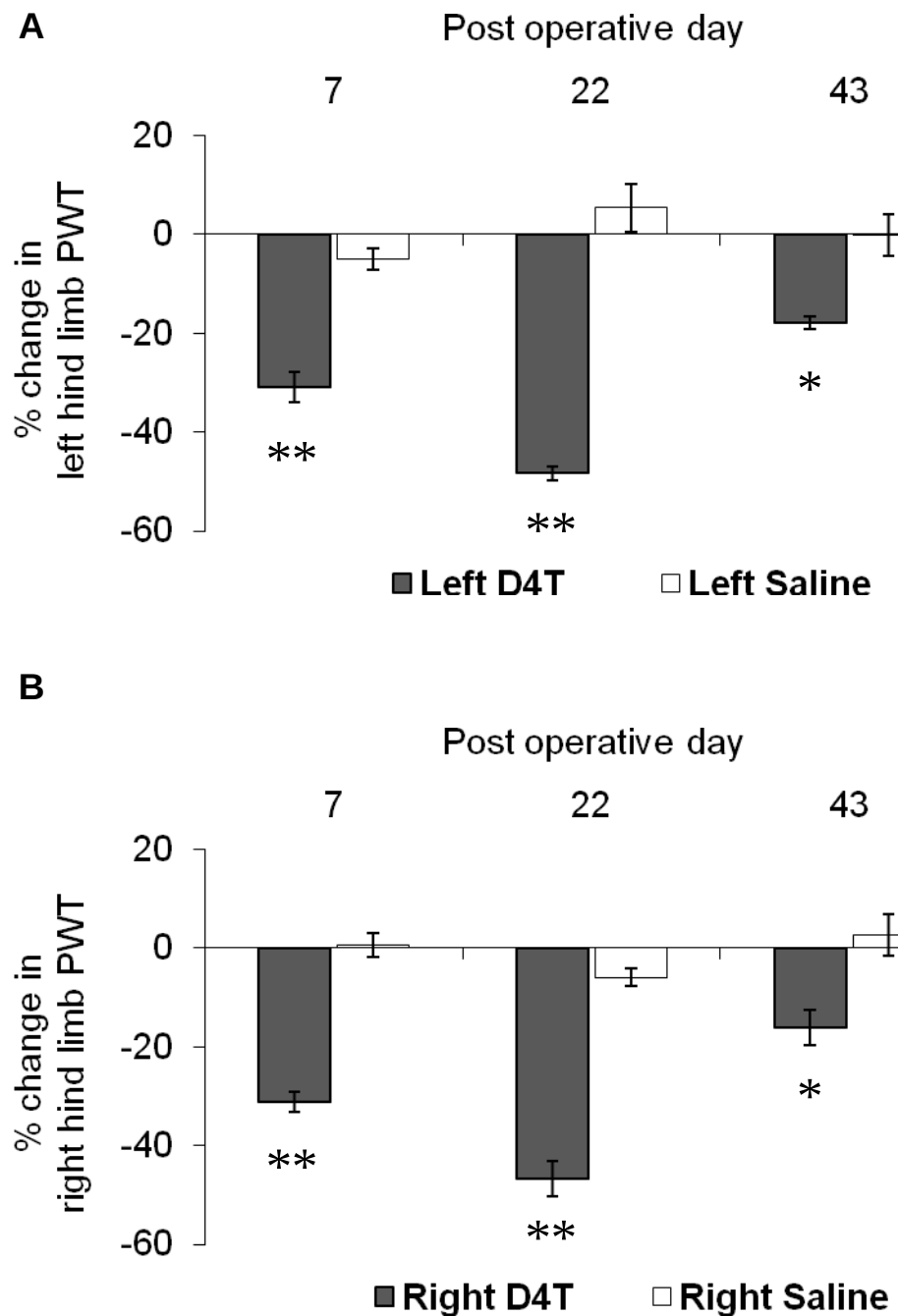


Figure 4.3 Mean percentage change in PWTs from baseline at various time points post d4T/saline injection in (A) the left paw and (B) the right paw using an electronic von Frey device. ** $P < 0.001$ and * $P < 0.05$ vs. respective baseline value using a Student's paired t test.

4.2.2 Temporal profile expression of ATF-3 in left L5 DRG

ATF-3 has been described as a marker of neuronal injury, and is not normally expressed or is present in low levels in uninjured animals (Tsujino et al., 2000). ATF-3 was not detected in the DRG of naïve animals (see Figure 4.4 and Table 3.2). Statistical analysis revealed that there were no significant differences in the temporal profile expression of ATF-3 in peripherin and NF-200 characterised cells ($p > 0.05$, one way ANOVA). No significant differences were observed with sham DRG at respective time points ($p > 0.05$, two way ANOVA). Following the administration of d4T to rats, the mean percentage ATF-3 immunoreactivity in peripherin and NF-200 characterised cells was $<1\%$ across all time points (Figure 4.4); a figure comparable to that of saline treated rats. Seven days following d4T administration, ATF-3 was expressed in few DRG cells (Figure 4.5) - ATF-3 immunoreactivity was $2.1 \pm 1.2 \%$ and $1.6 \pm 0.6\%$ in NF-200 and peripherin characterised cells respectively. At 22 days following d4T administration, ATF-3 was expressed in $1.7 \pm 0.5 \%$ and $0.1 \pm 0.1 \%$ of NF-200 and peripherin characterised cells respectively. Lastly, at 43 days post d4T administration, ATF-3 immunoreactivity was $0.45 \pm 0.45 \%$ and $0.9 \pm 0.5 \%$ in NF-200 and peripherin characterised cells respectively. ATF-3 immunoreactivity was only observed in neuronal cells only, and the few cells that were ATF-3 immunoreactive were observed in large size cells ($>900\mu\text{m}^2$) of which the immunoreactivity was nuclear. The cell size distribution analysis can be viewed across the various time points for ATF-3 immunoreactivity in Figure 4.6 for peripherin cells and Figure 4.7 for NF-200 cells. See Table 4.1 for mean cell size areas of ATF-3 immunoreactive cells across time in peripherin/NF-200 cells.

ATF-3

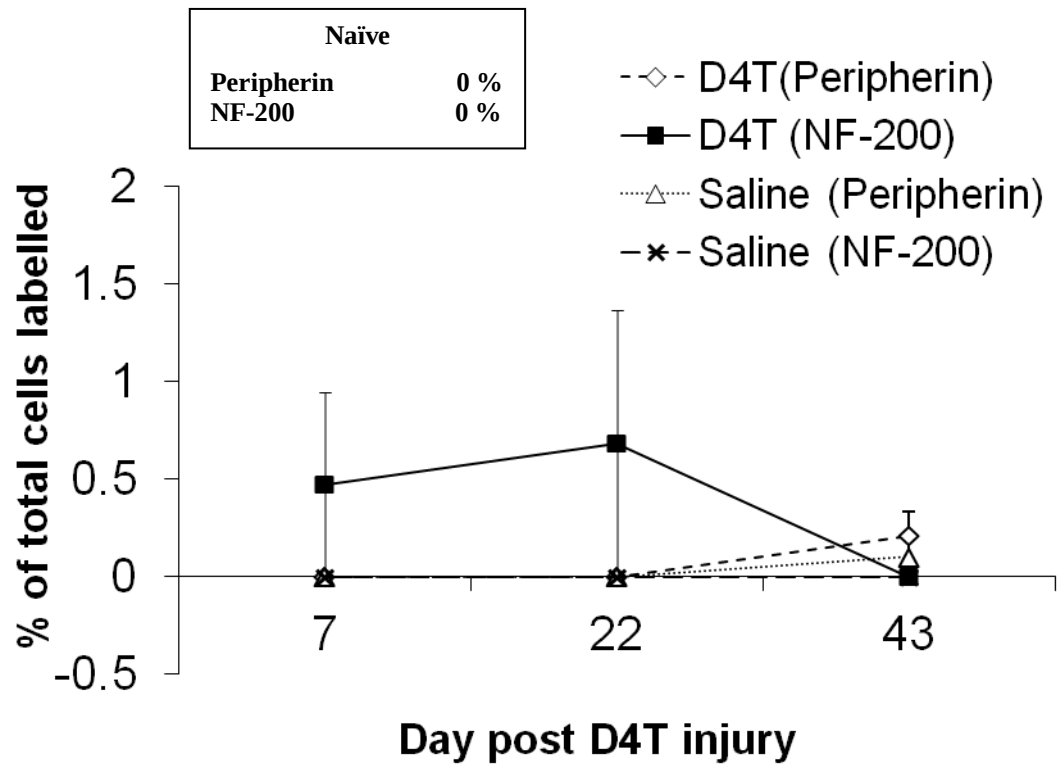


Figure 4.4 Mean percentage of total peripherin and NF-200 immunoreactive cells expressing ATF-3 in d4T, saline, and naive animals (Left L5 DRG n=4 animals per group). Data presented as mean $\pm$ SEM.

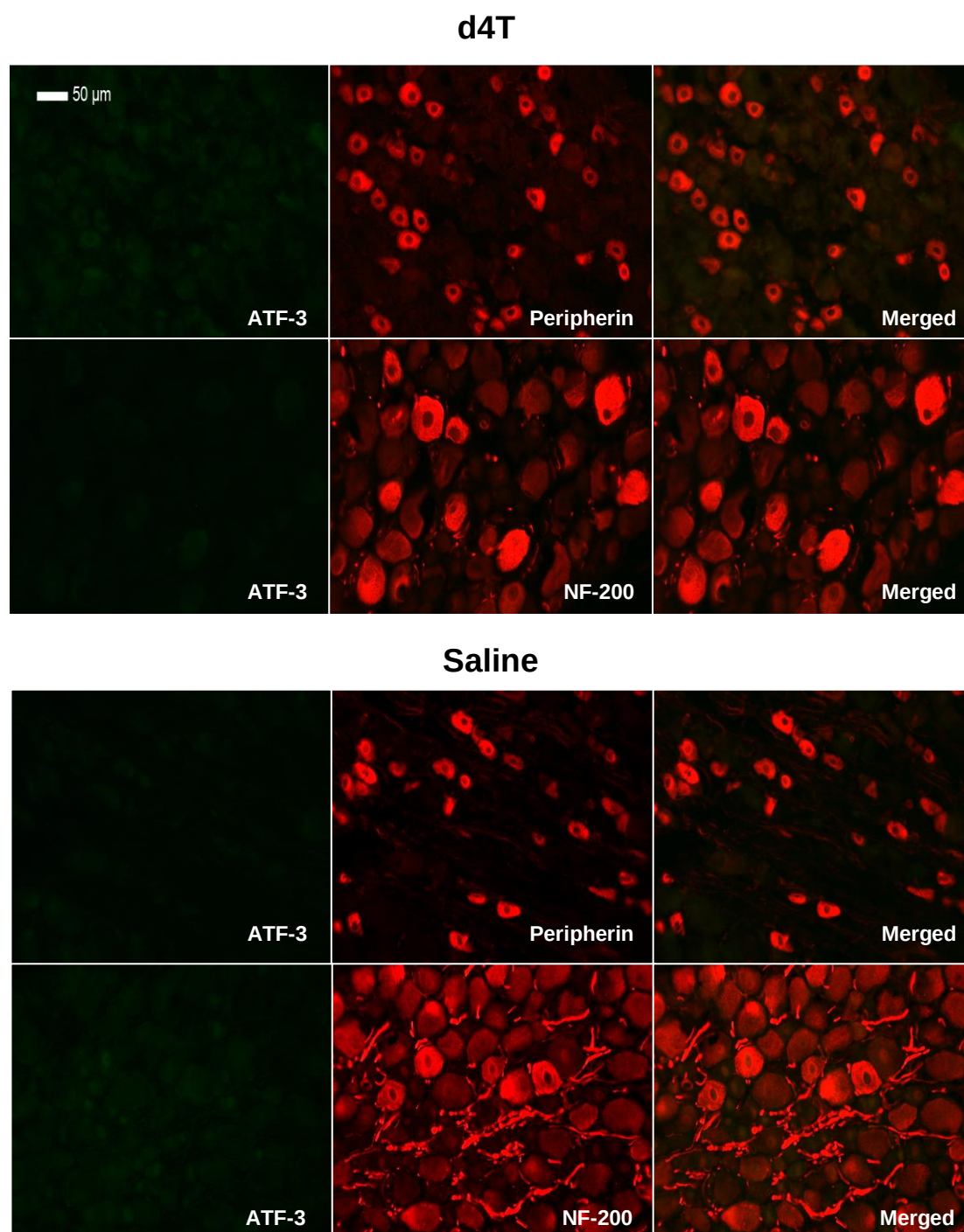


Figure 4.5 Representative images of ATF-3 immunohistochemistry in L5 DRG of d4T/saline treated animals (day 7) in peripherin and NF-200 neuronal sub-populations, magnification x20.

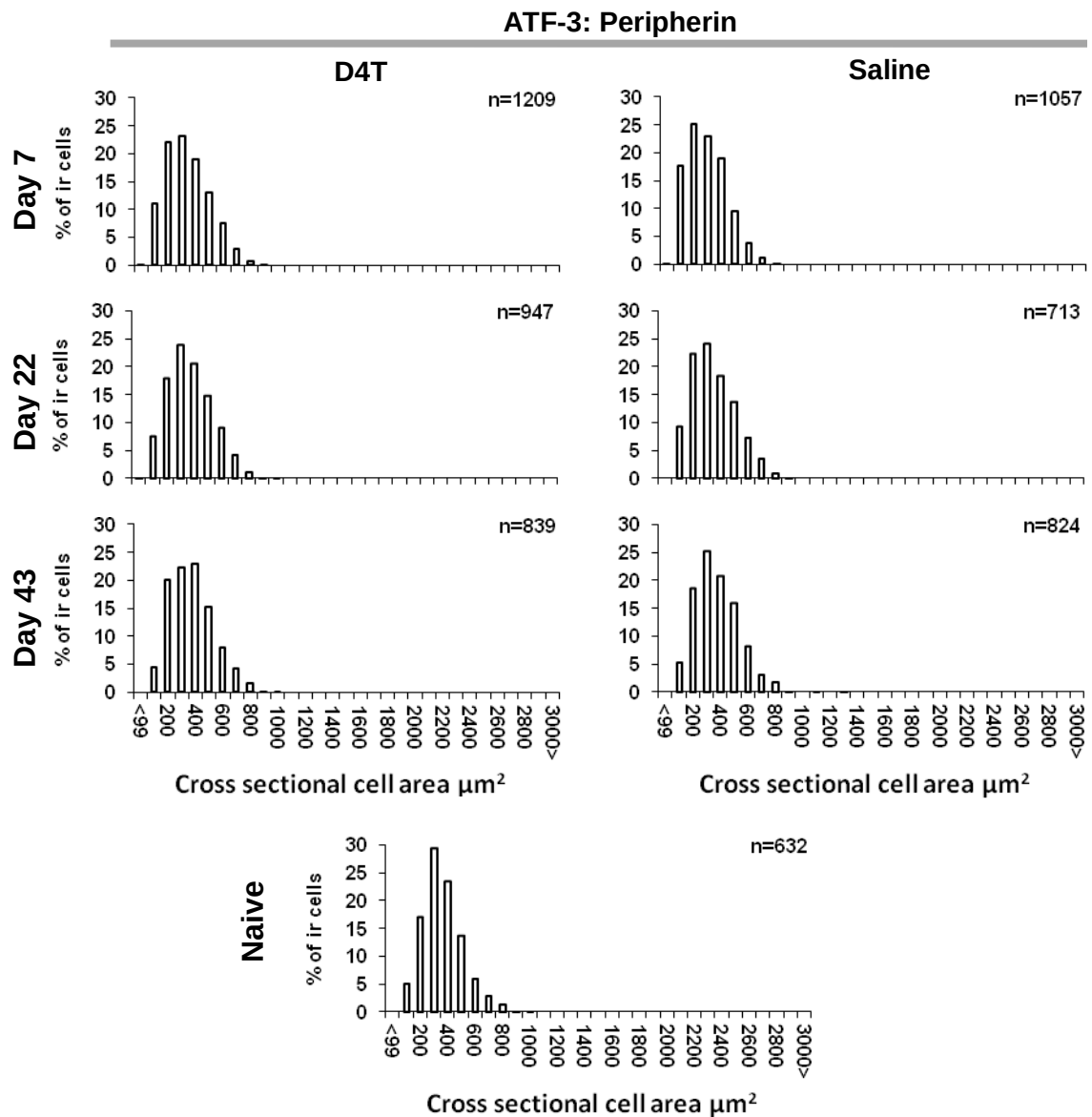


Figure 4.6 Percentage cell size area distribution analysis of ATF-3 & peripherin immunoreactivity (ir) in the left L5 DRG at 7, 22, and 43 days post initial d4T/saline injections, and in naive animals, (n= total number of nucleated peripherin immunoreactive cells).

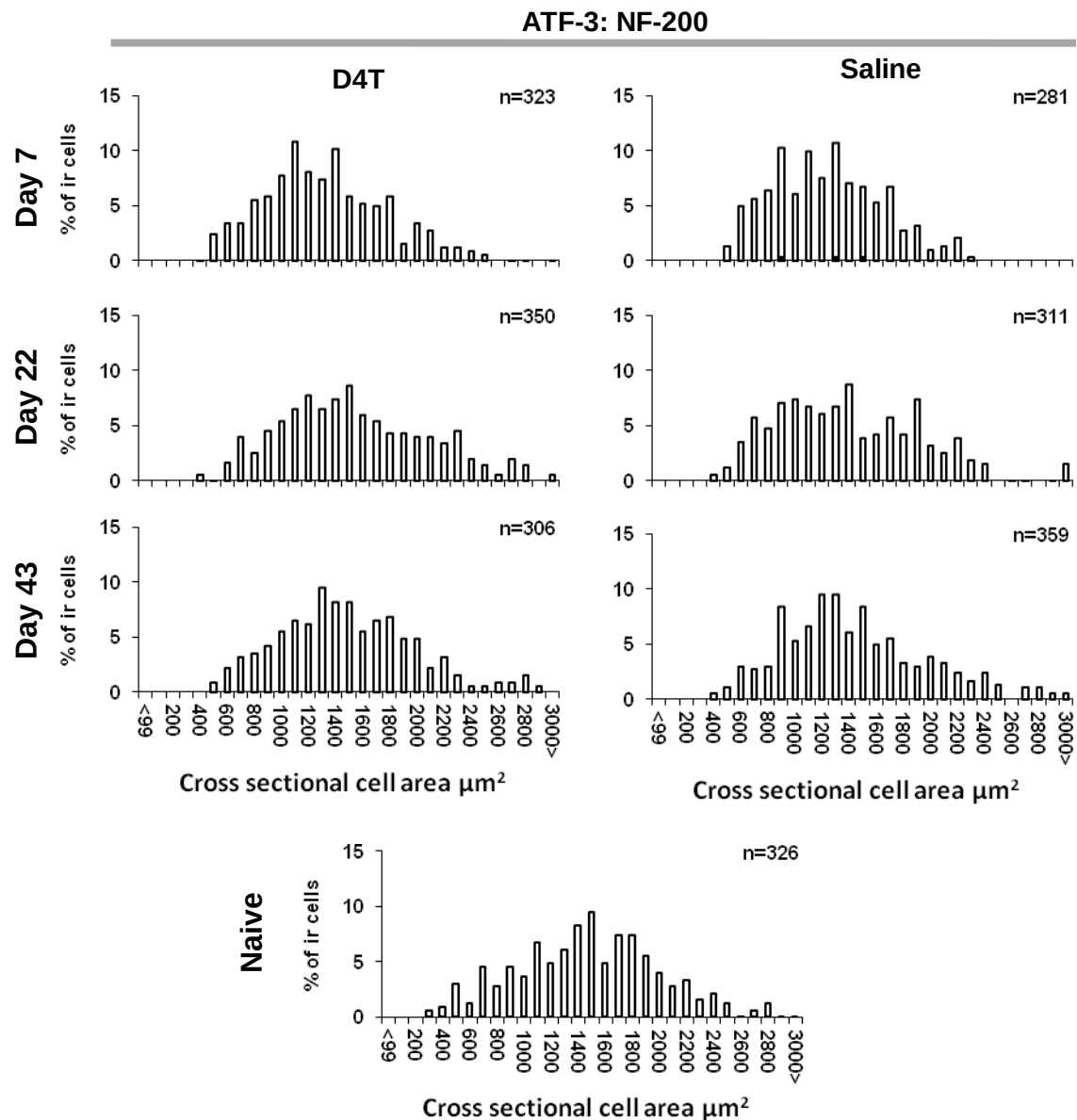


Figure 4.7 Percentage cell size area distribution analysis of ATF-3 & NF-200 immunoreactivity (ir) in the left L5 DRG at 7, 22, and 43 days post initial d4T/saline injections and in naive animals, (n= total number of nucleated NF-200 immunoreactive cells).

4.2.3 Temporal profile expression of GAP-43 in left L5 DRG

GAP-43 immunoreactivity in the L5 DRG of naïve animals only co-localised with peripherin immunoreactive cells (1.73 ± 1.73 %), and no GAP-43 immunoreactivity was observed in the NF-200 characterised population (see Figure 4.8 and Table 3.2). Following administration of d4T, a significant temporal pattern was observed in peripherin characterised cells but not in NF-200 cells ($p < 0.05$, one way ANOVA). A significant temporal pattern was also observed in NF-200 cells of saline treated rats ($p < 0.05$, one way ANOVA). There were no significant differences in GAP-43 immunoreactivity in d4T treated animals with respect to saline controls (Figure 4.8). GAP-43 immunoreactivity in the left L5 DRG of d4T and saline treated rats was variable, but the temporal expression remained $< 6\%$ in both peripherin and NF-200 characterised cells. Seven days following d4T administration, GAP-43 immunoreactivity was in 2.0 ± 1.2 % and 1.6 ± 0.6 % of NF-200 and peripherin characterised cells respectively. At 22 days following d4T administration, GAP-43 immunoreactivity was 1.1 ± 0.5 % and 0.1 ± 0.1 % in NF-200 and peripherin characterised cells respectively. Lastly, at 43 days post d4T administration, GAP-43 immunoreactivity was observed in 0.45 ± 0.45 % and 0.9 ± 0.5 % of NF-200 and peripherin characterised cells respectively. GAP-43 immunoreactivity was present mainly in the cell soma, but immunoreactivity was also observed in axons (Figure 4.9). The cell size distribution analysis across the various time points can be viewed for GAP-43 immunoreactivity in Figure 4.10 for peripherin cells and Figure 4.11 for NF-200 cells. See Table 4.1 for mean cell size areas of GAP-43 immunoreactive cells across time in peripherin/NF-200 cells.

GAP-43

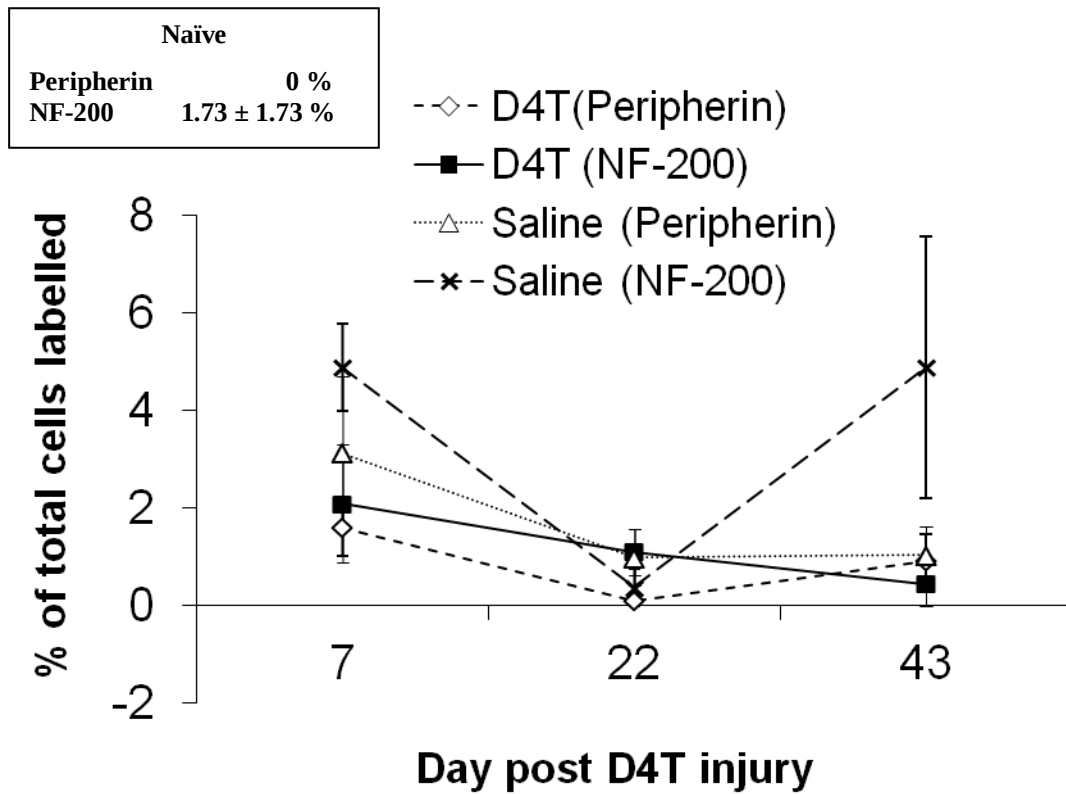


Figure 4.8 Mean percentage of total peripherin and NF-200 immunoreactive cells expressing GAP-43 in d4T, saline, and naïve animals (Left L5 DRG n=4 animals per group). Data presented as mean ± SEM.

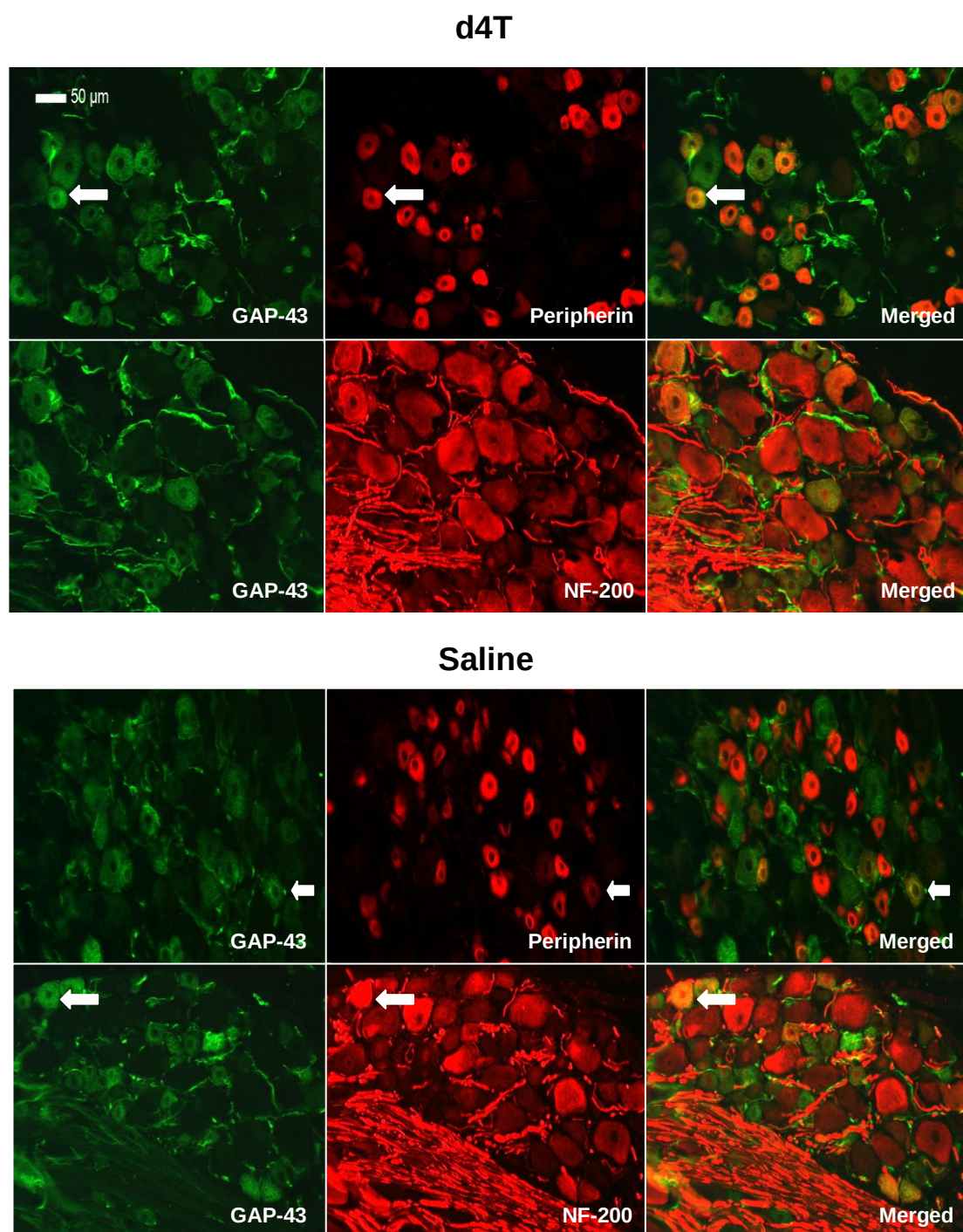


Figure 4.9 Representative images of GAP-43 immunohistochemistry in L5 DRG of d4T/saline treated animals (day 7) in peripherin and NF-200 neuronal sub-populations, magnification x20. The arrowheads represent co-localisation of immunoreactive cells.

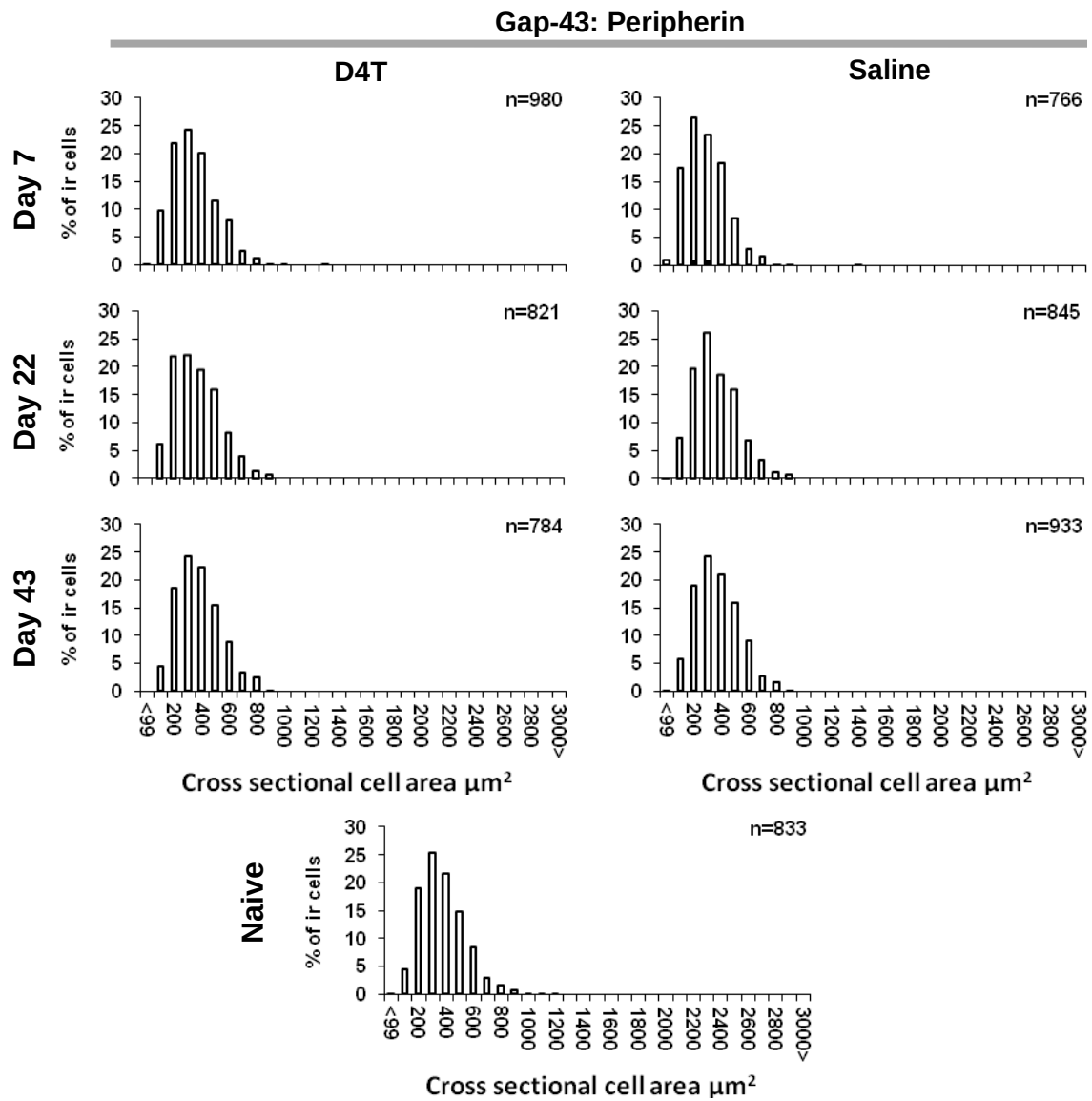


Figure 4.10 Percentage cell size area distribution analysis of GAP-43 & peripherin immunoreactivity (ir) in the left L5 DRG at 7, 22, and 43 days post initial d4T/saline injections and in naive animals, (n= total number of nucleated peripherin immunoreactive cells).

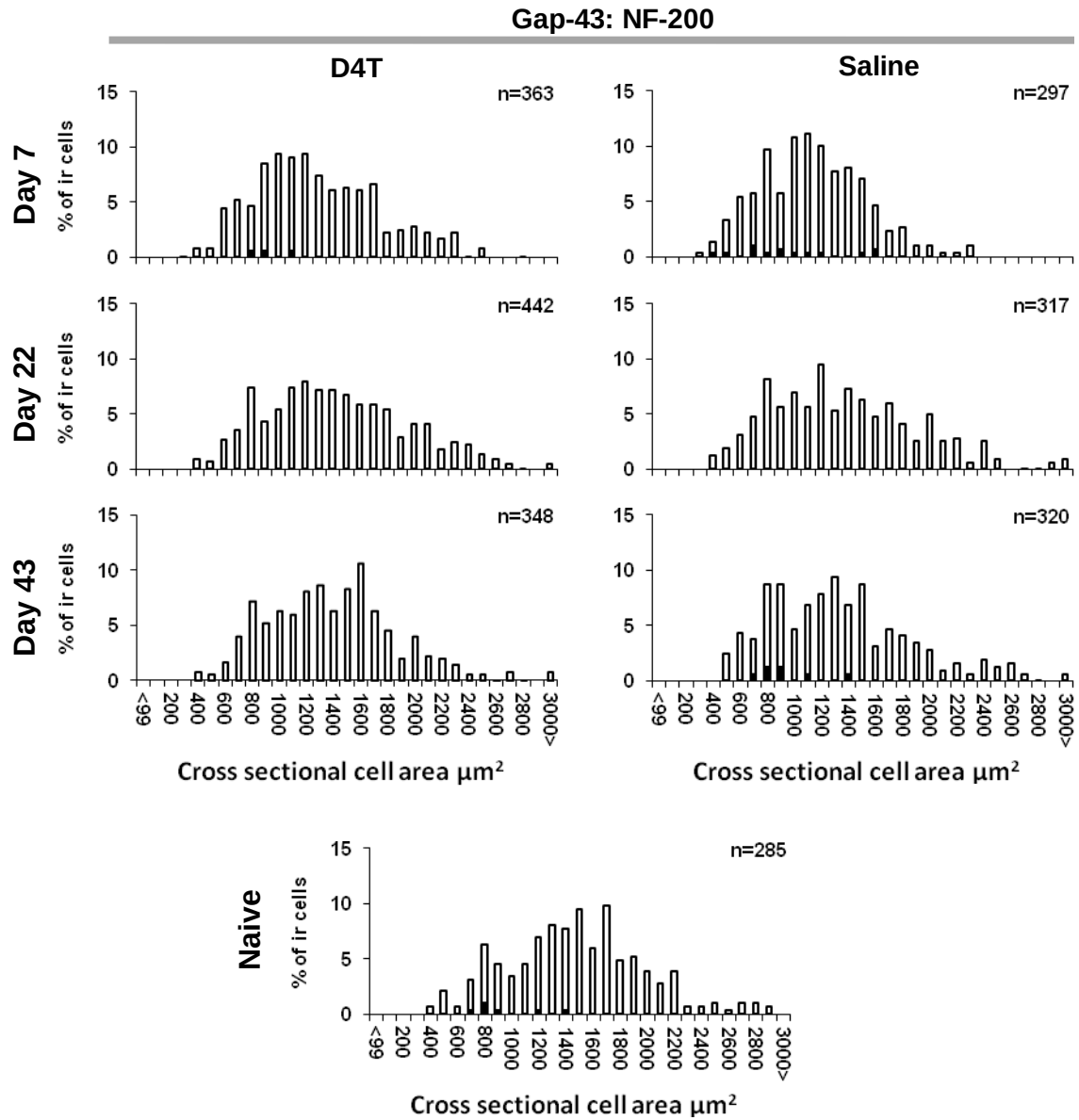


Figure 4.11 Percentage cell size area distribution analysis of GAP-43 & NF-200 immunoreactivity (ir) in the left L5 DRG at 7, 22, and 43 days post initial d4T/saline injections and in naive animals, (n= total number of nucleated NF-200 immunoreactive cells).

4.2.4 Temporal profile expression of NPY in left L5 DRG

NPY immunoreactivity in the L5 DRG of naïve animals only co-localised with NF-200 immunoreactive cells (0.28 ± 0.28 %), and no NPY immunoreactivity was observed in the peripherin characterised population (see Figure 4.12 and Table 3.2). Following administration of d4T, no significant differences in the temporal profile expression of NPY were observed in both NF-200 and peripherin cells ($p > 0.05$, one way ANOVA). No significant differences were observed compared with sham DRG at respective time points ($p > 0.05$, two way ANOVA). NPY immunoreactivity remained low across all time points in both d4T and saline treated rats ($< 0.5\%$), a figure comparable to that observed in saline treated rats ($< 0.2\%$) and also naïve rats (Figure 4.12). Seven days following d4T administration, NPY immunoreactivity was present in $0.15 \pm 0.15\%$ of NF-200 characterised cells, and no NPY immunoreactivity was observed in peripherin characterised cells (Figure 4.13). At 22 and 43 days following d4T injury, there were no NPY immunoreactive cells in both NF-200 and peripherin characterised cells. NPY immunoreactivity was cytoplasmic and was identified mainly in the cell soma however some immunoreactivity was observed in axons. The cell size distribution analysis across the various time points can be viewed for NPY immunoreactivity in Figure 4.14 for peripherin cells and Figure 4.15 for NF-200 cells. See Table 4.1 for mean cell size areas of NPY immunoreactive cells across time in peripherin/NF-200 cells.

NPY

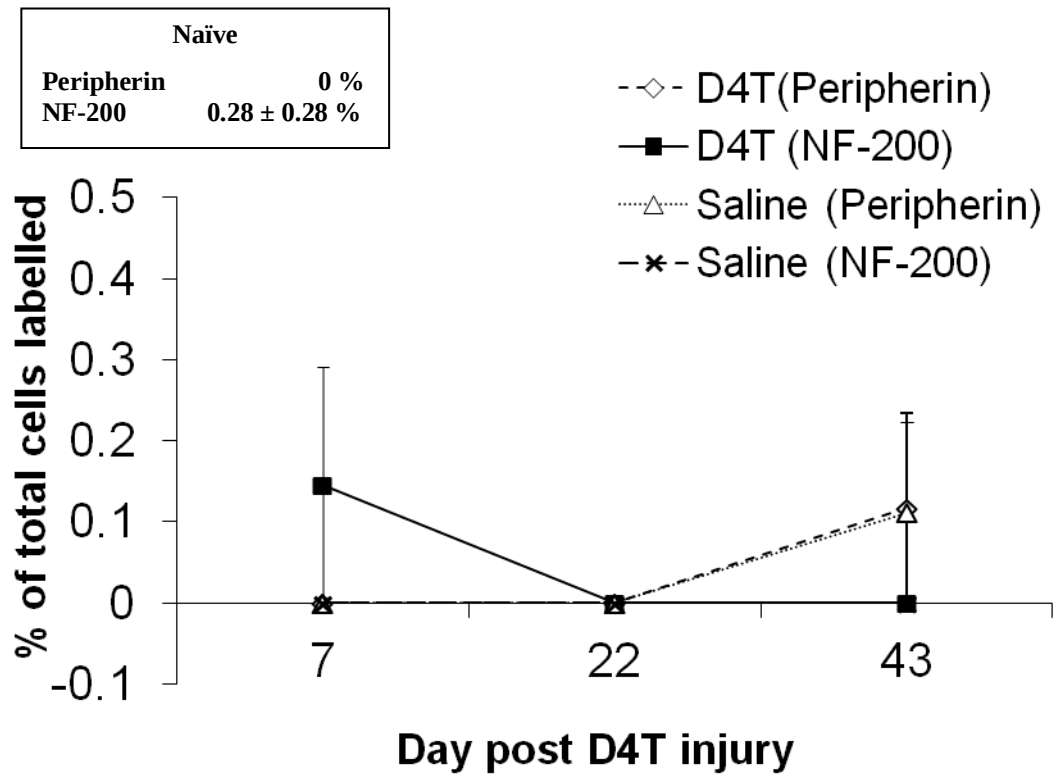
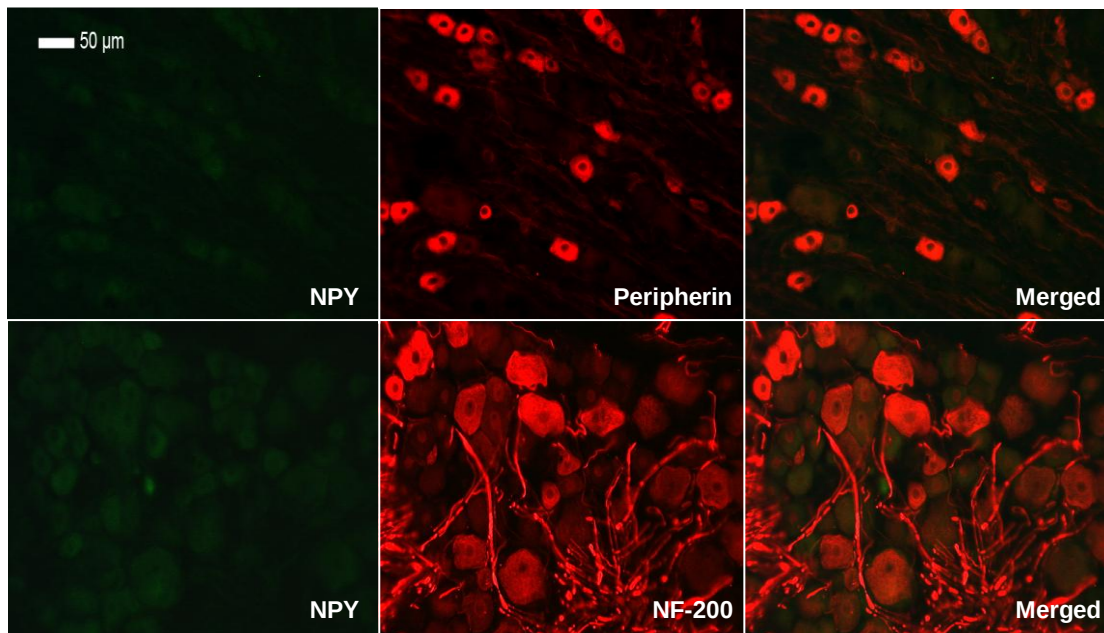


Figure 4.12 Mean percentage of total peripherin and NF-200 immunoreactive cells expressing NPY in d4T, saline, and naive animals (Left L5 DRG n=4 animals per group). Data presented as mean $\pm$ SEM.

D4T



Saline

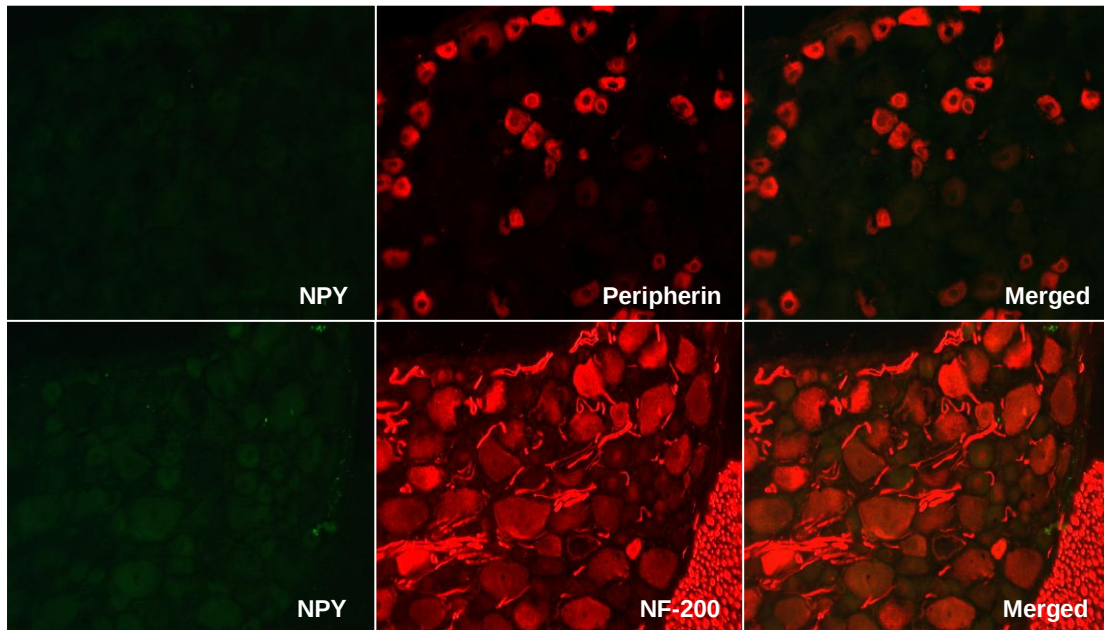


Figure 4.13 Representative images of NPY immunohistochemistry in L5 DRG of d4T treated animals (day 7) in peripherin and NF-200 neuronal sub-populations, magnification x20.

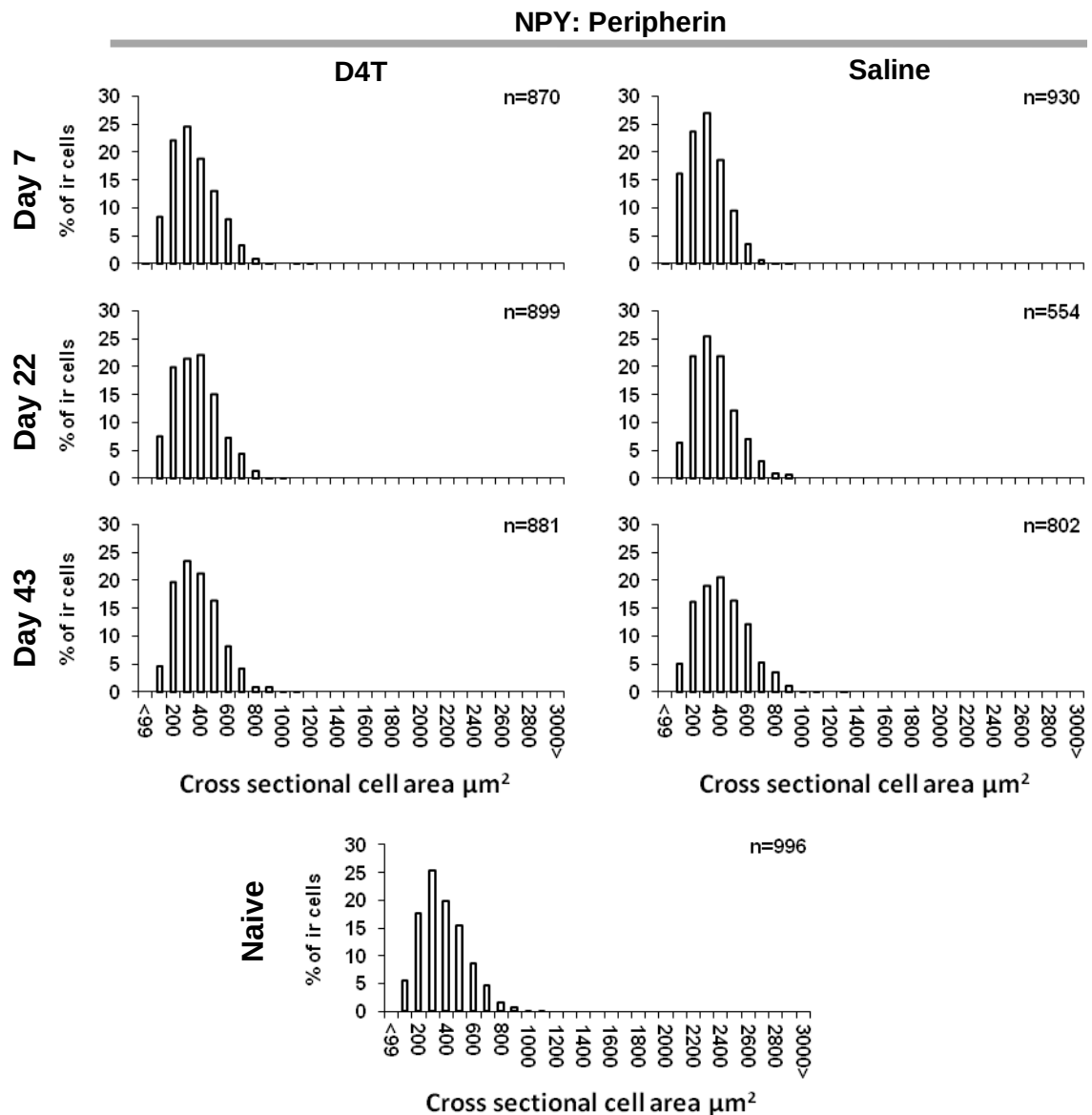


Figure 4.14 Percentage cell size area distribution analysis of NPY & peripherin immunoreactivity (ir) in the left L5 DRG at 7, 22, and 43 days post initial d4T/saline injections and in naive animals, (n= total number of nucleated peripherin immunoreactive cells).

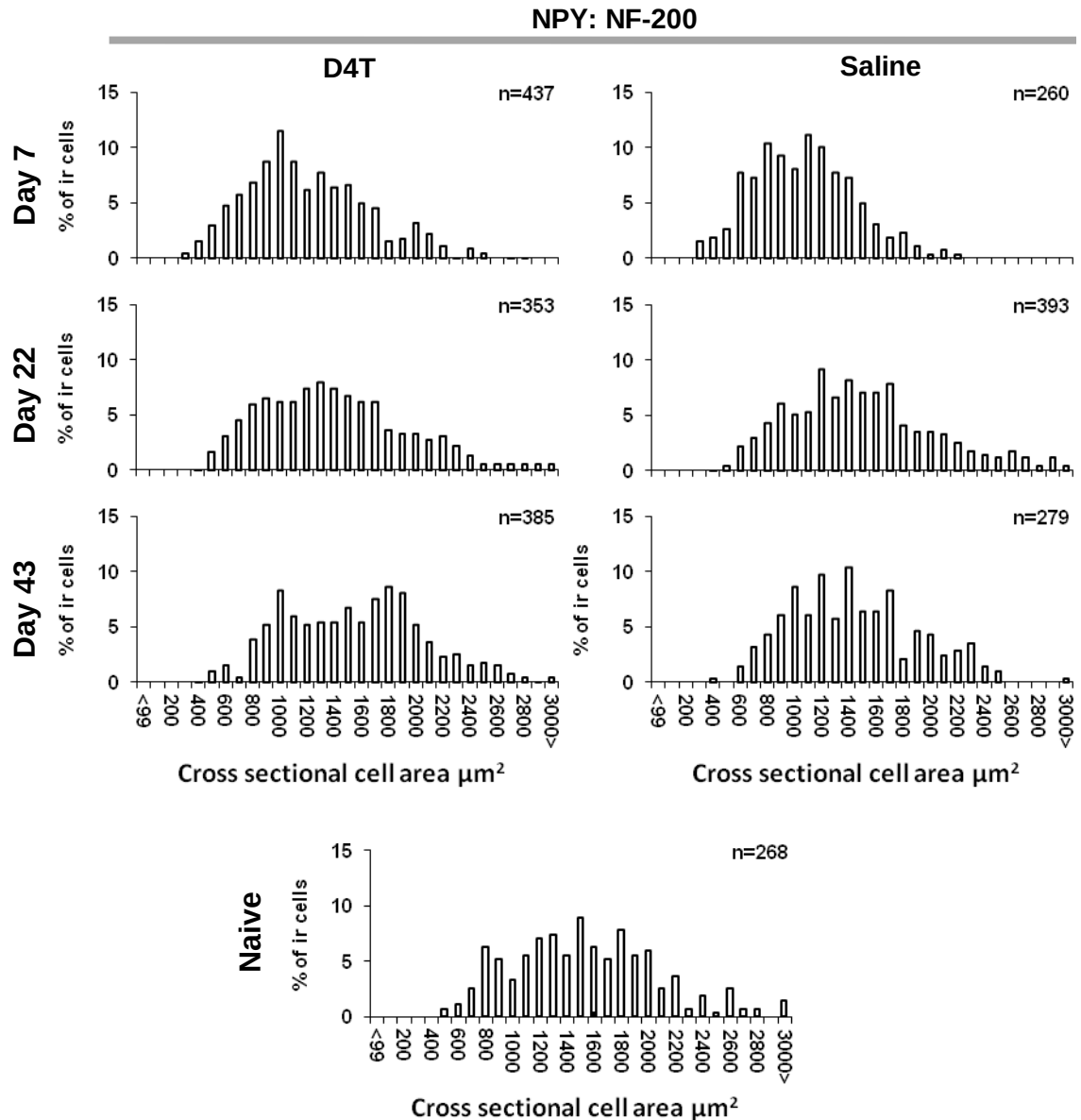


Figure 4.15 Percentage cell size area distribution analysis of NPY & NF-200 immunoreactivity (ir) in the left L5 DRG at 7, 22, and 43 days post initial d4T/saline injections and in naive animals, (n= total number of nucleated NF-200 immunoreactive cells).

4.2.5 Temporal profile expression of galanin in left L5 DRG

Galanin immunoreactivity in the L5 DRG of naïve animals was relatively low with 0.56 ± 0.56 % of galanin immunoreactivity in peripherin immunoreactive cells and no labelling present in NF-200 immunoreactive cells (see Figure 4.16 and Table 3.2). Following d4T administration, no significant differences was observed in the temporal profile expression of galanin in peripherin or NF-200 cells ($p > 0.05$, one way ANOVA). No significant differences were observed with sham DRG at respective time points ($p > 0.05$, two way ANOVA). The mean percentage galanin immunoreactivity in peripherin or NF-200 cells remained low ($< 2\%$) in all experimental time groups (Figure 4.16). The few cells with galanin immunoreactivity exhibited granular appearance. Seven days following d4T administration, galanin immunoreactivity was in $0.94 \pm 0.74\%$ of peripherin characterised cells, and no galanin immunoreactivity was observed in NF-200 characterised cells (Figure 4.17). At 22 days following d4T administration, galanin immunoreactivity was present in $1.3 \pm 0.8\%$ of peripherin characterised cells, and no galanin immunoreactivity was observed in NF-200 characterised cells. Lastly, at 43 days post d4T administration, galanin immunoreactivity was present in $1.7 \pm 0.3\%$ of peripherin characterised cells, and no galanin immunoreactivity was observed in NF-200 characterised cells. The cell size distribution analysis across the various time points can be viewed for galanin immunoreactivity in Figure 4.18 for peripherin cells and Figure 4.19 for NF-200 cells. See Table 4.1 for mean cell size areas of galanin immunoreactive cells across time in peripherin/NF-200 cells.

Galanin

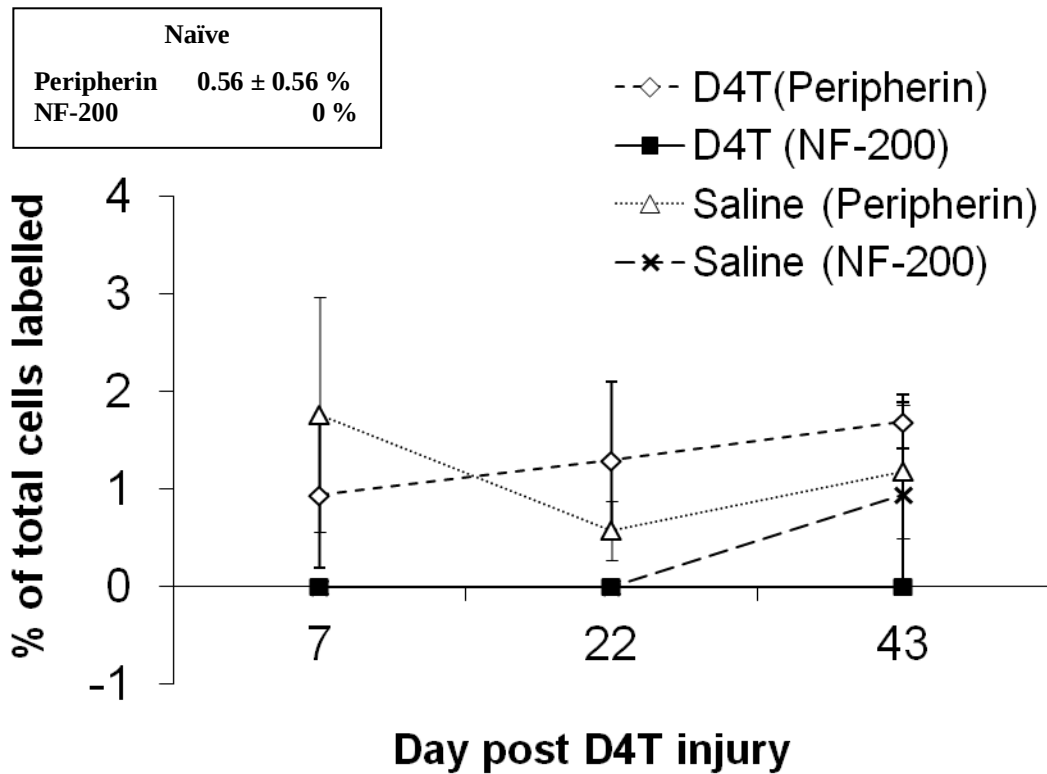


Figure 4.16 Mean percentage of total peripherin and NF-200 immunoreactive cells expressing galanin in d4T, saline, and naive animals (Left L5 DRG n=3-4 animals per group). Data presented as mean $\pm$ SEM.

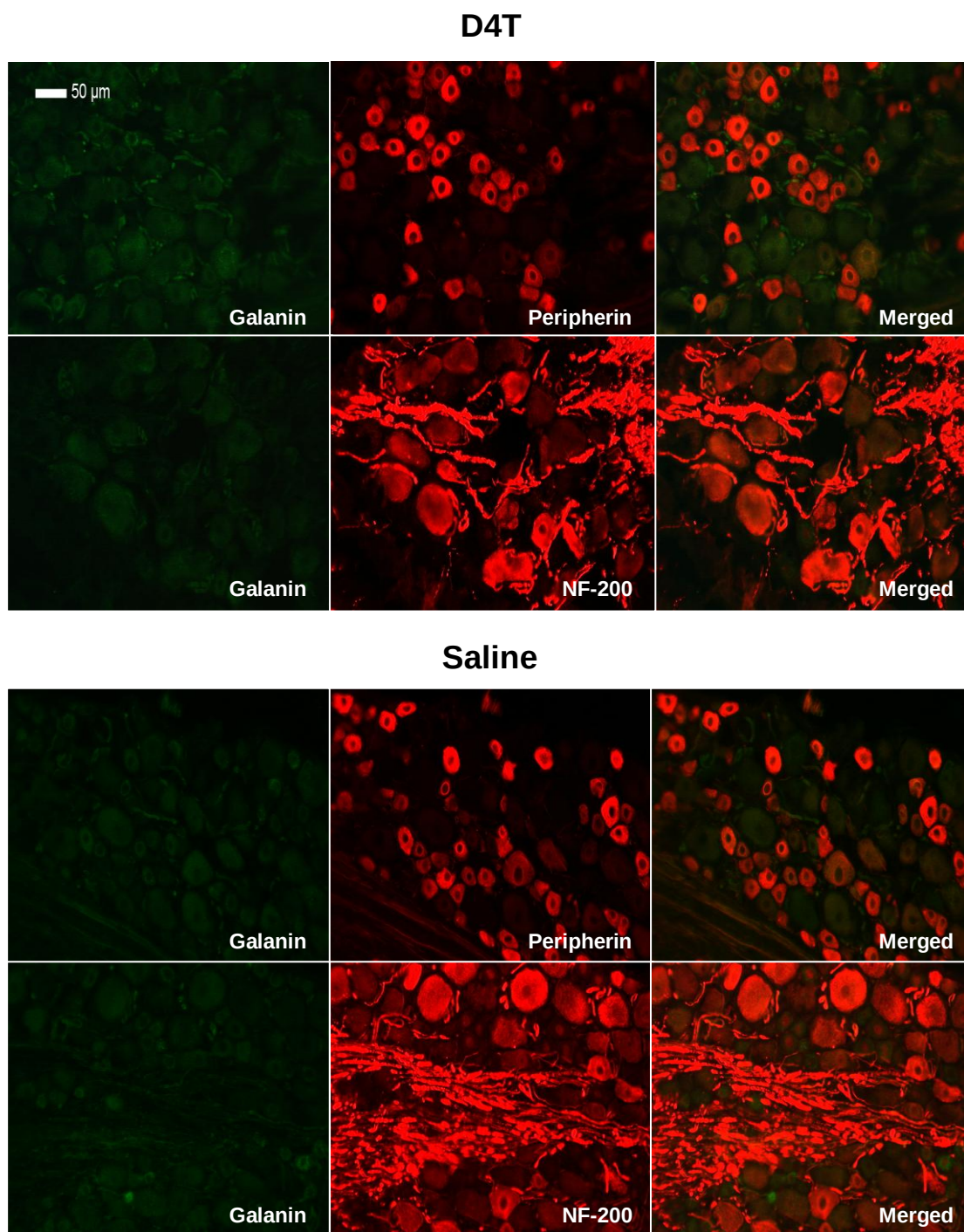


Figure 4.17 Representative images of galanin immunohistochemistry in L5 DRG of d4T treated animals (day 7) in peripherin and NF-200 neuronal sub-populations, magnification x20.

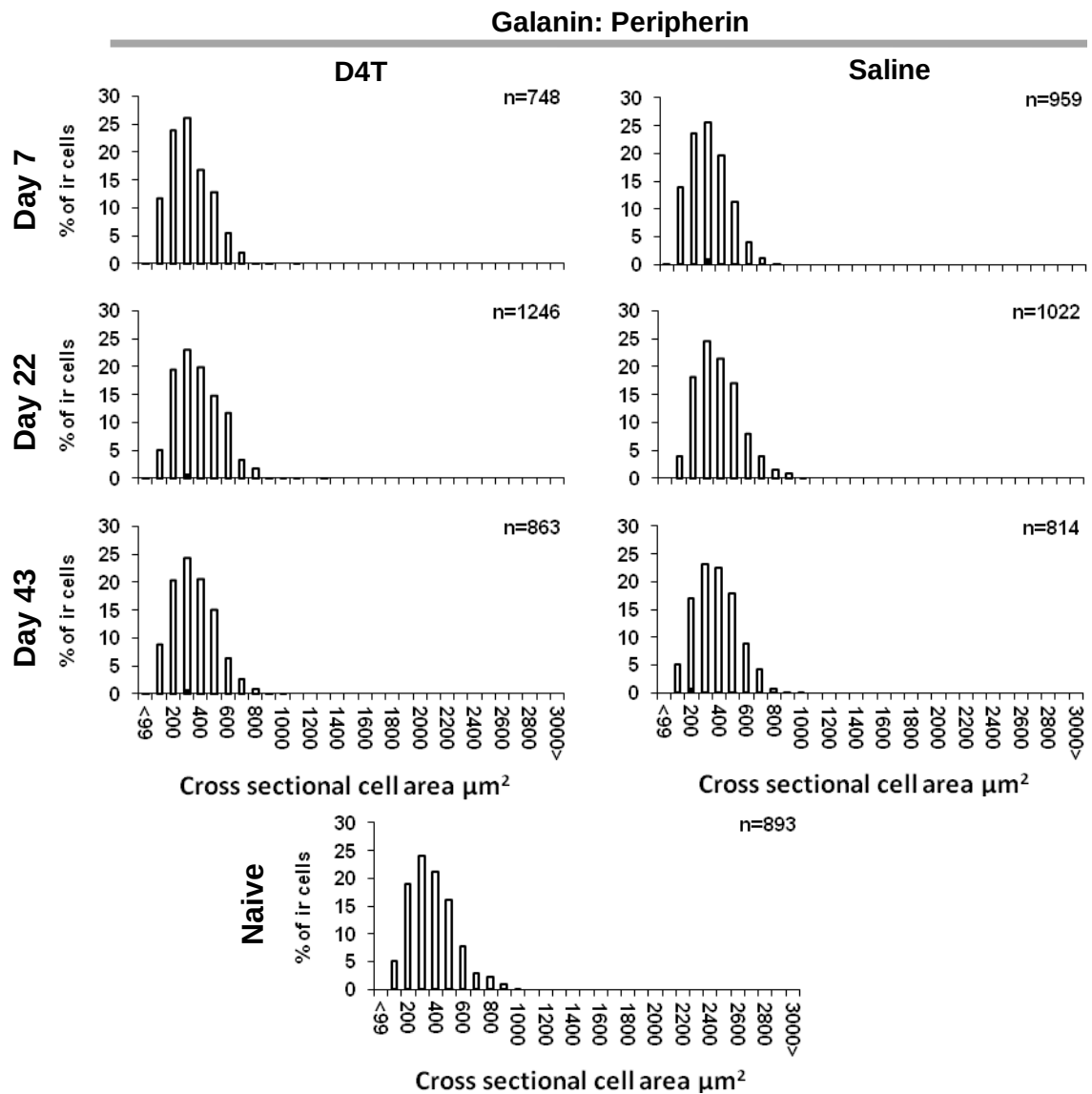


Figure 4.18 Percentage cell size area distribution analysis of galanin & peripherin immunoreactivity (ir) in the left L5 DRG at 7, 22, and 43 days post initial d4T/saline injections and in naive animals, (n = total number of nucleated peripherin immunoreactive cells).

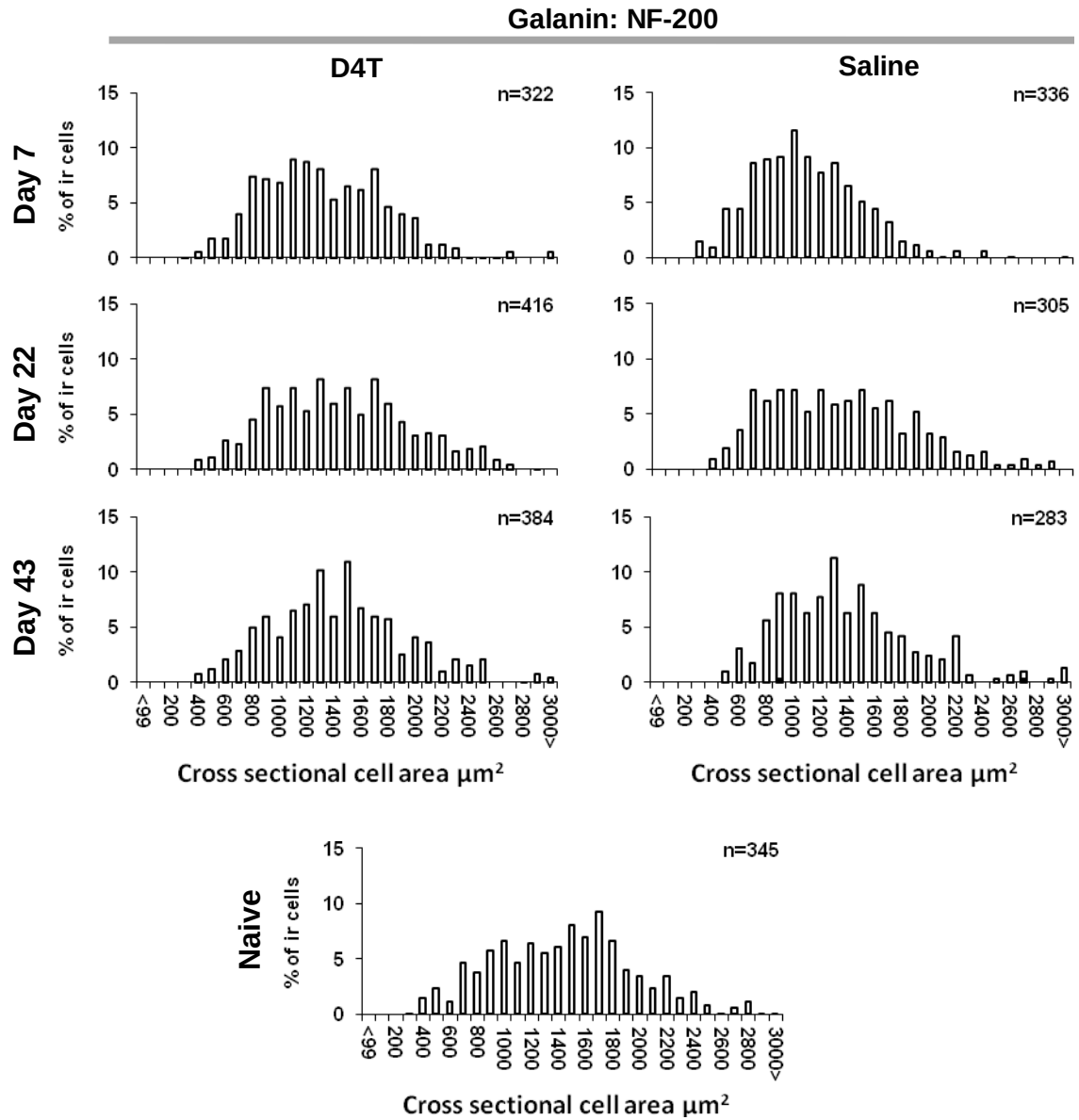


Figure 4.19 Percentage cell size area distribution analysis of galanin & NF-200 immunoreactivity (ir) in the left L5 DRG at 7, 22, and 43 days post initial d4T/saline injections and in naive animals, (n= total number of nucleated NF-200 immunoreactive cells).

4.2.6 Summary of cell size analysis

Since few immunoreactive cells were observed regarding ATF-3, GAP-43, NPY, and galanin, the cell size distribution data remained inconclusive. Table 4.1 lists the cell size analysis data for the few immunoreactive cells observed regarding ATF-3, GAP-43, NPY, and galanin in peripherin and NF-200 cells.

	d4T/saline treated animals [mean cell area $\mu\text{m}^2 \pm$ standard error of mean]		
Markers	Day 7	Day 22	Day 43
<u>ATF-3</u>			
d4T			
Peripherin	528.3 (1 of 1209)	n/a (0 of 947)	593.8 $\pm$ 220.8 (2 of 839)
NF-200	1376.9 $\pm$ 26.2 (1 of 323)	1232.0 $\pm$ 74.6 (2 of 350)	n/a (0 of 306)
Saline			
Peripherin	n/a (0 of 1057)	n/a (0 of 713)	201.6 (1 of 824)
NF-200	1276.4 $\pm$ 184.4 (3 of 281)	n/a (0 of 311)	n/a (0 of 359)
<u>GAP-43</u>			
d4T			
Peripherin	389.8 $\pm$ 35.4 (17 of 980)	278 (1 of 821)	477.0 $\pm$ 69.8 (7 of 784)
NF-200	1038.0 $\pm$ 58.5 (10 of 363)	1311.9 $\pm$ 204.2 (5 of 442)	2341.1 $\pm$ 661.4 (2 of 348)
Saline			
Peripherin	400.7 $\pm$ 40.4 (21 of 766)	451.3 $\pm$ 53.9 (6 of 825)	438.7 $\pm$ 53.4 (9 of 933)
NF-200	1029.4 $\pm$ 102.4 (14 of 297)	1014.9 $\pm$ 100.3 (3 of 317)	1011.7 $\pm$ 59.4 (15 of 320)
<u>NPY</u>			
d4T			
Peripherin	n/a (0 of 1057)	n/a (0 of 899)	373.0 (1 of 881)
NF-200	840.8 (1 of 437)	n/a (0 of 353)	n/a (0 of 385)
Saline			
Peripherin	n/a (0 of 1057)	n/a (0 of 554)	333.8 (1 of 802)
NF-200	n/a (0 of 260)	n/a (0 of 393)	n/a (0 of 279)
<u>Galanin</u>			
d4T			
Peripherin	306.5 $\pm$ 29.4 (7 of 748)	361.5 $\pm$ 26.1 (17 of 1246)	346.7 $\pm$ 24.9 (14 of 863)
NF-200	1038.0 $\pm$ 58.5 (10 of 363)	n/a (0 of 416)	n/a (0 of 384)
Saline			
Peripherin	351.4 $\pm$ 20.0 (17 of 959)	345.6 $\pm$ 25.7 (5 of 1022)	327.8 $\pm$ 40 (11 of 814)
NF-200	1029.4 $\pm$ 102.4 (14 of 297)	n/a (0 of 305)	1851.1 $\pm$ 923.5 (2 of 283)

Table 4.1 Mean cell area (μm^2) of ATF-3, GAP-43, NPY, and galanin immunoreactivity in NF-200 and peripherin characterised cell populations in left L5 DRG of d4t/saline injected animals at 7, 22, and 43 days post initial injection. Data are presented as means $\pm$ SEM. The numbers of ATF-3, GAP-43, NPY, and galanin immunoreactivity cell profiles out of the total number of peripherin/NF-200 DRG immunoreactive cells are indicated in brackets (n/a = not applicable as no data was available).

4.3 Discussion

This study was undertaken to investigate whether neurochemical markers known to be dysregulated in traumatic nerve injury exhibit similar expression patterns in a model of d4T induced peripheral neuropathy, and therefore reveal shared mechanisms. The mechanisms regarding ATN have not been fully elucidated and this toxicity presents a significant clinical problem. The results of the study demonstrated that those rats treated with d4T developed a robust bilateral hind paw mechanical hypersensitivity, during which, there was limited expression of ATF-3, NPY, galanin, and GAP-43 in both peripherin and NF-200 characterised L5 DRG cells. The pattern of responses to nerve damage are different in nerve trauma compared to NRTI related injury where the responses are observed in the DRG (i.e. biochemical changes), however with d4T related injury, such responses occur at the peripheral and central terminals of axons where there is a 'dying back' and no such biochemical changes in DRG (Calvo et al., 2012a;Wallace et al., 2007b). This is an important finding which indicates that the expression of neurochemicals ATF-3, GAP-43, NPY, and galanin in the DRG are more associated with nerve trauma processes and not necessarily behavioural hypersensitivity. This is further supported by the lack of ATF-3, GAP-43, NPY, and galanin expression in the DRG of d4T treated rats, despite the development of hind paw mechanical hypersensitivity in these rats.

Dosage

One of the main consequences of NRTI treatment in those with HIV is the development of neuropathic pain. Animal studies have revealed dose-dependent effects regarding neuronal toxicity and development of pain-related behaviours following NRTI treatment. A dose of 2-5 mg/kg has no effect on sensory thresholds (Ferrari and Levine, 2010). A slightly higher dose of 10mg/kg and 25mg/kg can produce behavioural mechanical hypersensitivity (Joseph et al., 2004). However this finding (the use of a slightly higher dose) disagrees with a study that has reported a decreasing but not significant trend in mechanical PWTs regarding the 25mg/kg dose (Wallace et al., 2007b). Studies involving doses of 50mg/kg have unequivocally demonstrated robust behavioural effects indicating a neuropathic pain-like state and therefore this dose was chosen for all d4T experimental studies.

4.3.1 Development of ATN-associated mechanical hypersensitivity

Upon the intravenous treatment of d4T (50mg/kg), rats demonstrated a bilateral decrease in PWTs that was evident at day 7 (~30%), peaking at day 22 (~47%) before reducing at day 43 (~17%). This d4T induced hypersensitivity is consistent with what is observed clinically (Dalakas, 2001; Reliquet et al., 2001) and in animal studies (Kamerman et al., 2012). However significant hypersensitivity in saline treated animals was observed at day 22 in the right hind limb. Although the percentage decrease from baseline was small ($5.9 \pm 1.8\%$), such a decrease is likely to be an anomaly and not necessarily a result of unintended nerve damage. No overt motor deficits were observed in d4T injected animals, an observation supported by previous study in ddC treated rats following assessment with an accelerating rotor rod device (Joseph et al., 2004). The peak level of mechanical hypersensitivity in d4T treated animals was greater than what has previously been reported in rats. Wallace *et al.*, 2007b and Joseph *et al.*, 2004, using ddC (50mg/kg), both reported a ~30% (day 21) and 20-40% (day 14) decrease in PWTs from baseline respectively. Similar peak levels of mechanical hypersensitivity have been noted with ddI and ddC (20-40%) (Joseph et al., 2004). The early onset of mechanical hypersensitivity following exposure to d4T is comparable with previous studies using ddC (50mg/kg), which observed a ~30% decrease from baseline 5-8 days following initial injection (Joseph and Levine, 2004; Joseph and Levine, 2006). The long lasting nature of the d4T hypersensitivity is also comparable to a previous study where behavioural hypersensitivity was evident at 43 days post initial injection (Wallace et al., 2007b). It is to be noted that the treatment regimes have differed amongst the various studies. In the present study, animals were treated with two doses of d4T separated by 4 days, whereas Wallace et al., 2007b used a treatment regime consisting of 3 doses per week. Joseph et al., 2004 and Zheng et al., 2011b regime consisted of a single bolus dose (50mg/kg). Taken together, these results suggest that the acute administration of NRTIs can induce long lasting mechanical hypersensitivity which does not alter upon repeated doses. Indeed this is comparable to epidemiological data showing that even acute exposures to NRTIs can increase the risk of developing ATN (Cherry et al., 2009; Smyth et al., 2007). The greater magnitude of behavioural hypersensitivity observed may reflect the differences between the methods used to analyze responses to mechanical stimuli.

Experiments by Wallace et al., 2007b used an electronic von Frey device to measure PWTs whereas experiments involving Joseph and colleagues had used an Analgesymeter which measures paw pressures according to the method of Randall-Selitto (Randall and Selitto, 1957). To describe briefly, a force is applied to the paw, which is placed on a small plinth under a cone-shaped pusher with a rounded tip, and represents a common method for assessing mechanically-related pain behaviours in rodents. A major drawback of the Analgesymeter however is the requirement of the animal to be restrained during testing which may engage stress-induced analgesia mechanisms that are known to affect behavioural outcome measures (Bardin et al., 2009). The nature of the experiments which involved the injection of pharmacological agents into the paw meant that it was not practical to use conventional von Frey techniques for nociceptive testing. This was not an issue in reports by Zheng et al., 2011b and Siau et al., 2006 (Bennett and colleagues) where mechanical hypersensitivity following ddC (50mg/kg) treatment was assessed via the response rate towards a 4g and 15g von Frey hair, indicating ‘allodynia’ and ‘hyperalgesia’ respectively. These animals demonstrated a ~30% and ~50% response rate towards the 4g and 15g von Frey respectively, suggestive of a neuropathic pain-like state. This behavioural analysis only ascertains the presence but not the degree of mechanical hypersensitivity. Such methods have been used to assess pain-like behaviours in ddC, d4T, and ddI models using 10, 27.5, and 75.8 mN von Frey hairs, obtaining similar results (30-40% response rate) (Joseph et al., 2004). Although it is to be noted that hyperalgesia and allodynia are terms used to describe pain in humans and cannot be extrapolated to infer evoked or spontaneous pain in rats since there are no tools that can actually measure pain in rodents. Nonetheless these behavioural assessments only further reinforce the neuropathic nature of the model. Recently, a study has been described using a mouse model of d4T-induced peripheral neuropathy, where animals develop a robust mechanical hypersensitivity (>80% drop from baseline) which lasts for at least 28 days (Renn et al., 2011). I believe this high level of hypersensitivity, which somewhat contradicts presented results, may be explained by a fundamental difference between the species of animal chosen for study. Although it has been argued the degree of behavioural hypersensitivity following trauma nerve injury are similar in mice and rats (Rigaud et al., 2008), this may not necessarily be the case in NRTI induced neuropathies where the nature of the nerve damage differs.

4.3.2 Temporal profile expressions of investigative markers

Tsujino et al., 2000 first reported that the immediate early gene protein ATF-3 was a reliable and sensitive marker for injured neurons following traumatic nerve injury. ATF-3 now represents one of the most commonly used markers of DRG neuronal injury. The results of the study indicate that there is limited expression of ATF-3 within L5 DRG following d4T administration. The lack of ATF-3 expression would indicate that there is no apparent neuronal injury; however this may not necessarily be the case. This is because the treatment of NRTIs to rats has previously been shown to initiate a loss of IENF density which would suggest a toxicity of C-fibres and a degenerative process (Calvo et al., 2012a;Wallace et al., 2007b). This toxicity may not be sufficient to induce ATF-3 expression in the DRG. Since there is an apparent toxicity to peripheral nerve fibres, investigations examined if there were evidence of axonal regeneration. There was no upregulation of the axonal regeneration marker GAP-43 (Schreyer and Skene, 1991) in the L5 DRG. This observation ties in with the lack of ATF-3 expression, a marker closely related to the regenerative process and the expression of GAP-43. Although the lack of GAP-43 does not rule out the possibility of a regenerative process since other growth associated proteins may be involved e.g. small proline rich protein 1A (SPRR1A), which is the case in trauma injury models (Seijffers et al., 2007;Starkey et al., 2009). The neuropeptides NPY and galanin which are upregulated in trauma models (Ma and Bisby, 1997;Ma and Bisby, 1998), did not exhibit any changes in their expression following treatment with d4T, even during peak mechanical hypersensitivity. The biochemical changes that occur in the DRG following peripheral nerve injury appear to be related to nerve trauma and not necessarily mechanical hypersensitivity since such reflex behaviours are apparent in the NRTI models where there is nerve damage but no such biochemical changes in the DRG. A similar argument can be made for the spinal microglial response that occurs following nerve trauma, which is not apparent in ddC treated animals (Blackbeard et al., 2012;Zheng et al., 2011a).

A previous study conducted by Wallace et al., 2007b using the ddC model analyzed the expression of pathological markers of immune cells known to be expressed in the sciatic nerve/DRG following traumatic nerve injury. The study reported no sciatic nerve

macrophage infiltration or DRG expression of ATF-3 and the neuropeptides NPY & galanin (21 days following initial injection). However an increase in the expression of CCL2 and CD68 were reported. Recently, there has been increasing interest on the role of CCL2, which is involved in macrophage recruitment, but can also stimulate nociceptors, and therefore suggestive of a role in pain modulation (Sun et al., 2006;Zhang and De Koninck, 2006). Under naïve conditions, within the DRG, CCL2 is constitutively expressed in small-medium diameter neurons (Tanaka et al., 2004) of the peptidergic class (Dansereau et al., 2008). However, following nerve injury CCL2 is upregulated in both damaged and undamaged primary sensory neurons (White et al., 2005b). The reported upregulation of CCL2 in ddC treated animals may be linked to the macrophage infiltration. CD68 (also known as ED-1) is protein that is expressed on the cell surface of macrophages and is commonly used to identify them in immunohistochemistry studies. The upregulation of CD68 in ddC treated animals indicates macrophage recruitment which is a likely consequence of the CCL2 upregulation. Indeed this is the case following trauma nerve injury, where the release of CCL2 from the DRG (Morin et al., 2007) can initiate the recruitment of resident macrophages by acting on the CCR2 receptor expressed on macrophages (Perrin et al., 2005). Taken together, the results from the current and previous studies indicate that other mechanisms are responsible for the development of pain like behaviour in ATN.

4.3.3 Mechanisms regarding NRTI toxicity and associated pain behaviours

There is a large body of evidence in both human and animal model studies implicating mitochondrial toxicity as a main mechanism regarding ATN. (Brinkman et al., 1999;Fleischer et al., 2004;Lehmann et al., 2011;Zhu et al., 2007). Mitochondrial activity plays an important role in cells, providing energy in the form of adenosine triphosphate (ATP). Antiretroviral drugs can alter mtDNA content, or fidelity of DNA synthesis via inhibition of DNA polymerase γ by incorporation into newly synthesized strands or by causing chain termination thus leading to mitochondrial dysfunction (Lewis et al., 2003). It is likely that all downstream effects are a result of this primary toxicity. The NRTI-induced mitochondrial dysfunction is associated with apoptosis, and draws parallels with other drug induced neuropathies involving mitochondrial dysfunction (e.g. cancer chemotherapy

agents: vincristine, cisplatin, paclitaxel), and metabolic diseases (e.g. diabetes), indicating possible shared mechanisms. Sustained exposure to antiretrovirals can result in oxidative stress (Opie et al., 2007) which may result in reduced ATP production, and an increased amount of reactive oxygen species (ROS) and nitric oxide in neurons. Mitochondria-dependent apoptosis is known to be initiated by a number of factors that include ROS, ceramide, and nitric oxide (Kirkland et al., 2002), which results in a release of cytochrome c and the eventual formation of a complex involving apoptotic protease activating factor-1 and caspase-9, leading to the activation of downstream caspases (Nomura, 2004). Inhibitors of caspase 1, 2, 8, and 9 have all been shown to attenuate mechanical behavioural hypersensitivity associated with ddC (and vincristine) treatment, thus demonstrating a link between apoptosis and neuropathic pain-like behaviour (Joseph and Levine, 2004). The increased ROS as well as activation of apoptotic pathways may also exacerbate pain behaviours by altering GABAergic transmission in the spinal cord, which can occur following peripheral nerve injury (Yowtak et al., 2011). Indeed, the removal of ROS by scavengers (e.g. tempol) has been shown to reverse mechanical hypersensitivity in ddC treated animals (Zheng et al., 2011b), which further supports an important role of ROS systems in ATN related pain. The increase in ROS might be explained by a decrease in endogenous superoxide dismutase-2 (SOD-2) that is additionally observed following ddC treatment. SOD-2 plays an important role in regulating ROS by binding to superoxide by products of the mitochondrial electron transport chain, converting them to hydrogen peroxide and oxygen. Failure of SOD-2 to remove the superoxide by products leads to an increase in mitochondrial ROS (Melov et al., 1998). The rise in ROS may be countered by inhibitors of the mitochondrial electron transport chain complexes I, II, III, IV, and V, all of which have been shown to attenuate neuropathic pain-related behavior in rats (Joseph and Levine, 2004). Mitochondrial related apoptosis may also be activated via tumor necrosis factor (TNF α) (Micheau and Tschopp, 2003) and has been shown to be upregulated in DRG and spinal cord following NRTI treatment (Zheng et al., 2011c). TNF α has previously been described to contribute towards peripheral pain mechanisms (Joseph and Levine, 2004; Schafers et al., 2003b; Schafers et al., 2003c). The acute administration of a soluble TNFR1 to scavenge TNF α can temporarily reduce mechanical hypersensitivity (effects lasting 1-4 hours) (Zheng et al., 2011c). The Mitochondria is an important regulator of

neuronal intracellular calcium concentrations (Svichar et al., 1997). Chronic exposure of ddC (7 μ M) to DRG cultures results in altered mitochondrial-related calcium homeostasis (Werth et al., 1994). These observations mirror experiments by Keswani et al., 2003a where ddC, ddI, and d4T (10 μ M) treated cultures initiated a reduction in the mitochondrial membrane potential differential that was partially reversed by FK-506 - an inhibitor of calcineurin; a protein sensitive to intracellular calcium concentrations. This observation is suggestive of abnormal calcium buffering, further highlighted by Joseph et al., 2004, which demonstrated a reduction in ddC-induced mechanical hypersensitivity following administration of calcium buffers TMB-8 and Quin-2.

Electrophysiological experiments have revealed that d4T treated mice (after 3 days) exhibit higher numbers of action potentials per second in WDR spinal neurons upon stimulation with brush, pressure, and noxious pinch (Renn et al., 2011). Alterations in the distribution of interspike intervals (ISIs) of C-fibres in ddC-treated rats during suprathreshold stimulus have been observed with increased percentages of ISIs between 0.1 and 0.3 seconds (Joseph et al., 2004). Such changes in firing properties may transcend into exacerbated nociception or pain responses.

The induction of pain-related behaviours in d4T treated animals may be in part related to an increase of BDNF growth factor in the superficial dorsal horn that is observed at 1, 3 and 7 days post injection, (that returns to control levels by day 14) (Renn et al., 2011). BDNF is a known modulator of sensory transmission, acting on the trkB receptor, and plays a key role in central sensitization (Pezet and McMahon, 2006). The sequestering of BDNF by intrathecal treatment of trkB-Fc attenuates the d4T associated mechanical hypersensitivity (effect lasting 24 hours) (Renn et al., 2011).

In summary, many potential mechanisms may contribute to NRTI-related neuropathic pain that may explain the behavioural observations in the current study. These include defects in mitochondrial function, production of ROS, and alterations in calcium homeostasis. The pre-existing HIV infection in affected individuals may only serve to exacerbate the effects of NRTIs (Keswani et al., 2006;Wallace et al., 2007b). The possibility of genetic susceptibility

and environmental factors cannot be excluded, and has been proposed as factors that can influence the quality of the pain (Young et al., 2012) that maybe relevant in ATN. For instance, known polymorphisms in the gene encoding mitochondrial SOD-2 affect its activity (Pociot et al., 1993). Another example is alcohol abuse, a common incidence in acquired immunodeficiency syndrome (AIDS) patients (Welch, 2000) that can increase pain, and is associated with abnormal primary afferent function (Ferrari and Levine, 2010).

4.3.4 Limitations and future studies

The current study only examined mechanical behavioural hypersensitivity following d4T treatment and thermal nociception was not evaluated. There have been conflicting reports regarding the onset of thermal hypersensitivity in the ATN model. Joseph et al., 2004 demonstrated thermal hypersensitivity in ddC-treated rats at 5 days post injection (~50% decrease from baseline). In contrast, Renn et al., 2011 observed a trend towards thermal hypersensitivity in d4T-treated mice over a 28 day period; however there were no significant difference in PWLs when compared to the saline controls. These findings are shared by Wallace et al., 2007 indicating that thermal hypersensitivity is not a significant consequence of NRTI treatment. This view is consistent with clinical data showing that patients with HIV-related pain do not usually present with thermal hypersensitivity (Martin et al., 2003). Nevertheless it would be worth perusing whether there is thermal hypersensitivity associated with d4T in rats, rather than in mice, which has been the common species of choice regarding ATN modeling. A possible drawback of the study is other tissues (e.g. sural & sciatic nerve, spinal cord, and paw skin) were not examined. Sural nerve biopsies from patients with ddC-neuropathy have shown severe axonal destruction, prominent in unmyelinated fibres (Lewis and Dalakas, 1995). This could be examined in d4T treated rats via immunohistochemical staining of β -amyloid precursor protein (β -APP) - an axonal injury marker that accumulates in the axon (Medana and Esiri, 2003). Moreover, the extent of any morphological damage observed can be correlated to the loss of small fibres observed in paw skin (Wallace et al., 2007b). The study could additionally be improved by investigating the expression profiles of other markers that have been implicated in ATN, particularly CCL2, which is upregulated in the DRG following ddC treatment (Wallace et al., 2007b).

Macrophage recruitment in the DRG of ddC treated animals (Wallace et al., 2007b) could be further investigated by characterizing CD-68 expression over time and could be correlated with the CCL2 expression. Since TNF α is found in the spinal cord of ddC treated animals (Zheng et al., 2011c), it would be interesting to investigate similar events in DRG, especially given the presence of macrophages, that can release TNF α (Holguin et al., 2004), thus altering the excitability of neurons (Schafers et al., 2003b).

4.3.5 Conclusion

In this chapter, I examined the effect of d4T induced nerve injury on the development of mechanical hypersensitivity and have characterized the temporal profile expressions of markers known to be dysregulated after nerve injury in the L5 DRG. Rats treated with d4T demonstrated mechanical hypersensitivity indicating a pain-like state but did not demonstrate any evidence of injury (ATF-3 expression) or nerve regeneration (GAP-43) in the DRG. Furthermore, there were no changes in the expression of the neuropeptides galanin and NPY. The results of this study therefore suggest that other mechanisms are responsible for ATN.

Chapter 5

- 5 Evaluation of hind paw sensory behaviour and the DRG macrophage response following chronic administration of docosahexaenoic acid (DHA) in an animal model of TNT induced peripheral nerve trauma**

5.1 Introduction

5.1.1 PUFAs

Poly unsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond in their chemical composition and are structural components and main constituents of cell membranes (Yaqoob and Calder, 2007). PUFAs are categorised according to the position of the first double bond from the n-terminal of the molecule (Shapiro, 2003). There are different types of PUFAs that includes the methylene-interrupted (i.e. ω -3, ω -6, and ω -9 fatty acids) and conjugated fatty acids (e.g. ruminic acid, calendic acid), but the most frequently described fatty acids in the literature are the ω -3 fatty acids due to its associated health benefits and the ω -6 fatty acids due to its negatively associated health effects (Holub and Holub, 2004). High concentrations of the ω -6 PUFAs linoleic are (18:2n-6)² are found in vegetable oils (e.g. corn, sunflower and soybean), and is required for the production of arachidonic acid which is present in phospholipids of cell membranes. The activity of ω -6 PUFAs such as arachidonic acid (AA) are considered to be proinflammatory whereas ω -3 PUFAs such as eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) have anti-inflammatory properties. The ω -3 PUFAs EPA (20:5n-3), DHA (22:6n-3), and α linolenic acid (ALA 18:3n-3), are all major constituents of fish oil and are essential components of the human diet that must be ingested since the body cannot synthesize them de novo, and are required for good health. For example, DHA is required for the normal function of nervous tissue, and modulates an array of biochemical and cellular processes including gene expression and neuronal growth and survival (Kitajka et al., 2004). High concentrations of DHA are found in the retina where it aids in photoreceptor biogenesis (Scott and Bazan, 1989).

The ω -3 PUFAs are likely to be beneficial in the prevention and management of various human inflammatory conditions including joint pain, irritable bowel syndrome (IBS), and

² (x:an-6) x= number of carbon atoms, a= number of double bonds, n= position of first double bond from methyl end of fatty acid molecule

coronary artery disease (Goldberg and Katz, 2007). The the improved health outcomes regarding ω -3 PUFAs may be attributed to the neuroprotective and anti-inflammatory effects. Such properties have been well documented especially in pre clinical studies, most notably in the stroke field (Bazan, 2005;Bazan, 2006;Bazan, 2007) and spinal cord injury (SCI) field (Huang et al., 2007;King et al., 2006;Ward et al., 2010).

5.1.2 Anti-inflammatory and neuroprotective actions of ω -3 PUFAs

Brain ischemia-reperfusion injury is often used as a model of stroke (Carmichael, 2005). Such injury in rats triggers the release of DHA from the membrane (Yoshida et al., 1984), which possibly acts to reduce inflammation since the exogenous administration of DHA to brain ischemia-reperfusion treated rats is associated with a reduced inflammatory response (Pan et al., 2009). Other ω -3 PUFAs such as ALA (i.v) administered either before or after a global ischemic insult have been shown to significantly reduce neuronal death (indicated by increased density of cresyl violet stained hippocampal sections) (Blondeau et al., 2002;Lauritzen et al., 2000). The intravenous administration of mixed ω -3 PUFAs has been shown to inhibit age related apoptosis of cortical neurons in rats (Martin et al., 2002). The anti-inflammatory properties of DHA may be further enhanced by the production of metabolites such as resolvins and neuroprotectin D1 (NPD1) which have even greater anti-inflammatory properties and these compounds are present in the resolving phase of inflammation (Serhan et al., 2008).

Resolvins

Resolvins were first identified in the exudates of mice that had been administered aspirin and DHA (Serhan et al., 2008), and have been subsequently shown to exhibit potent anti-inflammatory and pro-resolution actions. DHA and EPA are metabolized by P450 cytochrome enzymes in the liver, as well as lipoxygenase and cyclooxygenase-2 (COX-2) to form D and E series resolvins respectively (Figure 5.1) (Arnold et al., 2010). The intraplantar or intrathecal administration of resolvin D1 (or E1) in mice potently reverses the reduction in paw withdrawal thresholds (PWTs) induced by intraplantar injection of

formalin, carrageenan, or complete Freund's adjuvant, without affecting basal thresholds (Xu et al., 2010). This observation may be explained by the decreased production of the proinflammatory cytokines TNF α and IL-1 β in the rat hind paw associated with resolvin D1 administration (Hong et al., 2003; Lima-Garcia et al., 2011). Resolvin D1 has also been shown to inhibit neutrophil infiltration in a dose dependent fashion (at nanogram doses) in a mouse model of peritonitis (Sun et al., 2007). Resolvin D1 can initiate CCR5 expression in dying neutrophils (Ariel et al., 2006), and this receptor expression helps clear up the proinflammatory ligands of CCR5 (CCL3 and CCL5) from the inflammatory site.

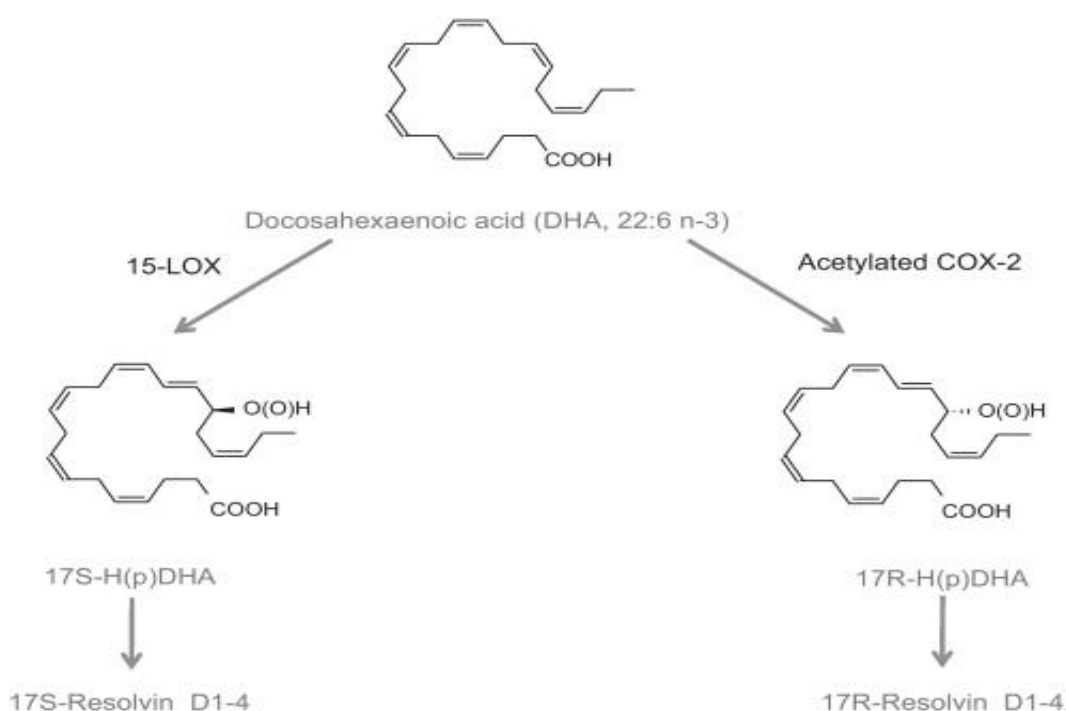


Figure 5.1 DHA is metabolised by 15-lipoxygenase action or acetylated COX-2 to form intermediate 17S- or 17R-H (p)DHA. Subsequent processing through epoxide intermediates leads to the formation of 17S-resolvins D1–D4 (15-lipoxygenase pathway) and 17R-Resolvins D1–D4 (aspirin-triggered acetylated COX-2 pathway) respectively. Image reproduced from Weylandt et al., 2012.

Neuroprotectin D1

The metabolism of DHA via lipoxygenase leads to the formation of NPD1 (see Figure 5.2) (Niemoller et al., 2009). NPD1 was first discovered in a human retinal pigment epithelial cell line following oxidative stress where it was associated with neuroprotection (Mukherjee et al., 2004). Research by Bazan and colleagues have identified NPD1 as a compound that has both potent anti-inflammatory and neuroprotective properties (Bazan, 2005;Bazan, 2009;Belayev et al., 2009). For example, NPD1 has been shown to inhibit T cell migration into the peritoneum in a model of zymosan A induced murine peritonitis (Ariel et al., 2005). Leucotriene B4 induced neutrophil migration in human microvascular endothelial cells has also been shown to be inhibited by NPD1 (Serhan et al., 2006). NPD1 has additionally been shown to inhibit apoptotic DNA damage, upregulate anti-apoptotic, and downregulate pro-apoptotic proteins, as well as bind toxic peroxides (Bazan, 2006;Bazan, 2007;Mukherjee et al., 2004). Previous experiments in rats have shown that NPD1 is present in the hippocampus at least 1 hour following middle cerebral artery occlusion (MCA-O) injury (1 hour occlusion, 2 hour reperfusion) in mice, and is still present even at 25 hours post injury (Marcheselli et al., 2003). Following brain ischemia injury, DHA is released from the cell membrane (Yoshida et al., 1984) and is metabolized into NPD1 (Marcheselli et al., 2003). It is possible that a similar process occurs after peripheral nerve injury and the intravenous administration of DHA may lead to further production of NPD1 which can act to minimize inflammation, ensuring a suitable environment for neuronal survival. The mechanism behind the anti-inflammatory actions is thought to be the inhibition of NF κ B and COX-2 expression, which has been demonstrated in cultured human neural progenitor cells and in the hippocampus of MCA-O rats (Marcheselli et al., 2003).

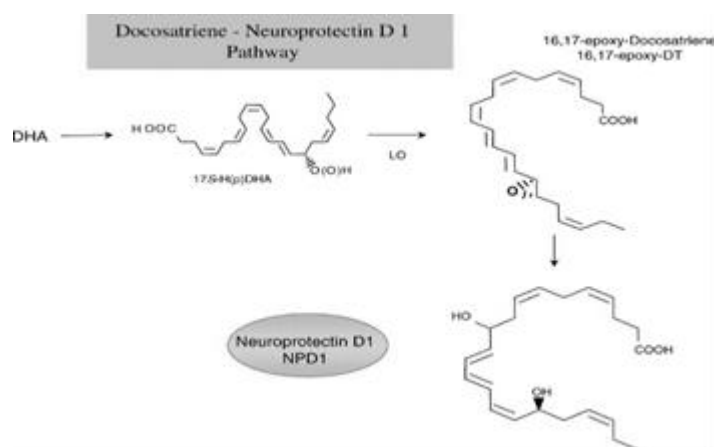


Figure 5.2 An illustration of NPD1 biosynthesis: DHA is hydrolyzed by phospholipase A2 (PLA2) where it undergoes lipoxygenation, followed by epoxidation, and then hydrolysis to generate NPD1. Image reproduced from Serhan et al., 2004.

Neuroprotection in SCI

Traumatic injury of the spinal cord leads to apoptosis of both neuronal and glial cells and degeneration of ascending and descending tracts. The collective effects of the injury leads to the development of mechanical and thermal hypersensitivity in the hind paws (Bruce et al., 2002; Christensen et al., 1996). The acute administration of DHA (i.v) following SCI (within 30 minutes) has been associated with improved locomotion (from day 4 up to cessation of the study - 6 weeks) (Huang et al., 2007). Moreover, improvements in the level of oxidative stress, neuronal survival, and histological outcomes, including reduced inflammatory responsiveness, axonal injury, and cavity formation were observed. The acute administration of DHA (i.v.) also reduces axonal dysfunction, (indicated by a reduced accumulation of β -amyloid precursor protein compared to saline controls) in the spinal dorsal columns (Ward et al., 2010). Histological outcomes were additionally improved with the combination of an acute DHA injection and an enriched DHA diet (Huang et al., 2007). Taken together, the results of these studies indicate that DHA is neuroprotective following nerve injury. Since ω -3 PUFAs have been shown to be both anti-inflammatory and neuroprotective in a variety of nerve injury models (Bazan, 2007; Belayev et al., 2009; Gladman et al., 2012; Huang et al., 2007; Ward et al., 2010), it raises the possibility that they could have therapeutic potential in peripheral neuropathic pain conditions.

5.1.3 ω -3 PUFAs in neuropathic pain

Few studies have investigated the effects of ω -3 PUFAs on outcome measures of neuropathic pain in rodents. In a rat model of trigeminal neuralgia which involved chronic constriction of the infraorbital nerve, a diet enriched with linoleic acid was able to delay (for up to 2 weeks post nerve injury) the development of facial mechanical hypersensitivity associated with this type of nerve injury (Martin and Avendano, 2009). Although the injury induced mechanical hypersensitivity was not prevented, the delay in its development could be attributed to the neuroprotective effects of the ω -3 PUFAs. A previous study has indicated that the consumption of corn and soy fats (containing both ω -3 and ω -6 PUFAs) suppresses the level of hind paw mechanical and thermal hypersensitivity associated with partial sciatic nerve injury (Perez et al., 2004). However, a similar study within the same research group later concluded that ω -3 fatty acids may be associated with increased neuropathic ‘pain-like’ behaviours in sciatic nerve injured rats (Heller et al., 2006; Perez et al., 2005). Such observations may be explained by a compromised methodology that did not account specifically the composition of the various oils examined (i.e. canola, corn, hemp, soy, and sunflower oil), all of which differ in their ω -3 and ω -6 PUFA content. This consideration is important, given the proinflammatory effects of ω -6 PUFA (Serhan et al., 2008); although the actual ratio of ω -3 to ω -6 PUFAs is the crucial factor (Russo, 2009). EPA and DHA both compete with AA as a substrate for COX-2 and lipoxygenase. These enzymes are responsible for the formation of important biological mediators such as prostaglandins and protacyclins (Funk, 2001). If the concentration of ω -3 PUFAs increases in the membrane, this can lead to a reduction in synthesis of inflammatory eicosanoids, which has been demonstrated in glial cells *in vitro* (Petroni et al., 1994). Therefore it is possible that the effects of high ω -6 PUFAs could be overcome via a larger proportion/concentration of ω -3 PUFAs present *in vivo* since they compete for the same enzymes (Shapiro, 2003).

A recent transgenic study has demonstrated that mice expressing the fat-1 gene (encoding for ω -3 fatty acid desaturase) have increased endogenous ω -3 PUFAs (and a concomitant decrease in ω -6 PUFAs) that is associated with an accelerated improved outcome measures of sensory behavioural testing 7 days after sciatic nerve crush injury (Gladman et al., 2012).

The injury-induced expression of neuronal injury marker activating transcription factor-3 (ATF-3) was also decreased in the DRG of fat-1 mice (Gladman et al., 2012). DRG cultures taken from fat-1 mice have demonstrated neuroprotection following mechanical strain or hypoxic injury (Gladman et al., 2012). In addition to the neuroprotection, DHA may encourage growth of neurons. Cultured DRG neurons when treated with DHA has been shown to increase neurite outgrowth, length of outgrowth, and the number of neurons with growth cones (Robson et al., 2010). In addition, the amplitude of the effects was comparable to that of nerve growth factor (NGF) and even greater in EPA treated cultures. In another study using cultured cortical neurons from rat pups, significant increases in the percentage of neurite outgrowth was associated with an increase in the level of growth associated protein-43 (GAP-43) immunoreactivity upon treatment with DHA (Cao et al., 2005). Following peripheral nerve trauma there may be a loss of NGF from neurons and thus trophic support leading to a rise in neuropeptide expression (Averill et al., 2004;Verge et al., 1995). Since NGF is a well known regulatory factor of DRG neuropeptide expression and ATF-3, and can reduce their expression (Averill et al., 2004;Verge et al., 1995), their effects could be mimicked by DHA administration. Collectively this evidence suggests that DHA may reduce nerve damage (ATF-3 expression) associated with trauma injury and aid in the regenerative process that may have beneficial consequences in neuropathic pain.

5.1.4 DHA regulation of the macrophage inflammatory response

Data from *in vitro* experiments supports a dose dependent inhibition of IFN γ /lipopolysaccharide (LPS) stimulated macrophage activation when exposed to DHA (Ajmone-Cat et al., 2011;Dustin et al., 1990;Lu et al., 2010). This inhibition is associated with a reduction in NF κ B activation, and the secretion of IL-1 β , TNF α , and IL-6, all key cytokines involved in the inflammatory response (Mullen et al., 2010;Oliver et al., 2012). The reduction of proinflammatory cytokine release from stimulated macrophages has also been observed in animals administered DHA through diet supplementation – investigations by Lokesh et al., 1988 reported a reduction in the synthesis of leukotrienes (C4 and B4) and prostaglandins (E2 and F1 α) following a diet containing 0.5% wt of EPA, DHA, and linolenic acid. A previous *in vivo* study demonstrated a reduction in the release of IL-6

from peritoneal macrophages in mice (Shi and Pestka, 2009). Such anti-inflammatory properties have been demonstrated in Alzheimer disease patients treated with DHA (1700mg) – these patients demonstrated a reduction in IL-1 β , IL-6, and granulocyte colony-stimulating factor from peripheral blood mononuclear cells (PBMCs) (Vedin et al., 2008). The mechanism by which DHA inhibits macrophage activation is thought to occur via the non-covalent binding to the peroxisome proliferator-activated receptors (PPARs) (Forman et al., 1995). PPARs are ligand activated transcription factors involved in the regulation of biological processes including lipid and glucose metabolism, and overall energy homeostasis (Stienstra et al., 2007). PPARs can form heterodimers with the retinoic-x-receptor (RXR) (Abdelrahman et al., 2005) which additionally binds DHA (de Urquiza et al., 2000;Lengqvist et al., 2004), and may also be involved in regulating the inflammatory response (Chung et al., 2000). Of interest is the PPAR γ isoform that is expressed in adipose tissue but also on monocytes/macrophages (Braissant et al., 1996). This isoform is involved in regulating the production of proinflammatory mediators (e.g. TNF α and IL-1 β) (Jiang et al., 1998) by interfering with the binding of transcription factors including STAT, NF- κ B, and activating protein-1 (AP-1) (Ricote et al., 1998). In addition, activated PPAR γ can interact directly with NF κ B, preventing its translocation to the nucleus (Vanden Berghe et al., 2003), or indirectly by stimulating the expression of the inhibitory protein I κ B α , which reduces the effect of NF- κ B, leading to suppression of NF- κ B DNA-binding activity (Delerive et al., 2000).

There is evidence to support that DHA is more potent than EPA. The pretreatment of LPS stimulated macrophages with 100 mM of EPA and DHA can significantly decrease TNF α , IL-1 β , and IL-6 production compared to control cells, with a more profound effect observed in macrophages treated with DHA (Weldon et al., 2007). In a separate study also using LPS stimulated macrophages pretreated with EPA or DHA (50 μ M for 5 days), there was an enhanced secretion of the anti-inflammatory cytokine IL-10 and reduced expression of the ‘proinflammatory’ M1 macrophage phenotype (Oliver et al., 2012). In light of this evidence, and the accumulative evidence regarding neuroprotection in SCI, I hypothesized whether DHA could modulate the macrophage response following peripheral trauma nerve injury.

5.1.5 Aim of experiments

- ❖ Investigate whether the administration of DHA (i.v.)³ at and every 3 days⁴ post TNT injury can reverse the development of mechanical hypersensitivity associated with such injury within a 4 week period.
- ❖ Determine if DHA can modulate macrophage infiltration in the L5 DRG following TNT injury

³ The intravenous route was taken because this route ensured maximum bioavailability and therefore maximum exposure of DHA to the injured tibial nerve.

⁴ The 3 day administration regime was deemed optimal since this was an appropriate time frame for the tail vein to recover while additionally reducing the stress on the rats, and ensuring the stability of plasma DHA levels.

5.2 Results

DHA (500 nmol/kg) or saline (0.9%) was administered through the tail vein 30 minutes following TNT injury and every 3 days thereafter until day 28 when animals were culled. Figure 5.3 indicates the breakdown of the total number of animals used in the study and explanations of the relevant exclusions.

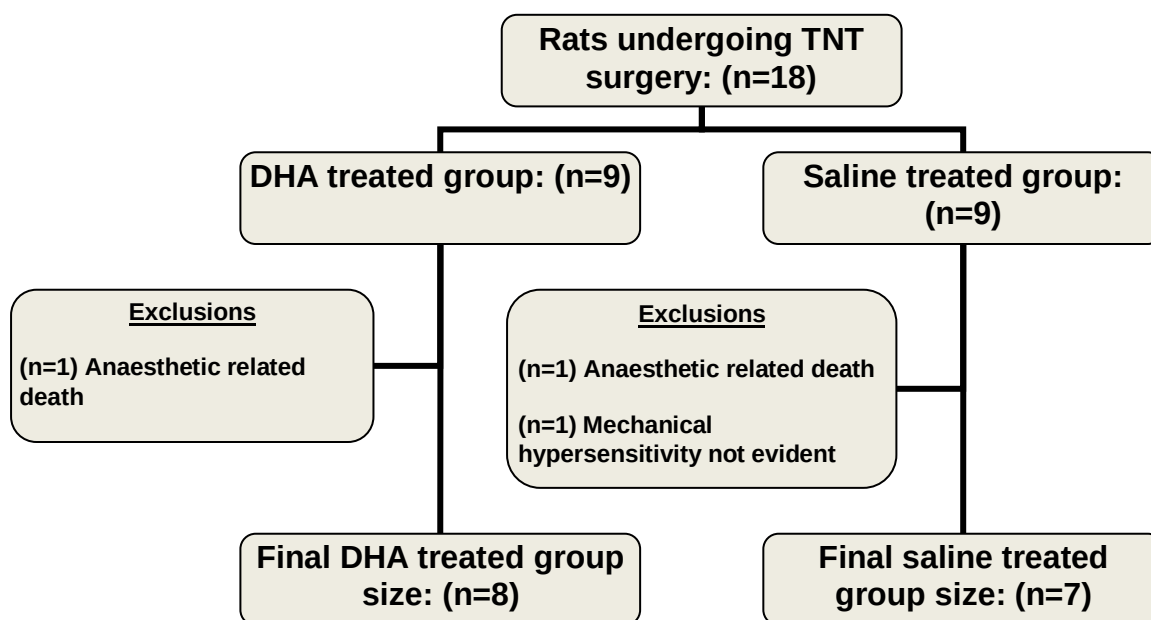


Figure 5.3 Breakdown of the total number of animals used within the study. All animals (n=18) underwent TNT surgery and were divided into two groups: DHA treated (n=9) and saline treated groups (n=9). All animals were checked post mortem to identify transection of tibial nerve with aid of a dissecting microscope. A total of three animals were excluded from the study due to anaesthetic overdose (n=2) and inability to demonstrate $\geq 30\%$ drop in baseline at any point within the 28 day behavioural testing period following TNT injury (n=1).

5.2.1 Effect of DHA on hind-paw mechanical sensory testing

Behavioural sensory testing in the mid-plantar paw was conducted using an electronic von Frey device every 7 days post injury (Figure 5.4). Following TNT injury, there were no significant differences regarding ipsilateral mechanical hypersensitivity between DHA (n=8) and saline (n=7) treated animals over the 28 days (analyzed using a one way ANOVA and Tukey's post hoc test). Saline and DHA treated animals exhibited baseline PWTs of 44.3 ± 3.8 g and 44.9 ± 3.1 g respectively. No differences in PWTs were observed with respect to baseline 7 and 14 days post surgery in both groups. From at least 21 days following TNT injury, animals treated with DHA exhibited significant differences in mechanical hypersensitivity with respect to baseline values ($p < 0.05$). The PWTs at 21 and 28 days post TNT injury in DHA animals were 28.3 ± 3.1 g ($\downarrow 34.5 \pm 6.8\%$) and 20.8 ± 1.5 g ($\downarrow 51.1 \pm 4.2\%$) respectively. Saline treated TNT animals exhibited significant mechanical hypersensitivity 28 days following surgery with respect to baseline, 30 ± 0.1 s ($\downarrow 43.6 \pm 8.1\%$) ($p < 0.05$).

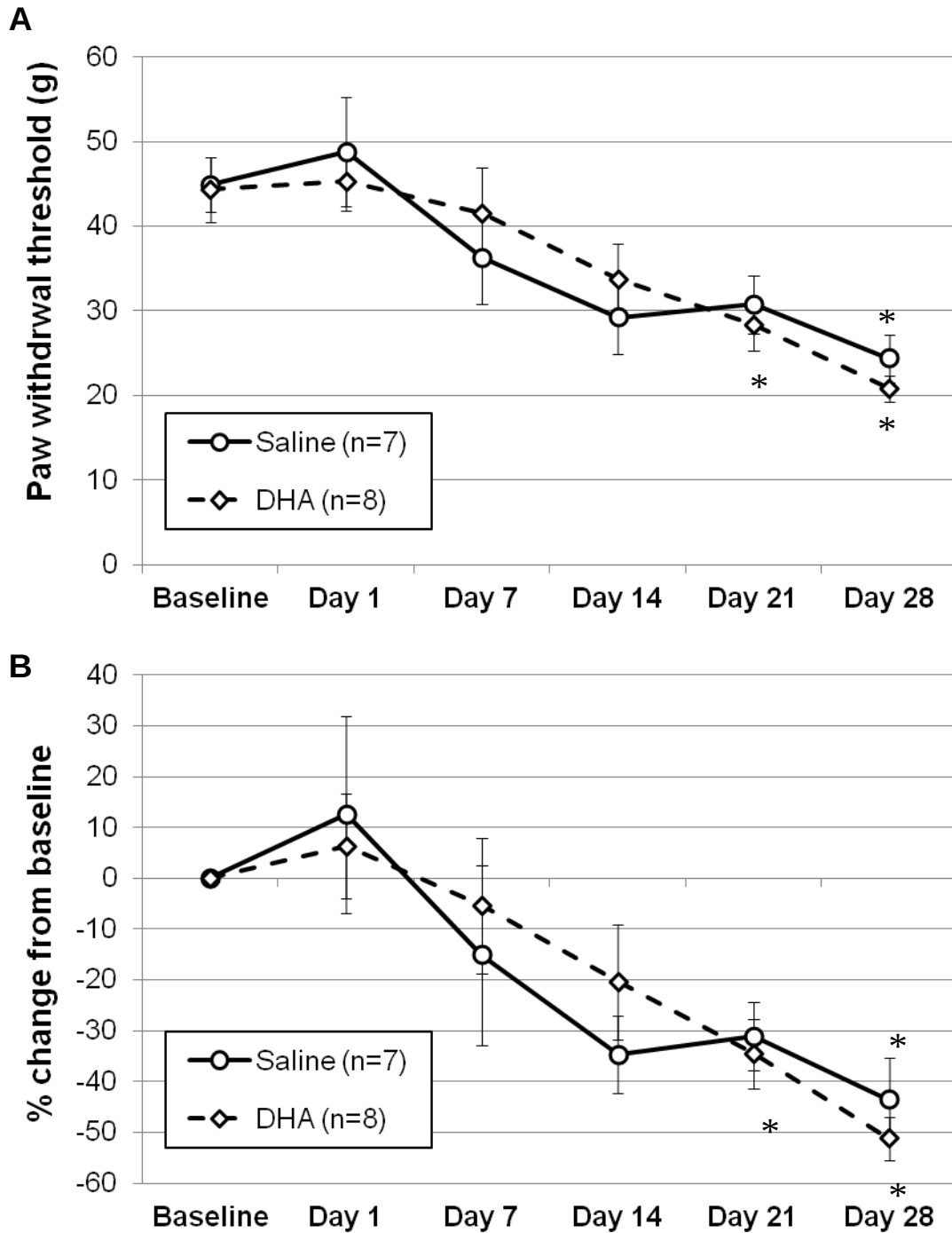


Figure 5.4 Mechanical behavioural responses using an electronic von Frey device over a 28 day period following TNT injury in DHA/saline treated rats. (A) Mean ipsilateral paw withdrawal latencies and (B) percentage change from baseline. * $P < 0.05$ vs. baseline value using one way ANOVA followed by Dunnett's test. A one way ANOVA followed by a Tukey's test revealed no significant differences between DHA and saline treated animals on respective days.

5.2.2 Effect of DHA on hind-paw thermal sensory testing

Behavioural sensory testing in the heel of the paw was conducted using a Hargreaves device every 7 days post injury. Following TNT injury, there were no significant differences regarding thermal paw withdrawal latencies between DHA and saline treated animals over the 28 day testing period (analyzed using a one way ANOVA and Tukey's post hoc test) (see Figure 5.5). Saline and DHA treated animals exhibited baseline PWLs of 11.0 ± 1.0 s and 8.2 ± 0.7 s respectively. At day one post TNT surgery saline and DHA treated animals exhibited PWLs of 13.2 ± 1.7 s and 10.9 ± 1.2 s respectively. At 7 days post TNT surgery saline and DHA treated animals exhibited PWLs of 9.7 ± 1.2 s and 7.8 ± 1.1 s respectively. At 14 days post TNT surgery saline and DHA treated animals exhibited PWLs of 7.7 ± 0.8 s and 6.6 ± 0.7 s respectively. At 21 days post TNT surgery saline and DHA treated animals exhibited PWLs of 10.2 ± 1.1 s and 7.3 ± 0.8 s respectively. At 28 days post TNT surgery saline and DHA treated animals exhibited PWLs of 8.9 ± 0.9 s and 7.0 ± 0.6 s respectively. There were no significant differences in post operative PWL values when compared to respective baseline in both DHA and saline treated TNT injured rats (analyzed using a one way ANOVA followed by Dunnett's test post hoc test).

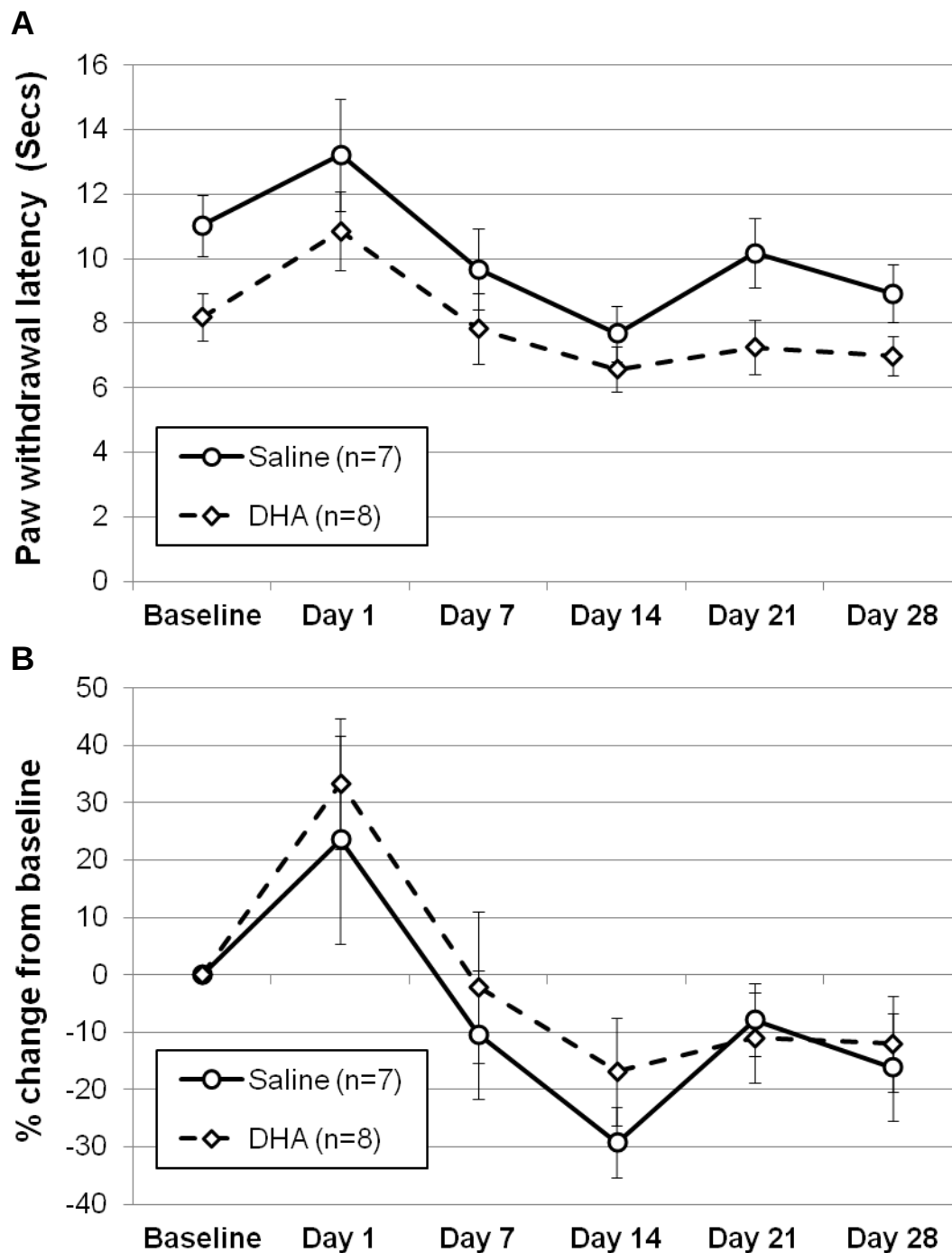


Figure 5.5 Thermal behavioural responses using a Hargreaves device over a 28 day period following TNT injury in DHA/saline treated rats. (A) Mean ipsilateral paw withdrawal latencies and (B) percentage change from baseline. A one way ANOVA with a Dunnett's test revealed no significant differences between baseline and post injury PWLs. A one way ANOVA with a Tukey's test revealed no significant differences between DHA and saline rats on respective days.

5.2.3 ED-1 labeling in the ipsilateral L5 DRG

Macrophages can be identified immunohistochemically via the cell surface marker ED-1. The labeling pattern of ED-1 was similar amongst DHA and saline treated rats 28 days post TNT injury (Figure 5.6A and B). In addition the macrophage morphology/labeling pattern was consistent with what is commonly observed after nerve trauma injury – a change into an amoeboid shape (Calvo and Bennett, 2012). There were no significant differences regarding the mean density of ED-1 immunoreactivity (per $250,000\mu\text{m}^2$) in the ipsilateral L5 DRG of rats treated with DHA or saline at 28 days post TNT injury. The mean density of ED-1 immunoreactivity in DHA treated rats was $0.12 \pm 0.03 \mu\text{m}^2$ compared to $0.15 \pm 0.03 \mu\text{m}^2$ in saline controls (see Figure 5.6C).

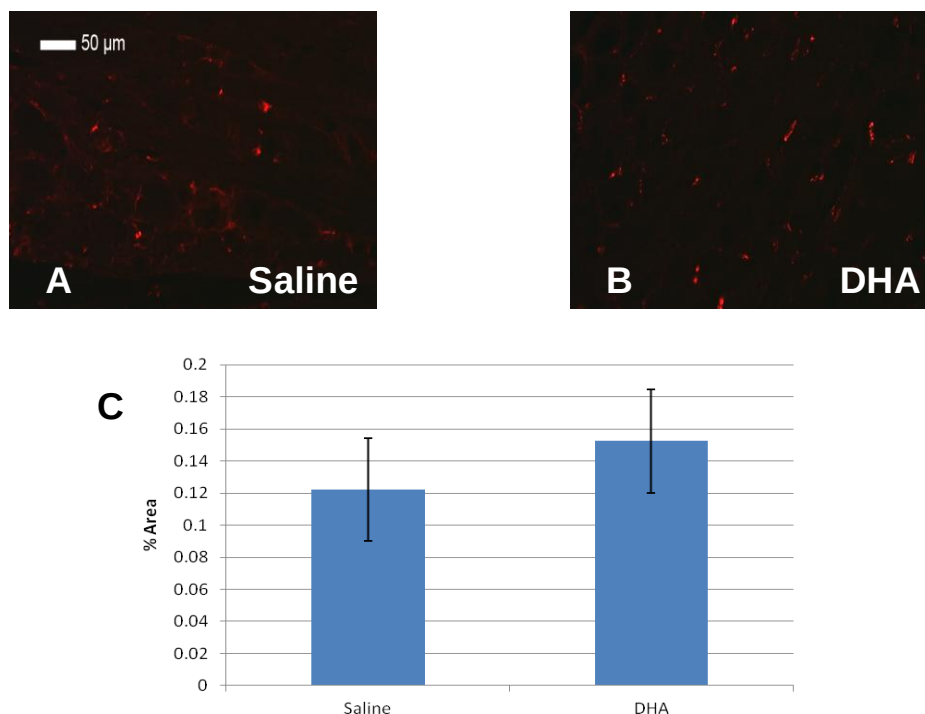


Figure 5.6 Representative images of ED-1 immunohistochemistry (macrophage marker) in TNT injured animals treated with (A) Saline and (B) DHA. Quantification of ED-1 was calculated via the mean percentage area per $250,000\mu\text{m}^2$ (C). Data presented as mean $\pm$ SEM. Magnification was at x20, and scale bar indicates $50\mu\text{m}$. TNT Rats treated with DHA did not differ in the amount of ED-1 immunohistochemistry in the L5 DRG when compared to saline treated TNT rats indicating a lack of an effect on macrophage infiltration in the DRG.

5.3 Discussion

5.3.1 Effect of DHA on pain related behaviours

The aim of the study was to investigate whether repeated intravenous injections of DHA (500nmol/kg, every 3 days) could reverse mechanical related hypersensitivity associated with TNT injury. The results of the study suggest that the administration of DHA in TNT injured animals does not affect the development of mechanical hypersensitivity associated with such injury. These results contrast previous studies that have demonstrated efficacy in reducing ‘pain’ related behaviours following intravenous administration of DHA in SCI (dose used 250nmol/kg) (Huang et al., 2007; King et al., 2006). The administration of DHA in the TNT model has provided a different result to that observed in SCI rats. In these previous studies, the effect of DHA was tested at a lower dose than the dose used in the current study. The lack of efficacy regarding DHA inability to reverse the TNT-associated hypersensitivity may perhaps be indicative of a sub optimal dosage regime. Although the dose used in the SCI studies (250 nmol/kg) demonstrated efficacy, it is possible that high dose used in the current study (500 nmol/kg) was detrimental to neurons in the DRG. As an exploratory study, only one dose of DHA was tested which was the 500nmol/kg dose. A previous study has demonstrated neuroprotective effects with a single bolus dose of intravenous DHA (500nmol/kg) administered 30 minutes after SCI (in mice) (Lim et al., 2010). This treatment led to a significant increase in neuronal and oligodendrocyte survival, as well as a marked reduction in the microglial response (Lim et al., 2010). In another study, the administration of linolenic acid 3 days before global ischemic insult (6 minutes duration) resulted in significant reductions in neuronal death 72 hours after insult using a similar high dose of 500nmol/kg (Blondeau et al., 2002). These observations, in conjunction with the robust nature of the TNT injury supported the use of the 500nmol/kg dose regime. It appears that there is a level of complexity regarding DHA mediated neuroprotection which may be influenced by the particular dose used and the timing of administration. In a previous study, the pre-treatment with DHA (500 nmol/kg, i.p.) at 1 hour and 3 days, or daily for 6 weeks before ischemia (90 minutes MCA-O), was accompanied by decreases in blood-brain barrier disruption, brain oedema, inflammatory cell infiltration, IL-6 expression, and caspase-3

activity in rats (Pan et al., 2009). However results from another study has demonstrated that the acute post treatment with DHA (500 nmols per rat (0.16 mg) i.p., 1 hour after reperfusion) following 90 minutes of MCA-O is associated with exacerbated cerebral ischemia-reperfusion injury (Belayev et al., 2009). In a similar study, using the same model with a 2 hour MCA-O, rats demonstrated significant neuroprotection following the administration of DHA-albumin after injury (Belayev et al., 2005). The lower DHA–albumin dose used in this study (0.63 g/kg) was highly neuroprotective, whereas the larger DHA–albumin dose (1.25 g/kg) was not. Such results indicate that high doses of DHA administered following trauma nerve injury could be accompanied by worsening outcomes of nerve damage (i.e. lowered PWTs), although it is to be noted that there were no significant differences in PWTs between DHA and saline treated rats on respective days in my study. The reason for the DHA related exacerbated cerebral ischemia-reperfusion injury reported by Belayev et al., 2009 is not entirely clear. DHA has been shown to be susceptible to oxidative stress *in vitro*, which may be a contributing factor (Cho et al., 1987). At high concentrations, DHA may become a substrate for lipid peroxidation essentially converting into a free radical which has multiple negative effects including loss of membrane fluidity, reduced membrane potential, and membrane rupture leading to release of intracellular contents (Gutteridge, 1995). Furthermore, products of DHA such as malondialdehyde and 4-hydroxynonenal may initiate further oxidative damage which is equally toxic that includes attacking amino acid side chains in proteins (Requena et al., 2003) and the fragmentation of DNA (Wang et al., 2006). It is thought that the acute administration of DHA within one hour of injury is a critical window for the neuroprotective effects to be observed (Huang et al., 2007). Dietary supplementation with DHA for 1 week, without an initial acute bolus has been shown to be ineffective for neuroprotection in a model of SCI (Huang et al., 2007). If the initial dose of DHA was not effective then it is likely that subsequent doses of DHA may not have been effective or a much longer time frame would have been required for the neuroprotective effects to be observed (i.e. effect on behavioural sensory testing). There is limited and weak evidence from a clinical case study report to suggested that ω -3 PUFAs are beneficial in peripheral neuropathic pain conditions when administered over a long period of time (months- years) (Ko et al., 2010), however this will need to be further investigated in a more robust fashion.

A second explanation for the lack of effect reported may be related to some fundamental properties of the TNT model which will be herein discussed. The TNT model was chosen because of its advantages over the more conventional nerve trauma models (e.g. SNT, CCI) due to its reproducibility and ease of surgical procedure. The nature of the injury restricts damage to one nerve region, so that the distribution of injured and uninjured (spared) populations can be standardized, therefore reducing nerve injury variability amongst animals. While the nerve damage is considered similar amongst animals, this uniformity may not translate into consistent PWTs upon mechanical sensory reflex testing. In addition, although the tibial and sural nerve territories of the plantar paw have been identified (Decosterd and Woolf, 2000), there might be possible overlap of innervation territories. This overlap in nerve territories could mean that upon testing of the midplantar paw, the probe-tip of the electronic von Frey device could be in contact with the more hypersensitive sural nerve region, in oppose to the intended tibial nerve region, resulting in either high or low PWTs. Such variability in the PWTs was not immediately obvious in the initial study (**Chapter 3: Evaluation of DRG neurochemical markers in an animal model of tibial nerve transection induced peripheral nerve trauma injury**). It was apparent that there were a number of inconsistent PWTs in TNT injured rats used in this particular study. Due to this variability, a Kolmogorov-Smirnov normality distribution test was conducted of all raw PWT's in DHA/saline treated rats (Table 5.1):

	Baseline	Day 1	Day7	Day 14	Day 21	Day 28
DHA						
1	✓	✓	✓	✓	✓	✓
2	✓	✓	✓	✓	✗	✓
3	✓	✓	✓	✓	✓	✓
4	✓	✗	✓	✓	✓	✓
5	✓	✓	✓	✓	✓	✓
6	✗	✓	✓	✓	✓	✓
7	✓	✓	✓	✓	✓	✓
8	✓	✓	✓	✓	✓	✓
Saline						
1	✓	✓	✓	✓	✗	✓
2	✗	✓	✓	✓	✓	✓
3	✓	✓	✓	✓	✓	✓
4	✓	✓	✗	✗	✓	✓
5	✓	✓	✓	✓	✓	✓
6	✓	✓	✓	✓	✓	✓
7	✓	✓	✓	✓	✓	✓

Table 5.1 Indication of normal distribution regarding PWTs in TNT rats treated with DHA/saline at baseline and at various time points post surgery following a Kolmogorov-Smirnov normality test. Normality was calculated for each animal using raw data values – 10 at baseline and 5 post surgery. 7.8% of data points failed to exhibit normal distribution; (✓) passed ($p>0.05$); (✗) failed ($p<0.05$).

The results of the normal distribution indicate that the majority of data points fitted a normal distribution (92.2%), and therefore I can conclude that the nature of the nerve injury was not likely to influence sensory reflex testing thresholds. Further discussion regarding the Kolmogorov-Smirnov normality distribution test is provided in the general discussion with comparisons to the TNT animals used in **Chapter 3**. The comparisons between the two sets of data are an important quality control step given that the same model was used for both studies.

5.3.2 Macrophage infiltration in the ipsilateral L5 DRG

The other aim of the study was to investigate whether DHA administration could modulate the macrophage response in the L5 DRG following TNT injury. Since DHA administration did not alter TNT induced mechanical hypersensitivity, key biochemical markers (i.e. ATF-3, GAP-43, NPY, and galanin) were not evaluated. Following traumatic peripheral nerve injury, Wallerian degeneration takes place; distal axons degenerate, Schwann cells differentiate, and macrophages become activated (Stoll et al., 1989). Activated macrophages will aid in the clearance of debris via phagocytosis at the site of injury and their presence normally contributes towards the healing and repair of injured tissue, returning it to a normal physiological state (Perry et al., 1987; Scholz and Woolf, 2007). However, macrophages also co-ordinate an immune response releasing proinflammatory mediators such as cytokines, and interleukins (Scholz and Woolf, 2007). The infiltration of macrophages into the DRG is associated with the development of behavioural paw mechanical hypersensitivity (Coyle, 1998; Liu et al., 2000b). The ED-1 antibody identifies CD68; a marker present on the surface of macrophages, and can be used to label macrophages in nerve tissue (Coyle, 1998; Hu and McLachlan, 2003; Scholz and Woolf, 2007). Analysis of ED-1 immunoreactivity was conducted using similar methodology to a previous report (Wallace et al., 2007b). To mention briefly, the mean percentage area of ED-1 immunoreactivity per $250,000\mu\text{m}^2$ ($500\mu\text{m} \times 500\mu\text{m}$) was calculated per animal using comparable levels of DRG representing the beginning, mid, and end sections of the DRG (3 DRG sections; 2 images per DRG), and a mean was determined for the respective groups. Quantitative analysis of ED-1 labeling in the ipsilateral L5 DRG of TNT rats (28 days post injury), treated with saline or DHA

(500nmol/kg, administered every 3 days post injury) revealed no differences in the expression levels between the two groups; a result consistent with the lack of behavioural effect observed (i.e. no reversal of paw mechanical hypersensitivity). The nature of the ED-1 staining pattern was consistent with literature reports depicting activated macrophages (Hu and McLachlan, 2003). No studies to date have examined the effects of DHA on macrophage infiltration into the DRG following peripheral nerve injury. Nonetheless, these findings contrast previous studies that propose a modulatory role for DHA on macrophages in experimental nerve injured animal models including stroke and SCI models. SCI initiates the recruitment of neutrophils, macrophages, and microglial cells at the injury site (Dusart and Schwab, 1994). The acute administration of DHA (250nmol/kg) within 30 minutes of SCI injury in rats has been shown to reduce ED-1 immunolabeling in the spinal cord at 6 weeks post injury (Huang et al., 2007). Similarly, in a rat model of acute ischemic stroke injury, intravenous DHA has been shown to attenuate ED-1 immunoreactivity in the brain and was associated with a reduced infarct size at 4 and 5 hours post injury (Belayev et al., 2011), thus indicating DHA has short and long term anti-inflammatory effects. However the results of the current study parallel a previous investigation that has demonstrated no significant differences in the density of ED1-positive macrophages at the lesion site following administration of DHA (250nmol/kg) 7 days after SCI in rats (King et al., 2006). The results of my study also adds to existing knowledge from *in vitro* experiments that demonstrates an inhibition of macrophage activity (IFN γ /LPS activated) when exposed to DHA (Ajmone-Cat et al., 2011;Dustin et al., 1990;Lu et al., 2010). There are no obvious explanations for this discrepancy, but it appears that there are multiple and complex factors that can determine the effects of DHA on macrophages *in vivo*. Such factors are likely to include the nature of nerve trauma injury, the timing of DHA administration (either before or after injury), and the production of potent metabolites.

5.3.3 Mechanisms of DHA mediated analgesia

There are numerous proposed mechanisms that may explain the neuroprotective and anti-inflammatory of ω -3 PUFAs. A growing and widely supported mechanism is the regulation of macrophages and inflammation (Yaqoob and Calder, 2007). DHA can bind to PPAR γ which can regulate the transcription of anti-inflammatory cytokines such as TNF α and IL-1 β (Jiang et al., 1998). Experiments in rodents have shown that the intraplantar or intrathecal administration of both TNF α and IL-1 β cytokines are associated with mechanical paw hypersensitivity in rodents (Cunha et al., 1992; Safieh-Garabedian et al., 1995; Youn et al., 2008). The reduction of TNF α and IL-1 β release from macrophages in response to DHA in peripheral nerve injury may reduce the sensitization of neurons that otherwise would lead to an increase in neuronal excitability and perhaps the development of pain (McMahon et al., 2005). Studies involving chronic ω -3 PUFAs supplementation suggest that after such intervention, the ω -3 fatty acid content (i.e. arachidonic acid content) of the cell membrane is likely to decrease, in parallel with an increasing DHA content. Since DHA competes with arachidonic acid for the action site of lipoxygenase, a higher membrane content of DHA may 'out compete' arachidonic acid, therefore reducing the production or effects of prostanoids and proinflammatory cytokines. Prostanoids have been shown to facilitate the release of neuropeptides from nerve terminals such as substance P (SP) and calcitonin gene related peptide (CGRP) (Martin et al., 1987), both of which participate in nociceptive signalling (Benemei et al., 2009; Snijdelaar et al., 2000). Furthermore, the EP1 prostanoid receptor, has been shown to increase in sensory neurons after injury which may additionally sensitise neurons (Durrenberger et al., 2006). DHA can further modulate the inflammatory responses via the production of metabolites such as NPD1 and resolvin D1 both of which exhibits significant anti-inflammatory and neuroprotective properties (Bazan, 2009; Hong et al., 2003; Marcheselli et al., 2010; Mukherjee et al., 2004; Serhan et al., 2008), and could have therapeutic potential. For example, the intrathecal or intraplantar resolvin D1 has been shown to reduce mechanical hypersensitivity in various models of inflammatory 'pain' (Xu et al., 2010). Inflammation is also associated with the production of reactive oxygen species (ROS) such as super oxide (Wang et al., 2004). ROS have been implicated in degenerative disease processes such as Alzheimer's disease and Parkinson's disease (Uttara et al., 2009).

Evidence from pre clinical studies have shown that the systemic injection of ROS scavenger phenyl-*N-tert*-butylnitron (PBN) in rats relieves spinal nerve ligation (SNL)-induced mechanical hypersensitivity in a dose-dependent manner (Kim et al., 2004). Such observations support a role of ROS in nerve trauma and perhaps neuropathic pain. The ω -3 PUFAs have been shown to reduce oxidative stress following neurological injury, thus limiting the potential for mitochondrial damage or apoptosis initiated by ROS (Dyall and Michael-Titus, 2008). Previous *in vitro* experiments demonstrate that the exposure of cultured hippocampal cells to DHA leads to an increase in antioxidant enzyme activity, including glutathione peroxidase and glutathione reductase (Wang et al., 2003b). Such effects on antioxidant processes have been observed *in vivo* – investigations by Songour et al., 2003 have shown that rats consuming an ω -3 PUFA diet (0.4 g/kg/day) for 30 days exhibit significantly less nitric oxide and SOD activity compared to respective control rats, in the hypothalamus. The effect of DHA on antioxidant enzyme activity could restrict the potential damage to DRG neurons caused by nerve trauma injury induced ROS, thus allowing the continuation of nerve regeneration and the likelihood of a quicker recovery.

It has been suggested that ω -3 PUFAs can bind and block both the voltage gated sodium ion channels (VGSCs) and voltage gated calcium ion channels (VGCCs) (Shapiro, 2003). Previous *in vitro* data has shown DHA can exert a dose-dependent suppression of voltage-activated sodium currents in rat ventricular cardiac myocytes (Xiao et al., 1995). DHA has also been shown to prevent toxin-induced fibrillation of cultured neonatal rat cardiac myocytes via blockade of L-type VGCCs (Leaf, 2001). Other *in vitro* data also supports the blockade of T-type VGCCs (Danthi et al., 2005). The interaction of ω -3 PUFAs with VGSCs is of interest because following peripheral nerve trauma there are complex changes in the type of VGSCs expressed as well as changes in channel function and number expressed in the DRG (Wood et al., 2004). These changes are thought to contribute to increased hyperexcitability and ectopic activity in neurons which may contribute towards neuropathic pain pathophysiology (Lai et al., 2004). It is possible that DHA may reduce the excitation of neurons following peripheral nerve trauma via blockade of VGSC's and VGCC's.

The neuroprotective effects of ω -3 PUFAs may also be attributed to their activation of a subset of K^+ channels that contain two pore-forming domains; namely, TREK-1 and TRAAK (Lesage and Lazdunski, 2000). These channels are abundant in the brain, particularly the hippocampus where they are located pre and postsynaptically (Lauritzen et al., 2000). The activation of these channels by ω -3 PUFAs might reduce the resting potential as well as in the firing properties of neurons (Lauritzen et al., 2000). The activation of TREK-1 prevents the excessive release of excitotoxic glutamate thus acting as a break on neuronal excitability (Blondeau et al., 2002) – such actions may also reduce hyperexcitability of damaged peripheral sensory neurons in the DRG. More research is needed to identify the potency of such actions since specific TREK-1 and TRAAK blockers have not yet been discovered (Lauritzen et al., 2000).

5.3.4 Limitations and future studies

A major limitation to the study is serum phospholipids levels before and at time points post injury was not measured and therefore the mean blood serum DHA levels were not determined. Additionally, the production of DHA metabolites (e.g. NPD1 and resolvins D1) was not measured and their likely effects cannot be ascertained. The blood serum levels of DHA and its metabolites could have been measured by taking routine blood samples at various time points post injury, and conducting a lipid analysis using high performance liquid chromatography (Marcheselli et al., 2003). Lipid analysis techniques has been used previously to quantify ω -3 PUFA content in the blood serum of rats with traumatic brain injury following dietary fish oil supplementation (Mills et al., 2011). Such data would also indicate whether the dosage regime was robust to ensure DHA serum levels remained at a dose deemed to be effective. The use of single dose regime to evaluate the effects of DHA is another limitation of the study. The explorative nature of the study allowed for the examination of a single dose regime, however a dose dependent study (using lower doses e.g. 250nmol/kg and 100nmol/kg) would strengthen the data and allow comparisons with other studies of nerve trauma injury that have used such doses. Furthermore, the use of a dose-response is essential in pharmacological studies in order to analyse the safety profile and identify the therapeutic window, and within the current study, to define an optimum

dose that would maximize the degree of neuroprotection. A more robust approach could have been the oral administration of DHA via powdered diet which would have allowed a sustained delivery of ω -3 PUFAs without the unnecessary stress of regular tail vein injections. Dietary DHA enrichment via powdered diet has been beneficial in other models of traumatic nerve injury such as SCI (although with an initial acute bolus of DHA following injury) (Huang et al., 2007; King et al., 2006; Mills et al., 2011). A future experiment could involve analyzing the effect of pre injury administration of DHA and would focus on the likelihood of pre-emptive strategies. A recent study have shown that the pretreatment of DHA (250nmol/kg, $\sim$ 82 μ g/kg) 7 days and 1 hour before SCI results in axonal preservation and increased white matter sparing compared to the vehicle injections 7 days after nerve injury (Figuerola et al., 2012), such a treatment regime is worth exploring for peripheral nerve injury. The administration of other ω -3 PUFAs such as EPA could have been tested since they also have neuroprotective and anti-inflammatory properties similar to DHA, and can be metabolized to form E series resolvins which have enhanced anti-inflammatory properties (Serhan et al., 2008). The phenotype of microglia in the spinal cord could have been examined since their activation has been recognized as a key contributor towards neuropathic pain mechanisms (Coull et al., 2005). Macrophages/microglia continuously alter the expression of surface markers in response to local stimuli and can be characterised accordingly as either 'activated' proinflammatory (M1) or 'alternative' immunomodulatory or anti-inflammatory (M2) cells (Kigerl et al., 2009; Shapiro et al., 2011). Further investigations could have examined the phenotype of the macrophages present in the DRG or spinal cord in DHA treated animals via double labeling experiments using surface receptors associated with M1 macrophages (CD86, CD16, and CD32) or M2 macrophages (CD206 and argenase-1) (Kigerl et al., 2009). It would be interesting to discover if DHA initiates a switch to the M2 phenotype which typically promotes tissue repair/remodeling (Shapiro et al., 2011).

It is possible that the TNT model could have been an inappropriate choice for this study due to an insufficient macrophage response that is attributed to the limited surgery of TNT, and therefore inflammation and macrophages could play less of a role in paw related mechanical hypersensitivity compared to other trauma nerve injury models. This could be investigated

by using the more robust nerve damage SNT model as a control group and comparing the ED-1 immunoreactivity. The activation state of spinal microglia could have been assessed via double labeling experiments using MAPKs such as p38 or ERK, both of which are both upregulated in the spinal cord after peripheral and spinal nerve injury (Calvo et al., 2012b; Ji and Suter, 2007) and are thought to be reflective of an M1 phenotype (Jin et al., 2003; Tsuda et al., 2004). DHA has been shown to impair p38 MAPK signaling in cultured macrophages (Ajmone-Cat et al., 2011); therefore investigations could reveal similar findings *in vivo*.

This study could have considered the use of a naive group for the ED-1 immunohistochemistry as an additional control to make a comparison to baseline levels. Additionally a negative control could have been conducted to assert the validity of the ED-1 immunohistochemistry.

5.3.5 Conclusions

To conclude, in this chapter I have examined the effects of DHA on the development of mechanical hypersensitivity following TNT injury. There were no differences in mechanical or thermal behavioural hypersensitivity between DHA and saline treated animals following TNT injury over 28 days. Additionally, no differences were observed regarding macrophage infiltration in the injured L5 DRG between DHA and saline treated TNT animals. I have raised some concerns regarding the dose of DHA used in the study and the inconsistencies in the behaviour that may have contributed to the reported outcomes.

Chapter 6

6 General discussion

General discussion

Peripheral nerve injury results in biochemical changes and neuroinflammation within the DRG (Lacroix-Fralish et al., 2011; Woolf and Salter, 2000; Zimmermann, 2001). In this thesis, I attempted characterize such changes using immunohistochemical techniques and therefore further our understanding of neuropathic pain mechanisms.

Research aims:

- (1) To provide temporal profile expressions of commonly dysregulated neurochemical markers (i.e. ATF-3, GAP-43, NPY, and galanin) in traumatic and non-traumatic nerve injury using the TNT and d4T induced models of peripheral nerve injury respectively.
- (2) To compare and contrast these changes in both models.
- (3) Characterize, compare, and contrast evoked sensory parameters (limb withdrawal responses) in both models.
- (4) Investigate whether DHA (ω -3 PUFA) can reverse mechanical and thermal hypersensitivity associated with TNT injury and alter the expression of neurochemical markers in the DRG. Since no behavioural changes were observed upon sensory testing in DHA treated animals, investigations focused on the potential of DHA to modulate the DRG macrophage response.

Research hypothesis:

Peripheral nerve damage initiates neurochemical changes in the dorsal root ganglion, and the development pain-like behaviours, which can be modulated via the administration of ω -3 polyunsaturated fatty acids.

Forwarding remarks

This thesis has demonstrated completely different expression patterns of neurochemical markers in the DRG following TNT injury and d4T induced nerve damage in rats which highlights the mechanistic difference between nerve trauma and antiretroviral associated neuropathic pain.

6.1 Summary of the DRG neurochemical response to TNT injury and d4T induced nerve damage

The majority of neuropathic pain studies focus on the use of trauma nerve models, which is not wholly representative of the clinical situation that includes other aetiologies relating to diabetes, herpes zoster, and HIV. Therefore my experiments addressed this issue by investigating mechanisms of nerve trauma and NRTI related neuropathy using the TNT and d4T model respectively (

Table 6.1).

	TNT	d4T
ATF-3	• Greater proportion of ATF-3 expression in NF-200 immunoreactive cells (71.1 ± 7.2 % [also the peak]) compared to peripherin immunoreactive cells ($11.9. \pm 5.6$ %) 1 day after injury • Expression downregulated in NF-200 cells 1 day post injury • Expression levels remained consistent at all other time points in peripherin (peak; $25.4. \pm 8.3$ %) and NF-200 immunoreactive cells	• Low expression across all time points ($<0.1\%$) in peripherin and NF-200 immunoreactive cells. No significant temporal trends were observed.
NPY	• Distinct expression patterns in small vrs. Large cells • Expression observed at 7, 14, 28, and 43 days post surgery, mainly in NF-200 cells (peak; 51.7 ± 8.3 %) • An increasing temporal trend was observed in peripherin immunoreactive cells but expressions remained low ($<6.5\%$)	• Low expression across all time points ($<0.5\%$) in peripherin and NF-200 immunoreactive cells. No significant temporal trends were observed.
GAP-43	• Expression observed at all time points and temporal profile expressions were similar in peripherin and NF-200 immunoreactive cells • Peak observed day 14 in both peripherin (15.7 ± 1.7 %) and NF-200 (15.8 ± 4.8 %) immunoreactive cells	• Low expression across all time points ($<2\%$) in peripherin and NF-200 immunoreactive cells No significant temporal trends were observed.
Galanin	• Expression observed at all time points and temporal profile expressions were similar in peripherin and NF-200 immunoreactive cells • Low expression 1 day post injury ($<2\%$) in peripherin and NF-200 immunoreactive cells • Peak observed day 14 in both peripherin (19.3 ± 4.8 %) and NF-200 (25.8 ± 4.2 %) immunoreactive cells	• Low expression across all time points ($<2\%$) in peripherin and NF-200 immunoreactive cells time point. No significant temporal trends were observed.

Table 6.1 Summary of DRG neurochemical marker expression in the TNT and d4T models of peripheral nerve damage

6.2 DRG neurochemical response to peripheral nerve trauma

Results outlined in **Chapter 3** demonstrate that TNT injury results in a robust upregulation of axonal injury marker ATF-3, nerve regeneration marker GAP-43, and the neuropeptides NPY & galanin. Evidence from the literature parallels with the observation of ATF-3 expression in the DRG following peripheral nerve damage at various time points post injury (Averill et al., 2004; Averill et al., 2002; Djouhri et al., 2006; Fukuoka et al., 2012; Obata et al., 2003; Peters et al., 2005; Shortland et al., 2006; Tsujino et al., 2000; Wright et al., 2004). Of particular interest is the novel finding of disproportionate upregulation of ATF-3 in large DRG cells one day post TNT injury (acute stage of nerve trauma) compared to small DRG cells. Such observations parallel with previous studies that have demonstrated microscopic damage and degeneration in large myelinated fibres with less severe changes evident in smaller unmyelinated fibres (Basbaum et al., 1991; Munger et al., 1992), although the proportion of ATF-3 immunoreactive DRG cells after this time remains consistent in both small and large sized cells. The function of ATF-3 is not clear, however its presence is linked to cell stress and cancer (Thompson et al., 2009), and in neurons has been associated with regeneration of peripheral sensory neurons (Hunt et al., 2004; Saito and Dahlin, 2008). The over expression of ATF-3 in cancer cell lines has been found to be associated with increased proliferation and increased transition of the G1 (growth phase) to the S-phase (DNA synthesis) of the cell cycle (Pelzer et al., 2006). Another study has found ATF-3 to be protective against apoptosis in a HeLa cells (Wolfgang et al., 1997). In light of this evidence, it is likely that ATF-3 expression may be protective against neuronal cell death following nerve damage. Such neuroprotection (or reduced cell death) has been demonstrated in superior nerve ganglion neurons in which ATF-3 has been overexpressed via a viral vector (Nakagomi et al., 2003).

The upregulation of GAP-43 has been associated with nerve regeneration (Woolf et al., 1990). Previous studies have identified GAP-43 in the DRG following peripheral nerve injury (Nahin et al., 1994; Schreyer and Skene, 1991; Schreyer and Skene, 1993; Woolf et al., 1990). Following TNT injury, GAP-43 was upregulated in both peripherin and NF-200 cells, with both populations exhibiting similar but not significant temporal profiles. This

observation indicates nerve regeneration processes occurring after TNT injury. Although nerve regeneration has been recognized as a likely role for GAP-43, it has been proposed that GAP-43 may be involved in neuronal remodeling (Benowitz and Routtenberg, 1997; Neve et al., 1987). GAP-43 could also be important in the maintenance and structure of the soma, and axonal fibres including their synaptic terminals. The regenerative process may represent an important event that enables primary sensory neurons to recover and regain normal physiology. However, it could be that those damaged neurons that have 'regenerated' could maintain abnormal physiology that could perpetuate neuropathic pain processes e.g. ectopic activity.

The upregulation of galanin is a known consequence of peripheral nerve trauma and has been observed at various time points post injury, mainly in small diameter DRG neurons (Averill et al., 2002; Fukuoka et al., 1998; Hokfelt et al., 1987; Hokfelt et al., 1994; Holmes et al., 2003; Ma and Bisby, 1997; Shi et al., 1999; Shortland et al., 2006; Villar et al., 1989). This thesis has examined the temporal nature of galanin expression in the L5 DRG after nerve trauma injury and has identified a similar dynamic expression profile that occurs in both peripherin/NF-200 cells (peak at 14 days in both peripherin/NF-200 cells). The percentage expression observed in both populations was lower than that observed in aforementioned studies and is likely explained via the use of arguably more robust models of peripheral nerve trauma (i.e. SNT, CCI, and complete sciatic nerve transection (CSNT)). The idea that the degree of nerve damage influences expression of galanin is supported by a previous study using the single ligature nerve constriction model where animals were grouped according to the degree of ligation to represent light (10-30%), medium (40-80%) and strong ligature (90%). After light ligation, a modest upregulation of galanin was observed, mainly in small-sized neurons. However, following medium or strong ligatures, there was a larger increase in the number of galanin immunoreactive neurons, involving also medium and large sized cells (Coronel et al., 2008).

The results in this thesis agrees with the observations of others that have observed the upregulation of NPY following peripheral nerve trauma, mainly in large sized neurons (Ma and Bisby, 1997; Ohara et al., 1994; Wakisaka et al., 1991; Wakisaka et al., 1992). The

temporal profile indicates a robust downregulation after 28 days in large sized cells. In addition to this, the results demonstrate that small sized cells also upregulate NPY, but to a much lesser extent compared to large diameter cells.

The release of neuropeptides that are associated with antinociception such as NPY (Hua et al., 1991;Intondi et al., 2008) or galanin (Hua et al., 2004) may reflect the nervous system attempts to restore normal nociceptive processing following nerve trauma. Their downregulation after peripheral nerve trauma suggests a partial recovery from nerve injury and is associated with the downregulation of ATF-3.

In order to determine if there were a relationship between the temporal expression of investigative markers in both cell populations (peripherin and NF-200) and the degree of behavioural mechanical hypersensitivity, a Pearson's correlation co-efficient was calculated. Although there was a noticeable trend towards an association in both peripherin and NF-200 cells with regards to GAP-43, NPY, and galanin immunoreactivity and also with ATF-3 expression in peripherin characterized cells. The likelihood of a significant association could have been investigated through the use of increased experimental group sizes. No correlations were observed and this suggests that these markers may be indicators of nerve injury and less likely to be markers of neuropathic pain. The lack of correlation between mechanical hypersensitivity and expression of neurochemical markers ATF-3 and GAP-43 parallels with that of previous findings (Jaken et al., 2011;Shortland et al., 2006).

A proposed mechanism for nerve trauma injury is the loss of target derived trophic support (e.g. NGF, BDNF, NT-3) leading to a dysregulation of neuropeptide expression, and associated 'pain' (Ohara et al., 1995). Previous work have demonstrated that the intrathecal administration of NGF via an osmotic pump to sciatic nerve injured rats can partially or fully prevent the upregulation of NPY, galanin, and ATF-3 in the DRG (Averill et al., 2004;Fitzgerald et al., 1985) and additionally prevent the development of pain-related behaviours (Wang et al., 2003a). Since sensory neurons depend on the presence of NGF for viability (Molliver et al., 1997), the concept of NGF treatment relieving neuropathic pain seems a plausible hypothesis. However, evidence from animal studies suggests NGF induces

hypersensitivity through the activation of trkA receptors (McMahon et al., 1995), which correlates with clinical observations regarding pain at site of injection, or widespread pain upon systemic administration (Svensson et al., 2003). This contradiction likely reflects differences in dose surrounding the affected nerve, and the dynamic responses to nerve injury. Further evidence regarding the role of NGF in pain derives from humans that have mutations in NGF/trkA signaling, in which they consequently demonstrate congenital analgesia (Indo et al., 2001). Various clinical trials have now provided support towards anti-NGF therapies that include the sequestration of NGF via antisera or soluble receptors (Lane et al., 2010;Pezet and McMahon, 2006). However concerns regarding safety (i.e. autoimmune reactions) will need to be addressed since side effects/worsening of condition have been observed in osteoarthritis patients leading to temporary suspension of the clinical trial (Wood, 2010).

6.3 DRG neurochemical response to antiretroviral induced nerve damage

This study revealed that d4T treated animals do not exhibit altered expressions of axonal injury marker ATF-3, nerve regeneration marker GAP-43, and the neuropeptides NPY & galanin. Such observations corroborate with a previous study examining such markers following administration of a similar antiretroviral ddC in rats (Wallace et al., 2007b). The lack of altered expression of all investigated markers in d4T treated rats suggests that there is no alteration in trophic support in NRTI neuropathy models or it could be that any putative alterations are compensated by other, perhaps unknown trophic factors. However the effects of neurotrophins may still play a role in NRTI related neuropathy. In a recent study, the systemic administration of d4T in mice (at similar doses used in the thesis) induced mechanical, but not thermal hypersensitivity that was associated with an increase in neuronal activity and BDNF expression in the spinal dorsal horn (Renn et al., 2011). Exogenous intrathecal BDNF exacerbated these observations, and the pretreatment with intrathecal trkB-Fc or a tyrosine kinase inhibitor reduced the d4T-induced mechanical hypersensitivity. BDNF, like NGF is a known modulator of sensory transmission, acting on the trkB receptor, and plays a key role in central sensitization (Pezet and McMahon, 2006). It is possible that the presence of BDNF in the cord, in d4T animals is enough to precipitate the changes in

sensory behaviour without necessarily inducing the changes in neuropeptide expression that is observed in peripheral nerve trauma injury.

6.4 Comparison and contrast between nerve trauma and antiretroviral associated neuropathy

This thesis has revealed that there are distinct differences in the neurochemical responses to nerve damage in the DRG in two different types of nerve damage that are both associated with neuropathic pain. This is an important finding that is only beginning to transcend throughout the neuropathic pain field. In both animal models and patients, there are critical differences between the responses to different types of nerve damage that are associated with neuropathic pain. Both the d4T and TNT models fit the definition for nerve damage (Jensen et al., 2011). Although neurochemical changes were not apparent in the DRG of d4T treated rats, evidence from previous studies have demonstrated a clear neurotoxicity that manifests in the retraction of C-fibres from the paw skin that is analogous to the ‘die back’ phenomenon that occurs in ATN patients (Calvo et al., 2012a;Wallace et al., 2007b). In contrast to the d4T model, TNT injured rats demonstrated an apparent DRG neurochemical response that manifested in the expression of axonal injury marker ATF-3, neuropeptides NPY & galanin, and axonal regeneration marker GAP-43; all findings consistent with literature reports. This result suggests that these markers are possibly markers of nerve injury and not to be interpreted as generic markers for neuropathic pain since evidence for nerve damage is apparent in d4T treated rats, as aforementioned (retraction of C-fibres from paw skin). Such distinct responses to nerve damage between these two models are also observed at gene level within the DRG (Maratou et al., 2009) and with regards to spinal microglia infiltration which is only evident following nerve trauma (Blackbeard et al., 2012;Zheng et al., 2011a). My research also supports the argument that there different mechanisms for neuropathic pain, and that the use of a diverse range of animal models that reflect different clinical antilogies can play an important role in understanding these mechanisms. Evidence from clinical studies have indicated that various mechanisms may be at play and that neuropathic pain patients can be grouped according to their symptoms and sensory profiles using quantitative sensory testing (QST) (Baron et al., 2009;Maier et al., 2010).

Much of the evidence regarding NRTI associated pain relates to mitochondrial toxicity; a feature that may not be relevant in nerve trauma associated pain. The neuropathic changes in NRTI treated animals originate from mitochondrial dysfunction, leading to oxidative stress, apoptosis of neurons, altered calcium homeostasis, and axonal degeneration (Joseph and Levine, 2004; Opii et al., 2007; Wallace et al., 2007b). Although this thesis has revealed potentially differing mechanisms between d4T and nerve trauma associated nerve damage, some of the end processes are similar. For example, activated caspase-3 (the critical executioner of cell death) is induced in the L5 DRG following L5 spinal nerve crush injury (Sekiguchi et al., 2009) and is associated with mechanical hypersensitivity. Inhibitors of both activator (1,2,8, and 9) and effector (3) caspases have all been shown to attenuate mechanical behavioural hypersensitivity associated with the ddC induced neuropathy model (Joseph and Levine, 2004). Another example of similar process between trauma and NRTI nerve damage is retraction of C-fibres in paw skin. Immunostaining of skin biopsies using neuronal marker protein gene product 9.5 (PGP 9.5, a ubiquitin carboxyl hydrolase), can be used to quantify the IENF density in rats (Smith et al., 2005) and in humans (McCarthy et al., 1995). A reduced IENF density is observed in ddC treated rats (Wallace et al., 2007b) but also in nerve trauma models (Hsieh et al., 2009) and chemotherapy associated neuropathies (Zheng et al., 2011a) – a phenomenon that may be relevant to neuropathic pain mechanisms.

Inflammation may be more relevant in trauma related pain. After traumatic nerve injury, microglia become activated, and there is an increase in the total number infiltrating the spinal cord (Blackbeard et al., 2007). Activated microglia can release BDNF that can modify chloride currents and alter neuronal excitability in the cord of nerve trauma rats (Coull et al., 2005). This microglial infiltration/activation response is not observed in the ddC antiretroviral model (Blackbeard et al., 2012; Zheng et al., 2011a), and the administration of minocyclin (a microglia inhibitor) has no effect on the associated mechanical hypersensitivity (Wallace et al., 2007b). Moreover, there is no macrophage infiltration in the sciatic nerve and collectively, the evidence suggests that inflammation is not a mitigating factor for ATN neuropathy. However, contradictory to this is the presence of CCL2 (a macrophage chemoattractant) in the DRG of ddC treated rats, which does not entirely rule out the possibility that inflammation may have some role to play, and could be investigated.

6.5 Evaluation of ω -3 PUFAs role in nerve damage

Data from this thesis has only proven part of the hypothesis. The administration of DHA to TNT injured rats did not alter outcomes of behavioural sensory testing or the infiltration of ED-1 immunoreactive cells in the DRG, a result that contradicts the recognized neuroprotective and anti-inflammatory properties of this compound.

The intake of ω -3 PUFAs (e.g. DHA or EPA) in the diet can improve joint pain and reduce the risk of cardiovascular disease (Goldberg and Katz, 2007). Evidence from preclinical studies suggests ω -3 PUFA supplementation in the diet can reduce both the histological outcomes and sensory behavioural outcome measures associated with SCI (Huang et al., 2007). I sought to examine the effect of DHA (administered every 3 days following nerve injury) at a dose comparable to previous studies in which an effect was observed (Lim et al., 2010). Data from earlier experiments (**Chapter 3**) revealed no correlations between TNT associated mechanical hypersensitivity and the temporal expression profiles of investigative markers (i.e. ATF-3, Gap-43, NPY, and galanin). There were also no observed differences in the development of mechanical hypersensitivity between DHA and saline treated rats following TNT injury. Therefore histological analysis in the DRGs of DHA/saline treated rats did not focus on the expression profiles of neurochemical makers associated with nerve trauma, but rather the potential modulation of the macrophage response. Following peripheral nerve injury, macrophages infiltrate the DRG, where they are likely to release an array of cytokines (e.g. TNF α and IL-1 β) that can promote inflammation which can sensitize neurons and exacerbate outcome measures of sensory behavioural responses. Consequently, I sought to investigate whether DHA administered to TNT injured rats could decrease the macrophage response in the ipsilateral DRG compared to saline treated rats. The results demonstrated no differences in the percentage area of ED-1 immunoreactivity (macrophage marker), a finding consistent with the lack of behavioural change observed. This result may be reflective of a sub optimal dosage regime that could have been detrimental to the DRG neurons. Although the dose used in a previous SCI study (250 nmol/kg i.v.) demonstrated efficacy (Huang et al., 2007), it is possible that at higher doses, there is the possibility of tipping the balance between the detrimental effects of lipid peroxidation that can occur with

ω -3 PUFAs (Gutteridge, 1995) and the benefits of neuroprotection. A previous identical study using the dose used in the current study (500 nmol/kg) identified neuroprotection at this dose (Lim et al., 2010). The nature of this balance will need to be understood to fully realize the potential of DHA as a neuroprotective agent. Since inconsistent and large standard deviation values were observed in some animals regarding PWTs (especially during the early stages of nerve injury) a Kolmogorov-Smirnov normality distribution test was conducted of all DHA/saline treated rats. Results of this analysis revealed a small percentage of data points that reflect an abnormal distribution (see Table 6.2). A normality distribution test was also conducted for all data points regarding TNT/sham animals (data acquired in **Chapter 3**), in order to demonstrate validity of earlier results (see Table 6.3). Analysis revealed few rats exhibiting abnormal distribution of PWT values, a result comparable to that of TNT rats treated with DHA/Saline and therefore I can conclude that the majority (>90%) of data sets exhibits normal distribution.

	Baseline	Day 1	Day7	Day 14	Day 21	Day 28
DHA						
1	✓	✓	✓	✓	✓	✓
2	✓	✓	✓	✓	✗	✓
3	✓	✓	✓	✓	✓	✓
4	✓	✗	✓	✓	✓	✓
5	✓	✓	✓	✓	✓	✓
6	✗	✓	✓	✓	✓	✓
7	✓	✓	✓	✓	✓	✓
8	✓	✓	✓	✓	✓	✓
Saline						
1	✓	✓	✓	✓	✗	✓
2	✗	✓	✓	✓	✓	✓
3	✓	✓	✓	✓	✓	✓
4	✓	✓	✗	✗	✓	✓
5	✓	✓	✓	✓	✓	✓
6	✓	✓	✓	✓	✓	✓
7	✓	✓	✓	✓	✓	✓

Table 6.2 Indication of normal distribution regarding PWTs in TNT rats treated with DHA/saline at baseline and at various time points post surgery following a kolmogorov-Smirnov normality test. Normality was calculated for each animal using raw data values – 10 at baseline and 5 post surgery. 7.8% of data points failed to exhibit normal distribution; (✓) passed ($p>0.05$); (✗) failed ($p<0.05$).

	BL 1	Day 1	BL 7	Day 7	BL 14	Day 14	BL 28	Day 28	BL 43	Day 43
TNT										
1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
4	N/A	N/A	N/A	N/A	N/A	N/A	✓	✓	✗	✓
Sham										
1	✓	✓	✓	✓	✗	✓	✓	✓	✓	✓
2	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓
3	✓	✓	✓	✓	✓	✓	✗	✓	✓	✗
4	✓	✓	✓	N/A	N/A	✓	✓	✓	✓	✓

Table 6.3 Indication of normal distribution regarding mechanical PWTs in TNT/sham operated rats at baseline (BL) and at the various time points post surgery following a kolmogorov-Smirnov normality test. Normality was calculated for each animal using raw data values – 10 at baseline and 5 post surgery. 6.9% of data points failed to exhibit normal distribution; (✓) passed ($p>0.05$); (✗) failed ($p<0.05$).

6.6 Study limitations and further studies

6.6.1 Assessment of mechanical hypersensitivity

The use of the electronic von Frey device offers many advantages and was originally used to overcome some of the issues surrounding the use of the graded von Frey monofilaments which are mechanically unstable and difficult to standardize (Moller et al., 1998). The size of the electronic von Frey probe tip is consistent and therefore limits the response bias upon application. Graded von Frey filaments alter performance in response to varying humidity and temperature, in a way that the electronic device is not, and this could have a bearing on withdrawal responses (Andrews, 1993). The electronic von Frey device also allows continuous measurements of PWTs as opposed to incremental measurements of the graded von Frey filaments. The electronic von Frey setup is computerized, and there is the advantage of a real time display of withdrawal responses. Copies of the traces can be obtained and referred to for future reference. The results of the graded von Frey test is commonly converted from Newton's to grams and the scale is log rhythmic, and therefore its use is attached with an inheritable degree of error. Finally, the electronic von Frey has been shown to have a high interobserver reliability, both between PWT's obtained and between rates of application used (Moller et al., 1998). Despite the advantages of the electronic von Frey, some essential prerequisites must be understood. Firstly, the probe tip of the electronic von Frey device must be held perpendicular to the hind paw skin surface to ensure an appropriate delivery of the presenting stimulus. Secondly, the rate of application needs to remain constant every time, anything contrary may change the nature of the force applied. If the application is too fast, the animal may lift its paw abruptly before there is a chance to measure the withdrawal threshold. Too slow an application, and a withdrawal response may not be obtained since the force applied was not enough to reach threshold. Lastly, the region of the paw stimulated must remain consistent since different regions of the paw may react differently when presented with the same stimulus. The electronic von Frey device allows standardization of testing, detailed documentation of each experiment, and provides an objective and accurate method for measuring PWTs to mechanical stimuli and was suitable for experiments detailed in this thesis.

Examination of PWTs in TNT animals were conducted in the mid-plantar region of the paw which is innervated by the tibial nerve. Other studies using the TNT model have examined the lateral part of the paw which receives sural nerve innervations and is reported to be the hypersensitive part of the paw in the acute stages of nerve injury (Lee et al., 2000; Unal-Cevik and Oaklander, 2011). The sural nerve is one of the branches of the sciatic nerve that conveys mainly sensory information whereas the tibial transmits both sensory and motor information. Following chronic exposure to ddC in humans, there is a disproportionate degree of injury to the sural nerve in particular (Lewis and Dalakas, 1995). Alternatively the lateral part of the paw could be examined in TNT injured animals and subsequently compared to the PWT responses in d4T injured animals.

Increases in PWTs

An increase was observed in the ipsilateral PWTs of sham operated animals across time that at 43 days post injury. An increase was also observed in contralateral paw of TNT operated animals at day 28 post injury. While somewhat unusual, this might be explained by an increase in weight over time, thus leading to larger PWTs. This may not be a frequent occurrence in most peripheral nerve injury animal models studies since most studies rarely examine time points beyond 14-21 days, and therefore such increases in PWTs are generally not observed. This phenomenon could be further examined and accounted for by measuring the PWTs in a naïve control group of standard adult weight (200-250g) to normalise post surgery PWTs of all experimental animals.

6.6.2 Assessment of thermal hypersensitivity

The Hargreaves device was used to assess reflex withdrawal responses to noxious thermal stimuli (Hargreaves et al., 1988). This device has advantages over other assays such as the hot plate test. Firstly, the Hargreaves device has an automated reflex withdrawal detector that is sensitive to the nearest 0.1 second. Secondly, the intensity of the stimulus can be altered; additionally there is a cut-off limit of 21.4 seconds if the animal is still in contact with the stimulus to prevent any potential cutaneous injury. Lastly, the heat beam can be

focused to different parts of the paw allowing for testing in different regions of the paw. There are however some disadvantages, some of which are not directly related to the function of the device. There is the potential for the animal to acclimatise to the device which can also be interpreted as ‘habitual learning’. The animal may also urinate and/or defecate on the Plexi glass surface of the chamber which if not cleaned up can interfere with testing. Finally, the Hargreaves device may be vulnerable to experimental bias in which the experimenter switches the part of the paw that is being stimulated, therefore the proper use of the device is dependent on the integrity of the user. One of the main facets of nerve trauma models is the altered posture of the paw so that it is dorsi-flexed. This abnormal placement of the paw means that upon testing the heat stimulus may not be in full contact with the mid-plantar region of the paw, which can lead to an inaccurate PWL reading. As a result, the presentation of the thermal stimulus to the heel of the paw which was found to be more often than not in contact with the glass surface of the testing chamber. Thermal hypersensitivity (i.e. drop in PWL’s) was evident in TNT animals at 28 days post surgery, however such hypersensitivity has not been observed in some neuropathic pain models where the nature of the injury is similar (Decosterd and Woolf, 2000).

The current study only examined mechanical behavioural hypersensitivity following d4T treatment and thermal nociception was not evaluated. There have been conflicting reports regarding the onset of thermal hypersensitivity in the NRTI peripheral neuropathy model. Joseph et al., 2004 demonstrated thermal hypersensitivity in ddC-treated rats at 5 days post injection (~50% decrease from baseline). In contrast, Renn et al., 2011 observed a trend towards hypersensitivity in d4T-treated mice over a 28 day period; however there were no significant differences in PWLs when compared to the saline controls. These findings are shared by Wallace et al., 2007 indicating that thermal hypersensitivity is not a significant consequence of NRTI treatment. This view is consistent with clinical data showing that patients with HIV-related pain do not usually present with thermal hypersensitivity (Martin et al., 2003). Nevertheless it would be worth perusing whether there is thermal hypersensitivity associated with d4T in rats, rather than in mice, since this has been the common species of choice regarding ATN modeling.

Decosterd and Woolf, 2000, using the SNI and sciatic nerve crush injury models revealed no changes in PWL in response to a radiant heat stimulus (using a Hargreaves device). However the duration of withdrawal in response to the heat stimulus increased significantly 14 days post injury. Perhaps it would have been more appropriate to measure the duration of response when assessing thermal behavioural responses in TNT injured rats. The measurement of PWLs represents the most widely used outcome measure in pain studies, but this is not a feature typical in neuropathic pain patients (Koltzenburg, 1998). In fact, in certain neuropathic states such as PHN, there is a paradoxical phenomenon of thermal sensory loss in areas where extreme pain is experienced (Rowbotham and Fields, 1996).

6.6.3 Oversimplification of DRG cellular phenotype markers

It is widely acknowledged that small sized DRG cells are the unmyelinated C-fibre population which encode nociceptive information and the larger sized DRG cells are the myelinated A β fibre population which encode non-noxious stimuli (Snider and McMahon, 1998). The size of a DRG cell correlates with both its physiological and electrophysiological properties. Large diameter DRG cells have fast conduction velocities whereas the smaller C-fibres have slow conduction velocities (Lawson and Waddell, 1991). Various markers can be used to identify different subpopulations of sensory neurons (see Figure 1.5, **Chapter 1**). However the notion that there are distinct populations of sensory neurons in the DRG is somewhat oversimplified. For instance, there is ~10% crossover between the small diameter IB4 and CGRP cell populations (see Figure 1.5, **Chapter 1**). Furthermore, crossover is evident between the large myelinated NF-200 and the CGRP population and also the peripherin population (<8%) (Quick et al., 2012). In this thesis, immunohistochemical analysis did not account for potential cross over between peripherin and NF-200 immunoreactive neurons which could have been identified via specific double labeling control experiments. However it is to be noted that the cell size distribution analysis of peripherin and NF-200 immunoreactive neurons provided an important safeguard, and would have revealed any potential inconsistencies in the cell size. Another limitation of the study was the analysis of investigative markers did not account for the possibility of such labeling not occurring in either the peripherin (small) or NF-200 (large) populations examined.

Another limitation of the study, which was not previously considered, was the possibility of changes in the expression of peripherin and NF-200 in the DRG after nerve injury which could potentially have affected the reported percentage temporal profiles of ATF-3, GAP-43, NPY, and galanin. There is evidence to suggest that the mRNA for NF-200 is decreased after nerve crush injury within the first 4 weeks and only start to recover after this time (Wong and Oblinger, 1990). Another study using insitu hybridization has revealed that the mRNA for peripherin (type III) in small sized neurons is not significantly different from that observed in normal uninjured rats 2 weeks after axotomy. At this time medium and large sized DRG increased their mRNA for peripherin (type III) (Oblinger et al., 1989). This evidence suggests that there is a possibility that the protein levels of peripherin and NF-200 may not been consistent throughout the study, but the extent to which the results are affected is yet to be determined. This aspect of the study could be further investigated by counting the total number nucleated peripherin and NF-200 cells in the L5 DRG across the various study time points to correct for any potential variability.

6.6.4 Co-localization of investigative markers

The study could be further extended by completing double labeling experiments between ATF-3 and other investigative markers (i.e. NPY, galanin, GAP-43) in the DRG - this will identify if those neurons exhibiting evidence of regeneration or altered neuropeptide expression are axotomized (injured) or intact neurons. Previous experiments using different trauma models have shown NPY co-localises with other neuropeptides that have been associated with nerve injury such as vasoactive intestinal peptide (VIP) & galanin (Ma and Bisby, 1998), reg-2 (Averill et al., 2002), and ATF-3 (Shortland et al., 2006). It would also be of interest if the sciatic nerve could be examined for GAP-43 expression which will indicate nerve regeneration occurring in the tibial nerve.

6.6.5 Characterization of other pathological/dysregulated proteins

In recent years, a number of other neuropeptides have been associated with the response to nerve injury. Reg-2 (also known as PAP1) is a macrophage chemoattractant that has been

reported to exhibit proregenerative properties in motor and sensory neurons after peripheral nerve injury by stimulating Schwann cell growth (Livesey et al., 1997; Namikawa et al., 2006). Reg-2 is not normally expressed in the adult rat nervous system (Livesey et al., 1997). However 24 hours after peripheral nerve injury, reg-2 is upregulated, predominately in small diameter cell neurons, with expression switching to larger cells (within 5 days); an observation suggestive of differing regeneration mechanisms/factors in various DRG subpopulations (Averill et al., 2002). The upregulation of reg-2 mRNA has been additionally observed in microarray experiments involving the DRG 14 days following SNL (Maratou et al., 2009).

Of interest is VGF, a neuropeptide that is associated with the regulation of metabolism (Hahm et al., 1999) and synaptic plasticity (Alder et al., 2003), but its role in neuropathic pain is unknown. The upregulation of VGF mRNA in the L5 DRG of SNL rats 14 days after injury is suggestive of an associative role in nerve damage (Maratou et al., 2009), and perhaps neuropathic pain, and could have been further investigated. VGF, like other neuropeptides, maintains low expression levels in normal conditions, but peripheral nerve injury results in increased VGF expression in both small and large diameter L4/5 DRG neurons following SNI (Moss et al., 2008). VGF gene transcription is driven by the neurotrophic factors NGF and BDNF, which are released by macrophages, mast cells, and Schwann cells following peripheral nerve injury, and could have been additionally investigated.

VIP - another neuropeptide that is upregulated in nerve injury (Ma and Bisby, 1998) (predominately in small diameter neurons) could be investigated. The intrathecal administration of VIP decreases reaction time of the tail flick test, and reveals a potentially excitatory role in nociception and perhaps neuropathic pain (Cridland and Henry, 1988). Lastly, immunohistochemical analysis for caspase-3 in the DRG (an indicator of apoptotic processes) would particularly be of interest especially in the acute stages of nerve trauma, given the high level of ATF-3 expression present at this stage following nerve trauma.

One way to identify potential mechanisms regarding neuropathic pain, and specifically NRTI related neuropathy, is to conduct a gene microarray of the DRG. Such studies have been performed using peripheral nerve trauma animals (Costigan et al., 2002; Maratou et al., 2009; Valder et al., 2003; Wang et al., 2002) but not in NRTI treated animals. A similar approach has been used to describe dysregulated genes involving the combined ddC + gp120 model (Maratou et al., 2009), but such data is complicated by the toxic effects of the gp120. A gene microarray and proteomic study could identify dysregulated proteins in the DRG of trauma and NRTI treated animals, and comparisons could be made between the two model types. The advantage of proteomics is that further information regarding protein isoforms, modifications, and the interactions between them can be obtained (Tyers and Mann, 2003); this can provide targets for conventional immunohistochemistry studies.

6.6.6 Analysis of other nervous tissue

A possible drawback of the study is other tissues (e.g. L4 DRGs, contralateral L5 DRGs, sural & sciatic nerve, spinal cord, and paw skin) were not examined. Based on observations from the Fluorogold tract tracing study, it would appear that the majority of damage following TNT injury occurs in the L4 DRG, and therefore it would have been of interest to examine the expression of biochemical markers here to make comparisons with the L5 DRG. Evidence from previous studies using trauma models indicate either no or minimal changes in expression of neuropeptides in contralateral DRGs (Ma and Bisby, 1997), however this has not been confirmed in the TNT model. Since d4T injury is bilateral, any altered expression of investigative makers in the opposing DRG would have been expected to be identical. Neuropeptide expression has been observed in the gracilis tract of the spinal cord of trauma animals, and it is likely that in this context NPY is transported from the DRG into the spinal cord where it can exert its anti nociceptive effects. Neuropeptide immunoreactivity could have been examined in the spinal cord.

Sural nerve biopsies from patients with ddC-neuropathy have shown severe axonal destruction, prominent in unmyelinated fibres (Lewis and Dalakas, 1995). This could be examined in d4T treated rats via immunohistochemical staining of β -amyloid precursor

protein (β -APP) - an axonal injury marker that accumulates in the axon (Medana and Esiri, 2003). Moreover, the extent of any morphological damage observed can be correlated to the loss of small fibres observed in paw skin (Wallace et al., 2007b). The study could additionally be improved by investigating the expression profiles of other markers that have been implicated in NRTI associated neuropathy, particularly CCL2, which is upregulated in the DRG following ddC treatment (Wallace et al., 2007b). Macrophage recruitment in the DRG of ddC treated animals (Wallace et al., 2007b) could be further investigated by characterizing CD-68 expression over time and could be correlated with the CCL2 expression. Since TNF α is found in the spinal cord of ddC treated animals (Zheng et al., 2011c), it would be interesting to investigate similar events in DRG, especially given the presence of macrophages, that are capable of releasing TNF α (Holguin et al., 2004), thus altering the excitability of neurons (Schafers et al., 2003b).

6.6.7 Additional immunohistochemistry control experiments for ED-1 in saline/DHA treated rats

This study could have considered the use of a naive group for the ED-1 immunohistochemistry as an additional control to make a comparison to baseline levels since both saline and DHA treated rats underwent TNT surgery. Additionally a negative control could have been conducted to assert the validity of the ED-1 immunohistochemistry.

6.6.8 Measuring co-morbidities of pain

Patients suffering from neuropathic pain often experience co-morbidities such as sleep disturbances, physical inactivity, stress, anxiety and depression (Daniel et al., 2008; Meyer-Rosberg et al., 2001; Nicholson and Verma, 2004). Other co-morbidities can be associated with particular chronic pain syndromes such as complex regional pain syndrome (CRPS) such as asthma, osteoporosis, and asthma (de Mos et al., 2008). To extend the current study, more outcome measures of 'neuropathic pain' behaviour could have been included to address these co-morbidities. For example, the open field or elevated plus maze could measure anxiety-like behaviours in TNT injured or d4T treated rats. These assays are

ethologically valid, and utilizes the conflict between the rats desire to explore new environments and their natural aversion to exposed well-lit areas. Such anxiety-like behaviours have been demonstrated in peripheral nerve trauma and ddC-treated rats (Hasnie et al., 2007;Roeska et al., 2008;Seminowicz et al., 2009;Wallace et al., 2007c;Wallace et al., 2007b). Other researchers have focused on assessing depressive-like behaviours, using assays such as the FST or the tail suspension test which additionally could be used in a future study (Cryan et al., 2005;Cryan and Holmes, 2005).

6.7 Conclusion

The past 20 years has seen a number of advances in technology and neuropathic pain research that has furthered our understanding and led to the identification of potential targets for drug development. For instance, gene microarrays have allowed the analysis of global gene expression in the DRG following nerve injury.

In this thesis I have:

1. Demonstrated that there are distinct differences in the neurochemical responses to nerve damage in the DRG in two different types of nerve damage that are both associated with neuropathic pain; TNT nerve trauma and d4T associated neuropathy. This may have implications for neuropathic pain research in terms of how potential mechanisms are interpreted since the expression of a given marker after nerve damage may not always be necessarily indicative of behavioural hypersensitivity or neuropathic pain.
2. Shown no significant changes in the expression of ATF-3, GAP-43, NPY and galanin in the L5 DRG following d4T administration in rats.
3. Demonstrated that in the acute stages of nerve trauma damage (one day post TNT injury), large diameter fibres are more proportionally affected, which is indicated by

a greater percentage of ATF-3 expression in NF-200 immunoreactive neurons compared to peripherin immunoreactive neurons in the ipsilateral L5 DRG.

4. Shown dynamic expression patterns of ATF-3, NPY, galanin, and GAP-43 following TNT injury. While the expression of these markers generally peaked at post operative day 14 (with exception of ATF-3), this did not correspond with the peak of mechanical hypersensitivity which was observed 28 days post TNT injury.
5. Demonstrated no correlations between TNT associated mechanical hypersensitivity and the expression of ATF-3, NPY, GAP-43, and galanin, suggesting that their expression is not critical towards the magnitude of neuropathic pain states. This further supports the argument that these markers are markers of nerve trauma and not neuropathic pain.
6. Raised concerns regarding the dose of DHA used to achieve neuroprotection following peripheral trauma nerve injury since the dose used in the study may have been sub-optimal which possibly explains the lack of effect. Further research is warranted to understand the mechanisms of the neuroprotection, with the first step being an appropriate dose response of DHA to peripheral trauma nerve injury.

Reference List

- Abbadie, C., Lindia, J.A., Cumiskey, A.M., Peterson, L.B., Mudgett, J.S., Bayne, E.K., Demartino, J.A., Macintyre, D.E., and Forrest, M.J. (2003). Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proc. Natl. Acad. Sci. U. S. A* 100, 7947-7952.
- Abdelrahman, M., Sivarajah, A., and Thiernemann, C. (2005). Beneficial effects of PPAR- α ligands in ischemia/reperfusion injury, inflammation and shock. *Cardiovascular Research* 65, 772-781.
- Abdi, S., Lee, D.H., and Chung, J.M. (1998). The anti-allodynic effects of amitriptyline, gabapentin, and lidocaine in a rat model of neuropathic pain. *Anesthesia and Analgesia* 87, 1360-1366.
- Abdulla, F.A., and Smith, P.A. (1999). Nerve injury increases an excitatory action of neuropeptide Y and Y2-agonists on dorsal root ganglion neurons. *Neuroscience* 89, 43-60.
- Abdulla, F.A., Stebbing, M.J., and Smith, P.A. (2001). Effects of substance P on excitability and ionic currents of normal and axotomized rat dorsal root ganglion neurons. *Eur. J. Neurosci.* 13, 545-552.
- Affandi, J.S., Price, P., Imran, D., Yuniastuti, E., Djauzi, S., and Cherry, C.L. (2008). Can we predict neuropathy risk before stavudine prescription in a resource-limited setting? *AIDS Res. Hum. Retroviruses* 24, 1281-1284.
- Aigner, L., Arber, S., Kapfhammer, J.P., Laux, T., Schneider, C., Botteri, F., Brenner, H.R., and Caroni, P. (1995). Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. *Cell* 83, 269-278.
- Ajmone-Cat, M.A., Salvatori, M.L., De Simone, R., Mancini, M., Biagioni, S., Bernardo, A., Cacci, E., and Minghetti, L. (2011). Docosahexaenoic acid modulates inflammatory and antineurogenic functions of activated microglial cells. *J. Neurosci. Res.*
- Alder, J., Thakker-Varia, S., Bangasser, D.A., Kuroiwa, M., Plummer, M.R., Shors, T.J., and Black, I.B. (2003). Brain-derived neurotrophic factor-induced gene expression reveals novel actions of VGF in hippocampal synaptic plasticity. *J. Neurosci.* 23, 10800-10808.
- Aldskogius, H., and Kozlova, E.N. (1998). Central neuron-glial and glial-glial interactions following axon injury. *Prog. Neurobiol.* 55, 1-26.
- Amir, R., and Devor, M. (1996). Chemically mediated cross-excitation in rat dorsal root ganglia. *J. Neurosci.* 16, 4733-4741.
- Anderson, T.D., Davidovich, A., Arceo, R., Brosnan, C., Arezzo, J., and Schaumburg, H. (1992). Peripheral neuropathy induced by 2',3'-dideoxycytidine. A rabbit model of 2',3'-dideoxycytidine neurotoxicity. *Lab Invest* 66, 63-74.
- Andreatini, R., and Bacellar, L.F. (1999). The relationship between anxiety and depression in animal models: a study using the forced swimming test and elevated plus-maze. *Braz. J. Med. Biol. Res.* 32, 1121-1126.
- Andrews, K. (1993). The effect of changes in temperature and humidity on the accuracy of von Frey hairs. *J. Neurosci. Methods* 50, 91-93.
- Andrews, N., Legg, E., Lisak, D., Issop, Y., Richardson, D., Harper, S., Huang, W., Burgess, G., Machin, I., and Rice, A.S. (2011). Spontaneous burrowing behaviour in the rat is reduced by peripheral nerve injury or inflammation associated pain. *Eur. J. Pain.*

- Ariel, A., Fredman, G., Sun, Y.P., Kantarci, A., Van Dyke, T.E., Luster, A.D., and Serhan, C.N. (2006). Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. *Nat. Immunol.* 7, 1209-1216.
- Ariel, A., Li, P.L., Wang, W., Tang, W.X., Hong, S., Gotlinger, K.H., and Serhan, C.N. (2005). The novel docosatriene, Protectin D1, produced by T(H)2-polarization promotes human T cell apoptosis via lipid-raft clustering. *Clinical Immunology* 115, S263.
- Arnold, C., Konkel, A., Fischer, R., and Schunck, W.H. (2010). Cytochrome P450-dependent metabolism of omega-6 and omega-3 long-chain polyunsaturated fatty acids. *Pharmacol. Rep.* 62, 536-547.
- Asbury, A.K., and Fields, H.L. (1984). Pain due to peripheral nerve damage: an hypothesis. *Neurology* 34, 1587-1590.
- Averill, S., Davis, D.R., Shortland, P.J., Priestley, J.V., and Hunt, S.P. (2002). Dynamic pattern of reg-2 expression in rat sensory neurons after peripheral nerve injury. *J. Neurosci* 22, 7493-7501.
- Averill, S., Michael, G.J., Shortland, P.J., Leavesley, R.C., King, V.R., Bradbury, E.J., McMahon, S.B., and Priestley, J.V. (2004). NGF and GDNF ameliorate the increase in ATF3 expression which occurs in dorsal root ganglion cells in response to peripheral nerve injury. *Eur. J. Neurosci* 19, 1437-1445.
- Bacellar, H., Munoz, A., Miller, E.N., Cohen, B.A., Besley, D., Selnes, O.A., Becker, J.T., and McArthur, J.C. (1994). Temporal trends in the incidence of HIV-1-related neurologic diseases: Multicenter AIDS Cohort Study, 1985-1992. *Neurology* 44, 1892-1900.
- Backonja, M.M., Miletic, G., and Miletic, V. (1995). The Effect of Continuous Morphine Analgesia on Chronic Thermal Hyperalgesia Due to Sciatic Constriction Injury in Rats. *Neuroscience Letters* 196, 61-64.
- Bahia, P.K., Suzuki, R., Benton, D.C., Jowett, A.J., Chen, M.X., Trezise, D.J., Dickenson, A.H., and Moss, G.W. (2005). A functional role for small-conductance calcium-activated potassium channels in sensory pathways including nociceptive processes. *J. Neurosci* 25, 3489-3498.
- Barclay, M., Noakes, P.G., Ryan, A.F., Julien, J.P., and Housley, G.D. (2007). Neuronal expression of peripherin, a type III intermediate filament protein, in the mouse hindbrain. *Histochem. Cell Biol.* 128, 541-550.
- Bardin, L., Malfetes, N., Tancredi, A.N., and Depoortere, R. (2009). Chronic restraint stress induces mechanical and cold allodynia, and enhances inflammatory pain in rat: Relevance to human stress-associated painful pathologies. *Behavioural Brain Research* 205, 360-366.
- Baron, R. (2006). Mechanisms of disease: neuropathic pain--a clinical perspective. *Nat Clin. Pract. Neurol.* 2, 95-106.
- Baron, R., Tolle, T.R., Gockel, U., Brosz, M., and Freynhagen, R. (2009). A cross-sectional cohort survey in 2100 patients with painful diabetic neuropathy and postherpetic neuralgia: Differences in demographic data and sensory symptoms. *Pain* 146, 34-40.
- Barrette, B., Hebert, M.A., Filali, M., Lafortune, K., Vallieres, N., Gowing, G., Julien, J.P., and Lacroix, S. (2008). Requirement of myeloid cells for axon regeneration. *Journal of Neuroscience* 28, 9363-9376.
- Basbaum, A.I., Gauthron, M., Jazat, F., Mayes, M., and Guilbaud, G. (1991). The spectrum of fiber loss in a model of neuropathic pain in the rat: an electron microscopic study. *Pain* 47, 359-367.
- Bauer, C.S., Rahman, W., Tran-Van-Minh, A., Lujan, R., Dickenson, A.H., and Dolphin, A.C. (2010). The anti-allodynic alpha(2)delta ligand pregabalin inhibits the trafficking of the calcium channel alpha(2)delta-1 subunit to presynaptic terminals in vivo. *Biochem. Soc. Trans.* 38, 525-528.

- Bazan,N.G. (2005). Neuroprotectin D1 (NPD1): A DHA-derived mediator that protects brain and retina against cell injury-induced oxidative stress. *Brain Pathology* 15, 159-166.
- Bazan,N.G. (2006). Cell survival matters: docosahexaenoic acid signaling, neuroprotection and photoreceptors. *Trends in Neurosciences* 29, 263-271.
- Bazan,N.G. (2007). Omega-3 fatty acids, pro-inflammatory signaling and neuroprotection. *Current Opinion in Clinical Nutrition and Metabolic Care* 10, 136-141.
- Bazan,N.G. (2009). Neuroprotectin D1-mediated anti-inflammatory and survival signaling in stroke, retinal degenerations, and Alzheimer's disease. *J. Lipid Res.* 50 *Suppl*, S400-S405.
- Beart,P.M., and O'Shea,R.D. (2007). Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. *Br. J. Pharmacol.* 150, 5-17.
- Beck,E.J., Mandalia,S., Williams,I., Power,A., Newson,R., Molesworth,A., Barlow,D., Easterbrook,P., Fisher,M., Innes,J., Kinghorn,G., Mandel,B., Pozniak,A., Tang,A., and Tomlinson,D. (1999). Decreased morbidity and use of hospital services in English HIV-infected individuals with increased uptake of anti-retroviral therapy 1996-1997. National Prospective Monitoring System Steering Group. *AIDS* 13, 2157-2164.
- Bee,L.A., and Dickenson,A.H. (2008). Descending facilitation from the brainstem determines behavioural and neuronal hypersensitivity following nerve injury and efficacy of pregabalin. *Pain* 140, 209-223.
- Belayev,L., Khoutorova,L., Atkins,K.D., and Bazan,N.G. (2009). Robust docosahexaenoic acid-mediated neuroprotection in a rat model of transient, focal cerebral ischemia. *Stroke* 40, 3121-3126.
- Belayev,L., Khoutorova,L., Atkins,K.D., Eady,T.N., Hong,S., Lu,Y., Obenaus,A., and Bazan,N.G. (2011). Docosahexaenoic Acid Therapy of Experimental Ischemic Stroke. *Transl. Stroke Res.* 2, 33-41.
- Belayev,L., Marcheselli,V.L., Khoutorova,L., Rodriguez de Turco,E.B., Busto,R., Ginsberg,M.D., and Bazan,N.G. (2005). Docosahexaenoic acid complexed to albumin elicits high-grade ischemic neuroprotection. *Stroke* 36, 118-123.
- Benemei,S., Nicoletti,P., Capone,J.G., and Geppetti,P. (2009). CGRP receptors in the control of pain and inflammation. *Curr. Opin. Pharmacol.* 9, 9-14.
- Benn,S.C., and Woolf,C.J. (2004). Adult neuron survival strategies--slamming on the brakes. *Nat. Rev. Neurosci.* 5, 686-700.
- Bennett,D.L., French,J., Priestley,J.V., and McMahon,S.B. (1996a). NGF but not NT-3 or BDNF prevents the A fiber sprouting into lamina II of the spinal cord that occurs following axotomy. *Mol. Cell Neurosci* 8, 211-220.
- Bennett,D.L.H., Groves,M., Blake,J., Holton,J.L., King,R.H.M., Orrell,R.W., Ginsberg,L., and Reilly,M.M. (2008). The use of nerve and muscle biopsy in the diagnosis of vasculitis: a 5 year retrospective study. *Journal of Neurology Neurosurgery and Psychiatry* 79, 1376-1381.
- Bennett,D.L.H., Dmietrieva,N., Priestley,J.V., Clary,D., and McMahon,S.B. (1996b). trkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. *Neuroscience Letters* 206, 33-36.
- 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, 87-107.

- Benoliel,R., Eliav,E., and Iadarola,M.J. (2001). Neuropeptide Y in trigeminal ganglion following chronic constriction injury of the rat infraorbital nerve: is there correlation to somatosensory parameters? *Pain* 91, 111-121.
- Benowitz,L.I., and Routtenberg,A. (1997). GAP-43: An intrinsic determinant of neuronal development and plasticity. *Trends in Neurosciences* 20, 84-91.
- Bergman,E., Carlsson,K., Liljeborg,A., Manders,E., Hokfelt,T., and Ulfhake,B. (1999). Neuropeptides, nitric oxide synthase and GAP-43 in B4-binding and RT97 immunoreactive primary sensory neurons: normal distribution pattern and changes after peripheral nerve transection and aging. *Brain Res.* 832, 63-83.
- Bertoglio,L.J., and Carobrez,A.P. (2000). Previous maze experience required to increase open arms avoidance in rats submitted to the elevated plus-maze model of anxiety. *Behav. Brain Res.* 108, 197-203.
- Besse,D., Lombard,M.C., and Besson,J.M. (1991). Autoradiographic distribution of mu, delta and kappa opioid binding sites in the superficial dorsal horn, over the rostrocaudal axis of the rat spinal cord. *Brain Res.* 548, 287-291.
- Besse,D., Lombard,M.C., and Besson,J.M. (1992a). Time-related decreases in mu and delta opioid receptors in the superficial dorsal horn of the rat spinal cord following a large unilateral dorsal rhizotomy. *Brain Res.* 578, 115-127.
- Besse,D., Lombard,M.C., Perrot,S., and Besson,J.M. (1992b). Regulation of opioid binding sites in the superficial dorsal horn of the rat spinal cord following loose ligation of the sciatic nerve: comparison with sciatic nerve section and lumbar dorsal rhizotomy. *Neuroscience* 50, 921-933.
- Besson,M., Brook,P., Chizh,B.A., and Pickering,A.E. (2005). Tactile allodynia in patients with postherpetic neuralgia: Lack of change in skin blood flow upon dynamic stimulation. *Pain* 117, 154-161.
- Bhangoo,S.K., Ren,D., Miller,R.J., Chan,D.M., Ripsch,M.S., Weiss,C., McGinnis,C., and White,F.A. (2007). CXCR4 chemokine receptor signaling mediates pain hypersensitivity in association with antiretroviral toxic neuropathy. *Brain Behav. Immun.* 21, 581-591.
- Biel,M., Wahl-Schott,C., Michalakis,S., and Zong,X. (2009). Hyperpolarization-activated cation channels: from genes to function. *Physiol Rev.* 89, 847-885.
- Birklein,F., Schmelz,M., Schifter,S., and Weber,M. (2001). The important role of neuropeptides in complex regional pain syndrome. *Neurology* 57, 2179-2184.
- Bisby,M.A., and Chen,S. (1990). Delayed wallerian degeneration in sciatic nerves of C57BL/Ola mice is associated with impaired regeneration of sensory axons. *Brain Res.* 530, 117-120.
- Blackbeard,J., O'Dea,K.P., Wallace,V.C., Segerdahl,A., Pheby,T., Takata,M., Field,M.J., and Rice,A.S. (2007). Quantification of the rat spinal microglial response to peripheral nerve injury as revealed by immunohistochemical image analysis and flow cytometry. *J. Neurosci Methods* 164, 207-217.
- Blackbeard,J., Wallace,V.C.J., O'Dea,K.P., Hasnie,F., Segerdahl,A., Pheby,T., Field,M.J., Takata,M., and Rice,A.S.C. (2012). The correlation between pain-related behaviour and spinal microgliosis in four distinct models of peripheral neuropathy. *European Journal of Pain* n/a.
- Blackburn-Munro,G., and Jensen,B.S. (2003). The anticonvulsant retigabine attenuates nociceptive behaviours in rat models of persistent and neuropathic pain. *Eur. J. Pharmacol.* 460, 109-116.

- Blakeman, K.H., Hao, J.X., Xu, X.J., Jacoby, A.S., Shine, J., Crawley, J.N., Iismaa, T., and Wiesenfeld-Hallin, Z. (2003). Hyperalgesia and increased neuropathic pain-like response in mice lacking galanin receptor 1 receptors. *Neuroscience* 117, 221-227.
- Bleakman, D., Alt, A., and Nisenbaum, E.S. (2006). Glutamate receptors and pain. *Semin. Cell Dev. Biol.* 17, 592-604.
- Blondeau, N., Widmann, C., Lazdunski, M., and Heurteaux, C. (2002). Polyunsaturated fatty acids induce ischemic and epileptic tolerance. *Neuroscience* 109, 231-241.
- Blumenkopf, B., and Lipman, J.J. (1991). Studies in autotomy: its pathophysiology and usefulness as a model of chronic pain. *Pain* 45, 203-209.
- Bodner, A., Toth, P.T., and Miller, R.J. (2004). Activation of c-Jun N-terminal kinase mediates gp120IIIB- and nucleoside analogue-induced sensory neuron toxicity. *Experimental Neurology* 188, 246-253.
- Boyce, S., Wyatt, A., Webb, J.K., O'Donnell, R., Mason, G., Rigby, M., Sirinathsinghji, D., Hill, R.G., and Rupniak, N.M. (1999). Selective NMDA NR2B antagonists induce antinociception without motor dysfunction: correlation with restricted localisation of NR2B subunit in dorsal horn. *Neuropharmacology* 38, 611-623.
- Braissant, O., Fougère, F., Scotto, C., Dauca, M., and Wahli, W. (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs): Tissue distribution of PPAR- α , - β , and - γ in the adult rat. *Endocrinology* 137, 354-366.
- Braz, J.M., and Basbaum, A.I. (2010). Differential ATF3 expression in dorsal root ganglion neurons reveals the profile of primary afferents engaged by diverse noxious chemical stimuli. *Pain* 150, 290-301.
- Braz, J.M., Nassar, M.A., Wood, J.N., and Basbaum, A.I. (2005). Parallel "pain" pathways arise from subpopulations of primary afferent nociceptor. *Neuron* 47, 787-793.
- Brewer, A., Langel, U., and Robinson, J.K. (2005). Intracerebroventricular administration of galanin decreases free water intake and operant water reinforcer efficacy in water-restricted rats. *Neuropeptides* 39, 117-124.
- Bridges, D., Ahmad, K., and Rice, A.S. (2001a). The synthetic cannabinoid WIN55,212-2 attenuates hyperalgesia and allodynia in a rat model of neuropathic pain. *Br. J. Pharmacol.* 133, 586-594.
- Bridges, D., Thompson, S.W., and Rice, A.S. (2001b). Mechanisms of neuropathic pain. *Br. J. Anaesth.* 87, 12-26.
- Brinkman, K., Delnoy, P.P., and de, P.B. (1998). Highly active antiretroviral therapy (HAART) and prolonged survival of a patient with an HIV-related Burkitt lymphoma, despite an intracardiac relapse. *Int. J. STD AIDS* 9, 773-775.
- Brinkman, K., Smeitink, J.A., Romijn, J.A., and Reiss, P. (1999). Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. *Lancet* 354, 1112-1115.
- Brooks, J., and Tracey, I. (2005). From nociception to pain perception: imaging the spinal and supraspinal pathways. *J. Anat.* 207, 19-33.
- Bruce, J.C., Oatway, M.A., and Weaver, L.C. (2002). Chronic pain after clip-compression injury of the rat spinal cord. *Exp. Neurol.* 178, 33-48.

- Brumovsky,P.R., Bergman,E., Liu,H.X., Hokfelt,T., and Villar,M.J. (2004). Effect of a graded single constriction of the rat sciatic nerve on pain behavior and expression of immunoreactive NPY and NPY Y1 receptor in DRG neurons and spinal cord. *Brain Res.* 1006, 87-99.
- Burgess,S.E., Gardell,L.R., Ossipov,M.H., Malan,T.P., Jr., Vanderah,T.W., Lai,J., and Porreca,F. (2002). Time-dependent descending facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain. *J. Neurosci.* 22, 5129-5136.
- Calcutt,N.A., and Chaplan,S.R. (1997). Spinal pharmacology of tactile allodynia in diabetic rats. *Br. J. Pharmacol.* 122, 1478-1482.
- Calvo,M., and Bennett,D.L.H. (2012). The mechanisms of microgliosis and pain following peripheral nerve injury. *Experimental Neurology* 234, 271-282.
- Calvo,M., Huang ,W., and Bennett,D.L.H. An animal model of stavudine (d4t)-induced painful peripheral sensory neuropathy is associated with 'dying back' of of unmyelinated axons. International Association for the Study of Pain, 14th World Congress on Pain, August 27-31, 2012, Milan, Italy . 2012a.
Ref Type: Abstract
- Calvo,M., Dawes,J.M., and Bennett,D.L. (2012b). The role of the immune system in the generation of neuropathic pain. *The Lancet Neurology* 11, 629-642.
- Cao,D., Xue,R., Xu,J., and Liu,Z. (2005). Effects of docosahexaenoic acid on the survival and neurite outgrowth of rat cortical neurons in primary cultures. *J Nutr. Biochem.* 16, 538-546.
- Cao,L., and Deleo,J.A. (2008). CNS-infiltrating CD4+ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain. *Eur. J. Immunol.* 38, 448-458.
- Carmichael,S. (2005). Rodent models of focal stroke: Size, mechanism, and purpose. *Neurotherapeutics* 2, 396-409.
- Carr,D.B., and Goudas,L.C. (1999). Acute pain. *Lancet* 353, 2051-2058.
- Caterina,M.J., Leffler,A., Malmberg,A.B., Martin,W.J., Trafton,J., Petersen-Zeit,K.R., Koltzenburg,M., Basbaum,A.I., and Julius,D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 288, 306-313.
- Chaplan,S.R., Guo,H.Q., Lee,D.H., Luo,L., Liu,C., Kuei,C., Velumian,A.A., Butler,M.P., Brown,S.M., and Dubin,A.E. (2003). Neuronal hyperpolarization-activated pacemaker channels drive neuropathic pain. *J. Neurosci.* 23, 1169-1178.
- Chaplan,S.R., Pogrel,J.W., and Yaksh,T.L. (1994). Role of voltage-dependent calcium channel subtypes in experimental tactile allodynia. *J. Pharmacol. Exp. Ther.* 269, 1117-1123.
- Chen,B.P., Liang,G., Whelan,J., and Hai,T. (1994). ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms. *J. Biol. Chem.* 269, 15819-15826.
- Chen,B.P., Wolfgang,C.D., and Hai,T. (1996). Analysis of ATF3, a transcription factor induced by physiological stresses and modulated by gadd153/Chop10. *Mol. Cell Biol.* 16, 1157-1168.
- Chen,C.H., and Cheng,Y.C. (1989). Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2',3'-dideoxycytidine. *J. Biol. Chem.* 264, 11934-11937.

- Chen, C.H., Vazquez-Padua, M., and Cheng, Y.C. (1991). Effect of anti-human immunodeficiency virus nucleoside analogs on mitochondrial DNA and its implication for delayed toxicity. *Mol. Pharmacol.* 39, 625-628.
- Chen, X., and Levine, J.D. (2007). Mechanically-evoked C-fiber activity in painful alcohol and AIDS therapy neuropathy in the rat. *Mol. Pain* 3, 5.
- Cherry, C.L., Affandi, J.S., Imran, D., Yuniastuti, E., Smyth, K., Vanar, S., Kamarulzaman, A., and Price, P. (2009). Age and height predict neuropathy risk in patients with HIV prescribed stavudine. *Neurology* 73, 315-320.
- Cherry, C.L., and Wesselingh, S.L. (2003). Nucleoside analogues and HIV: the combined cost to mitochondria. *Journal of Antimicrobial Chemotherapy* 51, 1091-1093.
- Cherry, C.L., Kamerman, P.R., Bennett, D.L., and Rice, A.S. (2012). HIV-associated sensory neuropathy: still a problem in the post-stavudine era? *Future Virology* 7, 849-854.
- Cheunsuang, O., and Morris, R. (2000). Spinal lamina I neurons that express neurokinin 1 receptors: morphological analysis. *Neuroscience* 97, 335-345.
- Cho, S.Y., Miyashita, K., Miyazawa, T., Fujimoto, K., and Kaneda, T. (1987). Autoxidation of ethyl eicosapentaenoate and docosahexaenoate. *Journal of the American Oil Chemists' Society* 64, 876-879.
- Christensen, M.D., Everhart, A.W., Pickelman, J.T., and Hulsebosch, C.E. (1996). Mechanical and thermal allodynia in chronic central pain following spinal cord injury. *Pain* 68, 97-107.
- Chung, S.W., Kang, B.Y., Kim, S.H., Pak, Y.K., Cho, D., Trinchieri, G., and Kim, T.S. (2000). Oxidized low density lipoprotein inhibits interleukin-12 production in lipopolysaccharide-activated mouse macrophages via direct interactions between peroxisome proliferator-activated receptor-gamma and nuclear factor-kappa B. *Journal of Biological Chemistry* 275, 32681-32687.
- Clatworthy, A.L., Illich, P.A., Castro, G.A., and Walters, E.T. (1995). Role of Peri-Axonal Inflammation in the Development of Thermal Hyperalgesia and Guarding Behavior in A Rat Model of Neuropathic Pain. *Neuroscience Letters* 184, 5-8.
- Coderre, T.J., Grimes, R.W., and Melzack, R. (1986). Deafferentation and chronic pain in animals: an evaluation of evidence suggesting autotomy is related to pain. *Pain* 26, 61-84.
- 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, 233-242.
- Cohrs, R.J., Randall, J., Smith, J., Gilden, D.H., Dabrowski, C., van Der, K.H., and Tal-Singer, R. (2000). Analysis of individual human trigeminal ganglia for latent herpes simplex virus type 1 and varicella-zoster virus nucleic acids using real-time PCR. *J. Virol.* 74, 11464-11471.
- Corness, J., Stevens, B., Fields, R.D., and Hokfelt, T. (1998). NGF and LIF both regulate galanin gene expression in primary DRG cultures. *Neuroreport* 9, 1533-1536.
- Coronel, M.F., Brumovsky, P.R., Hokfelt, T., and Villar, M.J. (2008). Differential galanin upregulation in dorsal root ganglia and spinal cord after graded single ligature nerve constriction of the rat sciatic nerve. *J. Chem. Neuroanat.* 35, 94-100.
- Costigan, M., Befort, K., Karchewski, L., Griffin, R.S., D'Urso, D., Allchorne, A., Sitarski, J., Mannion, J.W., Pratt, R.E., and Woolf, C.J. (2002). Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC. Neurosci* 3, 16.

- Costigan,M., Moss,A., Latremoliere,A., Johnston,C., Verma-Gandhu,M., Herbert,T.A., Barrett,L., Brenner,G.J., Vardeh,D., Woolf,C.J., and Fitzgerald,M. (2009). T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. *J. Neurosci.* 29, 14415-14422.
- Coull,J.A., Beggs,S., Boudreau,D., Boivin,D., Tsuda,M., Inoue,K., Gravel,C., Salter,M.W., and De,K.Y. (2005). BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* 438, 1017-1021.
- Courteix,C., Eschalier,A., and Lavarenne,J. (1993). Streptozocin-induced diabetic rats: behavioural evidence for a model of chronic pain. *Pain* 53, 81-88.
- Coward,K., Plumpton,C., Facer,P., Birch,R., Carlstedt,T., Tate,S., Bountra,C., and Anand,P. (2000). Immunolocalization of SNS/PN3 and NaN/SNS2 sodium channels in human pain states. *Pain* 85, 41-50.
- Cox,J.J., Reimann,F., Nicholas,A.K., Thornton,G., Roberts,E., Springell,K., Karbani,G., Jafri,H., Mannan,J., Raashid,Y., Al-Gazali,L., Hamamy,H., Valente,E.M., Gorman,S., Williams,R., McHale,D.P., Wood,J.N., Gribble,F.M., and Woods,C.G. (2006). An SCN9A channelopathy causes congenital inability to experience pain. *Nature* 444, 894-898.
- Coyle,D.E. (1998). Partial peripheral nerve injury leads to activation of astroglia and microglia which parallels the development of allodynic behavior. *Glia* 23, 75-83.
- Craig,A.D. (2003). Interoception: the sense of the physiological condition of the body. *Current Opinion in Neurobiology* 13, 500-505.
- Cridland,R.A., and Henry,J.L. (1988). Effects of intrathecal administration of neuropeptides on a spinal nociceptive reflex in the rat: VIP, galanin, CGRP, TRH, somatostatin and angiotensin II. *Neuropeptides* 11, 23-32.
- Crucchi,G., Sommer,C., Anand,P., Attal,N., Baron,R., Garcia-Larrea,L., Haanpaa,M., Jensen,T.S., Serra,J., and Treede,R.-D. (2010). EFNS guidelines on neuropathic pain assessment: revised 2009. *European Journal of Neurology* 17, 1010-1018.
- Cryan,J.F., Mombereau,C., and Vassout,A. (2005). The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci. Biobehav. Rev.* 29, 571-625.
- Cryan,J.F., and Holmes,A. (2005). The ascent of mouse: advances in modelling human depression and anxiety. *Nat Rev Drug Discov* 4, 775-790.
- Cui,L., Locatelli,L., Xie,M.Y., and Sommadossi,J.P. (1997). Effect of nucleoside analogs on neurite regeneration and mitochondrial DNA synthesis in PC-12 cells. *J. Pharmacol. Exp. Ther.* 280, 1228-1234.
- Cummins,T.R., Dib-Hajj,S.D., and Waxman,S.G. (2004). Electrophysiological properties of mutant Nav1.7 sodium channels in a painful inherited neuropathy. *J. Neurosci.* 24, 8232-8236.
- Cunha,F.Q., Poole,S., Lorenzetti,B.B., and Ferreira,S.H. (1992). The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia. *Br. J. Pharmacol.* 107, 660-664.
- Curtis,R., Stewart,H.J.S., Hall,S.M., Wilkin,G.P., Mirsky,R., and Jessen,K.R. (1992). Gap-43 Is Expressed by Nonmyelin-Forming Schwann-Cells of the Peripheral Nervous-System. *Journal of Cell Biology* 116, 1455-1464.
- D'Mello,R., and Dickenson,A.H. (2008). Spinal cord mechanisms of pain. *Br. J. Anaesth.* 101, 8-16.

- Dalakas,M.C., Semino-Mora,C., and Leon-Monzon,M. (2001). Mitochondrial alterations with mitochondrial DNA depletion in the nerves of AIDS patients with peripheral neuropathy induced by 2'3'-dideoxycytidine (ddC). *Lab Invest* 81, 1537-1544.
- Dalakas,M.C. (2001). Peripheral neuropathy and antiretroviral drugs. *Journal of the Peripheral Nervous System* 6, 14-20.
- Dalziel,R.G., Bingham,S., Sutton,D., Grant,D., Champion,J.M., Dennis,S.A., Quinn,J.P., Bountra,C., and Mark,M.A. (2004). Allodynia in rats infected with varicella zoster virus--a small animal model for post-herpetic neuralgia. *Brain Res. Brain Res. Rev* 46, 234-242.
- Daniel,H.C., Narewska,J., Serpell,M., Hoggart,B., Johnson,R., and Rice,A.S.C. (2008). Comparison of psychological and physical function in neuropathic pain and nociceptive pain: Implications for cognitive behavioral pain management programs. *European Journal of Pain* 12, 731-741.
- Dansereau,M.A., Gosselin,R.D., Pohl,M., Pommier,B., Mechighel,P., Mauborgne,A., Rostene,W., Kitabgi,P., Beaudet,N., Sarret,P., and Melik-Parsadaniantz,S. (2008). Spinal CCL2 pronociceptive action is no longer effective in CCR2 receptor antagonist-treated rats. *J. Neurochem.* 106, 757-769.
- Danthi,S.J., Enyeart,J.A., and Enyeart,J.J. (2005). Modulation of native T-type calcium channels by omega-3 fatty acids. *Biochemical and Biophysical Research Communications* 327, 485-493.
- de Mos,M., Huygen,F.J., Dieleman,J.P., Koopman,J.S., Stricker,B.H., and Sturkenboom,M.C. (2008). Medical history and the onset of complex regional pain syndrome (CRPS). *Pain* 139, 458-466.
- de Mos,M., Huygen,F.J.P.M., Stricker,B.H.C., Dieleman,J.P., and Sturkenboom,M.C.J.M. (2009). The association between ACE inhibitors and the complex regional pain syndrome: Suggestions for a neuro-inflammatory pathogenesis of CRPS. *Pain* 142, 218-224.
- de Urquiza,A.M., Liu,S.Y., Sjoberg,M., Zetterstrom,R.H., Griffiths,W., Sjovall,J., and Perlmann,T. (2000). Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science* 290, 2140-2144.
- De Vry,J., Kuhl,E., Franken-Kunkel,P., and Eckel,G. (2004). Pharmacological characterization of the chronic constriction injury model of neuropathic pain. *European Journal of Pharmacology* 491, 137-148.
- Deacon,R.M.J. (2006). Housing, husbandry and handling of rodents for behavioral experiments. *Nat. Protocols* 1, 936-946.
- Decosterd,I., Ji,R.R., Abdi,S., Tate,S., and Woolf,C.J. (2002). The pattern of expression of the voltage-gated sodium channels Na(v)1.8 and Na(v)1.9 does not change in uninjured primary sensory neurons in experimental neuropathic pain models. *Pain* 96, 269-277.
- Decosterd,I., and Woolf,C.J. (2000). Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 87, 149-158.
- DeLeo,J.A., Tanga,F.Y., and Tawfik,V.L. (2004). Neuroimmune activation and neuroinflammation in chronic pain and opioid tolerance/hyperalgesia. *Neuroscientist.* 10, 40-52.
- Delerive,P., Gervois,P., Fruchart,J.C., and Staels,B. (2000). Induction of I kappa B alpha expression as a mechanism contributing to the anti-inflammatory activities of peroxisome proliferator-activated receptor-alpha activators. *Journal of Biological Chemistry* 275, 36703-36707.
- Detke,M.J., Rickels,M., and Lucki,I. (1995). Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology (Berl)* 121, 66-72.

- Devor, M. (2009). Ectopic discharge in Abeta afferents as a source of neuropathic pain. *Exp. Brain Res.* 196, 115-128.
- Dib-Hajj, S.D., Rush, A.M., Cummins, T.R., Hisama, F.M., Novella, S., Tyrrell, L., Marshall, L., and Waxman, S.G. (2005). Gain-of-function mutation in Nav1.7 in familial erythromelalgia induces bursting of sensory neurons. *Brain* 128, 1847-1854.
- Dib-Hajj, S.D., Tyrrell, L., Black, J.A., and Waxman, S.G. (1998). Na_v1.7, a novel voltage-gated Na channel, is expressed preferentially in peripheral sensory neurons and down-regulated after axotomy. *Proc. Natl. Acad. Sci. U. S. A* 95, 8963-8968.
- 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, 1235-1238.
- 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, 1281-1292.
- Dominguez, E., Rivat, C., Pommier, B., Mauborgne, A., and Pohl, M. (2008). JAK/STAT3 pathway is activated in spinal cord microglia after peripheral nerve injury and contributes to neuropathic pain development in rat. *J. Neurochem.* 107, 50-60.
- Dorsey, S.G., Leitch, C.C., Renn, C.L., Lessans, S., Smith, B.A., Wang, X.M., and Dionne, R.A. (2009). Genome-wide screen identifies drug-induced regulation of the gene giant axonal neuropathy (Gan) in a mouse model of antiretroviral-induced painful peripheral neuropathy. *Biol. Res. Nurs.* 11, 7-16.
- Durrenberger, P.F., Facer, P., Casula, M.A., Yiangou, Y., Gray, R.A., Chessell, I.P., Day, N.C., Collins, S.D., Bingham, S., Wilson, A.W., Elliot, D., Birch, R., and Anand, P. (2006). Prostanoid receptor EP1 and Cox-2 in injured human nerves and a rat model of nerve injury: a time-course study. *BMC. Neurol.* 6, 1.
- Dusart, I., and Schwab, M.E. (1994). Secondary Cell Death and the Inflammatory Reaction After Dorsal Hemisection of the Rat Spinal Cord. *European Journal of Neuroscience* 6, 712-724.
- Dustin, L.B., Shea, C.M., Soberman, R.J., and Lu, C.Y. (1990). Docosahexaenoic acid, a constituent of rodent fetal serum and fish oil diets, inhibits acquisition of macrophage tumoricidal function. *J. Immunol.* 144, 4888-4897.
- Dworkin, R.H. (2002). An overview of neuropathic pain: syndromes, symptoms, signs, and several mechanisms. *Clin. J. Pain* 18, 343-349.
- Dworkin, R.H., Backonja, M., Rowbotham, M.C., Allen, R.R., Argoff, C.R., Bennett, G.J., Bushnell, M.C., Farrar, J.T., Galer, B.S., Haythornthwaite, J.A., Hewitt, D.J., Loeser, J.D., Max, M.B., Saltarelli, M., Schmader, K.E., Stein, C., Thompson, D., Turk, D.C., Wallace, M.S., Watkins, L.R., and Weinstein, S.M. (2003). Advances in neuropathic pain: diagnosis, mechanisms, and treatment recommendations. *Arch. Neurol.* 60, 1524-1534.
- Dworkin, R.H., O'Connor, A.B., Backonja, M., Farrar, J.T., Finnerup, N.B., Jensen, T.S., Kalso, E.A., Loeser, J.D., Miaskowski, C., Nurmikko, T.J., Portenoy, R.K., Rice, A.S., Stacey, B.R., Treede, R.D., Turk, D.C., and Wallace, M.S. (2007). Pharmacologic management of neuropathic pain: evidence-based recommendations. *Pain* 132, 237-251.
- Dyall, S., and Michael-Titus, A. (2008). Neurological Benefits of Omega-3 Fatty Acids. *NeuroMolecular Medicine* 10, 219-235.

- Earnshaw, W.C., Martins, L.M., and Kaufmann, S.H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* 68, 383-424.
- Eisenach, J.C., De, K.M., and Klimscha, W. (1996). $\alpha(2)$ -adrenergic agonists for regional anesthesia. A clinical review of clonidine (1984-1995). *Anesthesiology* 85, 655-674.
- Elliott-Hunt, C.R., Marsh, B., Bacon, A., Pope, R., Vanderplank, P., and Wynick, D. (2004). Galanin acts as a neuroprotective factor to the hippocampus. *Proceedings of the National Academy of Sciences of the United States of America* 101, 5105-5110.
- Farquhar-Smith, W.P., Egertova, M., Bradbury, E.J., McMahon, S.B., Rice, A.S., and Elphick, M.R. (2000). Cannabinoid CB(1) receptor expression in rat spinal cord. *Mol. Cell Neurosci.* 15, 510-521.
- Ferrari, L.F., and Levine, J.D. (2010). Alcohol consumption enhances antiretroviral painful peripheral neuropathy by mitochondrial mechanisms. *Eur. J. Neurosci.* 32, 811-818.
- Ferreira, J., Beirith, A., Mori, M.A., Araujo, R.C., Bader, M., Pesquero, J.B., and Calixto, J.B. (2005). Reduced nerve injury-induced neuropathic pain in kinin B1 receptor knock-out mice. *J. Neurosci.* 25, 2405-2412.
- Field, M.J., Bramwell, S., Hughes, J., and Singh, L. (1999). Detection of static and dynamic components of mechanical allodynia in rat models of neuropathic pain: are they signalled by distinct primary sensory neurones? *Pain* 83, 303-311.
- Fields, H.L., Malick, A., and Burstein, R. (1995). Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. *J. Neurophysiol.* 74, 1742-1759.
- Figuerola, J.D., Cordero, K., Baldeosingh, K., Torrado, A.I., Walker, R.L., Miranda, J.D., and Leon, M.D. (2012). Docosahexaenoic acid pretreatment confers protection and functional improvements after acute spinal cord injury in adult rats. *J. Neurotrauma* 29, 551-566.
- Finnerup, N.B. (2008). A review of central neuropathic pain states. *Curr. Opin. Anaesthesiol.* 21, 586-589.
- Finnerup, N.B., Sindrup, S.H., and Jensen, T.S. (2010b). Recent advances in pharmacological treatment of neuropathic pain. *F1000. Med. Rep.* 2, 52.
- Finnerup, N.B., Sindrup, S.H., and Jensen, T.S. (2010a). The evidence for pharmacological treatment of neuropathic pain. *Pain* 150, 573-581.
- 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, 131-141.
- Flatters, S.J.L., and Bennett, G.J. (2004). Ethosuximide reverses paclitaxel- and vincristine-induced painful peripheral neuropathy. *Pain* 109, 150-161.
- Fleetwood-Walker, S.M., Quinn, J.P., Wallace, C., Blackburn-Munro, G., Kelly, B.G., Fiskerstrand, C.E., Nash, A.A., and Dalziel, R.G. (1999). Behavioural changes in the rat following infection with varicella-zoster virus. *J. Gen. Virol.* 80 (Pt 9), 2433-2436.
- Fleischer, R., Boxwell, D., and Sherman, K.E. (2004). Nucleoside analogues and mitochondrial toxicity. *Clinical Infectious Diseases* 38, E79-E80.
- Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M., and Evans, R.M. (1995). 15-Deoxy-Delta(12,14)-Prostaglandin J(2) Is A Ligand for the Adipocyte Determination Factor Ppar-Gamma. *Cell* 83, 803-812.

- Fossat,P., Dobremez,E., Bouali-Benazzouz,R., Favereaux,A., Bertrand,S.S., Kilk,K., Leger,C., Cazalets,J.R., Langel,U., Landry,M., and Nagy,F. (2010). Knockdown of L calcium channel subtypes: differential effects in neuropathic pain. *J. Neurosci* 30, 1073-1085.
- Fox,A., Eastwood,C., Gentry,C., Manning,D., and Urban,L. (1999). Critical evaluation of the streptozotocin model of painful diabetic neuropathy in the rat. *Pain* 81, 307-316.
- Fox,A., Wotherspoon,G., McNair,K., Hudson,L., Patel,S., Gentry,C., and Winter,J. (2003). Regulation and function of spinal and peripheral neuronal B1 bradykinin receptors in inflammatory mechanical hyperalgesia. *Pain* 104, 683-691.
- Fukuoka,T., Kondo,E., Dai,Y., Hashimoto,N., and Noguchi,K. (2001). Brain-derived neurotrophic factor increases in the uninjured dorsal root ganglion neurons in selective spinal nerve ligation model. *J. Neurosci.* 21, 4891-4900.
- Fukuoka,T., Tokunaga,A., Kondo,E., Miki,K., Tachibana,T., and Noguchi,K. (1998). Change in mRNAs for neuropeptides and the GABA(A) receptor in dorsal root ganglion neurons in a rat experimental neuropathic pain model. *Pain* 78, 13-26.
- Fukuoka,T., Yamanaka,H., Kobayashi,K., Okubo,M., Miyoshi,K., Dai,Y., and Noguchi,K. (2012). Re-evaluation of the phenotypic changes in L4 dorsal root ganglion neurons after L5 spinal nerve ligation. *Pain* 153, 68-79.
- Funk,C.D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294, 1871-1875.
- Gao,Y.J., and Ji,R.R. (2010). Targeting astrocyte signaling for chronic pain. *Neurotherapeutics.* 7, 482-493.
- Garry,E.M., Delaney,A., Anderson,H.A., Sirinathsinghji,E.C., Clapp,R.H., Martin,W.J., Kinchington,P.R., Krah,D.L., Abbadie,C., and Fleetwood-Walker,S.M. (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, 97-111.
- Gaudet,A.D., Popovich,P.G., and Ramer,M.S. (2011). Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. *J. Neuroinflammation.* 8, 110.
- Gebhart,G.F. (2004). Descending modulation of pain. *Neurosci. Biobehav. Rev.* 27, 729-737.
- Gehrmann,J., Matsumoto,Y., and Kreutzberg,G.W. (1995). Microglia: intrinsic immuneffector cell of the brain. *Brain Res. Brain Res. Rev.* 20, 269-287.
- Gibson,S.J., Polak,J.M., Allen,J.M., Adrian,T.E., Kelly,J.S., and Bloom,S.R. (1984). The distribution and origin of a novel brain peptide, neuropeptide Y, in the spinal cord of several mammals. *J. Comp Neurol.* 227, 78-91.
- Gilden,D.H., Cohrs,R.J., and Mahalingam,R. (2003). Clinical and molecular pathogenesis of varicella virus infection. *Viral Immunol.* 16, 243-258.
- Gilks,C.F., Crowley,S., Ekpini,R., Gove,S., Perriens,J., Souteyrand,Y., Sutherland,D., Vitoria,M., Guerma,T., and De,C.K. (2006). The WHO public-health approach to antiretroviral treatment against HIV in resource-limited settings. *Lancet* 368, 505-510.
- Gladman,S.J., Huang,W., Lim,S.N., Dyllal,S.C., Boddy,S., Kang,J.X., Knight,M.M., Priestley,J.V., and Michael-Titus,A.T. (2012). Improved outcome after peripheral nerve injury in mice with increased levels of endogenous omega-3 polyunsaturated fatty acids. *J. Neurosci.* 32, 563-571.

- Gold,M.S., Weinreich,D., Kim,C.S., Wang,R., Treanor,J., Porreca,F., and Lai,J. (2003). Redistribution of Na(V)1.8 in uninjured axons enables neuropathic pain. *J. Neurosci.* 23, 158-166.
- Goldberg,R.J., and Katz,J. (2007). A meta-analysis of the analgesic effects of omega-3 polyunsaturated fatty acid supplementation for inflammatory joint pain. *Pain* 129, 210-223.
- Goldstein,M.E., House,S.B., and Gainer,H. (1991). NF-L and peripherin immunoreactivities define distinct classes of rat sensory ganglion cells. *J Neurosci. Res.* 30, 92-104.
- Gonçalves,L., Silva,R., Pinto-Ribeiro,F., Pêgo,J.M., Bessa,J.M., Pertovaara,A., Sousa,N., and Almeida,A. (2008). Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. *Experimental Neurology* 213, 48-56.
- Graeber,M.B., Bise,K., and Mehraein,P. (1994). CR3/43, a marker for activated human microglia: application to diagnostic neuropathology. *Neuropathol. Appl. Neurobiol.* 20, 406-408.
- Graeber,M.B., and Christie,M.J. (2012). Multiple mechanisms of microglia: A gatekeeper's contribution to pain states. *Exp. Neurol.*
- Graeber,M.B., Streit,W.J., and Kreutzberg,G.W. (1988). Axotomy of the rat facial nerve leads to increased CR3 complement receptor expression by activated microglial cells. *J. Neurosci. Res.* 21, 18-24.
- Griffin,J.W., George,R., and HO,T. (1993). Macrophage Systems in Peripheral Nerves. A Review. *Journal of Neuropathology & Experimental Neurology* 52.
- Guimaraes,N.N., de Andrade,H.H., Lehmann,M., Dihl,R.R., and Cunha,K.S. (2010). The genetic toxicity effects of lamivudine and stavudine antiretroviral agents. *Expert. Opin. Drug Saf* 9, 771-781.
- Gundlach,A.L. (2002). Galanin/GALP and galanin receptors: role in central control of feeding, body weight/obesity and reproduction? *European Journal of Pharmacology* 440, 255-268.
- Gutteridge,J.M. (1995). Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin. Chem.* 41, 1819-1828.
- Haanpää,M., Attal,N., Backonja,M., Baron,R., Bennett,M., Bouhassira,D., Cruccu,G., Hansson,P., Haythornthwaite,J.A., Iannetti,G.D., Jensen,T.S., Kauppila,T., Nurmikko,T.J., Rice,A.S., Rowbotham,M., Serra,J., Sommer,C., Smith,B.H., and Treede,R.D. (2011). NeuPSIG guidelines on neuropathic pain assessment. *Pain* 152, 14-27.
- Hahn,S., Mizuno,T.M., Wu,T.J., Wisor,J.P., Priest,C.A., Kozak,C.A., Boozer,C.N., Peng,B., McEvoy,R.C., Good,P., Kelley,K.A., Takahashi,J.S., Pintar,J.E., Roberts,J.L., Mobbs,C.V., and Salton,S.R. (1999). Targeted deletion of the Vgf gene indicates that the encoded secretory peptide precursor plays a novel role in the regulation of energy balance. *Neuron* 23, 537-548.
- Hahn,K., Robinson,B., Anderson,C., Li,W., Pardo,C.A., Morgello,S., Simpson,D., and Nath,A. (2008). Differential effects of HIV infected macrophages on dorsal root ganglia neurons and axons. *Exp. Neurol.* 210, 30-40.
- Hai,T., and Curran,T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. U. S. A* 88, 3720-3724.
- Hai,T.W., Liu,F., Coukos,W.J., and Green,M.R. (1989). Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* 3, 2083-2090.

- Hains,B.C., Klein,J.P., Saab,C.Y., Craner,M.J., Black,J.A., and Waxman,S.G. (2003). Upregulation of sodium channel Nav1.3 and functional involvement in neuronal hyperexcitability associated with central neuropathic pain after spinal cord injury. *J. Neurosci.* 23, 8881-8892.
- Hall,S.M., Kent,A.P., Curtis,R., and Robertson,D. (1992). Electron-Microscopic Immunocytochemistry of Gap-43 Within Proximal and Chronically Denervated Distal Stumps of Transected Peripheral-Nerve. *Journal of Neurocytology* 21, 820-831.
- 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, 77-88.
- Hasnie,F.S., Breuer,J., Parker,S., Wallace,V., Blackbeard,J., Lever,I., Kinchington,P.R., Dickenson,A.H., Pheby,T., and Rice,A.S.C. (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, 1495-1508.
- Heinricher,M.M., McGaraughty,S., and Tortorici,V. (2001). Circuitry underlying antinociceptive actions of cholecystokinin within the rostral ventromedial medulla. *J. Neurophysiol.* 85, 280-286.
- Heller,A.R., Stehr,S.N., and Litz,R.J. (2006). Omega-3 Alpha Linolenic Acid Does Not Reflect the Entire Omega-3 Fatty Acid Family. *Anesthesia & Analgesia* 102, 1289-128a.
- Hempnall,K., Nurmikko,T.J., Johnson,R.W., A'Hern,R.P., and Rice,A.S. (2005). Analgesic therapy in postherpetic neuralgia: a quantitative systematic review. *PLoS. Med.* 2, e164.
- Hendry,I.A., Murphy,M., Hilton,D.J., Nicola,N.A., and Bartlett,P.F. (1992). Binding and retrograde transport of leukemia inhibitory factor by the sensory nervous system. *J. Neurosci.* 12, 3427-3434.
- Herdegen,T., Leah,J.D., Manisali,A., Bravo,R., and Zimmermann,M. (1991). c-JUN-like immunoreactivity in the CNS of the adult rat: basal and transynaptically induced expression of an immediate-early gene. *Neuroscience* 41, 643-654.
- Herkenham,M. (1991). Characterization and localization of cannabinoid receptors in brain: an in vitro technique using slide-mounted tissue sections. *NIDA Res. Monogr* 112, 129-145.
- Herzberg,U., and Sagen,J. (2001). Peripheral nerve exposure to HIV viral envelope protein gp120 induces neuropathic pain and spinal gliosis. *J. Neuroimmunol.* 116, 29-39.
- Hickey,W.F., Hsu,B.L., and Kimura,H. (1991). T-lymphocyte entry into the central nervous system. *J. Neurosci. Res.* 28, 254-260.
- Hobson,S.A., Holmes,F.E., Kerr,N.C., Pope,R.J., and Wynick,D. (2006). Mice deficient for galanin receptor 2 have decreased neurite outgrowth from adult sensory neurons and impaired pain-like behaviour. *J. Neurochem.* 99, 1000-1010.
- Hofmann,H.A., De,V.J., Siegling,A., Spreyer,P., and Denzer,D. (2003). Pharmacological sensitivity and gene expression analysis of the tibial nerve injury model of neuropathic pain. *Eur. J. Pharmacol.* 470, 17-25.
- Hokfelt,T., Wiesenfeld-Hallin,Z., Villar,M., and Melander,T. (1987). Increase of galanin-like immunoreactivity in rat dorsal root ganglion cells after peripheral axotomy. *Neurosci. Lett.* 83, 217-220.
- Hokfelt,T., Xu,Z.Q., Shi,T.J., Holmberg,K., and Zhang,X. (1998). Galanin in ascending systems. Focus on coexistence with 5-hydroxytryptamine and noradrenaline. *Ann. N. Y. Acad. Sci.* 863, 252-263.

- Hokfelt, T., Zhang, X., and Wiesenfeld-Hallin, Z. (1994). Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends Neurosci* 17, 22-30.
- Holguin, A., O'Connor, K.A., Biedenkapp, J., Campisi, J., Wieseler-Frank, J., Milligan, E.D., Hansen, M.K., Spataro, L., Maksimova, E., Bravmann, C., Martin, D., Fleshner, M., Maier, S.F., and Watkins, L.R. (2004). HIV-1 gp120 Stimulates proinflammatory cytokine-mediated pain facilitation via activation of nitric oxide synthase-I (nNOS). *Pain* 110, 517-530.
- Holmes, A., and Rodgers, R.J. (1998). Responses of Swiss-Webster mice to repeated plus-maze experience: further evidence for a qualitative shift in emotional state? *Pharmacol. Biochem. Behav.* 60, 473-488.
- Holmes, F.E., Bacon, A., Pope, R.J., Vanderplank, P.A., Kerr, N.C., Sukumaran, M., Pachnis, V., and Wynick, D. (2003). Transgenic overexpression of galanin in the dorsal root ganglia modulates pain-related behavior. *Proc. Natl. Acad. Sci. U. S. A* 100, 6180-6185.
- Holmes, F.E., Mahoney, S., King, V.R., Bacon, A., Kerr, N.C., Pachnis, V., Curtis, R., Priestley, J.V., and Wynick, D. (2000). Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. *Proc. Natl. Acad. Sci. U. S. A* 97, 11563-11568.
- Holub, D.J., and Holub, B.J. (2004). Omega-3 fatty acids from fish oils and cardiovascular disease. *Mol. Cell Biochem.* 263, 217-225.
- Holzer, P. (1998). Neurogenic vasodilatation and plasma leakage in the skin. *Gen. Pharmacol.* 30, 5-11.
- Hong, S., Gronert, K., Devchand, P.R., Moussignac, R.L., and Serhan, C.N. (2003). Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. *J. Biol. Chem.* 278, 14677-14687.
- Hsieh, C.H., Jeng, S.F., Lu, T.H., Yang, J.C., Hsieh, M.W., Chen, Y.C., and Rau, C.S. (2009). Correlation between skin biopsy with quantification of intraepidermal nerve fiber and the severity of sciatic nerve traction injury in rats. *J. Trauma* 66, 737-742.
- Hu, B., Doods, H., Kremer, A., Roeska, K., Treede, R.D., and Ceci, A. (2007). 151 Mononeuropathic animals displayed depressive-like behaviour in the model of forced swimming test. *European Journal of Pain* 11, 65-66.
- Hu, B., Doods, H., Treede, R.D., and Ceci, A. (2009). Depression-like behaviour in rats with mononeuropathy is reduced by the CB2-selective agonist GW405833. *Pain* 143, 206-212.
- Hu, J.Y., Li, C.L., and Wang, Y.W. (2012). Intrathecal administration of triptolide, a T lymphocyte inhibitor, attenuates chronic constriction injury-induced neuropathic pain in rats. *Brain Res.* 1436, 122-129.
- Hu, P., and McLachlan, E.M. (2003). Distinct functional types of macrophage in dorsal root ganglia and spinal nerves proximal to sciatic and spinal nerve transections in the rat. *Exp. Neurol.* 184, 590-605.
- Hua, X.Y., Boublik, J.H., Spicer, M.A., Rivier, J.E., Brown, M.R., and Yaksh, T.L. (1991). The antinociceptive effects of spinally administered neuropeptide Y in the rat: systematic studies on structure-activity relationship. *J. Pharmacol. Exp. Ther.* 258, 243-248.
- Hua, X.Y., Hayes, C.S., Hofer, A., Fitzsimmons, B., Kilk, K., Langel, U., Bartfai, T., and Yaksh, T.L. (2004). Galanin acts at GalR1 receptors in spinal antinociception: synergy with morphine and AP-5. *J. Pharmacol. Exp. Ther.* 308, 574-582.
- Hua, X.Y., Salgado, K.F., Gu, G., Fitzsimmons, B., Kondo, I., Bartfai, T., and Yaksh, T.L. (2005). Mechanisms of antinociception of spinal galanin: how does galanin inhibit spinal sensitization? *Neuropeptides* 39, 211-216.

- Huang,L.Y., and Neher,E. (1996). Ca(2+)-dependent exocytosis in the somata of dorsal root ganglion neurons. *Neuron* 17, 135-145.
- Huang,W.L., King,V.R., Curran,O.E., Dyllal,S.C., Ward,R.E., Lal,N., Priestley,J.V., and Michael-Titus,A.T. (2007). A combination of intravenous and dietary docosahexaenoic acid significantly improves outcome after spinal cord injury. *Brain* 130, 3004-3019.
- Huang,Y.H., and Bergles,D.E. (2004). Glutamate transporters bring competition to the synapse. *Curr. Opin. Neurobiol.* 14, 346-352.
- Hudson,L.J., Bevan,S., Wotherspoon,G., Gentry,C., Fox,A., and Winter,J. (2001). VR1 protein expression increases in undamaged DRG neurons after partial nerve injury. *Eur. J. Neurosci.* 13, 2105-2114.
- 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 laminae of the spinal cord dorsal horn after nerve section. *J. Neurosci* 23, 9491-9499.
- Hulse,R.P., Donaldson,L.F., and Wynick,D. (2012). Peripheral galanin receptor 2 as a target for the modulation of pain. *Pain Res. Treat.* 2012, 545386.
- Hunt,D., Hossain-Ibrahim,K., Mason,M.R., Coffin,R.S., Lieberman,A.R., Winterbottom,J., and Anderson,P.N. (2004). ATF3 upregulation in glia during Wallerian degeneration: differential expression in peripheral nerves and CNS white matter. *BMC. Neurosci.* 5, 9.
- Hwang,J.H., and Yaksh,T.L. (1997). The effect of spinal GABA receptor agonists on tactile allodynia in a surgically-induced neuropathic pain model in the rat. *Pain* 70, 15-22.
- Ibrahim,M.M., Deng,H., Zvonok,A., Cockayne,D.A., Kwan,J., Mata,H.P., Vanderah,T.W., Lai,J., Porreca,F., Makriyannis,A., and Malan,T.P., Jr. (2003). Activation of CB2 cannabinoid receptors by AM1241 inhibits experimental neuropathic pain: pain inhibition by receptors not present in the CNS. *Proc. Natl. Acad. Sci. U. S. A* 100, 10529-10533.
- Idanpaan-Heikkila,J.J., and Guilbaud,G. (1999). Pharmacological studies on a rat model of trigeminal neuropathic pain: baclofen, but not carbamazepine, morphine or tricyclic antidepressants, attenuates the allodynia-like behaviour. *Pain* 79, 281-290.
- Immke,D.C., and Gavva,N.R. (2006). The TRPV1 receptor and nociception. *Seminars in Cell & Developmental Biology* 17, 582-591.
- Indo,Y., Mardy,S., Miura,Y., Moosa,A., Ismail,E.A., Toscano,E., Andria,G., Pavone,V., Brown,D.L., Brooks,A., Endo,F., and Matsuda,I. (2001). Congenital insensitivity to pain with anhidrosis (CIPA): novel mutations of the TRKA (NTRK1) gene, a putative uniparental disomy, and a linkage of the mutant TRKA and PKLR genes in a family with CIPA and pyruvate kinase deficiency. *Hum. Mutat.* 18, 308-318.
- Intondi,A.B., Dahlgren,M.N., Eilers,M.A., and Taylor,B.K. (2008). Intrathecal neuropeptide Y reduces behavioral and molecular markers of inflammatory or neuropathic pain. *Pain* 137, 352-365.
- Isacsson,A., Kanje,M., and Dahlin,L.B. (2005). Induction of activating transcription factor 3 (ATF3) by peripheral nerve compression. *Scand. J. Plast. Reconstr. Surg. Hand Surg.* 39, 65-72.
- Jaken,R.J., van,G.S., Joosten,E.A., Losen,M., Martinez-Martinez,P., De,B.M., Marcus,M.A., and Deumens,R. (2011). Neuropathy-induced spinal GAP-43 expression is not a main player in the onset of mechanical pain hypersensitivity. *J. Neurotrauma* 28, 2463-2473.
- Jensen,T.S., Gottrup,H., Sindrup,S.H., and Bach,F.W. (2001). The clinical picture of neuropathic pain. *Eur. J. Pharmacol.* 429, 1-11.

- Jensen,T.S., Baron,R., Haanpää,M., Kalso,E., Loeser,J.D., Rice,A.S.C., and Treede,R.D. A new definition of neuropathic pain. *Pain* 152[10], 2204-2205. 2011.
Ref Type: Abstract
- Jensen,T.S., and Lenz,F.A. (1995). Central post-stroke pain: a challenge for the scientist and the clinician. *PAIN* 61, 161-164.
- 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, 1114-1119.
- Ji,R.R., and Strichartz,G. (2004). Cell signaling and the genesis of neuropathic pain. *Sci. STKE.* 2004, reE14.
- Ji,R.R., and Suter,M.R. (2007). p38 MAPK, microglial signaling, and neuropathic pain. *Mol. Pain* 3, 33.
- 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, 1-10.
- Ji,R.R., Zhang,Q., Bedecs,K., Arvidsson,J., Zhang,X., Xu,X.J., Wiesenfeld-Hallin,Z., Bartfai,T., and Hokfelt,T. (1994a). Galanin antisense oligonucleotides reduce galanin levels in dorsal root ganglia and induce autotomy in rats after axotomy. *Proc. Natl. Acad. Sci. U. S. A* 91, 12540-12543.
- Ji,R.R., Zhang,X., Wiesenfeld-Hallin,Z., and Hokfelt,T. (1994b). Expression of neuropeptide Y and neuropeptide Y (Y1) receptor mRNA in rat spinal cord and dorsal root ganglia following peripheral tissue inflammation. *J Neurosci.* 14, 6423-6434.
- Jiang,C.Y., Ting,A.T., and Seed,B. (1998). PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* 391, 82-86.
- 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. *J. Neurosci.* 23, 4017-4022.
- Jones,G., Zhu,Y., Silva,C., Tsutsui,S., Pardo,C.A., Keppler,O.T., McArthur,J.C., and Power,C. (2005). Peripheral nerve-derived HIV-1 is predominantly CCR5-dependent and causes neuronal degeneration and neuroinflammation. *Virology* 334, 178-193.
- Joseph,E.K., Chen,X., Khasar,S.G., and Levine,J.D. (2004). Novel mechanism of enhanced nociception in a model of AIDS therapy-induced painful peripheral neuropathy in the rat. *Pain* 107, 147-158.
- Joseph,E.K., and Levine,J.D. (2006). Mitochondrial electron transport in models of neuropathic and inflammatory pain. *Pain* 121, 105-114.
- Joseph,E.K., and Levine,J.D. (2004). Caspase signalling in neuropathic and inflammatory pain in the rat. *Eur. J. Neurosci* 20, 2896-2902.
- Julius,D., and Basbaum,A.I. (2001). Molecular mechanisms of nociception. *Nature* 413, 203-210.
- Kajander,K.C., Pollock,C.H., and Berg,H. (1996). Evaluation of hindpaw position in rats during chronic constriction injury (CCI) produced with different suture materials. *Somatosensory and Motor Research* 13, 95-101.
- Kakuda,T.N. (2000). Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. *Clin. Ther.* 22, 685-708.

- Kamerman,P.R., Moss,P.J., Weber,J., Wallace,V.C.J., Rice,A.S.C., and Huang,W.L. (2012). Pathogenesis of HIV-associated sensory neuropathy: evidence from in vivo and in vitro experimental models. *Journal of the Peripheral Nervous System* 17, 19-31.
- Kataoka,K., Kanje,M., and Dahlin,L.B. (2007). Induction of activating transcription factor 3 after different sciatic nerve injuries in adult rats. *Scand. J. Plast. Reconstr. Surg. Hand Surg.* 41, 158-166.
- Kaul,M., and Lipton,S.A. (1999). Chemokines and activated macrophages in HIV gp120-induced neuronal apoptosis. *Proc. Natl. Acad. Sci. U. S. A* 96, 8212-8216.
- Kaul,S., Dandekar,K.A., Schilling,B.E., and Barbhuiya,R.H. (1999). Toxicokinetics of 2',3'-dideoxy-3'-deoxythymidine, stavudine (D4T). *Drug Metabolism and Disposition* 27, 1-12.
- Kawamata,M., and Omote,K. (1996). Involvement of increased excitatory amino acids and intracellular Ca^{2+} concentration in the spinal dorsal horn in an animal model of neuropathic pain. *Pain* 68, 85-96.
- Keilbaugh,S.A., Hobbs,G.A., and Simpson,M.V. (1997). Effect of 2',3'-dideoxycytidine on oxidative phosphorylation in the PC12 cell, a neuronal model. *Biochem. Pharmacol.* 53, 1485-1492.
- Kennedy,P.G., Grinfeld,E., Bontems,S., and Sadzot-Delvaux,C. (2001). Varicella-Zoster virus gene expression in latently infected rat dorsal root ganglia. *Virology* 289, 218-223.
- Kerekes,N., Mennicken,F., O'Donnell,D., Hokfelt,T., and Hill,R.H. (2003). Galanin increases membrane excitability and enhances Ca^{2+} currents in adult, acutely dissociated dorsal root ganglion neurons. *Eur. J. Neurosci.* 18, 2957-2966.
- Keswani,S.C., Chander,B., Hasan,C., Griffin,J.W., McArthur,J.C., and Hoke,A. (2003a). FK506 is neuroprotective in a model of antiretroviral toxic neuropathy. *Ann. Neurol.* 53, 57-64.
- Keswani,S.C., Jack,C., Zhou,C., and Hoke,A. (2006). Establishment of a rodent model of HIV-associated sensory neuropathy. *J. Neurosci.* 26, 10299-10304.
- Keswani,S.C., Polley,M., Pardo,C.A., Griffin,J.W., McArthur,J.C., and Hoke,A. (2003b). Schwann cell chemokine receptors mediate HIV-1 gp120 toxicity to sensory neurons. *Ann. Neurol.* 54, 287-296.
- Kieburz,K., Simpson,D., Yiannoutsos,C., Max,M.B., Hall,C.D., Ellis,R.J., Marra,C.M., McKendall,R., Singer,E., Dal Pan,G.J., Clifford,D.B., Tucker,T., and Cohen,B. (1998). A randomized trial of amitriptyline and mexiletine for painful neuropathy in HIV infection. AIDS Clinical Trial Group 242 Protocol Team. *Neurology* 51, 1682-1688.
- Kigerl,K.A., Gensel,J.C., Ankeny,D.P., Alexander,J.K., Donnelly,D.J., and Popovich,P.G. (2009). Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. *J. Neurosci.* 29, 13435-13444.
- Kim,H.K., Park,S.K., Zhou,J.L., Taglialatela,G., Chung,K., Coggeshall,R.E., and Chung,J.M. (2004). Reactive oxygen species (ROS) play an important role in a rat model of neuropathic pain. *Pain* 111, 116-124.
- Kim,K.J., Yoon,Y.W., and Chung,J.M. (1997). Comparison of three rodent neuropathic pain models. *Exp. Brain Res.* 113, 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, 355-363.

- Kim,S.H., Nam,J.S., Choi,D.K., Koh,W.W., Suh,J.H., Song,J.G., Shin,J.W., and Leem,J.G. (2011). Tumor Necrosis Factor-alpha and Apoptosis Following Spinal Nerve Ligation Injury in Rats. *Korean J. Pain* 24, 185-190.
- Kinchington,P.R., and Goins,W.F. (2011). Varicella zoster virus-induced pain and post-herpetic neuralgia in the human host and in rodent animal models. *J. Neurovirol.* 17, 590-599.
- King,V.R., Huang,W.L., Dyllal,S.C., Curran,O.E., Priestley,J.V., and Michael-Titus,A.T. (2006). Omega-3 fatty acids improve recovery, whereas omega-6 fatty acids worsen outcome, after spinal cord injury in the adult rat. *J. Neurosci* 26, 4672-4680.
- Kinloch,R.A., and Cox,P.J. (2005). New targets for neuropathic pain therapeutics. *Expert Opinion on Therapeutic Targets* 9, 685-698.
- Kirkland,R.A., Windelborn,J.A., Kasprzak,J.M., and Franklin,J.L. (2002). A Bax-induced pro-oxidant state is critical for cytochrome C release during programmed neuronal death. *Journal of Neuroscience* 22, 6480-6490.
- Kitajka,K., Sinclair,A.J., Weisinger,R.S., Weisinger,H.S., Mathai,M., Jayasooriya,A.P., Halver,J.E., and Puskas,L.G. (2004). Effects of dietary omega-3 polyunsaturated fatty acids on brain gene expression. *Proceedings of the National Academy of Sciences of the United States of America* 101, 10931-10936.
- Ko,G.D., Nowacki,N.B., Arseneau,L., Eitel,M., and Hum,A. (2010). Omega-3 fatty acids for neuropathic pain: case series. *Clin. J. Pain* 26, 168-172.
- Kobbert,C., Apps,R., Bechmann,I., Lanciego,J.L., Mey,J., and Thanos,S. (2000). Current concepts in neuroanatomical tracing. *Prog. Neurobiol.* 62, 327-351.
- Koltzenburg,M. (1998). Painful neuropathies. *Current Opinion in Neurology* 11, 515-521.
- Kovelowski,C.J., Ossipov,M.H., Sun,H., Lai,J., Malan,T.P., and Porreca,F. (2000). Supraspinal cholecystokinin may drive tonic descending facilitation mechanisms to maintain neuropathic pain in the rat. *Pain* 87, 265-273.
- Kozai,T., Yamanaka,H., Dai,Y., Obata,K., Kobayashi,K., Mashimo,T., and Noguchi,K. (2007). Tissue type plasminogen activator induced in rat dorsal horn astrocytes contributes to mechanical hypersensitivity following dorsal root injury. *Glia* 55, 595-603.
- Kremeyer,B., Lopera,F., Cox,J.J., Momin,A., Rugiero,F., Marsh,S., Woods,C.G., Jones,N.G., Paterson,K.J., Fricker,F.R., Villegas,A., Acosta,N., Pineda-Trujillo,N.G., Ramirez,J.D., Zea,J., Burley,M.W., Bedoya,G., Bennett,D.L., Wood,J.N., and Ruiz-Linares,A. (2010). A gain-of-function mutation in TRPA1 causes familial episodic pain syndrome. *Neuron* 66, 671-680.
- Kreutzberg,G.W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 19, 312-318.
- Kuraishi,Y., Kawamura,M., Yamaguchi,T., Houtani,T., Kawabata,S., Futaki,S., Fujii,N., and Satoh,M. (1991). Intrathecal Injections of Galanin and Its Antiserum Affect Nociceptive Response of Rat to Mechanical, But Not Thermal, Stimuli. *Pain* 44, 321-324.
- Lacroix-Fralish,M.L., Austin,J.S., Zheng,F.Y., Levitin,D.J., and Mogil,J.S. (2011). Patterns of pain: meta-analysis of microarray studies of pain. *Pain* 152, 1888-1898.
- Lai,J., Gold,M.S., Kim,C.S., Bian,D., Ossipov,M.H., Hunter,J.C., and Porreca,F. (2002). Inhibition of neuropathic pain by decreased expression of the tetrodotoxin-resistant sodium channel, NaV1.8. *Pain* 95, 143-152.

- Lai, J., Porreca, F., Hunter, J.C., and Gold, M.S. (2004). Voltage-gated sodium channels and hyperalgesia. *Annu. Rev Pharmacol. Toxicol.* 44, 371-397.
- Landry, M., Aman, K., Dostrovsky, J., Lozano, A.M., Carlstedt, T., Spenger, C., Josephson, A., Wiesenfeld-Hallin, Z., and Hokfelt, T. (2003). Galanin expression in adult human dorsal root ganglion neurons: initial observations. *Neuroscience* 117, 795-809.
- Landry, M., Holmberg, K., Zhang, X., and Hokfelt, T. (2000). Effect of axotomy on expression of NPY, galanin, and NPVY1 and Y2 receptors in dorsal root ganglia and the superior cervical ganglion studied with double-labeling in situ hybridization and immunohistochemistry. *Experimental Neurology* 162, 361-384.
- Lane, N.E., Schnitzer, T.J., Birbara, C.A., Mokhtarani, M., Shelton, D.L., Smith, M.D., and Brown, M.T. (2010). Tanezumab for the treatment of pain from osteoarthritis of the knee. *N. Engl. J. Med.* 363, 1521-1531.
- Lang, R., Gundlach, A.L., and Kofler, B. (2007). The galanin peptide family: Receptor pharmacology, pleiotropic biological actions, and implications in health and disease. *Pharmacology & Therapeutics* 115, 177-207.
- Lauritzen, I., Blondeau, N., Heurteaux, C., Widmann, C., Romey, G., and Lazdunski, M. (2000). Polyunsaturated fatty acids are potent neuroprotectors. *EMBO J.* 19, 1784-1793.
- Lawson, S.N., and Waddell, P.J. (1991). Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. *J. Physiol* 435, 41-63.
- Leaf, A. (2001). The electrophysiological basis for the antiarrhythmic actions of polyunsaturated fatty acids. *European Heart Journal Supplements* 3, D98-D105.
- Ledeboer, A., Sloane, E.M., Milligan, E.D., Frank, M.G., Mahony, J.H., Maier, S.F., and Watkins, L.R. (2005). Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. *Pain* 115, 71-83.
- Lee, B.H., Won, R., Baik, E.J., Lee, S.H., and Moon, C.H. (2000). An animal model of neuropathic pain employing injury to the sciatic nerve branches. *Neuroreport* 11, 657-661.
- Lee, S.H., Kayser, V., Desmeules, J., and Guilbaud, G. (1994). Differential Action of Morphine and Various Opioid Agonists on Thermal Allodynia and Hyperalgesia in Mononeuropathic Rats. *Pain* 57, 233-240.
- Legg, E.D., Novejarque, A., and Rice, A.S. (2009). The Three Ages of Rat: the influence of rodent age on affective and cognitive outcome measures in peripheral neuropathic pain. *Pain* 144, 12-13.
- Lehmann, H.C., Chen, W., Borzan, J., Mankowski, J.L., and Hoke, A. (2011). Mitochondrial dysfunction in distal axons contributes to human immunodeficiency virus sensory neuropathy. *Ann Neurol.* 69, 100-110.
- Lengqvist, J., de Urquiza, A.M., Bergman, A.C., Willson, T.M., Sjoval, J., Perlmann, T., and Griffiths, W.J. (2004). Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X receptor alpha ligand-binding domain. *Molecular & Cellular Proteomics* 3, 692-703.
- Lesage, F., and Lazdunski, M. (2000). Molecular and functional properties of two-pore-domain potassium channels. *Am. J. Physiol Renal Physiol* 279, F793-F801.
- Levy, D., and Zochodne, D.W. (2000). Increased mRNA expression of the B1 and B2 bradykinin receptors and antinociceptive effects of their antagonists in an animal model of neuropathic pain. *Pain* 86, 265-271.
- Lewis, W., and Dalakas, M.C. (1995). Mitochondrial Toxicity of Antiviral Drugs. *Nature Medicine* 1, 417-422.

- Lewis, W., Day, B.J., and Copeland, W.C. (2003). Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. *Nat. Rev. Drug Discov.* 2, 812-822.
- Li, C.Y., Zhang, X.L., Matthews, E.A., Li, K.W., Kurwa, A., Boroujerdi, A., Gross, J., Gold, M.S., Dickenson, A.H., Feng, G., and Luo, Z.D. (2006). Calcium channel $\alpha_2\delta_1$ subunit mediates spinal hyperexcitability in pain modulation. *Pain* 125, 20-34.
- Lichtman, A.H., Cook, S.A., and Martin, B.R. (1996). Investigation of brain sites mediating cannabinoid-induced antinociception in rats: evidence supporting periaqueductal gray involvement. *J. Pharmacol. Exp. Ther.* 276, 585-593.
- Lim, S., Huang, W., Hall, J.C.E., Priestley, J.V., and Michael-Titus, A. Docosahexaenoic Acid is Neuroprotective After Spinal Cord Compression Injury in the Mouse. FENS meeting, Amsterdam, July 2010 . 2010.
Ref Type: Abstract
- Lima-Garcia, J.F., Dutra, R.C., da, S.K., Motta, E.M., Campos, M.M., and Calixto, J.B. (2011). The precursor of resolvin D series and aspirin-triggered resolvin D1 display anti-hyperalgesic properties in adjuvant-induced arthritis in rats. *Br. J. Pharmacol.* 164, 278-293.
- Linda, H., Skold, M.K., and Ochsmann, T. (2011). Activating transcription factor 3, a useful marker for regenerative response after nerve root injury. *Front Neurol.* 2, 30.
- Lindia, J.A., McGowan, E., Jochowitz, N., and Abbadie, C. (2005). Induction of CX3CL1 expression in astrocytes and CX3CR1 in microglia in the spinal cord of a rat model of neuropathic pain. *J. Pain* 6, 434-438.
- Lipton, S.A. (1991). HIV-related neurotoxicity. *Brain Pathol.* 1, 193-199.
- Litvak, V., Ramsey, S.A., Rust, A.G., Zak, D.E., Kennedy, K.A., Lampano, A.E., Nykter, M., Shmulevich, I., and Aderem, A. (2009). Function of C/EBP[δ] in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. *Nat Immunol* 10, 437-443.
- Liu, H., Liu, Z., Yang, X., Huang, F., Ma, C., and Li, Z. (2008). Neurotoxicity caused by didanosine on cultured dorsal root ganglion neurons. *Cell Biol. Toxicol.* 24, 113-121.
- Liu, T., van Rooijen, N., and Tracey, D.J. (2000a). Depletion of macrophages reduces axonal degeneration and hyperalgesia following nerve injury. *Pain* 86, 25-32.
- Liu, T., van, R.N., and Tracey, D.J. (2000b). Depletion of macrophages reduces axonal degeneration and hyperalgesia following nerve injury. *Pain* 86, 25-32.
- Livesey, F.J., O'Brien, J.A., Li, M., Smith, A.G., Murphy, L.J., and Hunt, S.P. (1997). A Schwann cell mitogen accompanying regeneration of motor neurons. *Nature* 390, 614-618.
- Loeser, J.D., and Melzack, R. (1999). Pain: an overview. *Lancet* 353, 1607-1609.
- Lokesh, B.R., Black, J.M., German, J.B., and Kinsella, J.E. (1988). Docosahexaenoic acid and other dietary polyunsaturated fatty acids suppress leukotriene synthesis by mouse peritoneal macrophages. *Lipids* 23, 968-972.
- Lopez-Rubalcava, C., and Lucki, I. (2000). Strain differences in the behavioral effects of antidepressant drugs in the rat forced swimming test. *Neuropsychopharmacology* 22, 191-199.

Lu,D.Y., Tsao,Y.Y., Leung,Y.M., and Su,K.P. (2010). Docosahexaenoic acid suppresses neuroinflammatory responses and induces heme oxygenase-1 expression in BV-2 microglia: implications of antidepressant effects for omega-3 fatty acids. *Neuropsychopharmacology* 35, 2238-2248.

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

Ma,Q.P. (2001). The expression of bradykinin B(1) receptors on primary sensory neurones that give rise to small caliber sciatic nerve fibres in rats. *Neuroscience* 107, 665-673.

Ma,W., and Bisby,M.A. (1998). Partial and complete sciatic nerve injuries induce similar increases of neuropeptide Y and vasoactive intestinal peptide immunoreactivities in primary sensory neurons and their central projections. *Neuroscience* 86, 1217-1234.

Ma,W., and Bisby,M.A. (1997). Differential expression of galanin immunoreactivities in the primary sensory neurons following partial and complete sciatic nerve injuries. *Neuroscience* 79, 1183-1195.

Ma,W., Zhang,Y., Bantel,C., and Eisenach,J.C. (2005). Medium and large injured dorsal root ganglion cells increase TRPV-1, accompanied by increased ^{+12}C -adrenoceptor co-expression and functional inhibition by clonidine. *Pain* 113, 386-394.

Macleod,M.R., Fisher,M., O'Collins,V., Sena,E.S., Dirnagl,U., Bath,P.M., Buchan,A., van der Worp,H.B., Traystman,R., Minematsu,K., Donnan,G.A., and Howells,D.W. (2009). Good laboratory practice: preventing introduction of bias at the bench. *Stroke* 40, e50-e52.

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

Maione,S., Bisogno,T., de,N., V, Palazzo,E., Cristino,L., Valenti,M., Petrosino,S., Guglielmotti,V., Rossi,F., and Di,M., V (2006). Elevation of endocannabinoid levels in the ventrolateral periaqueductal grey through inhibition of fatty acid amide hydrolase affects descending nociceptive pathways via both cannabinoid receptor type 1 and transient receptor potential vanilloid type-1 receptors. *J. Pharmacol. Exp. Ther.* 316, 969-982.

Makinson,A., Moing,V.L., Kouanfack,C., Laurent,C., and Delaporte,E. (2008). Safety of stavudine in the treatment of HIV infection with a special focus on resource-limited settings. *Expert. Opin. Drug Saf* 7, 283-293.

Maratou,K., Wallace,V.C., Hasnie,F.S., Okuse,K., Hosseini,R., Jina,N., Blackbeard,J., Pheby,T., Orengo,C., Dickenson,A.H., McMahon,S.B., and Rice,A.S. (2009). Comparison of dorsal root ganglion gene expression in rat models of traumatic and HIV-associated neuropathic pain. *Eur. J. Pain* 13, 387-398.

Marcheselli,V.L., Hong,S., Lukiw,W.J., Tian,X.H., Gronert,K., Musto,A., Hardy,M., Gimenez,J.M., Chiang,N., Serhan,C.N., and Bazan,N.G. (2003). Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *Journal of Biological Chemistry* 278, 43807-43817.

Marcheselli,V.L., Mukherjee,P.K., Arita,M., Hong,S., Antony,R., Sheets,K., Winkler,J.W., Petasis,N.A., Serhan,C.N., and Bazan,N.G. (2010). Neuroprotectin D1/protectin D1 stereoselective and specific binding with human retinal pigment epithelial cells and neutrophils. *Prostaglandins Leukotrienes and Essential Fatty Acids* 82, 27-34.

- Markman,J.D., and Dworkin,R.H. (2006). Ion channel targets and treatment efficacy in neuropathic pain. *J. Pain* 7, S38-S47.
- Marrion,N.V. (1997). Control of M-current. *Annu. Rev Physiol* 59, 483-504.
- 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, 23-31.
- Martin,D.S.D., Lonergan,P.E., Boland,B., Fogarty,M.P., Brady,M., Horrobin,D.F., Campbell,V.A., and Lynch,M.A. (2002). Apoptotic changes in the aged brain are triggered by interleukin-1 beta-induced activation of p38 and reversed by treatment with eicosapentaenoic acid. *Journal of Biological Chemistry* 277, 34239-34246.
- Martin,H.A., Basbaum,A.I., Kwiat,G.C., Goetzl,E.J., and Levine,J.D. (1987). Leukotriene and prostaglandin sensitization of cutaneous high-threshold C- and A-delta mechanonociceptors in the hairy skin of rat hindlimbs. *Neuroscience* 22, 651-659.
- Martin,Y.B., and Avendano,C. (2009). Effects of removal of dietary polyunsaturated fatty acids on plasma extravasation and mechanical allodynia in a trigeminal neuropathic pain model. *Mol. Pain* 5, 8.
- Matsumura,S., Kunori,S., Mabuchi,T., Katano,T., Nakazawa,T., Abe,T., Watanabe,M., Yamamoto,T., Okuda-Ashitaka,E., and Ito,S. (2010). Impairment of CaMKII activation and attenuation of neuropathic pain in mice lacking NR2B phosphorylated at Tyr1472. *Eur. J. Neurosci.* 32, 798-810.
- Matsunaga,Y., Yamanishi,K., and Takahashi,M. (1982). Experimental infection and immune response of guinea pigs with varicella-zoster virus. *Infect. Immun.* 37, 407-412.
- Matzner,O., and Devor,M. (1994). Hyperexcitability at sites of nerve injury depends on voltage-sensitive Na⁺ channels. *J. Neurophysiol.* 72, 349-359.
- McCarthy,B.G., Hsieh,S.T., Stocks,A., Hauer,P., Macko,C., Cornblath,D.R., Griffin,J.W., and McArthur,J.C. (1995). Cutaneous innervation in sensory neuropathies: evaluation by skin biopsy. *Neurology* 45, 1848-1855.
- McMahon,S.B., Bennett,D.L., Priestley,J.V., and Shelton,D.L. (1995). The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule. *Nat. Med.* 1, 774-780.
- McMahon,S.B., Cafferty,W.B., and Marchand,F. (2005). Immune and glial cell factors as pain mediators and modulators. *Exp. Neurol.* 192, 444-462.
- Medana,I.M., and Esiri,M.M. (2003). Axonal damage: a key predictor of outcome in human CNS diseases. *Brain* 126, 515-530.
- Medhurst,S.J., Collins,S.D., Billinton,A., Bingham,S., Dalziel,R.G., Brass,A., Roberts,J.C., Medhurst,A.D., and Chessell,I.P. (2008). Novel histamine H3 receptor antagonists GSK189254 and GSK334429 are efficacious in surgically-induced and virally-induced rat models of neuropathic pain. *Pain* 138, 61-69.
- Meiri,K.F., Pfenninger,K.H., and Willard,M.B. (1986). Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. *Proc. Natl. Acad. Sci. U. S. A* 83, 3537-3541.
- Meiri,K.F., Willard,M., and Johnson,M.I. (1988). Distribution and Phosphorylation of the Growth-Associated Protein Gap-43 in Regenerating Sympathetic Neurons in Culture. *Journal of Neuroscience* 8, 2571-2581.

- Melli, G., Keswani, S.C., Fischer, A., Chen, W., and Hoke, A. (2006). Spatially distinct and functionally independent mechanisms of axonal degeneration in a model of HIV-associated sensory neuropathy. *Brain* 129, 1330-1338.
- Melov, S., Schneider, J.A., Day, B.J., Hinerfeld, D., Coskun, P., Mirra, S.S., Crapo, J.D., and Wallace, D.C. (1998). A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase. *Nature Genetics* 18, 159-163.
- Melzack, R., and Wall, P.D. (1965). Pain mechanisms: a new theory. *Science* 150, 971-979.
- Menard, C., Patenaude, C., Gagne, A.M., and Massicotte, G. (2009). AMPA receptor-mediated cell death is reduced by docosahexaenoic acid but not by eicosapentaenoic acid in area CA1 of hippocampal slice cultures. *J. Neurosci. Res.* 87, 876-886.
- Merskey, H., and Bogduk, N. (1994). Part III: Pain Terms, A Current List with Definitions and Notes on Usage. In *Classification of Chronic Pain*, pp. 209-214.
- Meyer, R.L., Miotke, J.A., and Benowitz, L.I. (1994). Injury-Induced Expression of Growth-Associated Protein-43 in Adult-Mouse Retinal Ganglion-Cells In-Vitro. *Neuroscience* 63, 591-602.
- Meyer-Rosberg, K., Kvarnstrom, A., Kinnman, E., Gordh, T., Nordfors, L.O., and Kristofferson, A. (2001). Peripheral neuropathic pain--a multidimensional burden for patients. *Eur. J. Pain* 5, 379-389.
- Michael, G.J., Averill, S., Shortland, P.J., Yan, Q., and Priestley, J.V. (1999). Axotomy results in major changes in BDNF expression by dorsal root ganglion cells: BDNF expression in large trkB and trkC cells, in pericellular baskets, and in projections to deep dorsal horn and dorsal column nuclei. *Eur. J. Neurosci.* 11, 3539-3551.
- Michael, G.J., and Priestley, J.V. (1999). Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. *J. Neurosci.* 19, 1844-1854.
- Michaels, J., Sharer, L.R., and Epstein, L.G. (1988). Human immunodeficiency virus type 1 (HIV-1) infection of the nervous system: a review. *Immunodef. Rev.* 1, 71-104.
- Micheau, O., and Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 114, 181-190.
- Michener, S.R., Aimone, L.D., Yaksh, T.L., and Go, V.L. (1990). Distribution of galanin-like immunoreactivity in the pig, rat and human central nervous system. *Peptides* 11, 1217-1223.
- Millan, M.J. (2002). Descending control of pain. *Prog. Neurobiol.* 66, 355-474.
- Milligan, E.D., Mehmert, K.K., Hinde, J.L., Harvey, L.O., Martin, D., Tracey, K.J., Maier, S.F., and Watkins, L.R. (2000). Thermal hyperalgesia and mechanical allodynia produced by intrathecal administration of the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp120. *Brain Res.* 861, 105-116.
- Milligan, E.D., Zapata, V., Chacur, M., Schoeniger, D., Biedenkapp, J., O'Connor, K.A., Verge, G.M., Chapman, G., Green, P., Foster, A.C., Naeve, G.S., Maier, S.F., and Watkins, L.R. (2004). Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. *Eur. J. Neurosci.* 20, 2294-2302.
- Mills, J.D., Bailes, J.E., Sedney, C.L., Hutchins, H., and Sears, B. (2011). Omega-3 fatty acid supplementation and reduction of traumatic axonal injury in a rodent head injury model. *J. Neurosurg.* 114, 77-84.

- Minami,T., Matsumura,S., Mabuchi,T., Kobayashi,T., Sugimoto,Y., Ushikubi,F., Ichikawa,A., Narumiya,S., and Ito,S. (2003). Functional evidence for interaction between prostaglandin EP3 and kappa-opioid receptor pathways in tactile pain induced by human immunodeficiency virus type-1 (HIV-1) glycoprotein gp120. *Neuropharmacology* 45, 96-105.
- Minthorby,C.A. (1994). Transcriptional Regulation of the Human Neuropeptide-y Gene by Nerve Growth-Factor. *Journal of Biological Chemistry* 269, 15460-15468.
- Mitsukawa,K., Lu,X., and Bartfai,T. (2010). Galanin, galanin receptors, and drug targets. *EXS* 102, 7-23.
- Mogil,J.S., and Cragg,S.E. (2004). What should we be measuring in behavioral studies of chronic pain in animals? *Pain* 112, 12-15.
- Mogil,J.S. (2009). Animal models of pain: progress and challenges. *Nat Rev Neurosci* 10, 283-294.
- Moller,K.A., Johansson,B., and Berge,O.G. (1998). Assessing mechanical allodynia in the rat paw with a new electronic algometer. *J. Neurosci. Methods* 84, 41-47.
- Molliver,D.C., Wright,D.E., Leitner,M.L., Parsadanian,A.S., Doster,K., Wen,D., Yan,Q., and Snider,W.D. (1997). IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* 19, 849-861.
- 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. *J. Neurosci* 22, 6724-6731.
- Mora,S., Dussaubat,N., and az-Veliz,G. (1996). Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. *Psychoneuroendocrinology* 21, 609-620.
- Morin,N., Owolabi,S.A., Harty,M.W., Papa,E.F., Tracy,T.F., Shaw,S.K., Kim,M., and Saab,C.Y. (2007). Neutrophils invade lumbar dorsal root ganglia after chronic constriction injury of the sciatic nerve. *Journal of Neuroimmunology* 184, 164-171.
- Moss,A., Ingram,R., Koch,S., Theodorou,A., Low,L., Baccei,M., Hathway,G.J., Costigan,M., Salton,S.R., and Fitzgerald,M. (2008). Origins, actions and dynamic expression patterns of the neuropeptide VGF in rat peripheral and central sensory neurones following peripheral nerve injury. *Mol. Pain* 4, 62.
- Moya,K.L., Benowitz,L.I., Jhaveri,S., and Schneider,G.E. (1988). Changes in rapidly transported proteins in developing hamster retinofugal axons. *J. Neurosci* 8, 4445-4454.
- Mueller,M., Wacker,K., Ringelstein,E.B., Hickey,W.F., Imai,Y., and Kiefer,R. (2001). Rapid response of identified resident endoneurial macrophages to nerve injury. *American Journal of Pathology* 159, 2187-2197.
- Mukherjee,P.K., Marcheselli,V.L., Serhan,C.N., and Bazan,N.G. (2004). Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc. Natl. Acad. Sci. U. S. A* 101, 8491-8496.
- Mullen,A., Loscher,C.E., and Roche,H.M. (2010). Anti-inflammatory effects of EPA and DHA are dependent upon time and dose-response elements associated with LPS stimulation in THP-1-derived macrophages. *The Journal of Nutritional Biochemistry* 21, 444-450.
- Munger,B.L., Bennett,G.J., and Kajander,K.C. (1992). An experimental painful peripheral neuropathy due to nerve constriction. I. Axonal pathology in the sciatic nerve. *Exp. Neurol.* 118, 204-214.

- Murphy,M., Reid,K., Brown,M.A., and Bartlett,P.F. (1993). Involvement of Leukemia Inhibitory Factor and Nerve Growth-Factor in the Development of Dorsal-Root Ganglion Neurons. *Development* 117, 1173-1182.
- Murphy,P.G., Ramer,M.S., Borthwick,L., Gauldie,J., Richardson,P.M., and Bisby,M.A. (1999). Endogenous interleukin-6 contributes to hypersensitivity to cutaneous stimuli and changes in neuropeptides associated with chronic nerve constriction in mice. *European Journal of Neuroscience* 11, 2243-2253.
- Myers,M.G., Duer,H.L., and Hausler,C.K. (1980). Experimental infection of guinea pigs with varicella-zoster virus. *J. Infect. Dis.* 142, 414-420.
- Myers,M.G., Stanberry,L.R., and Edmond,B.J. (1985). Varicella-zoster virus infection of strain 2 guinea pigs. *J. Infect. Dis.* 151, 106-113.
- Myers,R.R., Heckman,H.M., and Rodriguez,M. (1996). Reduced hyperalgesia in nerve-injured WLD mice: Relationship to nerve fiber phagocytosis, axonal degeneration, and regeneration in normal mice. *Experimental Neurology* 141, 94-101.
- Nadeau,S., Filali,M., Zhang,J., Kerr,B.J., Rivest,S., Soulet,D., Iwakura,Y., Vaccari,J.P.D., Keane,R.W., and Lacroix,S. (2011). Functional Recovery after Peripheral Nerve Injury is Dependent on the Pro-Inflammatory Cytokines IL-1 beta and TNF: Implications for Neuropathic Pain. *Journal of Neuroscience* 31, 12533-12542.
- Nahin,R.L., Ren,K., De,L.M., and Ruda,M. (1994). Primary sensory neurons exhibit altered gene expression in a rat model of neuropathic pain. *Pain* 58, 95-108.
- Nakagomi,S., Suzuki,Y., Namikawa,K., Kiryu-Seo,S., and Kiyama,H. (2003). Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation. *J. Neurosci.* 23, 5187-5196.
- Namikawa,K., Okamoto,T., Suzuki,A., Konishi,H., and Kiyama,H. (2006). Pancreatitis-associated protein-III is a novel macrophage chemoattractant implicated in nerve regeneration. *J. Neurosci* 26, 7460-7467.
- Nassar,M.A., Baker,M.D., Levato,A., Ingram,R., Mallucci,G., McMahon,S.B., and Wood,J.N. (2006). Nerve injury induces robust allodynia and ectopic discharges in Nav1.3 null mutant mice. *Mol. Pain* 2, 33.
- Naveilhan,P., Hassani,H., Lucas,G., Blakeman,K.H., Hao,J.X., Xu,X.J., Wiesenfeld-Hallin,Z., Thoren,P., and Ernfor,P. (2001). Reduced antinociception and plasma extravasation in mice lacking a neuropeptide Y receptor. *Nature* 409, 513-517.
- Neve,R.L., Perrone-Bizzozero,N.I., Finklestein,S., Zwiers,H., Bird,E., Kurnit,D.M., and Benowitz,L.I. (1987). The neuronal growth-associated protein GAP-43 (B-50, F1): neuronal specificity, developmental regulation and regional distribution of the human and rat mRNAs. *Brain Res.* 388, 177-183.
- Nicholson,B., and Verma,S. (2004). Comorbidities in chronic neuropathic pain. *Pain Med.* 5 Suppl 1, S9-S27.
- Niemoller,T.D., Stark,D.T., and Bazan,N.G. (2009). Omega-3 fatty acid docosahexaenoic acid is the precursor of neuroprotectin D1 in the nervous system. *World Rev. Nutr. Diet.* 99, 46-54.
- Nomura,Y. (2004). Neuronal apoptosis and protection: Effects of nitric oxide and endoplasmic reticulum-related proteins. *Biological & Pharmaceutical Bulletin* 27, 961-963.
- Novakovic,S.D., Tzoumaka,E., McGivern,J.G., Haraguchi,M., Sangameswaran,L., Gogas,K.R., Eglén,R.M., and Hunter,J.C. (1998). Distribution of the tetrodotoxin-resistant sodium channel PN3 in rat sensory neurons in normal and neuropathic conditions. *J. Neurosci.* 18, 2174-2187.

- Obata,K., Katsura,H., Miyoshi,K., Kondo,T., Yamanaka,H., Kobayashi,K., Dai,Y., Fukuoka,T., Akira,S., and Noguchi,K. (2008). Toll-like receptor 3 contributes to spinal glial activation and tactile allodynia after nerve injury. *J. Neurochem.* 105, 2249-2259.
- Obata,K., Yamanaka,H., Dai,Y., Mizushima,T., Fukuoka,T., Tokunaga,A., and Noguchi,K. (2004). Differential activation of MAPK in injured and uninjured DRG neurons following chronic constriction injury of the sciatic nerve in rats. *Eur. J. Neurosci.* 20, 2881-2895.
- Obata,K., Yamanaka,H., Fukuoka,T., Yi,D., Tokunaga,A., Hashimoto,N., Yoshikawa,H., and Noguchi,K. (2003). Contribution of injured and uninjured dorsal root ganglion neurons to pain behavior and the changes in gene expression following chronic constriction injury of the sciatic nerve in rats. *Pain* 101, 65-77.
- Oblinger,M.M., Wong,J., and Parysek,L.M. (1989). Axotomy-induced changes in the expression of a type III neuronal intermediate filament gene. *J Neurosci.* 9, 3766-3775.
- Oh,S.B., Tran,P.B., Gillard,S.E., Hurley,R.W., Hammond,D.L., and Miller,R.J. (2001). Chemokines and glycoprotein120 produce pain hypersensitivity by directly exciting primary nociceptive neurons. *J. Neurosci.* 21, 5027-5035.
- Ohara,S., Roth,K.A., Beaudet,L.N., and Schmidt,R.E. (1994). Transganglionic neuropeptide Y response to sciatic nerve injury in young and aged rats. *J. Neuropathol. Exp. Neurol.* 53, 646-662.
- Ohara,S., Tantuwaya,V., DiStefano,P.S., and Schmidt,R.E. (1995). Exogenous NT-3 mitigates the transganglionic neuropeptide Y response to sciatic nerve injury. *Brain Res.* 699, 143-148.
- Okamoto,M., Baba,H., Goldstein,P.A., Higashi,H., Shimoji,K., and Yoshimura,M. (2001). Functional reorganization of sensory pathways in the rat spinal dorsal horn following peripheral nerve injury. *J. Physiol* 532, 241-250.
- Oliver,E., McGillicuddy,F.C., Harford,K.A., Reynolds,C.M., Phillips,C.M., Ferguson,J.F., and Roche,H.M. (2012). Docosahexaenoic acid attenuates macrophage-induced inflammation and improves insulin sensitivity in adipocytes-specific differential effects between LC n-3 PUFA. *The Journal of Nutritional Biochemistry* 23, 1192-1200.
- Oomen,C.M., Feskens,E.J., Rasanen,L., Fidanza,F., Nissinen,A.M., Menotti,A., Kok,F.J., and Kromhout,D. (2000). Fish consumption and coronary heart disease mortality in Finland, Italy, and The Netherlands. *Am. J. Epidemiol.* 151, 999-1006.
- Opii,W.O., Sultana,R., Abdul,H.M., Ansari,M.A., Nath,A., and Butterfield,D.A. (2007). Oxidative stress and toxicity induced by the nucleoside reverse transcriptase inhibitor (NRTI)-2',3'-dideoxycytidine(ddC): Relevance to HIV-dementia. *Experimental Neurology* 204, 29-38.
- Palanza,P. (2001). Animal models of anxiety and depression: how are females different? *Neurosci. Biobehav. Rev* 25, 219-233.
- Pan,H.C., Kao,T.K., Ou,Y.C., Yang,D.Y., Yen,Y.J., Wang,C.C., Chuang,Y.H., Liao,S.L., Raung,S.L., Wu,C.W., Chiang,A.N., and Chen,C.J. (2009). Protective effect of docosahexaenoic acid against brain injury in ischemic rats. *J. Nutr. Biochem.* 20, 715-725.
- 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, 1026-1030.
- Pandya,R., Krentz,H.B., Gill,M.J., and Power,C. (2005). HIV-related neurological syndromes reduce health-related quality of life. *Can. J. Neurol. Sci.* 32, 201-204.

- Pardo,C.A., McArthur,J.C., and Griffin,J.W. (2001). HIV neuropathy: insights in the pathology of HIV peripheral nerve disease. *J. Peripher. Nerv. Syst.* 6, 21-27.
- Parkin,J., and Cohen,B. (2001). An overview of the immune system. *Lancet* 357, 1777-1789.
- Pearson,A.G., Gray,C.W., Pearson,J.F., Greenwood,J.M., During,M.J., and Dragunow,M. (2003). ATF3 enhances c-Jun-mediated neurite sprouting. *Brain Res. Mol. Brain Res.* 120, 38-45.
- Pelzer,A.E., Bektic,J., Haag,P., Berger,A.P., Pycha,A., Schafer,G., Rogatsch,H., Horninger,W., Bartsch,G., and Klocker,H. (2006). The expression of transcription factor activating transcription factor 3 in the human prostate and its regulation by androgen in prostate cancer. *J Urol.* 175, 1517-1522.
- Perez,J., Ware,M.A., Chevalier,S., Gougeon,R., Bennett,G.J., and Shir,Y. (2004). Dietary fat and protein interact in suppressing neuropathic pain-related disorders following a partial sciatic ligation injury in rats. *Pain* 111, 297-305.
- Perez,J., Ware,M.A., Chevalier,S., Gougeon,R., and Shir,Y. (2005). Dietary omega-3 fatty acids may be associated with increased neuropathic pain in nerve-injured rats. *Anesth. Analg.* 101, 444-8, table.
- Perrin,F.E., Lacroix,S., Aviles-Trigueros,M., and David,S. (2005). Involvement of monocyte chemoattractant protein-1, macrophage inflammatory protein-1 alpha and interleukin-1 beta in Wallerian degeneration. *Brain* 128, 854-866.
- Perry,M.J., Lawson,S.N., and Robertson,J. (1991). Neurofilament immunoreactivity in populations of rat primary afferent neurons: a quantitative study of phosphorylated and non-phosphorylated subunits. *J. Neurocytol.* 20, 746-758.
- Perry,V.H., Brown,M.C., and Gordon,S. (1987). The Macrophage Response to Central and Peripheral-Nerve Injury - A Possible Role for Macrophages in Regeneration. *Journal of Experimental Medicine* 165, 1218-1223.
- Peters,C.M., Ghilardi,J.R., Keyser,C.P., Kubota,K., Lindsay,T.H., Luger,N.M., Mach,D.B., Schwei,M.J., Sevcik,M.A., and Mantyh,P.W. (2005). Tumor-induced injury of primary afferent sensory nerve fibers in bone cancer pain. *Exp. Neurol.* 193, 85-100.
- Petersen,E.A., Ramirez-Ronda,C.H., Hardy,W.D., Schwartz,R., Sacks,H.S., Follansbee,S., Peterson,D.M., Cross,A., Anderson,R.E., and Dunkle,L.M. (1995). Dose-related activity of stavudine in patients infected with human immunodeficiency virus. *J. Infect. Dis.* 171 Suppl 2, S131-S139.
- Petit-Demouliere,B., Chenu,F., and Bourin,M. (2005). Forced swimming test in mice: a review of antidepressant activity. *Psychopharmacology (Berl)* 177, 245-255.
- Petroni,A., Salami,M., Blasevich,M., Papini,N., and Galli,C. (1994). Inhibition by n-3 fatty acids of arachidonic acid metabolism in a primary culture of astroglial cells. *Neurochem. Res.* 19, 1187-1193.
- Petrosino,S., Palazzo,E., de,N., V, Bisogno,T., Rossi,F., Maione,S., and Di,M., V (2007). Changes in spinal and supraspinal endocannabinoid levels in neuropathic rats. *Neuropharmacology* 52, 415-422.
- Pezet,S., and McMahon,S.B. (2006). Neurotrophins: mediators and modulators of pain. *Annu. Rev. Neurosci.* 29, 507-538.
- Phillips,T.J., Cherry,C.L., Cox,S., Marshall,S.J., and Rice,A.S. (2010). Pharmacological treatment of painful HIV-associated sensory neuropathy: a systematic review and meta-analysis of randomised controlled trials. *PLoS. One.* 5, e14433.

- Pociot,F., Lorenzen,T., and Nerup,J. (1993). Manganese Superoxide-Dismutase (Sod2) Gene Polymorphism in Insulin-Dependent Diabetes-Mellitus. *Disease Markers* 11, 267-274.
- Polomano,R.C., Mannes,A.J., Clark,U.S., and Bennett,G.J. (2001). A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel. *Pain* 94, 293-304.
- Porsolt,R.D., Le,P.M., and Jalfre,M. (1977). Depression: a new animal model sensitive to antidepressant treatments. *Nature* 266, 730-732.
- Prut,L., and Belzung,C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur. J. Pharmacol.* 463, 3-33.
- Quartilho,A., Mata,H.P., Ibrahim,M.M., Vanderah,T.W., Porreca,F., Makriyannis,A., and Malan,T.P., Jr. (2003). Inhibition of inflammatory hyperalgesia by activation of peripheral CB2 cannabinoid receptors. *Anesthesiology* 99, 955-960.
- Quick,K., Zhao,J., Eijkelkamp,N., Linley,J.E., Rugiero,F., Cox,J.J., Raouf,R., Gringhuis,M., Sexton,J.E., Abramowitz,J., Taylor,R., Forge,A., Ashmore,J., Kirkwood,N., Kros,C.J., Richardson,G.P., Freichel,M., Flockerzi,V., Birnbaumer,L., and Wood,J.N. (2012). TRPC3 and TRPC6 are essential for normal mechanotransduction in subsets of sensory neurons and cochlear hair cells. *Open Biology* 2.
- Raghavendra,V., Tanga,F., and Deleo,J.A. (2003). Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J. Pharmacol. Exp. Ther.* 306, 624-630.
- Rahman,W., Suzuki,R., Webber,M., Hunt,S.P., and Dickenson,A.H. (2006). Depletion of endogenous spinal 5-HT attenuates the behavioural hypersensitivity to mechanical and cooling stimuli induced by spinal nerve ligation. *Pain* 123, 264-274.
- Ramer,M.S., French,G.D., and Bisby,M.A. (1997). Wallerian degeneration is required for both neuropathic pain and sympathetic sprouting into the DRG. *Pain* 72, 71-78.
- Ramer,M.S., Murphy,P.G., Richardson,P.M., and Bisby,M.A. (1998). Spinal nerve lesion-induced mechanoallodynia and adrenergic sprouting in sensory ganglia are attenuated in interleukin-6 knockout mice. *Pain* 78, 115-121.
- Ramsey,I.S., Delling,M., and Clapham,D.E. (2006). An introduction to TRP channels. *Annu. Rev. Physiol* 68, 619-647.
- RANDALL,L.O., and Stelitto,J.J. (1957). A method for measurement of analgesic activity on inflamed tissue. *Arch. Int. Pharmacodyn. Ther.* 111, 409-419.
- Rao,M.S., Sun,Y., Escary,J.L., Perreau,J., Tresser,S., Patterson,P.H., Zigmond,R.E., Brulet,P., and Landis,S.C. (1993). Leukemia Inhibitory Factor Mediates An Injury Response But Not A Target-Directed Developmental Transmitter Switch in Sympathetic Neurons. *Neuron* 11, 1175-1185.
- 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. *Proc. Natl. Acad. Sci. U. S. A* 98, 13373-13378.
- Rashid,M.H., Inoue,M., Toda,K., and Ueda,H. (2004). Loss of peripheral morphine analgesia contributes to the reduced effectiveness of systemic morphine in neuropathic pain. *J. Pharmacol. Exp. Ther.* 309, 380-387.
- 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, 461-469.

Reilly,S.C., Cossins,A.R., Quinn,J.P., and Sneddon,L.U. (2004). Discovering genes: the use of microarrays and laser capture microdissection in pain research. *Brain Res. Brain Res. Rev* 46, 225-233.

Reliquet,V., Robillard,N., Perre,P., Muller,J.Y., Raffi,F., and Audrain,M. (2001). Immune response to antiretroviral treatment combining stavudine, didanosine and nevirapine. *Presse Medicale* 30, 1143-1147.

Renaud-Thery,F., Nguimfack,B.D., Vitoria,M., Lee,E., Graaff,P., Samb,B., and Perriens,J. (2007). Use of antiretroviral therapy in resource-limited countries in 2006: distribution and uptake of first- and second-line regimens. *AIDS 21 Suppl 4*, S89-S95.

Renn,C.L., Leitch,C.C., Lessans,S., Rhee,P., McGuire,W.C., Smith,B.A., Traub,R.J., and Dorsey,S.G. (2011). Brain-derived neurotrophic factor modulates antiretroviral-induced mechanical allodynia in the mouse. *J. Neurosci. Res.* 89, 1551-1565.

Requena,J.R., Levine,R.L., and Stadtman,E.R. (2003). Recent advances in the analysis of oxidized proteins. *Amino. Acids* 25, 221-226.

Reynolds,M.L., Fitzgerald,M., and Benowitz,L.I. (1991). GAP-43 expression in developing cutaneous and muscle nerves in the rat hindlimb. *Neuroscience* 41, 201-211.

Rice,A.S. (2008). Should cannabinoids be used as analgesics for neuropathic pain? *Nat. Clin. Pract. Neurol.* 4, 654-655.

Rice,A.S., Cimino-Brown,D., Eisenach,J.C., Kontinen,V.K., Lacroix-Fralish,M.L., Machin,I., Mogil,J.S., and Stohr,T. (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, 243-247.

Ricote,M., Li,A.C., Willson,T.M., Kelly,C.J., and Glass,C.K. (1998). The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 391, 79-82.

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

Robson,L.G., Dyall,S., Sidloff,D., and Michael-Titus,A.T. (2010). Omega-3 polyunsaturated fatty acids increase the neurite outgrowth of rat sensory neurones throughout development and in aged animals. *Neurobiology of Aging* 31, 678-687.

Rodin,B.E., and Kruger,L. (1984). Deafferentation in animals as a model for the study of pain: an alternative hypothesis. *Brain Res.* 319, 213-228.

Roeska,K., Doods,H., Arndt,K., Treede,R.D., and Ceci,A. (2008). Anxiety-like behaviour in rats with mononeuropathy is reduced by the analgesic drugs morphine and gabapentin. *Pain*.

Rolke,R., Baron,R., Maier,C., Tolle,T.R., Treede,R.D., Beyer,A., Binder,A., Birbaumer,N., Birklein,F., Botefur,I.C., Braune,S., Flor,H., Hugel,V., Klug,R., Landwehrmeyer,G.B., Magerl,W., Maihofner,C., Rolko,C., Schaub,C., Scherens,A., Sprenger,T., Valet,M., and Wasserka,B. (2006a). Quantitative sensory testing in the German Research Network on Neuropathic Pain (DFNS): standardized protocol and reference values. *Pain* 123, 231-243.

Rolke,R., Magerl,W., Campbell,K.A., Schalber,C., Caspari,S., Birklein,F., and Treede,R.D. (2006b). Quantitative sensory testing: a comprehensive protocol for clinical trials. *Eur. J. Pain* 10, 77-88.

- Rosenberger,C.M., Clark,A.E., Treuting,P.M., Johnson,C.D., and Aderem,A. (2008). ATF3 regulates MCMV infection in mice by modulating IFN-gamma expression in natural killer cells. *Proceedings of the National Academy of Sciences of the United States of America* 105, 2544-2549.
- Rowbotham,M.C., and Fields,H.L. (1996). The relationship of pain, allodynia and thermal sensation in post-herpetic neuralgia. *Brain* 119 (Pt 2), 347-354.
- Russo,G.L. (2009). Dietary α -6 and α -3 polyunsaturated fatty acids: From biochemistry to clinical implications in cardiovascular prevention. *Biochemical Pharmacology* 77, 937-946.
- Sadosky,A., McDermott,A.M., Brandenburg,N.A., and Strauss,M. (2008). A review of the epidemiology of painful diabetic peripheral neuropathy, postherpetic neuralgia, and less commonly studied neuropathic pain conditions. *Pain Pract.* 8, 45-56.
- Sadzot-Delvaux,C., Merville-Louis,M.P., Delree,P., Marc,P., Piette,J., Moonen,G., and Rentier,B. (1990). An in vivo model of varicella-zoster virus latent infection of dorsal root ganglia. *J. Neurosci. Res.* 26, 83-89.
- Safieh-Garabedian,B., Poole,S., Allchorne,A., Winter,J., and Woolf,C.J. (1995). Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. *Br. J. Pharmacol.* 115, 1265-1275.
- Saito,H., and Dahlin,L.B. (2008). Expression of ATF3 and axonal outgrowth are impaired after delayed nerve repair. *BMC. Neurosci.* 9, 88.
- Schafers,M., Brinkhoff,J., Neukirchen,S., Marziniak,M., and Sommer,C. (2001). Combined epineurial therapy with neutralizing antibodies to tumor necrosis factor-alpha and interleukin-1 receptor has an additive effect in reducing neuropathic pain in mice. *Neurosci Lett.* 310, 113-116.
- Schafers,M., Geis,C., Svensson,C.I., Luo,Z.D., and Sommer,C. (2003a). Selective increase of tumour necrosis factor-alpha in injured and spared myelinated primary afferents after chronic constrictive injury of rat sciatic nerve. *Eur. J. Neurosci.* 17, 791-804.
- Schafers,M., Lee,D.H., Brors,D., Yaksh,T.L., and Sorkin,L.S. (2003b). Increased sensitivity of injured and adjacent uninjured rat primary sensory neurons to exogenous tumor necrosis factor-alpha after spinal nerve ligation. *J. Neurosci.* 23, 3028-3038.
- Schafers,M., Sorkin,L.S., Geis,C., and Shubayev,V.I. (2003c). Spinal nerve ligation induces transient upregulation of tumor necrosis factor receptors 1 and 2 in injured and adjacent uninjured dorsal root ganglia in the rat. *Neuroscience Letters* 347, 179-182.
- Schmued,L.C., Albertson,C.M., Andrews,A., Sandberg,J.A., Nickols,J., and Slikker,W., Jr. (1996). Evaluation of brain and nerve pathology in rats chronically dosed with ddI or isoniazid. *Neurotoxicol. Teratol.* 18, 555-563.
- Schmued,L.C., and Fallon,J.H. (1986). Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. *Brain Res.* 377, 147-154.
- Scholz,J., Broom,D.C., Youn,D.H., Mills,C.D., Kohno,T., Suter,M.R., Moore,K.A., Decosterd,I., Coggeshall,R.E., and Woolf,C.J. (2005). Blocking caspase activity prevents transsynaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury. *J. Neurosci* 25, 7317-7323.
- Scholz,J., and Woolf,C.J. (2007). The neuropathic pain triad: neurons, immune cells and glia. *Nat. Neurosci.* 10, 1361-1368.

- Schreyer,D.J., and Skene,J.H. (1991). Fate of GAP-43 in ascending spinal axons of DRG neurons after peripheral nerve injury: delayed accumulation and correlation with regenerative potential. *J. Neurosci.* *11*, 3738-3751.
- Schreyer,D.J., and Skene,J.H.P. (1993). Injury-associated induction of GAP-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. *J. Neurobiol.* *24*, 959-970.
- Scott,B.L., and Bazan,N.G. (1989). Membrane Docosaehaenoate Is Supplied to the Developing Brain and Retina by the Liver. *Proceedings of the National Academy of Sciences of the United States of America* *86*, 2903-2907.
- Scott,R.L., Gurusinghe,A.D., Rudvosky,A.A., Kozlakivsky,V., Murray,S.S., Satoh,M., and Cheema,S.S. (2000). Expression of leukemia inhibitory factor receptor mRNA in sensory dorsal root ganglion and spinal motor neurons of the neonatal rat. *Neurosci. Lett.* *295*, 49-53.
- Seijffers,R., Allchorne,A.J., and Woolf,C.J. (2006). The transcription factor ATF-3 promotes neurite outgrowth. *Mol. Cell Neurosci.* *32*, 143-154.
- Seijffers,R., Mills,C.D., and Woolf,C.J. (2007). ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. *J. Neurosci.* *27*, 7911-7920.
- Sekiguchi,M., Sekiguchi,Y., Konno,S., Kobayashi,H., Homma,Y., and Kikuchi,S. (2009). Comparison of neuropathic pain and neuronal apoptosis following nerve root or spinal nerve compression. *Eur. Spine J.* *18*, 1978-1985.
- 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*, 205-218.
- Seminowicz,D.A., Laferriere,A.L., Millicamps,M., Yu,J.S.,Coderre,T.J., and Bushnell,M.C. (2009). MRI structural brain changes associated with sensory and emotional function in a rat model of long-term neuropathic pain. *Neuroimage.* *47*, 1007-1014.
- Serhan,C.N., Chiang,N., and Van Dyke,T.E. (2008). Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat. Rev. Immunol.* *8*, 349-361.
- Serhan,C.N., Gotlinger,K., Hong,S., and Arita,M. (2004). Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis. *Prostaglandins Other Lipid Mediat.* *73*, 155-172.
- Serhan,C.N., Gotlinger,K., Hong,S., Lu,Y., Siegelman,J., Baer,T., Yang,R., Colgan,S.P., and Petasis,N.A. (2006). Anti-inflammatory actions of neuroprotectin D1/protectin D1 and its natural stereoisomers: assignments of dihydroxy-containing docosatrienes. (vol 176, pg 1848, 2006). *Journal of Immunology* *176*, 3843.
- Seutin,V., Verbanck,P., Massotte,L., and Dresse,A. (1989). Galanin decreases the activity of locus coeruleus neurons in vitro. *Eur. J. Pharmacol.* *164*, 373-376.
- Sevcik,J., Finta,E.P., and Illes,P. (1993). Galanin receptors inhibit the spontaneous firing of locus coeruleus neurones and interact with mu-opioid receptors. *Eur. J. Pharmacol.* *230*, 223-230.
- Shafer,R.W., and Vuitton,D.A. (1999). Highly active antiretroviral therapy (HAART) for the treatment of infection with human immunodeficiency virus type 1. *Biomed. Pharmacother.* *53*, 73-86.
- Shakur,S.F., Bhansali,A., Mian,A.Y., and Rosseau,G.L. (2011). Neurosurgical treatment of trigeminal neuralgia. *Dis. Mon.* *57*, 570-582.

- Shapiro,H. (2003). Could n-3 polyunsaturated fatty acids reduce pathological pain by direct actions on the nervous system? *Prostaglandins Leukotrienes and Essential Fatty Acids* 68, 219-224.
- Shapiro,H., Lutaty,A., and Ariel,A. (2011). Macrophages, meta-inflammation, and immuno-metabolism. *ScientificWorldJournal*. 11, 2509-2529.
- Shepard,J.D., and Myers,D.A. (2008). Strain differences in anxiety-like behavior: association with corticotropin-releasing factor. *Behav. Brain Res.* 186, 239-245.
- Sherrington,C.S. (1906). Observations on the scratch-reflex in the spinal dog. *J. Physiol* 34, 1-50.
- Shi,T.J., Cui,J.G., Meyerson,B.A., Linderorth,B., and Hokfelt,T. (1999). Regulation of galanin and neuropeptide Y in dorsal root ganglia and dorsal horn in rat mononeuropathic models: possible relation to tactile hypersensitivity. *Neuroscience* 93, 741-757.
- Shi,T.J.S., Li,J., Dahlström,A., Theodorsson,E., Ceccatelli,S., Decosterd,I., Pedrazzini,T., and Hökfelt,T. (2006). Deletion of the neuropeptide Y Y1 receptor affects pain sensitivity, neuropeptide transport and expression, and dorsal root ganglion neuron numbers. *Neuroscience* 140, 293-304.
- Shi,Y.H., and Pestka,J.J. (2009). Mechanisms for suppression of interleukin-6 expression in peritoneal macrophages from docosahexaenoic acid-fed mice. *Journal of Nutritional Biochemistry* 20, 358-368.
- Shir,Y., and Seltzer,Z. (1991). Effects of sympathectomy in a model of causalgiform pain produced by partial sciatic nerve injury in rats. *Pain* 45, 309-320.
- Shlay,J.C., Chaloner,K., Max,M.B., Flaws,B., Reichelderfer,P., Wentworth,D., Hillman,S., Brizz,B., and Cohn,D.L. (1998). Acupuncture and amitriptyline for pain due to HIV-related peripheral neuropathy: a randomized controlled trial. *Terry Bein Community Programs for Clinical Research on AIDS. JAMA* 280, 1590-1595.
- Shortland,P.J., Baytug,B., Krzyzanowska,A., McMahon,S.B., Priestley,J.V., and Averill,S. (2006). ATF3 expression in L4 dorsal root ganglion neurons after L5 spinal nerve transection. *Eur. J. Neurosci.* 23, 365-373.
- 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, 507-514.
- Simmons,D.R., Spike,R.C., and Todd,A.J. (1995). Galanin is contained in GABAergic neurons in the rat spinal dorsal horn. *Neurosci. Lett.* 187, 119-122.
- Simpson,D.M., and Tagliati,M. (1995). Nucleoside analogue-associated peripheral neuropathy in human immunodeficiency virus infection. *J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol.* 9, 153-161.
- Sindrup,S.H., and Jensen,T.S. (1999). Efficacy of pharmacological treatments of neuropathic pain: an update and effect related to mechanism of drug action. *Pain* 83, 389-400.
- Smith,A.G., Howard,J.R., Kroll,R., Ramachandran,P., Hauer,P., Singleton,J.R., and McArthur,J. (2005). The reliability of skin biopsy with measurement of intraepidermal nerve fiber density. *J. Neurol. Sci.* 228, 65-69.
- Smyth,K., Affandi,J.S., McArthur,J.C., Bowtell-Harris,C., Mijch,A.M., Watson,K., Costello,K., Woolley,I.J., Price,P., Wesselingh,S.L., and Cherry,C.L. (2007). Prevalence of and risk factors for HIV-associated neuropathy in Melbourne, Australia 1993-2006. *HIV. Med.* 8, 367-373.
- Snider,W.D., and McMahon,S.B. (1998). Tackling pain at the source: new ideas about nociceptors. *Neuron* 20, 629-632.

- Snijdelaar,D.G., Dirksen,R., Slappendel,R., and Crul,B.J. (2000). Substance P. *Eur. J. Pain* 4, 121-135.
- Sommer,C., Petrasch,S., Lindenlaub,T., and Toyka,K.V. (1999). Neutralizing antibodies to interleukin 1-receptor reduce pain associated behavior in mice with experimental neuropathy. *Neurosci. Lett.* 270, 25-28.
- Sommerville,T., Reynolds,M.L., and Woolf,C.J. (1991). Time-dependent differences in the increase in GAP-43 expression in dorsal root ganglion cells after peripheral axotomy. *Neuroscience* 45, 213-220.
- Songur,A., Sarsilmaz,M., Sogut,S., Ozyurt,B., Ozyurt,H., Zararsiz,I., and Turkoglu,A.O. (2004). Hypothalamic superoxide dismutase, xanthine oxidase, nitric oxide, and malondialdehyde in rats fed with fish omega-3 fatty acids. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* 28, 693-698.
- Sotocinal,S.G., Sorge,R.E., Zaloum,A., Tuttle,A.H., Martin,L.J., Wieskopf,J.S., Mapplebeck,J.C.S., Wei,P., Zhan,S., Zhang,S.R., McDougall,J.J., King,O.D., and Mogil,J.S. (2011). The Rat Grimace Scale: A partially automated method for quantifying pain in the laboratory rat via facial expressions. *Molecular Pain* 7.
- Stam,F.J., MacGillavry,H.D., Armstrong,N.J., de Gunst,M.C., Zhang,Y., van Kesteren,R.E., Smit,A.B., and Verhaagen,J. (2007). Identification of candidate transcriptional modulators involved in successful regeneration after nerve injury. *Eur. J. Neurosci.* 25, 3629-3637.
- Starkey,M.L., Davies,M., Yip,P.K., Carter,L.M., Wong,D.J., McMahon,S.B., and Bradbury,E.J. (2009). Expression of the regeneration-associated protein SPRR1A in primary sensory neurons and spinal cord of the adult mouse following peripheral and central injury. *J. Comp Neurol.* 513, 51-68.
- Stefani,A., Spadoni,F., and Bernardi,G. (1998). Gabapentin inhibits calcium currents in isolated rat brain neurons. *Neuropharmacology* 37, 83-91.
- Sterne,G.D., Brown,R.A., Green,C.J., and Terenghi,G. (1998). NT-3 modulates NPY expression in primary sensory neurons following peripheral nerve injury. *J. Anat.* 193 (Pt 2), 273-281.
- Stienstra,R., Duval,C., Muller,M., and Kersten,S. (2007). PPARs, Obesity, and Inflammation. *Ppar Research*.
- Stoll,G., Griffin,J.W., Li,C.Y., and Trapp,B.D. (1989). Wallerian degeneration in the peripheral nervous system: participation of both Schwann cells and macrophages in myelin degradation. *J. Neurocytol.* 18, 671-683.
- Streit,W.J., Graeber,M.B., and Kreutzberg,G.W. (1989). Peripheral nerve lesion produces increased levels of major histocompatibility complex antigens in the central nervous system. *J. Neuroimmunol.* 21, 117-123.
- Suaudeau,C., Rinaldi,D., Lepicard,E., Venault,P., Crusio,W.E., Costentin,J., and Chapouthier,G. (2000). Divergent levels of anxiety in mice selected for differences in sensitivity to a convulsant agent. *Physiol Behav.* 71, 517-523.
- Sugimoto,T., Bennett,G.J., and Kajander,K.C. (1990). Transsynaptic degeneration in the superficial dorsal horn after sciatic nerve injury: effects of a chronic constriction injury, transection, and strychnine. *Pain* 42, 205-213.
- Sun,J.H., Yang,B., Donnelly,D.F., Ma,C., and LaMotte,R.H. (2006). MCP-1 enhances excitability of nociceptive neurons in chronically compressed dorsal root ganglia. *J. Neurophysiol.* 96, 2189-2199.
- Sun,Y., and Zigmond,R.E. (1996). Leukaemia inhibitory factor induced in the sciatic nerve after axotomy is involved in the induction of galanin in sensory neurons. *Eur. J. Neurosci.* 8, 2213-2220.
- Sun,Y.P., Oh,S.F., Uddin,J., Yang,R., Gotlinger,K., Campbell,E., Colgan,S.P., Petasis,N.A., and Serhan,C.N. (2007). Resolvin D1 and its aspirin-triggered 17R epimer. Stereochemical assignments, anti-inflammatory properties, and enzymatic inactivation. *J. Biol. Chem.* 282, 9323-9334.

- Sung,B., Lim,G., and Mao,J. (2003). Altered expression and uptake activity of spinal glutamate transporters after nerve injury contribute to the pathogenesis of neuropathic pain in rats. *J. Neurosci.* 23, 2899-2910.
- 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, 53.
- 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, 101-109.
- Suzuki,R., Morcuende,S., Webber,M., Hunt,S.P., and Dickenson,A.H. (2002). Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. *Nat Neurosci.* 5, 1319-1326.
- Suzuki,R., Rahman,W., Hunt,S.P., and Dickenson,A.H. (2004). Descending facilitatory control of mechanically evoked responses is enhanced in deep dorsal horn neurones following peripheral nerve injury. *Brain Res.* 1019, 68-76.
- Suzuki,R., Rahman,W., Rygh,L.J., Webber,M., Hunt,S.P., and Dickenson,A.H. (2005). Spinal-supraspinal serotonergic circuits regulating neuropathic pain and its treatment with gabapentin. *Pain* 117, 292-303.
- Svensson,P., Cairns,B.E., Wang,K., and rendt-Nielsen,L. (2003). Injection of nerve growth factor into human masseter muscle evokes long-lasting mechanical allodynia and hyperalgesia. *Pain* 104, 241-247.
- Svichar,N., Kostyuk,P., and Verkhatsky,A. (1997). Mitochondria buffer Ca^{2+} entry but not intracellular Ca^{2+} release in mouse DRG neurones. *Neuroreport* 8, 3929-3932.
- Sweitzer,S.M., Schubert,P., and Deleo,J.A. (2001). Propentofylline, a glial modulating agent, exhibits antiallodynic properties in a rat model of neuropathic pain. *J. Pharmacol. Exp. Ther.* 297, 1210-1217.
- Swett,J.E., Torigoe,Y., Elie,V.R., Bourassa,C.M., and Miller,P.G. (1991). Sensory neurons of the rat sciatic nerve. *Exp. Neurol.* 114, 82-103.
- Taiwo,O.B., and Taylor,B.K. (2002). Antihyperalgesic effects of intrathecal neuropeptide Y during inflammation are mediated by Y1 receptors. *Pain* 96, 353-363.
- Tanaka,T., Minami,M., Nakagawa,T., and Satoh,M. (2004). Enhanced production of monocyte chemoattractant protein-1 in the dorsal root ganglia in a rat model of neuropathic pain: possible involvement in the development of neuropathic pain. *Neurosci. Res.* 48, 463-469.
- Tang,H.L., Tang,H.M., Mak,K.H., Hu,S.M., Wang,S.S., Wong,K.M., Wong,C.S.T., Wu,H.Y., Law,H.T., Liu,K., Talbot,C.C., Lau,W.K., Montell,D.J., and Fung,M.C. (2012). Cell survival, DNA damage, and oncogenic transformation after a transient and reversible apoptotic response. *Molecular Biology of the Cell* 23, 2240-2252.
- Tanga,F.Y., Nutile-McMenemy,N., and Deleo,J.A. (2005). The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc. Natl. Acad. Sci. U. S. A* 102, 5856-5861.
- Tatemoto,K., Carlquist,M., and Mutt,V. (1982). Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* 296, 659-660.
- Tatemoto,K., Rokaeus,A., Jornvall,H., Mcdonald,T.J., and Mutt,V. (1983). Galanin - A Novel Biologically-Active Peptide from Porcine Intestine. *Febs Letters* 164, 124-128.

- Thacker, M.A., Clark, A.K., Bishop, T., Grist, J., Yip, P.K., Moon, L.D., Thompson, S.W., Marchand, F., and McMahon, S.B. (2009). CCL2 is a key mediator of microglia activation in neuropathic pain states. *Eur. J. Pain* 13, 263-272.
- Thompson, M., Xu, D., and Williams, B. (2009). ATF3 transcription factor and its emerging roles in immunity and cancer. *Journal of Molecular Medicine* 87, 1053-1060.
- Todd, A.J. (2002). Anatomy of primary afferents and projection neurones in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor. *Exp. Physiol* 87, 245-249.
- Tominaga, M., and Caterina, M.J. (2004). Thermosensation and pain. *J Neurobiol.* 61, 3-12.
- Tong, Y.G., Wang, H.F., Ju, G., Grant, G., Hokfelt, T., and Zhang, X. (1999). Increased uptake and transport of cholera toxin B-subunit in dorsal root ganglion neurons after peripheral axotomy: possible implications for sensory sprouting. *J. Comp Neurol.* 404, 143-158.
- Torebjork, H.E., Lundberg, L.E.R., and LaMotte, R.H. (1992). Central Changes in Processing of Mechanoreceptive Input in Capsaicin-Induced Secondary Hyperalgesia in Humans. *Journal of Physiology-London* 448, 765-780.
- Tozaki-Saitoh, H., Tsuda, M., Miyata, H., Ueda, K., Kohsaka, S., and Inoue, K. (2008). P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. *J. Neurosci.* 28, 4949-4956.
- Treede, R.D., Jensen, T.S., Campbell, J.N., Cruccu, G., Dostrovsky, J.O., Griffin, J.W., Hansson, P., Hughes, R., Nurmikko, T., and Serra, J. (2008). Neuropathic pain: redefinition and a grading system for clinical and research purposes. *Neurology* 70, 1630-1635.
- Treit, D., and Fundytus, M. (1988). Thigmotaxis as a test for anxiolytic activity in rats. *Pharmacol. Biochem. Behav.* 31, 959-962.
- Tsai, Y.J., Lin, C.T., and Lue, J.H. (2007). Characterization of the induced neuropeptide Y-like immunoreactivity in primary sensory neurons following complete median nerve transection. *J. Neurotrauma* 24, 1878-1888.
- Tsuda, M., Inoue, K., and Salter, M.W. (2005). Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. *Trends Neurosci* 28, 101-107.
- Tsuda, M., Kohro, Y., Yano, T., Tsujikawa, T., Kitano, J., Tozaki-Saitoh, H., Koyanagi, S., Ohdo, S., Ji, R.R., Salter, M.W., and Inoue, K. (2011). JAK-STAT3 pathway regulates spinal astrocyte proliferation and neuropathic pain maintenance in rats. *Brain* 134, 1127-1139.
- Tsuda, M., Mizokoshi, A., Shigemoto-Mogami, Y., Koizumi, S., and Inoue, K. (2004). Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. *Glia* 45, 89-95.
- Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M.W., and Inoue, K. (2003). P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 424, 778-783.
- Tsujino, H., Kondo, E., Fukuoka, T., Dai, Y., Tokunaga, A., Miki, K., Yonenobu, K., Ochi, T., and Noguchi, K. (2000). Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. *Mol. Cell Neurosci* 15, 170-182.
- Tyers, M., and Mann, M. (2003). From genomics to proteomics. *Nature* 422, 193-197.
- Unal-Cevik, I., and Oaklander, A.L. (2011). Comparing partial and total tibial-nerve axotomy: long-term effects on prevalence and location of evoked pain behaviors. *Pain Pract.* 11, 109-119.

- Uttara,B., Singh,A.V., Zamboni,P., and Mahajan,R.T. (2009). Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. *Current Neuropharmacology* 7, 65-74.
- Vaeroy,H., Tanum,L., Bruaset,H., Morkrid,L., and Forre,O. (2005). Symptoms of depression and anxiety in functionally disabled rheumatic pain patients. *Nord. J. Psychiatry* 59, 109-113.
- Valder,C.R., Liu,J.J., Song,Y.H., and Luo,Z.D. (2003). Coupling gene chip analyses and rat genetic variances in identifying potential target genes that may contribute to neuropathic allodynia development. *J. Neurochem.* 87, 560-573.
- Vallbo,A.B., Olausson,H., and Wessberg,J. (1999). Unmyelinated afferents constitute a second system coding tactile stimuli of the human hairy skin. *J Neurophysiol.* 81, 2753-2763.
- Van Steenwinckel, Brisorgueil,M.J., Fischer,J., Verge,D., Gingrich,J.A., Bourgoin,S., Hamon,M., Bernard,R., and Conrath,M. (2008). Role of spinal serotonin 5-HT_{2A} receptor in 2',3'-dideoxycytidine-induced neuropathic pain in the rat and the mouse. *Pain* 137, 66-80.
- Van,S.J., Brisorgueil,M.J., Fischer,J., Verge,D., Gingrich,J.A., Bourgoin,S., Hamon,M., Bernard,R., and Conrath,M. (2008). Role of spinal serotonin 5-HT_{2A} receptor in 2',3'-dideoxycytidine-induced neuropathic pain in the rat and the mouse. *Pain* 137, 66-80.
- Vanden Berghe,W., Vermeulen,L., Delerive,P., De Bosscher,K., Staels,B., and Haegeman,G. (2003). A paradigm for gene regulation: Inflammation, NF-kappa B and PPAR. *Peroxisomal Disorders and Regulation of Genes* 544, 181-196.
- Vedin,I., Cederholm,T., Levi,Y.F., Basun,H., Garlind,A., Irving,G.F., Joenahagen,M.E., Vessby,B., Wahlund,L.O., and Palmblad,J. (2008). Effects of docosahexaenoic acid-rich n-3 fatty acid supplementation on cytokine release from blood mononuclear leukocytes: the OmegAD study. *American Journal of Clinical Nutrition* 87, 1616-1622.
- Vellani,V., Zachrisson,O., and McNaughton,P.A. (2004). Functional bradykinin B1 receptors are expressed in nociceptive neurones and are upregulated by the neurotrophin GDNF. *J. Physiol* 560, 391-401.
- Vera-Portocarrero,L.P., Zhang,E.T., Ossipov,M.H., Xie,J.Y., King,T., Lai,J., and Porreca,F. (2006). Descending facilitation from the rostral ventromedial medulla maintains nerve injury-induced central sensitization. *Neuroscience* 140, 1311-1320.
- Verge,G.M., Milligan,E.D., Maier,S.F., Watkins,L.R., Naeve,G.S., and Foster,A.C. (2004). Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. *Eur. J. Neurosci.* 20, 1150-1160.
- Verge,V.M., Merlio,J.P., Grondin,J., Ernfors,P., Persson,H., Riopelle,R.J., Hokfelt,T., and Richardson,P.M. (1992). Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF. *J. Neurosci.* 12, 4011-4022.
- Verge,V.M., Richardson,P.M., Wiesenfeld-Hallin,Z., and Hokfelt,T. (1995). Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. *J. Neurosci.* 15, 2081-2096.
- Verge,V.M., Xu,X.J., Langel,U., Hokfelt,T., Wiesenfeld-Hallin,Z., and Bartfai,T. (1993). Evidence for endogenous inhibition of autotomy by galanin in the rat after sciatic nerve section: demonstrated by chronic intrathecal infusion of a high affinity galanin receptor antagonist. *Neurosci. Lett.* 149, 193-197.

Verma S, Estanislao L, and Simpson D. HIV-associated neuropathic pain: epidemiology, pathophysiology and management. *CNS Drugs* 19[4], 325-334. 2007.

Ref Type: Generic

Verma,S., Micsa,E., Estanislao,L., and Simpson,D. (2004). Neuromuscular complications in HIV. *Curr. Neurol. Neurosci. Rep.* 4, 62-67.

Veronesi,B., and Oortgiesen,M. (2006). The TRPV1 receptor: target of toxicants and therapeutics. *Toxicol. Sci.* 89, 1-3.

Vikman,K.S., Hill,R.H., Backstrom,E., Robertson,B., and Kristensson,K. (2003). Interferon-gamma induces characteristics of central sensitization in spinal dorsal horn neurons in vitro. *Pain* 106, 241-251.

Vikman,K.S., Siddall,P.J., and Duggan,A.W. (2005). Increased responsiveness of rat dorsal horn neurons in vivo following prolonged intrathecal exposure to interferon-gamma. *Neuroscience* 135, 969-977.

Villar,M.J., Cortes,R., Theodorsson,E., Wiesenfeld-Hallin,Z., Schalling,M., Fahrenkrug,J., Emson,P.C., and Hokfelt,T. (1989). Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin. *Neuroscience* 33, 587-604.

Vriens,J., Owsianik,G., Hofmann,T., Philipp,S., Stab,J., Chen,X., Benoit,M., Xue,F., Janssens,A., Kerselaers,S., Oberwinkler,J., Vennekens,R., Gudermann,T., Nilius,B., and Voets,T. (2011). TRPM3 Is a Nociceptor Channel Involved in the Detection of Noxious Heat. *Neuron* 70, 482-494.

Wagner,R., Janjigian,M., and Myers,R.R. (1998). Anti-inflammatory interleukin-10 therapy in CCI neuropathy decreases thermal hyperalgesia, macrophage recruitment, and endoneurial TNF-alpha expression. *Pain* 74, 35-42.

Wakisaka,S., Kajander,K.C., and Bennett,G.J. (1991). Increased neuropeptide Y (NPY)-like immunoreactivity in rat sensory neurons following peripheral axotomy. *Neurosci. Lett.* 124, 200-203.

Wakisaka,S., Kajander,K.C., and Bennett,G.J. (1992). Effects of peripheral nerve injuries and tissue inflammation on the levels of neuropeptide Y-like immunoreactivity in rat primary afferent neurons. *Brain Res.* 598, 349-352.

Walf,A.A., and Frye,C.A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat. Protocols* 2, 322-328.

Walker,K., Perkins,M., and Dray,A. (1995). Kinins and kinin receptors in the nervous system. *Neurochemistry International* 26, 1-16.

Wall,P.D., Scadding,J.W., and Tomkiewicz,M.M. (1979). The production and prevention of experimental anesthesia dolorosa. *Pain* 6, 175-182.

Wallace,V.C., Blackbeard,J., Pheby,T., Segerdahl,A.R., Davies,M., Hasnie,F., Hall,S., McMahon,S.B., and Rice,A.S. (2007a). Pharmacological, behavioural and mechanistic analysis of HIV-1 gp120 induced painful neuropathy. *Pain* 133, 47-63.

Wallace,V.C., Blackbeard,J., Segerdahl,A.R., Hasnie,F., Pheby,T., McMahon,S.B., and Rice,A.S. (2007b). Characterization of rodent models of HIV-gp120 and anti-retroviral-associated neuropathic pain. *Brain* 130, 2688-2702.

Wallace,V.C., Segerdahl,A.R., Blackbeard,J., Pheby,T., and Rice,A.S. (2008). Anxiety-like behaviour is attenuated by gabapentin, morphine and diazepam in a rodent model of HIV anti-retroviral-associated neuropathic pain. *Neurosci. Lett.* 448, 153-156.

- Wallace,V.C., Segerdahl,A.R., Lambert,D.M., Vandevoorde,S., Blackbeard,J., Pheby,T., Hasnie,F., and Rice,A.S. (2007c). The effect of the palmitoylethanolamide analogue, palmitoylethanolamide (L-29) on pain behaviour in rodent models of neuropathy. *Br. J. Pharmacol.* *151*, 1117-1128.
- Wallace,V.C.J., and Rice,A.S.C. (2008). Applied physiology: neuropathic pain. In *Clinical Pain Management 2nd Edition: 4 Volume Set*, A.S. Rice, D. Justins, C. Miaskowski, and T. Newton-John, eds. pp. 3-17.
- Wang,G., Hong,Y., Johnson,M.K., and Maier,R.J. (2006). Lipid peroxidation as a source of oxidative damage in *Helicobacter pylori*: protective roles of peroxiredoxins. *Biochim. Biophys. Acta* *1760*, 1596-1603.
- Wang,H., Sun,H., Della,P.K., Benz,R.J., Xu,J., Gerhold,D.L., Holder,D.J., and Koblan,K.S. (2002). Chronic neuropathic pain is accompanied by global changes in gene expression and shares pathobiology with neurodegenerative diseases. *Neuroscience* *114*, 529-546.
- Wang,R., Guo,W., Ossipov,M.H., Vanderah,T.W., Porreca,F., and Lai,J. (2003a). Glial cell line-derived neurotrophic factor normalizes neurochemical changes in injured dorsal root ganglion neurons and prevents the expression of experimental neuropathic pain. *Neuroscience* *121*, 815-824.
- Wang,X., Zhao,X., Mao,Z.Y., Wang,X.M., and Liu,Z.L. (2003b). Neuroprotective effect of docosahexaenoic acid on glutamate-induced cytotoxicity in rat hippocampal cultures. *Neuroreport* *14*, 2457-2461.
- Wang,Z.Q., Porreca,F., Cuzzocrea,S., Galen,K., Lightfoot,R., Masini,E., Muscoli,C., Mollace,V., Ndengele,M., Ischiropoulos,H., and Salverini,D. (2004). A newly identified role for superoxide in inflammatory pain. *Journal of Pharmacology and Experimental Therapeutics* *309*, 869-878.
- Ward,R.E., Huang,W., Curran,O.E., Priestley,J.V., and Michael-Titus,A.T. (2010). Docosahexaenoic acid prevents white matter damage after spinal cord injury. *J. Neurotrauma* *27*, 1769-1780.
- Warner,W.A., Bregman,C.L., Comereski,C.R., Arezzo,J.C., Davidson,T.J., Knupp,C.A., Kaul,S., Durham,S.K., Wasserman,A.J., and Frantz,J.D. (1995). Didanosine (ddl) and stavudine (d4T): Absence of peripheral neurotoxicity in rabbits. *Food and Chemical Toxicology* *33*, 1047-1050.
- Watkins,L.R., Milligan,E.D., and Maier,S.F. (2001b). Spinal cord glia: new players in pain. *Pain* *93*, 201-205.
- Watkins,L.R., Milligan,E.D., and Maier,S.F. (2001a). Glial activation: a driving force for pathological pain. *Trends in Neurosciences* *24*, 450-455.
- Welch,K.J. (2000). Correlates of alcohol and/or drug use among HIV-infected individuals. *Aids Patient Care and Stds* *14*, 317-323.
- Weldon,S.M., Mullen,A.C., Loscher,C.E., Hurley,L.A., and Roche,H.M. (2007). Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. *Journal of Nutritional Biochemistry* *18*, 250-258.
- Werner,M.F., Kassuya,C.A., Ferreira,J., Zampronio,A.R., Calixto,J.B., and Rae,G.A. (2007). Peripheral kinin B(1) and B(2) receptor-operated mechanisms are implicated in neuropathic nociception induced by spinal nerve ligation in rats. *Neuropharmacology* *53*, 48-57.
- Wernersson,J., Johansson,I., Larsson,U., Minth-Worby,C., Pahlman,S., and Andersson,G. (1998). Activated transcription of the human neuropeptide Y gene in differentiating SH-SY5Y neuroblastoma cells is dependent on transcription factors AP-1, AP-2 alpha, and NGFI. *Journal of Neurochemistry* *70*, 1887-1897.
- Werth,J.L., Zhou,B., Nutter,L.M., and Thayer,S.A. (1994). 2',3'-Dideoxycytidine Alters Calcium Buffering in Cultured Dorsal-Root Ganglion Neurons. *Molecular Pharmacology* *45*, 1119-1124.

- Weylandt,K.H., Chiu,C.Y., Gomolka,B., Waechter,S.F., and Wiedenmann,B. (2012). Omega-3 fatty acids and their lipid mediators: Towards an understanding of resolvins and protectin formation. *Prostaglandins & Other Lipid Mediators* 97, 73-82.
- White,D.M. (1997). Intrathecal neuropeptide Y exacerbates nerve injury-induced mechanical hyperalgesia. *Brain Res.* 750, 141-146.
- White,F.A., Bhangoo,S.K., and Miller,R.J. (2005a). Chemokines: integrators of pain and inflammation. *Nat Rev Drug Discov* 4, 834-844.
- White,F.A., Jung,H., and Miller,R.J. (2007). Chemokines and the pathophysiology of neuropathic pain. *Proc. Natl. Acad. Sci. U. S. A* 104, 20151-20158.
- White,F.A., Sun,J., Waters,S.M., Ma,C., Ren,D., Ripsch,M., Steflik,J., Cortright,D.N., LaMotte,R.H., and Miller,R.J. (2005b). Excitatory monocyte chemoattractant protein-1 signaling is up-regulated in sensory neurons after chronic compression of the dorsal root ganglion. *Proc. Natl. Acad. Sci. U. S. A* 102, 14092-14097.
- White,J.D. (1993). Neuropeptide Y: a central regulator of energy homeostasis. *Regul. Pept.* 49, 93-107.
- Wickenden,A.D. (2002). Potassium channels as anti-epileptic drug targets. *Neuropharmacology* 43, 1055-1060.
- Wiese,U.H., Ruth,J.L., and Emson,P.C. (1992). Differential expression of growth-associated protein (GAP-43) mRNA in rat primary sensory neurons after peripheral nerve lesion: a non-radioactive in situ hybridisation study. *Brain Res.* 592, 141-156.
- Wiesenfeld-Hallin,Z., Villar,M.J., and Hokfelt,T. (1988). Intrathecal galanin at low doses increases spinal reflex excitability in rats more to thermal than mechanical stimuli. *Exp. Brain Res.* 71, 663-666.
- Wilkinson,M.F., Earle,M.L., Triggle,C.R., and Barnes,S. (1996). Interleukin-1 β , tumor necrosis factor- α , and LPS enhance calcium channel current in isolated vascular smooth muscle cells of rat tail artery. *FASEB J.* 10, 785-791.
- Williams,A.G., Hargreaves,A.C., Gunn-Moore,F.J., and Tavares,J.M. (1998). Stimulation of neuropeptide Y gene expression by brain-derived neurotrophic factor requires both the phospholipase C γ and Shc binding sites on its receptor, TrkB. *Biochemical Journal* 333, 505-509.
- Williams,K.C., Ulvestad,E., and Hickey,W.F. (1994). Immunology of multiple sclerosis. *Clin. Neurosci.* 2, 229-245.
- Wolfgang,C.D., Chen,B.P., Martindale,J.L., Holbrook,N.J., and Hai,T. (1997). gadd153/Chop10, a potential target gene of the transcriptional repressor ATF3. *Mol. Cell Biol.* 17, 6700-6707.
- Wong,J., and Oblinger,M.M. (1990). Differential regulation of peripherin and neurofilament gene expression in regenerating rat DRG neurons. *J Neurosci. Res.* 27, 332-341.
- Wood,J.N., Boorman,J.P., Okuse,K., and Baker,M.D. (2004). Voltage-gated sodium channels and pain pathways. *J. Neurobiol.* 61, 55-71.
- Wood,J.N. (2010). Nerve Growth Factor and Pain. *New England Journal of Medicine* 363, 1572-1573.
- Woolf,C.J. (1983). Evidence for a central component of post-injury pain hypersensitivity. *Nature* 306, 686-688.
- Woolf,C.J., and Mannion,R.J. (1999). Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet* 353, 1959-1964.

- Woolf, C.J., Reynolds, M.L., Molander, C., O'Brien, C., Lindsay, R.M., and Benowitz, L.I. (1990). The growth-associated protein GAP-43 appears in dorsal root ganglion cells and in the dorsal horn of the rat spinal cord following peripheral nerve injury. *Neuroscience* 34, 465-478.
- Woolf, C.J., and Salter, M.W. (2000). Neuronal plasticity: increasing the gain in pain. *Science* 288, 1765-1769.
- Woolf, C.J., Shortland, P., and Coggeshall, R.E. (1992). Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 355, 75-78.
- Wotherspoon, G., and Winter, J. (2000). Bradykinin B1 receptor is constitutively expressed in the rat sensory nervous system. *Neurosci. Lett.* 294, 175-178.
- Wright, D.E., Ryals, J.M., McCarson, K.E., and Christianson, J.A. (2004). Diabetes-induced expression of activating transcription factor 3 in mouse primary sensory neurons. *J. Peripher. Nerv. Syst.* 9, 242-254.
- Wynick, D., Thompson, S.W., and McMahon, S.B. (2001). The role of galanin as a multi-functional neuropeptide in the nervous system. *Curr. Opin. Pharmacol.* 1, 73-77.
- Xiao, Y.F., Kang, J.X., Morgan, J.P., and Leaf, A. (1995). Blocking effects of polyunsaturated fatty acids on Na⁺ channels of neonatal rat ventricular myocytes. *Proc. Natl. Acad. Sci. U. S. A* 92, 11000-11004.
- Xu, I.S., Hao, J.X., Xu, X.J., Hokfelt, T., and Wiesenfeld-Hallin, Z. (1999). The effect of intrathecal selective agonists of Y1 and Y2 neuropeptide Y receptors on the flexor reflex in normal and axotomized rats. *Brain Res.* 833, 251-257.
- Xu, X.J., Dalsgaard, C.J., Maggi, C.A., and Wiesenfeld-Hallin, Z. (1992). NK-1, but not NK-2, tachykinin receptors mediate plasma extravasation induced by antidromic C-fiber stimulation in rat hindpaw: demonstrated with the NK-1 antagonist CP-96,345 and the NK-2 antagonist Men 10207. *Neurosci Lett.* 139, 249-252.
- Xu, X.J., Hao, J.X., Olsson, T., Kristensson, K., van der Meide, P.H., and Wiesenfeld-Hallin, Z. (1994). Intrathecal interferon-gamma facilitates the spinal nociceptive flexor reflex in the rat. *Neurosci. Lett.* 182, 263-266.
- Xu, Z.Z., Zhang, L., Liu, T., Park, J.Y., Berta, T., Yang, R., Serhan, C.N., and Ji, R.R. (2010). Resolvins RvE1 and RvD1 attenuate inflammatory pain via central and peripheral actions. *Nature Medicine* 16, 592-U129.
- Yamaguchi-Sase, S., Hayashi, I., Okamoto, H., Nara, Y., Matsuzaki, S., Hoka, S., and Majima, M. (2003). Amelioration of hyperalgesia by kinin receptor antagonists or kininogen deficiency in chronic constriction nerve injury in rats. *Inflamm. Res.* 52, 164-169.
- Yang, Y., Wang, Y., Li, S., Xu, Z., Li, H., Ma, L., Fan, J., Bu, D., Liu, B., Fan, Z., Wu, G., Jin, J., Ding, B., Zhu, X., and Shen, Y. (2004). Mutations in SCN9A, encoding a sodium channel alpha subunit, in patients with primary erythromalgia. *J. Med. Genet.* 41, 171-174.
- Yaqoob, P., and Calder, P.C. (2007). Fatty acids and immune function: new insights into mechanisms. *Br. J. Nutr.* 98 Suppl 1, S41-S45.
- Yoshida, S., Harik, S.I., Busto, R., Santiso, M., Martinez, E., and Ginsberg, M.D. (1984). Free Fatty-Acids and Energy Metabolites in Ischemic Cerebral-Cortex with Noradrenaline Depletion. *Journal of Neurochemistry* 42, 711-717.
- Youn, D.H., Wang, H., and Jeong, S.J. (2008). Exogenous tumor necrosis factor-alpha rapidly alters synaptic and sensory transmission in the adult rat spinal cord dorsal horn. *J. Neurosci. Res.* 86, 2867-2875.

- Young,E.E., Lariviere,W.R., and Belfer,I. (2012). Genetic basis of pain variability: recent advances. *Journal of Medical Genetics* 49, 1-9.
- Yowtak,J., Lee,K.Y., Kim,H.Y., Wang,J., Kim,H.K., Chung,K., and Chung,J.M. (2011). Reactive oxygen species contribute to neuropathic pain by reducing spinal GABA release. *Pain* 152, 844-852.
- Yu,L., Yang,F., Luo,H., Liu,F.Y., Han,J.S., Xing,G.G., and Wan,Y. (2008). The role of TRPV1 in different subtypes of dorsal root ganglion neurons in rat chronic inflammatory nociception induced by complete Freund's adjuvant. *Mol. Pain* 4, 61.
- Zelev,T., Sketelj,J., and Bajrovic,F.F. (2010). Efficacy of fluorescent tracers in retrograde labeling of cutaneous afferent neurons in the rat. *Journal of Neuroscience Methods* 191, 208-214.
- Zenonos,G., and Kim,J.E. (2010). A T cell-orchestrated immune response in the adult dorsal spinal cord as a cause of neuropathic pain-like hypersensitivity after peripheral nerve damage: a door to novel therapies? *Neurosurgery* 66, N24-N25.
- Zhang,J., and De Koninck,Y. (2006). Spatial and temporal relationship between monocyte chemoattractant protein-1 expression and spinal glial activation following peripheral nerve injury. *Journal of Neurochemistry* 97, 772-783.
- Zhang,K., Rana,F., Silva,C., Ethier,J., Wehrly,K., Chesebro,B., and Power,C. (2003). Human immunodeficiency virus type 1 envelope-mediated neuronal death: uncoupling of viral replication and neurotoxicity. *J. Virol.* 77, 6899-6912.
- Zhang,W., Gardell,S., Zhang,D., Xie,J.Y., Agnes,R.S., Badghisi,H., Hruby,V.J., Rance,N., Ossipov,M.H., Vanderah,T.W., Porreca,F., and Lai,J. (2009). Neuropathic pain is maintained by brainstem neurons co-expressing opioid and cholecystokinin receptors. *Brain* 132, 778-787.
- Zhang,X., Bao,L., Shi,T.J., Ju,G., Elde,R., and Hokfelt,T. (1998). Down-regulation of mu-opioid receptors in rat and monkey dorsal root ganglion neurons and spinal cord after peripheral axotomy. *Neuroscience* 82, 223-240.
- Zhang,X., Ju,G., Elde,R., and Hokfelt,T. (1993a). Effect of Peripheral-Nerve Cut on Neuropeptides in Dorsal-Root Ganglia and the Spinal-Cord of Monkey with Special Reference to Galanin. *Journal of Neurocytology* 22, 342-381.
- Zhang,X., Meister,B., Elde,R., Verge,V.M., and Hokfelt,T. (1993b). Large calibre primary afferent neurons projecting to the gracile nucleus express neuropeptide Y after sciatic nerve lesions: an immunohistochemical and in situ hybridization study in rats. *Eur. J. Neurosci.* 5, 1510-1519.
- Zhang,X., Nicholas,A.P., and Hokfelt,T. (1993c). Ultrastructural studies on peptides in the dorsal horn of the spinal cord--I. Co-existence of galanin with other peptides in primary afferents in normal rats. *Neuroscience* 57, 365-384.
- Zhang,X., Shi,T., Holmberg,K., Landry,M., Huang,W., Xiao,H., Ju,G., and Hokfelt,T. (1997). Expression and regulation of the neuropeptide Y Y2 receptor in sensory and autonomic ganglia. *Proc. Natl. Acad. Sci. U. S. A* 94, 729-734.
- Zheng,F.Y., Xiao,W.H., and Bennett,G.J. (2011a). The response of spinal microglia to chemotherapy-evoked painful peripheral neuropathies is distinct from that evoked by traumatic nerve injuries. *Neuroscience* 176, 447-454.
- Zheng,W., Zheng,x., Liu,S., and Hao,S. ROS in the spinal cord is involved in the neuropathic pain induced by antiretroviral drug in rats. *Society for Neuroscience Meeting* . 2011b.
- Ref Type: Abstract

Zheng,X., Ouyang,H., Liu,S., Mata,M., Fink,D.J., and Hao,S. (2011c). TNF+¹ is involved in neuropathic pain induced by nucleoside reverse transcriptase inhibitor in rats. *Brain, Behavior, and Immunity* 25, 1668-1676.

Zhu,Y., Antony,J.M., Martinez,J.A., Glerum,D.M., Brussee,V., Hoke,A., Zochodne,D., and Power,C. (2007). Didanosine causes sensory neuropathy in an HIV/AIDS animal model: impaired mitochondrial and neurotrophic factor gene expression. *Brain* 130, 2011-2023.

Zhuang,Z.Y., Gerner,P., Woolf,C.J., and Ji,R.R. (2005). ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* 114, 149-159.

Zhuo,M., and Gebhart,G.F. (1997). Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. *J. Neurophysiol.* 78, 746-758.

Ziegler,E.A., Magerl,W., Meyer,R.A., and Treede,R.D. (1999). Secondary hyperalgesia to punctate mechanical stimuli - Central sensitization to A-fibre nociceptor input. *Brain* 122, 2245-2257.

Ziff,E.B. (1990). Transcription factors: a new family gathers at the cAMP response site. *Trends Genet.* 6, 69-72.

Zimmermann,M. (2001). Pathobiology of neuropathic pain. *Eur. J. Pharmacol.* 429, 23-37.